Characterization of the Nuclear Export Signal of Human Papillomavirus 16 L2 Minor Capsid Protein

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Boston College College of Arts and Sciences Department of Biology

Characterization of the Nuclear Export Signal of Human Papillomavirus 16 L2 Minor Capsid Protein

> Courtney Halista Biology Honors Thesis Advisor: Dr. Junona Moroianu May 2011

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Abstract_

The L2 minor capsid protein of human papillomavirus is one of two structural proteins that comprise the icosahedral shell. The icosahedron contains 72 pentameric capsomeres, each of which is stabilized by the formation of hydrophobic, intermolecular bonds between the n- and c-terminal domains of the L2 minor capsid protein and the L1 major capsid proteins. In addition to its structural role, L2 has also been implicated in the cellular entry of the virion and the nuclear localization of the viral genome. During the late phase of viral infection, the L1 and L2 proteins are synthesized in the cytoplasm of the differentiated epithelial cells. From the cytoplasm, the structural proteins are then transported through the nuclear pore complex into the cell nucleus where they encapsulate the replicated viral DNA in order to form new virions. Previous experiments conducted in the Moroianu lab by Shahan Mamoor suggest that high risk HPV16 L2 minor capsid protein may also undergo nuclear export. Two potential, leucine-rich nuclear export signals (NESs) have been identified in the HPV16 L2 sequence, one in the nterminus (51MGVFFGGLGI60) and one in the c-terminus (462LPYFFDSVSL471). The purpose of this project was to characterize the functional NES involved in nuclear export of HPV16 L2. DNA primers for mutant L2 proteins were designed to specifically target the two potential NES regions. Two primers had mutations in the n-terminal located NES (nNES), while the other two primers had mutations in the c-terminal NES (cNES). L2 nuclear retention mutants, RR297AA ("MS4") and RTR313AAA ("MS5"), served as the templates for these NES mutations. Previous analyses conducted by Shahan Mamoor demonstrated that L2 proteins containing the "MS4" or "MS5" mutations were localized pancellularly. Using mutagenesis, the desired secondary mutations were introduced into the mutant L2 genes in order to create four, distinct mutants: RR297AA + P463_ ("MS4 T1"), RR297AA + V469_ ("MS4 T2), RTR313AAA + P463_ ("MS5 T1"), and RTR₃₁₃AAA + V_{469} ("MS5 T2"). In order to visualize the localization of the mutant L2 proteins, transfection assays in HeLa cells followed by fluorescence microscopy were performed. In contrast to the pancellular localization of the MS4 and MS5 L2 mutants, the "MS4 T1," "MS4 T2," "MS5 T1", and "MS5 T2" mutants were all localized nuclearly. These results suggest that deletion of the cNES inhibits nuclear export of the HPV16 L2 minor capsid protein.

Acknowledgements

I would like to begin by thanking those individuals who made my work in the lab rewarding and memorable. First and foremost, my Laboratory Director, Professor Junona Moroianu. Under her tutelage, I have learned the value of persistence and the importance of patience. Whereas I would have considered inconclusive results and/or failed experiments acceptable at the beginning of my research career, I am now determined to redesign and repeat those same experiments until I achieve success. For example, after over a dozen failed attempts to visualize the HPV16 L2 protein using an immunoblot assay, I was convinced that my goal was unattainable. Professor Moroianu, however, was determined. She encouraged me to repeat the experiment one last time and, as she predicted, it worked! I have never before felt such a profound sense of accomplishment. Following this experience, I developed an appreciation for the incredible level of commitment that is required to achieve success in the field of scientific research. Without the guidance, patience, and continual support of Professor Moroianu, this project would never have reached completion.

Secondly, I am forever indebted to my laboratory partner, Shahan Mamoor. In the Fall of my Junior year, I was assigned to work with Shahan on the HPV16 L2 protein. For the next year and a half, Shahan and I worked tirelessly on what was often described as a "stubborn" protein. For me, Shahan exemplifies what it means to be a scientist. His passion for research, attention to detail, and thorough investigative techniques are inspiring. As my teacher, lab partner, and friend, Shahan ensured that my laboratory experience was not only enlightening, but enjoyable. He eagerly explained unfamiliar experiments and readily demonstrated novel techniques. Shahan provided me with the tools and knowledge necessary to continue with the project in his absence. I would not have had the confidence to assume an individual research project were it not for him.

I would also like to thank laboratory technician, Lauren Crosby, and graduate student, Zeynep Onder, for their collaborative roles on this project. When I first joined the laboratory, Lauren served as my mentor. She taught me how to purify proteins, prepare solutions, and perform basic experiments. After Shahan matriculated at the end of my junior year, Lauren assumed the role of research assistant. Although she only worked with HPV16 L2 for a short period of time, her contributions are invaluable to my thesis. Zeynep, likewise, has adopted L2 as her protein of study. Meticulous and dedicated, I could not have asked for a more dependable colleague.

Lastly, I must thank all of the graduate and undergraduate students with whom I have had the pleasure of working. Jeremy, Ben, Courtney, Katherine, Heather, Adi, Danielle, Erin, and Erin – thank you for your guidance and, more importantly, your friendship. I will truly miss you all.

