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ABSTRACT

Human papillomaviruses (HPVs) are small dsDNA tumor viruses, which are the etiologic agents of most cervical cancers and are associated with a growing percentage of oropharyngeal cancers. The HPV capsid is non-enveloped, having a T=7 icosahedral symmetry formed *via* the interaction among 72 pentamers of the major capsid protein, L1. The minor capsid protein L2 associates with L1 pentamers, although it is not known if each L1 pentamer contains a single L2 protein. The HPV life cycle strictly adheres to the host cell differentiation program, and as such, native HPV virions are only produced *in vivo* or in organotypic “raft” culture. Research producing synthetic papillomavirus particles—such as virus-like particles (VLPs), papillomavirus-based gene transfer vectors, known as pseudovirions (PsV), and papillomavirus genome-containing *quasivirions* (QV)—has bypassed the need for stratifying and differentiating host tissue in viral assembly and has allowed for the rapid analysis of HPV infectivity pathways, transmission, immunogenicity, and viral structure.

KEY WORDS: papillomavirus, assembly, structure, cancer, life cycle.

Replication and Assembly of Human Papillomaviruses

INTRODUCTION

Over 200 human papillomavirus (HPV) types have been identified. All HPV types are epitheliotropic viruses that can be subdivided based on their ability to infect either mucosal or cutaneous keratinocytes. Mucosal-infecting HPV types can be further subdivided into low-risk and high-risk types. Low-risk HPV types lead to the development of benign neoplasms, such as warts and condyloma acuminatum, whereas high-risk HPV types can lead to malignant neoplasms, such as cervical cancer (de Villiers *et al.*, 2004; Longworth and Laimins, 2004). A very strong association has been made between malignant progression and certain HPV types such as HPV16, HPV18, HPV31, HPV45, etc. (de Villiers *et al.*, 2004; IARC, 2007; Longworth and Laimins, 2004). Infection from any of the high-risk HPV types is considered the most significant risk factor regarding the development of cervical cancer (Franquemont *et al.*, 1989; Morrison *et al.*, 1991; Madsen *et al.*, 2008). In the case of infections of the oral cavity and/or oropharynx, HPV6, HPV11, HPV16, and HPV18 are common, with approximately 40-60% of head and neck cancers containing oncogenic HPV types such as HPV16 and HPV18 (zur Hausen, 1996; Herrero *et al.*, 2003; Gillison, 2004; D'Souza *et al.*, 2007; Anaya-Saavedra *et al.*, 2008; Fakhry *et al.*, 2008). While HPV16 is found in approximately 90% of HPV-containing head and neck cancers, several other high-risk HPV types have been detected in these lesions (Hoshikawa *et al.*, 1990; Niedobitek *et al.*, 1990; Ostwald *et al.*, 1994; Shindoh *et al.*, 1995; zur Hausen, 1996; Mellin *et al.*, 2000; Venuti *et al.*, 2000; Mork *et al.*, 2001).

HPV virions are non-enveloped, consisting of dsDNA-containing, icosahedral capsids. HPV viral genomes are approximately 8 kb, circular, and are replicated within the nuclei of host cells. Within the capsids, viral genomes are associated with cellular histones, forming chromatin-like structures (Larsen *et al.*, 1987; Doorbar, 2005). Viral genomes from all HPV types harbor an average of 8 open reading frames (ORFs), and these ORFs are expressed from polycistronic mRNAs which are transcribed from a single DNA strand (Favre *et al.*, 1975; Zheng and Baker, 2006). The 8 ORFs can be subdivided into early and late genes, as well as a non-coding region called the upstream regulatory region (URR) (Fig. 1) (Zheng and Baker, 2006; Buck *et al.*, 2008). The early (E) ORFs encode the E1, E2, E4, E5, E6, and E7 proteins (Doorbar *et al.*, 1986; Zheng and Baker, 2006; Buck *et al.*, 2008). E1 and E2 proteins have been shown to regulate viral replication as well as the coordinated expression of the other early viral genes (Cripe *et al.*, 1987; Gloss and Bernard, 1990; Mohr *et al.*, 1990; Meyers *et al.*, 1992; Conger *et al.*, 1999). The spliced E1^{E4} protein may affect the replication of viral genomes through the inhibition of the G2-to-M transition, while also interacting with cellular keratin networks, causing their collapse, thus allowing mature virions to escape from the cornified envelope (White *et al.*, 1999; Bryan and Brown,

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Figure 1. Genomic organization of high-risk HPV16 in linear fashion. Early ORFs are indicated in grey, while late ORFs are indicated in black. The early (p97) and late (p670) promoters are designated by black arrows. The upstream regulatory region (URR) is depicted.

2000; Brown *et al.*, 2006; Knight *et al.*, 2006; Mach *et al.*, 2006; Gambhira *et al.*, 2007). E5, E6, and E7 proteins that are coded by the high-risk HPVs are oncogenic, since they are able to transform and stimulate the growth of cells (Androphy *et al.*, 1987; Phelps *et al.*, 1988; Bedell *et al.*, 1991). The late (L) ORFs encode the L1 and L2 proteins. L1 serves as the major capsid protein, while L2 serves as the minor capsid protein (Favre, 1975; Doorbar and Gallimore, 1987; Chen *et al.*, 2000; Buck *et al.*, 2008).

