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Analysis of Human Papillomavirus Capsid Proteins: Insights into Capsid Assembly

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Analysis of Human Papillomavirus Capsid Proteins: Insights into Capsid Assembly

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Analysis of Human Papillomavirus Capsid Proteins: Insights into Capsid Assembly

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***Abstract*:** Papillomaviruses (PVs) are double stranded (ds)-DNA viruses (~8-kbp), which infect mucosal and cutaneous epithelial cells from various mammalian species, causing tumors in both epithelial cell-types [22, 48-50, 64, 65]. During the early-phase of an HPV infection; the genome is maintained within the basal epithelium establishing the primary infection, while during the late-phase, the capsid proteins (L1 and L2), are expressed to encapsidate the viral genome generating infectious virion particles required for PV propagation [49, 50, 52-55, 64, 67]. Natural PV infections produce morphologically homogenous progeny virions 55-nm in diameter [15, 22-24, 26, 68]. Transient transfection systems allow individual expression of the capsid proteins, which are able to produce low-levels of infectious virion-like particles (VLPs) and non-infectious VLPs that have the capacity to resemble and function as wild-type virions [5, 8, 15-24, 30, 32, 51, 68].

***Results*:** The research herein demonstrates that the L2 protein is highly conserved amongst all PV genera. The conserved sequence homology amongst the variety of PV L2 proteins reveals its necessity and conserved role during PV infections. The L1 and L2 DNA-binding domains (DNA-BD) were bioinformatically analyzed and the results showed amino acid sequence homology amongst all PV genera. The L2 DNA-BDs have sequence homology in comparison to the DNA-binding proteins from three species within the Microviridae family (ex. ϕX174, alpha-3, and G4 bacteriophages). Previously, reports demonstrate that the L2 protein interacts with the PV encoded E2, trans-regulatory, protein [2, 3]. The studies reported herein discover a novel interaction between the E2 and L1 protein of HPV16. The L1-to-E2 interaction is highly dynamic and may be dependent upon the spontaneous oligomerization of the L1 protein. Use of the Cluspro docking software predicts E2 to interact with the BC-loop (49-61 a.a.) and HI-loop (341-362 a.a) of the L1 protein. The locale of the BC- and HI-loops, within the valley of pentamer-to-pentamer interactions, and the observed interaction of the L1 and E2 proteins may function to preserve T=7 capsid uniformity during PV capsid assembly.

DEDICATION

I, Willie Andrew Hughes, would prefer to dedicate this master's thesis to the realm of science, "for in its purity: genuine understanding, knowledge, and human appreciation are truly realized and accepted."

Willie Andrew Hughes, 2013

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Overview of Human Papillomavirus Type 16

HPV16 is a small-DNA virus composed of a 7904-bp genome having 8 well-defined open reading frames (ORFs) (Figure 1). The HPV16 genome is circular and packaged along with cellular histones [22, 32, 33]. The lifecycle of HPVs are dependent on the differentiation status of the infected epithelial cell-type [22, 24, 32, 39, 48-50, 52-55, 63, 64, 67, 69]. Epithelial HPV infections can be categorized into two types: mucosal or cutaneous infections. HPV16 has the capacity to induce carcinoma within infected epithelial tissues [66].

Cutaneous infections can produce the common wart at the site of the infection on the hands and feet of affected individuals [22-24, 26, 32]. Cutaneous HPV-infections have not been definitely proven to have a role in carcinogenesis, unlike mucosal HPV-infections. None-the-less, a wart is an HPV-positive abnormal growth that by definition is an HPV-positive benign hyperplasia.

A mucosal infection occurs within epithelial cells lining the mucus-secreting tissues of the body (for example, tissues of the: mouth, esophagus, anus, penis, and vaginal tract) and the onset of disease or carcinoma can be attributed to high-risk (HR) and/or low-risk (LR) HPVs [66, 69]. The subsets of risk-associated HPV strains are classified by the accumulated epidemiological evidence of the relative association of certain HPV strains with cancers: HR-HPV strains (types 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68a, 73, 82, 82-subtype) and LR-HPV strains (types 6, 6a, 6b, 11, 40, 42, 43, 44, 54, 61, 70, 72 and 81) [71-74].

In either a mucosal or a cutaneous HPV infection, the non-productive phase of the lifecycle occurs within keratinocytes at the basal layer, whereas the productive phase occurs within the upward migrating terminally differentiated keratinocytes. In the late phase of the viral lifecycle, the HPV viral DNA becomes encapsidated in a protein-shell resembling an icosahedron-geometric shape [26, 68, 84]. The protective protein-shell is composed of two HPV-encoded proteins termed the Major (L1) and the Minor (L2) capsid proteins (4).

Late-Phase of Papillomavirus Lifecycle

During keratinocyte differentiation, the cells regulate their RNA-splicing mechanisms [53-55, 64]. HPV utilizes the alternative-splicing mechanisms throughout the differentiation program of the infected cell [55] and facilitates regulation of the polyadenylation machinery promoting the switch from early viral mRNA transcription (ex. E6, E7, E1, E2, E4, & E5 transcripts) to late

viral mRNA transcription (ex. E2, E1^E4, L1, & L2 transcripts). The progression from early to late transcription corroborates with the transition from the early-phase to the late-phase of the HPV lifecycle and leads to the production of the capsid proteins (L1 and L2), virus assembly, and release of infectious virus (Figure 1) [64, 67]. The switch between the early and late HPV infections can be detected from the positive immuno-reactivity with antibodies specific for the presence of the capsid proteins, L1 and L2, within the terminally differentiated keratinocytes [35-37, 69].

Packaging Enhancement Signal of Papillomavirus

During a natural papillomavirus infection, encapsidation has been speculated to be mediated by a particular *cis*-element sequence, termed a "packaging enhancement signal" (PES). Even though, in the absence of any PES HPV16 VLPs do efficiently transfer target genes [28]. The putative PES is a 120-bp *cis*-element within the E1-open reading frame (ORF) (nt. 1506-1625) from the Bovine Papillomavirus type 1 (BPV1) genome. This 120-bp *cis*-element has been shown to have a 2 to 3-fold increase/enhancement of target gene transfer when included within the target plasmid during encapsidation analysis using Rabbit Hemorrhagic Disease Virus (RHDV)-VP60 capsids utilizing the HPV16 DNA-binding termini from L1, and not from L2 [28]. Also, HPV6b VLPs have been shown to recognize the BPV1 PES [90]. The differences observed in gene transfer when using the RHDV-VP60 –L1BS versus the RHDV-VP60-L2BS is not attributed to specific DNA-binding activity but rather to the intrinsic heparin-sulfate binding sequence located within the full-length L1-COOH terminus used to create the RHDV-VP60-L1BS chimera VLPs. The heparin-sulfate binding sequence would allow the VP60-L1BS chimeras to attach more effectively to the cell surface, which would facilitate an increased uptake of the target plasmid [28, 75, 88].

The PES mediated-encapsidation hypothesis has not been observed during pseudovirus production utilizing the complete PV genome compared to a PV genome void of the 120-bp *cis*-element, although the enhancement has been observed in the target plasmid with the BPV1 PES compared to the target plasmid without the BPV1 PES. Further experimentations are needed to substantiate the PES and to understand more about the biological significance of the PES within the encapsidation process of HPVs.

Papillomavirus Capsid Synthesis, Structure, and Function

HPV16 encapsidation occurs within the nucleus near PML sites within the nucleus that immuno-react with ND-10 antibodies [38-42, 63]. The viral genome is packaged within the major

(L1) and minor (L2) capsid proteins that are produced through alternative-splicing mechanisms, which regulates the capsid-ORF transcriptional program [4, 6, 15, 19, 21, 39, 55, 67].

The major capsid protein is responsible for cellular entry during an initial infection, by recognizing cellular heparin sulfate-proteoglycan (HSPG) receptors or alpha-6 integrins depending, presumably dependent upon cell-type [75, 86, 87, 88]. The resulting capsid-to-cellular membrane attachment facilitates endosomal cellular entry via the L1 interacting with HSPG followed by capsid re-organization to expose the amino-terminus of L2 to cleavage by furin, respectively [75, 88, 89]. The attached virion enclosed within a transport vesicle is guided through the cytoplasmic space to the nucleus and at this point L1 is presumably degraded, and the resultant L2-to-HPV16 DNA complex remains [88, 89]. The L2 protein protects the HPV16 DNA from intracellular degradation and guides the DNA to the nucleus of the infected cell for nuclear-translocation, thus establishing the infection [9-14, 40-41].

The structure of the L2-protein has not been crystallized as of to-date, although through bioinformatic analysis a putative structure for the minor capsid protein has been proposed [70]. Cryo-EM analyses have allowed for the re-construction of a virion particle to deduce L2's localization within the L1-capsid, suggesting that L2 is localized underneath the L1-capsid, which would be consistent with L2 functioning as a sub-scaffold within the intact virion [68, 75].

The crystal structure of the L1-protein is available [5] and used in determining the hypothetical localization of L2 within the L1-pentameric shaft through the Cluspro docking software [70]. The *in silico* analysis confirms the reasoning deduced from the known interactions of L2 with β-actin [9] and the nuclear pore complex proteins [10-14], and from the observation that L2 is required for an infectious virion [83].

Observations of the two structures (L1 and L2) leaves open the possibility that the non-structured terminal-amino acids may be involved in non-specific DNA encapsidation due to the amino acid sequences that are conserved in these termini compared with other non-specific DNA-BPs [5, 27-31, 68, 70]. However, these termini have demonstrated inconsistent DNA-binding results from Southwestern *in vitro* analysis.

Positive L1 DNA-binding was demonstrated using exogenous plasmid DNA [20, 29], but does not appear to specifically bind to HPV DNA under the condition used [27]. L2 DNA-binding has been demonstrated using HPV DNA and non-specific bacteriophage DNA [27]. Mehdaoui et al, has demonstrated a conserved function of the proposed DNA-binding domains (DNA-BD) from HPVs capsid termini by transferring the termini onto the capsid protein (ex. VP60) from rabbit hemorrhagic disease virus (RHDV), which is unable to bind to its own viral-DNA natively [28]. The transfer of the HPV16 capsid DNA-binding domains onto the RHDV

capsids allowed the RHDV capsids to bind DNA, encapsidate DNA, and to efficiently deliver the encapsidated gene into target cells in contrast to the wild-type RHDV capsid proteins [28].

Electron-micrographs of HPV virions extracted from wart-tissues demonstrate that wild-type virions produced naturally are uniform in shape and in size with no evidence of incomplete or defective particles [15, 22-24, 26, 32]. Comparatively, the VLP-HPV particles expressed from transient transfection systems do not produce VLPs that definitively resemble wild-type HPV-virions. The VLP systems produce a milieu of capsid structures with varying morphology than authentic wild-type HPV-virions [5, 7, 8, 16-18, 20, 25, 51, 68]. L1 and L2 capsid proteins have the capacity to spontaneously oligomerize, which is the driving force during capsid assembly [76, 77].

The contentious hypothesis regarding regulated versus non-regulated HPV capsid assembly does not intend to dispel the fact that HPV capsids self-oligomerize, but addresses several lines of evidence suggesting that oligomerization may be regulated in order to generate uniform virions of 55-nm diameter. The ramifications of regulated spontaneous oligomerization can be assessed by studying the infectivity of the resulting VLP-HPV particles compared to wild-type HPV virions. Also, regulated spontaneous oligomerization could be analyzed indirectly through the quantification of specific-neutralizing antibodies that are raised against the major neutralizing (conformational) epitopes versus non-neutralizing antibodies that are raised against non-neutralizing (linear) epitopes of the resulting capsids from VLP-HPV particles versus wild-type HPV virions.

Papillomavirus Variable Regions or Surface Loops

The capsids of HPVs have five conformational immuno-reactive epitope regions, which varies amidst each HPV-type and amongst the PVs capable of infecting other species (for ex. Bovine-PV and Canine Oral-PV). There is a conformational-dominant immuno-reactive epitope region within all PVs, which are located within the hollow pentameric-shaft space, which forms via the oligomerization of five-L1 monomer (L1x5) protein molecules and is composed of the DE- and FG-loops of L1 [5, 78-82]. The L1x5 molecules are connected via intra-strand tethering, where the terminal amino- and carboxyl- amino acids function as arms that protrude and interacts with an adjacent L1 protein forming the interactions necessary for pentamerization and ultimately total capsid formation [5].

Objective

The major objective of this thesis work is to amalgamate the known information generated from papillomavirus research and to discern the caveats pertaining to wild-type virion formation compared to *in vitro* system virion production and virion encapsidation (specific versus non-specific encapsidation).

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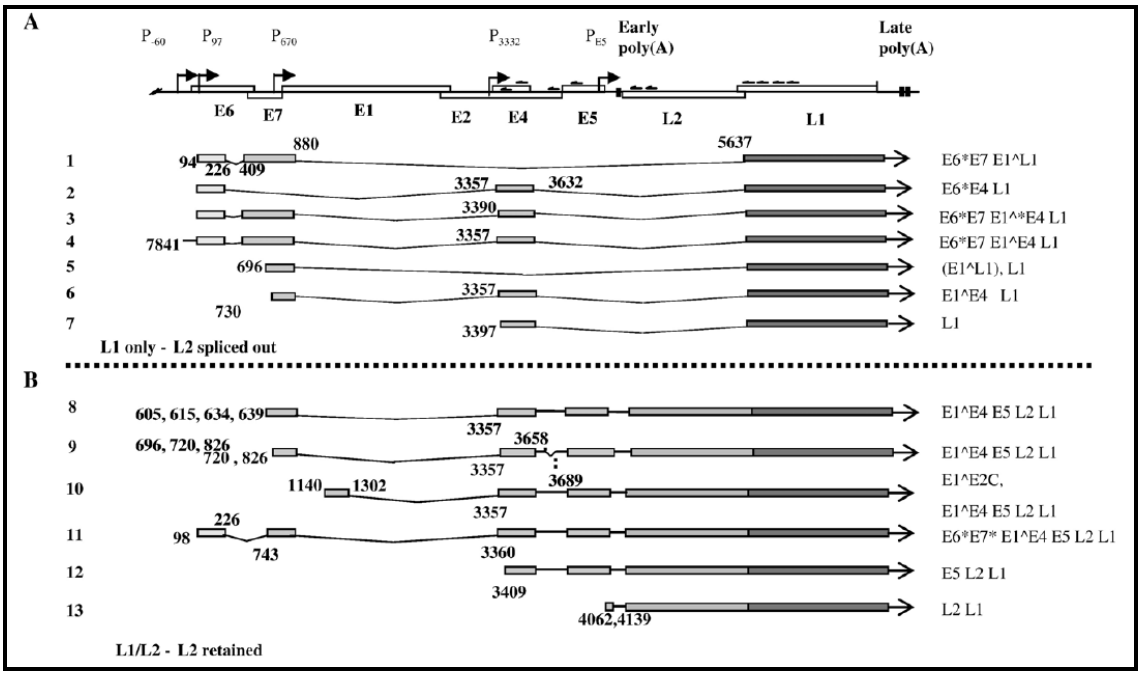
A
P-60
P97
P670
P3332
PE5
Early poly(A)
Late poly(A)
E6
E7
E1
E2
E4
E5
L2
L1
1
2
3
4
5
6
7
880
94
226
409
5637
E6*E7 E1^L1
3357
3632
E6*E4 L1
3390
E6*E7 E1^*E4 L1
7841
3357
E6*E7 E1^E4 L1
696
(E1^L1), L1
3357
E1^E4 L1
730
3397
L1
L1 only - L2 spliced out
B
8
9
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11
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605, 615, 634, 639
3357
E1^E4 E5 L2 L1
696, 720, 826
720 , 826
3658
3357
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1140
1302
3689
E1^E2C,
3357
E1^E4 E5 L2 L1
226
98
743
3360
E6*E7* E1^E4 E5 L2 L1
E5 L2 L1
3409
L2 L1
4062,4139
L1/L2 - L2 retained

Figure 1.

Schematic diagram of the transcripts identified by 5'RACE.

(A) A diagram of the linearised virus genome. Open boxes; coding regions, arrows; promoter positions, chevrons; approximate positions of the primers used in 5'RACE. Early poly(A) and vertical bar: early polyadenylation site, late poly(A) and vertical bars: late polyadenylation sites. (B) Structures of the cDNA species identified. Grey bars: coding sequences, lines: introns spliced out. Numbers indicate the genomic positions of the 5' ends of the cDNAs and the splice donor and acceptor sites [64].

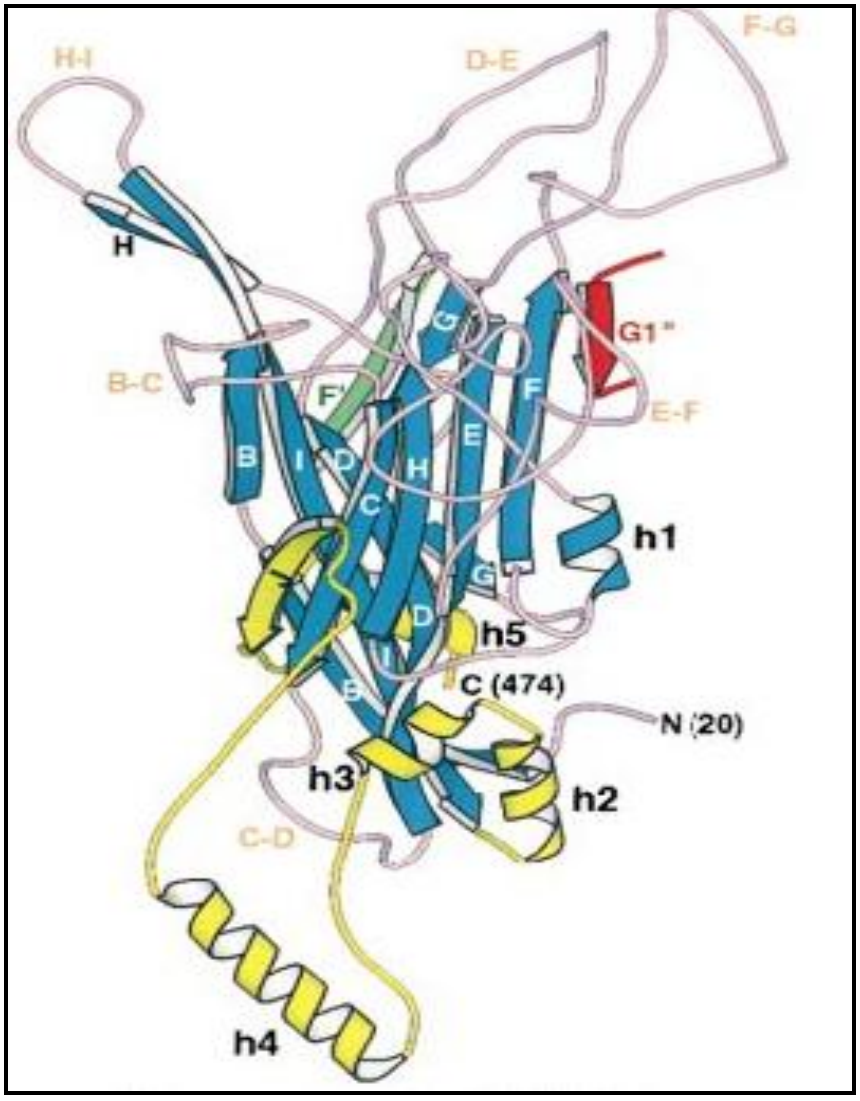
H-I
D-E
F-G
H
G1"
B-C
F'
F
E-F
B
I
D
H
E
C
h1
G
D
h5
I
B
C (474)
N (20)
h3
h2
C-D
h4

Figure 2.