Abbreviations_

BME: β-mercaptoethanol cNES: Carboxyl terminal nuclear export signal cNLS: Carboxyl terminal nuclear localization signal **DAPI:** 4,6,-diamidinophenylindole **DMEM:** Dulbecco's Modified Eagle Medium **dNTP:** Deoxyribonucleotide triphosphate **EGFP:** Enhanced Green Fluorescent Protein HeLa: Henrietta Lack's cervical cancer-derived cells HPV16 L2 "MS4": RR297AA HPV16 L2 "MS4 S1": RR297AA + FF54AA **HPV16 L2 "MS4 S2":** RR297AA + LGI58AAA HPV16 L2 "MS4 T1": RR297AA + P463_ HPV16 L2 "MS4 T2": RR297AA + V469_ HPV16 L2 "MS5": RTR313AAA HPV16 L2 "MS5 T1": RTR313AAA + P463_ HPV16 L2 "MS5 T2": RTR313AAA + V469_ **HRP:** Horse radish peroxidase **HSPG:** Heparan sulfate proteoglycan NLS: Nuclear localization signal **nNES:** Amino terminal nuclear export signal nNLS: Amino terminal nuclear localization signal **NPC:** Nuclear pore complex **ORF:** Open reading frame **PCR:** Polymerase chain reaction **PBS:** Phosphate-buffered saline **SDS:** Sodium dodecyl sulfate

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

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Introduction_

Human Papillomaviruses

Human papillomaviruses (HPVs) are small, nonenveloped, icosahedral DNA viruses that specifically infect basal squamous epithelial cells. Over 200 types of HPV have been characterized, about 40 of which are known to infect the mucosal cells of the anogenital and oropharyngeal tracts (Ili et al., 2011). Depending on their oncogenic potential, these 40 HPVs are further classified into high-risk and low-risk types. High-risk types, including HPV16, 18, 31, and 45, have been implicated in the development of cervical and other anogenital cancers (zur Hausen, 2000). Low-risk types are associated with benign genital warts and include HPV6 and 11.

HPV is considered to be the most common sexually transmitted disease in the United States. Approximately 26% of females ages 14-59 are currently infected. Women ages 20-24 have the highest rate of infection at approximately 44% (Dunne et al., 2007). For most individuals, the infection is cleared by the immune system within 18 months. However, those individuals suffering from a persistent, high-risk HPV infection are at risk for developing cancer of the mucosal tissue. More than 99% of cervical cancers and 20% of oropharyngeal cancers test positive for HPV DNA. Significantly, cervical cancer is the second most common cancer and the fifth leading cause of death in women worldwide. With over 500,000 new cases and 250,000 deaths reported annually, cervical cancer remains an international public health concern (Longworth and Laimins, 2004).

Human Papillomavirus Genome and Life Cycle

The HPV genome consists of a single molecule of 8-kb double-stranded, circular DNA enclosed within an icosahedral capsid (Longworth and Laimins, 2004). The genome can be divided into three distinct regions: an early (E) region, a late (L) region, and an upstream regulatory region (URR). The early region contains 6 open reading frames (ORFs) that encode 6 non-structural proteins (E1, E2, E4, E5, E6 and E7). These genes are expressed early in the viral life cycle and facilitate viral transcription, DNA replication, and cell transformation. E1 functions as the viral helicase. In complex with E1, E2 binds the viral origin of replication and recruits cellular polymerases (Longworth and Laimins, 2004). E2 has also been implicated in the regulation of E6 and E7 expression. E4 is responsible for arresting the cell in G2 phase

(Longworth and Laimins, 2004), while E5 appears to affect membrane signaling. E6 and E7 act as oncogenes in high-risk HPVs. By forming a complex with tumor suppressor protein p53 and the ubiquitin ligase E6AP, E6 promotes the ubiquitination and subsequent degradation of p53 (Longworth and Laimins, 2004). Without p53, the infected cell is unable to induce cell cycle arrest in response to DNA damage. E7 has equally deleterious effects in that it binds the retinoblastoma protein (pRB) and prevents it from binding the E2F/DP1 transcription factors. This interference induces the transcription of the S-phase genes, thereby enabling viral replication (McLaughlin,-Drubin and Munger, 2009).

The late region contains 2 open reading frames (ORFs) that encode 2 structural proteins (L1 and L2). In combination, the L1 and L2 proteins form the 55 nm icosahedral shell characteristic of HPVs. 360 L1 major capsid proteins assemble into 72 pentameric capsomeres. These capsomeres are held together via disulfide bonds between c-terminal arms of neighboring L1 proteins. A single L2 protein is located at the center of each pentameric capsomere and provides structural stability. Studies indicate that hydrophobic, intermolecular interactions exist between the c-terminal domain of one molecule of L2 at the center of a pentamere and the n-terminal domain of a second molecule of L2 on a neighboring pentamere. Although formation of L1-only capsomeres is possible, the existence of intermolecular interactions between L2 molecules aids in capsid formation and provides additional structural stability (Pereira, 2009).

