

Human papillomavirus tropism

Determinants of viral tissue specificity

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Front cover: A BPV virion half bound by a neutralizing monoclonal antibody.
BPV virion made by Benes Trus, NIH. With permission from Schiller, J.
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Till mamma & pappa

Yada yada hi dharmasya
Glanir bhavati bhārata
Abhyutthanam adharmasya
Tadatmanam srijamy aham
paritrānaya sādhanam
vīnasaya ca duskṛtam
dharma-samsthāpanārthaya
sambhavami yuge yuge



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Abstract

Cervical cancer is the second most common cancer among women worldwide and human papillomavirus (HPV) is a prerequisite for the development of this cancer. HPV belongs to the *Papillomaviridae* family and infects the basal layer of epithelial cells where it generally progresses into warts or condylomas. HPV can only reproduce in differentiating epithelia and it is therefore difficult to study the natural infection of HPV. More than 100 HPV types exist and they are divided into different genera based on their L1 open reading frame sequence. Most of the HPV types in the alpha-papillomavirus genus infect the mucosal epithelium while HPVs from the beta-papillomavirus genus usually infect cutaneous epithelial cells. Presently, it is not known what decides the anatomical tropism and our aim was to study determinants of this tropism.

By using HPV virus like particles (VLP) and pseudovirus we found that VLPs from the two alpha-papillomaviruses HPV-6 and HPV-16 interacted with cell-surface heparan sulfate (HS) for initial attachment. When we labelled HPV VLPs with a fluorescent dye to study internalization HPV-6 was more strongly inhibited than HPV-16. Furthermore, a pseudovirus infection assay demonstrated that the beta-papillomavirus HPV-5 was less dependent on HS for infection than HPV-16. By analyzing the isoelectric point (pI) of the HPV L1 capsid protein we found that alpha HPV types were more positively charged than beta HPV types. Also, HPV-6 had a higher positive charge than HPV-16. Thus, the inhibition of the negatively charged heparin against HPV infection was clearly related to the charge of the HPV L1 capsid. This suggested that the initial interaction could be one of the determinants of tropism although not the sole factor.

Lactoferrin is a protein found in milk, saliva, semen, tear fluid and endocervical secretions that has antiviral activities. Both human and bovine lactoferrin inhibited HPV infection but we found no significant differences in inhibition of alpha- and beta-papillomavirus infection. We could however demonstrate that different lactoferricins, small peptide derivatives from the N-terminal part of lactoferrin, were able to inhibit HPV infection. This antiviral activity depended on lactoferricin peptide, HPV type and cell origin.

The regulation of HPV gene expression in the host cell could also determine HPV tropism. The HPV long control region (LCR) contains cis-responsive elements that regulate HPV transcription and the epithelial tropism of HPV is determined by epithelial specific constitutive enhancers in the LCR. It has been hypothesized that the combination of transcription factors in the host cell determines the cell-type-specific expression. In cells with a skin origin the HPV-5 LCR was twice as efficient in transcriptional activation compared to HPV-16 LCR, while in cervical cells the HPV-16 LCR was almost twice as effective in activating transcription compared to HPV-5 LCR.

To conclude, alpha- and beta-papillomaviruses differed regarding their ability to infect cells and regulate viral gene expression. These abilities corresponded with their natural host cells and suggested that HPV anatomical tropism could be determined at several steps in the HPV life cycle.

Abbreviations

bLf	Bovine lactoferrin
bLfcin	Bovine lactoferricin
BPV	Bovine papillomavirus
bp	Base pair
CDK	Cyclin-dependent kinases
CFDA-SE	Carboxy-fluorescein diacetate, succinimidyl ester
CRPV	Cottontail rabbit papillomavirus
DNA	Deoxyribonucleic acid
ECM	Extra cellular matrix
EGFR	Epidermal growth factor receptor
EV	Epidermodysplasia verruciformis
GAG	Glycosaminoglycan
HCV	Hepatitis C virus
hLf	Human lactoferrin
hLfcin	Human lactoferricin
HPV	Human papillomavirus
HSV	Herpes simplex virus
hTERT	Human telomerase reverse transcriptase
IC ₅₀	50% inhibitory concentration
kDa	Kilodalton
LCR	Long control region
Lf	Lactoferrin
Lfcin	Lactoferricin
NLS	Nuclear localization signal
ORF	Open reading frame
PCR	Polymerase chain reaction
pI	Isoelectric point
PsV	Pseudovirus
PV	Papillomavirus
Rb	Retinoblastoma protein
RNA	Ribonucleic acid
VLP	Virus-like particle

List of papers

Paper I

Carboxy-fluorescein diacetate, succinimidyl ester labeled papillomavirus virus-like particles fluoresce after internalization and interact with heparan sulfate for binding and entry.

Drobni P, Mistry N, McMillan N and Evander M. (2003) *Virology*, 310: 163-72

Paper II

Entry of human Papillomavirus type 5 is only partly dependent of a heparan sulfate receptor.

Mistry N, Drobni P, Wibom C and Evander M. *Manuscript*.

Paper III

The anti-papillomavirus activity of human and bovine lactoferricin.

Mistry N, Drobni P, Näslund J, Sunkari V.G., Jenssen H and Evander M. In press.

Antivir. Res. (2007) doi: 10.1016/j.antiviral.2007.03.012

Paper IV

Transcriptional activation of the human papillomavirus type 5 and 16 long control region in cells from cutaneous and mucosal origin.

Mistry N, Simonsson M and Evander M. (2007) *Virology Journal*, 4:27

Summary of papers

Paper I

Carboxy-fluorescein diacetate, succinimidyl ester labeled papillomavirus virus-like particles fluoresce after internalization and interact with heparan sulfate for binding and entry. Drobni P, Mistry N, McMillan N and Evander M. (2003) *Virology*, 310: 163-72

In this paper we studied the binding and internalization of two alpha human papillomaviruses with predominantly mucosal tropism (HPVs). We pretreated HPV virus-like particles (VLPs) with several different types of glycosaminoglycans (GAGs) and added them to cells. We found that heparin caused a dose-dependent inhibition of HPV binding and was the most effective GAG tested. Heparan sulfate from a mucosal source also reduced binding of HPV-16 at low concentrations. Additionally, we showed that cell lines lacking GAGs were incapable of binding HPV-16 VLPs. Pretreating cells with heparinase III to eliminate heparan sulfate from the cell surface reduced attachment of HPV-16. By labelling our HPV VLPs with carboxy-fluorescein diacetate, succinimidyl ester (CFDA-SE) we analyzed HPV internalization. We could demonstrate dose-dependent heparin inhibition of HPV-6 and HPV-16 internalization. This suggested a role for heparan sulfate as an attachment receptor for HPV-6 and -16.

Paper II

Entry of human Papillomavirus type 5 is only partly dependent of a heparan sulfate receptor. Mistry N, Drobni P, Wibom C and Evander M. *Manuscript*.

By calculating the pI of the L1 capsid protein we showed that almost all alpha HPVs had a positive charge at physiological pH, while beta HPVs with predominantly cutaneous

tropism had a negative charge at the same pH. This suggested that attachment to the negatively charged heparan sulfate might be charge dependent. To compare if the infection of the beta HPV-5 and the alpha HPV-16 differed in their use of heparan sulfate as an attachment receptor we incubated HPV pseudovirus (PsV) with different concentrations of heparin together with cells from two different origins. We showed that infection of HPV-16 PsV was inhibited by heparin in both skin and mucosal cell lines in a dose-dependent manner. HPV-5 was only mildly inhibited by heparin in cutaneous cells and no inhibition was detected in mucosal cell lines, suggesting that HPV-5 was less dependent of heparan sulfate for infection than HPV-16. Infection of both HPV-5 and HPV-16 was blocked by an inhibitor of clathrin dependent endocytosis, chlorpromazine, implying that they used the same endocytotic pathway for internalization.

Paper III

The anti-papillomavirus activity of human and bovine lactoferricin.

Mistry N, Drobni P, Näslund J, Sunkari V.G., Jenssen H and Evander M. In press.

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Lactoferrin (Lf) and lactoferricin (Lfcin) are found in body secretions and they show excellent antiviral activities against many viruses. Here we added Lf and Lfcin to cells lines from different origin followed by incubation with beta HPV-5 and alpha HPV-16 PsV. Both bovine (bLf) and human lactoferrin (hLf) showed antiviral properties and inhibited HPV-5 and HPV-16 infection. Lfcin also demonstrated potent antiviral activities but here the effect differed depending on cell type, Lfcin and HPV type. We could also detect a weak binding of Lf and Lfcin to HPV-16 VLPs. However, western blot analysis demonstrated that Lf did not have any protease activity on the HPV-16 capsid. We now know more about the Lfcin *in vitro* effects on HPV infection but further studies are needed to analyze the *in vivo* effects of the peptide and the potential synergy effect of Lf or Lfcin with treatments currently in use today.

Paper IV

Transcriptional activation of the human papillomavirus type 5 and 16 long control region in cells from cutaneous and mucosal origin. Mistry N, Simonsson M and Evander M. (2007) *Virology Journal*, 4:27

The genome of HPV contains a long control region (LCR) that includes cis-responsive elements, promoters and replication origin. The cis-responsive elements are epithelial specific enhancers that regulate the transcription of HPV. By inserting the complete LCR into a reporter plasmid we analyzed the transcriptional activity of LCR in different cell lines. We found that the LCR of the alpha HPV-16 was more active in a mucosal cell line than the beta HPV-5. We could also detect that the LCR of HPV-5 was a better transcriptional activator in cell lines with cutaneous origin compared to HPV-16 LCR. This suggested that transcriptional activation could be important for determining HPV tropism.