The crystal structure of the major capsid protein (L1) of HPV16.

The image above depicts an individual L1 protein molecule expressed from the HPV16 L1-ORF [5]. Five L1 molecules interact together to produce the L1-pentamer, of which 72-pentamers constructs an intact HPV16 virion particle. The variable regions of interest to this thesis are the DE- and FG-loops known to interact with the L2 protein [6, 7, 70] and are capable of producing conformational neutralizing antibodies [35, 37, 78-80, 82, 85]. Also of interest, the variable regions BC- and HI-loops, which are predicted herein this thesis as being involved in interacting with the E2, trans-regulatory protein.

Chapter 2: Evolutionary and structural analyses of alpha-papillomavirus capsid proteins yields novel insights into L2 structure and interaction with L1.

The work herein Chapter 2 was published in the Virology Journal*

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Authors' Contributions

JL assembled HPV sequences and performed MUSCLE and Clustal analyses. DP did further data and literature research and helped write the manuscript. SR performed 3D protein modeling and docking analyses. TJ, **WAH** helped in analysis of 3D structures and conserved regions. FT helped in writing the manuscript and data analysis. PCA conceived of the study and coordinated the work, performed 3D rendering of structures and using Pymol, and edited the manuscript.

Abstract

Background: PVs (PV) are small, non-enveloped, double-stranded DNA viruses that have been identified as the primary etiological agent for cervical cancer and their potential for malignant transformation in mucosal tissue has a large impact on public health. The PV family, Papillomaviridae, is organized into multiple genus based on sequential parsimony, host range, tissue tropism, and histology. We focused this analysis on the late gene products, major (L1) and minor (L2) capsid proteins from the family Papillomaviridae genus Alpha-papillomavirus. Alpha-PVs preferentially infect oral and anogenital mucosa of humans and primates with varied risk of oncogenic transformation. Development of evolutionary associations between PVs will likely provide novel information to assist in clarifying the currently elusive relationship between PV and its microenvironment (i.e., the single infected cell) and macro environment (i.e., the skin tissue). We attempt to identify the regions of the major capsid proteins as well as minor capsid proteins of alpha-papillomavirus that have been evolutionarily conserved, and define regions that are under constant selective pressure with respect to the entire family of viruses.

Results

This analysis shows the loops of L1 are in fact the most variable regions among the alpha-PVs. We also identify regions of L2, involved in interaction with L1, as evolutionarily conserved among the members of alpha- PVs. Finally, a predicted three-dimensional model was generated to further elucidate probable aspects of the L1 and L2 interaction.

Introduction

Papillomaviruses (PVs) are small, non-enveloped, double-stranded DNA viruses identified as the primary etiological agent in cervical cancer and their potential for malignant transformation in mucosal tissue is a major health concern. Papillomaviruses (PVs) have also been linked to benign cutaneous lesions and with some non-melanoma skin cancers. These viruses are very common pathogens of epithelial surfaces that account for a variety of proliferating lesions in humans and animals. In the past few years, the available number of complete HPV genomic sequences has increased substantially to comprise more than 150 GenBank entries (2007). The PV family, *Papillomaviridae*, is organized into multiple genera based on sequential parsimony, host range, tissue tropism, and histology. We focused this analysis on the late gene products, major (L1) and minor (L2) capsid proteins of the family *Papillomaviridae* genus *Alpha-papillomavirus*. Alpha-PVs preferentially infect oral and anogenital mucosa of humans and primates with varied risk of oncogenic transformation. Two members of the genus *Alpha-papillomavirus* are also associated with cutaneous lesions, Human papillomavirus (HPV) 2 and HPV10. The alpha genus includes twelve cutaneous HPV types and two simian PVs [1]. HPVs of the alpha genus are also sub-categorized based on associated risk of oncogenic transformation into Low Risk (LR) and High Risk (HR) genotypes. The HR group includes 19 HPV genotypes (types 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68a, 73, 82, 82subtype) and 13 are grouped as LR (types 6, 6a, 6b, 11, 40, 42, 43, 44, 54, 61, 70, 72 and 81) according to epidemiological evidence [2]. Infection with HR HPV genotypes such as HPV16 and HPV18 has been directly related to the subsequent development of cervical cancer [3, 4].

PV genomes are characterized by eight well-defined open reading frames (ORFs), which are all transcribed from the same DNA strand and orientation. The translated proteins are classified as "early" (E) or "late" (L) based on their temporal expression. The viral ORFs include 3 regulatory genes involved in transcription and replication (E1, E2, and E4), 3 oncogenes (E5,

E6, and E7), and 2 genes encoding for self-assembling proteins which constitute the viral capsid (L1 and L2) [5]. PV capsids are approximately 600 A° (50 nm) in diameter and composed of 72 pentameric capsomeres arranged in a T = 7 icosahedral lattices [6]. The PV capsid proteins L1 and L2 are synthesized late in the infection cycle and function to encapsidate the closed circular double-stranded DNA mini-chromosome [7]. The 72 viral capsomeres are composed of L1 protein pentamers, and the capsomeres are associated with 12 or more copies of the L2 protein. Recombinant L1 or L1 and L2 can be generated in a variety of expression systems to produce self-assembled virus-like particles (VLPs), which approximate the structure of native virions [8, 9]. The structure of "small," T = 1 VLPs assembled from HPV16 L1 expressed in *Escherichia coli* (*E. coli*) has recently been resolved at a resolution of 3.5-A° [10]. Moreover, the crystal structure of L1 major capsid protein provides insights into the conformation of neutralizing epitopes, potential receptor binding sites, the nature of inter-capsomeric contacts [6] and interactions with L2. High levels of neutralizing antibodies can be generated after immunization with HPV L1 VLPs producing highly type-specific neutralization activity [11, 12].

Conformational epitopes and the location of epitopes are critical for the production of neutralizing antibodies [13, 14]. It has been suggested that L1 loops extending toward the outer surface of the capsomere contain type specific epitopes [6]. Studies with monoclonal antibodies suggest epitopes composed of FG and HI loops are important for HPV 16 [15] neutralization whereas BC, DE, and HI loops are important for neutralization of HPV 6 and 11 [16]. It has also been recently reported that different PV types display distinct features on their surfaces [11]. Analysis of the HPV 11 L1 protein implicated the C-terminus in both DNA binding, as well as inter-capsomere binding [17]. However, less is known about other alpha-PVs. Within a virion, L2 forms contacts with the viral genome, in addition to contacts with L1 pentamers functioning to encapsidate the genome [18]. Comparison of HPV L2 with the polyomavirus major and minor capsid protein suggests that L2 may interact with residues located within the central cavity of L1 pentamer [19]. The carboxy-terminal 44 amino acid region of L2 has been shown to facilitate the interaction of L2 with L1 [19]. Among these 44 amino acids, residues 413–419 are important, since they contain conserved proline residues. It was further demonstrated that heterologous L1-L2 complexes for some PVs can be produced inside the bacteria. Taking these facts into consideration, we hypothesized that the interaction domains of L1 and L2 should be fairly conserved among alpha- PVs. Some L2 domains may be exposed at the virion surface, thus enabling recognition of a specific epitope for immune recognition [20, 21]. This surface-exposed region of L2 would also be able to interact with cellular receptors to facilitate uptake of virions [22]. Moreover, it has been suggested with bovine papillomavirus (BPV) that L2, amino acids

61–123 are exposed on the surface of the virion and can be recognized by monoclonal antibodies while the majority of the residues appear to be buried inside the surface [23]. The association of HPVs with benign and malignant neoplasia has led to research efforts focused toward improvement on the current understanding of diversity within this virus group, so that diagnosis, treatment, and control of HPV infections may be optimized. Many aspects about evolution of PVs are still relatively poorly understood.

Therefore, probing of evolutionary and structural relationships between PVs will likely provide novel insight to assist in clarifying the functional differences between PVs and their tropic microenvironment; cutaneous cells or mucosal epithelial cells. To date, a broad range of bioinformatics tools have been applied to analyze the complete PV genome (or at least properly alignable genomic regions).

In this paper, we identify the regions of the major capsid proteins as well as minor capsid proteins of alpha-papillomavirus that have been evolutionarily conserved, and define regions that are under constant selective pressure with respect to the entire family of viruses. Here we show that the loops of L1 are, in fact, the most variable regions among the alpha-PVs. We also identify regions of L2 involved in interaction with L1 as evolutionarily conserved among the members of alpha-PVs. Finally, we generated a predicted three-dimensional model to further elucidate probable aspects of the L1 and L2 interaction.

Materials and Methods

Alpha-papillomaviruses: The Seventy-six alpha-PV sequences obtained for this analysis were retrieved from the NCBI protein database according to the reference list of alpha-PVs published on the Universal Virus Database [24]. In addition to the list of PV species in the alpha genus provided by ICTV we also included six characterized variant HPV16 sequences in this analysis. Corresponding GenBank accession numbers are included in the additional data file [See Additional file 1].

Alignment: The compiled protein sequence sets were aligned using MUltiple Sequence Comparison by Log-Expectation, MUSCLE [25, 26]. MUSCLE alignment was selected as it has been shown to be one of the most accurate multiple alignment tools currently available [26]. MUSCLE utilizes a 3-stage algorithm 1. Generate a progressive alignment 2. Increase the accuracy of the progressive alignment by reconstructing a tree with the Kimura matrix and the

clustering method 3. Iterative refinement of progressive alignment MUSCLE outputs were then loaded into the CLUSTAL-X user interface for graphical representation of residue conservation and analysis [27]. Sequence logo representation of MUSCLE alignments were generated using WebLogo 3 [28]. The complete output of the L1 and L2 alignments can be viewed in the Additional Data file [See Additional file 1].

3D prediction of L2 and L1-L2 interaction: The HPV16 L1 protein structure was obtained from the RCSB Protein Data Bank [29]. The secondary structure of HPV16 L2 protein was predicted by submission of the L2 amino acid sequence into 3D-Jigsaw [30] and these data refined using the Swiss Model Server http://swissmodel.expasy.org. The L2 amino acid sequence data was then submitted to SAM-T09, Sequence Alignment and Modeling System, for tertiary structure prediction [31-34]. The SAM predicted L2 structure was then further refined using AL2TS to predict side chains [35]. HPV16 L1 protein structure and the N-terminus of the L2 predicted structure were then submitted to ClusPro, a Protein-Protein Docking Web Server [36-40]. The L1 PDB crystal structure and predicted L2 structure were submitted as ligand and receptor, respectively. PyMOL, a molecular visualization program, was used to view and manipulate both the predicted L2 model and the predicted protein-protein interaction models of HPV16 L1-L2.

Results

Variable regions coincide with surface loops of L1 protein

We found the external loop regions of alpha-PVs correlate to the least conserved regions in our alignment (Figure 1). The external loop regions: DC loop (AA 50–69), DE loop (AA 110–153), EF loop (AA 160–189) and FG loop (AA 262–291) and the HI loop (AA 348–360) have been characterized as being antigenic in the HPV16 model [15]. The regions, which have been previously characterized as showing antigenicity, and have characterized monoclonal antibodies, are L1 residues F50, 1–173, 111–130, A266, 268–281 and 427–445 [19,20]. It has been suggested that these regions are less conserved than other L1 regions because they are under constant immunogenic selective pressure. Our sequence analysis of L1 shows high degree of similarity among all the genotypes [1,2] [Additional file 1]. Despite being classified into different genotypes, identical variable regions are clearly present within the HPV L1 protein (Fig. 1).

L1 conserved cysteines and lysines

HPV16 cysteine 201 and 454 are conserved across the entire alpha-papillomavirus family alignment (Fig. 1). This is in good agreement with previous studies that found these regions were required for interaction between the L1 monomers to form trimers [18]. These trimers are believed to be required to form the capsomer, and thus the virion. There are also three lysines residues (278, 356 and 361) that are moderately conserved and highly conserved when viewed from the fact that in each alpha-PV, at least one of these three residues was a lysine. It has also been shown that these residues are involved in cellular binding to host heparan sulfate chains [14].

Conserved C-terminus DNA binding region

Our alignment shows that the C-terminal DNA binding domain, rich in lysines, from HPV16 AA 500–531, is highly conserved for alpha-PVs (Fig. 2). The specific location of lysines in the sequence is somewhat variable especially upstream away from the C-terminus. At the extreme C-terminus there are almost completely lysine residues, which are conserved across the alpha-PV family.

H4 helix region is conserved

The H4 region (AA 413–428, [19]) is in a region of conservation with 5 amino acids being universally conserved (414L, 418Y, 419R, 425A, and 428C4) and four being close to universal (413T, 416D, 420F and 421L). This conservation is, mostly but not wholly, at the N terminus side of the helix.

L1 regions of interaction with L2 conserved

Upon analysis of the 3d docking prediction between L1 and L2, we targeted regions of L1 that were in prime positions to be involved in the protein interaction with L2, specifically the interaction in the region of 247–269 and the region of 113 to 130 (Fig. 3). These regions have a fair amount of conservation (supplemental Fig.), which is probably due to the protein interaction being critical for infectious virion formation.

L1 interaction domain of L2 is highly conserved

We analyzed the L1 carboxy-terminal binding domain of L2 among alpha-PVs. We observed a moderate degree of conservation exists for these domains among alpha-PVs. Interestingly, proline residues are conserved in many genotypes and occur frequently in this

region compared to other regions of the L2 protein. L2 is hypothesized to have at least two L1 interaction domains and the second domain has been suggested to be located in the N terminal portion of L2. Our results show that such repetitive proline residues are not highly conserved in the amino terminal portion of L2, but to some extent the repetitive proline motifs are found in region corresponding with HVP16 aa 97–150 (Fig. 2a & 2b). The alpha-papillomavirus L2 alignment results did not verify a conserved amino acid region corresponding to the hypothesized second amino terminal L1 interaction domain of L2 as found in BPV1.

N-terminal L1 binding domain of L2

We attempted to identify possible conserved neutralizing epitope domains of L2 that would provide valuable direction for development of cross-protective therapeutics against alpha-PVs. Our data suggests residues corresponding to HPV16 aa108–120 are moderately conserved and a specific subset of 8 residues are highly conserved (Fig. 2d). Other domains of L2 responsible for neutralizing antibody response have been suggested corresponding to amino-terminal 88 residues [41] more specifically 17–36 amino acid region might be responsible for neutralizing antibody response [42,43]. Our alignment shows the amino-terminal residues are mostly conserved among alpha-PVs. Two alignments of alpha-PVs, grouped into high risk and low risk, depicted a similar pattern of conservation at amino terminus as well as for the residues corresponding to HPV16 L2 aa 108–120.

DNA binding domains of L2

Positively charged arginine and lysine residues of the extreme carboxy-terminus DNA binding domain of L2 appear highly conserved among alpha-PVs (Fig. 2a). The evolutionarily conservation of the L2 amino-terminus including the DNA binding domain suggests the function of DNA binding for capsid formation and viral DNA transport upon cellular entry has remained relatively stable over the divergence of these PVs.

Predicted 3D model of the L1:L2 interaction

Data from the predicted secondary structure of HPV16 L2 was compared with the tertiary structure model of L2 confirming similarity. The L1 binding sites on L2 were confirmed to be within the N-terminal region. Specific interactions predicted between L1 and L2 include the DE loop and the FG loop of L1 and specific proline-rich regions of L2 (Fig. 3). Amino acids 105–120 within the L1 DE loop interact with one highly conserved and one completely conserved proline within L2 at amino acids 53–59. The FG loop of L1, consisting of amino acids 247–269, also is

predicted to interact with one highly conserved and one completely conserved proline of L2. These regions of L2 consist of residues 24–30 and 260–264. These proline residues range from highly conserved to completely conserved among all alpha-PVs. Based on the protein-protein interaction model of the L1 and L2 monomers, we conclude that L2 binds within the center of the L1 pentamer. The position of the L2 antigenic region, therefore, is predicted to face outward when bound to the L1 pentamer (Fig. 3).

Discussion

Analysis of the alpha-PV family L1 and L2 proteins provided evolutionary information to assist in understanding the predicted interaction domains and their roles in virion assembly. Particularly of value is our L2:L1 structural interaction model, which has similarities with the manner in which Polyomavirus VP2 interacts with VP1 [44]. Analysis of the sequence alignments, suggested that the variable regions of L1 are mainly located within the surface loops and comprise several neutralizing epitopes. Numerous groups have identified neutralizing epitopes within the L1 surface loops, strongly suggesting that these regions are the major targets for neutralizing antibodies [14-16, 36, 45-49]. Conversely, only a few CTL epitopes have been identified within L1 protein and targeting of these CTL epitopes could be linked to individual HLA allele expression [50-52]. Indeed, our sequence analysis indicated a strong correlation between these immune epitopes and variable regions of L1 (Fig. 1). We conclude that immune selection as the main driving force for diversity of surface loops on HPV L1 protein, but that the overall structures of the loops are conserved. It is possible that there are other, yet to be identified, epitopes downstream of the HI loop, as our analysis shows that some of these regions are relatively variable (Supplemental Fig.). The caveat to our analysis is that we had only compared linear epitopes with variable regions, as data on discontinuous epitopes is unavailable. It is conceivable that some variable regions could also comprise of discontinuous epitopes.

Nevertheless, comparison and identification of L1 protein variable regions could provide beneficial information for development of broadly neutralizing antibodies against HPV.
Along with the interaction loops, several other features or regions of HPV L1 are relatively conserved within the multiple alignments. There are also 3 lysine amino acids (278, 356 and 361) that are thought to bind to heparin-sulfate side chains on the cell surface and facilitate cellular entry. Mutations of these residues to alanine is known to cause a reduction cell binding and infection of pseudovirions [53]. Residue 278 is within the FG loop, while the other two are

contained in the HI loop. These residues are some-what well conserved within alpha-papillomavirus family. With residue 361 being the highest conservation and 356 being the lowest and not very well conserved. There is most likely an evolutionary selective pressure to change these loops and looking at the amino acid conservation it appears that all of these amino acids occur at or right next to regions of low conservation. This presumably is due to the selective pressure placed on the antigenic loops by the host adaptive immunity. The function of cellular binding and entry to the cell is absolutely required for viral replication and existence, and so there should be selective pressure to maintain amino acids that are required for cell entry. If these residues are indeed important for cellular binding and entry, then these residues are probably experiencing both of these pressures and this may be an explanation of why some are less conserved than others, while each sequence tested have at least one lysine at one of these positions. The results of the previous experiments [53] suggest that there is an additive effect with these residues, suggesting that they may not all be at the same selective pressure, which would support the idea that alpha-PVs can withstand some changes in these residues. Also there is a region, the H4 helix that is thought to be involved in pentamer-pentamer interaction [6]. H4 is the helix that is thought to be on the outer rim, deep within the pentamer interaction. It is the most distal part of the protein. Deleting this region causes loss of interaction between pentamers, however the pentamers still form. This region, while not being the best conserved, contains 5 amino acids that are universally conserved in the alpha-PV family. These amino acids are probably critically important in the pentamer interaction, and may constitute conserved interaction points.

We have shown that the conserved region where the final 11 AA of the C-terminus are involved in DNA interaction [54] is fairly well conserved across the genomes, albeit not exactly the same residues positions along the sequence, but the region is holistically conserved (Fig. 1f). Most likely this region is involved in packaging DNA into the virion. Since DNA packaging universally needs to be accomplished between alpha-PVs, conservation of this region is probably evolutionarily favored.