HPV infection begins with a microtrauma in the epithelium. The virion binds a heparinsulfate receptor on the surface of a basal stem cell and is endocytosed through either a clathrin or caveolin mediated pathway. Following disassembly in either a late endosome or lysosome, the viral genome is transported into the cell nucleus. Initially, the virus is maintained as an extrachromosomal nuclear plasmid at 20-100 copies per cell. During this time, the early stage proteins are expressed. As the infected basal cell replicates, daughter cells migrate from the basal layer to the suprabasal layer and differentiate into epithelial cells. It is at this time that the late stage proteins are expressed and the viral genome copy number increases to 500-1000 copies per cell (Longworth and Laimins, 2004; Fang et al., 2006). To complete the cycle, new virions are assembled and shed into the surrounding environment at the epithelial surface (Jones and Münger, 1996).

Human Papillomavirus Vaccines

The currently available vaccines, Gardasil and Cervarix, are primarily recommended for prophylactic use. Gardasil is a quadrivalent vaccine containing recombinant virus-like particles (VLPs) assembled from L1 proteins of HPV types 6, 11, 16, and 18. HPV-6 and HPV-11 are associated with the majority of anogenital warts, while HPV-16 and HPV-18 are responsible for approximately 70% of the high-grade cervical lesions associated with cervical cancer (FUTURE II Study Group, 2007). In a study conducted by Merck in 2007, 12,167 women were vaccinated with Gardasil and followed for a period of 3 years. The primary composite endpoint included cervical intraepithelial neoplasia (Grade 2 or 3), adenocarcinoma in situ, or invasive carcinoma of the cervix (FUTURE II Study Group, 2007). While the vaccine demonstrated 98% efficacy for the prevention of the primary composite endpoint, it achieved only 44% efficacy in the intention-to-treat population (FUTURE II Study Group, 2007). In contrast to Gardasil, Cervarix is a bivalent vaccine containing recombinant VLPs assembled from L1 proteins of HPV types 16 and 18. Cervarix is 71% effective in the prevention of cervical intraepithelial neoplasias, grades 2 or higher, in those individuals who have not been previously exposed to HPV. (Schauner and Lyon, 2010).

The major limitation of the L1 VLP vaccines, Gardasil and Cervarix, is that they are type-specific. They are directed against the 70% of cervical cancers that are caused by HPV types 16 and 18. In order to provide protection against more than 90% of cervical cancers, it would be necessary to generate a vaccine that incorporates VLPs from nine oncogenic HPV types (Jagu, et al., 2007).

Researchers at Johns Hopkins University are currently investigating the possibility of using the L2 minor capsid protein as an alternative to the L1 major capsid protein for HPV vaccine development. Preclinical studies demonstrate that vaccination of rabbits with amino-terminal L2 polypeptides induces protection against both homologous and heterologous papillomavirus types (Jagu, et al., 2007). For example, Gambhira, et al. observed that vaccination of rabbits with HPV-16 L2 11-200 provides protection against both cutaneous and mucosal infection by cottontail rabbit papillomavirus (CRPV) and rabbit oral papillomavirus, even though CRPV and rabbit oral papillomavirus are evolutionarily divergent from of HPV-16 (Gambhira, et al. and Jagu, et al., 2007). However, the neutralizing titers and degree of protection conferred by monovalent L2 immunogens is greater for the homolgous-type virus than

for a heterologous-type virus (Jagu, et al.). Essentially, vaccination with an HPV-16 L2-derived immunogen would induce a stronger, neutralizing response to an HPV type 16 infection than to an infection with another type of HPV.

In order to circumvent this problem, Jagu et al. designed a concatenated multitype L2 fusion protein that contains cross-protective L2 epitopes from several human papillomavirus types (Jagu et al.). Unlike the monovalent L2 immunogen, the concatenated multitype L2 fusion protein induced high neutralizing antibody titers against all heterologous HPVs tested when injected into mice. Although an L2 multitype vaccine has yet to be tested in humans, these results suggest that the generation of a pan-oncogenic HPV vaccine is possible (Jagu et al.).

HPV16 L2 Minor Capsid Protein

L2 is the minor capsid protein of human papillomavirus. It is one of two structural proteins that is expressed late in the viral life cycle. The role of L2 in HPV infection is multifaceted. In addition to structural stability, it has been demonstrated that L2 facilitates viral entry into the cell and assists in nuclear localization of viral DNA. (Bordeaux et al., 2006)

HPV internalization into the host cell begins when L1 binds to a heparan sulfate proteoglycan (HSPG) receptor in the extracellular matrix. The binding of L1 to HSPG is thought to trigger a conformational change that ultimately exposes the n-terminal region of the L2 protein. The first 9 amino acid residues of the n-terminal region are then cleaved by the proprotein convertase, furin. Precleavage of the n-terminal domain of L2 enables HPV virions to infect cells lacking HSPG receptors (Richards et al., 2006). This observation suggests that furin cleavage of L2, and not HSPG binding, is essential for viral entry. Following L2 cleavage at the cell surface, the virion is internalized via either a clathrin-dependent or calveolin-dependent pathway. The virion is the then transferred to an endosome, from which it is released via a membrane-destabilizing peptide located in the c-terminal region of the L2 protein (Kamper et al., 2006). Viral progression of the genome toward the nucleus is aided by the binding of the Cterminal region of L2 with the microtubule motor protein, dynein. Lastly, the presence of L2 promotes the entrance of the HPV genome into the cell nucleus. Nuclear localization signals on the L2 protein interact with several karyopherin β (Kap β) nuclear import receptors. This interaction enables the transport of the HPV genome from the cytoplasm, through the nuclear pore complex, and into the cell nucleus where viral replication can begin (Pereira, 2009).

