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Current understanding of the mechanism of HPV infection

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Abstract

HPVs (human papillomaviruses) and other papillomaviruses have a unique mechanism of infection that has likely evolved to limit infection to the basal cells of stratified epithelium, the only tissue in which they replicate. Recent studies in a mouse cervicovaginal challenge model indicate that, surprisingly, the virus cannot initially bind to keratinocytes *in vivo*. Rather it must first bind via its L1 major capsid protein to heparan sulfate proteoglycans (HSPGs) on segments of the basement membrane (BM) exposed after epithelial trauma and undergo a conformational change that exposes the N-terminus of L2 minor capsid protein to furin cleavage. L2 proteolysis exposes a previously occluded surface of L1 that binds an as yet undetermined cell surface receptor on keratinocytes that have migrated over the BM to close the wound. Papillomaviruses are the only viruses that are known to initiate their infectious process at an extracellular site. In contrast to the *in vivo* situation, the virions can bind directly to many cultured cell lines through cell surface HSPGs and then undergo a similar conformational change and L2 cleavage. Transfer to the secondary receptor leads to internalization, uncoating in late endosomes, escape from the endosome by an L2-dependent mechanism, and eventual trafficking of an L2–genome complex to specific subnuclear domains designated ND10 bodies, where viral gene transcription is initiated. The infectious process is remarkably slow and asynchronous both *in vivo* and in cultured cells, taking 12–24 h for initiation of transcription. The extended exposure of antibody neutralizing determinants while the virions reside on the BM and cell surfaces might, in part, account for the remarkable effectiveness of vaccines based on neutralizing antibodies to L1 virus-like particles or the domain of L2 exposed after furin cleavage.

Keywords

HPV infection cycle; HPV binding; HPV entry; HPV intracellular trafficking; HPV antibodies

Introduction

Papillomaviruses (PVs) have an interesting and, in some ways, unique process of infection. Emerging insights into this process suggest that many of its unusual aspects are adaptations to characteristic features of the viral lifestyle, namely the restriction of the productive life cycle to terminally differentiating stratified squamous epithelium and the ability to delay induction of an effective immune response for an extended time. The inability to productively infect replicating cells in culture has hampered studies of PV infection. Insights

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into the infectious process have therefore been dependent on a succession of technological advances enabled by the advent of modern molecular biology. These advances have, in turn, allowed successively more sophisticated analyses of the process.

Early studies mostly involved non-infectious virus-like particles (VLPs) (that can be generated by expression of solely the L1 major capsid protein) [1]. VLPs enabled cell surface interaction studies, but it was impossible to distinguish between infectious and non-infectious uptake of the particles. Subsequent studies mostly utilized either virions, usually generated in organotypic raft culture, or infectious pseudoviruses (PsVs) that transduce genes easily monitored for infectious events [2,3]. PsVs are generated by co-expression of L1 and the minor capsid protein L2 in replicating mammalian cells containing autonomous replicons that can be encapsidated by the assembling particles. Recent experiments have begun to examine PsV infection of epithelial tissues *in vivo* and have revealed unique features of infection that were not observed in the examination of cultured cells [4]. An understanding of PV infection may contribute to the development and evaluation of strategies to prevent infection by human papillomaviruses (HPVs), the causative agents of essentially all cervical cancers, a number of other carcinomas, and cutaneous and mucosal papillomas.

The recent demonstration of the remarkable effectiveness of prophylactic HPV vaccines has generated increased interest in understanding how the vaccines prevent HPV infection. This review focuses on events of PV infection from the initial contact with the cell or tissue through the steps leading to the expression of the viral genome in the nucleus. It also discusses how vaccine-induced neutralizing antibodies are able to prevent infection.

Attachment

Initial studies using VLPs established that PVs bind to many epithelial and other cultured cell lines through an evolutionary conserved proteinaceous receptor abundantly displayed on the cell surface [5]. VLPs composed of L1 alone or both L1 and L2 bound similarly, implying that L1 contains the major determinant(s) for initial attachment. Most investigators now agree that heparan sulfate proteoglycans (HSPGs) are the critical primary attachment factors, at least for epithelial cells. Findings that support this conclusion include inhibition of binding and infection by heparinase treatment or by heparin (a soluble form of heparan sulfate [HS]) [6,7].