High-risk HPV types initiate the majority of transcription *via* 2 major viral promoters (Smith *et al.*, 2007). The first, or early, promoter initiates upstream from the E6 ORF, and synthesizes transcripts that are translated early in the viral life cycle. Since the early promoter functions independently from differentiation, it can initiate prior to the productive phase of the viral life cycle. In HPV16 the early promoter is referred to as p97, in HPV31 the early promoter is referred to as p99, and in HPV18 it is referred to as p105 (Fig. 1) (Hirochika *et al.*, 1987; Gloss and Bernard, 1990; Romanczuk *et al.*, 1990; Kyo *et al.*, 1995; Kanaya *et al.*, 1997; Cumming *et al.*, 2004; Smith *et al.*, 2007). The second, or late, promoter is initiated in a differentiation-dependent manner, and thus is activated only when cells are grown in the host's stratifying/differentiating tissue, or *in vitro* through the use of methylcellulose or organotypic culture techniques. Once activated, the late promoter directs transcription from a heterogeneous set of start sites which are clustered around nucleotide 742 (p742) in HPV31 (Ozbun and Meyers, 1998, 1999; Smith *et al.*, 2007). A late promoter has been identified in HPV16 (p670), and evidence suggests that a late promoter exists in HPV18 as well (Hummel *et al.*, 1992; Meyers *et al.*, 1992; Grassmann *et al.*, 1996; Parker *et al.*, 1997). The late promoter specifically serves to produce a set of transcripts that facilitate the translation of L1 and L2 proteins.

The life cycle of HPV is strictly linked to the differentiation program of the host keratinocyte, whereby the assembly of mature virions is restricted to terminally differentiated suprabasal cells, limited in part by the differentiation-dependence of the late promoter (Bedell *et al.*, 1991; Hummel *et al.*, 1992; Meyers *et al.*, 1992; Barksdale and Baker, 1993; Grassmann *et al.*, 1996; Ozbun and Meyers, 1997; Smith *et al.*, 2007). Initial infection by HPVs is thought to occur through micro-abrasions of the epithelial tissue, thus allowing entry of the HPV particle into

cells of the basal layer (Belnap *et al.*, 1996). Basal layer cells consist mainly of stem cells and transit-amplifying cells, and it is the epithelial stem cells that must be infected for a lesion to be maintained (Kaur and Li, 2000; Egawa, 2003). These cells continuously divide and replenish cells that are lost due to desquamation (Kaur and Li, 2000). Controversies exist as to the primary and/or secondary receptors for HPV entry, with alpha integrin and heparin sulfate as key possibilities, in addition to a role for laminin 5 (Giroglou *et al.*, 2001; Yoon *et al.*, 2001; Culp and Christensen, 2004; Doorbar, 2005; Patterson *et al.*, 2005). Heparin sulfate appears important for adsorption and infection of a variety of HPV-typed VLPs, PsV, and native virions in non-host and transformed cell lines, whereas HPV31b organotypic culture-derived native virions (OTNV) do not require heparin sulfate for infection of HaCat or N/Tert-1 human keratinocytes, but do require it for infection of non-host and transformed cell lines (Giroglou *et al.*, 2001; Yoon *et al.*, 2001; Culp and Christensen, 2004; Doorbar, 2005; Patterson *et al.*, 2005). Post-adsorption to the cell surface, internalization of virions has been shown to take many hours, and, depending on the papillomavirus type, virions can enter *via* clathrin-coated pits or caveolae (Fligge *et al.*, 2001; Bousarghin *et al.*, 2003; Day *et al.*, 2003; Hindmarsh and Laimins, 2007; Smith *et al.*, 2007). Recent studies involving entry pathways (*e.g.*, clathrin- vs. caveolar-mediated endocytosis) and entry kinetics (*e.g.*, few vs. many hours) of HPV have suffered due to a lack of consistency (Bousarghin *et al.*, 2003; Culp and Christensen, 2004; Hindmarsh and Laimins, 2007; Smith *et al.*, 2007; Laniosz *et al.*, 2008). This may be due to the use of multiple types of synthetic papillomavirus particles and cell lines, in addition to the use of native virions, and cross-talk between clathrin and caveolar pathways (Fligge *et al.*, 2001; Bousarghin *et al.*, 2003; Hindmarsh and Laimins, 2007; Schelhaas *et al.*, 2007; Laniosz *et al.*, 2008). Recent discoveries with polyomaviruses suggest that controversies over papillomavirus entry may be from the initial usage of clathrin-mediated endocytosis and later exploitation of caveolar endocytic machinery within the cell (Bousarghin *et al.*, 2003; Querbes *et al.*, 2006; Laniosz *et al.*, 2008).

While many details are lacking regarding the delivery of the viral genome into the nucleus, it is thought that the N-terminus of L2 is cleaved within the endosomal compartment *via* the cellular protease, furin, thus releasing an L2/genome complex into the cytosol (Day *et al.*, 2003; Richards *et al.*, 2006). The L2/genome complex may then interact with syntaxin 18, which ferries the complex to a perinuclear site (Bossis *et al.*, 2005; Richards *et al.*, 2006; Laniosz *et al.*, 2008). L2 may then translocate the genome into the nucleus through its NLS (Fay *et al.*, 2004; Bordeaux *et al.*, 2006). Once in the nucleus, a sophisticated cascade of viral gene expression occurs which serves to maintain a minimum number of viral DNA copies *per cell* (Doorbar, 2005).

E1 and E2 are among the first early proteins that are expressed, assisting in the establishment of 20 to 100 episomal copies *per* basal cell (Mohr *et al.*, 1990; Meyers *et al.*, 1992; Conger *et al.*, 1999; Smith *et al.*, 2007). These 2 proteins form a complex with the viral origin of replication and recruit cellular polymerases and the necessary accessory proteins to facilitate replication (Mohr *et al.*, 1990). E2 is a DNA-binding protein

that recruits E1 to the origin of replication, but also serves to regulate transcription of E6 and E7 from the early promoter (Cripe *et al.*, 1987; Gloss and Bernard, 1990; Mohr *et al.*, 1990; Meyers *et al.*, 1992). The E6 and E7 proteins from high-risk, but not low-risk, HPV types are oncoproteins. High-risk E6 is able to bind the tumor suppressor protein, p53, forming a trimeric complex along with the cellular ubiquitin ligase, E6AP. The ubiquitin ligase activity of E6AP leads to the rapid turnover of p53 (Rolfe *et al.*, 1995). High-risk E7 is able to bind to and modulate a variety of proteins, such as the retinoblastoma (Rb) family of tumor suppressors, and cell-cycle regulatory proteins, leading to enhanced cellular replication (Dyson *et al.*, 1989; Neary and DiMaio, 1989).