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Additionally, Pereira observed that, as the level of L2 expression increases, the viral components necessary for replication localize to nuclear domain 10 (ND10) (Pereira, 2009). These results suggest that the minor capsid protein of HPV is not only essential for capsid stabilization and viral entry, but also for genome replication within the host cell nucleus.

Nuclear Import of HPV16 L2 Minor Capsid Protein

Karyopherins are soluble import receptors belonging to the karyopherin β / importin β (Kap β /Imp β) superfamily. Interaction between karyopherins and nucleoporins at the nuclear pore complex facilitates protein transport into the nucleus. Following nuclear entry, the binding of Ran-GTP to Kap β importins triggers protein release (Moroianu, 1999).

Two independent nuclear localization signals (NLSs) have been identified in the HPV16 L2 protein. One NLS is located in the n-terminal region (nNLS) (1MRHKRSAKRTKR13), while the second NLS is located in the c-terminal region (cNLS) (454LRKRRKRL462). These NLSs play an essential role in nuclear import of the L2 minor capsid protein of HPV16 (Darshan et al., 2004). The nNLS of HPV16 L2 forms a complex with Kap β_2 , Kap β_3 , and Kap $\alpha_2\beta_1$ heterodimers via the Kap α_2 adapter. The cNLS also interacts with Kap $\alpha_2\beta_1$, however with lower affinity that the nNLS. Significantly, the nNLS alone was unable to mediate nuclear import of a GST fusion protein via Kap β_2 and Kap β_3 . These results suggest that the nNLS requires other residues for nuclear import of HPV16 L2 (Darshan et al., 2004).

Disruption of Nuclear Retention of HPV16 L2 Minor Capsid Protein

Research conducted in the Moroianu lab by graduate student Shahan Mamoor has demonstrated that nuclear retention of HPV16 L2 minor capsid protein can be disrupted by the introduction of mutations into the middle region of the L2 gene. When analyzed independently, both the "MS4" mutation (RR₂₉₇AA) and the "MS5" mutation (RTR₃₁₃AAA) exhibited pancellular localization of L2. Pancellular localization of HPV16 L2 implies that the protein is being both imported and exported from the cell nucleus. Whereas previous analyses had demonstrated that nuclear import of the HPV16 L2 protein is karyopherin-dependent, the nuclear export mechanism of HPV16 L2 had not yet been determined.

Objectives and Hypothesis

The characterization of the HPV16 L2 nuclear export signal became the focus of my research. Using the "MS4" and "MS5" nuclear retention mutants as templates, my goal was to re-establish nuclear localization of the HPV16 L2 minor capsid protein by disrupting the nuclear export signal(s) (NESs). Two regions that could potentially influence nuclear export were identified, one in the n-terminus $({}_{51}MGVFFGGLGI_{60})$ and one in the c-terminus $(_{462}LPYFFDSVSL_{471})$. Primers were designed to target these two, potential NESs. Two primers contained mutations in the n-terminal NES (nNES) (S1 and S2). The other two primers contained mutations in the c-terminal NES (cNES) (T1 and T2). First, these four, secondary mutations were introduced into the L2 gene containing the "MS4" primary mutation. Using fluorescence microscopy, the cellular localization of the resultant double mutants was analyzed. Nuclear localization of "MS4 T1" and "MS4 T2" suggested that the c-terminal region serves as a functional NES. In order to confirm these results, the "T1" and "T2" secondary mutations in the c-terminal region were introduced into the L2 gene containing the "MS5" primary mutation. Nuclear localization of the HPV16 L2 double mutants "MS5 T1" and "MS5 T2" would indicate that the nuclear export signal of HPV16 L2 is located in the c-terminal region (462LPYFFDSVSL471).

Materials and Methods

Site-directed Mutagenesis

HPV16 L2 primary mutants, "MS4" and "MS5", were generated using either 50 or 100 ng of plasmid DNA in combination with 5 μ l 10x reaction buffer, 125 ng each forward and reverse primer, 1 μ l dNTP mix, and 1 μ l *PfuTurbo* DNA polymerase. Mutant DNA was synthesized in a PCR thermocycler according to the following parameters: 95°C for 30 seconds, 95°C for 30 seconds (denaturation), 55°C for 60 seconds (annealing), 68°C for 6.5 minutes (extension), and 68°C for 10 minutes (final extension). The denaturation, annealing, extension, and final extension segments were repeated for a total of 18 cycles. Following temperature cycling, the reactions were placed on ice for 2 minutes and treated with 1 μ l *Dpn* I restriction enzyme in order to digest the parental, methylated DNA. After 1 hour of incubation at 37°C, mutant plasmids were transformed into XL1-Blue supercompetent cells. Cultures were grown overnight at 37°C with shaking. Mutant plasmids were then extracted using a Quantum Miniprep Kit and sent for sequencing (Eurofins MWG) in order to confirm the success of the mutagenesis.

