Published in final edited form as:

Biochim Biophys Acta. 2012 July ; 1819(7): 820–825. doi:10.1016/j.bbagr.2012.01.011.

Hitchhiking on Host Chromatin: how Papillomaviruses Persist

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Abstract

Persistent viruses need mechanisms to protect their genomes from cellular defenses and to ensure that they are efficiently propagated to daughter host cells. One mechanism by which papillomaviruses achieve this is through the association of viral genomes with host chromatin, mediated by the viral E2 tethering protein. Association of viral DNA with regions of active host chromatin ensures that the virus remains transcriptionally active and is not relegated to repressed heterochromatin. In addition, viral genomes are tethered to specific regions of host mitotic chromosomes to efficiently partition their DNA to daughter cells. Vegetative viral DNA replication also initiates at specific regions of host chromatin, where the viral E1 and E2 proteins initiate a DNA damage response that recruits cellular DNA damage and repair proteins to viral replication foci for efficient viral DNA synthesis. Thus, these small viruses have capitalized on interactions with chromatin to efficiently target their genomes to beneficial regions of the host nucleus.

Keywords

virus; HPV; papillomavirus; replication; chromatin; mitosis; DNA damage response; ND10; PML

Papillomavirus life cycle and persistence

Papillomaviruses are ubiquitous. They infect the epithelium of mammals, birds and reptiles and give rise to benign warts or papillomas. Each viral type is species specific but also has a tropism for a specific region of the host's cutaneous or mucosal epithelium. Papillomas are normally benign and self-limiting, but a small percentage of those infected with "high risk" HPVs can become cancerous. In fact, HPV infection is the causative agent of almost all cervical carcinomas and about 25% of head and neck cancers [14,40].

The papillomavirus life cycle is well adapted to develop a long term, persistent relationship with its host (reviewed in [3]). The virus enters the epithelium through an abrasion and infects the basal dividing layer of cells. The virus immediately has to surmount the challenges of delivering its genome to the nucleus and ensuring that it escapes the host's intrinsic defense against invading pathogens and foreign DNA. It must next establish the genome in a region of the nucleus where it can initiate its transcription and replication program unimpeded by the host. The overall tactic of papillomaviruses is to persistently infect the proliferating cells in a differentiating tissue; only low level transcription and DNA replication occurs in these cells but the virus is maintained indefinitely (see figure 1). As

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daughter cells begin the differentiation process, late viral functions are activated and the virus produces high numbers of progeny genomes and viral particles. Thus, the virus has developed a strategy of persistence whereby it maintains its genome in long-lived dividing cells and reserves high levels of replication and expression to cells that are not under strict immune surveillance because they are destined to terminally differentiate and slough off the surface of the epithelium. Remarkably, the virus is able to do this with only a handful of gene products that target key processes in the cell (see figure 2). Nevertheless, most papillomavirus infections are eventually cleared by the host and usually only individuals with immune deficiencies have life-long infections that continuously produce virus.

The type of persistent infection described above is characteristic of papillomaviruses that cause benign papillomas or warts. It should not be confused with the persistence of cervical infection by high-risk HPVs. In the latter situation, the virus causes progressive genomic instability of the host and this is thought to underlie malignant progression [5]. With progression, the infected cells differentiate less and less and the viral genomes often become integrated. Thus, this is a dead-end for the virus and not similar to the strategy of persistence described above.

Cell Entry and Overcoming Intrinsic Host Defenses

Papillomaviruses are small, non-enveloped 50nm particles composed of the major (L1) and minor (L2) capsid proteins surrounding a circular 8kbp DNA genome (figure 2). The genome is packaged in nucleosomes derived from cellular histones [11]. Tissue abrasion of the host epithelium exposes heparin sulfate polysaccharides that are incorporated in the basement membrane. Papillomavirus particles initially bind to these proteoglycans. The capsid proteins, L1 and L2, subsequently undergo proteolytic cleavage and conformational change that enable them to attach to the primary receptor on the surface of keratinocytes adjacent to the wound (reviewed in [33]). The virus particle is internalized by endocytosis (reviewed in [32]) and is uncoated and escapes the late endosomes by an L2-dependent mechanism [19]. The viral genome, in complex with the L2 minor capsid protein, traffics to the nucleus [6]. However, contrary to earlier thoughts, the virus is not imported through the nuclear membrane but likely relies on nuclear envelope breakdown at mitosis to enter the nucleus [30].