As HPV-infected basal cells divide, each new daughter cell is replete with a set of viral genomes as the genomes are equally partitioned during mitosis. Recent studies suggest that both Brd4-dependent and independent partitioning of viral genomes into daughter cells can occur, depending on the papillomavirus type studied (You *et al.*, 2004; McPhillips *et al.*, 2006). Post-mitosis, one cell remains attached to the basal layer, while the other cell is detached from the basal layer and begins to migrate up through the suprabasal layers (Lehman and Botchan, 1998; Bastien and McBride, 2000). During migration of the cell up through the strata, the cell begins a process of terminal differentiation (Fig. 2) (Pang *et al.*, 1993). In non-HPV-infected epithelia, cells normally exit the cell cycle once they detach from the basal layer, and this is often accompanied by the loss of nuclei in differentiating suprabasal cells (Pang *et al.*, 1993). In the case of HPV-infected epithelia, detached cells remain mitotically active due to the oncogenic properties of the E7 protein (Morris *et al.*, 1993). Because of this, infected cells that have already differentiated can re-enter the S phase and enhance the expression of cellular replication factors that are required for concomitant viral genome amplification, and late gene expression (Bedell *et al.*, 1991; Barksdale and Baker, 1993; Swindle *et al.*, 1999; Banerjee *et al.*, 2006). While designated 'early proteins', E1^{E4} and E5 are both translated in a differentiation-dependent manner, both of which may affect the replication of viral genomes through the inhibition of G2-to-M transition, and stimulation of cell-cycle progression, respectively (Doorbar *et al.*, 1997; White *et al.*, 1999; Fehrman *et al.*, 2003; Knight *et al.*, 2006). Similarly, the L1 and L2 capsid proteins are expressed only in terminally differentiated keratinocytes (Barksdale and Baker, 1993). Studies with both papillomaviruses and polyomaviruses suggest that, once expressed, capsid proteins assemble into icosahedral capsids *via* assistance from chaperone proteins (Buck *et al.*, 2005; Chromy *et al.*, 2003, 2006; Bird *et al.*, 2008). It is unknown if encapsidation of the viral genome takes place during capsid assembly or after; however, encapsidation is assisted by L2 and may be facilitated by E2 proteins (Heino *et al.*, 2000; Gu *et al.*, 2004; Holmgren *et al.*, 2005). In the cornified envelope, E1^{E4} proteins are thought to interact with cellular keratin networks, causing their collapse, thus allowing mature virions to escape from the cornified cells (Doorbar *et al.*, 1986; Bryan and Brown, 2000; Brown *et al.*, 2006; Mach *et al.*, 2006; Gambhira *et al.*, 2007). Mechanistic details concerning the host cell differentiation program that leads to the productive phase of the HPV life cycle are unclear.

Figure 2. HPV virions establish an infection in basal epithelial cells by gaining access through micro-abrasions within the tissue. Upon establishment, viral genomes are maintained at approximately 20–100-episomal copies *per* cell. Once maintained, viral genomes transit up through the differentiating strata as the mitotically active basal cells divide. In the suprabasal layers, concomitant late gene expression and viral genome amplification allow for assembly of progeny virions. Progeny virions escape with the highly keratinized cornified envelope, allowing the viral life cycle to continue.

It is also unclear as to the regulated molecular interactions that must take place for individual capsid proteins to assemble into higher-ordered structures over the time-course of stratification and differentiation of the host keratinocyte.

To study aspects of the HPV life cycle, including HPV infectivity pathways, transmission, immunogenicity, and viral structure, recent research predominantly utilizes synthetic papillomavirus particles, such as virus-like particles (VLPs), papillomavirus-based gene transfer vectors, known as pseudovirions (PsV), and papillomavirus genome-containing *quasivirions* (QV), which bypasses the need for stratifying and differentiating tissue in the production of native HPV and facilitates rapid scientific discovery.

STRUCTURE

Background

HPV virions are non-enveloped, icosahedral particles approximately 50 to 60 nm in diameter (Hagensee *et al.*, 1994; Chen *et al.*, 2000; Modis *et al.*, 2002). Each particle is composed of 72 pentamers of the L1 capsid protein. L1 pentamers are thought to form a network of intra- and interpentameric disulfide interactions which serve to stabilize the capsid (Sapp *et al.*, 1998; Fligge *et al.*, 2001; Buck *et al.*, 2005; Kondo *et al.*, 2007). In addition to L1, an unknown amount of the minor capsid protein, L2, exists within the virion. Previous cryoelectron microscopy studies of native bovine papillomavirus type 1 (BPV1) virions suggested that one L2 protein occludes the center of each pentavalent capsomere, for a total of 12 L2 proteins *per* virion (Trus *et al.*, 1997). In support of this, SDS-PAGE analyses of L1/L2 VLPs have depicted a 30:1 ratio of L1 to L2, for a total of 12 L2 proteins *per* virion (Hagensee *et al.*, 1993; Kirnbauer *et al.*, 1993; Volpers *et al.*, 1994; Bossis *et al.*, 2005). However, SDS-PAGE analyses of native HPV1 virions, biochemical analyses of HPV11 L1/L2 proteins, and recent cryoelectron microscopy

images of HPV16 pseudovirions (PsV) suggest that papillomavirus particles can contain up to 36 and as much as 72 L2 proteins *per* particle (Doorbar and Gallimore, 1987; Finnen *et al.*, 2003; Buck *et al.*, 2008). While L1 is sufficient to produce synthetic papillomavirus particles, L2 has been shown to affect the final structure of the virion, in addition to enhancing infectivity and DNA encapsidation of the particle (Kirnbauer *et al.*, 1992, 1993; Holmgren *et al.*, 2005; Kondo *et al.*, 2007). Also housed within the virion is a histone-associated, 8-kb, circular viral genome which has been shown to alter the final structure of the virion (Sapp *et al.*, 1998; Fligge *et al.*, 2001). In addition to L2 and viral DNA, a minority population of unknown viral and/or cellular proteins/factors such as molecular chaperones and karyopherins may also exist which influence the final structure of native virions (Chromy *et al.*, 2003, 2006; Bird *et al.*, 2008).

