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FEMS Microbiology Letters 189 (2000) 121–127

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

The nine C-terminal amino acids of the major capsid protein of the human papillomavirus type 16 are essential for DNA binding and gene transfer capacity

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Received 19 February 2000; received in revised form 27 April 2000; accepted 26 May 2000

Abstract

Four C-terminal deletion mutants of the human papillomavirus 16 L1 protein were expressed in the baculovirus expression system. They consist of the deletion of amino acids 497–505, 477–505, 403–505 and 302–505 (ΔC9, ΔC31, ΔC103 and ΔC204 respectively). Only two of the C-terminally deleted proteins, ΔC9 and ΔC31, retained the ability to form virus-like particles (VLPs) resembling those obtained with the full length L1 protein. Analysis of deleted L1 proteins and corresponding VLPs indicated that the C-terminus was necessary both for DNA binding and DNA packaging. These results were corroborated by the loss of the gene transfer capacities of C-terminal deleted VLPs.

Keywords: Human papillomavirus type 16; L1 Protein; Virus-like particle; DNA binding; DNA protection; Gene transfer; Nuclear localization signal peptide

1. Introduction

Human papillomaviruses (HPVs) are small non-enveloped DNA viruses with an icosahedral structure composed of 72 pentameric capsomers. Virion particles contain a double stranded circular DNA genome of about 8000 bp. These particles have a 55 nm diameter and a capsid which is composed of major L1 and minor L2 structural proteins. Both proteins, L1 and L2, are synthesized in the cytoplasm of the host cell during the late phase of infection before being imported into the nucleus where virion assembly takes place [1]. DNA binding activity has already been reported for these two proteins which would direct viral genome packaging. The first 12 N-terminal amino acids of the HPV-16 L2 and the last C-terminal amino acids of HPV-11 L1 have been identified as responsible for the DNA binding of these proteins [2–4].

Most HPV structural studies have been hampered by the difficulties in obtaining infectious virus stocks. Several animal and cell culture systems have generated only limited quantities of virion particles of the high-risk HPV types for such studies [5]. Recombinant expression systems have then been developed to express L1 and L2 proteins and led to the finding that the major capsid protein L1 alone can self-assemble into virus-like particles (VLPs) when expressed in heterologous systems [6]. These VLPs are structurally similar to authentic virions, and present conformational epitopes that generate neutralizing antibodies [7–10]. Moreover, it has been shown that HPV VLPs composed of L1 or L1/L2 have the ability to package irrelevant plasmid DNA containing reporter genes by using cellular [11,12] or non-cellular systems [9]. The pseudovirions are able to transfer the plasmid DNA into cells, where the reporter gene is expressed. Entry of these artificial gene delivery vehicles into cells is dependent on the interaction between VLPs and the cell surface, and two candidate cell receptors have recently been identified. The first is the α-6 integrin [13,14] which interacts with an as yet unrecognized L1 region and the second is con-

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0378-1097/00/$20.00
PII: S0378-1097(00)00248-2

stituted of the cell-surface glycosaminoglycans which interact with the C-terminal portion of HPV L1 [15].

In order to achieve better understanding of the mechanisms of papillomavirus assembly, DNA packaging and generation of pseudovirions suitable for gene transfer, we generated deletion mutants in the C-terminal parts of HPV-16 L1 protein. Such mutants were first cloned and expressed using recombinant baculoviruses in insect cells and then tested for VLP assembly, DNA binding, DNA packaging and for their ability to transfer genes into mammalian cells.

2. Materials and methods

2.1. Molecular cloning and expression of HPV 16 L1 truncated genes

HPV 16 L1 truncated genes were amplified by PCR from a full length HPV 16 L1 gene [16]. The synthetic oligonucleotide primers used in PCR experiments are described in Table 1. Following amplification, the PCR products were cloned into the pCRII-1 Topo vector (Topo TA cloning, Invitrogen, San Diego, CA, USA) and then subcloned into the pBlueBacIII vector (Invitrogen) or the pFastBacI vector (Life Technologies) after digestion with *Hin*dIII and partial digestion with *Bam*HI restriction enzymes.

