INSIGHTS INTO THE INTERACTION AND NUCLEAR CAPSID-ASSOCIATION OF HERPES SIMPLEX VIRUS TYPE 1 TEGUMENT PROTEINS UL36 AND UL37

A Dissertation in
Microbiology and Immunology

by
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Submitted in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy

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ABSTRACT

Herpesvirus virions are composed of three morphologically distinct structures: the capsid, tegument and envelope. The icosahedral capsid encloses the viral DNA genome. Virions are bounded by a host-derived lipid envelope embedded with viral glycoproteins and other transmembrane proteins. The tegument is the proteinaceous region that lies between the capsid and envelope. More than 25 virus-encoded proteins, as well as host proteins, are packaged into the tegument region. Incorporation of tegument proteins may occur at multiple sites within the cell and is governed by a complex series of protein interactions. Tegument proteins may associate with capsids within the nucleus, as the capsid exits the nucleus through budding and fusion with the nuclear membrane, during microtubule-mediated transport in the cytoplasm and during final envelopment at vesicles derived from the trans-Golgi network (TGN). The studies described in this dissertation were initiated to identify tegument proteins associated with capsids isolated from the nucleus of herpes simplex virus type 1 (HSV-1)-infected cells.

Several lines of evidence led us to investigate if the conserved, essential tegument protein, UL36, is associated with capsids in the nucleus. UL36 is tightly associated with capsids, coimmunoprecipitates with the major capsid protein, VP5, and is present in the nucleus of HSV-1-infected cells. Furthermore, viruses lacking UL36 exhibit a severe defect in tegumentation and do not undergo final envelopment, suggesting that incorporation of UL36 may be required for subsequent acquisition of other tegument proteins. To address this issue, capsids were isolated and purified from the nuclear fraction of HSV-1-infected cells and analyzed for the presence of UL36 by Western blotting. As hypothesized, detectable amounts of UL36 associate with capsids from the
nuclear fraction. Interestingly, UL36 associates only with DNA-filled C capsids and is not detected in association with A capsids or B capsids, which do not mature into infectious virions. Further analysis of nuclear capsids revealed that the UL36 binding partner, UL37, also associates with capsids isolated from the nuclear fraction. These results suggest that UL36 and UL37 are among the initial tegument proteins incorporated into virions and therefore may function to facilitate the incorporation of other tegument proteins into the virus particle.

The interaction of UL36 and UL37 homologues is conserved across the herpesvirus family. The region and individual residues of UL36 critical for interaction with UL37 have been identified. In contrast, the region of UL37 necessary for binding UL36 was unknown. To fill this gap of knowledge, coimmunoprecipitation assays were used to identify the region of UL37 necessary for binding UL36. Deletion of amino acids 568-809 of UL37 reduced coimmunoprecipitation of UL36 to near background levels. The carboxy-terminal half of UL37, residues 568-1123, was sufficient for interaction with UL36. Further deletion of this region of UL37 caused deleterious effects on UL36 binding. These studies revealed that amino acids 568-809 are necessary and the carboxy-terminal half of UL37 is sufficient for interaction with UL36.

To expand the identification of interaction domains of UL37, studies were also performed to identify the regions of UL37 involved in self-association. Coimmunoprecipitation experiments revealed that two regions of UL37, amino acids 1-300 and 568-1123, are independently capable of self-association. Because the carboxy-terminal half of UL37 serves dual roles of self-association and UL36 binding, studies were performed to determine if this region is capable of interacting with both proteins.
simultaneously, thus forming a UL37-UL37-UL36 complex. Full length UL37 appears
to self-associate only under conditions when UL36 is not present, or is present in low
amounts. This result suggests that UL36 competes with UL37 for interaction with
another molecule of UL37. Therefore UL36 appears to regulate the self-association of
UL37.

Expression of UL37 is necessary for tegumentation and envelopment of infectious
HSV-1 virions. The functional role(s) of UL37 that is critical during infection is
unknown. To identify regions of UL37 necessary for production of infectious virions, a
trans-complementation assay was utilized. The carboxy-terminal region of UL37, amino
acids 568-1123, partially rescues the UL37 deletion virus, KΔUL37. These results
suggest that the carboxy-terminus of UL37 may contribute to its essential functional role
within the virus-infected cell. In conclusion, the carboxy-terminal half of UL37, amino
acids 568-1123, is involved in UL37 self-association, interaction with UL36 and plays a
critical role during virus assembly.

Collectively, the studies described in this dissertation provide novel insights
regarding the interaction of tegument proteins UL36 and UL37 with capsids and the
regions of UL37 involved in the conserved interaction with UL36. These studies may
provide the foundation for future investigations to elucidate the functional role(s) of
UL36 and UL37 critical for assembly of infectious virions.
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<tr>
<td>Ac</td>
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<td>aromatic amino acid</td>
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<td>ARR</td>
<td>arginine rich region</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
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<td>βME</td>
<td>β-mercaptoethanol</td>
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<tr>
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<td>bovine herpesvirus</td>
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<td>enhanced chemiluminescence</td>
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<td>ethylenediaminetetraacetic acid</td>
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<td>HCMV</td>
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<td>hpi</td>
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<td>infected cell protein</td>
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<td>ONM</td>
<td>outer nuclear membrane</td>
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<td>polyacrylamide gel electrophoresis</td>
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<td>Y</td>
<td>tyrosine</td>
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PREFACE

Successful scientific research is often the result of collaborative efforts. Throughout my graduate education my scientific efforts were aided by intellectual discussions with my laboratory colleagues. Their intellectual contribution is acknowledged by inclusion of their names in the multi-authored chapters. However, all of the experiments presented in this dissertation were conducted by myself.
ACKNOWLEDGMENTS

I would describe my graduate education as a journey. This long scientific journey has been filled with positive experiences and challenges, some anticipated and others unexpected. My graduate experience has not only developed me as a scientist but has also shaped me as a person. Along this journey I learned many scientific lessons, life lessons and made a lot of good memories. It is my privilege to thank those who have selflessly supported me.

I would like to thank Dr. Courtney for his patience and sincere concern for my professional development. You have given me the independence and opportunity to both struggle and succeed in my scientific endeavors and you have challenged me to become a better scientist. I am sincerely grateful for your wisdom, support and encouragement throughout the years.

I would like to recognize my graduate committee: Drs. Neil Christensen, Kristin Eckert, David Spector, Judith Tevethia and John Wills for fostering my scientific development. Your critical analyses and passion for science have made my committee meetings productive and encouraging. I thank you for challenging me to do better.

I am proud to be a part of the Microbiology and Immunology department that is composed of supportive students, faculty and staff. I am grateful for the many Penn State friends whom I have met during my graduate school journey. The scientific discussions and debates, encouragement and friendship that you all have shared are truly appreciated. I’d like to thank two guys, Mike and Kevin, who in many ways were like the older brothers that I never really wanted. You’ve helped make the many hours working in lab entertaining, productive and fun. I would especially like to thank two amazing friends,
Jodi and Missy. You both have been shoulders to lean on, encouraged me when I was most challenged and have celebrated the good times with me. I cherish our friendships and all of your support.

Finally I want to express my gratitude to my family. Without their love and support I could not have attained this goal. I am particularly grateful to my Mom for all of her love, patience and sacrifices throughout my childhood. During the last year of my graduate studies I was blessed with a beautiful baby boy, Levi. I thank you Levi for bringing me joy and making me smile during the challenge of thesis writing. You have shown me my own strength. You give me hope and make me believe in all that is good in this world. You constantly amaze me and I am totally in love with you.

My deepest appreciation goes to my husband and best friend Steve. I thank you for your unwavering patience, support and faith in me. Your unconditional love and encouragement have given me the strength to accomplish this goal. You are an incredible husband and father. I love you more than words can express and offer you my most heartfelt thanks.
Herpesvirus virions are composed of three distinct structures: an icosahedral capsid enclosing the viral genome, the tegument and an envelope (Fig. 1.1). The virion is bounded by the host-derived lipid envelope embedded with viral glycoproteins. The proteinaceous region between the capsid and envelope is the tegument. Assembly of virus particles begins in the nucleus where the viral genome is packaged into assembled capsids (Fig. 1.2). The capsid undergoes an envelopment-deenvelopment event by budding into the inner nuclear membrane and subsequently fusing with the outer nuclear membrane resulting in capsid release into the cytoplasm. After exiting the nucleus, the capsid travels through the cytoplasm to the site of final envelopment at vesicles derived from the \textit{trans}-Golgi network (TGN). After budding into TGN vesicles, enveloped virions are transported to the cell surface via the secretory pathway and are released from the cell.