Aim of this thesis

HPV is a causative agent for warts, condylomas, recurrent respiratory papillomatosis and cervical cancer. HPV infects both cutaneous and mucosal epithelial cells but not much is known regarding the mucosal versus cutaneous tissue tropism. The attachment and internalization steps are tropism determinants for several viruses. Our aim of this thesis was to investigate determinants of HPV tropism by studying HPV attachment to the cell surface, HPV infection and HPV transcriptional regulation.

Populärvetenskaplig sammanfattning på svenska

Livmoderhalscancer är idag den näst vanligaste cancerformen hos kvinnor i världen och man hittar närmare 500 000 nya fall varje år. Man vet idag att humana papillomvirus (HPV) är orsaken till livmoderhalscancer. Det finns en mängd olika typer av HPV och de orsakar vanligen vårtor eller kondylom, men vissa HPV-typer har förmågan att utveckla livmoderhalscancer. HPV kan infektera celler i både hud och slemhinnor beroende på vilken HPV-typ det är och i nuläget vet man inte hur detta styrs. Vi har i våra studier kunnat visa på intressanta skillnader mellan olika HPV-typer.

För att HPV ska kunna infektera sin värdcell så måste den först binda till den cellen. Genom vår forskning har vi kunnat visa att HPV-typer som infekterar slemhinna binder till en minusladdad sockerstruktur på cellens yta, medan HPV-typer som infekterar huden binder sämre till samma sockerstruktur. När HPV väl fäst och tagit sig in i en cell så måste den föröka sig för att sedan kunna infektera fler celler. Förökningen av HPV regleras via dess arvs massa och den kan vara mer aktiv beroende på vilken celltyp det är. Våra studier visar att arvs massan från HPV-typer som infekterar hudceller var mer aktiva i hudceller, och HPV-typer som infekterar slemhinnan var istället mer aktiva i slemhinne celler.

Vi har också försökt att blockera HPV infektionen med hjälp av lactoferrin, ett protein som ingår i vårt medfödda immunförsvar och finns naturligt i kroppen. Våra resultat visar att HPV infektionen minskar betydligt i närvaro av lactoferrin. Vi studerade också ett fragment av lactoferrin som kallas lactoferricin. Vi har för första gången kunnat påvisa att detta proteinfragment blockerade HPV infektion. Effekten mot HPV infektion varierade beroende på celltyp, HPV-typ och strukturen på olika lactoferricin.

Eftersom det finns skillnader mellan olika HPV-typer kan det vara svårt att framställa ett bra botemedel mot HPV. Det är därför viktigt att studera hur olika HPV-typer infekterar celler. Med våra studier har vi kunnat se att en del av skillnaden i infektionen ligger i bindning av virus till den cell den infekterar och hur reglering av arvs massan sker.

Introduction

History

In 1884, Licht reported that a wart, removed from his brother, could be transmitted and grow into warts on his skin (Licht, 1894). Ciuffo later demonstrated that warts could be transmitted between humans from cell free extracts (Ciuffo, 1907). In 1933, Shope discovered that a virus was the causative agent of benign papillomas on cottontail rabbits (Shope, 1933), and that cottontail rabbit papillomavirus (CRPV) had a tropism for cutaneous epithelium. Papillomavirus (PV) was later associated with cancer when CRPV lesions on cottontail rabbits was discovered to be of a tumorous nature (Rous, 1934; Syverton, 1935). It was long believed that human lesions associated with human papillomavirus (HPV) were caused by the same HPV type and virus particles were visualized by electron microscopy (Almeida et al., 1962). In the 1970's, when essential molecular procedures became known, the variability and plurality of PV was discovered. Several different HPV DNA was then found in lesions from both cutaneous and mucosal epithelium (Favre et al., 1975; Gissmann and zur Hausen, 1980; Orth et al., 1977). The DNA of HPV-1 was found in plantar wart (Favre et al., 1975) and HPV-6 was found in genital warts (condylomata acuminata)(Gissmann and zur Hausen, 1980). It was first suggested that HPV could contribute to tumour development in a study of HPV-5 infected patients that displayed a high susceptibility to cutaneous cancer (Lutzner et al., 1980). Meisels and coworkers were the first to associate HPV and cervical carcinoma by cytological and histological examinations (Meisels and Fortin, 1976; Meisels et al., 1977). Detection of HPV DNA in low grade and high grade squamous and glandar intraepithelial lesions of cervix showed a relation between HPV and cervical carcinoma (zur Hausen, 1976). In 1983 the first HPV, HPV-16, was cloned from an invasive cervical cancer (Durst et al., 1983) and later it was shown that over 90% of cervical cancers worldwide contained HPV DNA. Today it is clear that infection with certain HPVs is the main etiological agent responsible for development of cervical cancer (Bosch et al., 1995; Walboomers et al., 1999).

Recently, a vaccine against infection with two of the most common HPV types causing cervical cancer has been approved (Bryan, 2007).

Taxonomy and classification

Papillomavirus was previously grouped together with polyomavirus and simian vacuolating virus (SV40) into a family named *papovaviridae* because of the similarity of their icosahedral capsids and circular double-stranded DNA genomes. When molecular cloning came into use investigators found some major differences between these viruses. Polyomavirus and papillomavirus differed in the size of their capsid and genome. Also, their genomes had diverse amounts of open reading frames (ORF) and differed in organization. This divided *papovaviridae* into *papillomaviridae* and *polyomaviridae* according to the International Committee on the Taxonomy of Viruses (ICTV). Through the years papillomavirus has been found in many mammals and based on the variability of one HPV type found in humans from different continents a coevolutionary and prehistoric spread of the virus together with mankind was suggested (Ho et al., 1993). Up to now there have been over 100 reported HPVs, and they have a strict host and tissue specificity. PVs that are isolated and completely sequenced are grouped according to differences in ORF of the highly conserved L1 major capsid protein. L1 sequences that share less than 60% nucleotide sequence identity are divided into different genus. The PV types within a species share between 71% and 89% nucleotide identity within the complete L1 ORF (Fig. 1) (de Villiers et al., 2004).

Tropism

The many PV types identified can roughly be divided into two groups of tropism, those infecting cutaneous or mucosal epithelium. PV types specified to infect cutaneous epithelium are rarely detected in mucosal epithelial cells and those PV types that infect mucosal epithelium are infrequently detected in cutaneous cells. The main genera of

papillomaviridae are the alpha and beta PV since they include most of the identified PVs. The largest genus is alpha papillomavirus and it contains HPV types associated with mucosal lesions. HPV types in the beta genus are generally associated with cutaneous lesions. However there are exceptions, e.g. HPV species 4 within the alpha genus is frequently found in cutaneous lesions similar to those caused by HPV-4 in the gamma papillomavirus genus and therefore terms like mucosal or cutaneous HPVs are now replaced by genus and species (Bernard, 2005; de Villiers et al., 2004).

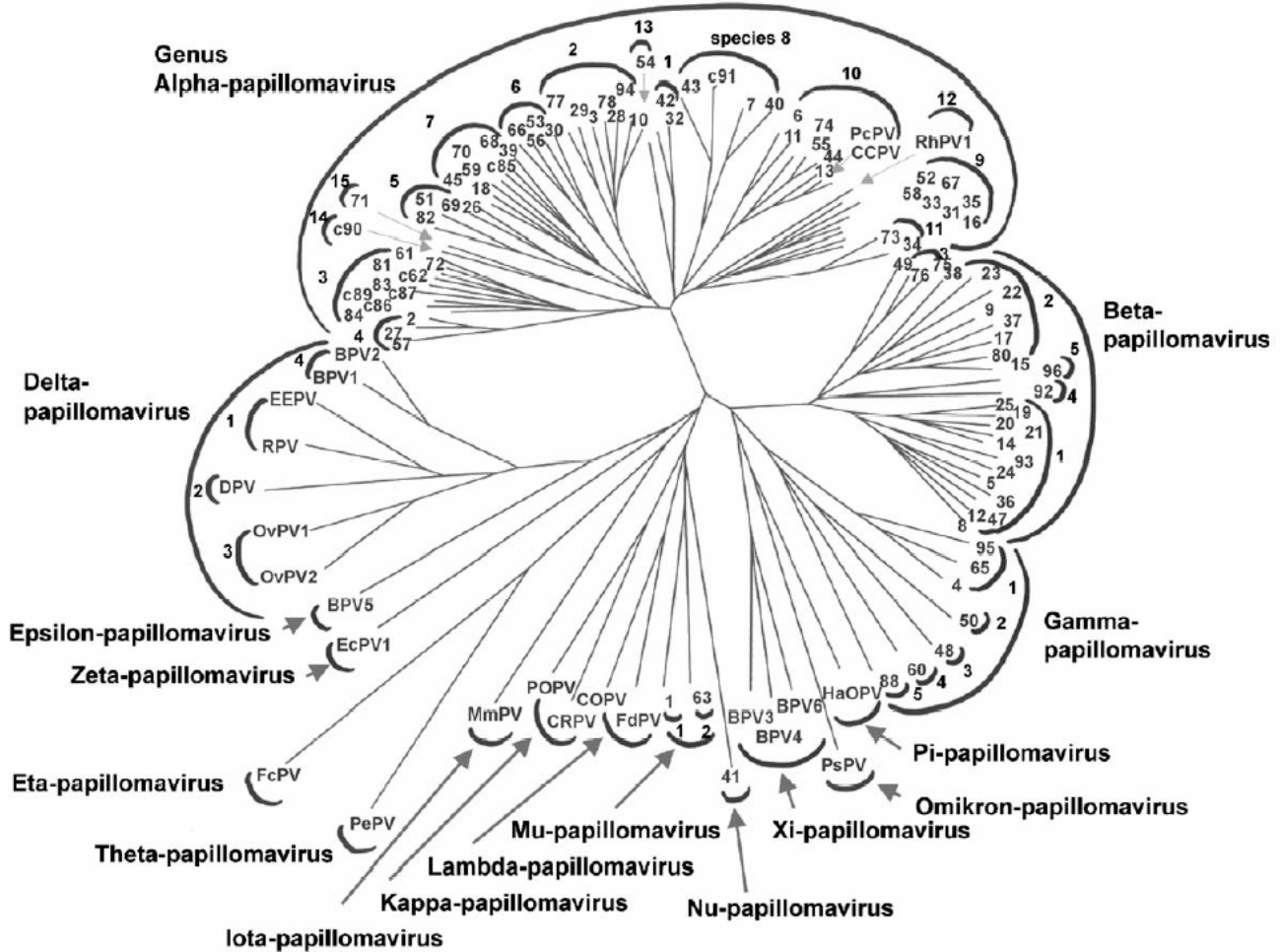


Fig. 1. Phylogenetic tree based on L1 ORF sequence from 118 papillomavirus. From de Villiers 2004 with permission.