Recombinant baculoviruses encoding HPV-16 L1-ΔC9, HPV-16 L1-ΔC31 were generated by using the Bac-to-Bac baculovirus expression system (Life Technologies). HPV-16 L1-ΔC103 and HPV-16 L1-ΔC204 were generated by using the Linear AcMNPV transfection kit (Invitrogen).

2.2. Expression and characterization of deleted proteins

Sf21 cells, maintained in supplemented Grace's insect medium with 10% fetal calf serum (FCS), were infected with the different recombinant baculoviruses at a multiplicity of infection of 10. Four days post-infection, cells were harvested and resuspended in SDS-PAGE loading buffer. Cellular extracts were separated on 12% SDS-PAGE, electroblotted onto Protran BA 83 nitrocellulose (Schleicher and Schuell, Dassel, Germany), and probed with anti-HPV-16 VLP mouse polyclonal serum [17].

Table 1
Sequences of oligonucleotide primers used in PCR experiments

Primer	Sequence
Forward wt L1	5′-**ATG**TCTCTTTGGCTGCCTAGT-3′
Reverse ΔC9	5′-TTATGTAGAGGTAGATGAGGTG-3′
Reverse ΔC31	5′-CCTACAATCCTGCTTGTAGTA-3′
Reverse ΔC103	5′-TTACCAGTCCTCCAAAATAGTG-3′
Reverse ΔC204	5′-TTAGGTAACCATAGAACCACTAG-3′

Start codons are in bold face and stop codons are underlined.

2.3. Purification of HPV VLPs

VLPs were purified as previously described for HPV-16 L1 VLPs [16,17]. Four days post-infection, Sf21 cells infected with respective recombinant baculoviruses were collected by centrifugation, resuspended in ice-cold PBS and sonicated by three bursts of 15 s each at 60% maximal power (Vibra-cell, Bioblock Scientific, Strassbourg, France). Cellular lysates were loaded on a CsCl gradient and centrifuged to equilibrium in a Beckman SW28 rotor (20 h, 27 000 rpm, 4°C). CsCl gradient fractions were harvested and investigated for density by refractometry and for the presence of VLPs by electron microscopy. CsCl fractions containing VLPs were pooled, diluted in PBS and ultracentrifuged in a Beckman SW 28 rotor (28 000 rpm, 4°C). After centrifugation, the VLP pellets were resuspended in a buffer containing 50 mM Tris pH 7.5, 150 mM NaCl.

2.4. Electron microscopy

VLP preparations were applied to carbon-coated grids, negatively stained with 1.5% uranyl acetate. Observations were performed at 50 000× nominal magnification with a JEOL 1010 electron microscope. All the electron micrographs were taken at 50 000×.

2.5. Detection of DNA binding

The South-Western assay, which allows the detection of DNA–protein interactions, was based on previously published procedures with some modifications [4,18]. Briefly, purified VLPs were boiled in the presence of 1% SDS and the denatured L1 proteins were separated on 10% SDS-PAGE and transferred to BA 83 nitrocellulose by electroblotting (Hoefer Semiphor, Pharmacia). After a blocking step with 0.2% BSA for 30 min at 25°C, a renaturation step was carried out by overnight incubation at 4°C in 50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 200 mM NaCl, 0.1% Nonidet P40 and 10% glycerol. The DNA binding assay was performed for 30 min at room temperature in binding buffer containing 30 mM HEPES (pH 7.4), 5 mM $MgCl_2$, 50 mM NaCl using a digoxigenin-labelled plasmid (Dig DNA labeling and detection kit, Positive control, Boehringer-Mannheim, Meylan, France). The membranes were then washed four times with binding buffer and bound DNA was revealed by an anti-digoxigenin alkaline phosphatase conjugate antibody (Boehringer-Mannheim) and NBT, BCIP as substrates.