Within the cell, the virus encounters and must counteract intrinsic host defense mechanisms. The endosomes contain pattern recognition receptors that can sense nucleic acids of foreign pathogens, but HPV16 can down regulate TLR9, the Toll-like receptor that detects viral DNA [15]. Nuclear antiviral defenses present viruses with another challenge and HPV must battle with these processes to establish a persistent and productive infection. The best studied nuclear, anti-viral defense mechanisms are mediated by nuclear structures alternatively called ND10 (nuclear domain 10) or PML (promyelocytic leukemia) bodies. Most cells contain between five and thirty ND10 bodies and they have been implicated in many cellular functions such as transcription, DNA replication and repair (reviewed in [21]). However, they are perhaps best known for their role in anti-viral defense. Upon entry into the nucleus, the genome of most DNA viruses accumulates adjacent to or within ND10 bodies [39]. Some of the main components of ND10 bodies are a series of PML isoforms (TRIM tripartite motif proteins), a series of SP100 variants that probably function in chromatin-mediated gene regulation (they contain SAND and HMG DNA binding domains and PHD and bromo- chromatin binding domains) and Daxx, a transcriptional repressor. Each of these components is interferon inducible and heavily sumoylated and collectively are capable of repressing viral replication and gene expression. Moreover, many viruses encode proteins that disrupt or modify the components or composition of ND10 bodies,

leading to the hypothesis that ND10 bodies constitute an antiviral defense that must be destroyed by the virus (reviewed in [10]).

In the case of papillomaviruses, the L2 capsid protein and viral genome become associated with the ND10 bodies upon nuclear entry [6] and, while there is some reorganization of the ND10 components [12], the nuclear bodies remain intact. In fact, viral DNA replication and transcription are greatly reduced in PML^{-/-} mouse fibroblasts suggesting that ND10 bodies are actually required for efficient papillomavirus infection [6]. A common strategy of viruses is to exploit host defenses and use cellular responses to their advantage. So while papillomaviruses might initially be recognized as foreign intruders by ND10 bodies, they may have subverted these responses and be using the ND-10 chromatin-associated factors to establish an efficient and long-term infection. Figure 3 shows the ND10 localization of PML, Sp100 and Daxx in primary human keratinocytes. In our laboratory, we have used siRNA technology to down-regulate PML, Sp100 and Daxx expression in primary human keratinocytes and find that all three factors modulate HPV infection (Stepp, Meyers and McBride, unpublished data). The viral transcriptional regulatory E2 protein is also recruited to ND10 domains in the presence of L2 [7] and this could facilitate the establishment of a persistent infection.

Maintaining a Persistent Infection

When acute viruses successfully enter the cell and initiate infection, there is a cascade of immediate early, early and late gene expression and progeny genomes are rapidly generated in large numbers and virions are released from the cell. In contrast, after having escaped the initial host defense system, chronic or persistent viruses must establish a safe, long term niche within the host nucleus. Some viruses achieve this by integrating into the host genome but others, such as the papillomaviruses and gamma-herpesviruses, achieve this by maintaining their genome as low copy extrachromosomal circular plasmid. Nevertheless, these genomes are still vulnerable to transcriptional silencing (DNA methylation and packaging in heterochromatin) and elimination from the cell.

The papillomavirus E2 protein is the major transcriptional regulator of the virus. E2 can both activate and repress viral transcription by binding to specific binding sites within the HPV promoter and enhancer sequences (see Figure 2). E2 also binds specifically to the viral replication origin to assist in loading the E1 protein, an origin binding helicase. Moreover, E2 interacts with host chromatin. This is important in interphase cells to retain the E2 protein and viral DNA in specific regions of the nucleus [18,20] and in mitotic cells to partition the viral genomes to daughter cells [1,16,37]. Finally, in differentiating cells E2 directs the formation of specific viral replication foci in which viral DNA is amplified to high levels [31].