Disease

To initiate an infection PV needs to reach and enter the dividing cells at the basal layer of the epithelium. A broad range of different HPV types are found in healthy skin of humans (Antonsson et al., 2003; Hazard et al., 2007). Infection of cutaneous epithelium by the PV in the beta papillomavirus genus and some alpha papillomavirus such as HPV-2,-3 and -10 could progress into skin warts. Skin warts are a benign proliferation of epithelial keratinocytes and they are often found on hands and feet, but infection can take place anywhere on the skin. Infections are generally self-limited and regress within 1-2 years and they are transmitted by skin to skin contact as well as through highly contaminated areas such as swimming pools (Jablonska et al., 1997). Some HPV types are also associated with individuals suffering of *epidermodysplasia verruciformis* (EV). EV is a rare cutaneous disease that is hereditary and persists through life. The individuals with EV are susceptible to persistent HPV infection because of a deficiency in cutaneous immunity (Orth, 2006). Benign lesions appear as plane warts or reddish or brownish plaques and can converse to malignances (Majewski et al., 1997). Among the HPVs that are related to EV patients the beta PV HPV-5 and HPV-8 have been associated with invasive tumours (Jablonska and Majewski, 1994; Majewski et al., 1997).

PVs infecting the basal layer of genital epithelium often results in condyloma. Condylomas are generally described as painless, flesh-coloured or greyish-white warts on the vulva, penis or anus. The infection is transmitted through sexual contacts. Condylomas are caused by PV in the alpha-papillomavirus genus. HPV-6 and -11 are the most frequent found types in genital warts (condylomas) and since they are not associated with genital cancer, they are designated as low-risk types. Similar to skin warts, condylomas are self-limiting and regress spontaneously but treatments may be required. Although destructive therapy is often used to treat these lesions, two topical treatments can be used for the clearance of genital warts, imiquimod and podophyllotoxin. For imiquimod the clearance rate is 50 % for women and recurrences occur in up to 20% of patients. Podophyllotoxin, a caustic agent, is also used to treat warts caused by HPV and the short-term clearance rate for podophyllotoxin is 60-80%, but unfortunately 40-60% of

recurrences are reported (Scheinfeld and Lehman, 2006; Snoeck, 2006) . HPV-6 and -11 are also found in juvenile recurrent respiratory papillomatis. This is a rare disease were wart-like growth occurs in the aerodigestive tract (Stamataki et al., 2007). The high-risk HPVs develop flat condylomas and they are necessary agent for the development of cervical cancer. There are approximately fifteen HPV types that are associated with cervical cancer, and HPV-16 is the high-risk type that is most prevalent in cervical cancers (Munoz et al., 2003; Walboomers et al., 1999; Wiley and Masongsong, 2006). Cervical cancer is globally the second leading cause of cancer among women with almost 500.000 new cases in year 2000 (Parkin, 2001).

Biology

The viral genome

Inside the HPV capsid there is a circular, double stranded DNA of approximately 8000 base pairs (bp) that is histone-associated (Fig. 2). Generally, there are six early genes, two late genes and one non coding region also known as long control region (LCR). Early genes encode proteins that are involved in viral replication, transcription and cell transformation. Two late genes, L1 and L2, encode viral capsid proteins. The LCR, located between L1 gene and E6 gene (Fig. 2) include several binding sites for transcription factors, viral proteins E1, E2 and contains the origin of DNA replication (Desaintes and Demeret, 1996).

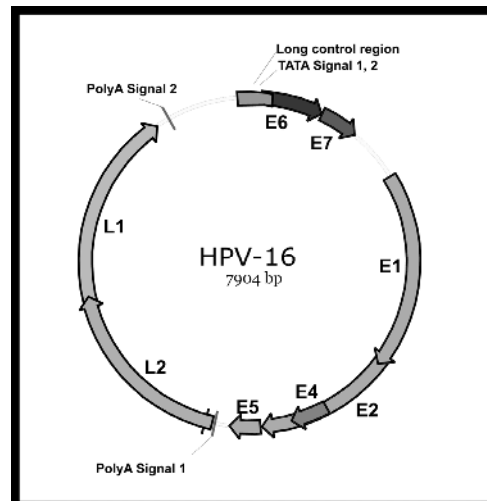


Fig.2. Genome organization of HPV-16.

Transcription and replication

Once HPV has entered the cell, the viral DNA is transported to the nucleus. The transcription of HPV DNA is an intricate process where several epithelial specific enhancers, alternative splicing and RNA processing is involved. The location of transcription initiation is found in the LCR of all PV DNA. LCR is 400-850 bp long and within this region there are binding sites for transcription factors, polyadenylation sites for late proteins, epithelial specific enhancers, several E2 binding sites and promoters (Fig. 3) (O'Conner et al., 1995). The number of promoters varies depending on PV type, but all HPV appear to have a promoter in front of the E6 gene in common (Fig. 3) (Stubenrauch et al., 1992; Thierry et al., 1987). For alpha HPV types, transcription starts with the interaction of transcription factors with the TATA-box and the SP1 binding site on the E6 promotor (Apt et al., 1996). Further, epithelial specific enhancers located on the central part of LCR bind to transcription factors and promote the poorly activated E6 promoter (Cripe et al., 1987). Depending on the PV type these enhancers differ in quantity, arrangement and type (Fig. 3). The nature of the epithelial cells could decide how much the E6 promoter is activated by enhancers (Sailaja et al., 1999). A synergism between several epithelial specific enhancers may contribute to the regulation of HPV transcription (Bernard, 2002). Among the many epithelial specific enhancers, AP-1 seems to play an important role for HPV transcription in epithelial cells (Prusty and Das, 2005).

E2 is a virally encoded protein and it can regulate the transcription from adjacent promoters by interacting with transcription factors like the TATA-box binding protein and SP1 (Dong et al., 1994; Lewis et al., 1999). The E2 protein recognizes a palindrome sequence positioned at various sites in the LCR and two E2 binding sites are noticed adjacent to the E6 promoter in many HPV-types (fig. 3). It is most likely that in the early phase of viral life cycle there are only low levels of E2 expressed boosting the transactivation of transcription, while in the late phase high levels of E2 repress transcription by blocking transcription factors from binding to its promoter (Bouvard et

al., 1994). The transcription generates a polycistronic mRNA that codes for several different viral proteins through the infection (Hummel et al., 1995).

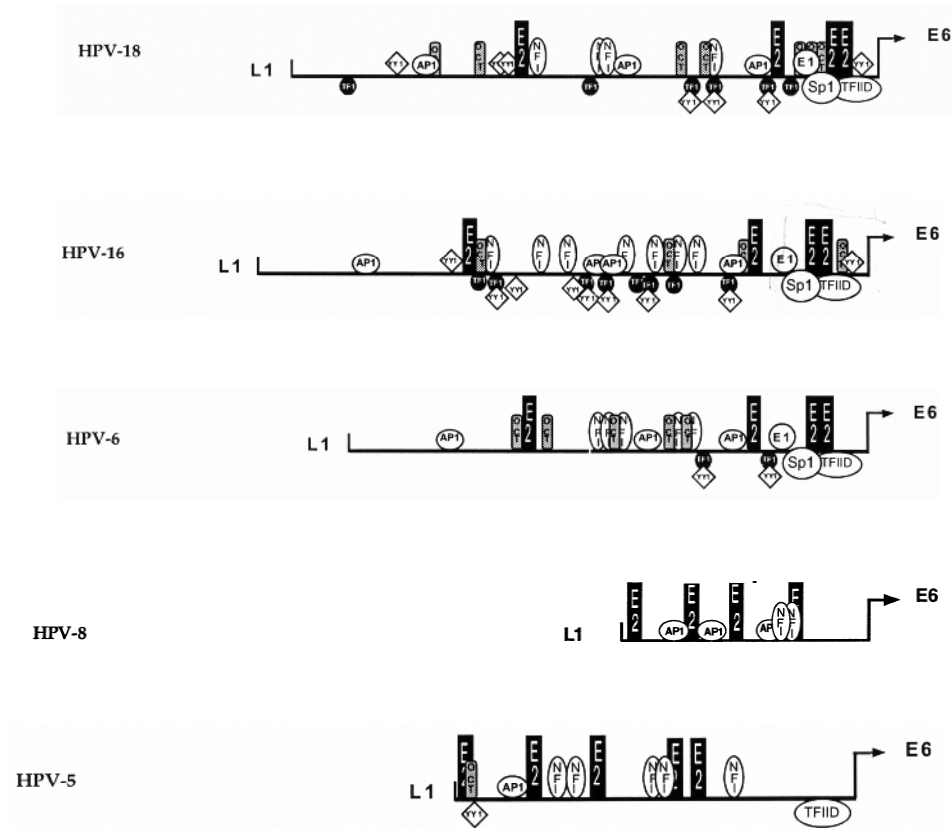


Fig 3. Arrangement of enhancing factors in the LCR of alpha (HPV-18, -16 and -6) and beta (HPV-8 and -5) papillomavirus. From Sailaja et al 1999, with permission.

