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The nuclear retention signal of HPV16 L2 protein is essential for incoming viral genome to transverse the trans-Golgi network

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ABSTRACT

The Human papillomavirus (HPV) capsid is composed of the major and minor capsid proteins, L1 and L2, respectively. Infectious entry requires a complex series of conformational changes in both proteins that lead to uptake and allow uncoating to occur. During entry, the capsid is disassembled and host cyclophilins dissociate L1 protein from the L2/DNA complex. Herein, we describe a mutant HPV16 L2 protein (HPV16 L2-R302/5A) that traffics pseudogenome to the *trans*-Golgi network (TGN) but fails to egress. Our data provide further evidence that HPV16 traffics through the TGN and demonstrates that L2 is essential for TGN egress. Furthermore, we show that cyclophilin activity is required for the L2/DNA complex to be transported to the TGN which is accompanied by a reduced L1 protein levels.

Introduction

Human Papillomaviruses (HPV) are non-enveloped epitheliotropic viruses, which are associated with malignancies of the epithelium including cervical, penile, anogenital, and oropharyngeal carcinoma. The viral capsid is composed of 360 copies of the major capsid protein, L1, organized into 72 pentamers, which are also called capsomeres. Sixty capsomeres are pentavalent, *i.e.* have five nearest neighbors, whereas 12 capsomeres are hexavalent (Chen et al., 2000; Finch and Klug, 1965). Capsomeres are interconnected by invading C-terminal arms, which are stabilized by intercapsomeric disulfide bonds resulting in covalently linked L1 dimers and trimers (Li et al., 1998; Mejia et al., 2006; Sapp et al., 1998; Wolf et al., 2010). In addition, up to 72 copies of the minor capsid protein, L2, are present within the capsid, mostly hidden inside with only a short N-terminal portion accessible (Buck et al., 2008; Liu et al., 1997; Modis et al., 2002). The protein shell encapsidates a circular, double-stranded DNA viral genome of about 8000 bp, which is organized into chromatin (Favre et al., 1977).

Recent advances in understanding the HPV16 internalization process was based on the development of systems to generate pseudovirions, which mimic authentic particles (Buck et al., 2004, 2005a) that also encapsidate a pseudogenome. Using these particles, it was demonstrated that HPV16 particles attach to the extracellular matrix via primary heparin sulfate proteoglycan receptors using L1 lysine residues 278 and 361 present on capsomeres (Knappe et al., 2007). Attachment triggers conformational changes in both capsid proteins allowing transfer to unknown secondary receptors and infectious internalization (Day et al., 2008; Richards et al., 2013; Selinka et al., 2007; Spoden et al., 2008). These processes are extremely slow and asynchronous, taking several hours to complete (Christensen et al., 1995; Giroglou et al., 2001; Smith et al., 2007). Infectious internalization of HPV16 occurs via a clathrin- and caveolae-independent pathway (Spoden et al., 2008, 2013; Schelhaas et al., 2012). Acidification of the endocytic compartment is essential for successful infection (Day et al., 2003; Selinka et al., 2002) and is accompanied or followed by uncoating and egress from endosomes (Kamper et al., 2006), and subsequent accumulation of incoming pseudogenomes at PML nuclear bodies (Day et al., 2004). Successful delivery of the pseudogenome to the nucleus depends on the activity of numerous host cell factors. One such factor, cell surface cyclophilin B, a chaperone peptidyl-prolyl *cis*/*trans* isomerase, facilitates exposure of a hidden cleavage site on the N-terminus of the L2 protein that is subsequently cleaved by furin convertase, both events that have been shown to be required for pseudogenome delivery (Bienkowska-Haba et al., 2009; Richards et al., 2006). Our lab has demonstrated that host cell cyclophilins (CyPs) are also required at a secondary, post-internalization step which mediates the dissociation of the L1 protein from the L2/DNA complex prior to nuclear entry (Bienkowska-Haba et al., 2012). The L2 protein mediates egress of the pseudogenome from endosomes (Kamper et al., 2006) and

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retrograde transport of pseudogenome along microtubules to the nucleus (Florin et al., 2006; Schneider et al., 2011).

Recently, a genome-wide siRNA screening identified cellular factors involved in retrograde trafficking of HPV. Among these factors, components of the retromer complex and Rab GTPases were found to be required for retrograde transport of HPV16 pseudovirions to the *trans*-Golgi network (TGN) (Day et al., 2013; Lipovsky et al., 2013). It was demonstrated that HPV16 virions enter the TGN in a retromer-dependent manner during infectious entry. Additionally, it was also shown that TGN localization is dependent on furin-cleaved L2 protein (Day et al., 2013). Following TGN egress, the L2 protein accompanies the viral genome to PML nuclear bodies (PML NBs) (Day et al., 2004). Evidence indicates that nuclear import of viral DNA requires nuclear envelope breakdown (Pyeon et al., 2009) suggesting independence of active nuclear import pathways despite the existence of redundant nuclear localization signals (NLS) in L2 (Becker et al., 2003).

The L2 protein has two canonical nuclear localization signals on both the N- and C-terminal ends. Recently, a third element was identified located between amino acids 291–315, which we named the mNLS, that contain a stretch of arginine residues that were shown to mediate nuclear retention *in vitro* (Mamoor et al., 2012). To further investigate the role of the mNLS in nuclear delivery, our lab has generated NLS mutants with exchanges in bases within mNLS and deletions in the N- and C-terminal NLSs. Using transfection and over-expression, we show that neither NLS is required for nuclear translocation. Additionally, we confirm that the mNLS functions as a nuclear retention signal *in vitro*, results that are consistent with a previous report (Mamoor et al., 2012). In the context of infectious entry, we show that pseudovirions with mutations in the mNLS accumulate in the TGN. Our data presented herein provide further evidence that HPV genomes traffic through the TGN and confirm that CyPs are essential for transport to the TGN.

