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A deletion and point mutation study of the human papillomavirus type 16 major capsid gene

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Abstract

Recombinant human papillomavirus (HPV) virus-like particles (VLPs) made from the major capsid protein L1 are promising vaccine candidates for use as vaccines against genital and other HPV infections, and particularly against HPV-16. However, HPV-16 genotype variants have different binding affinities for neutralising mouse Mabs raised against HPV-16 L1 VLPs. This paper analyses, using a panel of well-characterised Mabs, the effects on the antigenicity of various C- and N-terminal deletants of HPV-16 L1 made in insect cells via recombinant baculovirus, of an A → T mutation at residue 266 (A266T), and of a C → G mutation at conserved position 428 (C428G). The effects of these changes on assembly of the variant L1s were studied by electron microscopy. Binding of Mab H16:E70 to A266T was reduced by almost half in comparison to wild type L1. Retention of the C-terminal region 428–483 was critical for the binding of conformation-specific Mabs (H16:V5, H16:E70, H16:U4 and H16:9A) whereas deletion of the nuclear localisation signal (NLS) or the C428G mutation or an N-terminal deletion (residues 2–9) did not affect the antigenicity. The N-terminal deletion resulted in a mixed population of 30 and 55 nm VLPs, which differs from the same construct expressed in *Escherichia coli*, whereas pentamer aggregates resulted from deletion of the 428–465 region or the C428G mutation. The results have implications both for considering use of single-genotype HPV vaccines, and for design of novel second-generation vaccines.

Keywords: Papillomavirus; HPV-16; VLP; L1; Deletion; Antigenicity

1. Introduction

Papillomaviruses (family: *Papillomaviridae*) are widespread in nature in higher vertebrates, but are highly species-specific (de Villiers et al., 2004). In humans they infect epithelial tissue from the skin, mouth, oesophagus, larynx and anogenital tract (Steller, 2002). To date over 80 different HPV types have been sequenced and there exist more than 100 additional HPV types that are less well-characterised. HPVs are a heterogeneous group of DNA viruses with double stranded closed circular genomes of ∼8 kilobases (kb) (Seedorf et al., 1985). The HPV genome encodes six early open reading frame (ORF) proteins (E1, E2, E4, E5, E6 and E7) with a variety of regulatory functions, and two late ORF proteins (major capsid protein L1 and the minor capsid protein L2). A subset of genital HPVs – most notably HPV-16 – are termed "high risk" as they cause cervical cancer (Clifford et al., 2003; IARC, 1999).

Papillomavirus-like particles (VLPs), made from the major capsid protein L1 alone, or by co-expression of L1 and the minor L2 capsid protein, induce protective immunity in animal models (Breitburd et al., 1995; Jansen et al., 1995; Suzich et al., 1995). Candidate *Saccharomyces cerevisiae*- and insect cell-produced HPV VLP vaccines used in recent clinical trials elicited ∼100% seroconversion and protection from infection and disease caused by types included in the vaccines (Harper et al., 2004; Koutsky et al., 2002; Villa et al., 2005).

However, viruses that are controlled efficiently by neutralising antibodies may mutate to give rise to serotypes or strains that do not necessarily react with neutralising antibodies elicited by other strains (Bachmann and Zinkernagel, 1996). Numerous

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HPV variants have been isolated from different geographical locations and evolution of the variants correlates with migration patterns of early humans (Ho et al., 1991). The variants have been grouped according to homology into five major phylogenetic branches: European, African 1, African 2, North American and Asian American (Chan et al., 1992; Yamada et al., 1995). Amino acid changes in L1 map closely to the principal neutralisation epitopes of the well-characterised Mabs H16:V5 and H16:E70 (Chen et al., 2000), suggesting that the variants evolved to escape neutralisation (Pastrana et al., 2001). In support of this hypothesis, the H16:E70 Mab neutralised a European (114K) but not an Africa 2 (Z-1194) pseudotype virion (Roden et al., 1997) and a single amino acid change caused loss of H16:V5 binding (White et al., 1999). The H16:V5 Mab has previously been shown to block much of the neutralising activity of human immune serum (Wang et al., 1997), and immunodominant neutralising serum or monoclonal antibodies mainly recognise conformational epitopes (Christensen et al., 1994; Christensen and Kreider, 1990; Roden et al., 1994). Thus, point mutations could potentially seriously affect the efficacy of single-serotype HPV-16 vaccines, and it is therefore important to determine the effects of mutations on neutralisation.

In the search for novel vaccines against HPV types, it is important to explore the effects of deletions on the assembly properties of the L1 protein, as more efficiently assembling L1 or more stable polymers may make better vaccines. Chen et al. (2000) showed that a 10 residue N-terminal deleted L1 (ΔN10) expressed in *Escherichia coli* forms T = 1 particles, and addition of a glycine to ΔN10 causes reversion to T = 7 particle assembly. The ΔN10 T = 1 and full length L1 T = 7 particles were stable at high ionic strength over a pH range of 4–7.5; the L1 T = 7 particles were slightly unstable at pH 7.5. The PVs have a bipartite nuclear localisation signal (NLS; KRK1atpttssttaKRKKRKL2); the HPV-16 L1 NLS is between residues 484 and 505 (Zhou et al., 1991). Deletion of 30 C-terminal residues from L1 does not affect assembly into T = 7 particles (Chen et al., 2001). The cysteine at residue 428 is highly conserved among all papillomaviruses and forms disulphide bonds with residue 175 in T = 7 particles. This is not the case in T = 1 particles since the two cysteines are too far apart: interpentameric interaction here is attributed to helices h3, h4 and h5. Mutations of these cysteines results in the formation of capsomers rather than VLPs (Fligge et al., 2001; Li et al., 1998; McCarthy et al., 1998; Sapp et al., 1998).

A study on canine oral papillomavirus (COPV) L1 indicated that a C-terminal deletion of 67 residues and an N-terminal deletion of 25 residues allowed for VLP formation, but that conformation-specific antibodies only bound particles resulting from C-terminal deletion of 26 residues or less (Chen et al., 1998). Chen et al. (2000), who solved the crystal structure of HPV-16 L1, reported that N-terminal deletions of 15 and 20 residues and C-terminal deletions of 46 and 86 residues rendered the gene product sensitive to trypsin, therefore more prone to proteolytic degradation. Digestion of the recombinant HPV-11 L1 and protein with trypsin yielded two major cleavage products: a ∼42 kDa product resulting from the cleavage at R416 (R420 for HPV-16) and an intermediate species of ∼48 kDa likely to result from the cleavage at R462 (R466 for HPV-16) (Li et al., 1997). Thiol reduction renders the HPV VLPs trypsin sensitive where only a 42 kDa cleavage product is observed.

This paper reports an investigation of the antigenicity (the ability to bind various well-charaterised monoclonal antibodies) of HPV-16 L1 variants produced by mutation and/or deletion of a native HPV-16 L1 gene. The antigenicity characterisation indicates the display of the various epitopes that the monoclonal antibodies bind. The HPV-16 L1 sequence used for this work (GenBank accession number AY177679) is identical in amino acid sequence to variant 114K and differs from the Phi sequence published by Touze et al. (1998) at residue 266 (Ala in 114K; Thr in Phi1). Since Phi1 has been demonstrated to yield more VLP product than 114K, it was necessary to investigate the antigenicity of this variant.