The LCR of HPV is also required for replication of HPV. For efficient replication of HPV DNA the viral protein E1 and E2 must cooperate (Sverdrup and Khan, 1994). Near the E2 binding site within the LCR of alpha HPVs there is an E1 binding site, the replication ori (Fig. 3). The E2 protein binds to E1 and can recruit E1 to the origin of replication. An E2 mutant that lack the ability to bind to LCR do not replicate since the attachment of E1 alone to LCR is too weak (Liu et al., 1995; Russell and Botchan, 1995;

Ustav and Stenlund, 1991). When bound to ori, the E1 helicase uncoil the viral DNA and recruit cellular replication proteins including DNA polymerase α /primase and RPA (single-stranded DNA binding protein, replication protein A) (Amin et al., 2000; Loo and Melendy, 2004). The nuclear compartment for replication is suggested to be near ND10 (nuclear domain 10). Using immunofluorescence E1 and E2 of HPV-11 and cellular RPA were located near ND10 compartments in the nucleus (Swindle et al., 1999).

The viral proteins

The structure proteins L1 and L2

The PV DNA is surrounded by a capsid, which consists of two proteins, the major capsid protein L1 and the minor capsid protein L2. The L1 proteins are highly conserved among alpha and beta HPV-types and form five-fold (star-shaped) capsomers, which are arranged into 60 hexamers and 12 pentamers. Together these 72 capsomers form a 55nm icosahedral capsid (Fig.4) (Baker et al., 1991). Each L1 protein has a molecular weight of 55kD and the interaction with another L1 protein is by disulfide cross linking (Doorbar and Gallimore, 1987). The L1 sequence is around 510 amino acids long and can self-assemble into a virus-like particle (VLP) when produced in a recombinant expression system (Hagensee et al., 1993; Kirnbauer et al., 1993; Zhou et al., 1991). These VLPs are morphologically very similar to HPVs extracted from warts. VLPs can be produced with both L1 and L2 proteins or with L1 alone (Hagensee et al., 1993).

L2 is a viral protein around 78kD in size and is essential for the initial infection (Doorbar and Gallimore, 1987). The amino acids near the C-terminal part of the L2 protein are believed to bind into L1 capsomers on the HPV capsid (Finnen et al., 2003). Antibodies against a small part of L2 inhibited HPV infection, suggesting that L2 could be exposed on the surface of the capsid (Kawana et al., 1999). L2 also binds viral DNA and it has been shown to be important for the encapsidation of the PV genome (Zhou et al., 1994). To establish an infection the HPV genome has to be uncoated and escape the endosome

for further transport to the nucleus. When the furin site of the L2 protein is cleaved, this minor capsid protein becomes involved in endosomal exit (Richards et al., 2006) and the L2 C-terminus have been shown to facilitate escape of viral genomes from the endocytic compartment (Kamper et al., 2006). The L2 protein contains a nuclear localization signal (NLS) and has the ability to interact with the motor protein dynein in the microtubule network (Florin et al., 2006). It moves to the nucleus by hitch-hiking on dynein after endosomal escape and together with the ability to bind viral DNA, L2 has a major role in transporting the HPV genome to the nucleus which is essential for an HPV infection.

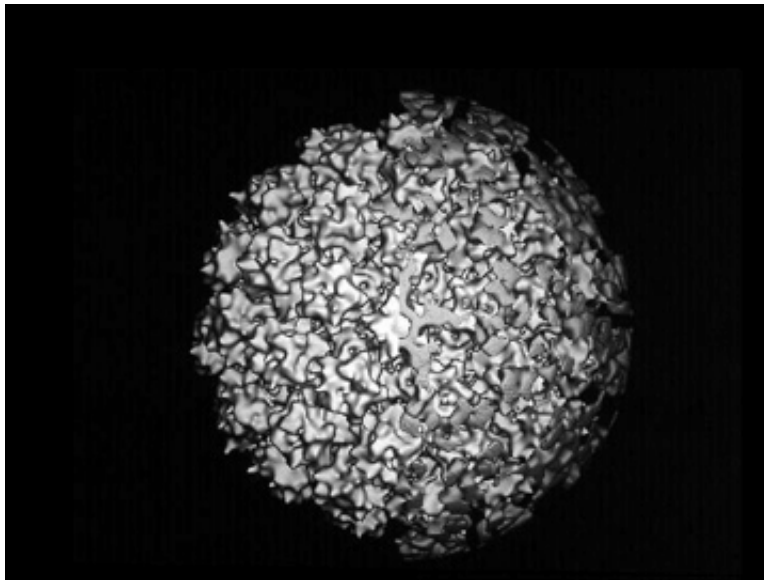


Fig. 4. A BPV virion half bound by a neutralizing monoclonal antibody, Benes Trus, NIH is acknowledged. From <http://ccr.cancer.gov/Staff/gallery.asp?profileid=5637>. With permission from J. Schiller, NIH.

The replication proteins E1 and E2

Many DNA viruses take advantage of the host cell replication machinery since they do not encode all proteins necessary for their replication. Papillomaviruses encode two viral proteins, E1 and E2, which are important for viral replication and maintenance of PV DNA (Ustav and Stenlund, 1991). The E1 protein is about 68 kDa in size and is highly conserved among PVs. This protein recognizes the replication ori (Frattini and Laimins, 1994b) and possesses both helicase and ATPase activity (Hughes and Romanos, 1993). Like other helicase proteins, E1 move along the ds DNA and unwinds the double helix (Frattini and Laimins, 1994a). E1 also forms a complex with the E2 protein and this complex facilitates E1 binding to PV DNA. When E1 is bound to PV DNA it can attract and also bind the cellular DNA polymerase α -primase in order to start replication of the viral DNA with other cellular proteins (Amin et al., 2000; Park et al., 1994).

The E2 protein binds as a dimer to a palindrome sequence (ACCGN₄CGGT) which is repeated several times along the LCR. E2 is of importance for the viral lifecycle as it functions as a trans-activator and regulates transcription (Dostatni et al., 1988; Hou et al., 2002). The C-terminal functions as a DNA binding domain and the N-terminal is responsible for transactivation. These two regions are connected with a hinge region that is less conserved among the different HPV types (McBride et al., 1989; McBride et al., 1988). PV is known to cause persistent infections in epithelial cells of many mammals and it is important for HPV to preserve a stable episome of the PV genome in the nuclei during cell division. To prevent loss of the episome, the E2 protein produced by some PV types binds to a cellular protein called Brd4 and tethers the viral genomes to mitotic chromosomes, leading to maintenance of the viral genome through the process of breakdown and reassembly of the nuclear membrane during cell proliferation (You et al., 2004). Binding to Brd4 has also been shown to be important for E2 mediated transcriptional activation (McPhillips et al., 2006).

The E4 and E5 proteins

During PV infection the E4 protein is expressed in large quantities late after infection and it can bind and promote the collapse of the cyokeratin network in the cell (Doorbar et al., 1991). The ORF of E4 lacks the initiator codon AUG and is therefore translated from a spliced transcript resulting in an E1^{E4} fusion protein (Nasseri et al., 1987). The E1^{E4} protein is abundantly expressed in the upper layers of epithelial cells where it induce G₂ arrest (Davy et al., 2005; Davy et al., 2002). The C-terminal part of E1^{E4} HPV associates with E4-DBD (E4-DEAD box protein), which is an RNA helicase involved in RNA processing such as RNA splicing, transport and translation initiation, suggesting a role in regulation of gene expression for E4 (Doorbar et al., 2000).

The HPV E5 ORF encodes a small transmembrane protein consisting of nearly 90 amino acids that is associated with endosomal membranes and can be located in the Golgi apparatus and the endoplasmic reticulum (Conrad et al., 1993). The E5 protein of bovine papillomavirus-1 (BPV) interacts with the platelet-derived growth factor β receptor in fibroblasts and becomes tyrosine phosphorylated, a reaction leading to cell transformation and proliferation (Goldstein et al., 1994; Klein et al., 1998). The E5 protein of HPV has been shown to cooperate with epidermal growth factor receptor (EGFR), thus stabilizing and prolonging the activation of EGFR, consequently inducing cell mitogenesis (Straight et al., 1993). There are conflicting data whether cooperation of the E5 protein of high-risk HPV types with EGFR increases cell proliferation.

The oncoproteins E6 and E7

The E6 and E7 proteins are expressed early in the life cycle of HPV but can also be found throughout the layers of differentiated epithelial cells. These proteins from high-risk HPV types like HPV-16, -18 and -31 can function as oncoproteins when transfected into cells. The E6 and E7 proteins of low-risk HPV types do not have the same oncogenic character. When HPV infects the basal layer of epithelial cells, expression of HPV E6 and E7 keeps

keratinocytes in the cell cycle during the differentiation process, while uninfected epithelial cells differentiate but exit the cell cycle and stop dividing (Munger et al., 1989). The E6 protein is made of 151 amino acids and carries 4 metal binding motifs (Cys-X-X-Cys) and is able to form 2 zinc fingers (Barbosa et al., 1989). E6 has been shown to interact with several cellular proteins. Early studies show that E6 binds the tumor suppressor p53 (Scheffner et al., 1990; Werness et al., 1990). p53 is involved in cell cycle arrest or cellular apoptosis upon DNA damage and stops viral infection from spreading to adjacent cells (Chen et al., 1996). To prevent p53 triggered apoptosis E6 forms a complex with E6-AP which is an ubiquitin ligase and degrades p53 (Huibregtse et al., 1993). E6 is also able to push cells into S-phase in presence of the cell cycle inhibitor INK^{4A}. This leads to duplications and instability of chromosomes (Malanchi et al., 2002). Another form of E6 has also been found in cervical neoplasias, called E6*, which is a result of alternate splicing. E6* inhibits degradation of p53 by interacting with the full length E6 and E6-AP and HPV is then unable to immortalize cells (Pim and Banks, 1999). Another important E6 function is the ability to activate expression of hTERT and together with the inhibition of INK^{4A} immortalize human epithelial cells (Kiyono et al., 1998; Klingelutz et al., 1996). The E6 protein from some HPV types infecting the cutaneous epithelium targets a pro-apoptosis protein, Bak. This protein is up-regulated in cells exposed to UV-B radiation to induce apoptosis. The E6 has shown to degrade this protein and inhibits apoptosis of cells exposed to UV-B radiation (Jackson et al., 2000).