More than 25 virus-encoded proteins, as well as host-derived proteins, are contained within the tegument. Throughout the literature the tegument region has been described as amorphous, unsymmetrical and even unstructured. These descriptions result from the complexity and incomplete understanding of the tegument architecture. With the exception of the tegument proteins that interact directly with the penton vertices of the capsid, much of the tegument region appears to lack icosahedral structure (334, 366). Recently, there has been much interest in determining the cellular sites where tegument proteins localize and associate with virions. It is possible that tegument proteins are incorporated into the assembling virion at several stages during virus assembly (Fig. 1.2). First, tegument proteins may associate with capsids located within the nucleus. Next, tegument proteins may interact with capsids as they bud into the inner nuclear membrane.
Figure 1.1. Basic Structure of an HSV-1 Virion. The linear double stranded viral genome is enclosed within the icosahedral capsid. The virion is bounded by the host-derived lipid envelope containing more than a dozen different virus-encoded glycoproteins (orange spikes). The protein rich region that lies between the capsid and the envelope is known as the tegument. At least 25 different virus-encoded proteins are incorporated into the tegument. Populations of tegument proteins (red and yellow) are known to interact tightly with the capsid, whereas other populations of tegument proteins (blue) are membrane-associated or interact with membrane-associated proteins. Other tegument proteins (green) appear to link the capsid-associated tegument with membrane-associated tegument proteins.
Figure 1.2. **HSV-1 Virion Assembly Pathway.** Spherical procapsids are assembled within the nucleus of the infected cell. The viral genome is packaged into the capsid through the UL6 portal complex as scaffold proteins simultaneously exit and the capsid matures into a stable icosahedron. The capsid buds into the inner nuclear membrane, gaining a primary envelope. The enveloped perinuclear capsid exits the perinuclear space by fusing with the outer nuclear membrane, thus losing the primary envelope. The capsid travels to TGN-derived vesicles via microtubule-dependent transport. The capsid acquires a lipid envelope by budding into TGN-derived vesicles. The enveloped virion is transported within a vesicle to the cell surface. The vesicle fuses with the plasma membrane, releasing the virion into the extracellular space.
during nuclear egress. Tegument proteins may also bind to capsids as they are transported via microtubules in the cytoplasm. The final subset of tegument proteins is incorporated into virions during acquisition of the final envelope as the capsid buds into TGN-derived vesicles.

The studies described in this dissertation were initiated with the goal of identifying tegument proteins that may associate with capsids in the nucleus. The large tegument protein UL36 is considered a capsid-associated or “inner tegument protein” based upon its tight interaction with capsids and coimmunoprecipitation with the major capsid protein VP5 (103, 207, 313). HSV-1 UL36 is present in the nucleus of infected cells and is required for tegumentation and envelopment of capsids (64, 207, 223, 278). Therefore, we hypothesized that UL36 associates with capsids in the nucleus to facilitate subsequent incorporation of tegument proteins into the assembling virion. Chapter III describes the purification of capsids from the nuclear fraction. As we hypothesized, UL36 was associated with nuclear capsids. Curiously, UL36 selectively associates with a subset of capsids, DNA-filled C capsids. The UL36 binding partner, UL37, is also essential for tegumentation and envelopment of HSV-1 virions (63, 278). We extended our investigation to show that UL37 also associates with capsids isolated from the nuclear fraction. In conclusion, UL36 and UL37 are essential for tegumentation and associate with capsids in the nucleus. These studies suggest that UL36 and UL37 are among the initial tegument proteins to associate with capsids and serve as a platform to facilitate the addition of other tegument proteins.

The interaction of UL36 and UL37 homologues is conserved across the herpesvirus family (25, 81, 171, 285, 336, 338). The region of HSV-1 UL36 sufficient
for binding UL37 encompasses amino acids 512-767 (338). Residues F593 and E596 of UL36 are critical for interaction with UL37 (216). In contrast, when these studies were initiated the domain of UL37 involved in the UL36-UL37 interaction was unknown. To fill this gap of knowledge, we utilized coimmunoprecipitation assays to identify the region of UL37 involved in binding to UL36. The results in Chapter IV show that the carboxy-terminal half of UL37, amino acids 568-1123, mediates binding to UL36. We also determined that this region is involved in self-association of UL37. In addition, a second self-association domain lies within the amino-terminal 300 amino acids of UL37. We utilized a trans-complementation assay to identify regions of UL37 that rescue a UL37 deletion virus, KΔUL37 (63). The carboxy-terminal half of UL37 enables release of infectious virus particles from cells infected with the mutant virus, suggesting that this region of UL37 plays an important role in assembly.

The carboxy-terminal half of UL37 binds UL36, binds other molecules of UL37 and facilitates virus assembly. Because this region of UL37 serves multiple roles, we set out to determine if UL37 can interact with UL36 and UL37 simultaneously, resulting in a UL37-UL37-UL36 protein complex. The final observations presented in Chapter IV provide evidence that UL37 does not self-associate when UL36 is abundant. Therefore, self-association of UL37 appears to be regulated by UL36. A discussion of the potential roles of UL37 self-association and regulation by UL36 is presented in Chapter V.

UL36 and UL37 are essential conserved tegument proteins that serve multiple functions during infection. A considerable amount of effort has recently been extended by numerous laboratories to elucidate the functional roles of these proteins during infection. A thorough discussion of the current knowledge regarding UL36 and UL37,
and UL36 and UL37 homologues, is presented in Chapter II. In addition, a review of the
HSV-1 assembly pathway is presented so that one may consider the roles UL36 and
UL37, and their interactions, may play in this process.
CHAPTER II

REVIEW OF THE LITERATURE
CLASSIFICATION OF HERPESVIRUSES

Herpesviruses are widely distributed throughout the animal kingdom and at least one herpesvirus has been isolated from nearly every animal species examined. Eight herpesviruses infect humans as their primary host, including: herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), varicella-zoster virus (VZV), Epstein-Barr virus (EBV), human cytomegalovirus (HCMV), human herpesvirus 6 (HHV-6), human herpesvirus 7 (HHV-7) and Kaposi’s sarcoma-associated herpesvirus (KSHV). Several other animal herpesviruses have also been studied extensively, including pseudorabies virus (PRV), equine herpesvirus type 1 (EHV-1), Marek’s disease virus (MDV) and bovine herpesvirus type 1 (BHV-1), due to their economic impact on agriculture and/or conservation with human herpesviruses.

All herpesviruses share common architectural and biological properties. Historically, viruses were classified into the Herpesviridae family based upon virion morphology. All herpesvirus virions share distinctive features including: the DNA genomic core, an icosahedral capsid, tegument and a viral envelope (Fig. 1.1). The icosahedral capsid has a triangulation number of 16 (T=16) and is composed of 162 capsomers, including 150 hexons and 12 pentons. Each capsid contains a portal complex at one pentameric position, through which the viral genome is inserted (240). The double stranded linear viral DNA is densely packed within the icosahedral capsid. Surrounding the capsid is an electron dense, amorphous region known as the tegument. The tegument is composed of over 25 virus-encoded proteins, as well as several host-derived proteins (reviewed in 140). The virion is bounded by a host-derived lipid envelope that contains several different embedded viral glycoproteins and other transmembrane proteins. The
overall virus diameter of herpesvirus virions is relatively large, ranging from 120 to 260 nm (259). Cryoelectron microscopy studies estimate the diameter of HSV-1 virions to be about 225 nm (117).

In addition to structural similarities, all herpesviruses also share four distinct biological properties. Perhaps the most notable biological property of herpesviruses is their ability to establish life-long infections in their hosts through latency. In a latently-infected cell, viral genomes exist as closed circular molecules. During latency, viral genes involved in lytic replication are not expressed, but viral latency proteins are expressed. Triggers such as psychological stress, ultraviolet (UV) radiation, and low immune defenses can cause reactivation of the virus resulting in lytic infection and the production of progeny virions. The frequency and severity of reactivation vary among the different herpesviruses. A second common feature of herpesviruses is expression of numerous virus-encoded enzymes involved in nucleic acid metabolism (i.e. thymidine kinase, ribonucleotide reductase), DNA synthesis (i.e. DNA polymerase, helicase) and protein modification (i.e. protein kinases). Furthermore, herpesviruses universally rely upon the nucleus as the site of DNA replication and capsid assembly. After the DNA filled capsid exits the nucleus, final tegumentation and envelopment occur in the cytoplasm. The final common biological feature of herpesvirus members is that lytic infection and the production of virus particles leads to destruction of the host cell, termed cytopathic effect (CPE).

Before herpesvirus genomes were sequenced, the Herpesviridae family was categorized into three subfamilies, the Alphaherpesvirinae, Betaherpesvirinae and Gammaherpesvirinae, on the basis of biological properties including host range, length of
replication cycle and site of viral latency (Table 2.1) (259). Three alphaherpesviruses have been isolated from humans including: HSV-1, HSV-2 and VZV. In addition, PRV, BHV-1, EHV-1 and MDV, which naturally infect swine, cattle, horses and chickens respectively, are also members of the Alphaherpesvirinae. Alphaherpesviruses have a characteristic variable host range, a relatively short reproductive cycle (compared to beta- and gammaherpesviruses), spread rapidly in cell culture and efficiently destroy infected cells. Members of Alphaherpesvirinae primarily undergo latency in sensory ganglia.

Human betaherpesviruses include HCMV, HHV-6A, HHV-6B and HHV-7. Betaherpesviruses are characterized by a restricted host range and long reproductive cycle. Infections with betaherpesviruses progress slowly in cell culture and result in the formation of cytomegalia (enlarged cells). Betaherpesviruses establish latency in secretory glands, kidneys and lymphoreticular cells.

Members of the Gammaherpesvirinae that infect humans include KSHV and EBV. Gammaherpesviruses have a restricted host range in cell culture that is limited to the family or order of the natural host. Some gammaherpesviruses can replicate in vitro in epithelial and fibroblast cells, but all members can replicate in lymphoblastoid cells, usually specific for either T or B lymphocytes. The gammaherpesviruses also establish latency in T and B lymphocytes.