Second, we report an investigation of the effects of various deletions, both C- and N-terminal, together with mutation of the conserved cysteine at residue 428 specifically for HPV-16, on the L1 antigenicity. The various deletion mutants were designed based on studies by Zhou et al. (1991; for Δ483), Li et al. (1997; for mutant Δ465, 1998), Fligge et al. (2001) and Sapp et al. (1998; for mutants Δ428). In addition, the mutation of the cystein at residue 428 to glycine was based on studies by Fligge et al. (2001), Li et al. (1998) and Sapp et al. (1998) and the ΔN10 deletion mutants were based on studies by Chen et al. (2000, 2001). However, these reported studies were for HPV-16 (Chen et al., 2000, 2001; Zhou et al., 1991), HPV-11 (Li et al., 1998), HPV-33 (Fligge et al., 2001; Sapp et al., 1998) and COPV (Li et al., 1997). This paper describes the investigation of all these mutants in HPV-16 L1 in various combinations.

Both these variant and deletion/mutation studies were carried out to prospect for suitable HPV-16 L1 gene sequences for use as new candidates for vaccine development where the capsid proteins in their folded states maintained the binding of Mab H16:V5, e.g. chimaeric vaccines (Greenstone et al., 1998; Liu et al., 2000, 2002; Muller et al., 1997; Nieland et al., 1999; Slupetzky et al., 2001; Varsani et al., 2003a,b). In addition our lab in particularly interested in plant-produced vaccines and we get recombinational deletion of the L1 gene during transient expression with tobacco mosaic virus (TMV; Varsani et al., 2006) in the later stages on infection. This is partly due large gene sizes that are not well-tolerated by the TMV and hence shorter genes that are potentially immunogenic could be used in such applications.

2. Materials and methods

2.1. Synthesis of deletion and mutant constructs

The HPV-16 L1 gene used here (South African isolate; GenBank accession number AY177679) has been described previously (Varsani et al., 2003a,b). The native HPV-16 L1 gene (referred to as L1 505 throughout) in pSK plasmid (pSK-L1-SA) was mutated so as to change amino acid residue 266 from an alanine to a threonine (A266T), by polymerase chain reaction (PCR) mutagenesis using overlapping primers described in Table 1. Deletion constructs were prepared by PCR amplification of the appropriate region of the HPV-16 L1 gene in the pSK-L1-

Table 1
Summary of neutralising monoclonal antibodies (Mabs) raised to HPV-16 capsid proteins and their characterised epitopes

Mab name	Capsid protein/epitopes	Epitope type	Reference
H16:V5	L1: F50, A266, S282	Conformational/neutralising	Christensen et al. (1996); Roden et al. (1997); White et al. (1999); Combita et al. (2002)
H16:E70	L1: F50, A266, S282	Conformational/neutralising	Christensen et al. (1996); Roden et al. (1997); White et al. (1999); Combita et al. (2002)
H16:U4	L1: unknown	Conformational/neutralising	Christensen et al. (1996); Roden et al. (1997); White et al. (1999); Combita et al. (2002); Carter et al. (2003)
H16:J4	L1: 268–281	Linear/neutralising	Christensen et al. (1996); Combita et al. (2002)
H16:I23	L1: 111–130	Linear/neutralising	Christensen et al. (1996); Combita et al. (2002)
H16:9A	L1: 1–173	Conformational/neutralising	Christensen et al. (2001)
H16:D9	L1: unknown	Linear	Christensen et al. (1996)

SA and cloning into a pGEM® T Easy vector system (Promega). C-terminal deletants are designated according to amino acid site adjacent to the deletion, and end deleted: thus, ΔC483 indicates a C-terminal truncation up to aa 483. N-terminal truncations all retained an N-terminal Met residue: these are designated as (e.g.) M-ΔN10. The mutation of residue 428 from cysteine to glycine of the various deletion mutants in pGEM® T Easy vector and of L1 505 in pSK vector were carried out by PCR using overlapping primers. As this abolishes VLP assembly while allowing pentamer formation, mutants were designated as pen (e.g. pen505 and penΔC483). Deletion and mutant constructs prepared for this study are summarised in Fig. 1.

2.2. Baculovirus expression of the deletant and mutant HPV-16 L1s in Sf21 insect cells

The deletion and mutation products ΔC483, ΔC465, ΔC427, M-ΔN10, M-ΔN10ΔC483, M-ΔN10ΔC465, M-ΔN10ΔC427, penΔC483, penΔC465, penM-ΔN10, penM-ΔN10ΔC483 and penM-ΔN10ΔC465 (see Fig. 1) cloned in the pGEM® T Easy vector were directionally subcloned into pFastBac1® vector (Life Technologies) into the *Not*I restriction site. The full length HPV-16 L1 (505), pen505 and A266T were directionally cloned into pFastBac1 using *Sal*I/*Xho*I restriction

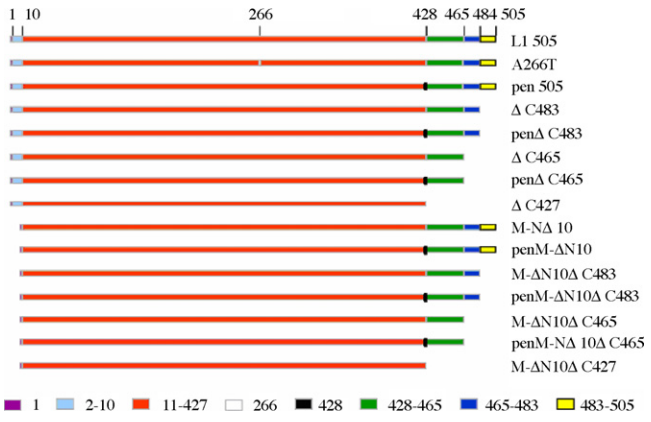

Fig. 1. Illustration of the HPV-16 L1 deletant and mutant constructs. pen, mutation of cysteine at 428 to glycine; M-ΔN, N-terminal deletion of residues 2–10; ΔC, C-terminal deletions; L1 504, South African HPV-16 isolate L1; A266T, mutation of alanine at residue 266 in L1 504 to threonine.

sites. The resulting pFastBac1® constructs were used to transfect DH10bac *E. coli* cells for the preparation of bacmid clones. Bacmid DNA was transfected into *Spodoptera frugiperda* (*Sf*21) cells (Life Technologies) using Cellfectin (Life Technologies).

The Bac-to-Bac® protocol was followed to amplify the recombinant virus. For analysis of the variant L1 gene products, five insect cell tissue culture 75 cm^2 flasks on average per recombinant baculovirus construct were seeded with 6×10^6 *Sf*21 cells and infected with 40 μl of the respective recombinant baculovirus amplified stock in a total of 10 ml of complete media. The flasks were incubated at 27 °C for 48–72 h. The infected cells were harvested by washing and centrifugation at ~4000 × *g*.

2.3. Purification of deletion and mutant HPV-16 L1s expressed in Sf21 cells

The harvested infected *Sf*21 cells were resuspended in high salt phosphate-buffered saline (PBS/0.5 M NaCl) and sonicated four times at 5 s intervals. The sonicated material was overlaid onto a 40% sucrose cushion and pelleted at 100,000 × *g* for 3 h. The pellet was resuspended in PBS with 0.4 g/ml CsCl with sonication (four times with 5 s intervals). The suspension was centrifuged at 100,000 × *g* at 10 °C for 24 h in a Beckman SW50.1 rotor. Two distinct bands were observed in most cases and the top bands were extracted and dialysed overnight against PBS at 4 °C. In cases where no clear band but a diffused zone was observed, 500 μl fractions were collected and analysed by ELISA using Mab H16:J4 (binds linear epitope 261–280), the positive fractions were pooled and dialysed as above.