We found the carboxy terminal L1 binding domain of L2 to be conserved among the alpha-PVs irrespective of high or low risk group. However, the structural interaction of L1 and L2 and formation of capsid is still not clear. Minor capsid protein (L2) binds the L1 capsomers but not to the VLP, suggesting that L2 co-assembles with L1 rather than being inserted into a pre-formed capsid [19]. L2 is required for efficient genome encapsidation, suggesting the capsid assembles around histone-bound genome rather than by injection of the genome into the capsid via a portal

vertex. The involvement of L2 in genome encapsidation coupled with the DNA-binding properties of L2 suggests that, within a virion, L2 forms multiple contacts with the viral genome in addition to contacts with L1 pentamers [18, 55]. Our results show that both DNA binding domains of L2 are highly conserved among alpha-PVs. The level of conservation of the L2 DNA binding domains indicates the maintenance of this binding function has been vital to the virus from an evolutionary standpoint. Two distinct L1 binding domains have been described for BPV1 L2; a C-terminal L1 binding domain (BPV L2 aa 384–460) that interacts with L1 capsomers *in vitro*, and a central region (BPV L2 aa129–246) that fails to interact with capsomeres [56]. These authors described the interaction between BPV1 L2 aa129–246 and L1 on the basis of co-immunoprecipitation and co-localization studies. However, when we aligned the N-terminal interaction domain of BPV with HPV16, only 20% similarity was observed. This region is furthermore not conserved among the members of alpha-PVs. Our data revealed that the N-terminal 100–150 amino acids of L1 are moderately conserved among alpha-PVs and there is occurrence of proline residues more frequently than other region of HPV. We hypothesize that this L2 region is likely to contain the second L1 interaction domain. However, further experimental evidence is required to support this hypothesis. The carboxy-terminus of the L1 binding domain described between residues 396–439 of HPV11 L2, is consistent with the C-terminal L1 binding domain in residues 384–460 of BPV1 L2 [56]. Our results confirmed that the C-terminal L1 interaction domain of L2 is highly conserved throughout the members of alpha-PVs. It seems that the C-terminus of L2 composed of many hydrophobic residues neutralizes charges on L1 which further leads to changes in conformation in L1, thereby permitting the assembly of T = 1 VLPs at neutral pH. Moreover the assembly of L1 and L2 into full-size T = 7 VLPs at neutral pH may require further modification of the *in vitro* assembly buffer conditions, different lengths of L2 or a combination of L1 and L1-L2 containing capsomere. For the important mechanism of capsid assembly, PVs have maintained an evolutionarily conserved L1 binding domain at the C-terminus of L2. The location of the primary L1-binding site on the carboxy-terminus of L2, the structural complexity, and hydrophobicity of the L1-L2 interaction have interesting parallels to the mouse polyomavirus VP1-VP2 interface [57]. However, a certain degree of difference in capsomere organization between PVs and polyomaviruses exists due to the amino acid variation between theses two viruses [6].

Recently much focus has been given toward the development of potential vaccines against HPV. Anti-L1 antibodies obtained by immunizing mice or rabbits with the L1 capsids have been shown to have primarily type specific neutralizing activity. Limited cross-neutralizing activity has been observed between closely related types such as HPV18 and 45, and HPV6 and HPV11 [58].

Moreover, anti-L1 antibodies can protect animals against challenge with animal PVs [59, 60]. The L1 capsids of HPV6, 11, 16, and 18 were used in recent clinical trials as prophylactic vaccines, which successfully induced type-specific neutralizing antibodies in recipients [61, 62]. However, there is no general consensus regarding the epitope at the amino terminus of L2 responsible for production of neutralizing antibody response. One group showed amino acids from 108–120 are conserved between HPV16 and HPV18, which have at least 46% similarity in this region [20].

Our results depict conservation of the first half of this region (aa108–120) among alpha-PVs and this might be the epitope associated with production of neutralizing antibody response. It is important to note that the second half of this region (108–120) is highly variable and the cause of this variability is currently unclear. Other domains of L2 responsible for neutralizing antibody response have been established as well [41, 42]. These groups suggested that amino-terminal 88 residue more specifically 17–36 amino acid region might be responsible for neutralizing antibody response. Our results correlated with the previous published results [20, 41, 63]. When separated and group by HR and LR, the alpha-PVs produced a similar pattern of conservation at the amino terminus as well as for HPV16 residues 108–120. These results suggest that both regions may be involved in production of neutralization antibody and cross protection against different types. A recent study reported that the amino terminal 18–144 is conserved in some of the papillomavirus and our results are also in good agreements this observation [63]. Furthermore, we show that the extreme N-terminal region is highly conserved for the alpha-PVs. The N-terminal region is also the location of a DNA binding domain and it is still unclear how the N-terminal epitope is exposed on the surface of the virion. Recently Buck et al 2007 proposed a model of assembly for L2 and L1 capsomeres suggesting there may be changes in conformation of capsid in order to extrude the terminal epitopes. Several studies have attempted to identify the nature of both neutralizing epitopes of both L1 and L2 using L1/L2 VLP to better define the topology of L2. All these data suggest that HPV16 L2 residues 108–120 and 69–81 are epitopes displayed on the surface of VLPs and virions [20, 22]. Clearly our knowledge of L2's topology in the capsid is limited but perhaps the L1 capsomer-L2 complex or pseudovirions might be suitable for X-ray crystallographic studies. Unlike structures of VLPs or capsomers, analysis of pseudovirion or true virion preparations would also clarify the interaction between the capsid and the nucleo-histone core. Studies with purified capsid proteins or VLPs indicate that the C-terminal positively charged tail of L1 that includes a nuclear localization signal is also critical for binding to and packaging DNA. Similar sequences on both termini of L2 may also play a role in encapsidation of the viral genome as well as infection. In the present study we attempt to predict the secondary

structure of L2. We also mapped the interaction domain of L2 within the monomer of L1. Our data shows that the amino terminus of L2 is involved in interaction with L1. Our data is unique from previous results in which the second independent L1 interaction domain of L2 has been shown to be amino acid 129–246 for BPV [56]. Analysis of the corresponding region of BPV with alpha-PVs we only 20% similarity suggesting that other regions of L2 may be involved in interaction with L1. Nonetheless, our L2:L1 structural interaction model had distinct similarities with the Polyomavirus VP2 interaction with VP1 [44]. The Polyomavirus VP2 protein is predicted to be inserted at the center of VP1 pentamers, just as we predict PV L2 to be positioned in L1 pentamers. The alignment of L2 for the alpha-PVs, the amino terminus 100–150 aa is rich in proline. Previous studies have also suggested that the proline rich regions are involved in protein-protein interaction [64]. Moreover, the carboxy terminus region of L2 contains repetitive prolines which are highly conserved in the alpha-PVs [19]. However, our computer-predicted L2 structure should be considered hypothetical. Nevertheless this interaction is representative of L1 and L2 interaction domains. Two large limitations of the predicted 3D interaction model are the absence of DNA bound to L2 and the difficulty in determining L2 flexure within the pentameric form of L1. In this model the DE and FG loop of L1 are involved in interaction with L2 and these loops are also outside the structure. According to one proposed model, L2 drives the formation of capsid by recruiting the L1 pentamers [65, 66] and it has been suggested that both the L1 interaction domain of L2 are necessary for efficient virus encapsidation [56]. Studies utilizing VLPs and purified capsid proteins coupled with detailed virion mutagenesis and structural studies are necessary for confirmation of these results.

Competing interests

The authors declare that they have no competing interests.

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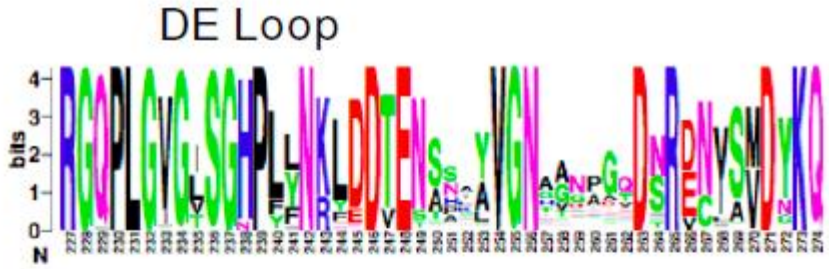
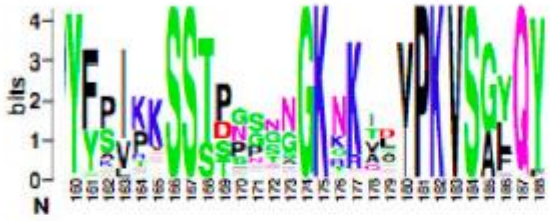
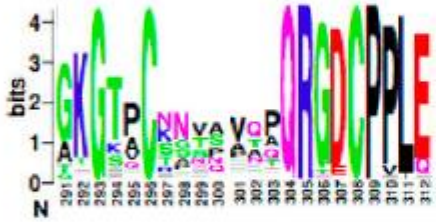
1a



1b



1c



1d

1e

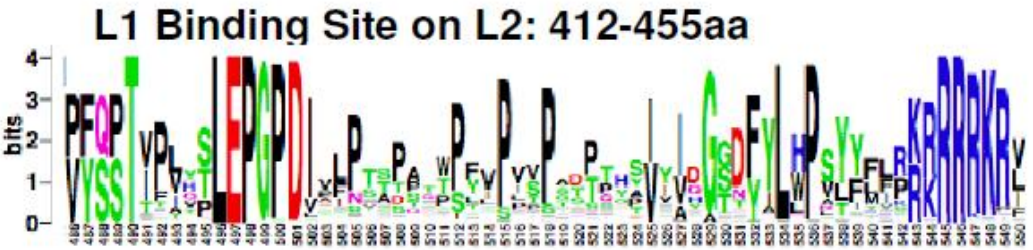
1f

Figure 1.

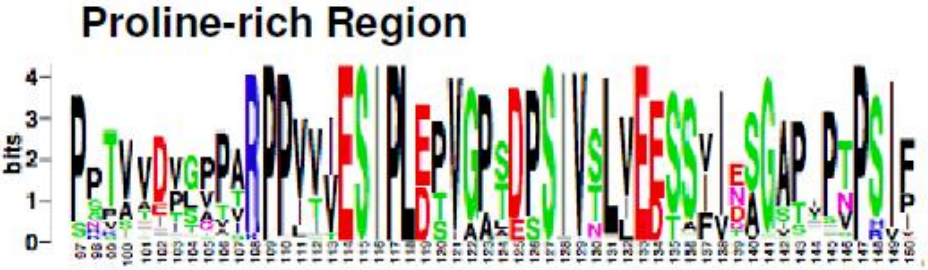
Analysis of conserved regions within the L1 protein.

Seventy-six alpha-HPV L1 sequences were obtained from the NCBI protein database under the Universal Virus Database, as well as six known HPV16 L1 variant sequences provided by the ICTV. The sequences were aligned using the MUltiple Sequence Comparison by Log-Expectation (MUSCLE). MUSCLE outputs were loaded into CLUSTALX user interface for graphical representation of residue conservation and analysis. Sequence conservation is by the height of residue logos (indicated in bits), as generated by WebLogo 3. The consensus sequences resulting from the alignments of the external loop regions are as follows: DE loop (aa 227–274) (1a), BC (aa 160–188) (1b), EF loop (aa 291–312) (1c), FG loop (aa 385–417) (1d), the HI loop (aa 475–490) (1e) antigenic determinants, and the conserved DNA binding regions (1f). The HPV16 cysteines at residues 201 and 454, which are involved with disulfide linkages, are conserved across the entire alpha-papillomavirus family.

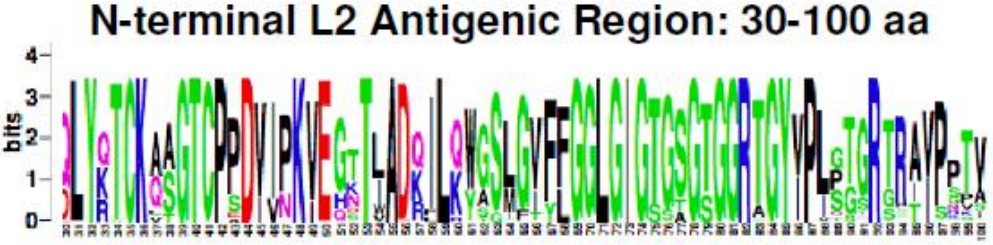
2a


L1 Binding Site on L2: 412-455aa
bits

2b


Proline-rich Region
bits

2c


N-terminal L2 Antigenic Region: 30-100 aa
bits

2d

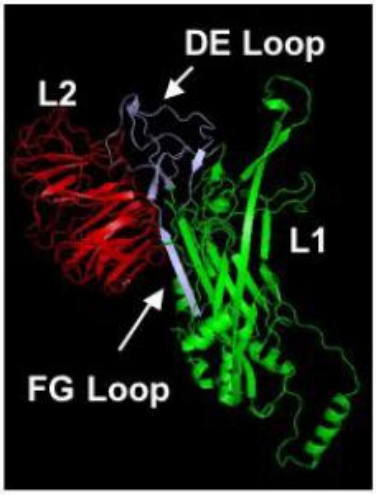

L2 Antigenic Region: 108-120aa
bits

Figure 2.

Analysis of conserved regions within the L2protein.

Similar to the L1 analysis, L2 protein sequences derived from NCBI and ICTV databases were aligned using MUSCLE and visualized with CLUSTALX interface for graphical representation of residue conservation and analysis. Sequence conservation is by the height of residue logos (indicated in bits), as generated by WebLogo 3. The known L1 interaction domain of L2 (486–550 aa in the alignment, corresponding to 412–455 aa in HPV16 L2) (2a), conserved proline rich regions (2b), well conserved N-terminal antigenic regions (30–100 aa) (2c), and (aa 108–120) (2d) are shown. The C-terminal DNA binding domain, rich in lysines, from HPV16 AA 500–531, is highly conserved for alpha HPVs.

3a



3b

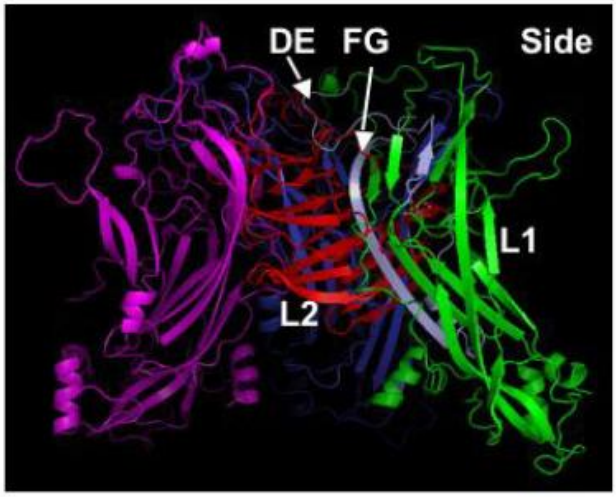


Figure 3.

Predicted 3D model of the L1:L2 interaction.

The HPV16 L1 protein structure was obtained from the RCSB Protein Data Bank. The secondary structure of HPV16 L2 protein was predicted with using the 3D-Jigsaw and the Swiss Modelling Server http://swissmodel.expasy.org. The data was then analyzed with the SAM-T09 program (Sequence Alignment and Modeling System, for tertiary structure prediction) which was further refined using AL2TS. The docking position of L2 to L1 was predicted with ClusPro, (Protein-Protein Docking Web Server). The L1 and L2 structures were then visualized using PyMOL (a molecular visualization program). The predicted L2 structure in its docking position on the L1 monomer (3a). The predicted orientation of the L2 protein within the L1 pentamer structure; two L1 monomers of L1 have been removed to clearly show the alignment of L2 within the structure (3b).

Additional material

Additional file 1

*Multiple sequence alignment of L1 and L2 proteins. Seventy-six alpha-HPV L1 and L2 sequences were obtained from the NCBI protein database under the Universal Virus Database, as well as six known HPV-16 L1 and L2 variant sequences provided by the ICTV. The sequences were aligned using the MUltiple Sequence Comparison by Log-Expectation (MUSCLE). MUSCLE outputs were loaded into CLUSTALX user interface for graphical representation of residue conservation and analysis. Sequence conservation is by the height of residue logos (indicated in bits), as generated by WebLogo 3. All outputs for both sequences are shown.*Click here for file [http://www.biomedcentral.com/content/supplementary/1743-422X-5-150-S1.pdf]

Chapter 3: Analysis of HPV16 Major and Minor Capsid Protein's DNA Binding Domains

Introduction

The encapsidation-strategy used by PVs to package their specific-genome has been argued to occur through non-specific interactions between the capsid proteins and the HPV genome [1, 34]. Furthermore, there are antagonizing results observed within experiments trying to demonstrate the DNA-binding capacity of the HPV major capsid proteins [1-4].

The theory of non-specific protein-to-DNA binding is an accepted theory in molecular biological research [15, 25, 30- 32, 34, 40]. The research conducted using histones has revealed that non-specific DNA interactions allow for the various regulatory functions of the histones when complexed with chromosomal genomic DNA [40]. The gene-delivery strategies employed in molecular biology today, when utilizing the HPV major and minor capsid proteins, do not include any HPV-specific genome *cis*-acting element(s) [13, 18] and thus would appear that the capsid proteins individually or together bind to DNA. However, the HPV capsid-to-DNA binding is mediated by non-specific protein-to-DNA interactions.

During a natural infection, it is known that L1 is not produced without L2 [15] and it is also known that L2 is required for the infectivity of an HPV virion [22]. Also, L2 has a role during the entry phase of an HPV infection, while L1 is docked at the cellular surface, and L2 transports the HPV-genome to the nucleus [21, 35-39]. Therefore; it is reasonable to deduce that L2 is the primary HPV-capsid protein responsible for binding to the HPV-genome during *de novo* virion production, when analyzing the HPV lifecycle from the entry phase perspective.

Animal viruses, plant viruses, and bacteriophages utilize the icosahedron structure to protect their genomic material from degradation by the surrounding environment, for infection, and for virion propagation. The exception is that not all isometric-structured viruses utilize a tethering-like protein via non-specific binding to encapsidate its specific-genome; on the contrary, specific binding proteins appear to fulfill the function for these viruses that utilizes a specific-encapsidating virion production system (25-27, 32, 33).

The bacteriophages, ϕX174 and G4, are two examples of viruses belonging to the Microviridae family utilizing the DNA encapsidation/condensing protein, J-protein (J), to non-specifically stabilize their single-stranded DNA (ssDNA) viral genome during encapsidaton [44]. The J-proteins belong to viruses within the Microviridae family [43, 47]. These bacteriophages are primitive icosahedral virions having a T=1 lattice. The HPV16 crystal structure produced is a T=1 virion [41], although the native HPV16 72-pentamer virion is T=7. The ability of the HPV16

virion to acquire the T=1 virion form suggests that the icosahedral shape may be evolutionarily conserved and functionally redundant to encapsidate viral-DNA from degradation. Protein J functions as a tether between the viral genome and the immature capsid protein necessary for morphogenesis (Figure 1) [45-47]. Protein J from the G4-phage has two functional domains composed of a protein interaction domain and a DNA-binding domain, while the J-protein from ϕX174 contains an additional 13-a.a. central hinge-like region (Figure 2) [43, 44, 47]. Both proteins have the capacity to re-capitulate DNA encapsidation during *in vitro* morphogenesis-complementation assays [44]. The J-protein is critical and necessary for the production of infectious virion particles, non-infectious particles are produced in the absence of the J-protein [45], which may serve in a similar capacity to the minor (L2) capsid protein of PVs [1, 22, 36].

As well, the capsid termini from HPV16 capsids contain DNA-binding potential and is able to confer their DNA-binding potential to the capsid proteins from an animal virus naturally unable to bind to DNA [18]. Also, the L2 protein from HPV16, and not L1, interacts with bacteriophage genomic DNA [1].