HSV-1 REPLICATION

INITIATION OF INFECTION

Herpesvirus replication is a complex process. This section of the literature review will discuss early events of infection, including virus attachment and entry, delivery of
### Table 2.1. Human Herpesviruses

<table>
<thead>
<tr>
<th>Subfamily</th>
<th>Name</th>
<th>Designation</th>
<th>Target Cell</th>
<th>Site of Latency</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>Herpes Simplex Virus Type 1</td>
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<td>Mucoepithelia</td>
<td>Sensory Ganglia</td>
</tr>
<tr>
<td></td>
<td>Herpes Simplex Virus Type 2</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Varicella-Zoster Virus</td>
<td>3</td>
<td>Mucoepithelia, T lymphocytes, liver, spleen</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Human Cytomegalovirus</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β</td>
<td>Roseola infantum/</td>
<td>6A</td>
<td>T lymphocytes, monocytes, macrophages</td>
<td>Secretory glands, Kidneys and Lymphoreticular Cells</td>
</tr>
<tr>
<td></td>
<td>Sixth disease</td>
<td>6B</td>
<td></td>
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<tr>
<td></td>
<td>Human Herpesvirus 7</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Epstein-Barr Virus</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>γ</td>
<td>Kaposi's sarcoma-associated virus</td>
<td>8</td>
<td>B lymphocytes, epithelia and fibroblasts</td>
<td>Lymphoid Tissue</td>
</tr>
</tbody>
</table>
the genome to the nucleus, DNA replication and gene expression. Infection begins with
binding of the virus to cell surface receptors. After attachment to the cell surface, fusion
of the viral envelope with the plasma membrane occurs quickly, resulting in capsid
release into the cytoplasm. After the capsid is deposited into the cytoplasm many
tegment proteins dissociate from the capsid, but a few tegument proteins (the “inner
tegument”) remain bound to the capsid. The capsid is then transported in a microtubule-
mediated fashion to the nucleus. The capsid binds to a nuclear pore and the viral genome
is released into the nucleus. Viral DNA replication occurs within the nucleus and gene
expression follows in a cascade fashion.

**Cellular Attachment and Fusion**

The first step in infection is virion binding to the cell surface. To accomplish this
step, the virion contains more than 12 virus-encoded glycoproteins embedded in its lipid
envelope. Two of these glycoproteins, glycoprotein C (gC) or glycoprotein B (gB),
independently mediate binding of the virion to cell surface glycosaminoglycans such as
heparan sulfate or chondroitin sulfate (19, 116, 123, 303, 314, 357). Virus particles
lacking gC are infectious (122). Similarly, virions containing mutant gB molecules that
are defective in heparan sulfate binding are also infectious (168). However, in the
absence of both gC and gB, cellular attachment is severely reduced (122). Cellular
attachment is mediated primarily through binding to heparan sulfate on the cell surface,
but in the absence of heparan sulfate, virion attachment can occur through chondroitin
sulfate binding (19, 116).
After the virion attaches to the cell, gD interacts with one of several cell entry receptors to trigger fusion of the viral envelope with the cell membrane. The interaction of gD with entry receptors is essential for virion entry into cells, thus virions lacking gD are not infectious (178). gD interacts with entry receptors that fall into three classes. The first class includes herpesvirus entry mediator (HVEM), a member of the tumor necrosis factor (TNF) receptor family (222, 350). gD also binds to the intercellular adhesion molecules, nectin-1 and nectin-2 (100). Lastly, gD interacts with 3-O-sulfated heparan sulfate (304). The three classes of cell entry receptors are differentially expressed on specific cell types (72, 162, 192, 222, 319, 328, 343). Therefore, herpesviruses may utilize several different receptors to enable entry into many different cell types (26, 100, 312).

The binding of gD to a cellular entry receptor induces a conformational change in gD that enables the interaction with three other glycoproteins: gB and/or gH/gL heterodimers (158). In addition to gD, the glycoproteins gB, gH and gL are also required for fusion (42, 43, 52, 158). The specific mechanism and the identity of the glycoprotein(s) that acts as the fusion protein are not clear. Current literature suggests that gD and gL do not directly participate in the fusion mechanism. gL is necessary for proper folding and localization of gH on the cell surface; however gL does not appear to play a direct role in fusion (128, 260). Furthermore, gD does not mediate fusion in the absence of gB, gH or gL. These observations suggest that gB and/or gH act as fusion molecules (reviewed in 120). Both gB and gH are conserved across the Herpesviridae family and contain structural similarities to known viral fusion proteins (262). gH contains an alpha helix and two heptad repeats in the ectodomain that are characteristic of
fusion peptides (101). Mutation or deletion of these motifs of gH abrogates the ability of virions to enter cells (102). Similarly, the crystal structure of gB reveals structural homology with the fusion peptide glycoprotein G of vesicular stomatitis virus (46, 121, 280). Interestingly, mutation of hydrophobic residues in the putative fusion loops of gB resulted in decreased membrane fusion (118).

Early studies investigating virus entry into Vero or HEp-2 cells suggested that after HSV-1 virions attach to cells they enter the cell by direct fusion at the plasma membrane (93, 94, 95, 354). Virus entry into neuronal cell lines also occurs through direct fusion (188, 244). It was assumed that HSV-1 entered all cell types by direct fusion. Recent work, however, has shown that virions enter some cell types through pH-dependent or pH-independent endocytosis. The endocytic entry route is similar to direct fusion in that both processes utilize gB and/or gC for cellular attachment, necessitate binding of gD to a cellular receptor, and require gB, gC, gH and gL for fusion. In the endocytic entry route, however, the viral envelope fuses with the endocytic vesicle instead of the plasma membrane. It was recently shown that virions enter keratinocytes and HeLa cells through pH-dependent endocytosis (243). Other studies reported that virions enter a murine melanoma cell line, C10, through endocytosis, and upon internalization, fusion occurs in a pH-independent mechanism (217).

Delivery of the Genome to the Nucleus

After fusion of the viral envelope with the cell membrane or endocytic vesicle, the deenveloped virion is released into the cytoplasm. Upon liberation into the cytoplasm, several events result in the deposition of the viral genome into the nucleus.
Figure 2.1. Virion Uncoating and Delivery of the Viral Genome to the Nucleus. The virion attaches to heparan sulfate on the cell surface via gB or gC. Following attachment, ligation of gD with a cell surface receptor triggers fusion of the viral envelope with the plasma membrane or an endocytic vesicle. Upon release of the capsid into the cytoplasm, phosphorylation of tegument proteins causes their release from the capsid. VP16 localizes to the nucleus where it functions to transactivate viral immediate-early gene expression. vhs functions to degrade mRNA, resulting in host gene-expression shut-off. Tegument proteins that remain bound to the capsid (yellow and red) facilitate microtubule-mediated transport of the capsid to the nucleus. After the capsid docks at a nuclear pore complex UL36 is cleaved, resulting in the release of the viral genome into the nucleus.
(Fig. 2.1). First, much of the tegument is shed from the capsid. Second, the capsid travels to the nucleus in a microtubule-mediated manner. Lastly, the capsid docks at a nuclear pore and the viral genome is released upon cleavage of the capsid-associated tegument protein UL36 (136).

After fusion of the viral envelope with a host membrane, the deenveloped capsid is released into the cytoplasm. Electron microscopic studies indicate that shortly after release into the cytoplasm many of the tegument proteins dissociate from the capsid (110, 111, 186). An in vitro assay demonstrated that several tegument proteins contained within the virion are phosphorylated by viral and/or cellular kinases (224). These data suggest that phosphorylation of tegument components triggers their release from the capsid. In vitro assays also demonstrated the propensity of certain tegument proteins to remain associated with the capsid upon detergent treatment of the virion and exposure to physiological concentrations of ATP and magnesium, while other tegument proteins dissociated from the capsid (224). Tegument proteins packaged in the virion serve a variety of functions that enhance infection. VP16, encoded by the UL48 ORF, dissociates from the capsid and localizes to the nucleus where it transactivates viral immediate-early gene expression (22, 266). VP16-mediated transcriptional transactivation is enhanced by other tegument proteins, UL46 and UL47 (365). The tegument protein encoded by the UL41 gene, vhs, functions to degrade mRNA, thus limiting expression of host proteins (163).

To release the viral genome into the nucleus the viral capsid must migrate from the cell periphery to the nucleus. To accomplish its cytoplasmic journey, the capsid utilizes microtubules for active transport toward the nucleus (157, 311, 329, 330). In
*in vitro* studies showed that capsids containing the outer tegument proteins have reduced microtubule motility compared to capsids that were stripped of outer tegument to expose the inner tegument proteins (355). Several tegument proteins remain associated with the capsid, including UL36, UL37 and US3, and appear to facilitate microtubule-based transport toward the nucleus through interaction with dynein motor proteins (55, 110, 156, 186, 187, 224, 296). Furthermore, the capsid protein VP26 may also play a role in microtubule-based transport, since it interacts with dynein light chains in yeast two-hybrid assays (71).

Electron microscopy showed that after transport to the nucleus the capsid docks at nuclear pore complexes (21). *In vitro* studies have shown that Importin β and Ran-GTP are required for capsid binding to nuclear pores (250). The arrangement of the viral DNA within the capsid and the structure and location of the UL6 portal complex have been compared to that of dsDNA bacteriophages (32, 47, 176, 240, 306, 333). The capsid is positioned such that the portal complex, located at a unique capsid vertex, is inserted into the nuclear pore (233). Recent studies have begun to elucidate the specific viral and cellular proteins involved in capsid-nuclear pore binding. Antibodies or small interfering RNA (siRNA) against nucleoporin Nup358 attenuate the ability of capsids to bind to the nucleus (55). Furthermore, the capsid-associated protein UL25 binds to nucleoporins CAN/Nup214 and hCG1 (254). UL25 also binds to the UL6 capsid portal protein and the capsid-associated tegument protein UL36; thus UL25 may serve as an interface between the incoming capsid and the nuclear pore complex (254).