Due to previous difficulties in propagating native virions in organotypic culture (*i.e.*, technical constraints and low productivity), methods have been utilized to make synthetic virus *in vitro* (Kirnbauer *et al.*, 1992, 1993; Gambhira *et al.*, 2007). The most efficient synthetic techniques involve the overexpression of L1 with or without L2 *via* codon-optimized plasmids within monolayers of insect or mammalian cells, although less efficient techniques have also been developed to assemble virus-like particles (VLPs) and PsV *in vitro* (Kirnbauer *et al.*, 1992; Fligge *et al.*, 2001; Gambhira *et al.*, 2007). L1 can spontaneously assemble into VLPs alone, or in tandem with L2. Expression plasmids or full-length HPV genomes can be incorporated into VLPs to produce PsV and *quasivirions* (QV), respectively (Buck *et al.*, 2004; Culp and Christensen, 2004; Pyeon *et al.*, 2005; Gambhira *et al.*, 2007). Assembly within the reducing environment of the cytosol/lysates of these cells is insufficient to form the much more extensive disulfide bonding network that is formed in native virions (Sapp *et al.*, 1998). In fact, overnight incubation of PsV allows for the formation of additional disulfide bonds in a redox-dependent and cellular lysate-dependent fashion (Buck *et al.*, 2005). Similarly, stabilization of VLPs has been reported through the removal of reducing agents, lowering of pH, and increasing of ionic strength (Mach *et al.*, 2006). These experiments suggest that differences in the redox states of monolayer *vs.* organotypic culture may play a role in the structural discrepancies noted between immature synthetic papillomavirus particles and native virions.

Structural studies of synthetic papillomavirus particles may or may not elucidate the native interactions that are necessary for the assembly of monomeric/pentameric L1. In fact, the crystal structure of an HPV16, 12-pentamer VLP lacks disulfide bonds altogether (Chen *et al.*, 2000). The potential differences between synthetic and native virions stress the need for caution in the analysis of HPV structure and life-cycle strategies, and call for the development of novel techniques to produce native virions efficiently for comparative studies.

Production of Synthetic and Native Papillomavirus Particles

Virus-like Particles (VLPs)

VLPs have been produced by a variety of similar methods (Kirnbauer *et al.*, 1992, 1993; Hagensee *et al.*, 1993; Rose *et al.*,

1993; Zhang *et al.*, 1998; Chen *et al.*, 2000; Querbes *et al.*, 2006) (Fig. 3). Generally, expression plasmids or viral vectors that express L1 alone, or L1 plus L2, are transfected or used to infect the following eukaryotic or prokaryotic cell types: 293T, BSC-1, *E. coli*, yeast, or Sf9 cells (Kirnbauer *et al.*, 1992, 1993; Hagensee *et al.*, 1993, 1994; Rose *et al.*, 1993; Belnap *et al.*, 1996; Zhang *et al.*, 1998; Chen *et al.*, 2000; Querbes *et al.*, 2006). Upon expression of L1, structures which retain the native T=7 icosahedral symmetry of the native virions are intracellularly self-assembled. VLPs are antigenically similar to native virions, suggesting gross structural similarities. In fact, VLPs can induce high titers of type-specific neutralizing antibodies, which made them good candidates for first-generation HPV vaccines (Rose *et al.*, 1994; Belnap *et al.*, 1996; Harper *et al.*, 2004). While not necessary for the production of VLPs, one advance in VLP technology incorporates L2 into the particle, which has been suggested to increase their overall stability (Yang *et al.*, 2005; Gambhira *et al.*, 2007; Kondo *et al.*, 2007). Novel findings into the cross-neutralization potential of the N-terminal peptide of L2 highlight the potential utility of L1/L2 chimeric VLPs in eliciting an optimized immune response (Gambhira *et al.*, 2007; Kondo *et al.*, 2007). Advancements in VLP technology have also increased the disulfide-bonding-mediated stabilization of VLPs *via* the manipulation of buffers, by removing reducing agents, lowering pH, and increasing ionic strength (Mach *et al.*, 2006). Most novel VLP production techniques seek to produce VLPs which appear more like native virions.

Pseudovirions (PsV)

While many protocols exist, with various degrees of efficacy, to produce PsV, the most efficient PsV production protocol utilizes transfection of codon-optimized, L1- and L2-expression plasmids, in addition to reporter-gene-expressing plasmids into 293T or 293TT cells, which allows for efficient intracellular production of papillomavirus gene transfer vectors (Buck *et al.*, 2005; Gambhira *et al.*, 2007) (Fig. 3). PsV are self-assembled within the transfected producer cells, allowing for the production of several billion transducing units *per* milliliter when a 5.9-kb plasmid is encapsidated, although 10-fold fewer transducing units *per* milliliter are obtained when an 8-kb plasmid is encapsidated (Buck *et al.*, 2004).

The utility of PsV is evidenced by the sheer volume of recent publications that report on the utilization of PsV for structural and neutralization analyses, and studies of papillomavirus entry pathways (Fligge *et al.*, 2001; Day *et al.*, 2003; Gu *et al.*, 2004; Buck *et al.*, 2005, 2006, 2008; Yang *et al.*, 2005; Gambhira *et al.*, 2007; Kondo *et al.*, 2007). In addition to the enhanced efficiency of PsV production/purification in comparison with native virions, PsV are structurally similar by cryoelectron microscopy, and have similar immunoreactive profiles against conformation-dependent neutralizing antibodies, suggesting that some, if not all, surface conformational epitopes are retained in PsV (Day *et al.*, 2003; Embers *et al.*, 2004; Yang *et al.*, 2005; Gambhira *et al.*, 2007; Buck *et al.*, 2008).