The predicted structure of the L2-to-L1 interaction, in correlation with the known crystal structures of L1 [41], suggests that the L2 protein is lodged within the conical-shape pentameric shaft composed of five-oligomerized L1-proteins and that both capsid proteins may have their amino-terminal and/or their carboxyl-terminal amino acids protruding into the interior of the viral particle core [20].

The accumulation of DNA-binding analysis performed in various research labs have only been examined in *in vitro* systems, which would not allow many conclusions to be revealed about the relevant biological events occurring during the HPV16 encapsidation process [1-4]. Thus, it is important to investigate the biological significance of the HPV DNA-binding domains in regards to DNA-binding, encapsidation, and capsid assembly/morphology in a concise system via a re-capitulation as can be performed using the ϕX174 encapsidation-complementation system. It is possible that such a mechanism is evolutionarily conserved.

Direct amino acid sequence comparison of the HPV capsid termini and of the J protein formed the hypothesis that icosahedral viruses utilizing non-specific DNA-binding proteins extrinsically (ex. ϕX174/G4) may have evolved over time to have this function intrinsically (HPV16) integrated at the termini of L2 capsid proteins. The goal of this research was to further examine HPV encapsidation: for example the protein interactions between L1 and L2 and their association with DNA.

Materials and Methods

In silico Analysis: The National Centers for Biotechnology Information (NCBI) server provided the sequence information used to perform amino acid sequence alignments with Clustal-W (www.ebi.ac.uk/Tools/msa/clustalw2). The alignment data was used to perform evolutionary tree-plots. The Protein Database (PDB) provided the crystal structures of the J-proteins: ϕX174 (**pdb id:** 1RB8) and G4 (**pdb id:**1GFF). The two Js were accessed from the NCBI server using the pFam accession (PF-0426) for the group and/or the InterPro accession (IPR-006815) code. The ϕX174 DNA was accessed on the NCBI server using NC_001422, the GI (216027), and/or the GeneID (2546404) accession codes.

Primers used to create chimeric proteins: The plasmid constructs were generated with standard molecular cloning techniques [48]. Figure-4 displays the amino and/or the carboxyl-terminal ends of HPV16 capsid proteins and how they were fused in-frame with Domain-II of the J-protein of ϕX174. The primers were created to ligate within the NcoI-site within the pSE420 vector MCS. Internal plasmid-specific Trc-His primers (IDT) verified the pSE420 vector and used to sequence the plasmid for the inserts.

Direct Sequencing of pSE420 clones: The ABI Prism Big Dye Terminator v3.1 cycle sequencing was used to verify clone insert into the parent pSE420 plasmid. The internal forward primer, Trc_His, was used to direct the sequencing reaction. The PCR reaction system parameters included a five-minute hot start at 95^{o}C, followed by 35-cycles of: 1) 95^{o}C for 30-seconds 2) 42^{o}C for 15-seconds and 3) 60^{o}C for 4-minutes, and ending with a final hold at 4^{o}C. The sequencing PCR product was precipitated as follows: 1) adding 5-µL of EDTA [125-mM] and 60-µL of 100% ice-cold ethanol with room temperature incubation for 15-min, next the samples were centrifuged at 1600-rcf at 4^{o}C for 45-min. The sample tubes were then inverted onto a kim-wipe and pulse centrifuged at 180-rcf for 17-seconds. Then, 60-µL of 70%-ethanol was added to the samples and centrifuged at 1600-rcf for 15-minutes at 4^{o}C. The sample tubes were inverted and pulse-centrifuged as before at 180-rcf. Finally, the samples were dried in a speed-vacuum for 5-minutes and used for direct-sequencing.

Plasmids: The cloning strategy was designed using standard molecular biological techniques [48]. The pSE420 plasmid is a 4613-bp protein expression plasmid. Cloning of the inserts into

this plasmid were facilitated by the *Nco*I restriction enzyme, which provides an intrinsic start codon. The promoter for this plasmid is IPTG-inducible via the "trc" promoter.

Results

An amino acid sequence homology BLAST analysis using only the HPV capsid termini of both L1 and L2 proteins, not including any portion of the structural amino acids giving each protein its structure, and this approach revealed sequence similarity between the HPV16 capsid proteins' termini and the J proteins of the Microviridae, specifically from ϕX174 and G4 (Figure 3).

Further investigation into the lifecycles of the Microviridae, ϕX174 and G4, revealed the unique opportunity to analyze the HPV16 capsid termini in a system in which the DNA tethering protein is known to be encapsidated within the viral particle due to its dual function of protein-to-protein and protein-to-DNA interactions at opposite termini of the J protein (Figure 1) [44-47].

Discussion

The terminal regions of the HPV16 and HPV18 capsid proteins: L1-COOH (35-a.a.), L2-NH2 (15-a.a.), and L2-COOH (25-a.a.), are suspected to intermittently interact with HPV DNA via non-specific interactions during virion assembly and these studies have been ambiguous during attempts to demonstrate the capsid-to-DNA interactions [1-4]. A bioinformatic comparison of the HPV terminal capsid regions revealed greater sequence homology with the J protein expressed by the phage ϕX174 than to bacterial, eukaryotic, and various viral DNA-binding proteins (Figure 3). The sequence homology amongst DNA-binding proteins from viruses infecting hosts in different kingdoms of organizational and hierarchal life at first was trivial. Examination of the ϕX174 lifecycle has revealed the J-protein's function, which is to mediate the tethering of ϕX174 genome to the newly forming capsid particle via non-specific genomic interactions [45, 47].

The impact of the J-protein's function is demonstrated via complete loss of virion formation in its absence, even though DNA replication occurred normally [47]. The capsid-termini from the L2-protein of HPV16 is analogous in its necessity for encapsidation and viral infectivity [13, 18,

19, 22]. Therefore, the goal of this research was to create chimeric J-like proteins composed of the HPV16 DNA-binding domains and the J-protein's protein-interaction domain (Figure 4) and to assess the capacity for the HPV16 capsid protein's ability to re-capitulate infectivity to the ϕX174 virus.

The idea for this research study progressed from data-mining the known DNA-binding domains from the NCBI website and the proposed hypothesis is to discover whether or not HPV16 capsid-DBDs participate in non-specific binding activity and will complement the function of ϕX174's J-protein. Previous research suggested that HPV capsid proteins utilize non-specific genomic interactions during the HPV encapsidation process, due to *in vitro* observations of the capsid proteins being able to encapsidate non-HPV DNA [5-8] and from the idea that L2 interacts with HPV and non-HPV DNA *in vitro* [1, 13, 18]. The inability to demonstrate L1 DNA-binding *in vitro* may be attributed to the system conditions, while the capacity for L2 to demonstrate DNA-binding may be a result of the same conditions (i.e. buffer effect).

The analysis concluded in previous reports were performed under *in vitro* conditions that do not mimic *in vivo* conditions. The ideas for "specific capsid-to-DNA interactions" take root from the cell's perspective in that amongst degrading host genomic DNA, there is HPV-16 episomal DNA, and the contention is: how are "non-specific capsid-to-DNA interactions" supposed to account for the efficient encapsidation of HPV-specific genomes resulting in the production of highly infectious virions. In theory, non-specific capsid-to-DNA interactions would result in non-specific encapsidation that would result in a mixed-population of virions containing HPV-specific DNA and virions containing degraded cellular DNA, which has been observed in murine-derived HPV pseudovirions [34].

The contradictions about HPV-encapsidation are relevant theories from the cellular perspective and from HPV's perspective, therefore; an alternative approach to assessing HPV-encapsidation is an important caveat in designing an experiment to approach a reasonable and definitive conclusion regarding this process. Due to the lack of clarity revealed from *in vitro* DNA-to-capsid Southwestern experiments, regarding HPV encapsidation, the ϕX174 encapsidation J protein complementation assays were designed to determine if HPV16 capsid-DBDs have the capacity to non-specifically encapsidate ϕX174 or G4 genomic DNA.

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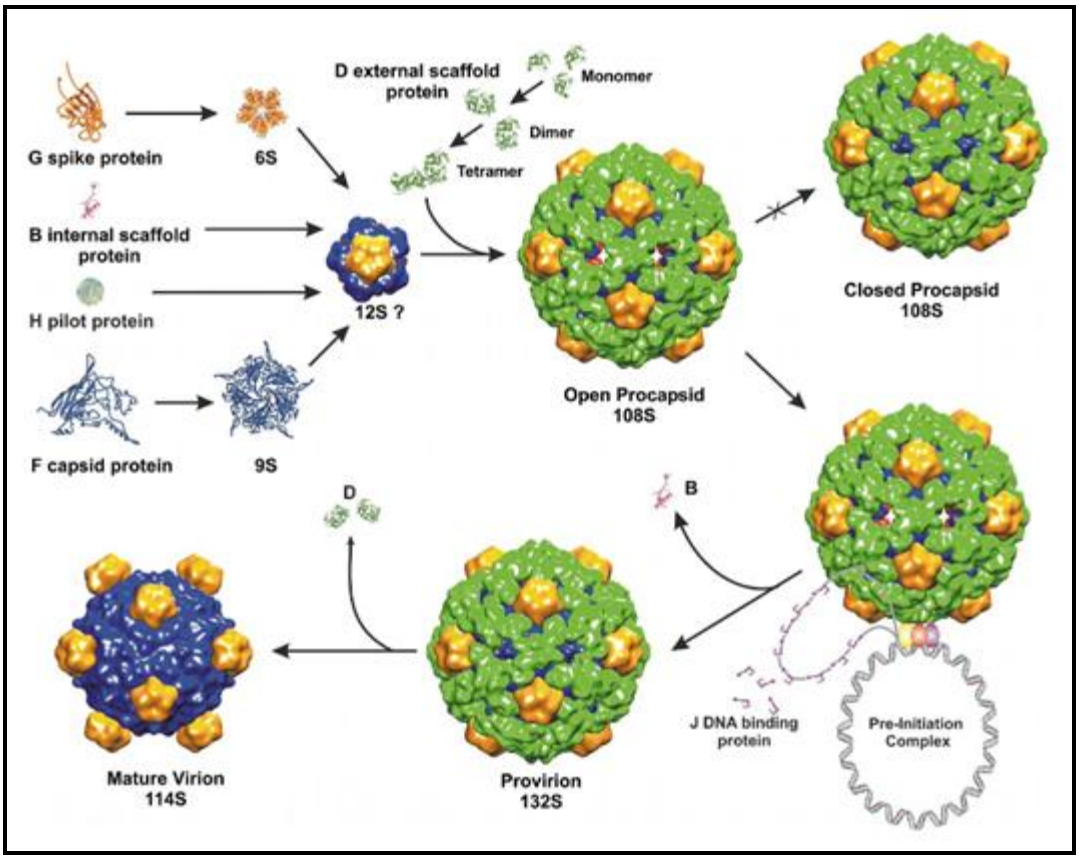
D external scaffold protein
Monomer
Dimer
Tetramer
G spike protein
6S
B internal scaffold protein
H pilot protein
12S ?
F capsid protein
9S
Closed Procapsid 108S
Open Procapsid 108S
D
B
Mature Virion 114S
Provirion 132S
J DNA binding protein
Pre-Initiation Complex

Figure 1.

Overview bacteriophage capsid assembly pathway: utility of the J protein.

Microviridae assembly pathway reprinted from Bernal *et al.*, 2003 with permission from Elsevier. A general depiction of the overall lifecycle for bacteriophages within the Microviridae family (ex.ϕX174, α-4, and G4), specifically the DNA-binding function of the J protein with the ss-DNA genome, is depicted (lower right corner). Also, the interaction of multiple copies of the J protein and ss-DNA genome being incorporated into the 108S capsid (open procapsid) transitions the 108S capsid into the 132S (provirion) capsid during capsid assembly, indirectly demonstrates the protein-to-protein dual capacity of the J protein [49, 50].

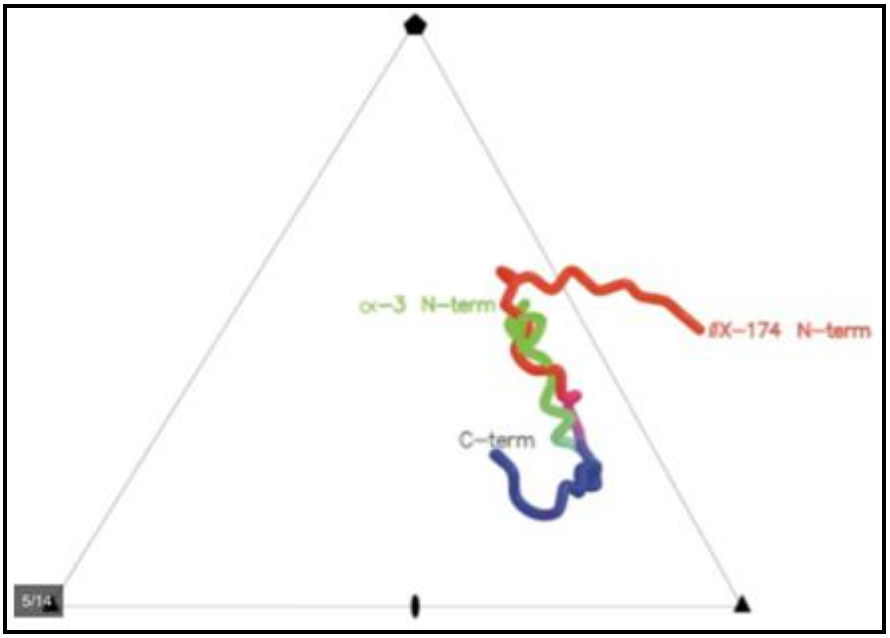
α-3 N-term
ØX-174 N-term
C-term
5/14

Figure 2.

Comparison and localization of bacteriophage DNA-binding J protein ϕX174 and α-3.

A hypothetical interior localization of the J protein, is depicted, within a single capsid monomeric-unit from either ϕX174 (red color) or α-3 (green color). The J protein of ϕX174 (red color) has an additional 13-a.a. compared to α-3 (green color), as shown. Both J proteins have a common C-terminus (blue color). The localization highlights the protein-to-protein interaction capacity (C-terminus) of the J proteins as well as their DNA-binding capacity (N-terminus). The J protein of G4 (not shown) is comparable to the α-3 J protein depicted [49].

Table 1. Summary of the *Microviridae* J proteins and the HPV16 Capsid Termini.

Parent Virus	**Protein Name**	**Amino Acid (a.a)**	**Amino Acid Composition**	*** Function**
ϕX174	J protein	38-a.a.	5'-MSKGKKRSGARPGRPQPLRGTKGKRKGARLWYVGGQQF-3'	protein / protein protein / DNA (n/s)
α-3	J protein	24-a.a.	5'-MKKARRSPSRRKGARLWYVGGSQF-3'	Protein / protein protein / DNA (n/s)
G4	J protein	25-a.a.	5'-MKKSIRRSGGKSKGARLWYVGGTQY-3'	protein / protein protein / DNA (n/s)
HPV16	L1 (major) Capsid: COOH-termini	35-a.a.	5'-FLLQAGLKAKPKFTLGKRKATPTTSSTSTTAKRKKRKL-3'	Heparin-Sulfate Binding Sequence NLS DNA-binding (n/s)
HPV16	L2 (minor) Capsid: NH2-termini	15-a.a.	5'-MRHKRSAKRTRASAT-3'	Furin Cleavage Site DNA-binding (n/s)
HPV16	L2 (minor) Capsid: COOH-termini	25-a.a.	5'-HPSYYMLRKRRKRLPYFFSDVSLAA-3'	NLS DNA-binding (n/s)

***Key**: protein / protein = protein-to-protein interaction, protein / DNA = protein-to-DNA interaction,

NLS = nuclear localization signal, (n/s) = non-specific

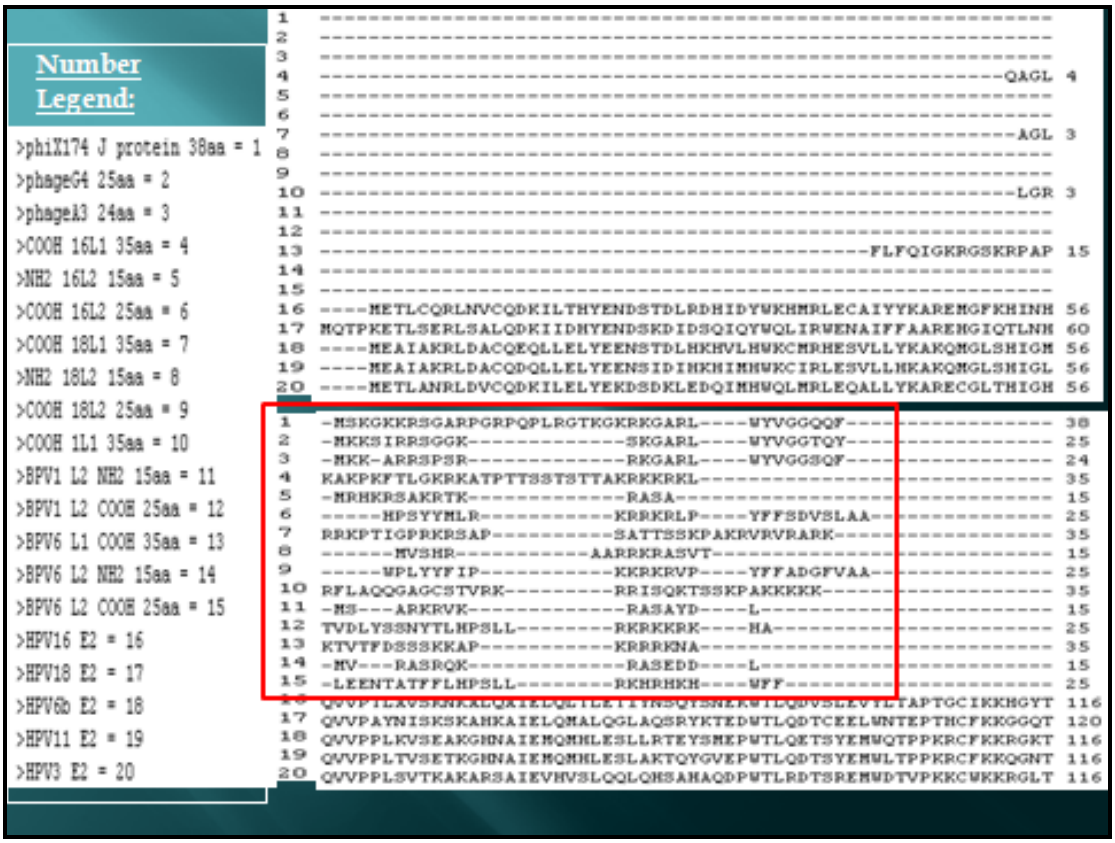
Number Legend:
>phiX174 J protein 38aa = 1
>phageG4 25aa = 2
>phageA3 24aa = 3
>COOH 16L1 35aa = 4
>NH2 16L2 15aa = 5
>COOH 16L2 25aa = 6
>COOH 18L1 35aa = 7
>NH2 18L2 15aa = 8
>COOH 18L2 25aa = 9
>COOH 1L1 35aa = 10
>BPV1 L2 NH2 15aa = 11
>BPV1 L2 COOH 25aa = 12
>BPV6 L1 COOH 35aa = 13
>BPV6 L2 NH2 15aa = 14
>BPV6 L2 COOH 25aa = 15
>HPV16 E2 = 16
>HPV18 E2 = 17
>HPV6b E2 = 18
>HPV11 E2 = 19
>HPV3 E2 = 20

Figure 3.

Amino acid sequence alignment of proposed DNA-Binding Domains.

