Arch Virol (2008) 153:585–589
DOI 10.1007/s00705-007-0025-2

BRIEF REPORT

An investigation into the use of human papillomavirus type 16 virus-like particles as a delivery vector system for foreign proteins: N- and C-terminal fusion of GFP to the L1 and L2 capsid proteins

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Received: 2 October 2007 / Accepted: 17 December 2007 / Published online: 4 January 2008

Abstract Development of vaccine strategies against human papillomavirus (HPV), which causes cervical cancer, is a priority. We investigated the use of virus-like particles (VLPs) of the most prevalent type, HPV-16, as carriers of foreign proteins. Green fluorescent protein (GFP) was fused to the N or C terminus of both L1 and L2, with L2 chimeras being co-expressed with native L1. Purified chimaeric VLPs were comparable in size (~55 nm) to native HPV VLPs. Conformation-specific monoclonal antibodies (Mabs) bound to the VLPs, thereby indicating that they possibly retain their antigenicity. In addition, all of the VLPs encapsidated DNA in the range of 6–8 kb.

Human papillomaviruses (HPVs; family *Papillomaviridae*) are non-enveloped viruses, of which over 100 types have been classified. Of these, genital HPVs fall into two groups: low-risk, causing benign anogenital warts, and high-risk, causing cervical cancers. Of the high-risk group, HPV type 16 (HPV-16) alone is responsible for up to 50% of diagnosed cervical cancers worldwide [3], making it an important model from which to develop appropriate prophylactic or therapeutic vaccination strategies.

The HPV virion consists of an ~8 kb double-stranded circular DNA genome encoding six early (E) proteins involved in viral replication and host cell malignant transformation, and two late (L) structural proteins, L1 (major capsid protein, making up the capsid) and L2 (minor capsid protein, incorporated within the capsid). Analysis of various L2 mutant constructs has localized the carboxyl region of L2 (residues 396–439) as a critical L1–L2 interaction domain which allows incorporation of L2 into the capsid [2]. The presence of L2 within the capsid is also suggested as a prerequisite for DNA encapsulation [9]. Recombinant expression of L1, either on its own or with L2, results in the formation of virus-like particles (VLPs) that resemble native papillomavirus (PV) particles, both morphologically and immunologically [1, 5, 16].

Despite the development of native HPV VLPs as candidate prophylactic vaccines, chimaeric VLPs are increasingly being researched and synthesised to accommodate more viral antigen targets. An extensive study investigating chimaeras of HPV-16 L1 and E7 (oncoprotein) was conducted [10] where either the 34-residue C-terminus of L1 was replaced with various segments of E7 or the E7 segments were inserted into the L1 residue region 295–302. Most of the chimaeric VLPs were able to induce a neutralising immune response [10]. HPV-16 E7 chimaeras of both L1 and L2 were shown to elicit a CD8+ CTL response against E7 in mice and to protect against E7-expressing tumors [4]. Similar results were obtained with chimaeric HPV-16 L1 fused at the C-terminus with a truncated E7 protein [15], E7 peptides [12] and a string of HIV CTL epitopes [7]. In addition, there are a number of reports describing the use of PV-like particles to deliver/display foreign epitopes [4, 7, 8, 10, 11, 19].

Electronic supplementary material The online version of this article (doi:10.1007/s00705-007-0025-2) contains supplementary material, which is available to authorized users.

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Despite such research on using chimaeric PV VLPs as a vaccine/epitope delivery system, there have been no systematic studies comparing the fusion of a foreign protein to the N- and C-termini of L1 and L2. Our study uses GFP fused to the N- and C-termini of both the HPV-16 L1 and L2 (GenBank accession # AY177679 and EU118173, respectively), with L2 chimeras being co-expressed with native L1, to compare the effects of N- and C-terminal fusions on VLP formation.

