Published in final edited form as:

Future Microbiol. 2009 December ; 4: 1255–1262. doi:10.2217/fmb.09.86.

The role of furin in papillomavirus infection

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Abstract

Papillomaviruses represent a medically important virus family. Infection with a high-risk human papillomavirus type is a prerequisite for cervical carcinoma development. Infection by low-risk types may result in the generation of benign skin warts. It was recently found that infectious entry of these viruses is dependent upon a specific proteolytic event that occurs prior to viral endocytosis. Specifically, a proprotein convertase, furin or proprotein convertase 5/6, must cleave the minor capsid protein for infection to proceed. Here, an overview of what is currently known about this process is presented, and what we have learned about the papillomavirus lifecycle from these studies discussed. This work also has implications for further advances in papillomavirus vaccine development.

Keywords

basement membrane; extracellular matrix; furin; heparan sulfate proteoglycan; papillomavirus; proprotein convertase

Papillomaviruses (PVs) comprise a family of nonenveloped icosahedral viruses that include over 100 human PV (HPV) types [1]. PVs replicate exclusively in the stratified epithelial keratinocytes of skin and mucosa. A 'high-risk' subset has been shown to cause cervical cancer and contribute to several other carcinomas, including penile, anal and oropharyngeal [2]. Other HPV types cause skin or genital warts. The viral capsids are composed of only two proteins; 72 pentamers of the major capsid protein, L1, and up to 72 copies of the minor capsid protein, L2 [3]. The L1 protein has the ability to spontaneously self-assemble in the absence of L2. This yields symmetrical but noninfectious particles known as virus-like particles (VLPs) [4]. These VLPs are the basis for the current prophylactic HPV vaccines. L1-only VLPs bind and enter cultured epithelial cells in a manner that is indistinguishable from infectious L1- and L2-containing virions [5]. Initial binding in both cases is predominantly to cell-surface heparan sulfate proteoglycans (HSPGs) [6,7]. Despite the minor structural role of L2, it is essential for infectivity and has been shown to play a number of indispensable roles during later stages of the infectious process (reviewed in [8]).

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Financial & competing interests disclosure

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Our laboratory recently discovered that a broadly active proprotein convertase (PC) inhibitor (decanoyl-RVKKR-cmk) effectively blocks PV infection [9]. PCs are a seven-member family of cellular endoproteases that activate proproteins by cleavage at a motif typified by basic motifs. The consensus cleavage motif for furin, the prototype PC, is R-X-K/R-R. Various cellular substrates rely upon activation by PCs including neuropeptides, peptide hormones, growth and differentiation factors, receptors, enzymes and adhesion molecules (reviewed in [10,11]). Infectious agents also utilize PC cleavage as a requisite step of their infectious cycle. Numerous bacterial toxins are activated by PC cleavage on the cell surface, as occurs for anthrax toxin, or during their endocytic entry, as occurs for *Pseudomonas* exotoxin. Cleavage of some viral envelope proteins, including those of avian influenza virus, HIV-1 and measles virus, is required for infectious virion assembly, with cleavage yielding the mature, fusogenic form prior to insertion into the cell membrane and viral exocytosis (reviewed in [12]). By contrast, PCs are required during PV infection, but not for assembly of infectious PVs. This represents the first, and so far only, example in which activation by a PC is inextricably linked to the infectious entry process of a virus. However, it has been demonstrated that Semliki Forest virus can be activated by furin on the cell surface during entry, if its normal cleavage during virus production is prevented [13]. Furin cleavage during infection may also occur to a minor extent during dengue virus entry [14].

To determine whether, in the case of PV, the virion or a cellular protein was the essential target of PC cleavage, we examined the PV capsid sequences for consensus furin cleavage sites. This revealed that a remarkably conserved multibasic motif characteristic of the consensus furin site close to the amino terminus of L2 was present in all PV sequences described in the GenBank database (subset shown in Table 1). By contrast, L1 has no conserved consensus cleavage site. We confirmed that the amino terminus of L2 could be cleaved by furin *in vitro*, and that all PV types for which a quantitative infectivity assay was available (described later) were sensitive to PC inhibition in cultured cells.