Nuclear retention mutant "MS4" (pEGFP-HPV16 L2 RR₂₉₇AA) served as the template for a series of secondary mutations. The first set of secondary mutations targeted a potential, leucine-rich nuclear export signal in the n-terminal region (nNES) of the HPV16 L2 gene (51MGVFFGGLGI₆₀). Site-directed mutagenesis, as outlined above, was utilized in order to introduce the secondary mutations into the "MS4" template. The two, resultant double-mutants were identified as "MS4 S1" (pEGFP-HPV16 L2 RR₂₉₇AA + FF₅₄AA) and "MS4 S2" (pEGFP-HPV16 L2 RR₂₉₇AA + LGI₅₈AAA).

Nuclear retention mutant "MS4" served as the template for two, additional secondary mutations. These secondary mutations targeted a potential, leucine-rich nuclear export signal in the c-terminal region (cNES) of the HPV16 L2 gene (462LPYFFDSVSL471). First, the L2 protein was truncated at proline residue 463. The resultant double-mutant was identified as "MS4 T1" (pEGFP-HPV16 L2 RR297AA + P463_). Then, the L2 protein was truncated at valine residue 469. The resultant double-mutant was identified as "MS4 T2" (pEGFP-HPV16 L2 RR297AA + V469_). Site-directed mutagenesis, as outlined above, was utilized in order to introduce the secondary mutations into the "MS4" template.

The effectiveness of the "T1" and "T2" NES mutations were also analyzed in the context of the "MS5" template (pEGFP-HPV16 L2 RTR₃₁₃AAA). Like "MS4", the "MS5" primary mutation disrupted nuclear localization of the L2 minor capsid protein (**Figure 8**). Again, the L2 protein was truncated, first at proline residue 463 to create the resultant double-mutant "MS5 T1" (pEGFP-HPV16 L2 RTR₃₁₃AAA + P₄₆₃) and next at valine residue 469 to create the resultant double-mutant "MS5 T2" (pEGFP-HPV16 L2 RTR₃₁₃AAA + V₄₆₉). Site-directed mutagenesis, as outlined above, was utilized in order to introduce the secondary mutations into the "MS5" template.

Graduate student, Shahan Mamoor, prepared the "MS4" and "MS5" primary mutants. Laboratory technician, Lauren Crosby, prepared the "MS4 S1" and "MS4 S2" double-mutants. Graduate student, Zeynep Onder, prepared the "MS4 T1", "MS4 T2" and "MS5 T2" doublemutants. Undergraduate research assistant, Courtney Halista, prepared the "MS5 T1" doublemutant.

Cell Culture

The ATCC HeLa cell line was cultured in Dulbeccos Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated Fetal Bovine Serum and 1% Penicillin/Streptomyocin solution (Penstrep) at 37°C and 5% CO₂.

Transfection Assays

ATCC HeLa cells were plated on 12 mm poly-L-lysine-coated glass coverslips 24 hours prior to transfection and grown overnight to 50-70% confluency. Transfection reactions for each well were prepared using 4 μ l Fugene 6 Reagent (Roche Applied Science), 96 μ l DMEM(-), and a concentration-dependent volume of the appropriate plasmid. Following a 20 minute incubation period, cells were washed with 500 μ l of DMEM(-). 100 μ l of the plasmid preparation was then added to the appropriate well. After 6 hours, the media was changed to 500 μ l DMEM(+) (1% penicillin-streptomycin solution and 10% fetal bovine serum). 24 hours after the initial transfection, cells were washed three times with chilled PBS and fixed with 10% paraformaldehyde for 10 minutes. Cells were then washed an additional three times with PBS. Coverslips were mounted on glass slides using Vectashield mounting medium (Vector Labs, CA) and DAPI (4,6,-diamidinophenylindole) in order to visual the cell nuclei. Imaging and quantitations of EGFP-fusion proteins were performed on a Zeiss Axioplan 2IE Microscope. A minimum of four experiments were used for quantitative analysis. The resultant quantitations are represented graphically with standard deviation (Figure 7 and Figure 11).