Certain other sulfated polymers, such as carrageenans, are even more potent infection inhibitors, but it has been difficult to predict relative activities based on structural considerations [8]. One study concluded that HPV-31 was exceptional in not requiring HSPGs for infection of cultured epithelial cells [9]. In addition to cell surfaces, PV capsids also bind to the extracellular matrix (ECM) that is deposited by many epithelial cell lines grown *in vitro* [10]. Both HS and laminin-5 may contribute to ECM binding of the capsids [11,12].

In contrast to most established epithelial cell lines, L1/L2 PsVs do not efficiently bind or infect primary cultured keratinocytes [13]. Quite remarkably, they also do not efficiently bind or infect intact epithelial tissues *in vivo*: neither stratified squamous nor simple columnar epithelium of the cervicovaginal tract or other organs [4]. In a mouse model, initial binding of HPV PsVs was shown to be limited to the basement membrane (BM), which underlies the epithelium, separating it from the dermis. The PsVs bound efficiently to regions of the BM only after these regions were exposed by mechanical or chemical trauma to the epithelium. Several hours after initial binding to the BM, the capsids were detected on the surfaces of epithelial cells in the vicinity of the “wound,” presumably due to transfer from the BM [4].

Instillation of heparin or heparinase into the vaginal tract prevented BM binding and PsV infection in the mouse cervicovaginal challenge model, implying that HS binding on the BM is an obligate initial step in infection in vivo [14]. In contrast to in vitro results, HPV-31 infection was clearly HSPG-dependent in the murine cervicovaginal challenge model. The ECM in vitro and the BM in vivo may not be entirely analogous since laminin-5 does not appear to have a role in binding the BM [14] and HSPGs apparently play a larger role in vivo. Our model of the in vivo events that precede uptake by the keratinocytes is illustrated in Fig. 1.

The findings outlined above suggest the following schema. Many different patterns of N- and O-sulfation are known to exist on HSPGs, and PV capsids preferentially bind to a specific subset [15]. PVs may have evolved to attach to HS modification patterns that are uniquely enriched on the BM in vivo. The surfaces of intact epithelia apparently contain sulfation patterns that do not bind PV capsids. Binding to the BM may have evolved to promote the preferential interaction with basal keratinocytes that are migrating over the exposed BM to close the wound. Interaction with these cells would benefit the virus because productive infection appears to be dependent on the full programme of keratinocyte terminal differentiation; therefore, interaction with or infection of suprabasal keratinocytes would be non-productive. Infection of these cells might even be detrimental by promoting an earlier or more robust immune response to the virus. Since epithelial cells normally divide when associated with the BM, in vitro passage of cells in culture may often select for sulfation patterns on cell surfaces that mimic those normally found on the BM, thus accounting for the more promiscuous binding of PsVs to cultured cell lines.

Several types of immunocytes bind and internalize PV capsids, including dendritic cells (DCs), Langerhans cells (LCs), monocytes, macrophages, and B cells [16–19]. While these interactions are likely to be important for immune recognition of the virion proteins after infection or VLP vaccination, there is no evidence that the interaction results in infection of these cell types either in vitro or in vivo. As with keratinocytes, the binding appears to be primarily L1-mediated. Binding by some cells, e.g., DCs, probably involves HSPGs, but other molecules, such as Fcγ receptors or langerin on LCs, may be involved in binding to other immunocytes.

Entry

There is a remarkably long delay between initial capsid binding and viral genome (or pseudogenome) expression. Spliced viral mRNA using a sensitive nested RT-PCR technique was first detected at 12 h post-infection with authentic bovine PV type 1 (BPV-1) [20]. In most assay systems, infection is not robustly detected until at least 24 h after capsid binding. This is the case for both cultured cells and keratinocytes in vivo. The first slow phase in infection is internalization, which usually takes 2–4 h after cell surface binding [21,22]. Several distinct pre-entry steps have been identified. Binding of HSPGs to the BM in vivo, or to the cell surface in vitro, induces a conformational change in the capsid that exposes the N-terminus of L2 to cleavage by furin, or the closely related proprotein convertase (PC) 5/6 [23]. The furin cleavage site is absolutely conserved among all PVs and cleavage is required for infection. In the mouse cervicovaginal challenge model, furin inhibition does not affect BM binding but prevents subsequent binding to keratinocytes. Immunohistochemical studies indicated that both furin and PC 5/6 are abundant at sites of disruption of the murine cervicovaginal tract, so both proteases may contribute to L2 cleavage of capsids bound to the BM [24].