ELISA was used for the detection of the DNA-binding activity of L1 peptides, P1, P2 and P3 (Fig. 1). Ten microgram of each peptide or 5 μg of disrupted HPV-16 VLPs in carbonate buffer (pH 9.6) were seeded per well and incubated overnight at 4°C. Bovine serum albumin (10 μg) and an HPV-16 E4 peptide (NH_2-RLGSEH-VDRPLTTP-COOH, 10 μg) were used as controls. After

a blocking step with 1% BSA in PBS for 1 h 30 min at 37°C, digoxigenin-labeled plasmid DNA (Boehringer-Mannheim) diluted in binding buffer was added. After 1 h of incubation at room temperature and four washes with binding buffer, bound DNA was revealed by anti-digoxigenin alkaline peroxidase conjugate antibody (Boehringer-Mannheim) diluted 1/1000 in binding buffer. After 1 h at 37°C and four washes with binding buffer, bound antibodies were revealed using 2,2′-azinobis (3-ethylbenzthiazoline-sulfonic acid) and H_2O_2. Absorbance was read at 405 nm and the results are the means of two independent experiments.

Peptide–DNA interactions were further characterized by gel retardation assay. Peptides were mixed with 500 ng of pCMV-β DNA in an electrical charge ratio of 10 positive per negative charge in 50 mM NaCl. After 30 min of incubation at room temperature, preparations were analyzed by electrophoresis on a 1% agarose gel in TAE 1× buffer, followed by ethidium bromide.

2.6. Disruption and refolding of VLPs

Disassembly and reassembly of VLPs were performed according to a previously described procedure [9]. Ten micrograms of purified VLPs were incubated in 50 mM Tris–HCl buffer (pH 7.5) containing 150 mM NaCl, 1 mM EGTA, and 20 mM DTT in a final volume of 50 μl at room temperature for 30 min. At this step, 1 μg of pCMV-β plasmid, 7.2 kbp, (Clontech, Ozyme, Montigny le Bretonneux, France) in 50 mM Tris–HCl buffer (pH 7.5) 150 mM NaCl was added to the disrupted VLPs in the presence of 1% DMSO. The preparation was then diluted in 50 mM Tris–HCl buffer (pH 7.5) and 150 mM NaCl. $CaCl_2$ molarity was increased stepwise from 0 to 5 mM with an increment of 1 mM h^{-1} at 20°C to obtain a final volume of 500 μl.

In order to evaluate the amounts of packaged plasmidic DNA, the encapsidation experiments were performed using 50 μg of VLPs and 5 μg of plasmid. After refolding, the preparation was treated for 1 h at 20°C with 100 IU of Benzonase® (Merck, Darmstadt, Germany). The Benzonase® was heat-inactivated for 10 min at 65°C in the presence of 25 mM EDTA and then the mixture was incubated in the presence of 3% SDS and 1 mg ml^{-1} of proteinase K (Appligene, Illkirch, France) for 2 h at 56°C. Plasmidic DNA was phenol-extracted and ethanol-precipitated. Purified DNA was linearized using *Hin*dIII restriction enzyme, diluted by two-fold dilutions in TE and electrophoresed in 1% agarose gels. The same amount of plasmidic DNA was processed in the same manner with and without Benzonase® treatment, as a control.

2.7. Transfection experiments

Cos-7 cells grown in monolayers in D-MEM/Glutamax (Life Technologies) supplemented with 10% FCS, 100 IU ml^{-1} penicillin and 100 μg ml^{-1} streptomycin were seeded in six-well plates (Nunc, Life Technologies) and grown to 80% of confluence. Cells were washed twice with D-MEM/Glutamax. pCMV-β-VLPs diluted in 1 ml of culture medium were added to each well. After incubation for 1 h at 37°C, the VLPs were removed and 3 ml of D-MEM/Glutamax supplemented with 10% FCS were added. The cells were then incubated for 48 h at 37°C. At this step, cells were washed with PBS and fixed with a mix of 0.2% glutaraldehyde/2% formaldehyde in PBS. Cells were then incubated at 37°C with a solution containing 1 mg ml^{-1} X-Gal, 4 mM potassium ferricyanide, 4 mM potassium ferrocyanide and 2 mM magnesium chloride in PBS for 4 h. Blue cells were counted and the results are the means of four independent experiments.