The E2 protein interacts with many cellular proteins. These proteins are primarily involved in nuclear processes such as DNA replication and repair, transcriptional regulation and chromatin binding, modifying and remodeling. One notable and major interacting protein is the cellular bromodomain protein, Brd4. Brd4 binds to acetylated lysines on histone tails and promotes transcriptional initiation and elongation (reviewed in [42]). The E2 proteins from all papillomaviruses interact with Brd4 to regulate viral transcription [2,17,23,34,36]. In addition, the E2 proteins from many, but not all viruses, bind with high affinity to Brd4 and stabilize its association with host chromatin [24]. In these viruses the E2-Brd4 complex is stable and persists throughout mitosis and is thought to be important for partitioning the viral genome to daughter cells [23,26].

To determine whether the E2-Brd4 complex was bound to specific regions of host chromatin, a genome-wide chromatin immunoprecipitation-on-chip analysis was carried out

in our laboratory using human promoter sequences [18]. In interphase cells both E2 and Brd4 were bound to most of the cellular transcriptionally active promoters, as evidenced by RNA polymerase II occupancy and histone H3K4me3 modification (see figure 4). The E2 protein did not change the activity of these promoters but this association ensures that the viral genome is retained in transcriptionally active nuclear domains [18]. The E2 protein is associated with these regions of chromatin through protein-protein interactions, which allows it to also bind and tether viral DNA to these regions [18,20].

Maintenance Replication and Tethering on Host Mitotic Chromosomes

With the exception of the viral E1 and E2 proteins, cellular proteins are required for papillomavirus DNA replication (reviewed in [22]). There are thought to be three different phases of DNA replication in the papillomavirus life cycle. The first is an initial burst of replication that occurs when the virus first enters the cell and which results in a low copy number of viral genomes per cell. The second phase of viral replication is maintenance replication and this takes place at the same time that infected cells replicate their own DNA. The replicated viral genome is partitioned to daughter cells in mitosis resulting in a constant copy number. The third and last phase is vegetative amplification, which occurs only in differentiated cells. This results in high copy numbers of progeny genomes that are destined to be packaged in new viral particles.

Viral DNA replication requires the E1 and E2 proteins and the replication origin and this is sufficient for both the initial and vegetative phases of viral replication. The maintenance phase however occurs in proliferating cells and requires, in addition, a partitioning mechanism to segregate viral genomes to daughter cells. This partitioning requires both the E2 protein in trans and multiple E2 binding sites in cis to the viral replication origin [28]. As shown in figure 5, the E2 protein associates with host chromatin and by binding to the E2 binding sites in the viral genome, tethers the viral genomes to host mitotic chromosomes [1,16,37].

The E2 proteins of different papillomaviruses appear to target different regions of mitotic chromosomes and consequently different cellular chromosomal proteins [26]. The best characterized of these proteins is Brd4. As described above, all E2 proteins interact with Brd4 to regulate viral transcription [23,36,38], but a subset of viruses also interact with Brd4 on host mitotic chromosomes [17,23,24,43]. These viruses include BPV1, HPV1 and a number of animal papillomaviruses. Brd4 is a dynamic protein that is not normally bound tightly to interphase or mitotic chromatin [8] but expression of E2 stabilizes this association [24]. The dimerization function of the E2 protein is at least partially responsible for the increased affinity of E2 for chromatin as it enables the formation of higher order complexes of E2 and Brd4 [4]. The E2 proteins from the alpha and beta HPVs are not detected in complex with the Brd4 protein on mitotic chromosomes [23,26]. Instead, the beta papillomavirus E2 proteins associate with the pericentromeric ribosomal DNA loci on the short arms of acrocentric chromosomes [29]. The alpha HPV E2 proteins are not as stably bound to mitotic chromosomes throughout mitosis as are other E2 proteins, but can also bind to the same pericentromeric regions of the host chromosomes as the beta HPV E2 proteins [26,29]. Brd4 interacts with the transactivation domain of E2 and mutation of specific residues within this domain can disrupt the E2-Brd4 interaction, E2-mediated transcriptional regulation, association of E2 with mitotic chromosomes and viral genome partitioning [2,23,36,43,44]. On the other hand, sequences from the E2 hinge region and DNA binding domain are necessary for interaction of the beta papillomavirus E2 proteins with pericentromeric regions of mitotic chromosomes [29,35]. Other factors that seem to be involved in the association of E2 with host chromatin are the DNA helicase, ChlR1 [27] and TopBP1, a protein involved in DNA replication and checkpoint control [9].