The second oncoprotein important for immortalization and cell cycle activation is E7. E7 contains 2 metal binding motifs that can form a zinc finger (Barbosa et al., 1989). The key functions of E7 is its binding to the retinoblastoma protein (Rb) and its ability to interact with a protein called histone deacetylase (HDAC) which disturbs the recruitment of Rb to the cellular E2F factor (Dyson et al., 1989; Longworth and Laimins, 2004). The Rb protein is phosphorylated by cyclin-dependent kinases (CDK) upon mitogenic signals and E7 disrupts the association of Rb with various E2F family members in late G₁ phase. This release of E2F turns on transcription of genes essential for entry into the S phase (Sherr and Roberts, 1999). The E7 binding to Rb forces the cell into S phase, independent of mitogenic signals (fig. 5) (Dyson et al., 1989; Edmonds and Vousden, 1989).

Furthermore, E7 enhances the activity of cyclin A and E and can also block CDK inhibitors and boost the progression of cell cycle (Jones et al., 1997; Zerfass-Thome et al., 1996; Zerfass et al., 1995).

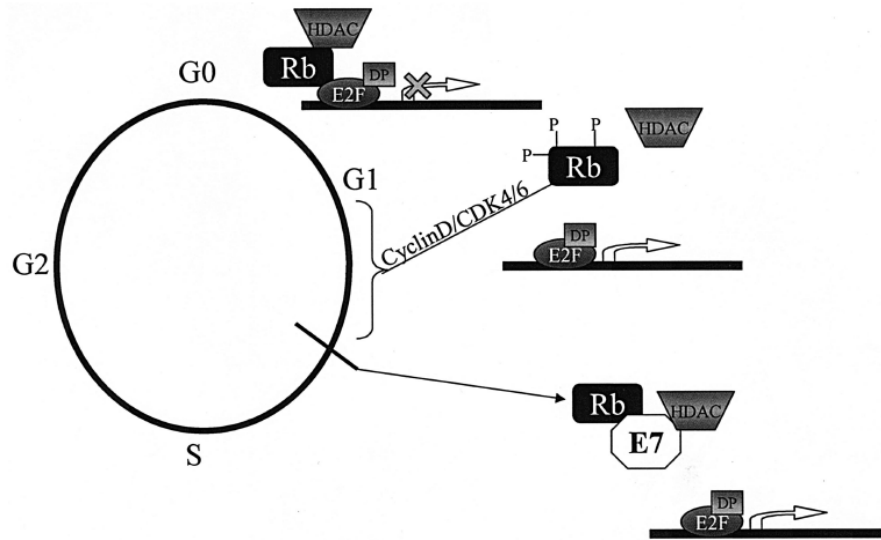


Fig. 5. Illustration of cell cycle regulation. From Longworth and Laimins 2004 with permission.

Life cycle

Epidermis is the upper epithelial layer of skin and mucosa and it consists of several stratum of cells known as stratified squamous epithelium. Underneath epidermis lies dermis separated from the upper layer by the basement membrane. The basal layer of epithelial cells in the epidermis is the site of infection for HPV and can be accessed when a micro lesion or minor trauma occurs on the skin. Entry of HPV has also been suggested to occur through hair follicle stem cells (Schmitt et al., 1996). When HPV reaches the basal layer it enters these cells, escapes from the endosome and the viral DNA is

transported to the nucleus. As the basal cells of the epithelium layer divides and the daughter cells begin to differentiate, the early genes are transcribed and early proteins are expressed (Doorbar, 2006). Once the HPV infected cells get closer to the uppermost layer of the epithelium an increase in DNA replication is found, the late proteins are expressed and L1 and L2 can self-assemble into icosahedral capsids and encapsidate the viral DNA. Thus, the differentiation of epithelial cells is vital for HPV infection and infectious HPVs are finally released on the surface of skin or mucosa by cell lysis (Doorbar, 2006)(fig. 6).

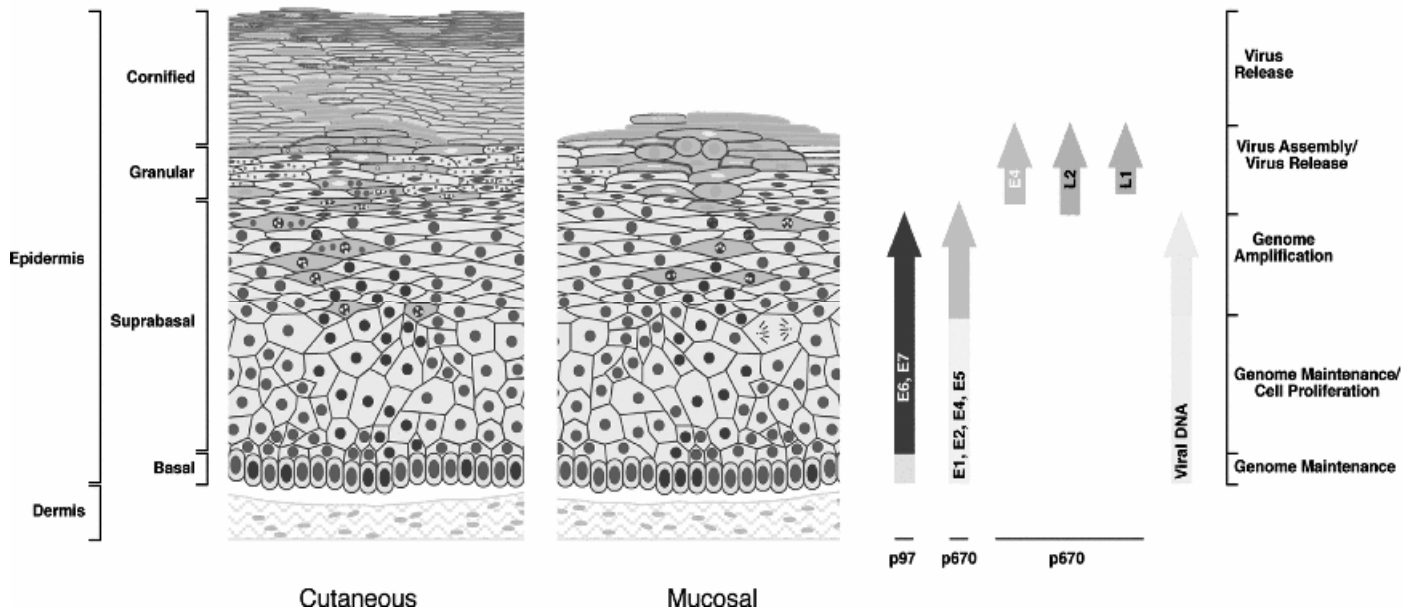


Fig. 6. Productive life cycle of HPV. From John Doorbar 2006, with permission.

Artificial HPVs

Since reproduction of HPV is tightly linked to the epithelial cell differentiation, generating HPV particles by infecting ordinary monolayer cell cultures is not possible. In order to produce virus particles, the L1 capsid protein of HPV was expressed in a recombinant vaccinia system and was shown to self-assemble into virus-like particles

(VLP) (Hagensee et al., 1993; Zhou et al., 1991). VLPs can be composed of both the L1 and the L2 capsid proteins or only the L1 protein. VLPs have similar size and the same icosahedral structure as an authentic virus (Hagensee et al., 1993). Several other systems have been developed to produce VLPs including yeast (Sasagawa et al., 1995), bacteria (Zhang et al., 1998) or insect cells (Kirnbauer et al., 1993). HPV particles can also be produced in organotypic raft culture systems and provide infectious HPV virions (Meyers et al., 1992). This technique mimics the *in vivo* viral production but it is time consuming and yield low virion quantities. Using recombinant systems to generate high yield of VLPs, have been significant tools for studying the early part of the HPV life cycle. To study binding and internalization, investigators have modified the VLPs by labeling them with e.g. isotopes (Roden et al., 1994) or a fluorescent dye (paper I) (Drobni et al., 2003) or attached a plasmid marker on the outside of VLP capsid (Combita et al., 2001).

VLPs are devoid of HPV DNA and consequently incapable of replication and expression of HPV genes but they generate neutralizing antibodies (Christensen et al., 1994). These empty capsids have therefore been used for a vaccine against certain low-risk (HPV-6, -11) and high-risk (HPV-16, -18) HPV (Villa et al., 2005). A 5 year follow-up study shows that the quadrivalent HPV vaccine against HPV-6, -11, -16 and -18 is effective in preventing HPV infection by these types (Lambert, 2007).

Despite the advantage of VLPs, they are now being replaced by the pseudovirus (PsV) system for studying the HPV life cycle. Genes of L1 and L2 capsid proteins were codon modified to eliminate codons that may inhibit RNA processing and translation. The modified capsid genes along with reporter plasmids were transfected into 293TT cells, a human embryonic kidney cell line that overexpress SV40 T, which results in high yield production of HPV capsid proteins that enclose a reporter plasmid (Buck et al., 2004). These PsVs will enter the cell and the reporter gene is expressed when the virus has delivered the DNA to the nucleus, similar to a real HPV infection. Interestingly, this method has also been used to generate infectious HPV particles. The full length HPV-16 genome was excised from its plasmid vector and recircularized by ligation. It was then

packaged into HPV capsids when transfected into cells together with codon modified L1 and L2 plasmids (Pyeon et al., 2005).