3. Results

3.1. Design and expression of recombinant proteins

The constructs encoding the various end truncated HPV-16 L1 proteins were obtained by introducing a stop codon at positions 1489, 1429, 1207 and 904 in the L1 coding sequence in order to generate C-terminal deletion mutants, ΔC9, ΔC31, ΔC103 and ΔC204 lacking 9, 31, 103 and 204 amino acids, respectively (Fig. 1). These deletions were designed to eliminate C-terminal sequences which partially or entirely remove the Lys/Arg rich region, which corresponds to the bipartite nuclear localization signal (NLS) identified by Zhou et al. [19] in HPV-16 L1.

The ΔC9, ΔC31, ΔC103, ΔC204 genes were amplified by PCR and subsequently cloned into a baculovirus transfer vector, pFastBacI or pBlueBacIII, under the control of the polyhedrin promoter. The recombinant baculoviruses were further amplified and titered. The synthesis of the recombinant proteins in Sf21 cells infected with recombinant viruses was analyzed by Western blotting. A band of L1-derived protein was identified in each case, and the modi-

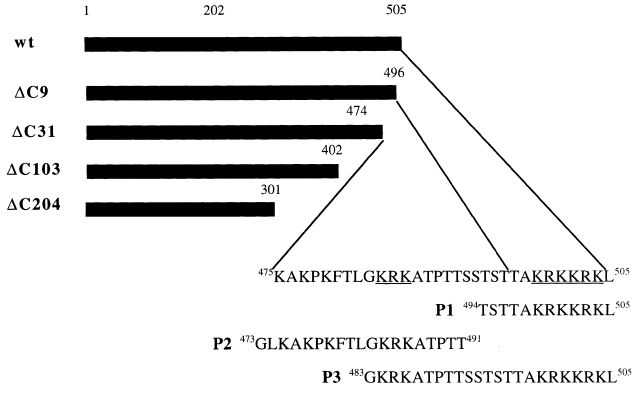

Fig. 1. Schematic maps of HPV-16 L1 protein and truncated forms of L1. The C-terminal Lys/Arg-rich region is detailed and amino acids composing the bipartite NLS are underlined.