2.4. Production of cottontail rabbit papillomavirus (CRPV) L1

The CRPV L1 gene (kindly provided by Dr. N. Christensen, Penn. State University) was PCR amplified using the following set of primers: forward 5′-TTAATTAAATGGCAGTGTGGCTGTCTACG-3′ (TTAATTAA—*Pac*I); reverse 5′-CTCGAGTTAAGTACGTCTCTTGCGTTTAGATGATTTC-3′ (CTCGAG—*Xho*I).

The amplified gene was cloned into pGEM® T Easy vector and sequenced. The CRPV L1 gene was directionally cloned

into pFastBac1® vector into the *Eco*RI restriction sites and the procedure outlined above was followed to amplify the recombinant baculovirus and purify the CRPV L1 VLPs.

2.5. Indirect immunofluorescence microscopy

Glass sides were soaked overnight in 70% ethanol/1% HCl, washed thoroughly with distilled water and baked for 20 min at 100 °C. The slides were transferred to sterile Petri dishes and seeded with 0.5 × 106 *Sf*21 cells in 500 μl complete media for 1 h; the media was replaced with 200 μl of amplified virus stock and incubated for a further hour at room temperature. The inoculum was removed and replaced with 500 μl of complete media and incubated overnight at 27 °C in a moist environment.

Uninfected cells and cells infected with recombinant baculovirus expressing GUS (Eric van der Walt, this laboratory) were used as controls. The immobilised *Sf*21cells were fixed in methanol at −20 °C for 5 min. The slides were washed with PBS and incubated for 1 h at room temperature in a moist environment with Mabs H16:V5 and H16:J4 (500 μl per slide) diluted 1:250 in 1% blocking solution (non-fat milk powder in PBS). Slides were washed for 10 min in PBS and incubated with FITC labelled secondary goat anti-mouse antibody (150 μl per slide) diluted 1:40 in blocking solution with Evans Blue dye (1:20 dilution in blocking solution) for 1 h in a dark moist environment. The slides were washed three times with PBS over 20 min and mounted with a drop of Vector Shield (Vector Laboratories). The slides were viewed with a Diaphot (TMD) inverted microscope using the Nikon DM510 B2 filter and Fluor 20 Ph3DL lens. Images were captured using an Zeiss AxioCam digital camera. See supplementary data for results.

2.6. Characterisation of the HPV-16 L1s by ELISA and Western blot

A dilution series in PBS of purified L1 variants 505, pen505, ΔC483, penΔC483, M-ΔN10, penM-ΔN10, M-ΔN10ΔC483, ΔC465, penΔC465, M-ΔN10ΔC465 and ΔC427 were tested against Mab H16:J4, and dilutions that gave similar absorbances by ELISA were selected in order to normalise the concentrations. The normalised dilutions were used for further antibody characterisation by ELISA. The protein products were coated onto ELISA plates overnight at 4 °C. The plates were washed four times with PBS and blocked with 1% non-fat milk. The monoclonal antibodies (binding sites summarised in Table 1) were diluted at 1:500 in PBS/milk and allowed to bind to the protein products for 2 h at room temperature followed by four stringent washes. The bound primary antibody was probed with the alkaline phosphatase labelled goat anti-mouse secondary antibody. The binding was detected using *p*-nitrophenyl phosphate (Sigma) and the ELISA plate absorbance was read at 405 nm using a Titrex ELISA plate reader.

The cell lysates from the expression of L1s 505, ΔC483, ΔC465 and ΔC427 in addition to a negative control (ROPV L2) were denatured for 10 min at 100 °C in SDS disruption solution. The denatured lysates (10 μl) were resolved on a 10% SDS-PAGE gel. The resolved gel was transferred onto a nitrocellulose membrane by semi-dry electrophoresis (Bio-Rad) for 25 min at 25 V. The membrane was blocked using 1% non-fat milk for 2 h and incubated with Mab H16:D9 at a dilution of 1:250 overnight at 4 °C. The membrane was washed with PBS/0.05% Tween-20 and probed with alkaline phosphatase-labelled secondary goat anti-mouse antibody diluted 1:2000, for 1 h at room temperature. Reaction was detected colorimetrically using 5-bromo-4-chloro-3-indoyl-phosphate (BCIP) and 4-nitro blue tetrazolium chloride (NBT) substrates.

2.7. Transmission electron microscopy

The various L1 preparations were viewed either after direct adsorption onto carbon coated copper grids or after immunotrapping with preadsorbed H16:J4 Mab (binds linear epitope residues 261–280) at a dilution of 1:50 onto carbon coated copper grids. The Mab was incubated with the carbon coated copper grids for 15 min, washed and the incubated with the purified gene products for 10 min. The grids were washed with water and stained with 2% uranyl acetate and viewed using a JEOL 200CX transmission electron microscope.

3. Results

3.1. HPV-16 L1 505 and A266T gene expression and antigenicity analysis

The VLPs resulting from the expression of the L1 proteins 505 and A266T in insect cells by means of recombinant baculovirus were analysed for antigenicity using a panel of well-characterised Mabs that bind neutralising, conformational and linear epitopes by direct ELISA assays (Fig. 2). Specificities of the Mabs are as follows: conformational epitope binding and neutralising Mabs H16:V5 and H16:E70 bind L1 aa residues F50, A266, S282; H16:U4 binds between aa 427 and 445;

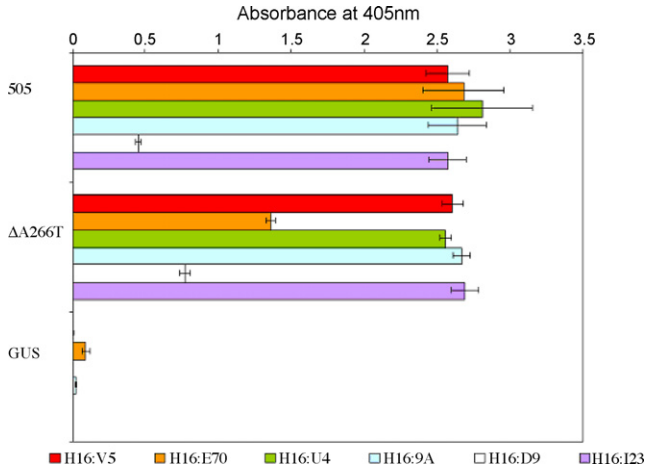

Fig. 2. Antibody characterisation by ELISA of VLPs resulting from the expression on of HPV-16 L1 504 and A266T in insect cells by recombinant baculovirus using a panel of well-characterised Mabs. A two-fold higher binding to H16:E70 is observed for 504 compared to A266T.