Virus binding to the cell surface

Prior to internalization, HPV needs to attach to cell receptors expressed on the surface of the epithelium. Studies have shown that binding of infectious BPV-1 to the cell surface was equally inhibited by BPV VLPs consisting of L1 alone or both L1 and L2, suggesting that the major capsid protein L1 is involved in the first interaction with cell surface receptors (Roden et al., 1994). VLPs have been shown to bind many cells lines of different origins indicating that the PV receptor is a common and widely expressed protein on the surface of cells (Muller et al., 1995; Roden et al., 1994).

Heparan sulfate

The sulfated sugar heparan sulfate was suggested as an initial attachment receptor for HPV-11 L1 VLPs (Joyce et al., 1999). Later studies using HPV-16 and HPV-33 L1/L2 VLPs enclosing a GFP marker plasmid revealed that heparan sulfate is required for HPV infection of COS-7 cells (Giroglou et al., 2001) and HPV-16 L1 VLPs used heparan sulfate for binding to HaCaT cells (Drobni et al., 2003). Additionally, several other alpha PV VLPs were shown to require heparan sulfate for attachment (Combita et al., 2001). Heparan sulfate belongs to a group of large molecules named glycosaminoglycans (GAGs), which are linear, repeating, disaccharides that are highly sulfated (Fig. 7). For binding inhibition experiments, heparin is often used because of its availability and similarity to heparan sulfate. However, the disaccharide chain of heparin is more sulfated (2.7 sulfate groups per disaccharide compared to heparan sulfate with an average of 1 sulfate group per disaccharide) and it contains more idouronic acid than heparan sulfate (Bernfield et al., 1999; Hileman et al., 1998). Heparin is an anti-blood coagulant and unlike heparan sulfate, heparin has intracellular location in mast cells (Hileman et al.,

1998). Heparan sulfate is frequently found in the extra cellular matrix (ECM) and on the surface of most cells in the body. It is involved in several biological functions such as cell proliferation, migration and wound healing. Because of its location it is a desirable molecule for viral infection (Liu and Thorp, 2002) and viruses such as cytomegalovirus (Compton et al., 1993), herpes simplex virus (HSV) (Spear et al., 1992; WuDunn and Spear, 1989), dengue virus (Chen et al., 1997) and foot and mouth disease virus (Fry et al., 1999) use heparan sulfate to establish viral infection. The sulfate groups of heparan sulfate contributes to the high negative charge of the molecule and interaction with heparan sulfate by viruses and other ligands can be dependent on the number of sulfate groups (Thompson et al., 1994; Trybala et al., 2000).

Syndecans

Heparan sulfate is often found on two membrane-bound proteoglycans, syndecans and glypicans. Glypicans are predominantly expressed in the central nervous system, whereas syndecans are the predominant heparan sulfate proteoglycan in epithelial cells, the target cells of HPV (Bernfield et al., 1999). HPV-11 infection was found to be most efficient in cells expressing syndecan-1 compared to syndecan-4 and glypican-1 suggesting syndecan-1 as an important proteoglycan in HPV entry (Shafti-Keramat et al., 2003).

N-sulfo-alpha-D-glucosaminopyrano-6-O-sulfatosyl-(1->4)-
2-O-sulfato-beta-L-iduronopyranan

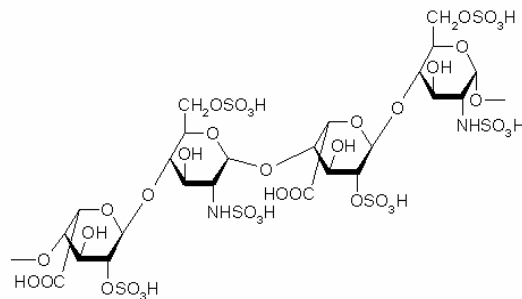


Fig. 7. Structure of heparan sulfate.

Alpha-6 integrin

$\alpha 6$ integrin was initially suggested as a candidate receptor for HPV (Evander et al., 1997; McMillan et al., 1999; Yoon et al., 2001). Integrins are cell adhesion receptors and they are involved in cell to cell interactions. One of the main functions of integrins is to bind and react to the ECM. In the cell membrane, integrins are heterodimeres and consist of two transmembrane glycoproteins, an alpha (α) unit and a beta (β) unit. The different combinations of $\alpha\beta$ units have its own binding and signaling properties. The $\alpha 6$ integrin can form a complex with a $\beta 1$ or $\beta 4$ integrin in primary keratinocyte cells. The $\alpha 6\beta 1$ is found in the parabasal layers, while $\alpha 6\beta 4$ is mainly found in the hemidesmosomes facilitating attachment to the basement membrane (Sonnenberg et al., 1991).

Laminin-5

Furthermore, HPV-11 was found to bind to a receptor in the ECM. This receptor is secreted by keratinocyte-derived cell lines, the natural host cell for PV (Culp et al., 2006a). The HPV-11 receptor in the ECM was identified as laminin-5 (Culp et al., 2006b). Interestingly, laminin-5 is a natural ligand for $\alpha 6\beta 4$ integrin at hemidesmosomes and keratinocytes lacking $\alpha 6$ integrin could not be infected by HPV-11, again supporting $\alpha 6$ integrin as a potential PV receptor (Culp et al., 2006b). Laminin 5, a component of ECM, is secreted by epithelial cells in tissue and in culture, and it is a major adhesive component of the basement membrane in many epithelial cells. During a trauma on the skin laminin-5 is expressed and secreted into the basement membrane by keratinocytes of the epidermis that migrates into the wound bed (Nguyen et al., 2000b). Interactions of $\alpha 6\beta 4$ integrin with laminin-5 assemble hemidesmosomes and generate an anchorage of keratinocytes. This interaction can also cause a signal reaction that regulates the cell cycle and cell invasion (Nguyen et al., 2000a). Upon ligation of laminin-5 to $\alpha 6\beta 4$ integrin, Ras is activated through a PI3 kinase signal and induce a growth signal to cells (Giancotti and Ruoslahti, 1999). A recent study shows that PV VLPs activate PI3 kinase

though $\alpha 6\beta 4$ integrin upon binding to cells (Fothergill and McMillan, 2006). This signal was activated by both L1 and L1/L2 VLPs but the correct conformation of the capsid was required (Fothergill and McMillan, 2006).

Virus internalization pathways

After binding to cell surface receptors HPV must be internalized into the cell to establish an infection. Nutrients are taken up by cells via endocytic pathways and this system is used by many pathogens to enter the cell. Several endocytic pathways have been described and clathrin-mediated and caveolae-mediated endocytosis are two pathways used by viruses to infect cells (Pelkmans and Helenius, 2003; Smith and Helenius, 2004). HPVs were shown to use the endocytotic pathway when cells were incubated with chlorpromazine to block clathrin-dependent receptor mediated endocytosis. This inhibited internalization of BPV-1, HPV-16 (Day et al., 2003) and HPV-58 (Bousarghin et al., 2003). Previously, internalization of HPV-33 was first thought to work through small pinocytosis that was devoid of clathrin proteins (Volpers et al., 1995). However, the HPV particle in the small endosome was not transported to the nucleus. Also, HPV-31 was suggested to use a caveolae-mediated endocytosis (Bousarghin et al., 2003). This is contradictory to another study which suggests a clathrin-dependent pathway for HPV-31 (Hindmarsh and Laimins, 2007). Several different inhibitors have been used to block the internalization of HPV. Bafilomycin A blocks acidification of endosomes and nocodazole disrupts microtubule formation. Both inhibitors blocked infection of BPV-1, HPV-16 and HPV-33 and point towards a dependency of low pH for uncoating of PV and trafficking to late endosome with microtubules (Day et al., 2003; Selinka et al., 2002). Moreover, L2 is of importance for the endosomal escape of HPV. The N-terminus of L2 is cleaved at a furin motif site in the endosomal compartment or at the cell surface after HPV has attached to its receptor (Richards et al., 2006). The capsid of HPV undergoes a conformational change postattachment (Selinka et al., 2003). Which may expose the N-terminus of L2 and make it accessible for cleavage.

Lactoferrin

Lactoferrin (Lf) is an 80 kDa, positively charged glycoprotein of approximately 680 amino acids folded into two lobes that are connected with an 11 amino acid α -helix region (fig. 8). It is a component of the innate immune defence and the structure is well conserved and with 69% sequence homology between bovine lactoferrin (bLf) and human lactoferrin (hLf) (Baker et al., 2000; Pierce et al., 1991). The N-terminal part is positively charged and responsible for binding to glycosaminoglycans such as heparan sulfate (Iyer and Lonnerdal, 1993; Wu et al., 1995). In addition to binding GAGs, Lf binds to a 105 kDa receptor with high affinity (Mazurier et al., 1989; Spik et al., 1994). Lf has also been shown to bind to several other cell surface receptors such as lipoprotein receptor-related protein (LPR) and intelectin (Suzuki et al., 2005). The biological activity of Lf is suggested to depend on the target cell and the presence of specific receptors (Suzuki et al., 2005).