Owing to the extreme reliance upon terminal keratinocyte differentiation for PV production, authentic virions cannot be generated in replicating cultured cells. However, a system exists that can efficiently produce infectious pseudovirions. In the pseudovirus production system, a plasmid encoding the two capsid proteins is cotransfected with a plasmid that encodes a quantifiable marker of infection, typically green fluorescent protein, secreted alkaline phosphatase or luciferase [15]. The capsid proteins assemble in the nucleus and encapsidate the circular marker plasmid. The resultant pseudovirions are structurally indistinguishable from authentic virions and deliver the marker plasmid, permitting a straightforward assessment of the efficiency of infection.

When pseudoviruses are initially extracted from the producer cells, they are in an immature form. These capsids are infectious, but have a more open conformation. Similarly to mature pseudovirus, infection is dependent upon cellular furin. When exposed to oxidizing conditions, they undergo a maturation process in which inter-L1 disulfide bond formation is associated with compaction and stabilization of the capsid. Fully mature capsids exhibit improved regularity and resistance to proteolytic digestion [16]. In solution, the amino terminus of L2 of immature, but not mature, virions is accessible to furin cleavage [9]. However, we found that a conformational change occurring after cell-surface HSPG binding exposes the L2 amino terminus of mature virions to furin cleavage [17]. Furin cleavage, in turn, was shown to expose broadly cross-neutralizing epitopes on L2 that lie immediately downstream of the furin recognition site. Therefore, the conformational change in the viral capsid on the cell surface and furin cleavage can be monitored by the binding of L2 cross-neutralizing antibodies [17]. These cell-surface events are illustrated in Figure 1a.

We found that addition of furin to HPV16 pseudovirions during the maturation process allowed us to obtain particles in which the mature capsids contained L2 with proteolytically processed N-termini. These furin precleaved (FPC) pseudovirions were infectious in cells lacking furin or in the presence of the PC inhibitor [18]. This finding established that L2 cleavage is the only furin-dependent activity required for pseudovirus infection. Unexpectedly, infection of FPC pseudovirions was not dependent upon cell-surface HSPG molecules (Figure 1b). Furthermore, it was possible to visualize FPC capsids bound to the surface of HSPG-deficient cells. Although previous studies had suggested the existence of an additional non-HSPG cell-surface receptor [7,17,19], this provided the first direct evidence for such a moiety. These findings led us to postulate that the initial interaction of the capsid with HSPG primarily functions to facilitate furin cleavage on the cell surface and, following this reaction, a secondary receptor is engaged. However, previous data have shown that L1-only and L1/L2 viral particles have indistinguishable cell-binding and internalization characteristics. Therefore, we concluded that L1-only VLPs must be in a conformation similar to FPC virions, which would have a hybrid character in that they are mature, but have undergone furin cleavage of L2. This was confirmed by data showing that L1-only particles can also bind to cells that lack HSPG [DAY PM, UNPUBLISHED DATA]. Therefore, although furin cleavage exposes a conserved critical region of L2 at the time of the capsid's residence on the cell surface, our model suggests that this site does not functionally participate in binding the secondary cell-surface receptor involved in virion internalization. Rather, furin cleavage of L2 leads to exposure of a cell-surface receptor binding site on L1.

Analysis of point mutants in the furin recognition sequence in the *L2* gene revealed a critical downstream L2 function in infection. The pseudoviruses incorporating the furin-recognition defective L2s contained wild-type amounts of L2 and the encapsidated marker plasmid, but were noninfectious. The mutant capsids had no gross changes in cell-surface binding, entry or initial intracellular trafficking kinetics [9]. This would not be predicted if cleavage of L2 were required for exposure of the binding site for the secondary L1 receptor. However, these mutants, perhaps owing to interruption of charge interactions between the capsid proteins, expose the cross-neutralizing L2 epitopes even in the fully mature state [DAY PM, UNPUBLISHED DATA]. Thus, they may be able to bind the secondary L1 receptor owing to a structural similarity to FPC capsids, in that the N-terminus of L2 is in an extended conformation. Alternatively, these findings are consistent with a model in which the initial conformational change in L1 (in L2-containing capsids) induced by HSPG binding independently exposes the secondary receptor binding site and the L2 furin cleavage site. The latter hypothesis is consistent with the observation that, in the presence of a PC inhibitor, wild-type L1/L2 capsids are internalized to a Lamp1⁺ compartment similarly to untreated capsids. However, it is inconsistent with more recent *in vivo* results, which are described in a following section. Therefore, we favor the interpretation that, in the presence of the PC inhibitor, virions are endocytosed through an aberrant pathway via the cell-surface HSPGs. It is well described that HSPG can be internalized into the endosomal/lysosomal compartments [20,21]. Noninfectious uptake of PV complexed with HSPGs was described by Selinka *et al.* [22]. It is presently unclear if these observations are related.