Table 2
List of primers used to synthesise the deletion and mutation constructs pf HPV-16 L1

Construct	Primers
505[a]	Forward: 5′-*CCCGGG*ATGTCTTTGGCTGCCTAG-3′ Reverse: 5′-*GCCTCGAC*TTACAGCTTACGTTTTTGC-3′
A266T[b]	Forward: 5′-TTATAGGGCTGGTACTGTTGGTGAAAATGTACCAGACG-3′ Reverse: 5′-*GGTACA*TTTTCACCAACAGTACCAGCCCTATTAAATAAATG-3′
ΔC483	Forward: 5′-*TTAATTAA*ATGTCTCTTTGGCTGCCTAGTGAGG-3′ Reverse: 5′-*CTCGAG*TTATCCTAATGTAAATTTTGGTTTGGCC-3′
ΔC465	Forward: 5′-*TTAATTAA*ATGTCTCTTTGGCTGCCTAGTGAGG-3′ Reverse: 5′-*CTCGAG*TTATCCTAAAGGAAACTGATCTAGGTCTGC-3′
ΔC427	Forward: 5′-*TTAATTAA*ATGTCTCTTTGGCTGCCTAGTGAGG-3′ Reverse: 5′-*CTCGAG*TTAAGCAATTGCCTGGGATGTTACAAACC-3′
M-ΔN10	Forward: 5′-*TTAATTAA*ATGGTCTACTTGCCTCCTGTCCCAG-3′ Reverse: 5′-*CTCGAG*TTACAGCTTACGTTTTTTGCGTTTAGCAGTTGTAG-3′
M-ΔN10ΔC483	Forward: 5′-*TTAATTAA*ATGGTCTACTTGCCTCCTGTCCCAG-3′ Reverse: 5′-*CTCGAG*TTATCCTAATGTAAATTTTGGTTTGGCC-3′
M-ΔN10ΔC465	Forward: 5′-*TTAATTAA*ATGGTCTACTTGCCTCCTGTCCCAG-3′ Reverse: 5′-*CTCGAG*TTATCCTAAAGGAAACTGATCTAGGTCTGC-3′
M-NΔ10CΔ427	Forward: 5′-*TTAATTAA*ATGGTCTACTTGCCTCCTGTCCCAG-3′ Reverse: 5′-*CTCGAG*TTAAGCAATTGCCTGGGATGTTACAAACC-3′
pen[c]	Forward: 5′-GTAACATCCCAGGCAATTGCT**GGA**CAAAAACATACACCTC-3′ Reverse: 5′-GCTGGAGGTGTATGTTTTTG**TCC**AGCAATTGCCTGGGATG-3′

CTCGAG, *Xho*I; *TTAATTAA*, *Pac*I; *CCCGGG*, *Xma*I/*Sma*I; *CTCGAC*, *Sal*I; 5′-**GGA**-3′/5′-**TCC**-3′, glycine.
[a] Primers used to amplify the HPV-16 L1 from a patient biopsy for cloning into pSK vector.
[b] Mutation of alanine residue 266 to threonine.
[c] Mutation of cysteine residue 428 to glycine.

H16:9A between 1 and 173; linear epitope binding and neutralising Mabs H16:J4 between 268 and 281and H16:I23 between 111 and 130; H16:D9 binds an unknown epitope (Carter et al., 2003; Christensen et al., 1996, 2001; Combita et al., 2002; Roden et al., 1997; White et al., 1999).

The respective binding affinities of Mabs H16:V5, H16:U4, H16:9A and H16:I23 to 505 and A266T VLPs were almost identical. However, Mab H16:E70 had almost twice the binding affinity for L1 505 than for A266T VLPs. The higher affinity of Mab H16:D9 for A266T preparations indicates that there was probably a larger amount of monomeric/denatured L1 in this extract as H16:D9 binds only to denatured L1 (Christensen et al., 1996).

No significant difference in the particle morphology was seen for L1 505 and A266T VLPs by electron microscopy. Due to the greater binding affinity for H16:E70, it was decided to use the HPV-16 L1 505 gene sequence for the deletion and point mutation study.

3.2. Synthesis and expression of deletion and point mutation constructs of HPV-16 L1

Four potential deletion regions were identified, one at the N-terminus and three at the C-terminus. Based on some of these key features of the HPV-16 L1, seven deletion constructs were synthesised, covering various permutations and combinations of deletion, by PCR amplification using various sets of primers. These are shown in Table 2.

All the deletion and mutation constructs were successfully expressed in insect cells using recombinant baculoviruses: expression was confirmed by indirect immunofluorescence using Mabs H16:J4 and H16:V5. Results of the indirect immunofluorescence of the deletion and mutant constructs are summarised in Table 3. Constructs 505, ΔC483, M-ΔN10,

Table 3
Indirect immunofluorescence microscopy of the deletion and mutant HPV-16 L1s expressed in insect cells

Construct	Mabs		Localisation	
	H16:J4	H16:V5	Nuclear	Cytoplasmic
505	+	+	+	−
pen505	+	+	+	−
M-ΔN10	+	+	+	−
penM-ΔN10	+	+	+	−
Δ483	+	+	−	+
penΔ483	+	+	−	+
M-ΔN10Δ483	+	+	−	+
penM-ΔN10Δ483	+	+	−	+
Δ465	+	−	−	+
penΔ465	+	−	−	+
M-ΔN10Δ465	+	−	−	+
penM-ΔN10Δ465	+	−	−	+
Δ427	+	−	−	+
M-ΔN10Δ427	+	−	−	+
GUS	−	−	n/a	n/a
Negative	−	−	n/a	n/a

n/a: not applicable.

M-ΔN10ΔC483, pen505, penΔC483, penM-ΔN10 and penM-ΔN10ΔC483 bound both the conformationally dependent neutralising Mab H16:V5 and Mab H16:J4 (recognises a linear epitope residues 261–280). The rest of the constructs bound only H16:J4. This data indicated that the C-terminal region residues 428–483 are important for maintaining the correct conformation of the particles formed, if any, as a result of the C-terminal deletions. Since the binding of H16:V5 is retained by the mutation of the cysteine at residue 428 in the constructs 505, ΔC483, M-ΔN10, M-ΔN10ΔC483, pen505, penΔC483, penM-ΔN10 and penM-ΔN10ΔC483, this indicates that the quaternary structure, probably at pentameric level, is not altered by this mutation.

The L1s were purified using CsCl gradient centrifugation and used in the antibody characterisation and electron microscopy analysis. Indirect immunofluorescence showed that the L1 product in the case of 505, M-ΔN10, pen505 and penM-ΔN10 was localised in the centre of the *Sf*21cells, presumably in the nucleus (not shown). For the constructs that did not have the NLS, the L1 product seemed to be diffused throughout the cell.

3.3. Characterisation of the deletant and mutant HPV-16 L1s using a panel of Mabs

The indirect immunofluorescence (see supplementary data) gave baseline data of the antigenicity of the various HPV-16 L1 gene products as well as of localisation in the *Sf*21 insect cells. It was nonetheless important to determine the full antigenicity profile of some of these products. The ELISA binding results with a panel of Mabs are summarised in Fig. 3. Gene products from constructs 505, ΔC483, M-ΔN10, M-ΔN10ΔC483, pen505, penΔC483, penM-ΔN10 and M-ΔN10ΔC483 bound all the Mabs used in this study whereas ΔC465, penΔC465 and M-ΔN10ΔC465 bound only the Mabs that recognise linear epitopes (H16:D9, H16:J4 and H16:I23). ΔC427 only bound two, H16:J4 and H16:I23, of the three Mabs that recognise a linear epitopes. Furthermore, the significant binding of H16:D9 to all the deletion and full length constructs except ΔC427 indicates the presence of significant quantities of unassembled L1 protein in the preparations.

The ΔC427 protein bound Mab H16:D9 to a barely detectable level; thus it is possible that the as yet undetermined binding region of Mab H16:D9 is between residues 428 and 465. Since H16:D9 apparently binds only to denatured HPV-16 L1 protein, Western blot analysis of the cell lysate from the expression of 505 (56.2 kDa), ΔC483 (53.7 kDa), ΔC465 (51.7 kDa) and ΔC427 (47.3 kDa) genes in *Sf*21 cells using recombinant baculovirus was performed. The Western blot results show the loss in binding for H16:D9 ΔC427 protein (Supplementary data, Fig. II).

