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Preparation and properties of a papillomavirus infectious intermediate and its utility for neutralization studies

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ABSTRACT

We show that minor capsid protein L2 is full length in clinical virion isolates and prepare furin-cleaved pseudovirus (fcPsV) as a model of the infectious intermediate for multiple human papillomavirus (HPV) types. These fcPsV do not require furin for *in vitro* infection, and are fully infectious *in vivo*. Both the γ-secretase inhibitor XXI and carrageenan block fcPsV infection *in vitro* and *in vivo* implying that they act after furin-cleavage of L2. Despite their enhanced exposure of L2 epitopes, vaccination with fcPsV particles fails to induce L2 antibody, although L1-specific responses are similar to PsV with intact L2. FcPsV can be applied in a simple, high-throughput neutralization assay that detects L2-specific neutralizing antibodies with >10-fold enhanced sensitivity compared with the PsV-based assay. The PsV and fcPsV-based assays exhibit similar sensitivity for type-specific antibodies elicited by L1 virus-like particles (VLP), but the latter improves detection of L1-specific cross-type neutralizing antibodies.

Introduction

Genital human papillomavirus (HPV) is one of the most common sexually transmitted infections, and persistent infection with ~15 'high risk' HPV genotypes (most often HPV16, HPV18, HPV31, HPV45) frequently can cause high-grade intraepithelial neoplasia. Left untreated, these high-grade lesions can progress to invasive carcinoma of the cervix and other anogenital regions and oropharynx. Indeed, HPV is the etiologic agent responsible for 5% of all cancer deaths worldwide, including 99% of cervical cancers (de Villiers et al., 2004; Parkin and Bray, 2006). As the two prophylactic HPV vaccines, Gardasil and Cervarix, are licensed for protection against only two oncogenic HPV types (HPV16 and HPV18), the development of more broadly acting inhibitors and second generation HPV vaccines has continued.

The study of papillomavirus has been technically difficult because completion of the PV life cycle requires squamous differentiation of the infected keratinocyte that is not replicated by standard tissue culture conditions. However, organotypic raft culture causes infected keratinocytes to undergo squamous differentiation and thus generates infectious PV (Conway et al., 2009b; McLaughlin-Drubin et al., 2004; Meyers et al., 1992). This method produces limited quantities of virions containing the authentic viral genome for which there is no simple per cell infectivity assay. An alternative approach is the production of PV pseudovirion (PsV) by the co-transfection of the 293TT cell line with codon-modified L1 and L2 expression vectors and a reporter plasmid genome (Buck et al., 2004). The cells are lysed 48 h later, and incubated overnight at 37 °C (known as the maturation step) before being purified by density gradient ultra-centrifugation. These purified PsV can be readily used for surrogate infectious studies both *in vitro* and *in vivo* because they deliver a reporter construct, typically expressing luciferase or GFP, or alternatively the PV genomes can be encapsidated in this system to produce quasivirions (QV) (Buck et al., 2004; Culp et al., 2006; Pastrana et al., 2004; Pyeon et al., 2005; Roberts et al., 2007).

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Residues 17-36 of minor capsid protein L2 are buried below the capsid surface of HPV16 PsV, inaccessible to the neutralizing monoclonal antibody RG1 (Gambhira et al., 2007), but become accessible to RG1 as early as 4 h in the infectious process (Kines et al., 2009). For exposure of the RG1 epitope, PV must first undergo a conformational change and adopt an intermediate structure. This is triggered by binding of virions to heparan sulfate proteoglycans (HSPG) on the basement membrane (that has been revealed upon wounding the epithelium) and cleavage of the very amino terminus of L2 by furin at a conserved site. This conformational change in the capsid is also modeled *in vitro* by the association of PsV with extracellular matrix (ECM) produced by certain cell lines, *e.g.* HaCaT and MCF7, although not 293TT cells to which the PsV bind directly via HSPGs (Johnson et al., 2009; Kines et al., 2009). Importantly this difference in mechanism of L2 exposure upon binding of PsV to 293TT cells has been linked to poor sensitivity in L2-, but not L1 VLP-specific antibody-dependent *in vitro* neutralization assays using this cell line (Day et al., 2008a, 2012a). Indeed, the discord between the low or undetectable neutralization titers measured using this system despite robust ELISA reactivity and protection upon passive transfer and PsV challenge of mice with the same L2-vaccinated sera, suggest the need for improved assays that use target cells other than 293TT to better replicate the uncloaking of L2 observed during infection *in vivo*.

