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Papillomavirus assembly: an overview and perspectives

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Abstract

Papillomavirus life cycle is tightly coupled to epithelial cell differentiation, which has hindered the investigation of many aspects of papillomavirus biology, including virion assembly. The development of in vitro production methods of papillomavirus pseudoviruses, and the production of "native" virus in raft cultures have facilitated the study of some aspects of the assembly process. In this paper we review the current knowledge of papillomavirus assembly, directions for future research, and the implications of these studies on new therapeutic interventions.

Keywords

papillomavirus; virion assembly; pseudovirus

1. Introduction

The main evolutionary driving force for a virus is to infect a cell in order to replicate and spread. To accomplish this, viruses need to produce infectious virions, and therefore assembly and genome packaging are often highly regulated processes. The capsid assembly reaction must be orchestrated from a limited number of virion proteins and result in the encapsidation of the viral genome. Viruses with DNA genomes have the specific problem of selectively packaging their genomic DNA in the presence of a vast excess of cellular DNA. Virus assembly is an important subject, not only for the understanding of the basic molecular mechanisms that underlie the process, but also to provide insight into ways that pharmacological inhibitors might interrupt the process, thereby inhibiting viral replication and transmission. The study of virus assembly has also been valuable in the development of gene delivery techniques, since viruses can be used as vectors to selectively package specific nucleic acids and efficiently transfer them into cells.

In vivo, papillomavirus replication and assembly is dependent on and integrated with the terminal differentiation of stratified squamous epithelium, making the study of the process particularly challenging. However, considerable progress had been gained during the past

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years, primarily by employing simplified in vitro experimental systems. These systems include pseudoviruses, papillomavirus capsids that have encapsidated reporter genes in monolayer culture, and also production of “native” papillomaviruses in organotypic raft cultures. Herein we review the current knowledge on the assembly of papillomaviruses.

2. Papillomavirus structure

Papillomaviruses are small non-enveloped viruses that package an approximately 8kb circular dsDNA genome. The viral capsid is composed by only two proteins, L1, the major capsid protein and L2, the minor capsid protein.

The T=7 icosahedral viral capsid is primarily comprised of 360 copies of L1 that are arranged in 72 pentamers, of which 12 are pentavalently coordinated and 60 pentamers are hexavalently coordinated (Baker et al., 1991; Klug and Finch, 1965; Trus et al., 1997). Interpentameric disulfide bonds, and also intrapentameric in the case of bovine papillomavirus 1 (BPV1), between L1 molecules stabilize the papillomavirus capsids (Buck et al., 2005a; Cardone et al., 2014; Conway et al., 2011; Ishii et al., 2003; Li et al., 1998; Modis et al., 2002; Sapp et al., 1998; Wolf et al., 2010). The capsid is also stabilized by C-terminal arms that invade the neighboring pentamers and fold back to the original pentamer (Wolf et al., 2010). L1 can self-assemble into virus like particles (VLPs) in the absence of L2 (Kirnbauer et al., 1992).

Atomic level structures of L1/L2 capsids have not been generated. However, cryoelectron microscopy reconstructions and antibody binding studies suggest that each L1 pentamer interacts with a single molecule of L2 (i.e., 72 copies of L2 per virion) (Buck et al., 2008; Cardone et al., 2014). L2 fills the central lumen of the L1 pentamer and a stretch of about 60 N-terminal amino acids may be exposed on the capsid surface (Kawana et al., 2001; Kondo et al., 2007; Liu et al., 1997; Roden et al., 1994). Whether this portion of L2 protrudes from the central lumen of the pentamer or from elsewhere in the capsid shell is unknown. The interactions between the two viral capsid proteins appear to be mostly hydrophobic (Finnen et al., 2003; Okun et al., 2001). L2 plays key roles in the infectious entry process (Aydin et al., 2014; Day et al., 2004; Holmgren et al., 2005; Unckell et al., 1997) and, for some papillomavirus types, for genome packaging (Buck et al., 2004; Holmgren et al., 2005; Zhao et al., 1998; Zhou et al., 1993).