To create the fusion constructs GFP-L1 and GFP-L2 (GFP fused to the N-terminus of L1 and L2, respectively); L1-GFP and L2-GFP (GFP fused to the C-terminus of L1 and L2, respectively), the capsid protein genes were PCR-amplified from pSKSAL1 and pSKSAL2 plasmids [native L1 and L2 in pSK (Stratagene), respectively] using the proofreading DNA polymerase Accuzyme™ (Bioline) and relevant primers (Supplementary Table 1). The PCR products were A-tailed and inserted into either of the vectors of the pdDNA3.1/NT-GFP-TOPO® TA (N-terminal fusions) and pcDNA3.1/CT-GFP-TOPO® TA (C-terminal fusions) expression kit (Invitrogen) to create the fusion constructs. The fusion constructs were amplified using a second set of primers (Supplementary Table 1) and subcloned into the suicide vector of the Gene JET™ (Fermentas). GFP-L1 and L1-GFP were then inserted into the *Xba*I and *Xba*I/*Pst*I site, respectively, of the pFastBac™ 1 vector (Invitrogen). For the L2-GFP chimaeras to be co-expressed with L1, the native full-length L1 sequence was excised from pSKSAL1 using *Sal*I/*Xba*I and inserted into the same sites of the primary multiple cloning site of the pFastBac™ Dual vector (Invitrogen), creating L1-pFastBacDual. The L2-GFP fusion constructs were then inserted into the *Nhe*I/*Nsi*I and *Nco*I/*Nsi*I site, respectively, of the secondary multiple cloning site of L1-pFastBacDual, creating L1:GFP-L2 and L1:L2-GFP fusion constructs. DH10Bac *E. coli* cells (Invitrogen) were transfected with the four pFastBac1® clones, and *Spodopetra fungiperda* (*Sf*21) (Invitrogen) cells were transfected with the resulting bacmid DNA using Cellfectin (Life Technologies). The Bac-to Bac® manufacturer-recommended protocol was followed to amplify the recombinant virus.

For each construct, 10 µl ml^{-1} of the amplified virus was used to infect five 75 cm^2 flasks of 6×10^6 *Sf*21 cells seeded in 10 ml of complete TC100 and incubated at 27°C for 48–72 h. Infected cells were harvested as described [19, 20]. To isolate the expressed constructs using a CsCl gradient, *Sf*21 cell pellets were resuspended in high-salt phosphate-buffered saline (PBS) with 0.5 M NaCl, 0.4 g ml^{-1} CsCl and protease EDTA-free inhibitor cocktail (Roche diagnostics). The suspension was sonicated for 4 min with 15 s treatments interspersed with 15 s intervals. The sonicated material was matured overnight at 4°C and then centrifuged at 35,000 rpm for 24 h at 16°C in a SW-55 Ti Beckman rotor. Two distinct bands were observed for constructs GFP-L1 and L1-GFP, and three bands for L1:GFP-L2 and L1:L2-GFP. Only the GFP-containing band that fluoresced upon excitation with a long-wave UV lamp was extracted for each of the constructs (Supplementary Figure 1). The extracted bands were verified by western blot analysis as described previously [20], using primary antibody H16:J4 (courtesy of Dr. Neil Christensen) and anti-GFP (Roche), secondary antibody anti-mouse conjugated to glutathione peroxidase (Sigma), and detected using SIGMA *Fast* NBT/BCIP. The L1 and L2 GFP fusion constructs were confirmed by the ca. 95 kDa products that bound H16:J4 and anti-GFP antibodies, respectively (Fig. 1). In addition, the native L1 in the dual expression vector was confirmed by the ca. 60 kDa product. Further confirmation was achieved using an ELISA protocol described by Varsani et al. [19, 20]. First, the L1 concentration in each sample was normalized using the monoclonal antibody H16:J4, which recognizes a linear epitope of L1, independent of protein conformation. The four normalised samples were then analysed by ELISA using a host of well-characterized monoclonal antibodies (Mabs) recognizing either linear or conformation-specific epitopes (courtesy of Dr Neil Christensen). This was done in order to investigate the structural integrity of L1 with GFP fused to the different termini of the two capsid proteins. An ELISA was performed in triplicate for each Mab on all four fusion constructs as well as positive (native HPV L1 VLPs) and negative (cell lysate) controls, and average values were calculated for each triplet (Fig. 2). The analysis indicates that L1/GFP fusions not only bind Mabs recognizing linear epitopes (H16:J4 and H16:I23) but also bind conformation-specific Mabs (H16:U4, H16:V5, H16:E70 and H16:9 A), indicating that L1 maintained its structural conformation in the presence of all types of GFP fusion chimeras. This indicates that the fused GFP did not alter or interfere with the VLP conformational epitopes.