Immunoblot Assays

ATCC HeLa cells were plated without coverslips 24 hours prior to transfection and grown overnight to 50-70% confluency. Transfection reactions for each well were prepared and distributed as outlined above. 24 hours after the initial transfection, cells were placed on ice and the DMEM(+) media was removed. 20 µl 2X SDS-PAGE loading buffer with BME was added to each well to form a lysate. Wells were scraped using the tip of a pipet for approximately 30 seconds. Lysates containing the same plasmid were combined in 0.7 µl tubes. Tubes were vortexed and then boiled on a heat block at 99.5°C for approximately 10 minutes. After microcentrifugation, 40 µl of each sample was subjected to SDS-PAGE and then transferred to nitrocellulose membrane. Ponceau staining was used to visualize the proteins. Lanes were then divided, split, and blocked overnight in 5% non-fat milk in PBS (pH 7.4) with 0.1% Tween-20 at 4°C. The following day, half of the lanes were incubated at room temperature for 1 hour with 1 ml anti-L2 antibody solution containing RG-1 Mab Tissue Culture Sup in a 1:2000 dilution. RG-1 Mab Tissue Culture Sup was used to detect the presence of L2 protein. The other half of the lanes were incubated at room temperature for 1 hour with 1 ml anti-GFP solution containing A.V. Monoclonal Antibody JL-8 in a 1:1000 dilution. A.V. Monoclonal Antibody JL-8 was used to detect the presence of enhanced green fluorescent protein (EGFP). Both sets of lanes were washed three times with 1X PBS and incubated at room temperature for 1 hour with the Goat anti-mouse HRP-conjugated secondary antibody in a 1:1000 dilution. The signal was detected using an ECL Detection Kit (Amersham Biosciences) and exposure to x-ray film (Denville Scientific Inc.).

<u>Results</u>

Pancellular localization of HPV16 L2 "MS4" unaffected by secondary mutations in the nterminal region

Transfection assays of double-mutants "MS4 S1" (pEGFP-HPV16 L2 RR₂₉₇AA + FF54AA) and "MS4 S2" (pEGFP-HPV16 L2 RR₂₉₇AA + LGI58AAA) were conducted using primary mutant "MS4" (pEGFP-HPV16 L2 RR₂₉₇AA) as the positive control and enhanced green fluorescent protein (EGFP) as the negative control. As predicted, both the "MS4" primary mutant and EGFP control were localized pancellularly (**Figure 4**). Both the "MS4 S1" and "MS4 S2 mutant proteins were also localized pancellularly (**Figures not shown**). Neither the "S1" nor "S2" secondary mutations restored nuclear localization. Experiments were performed by laboratory technician Lauren Crosby.

Nuclear localization of HPV16 L2 "MS4" restored by secondary mutation in cNES

Transfection assays of double-mutants "MS4 T1" (pEGFP-HPV16 L2 RR₂₉₇AA + P_{463_}) and "MS4 T2" (pEGFP-HPV16 L2 RR₂₉₇AA + V_{469_}) were conducted using primary mutant "MS4" (pEGFP-HPV16 L2 RR₂₉₇AA) as the positive control and enhanced green fluorescent protein (EGFP) as the negative control. As predicted, both the "MS4" primary mutant and EGFP control were localized pancellularly (**Figure 4**). Both the "MS4 T1" and "MS4 T2" mutant proteins were localized nuclearly (**Figure 5 and Figure 6**).

Nuclear localization of HPV16 L2 "MS5" restored by secondary mutation in cNES

The effectiveness of the "T1" and "T2" NES mutations were analyzed in the context of the "MS5" template (pEGFP-HPV16 L2 RTR₃₁₃AAA). Transfection assays of double-mutants "MS5 T1" (pEGFP-HPV16 L2 RTR₃₁₃AAA + P₄₆₃) and "MS5 T2" (pEGFP-HPV16 L2 RTR₃₁₃AAA + V₄₆₉) were conducted using primary mutant "MS5" as the positive control and enhanced green fluorescent protein (EGFP) as the negative control. As predicted, both the "MS5" and EGFP controls were localized pancellularly (**Figure 8**). Like the "MS4 T1" and "MS4 T2" double-mutants, the "MS5 T1" and "MS5 T2" double-mutants were localized nuclearly (**Figure 9 and Figure 10**).

Discussion

Experiments conducted by graduate student Shahan Mamoor in the Moroianu lab suggested that high risk HPV16 L2 minor capsid protein undergoes nuclear export. The focus of my research was to characterize the associated nuclear export signal. Previous data showed that nuclear localization of HPV16 L2 can be disrupted by introducing point mutations into the middle region of the HPV16 L2 sequence. Using site-directed mutagenesis, the two arginine residues located at positions 297 and 298 were replaced by two alanine residues to create the "MS4" mutant (RR₂₉₇AA). Separately, the arginine-threonine-arginine residues located at positions 313-315 were replaced by three alanine residues to create the "MS5" mutant (RTR₃₁₃AAA). When analyzed independently, both HPV16 L2 "MS4" and "MS5" mutants were localized pancellularly in HeLa cells.

Interestingly, the RJA nuclear export inhibitor partially restored nuclear localization of the MS4 and MS5 nuclear retention mutants. These data suggested that the HPV16 L2 protein undergoes nuclear export mediated by a leucine-rich nuclear export signal (NES). Motif analysis revealed the presence of two potential NESs, one in the n-terminal region and one in the c-terminal region.