We believe that the combination of the conformational change and furin cleavage of L2 exposes the binding site for the cell surface receptor that is involved in infectious

internalization. There are several lines of evidence that support this conjecture. Perhaps the best evidence comes from studies of furin-precleaved (FPC) PsVs. When PsVs are initially liberated from producer cells they are in an “immature” state characterized by a more open structure with few intercapsomeric disulfide bonds [25]. Unlike mature PsVs or authentic virions from papillomas, the immature capsids are susceptible to furin cleavage in solution [23]. Unlike normal PsVs and virions, FPC capsids are able to bind and infect cells that are devoid of HSPGs or contain HS modifications that are not normally recognized by the capsids, e.g., primary keratinocytes in culture [13]. Because L1 VLPs also bind these same cell types, we speculate that the conformational change induced by HSPG binding and subsequent furin cleavage of L2 exposes a secondary receptor binding site on L1 that is obscured in L1/L2 mature particles.

In the presence of a furin inhibitor, PsVs initially bind to the BM in vivo but are subsequently lost [24]. Therefore, we further speculate that the initial conformational change that exposes the furin cleavage site also reduces the affinity of the capsid for HS and thereby facilitates transfer to the keratinocyte-specific receptor. The identity of the keratinocyte-specific receptor is unknown. One candidate that has been suggested based on in vitro studies is $\alpha 6$ -integrin, an epithelial cell adhesion molecule [26]. However, some cell lines devoid of $\alpha 6$ -integrin are readily infected, so it certainly is not an obligatory cell surface receptor for in vitro infection [27,28].

Microscopy studies of individual capsid movement on the surface of cultured cells has revealed that the capsids preferentially bind to filopodia at the leading edge of migrating cells and then rapidly “surf” toward the cell body in an actin-dependent manner [29,30]. The particles then coalesce and become fixed in discrete punctate areas prior to internalization. It is uncertain whether in vitro surfing is in association with an HSPG receptor or secondary receptor. Nevertheless, these in vitro observations can easily be integrated into a model of in vivo infection in which the capsids bound to the exposed BM transfer to the leading edge of keratinocytes that are migrating over it during the wound healing process and subsequently surf towards the cell body. At this site, the capsids are internalized via the keratinocyte-specific receptor.

Intracellular trafficking

The endocytic pathways involved in internalization and intracellular trafficking of the PV capsid have been extensively investigated. However, little consensus has emerged. In part, this might be due to various genotypes using different pathways. However, disparate conclusions have also been reached in investigations of the same genotype. Differences in the nature of capsid (VLP, PsV, or virion) employed, the maturation state of the capsid, the specific experimental manipulations, and the end-points analysed (e.g., internalization versus infection) could all contribute to the discrepancies. Regardless of genotype, internalization occurs slowly and asynchronously over the span of several hours. In contrast, most other virus types are internalized within minutes of cell surface binding. The general scheme of internalization and intracellular trafficking is illustrated in Fig. 2.

Most studies have implicated a clathrin-mediated endocytosis pathway for the majority of PV types that have been studied, including BPV-1 and HPV-16 [20,31–33]. Uptake and infection are blocked by inhibitors of clathrin-mediated uptake, such as chlorpromazine. In addition, the capsids co-localize with well-established markers of the clathrin-mediated pathway, e.g., adaptor protein complex 2, transferrin receptor, and early endosome antigen 1. However, the slow kinetics of internalization are atypical for this pathway. Therefore, it is possible that these characteristics represent those of a previously undescribed endocytic pathway. In contrast, several, but not all, studies have concluded that HPV-31, which is

closely related to HPV-16, can enter through a caveolae-mediated pathway and not via clathrin-mediated endocytosis [33,34]. Other studies have suggested that BPV-1 and HPV-16 initially enter via clathrin-coated pits but then traffic through caveosomes to eventually reach the endoplasmic reticulum [32]. However, other laboratories have failed to detect inhibition of infection by caveolar inhibitors such as filipin and nystatin.