Fig. 8. Structure of human lactoferrin. From J. L. Gifford 2005,

Lf is a remarkable multifunctional protein that has antibacterial, antiviral and antifungal activity. By binding iron it keeps body fluids clear from free radicals that could damage the cells (Baldwin et al., 1984) and inhibits bacterial growth, since iron is important for bacterial metabolism (Orsi, 2004). It can also deliver iron to T-cells and enhance Th-1

response, thus showing a role in regulation of the immune system (Guillen et al., 2002). Furthermore, a bactericide activity independent of iron saturation has been described, where the N-terminal part of Lf can disrupt or penetrate the cell membrane of both gram-negative and -positive bacteria (Valenti and Antonini, 2005). It has also shown protease activity that could restrain pathogenic microbes (Hendrixson et al., 2003). Many viruses have been shown to be affected by Lf, including nonenveloped viruses such as adenovirus (Arnold et al., 2002), poliovirus (Marchetti et al., 1999), rotavirus (Superti et al., 1997), HPV (Drobni et al., 2004) and enveloped viruses such as herpes simplex virus type 1 (HSV-1) (Hasegawa et al., 1994) and hepatitis C virus (HCV) (Ikeda et al., 1998). Both hLf and bLf could inhibit virus infection by binding to a viral attachment receptor on cell surface and thereby inhibit viral attachment as shown for HSV (Andersen et al., 2004; Jenssen et al., 2004; Marchetti et al., 1996; Marchetti et al., 2004). Lactoferrin could also inhibit attachment by direct binding to the virus (HCV) and prevent adsorption to cells (Yi et al., 1997). Interestingly, Lf has also been shown to promote binding of adenovirus-5 and it was suggested that lactoferrin adenovirus-5 captures Lf and thereby linking the virions to the cell surface (Johansson et al., 2007).

Lf is secreted into milk, saliva, tears and mucosal fluids. It is secreted by exocrine glands and by polymorphonuclear neutrophil granulocytes. The highest concentration of Lf is found in colostrums, postpartum milk (7g/l). In normal conditions only low concentration is found in plasma, but levels increase during inflammation (Baynes and Bezwoda, 1994; van der Strate et al., 2001). In vaginal mucosa, the highest concentration of Lf is found after menses. Concentration is low before menses and in women taking oral contraceptives, indicative of hormonal control for lactoferrin in vaginal mucosa (Cohen et al., 1987).

Lactoferricin

Lactoferricin (Lfcin) (Fig. 9A) is a peptide, released from the N-terminus sequence of Lf after digestion by acidic pepsin. This peptide possesses similar bactericidal effect as the

parent protein LF (Yamauchi et al., 1993). Bovine lactoferricin (blfcin) is produced by pepsin cleavage of Lf, resulting in a peptide with residues 17-41 (Bellamy et al., 1992a) or 17-42 (Dionysius and Milne, 1997) (Fig. 9B). Human lactoferricin (hLfcin) was believed to contain two peptides, amino acid (a.a.) residues 1-11 and 12-47 linked with a disulfide bridge (Bellamy et al., 1992b). However, recent studies suggest a peptide chain that contains 49 residues (a.a. 1-49) (Fig.9B) (Hunter et al., 2005). Both human (hLfcin) and bLfcin form a loop structure held together by a disulfide bond. The disulfide bridge links a.a. 19 to 36 in bLfcin, and links a.a. 10 to 46 and 20 to 37 in hLfcin (fig. 9B). These looped peptides have antiviral activity against adenovirus (Di Biase et al., 2003), HSV (Jenssen, 2005; Siciliano et al., 1999), cytomegalovirus (Andersen et al., 2001) and HPV (paper III) (Mistry et al., 2007, in press).

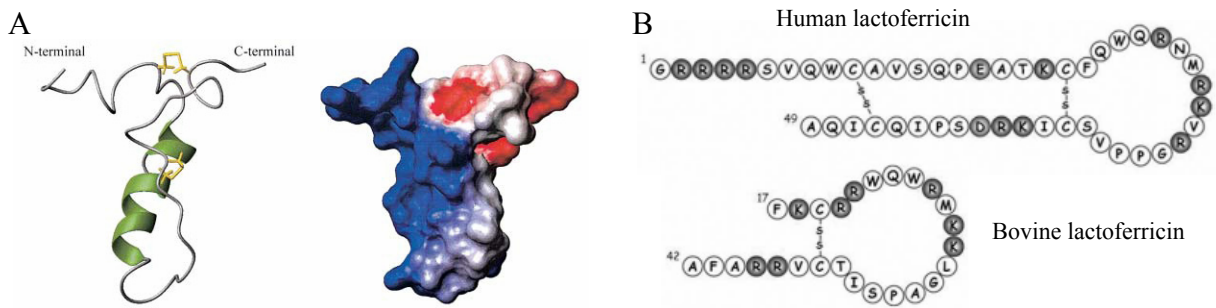


Fig. 9A. 3D Structure of human lactoferricin. From J. L. Gifford 2005. B. Amino acid composition of lactoferricins. From H. Jenssen 2005 with permission.

Results and discussion

Tropism and binding

HPVs infect either the basal layer of skin or the mucosal epithelium of the body and the individual HPV types exhibit a marked degree of specificity with respect to lesion morphology (Croissant et al., 1985). The most prevalent type in plantar warts is HPV-1, in common warts it is HPV-2 and in flat cutaneous warts it is HPV-3 and HPV-10 (Croissant et al., 1985). HPV-6 and -11 are found in exophytic condylomas and HPV-16 and HPV-18 usually cause flat condylomas in mucosal regions and have oncogenic potential (Croissant et al., 1985). It is not known how this anatomical tissue specificity of HPV is determined.

The early steps of the viral life cycle have been shown to be important for the tropism for several viruses. HSV (Manoj et al., 2004; Spear, 2004) and HIV (Broder and Collman, 1997; Zaitseva et al., 2003) require multiple interactions to cell receptors before entering their host cells. In the phylogenetic tree based on the ORF sequence of major capsid protein L1, all HPV types are divided into genus and species (de Villiers et al., 2004). When the L1 nucleotide sequence has less than 60% homology between two PV types they are divided into different genus. The PV types in the alpha-papillomavirus (alpha-PV) genus are mostly infecting mucosal surfaces while the beta-papillomavirus (beta-PV) types are rarely detected in mucosal cells but frequently infect the skin (Orth, 2004). Since PVs form genera based on their L1 sequence the capsid protein has a potential to be involved in determination of PV tropism, since L1 proteins are the first to interact with the host cell.

We studied binding and uptake of PV by using HPV-6 and -16 from the alpha PV-genus and HPV-5 from the beta-PV genus. First, HPV-16 VLPs were preincubated with different concentrations of GAGs. Our results showed a dose-dependent inhibition of HPV-16 VLP binding to HaCaT cells (originally from adult trunk skin) by GAGs such as dextran sulfate and heparin and also heparan sulfate from bovine intestinal mucosa and

porcine intestinal mucosa (paper I). The most effective GAG was heparin that showed a 50% inhibition (IC_{50}) at 0.5 $\mu\text{g/ml}$ followed by dextran sulfate ($IC_{50} = 1\mu\text{g/ml}$) and the two heparan sulfate from intestinal mucosa ($IC_{50} = 5-10 \mu\text{g/ml}$) (paper I). Interestingly, heparan sulfate from bovine kidney was not good at inhibiting HPV-16 VLP binding. Heparin contains more sulfate groups than heparan sulfate and that increases its net negative charge (Sasisekharan and Venkataraman, 2000), which could make heparin a better inhibitor than heparan sulfate for HPV binding. For some viruses (HSV, HCV) the degree of high sulfation increases the inhibition of virus binding (Barth et al., 2006; Trybala et al., 2000). This suggests that initial attachment to heparan sulfate could be through electrostatic forces.

Tropism and internalization

To study internalization we developed an assay where we labeled our VLPs with carboxy-fluorescein diacetate, succinimidyl ester (CFDA-SE) (paper I). CFDA-SE only fluoresce when cleaved by intracellular esterases and functions as an assay to study internalization of HPV VLPs. A reduced internalization into HaCaT cells was observed when preincubating CFDA-SE labeled HPV-6 and HPV-16 VLPs with heparin. HPV-6 showed an IC_{50} at 40 $\mu\text{g/ml}$ heparin and for HPV-16 $IC_{50} = 90\mu\text{g/ml}$ heparin (paper I). In paper II we continued our studies of HPV internalization using HPV pseudovirus (PsV). HPV-16 PsV had an IC_{50} of 24 $\mu\text{g/ml}$ for heparin inhibition in HaCaT cells while HPV-5 PsV was not inhibited to more than 43% in HaCaT cells (paper II). In C33A cells (from cervical mucosa), HPV-16 was inhibited by heparin while HPV-5 was not inhibited at all (paper II). It was clear that HPV-16 was more affected by heparin than HPV-5 and it was therefore reasonable to assume that heparin had a higher binding affinity for HPV-16 than HPV-5 (paper II). When we calculated the pI of the L1 capsid protein, we found that alpha-HPVs, predominantly infecting the mucosal epithelium, were positively charged at pH 7.4 and beta-HPVs, usually infecting skin, were negatively charged at the same pH (paper II). Interestingly, the internalization of alpha HPV-6 was more inhibited by heparin than alpha HPV-16 (paper I), which could be explained by the higher positively

charged HPV-6 L1 capsid protein (paper II). When we predicted the surface of the HPV capsid we found that HPV-5 exposed more negatively charged amino acids than HPV-16 (paper II). Heparin is a highly negatively charged protein and it is possible that the difference in heparin inhibition was determined by the higher affinity of heparin for the alpha-HPVs compared the beta-HPVs. However, there could also be an interaction with a second receptor. Recent studies show that a protein found in the ECM, laminin-5, interacted with HPV-11 (Culp et al., 2006a; Culp et al., 2006b). Laminin-5 is secreted by migrating and basal keratinocytes and is believed to function as a transreceptor to $\alpha 6$ integrin for efficient infection by HPV virions and pseudovirions (Culp et al., 2006a; Culp et al., 2006b). Some viruses such as HSV require a second cell receptor for infection but HSV types also differ in their use of heparan sulfate variants. HSV-1 interacted stronger to heparan sulfate with O-sulfation than HSV-2 (Trybala et al., 2000) and in a similar way O-sulfation of heparan sulfate was important for HPV-33 binding and infection (Selinka et al., 2003). Interestingly, a conformational change of PV has been suggested after initial binding (Selinka et al., 2003) and in order to infect the cell, HPV might need a conformation change to be internalized into its host cell. We do not know if this could explain any of the differences between HPV-5 and HPV-16.