3.4. Electron microscopy of the HPV-16 L1 variants

The purified L1 proteins were viewed under the electron microscope by immunotrapping with H16:V5 and H16:J4 Mab (Fig. 4). In general H16:J4 was found to be better for immunotrapping. VLPs of ∼55 nm diameter (presumably T = 7) were the predominant populations seen for proteins 505 and ΔC483. Mixed population of 55 nm VLPs and 30 nm (presumptive

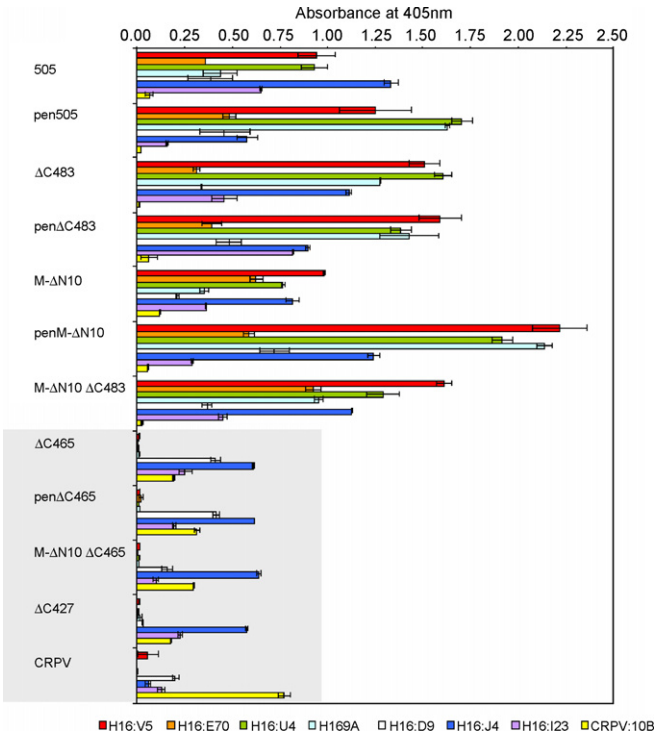

Fig. 3. Antibody binding characterisation by ELISA of some of the purified deletant and mutant HPV-16 L1 proteins, using a panel of Mabs. CRPV L1 was used as a negative control. ELISA data highlighted by the grey box show the constructs that did not bind any of the conformational-specific Mabs (H16:V5, H16:E70, H16:U4 and H16:9A).

T = 1) diameter particles were seen for M-ΔN10 and M-ΔN10ΔC483. Most of the particles observed for ΔC465 and M-ΔN10ΔC465 were aggregates of pentamers and/or partially assembled particles. Predominantly aggregates of pentamers were observed for ΔC427 and M-ΔN10ΔC427 (Fig. 4B). The mutation of the cysteine at residue 428 favoured the formation of capsomers (∼10 nm diameter) for pen505, penM-ΔN10, penΔC483 and penM-ΔN10ΔC483 whereas for pen465 and penM-ΔN10ΔC465 aggregates of pentamers were observed. Results are summarised in Table 3.

4. Discussion

The primary objective of a prophylactic HPV VLP-based vaccine is to elicit a protective immune response as a result of the induction of virus neutralising antibodies. A variety of neutralising epitopes of the HPV-16 major capsid protein L1 have been characterised: Christensen et al. (1996) in particular have generated a panel of monoclonal antibodies against HPV-16 to analyse surface and buried capsid epitopes. Most of the linear epitopes were identified using synthetic peptides. Mabs H16:V5, H16:E70 and H16:U4 were found to be type-specific and conformation-dependent and neutralised pseudotype HPV-16 virions (Combita et al., 2002; Roden et al., 1997). Mab H16:V5 completely blocked more than 75% of the serological reactivity of human immune sera (HPV-16 infected) to HPV-16 L1/L2 VLPs, whereas H16:E70 blocked considerably less well (Wang et al., 1997). This suggested that the binding site of the

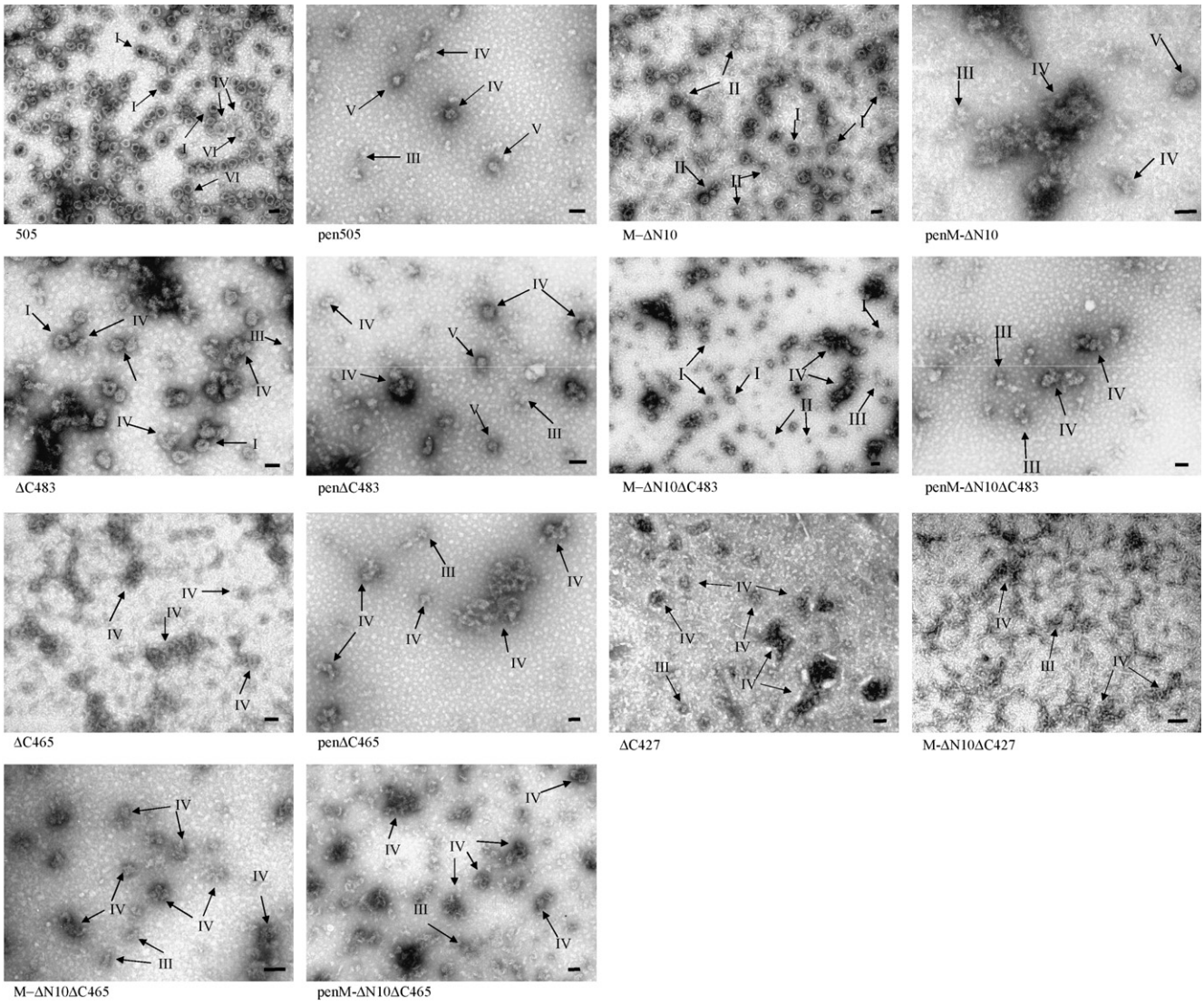

Fig. 4. Electron micrographs of purified proteins of L1s 505, pen505, ΔC483, penΔC483 M-ΔN10, penM-ΔN10, M-ΔN10ΔC483, penM-ΔN10ΔC483 ΔC465 and penΔC465, M-ΔN10ΔC465 penM-ΔN10ΔC465, M-ΔC427 and M-ΔN10ΔC427 immunotrapped with Mab H16:J4. Bar = 50 nm. (I) VLPs (55 nm diameter); (II) small VLPs (30 nm diameter) characterised by Chen et al. (2000); (III) free pentamers (approximately 10 nm diameter); (IV) pentamer aggregates/partially assembled particles; (V) capsomers of approximately 30 nm diameter; (VI) vlps in a state of disassembly/assembly.