Utilizing the point mutants, we found that furin cleavage was not necessary for uncoating of the viral capsid in the endosome, as BrdU-labeled pseudoviral genome was readily detected with BrdU-specific antibodies. Detection of the genome is utilized as a measure of capsid disassembly [5]. However, the genome and L2 were retained within the endosomal compartment. By contrast, the genome and L2 of the wild-type virus were detectable both in endosomes and the nucleus by 18–24 h postinfection. The ability to detect viral components in the nucleus is an indication that endosome escape must occur. It is unclear how the virus traverses the distance between the endosome and the nucleus. A similar retention was observed when the entry of wild-type virus was performed in the presence of a PC inhibitor. As

mentioned earlier, the infectious PV entry pathway traverses the endocytic pathway, and the vesicles containing uncoated capsids colocalized with Lamp-1, indicating localization in the late endosomal/lysosomal compartment. It is possible that aberrant HSPG-mediated internalization leads to nonproductive capsid degradation, which differs from programmed uncoating resulting from internalization via the legitimate secondary cell-surface receptor, despite leading to the same compartment. Alternatively, furin cleavage of L2 may be essential for escape from the endosome prior to the transit of the L2/genome complex into the nucleus. Interestingly, a C-terminal peptide of L2 required for infection has been shown to have intrinsic membrane-lysing activity and, in the context of L1/L2 capsids, is required for endosomal escape of the viral genome [23]. It is possible that furin cleavage of the N-terminus of L2 is mechanistically tied to exposure of this carboxyl region. The N-terminus of L2 proteins is highly conserved among types, possibly owing to a constraint to maintain an interaction with an intracellular receptor or chaperone necessary for endosomal escape or the subsequent trafficking into the nucleus. L2 contains two defined nuclear localization sequences, one that encompasses the furin cleavage site and a second at the carboxyl terminus [24]. Therefore, it is likely that only the latter sequence would participate in nuclear entry during this stage of the lifecycle.

A cervicovaginal murine model of PV infection was recently developed by our laboratory [25]. This model established that disruption of the epithelium and exposure of the underlying basement membrane (BM) are critical for infection. Initially, the pseudovirions bind almost exclusively to the BM and are then transferred to the epithelial cells that migrate over the wounded area. We are utilizing this model to examine host requirements for *in vivo* infection. We have found that *in vivo* infection requires attachment to HSPG moieties on the BM. Both heparin pretreatment of HPV16 pseudovirions and *in vivo* heparinase treatment of the genital tract prior to pseudovirus instillation abrogated BM binding and subsequent infection [26]. We have found that, similar to findings in cultured cells, FPC pseudovirions can bind efficiently to the epithelial cell surface after heparinase treatment, confirming the HSPG independence of epithelial-cell binding and infection *in vivo* [27].

We are currently investigating the requirement for furin cleavage in this *in vivo* model. The BM-bound virions expose the L2 cross-neutralizing epitopes, indicating that *in vivo* the initial conformational change and furin cleavage can take place at this site. We have also found that *in vivo* treatment with the PC inhibitor decanoyl-RVKR-cmk prior to virus instillation significantly inhibits *in vivo* infection [27]. In the presence of the PC inhibitor, the virus is initially found on the BM as usual, but is progressively lost over a time course that mirrors the exposure of the L2 neutralization epitope. The virus is not found associated with the epithelial cell surface. This feature of the *in vivo* pathway is inconsistent with the idea presented earlier that the secondary cell receptor can be engaged under conditions of PC inhibition in cultured cells. However, it is consistent with the model that aberrant endocytosis occurs via cell-surface HSPG in cultured cells, as the virion/cell surface interaction is not HSPG dependent *in vivo*. What is clear, to the extent determined so far, is that furin cleavage appears to be a requirement for PV infection *in vivo* in the murine system and also appears to be necessary for the transfer of virions to an epithelial cellular receptor. We postulate that the conformational change induced by HSPG binding reduces the affinity of the capsid for HSPGs. Since the capsids cannot bind the keratinocytes without L2 cleavage, reduced BM binding could explain the loss of the capsids from the tissue in the presence of the PC inhibitor.