While the efficient production of PsV allows for rapid analysis of many aspects of the papillomavirus life cycle, a thorough structural comparison between PsV and native virions is lacking;

thus, experimental results should be verified with native virions (Sapp *et al.*, 1998; Fligge *et al.*, 2001; Buck *et al.*, 2005). While the incorporation of plasmids into VLPs has been shown to enhance the amount of disulfide interactions that occur within each particle, it is apparent that further maturation events are required for further stabilization of PsV particles (Buck *et al.*, 2005). Such stabilization, which has been depicted *via* TEM and through biochemical studies, would predict global conformational changes throughout the PsV which may lead to altered immunoreactivities with neutralizing antibodies.

Maturation of PsV takes

advantage of a simple overnight incubation at 37°C. This step further orders these particles so they appear more like native virions by TEM and cryoelectron microscopy, presumably by exposing these particles to an oxidizing environment for extended periods of time (Buck *et al.*, 2005, 2008). At this point, it is unknown if the correct disulfide interactions are formed during PsV and VLP assembly/maturation. It is also unclear if additional factors such as the native, 8-kb, histone-associated genome, in addition to unknown cellular factors within stratified/differentiating tissue may further enhance the similarities among mature PsV, VLPs, and native virions.

Quasivirions (QV)

QV utilize technology similar to that of PsV, in that transfection of L1 and L2 codon-optimized expression plasmids, in addition to full-length, recircularized HPV genomes, into 293T or 293TT cells allows for efficient intracellular production of the native virion-like particles (Culp and Christensen, 2004; Pyeon *et al.*, 2005; Smith *et al.*, 2007) (Fig. 3). Optiprep-purified cottontail rabbit papillomavirus (CRPV) QV have been shown to have infectious titers similar to those of native virions *in vitro* and *in vivo*, are neutralizable by anti-L1 antibodies, and induce papilloma formation (Culp and Christensen, 2004; Pyeon *et al.*, 2005). These attributes suggest that QV are similar, if not identical, to native virions. The most beneficial aspect of the production of QV is that it employs a simple DNA preparation and transfection protocol, utilizing a single 10-cm dish of 293T or 293TT cells; however, similar yields of QV and native virions can be obtained with the more labor-intensive, organotypic

Figure 3. Virus-like particles (VLPs), pseudovirions (PsV), and quasivirions (QV) are all synthetically derived papillomavirus particles which are made via transfection and/or infection-based techniques that overexpress L1 alone, or in tandem with L2. The differentiating factor among the various forms of synthetic papillomavirus particles is the presence and/or absence of plasmid-based reporter genes or full-length papillomavirus genomes. VLPs are empty, while PsV contain plasmid-based reporter genes, and QV contain full-length papillomavirus genomes. A maturation step has been characterized with PsV which make them appear more like native virions *via* TEM and biochemical assays. *Transfection is used interchangeably with co-transfections and infection, since all permutations of transfections, co-transfections, and infections with viral vectors have been utilized to produce synthetic papillomavirus particles.

culture. While the QV technique is an enormous advance in papillomavirus research, it remains uncertain whether QV are structurally and functionally identical to native virions. Replication strategies of papillomavirus genomes, for instance, have revealed a differentiation-dependent switch from theta replication in monolayer culture to rolling-circle replication in stratifying and differentiating tissue (Flores and Lambert, 1997). Rolling-circle replication has been shown to be intimately involved in the formation of mature capsids in a variety of virus systems, and this possibility has yet to be explored in HPV (Koths and Dressler, 1980; Vlazny *et al.*, 1982; Flores and Lambert, 1997). Until the details of QV and native virions have been studied comprehensively, it is premature to treat QV and native virions identically, and thus interpretation of future studies that utilize QV should consider the potential structural and functional differences.

Native Virions

Native virions are produced only in stratified and differentiated epithelia and are thus synthesized only during a natural infection or in organotypic culture (Meyers *et al.*, 1992; Ozbun and Meyers, 1997; McLaughlin-Drubin *et al.*, 2003, 2004; Gu *et al.*, 2004; McLaughlin-Drubin and Meyers, 2005). While direct structural comparisons are lacking between organotypic culture-derived native virions (OTNV) and native virions derived from xenograft tissue or natural infection, the strict differentiation-dependence of OTNV highlights their novelty. For years, the ability to synthesize native virions *in vitro* was hindered due to the lack of a reproducible tissue culture system which allowed

Figure 4. Atomic structure of HPV16 major capsid protein L1. **(A)** Atomic resolution of monomeric HPV16 L1 from an N-terminally truncated form of L1. No disulfide bonds were formed in this particle. **(B)** Comparison of an HPV16, T=1, 12-pentamer particle composed of N-terminally truncated forms of L1, and a native, T=7, 72-pentamer, particle composed of full-length L1. Interpentameric and intrapentameric disulfide bonds were formed in this particle. Molecular modeling in (A) was rendered with Chimera (UCSF) (Chen *et al.*, 2000). Structures in (B) were obtained from <http://viperdb.scripps.edu> (Shepherd *et al.*, 2006).

for virion morphogenesis in a differentiation-dependent manner. Our laboratory has established a system utilizing organotypic “raft” cultures which allows for the completion of the HPV life cycle and allows us to examine all aspects of this life cycle, including, but not limited to, viral DNA amplification, late gene expression, virion morphogenesis, and infectivity. We have successfully depicted propagation of HPV16, HPV18, HPV31, HPV33, HPV39, HPV45, HPV11, and a variety of chimeric and mutant OTNV (Meyers *et al.*, 1992; Ozbun and Meyers, 1997; McLaughlin-Drubin *et al.*, 2003, 2004; Gu *et al.*, 2004; McLaughlin-Drubin and Meyers, 2004, 2005). Previously, the low productivity of organotypic culture garnered too few virions for many low-sensitivity analyses. However, advances in PCR, qPCR, mass spectroscopy, and purification techniques have afforded novel opportunities to examine aspects of the viral life cycle that were previously too difficult to explore.