Early studies showed that a temperature sensitive mutant virus, tsB7, containing a mutation mapping to the UL36 ORF, fails to release the viral genome after the capsid is
transported to the nucleus (21). The specific role of UL36 in genome release has recently been elucidated. Roizman’s group discovered that the amino terminus of UL36 is cleaved after capsid binding to the nuclear pore complex, resulting in the release of the viral genome (136). The specific cleavage site within UL36 and the protease responsible for the cleavage have not yet been identified, but the cleavage of UL36 is required for release of the viral genome (136). Furthermore, antisera against UL36 blocks capsid binding to the nucleus, suggesting that UL36 plays a role in the migration of the capsid to the nucleus and/or binding of the capsid to the nuclear pore complex (55).

In addition to cleavage of UL36, a second proteolytic cleavage event may be involved in the release of the viral genome from the capsid. Studies in vitro showed that release of DNA from purified capsids correlates with cleavage of a small proportion of the UL6 portal protein (233). Electron microscopy studies revealed that the DNA is ejected from the nucleus at a single vertex, presumed to be the portal, in the form of a single double helix (233).

Phases of Gene Expression

During herpesvirus infection, over 80 viral genes are expressed in a regulated cascade fashion (282). Transcription of viral genes is performed in the nucleus by cellular RNA polymerase II and translation occurs in the cytoplasm. All herpesvirus genes are classified into one of three gene classes (α, β or γ) based upon the time of peak gene expression after infection and the dependence on viral DNA synthesis for expression. The α, or immediate early genes, are synthesized approximately 2-4 h post-infection. Expression of α genes does not require any prior protein synthesis, however,
expression is enhanced by the transcriptional transactivator VP16 carried into the cell as part of the virion tegument. The α gene products stimulate expression of the β, or early genes. Peak expression of the β genes occurs at approximately 4-8 h post-infection. The β gene products are involved in the replication of the viral DNA and nucleotide metabolism. Next, viral DNA replication promotes expression of the γ or late genes. In general, the γ genes encode virion structural components. The γ genes are further categorized into γ₁ (leaky-late) and γ₂ (true-late) genes, with γ₂ genes absolutely dependent on DNA replication for expression.

Replication of the Viral Genome

Seven HSV-1 proteins are required for replication of the 152 kbp genome, including: UL5, UL8, UL9, UL29, UL30, UL42 and UL52 (175). Genome replication begins with the binding of the origin binding protein, UL9, to one of three origins of replication (OriL or two copies of OriS) (78, 190, 251, 316, 348). UL9 has ATP-binding and DNA helicase activities (190). Binding of UL9 to origin sequences causes a bend in the DNA, resulting in the formation of a stem loop structure, and localized unwinding of the DNA. The ssDNA-binding protein, ICP8, binds to UL9 and facilitates the helicase activity of UL9 (27, 29, 28). Through interactions with ICP8 and/or UL9, the helicase-primase complex is recruited to the origin. The helicase-primase complex is composed of UL5, UL8 and UL52 and functions to unwind the DNA and synthesize an oligonucleotide primer (69, 204). The DNA polymerase, UL31, and its processivity factor, UL42, are recruited to the origin (97, 107, 108). The initial phase of genome replication is thought to occur by a theta mechanism (99). However, later during
replication DNA replication must switch to a rolling circle mechanism to generate head-to-tail concatamers that are cleaved and packaged into capsids (295, 363).

HSV-1 also encodes enzymes that are involved in nucleotide metabolism. These viral enzymes include: alkaline endo-exonuclease (UL12), uracil DNA-glcosylase (UL2), deoxyuridine triphosphatase (UL50), thymidine kinase (UL23) and ribonucleotide reductase (UL39 and UL40) (30). Although these enzymes are not essential for replication in cell culture, they may play an essential role for DNA replication in resting cells such as neurons (282). The broad substrate specificity of the viral thymidine kinase is the basis of the primary therapeutic agent, acyclovir (or acyclovir derivatives), used to treat herpesviral infections. Thymidine kinase phosphorylates acyclovir to generate acyclovir diphosphate, which is then phosphorylated by cellular kinases to produce acyclovir triphosphate (30). The DNA polymerase binds acyclovir triphosphate, resulting in the inhibition of viral replication through chain termination and inactivation of the DNA polymerase (270).

**VIRION ASSEMBLY AND EGRESS**

**Capsid Structure and Assembly**

Herpesviruses package their genome into preformed capsids. Each capsid displays T=16 symmetry and consists of 162 capsomers, including 12 pentons and 150 hexons. The outer capsid shell is made up of 5 different viral proteins: VP5 (UL19), VP26 (UL35), UL6, VP23 (UL18) and VP19c (UL38). During capsid assembly, scaffold proteins, VP22a (UL26.5) and VP21 (UL26) and scaffold protease, VP24 (UL26) are located on the interior of the capsid. The pentameric and hexameric capsomers are
composed of 5 and 6 molecules, respectively, of the major capsid protein, VP5 (Fig. 2.2). The pentons are located at the capsid vertices. The hexons form the faces and edges of the capsid and are decorated with six molecules of VP26 in a continuous ring around the top (368, 367). The VP5 capsomers are linked by triplex structures consisting of 2 molecules of VP23 and one molecule of VP19c. A dodecameric ring of UL6 forms a portal complex at a unique pentameric vertex, through which the viral DNA is inserted after assembly of the capsid (240). Although UL6 is not required for capsid formation, it is necessary for genome packaging (165, 257, 301, 302). Capsid assembly requires the major capsid protein, VP5, the triplex proteins, VP23 and VP19c, and a scaffold protein (VP22a or VP21) encoded by U_{L}26.5 or U_{L}26 (282).

Scaffold proteins and the maturational protease, encoded by the U_{L}26.5 and U_{L}26 genes, play important roles during capsid formation and maturation (Fig. 2.3). The product of the U_{L}26 ORF functions as the maturational protease and cleaves itself at two sites to create VP24 (the protease), VP21 (a scaffold protein) and a C-terminal 25 amino acid peptide (179, 346). The product of the U_{L}26.5 ORF overlaps in frame with the U_{L}26 gene products, such that the C-terminal 329 amino acids of the major scaffold protein, VP22a, are identical to the C-terminal 329 amino acids of the protease (Fig. 2.3) (268). The protease also cleaves the C-terminal 25 amino acids of preVP22a to create the mature form of the major scaffold protein, VP22a (268).

Capsid assembly occurs in the nucleus of the infected cell; however, synthesis of viral proteins occurs in the cytoplasm. Therefore, capsid proteins must localize to the nucleus by way of an inherent nuclear localization signal (NLS) or through interaction with a protein containing an NLS. VP5, VP26, and VP23 are transported to the nucleus
Figure 2.2. **Surface Structure of an HSV-1 Capsid.** Electron cryomicroscopy and 3D reconstruction depicts the surface structure of HSV-1 B capsids. Herpesvirus capsids contain 12 pentons (red), 150 hexons (blue), and 320 triplexes (green). Pentons and hexons are composed of 5 or 6 molecules of VP5, respectively. Triplexes consist of one molecule of VP19c and two molecules of VP23. Adapted with permission from Zhou et al. *Science.* 2000 (367).
Figure 2.3. Processing of the HSV-1 Protease and Scaffold Proteins. The scaffold protease is encoded by UL26. The protease cleaves itself to create VP24 and uncleaved VP21. The protease also cleaves the C-terminal 25 amino acids of VP21. A downstream methionine, corresponding to amino acid 307 of UL26, is the first amino acid of the UL26.5 encoded preVP22a. The C-terminal 25 amino acids of preVP22a are cleaved by the protease, to yield the scaffold protein VP22a.
through interactions with NLS-containing proteins. In contrast, VP19c, preVP22a and UL6 localize to the nucleus in the absence of other viral proteins (256, 257). Two molecules of VP23 interact with a molecule of VP19c in the cytoplasm, then the triplex complexes localize to the nucleus through an NLS within VP19c (238, 275). The major capsid protein, VP5, localizes to the nucleus through interactions with triplex complexes or the uncleaved scaffolding protein, preVP22a (242, 275). In addition, VP26 is carried into the nucleus through its interaction with VP5 (61, 275).

Understanding of the capsid assembly process has been greatly enhanced by analysis of capsid formation in insect cells infected with baculoviruses expressing capsid and scaffold proteins (239, 321, 325). Capsid formation begins with the formation of VP5-preVP22a complexes that are mediated by the self-interaction of preVP22a. The VP23-VP19c triplex structures assemble around the VP5 capsomers. The triplexes link the capsomers together to form partial capsids that come together to form enclosed spherical procapsids (325). Incorporation of the UL6 portal complex into capsids occurs through interaction of UL6 with VP22a (241). The presence of portal complexes is thought to initiate capsid assembly (127, 236, 359). If UL6 is not present at the beginning steps of capsid assembly it fails to become incorporated (236). Capsid assembly can occur in the absence of UL6, suggesting that partial capsids can come together to form enclosed procapsids (239, 321, 325, 347).