An amino acid BLAST generated using specific and non-specific DNA-binding proteins from Human Papillomavirus16 and 18, Bovine Papillomavirus1 and 6, and of the J proteins from members of the Microviridae family (ex. ϕX174, α-3, and G4). The specific *cis*-element binding protein, full-length E2, of papillomaviruses is used during the BLAST in order to demonstrate robustness of the BLAST alignment and does not reveal any similarity to the non-specific binding proteins encompassed within the red box. The alignment does reveal the homology existing between the E2 proteins from varying papillomaviruses, numbers 16 – 20. The red box, encompasses the homologous regions from the known non-specific binding-proteins of the full-length J proteins and the known Papillomavirus's DNA-binding termini from the major (L1) and minor (L2) capsid proteins, numbers 1 - 15. The Papillomavirus DNA-binding termini used were: L1-COOH (35-a.a), L2-NH2 (15-a.a) and L2-COOH (25-a.a). The legend is in numerical order for simplicity: **1)** ϕX174-J, **2)** G4-J, **3)** α-3-J, **4)** HPV16 L1-COOH, **5)** HPV16 L2-NH2, **6)** HPV16 L2-COOH, **7)** HPV18 L1-COOH, **8)** HPV18 L2-NH2, **9)** HPV18 L2-COOH, **10)** BPV1 L1-COOH, **11)** BPV1 L2-NH2, **12)** BPV1 L2-COOH, **13)** BPV6 L1-COOH, **14)** BPV6 L2-NH2, **15)** BPV6 L2-COOH, **16)** HPV16 E2, **17)** HPV18 E2, **18)** HPV6b E2, **19)** HPV11 E2 , and **20)** HPV3 E2.

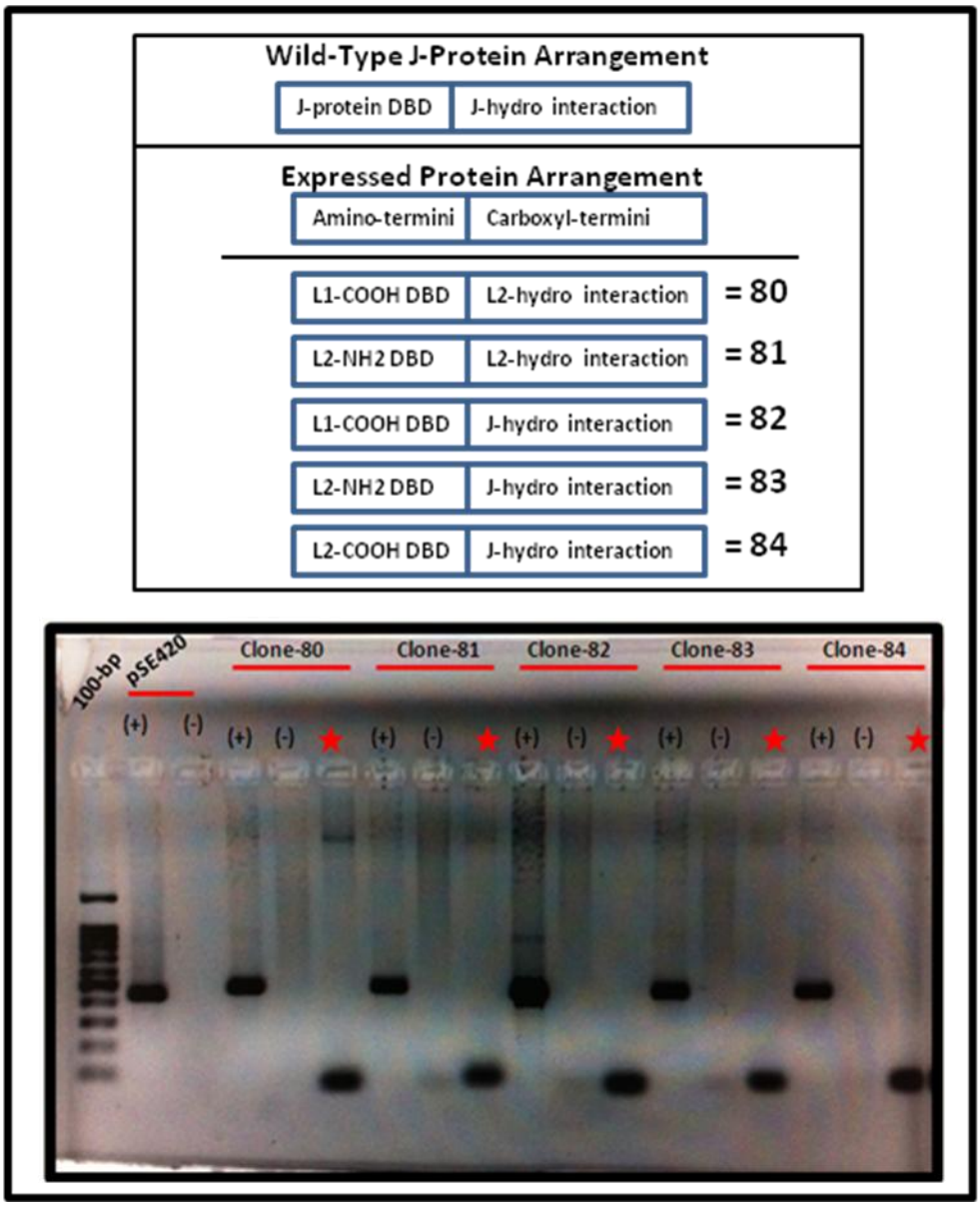
Wild-Type J-Protein Arrangement
J-protein DBD
J-hydro interaction
Expressed Protein Arrangement
Amino-termini
Carboxyl-termini
L1-COOH DBD
L2-hydro interaction
= 80
L2-NH2 DBD
L2-hydro interaction
= 81
L1-COOH DBD
J-hydro interaction
= 82
L2-NH2 DBD
J-hydro interaction
= 83
L2-COOH DBD
J-hydro interaction
= 84
100-bp
PSE420
Clone-80
Clone-81
Clone-82
Clone-83
Clone-84
(+)
(-)

Figure 4.

Depiction of the J-protein chimeras used during an established *in vitro* bacteriophage encapsidation analysis.

Organizational arrangement of the wild-type J-protein from alpha-3 and G4 bacteriophages, compared t the arrangement of the J-protein chimeras composed of the HPV16 capsid protein's DBDs with or without the J-protein's hydrophobic interaction domain (**A**). PCR analysis verified the insertion of the nucleotide-sequence encoding the chimeric proteins that indicated as clone-80 thru clone-84 and by ABI Big Dye terminator nucleotide sequencing (**B**). Compared to the negative-lanes, the specific primers for each clone were able to amplify the region of interest from the pSE420 constitutive expression vector (**red star**). Intrinsic primers, Trc-His primers, verify the presence of the pSE420 vector after bacterial transformation and plasmid mini-DNA preparation, indicated as positive in each sample.

Chapter 4: HPV16 Capsid Morphological Considerations: HPV Capsid Morphology or Capsid Formation Theory

Introduction

During keratinocyte differentiation, the cells begin to produce keratin and provide human skin with the physical properties that acts as a barrier against physical damage and micro-organismal infections. However, during an HPV-infection, keratinocyte terminal differentiation aids in the induction of the late-phase of the HPV's lifecycle [24-26]. The late-phase of the HPV lifecycle is marked by: 1) the production and presence of capsid protein, 2) an increase in HPV-specific genomic amplification, 3) subsequent virion/particle assembly, and 4) production of an infectious virus particle. These viral-specific events are highly orchestrated and dependent upon the differentiation-status of the upwardly migrating epithelium [27, 28].

Consequently, there are many intracellular molecular changes (via alternative splicing and the polyadenylation machinery/mechanisms) aiding keratinocyte differentiation and the regulation of HPV genomic transcription within HPV-infected keratinocytes facilitating HPV's late-phase [24-26].

The transfection of HPV16 E2 has demonstrated *in vivo* via transient transfections in primary human keratinocytes to mediate the transcription of the L1- and L2-mRNAs from the HPV16 genome [28]. The E2 regulated transcription of the capsid mRNAs is dependent upon the E1:E2 molar ratio; for example high concentrations of E1 (occurring in the stratum basale up to the stratum spinosum) favors HPV-genomic replication due to the helicase capacity of E1 [30, 33, 34] and the known interaction with E2 induces HPV genomic replication [29-32]. The same study, has revealed that high concentrations of E2 (occurring in the stratum spinosum up to the stratum corneum) favors the transcription of capsid mRNAs [28].

The research herein demonstrates that the early master regulatory protein (E2) interacts with HPV16's major (L1) capsid protein (Figure 1). HPV16 E2 is a known modular functioning protein with the capacity to facilitate viral transcription in conjunction with host-transcription factors [29-32]. E2 mediates HPV-DNA replication via interacting with the HPV16 E1 helicase-like protein [30, 32-34]. The E2 protein also interacts with the minor (L2) capsid protein and causes its degradation [35, 36]. The biological significance of the L2-to-E2 interaction maybe deduced as a result from research demonstrating that L2 is required for an infectious HPV-virion [37-39], thus the L2-to-E2 interaction may occur as a fail-safe mechanism to prevent premature capsid construction/formation. Also, it has been shown that L2 proteasomal-dependent

degradation is induced by the interaction with the E2 protein is observed in HPV and BPV analysis [35, 36].

The hypothesis of this research is that the observed interaction between HPV16 E2 with the major (L1) capsid protein maybe involved in the morphological spatial arrangement of the spontaneously forming HPV capsid *in vivo*. Therefore, this novel interaction may influence E2-mediated HPV-capsid assembly relative to non-regulated spontaneous HPV-capsid assembly.

Materials and Methods

Plasmids: The expression plasmid, pCDNA-3.1^{+}, transfected into HEK-293 cells utilizes the cytomegalovirus (CMV) promoter to induce the expression of exogenous proteins, due to cellular machinery able to *trans*-activate the CMV promoter. The pGEX-3T expression plasmid contains the T7 promoter allowing for the induced expression of the inserted open reading frame (ORF) within *E. coli* cells BL-21 (DE). Exogenous protein induction/expression occurred in the presence of 2-mM isopropyl β-D-1-thiogalactopyranoside (IPTG).

Cell-Lines: Bacterial, *Escherichia coli (E. coli*) BL21 (DE-3) cells were transformed with the pGEX-3T vector to express exogenous proteins labeled with a GST moiety at the NH_2-terminus, according to standard molecular cloning procedures [40]. The *E.* coli BL21 (DE-3) cell-line expresses the T7 phage RNA polymerase in order to induce exogenous proteins regulated by a T7-promoter. Human embryonic kidney cells (HEK-293) were transfected, using the Dreamfect reagent [**Oz Biosciences**], with expression plasmids to produce fusion or non-fusion exogenous proteins according to manufacturer's protocol. Post-transfection and incubation for 24-hours, for optimal protein expression, the cell-culture medium was replaced with fresh Dulbecco's Modified Eagle Media (DMEM) and supplemented with 10%-fetal bovine serum (10%-FBS). Mammalian cells were maintained within a 37^{o}C incubator at 5%-humidity and with 5%-CO_2.

S.D.S. /P.A.G.E or non-SDS/PAGE: Cells (*E. coli* or HEK-293) expressing exogenous proteins with or without a heterogenous peptide were prepared into W.C.L by centrifuging the cells away from the growth media. Then the cells were washed in 1x PBS and re-suspended into an ice-cold NP-40 lysis buffer solution, and incubated for 20-min on ice with intermittent vortexing. The samples were then spun in a refrigerated micro-centrifuge at 4^{o}C for 20-min and the supernatant transferred to a fresh micro-centrifuge tube. The W.C.L were quantified by the Nano-Drop

spectrophotometer and approximately equal aliquots were loaded onto SDS/PAGE or non-SDS/PAGE gels for protein analysis as indicated.

DENATURED (Reduced): The whole cell lysates (W.C.L) analyzed under denaturing conditions were dissolved into a 6x-sample/loading buffer containing sodium dodecyl-sulfate (SDS) and the known protein-denaturing reagents, dithiothreitol (DTT) and β-mercaptoethanol (β-Mercap). The protein samples were then boiled for 10-min, allowed to cool to room temperature, quickly micro-centrifuged, and loaded onto a 8%-PAGE gel supplemented with SDS, according to standard molecular analytical procedures [40]. The conditions presented herein, resolves proteins into there monomeric-state or into individual proteins.

INTACT (Non-Reduced): The W.C.L analyzed via non-reducing conditions were dissolved into the 6x-sample/loading buffer containing SDS and without the denaturing reagents as above. The protein samples were then boiled for 10-min, allowed to cool to room temperature, quickly micro-centrifuged, and loaded onto a 5%-non-SDS/PAGE gel supplemented with 80%-glycerol. The conditions presented herein, resolves intact proteins retaining their oligomeric-state, if applicable.

Western Blot Analysis: Post-electrophoresis; protein samples are transferred to a PVDF-membrane and then directly probed with specific antibodies as indicated within each figure, by the designation "**WB**". Post-transfer of proteins, the locales on the membrane devoid of transferred proteins are blocked with non-specific milk proteins in a 5%-milk T-TBS solution for at least 2-hours. The primary antibody is diluted and dissolved into a blotting solution containing 5%-milk and 1x T-TBS and incubated with the membrane containing the transferred proteins at 4^{o}C for 16-24-hrs. Next, the membranes are washed (3x) for 15-min each wash at room temperature to remove any un-bound antibody. The secondary antibody, prepared similarly as to the primary antibody, then incubates with the membrane at 4^{o}C for 8-12-hrs and washed (3x) as mentioned above. The membrane is incubated with a solution for chemi-illuminescence detection, according to the manufacturer's protocol [**Pierce**] and the membrane is visualized via X-ray film development. The secondary antibody is conjugated to horse-radish peroxidase (HRP), which is used to activate the chemi-illuminescence solution yielding the exact locale of the interacting: membrane-bound protein-to-primary antibody-to-secondary antibody complex.

Far-Western Protein-Interaction Assay: An adaptation of the traditional Western Blot procedure from above, the Far-western (**FW**) analysis, probes membrane-bound proteins with a blotting

solution containing the desired protein-probe of interest. Next, while the primary antibody during a traditional **WB** is specific for the known membrane-bound, the primary antibody used during the **FW** assay would be specific for the probing protein, and the secondary antibody used would be directed to the primary antibody used to detect the probing protein. The procedures during the FW are exactly similar to the traditional-**WB**, while the deviation is that the primary antibody used is specific for the probing protein. The probing protein upon comparison with a traditional-**WB** would yield information regarding the interaction capabilities of the two proteins of interest.

The **FW assay** is an analysis directed at determining whether self-oligomerizing proteins of interest (membrane-bound via Reducing or non-Reducing conditions) have modal circumstances for interactions with a probing protein (non-self oligomerizing).

ImmunoPrecipitation (IP) Protein-Interaction Assay: Proteins of interest are expressed within separate cell-lines and the cellular lysates (W.C.L) were prepared as mentioned above. Initially, the cellular lysates are pre-cleared with the isotype-control antibody to bind and remove non-specific binding proteins from contaminating the specific-immunoprecipitation's PAGE analysis. Pre-clearing occurs at 4^{o}C for 1-hr, the samples are then micro-centrifuged to remove the isotype-control antibody and any bound non-specific protein, and finally the supernatant is ready for immunoprecipitation. After pre-clearing, W.C.L expressing the proteins of interest are intermixed together in 1xT-TBS or 1xT-PBS solution and allowed to incubate for 16-hrs to overnight at 4^{o}C, while rocking and tumbling. Equal amounts of the samples are aliquotted, as indicated in the particular figure and the specific primary antibody is added to the aliquotted samples to allow the antibody to immunoprecipitate the protein of interest from the sample, along with any accompanying protein(s). The immunoprecipitating antibody incubates with the sample, with rocking and tumbling, for 16-hrs to overnight. Next, the samples are micro-centrifuged and the supernatant removed. The agarose-bound antibody and the immunoprecipitated proteins from the samples are then washed (3x) and allowed to settle, while on ice. The samples are then re-suspended in 6x-sample/loading buffer and loaded onto a SDS/PAGE gel for WB-analysis.

The **IP assay** is an analysis directed at determining whether proteins of interest are able to interact in a fluid/dynamic solution, dependent upon the quarternary structure of the proteins of interest.

Double-IP (D-IP) Protein-Interaction: After removal of the supernatant from the agarose-bound antibody-to-protein of interest, the supernatant is immunoprecipitated using an antibody specific for another protein of interest believed to be within the complex of proteins interacting together. The remaining protocol is the same as above.

3-D Prediction of the L1-E2 Interaction: The HPV16 L1 and the E2 protein structures were obtained from the RCSB Protein Data Bank [41]. These data were refined using the Swiss Model Server http://swissmodel.expasy.org. The full-length HPV16 L1 protein structure and the full-length E2 protein structure were then submitted to ClusPro, a Protein-Protein Docking Web Server [37-40]. The L1 PDB crystal structure (id: 1dzl) and the E2-TAD crystal structure (PDB id: 1dto) and the E2-DBD (PDB id: 1zzf) were submitted as ligand and receptor, respectively. PyMOL, a molecular visualization program, allowed for the visualization and for the manipulation of the protein-to-protein interaction models predicted for the HPV16 L1-E2.

Results

HPV16 E2 novel interaction with HPV-16 L1.

The HPV16 major capsid (L1) protein was expressed within HEK-293 and bacterial cells (Figure 1: panel-A, lanes-2, -3, and -5). The HPV16 E2 protein was expressed in bacterial cells as a fusion protein, with a Histidine-tag, at the NH_2-terminus of E2 (ex. His-E2) and used as a probing protein during the Far-western assay in Figure 1. The Far-western analysis (Figure 1, Panel-B, lanes-2, -3, and -5) demonstrates that the probing His-E2 protein interacts with the expressed L1 protein from the W.C.L. The L1 protein expressed within HEK-293 cells (Figure 1, panel-A, lane-2) demonstrates that L1-alone under reducing conditions remain in the disulfide-linked trimeric-form and in the monomeric-form, in agreement with the observations by Sapp et al, 1998 [22], also the His-E2 probe does interact with the monomer and trimer form of the L1 protein (Figure 1, panel-B, lane-2). The control HEK-293 cell-lysate does not have non-specific proteins interacting with the His-E2 probe (Figure 1, panel-B, lane-1). The His-E2 probe does not appear to interact with the GST-alone protein expressed without L1 (Figure 1, panel-B, lane-4). The Far-western experiment performed in reverse orientation, which uses GST-L1 as a probing protein, did not reveal an interaction between membrane-bound His-E2 after several attempts (results not shown). The results demonstrate for the first time an interaction between E2 and the L1 protein of HPV16.

Major Capsid (L1) Oligomeric Forms

Denatured (Reduced)

The HPV16 major capsid protein (L1) or the major and minor capsid proteins (L1/L2) were expressed within HEK-293 cells and WCL were prepared. As indicated, the W.C.L were subjected to immunoprecipitation analysis using the H16.V5 monoclonal antibody raised to the immuno-dominant (V5) epitope of the L1 protein (Figure 2, panel-A, lanes-2, -4, and -5) or with the IgG-isotype control antibody (Figure 2, panel-A, lanes-3 and -6). The H16.V5 antibody immune-precipitated L1-alone (Figure 2, panel-A, lane-4) and proteins presumably composed of L1/L2 (Figure 2, panel-A, lane-2). The H16.V5 antibody cross-reacted non-specifically with a cellular protein (Figure 2, panel-A, lane-5). The IgG-isotype control antibody does not precipitate any protein from the W.C.L (Figure 2, panel-A, lanes-3 & –6) revealing the H16.V5 specificity for the L1 protein. The HEK-293 control cell-lysate (Figure 2, panel-A, lane-1) does not cross-react with the H16.J4 antibody raised to detect denatured L1 proteins that were immunoprecipitated from each W.C.L in Figure 2, panel-A.