STRUCTURAL DETAILS OF PAPILLOMAVIRUS PARTICLES

High-resolution Structural Analyses of Synthetic Capsids

Unfortunately, the low productivity of organotypic culture previously made obtaining enough OTNV impossible for high-resolution, crystallographic studies. The production of synthetic papillomavirus particles has bypassed this setback. However, VLPs composed of full-length, HPV16 L1 are unable to be crystallized. Because of this, VLPs containing N-terminally truncated forms of HPV16 L1, which can be crystallized, have provided the only atomic resolution structures of HPV particles (Chen *et al.*, 2000). N-terminally truncated forms of L1 efficiently self-assemble into VLPs, as can full-length L1; however,

deletion of 10 amino acids starting from the second N-terminal methionine leads to the assembly of T=1 icosahedral lattices made from 12 L1 pentamers, rather than the native T=7 icosahedral lattice made from 72 L1 pentamers (Fig. 4B) (Chen *et al.*, 2000). Deleting fewer than 10 amino acids starting from the second N-terminal methionine retains the T=7 icosahedral lattice made from 72 L1 pentamers, but this native structure does not crystallize (Chen *et al.*, 2000). As mentioned above, atomic resolution has been obtained only from the T=1, 12-pentamer HPV16 capsids (Chen *et al.*, 2000). This structure is quite an accomplishment and allows our field to make further hypotheses regarding the structure of HPV; however, the ostensible small size of the T=1 particle and lack of disulfide bonds mean that this model may not be representative of all the detailed interactions that occur between and within L1 pentamers of native virions (Chen *et al.*, 2000).

Previous studies utilizing cryoelectron microscopy to resolve native BPV1 virions at a resolution of 9 Å have depicted protein protrusions that emanate from the pentavalent capsomeres into each of 5 neighboring hexavalent capsomeres (Trus *et al.*, 1997). These protrusions are morphologically similar to the C-terminal “arm-invading” model of polyomaviruses, which reinforce the invading arms *via* an interpentameric disulfide bridge(s) (Baker *et al.*, 1989, 1991; Stehle *et al.*, 1994, 1996; Sapp *et al.*, 1998; Jao *et al.*, 1999). While detailed information regarding the intra- and interpentameric molecular interactions between pentamers cannot be garnered from the current atomic structure of HPV16, assumptions can be made from this model. One such assumption is that if the T=1 particle expanded into a T=7 particle, the C-terminal h4 helix may reach into a neighboring pentamer, thus forming a critical interpentameric disulfide bridge between 2 Cys428 residues (Fig. 4A) (Chen *et al.*, 2000). This is a rational assumption, since the C-terminal “invading arm” of SV40 is stabilized by an N-terminal disulfide bridge (Sapp *et al.*, 1998; Jao *et al.*, 1999; Schelhaas *et al.*, 2007). However, analyses of recombinant VLPs suggest that a critical interpentameric disulfide bridge occurs between Cys428 and Cys175, and potentially Cys185 in HPV16, rather than between neighboring Cys428 residues (Sapp *et al.*, 1998; Chen *et al.*, 2000; Kondo *et al.*, 2007). In addition, biochemical analyses assessing the disulfide bonding patterns of HPV virions utilized mass spectroscopy of VLP capsid proteins, coupled with cryoelectron microscopy image reconstructions of native virions, to determine which cysteine residue(s) is/are critical in the formation of interpentameric disulfide bonds (Modis *et al.*, 2002). Briefly, HPV18 VLP L1 dimers were excised from a non-reducing polyacrylamide gel. L1 dimers were digested with trypsin and analyzed by liquid chromatography +/- tandem mass spectrometry (LC-MS/MS) (Modis *et al.*, 2002). LC-MS/MS analysis of

trypsin-digested HPV18 VLP L1 dimers revealed a tryptic product that corresponded to an 11-residue tryptic fragment that flanked the Cys428 equivalent of HPV18, Cys429 (Modis *et al.*, 2002). The tryptic fragment contained a 476-Da adduct on Cys429, which suggested that a tryptic peptide corresponding to Cys175 was linked to the Cys429 tryptic fragment *via* a disulfide bond (Modis *et al.*, 2002). These results are in contrast to the assumptions made from the atomic structure of the HPV16, T=1, 12-pentamer VLP, in that it seems that interpentameric interactions take place *via* Cys175 and Cys428 rather than the predicted Cys428 and Cys428. This result calls into question the accuracy to which VLPs assemble *in vitro*, or if the highly conserved cysteine residues Cys428, and Cys175 are arranged differently among various papillomavirus types. Because of the uncertain accuracy of VLP assembly in comparison with native virions, it is necessary that high-resolution studies of VLPs be coupled with the analysis of native virions.

Genetic and Biochemical Structural Analyses of Synthetic and Native Virions

Similar to the results obtained from current high-resolution structures of papillomavirus particles, which are unable to verify the exact disulfide bonding patterns necessary for intra- and interpentameric contacts, a variety of genetic and biochemical analyses has further complicated experimental structural analyses regarding the extent to which papillomavirus capsids are disulfide-bonded. Multiple reports have depicted PsV and native virions as having a much more extensively disulfide-bonded phenotype than VLPs, while matured PsV have a phenotype that mirrors native virions (Sapp *et al.*, 1998; Fligge *et al.*, 2001; Buck *et al.*, 2005). Through the use of non-reducing SDS-PAGE analysis, it is apparent that L1 from HPV1 native virions obtained from a hand wart form extensively disulfide-bonded multimers which migrate above 170 kDa, whereas 50% of L1 from HPV16, HPV18, and HPV18 VLPs predominantly migrate as the 50-kDa monomeric protein (Sapp *et al.*, 1998). Such multimerization of L1 from native virions seems to depend on components that make up the particle, in that the incorporation of nucleic acid alone into a VLP enhances the multimeric state of L1, based on similar non-reducing SDS-PAGE analyses of purified VLPs and PsV (Fligge *et al.*, 2001). These results suggest that VLPs, which lack certain components of native virions, such as a complete 8-kb viral genome, are less-ordered structures. Incorporation of nucleic acid into papillomavirus particles appears to reinforce and/or facilitate the formation of additional disulfide bonds which are absent in synthetic papillomavirus particles.

