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Concepts of papillomavirus entry into host cells

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Papillomaviruses enter basal cells of stratified epithelia. Assembly of new virions occurs in infected cells during terminal differentiation. This unique biology is reflected in the mechanism of entry. Extracellularly, the interaction of nonenveloped capsids with several host cell proteins, after binding, results in discrete conformational changes. Asynchronous internalization occurs over several hours by an endocytic mechanism related to, but distinct from macropinocytosis. Intracellular trafficking leads virions through the endosomal system, and from late endosomes to the trans-Golgi-network, before nuclear delivery. Here, we discuss the existing data with the aim to synthesize an integrated model of the stepwise process of entry, thereby highlighting key open questions. Additionally, we relate data from experiments with cultured cells to *in vivo* results.

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Introduction

Papillomaviruses (PV) are a large family of viruses with transforming potential, several of which are implicated in anogenital cancers and tumors of the head and neck [1]. PV particles are nonenveloped icosahedrons ($T = 7$) with a diameter of 50–55 nm. This capsid is formed by 72 pentamers of the major structural protein, L1, and variable amounts (up to 72 copies) of the minor structural protein, L2 [2,3]. The encapsidated genome is a circular, double-stranded DNA. These particles mediate transmission and entry through mechanisms that are currently unique amongst viruses. A particularly interesting feature is the protracted residence of viral particles on the cell surface before endocytic uptake and the extended time until infection is established [4•,5,6,7••,8]. Many of the atypical aspects of PV infections are likely adaptations due to the restriction of the productive life cycle to the terminally differentiating stratified squamous epithelium, and the ability to avoid induction of a host immune response [1]. The former issue has presented an experimental challenge as authentic viruses are not readily available for entry studies (see below).

A variety of *in vitro* systems have been used to produce surrogate viral particles. Non-infectious virus-like particles (VLPs) formed by L1 or L1 and L2 mimic the conformation of authentic virus [9]. They are the basis for the current vaccines, which attests to their authenticity at an immunologic level. Pseudovirions (PsV), harbor a plasmid, which encodes a reporter protein and serves as a viral pseudogenome [10]. Entry of VLPs can be followed by biochemical methods and microscopy, whereas expression of the PsV reporter indicates a successful ''pseudoinfection''. Therefore PsV are used for the majority of current PV research that is focused on entry, both *in vitro* and *in vivo*. In another *in vitro* PV production system the viral genome is transfected into primary keratinocytes, which are subsequently grown to differentiate into three-dimensional epithelium termed an organotypic raft. Virions are produced in the upper layers of the raft culture which mimics the natural situation [11]. However, with this method it is difficult to obtain particles of sufficient purity to adequately perform microscopic analyses of virus entry.

The range of methods to produce proxy PV virions poses a caveat to the comparison of different studies, as the purity and quality of the particles varies. If many defective or empty particles are added alongside legitimate particles, the high total dose may affect the outcome of infection. Many studies within the existing literature lack information on particle quality and quantity. We propose that subsequent work should document measures such as viral genome equivalents and viral protein amounts (or particle number) per cell.

In this review, we outline the emerging concepts of how incoming PV engage receptors, induce endocytosis, traffic intracellularly to the nuclear site of replication, and how structural alterations of the capsid may facilitate these processes and the release of the viral genome for eventual replication. Moreover, the data from *in vivo* studies are highlighted. Please refer to several recent reviews for a more detailed discussion on particular aspects of PV entry [1,12–20].

Binding

Several lines of evidence have established that PV initially bind to the glycosaminoglycan (GAG) chains

of heparan sulfate proteoglycans (HSPG). Early work showed that L1 VLPs interact with immobilized heparin, and that soluble heparin inhibits VLP binding to cells [21]. Later work demonstrated the importance of this interaction for PsV infection of cultured cells [6]. PV can also bind to the extracellular matrix (ECM) of cultured cells through interactions with HSPG and laminin-332 [22–24,25•]. Laminin-332 can serve as a transient binding receptor, but appears to be dispensable for infection of cultured cells.