We have examined the *in vivo* expression pattern of furin and found it to be localized throughout the epithelial layers above the BM [27]. Interestingly, furin levels appear to be higher at sites of trauma, especially in the basal cell layer.

We have also examined the localization of PC5/6, the only other known PC that could not be eliminated as playing a role in L2 cleavage from our *in vitro* studies. PC5/6 was also detected throughout all the epithelial layers, but additionally is found associated with the BM. This is intriguing given the exposure of the L2 cross-neutralizing epitopes at this site. Interestingly, there are increasing data on the PC cleavage of HSPG ligands, indicating a possible enrichment of convertases in the vicinity of the cellular glycocalyx [10,28-30]. Therefore, it is possible that interaction with HSPGs, in addition to inducing a conformational change in the virion, may serve to enhance PC cleavage of L2 by increasing the local concentration of the two reactants. It may be difficult to dissect the precise *in vivo* roles of furin versus PC5/6 as knockout mice for either gene have an embryonic-lethal phenotype (reviewed in [31]). Our model of *in vivo* infectious events is illustrated in Figure 2. A summary of the binding differences of the various pseudovirions' capsid preparations *in vivo* and in cultured cells is shown in Figure 3.

In summary, furin activation of the PV virion, through cleavage of L2, appears to be necessary for establishment of infection both *in vivo* and in cultured cells. Our results suggest that attachment to HSPG moieties primarily functions to facilitate the cell-surface cleavage of L2 by furin by enabling exposure of the furin cleavage site. Although the cleavage event occurs on the BM (*in vivo*) or cell surface (cultured cells), the infection deficit is not evident in cultured cells until the virus has been endocytosed and reached the late endosome. *In vivo* the deficit is detected earlier, since there is no keratinocyte binding. It is likely that cleaved L2 normally interacts with a specific intracellular receptor that is critical for the completion of endosome escape and/or nuclear entry. Interestingly, the binding site of one putative intracellular protein receptor, syntaxin 18, is immediately downstream of the cross-neutralizing 17–36 peptide [32]. However, there are currently no mechanistic insights into how removal of the N-terminus of L2 facilitates escape from the endosome. It is also unclear what percentage of L2 is cleaved during infection. Analysis of amino terminally tagged L2 incorporated into virions demonstrated that uncleaved L2 could be detected in the endosomes during productive infection, although no uncleaved L2 was found within the nucleus [9]. In addition, it was found that only 35% of the L2 within the FPC pseudovirus preparation is actually cleaved [18]. This level of cleavage was sufficient for furin-independent infection. However, in these experiments it is not clear if uncleaved L2 is distributed randomly among the pseudovirus population, or if some virions contain completely cleaved L2 and another population contains completely uncleaved.

It may be possible to further dissect what host factors, in addition to HSPG, govern the initial cell-surface events that lead to furin cleavage. Recent publications point to early signaling events in the PV infectious process prior to virus internalization [33]. It will be interesting to determine if these phenomena are mechanistically linked with the early conformational changes that are intertwined with furin cleavage. Furthermore, cysteine residues that are found within the L2 cross-neutralization epitope were shown to be disulfide linked and crucial for infection [34]. It is unclear presently if the reduction of these bonds during entry occurs prior to, or is dependent upon, furin cleavage.

It has also recently come to light that cyclophilin isomerases may mediate conformational changes in the PV capsid during entry, including exposure of the L2 major neutralization epitope. However, it has been found that FPC pseudovirus is equally susceptible to cyclophilin inhibition [BUCK C, PERS. COMM.]. Therefore, it is clear that cyclophilins play an important postfurin role in PV infection.

Papillomavirus capsids have also been shown to associate with tetraspanin-enriched microdomains on the cell surface [35]. This is intriguing as tetraspanins have been shown to interact with numerous cell-surface adhesion receptors. Additionally, signalling molecules and enzymes are also concentrated in these membrane microdomains (reviewed in [36]). However,

it is presently unclear how this association is related functionally or temporally to furin cleavage.