Recent studies suggest that nucleic acid alone, encapsidated within an L1/L2 capsid, is not sufficient to create a mature papillomavirus particle; thus, previous PsV protocols created papillomavirus particles which lacked the ordered structure of native virions (Buck *et al.*, 2004, 2005; Gambhira *et al.*, 2007). Since previous PsV protocols produced particles which lacked the disulfide bonds present in native virions, a maturation step was added to the PsV production protocol (Buck *et al.*, 2005). This maturation step allowed for increased disulfide bond formation, presumably since the virions were exposed to an oxidizing environment for an extended period of time. In comparison with

immature PsV, mature PsV do appear more resistant to proteolysis and chemical reduction, while also obtaining a more ordered structure, as seen *via* TEM; however, it is unknown if the disulfide bonds formed during PsV maturation are formed as they would *in vivo* (Buck *et al.*, 2005).

Genetic analyses of VLPs and PsV have also provided clues into the structural differences among the various papillomavirus particles, mostly by substituting the 12 highly conserved L1 cysteine residues for irrelevant amino acids. In one such example, a series of recombinant HPV16 VLPs, each containing a serine residue substituted for each of 12 conserved L1 cysteine residues, was produced and analyzed for their structural stabilities *via* their resistance to chemical reduction and tryptic proteolysis, in addition to their gross morphologic appearance *via* TEM (Kondo *et al.*, 2007). Analysis of the recombinant VLPs suggested that Cys428, Cys175, and Cys185 are pivotal residues, with Cys428Ser mutations leading to the complete dissociation of VLPs, Cys175Ser mutations leading to the production of large tube-like capsid structures, and Cys185Ser mutations leading to the production of small, approximately 40-nm capsids (Kondo *et al.*, 2007). Analysis of these data suggests a prominent role for Cys428 in the formation of interpentameric disulfide bonds, and reinforces previous assumptions made from the crystal structure of the HPV16, T=1 12-pentamer VLP (Chen *et al.*, 2000; Kondo *et al.*, 2007). These results contrast, however, with previous work using recombinant HPV33 VLPs, which depicted equivalent roles for Cys427 and Cys176 (*i.e.*, the equivalent of Cys428 and Cys175 in HPV16) in the formation of interpentameric disulfide bonds, which supports previous claims that pentamers from HPV18 VLPs form interpentameric disulfide bonds with each other (Sapp *et al.*, 1998; Modis *et al.*, 2002). These results call into question whether disulfide-bonding patterns differ between HPV16 VLPs, and HPV18 and HPV33 VLPs, or whether the various techniques utilized to produce synthetic papillomavirus particles lead to disulfide interactions that do not normally occur.

To complicate the structural analysis of papillomavirus particles further, recent studies have also suggested that HPV16 PsV containing the Cys428Ser mutation, which led to the complete dissociation of VLPs, retains residual infectivity. This suggests that PsV assembly can occur despite the loss of Cys428, which is thought to be critical in the formation of interpentameric disulfide bonds (Buck *et al.*, 2005). In this same study, HPV16 PsV which contain the Cys175Ser mutation retain 70-90% infectivity, suggesting that this residue does not form interpentameric disulfide linkages with Cys428 in HPV16 PsV (Buck *et al.*, 2005). How these interpentameric interactions form in native virions has not been examined.

STRUCTURAL ALTERATIONS AFFECTING FUNCTION

Many studies have detailed the viral components that comprise the papillomavirus capsid (L1, L2, viral DNA, and histones), in addition to viral and cellular components (*i.e.*, E2, E4, E5, E7, molecular chaperones, karyopherins, etc.) that may either directly or indirectly affect the structure of the capsid (Doorbar and Gallimore, 1987; Larsen *et al.*, 1987; Heino *et al.*, 2000; Fligge *et al.*, 2001; Chromy *et al.*, 2003, 2006; Gu *et al.*, 2004; Bordeaux *et al.*, 2006; Richards *et al.*, 2006; Kondo *et al.*, 2007; Bird *et al.*, 2008; Buck *et al.*, 2008). Many other unknown viral

and/or cellular factors are undoubtedly necessary for the proper assembly of native virions regarding regulation of redox states within the cell, proper cellular localization and initial interaction of capsid proteins, the correct formation/regulation of disulfide bonds, and regulation of capsid protein expression. Advances in synthetic papillomavirus particle technology have yielded information regarding the structural roles of various components, and as such, these synthetic technologies have adapted over time as investigators attempt to create a more native-like viral particle.

Incorporation of histone-associated plasmids, for example, has been shown to increase the infectivity of PsV 4- to 5-fold over PsV that contain histone-free plasmids (Fligge *et al.*, 2001). This increase in infectivity suggests that the presence of histones alone may alter the overall structure of PsV, rendering them more infectious, hindering post-entry endonuclease digestion of viral genomes, or facilitating early transcriptional events within the host cell. Earlier discoveries that the incorporation of plasmid DNA into VLPs increases the multimeric state of L1 support the idea that histones can alter the final conformation of the papillomavirus particle (Sapp *et al.*, 1998; Fligge *et al.*, 2001). Cryoelectron microscopy images of native BPV1 virions further support a role for chromatin in the final structure of native virions, in that the nucleohistone core was shown to have a thin link to the inner walls of hexavalent capsomere protrusions, and between capsomeres (Trus *et al.*, 1997). In either case, it is clear that the use of synthetic papillomavirus particles leads to many questions regarding the authenticity of the particles being tested.