The capsid is first assembled as a spherical procapsid (Fig. 2.4). The procapsid matures into an angular capsid quickly after assembly, undergoing structural and compositional changes (238, 332). Capsid assembly can occur in the absence of the scaffold protease, VP24, but protease activity is required for maturation of the capsid and
Figure 2.4. Capsid Maturation Pathway. During HSV-1 procapsid maturation, the viral protease (VP24) is released by autoproteolysis of UL26 and the protease processes the carboxy termini of the scaffolding proteins. The shape of the capsid then switches from round to polyhedral in shape and UL35 (VP26) binds to the outer tips of VP5 hexamers. Three types of icosahedral capsid species are observed in infected cells: A capsids, B capsids and C capsids. A capsids lack DNA and scaffolding proteins. B capsids contain scaffolding proteins but lack DNA. C capsids contain the viral genome. Adapted with permission from Trus et al. *Molecular Cell.* 2007 (334).
DNA packaging (98, 267, 279). Because of their transient nature and structural
instability, procapsids are difficult to isolate and are rarely captured in electron
micrographs (238). Prior to maturation, the VP24 protease cleaves the C-terminus of
preVP22a, thus abolishing the interaction of VP22a with VP5, allowing for exit of the
scaffold protein from the procapsid (98, 179, 267, 268). As part of the capsid maturation,
the capsid shape changes from spherical to polyhedral as the hexamers are remodeled
(124). The remodeling of the hexamers creates a continuous floor layer, exposing sites
on the hexameric tips for binding of VP26 (33, 368). The polyhedral capsid species are
much more stable than procapsids, with the VP5 subunits more tightly clustered in the
capsomers of angular capsids, creating closed capsid pores (238, 332).

Three different species of icosahedrally shaped capsids, designated A, B and C
capsids, can be isolated from nuclei of infected cells. All three angular capsid species
originate from a procapsid precursor (238). A, B and C capsids are distinguishable in
electron micrographs and can be separated from one another by sucrose gradient
fractionation due to their unique densities resulting from differences in protein and DNA
contents. C capsids contain the viral genome, exit the nucleus, and undergo final
tegumentation and envelopment. A and B capsids lack a viral genome and are considered
to be defective products of failed DNA packaging. B capsids contain the cleaved forms
of the viral scaffolding proteins, VP22a and VP21 and the scaffold protease, VP24. B
capsids are likely the result of unsuccessful initiation of DNA packaging. A capsids do
not contain the scaffolding proteins and are thought to arise due to failed attempts to
complete packaging of the viral genome (282).
Genome Packaging

Packaging of the herpesvirus genome involves cleavage of the concatameric DNA into unit length monomers and packaging of the genome into the capsid through the UL6 portal complex. Seven viral proteins are required for encapsidation of the viral DNA including: UL6, UL15, UL17, UL25, UL28, UL32 and UL33 (2, 9, 125, 166, 197, 257, 287, 288, 322, 323, 324). Many of the specific mechanisms of genome encapsidation remain to be defined (282). Packaging and cleavage of the viral DNA occur concomitantly. Signals for cleavage and packaging lie within the \( a \) sequences of the genome (337). Packaging elements within the \( a \) sequences have been mapped and designated \( pac1 \) and \( pac2 \) (58). Furthermore, cleavage sites have been identified within the DR1 sequences of the \( a \) sequences (220). As mentioned previously, the viral DNA is inserted into the capsid through the dodecameric UL6 portal complex located at a unique capsid vertex (240). As a monomer of genomic DNA is packaged into capsids, it is cleaved by the viral terminase complex composed of UL15, UL28 and UL33 (24, 351). Phenotypes of mutant viruses suggest that UL17 and UL32 are involved in targeting capsids to replication compartments, the site of DNA packaging (166, 288, 323). UL25 is thought to function as a plug for the portal complex, thus sealing the channel and preventing premature release of the viral DNA (205, 248).

Genome packaging and removal of the internal scaffold structure appear to be coordinated events (53, 267, 277). The entry of DNA into the procapsid is thought to decrease pH. The drop in pH abrogates the self-interaction of the scaffold and contributes to the exit of scaffold proteins through capsid channels (196). Scaffold
proteins are expelled from the capsid only under conditions that promote DNA packaging (57).

**Nuclear Egress of the Capsid**

Capsid maturation and genome packaging have been proposed to occur in nuclear structures, termed assemblons, located adjacent to nuclear matrix-associated ND10 sites (342). Herpesvirus capsids utilize actin for transport from the site of genome encapsidation to the inner nuclear membrane (INM) (79). Current evidence suggests that capsids exit the nucleus through an envelopment-deenvelopment model. In this process capsids gain a primary envelope by budding into the INM, and subsequently lose the primary envelope as they are translocated into the cytoplasm by fusing with the outer nuclear membrane (ONM).

Two virus-encoded proteins, UL31 and UL34, are structurally and functionally conserved among herpesviruses and are required for budding of capsids at the inner nuclear membrane (92, 148, 149, 272, 273, 274). UL34 and its homologues are predicted transmembrane proteins, and interact with UL31, enabling proper localization of both proteins at the inner nuclear membrane (92, 104, 185, 164, 272). At the nuclear membrane UL31 and UL34 bind nuclear lamins A/C or B and are required for the recruitment of cellular protein kinase C (PKC) (104, 229, 253, 272). PKC phosphorylates intranuclear lamins A/C and/or B, resulting in the local dissolution of the nuclear lamin network and underlying chromatin layer (229, 253). The US3 viral kinase also plays a role in phosphorylation of nuclear lamins A/C and/or B, as well as in the localization of the UL31-UL34 complex and recruitment of PKC to the nuclear rim (227,
The restructuring of the nuclear lamina by viral and cellular proteins gives capsids intimate access to the inner nuclear membrane, which is required for capsid egress from the nucleus. Interestingly, UL31 and UL34 are necessary and sufficient for the budding and fusion process at the nuclear membranes. Expression of UL31 and UL34 in stably transfected cells results in the formation of vesicles from the inner nuclear membrane which resemble primary envelopes (146).

The capsid associated protein UL25 is also conserved and necessary for primary envelopment (147, 159). In the absence of UL25, the budding process at the inner nuclear membrane does not occur (147, 159). These results suggest that capsid-associated UL25 may link the capsid with the inner nuclear membrane. Several viral proteins appear to be necessary for fusion of the primary enveloped capsid with the outer nuclear membrane. In the absence of US3, or gB and gH, primary enveloped capsids accumulate in the perinuclear space (77, 147, 274, 339). gB and gH appear to carry out similar fusogenic functions during fusion of the viral envelope with the plasma membrane during entry and fusion of perinuclear virions with the outer nuclear membrane during egress from the nucleus (356).

When the nuclear matrix has been properly disassembled and the capsid comes into contact with the nuclear membrane, capsids acquire a primary envelope by budding into the inner nuclear membrane. UL31 and UL34 are components of primary enveloped virus particles (often referred to as primary enveloped virions) located in the perinuclear space (92, 272). Several glycoproteins localize to the INM and have been reported to be present in primary enveloped capsids including gB, gC, gD and gM (15, 133, 331). UL31, UL34 and the above mentioned glycoproteins dissociate from the virus particle
when the primary enveloped virion fuses with the ONM, loses its primary envelope and is deposited into the cytoplasm. Many studies (including work presented in this dissertation) have investigated the association of tegument proteins with intranuclear and perinuclear capsids. The incorporation of tegument proteins into virus particles located in the nucleus and cytoplasm will be discussed in subsequent sections of this literature review.

**Tegumentation in the Nucleus**

After the capsid assembles in the nucleus, viral proteins interact with the capsid for purposes of DNA packaging, capsid transport and virion incorporation. Several viral proteins associate with the capsid in the nucleus. Because some of these proteins interact directly with the capsid and are added at an early point during assembly, it is debatable whether these proteins should be considered capsid proteins or tegument proteins. For the purpose of this dissertation, a protein is considered to be a capsid protein if it is incorporated into the virion during capsid assembly. According to this definition, capsid proteins include: VP5, VP19c, VP23, VP24, VP26, VP21, VP22a and UL6. Viral envelope proteins include the glycoproteins embedded in the viral envelope. In this dissertation, all other proteins that are packaged into extracellular virions, and not designated as part of the capsid or envelope, are considered tegument proteins. It is important to note, however, that throughout the literature many tegument proteins are not formally designated as part of the tegument, but instead are labeled according to their function or localization, with names such as packaging proteins, capsid-associated proteins, etc.
Over 25 virus-encoded tegument proteins are packaged into herpesvirus virions in a process known as tegumentation. The specific site in the virus infected cell where each of these proteins associates with, and becomes incorporated into, virions is largely unclear. Tegument proteins are thought to be incorporated into the maturing capsid in four possible locations: within the nucleus, at the inner nuclear membrane during primary envelopment, as the capsid is transported in the cytoplasm, and finally, as the capsid undergoes final envelopment at vesicles derived from the TGN. The suggested early events of tegumentation, those occurring within the nucleus, have been controversial. Data presented in this thesis provide evidence that the tegument proteins UL36 and UL37 are among the first subset of tegument proteins to associate with the capsid and are incorporated in the nucleus. This section of the literature review presents a summary of tegument proteins reported to associate with capsids in the nucleus or as the capsid leaves the nucleus through budding and fusion.