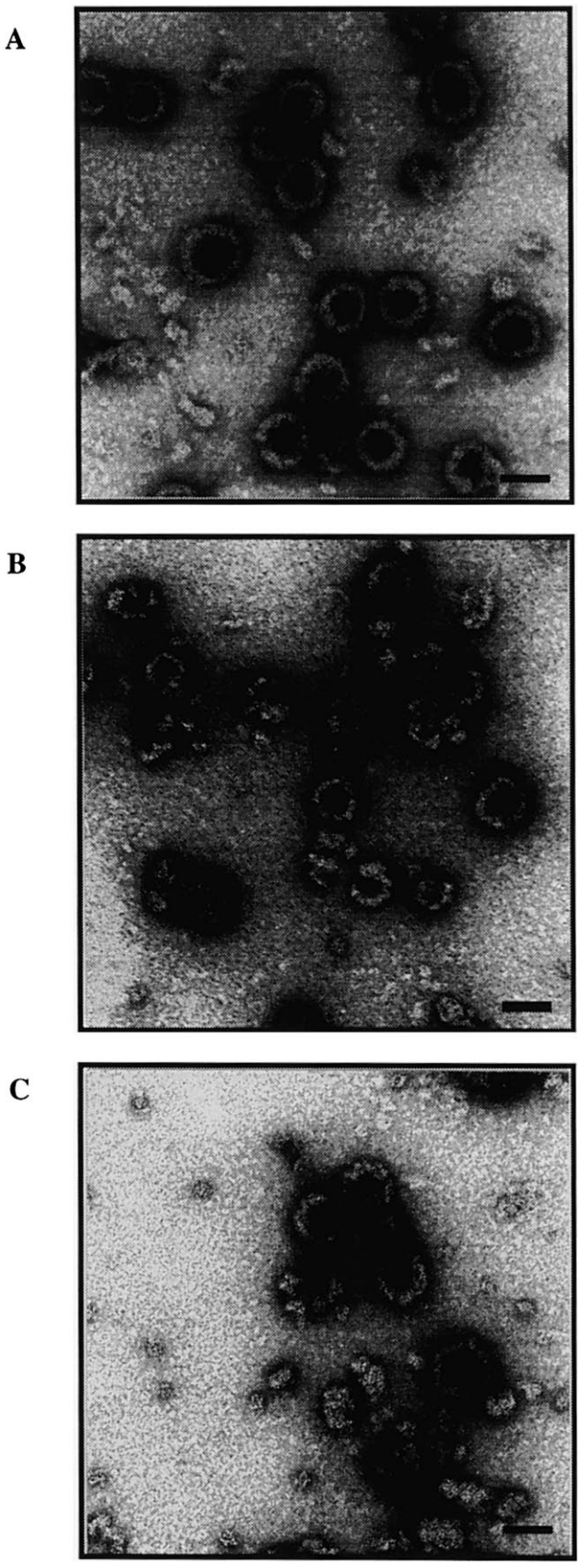

Fig. 2. Electron micrographs of VLPs obtained by expression of wt L1 (A), ΔC9 (B) and ΔC31 VLPs (C) truncated proteins. Preparations were negatively stained with 1.5% uranyl acetate and observed at 50 000× nominal magnification with a JEOL 1010 electron microscope. Electron micrographs were taken at 50 000×. Bars represent 50 nm.
←

fied products exhibited the expected reduction in size (data not shown).

3.2. Assembly of deleted L1s into VLPs

In order to determine the effects of mutations on particle assembly, we screened the L1 deletion mutants for their ability to form VLPs. Cell lysates from insect cells infected with the different recombinant baculoviruses were subjected to isopycnic banding on a CsCl preformed gradient. Following centrifugation, fractions were collected and analyzed by an ELISA using an anti-HPV-16 VLP mouse serum. The peak of reactivity for all deleted proteins corresponded to densities ranging from 1.27 to 1.29 g cm^{-3}, as previously observed for wt L1 (data not shown) [16,17]. Fractions composing the peak of reactivity were examined by transmission electron microscopy to detect the presence of papillomavirus VLP structures. In the case of the ΔC9, we observed empty virus capsids of about 50 nm and some free and aggregated capsomers. However, we observed a majority of irregular capsids with sizes ranging from 40 to 55 nm in diameter for the ΔC31 protein. As observed with wt L1 VLPs, ΔC9 and ΔC31 particles presented a central stain-filled hollow (Fig. 2). We did not observe VLPs or capsomers in the two largest C-terminal deletions, but only protein aggregates (Table 2).

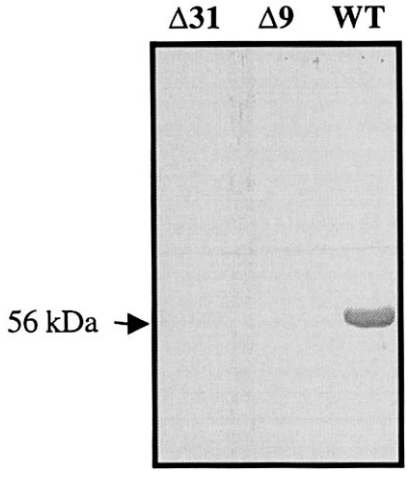

Fig. 3. Detection of DNA binding to wt HPV-16 L1 protein, ΔC9 and ΔC31 by South-Western blotting using a digoxigenin-labeled plasmid DNA. Bound DNA was revealed by an anti-digoxigenin alkaline phosphatase conjugate antibody and NBT/BCIP as substrates.