That the fusion constructs formed VLPs was confirmed using electron microscopy. Samples were prepared using immunoelectron microscopy and negative stain techniques. Carbon-coated copper grids coated with L1 H16:J4 antibody (diluted 1/50), were used to trap the constructs, after which they were stained with 2% uranyl acetate. Grids were visualized and photographed using a 2 × 2 k CCD camera using a LEO Omega 912 (Zeiss) transmission electron microscope. Figure 3 shows the VLPs present in the prominent fluorescing bands from the CsCl gradients. The VLPs in these samples looked well-formed and morphologically uniform. The chimaeric L1 VLPs (containing GFP-L1 and L1-GFP monomers) appeared to have added density in the capsid shell wall. L1-GFP VLPs particularly indicate the added density as being present on the inner surface of the capsid shell. Also, the overall L1-GFP VLP

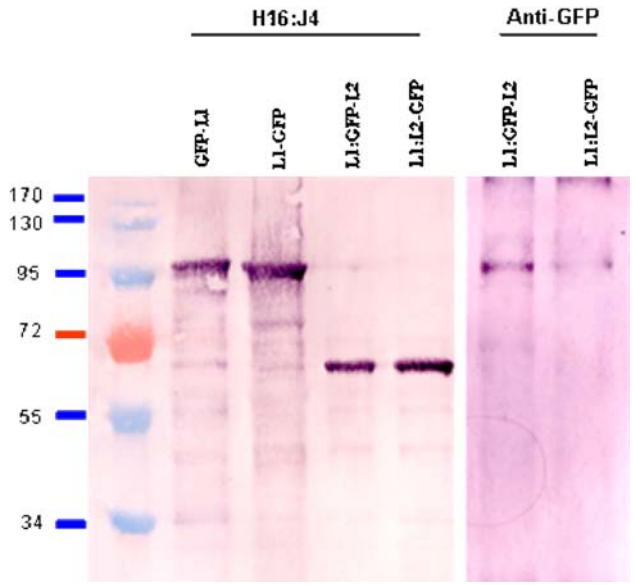

Fig. 1 Western blot analysis of fluorescing CsCl bands using HPV16:J4 for GFP-L1/L1-GFP (ca. 95 kDa) and L1 in the L1:GFP-L2/L1: L2-GFP (ca. 60 kDa) protein products

size remains comparable to other chimaeric VLPs (Fig. 3). In terms of VLP size, VLPs of all four chimaeric constructs were on average 55 nm in diameter, which is similar to the size determined previously for native VLPs.

The development of papillomavirus (PV) VLPs as DNA delivery vectors for genetic immunisations is at a relatively early stage. Bovine papillomavirus 1 (BPV-1) and HPV-33 VLPs, expressed by a recombinant vaccinia virus, have been shown to encapsidate a plasmid containing the β-galactosidase (β-Gal) reporter gene [18, 21]. BPV genomes autonomously replicating in a mammalian cell line have been encapsidated by BPV-1 and HPV-16 L1 protein expressed by recombinant Semliki forest virus [13]. DNA encapsidated in pseudovirions can also be generated from assembly of previously disassembled particles in the presence of different unrelated plasmids containing a reporter gene [6, 17]. Assembly of pseudovirions and encapsidation of plasmid DNA in *S. cerevisiae*-expressed HPV-16 L1/L2 has recently been shown, and in vitro infection of mammalian cells with these pseudovirions resulted in the delivery of the reporter gene [14]. These studies support the possibility of generating PV pseudovirions that package DNA in vitro and that such pseudovirions are able to deliver the packaged DNA to various cell lines.