Why have papillomaviruses evolved the elaborate and highly conserved mechanism of undergoing a conformational change and furin cleavage of L2 after HSPG binding? We believe that the most likely explanation is that this mechanism ensures that engagement of the cell-surface receptor and subsequent *in vivo* infection occurs only in basal keratinocytes. Given the close coordination of the viral lifecycle with squamous epithelial differentiation, it is likely that only infection of basal cells leads to productive infection. Basal keratinocytes would contact the virions bound to the BM during their migration to fill the wounded area (reviewed in [37]). It has recently been shown that cell division is necessary for PV infection of cultured cells [38]. If this requirement is also true for *in vivo* infection, it would provide additional evidence that wound healing over the BM is tied to PV infection. In addition, migrating keratinocytes extend filopodia into the wound [39]. Interestingly, two recent studies have shown that filopodia formation facilitates PV uptake into cultured keratinocytes [33,40]. Binding or infection of suprabasal cells would not only be nonproductive, but could potentially promote immune responses that might eliminate infection prior to propagation and transmission of the virus. The binding of HSPGs on immortalized epithelial cell lines, and subsequent L2 cleavage by furin on the cell surface, is likely due to an adaptation during passage in culture that results in cell-surface HSPG modifications that resemble the pattern of those found *in vivo* on the BM.

Interestingly, the mechanism outlined previously assures the delayed exposure of the cross-neutralization L2 epitope, and so may prevent the induction of broadly cross-neutralizing antibodies. Evolution into multiple genotypes, which are distinct serotypes, is a characteristic feature of PVs [1,41]. Many of the more than 100 HPV genotypes/serotypes represent common human infections. This type of speciation/distribution would not be expected if infection by one genotype generated neutralizing antibodies that could prevent infection by other types. Consistent with this hypothesis, L1-only VLPs and L1/L2 capsids both induce type-restricted neutralizing antibody responses [42]. Cross-neutralization is only seen among very closely related types and only after exposure of apparently super-physiological amounts of capsids to the systemic immune system. Even under these conditions, the induction of cross-type neutralizing antibodies is equivalent between L1/L2 capsids and L1-only VLPs. Therefore, in their normal context with the virion, the highly conserved and potentially cross-neutralizing L2 epitopes are not well exposed to B cells. However, when removed from the virion context and injected in isolation, full-length L2 or, more specifically, the conserved sequences immediately downstream of the furin cleavage site induced antibody-mediated protection from challenge with the homologous type in a bovine model [43]. L2 immunogens from this region have more recently been shown to induce antibodies with *in vitro* neutralizing activity against a broad panel of different PV types, including phylogenetically distant mucosal and cutaneous types that infect divergent mammalian species [42,44-47]. These immunogens also effectively prevent infection by heterologous types in the mouse cervicovaginal challenge model and in a rabbit PV challenge model, indicating that broad cross-neutralization of antibody to L2 immunogens is not simply an *in vitro* phenomenon [48,49]. Thus, there is experimental evidence to support the conjecture that exposure of these potentially broadly cross-neutralizing L2 epitopes occurs only after HSPG binding and furin cleavage and, thus, as a secondary consequence, these epitopes have limited exposure to B cells. This has likely allowed the evolution of PVs into many distinct types. However, this characteristic may prove to be the Achilles' heel of these viruses, as delivery of this peptide to the systemic immune system out of its normal context in the viral capsid may be the basis of an exceptionally effective cross-protective vaccine. The preclinical studies outlined earlier support this conjecture. Hopefully efficacy in humans will soon be tested.

Future perspective

As more detailed structural analyses of the PV capsid are performed, we expect that a greater understanding of the conformational changes induced by furin cleavage will follow. We anticipate that further studies will elucidate how these changes prevent efficient endosome escape in cultured cells. This work may indicate essential binding partners for L2 in this process. We also hope to utilize the FPC pseudovirus as a tool for isolation of the epithelial cell receptor. The identification of this receptor has eluded PV researchers for many years.