Finally, a recent study utilizing small-interfering-RNA-mediated downregulation of clathrin heavy chain and caveolin 1, and dominant negative mutants of proteins in these pathways, led to the conclusion that internalization of HPV-16 was both clathrin- and caveolin-independent. The authors suggested that the capsids might be internalized via a novel pathway involving tetraspanin-enriched microdomains [35]. In general, the results of inhibitor studies must be interpreted with caution, since the inhibition of a major endocytic pathway is likely to have many secondary effects on cell physiology, and inhibition of one endocytic pathway may lead to a default uptake by an alternative pathway.

Uptake and trafficking into Lamp-2-positive late endosomes, at least for HPV-16 and BPV-1, appears to exclusively involve L1-specific receptors, since L1 VLPs and authentic virions co-localize up to this point when initially bound to the same cell [20]. At least partial uncoating occurs in the late endosomes, as measured by the exposure of 5-bromo-2-deoxyuridine (BrdU)-labelled viral genomic DNA in this compartment [36]. Uncoating is not observed until approximately 8–12 h after cell surface binding. The genomes of L2-containing capsids escape from the late endosome, whereas the genomes of L1-only capsids do not. Consistent with a critical role of L2 in endosome escape is the finding that a conserved C-terminal L2 peptide has strong membrane-penetrating and disrupting activity in vitro [37]. L2 and the genome remain in a complex, as evidenced by co-localization of L2 and BrdU-specific antibodies [36].

After endosome escape, both the fate of L1 and the mechanism by which the L2–genome complex traffic through the cytoplasm and into the nucleus are poorly understood. Microtubule disruption inhibits PV infection at a late step [20,38], most likely the post-endosomal step of delivering the viral genome into the nucleus. Cytoplasmic transport along microtubules is mediated by motor protein complexes, and L2 has been found to interact with the microtubule network via the motor protein dynein during infectious entry [39]. There is good evidence that cell division is required for establishment and expression of the viral genome in the nucleus, at least in cultured cells [40]. Therefore, entry of the viral genome into the nucleus may follow nuclear membrane breakdown during mitosis rather than through active transport of the L2–genome complex via karyopherins [41]. Ultimately, the complexes predominantly localize in distinct punctate nuclear domains designated ND10 bodies or promyelocytic leukaemia (PML) oncogenic domains (PODs), as determined by their co-localization with PML, the ND10 defining protein [36].

Localization at ND10 promotes transcription of the viral genome. This positive function of ND10 domains in the PV life cycle contrasts with the evidence that herpes and other DNA viruses target PML for degradation because ND10s function to inhibit viral replication (reviewed in [42,43]). Reorganization of ND10 by L2 has been observed in productive lesions of the cervix [44]; so, although the role of ND10 in the establishment of infection in vivo has not been confirmed, the interaction of L2 with these nuclear bodies per se does not appear to be an in vitro artefact.

Antibody neutralization

Vaccines based on L1-only VLPs are highly effective at preventing PV infection and the neoplastic diseases they induce, both in preclinical trials involving animal PV challenge models and in HPV vaccine clinical trials evaluating anogenital infection in both women and

men (reviewed in [45]). Remarkably, transient infection is rarely detected in vaccinees, implying that the vaccines usually induce sterilizing immunity [46]. VLP vaccination induces high titres of genotype-restricted neutralizing antibodies, as measured using in vitro assays [47].