3. Papillomavirus replication

Papillomaviruses have a specific tropism for stratified squamous epithelial cells. Viral replication is tightly linked to the differentiation of the epithelium and has traditionally been divided into two phases: an early phase where replication of the viral genome occurs and a late phase where capsid protein production and assembly occurs (Doorbar et al., 2015). To establish a persistent infection, papillomavirus must infect the mitotically active basal cell layer. In these cells, steady state viral genome replication can occur, but vegetative genome replication, characterized by high copy number amplification, only occurs after the daughter of a dividing cell has moved away from the basement membrane into the upper layers of the epithelium and commenced the differentiation process. Capsid assembly occurs in the more

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differentiated keratinocytes and virus release is thought to be a passive process dependent on nuclear envelope breakdown and desquamation of dying terminally differentiated keratinocytes of the outermost layers of the stratified epithelium. Despite the absolute dependence on epithelial differentiation for virus production in vivo, most functional studies of papillomavirus assembly have been conducted in replicating cultured cells using heterologous vectors to express the capsid proteins and, in many cases, to replicate the target genome for packaging. Aspects of the assembly process that are controlled by the differentiation process cannot be addressed by these systems and may remain to be discovered. Differentiation-dependent mechanisms might be address using organotypic "raft" cultures (Ryndock et al., 2015).

4. Papillomavirus capsid assembly

Both papillomavirus structural proteins, L1 and L2, contain canonical nuclear localization sequences that facilitate translocation of the proteins into the nucleus where assembly occurs (Darshan et al., 2004; Rose et al., 1993; Zhou et al., 1991). For most high-risk human papillomaviruses (HPVs), L2 expression and nuclear localization in the suprabasal cells appears to precede L1 expression (Florin et al., 2002). In contrast, HPV1 L1 seems to be expressed and located in the nucleus throughout the epithelium, while L2 was detected only in the upper epidermal cell layers (Egawa et al., 2000). For *Mus musculus* papillomavirus 1 (MusPV1) L1 is expressed throughout the epithelium in the cytoplasm and only upon L2 expression in the upper layers it is transported to the nucleus (Handisurya et al., 2013). Therefore, in vivo, MusPV1 L2 appears to recruit L1 to the nucleus.

The transport of the L1 proteins of HPV11, 16 and 45 from the cytoplasm into the nucleus was shown to depend on karyopherins (Bird et al., 2008; Darshan et al., 2004; Merle et al., 1999; Nelson et al., 2000; Nelson et al., 2002; Nelson et al., 2003). L1 is imported into the nucleus as pentamers (Bird et al., 2008; Florin et al., 2002; Merle et al., 1999; Nelson et al., 2002), and assembly into capsids only occurs in the nucleus. Karyopherin binding has been implicated in inhibiting capsid assembly in the cytoplasm (Bird et al., 2008). Hsp70 family members can trigger disassembly of BPV capsids into pentamers (Chromy et al., 2006), which may also help prevent premature assembly in the cytoplasm. L2 nuclear localization is required for its incorporation into capsids (Becker et al., 2004). Karyopherins and Hsc70 have been suggested to be involved in the transport of L2 protein into the nucleus (Bordeaux et al., 2006; Florin et al., 2004). Hsc70 has also been proposed to play a role in L2 incorporation into the capsid or genome packaging (Florin et al., 2004). During assembly, the interaction between L1 and L2 appears to be regulated by sumoylation of L2, such that sumoylation inhibits L1 interactions (Marusic et al., 2010). Under some experimental condition, L2 also promotes L1 capsomer assembly into capsids (Ishii et al., 2005). The cellular protein nucleophosmin, also interacts with L2 protein to promote the correct assembly of infectious capsids, possibly providing a scaffold for the early processes, as it is displaced from L2 by L1 expression, but the precise molecular mechanisms involved are not understood (Day et al., 2015). Studies of raft-derived virions suggest that the L2 of some HPV types, e.g. HPV16 and 45, may be cleaved by furin during virion production in differentiating epithelium, rather than during infection, as has been observed for all types of

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pseudovirion produced in replicating cells that have been examined (Cruz et al., 2015; Richards et al., 2006)

The assembly kinetics of purified L1 pentamers into VLPs have been investigated in cell-free reactions. Investigations involving multi-angle light scattering concluded that the assembly reaction has a concentration-dependent lag phase with pentamer dimerization being the key nucleating event. This nucleation was followed by rapid sequential addition of single pentamers to form the complete VLP (Casini et al., 2004). It has not been possible to study the kinetics of intracellular assembly.