H16:V5 is similar to the immunodominant site recognised by the human sera following infection. Residues F50, A266 and S282 were found to be critical for the binding of Mabs H16:E70 and H16:V5 (Roden et al., 1997; White et al., 1999). However, residue F50 is not surface exposed and hence it is proposed that mutating this residue might result in conformational changes that alter the binding efficiency of Mab H16:V5 to HPV-16 L1 VLPs. Carter et al. (2003) discovered that Mab H16:U4 binds to the C-terminal arm of the L1 (residues 427–445).

Combita et al. (2002) demonstrated linear neutralising epitopes on the HPV-16 L1 capsid protein: these are the epitopes that bind the Mabs H16:J4 (residues 261–280) and H16:I23 (residues 111–130). Sera raised to the two linear epitopes were also found to be cross-neutralising: antibodies to 111–130 reacted with HPV-31, 33, 58 and 45 whereas antibodies to 261–280 reacted with HPV-45 and 59. The epitopes of Mabs H16:V5, H16:E70, H16:U4, H16:J4 and H16:I23 are all surface exposed on the head and crown of the pentamer.

In a study to determine the yield of VLPs from the expression of 6 HPV-16 variants by recombinant baculovirus in insect cells, Touze et al. (1998) showed that Phi1 sequence generated the greatest yield of VLPs, 79 times more relative to Fra25. Therefore, this indicates that there are certain elements in the HPV-16 variant gene sequences that play a key role in the assembly of major capsid particles. The HPV-16 L1-SA gene used here is identical to 114K in predicted amino acid sequences, and varied from the Phi1 sequence by one residue (A in South African isolate and T in Phi1) at position 266.

Roden et al. (1997) demonstrated that a HPV-16 variant (Z-1194) with seven residues differing from AY177679 (Y76, D176, T181, A266, P282, P353 and F475) lost Mab H16:E70 binding; further analysis of various mutants showed that the

serine at residue 282 was necessary to maintain the binding of H16:E70. It was therefore thought necessary to analyse the antigenicity of the VLPs resulting from the L1 505 and a mutant A266T which encodes identical amino acid sequence to Phi1. Residue 50 is not fully surface exposed, whereas the other key residues involved in the binding of neutralising Mabs H16:V5 and H16:E70 are surface exposed, and majority of the other amino acid variants are on the surface. This supports their notion that papillomaviruses may be subject to evolutionary pressure to escape neutralisation (Roden et al., 1997). The evolution of greater than 80 HPV genotypes is consistent with this hypothesis.

The comparison of the antigenicity (Fig. 2) of the two variants 505 and A266T clearly indicates that A266 enhances the binding of H16:E70 by almost two-fold compared to T266. White et al. (1999) carried out a study to map the H16:V5 and H16:E70 epitopes and showed that the variant GU-1 (differs from AY177379 at G41, Y76, N176, T181, P282, P353 and F474) was unable to bind Mab H16:E70, whereas GU-2 (differs from AY1773979 at L50, T266 and N380) was unable to bind both H16:E70 and H16:V5 Mabs. The mutation of L50 to F in GU-2 completely restored Mab H16:V5 and partially restored Mab H16:E70 binding; full binding of the latter was achieved by mutating the T 266 to A (White et al., 1999).

The indirect immunofluorescence data for *Sf*21 cells infected with recombinant baculoviruses provides a good basis in terms of antigenicity of the variant L1 products. One obvious result of the deletions and mutations of L1 genes is that the proteins ΔC465, M-ΔN10ΔC465, ΔC428 and M-ΔN10ΔC428 have disrupted H16:V5 binding sites. Another is that the N-terminal deletion had no affect on the binding of Mab H16:V5, and that the deletion of the NLS clearly prevents the localisation of the protein L1 protein product into the nucleus (Figs. 3 and 4) confirming previous work (Zhou et al., 1991). Further, mutation of the C 428 to G does not affect the Mab H16:V5 binding, indicating that capsomeric pentamers potentially maintain or share the same epitope conformation as intact VLPs. It is possible that these protein products could elicit a neutralising immune response similar to that reported for HPV-33 L1 capsomers (Fligge et al., 2001). The indirect immunofluorescence is unique tool for rapid qualitative analysis of gene expression since the gene products are not lost during purification.

The antibody characterisation of the various purified L1s (Fig. 3) revealed that the conformation of proteins ΔC465, penΔC465, penM-ΔN10Δ465 and ΔC428 is altered since none of the conformation-specific Mabs (H16:V5, H16:E70, H16:U4 and H16:9A) bind significantly to them. Our data also agrees with other published material (Carter et al., 2003) which suggests that the binding region for Mab H16:U4 is on the C-terminal arm (Fig. 4). These results, in addition to those from the indirect immunofluorescence of the infected *Sf*21 cells, suggest that the region 428–475 is critical for maintaining the correct conformation. In both the T = 1 and T = 7 models of HPV-16 L1, the residues 445–474 interact with the core of the pentamer, the interaction being intrapentameric for T = 1 and interpentameric for T = 7, thus providing stability to the pentamers in the former case. The C-terminal deletion ΔC465 results in the partial destruction of the h5 helix that docks into the intersubunit gap, and the loss of these important residues definitely seems to distort the overall stability of the pentamers.

Further C-terminal deletions up to residue 428 destabilise the pentamers to a greater extent since the disulphide bond between cysteine residues 175 and 428 is destroyed; the invading C-terminal arm in the T = 7 particles therefore has minimal contact with the neighbouring pentamer. As a result the pentamers are probably distorted, thus the loss in binding to all the conformation-specific Mabs.

A degree of antigenic cross-reactivity between the CRPV L1 VLPs, HPV-16 L1 VLPs and the deletion/mutant proteins was observed for the anti-HPV-16 L1 Mabs that bound linear epitopes and the anti-CRPV L1 Mab (Fig. 3). Multiple sequence alignments of various PV genotypes at the binding regions of Mabs H16:J4 and H16:I23 show conserved regions, thus possibly explaining the cross-reactivity. The epitopes for Mabs H16:D9 and CRPV:10B have not been characterised so far but the antibody binding data (Fig. 3) with support from the Western blot analysis (Supplementary data, Fig. II) suggests that the inability of ΔC427 to bind Mab H16:D9 and indicates that the epitope is located in the region 428–465.