Studies of the PsV production procedure show that HPV PsV particles which do not undergo the maturation step are more susceptible to neutralization by L2 antibodies, suggesting L2 is initially exposed during the early events of packaging and co-assembly with L1 capsomeres but is slowly "buried" in the capsid structure as the virus matures into a more stabilized form (Buck et al., 2005; Buck and Trus, 2012). In contrast, studies of organotypic raft culture-derived virions show more mature HPV virus particles (*i.e.* virions from a 20-day old raft) harvested from cornified layer, are more susceptible to neutralization by L2-specific antibodies compared to virions harvested from the suprabasal layer of the tissue rafts cultured for 10 days (Conway et al., 2011). These findings suggest differential exposure of L2 epitopes on the capsid during virion morphogenesis and elevation from the more reducing environment of the suprabasal layers to the upper oxidizing cornified layers in the differentiated tissue raft culture (Conway et al., 2009b, 2011). Whereas some studies analyzing L2 in virions purified from warts suggest L2 is full-length, others show L2 existing in a doublet by immunoblot (Doorbar and Gallimore, 1987; Jin et al., 1989; Rose et al., 1990). While the latter may reflect partial degradation during virion purification, it remains possible that a subset of wart-derived infectious virions exhibit variable degrees of L2 exposure and furin-cleavage.

There are important implications if a subset of L2 is already exposed and cleaved by furin even before transmission and encountering the host. Firstly, partially pre-cleaved HPV can infect both HSPG- or furin-deficient cell lines (Day et al., 2008b; Kines et al., 2009). This shows that changes in the conformation of the capsid associated with L2 exposure and cleavage allows HPV to become independent from cellular factors considered to be required for infection. Importantly, carrageenan (a type of sulfated polysaccharide extract from red algae which is used in sexual lubricants) as well as inhibitors of γ-secretase (*e.g.* XXI) (Huang et al., 2010; Karanam et al., 2010) were recently identified as potent inhibitors of mucosal trophic HPV types infection (Buck et al., 2006; Marais et al., 2011; Roberts et al., 2007). However, it is currently unknown if their potency would be compromised if a subset of, or the true infectious form of native virions are in the L2-cleaved conformation. A second implication is that potential differences in L2 cleavage within organotypic raft-derived virion and PsV preparations may account for reported differences in sensitivity to inhibitors and the impact of mutations in the RG1 epitope (Conway et al., 2009a; Cruz and Meyers, 2013).

Here we describe a method to generate milligram quantities of highly (~90% of L2) furin-cleaved pseudovirus (fcPsV), and examined their immunogenicity and the impact of carrageenan, and furin and γ-secretase inhibitors upon infectivity of the furin-cleaved intermediate. To confirm if furin cleavage of L2 represents an infectious intermediate, we examined the cleavage status of L2 in several wart-derived virions of divergent PV genotypes. Further, we assessed if use of fcPsV for *in vitro* neutralization studies could enhance the sensitivity for L2-specific neutralizing antibodies in a high throughput format without compromising measurement of L1 VLP-specific antibody.

Results

Generation of 293TTF, a clonal cell line that overexpresses enzymatically active furin

L2 in HPV16 PsV could be cleaved if furin is added during the maturation step of the standard HPV PsV protocol. However, the extent of L2 cleavage in the virions was only approximately 35% in these preparations (Day et al., 2008b). This prompted us to develop an alternative approach to reproducibly produce fully furin-cleaved PsV at high titer. To this end, a clonal 293TT cell line that over-expresses furin, termed 293TTF, was generated from the 293TT cell line that is used for conventional PsV production (Fig. 1).

Based upon functional analyses furin is present in 293TT cells but the amounts were below the limit of detection in our Western blot analysis (Fig. 1A, lane 1). This was consistent with the literature on furin being highly regulated and not readily detected via western blot methods (Bourne and Grainger, 2011; Thomas, 2002). However, Western blot analysis of 293TTF cells produced a prominent band of 90–100 kDa, a size consistent with an unresolved doublet of the endogenous immature/pro-furin (96 kDa) and mature furin (90 kDa) (Fig. 1A, lanes 3 and 4). Image analysis by densitometry showed that the total amount of furin expressed in 293TTF was at least 150-fold higher than in parental 293TT cells. Another band was observed at ~60 kDa which was reported in the antibody material data sheet provided by the manufacturer as a furin splice variant (Fig. 1A, lanes 1, 3, and 4). The level of secreted furin released by 293TTF cells was also 200-fold higher compared to 293TT cells (Fig. 1A, lane 5–7). Secreted furin has a lower molecular weight (~80 kDa) than cell-associated furin due to cleavage of the latter's C-terminal transmembrane region.

Production and analysis of HPV furin-cleaved pseudovirus (fcPsV)

To assess if 293TTF can act as a producer cell line for furin-cleaved pseudovirus (fcPsV), we performed the standard PsV production protocol using either 293TT or 293TTF. Particles purified from each preparation using Optiprep™ step gradients were morphologically indistinguishable when stained with uranyl acetate and viewed by transmission electron microscopy (Fig. 2A and B).

To examine HPV16 fcPsV functionally, we tested whether fcPsV produced from 293TTF could bypass the requirement for furin and thus infect furin-deficient cell lines such as FD11 (Chinese hamster ovary-CHO cells with furin gene knocked out) (Gordon et al., 1995) and LoVo (a human colon adenocarcinoma line) (Drewinko et al., 1976). In FD11 cells, the infectivity of HPV16 fcPsV was 2 logs higher than HPV16 PsV (Fig. 1B). Importantly, the infectivity of HPV16 PsV was restored to levels similar to that of fcPsV in FD11-F cells which are FD11 cells re-complemented for the wild type furin