Taken together, the findings suggest a general mechanism for papillomavirus assembly where (i) papillomavirus genome is replicated to high copy number in the nucleus (ii) L2 and L1 pentamers are recruited to the nucleus and (iii) the capsid assembles once all components are present in the nucleus. The major difference among papillomavirus types appears to be in the relative timing of L1 and L2 expression: for some papillomavirus types L2 seems to precedes L1 and for others L1 precedes L2. It is unclear why these different patterns of L1 and L2 expression have evolved.

After capsid assembly in the nucleus, the redox gradient in the upper layers of the differentiating epithelium in raft cultures allows disulfide bond formation between L1 molecules, leading to capsid maturation (Conway et al., 2009). Based upon studies of monolayer culture cell-derived capsids, this maturation is a slow process that does not seem to be dependent on L2 incorporation into the capsid or DNA packaging (Buck et al., 2005b). Maturation leads to a more compact and stable capsid that is resistant to protease digestion (Buck et al., 2005b).

5. Papillomavirus genome packaging

Viral genome incorporation into the papillomavirus capsid is not well understood at present. It is interesting to note that, when compared to other viruses, papillomaviruses are rather inefficient regarding the length of DNA that can be packaged. Phage P2, for example, has icosahedral capsids of 60nm (similar to papillomaviruses), but packages a linear dsDNA of about 33kb, which is considerably longer than the 8kb of papillomaviruses. In some dsDNA phages, for example phage P22, and for other viral families, such as adenoviruses and herpeviruses that have a tightly packaged DNA genome, the nucleic acids are generally “injected”, in an ATP-dependent manner, into the viral pro-capsid through a specialized portal vertex (Zhang et al., 2012). Other viruses with smaller genomes tend to directly assemble the capsid around the viral genome. Examples of this mechanism include SV40 and Tobacco Mosaic Virus (Butler, 1999; Coca-Prados and Hsu, 1979; Roitman-Shemer et al., 2007; Turner et al., 1988).

Most viral genomes contain packaging signals that are recognized by viral proteins during assembly to allow for selective encapsidation of the viral nucleic acids. For papillomaviruss no such sequence has been identified. Both L1 and L2 have positively charged sequences at their N- and/or C-termini that bind DNA, but they do so in a sequence independent manner, presumably by ionic interactions with the DNA’s phosphate backbone (El Mehdaoui et al.,

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2000; Li et al., 1997; Touze et al., 2000; Zhou et al., 1994). The only sequence postulated to enhance DNA packaging by papillomavirus capsids is nucleotides 1506-1625 of BPV1, inclusion of which enhances DNA packaging by BPV1 and HPV6b under specific in vitro conditions (Zhao et al., 1999). However, it is well established that papillomaviruses can efficiently package a variety of double stranded DNAs entirely unrelated to the papillomavirus genome, as long as the genome size of 8kb is not exceeded (Buck et al., 2004; Kawana et al., 1998; Stauffer et al., 1998), indicating that a specific viral packaging sequence is not required for nucleic acid encapsidation, at least in replicating cells. These findings imply that a different mechanism must be employed by papillomaviruss in order to achieve preferential packaging of their genomes.

A recent in vitro study suggested that papillomavirus genome incorporation may occur through a previously unrecognized size discrimination mechanism (Cerqueira et al., 2015). The viral capsid appears to undergo repeated rounds of assembly and disassembly in the cell nucleus sampling both cellular DNA loops and the viral genome. Consistent with this model, gentle lysis of cell nuclei in which L1 and L2 are expressed followed by endonuclease treatment, results in the extensive generation of capsids that have incorporated cellular DNA fragments of less than 8kb (Cardone et al., 2014). Since under normal physiological conditions cellular DNA would be too large to be completely contained within the particle, these protocapsids cannot complete the assembly reaction and so they remain unstable. This is evident experimentally, as the unstable assembly intermediates are subject to partial disassembly and disassociation from the DNA. In contrast, protocapsids that assemble around a viral genome-sized DNA can complete the full assembly reaction, leading to the formation of a stable virion that is no longer subject to disassembly. The nuclear factors that participate in the intracellular assembly and disassembly reactions have not been conclusively identified.