Incorporation of L2 into VLPs also ostensibly alters the conformation of the virus particle. *In vitro* studies of VLP assembly under reducing conditions have depicted an important role for L2 in the initial assembly of L1/L2 aggregates (Kondo *et al.*, 2007). L2 was also able to aggregate mutant HPV16 L1 proteins that lacked the critical interpentameric disulfide-bond-forming Cys428. L1/L2 aggregates, presumably, are the nucleation site for further capsid assembly, suggesting that L2 plays an important role in initial, regulated assembly of VLPs. Further linking the importance of L2 in native virion assembly is its ability to bind both L1 and DNA, suggesting that L2 is the mediator that facilitates assembly (Fligge *et al.*, 2001; Fay *et al.*, 2004; Bordeaux *et al.*, 2006). Further, the analysis of *in vitro* assembly of L1/L2 VLPs has shown that L2 is incorporated into VLPs only when co-expressed with L1, or with L1 capsomeres (Kondo *et al.*, 2007). L2 will not bind to intact VLPs, which suggests that L2 plays an early nucleation role in HPV assembly.

While the importance of L2 remains convincing in VLP assembly, and in binding assays, growth of HPV31 native virions containing 2 artificially inserted internal stop codons, which effectively shut down synthesis of L2 in organotypic culture, yields some infectious virus (Holmgren *et al.*, 2005). Although the L2-deficient native virions were more than 100-fold less infectious than their wild-type counterparts, such important roles ascribed to L2 *via* VLP- and PsV-dependent experiments—such as initial capsid assembly, DNA encapsidation, capsid disassembly, and ferrying DNA into the nucleus—unearth additional questions as to the redundancy of native virions. Are there other viral and/or cellular proteins that exist within stratifying/differentiating epithelia that can assist L2 in the above activities, and if so, how do these proteins interact with L1-only virions to

produce an infectious virus? These same experiments utilizing L2-deficient native virions depicted a role for L2 in DNA encapsidation and/or structural stability of the virions, since fewer DNase-resistant genomes were detected in the L2-deficient samples when compared with the wild-type native virions (Holmgren *et al.*, 2005). Over the years, many publications have cited the appearance of viral and/or cellular factors that co-purify with native virions, such as E2, E4, E5, E7, and histones (Doorbar and Gallimore, 1987; Larsen *et al.*, 1987; Heino *et al.*, 2000; Fligge *et al.*, 2001; Chromy *et al.*, 2003, 2006; Gu *et al.*, 2004; Bordeaux *et al.*, 2006; Richards *et al.*, 2006; Kondo *et al.*, 2007; Bird *et al.*, 2008; Buck *et al.*, 2008).

A recent report has suggested that structural alterations of HPV16 PsV dramatically decrease infectivity of these particles (Ishii *et al.*, 2007). Specifically, the addition of a variety of thiol-reactive reagents such as DTNB and NEM alkylate free C146, C225, and C229 sulfhydryls of L1 (Ishii *et al.*, 2007). Alkylation of these sulfhydryls leads to marked decreases in infectivity, while C146A, C225A, and C229A substitutions did not appreciably alter infectivity (Ishii *et al.*, 2007). The authors suggest that either local structural changes perturb entry of these particles, or that the free C146, C225, and C229 sulfhydryls interact with an as-of-yet unknown cellular protein involved in uncoating, since the loss of disulfide bonding *via* dithiothreitol or 2-mercaptoethanol-mediated reduction of disulfide bonds within PsV does not affect infectivity (Buck *et al.*, 2006; Ishii *et al.*, 2007). In either case, these results highlight the functional discrepancies that may arise given subtle conformational changes in the viral capsid.

Not only have structural alterations of HPV particles affected infectivity, but also they have altered the particle's function as an immunogen (Kirnbauer *et al.*, 1992; Breitburd *et al.*, 1995; White *et al.*, 1999; Christensen *et al.*, 2001; Yang *et al.*, 2005). Specifically, global conformational changes in VLPs can change the immunoreactivity profile against a panel of conformation-dependent neutralizing monoclonal antibodies (MAbs) (White *et al.*, 1999; Christensen *et al.*, 2001). For example, VLPs with a F50L substitution are not bound by the conformation-dependent MAbs, H16.V5 and H16.E70, while they are bound by MAbs that recognize surface linear epitopes (Christensen *et al.*, 2001). Further, vaccination of mice with HPV16 VLPs containing the assembly-incompetent D223G, N327S, and F446S L1 substitutions failed to induce neutralizing antibodies, while HPV16 VLPs containing the assembly-competent D202H substitution led to the activation of bone-marrow-derived dendritic cells (BMDCs) and a potent L1-specific humoral response, but also failed to induce neutralizing antibodies. Analysis of these data implies that structural alteration of VLPs can change the cellular immune response to the immunogen (Yang *et al.*, 2005). The elucidation of the correct structure of HPV is important for optimization of an HPV immunogen that will raise optimum neutralizing antibody titers, and an optimum cellular immune response against native HPV capsid structures.

CONCLUDING REMARKS

The efficiency by which synthetic papillomavirus particles are produced is invaluable in the efforts to uncover details regarding

HPV structure, infectivity, transmission, and immunogenicity, and the protocols developed utilizing synthetic papillomavirus particles will be invaluable in future studies of native virions. Novel techniques continue to develop to construct synthetic papillomavirus particles with ever-increasing complexity, which seems to tighten the structural gap between synthetic and native virions. This being the case, detailed side-by-side comparisons of the structural aspects between these groups of particles are necessary for confident interpretation of the data.

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