Results

Nuclear translocation of HPV16 L2 protein in vivo

Two sequence elements at the N- and C-terminus of HPV16 L2 protein termed nNLS and cNLS, respectively, have been extensively characterized as putative nuclear localization signals using a variety of *in vitro* techniques (Bordeaux et al., 2006; Darshan et al., 2004; Klucevsek et al., 2006; Mamoor et al., 2012; Sun et al., 1995). In order to identify NLSs that might be used *in vivo* during infection and after synthesis, we deleted these elements and tested the intracellular localization of mutant L2 following transfection of expression plasmids. The truncations were kept short to minimize potential effects on L2 protein folding. All transfected mutant L2 constructs were expressed in HeLa cells (Fig. 1F). As shown in Fig. 1A and G, deletion of both signals did not abrogate nuclear import of HPV16 L2 protein suggesting that neither NLS is essential for L2 nuclear translocation. We therefore concentrated on a third element, which was shown to influence intracellular localization of HPV6b (Sun et al., 1995) and HPV33 L2 (Becker et al., 2003). This region is highly conserved among members of the Papillomaviridae family Alphapapillomavirus genus (Fig. 1B). Located between HPV16 L2 amino acid residues 291 and 315, this region contains a number of conserved arginine residues. We introduced point mutations in this region in the context of full length and terminally truncated L2 protein focusing on but not restricted to basic amino acid residues. Replacement of arginine residues 297, 298, 302, and 305 for alanine affected nuclear localization when analyzed at 24 hours post-transfection (hptx) of full length and truncated L2. At this time, a significant percentage of mutant L2 protein was found in the cytoplasm. In addition, a varying fraction of mutant L2 localized to the nucleus in a punctate pattern co-localizing with PML protein (Fig. 1C). A quantification of the results is provided in Fig. 1G. Additional deletions of the terminal nNLS and cNLS only had a minor impact on subcellular localization as shown for 16L2-13-455-R297/8-302/5A. These data suggest that the contribution of nNLS and cNLS to nuclear import is rather negligible under our conditions. The strongest effect was observed when all four arginine residues were replaced. However, pronounced reductions in nuclear import of L2 protein were also found for double mutants R297/8A and R302/5A (Fig. 1D and G). Exchange of arg-291, lys-309, arg-315, ser-304, gly-307, or ser-295 and thr-296 for alanine did not affect nuclear accumulation of L2 protein (data not shown). We also exchanged arginine residues at positions 295 and 298 of HPV18 L2, which are homologous to arg-302 and arg-305 of HPV16 L2, and found that these mutant proteins mainly localize to the cytoplasm as well at 24 hptx (Fig. 1E and G). Looking at earlier times post-transfection, we observed wild-type HPV16 L2 localize in the nucleus as early as 6 hptx and remained nuclear (Fig. 2A) whereas HPV16 mutant L2 protein localized in the nucleus at earlier times post-transfection (Fig. 2B), but was found to be relocated to the cytoplasm after 12 hptx. The cytoplasmic relocalization can be abrogated by inhibiting CRM1-dependent nuclear export with leptomycin B (LMB) treatment (Fig. 2C). Taken together, these results confirm previous observations by others (Mamoor et al., 2012) that mutations in this motif did not abrogate nuclear import but rather affected nuclear retention.

Characterization of mutant pseudoviruses

In order to investigate the role of this sequence element in context of infectious HPV entry, pseudoviruses harboring mNLS mutant L2 protein were generated using the 293TT production cell line (Buck et al., 2004). All tested mutant L2 proteins were efficiently incorporated into pseudoviruses, and all pseudoviruses encapsidated the pEGFP-C1 pseudogenome used for our studies with comparable efficiency (Fig. 3A). We determined the infectivity of the L2 mutants by infecting 293TT and HaCaT cells with mutant pseudovirus for 72 h at 37 °C and quantified GFP transcript by real-time PCR (Fig. 3B). The exchange of arginine 302 and 305 with alanine in the L2 protein rendered the pseudovirions non-infectious (Fig. 3B). Mutants 16L2-R291A, -S304A, -G307A, -K309A, and -R315A fully supported infection (data not shown). Similarly, mutant HPV18 pseudovirus harboring 18L2-R295/8A was also strongly defective (data not shown) suggesting that the importance of this region may be conserved among genital high-risk Papillomaviruses. We assessed the integrity of the capsid structure of intact virions by measuring reactivity with L1-specific antibodies by the enzyme-linked immunosorbent assay (ELISA). Pseudoviruses were bound for 1 h at 37 °C to wells of an ELISA plate and incubated with the HPV16 VLP-specific polyclonal antibody K75, monoclonal antibody (MAb) 33L1-7 that recognizes a linear epitope of the L1 protein at amino acids 303 to 313 indicative of disassembly, and MAb H16.V5 that recognizes a conformationally-dependent epitope of the FG loop of the L1 protein when assembled into capsids (Sapp et al., 1994; White et al., 1999). We observed comparable reactivity with all three antibodies between wild-type and mutant pseudoviruses suggesting that these mutations do not disrupt the capsid structure (Fig. 3C).

Reduced infectivity is not due to deficiency in cell binding or uncoating

To show that the reduction in infectivity is not due to a defect in cell binding, we bound pseudovirus particles to HaCaT cells grown on coverslips for 1 h at 37 °C, then fixed and stained using MAb 33L1-7 following the Click-iT reaction cocktail, which has previously been shown to denature the capsid protein to make the linear L1-specific epitope accessible (Bienkowska-Haba et al., 2012). We did not observe any difference in the binding of mutant L2 pseudoviruses compared to the binding of wild-type