These antibodies are thought to be the primary, if not the only, immune effectors of protection following vaccination. Consistent with this idea, passive transfer of VLP-induced antibodies induced protection from experimental challenge in both animal PV challenge models [48,49] and in the mouse cervicovaginal HPV challenge model (our unpublished observation). The insights into the process of PV infection obtained in the studies outlined above provided the critical background for several recent studies to investigate how vaccine-induced antibodies prevent infection. One initial implication of the infection studies is that the selection of L1 VLPs, rather than L1/L2 VLPs, for the commercial vaccines may have been a fortunate choice. L1-only VLPs were selected over the physiologically more relevant L1/L2 VLPs because they were simpler to manufacture and generated titres of genotype-specific in vitro neutralizing antibodies similar to those of L1/L2 VLPs.

However, based upon subsequent insights into the infectious process, we now suspect that L1-only VLPs display both the HSPG and secondary receptor binding sites to the humoral immune system [24]. In contrast, soluble L1/L2 VLPs would display only the HSPG binding site because the secondary receptor binding site is occluded by the N-terminus of L2. With the potential to generate 2 classes of neutralizing antibodies, L1 VLP vaccination might be more effective at preventing infection in vivo.

Supporting the idea that L1 VLPs induce 2 classes of neutralizing antibodies was the finding that monoclonal antibodies raised to L1 VLPs could prevent infection of cultured cells by 2 distinct mechanisms [50]. One class, exemplified by H16.U4, blocked cell surface association but allowed ECM binding. The second class, exemplified by H16.V5 and H16.E70, allowed cell surface association but not ECM binding. However, V5- and E70-bound capsids were not internalized after cell surface binding. The failure to internalize correlated with a failure to expose an N-terminal epitope of L2 that is normally exposed only after the HSPG-dependent conformational change and furin cleavage [51]. Thus we hypothesized that the primary mechanism of inhibition by this second class of antibody is prevention of the initial conformational change, perhaps by binding bivalently across capsomers. The monoclonal antibodies of this class have very low 50% inhibitory concentrations (IC₅₀) of 2 pM and 40 pM, whereas the antibody of the first class has an IC₅₀ of 5 nM. This led to the conjecture that perhaps fewer bound antibody molecules might be needed to prevent the conformational change, which might occur as a concerted reaction across the capsid, than are needed to block cell association. Sera from VLP-vaccinated women behaved as the second class, in that they prevented internalization but not cell surface binding [51]. We are currently investigating the in vivo mechanisms of neutralization by VLP-induced antibodies using the murine cervicovaginal challenge model.

Vaccines based on L2 have the unexpected ability to induce broadly genotype cross-neutralizing antibodies, with cross-neutralization even extending across PV genus boundaries [52]. The epitopes that induce these cross-neutralizing antibodies are not exposed or are subdominant when L2 is in its normal context within the capsid, since L1/L2 VLPs induce no more cross-neutralizing antibodies than do L1 VLPs [53]. The results of mapping the major cross-neutralizing L2 epitopes provided an explanation for these observations. These highly conserved epitopes are centred on amino acids 17–36, a peptide that is immediately downstream of the conserved furin cleavage site at amino acid 13 (for HPV-16) [54,55]. Thus, these epitopes are not exposed until after HSPG binding and furin cleavage and are, therefore, not routinely subject to systemic B cell responses [51]. In fact,

binding of RG-1, a cross-neutralizing L2 monoclonal antibody that recognizes this sequence, has been an invaluable reagent for monitoring the HSPG-dependent conformational change and furin cleavage events. L2 vaccines induce strong protection against homologous and heterologous virus challenge in animal PV models [52,53,56–58].

As expected, L2 neutralizing antibodies did not block cell surface binding in vitro, since they do not interact with the capsids in solution. Because the first described L2-dependent activity during infection is endosome escape, we had anticipated that L2 neutralizing antibodies would block at this late stage of infection, following internalization. Unexpectedly, L2 antibodies induced the release of the capsid–antibody complexes from the surface of cultured cells and their accumulation on the ECM [51]. Based upon our current understanding of PV infection, these results suggest that binding of antibodies to the L2 terminus, exposed after furin cleavage, sterically hinders binding of the secondary receptor by L1. Loss of cell surface attachment is consistent with the previously mentioned idea that the conformational change that exposes L2 to furin also reduces the capsid's affinity for HSPGs. Accumulation on the ECM indicates that at least 1 of its receptors is distinct from the cell surface HSPGs and the secondary receptor. Laminin 5 is the most likely candidate [11]. We are presently performing studies to investigate the in vivo mechanisms of anti-L2 protection.