The membrane-bound protein blot (Figure 2, panel-A) was stripped of the immuno-detecting reagents (primary and secondary antibodies) using 0.2%-NaOH and incubated for 15-min. Subsequently, the membrane was washed (2x) with 1xT-TBS for 20-min and incubated in 5%-milk T-TBS solution. This step blocks the locations that may have protein removed during the stripping step and re-calibrates the membrane for the follow-up Far-western assay with the His-E2 probe.

The stripped membrane was probed with a His-E2 protein lysate and the Far-western assay reveals that the immunoprecipitated protein complexes detected with the H16.J4 antibody were able to interact with the His-E2 probe (Figure 2, panel-B, lanes-2 & -4). The protein complex that non-specifically interacted with the H16.V5 immunoprecipitating antibody did not interact with the His-E2 probing protein (Figure 2, panel-B, lane-5). Also, the primary antibody directed against E2 did not non-specifically interact with the control HEK-293 cell-lysate (Figure 2, panel-B, lane-1).

Intact (non-Reduced)

Aliquots of the W.C.L samples from Figure 2, were assessed on a non-SDS/PAGE gel under non-Reducing conditions (Figure 3). The protein complexes that were immunoprecipitated with the H16.V5 antibody were able to oligomerize in the absence of the disulfide-reducing agents (ex. DTT and β-mercapto-ethanol) (Figure 3, panel-A, lanes-2 & -4). The non-specifically

precipitated protein complexes formed higher-order protein complexes in the non-SDS/PAGE gel (Figure 3, panel-A, lane5 compared to Figure2, panel-A, lane 5). The protein product apparent in Figure 3, panel-A, lane-3 may have been residual sample from lane-4 due to comparable molecular weights of the proteins in the two lanes. There is a marked difference in the molecular weights of the protein complexes immunoprecipitated by the H16.V5 antibody (Figure 3, panel-A, lanes-2 & -4). The membrane-bound protein blot (Figure 3, panel-A) was stripped of the immuno-detecting reagents as above and re-calibrated for the follow-up Far-western assay with the His-E2 probe.

The stripped membrane was probed with the His-E2 probe, as before. The Far-western assay revealed that the His-E2 probe interacted with immunoprecipitated L1/L2 protein complexes rather than the L1-alone protein complexes (Figure 3, panel-B, lane-2). The His-E2 probe does not interact with the non-specific protein complexes (Figure 3, panel-B, lane-5) nor with the control HEK-293 cell-lysate (Figure 3, panel-B, lane-1). The interaction of the His-E2 probe with the L1/L2-VLP complexes and not with the L1-VLP complexes provides corroborative evidence that L1-alone VLPs are inefficient at oligomerization, in agreement with the observations seen by other laboratories [1, 14, 18, 22, 23, 64, 65]. Also, it has been observed that L1-VLPs and L1/L2-VLPs oligomerize into spontaneous structures that lead to tubular-structures (ex. 25-30 nm or 50-60 nm) and spherical-structures (ex. 50-60 nm) with varying lengths during self-assembly in mammalian cells and during vaccine production [22, 23, 42, 64, 66, 67]. These analyses may result, presumably, due to improper L1 self-oligomerization, which may be the reasoning that the E2 probes were unable to interact with the L1-VLP complexes, herein this report.

Minor Capsid (L2) Oligomeric Forms:

Denatured (Reducing Conditions)

HEK-293 cells were used to express the HPV16 minor capsid protein (L2) and WCL was prepared as above using ice-cold NP-40 lysis buffer. The W.C.L was immuno-precipitated with a conformationally-dependent specific antibody raised against the NH_2-terminus of the L2 protein [**Santa Cruz Biotechnology, Inc.**] and the samples processed as above (Figure 1, panel-A). The primary antibody was unable to interact and detect the L2 protein when the reducing agents DTT and β-mercapto-ethanol were present (Figure 4, panel-A). A trans-membrane prediction was performed for the L2 protein using the primary-a.a. as the input data in the Pred-TMR online

program at **[**http://athina.biol.uoa.gr/PRED-TMR/]. The *in silico* prediction revealed the conformational loop as a 20-a.a.stretch within the NH_2-terminus of L2 (45 – 65-a.a.).

The membrane-bound protein blot was stripped of the detecting reagents and the membrane was re-calibrated for the follow-up Far-western assay with His-E2 probe, as described above. The His-E2 probe was incubated with the stripped membrane blot as above (Figure 4, panel-B). The His-E2 probe did not interact with the denatured L2 protein on the SDS/PAGE gel (Figure 4, panel-B, lane-4). The His-E2 protein had a positive reaction with the L1 protein present in the dually-transfected cells expressing L1/L2 together (Figure 4, panel-A, lane-2), the same result is observed and described for L1 (Figure 2, panel-A, lane-2).

Intact (Non-Reducing Conditions)

The prepared W.C.L expressing L1/L2 or L2-alone was subjected to non-SDS/PAGE gel analysis to verify that the primary antibody to L2 does interact with the conformational loop predicted from the Pred-TMR software and as described previously. In the absence of the disulfide-bond reducing agents, the primary antibody raised to the NH_2-terminus of L2 protein detected the L2 protein precipitated from the W.C.L (Figure 5, panel-A, lanes-2 & -4). The L2-antibody and the IgG-isotype control antibody both cross-react and precipitates non-specific cellular protein(s) (Figure 5, panel-A, lane-3, -5, & -6) from the W.C.L but does not detect any non-specific proteins from the control HEK-293 cell-lysate (Figure 5, panel-A, lane-1).

The membrane-bound protein blot was stripped of the detecting reagents and a Far-western assay performed with the His-E2 probe, as above. The His-E2 probe positively interacted with the W.C.L expressing the L1/L2 proteins together (Figure 5, panel-B, lane-2) and W.C.L expressing L2-alone (Figure 5, panel-B, lane-4). The His-E2 probe did not interact with the non-specific proteins that were detected (Figure 5, panel-A, lanes-3, -5, & -6). The L2-to-E2 Far-western analysis results corroborated the hypothesized outcome and demonstrated the dynamic status for the L2 protein. In summary, the results suggested that L2 has an influence on L1 allowing the E2 probe to interact with L1. It is possible for the E2 protein to interact with a single L2 protein complexed with a trimer of L1 proteins, and it is possible that the E2 probe interacted with an L1 trimer complex.

Major and Minor Capsid Protein

Intact (Non-Reducing Conditions)

Fusion capsid proteins, GST-L1 and GST-L 2, were loaded onto a non-SDS/PAGE gel in order to determine whether the GST-moiety interferes with the capsid oligomerization process to modulate the dynamics of the E2 interaction as described above. The GST-L1 and the GST-L2 were not able to oligomerize into higher molecular weight protein complexes as compared to the native proteins expressed in HEK-293 cells. The absence of the disulfide-bond reducing reagents within the loading buffer demonstrates the His-E2 probe being capable of interacting with a non-denatured GST-L1 and GST-L2 proteins (Figure 7, panel-C, lanes-2 & -9). Comparing the western blot detection analysis of panel-A to panel-B, suggests that the conformation of the L1-protein during denaturing versus non-denaturing conditions has an affect on the presentation of the GST-moiety during western blot detection with GST-specific antibodies. Ultimately, the location and orientation of the GST-moiety at the amino-terminus of L1 has no affect on the L1-to-E2 interaction, as well as for the positive control (GST-L2). The conclusions from the results within Figures 1-6 suggested that E2 does interact with intact L1-monomer, denatured L1, and denatured L1-trimers. Also, Figures 1-6 reveal that E2 does not interact with intact L1-alone protein expressed within HEK-293 cells. The dynamics for the L1-to-E2 interaction strongly suggests that E2 may have a role during capsid morphogenesis, due to E2 being able to interact with intact GST-L1 as a monomer and E2 being unable to interact with intact L1-alone VLPs that have spontaneously formed.

Multi-expression of Capsid Protein(s) and the E2 protein

The capsid proteins (L1-alone or L1/L2 together) were expressed within HEK-293 cells along with E2 and W.C.L were prepared with NP-40 lysis buffer, as mentioned above. The W.C.L were immunoprecipitated and subsequently subjected to non-SDS/PAGE gel analysis (Panel-A) or to SDS/PAGE gel analysis (Panel-B & Panel-C). The non-SDS/PAGE analysis demonstrates that immunoprecipitation with antibodies to E2 precipitates a high-molecular weight complex with an increased sensitivity to the H16.V5 conformational-dependent antibody (Figure 6, panel-A, lanes-6 & -9). The L1 and/or L2 specific antibodies did not immunoprecipitate a H16.V5 detectable high-molecular weight complex, similarly to the immunoprecipitation by E2 (Figure 6, panel-A, lanes-2 – 5, and lanes-7 & -8). Upon further SDS/PAGE gel analysis of the indicated samples, Panel-B and Panel-C, both demonstrate that the L1-antibodies used during the W.C.L- immunoprecipitation were able to immunoprecipitate the

heterogeneously expressed L1-alone or L1/L2 proteins (Panel-B, lanes-2 & -5 and Panel-C, lanes-7). Although, the antibodies specific for the amino-terminus of L2 did not precipitate any capsid proteins from the L1/L2/E2 W.C.L (Figure 6, Panel-C, lane-8) nor from the L1/L2 W.C.L (not shown), confirming the results from non-SDS/PAGE gel analysis from above. Even though, L2 antibodies did not immuno-react with the capsid proteins expressed in the W.C.L, capsid expression did occur and re-immunoprecipitation, double-IP (D-IP), revealed identical immuno-reactive species as detected from the E2-immunoprecipitation of the L1/E2 and L1/L2/E2 W.C.L (Figure 6: panel-A, compare lane-9 to lane-10; panel-B, compare lanes-2 &-5 to lane-6; panel-C, compare lane-7 to lanes-9 & -10). The detected capsid proteins, co-expressed with E2, reveal that the L1 protein may be altered in some fashion mimicking wild-type and/or pseudovirion capsid production. The observations of L1-VLPs or L1/L2-VLPs herein this thesis does not deviate from what has been observed by many researchers in various laboratories, indicating pleomorphic-VLPs in shape and in size [23]. The results herein, report for the first time, altered L1-species within a same W.C.L preparation (Figure 6, panel-B, lane-6 and panel-C, lane-9 &- 10).

BioInformatic Prediction of the L1-to-E2 Interaction.

The analysis of L1 interacting with E2, as described above, were modeled with the Cluspro Docking on-line server (www.cluspro.com), while the images were visualized and manipulated with the DeepView Swiss-PDBViewer v3.7 (SP5) (www.expasy.org/spdbv) (Figure 8) [68]. Panel-A demonstrates the predicted interaction of the L1 (red) with the E2-DNA binding domain (E2-DBD) (blue), the E2-transactivation domain (E2-TAD) is in the confetti color scheme. The E2-TAD:DBD-to-L1 complex from panel-A is used as the ligand during the subsequent round of predictions with L1-a as the receptor, panel-B. Cluspro predicted that the E2-TAD from a single homodimer capable of interacting with the HI-loops from two-different donor L1-molecules, panel-B. Also, E2 the program predicted E2-DBD to interact with the BC-loop from L1-a. The BC-loop from L1-b is not readily viewable due to its frontward positioning. The predicted interactions suggest E2 to juxtaposition between two potential pentamers, eventually integrated into an eventual capsid or virion. The predicted structure agrees would account for the necessary restraints during spontaneous-capsid formation to restrict the HPV virion to a 72x L1-pentamer virion with a T=7 lattice. In the absence of any restraints during L1-oligomerization, the T=1 lattice is a readily common feature of spontaneous-capsid formation [1].

Discussion

HPVs express two capsid proteins capable of protecting the papillomaviral-DNA in the extracellular environment, providing cellular entry, and providing epitopes that interact with the hosts cellular immune system [1-12]. These proteins are the major (L1) and the minor (L2) capsid proteins present within all the PVs [1, 13-19, 67].

De novo synthesis of HPV L1 and L2 occurs during the late phase of the HPV lifecycle, which occurs towards the terminal differentiation stage of aging keratinocytes [38, 47-50]. As observed within *in vitro* capsid formation assays the results demonstrate HPV capsid proteins capable of oligomerization or assembly, spontaneously and free of regulation [1, 14, 15, 18-20, 23, 56-59, 64, 66, 67].

Although the possibility for E2 having a role during capsid assembly has never been ruled out [18, 55], there has been no evidence demonstrating that E2 interacts with the L1 protein, until recently (Figure 1). Furthermore, E2 does not enhance the encapsidation or the packaging of the HPV-specific DNA [18], even though E2 has specific binding sites within the long control region of the HPV genome. This conclusion further indicates that there are still important issues that have not been resolved, and these include: 1) whether non-specific HPV encapsidation, mediated by the L2 protein occurs [60, 61] and 2) does L1 interact with the encapsidated-genome within an infectious virion particle [62].

In silico data predicts E2 to interact with the BC-loop (49-58 a.a) and HI-loop (341-362 a.a) of a single L1-pentamer unit (Figure 9). Also, E2 appears to be capable of interacting with two-L1 monomers/capsomeres from different L1-pentamer units. This data suggests that E2 is the unidentified protein capable of restricting capsid assembly to just the T=7 icosahedron, as opposed to the crystallized T=1 HPV-VLP structure [1]. Evidence for the BC-loop's role for the HPV capsid's structural integrity and neutralizing epitope presentation has been revealed [3-6, 8, 51-53]. The insertion of a foreign peptide sequence into the BC-loop between positions, 56/57-a.a., produces VLPs/virions with an increased diameter (60-nm) compared to the wild-type HPV virion diameter (55-nm), while a insertion of a foreign peptide sequence into positions 266/267-a.a. within the FG-Loop produced smaller 40-nm diameter VLPs/virions [54]. Therefore, the presumption by previous labs [18, 55], that E2 could possibly have a role during HPV's late-phase agrees with the bioinformatic predictions revealing E2 to interact with the BC-loop shown to be necessary for overall capsid integrity through facilitating the presentation of the immuno-dominant H16.V5 epitope to the immune system being dependent upon preservation of the BC-loop [52]. These analyses would suggest that E2 may have a role during capsid assembly [18] rather than during HPV-genomic encapsidation [55].

The spontaneous oligomerization within L1-alone and L1/L2 VLP preparations leads to the formation of HPV-like VLPs and tubular-like VLPs, which are routinely replicated and observed within electron micrographs [18, 22, 23, 57, 59, 63]. Also, the observed data has revealed that about half of a VLP preparation is composed of capsomeres and the other half to be HPV-like and tubular-like VLPs, which breaks down into trimers and monomers [22]. Whereas within wart-derived HPV virions, nearly all of the capsomeres are employed for capsid assembly during wild-type capsid morphogenesis and the pseudovirions observed during electron microscopy are breaks down into trimers without any observance of L1 monomers, due to known reducing agents (ex. DTT and β–mercapto-ethanol) [22-23]. Also, Sapp et al, 1998 revealed differences in molecular weights of the L1 protein analyzed from VLP preparations when compared to a preparation of wart-derived pseudovirions of HPV. There is also a difference in the density between these VLPs (1.29-g/cm3) and the pseudovirions (1.32-g/cm3) analyzed after density gradient preparations [22].

The analysis from Figures 2-6 was from HEK-293 cell lysate transfected with plasmid or plasmids expressing the indicated protein(s). HEK-293 cells that were co-transfected with L1/L2 or L1/E2 were split into two aliquots, while cells transfected with three plasmids L1/L2/E2 were split into three aliquots. The aliquots were analyzed via immunoprecipitation in order to determine if E2 has an affect on L1-alone or L1/L2-VLPs. The immuno-precipitations of capsids from HEK-293 cell-lysates expressing: L1-alone, L1/L2, L1/E2, or L1/L2/E2, with antibodies specific to L1 (H16.V5) or E2 (TVG-261) reveals two different molecular weight VLP capsid structures from the W.C.L aliquots expressing: L1-alone, L1/L2 and L1/E2, L1/L2/E2 (Figure 6). This data agrees with the differences observed by Sapp, et al, 1998, when using pseudovirions from wart tissues compared to HPV16/18 VLPs as reagents and analysis under reducing conditions during PAGE analysis and within this report (Figure 1, Lane-2) [22].

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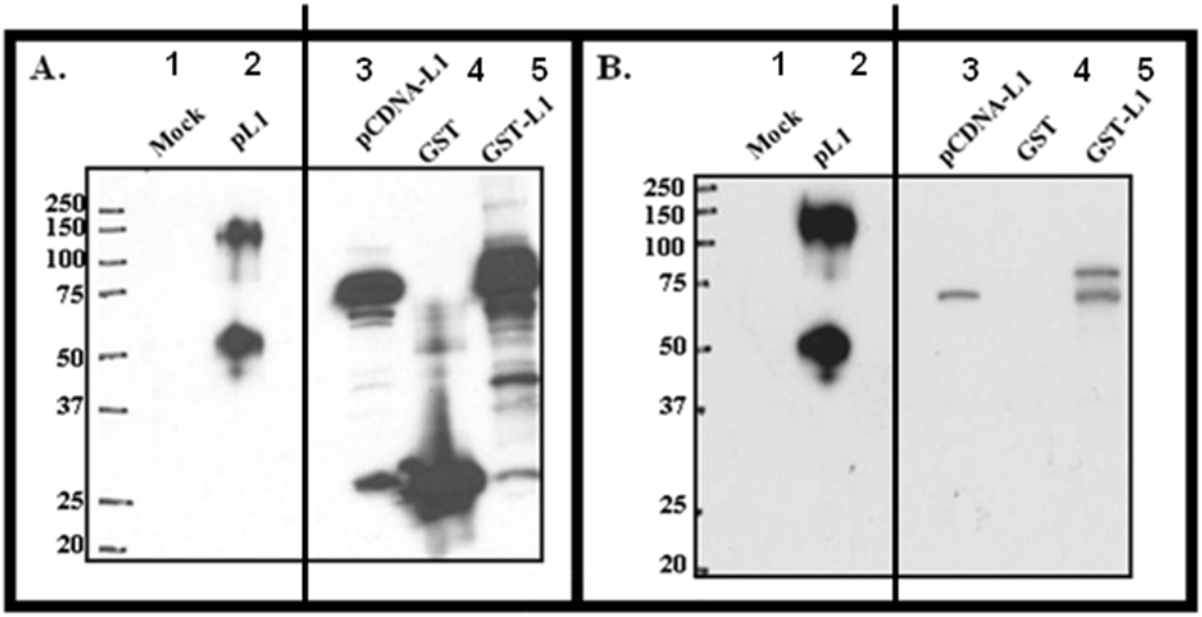
A.
1
2
3
4
5
Mock
pL1
pCDNA-L1
GST
GST-L1
250
150
100
75
50
37
25
20
B.
1
2
3
4
5
Mock
pL1
pCDNA-L1
GST
GST-L1
250
150
100
75
50
37
25
20

Figure 1.

HPV16 E2, trans-activating protein, interacts with the L1 protein of HPV16.

Far-western analysis of HEK-293 cell-lysate expressing the major capsid (L1) protein (**panel-A, lane 1**) or a bacterial cell-lysates expressing the L1 protein and GST-L1 (**panel-A, lanes 3 & 5**, respectively) demonstrates the HPV16 E2 protein capable of interacting with the major capsid protein from HPV16 (**panel-B, lanes 2, 3, & 5**). The HEK-293 control cell-lysate (**panel-A, lane 1**) and the bacterial cell-lysate expressing GST-alone (**panel-A, lane 4**) does not interact with the E2 protein. L1 and GST-L1 were detected with a cocktail of antibodies raised against the H16.J4 loop and GST-moiety, in panel-A.