To determine whether the VLPs had the potential to incorporate DNA into their structure during their assembly, VLP samples were electrophoresed through 0.6% agarose gels. Based on differential analyses using ethidium bromide staining, we distinguished GFP fluorescence at 254 nm from DNA with inter-chelated ethidium bromide. All four types of the chimaeric VLPs prepared by CsCl density gradient purification appeared to package random fragments of genomic DNA ranging from 6 to 8 kb in size (Supplementary Figure 2). This contradicts previous reports that suggest that L2 is a prerequisite for DNA incorporation [9], as we found DNA was also incorporated into VLPs composed purely of L1-GFP fusion proteins. However, Touze and Coursaget [17] reported the packaging in vivo of random plasmid DNA and its delivery to eukaryotic cells by HPV-16 L1 VLPs. The intrinsic capability of these VLPs to package DNA during their initial formation may predispose them towards development as DNA vaccine delivery vehicles.

In summary, L1, which is a major constituent of HPV VLPs, maintains its structural conformation in the presence of N- and C-terminal GFP fusions. In addition, L2 N- and

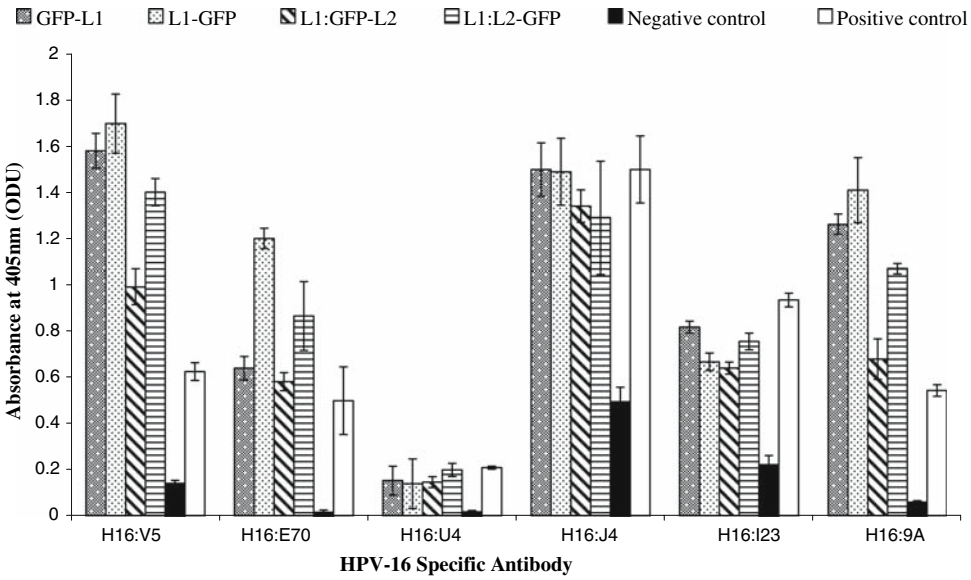

Fig. 2 Antibody-binding characterisation by ELISA of the purified GFP fusion products (confirmed by western blot analysis) using a panel of monoclonal antibodies. H16:V5, H16:E70, H16:U4 and H16:9 A are conformation-specific Mabs that bind neutralising epitopes. H16:J4 and H16:I23 bind linear eptiopes. *Error bars* represent standard deviation for each triplet