Targeting of vaccines to cryptic, broadly cross-reactive epitopes that are exposed only after primary receptor binding have been proposed for other viruses, including HIV. However, the performance of these types of vaccines has, for the most part, not matched their theoretical attractiveness. However, our recently obtained mechanistic insights into HPV infection in vivo provide explanations for the exceptional effectiveness of vaccines targeting cryptic L2 epitopes in preclinical models. First, the relationship between the primary attachment factor and the internalization receptor are unique in being topologically separated, with the former being on the BM and the latter on the cell surface. Second, internalization after cell binding occurs incredibly slowly; therefore, the crucial L2 epitopes are exposed for several hours. This situation is in marked contrast to HIV fusion intermediates, which are very transiently exposed structures (reviewed in [59]). These considerations encourage the further development and clinical testing of L2-based HPV vaccines.

Conclusion

The examination of the PV infectious process in the mouse cervicovaginal challenge model has revealed many similarities but also important differences between infection of an epithelial tissue and infection of cultured cell lines. In both cases, HSPGs are the primary attachment factor and infection is unusually slow and asynchronous. The major difference is that in vivo, the critical HSPGs involved in capsid binding are located on the acellular BM rather than on the cell surface, and the first set of conformational changes required for infection occur prior to cell surface binding. To our knowledge, PVs are the only viruses in which the infectious process is initiated at an extracellular site. The virions also bind to the ECM deposited by cultured cells, but it is not equivalent to the BM, because the initial conformation changes and furin cleavage do not occur there.

It is interesting to consider the possibility that PVs have actively evolved to have an extremely slow infectious process. In vivo, infection is limited to sites of epithelial disruption, and host immune response mechanisms would likely be focused on these sites. In our murine model, the epithelium is repaired within 1–2 days. Therefore, a delay of 1–2 days in the initiation of viral gene expression may facilitate the escape from the initial immune response to infection. However, this adaptation to escape natural immune surveillance by retarding infection may be the virus' Achilles heel with respect to vaccine

interventions. The prolonged exposure of targets of neutralizing antibodies during the infectious process probably contributes to the exceptional effectiveness of L1- and L2-based prophylactic vaccines.

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Keypoints

- HPV virions cannot bind the cell surface receptor involved in their internalization until they have undergone an HSPG-dependent conformational change and furin cleavage of L2.
- The HSPGs that serve as the critical attachment factor are on the basement membrane in epithelial tissues, whereas they are on the cell surface of immortalized cells in culture.
- HSPG and secondary receptor binding are L1-dependent.
- The first known role of L2 in infection is escape of the L2–genome complex from late endosomes.
- Association with ND10 in the nucleus facilitates viral genome transcription.
- The processes of internalization and intracellular trafficking are slow and asynchronous both in vivo and in vitro.
- The exceptional effectiveness of L1 and L2 neutralizing antibodies in preventing in vivo infection is likely due, at least in part, to the lengthy exposure of neutralizing epitopes while the virus resides on the BM and cell surface.

Fig. 1.

The virion first binds to HSPGs on the BM exposed after disruption (A). This induces a conformational change exposing a site on L2 susceptible to proprotein convertase (furin or PC 5/6) cleavage (B). After L2 cleavage, an L2 neutralizing epitope is exposed and a previously unexposed region of L1 binds to an unidentified secondary receptor on the invading edge of the epithelial cells (C). BM=basement membrane; HSPG=heparan sulfate proteoglycan.

Fig. 2.

After initial binding to HSPGs and furin cleavage, the virus is transferred to an unidentified receptor on the cell surface (A). The virus then enters the cell via an endocytic pathway (B) and within 4 h localizes in the early endosome (C). By 12 h, the virus uncoats within the late endosome, and the viral genome complexed with L2 is released (D). The L2-genome complex traffics through the cytoplasm, perhaps via microtubules, and enters the nucleus by 24 h (E). After nuclear entry, the complex co-localizes with ND10 and RNA transcription begins (F). HSPG=heparan sulfate proteoglycan.