Although they are not historically referred to as tegument proteins, proteins involved in DNA packaging, UL25 and UL17, by definition, are tegument proteins incorporated into the virus particle in the nucleus (334). Trus and colleagues used cryoelectron microscopy to analyze capsids isolated from the nuclear fraction. They observed a C-capsid-specific component (CCSC) on the capsid surface that is bound to triplexes located closest to pentons (334). The CCSC is present on C capsids but not on A or B capsids (334). Zhou and colleagues also observed tegument structures located on penton vertices and speculated their identity may be UL36 (366). Trus and colleagues analyzed the protein content of capsids by SDS-PAGE and Coomassie blue staining and observed protein bands unique to C capsids. The CCSC bands were identified by mass
spectrometry as a heterodimer of UL25 and UL17 (334). The CCSC is present at about 30 copies per C capsid (334). UL25 is known to play a role in stabilization of the DNA-filled capsid and egress of the capsid from the nucleus (147, 159, 237). Interestingly, fibers connecting the C capsid to the INM were seen by electron microscopy most frequently emanating from pentons (10). The UL17/UL25 complex may play a role in connecting the capsid to the INM. Furthermore, the CCSC may represent an initial layer of tegument and act as a signal for the DNA-filled capsid to exit the nucleus (147, 237).

The large tegument protein, UL36, binds the major capsid protein, VP5, and interacts tightly with the capsid in detergent extraction assays (103, 207, 313). In addition, UL36 binds the capsid-associated tegument protein UL25 (54, 254). It is widely accepted that UL36 is present in the deepest layers of the tegument, interacting intimately with the capsid (103, 313, 366). Cryoelectron microscopy by Zhou and colleagues also suggests that a large tegument protein, presumably UL36, interacts directly with VP5 at the capsid vertices (366). Data presented in this dissertation show the presence of UL36 and its binding partner, UL37, on capsids isolated from the nuclear fraction (Fig. 3.3). Interestingly, UL36 was present on C capsids, but was not detected on A and B capsids. The absence of UL36 or UL37 during virus assembly blocks tegumentation and envelopment of capsids (63, 64, 91, 150, 174, 278). Various assays including coimmunoprecipitation and yeast two-hybrid have shown that UL36 and UL37, and their homologues, interact with several capsid proteins (Tables 2.3 and 2.4) (25, 81, 171, 207, 285, 336). These interactions likely facilitate their association with the capsid and will be discussed in further detail in subsequent sections of this literature review.
UL36 and UL37 appear to represent an inner layer of tegument, which is added in the nucleus, and acts as a scaffold for addition of other tegument proteins.

The association of UL36 and UL37 with nuclear capsids is a controversial topic. Trus and colleagues isolated capsids from the nuclear fraction and analyzed them by SDS-PAGE and Coomassie blue staining and did not observe protein bands corresponding to the molecular weight of UL36 or UL37 (334). The authors estimated that there is less than one molecule of UL36 on nuclear C capsids (334). The reason for contrasting findings regarding the association of UL36 and UL37 with nuclear capsids is unclear but will be analyzed in further detail in the discussion section of this dissertation.

The product of the UL41 gene, vhs, is a minor component of the virion tegument and appears to be incorporated into the virion within the nucleus (269). Interestingly, two forms of vhs, which differ in the extent of phosphorylation, are present in the nucleus and are associated with nuclear B and C capsids (269). However, only the hypophosphorylated vhs species is present in virions, suggesting that the hyperphosphorylated species dissociates from the capsid before final envelopment (269). Additional quantities of vhs are also incorporated into the virion at later steps during assembly (269). vhs interacts with VP16, another tegument protein that associates with capsids early during assembly (the capsid-association of VP16 is discussed below) (309). Another tegument protein, UL21, has also been suggested to associate with nuclear capsids (320). However, the sedimentation profile of UL21 in the published sucrose gradients is spread among several fractions and fails to peak in capsid fractions, therefore it is difficult to interpret if UL21 is bound to nuclear capsids or sedimenting as part of
aggregate structures. The protein interactions that may mediate the association of UL21 with nuclear capsids are currently unknown.

Another step where tegument proteins may incorporate into the maturing virion is as the capsid exits the nucleus. It is possible that tegument proteins may localize to the inner nuclear membrane, interact with the capsid as it undergoes primary envelopment and become incorporated into the virion during this budding event. Perinuclear primary enveloped capsids quickly undergo fusion with the outer nuclear membrane and are difficult to isolate. Immunoelectron microscopy studies have been useful for revealing the presence of tegument proteins on capsids located between the INM and ONM. These studies provide insight into which proteins associate with capsids at an early time during virus assembly, however, they do not determine whether the tegument proteins are added before or during primary envelopment.

Immunoelectron microscopy studies have shown that the viral kinase, US3, is associated with perinuclear capsids (109, 274). Another tegument protein, VP16, was not detected on capsids isolated from the nuclear fraction (269, 360). However, VP16 was associated with capsids located in the perinuclear space when analyzed by immunoelectron microscopy (232). These results suggest that VP16 associates with the capsid as it undergoes primary envelopment. UL36 interacts with VP16 (81, 171, 338), possibly facilitating the association of VP16 with nuclear capsids. Wilson and colleagues recently developed a method to isolate and purify capsids from the perinuclear space (252). Mass spectrometry of isolated perinuclear capsids revealed the presence of the major tegument component, VP22 (252). The interaction of VP22 with capsids may be mediated by its binding partner, VP16 (74, 232).
It should be noted that other studies failed to detect some of the tegument proteins mentioned above in association with perinuclear capsids. Immunoelectron microscopy studies failed to detect VP16 on perinuclear capsids (109) and another study reported weak to moderate labeling of capsids with VP16 antisera (219). Similarly, VP22 was not detected with perinuclear capsids (109, 232) and showed weak antibody labeling in another immunoelectron microscopy study (219). Furthermore, UL36 and UL37 did not appear to be associated with perinuclear capsids when assayed by immunoelectron microscopy (109, 145, 221). The contrasting results may be explained by differences in affinity of antisera used and/or differences in the assembly of PRV and HSV-1. Finally, it is also difficult to determine if tegument proteins that interact with capsids in the nucleus or during primary envelopment remain associated with the capsid during and after fusion with the ONM, or if they dissociate from the capsid in a similar fashion to UL31 and UL34.

**Tegumentation in the Cytoplasm**

The majority of the tegument is thought to become incorporated by associating with capsids located in the cytoplasm. There are two possible mechanisms of tegumentation of cytoplasmic capsids: 1) tegumentation that occurs as the capsid traverses the cytoplasm through microtubule-mediated transport and 2) tegumentation of the capsid during final envelopment by budding into TGN-derived vesicles. The inner tegument is thought to consist of the tegument proteins that interact most proximally with the capsid and are likely incorporated early during assembly. The inner tegument not only acts as a scaffold to drive the tegumentation process, but also aids in transport of the
capsid to TGN-derived vesicles and ultimately leads the capsid to the budding site. The outer tegument is made up of tegument proteins that are generally not strongly associated with the capsid after packaging. Protein interactions between the inner tegument and outer tegument link the capsid to the site of final tegumentation and envelopment (Fig. 2.5). Outer tegument proteins (and possibly all tegument proteins) bind TGN-derived vesicles directly or indirectly to enable their incorporation during the final viral budding event. Viral glycoproteins and membrane-bound tegument proteins also play vital roles in cytoplasmic tegumentation and envelopment. Glycoproteins and membrane-associated tegument proteins function to bind tegument proteins, thus facilitating their incorporation during virus envelopment at the TGN. Virion assembly culminates as the capsid, decorated with inner tegument previously accumulated in the nucleus and during transport to the TGN, buds into TGN vesicles containing an accumulation of tegument and glycoproteins to acquire its final subset of tegument proteins and lipid envelope.

Upon release into the cytoplasm the capsid utilizes microtubules for transport to the site of final envelopment (70, 187, 218). During virus entry capsids utilize dynein, a minus-end-directed microtubule motor for transport to the nucleus (172). In contrast, during cytoplasmic egress capsids are shuttled along microtubules via the plus-end-directed motor protein, kinesin (66). Biochemical analysis reveals that outer tegument proteins are removed upon treatment of virions with detergent and high salt conditions, resulting in exposure of inner tegument proteins including UL36, UL37, US3 and VP16 (109, 231, 274, 293, 355, 364). The inner tegument proteins appear to facilitate microtubule-mediated transport in the cytoplasm (6, 71, 187, 355). The roles of UL36 and UL37 in microtubule-based transport will be discussed in greater detail in subsequent
Secondary envelopment of HSV-1 capsids is facilitated by interactions between tegument proteins bound to cytoplasmic capsids and tegument proteins associated with TGN-derived membranes. Several tegument proteins are associated with capsids located in the nucleus, in the perinuclear space and in the cytoplasm. Interactions between capsid-associated tegument proteins and membrane-associated tegument proteins have been reported and are represented by black lines. These interactions likely drive secondary envelopment.
sections of this review.

The HSV-1 tegument proteins VP16, UL37 and UL36 are essential for assembly of infectious virions (64, 63, 174, 226, 278, 345). In the absence of any one of these essential tegument proteins, capsids accumulate in the cytoplasm and do not undergo final envelopment. Populations of VP16, UL37 and UL36 are associated with capsids in the nucleus (41, 232). Together, these results suggest that populations of VP16, UL37 and UL36 are added early during virus assembly and play key roles in the progression of cytoplasmic tegumentation and envelopment. Protein interactions and incorporation of UL36 and UL37 will be discussed in detail in subsequent sections of this review.