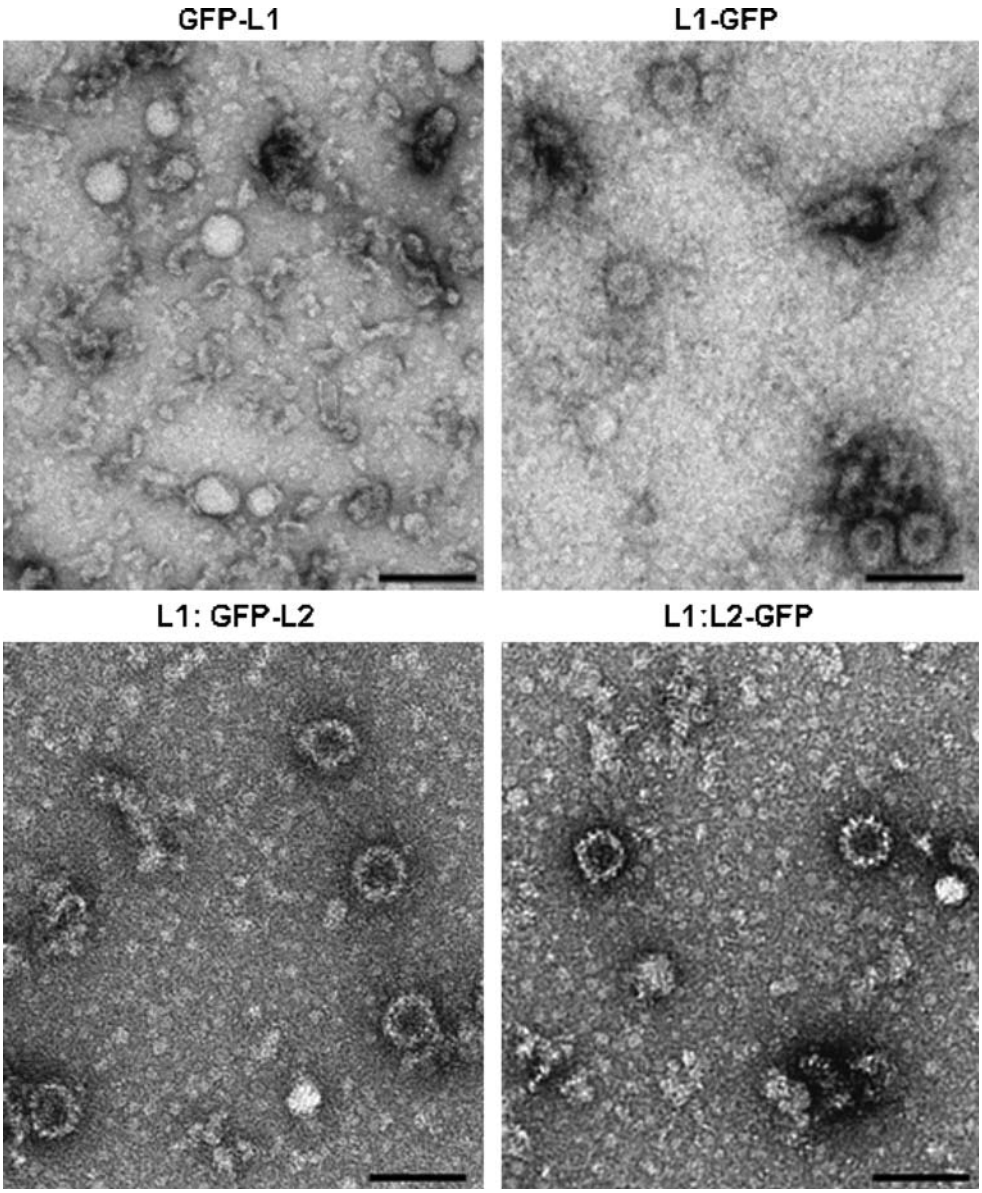

Fig. 3 Electron micrographs of VLPs of GFP-L1, L1-GFP, L1/GFP-L2 and L1/L2-GFP from the fluorescing band fractions of the CsCl gradients. *Bar* 100 nm

C-terminal GFP fusions were incorporated into VLPs made up of native L1. ELISA results suggest that the GFP does not interfere with or alter the conformation-specific epitopes of L1 in VLPs. HPV 16 VLPs exhibit the potential for the development of multivalent vaccines that are capable of presenting one or even more than one type of antigen concurrently to the immune system by potentially humoral or cellular means. Furthermore, the potential exists to use the system as a DNA vaccine delivery vector, possibly even coupling this strategy with the above-mentioned systems to generate vaccines capable of stimulating both arms of the immune system simultaneously.

Acknowledgments We would like thank to Dr. Neil Christensen for providing the monoclonal antibodies used in this study. This research was funded by the Poliomyelitis Research Foundation of South Africa (grant #04/25) and the University of Cape Town start up grant. AV is supported by the Carnegie Corporation of New York.

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