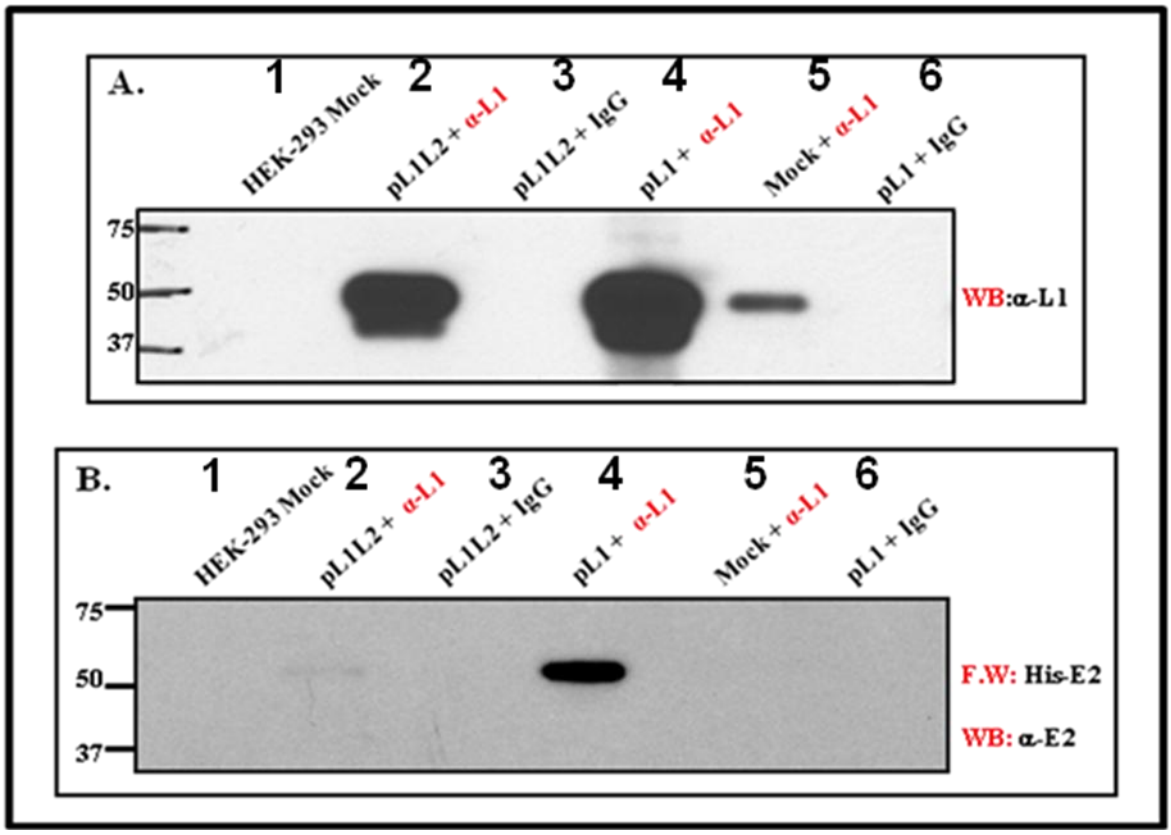
A.
1
HEK-293 Mock
2
pL1L2 + α-L1
3
pL1L2 + IgG
4
pL1 + α-L1
5
Mock + α-L1
6
pL1 + IgG
75
50
37
WB:α-L1
B.
1
HEK-293 Mock
2
pL1L2 + α-L1
3
pL1L2 + IgG
4
pL1 + α-L1
5
Mock + α-L1
6
pL1 + IgG
75
50
37
F.W: His-E2
WB: α-E2

Figure 2.

Qualitative assessment of L1 proteins expressed in HEK-293 cells *in vivo*, interacting with recombinant expressed E2 protein under reducing conditions.

An expression plasmid with a CMV-promoter was used to express the L1 protein (~55-KDa) within HEK-293 cells. The W.C.L were prepared under native conditions, without DTT nor beta-mercaptoethanol in the lysis buffer, and the commercial H16.V5-antibodies to the DE- and FG-loops were used to immunoprecipitate L1 proteins via the major neutralizing and conformational epitopes. **A)** The H16.J4 antibody (non-neutralizing and non-conformational epitope) detects the immunoprecipitated L1 protein when compared to the untransfected HEK-293 cells and compared to HEK-293 W.C.L immunoprecipitated by the H16.V5-L1 antibodies, which immunoprecipitates non-specific unknown cellular proteins. Isotype antibodies were unable to immunoprecipitate any non-specific cellular protein products. **B)** The western blot from panel-A was stripped and re-probed, FarWesterned (F.W.), with the bacterially expressed His-E2 protein (F.W: His-E2). The His-E2 protein was allowed to incubate at 4^{o}C with the L1 proteins on the western blot membrane and the His-E2 protein was detected by E2 antibodies (TVG-261). The analysis for figure is performed under reducing conditions in the presence of DTT and beta-mercapto-ethanol within the 6x protein loading buffer and the samples were loading onto an 8% SDS-PAGE gel for analysis.

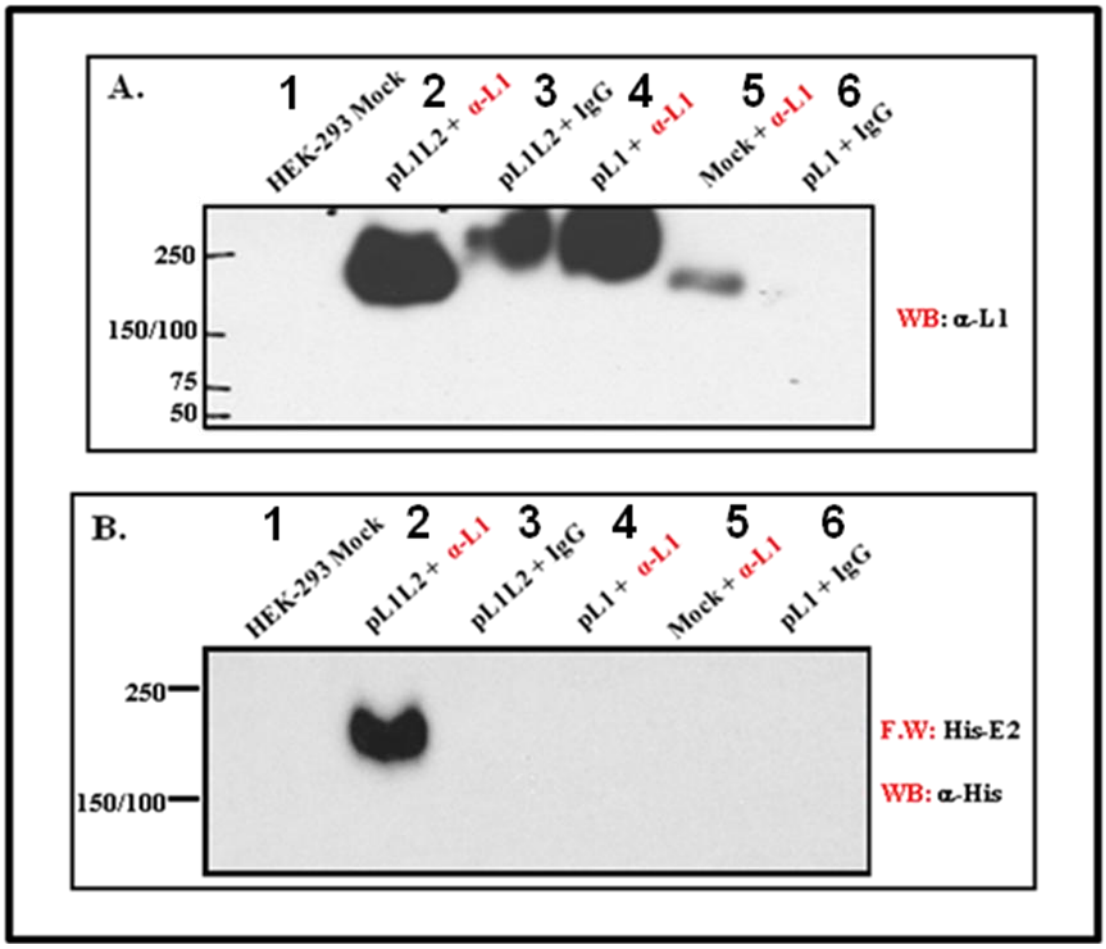
A.
1 HEK-293 Mock
2 pL1L2 + α-L1
3 pL1L2 + IgG
4 pL1 + α-L1
5 Mock + α-L1
6 pL1 + IgG
250
150/100
75
50
WB: α-L1
B.
1 HEK-293 Mock
2 pL1L2 + α-L1
3 pL1L2 + IgG
4 pL1 + α-L1
5 Mock + α-L1
6 pL1 + IgG
250
150/100
F.W: His-E2
WB: α-His

Figure 3.

Qualitative assessment of L1 proteins expressed in HEK-293 cells *in vivo,* interacting with recombinant expressed E2 protein under Non-Reducing conditions.

The samples within this figure are the same samples analyzed in Figure 2, above. The samples were loaded onto an SDS-PAGE gel using 6x-loading buffer without DTT and β-mercapto-ethanol. **Panel-A** reveals that the monomeric forms of L1 and L1/L2 are capable of oligomerizing under the listed conditions. Although, the His-E2 recombinant protein is only capable of interacting with the oligomerized capsid formations composed of L1 and L2 as opposed not interacting with the oligomerized capsid formations composed of L1-alone, **panel-B.** An expression plasmid with a CMV-promoter was used to express the L1 protein (~55-KDa) within HEK-293 cells. The W.C.L were prepared under native conditions, without DTT nor β-mercapto-ethanol in the lysis buffer, and the commercial H16.V5-antibodies to the DE- and FG-loops were used to immunoprecipitate L1 proteins via the major neutralizing and conformational epitopes. **A)** The H16.J4 antibody (non-neutralizing and non-conformational epitope) detects the immuno-precipitated L1 protein when compared to the untransfected HEK-293 cells and compared to HEK-293 W.C.L immunoprecipitated by the H16.V5 L1 antibodies, which immunoprecipitates non-specific unknown cellular proteins. Isotype antibodies were unable to immunoprecipitate any non-specific cellular protein products. **B)** The western blot from panel-A was stripped and re-probed, Far-westerned (F.W.), with the bacterially expressed His-E2 protein (F.W: His-E2). The His-E2 protein was allowed to incubate at 4^{o}C with the L1 proteins on the western blot membrane and the His-E2 protein was detected by E2 antibodies (TVG-261). The analysis for figure is performed under reducing conditions in the presence of DTT and β-mercapto-ethanol within the 6x protein loading buffer and the samples were loading onto an 8% SDS-PAGE gel for analysis.

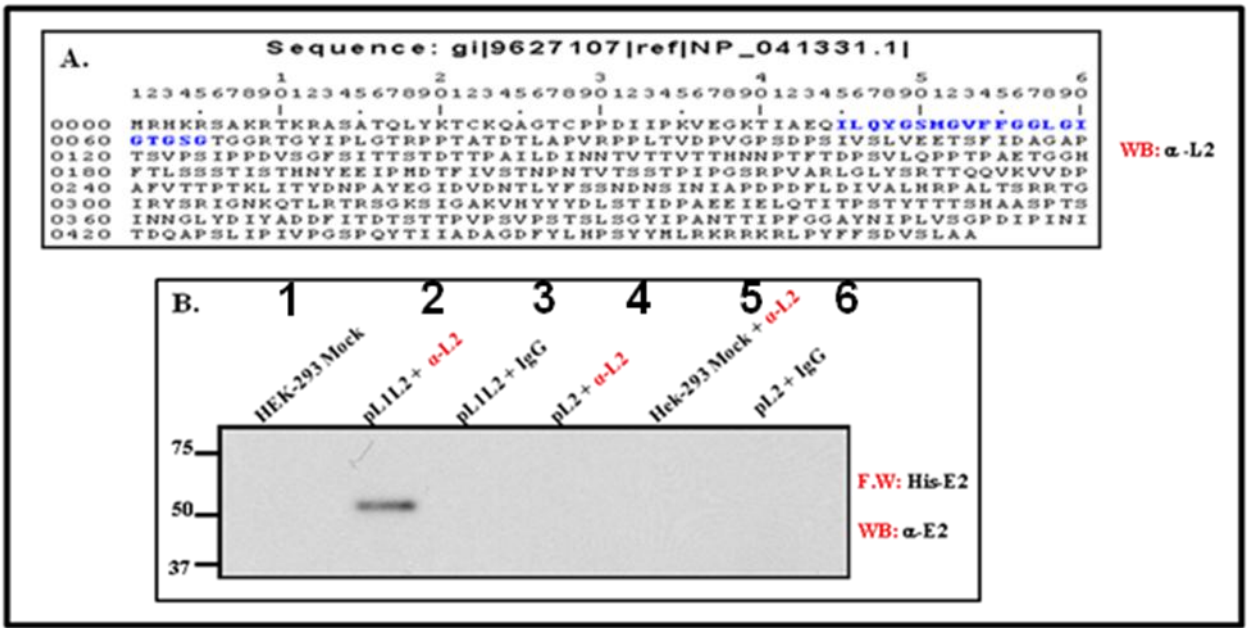
A.
Sequence: gi|9627107|ref|NP_041331.1|
MRHKRSAKRTKRASATQLYKTCKQAGTCPPDIIPKVEGKTIAEQILQYGSMGVFFGGLGI
GTGSGTGGRTGYIPLGTRPPTATDTLAPVRPPLTVDPVGPSDPSIVSLVEETSFIDAGAP
TSVPSIPPDVSGFSITTSTDTTPAILDINNTVTTVTTHNNPTFTDPSVLQPPTPAETGGH
FTLSSSTISTHNYEEIPMDTFIVSTNPNTVTSSTPIPGSRPVARLGLYSRTTQQVKVVDP
AFVTTPTKLITYDNPAYEGIDVDNTLYFSSNDNSINIAPDPDFLDIVALHRPALTSRRTG
IRYSRIGNKQTLRTRSGKSIGAKVHYYYDLSTIDPAEEIELQTITPSTYTTTSHAASPTS
INNGLYDIYADDFITDTSTTPVPSVPSTSLSGYIPANTTIPFGGAYNIPLVSGPDIPINI
TDQAPSLIPIVPGSPQYTIIADAGDFYLHPSYYMLRKRRKRLPYFFSDVSLAA
WB: α-L2
B.
1
2
3
4
5
6
HEK-293 Mock
pL1L2 + α-L2
pL1L2 + IgG
pL2 + α-L2
Hek-293 Mock + α-L2
pL2 + IgG
75
50
37
F.W: His-E2
WB: α-E2

Figure 4.

Qualitative assessment of L2 proteins expressed in HEK-293 cells *in vivo,* interacting with recombinant expressed E2 protein.

An expression plasmid with a CMV-promoter was used to express the L2 protein (~75-KDa) within HEK-293 cells. The W.C.L were prepared under native conditions, without DTT nor β-mercapto-ethanol in the lysis buffer, and the commercial antibodies directed against the NH_2-terminus of L2 were used to immunoprecipitate L2 proteins via the known conformational epitope. **A)** The L2 antibody (conformational epitope) does not detect the immunoprecipitated L2 protein when compared to the untransfected HEK-293 cells. In the place of the denaturing western blot, a trans-membrane prediction analysis suggests that the L2 protein has a trans-membrane like loop-structure that under denaturing condition does not interact with the conformational-dependent antibodies. **B)** The western blot from panel-A was stripped, re-probed, and Far-westerned (**F.W.**) with the bacterially expressed His-E2 protein (**F.W:** His-E2). The His-E2 protein was allowed to incubate at 4^{o}C with the L2 proteins on the western blot membrane and the His-E2 protein was detected by E2 antibodies (TVG-261). The analysis for this figure is performed under reducing conditions in the presence of DTT and β-mercapto-ethanol within the 6x-protein loading buffer and the samples were loading onto an 8% SDS-PAGE gel for analysis.

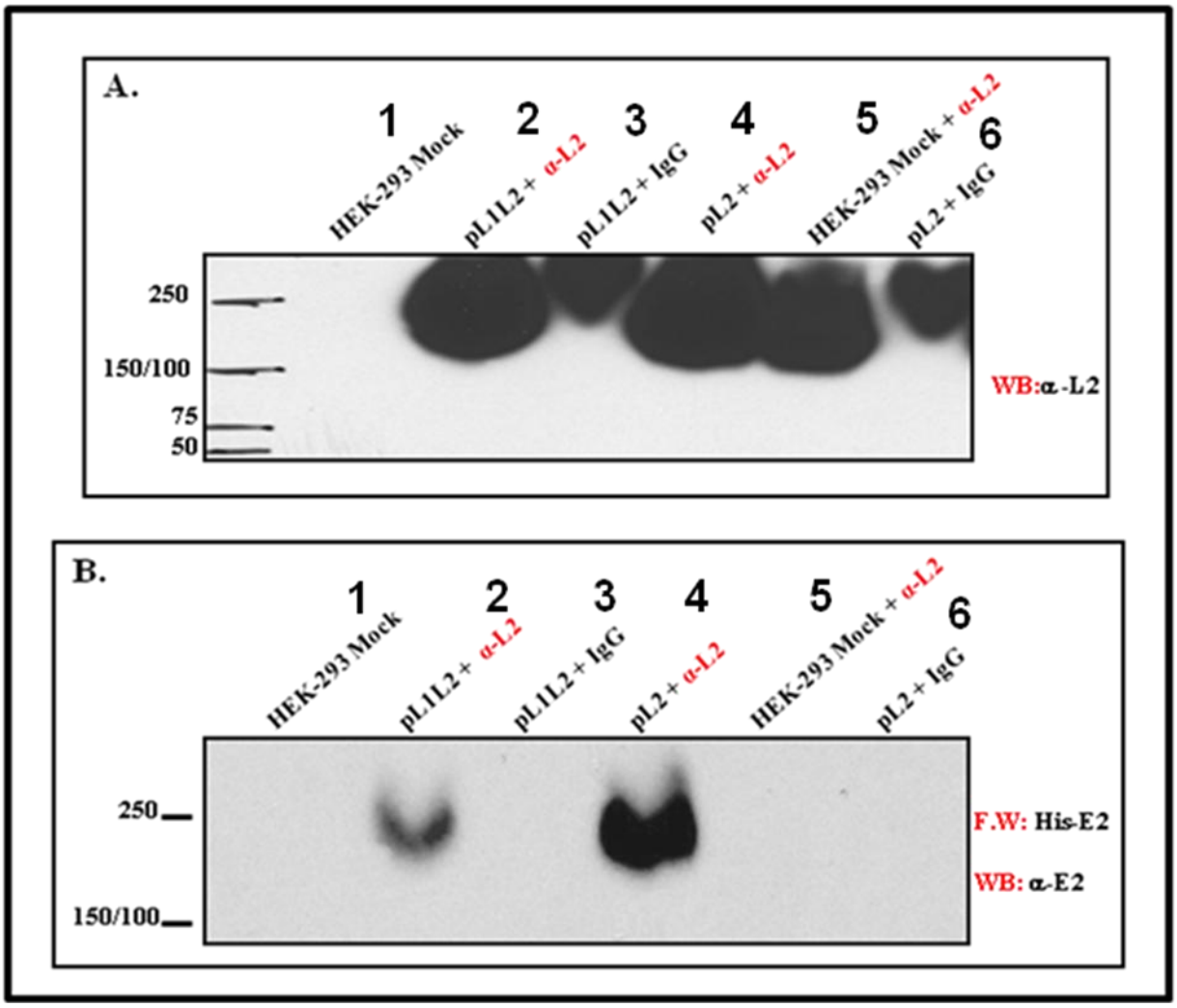
A.
1
HEK-293 Mock
2
pL1L2 + α-L2
3
pL1L2 + IgG
4
pL2 + α-L2
5
HEK-293 Mock + α-L2
6
pL2 + IgG
250
150/100
75
50
WB:α-L2
B.
1
HEK-293 Mock
2
pL1L2 + α-L2
3
pL1L2 + IgG
4
pL2 + α-L2
5
HEK-293 Mock + α-L2
6
pL2 + IgG
250
150/100
F.W: His-E2
WB: α-E2

Figure 5.