Hitchhiking of viral genomes on host chromosomes is not a unique strategy of papillomaviruses. Other persistent viruses that replicate as extrachromosomal plasmids use the same mechanism. Best studied are the gamma herpesviruses Epstein-Barr virus (EBV) and Kaposi's sarcoma associated herpesvirus (KSHV). These viruses encode proteins that are analogous to E2 and tether the large herpesvirus genomes to host mitotic chromosomes (reviewed in [22]). Notably, the herpesvirus tethering proteins EBV EBNA and KSHV LANA have DNA binding domains that have a very similar structure to that of the E2 protein. These proteins tether to host chromosomes through short basic peptides that are analogous to those required for chromosome binding of the beta HPV E2 proteins (reviewed in [22]).

Vegetative replication: papillomaviruses utilize the cellular DNA damage response for their own replication

Papillomavirus genomes are maintained long term in the proliferating basal cells of a papilloma. When uninfected basal cells divide, the daughter cells leave the basement membrane, exit the cell cycle and begin the process of programmed terminal differentiation (see figure 1). The various layers of the epithelium are designated stratum basale, stratum spinosum, stratum granulosum and stratum corneum. These names reflect the primary histological features that appear as the cells differentiate. In the first stage of differentiation (stratum spinosum) the cells have a spiny appearance that is due to the formation of desmosomes, which join cytoplasmic keratin filaments between cells and give the epithelium structural strength. In the next layer, the stratum granulosum, lipid-containing, basophilic keratohyalin granules provide the epithelium with an impermeable barrier. The stratum corneum provides an additional protective layer that contains dead or dying cells which are eventually shed from the epithelium. The papillomavirus life cycle is finely tuned to this differentiation program. The dividing basal cells act as a reservoir of infected cells containing viral genomes. As these cells progress through the differentiation process the virus switches to vegetative viral DNA amplification, late gene expression and virion assembly. Viral laden squames are exfoliated from the surface of the epithelium where they can make contact with their next host.

The advantage of this strategy is to allow the virus to persist, undetected by the immune system for very long periods of time. However, a disadvantage is that the virus, which is very reliant on host DNA replication machinery, must amplify its DNA in differentiated cells that would normally have exited the cell cycle. The papillomavirus E5, E6 and E7 regulatory proteins promote cellular proliferation and delay cell cycle exit and differentiation in the upper stratified layers so that the virus has the constituents necessary to synthesize large amounts of viral DNA. It was initially thought that papillomaviruses amplified their DNA in upper-layer cells that were in a pseudo-S phase. However, recent evidence indicates that DNA amplification occurs in cells in the G2-phase of the cell cycle [41]. Furthermore, research in our laboratory and others has shown that expression of the viral E1 and E2 replication proteins causes a cellular DNA damage response and cell cycle arrest [13,31]. Replication of the viral genome per se is not required for induction of the DNA damage response; instead the DNA binding and helicase functions of E1 protein are responsible. E1 may induce the DNA damage response by non-specifically initiating replication of host DNA. However, in the presence of the E2 protein, E1 and E2 form defined nuclear foci that recruit DNA damage response and repair proteins (see figure 6). These foci contain γ H2AX, pATM and Nbs1 and show evidence of both DNA synthesis and DNA ends [31]. The chromatin binding ability of the E2 protein is required for the formation of the E1/E2 foci. Binding of the E1/E2 foci at specific regions of host chromatin causes unscheduled replication of host DNA that leads to a DNA damage response and an influx of cellular proteins to both signal and repair the damage. This strategy is

advantageous to the virus because it recruits DNA repair enzymes to specific regions of the host nucleus resulting in a local concentration of enzymes to replicate the viral DNA. Furthermore, the virus is now able to replicate its DNA outwith S-phase in the absence of competition from cellular DNA synthesis. The fact that the cells are growth arrested is no longer a problem for the viral life cycle as these cells are differentiating and destined to die shortly. In support of this model, it has been shown that induction of the host ATM DNA damage response is essential for vegetative viral DNA replication [25].