The first series of experiments targeted the leucine-rich n-terminal region of the L2 protein (51MGVFFGGLGI₆₀). Using the HPV16 L2 "MS4" construct as a template, two separate secondary mutations were introduced into the L2 sequence using site-directed mutagenesis. In the first of two n-terminal secondary mutations, phenylalanine residues 54 and 55 were substituted with two alanine residues to create the resultant double-mutant RR₂₉₇AA + FF₅₄AA ("MS4 S1"). In the second of two n-terminal secondary mutations, the leucine-glycine-isoleucine residues at positions 58-60 were substituted with three alanine residues to create the resultant double-mutant RR₂₉₇AA + LGI₅₈AAA ("MS4 S2"). Transfection assays in HeLa cells followed by fluorescence microscopy demonstrated that both the "MS4 S1" double-mutant and the "MS4 M2" double-mutant were localized pancellularly and that nuclear localization had not been restored. These results suggested that the nuclear export signal was not located in the n-terminus of the HPV16 L2 sequence.

Next, the leucine-rich c-terminal region of the HPV16 L2 protein (462LPYFFDSVSL471) was targeted. Again, the HPV16 L2 "MS4" construct was used as a template for two separate secondary mutations. In the first of two c-terminal secondary mutations, the protein was

truncated at proline residue 463 to create the resultant double-mutant RR₂₉₇AA + P_{463_} ("MS4 T1"). In the second of two c-terminal secondary mutations, the protein was truncated at valine residue 469 to create the resultant double-mutant RR₂₉₇AA + V_{469_} ("MS5 T2"). Transfection assays in HeLa cells followed by fluorescence microscopy demonstrated that both the "MS4 T1" double-mutant and the "MS4 T2" double-mutant were localized nuclearly. L2 was localized nuclearly in 79.5% of the cells transfected with EGFP-16 L2 MS4 T1 and in 83.7% of the cells transfected with EGFP-16 L2 MS4 T2 (**Table 1**). These results suggested that the nuclear export signal of HPV16 L2 is located in the c-terminal region.

In order to confirm these results, the "T1" and "T2" truncations were introduced into the "MS5" construct. The resulting double-mutants were identified as "MS5 T1" (RTR₃₁₃AAA + P_{463_}) and "MS5 T2" (RTR₃₁₃AAA + V_{469_}). Transfection assays in HeLa cells followed by fluorescence microscopy demonstrated that both the "MS5 T1" double-mutant and the "MS5 T2" double-mutant were localized nuclearly. L2 was localized nuclearly in 87.6% of the cells transfected with EGFP-16 L2 MS5 T1 and in 94.5% of the cells transfected with EGFP-16 L2 MS5 T1 and in 94.5% of the transfections utilizing the "MS4" c-terminal double-mutants, these observations suggest that the nuclear export signal of HPV16 L2 is located in the c-terminal region.

The identification and characterization of both the nuclear localization signals (NLSs) and nuclear export signal (NES) of HPV16 L2 is critical for understanding the mechanism of HPV infection. Research conducted in the Moroianu lab concerning both the import and export pathways of HPV16 L2 could be helpful in the identification of specific drugs that could be used to inhibit nucleocytoplasmic trafficking of HPV16 L2 and, thus, prevent viral infection.



Figure 1: Schematic Representation of Human Papillomavirus Type 16 Genome

Image obtained from <http://www.microbiologybytes.com/virology/Papillomaviruses. html>.



Figure 2: Schematic Representation of Human Papillomavirus Icosahedral Capsid Image obtained from http://www.microbiologybytes.com/blog/tag/hpv/>.



Figure 3: Agarose Gel Electrophoresis of Human Papillomavirus Type 16 L2 Mutants

Agarose gel electrophoresis of HPV16 L2 mutants. Plasmids were prepared using the Quikchange Site-Directed Mutagenesis Kit (Stratagene). Lane 1 contains a 1 kb DNA ladder (New England Biolabs). Lane 2 contains the HPV16 L2 "MS5" nuclear retention mutant as a positive control. Lane 3 contains the HPV16 L2 "MS5 T1" double- mutant. Lane 4 contains the HPV16 L2 "MS5 T2" double-mutant. Lane 5 contains the HPV16 L2 "MS5 L462A" double-mutant. Lane 6 contains EGFP as a negative control.

HPV16 L2 RR297AA ("MS4")



EGFP





Figure 4: Localization of HPV16 L2 "MS4" Nuclear Retention Mutant

HeLa cells were transfected with EGFP-HPV16 L2 "MS4" plasmids and examined 24 hours post-transfection using fluorescence microscopy. EGFP fluorescence localizes the protein of interest. DAPI staining localizes the cell nuclei. Note the pancellular localization of the EGFP-HPV16 L2 "MS4" nuclear retention mutation. Images courtesy of Zeynep Onder.

HPV16 L2 RR297AA + P463_ ("MS4 T1")



Figure 5: Localization of HPV16 L2 "MS4 T1" Double Mutant

HeLa cels were transfected with EGFP-HPV16 L2 "MS4 TI" plasmids and examined 24 hours post-transfection using fluorescence microscopy. DAPI staining localizes the cell nuclei. EGFP fluorescence localizes the protein of interest. Note the nuclear localization of the EGFP-HPV16 L2 "MS4 T1" double-mutant. Images courtesy of Zeynep Onder.