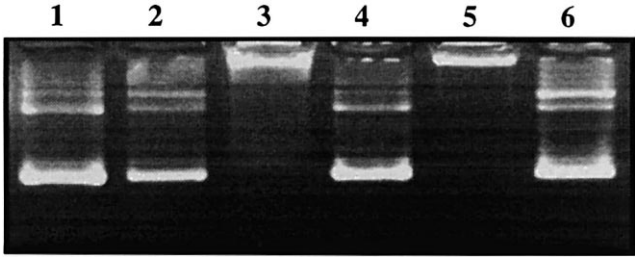

Fig. 4. Gel retardation assay. Peptide or protein–DNA complexes were analyzed by electrophoresis on agarose gel (1% w/v) stained with ethidium bromide. Lane 1: pCMV-β alone; lane 2: E4 peptide; lane 3: P1 peptide; lane 4: P2 peptide; lane 5: P3 peptide; lane 6: BSA.

3.3. DNA binding

DNA binding activity of ΔC9 and ΔC31 proteins was assessed using the South-Western blot assay. VLPs made of ΔC9 and ΔC31 deleted L1 protein and full length L1 as positive control [17] were separated by SDS-PAGE, electro-transferred to nitrocellulose and hybridized with digoxigenin-labelled plasmid DNA. Neither ΔC103, ΔC204 (data not shown) nor ΔC9 and ΔC31 proteins bound DNA (Fig. 3). To confirm that the C-terminus of L1 protein is sufficient for DNA binding, peptides P1, P2 and P3 (Fig. 1) located at the C-terminus of the HPV-16 L1 protein were studied by gel retardation for a qualitative approach and by ELISA for a semi-quantitative approach. The results obtained from the gel retardation assay (Fig. 4) indicated that P1, corresponding to the 12 ultimate amino acids of HPV-16 L1, interacted with pCMV-β whereas the upstream peptide P2, spanning the second part of the bipartite NLS, did not. However, the results obtained by ELISA suggest a synergistic effect between P1 and P2 sequences, since peptide P3, which contained the P1 sequence plus 11 amino acids containing the second part of the bipartite NLS, was shown to interact more strongly (OD = 1.824) than P1 (OD = 1.214) with plasmidic DNA (Table 3).

3.4. Packaging of plasmidic DNA and gene transfer

The larger C-terminally truncated L1s were not studied due to their inability to form VLPs. VLPs composed of ΔC9, ΔC31 and wt L1 were subjected to disassembly under reducing conditions in the presence of EGTA, and were refolded in the presence of pCMV-β using increasing Ca^{2+} concentrations. To establish the level of DNA protection obtained with ΔC9, ΔC31 and wt VLPs, refolded VLPs were subjected to Benzonase® treatment. After Benzonase® digestion, plasmid DNA was purified, linearized and electrophoresed on agarose gel. As shown in Table 2, DNA was not protected by VLPs from either deleted protein whereas VLPs composed of the wt L1 protected about 12.5% of the input plasmid DNA. This result was corroborated by the results of gene transfer into Cos-7 cells. We did not observe β-galactosidase-positive cells (blue cells) either in the case of ΔC9 or ΔC31 VLPs whereas 982 ± 625 blue cells were observed for wt VLPs.

4. Discussion

Four C-terminal truncation mutants of the HPV-16 L1 protein were generated to investigate VLP formation and the DNA packaging property of the L1 protein. We have confirmed that C-terminally deleted L1 proteins by 9 and 31 amino acids conserved the ability of the wt L1 protein of HPV-16 to self-assemble into VLPs, as shown by Müller et al. [20]. However, ΔC31 VLPs are more variable in size than wt VLPs, as observed by Chen et al. [21] for COPV deletion mutants. The ability of HPV-16 L1 protein deleted by up to 31 amino acids at its C-terminus to autoassemble into VLPs is in agreement with deletion mutagenesis studies of COPV and BPV-1 L1 protein which demonstrated that deletion of the C-terminal 24 or 26 amino acids of BPV-1 and COPV L1, respectively, generated a protein which could still form VLPs [21,22]. However, when an additional 72 amino acids (ΔC103) were deleted from the C-terminus of HPV-16 L1, the mutant protein failed to self-assemble into VLPs. This is in agreement with the fact that a 67-amino acid C-terminus deletion in the L1 of COPV [21] did not abolish formation of VLPs and conserved a PPS residue sequence corresponding to the PPP motif in the HPV 16 L1 sequence. The PPP motif observed at residue 408–410, is an analogue of the PYP sequence which was shown to be essential in polyomavirus inter-capsomer links [23].