Conclusions

Persistent viruses that establish a long term infection with the host can teach us a great deal about the inner workings of the cell. Papillomaviruses enter the cell nucleus and must escape intrinsic cellular defenses that eradicate or silence foreign DNA. They rely on host nuclear processes for transcription and replication and must establish themselves in beneficial regions of the cell nucleus and ensure that they are retained in daughter cells. Papillomaviruses have capitalized on host cell differentiation to switch to late gene expression and synthesize high levels of capsid proteins. But they have also taken advantage of the cell's DNA damage and repair response to nucleate viral DNA replication factories in differentiated cells. It is remarkable how these small viruses have evolved to target many of the key processes in the cell nucleus.

Acknowledgments

The work of the authors is funded by the Intramural Research Program of the NIAID, NIH.

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Highlights

- Papillomaviruses develop a long term, persistent relationship with their host
- The papillomavirus life cycle is tightly linked to keratinocyte differentiation
- Papillomaviruses must overcome intrinsic cellular defenses to establish an efficient infection
- Papillomaviruses retain their genomes in infected cells by tethering them to host chromatin
- Papillomaviruses utilize the cellular DNA damage response for their own replication

Figure 1. The papillomavirus life cycle is tightly linked to keratinocyte differentiation

The virus infects the dividing, basal layer of cells and becomes established as a low copy, autonomously replicating plasmid. When the daughter cells leave the basal layer, they progress through the process of differentiation and are pushed towards the surface of the epithelium. In concert, the viral DNA is amplified to high levels and late genes are expressed. Viral genomes are assembled into virion particles in the cornified layers of the epithelium. Viral-laden squames are shed from the surface of the epithelium.

Papillomaviruses have small, double-stranded circular genomes of about 7–8kbp. All papillomaviruses encode the replication proteins E1 and E2 and the capsid proteins L1 and L2. Most viruses also encode auxiliary proteins E5, E6 and E7, which manipulate host cell proliferation and cell cycle checkpoints and help conceal the presence of the virus from host immune defenses. Additionally, papillomavirus genomes have a long non-coding section that contains viral promoters, enhancers and the replication origin (ori). These elements contain specific binding sites for the E1 (black square) and E2 (black circle) proteins.

Figure 3. ND-10 bodies in keratinocytes

ND-10 bodies are thought to be involved in intrinsic anti-viral immunity. PML, Sp100 and Daxx are major components of the bodies. The images show immunofluorescent staining of PML and Sp100 (left panel) and PML and Daxx (right panel) in undifferentiated primary human keratinocytes. This is the classic pattern of ND-10 bodies observed in most mammalian cells.

Figure 4. Papillomavirus E2 protein interacts with Brd4 at active promoter regions in cellular chromatin

ChIP on chip analysis of BPV1 E2 and Brd4 binding to active human promoter regions. RNA polymerase II and H3K4me3 binding serve as markers of active chromatin. Shown is a small region of chromosome 3. TSS: transcriptional start site. Tiled region is the location represented on the microarray.

Figure 5. Papillomavirus genome partitioning model

Papillomavirus genomes are tethered to host mitotic chromosomes by the E2 protein. This ensures that they are partitioned to daughter cells, retained in the nucleus and associated with active chromatin.

Figure 6. Papillomavirus replication foci

Papillomavirus E1 and E2 proteins form nuclear foci that recruit DNA damage and repair proteins to host chromatin to amplify viral DNA. The images show immunofluorescent staining of HPV16 E1 and E2 proteins and phosphorylated ATM in a DAPI stained keratinocyte nucleus.