Executive summary

- The activity of furin, or a related proprotein convertase (PC), is required for papillomavirus infection.
- Attachment of papillomaviruses to heparan sulfate proteoglycans causes exposure of a furin cleavage site at the N-terminus of L2.
- *In vivo*, the critical heparan sulfate proteoglycans are located on the basement membrane.
- Furin cleavage of L2 mediates a conformational change allowing L1 to engage a secondary cell receptor.
- *In vivo*, PC inhibition prevents capsid attachment to the epithelial cell surface.
- PC inhibition in cultured cells results in nonproductive endocytosis.

Acknowledgments

Our research was supported by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research.

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Figure 1. Interaction of untreated and furin precleaved pseudovirions with the cell surface

(A) Cell-surface events during papillomavirus entry into cultured cells. Mature pseudovirions composed of both capsid proteins initially bind to HSPGs on the cell surface (1). This binding leads to a conformational change that exposes the N-terminus of L2, allowing access to, and cleavage of, the furin recognition site (2). This cleavage further exposes L2, including the cross-neutralization epitope encompassed by amino acids 17–36 (3). This capsid conformation is able to interact with a non-HSPG cell-surface receptor (4). (B) Furin precleaved virus can bind directly to the secondary receptor. Furin precleaved (FPC) virus can bypass the necessity of HPSG interaction and interact directly with the non-HSPG secondary receptor. However, the FPC virus is still able to interact with HSPGs, presumably owing to its maturation state. The binding characteristics of L1 virus-like particles are indistinguishable from those of the FPC virus. HSPG: Heparan sulfate proteoglycan.

Figure 2. Model of *in vivo* papillomavirus infection

Untreated, wild-type pseudovirions interact initially with heparan sulfate proteoglycans on the exposed BM. This causes a conformational change that allows exposure of the furin and/or PC5/6 cleavage site. Following PC cleavage, the L2 cross-neutralization epitope is exposed. These conformational changes allow transfer to a non-heparan sulfate proteoglycan epithelial cell receptor. The basal epithelial cells migrate over the BM to fill the wound. It is likely that transfer to the cells occurs during this migration. This model is based on experimental results obtained in the murine infection system. It is unclear how closely this mimics infection in humans. BM: Basement membrane; HPV: Human papillomavirus; PC: Protease convertase.

		Cultured cells			<i>In vivo</i>		
		HSPG	17/36+	Cell receptor	BM HSPG	17/36+	Cell receptor
L1/L2 mature	+	–	–	+	–	–
L1/L2 (+ furin inhibitor) ⁺	+	–	?	Transient	–	–
L1/L2 postfurin	+	+	+	?	+	+
FPC	+	+	+	+	+	+
L1-only VLPs	+	NA	+	+	NA	+

Figure 3. Summary of characteristics of pseudovirions in cultured cells and *in vivo*

Column headings are defined as follows: HSPG: binding to cell-surface HSPGs; 17/36+: exposure of the broadly cross-neutralizing L2 epitope encompassed by amino acids 17–36; Cell receptor: binding to the epithelial cell-surface receptor; BM HSPG: binding to the BM HSPGs. BM: Basement membrane; FPC: Furin-precleaved pseudovirus; HSPG: Heparan sulfate proteoglycan; NA: Not applicable; VLP: Virus-like particle.

Table 1
Alignment of the furin cleavage sites in L2 N-terminal sequences from phylogenetically diverse papillomavirus types

Type	Sequence	Genus/species
BPV1	M S A R K R V K R A S A...	$\delta/4$
CRPV	M V A R S R K R R A A P...	κ
HPV5	M A R A K R V K R D S V...	$\beta/1$
HPV6	M A H S R A R R R K R A S A...	$\alpha/10$
HPV16	M R H K R S A K R T K R A S A...	$\alpha/9$
HPV18	M V S H R A A R R K R A S V...	$\alpha/7$
HPV31	M R H K R S A K R T K R A S A ...	$\alpha/9$
	P4 P1	

A consensus furin cleavage site was found in every papillomavirus type examined. Some representative types are shown. All of these types were also proven to be sensitive to furin inhibition in infectivity assays performed in cultured cells. The P1 and P4 residues are indicated according to the standard nomenclature.

BPV: Bovine papilloma virus; CRPV: Cottontail rabbit papillomavirus; HPV: Human papillomavirus.