Many alpha-HPV types studied have been reported to use clathrin mediated endocytosis to enter cells (Day et al., 2003; Hindmarsh and Laimins, 2007). In our assay, we found that internalization of CFDA-SE labelled HPV-5 and HPV-16 VLPs was equally inhibited when cells were pretreated with chlorpromazine, an inhibitor of clathrin-dependent endocytosis (paper II). This strengthens other reports (Day et al., 2003; Hindmarsh and Laimins, 2007) stating that HPV use clathrin mediated endocytosis for internalization.

Tropism and lactoferrin

Lactoferrin (Lf) is found in body secretions at mucosal surfaces, but Lf has so far not been found at high levels in normal skin. The infection of several viruses have been

reported to be inhibited by Lf and its cleaved N-terminal peptide lactoferricin (Lfcin) (Arnold et al., 2002; Drobni et al., 2004; Hasegawa et al., 1994; Ikeda et al., 1998; Jenssen, 2005; Jenssen et al., 2004; Marchetti et al., 1999; Superti et al., 1997). One possibility is that the antiviral activity is in the early phase of viral infection. Both Lf and Lfcin are highly positively charged and may therefore interact with the negatively charged GAGs such as heparan sulfate and block attachment of the virus to cell.

The binding and internalization of the HPV-16 L1 VLPs has been shown to be inhibited by Lf (Buck et al., 2006; Drobni et al., 2004). In paper III preincubation of human Lf (hLf) or bovine Lf (bLf) in two different cell lines also reduced HPV-5 and HPV-16 infection by 70-95% at 1 μ M. Previously we showed that HPV-5 PsV infection was less inhibited by heparin than the HPV-16 (paper II). To study if Lf had antiviral activities and possibly could be important for HPV tropism we incubated to HaCaT cells and C33A cells with Lf before infecting them with HPV-5 and HPV-16 PsV (paper III). Both hLf and bLf was a potent inhibitor of HPV-5 and HPV-16 infection and there was no significant difference in inhibition detected between HPV-5 and HPV-16 infection (table 1) (paper III). We could not detect any difference in heparan sulfate expression on the surface of the cell lines used in our assay (paper II).

The derivative lactoferricin (Lfcin) peptide has also shown antiviral effects (Jenssen et al., 2004). We studied the antiviral activity of four Lfcin peptides in the same manner as for Lf (paper III). Interestingly, there was a difference between bovine lactoferricin (bLfcin) 17-42 and bLfcin 17-31. We observed a strong reduction of HPV-16 PsV infection with the linear blfcin 17-31 in the two cell lines studied but no HPV-16 antiviral effect was detected with the cyclic blfcin 17-42 within the range of concentrations tested (paper III). However, bLfcin 17-42 was capable of reducing infection of HPV-5 PsV in C33A cells but the bLfcin 17-31 was also a potent inhibitor against HPV-5 PsV in both HaCaT and C33A cell lines (table 1). Overall the bLfcin 17-31 peptide was a potent inhibitor of HPV PsV infection. Previously, a small peptide section within the bLfcin 17-31 (bLfcin 20-30) did not have any inhibitory effect against HPV-16 PsV infection in HeLa cells (Buck et al., 2006), which points to the importance of size and amino acid

composition for these inhibitory peptides in antiviral activity. The hLfcin 1-49 also showed an inhibitory effect against HPV-5 and HPV-16 PsV infection in both cell lines (paper III). Two peptides, hLfcin 1-49 and bLfcin 17-42, inhibited binding of HPV-16 VLPs to HaCaT cells by 35-50% at 30 μ M but no inhibition was detected by bLfcin 17-31 and hLfcin 18-42 peptides (paper III). The 10 amino acid shorter bLfcin 17-31 peptide is less positively charged than bLfcin 17-42 and has weaker affinity to heparan sulfate (Jenssen et al., 2004). This could explain the lower binding inhibition of HPV-16 VLPs by bLfcin 17-31. Also, hLfcin 18-42 do not possess the four cationic arginin residues present in hLfcin 1-49. This results in lower net positive charge and lower affinity to heparan sulfate (Mann et al., 1994). Another possibility for Lf to inhibit viral attachment to cells is by direct interaction with the viral particle (Ammendolia et al., 2007; Superti et al., 1997). We showed that bLf could interact with HPV-16 implying that the inhibition of HPV infection could be mediated both by Lf blocking of cell surface heparan sulfate and direct Lf interaction with the viral capsid.

Table 1. The mean and standard deviation of IC₅₀ of the inhibitory effect on HPV infection by lactoferrin and lactoferricin.

Inhibitor	HaCaT				C33A			
	HPV-5		HPV.16		HPV-5		HPV-16	
	IC ₅₀	SD	IC ₅₀	SD	IC ₅₀	SD	IC ₅₀	SD
bLf	0.14	0.046-0.24	0.28	0.00-0.60	0.35	0.20-0.50	0.26	0.20-0.31
hLf	0.26	0.19-0.32	0.41	0.33-0.50	0.34	0.32-0.36	0.26	0.17-0.32
bLfcin 17-31	7.3	4.2-10.3	4.05	3.3-4.8	10.8	10.3-11.3	2.6	1.7-3.2
bLfcin 17-42	17	7.8-26.4	---**	---	3.4	1.1-5.7	---	---
hLfcin 18-42	n.d.*	---	---	---	n.d.	---	n.d.	---
hLfcin 1-49	n.d.	---	---	---	n.d.	---	n.d.	---

* n.d= not done, ** IC₅₀ could not be calculated.

bLfcin 17-42 and hLfcin 1-49 are generated after tryptic digestion of Lf, and there was a difference in antiviral action between hLfcin 1-49 and bLfcin 17-42 (paper III) which can partly be explained by structural differences between the two peptides. When hLf is cleaved by pepsin, the N-terminal part that forms hLfcin keeps its α -helical domain but loses the β -sheet. In a different manner, the bLfcin N-terminal part is composed of only

an α -helical domain that after pepsin cleavage folds into a distorted β -sheet (Hwang et al., 1998). The loss of β -sheet in hLfcin seems to increase the inhibitory effect against HPV-5 and HPV-16 infection.

Tropism and transcription

Transcriptional regulation is another part of the viral life cycle that might determine HPV tropism. In the circular HPV DNA genome, between the L1 capsid ORF and the E6 ORF, the LCR is located. This interesting region does not code for any protein but contains several binding sites for epithelial specific enhancers. All HPV studied so far contain this LCR region that include transcription start site (O'Conner et al., 1995; Sailaja et al., 1999). This site can be either up-regulated by epithelial specific enhancers or down-regulated by silencers, depending on which proteins the infected cell express. It is therefore tempting to hypothesize that HPV tropism could be determined by differences in transcriptional regulation. To study this we cloned the complete LCR region from HPV-5 and HPV-16 into a plasmid expressing a reporter protein upon activation and transfected these constructs into cell lines from different origin (paper IV). Our results showed that the HPV-16 LCR was more activated in cells originally isolated from a human cervical lesion (W12E cells) than the HPV-5 LCR (paper IV). In the cutaneous cell line (HaCaT), the HPV-5 LCR was twice as active as the HPV-16 LCR (paper IV). In another study, both HPV-5 LCR and HPV-16 showed similar activity in HaCaT cells, however only the central part (containing only enhancers) of LCR was used in this study (Sailaja et al., 1999). Additionally, the same investigators found that some alpha HPV types have strong epithelial specific enhancers in both mucosal and cutaneous cells (Sailaja et al., 1999). Together, this suggests that HPV enhancers and silencers are important for the regulation of HPV transcription. This regulation may also differ depending on the differentiation state of the infected cells, but this needs to be further addressed.

Concluding remarks

The terminal differentiating cells of skin and mucosal epithelium is a protective barrier for microbes such as virus and bacteria. For productive infection, HPV needs to bind and enter the basal layer of this epithelium and reproduce in the differentiating epithelia. The HPV in the alpha genus contains mostly HPV types that infect mucosal epithelium and the cutaneous infecting HPV types are usually found within the beta genus. The aim of this thesis was to investigate possible determinants for this tropism.

The tropism for many viruses is determined by which cell surface receptor it binds. We demonstrated that infection of beta HPV-5 was not as dependent on heparan sulfate as alpha HPV-6 and -16, which could be due to charge differences on the capsid protein. We showed that beta-HPVs were more negatively charged than alpha-HPVs and the L1 protein of HPV-5 exposed more negatively charged amino acid residues than HPV-16 L1. Lactoferrin is a component of the innate immune defence and is found in relatively high amount in genital and saliva secretions. We could demonstrate that the infection of both HPV-5 and HPV-16 was reduced by bovine and human lactoferrin. When we inhibited infection with lactoferricin, a small peptide derivative of lactoferrin, we could show that it was dependent on size, charge and structure of the peptide as well as HPV type and cell origin.

We also investigated if the transcription of the HPV genome could determine the tropism. In our study HPV-5 LCR was more active in a cutaneous cell line than HPV-16, which suggests a better activation of HPV-5 transcription in these cell lines. Furthermore, the HPV-16 LCR was more effective than HPV-5 in a mucosal cell line. Thus, alpha- and beta-papillomaviruses differed regarding their ability to infect cells and regulate viral gene expression. These abilities corresponded with their natural host cells and suggested that HPV anatomical tropism could be determined at several steps in the HPV life cycle.

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