Detergent and salt treatments of purified virions suggest that VP16 is a component of both the inner and outer tegument layers (231, 293, 364). As such, VP16 participates in protein interactions with capsid-associated proteins, tegument proteins localized to the TGN and membrane-bound glycoproteins. The population of VP16 that is part of the inner tegument likely remains capsid-bound through its interaction with UL36 (81, 171, 338). Immunoelectron microscopy studies comparing the amounts of VP16 found on perinuclear capsids and extracellular virions provide evidence that additional copies of VP16 are incorporated into the virus in the cytoplasm (218, 232). Several protein interactions contribute to viral incorporation of VP16 in the cytoplasm. VP16 binds UL46 (VP11/12), an abundant tegument protein that is tightly associated with the capsid following detergent solubilization (138, 231, 338, 364). UL46 localizes to membranes of uninfected or virus-infected cells and is likely incorporated at TGN vesicles (231). VP16 also interacts with vhs, UL47 and VP22 (74, 247, 309, 338). A number of studies have identified interactions of VP16 with cytoplasmic tails of
glycoproteins gB, gD and gH (115, 137, 370). Through the interactions with membrane-associated vhs, UL46, VP22, gB, gD and gH, it is likely that a considerable amount of VP16 is incorporated during final envelopment.

The majority of the abundant tegument protein, VP22, is thought to be added to capsids during final envelopment at TGN-derived vesicles. VP22 associates with membranes in the infected cell and also in the absence of other viral proteins (39). VP22 interacts with the cytoplasmic tails of glycoproteins gE, gD and gM and the envelope protein US9 (51, 76, 89, 171, 246). The multiple interactions that facilitate the incorporation of VP22 are an example of many herpesvirus protein interactions that function redundantly. Although VP22 interacts with several viral proteins located at TGN membranes, the interactions are not essential for virus envelopment. The gene encoding HSV-1 VP22, UL49, is not essential for virus assembly (73, 265). In a similar fashion, a mutant virus containing simultaneous deletions of the genes encoding the major PRV tegument proteins UL46, UL47, VP22 and VP16 is viable (88). This mutant virus is another example of the redundancy of tegument interactions in virus assembly.

UL11 is a membrane-associated tegument protein that appears to play an important role in secondary envelopment. HSV-1, PRV and HCMV containing deletions of the UL11 homologue clearly display a defect in cytoplasmic virion assembly (13, 96, 153, 173, 182, 286, 294, 305). HSV-1 UL11 is considered a nonessential tegument protein, with viruses containing partial or complete deletions of UL11 producing 5-250-fold lower viral titers (13, 96, 173). UL11 binds directly to membranes via myristoyl and palmitoyl lipid side chains and is also found in lipid rafts (16, 155, 184). UL16 binds to UL11 and UL21, forming a conserved tripartite complex (144, 183, 338, 362). UL16,
UL11 and UL21 are likely incorporated during secondary envelopment, although studies suggest that populations of UL16 may also be incorporated before secondary envelopment after the capsid escapes the nucleus (209). The tripartite complex of UL16, UL11 and UL21 also plays a role in the recruitment of other tegument proteins to TGN vesicles. Deletion of UL11, UL16 or UL21 decreases the incorporation of various tegument proteins including UL36, UL46, UL49 and US3 (214, 215). Deletion of UL16 and UL21 does not block assembly of virus particles (12, 11). Additionally, UL11 interacts with the envelope protein UL56, and glycoproteins gD and gE (76, 154). In addition to VP22, UL46 and UL11, tegument proteins vhs and UL51 have also been shown to associate with membranes in the absence of other viral proteins (39, 169, 182, 228, 245).

UL46 is a membrane-associated tegument protein that is incorporated into virions in relatively abundant amounts (231, 364). UL46 associates with TGN membranes and is likely incorporated into virions during secondary envelopment. After packaging into the virion, UL46 remains tightly associated with capsids following detergent treatment of virions (231). Interactions of UL46 with a number of capsid and tegument proteins likely facilitate the tight association of UL46 with the capsid. Yeast two-hybrid analysis has indicated that UL46 interacts with the major capsid protein, VP5 and the triplex proteins, VP23 and VP19c (171). Furthermore, yeast two-hybrid studies also indicated that UL46 interacts with several tegument proteins including UL17, UL25, UL37, VP22, US3 and US10 (171, 338). UL46 also interacts with VP16 and enhances VP16-mediated transcription of IE genes (171, 199, 338). Membrane-bound UL46 may interact with capsid proteins and capsid-bound tegument proteins to promote the final budding event.
UL46 is not required for virus assembly (20). In the absence of UL46 there is a 2-fold increase in the amount of UL47 incorporated into virions (20). Similarly, in the absence of UL47 there is a 2.5-fold increase in the copy number of UL46 in virions. These observations suggest that UL46 or UL47 is not required for virus envelopment, but UL46 and UL47 may serve similar roles during assembly. Furthermore, simultaneous deletion of HSV-1 UL46 and UL47 does not significantly decrease virus production (364).

Noninfectious virus particles containing tegument and envelope proteins, but lacking a capsid, bud from TGN-derived vesicles and are secreted from infected cells. These noninfectious particles are commonly called light particles (L particles) due to their lower density than virions (4, 130, 201, 203, 276, 317). L particles form independently of virion assembly and contain a tegument and envelope protein composition and content similar to virions (201, 203, 276, 318). The formation of L particles suggests that tegument and envelope proteins are sufficient to induce the budding and fusion events necessary for final envelopment. The similar tegument protein composition and content of L particles and virions suggests that most (and possibly all) tegument proteins associate with TGN-derived membranes, either directly or indirectly, and are incorporated into virions during the final budding event. UL36 and UL37 are contained in L particles, suggesting that these proteins are added to the assembling virion in the cytoplasm as well as in the nucleus (278, 318).

Secondary Envelopment

As previously mentioned, at least 12 viral glycoproteins are embedded in TGN-derived vesicles. The cytoplasmic tails of membrane glycoproteins interact with
tegument proteins to facilitate tegument accumulation and incorporation at the TGN. In addition, several glycoproteins bind other glycoproteins and localize in membranes as a complex. During assembly viral glycoproteins serve to bridge the capsid and envelope and thus facilitate budding at the TGN. Glycoproteins gD, gB, gH and gL are necessary for virus infection due to roles during virus entry (42, 80, 178, 284). However, HSV-1 mutant viruses lacking individual glycoproteins gD, gB, gH or gL display no obvious defects in envelopment (42, 80, 178, 284). Furthermore, HSV-1 mutants lacking nonessential glycoproteins, including gE, gG, gI, gJ or gM, do not display any major defects in envelopment (12, 18, 68, 181, 284). The importance of several glycoprotein functions during envelopment has been highlighted by studies of mutant viruses containing deletions of two or more viral glycoproteins. Collectively, these studies suggest that several glycoproteins may serve redundant, but essential, functions during assembly, in a similar fashion to the redundant roles and interactions reported for several tegument proteins.

Glycoproteins gD and gE interact with membrane bound tegument proteins VP22 and UL11 (51, 76, 89, 171, 246). In the absence of either gD or gE tegumentation and envelopment proceed. However, a mutant virus lacking both gD and gE produces tegument-covered capsids that accumulate in the cytoplasm but do not undergo envelopment (75). These results suggest that gD and gE carry out similar functions during assembly to facilitate envelopment.

A PRV virus mutant lacking gM and gE is abrogated in secondary envelopment (38). However, an HSV-1 mutant containing deletions of gM and gE genes shows no significant impairment in envelopment (40). VP22 homologues bind HSV-1 gD and
PRV gM. In addition, the HSV-1 gD/gE deletion mutant, like the PRV gM/gE mutant, is abrogated in secondary envelopment. Together, these observations suggest that PRV gM and HSV-1 gD may perform similar functions during virus envelopment. Furthermore, simultaneous deletion of the PRV gM and UL11 genes results in large intracytoplasmic accumulations of tegument containing capsids that do not undergo envelopment (152). Simultaneous deletion of gM and UL11 in an HSV-1 mutant virus also has a similar effect (173). The phenotype of this mutant virus also demonstrates the importance of gM during HSV-1 secondary envelopment.

In addition to gE, gD and gM, other glycoproteins play important roles in envelopment and egress of HSV-1 virus particles. gE and gI interact to form heterodimers (134). Deletion of the genes encoding gE and gI is tolerated, however, the mutant virus is defective in cell-to-cell spread (67). Glycoproteins UL20 and gK are essential for envelopment and release of virions (14, 84, 90, 129). gK and UL20 interact for proper intracellular transport and localization to the TGN (82, 83, 86). Deletion of the UL20 gene results in an accumulation of unenveloped capsids in the cytoplasm (85). Furthermore, gK is required for transport of enveloped virions from the cytoplasm to the extracellular space (129, 132). Analysis of HSV-1 viruses expressing mutant UL20 proteins showed that UL20 carries out a function during envelopment that is independent of its role in localization of gK to membranes (211).

Several glycoproteins are complexed with other glycoproteins within membranes. HSV-1 glycoproteins gH and gL form a heterodimeric complex (128, 260, 284). Similarly, gM forms a complex with gN (135). As mentioned above, PRV gM appears to
play an important role during secondary envelopment as indicated by impairment of mutant viruses containing deletions of gM and gE or gM and UL11 (38, 152).