HPV16L2 RR₂₉₇AA + V₄₆₉ ("MS4 T2")



Figure 6: Localization of HPV16 L2 "MS4 T2" Double Mutant

HeLa cells were transfected with EGFP-HPV16 L2 "MS4 T2" plasmids and examined 24 hours posttransfection using fluorescence microscopy. DAPI staining localizes the cell nuclei. EGFP fluorescence localizes the protein of interest. Note the nuclear localization of the EGFP-HPV16 L2 "MS4 T2" double-mutant. Images courtesy of Zeynep Onder.

HPV16 L2 Mutant	Total # Nuclear	Total # Pancellular	Total # Cytoplasmic
MS4	442	1410	5
MS4 T1	1542	389	0
MS4 T2	1205	238	0

HPV16 L2 Mutant	% Nuclear	% Pancellular	% Cytoplasmic
MS4	23.8 +/- 4.82	75.9 +/- 5.12	0.5 +/- 1.25
MS4 T1	79.5 +/- 1.71	20.5 +/- 1.71	0
MS4 T2	83.7 +/- 7.54	16.3 +/- 7.54	0

Table 1: Quantitations of the Localization of HPV16 L2 "MS4" Mutants



Figure 7: Graphical Representation of Quantitations of HPV16 L2 "MS4" Mutants

Nuclear localization of HPV16 L2 is restored when the "T1" and "T2" secondary mutations are added to the "MS4" construct. The black error bars indicate standard deviation. Graph courtesy of Zeynep Onder.

HPV16 L2 RTR313AAA ("MS5")



Figure 8: Localization of HPV16 L2 "MS5" Nuclear Retention Mutant

HeLa cells were transfected with EGFP-HPV16 L2 "MS5" plasmids and examined 24 hours post-transfection using fluorescence microscopy. DAPI staining localizes the cell nuclei. EGFP fluorescence localizes the protein of interest. Note the pancellular localization of the EGFP-HPV16 L2 "MS5" nuclear retention mutant.

HPV16 L2 RTR₃₁₃AA A + P₄₆₃ ("MS5 T1")



Figure 9: Localization of HPV16 L2 "MS5 T1" Double Mutant

HeLa cells were transfected with EGFP-HPV16 L2 "MS5 T1" plasmids and examined 24 hours post-transfection using fluorescence microscopy. DAPI staining localizes the cell nuclei. EGFP fluorescence localizes the protein of interest. Note the nuclear localization of the EGFP-HPV16 L2 "MS5 T1" double-mutant.

HPV16 L2 RTR₃₁₃AA A + V₄₆₉ ("MS5 T2")



Figure 10: Localization of HPV16 L2 "MS5 T2" Double Mutant

HeLa cells were transfected with EGFP-HPV16 L2 "MS5 T2" plasmids and examined 24 hours posttransfection using fluorescence microscopy. DAPI staining localizes the cell nuclei. EGFP fluorescence localizes the protein of interest. Note the nuclear localization of the EGFP-HPV16 L2 "MS5 T2" double-mutant.

2.3.11	Nuclear	Pancellular	Cytoplasmic	% Nuclear	% Pancellular	% Cytoplasmic
HPV16 L2 MS5	2	26	0	7.1	92.9	0
HPV16 L2 MS5 T1	71	8	0	89.9	10.1	0
HPV16 L2 MS5 T2	79	5	0	94	6	0
2.8.11	Nuclear	Pancellular	Cytoplasmic	% Nuclear	% Pancellular	% Cytoplasmic
HPV16 L2 MS5	0	0	0	0	0	0
HPV16 L2 MS5 T1	18	2	0	90.0	10.0	0
HPV16 L2 MS5 T2	8	0	0	100.0	0.0	0
2.9.11	Nuclear	Pancellular	Cytoplasmic	% Nuclear	% Pancellular	% Cytoplasmic
HPV16 L2 MS5	0	28	0	0.0	100.0	0
HPV16 L2 MS5 T1	130	46	0	73.9	26.1	0
HPV16 L2 MS5 T2	9	1	0	90.0	10.0	0
2.17.11	Nuclear	Pancellular	Cytoplasmic	% Nuclear	% Pancellular	% Cytoplasmic
HPV16 L2 MS5	0	97	0	0.0	100.0	0.0
HPV16 L2 MS5 T1	193	7	0	96.5	3.5	0.0
HPV16 L2 MS5 T2	51	3	0	94.4	5.6	0.0

HPV16 L2 Mutant	Total # Nuclear	Total # Pancellular	Total # Cytoplasmic
MS5	2	153	0
MS5 T1	412	63	0
MS5 T2	147	9	0

HPV16 L2 Mutant	% Nuclear	% Pancellular	% Cytoplasmic
MS5	1.3 +/- 3.55	98.7 +/- 48.93	0
MS5 T1	86.7 +/- 9.63	13.3 +/- 9.63	0
MS5 T2	94.2 +/- 4.11	5.8 +/- 4.11	0

Table 2: Quantitations of the Localization of HPV16 L2 "MS5" Mutants



Figure 11: Graphical Representation of Quantiations of HPV16 L2 "MS5" Mutants

Nuclear localization of HPV16 L2 is restored when the "T1" and "T2" secondary mutations are added to the "MS5" construct. The black error bars indicate standard deviation.

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