The ability of the different recombinant proteins to bind DNA was assessed by South-Western blotting assay. In ΔC9 and ΔC31 proteins the deletion of the Lys/Arg-rich

Table 2
Summary of L1 proteins analyzed

	Structure adopted	DNA binding	DNA protection (%)	Gene transfer blue cells/well (mean ± S.D.)
wt L1	VLPs	+	12.5	982 ± 625
ΔC9	VLPs	−	0	0
ΔC31	VLPs	−	0	0
ΔC103	protein aggregates	−	NT	NT
ΔC204	protein aggregates	−	NT	NT

Structure adopted, DNA binding, DNA protection and gene transfer of the wt L1 and truncated L1 are described. NT: not tested

Table 3
Detection of DNA binding of wt HPV-16 L1 protein and three C-terminal synthetic peptides (P1, P2, P3) by ELISA

	P1	P2	P3	E4	BSA	WT L1
$OD_{405\ nm} \pm$ S.D.	1.214 ± 0.054	0.016 ± 0.007	1.824 ± 0.288	0.014 ± 0.070	0.061 ± 0.049	2.829 ± 0.029

HPV-16 E4 synthetic peptide (E4) and bovine serum albumin (BSA) were used as negative controls. Results are the mean of two independent experiments.

regions corresponding to the NLS dramatically reduced the L1 protein theoretical p*I* from 8.9 for wt to values of 7.43 and 5.93 for ΔC9 and ΔC31, respectively. In addition to the existence of a possible amino acid sequence specifically involved in DNA binding, the reduction in p*I* could also explain the loss of DNA binding for ΔC9 and ΔC31. Nevertheless, it was not obvious by South-Western blot assay that DNA packaging is only supported by DNA binding to L1 protein. In fact, in encapsidation experiments, ΔC9 and ΔC31 VLPs were not able to protect/package unrelated plasmid DNA or to transfer genetic information to target cells. However, since ΔC9 and more clearly ΔC31 VLPs appeared more irregular by electron microscopy examination, it is possible that these VLPs did not encapsidate plasmid DNA due to a modification in the VLP structure which affects their capacity to bind to cell receptor(s) or to encapsidate DNA.

In conclusion, we observed that the nine C-terminal amino acids of the HPV 16 L1 protein are not necessary for VLP formation but are essential for DNA binding and DNA packaging. For gene delivery and expression, HPV-16 VLPs must be capable of binding and packaging the DNA to be transferred, internalization, uncoating, nuclear transport and finally expression of the transgene. Our results and those of others suggest that the nine C-terminal amino acids of the L1 protein concentrate three of these properties: DNA binding, DNA packaging, and nuclear localization [4,19]. Moreover, as suggested by Joyce et al. [15], highly conserved amino acids composing the extreme C-terminus of HPV L1 proteins are involved in attachment to heparan sulfate.

The identification of this nine-amino acid sequence provides the possibility of modifying non-DNA binding capsid protein-forming VLPs by insertion of this sequence to build novel vectors for gene transfer.

Acknowledgements

This study was supported by the Association pour la Recherche sur le Cancer (Grant N° 9977), by MGEN/INSERM and the Ligue Contre le Cancer (Comité d'Indre et Loire). D.M., S.E., C.D., A.L.C.-R. and L.B. were supported by the Association pour la Recherche sur le Cancer, the Fondation de France, the Région Centre, Colciencias and the Ministère de l'Education Nationale et de la Recherche Technologique, respectively.

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