The electron microscopy data clearly indicates that for VLP assembly the C-terminal region 428–465 and the complete h5 helix are required. The majority of ΔC465, penΔC465, ΔC427, M-ΔN10ΔC465, penM-ΔN10ΔC465 and M-ΔN10ΔC427 deletion mutants (Fig. 4) form pentamers, and these pentamers, based on the antigenicity data, have an altered conformation. (Li et al., 1997) showed that HPV-11 L1 particles resulting from the cleavage of the R415 had a pentameric morphology but were unable to assemble further. This cleavage in the case of HPV-16 would be at residue R420 therefore supporting the observation of pentamers for deletants ΔC427 and M-ΔN10ΔC427.

Chen et al. (2000, 2001) demonstrated that a 10 residue N-terminal deletion in L1 protein expressed in *E. coli* and assembled at low pH and high ionic strength results in T = 1 particles of 30 nm diameter, and that the addition of either a glycine or a threonine residues to the NΔ10 results in T = 7 particles of 55 nm. In our study, the particles of M-ΔN10 and M-ΔN10ΔC483 – surprisingly – assemble in two different conformations, T = 1 and T = 7 (Fig. 4). This observation therefore apparently contradicts what was demonstrated by Chen et al. since the M-ΔN10, based on their data, should assemble into T = 1 particles. One explanations for this observation lies in the conditions under which the assembly takes place, and the expression systems: low pH (4–5.6) and high salt conditions were used to assemble the T = 1 particles which probably alters the conformation of the N- or C-terminal residues to accommodate for T = 1 assembly; moreover, *E coli* and baculovirus expression systems differ very considerably in their suitability for expression of eukaryotic proteins in terms of facilitation of folding and quaternary interactions, which could also affect final products. Sandalon and Oppenheim (1997) observed a heterogeneous population of particles (20–45 nm in diameter) of SV40 in insect cells. Hence, the second explanation for the size difference for the M-NΔ10 is the ‘mis-assembly’ of the particles. In this study assembly will have occurred under essentially physiological conditions.

The mutation of the cysteine to a glycine at residue 428 does not affect the antigenicity of the particles, thereby implying that the capsomers and pentamers are appropriately antigenic for elicitation of neutralising antibodies. This mutation probably destabilises the T = 7 particles, although does not prevent assembly since capsomers of approximately 10–30 nm in diameter were observed (Fig. 4).

To conclude, the deletion and mutation study has provided a very good understanding of the antigenicity of the various gene products. We have shown that a trade-off exists between higher yield versus appropriate antigenicity in relation to HPV-16 L1 variants. The 10 residue N-terminal region has no affect on the antigenicity of the particles as assessed by the panel of Mabs, but would result in a mixed population of VLPs. Large scale purification of these mixed population of 55 and 30 nm particles could be possibly laborious. However, deletion of the NLS has no impact on the assembly or the antigenicity and hence could potentially be used for further candidate HPV vaccine development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2006.07.012.

References

Bachmann, M.F., Zinkernagel, R.M., 1996. The influence of virus structure on antibody responses and virus serotype formation. Immunol. Today 17, 553–558.

Breitburd, F., Kirnbauer, R., Hubbert, N.L., Nonnenmacher, B., Trin, D.D., Orth, G., Schiller, J.T., Lowy, D.R., 1995. Immunization with viruslike particles from cottontail rabbit papillomavirus (CRPV) can protect against experimental CRPV infection. J. Virol. 69, 3959–3963.

Carter, J.J., Wipf, G.C., Benki, S.F., Christensen, N.D., Galloway, D.A., 2003. Identification of a human papillomavirus type 16-specific epitope on the C-terminal arm of the major capsid protein L1. J. Virol. 77, 11625–11632.

Chan, S.Y., Bernard, H.U., Ong, C.K., Chan, S.P., Hofmann, B., Delius, H., 1992. Phylogenetic analysis of 48 papillomavirus types and 28 subtypes and variants: a showcase for the molecular evolution of DNA viruses. J. Virol. 66, 5714–5725.

Chen, X.S., Garcea, R.L., Goldberg, I., Casini, G., Harrison, S.C., 2000. Structure of small virus-like particles assembled from the L1 protein of human papillomavirus 16. Mol. Cell. 5, 557–567.

Chen, X.S., Casini, G., Harrison, S.C., Garcea, R.L., 2001. Papillomavirus capsid protein expression in *Escherichia coli*: purification and assembly of HPV11 and HPV16 L1. J. Mol. Biol. 307, 173–182.

Chen, Y., Ghim, S.J., Jenson, A.B., Schlegel, R., 1998. Mutant canine oral papillomavirus L1 capsid proteins which form virus-like particles but lack native conformational epitopes. J. Gen. Virol. 79, 2137–2146.

Christensen, N.D., Kreider, J.W., 1990. Antibody-mediated neutralization in vivo of infectious papillomaviruses. J. Virol. 64, 3151–3156.

Christensen, N.D., Kirnbauer, R., Schiller, J.T., Ghim, S.J., Schlegel, R., Jenson, A.B., Kreider, J.W., 1994. Human papillomavirus types 6 and 11 have antigenically distinct strongly immunogenic conformationally dependent neutralizing epitopes. Virology 205, 329–335.

Christensen, N.D., Dillner, J., Eklund, C., Carter, J.J., Wipf, G.C., Reed, C.A., Cladel, N.M., Galloway, D.A., 1996. Surface conformational and linear epitopes on HPV-16 and HPV-18 L1 virus-like particles as defined by monoclonal antibodies. Virology 223, 174–184.

Christensen, N.D., Cladel, N.M., Reed, C.A., Budgeon, L.R., Embers, M.E., Skulsky, D.M., McClements, W.L., Ludmerer, S.W., Jansen, K.U., 2001. Hybrid papillomavirus L1 molecules assemble into virus-like particles that reconstitute conformational epitopes and induce neutralizing antibodies to distinct HPV types. Virology 291, 324–334.

Clifford, G.M., Smith, J.S., Aguado, T., Franceschi, S., 2003. Comparison of HPV type distribution in high-grade cervical lesions and cervical cancer: a meta-analysis. Br. J. Cancer 89, 101–105.

Combita, A.L., Touze, A., Bousarghin, L., Christensen, N.D., Coursaget, P., 2002. Identification of two cross-neutralizing linear epitopes within the L1 major capsid protein of human papillomaviruses. J. Virol. 76, 6480–6486.

de Villiers, E.M., Fauquet, C., Broker, T.R., Bernard, H.U., Zur Hausen, H., 2004. Classification of papillomaviruses. Virology 20, 17–27.

Fligge, C., Giroglou, T., Streeck, R.E., Sapp, M., 2001. Induction of type-specific neutralizing antibodies by capsomeres of human papillomavirus type 33. Virology 283, 353–357.

Greenstone, H.L., Nieland, J.D., de Visser, K.E., De Bruijn, M.L., Kirnbauer, R., Roden, R.B., Lowy, D.R., Kast, W.M., Schiller, J.T., 1998. Chimeric papillomavirus virus-like particles elicit antitumor immunity against the E7 oncoprotein in an HPV16 tumor model. Proc. Natl. Acad. Sci. U.S.A. 95, 1800–1805.

Harper, D.M., Franco, E.L., Wheeler, C., et al., 2004. Efficacy of a bivalent L1 virus-like particle vaccine in prevention of infection with human papillomavirus types 16 and 18 in young women: a randomised controlled trial. Lancet 364, 1757–1765.

Ho, L., Chan, S.Y., Chow, V., Chong, T., Tay, S.K., Villa, L.L., Bernard, H.U., 1991. Sequence variants of human papillomavirus type 16 in clinical samples permit verification and extension of epidemiological studies and construction of a phylogenetic tree. J. Clin. Microbiol. 29, 1765–1772.