Mutational analysis and X-ray crystallography of L1 capsomers indicated charge-based interactions of PV with heparin at minimally four different sites [26,27]. It appears that PV do not require a specific HSPG protein core for binding and infection [28,29•]. Since O-sulfation but not N-sulfation of HS moieties is required for infection [30,31], specificity for binding and entry is instead conferred by the sequence of GAGs and their sulfation pattern [22].

Although the majority of the PV literature stresses the importance of PV interaction with HS moieties for binding and infection, a few studies suggest that certain PV types or virion preparations may infect cells independent of HSPGs [32–35].

Extracellular structural changes

After initial binding, several discrete structural changes in human PV (HPV virions) have been described. Post-binding conformational changes were first suggested by a shift from a heparin-sensitive to a heparin-insensitive form of the virus [6]. PV interaction with HS-GAGs induces the exposure of a linear epitope located in the cleft between capsomers [22]. Additionally, a critical conformational change exposes the amino terminus of L2, which is originally buried within the capsid. This exposure appears to require extracellular cyclophilin B (CyPB), a cellular petidyl-prolyl cis/trans isomerase [36]. The exposed L2 N-terminus contains a furin/proprotein convertase cleavage site, which is conserved amongst most PV [37••]. *In vitro*, this site is proteolytically cleaved on the cell surface and cleavage is essential for successful infection. Importantly, these early, HSPG-dependent events have been shown to occur on the extracellular basement membrane (BM) in the murine cervico-vaginal model of HPV16 infection [38••].

Current data indicate that these structural alterations cause a reduced affinity of virus to HSPG that is required for subsequent engagement of a secondary receptor and primes the virus for later steps in entry, including uncoating and membrane penetration [31]. If HSPG release is blocked, the virus is channeled into a non-infectious uptake pathway [25•]. This suggests that, following HSPG interaction, the virus engages a second, HSPG-independent cellular receptor. Further support for a HSPG-independent receptor stems from the finding that virions in which L2 has been precleaved by furin can bind to and infect HSPG-deficient cells [39]. The interaction with the putative secondary receptor may trigger infectious uptake. The cleavage of L2 appears to further facilitate membrane penetration, a later step during entry [37••].

Internalization receptor

The identity of the putative secondary receptor has remained elusive despite extensive efforts to identify it. This could indicate that it is not a single molecule. The candidate receptors thus far include alpha 6 integrin, tetraspanin CD151, and annexin A2 heterotetramer [24,40–42,43••,44,45,56]. All of these have the attractive feature of high expression within the basal epithelium, the *in vivo* target cells for PV infection. All are also known to be associated with HSPG complexes. However, alpha 6 integrin and annexin A2 cannot be considered obligate receptors as deficient cell lines can be infected.

A rather unconventional mode of receptor engagement has been suggested, in which PVs and growth factors attached to HSPG mediate PV entry [46]. This idea entails attachment of particles to HSPG on cells, removal of the HSPG–virus complex by host metalloproteinases, and reattachment of these complexes to growth factor receptors *via* growth factors attached to the shed virus–HSPG complex. Although experimental evidence exists that cells can be infected *in vitro* by this mechanism, it is unclear to what extent this mechanism contributes to infection *versus* direct cell surface interaction and entry. It is not an obligate entry step as ECM-bound virus that is cleaved by furin can efficiently infect HSPG-deficient cells [39]. There is currently no evidence that this mechanism contributes to infection *in vivo*.

Dynamics of cell surface interactions

There is limited information on the dynamics of PV interactions before internalization. Data for virus ensembles suggest that binding is relatively quick but PV endocytose rather slowly [4–6,7••,8]. The reason for this protracted cell surface residence is undetermined. Single virus tracking revealed different types of lateral movement on the plasma membrane including diffusive and directed motions before confinement [47•]. The directed motion (surfing) is most prominent on filopodia [47•,48•]. Powered by actin retrograde flow, this motion propels bound virions toward the cell body [47•]. It is unclear how important this motion is for infection *in vivo*, but it is well established that filopodia play a major role in epithelial wound healing which appears to be critical for *in vivo* infection [49] A second study showed that single endocytic events can occur within two minutes of confinement in live cells [7••]. This suggested that the formation of an endocytic vesicle is quick, and that the slow uptake reflects an asynchronous mode of internalization is likely