6. Papillomavirus-based gene transfer vectors

The studies to develop efficient methods of producing papillomavirus pseudovirions have contributed substantially to our understanding of the fundamental aspects of papillomavirus assembly outlined above. Conversely, the emerging understanding of papillomavirus assembly has also informed the development of papillomavirus pseudoviruses as gene transfer vectors. Papillomavirus pseudoviruses have a number of realized and potential applications. Most prominently, they have been used to establish in vitro neutralization assays for evaluating prophylactic vaccines (Buck et al., 2005a; Day et al., 2012; Pastrana et al., 2004). Other applications include their use as critical reagents for mechanistic studies of papillomavirus infection in their target tissues in animal models (Day et al., 2010; Johnson et al., 2009; Kines et al., 2009; Roberts et al., 2007), as vaccines to generate intraepithelial T cell responses in mucosal tissues (Cuburu et al., 2012), and as cytotoxic gene transfer vectors for cancer therapy (Kines et al., 2016). These applications have been greatly facilitated by the promiscuous papillomavirus packaging characteristic, in that a wide variety of plasmids lacking viral sequences can be efficiently packaged (Buck et al., 2005a). However, not all plasmids can be packaged to equal efficiency, and it is unclear why some < 8kb plasmids are poor substrates of packaging even when present at high copy number in L1 and L2 expressing cells ((Buck et al., 2005a), unpublished observations). The most widely

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employed system for the generation of high titer pseudovirions involves the use of codon modified L1 and L2 genes, thereby eliminating negative regulatory elements that greatly limit expression in replicating cells. When the modified capsid genes are expressed under the control of strong heterologous promoters, the limiting factor in pseudovirion production becomes the number of pseudogenomes that are available for packaging in the nucleus (Buck et al., 2005a; Buck and Thompson, 2007; Buck et al., 2005b). Amplification of the pseudogenome to high copy number is achieved by inclusion of the SV40 origin of replication in the plasmid and production in 293TT cells, which express SV40 large T antigen (LT) and small T (ST) antigens. Maturation of the pseudovirions in crude lysates under oxidizing conditions is used to assure that stable preparations are produced. Using this strategy, titers of at least 10^{10} can routinely be produced for multiple HPV and animal types. However, there is considerable variability across types, and, for unknown reasons, some types produce almost no infectious virions, despite good capsid production and encapsidation of the pseudogenome (unpublished results).

The use of this strategy to produce pseudovirus for clinical applications could be curtailed by the presence of the LT and ST genes in the producer cells. Since pseudovirions of all papillomavirus types encapsidate cellular DNA to some extent, it can be predicted that LT and LST DNA will be detected in the pseudovirion preparation, if sensitive assays are employed. Since LT and ST are oncogenes, their presence in the pseudovirus preparations would raise safety concerns. To address this issue, we are in the process of developing strategies of producing the pseudovirions in cell-free systems in which high concentrations of purified L1/L2 protein and bacterially-derived peudogenome plasmids are used to drive the assembly reaction. In the first iteration, it was found that HPV16 L1/L2 proteins could generate high titers of peudovirions in the presence of purified bacterial plasmids, a nuclear extract and ATP (Cerqueira et al., 2015). In support of the size discrimination of genome packaging described above, assembly was efficient even when assembled VLPs were used in the reaction because the nuclear extracts were able to induce partial disassembly of the particles. Further refinement of this assembly strategy should result in high titer pseudovirus preparations that are suitable for clinical trials.

7. Future perspectives

Although some insights have been made into the papillomavirus assembly processes, many questions remain unanswered. How capsid assembly is regulated, which cellular factors are required for assembly and what is the sequence of events that occur during assembly are questions that remain mostly unknown. A complicating issue is that these features may vary among papillomavirus types.

The study of papillomavirus assembly has also become more important as the interest in papillomavirus capsids as gene delivery vectors has increased. Attractive features of papillomavirus pseudovirions in this regard include their ability to package non-viral DNA, their in vivo tropism for basal cells of mucosal and cutaneous epithelia and also their striking tropism for a wide variety of cancer cells (Kines et al., 2016). Generation of papillomavirus pseudovirions under defined cell-free reaction conditions will likely provide

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both GMP-compatible stocks for clinical applications and also mechanistic insides into assembly process.

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Highlights

- Review on papillomavirus assembly
- Directions for future research on papillomavirus assembly
- Possible application of papillomavirus vectors