IARC, 1999. The current status of development of prophylactic vaccines against human papillomavirus infection. Report of a technical meeting. Geneva, 16–18 February 1999, http://www.who.int/vaccines-documents/DocsPDF99/www9914.pdf.

Jansen, K.U., Rosolowsky, M., Schultz, L.D., Markus, H.Z., Cook, J.C., Donnelly, J.J., Martinez, D., Ellis, R.W., Shaw, A.R., 1995. Vaccination with yeast-expressed cottontail rabbit papillomavirus (CRPV) virus-like particles protects rabbits from CRPV-induced papilloma formation. Vaccine 13, 1509–1514.

Koutsky, L.A., Ault, K.A., Wheeler, C.M., Brown, D.R., Barr, E., Alvarez, F.B., Chiacchierini, L.M., Jansen, K.U., 2002. A controlled trial of a human papillomavirus type 16 vaccine. N. Engl. J. Med. 347, 1645–1651.

Li, M., Cripe, T.P., Estes, P.A., Lyon, M.K., Rose, R., Garcea, R.L., 1997. Expression of the human papillomavirus type 11 L1 capsid protein in *Escherichia coli*: characterization of protein domains involved in DNA binding and capsid assembly. J. Virol. 71, 2988–2995.

Li, M., Beck, D.L., Estes, P.A., Lyon, M.K., Garcea, R.L., 1998. Intercapsomeric disulfide bonds in papillomavirus assembly and disassembly. J. Virol. 72, 2160–2167.

Liu, W.J., Liu, X.S., Zhao, K.N., Leggatt, G.R., Frazer, I.H., 2000. Papillomavirus virus-like particles for the delivery of multiple cytotoxic T cell epitopes. Virology 273, 374–382 (in process citation).

Liu, X.S., Liu, W.J., Zhao, K.N., Liu, Y.H., Leggatt, G., Frazer, I.H., 2002. Route of administration of chimeric BPV1 VLP determines the character of the induced immune responses. Immunol. Cell. Biol. 80, 21–29.

McCarthy, M.P., White, W.I., Palmer-Hill, F., Koenig, S., Suzich, J.A., 1998. Quantitative disassembly and reassembly of human papillomavirus type 11 viruslike particles in vitro. J. Virol. 72, 32–41.

Muller, M., Zhou, J., Reed, T.D., Rittmuller, C., Burger, A., Gabelsberger, J., Braspenning, J., Gissmann, L., 1997. Chimeric papillomavirus-like particles. Virology 234, 93–111.

Nieland, J.D., Da Silva, D.M., Velders, M.P., de Visser, K.E., Schiller, J.T., Muller, M., Kast, W.M., 1999. Chimeric papillomavirus virus-like particles induce a murine self-antigen-specific protective and therapeutic antitumor immune response. J. Cell. Biochem. 73, 145–152.

Pastrana, D.V., Vass, W.C., Lowy, D.R., Schiller, J.T., 2001. NHPV16 VLP vaccine induces human antibodies that neutralize divergent variants of HPV16. Virology 279, 361–369.

Roden, R.B., Weissinger, E.M., Henderson, D.W., Booy, F., Kirnbauer, R., Mushinski, J.F., Lowy, D.R., Schiller, J.T., 1994. Neutralization of bovine papillomavirus by antibodies to L1 and L2 capsid proteins. J. Virol. 68, 7570–7574.

Roden, R.B., Armstrong, A., Haderer, P., Christensen, N.D., Hubbert, N.L., Lowy, D.R., Schiller, J.T., Kirnbauer, R., 1997. Characterization of a human papillomavirus type 16 variant-dependent neutralizing epitope. J. Virol. 71, 6247–6252.

Sapp, M., Fligge, C., Petzak, I., Harris, R., Streeck, R.E., 1998. Papillomavirus assembly requires trimerization of the major capsid protein by disulfides between two highly concerned cysteines. J. Virol. 72, 6186–6189.

Sandalon, Z., Oppenheim, A., 10-27-1997. Self-assembly and protein-protein interactions between the SV40 capsid proteins produced in insect cells. Virology 237, 414–421.

Seedorf, K., Krammer, G., Durst, M., Suhai, S., Rowekamp, W.G., 1985. Human papillomavirus type 16 DNA sequence. Virology 145, 181–185.

Slupetzky, K., Shafti-Keramat, S., Lenz, P., Brandt, S., Grassauer, A., Sara, M., Kirnbauer, R., 2001. Chimeric papillomavirus-like particles expressing a foreign epitope on capsid surface loops. J. Gen. Virol. 82, 2799–2804.

Steller, M., 2002. Cervical cancer vaccines: progress and prospects. J. Soc. Gynecol. Investig. 9, 254.

Suzich, J.A., Ghim, S.J., Palmer-Hill, F.J., White, W.I., Tamura, J.K., Bell, J.A., Newsome, J.A., Jenson, A.B., Schlegel, R., 1995. Systemic immunization with papillomavirus L1 protein completely prevents the development of viral mucosal papillomas. Proc. Natl. Acad. Sci. U.S.A. 92, 11553–11557.

Touze, A., El Mehdaoui, S., Sizaret, P.Y., Mougin, C., Munoz, N., Coursaget, P., 1998. The L1 major capsid protein of human papillomavirus type 16 variants affects yield of virus-like particles produced in an insect cell expression system. J. Clin. Microbiol. 36, 2046–2051.

Varsani, A., Williamson, A.L., Rose, R.C., Jaffer, M., Rybicki, E.P., 2003a. Expression of human papillomavirus type 16 major capsid protein in transgenic *Nicotiana tabacum* cv. Xanthi. Archiv. Virol. 148, 1771–1786.

Varsani, A., Williamson, A.L., de Villiers, D., Becker, I., Christensen, N.D., Rybicki, E.P., 2003b. Chimeric human papillomavirus type 16 (HPV-16) L1 particles presenting the common neutralizing epitope for the L2 minor capsid protein of HPV-6 and HPV-16. J. Virol. 77, 8386–8393.

Varsani, A., Williamson, A.L., Stewart, D., Rybicki, E.P., 2006. Transient expression of human papillomavirus type 16 L1 protein in *Nicotiana benthamiana* using an infectious tobamovirus vector. Virus Res. 120, 91–96.

Villa, L.L., Costa, R.L., Petta, C.A., et al., 2005. Prophylactic quadrivalent human papillomavirus (types 6, 11, 16 and 18) L1 virus-like particle vaccine in young women: a randomised double-blind placebo-controlled multicentre phase II efficacy trial. Lancet Oncol. 6, 271–278.

Wang, Z., Christensen, N., Schiller, J.T., Dillner, J., 1997. A monoclonal antibody against intact human papillomavirus type 16 capsids blocks the serological reactivity of most human sera. J. Gen. Virol. 78, 2209–2215.

White, W.I., Wilson, S.D., Palmer-Hill, F.J., et al., 1999. Characterization of major neutralizing epitope on human papillomavirus type 16 L1. J. Virol. 73, 4882–4889.

Yamada, T., Wheeler, C.M., Halpern, A.L., Stewart, A.C., Hildesheim, A., Jenison, S.A., 1995. Human papillomavirus type 16 variant lineages in United States populations characterized by nucleotide sequence analysis of the E6, L2, and L1 coding segments. J. Virol. 69, 7743–7753.

Zhou, J., Doorbar, J., Sun, X.Y., Crawford, L.V., McLean, C.S., Frazer, I.H., 1991. Identification of the nuclear localization signal of human papillomavirus type 16 L1 protein. Virology 185, 625–632.