Qualitative assessment of L2 proteins expressed in HEK-293 cells *in vivo*, interacting with recombinant expressed E2 protein under Non-Reducing conditions.

The samples within this figure are the same samples analyzed in Figure 4, above. The samples here within this figure were loaded onto an SDS-PAGE gel using 6x loading buffer without DTT and β-mercapto-ethanol. **Panel-A** reveals that L2-alone and L1/L2-VLPs are capable of oligomerizing under the listed conditions. Also, the His-E2 recombinant protein is capable of interacting with the oligomerized capsid VLPs composed of L1/ L2 and L2-alone, **Panel-B**.

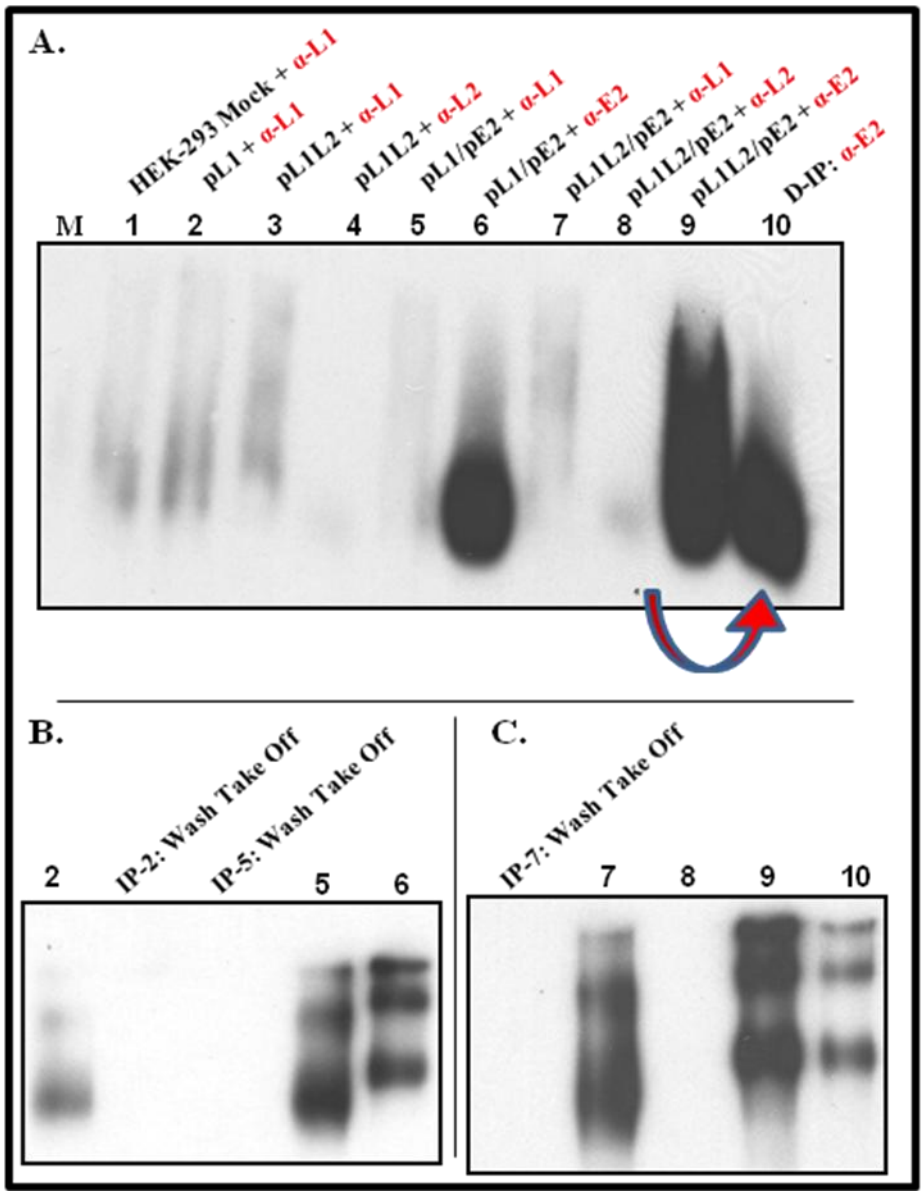
A.
HEK-293 Mock + α-L1
pL1 + α-L1
pL1L2 + α-L1
pL1L2 + α-L2
pL1/pE2 + α-L1
pL1/pE2 + α-E2
pL1L2/pE2 + α-L1
pL1L2/pE2 + α-L2
pL1L2/pE2 + α-E2
D-IP: α-E2
M 1 2 3 4 5 6 7 8 9 10
B.
IP-2: Wash Take Off
IP-5: Wash Take Off
2 5 6
C.
IP-7: Wash Take Off
7 8 9 10

Figure 6.

The expression of E2 with L1-alone or with L1/L2 capsid proteins.

The W.C.L from HEK-293 cells expressing the designated proteins from the respective expression plasmids (black lettering) were made and the specific antibody used during immunoprecipitation of the lysate (red) are indicated. Panel-A (lanes-6, -9, & -10), the non-SDS/PAGE gel (non-denaturing) analysis reveals an enriched protein species within the W.C.L that were immunoprecipitated with antibodies directed at E2 (TVG-261) and subsequently detected by Western Blotting with specific antibodies to L1 (H16.V5). SDS/PAGE analysis (denaturing) of the W.C.L expressing L1/E2, Panel-B, or L1/L2/E2, Panel-C, was conducted on the same lysates as in Panel-A. In the presence of the reducing agents (ex. DTT and β-mercapto-ethanol) the H16.V5 L1 antibody detects capsid proteins at differing molecular weights within the same WCL (panel-B, lane-2, -5 vs lane-6) (panel-C, lane-7 vs lane-9). During the immuno-precipitation with L2 antibodies, and subsequent detection with L1 specific-antibodies, no detectable protein was precipitated (panel-A & panel-C, lane-8). The supernatant, after immunoprecipitation with L2 antibodies, was re-immunoprecipitated with antibodies specific to E2 and the identical capsid proteins were precipitated and detected on the non-SDS/PAGE gel (panel-A & panel-C, lane-10), as seen in panel-A & -C, lane-9. The untransfected control HEK-293 cell lysate has no reactivity to H16.V5.

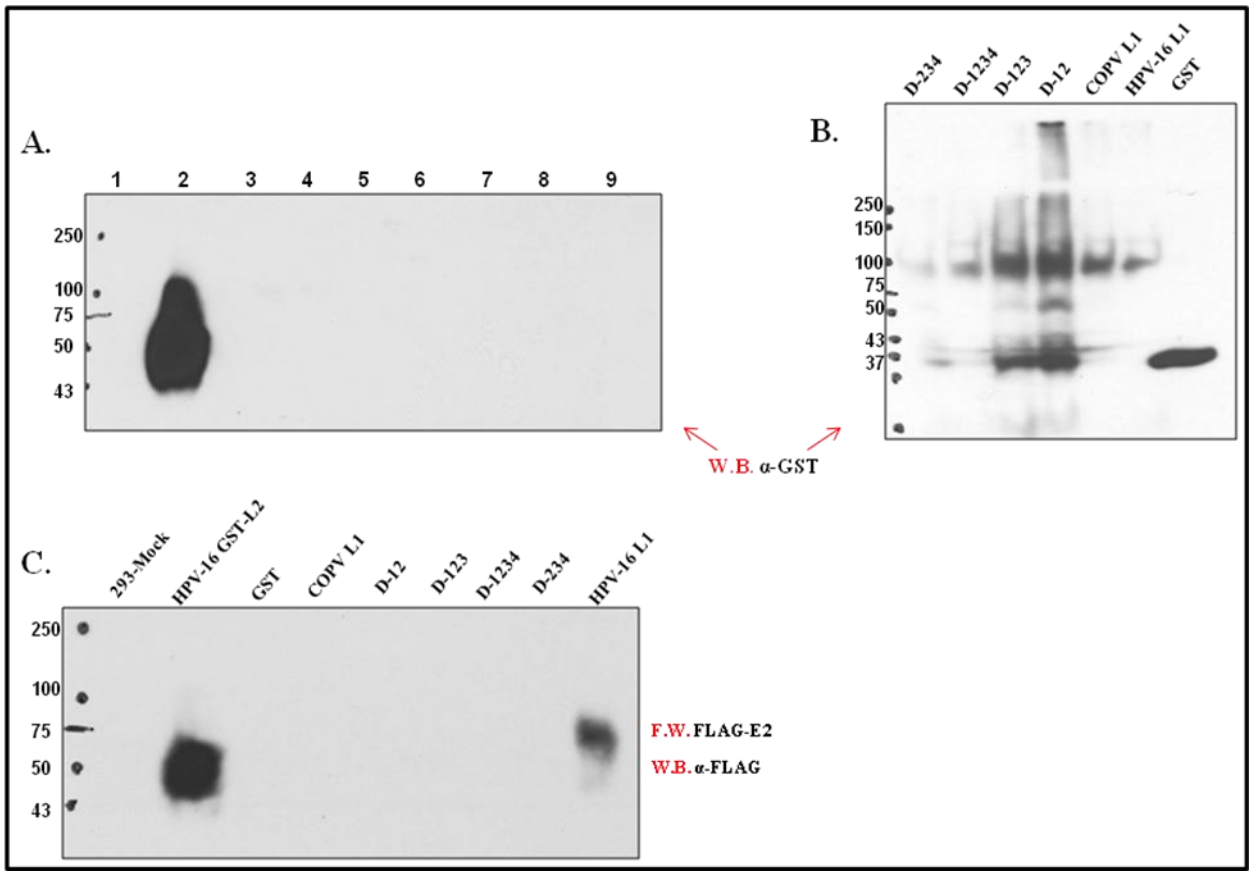
A.
1
2
3
4
5
6
7
8
9
250
100
75
50
43
B.
D-234
D-1234
D-123
D-12
COPV L1
HPV-16 L1
GST
250
150
100
75
50
43
37
W.B. α-GST
C.
293-Mock
HPV-16 GST-L2
GST
COPV L1
D-12
D-123
D-1234
D-234
HPV-16 L1
250
100
75
50
43
F.W. FLAG-E2
W.B. α-FLAG

Figure 7.

The hyper-variable loops from HPV16 L1 were inserted into the corresponding locale within the L1 of COPV and does not restore the E2-interacting capacity.

The protein designations for the chimeric proteins are listed in Table-1 provided. GST-fusion proteins were created, expressed, and detected with GST-specific antibodies on an SDS/PAGE gel (denaturing), panel-B, although no detection of the GST-fusion proteins were detected during non-SDS/PAGE gel analysis (non-denaturing), panel-A. The fusion protein, GST-L2, was detected on the non-SDS/PAGE as a positive control. Far-western (**F.W.**) analysis of (stripped and re-calibrated membrane from panel-A) with a FLAG-E2 probe is unable to interact with the chimeric L1-fusion proteins, while able to interact with the HPV16 GST-L1 fusion protein (panel-C) and detection with FLAG-specific antibodies. The NaCl concentration during the washing steps, after the FLAG-E2 probing, were progressively increased (ex. 1x, 5x, 10x) to reduce any contributing polar-interactions able to yield a false-positive result, panel-C. The GST-L2 fusion protein is used as a positive control for the interaction of L2-to-E2, panel-C, lane-2. The GST-L2 control in panel-A and panel-C serves as a control for the confirmation of the proteins, due to previous results demonstrating no interaction of E2 with denatured L2 protein (Figure 4, lane-4). The HEK-293 control cell lysate was used to demonstrate a non-reactivity of the cell-lysate used to create the FLAG-E2 probe.

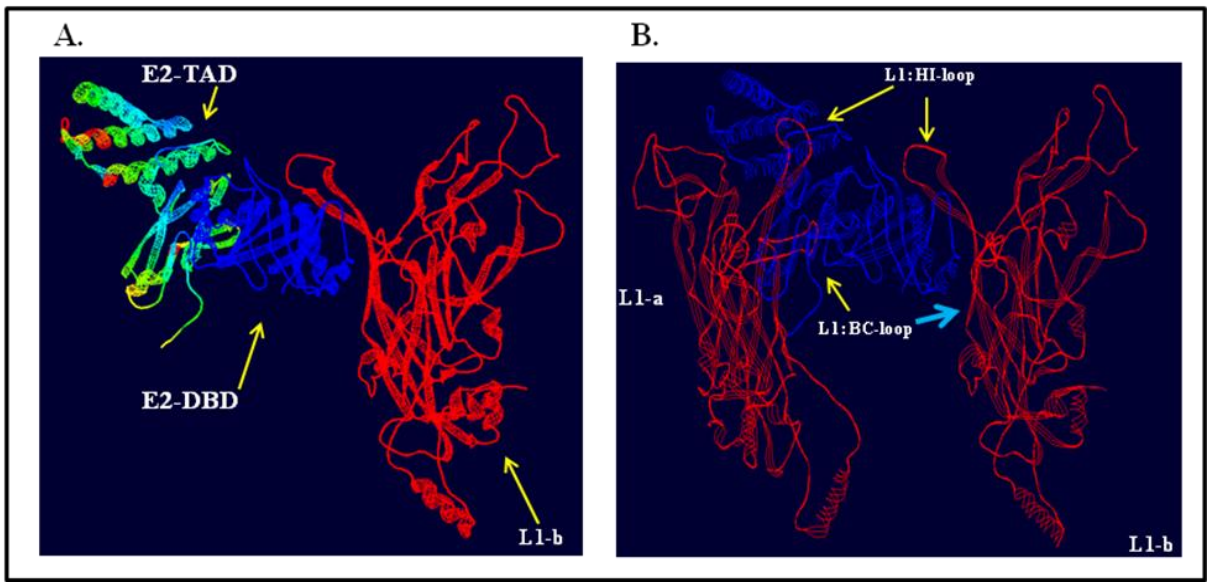
A.
E2-TAD
E2-DBD
L1-b
B.
L1:HI-loop
L1-a
L1:BC-loop
L1-b

Figure 8.

BioInformatic Prediction of the L1-to-E2 Interaction.

The functional E2 protein is composed of a homodimer of the crystal structures from the individual functional domains: trans-activation domain (E2-TAD, **pdb id**: 1dto) and the DNA-Binding Domain (E2-DBD, **pdb id**: 1zzf). Clupro accuracy was proven via remodeling the known interaction between the E2-TAD monomer (confetti) and the E2-DBD as a dimer (blue) as shown, Panel-A. The resulting interaction (E2-$TAD_{\mathbf{M}}$:$DBD_{\mathbf{D}}$) is used as a ligand during the interaction with a single L1-monomer (L1, **pdb id**: 1dzl) as the receptor, Panel-A. The entire resulting interaction, E2-TAD:DBD-to- $L1_{\mathbf{M}}$-b, is used as the ligand to another $L1_{\mathbf{M}}$ –a as the receptor (L1 with BC-Loop, red) in the Cluspro Docking program, Panel-B. The HI-loops from separate $L1_{\mathbf{M}}$ interacts with the E2-TAD from the homodimer, although the monomer is used for the clarity of these images. The $L1_{\mathbf{M}}$-a BC-loop interacts with the E2-DBD of the homodimer, the BC-loop from L1-b (turquoise arrow) is not visible due to its frontward positioning.

Conclusion

Overall:

The conclusions within this report are dependent upon bioinformatic analysis and actual laboratory analysis.

Chapter 2:

The bioinformatic analysis of the L1-to-L2 interaction domains are performed within this report, while the actual laboratory analysis has been performed by other laboratories previously. The prediction of the L2 protein is based upon a consensus sequence of the alpha-papillomavirus L2 sequences, and still needs to be actually crystallized through laboratory analysis in order to substantiate the bioinformatic prediction. Even though, the bioinformatic analysis preliminarily confirms the locale of L2 within the L1 pentameric-shaft.

Chapter 3:

The bioinformatic analysis assessing the DNA-binding termini of the HPV capsid proteins has revealed a similarity between the DNA-binding portions of the *Microviridae* J proteins. The J proteins, as does the HPV capsid proteins, have demonstrated non-specific DNA-binding capacity, and the laboratory analysis within the *Microviridae* encapsidation system will afford specific recognition of the DNA-binding domain versus nuclear localization signals within the HPV capsid proteins. The laboratory *in vitro* encapsidation analysis for this study is a collaborative effort between the HPV laboratory (molecular cloning of reagents) and the bacteriophage laboratory of Dr. Bentley Fane at the University of Arizona (performing the plaque assays).

Chapter 4:

The exploratory “capsid assembly” research reported herein reports bioinformatic analysis and laboratory analysis. The bioinformatic and the laboratory analysis conducted for this thesis are in agreement with each other, suggesting the probability and possibility for E2-mediated pentamer-spatial-arrangement during *de novo* HPV capsid formation. During a natural infection E2 is present, while the VLP systems in use lack E2 during the transient expression of L1- or L1/L2-VLPs. Further laboratory analysis needs to be performed in order to verify the actual region of L1 that E2 interacts at, in order to substantiate the dynamics of the observed interactions within this report. There are five sets of BC- and HI-loops per L1-pentamer. These observations

speculate that the E2 protein, as a homodimer, is the only HPV-specific viral protein with uniform physical dimensions necessary to maintain equi-distance between the L1-pentamers to preserve some sort of spatial arrangement to preserve the 72 L1-pentamer HPV virion.

The results herein reveal that E2 is capable of interacting with the L1 protein during cellular preparations expressing L1-alone or L1/L2 together, in the absence of DTT on a non-reducing PAGE gel via FarWestern blotting (Figures 2-5, panel-B, Lane-2). Also, Figure 3 (panel-B, lane-4), reveals that E2 does not interact with L1 post-spontaneous L1-oligomerization, when compared to the fusion protein of GST-L1 (Figure 7, panel-C, lane 9), which is unable to oligomerize due to the GST-moiety at the NH_2-terminus of L1. Also, E2 does interact with L1/L2-VLPs co-expressed together under non-reducing conditions (Figure 5, panel-B, lane-4). The interaction of E2 with an intact or denatured L2, follows the same dynamics as seen herein for the L1-to-E2 interaction and as observed for the antibody-mapping assays [3, 8] revealing that the production of antibodies recognizing intact (conformational) or denatured (linear) L1 proteins are dependent upon an intact or a denatured capsid.

The significance of re-creating the natural HPV capsid assembly process is deemed necessary to achieve at best the most ideal conditions for VLPs to mimick wild-type HPV virions and also to produce a more homogenous composition of neutralizing conformational antibodies (ex. H16.V5, H16.E70, or H16.U4) [10, 18-22]. The observation that the BC-Loop is necessary for structural integrity, is not exposed on the surface of an HPV virion, and is not used for epitope presentation demonstrates that the inclusion of E2 during the VLP expression systems may provide a more natural HPV virion assembly process to facilitate efficient antibody production.