In addition to viral proteins, recent studies have identified several cellular proteins involved in cytoplasmic envelopment of herpesvirus particles. Expression of a dominant-negative mutant of Vps4 has shown that this cellular enzyme is required for cytoplasmic envelopment of HSV-1 virions (56). Vps4 is essential for the formation of cellular multivesicular bodies (MVB) (8). Vps4 functions to disassemble and recycle the endosomal sorting complexes required for transport (ESCRT) machinery (7). In addition, Vps24/CHMP3, a component of the ESCRT-III complex, is also critical for HSV-1 envelopment and release (45). Expression of a dominant-negative ESCRT-III complex protein, CHMP1A, inhibits virion formation (258). Viruses are known to recruit ESCRT proteins from MVBs to other virus budding compartments (195). Furthermore, ESCRT-III proteins and Vps4 are present in purified virions (258). These results suggest that herpesviruses utilize the budding machinery of the ESCRT pathway to accomplish secondary envelopment at vesicles that are likely derived from the TGN.

Tegument-coated capsids acquire their final envelope by budding into a platform of glycoprotein-tegument complexes on membranes. The site of cytoplasmic envelopment is thought to be vesicles derived from the TGN. Studies also suggest that endosomes may play a role in secondary envelopment (335). Following successful secondary envelopment, virions are released from the cell in a manner similar to exocytosis, by fusion of the virion-containing vesicle with the plasma membrane. A summary of proteins contained in HSV-1 virions is shown in Table 2.2.
Dynamic Interactions within the Tegument

As described above, tegument assembly is a complex process involving a myriad of protein interactions resulting in packaging of over 25 viral and cellular proteins into the virion. Recent studies have revealed that structural changes occur in the tegument during and after complete assembly of the virion. Studies of the abundant tegument protein, UL46, suggest that the protein changes from a membrane-associated protein during envelopment to a capsid-proximal protein within the virion (231). Furthermore, recent studies suggest that a population of UL46 is cleaved during or after virion assembly, resulting in incorporation of a truncated form of the protein (230).

As another example of dynamic interactions of the tegument, the ability of UL16 to remain capsid-associated appears to decrease during assembly of the virion. UL16 remains capsid-associated following detergent treatment of cytoplasmic capsids, but not extracellular virions (209). The mechanism for the positional change appears to depend on modification of cysteines in UL16 (209). Furthermore, Meckes and Wills extended these studies to show that the natural mechanism for release of UL16 from the capsid is triggered by virion binding to an immobilized heparan receptor (210). These studies suggest that binding to the cellular receptor induces signaling across the viral membrane, resulting in structural changes in the tegument (210).

Recent electron microscopy studies highlight dramatic differences in the tegument architecture of extracellular and cell-associated virions. In electron micrographs
### Table 2.2. HSV-1 Virion Proteins and Functions

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Capsid</strong></td>
<td></td>
</tr>
<tr>
<td>UL6</td>
<td>Capsid portal protein</td>
</tr>
<tr>
<td>UL18 (VP23)</td>
<td>Triplex protein</td>
</tr>
<tr>
<td>UL19 (VP5)</td>
<td>Major capsid protein</td>
</tr>
<tr>
<td>UL26 (VP24)</td>
<td>Scaffold protease</td>
</tr>
<tr>
<td>UL35 (VP26)</td>
<td>Binds tips of hexons</td>
</tr>
<tr>
<td>UL38 (VP18c)</td>
<td>Triplex protein</td>
</tr>
<tr>
<td>UL7</td>
<td>Regulates mitochondrial function</td>
</tr>
<tr>
<td>UL11</td>
<td>Secondary envelopment</td>
</tr>
<tr>
<td>UL13 (PK)</td>
<td>Protein kinase, tegument dissociation</td>
</tr>
<tr>
<td>UL14</td>
<td>Nuclear import, nuclear targeting of capsids, cell to cell spread</td>
</tr>
<tr>
<td>UL15</td>
<td>ATPase subunit of terminase</td>
</tr>
<tr>
<td>UL16</td>
<td>Secondary envelopment</td>
</tr>
<tr>
<td>UL17</td>
<td>DNA cleavage and capsid localization within the nucleus</td>
</tr>
<tr>
<td>UL21</td>
<td>Secondary envelopment</td>
</tr>
<tr>
<td>UL23</td>
<td>Thymidine kinase, viral DNA replication</td>
</tr>
<tr>
<td>UL25</td>
<td>Caps the DNA portal after genome packaging, binds to nuclear pores</td>
</tr>
<tr>
<td>UL28</td>
<td>Subunit of terminase</td>
</tr>
<tr>
<td>UL33</td>
<td>Subunit of terminase</td>
</tr>
<tr>
<td>UL36 (VP1/2)</td>
<td>Capsid transport, secondary envelopment, viral DNA release, deubiquitination</td>
</tr>
<tr>
<td>UL37</td>
<td>Secondary envelopment</td>
</tr>
<tr>
<td>UL41 (vhs)</td>
<td>Degrades mRNA</td>
</tr>
<tr>
<td>UL46 (VP11/12)</td>
<td>Modulates transactivation activity of VP16</td>
</tr>
<tr>
<td>UL47 (VP13/14)</td>
<td>Modulates transactivation activity of VP16</td>
</tr>
<tr>
<td>UL48 (VP16)</td>
<td>Secondary envelopment, transactivation of α genes</td>
</tr>
<tr>
<td>UL49 (VP22)</td>
<td>Secondary envelopment</td>
</tr>
<tr>
<td>UL50</td>
<td>dUTPase, viral DNA replication</td>
</tr>
<tr>
<td>UL51</td>
<td>unknown</td>
</tr>
<tr>
<td>UL55</td>
<td>unknown</td>
</tr>
<tr>
<td>UL56</td>
<td>unknown</td>
</tr>
<tr>
<td>US2</td>
<td>unknown</td>
</tr>
<tr>
<td>US3</td>
<td>Protein kinase, primary deenvelopment, tegument dissociation</td>
</tr>
<tr>
<td>US10</td>
<td>unknown</td>
</tr>
<tr>
<td>US11</td>
<td>Regulates host translation, capsid transport</td>
</tr>
<tr>
<td>ICP34.5</td>
<td>Regulates host translation, viral DNA replication and immune response</td>
</tr>
<tr>
<td>ICP0</td>
<td>Regulates viral transcription</td>
</tr>
<tr>
<td>ICP4</td>
<td>Required for expression of majority of β and γ genes</td>
</tr>
<tr>
<td><strong>Tegument</strong></td>
<td></td>
</tr>
<tr>
<td>UL1 (gL)</td>
<td>Fusion of membranes</td>
</tr>
<tr>
<td>UL10 (gM)</td>
<td>Interacts with gN</td>
</tr>
<tr>
<td>UL20</td>
<td>Viral egress</td>
</tr>
<tr>
<td>UL22 (gH)</td>
<td>Fusion of membranes</td>
</tr>
<tr>
<td>UL27 (gB)</td>
<td>Fusion of membranes</td>
</tr>
<tr>
<td>UL43</td>
<td>unknown</td>
</tr>
<tr>
<td>UL44 (gC)</td>
<td>Cellular attachment</td>
</tr>
<tr>
<td>UL45</td>
<td>unknown</td>
</tr>
<tr>
<td>UL49A (gN)</td>
<td>unknown, interacts with gM</td>
</tr>
<tr>
<td>UL53 (gK)</td>
<td>Viral egress</td>
</tr>
<tr>
<td>US4 (gG)</td>
<td>Viral egress</td>
</tr>
<tr>
<td>US5 (gJ)</td>
<td>unknown</td>
</tr>
<tr>
<td>US8 (gD)</td>
<td>Entry, secondary envelopment</td>
</tr>
<tr>
<td>US7 (gF)</td>
<td>Cell to Cell spread</td>
</tr>
<tr>
<td>US8 (gE)</td>
<td>Cell to Cell spread</td>
</tr>
<tr>
<td>US9</td>
<td>unknown</td>
</tr>
</tbody>
</table>

Genes essential for HSV-1 growth in cell culture are underlined.

* vhs: virion host shutdown; PK: protein kinase
Newcomb and Brown observed that the tegument is symmetrically arranged around the capsid in cell associated virions; however, tegument is asymmetrically arranged in extracellular virions (235). In other words, the overall staining of extracellular virions appears “acorn-shaped”, and the images of cell-associated virions resemble “targets” (235). In addition, the tegument of extracellular virions, but not cell-associated virions, is resistant to removal with the nonionic detergent Triton X-100 (235). These results suggest that the virion tegument undergoes a time-dependent structural transformation (235).

THE TEGUMENT PROTEIN ENCODED BY THE UL37 OPEN READING FRAME

More than 25 virus-encoded proteins are packaged into the tegument of a herpesvirus virion. The proteins encoded by the UL36 and UL37 ORFs are the only two tegument proteins that are conserved across the alpha-, beta-, and gammaherpesvirinae subfamilies and are also essential for productive HSV-1 virion assembly and release in cell culture. In recent times there has been much interest in the roles these proteins play during infection. The work discussed in this dissertation provides insight into the interaction between these proteins and their incorporation into the virus particle.

EXPRESSION AND LOCALIZATION

UL37 is a 1,123 amino acid, 120 kDa protein encoded by the UL37 ORF (300). When infected cells are treated with the DNA synthesis inhibitor phosphonoacetic acid (PAA) UL37 protein is detected in the presence of PAA but protein expression is
increased upon DNA synthesis (300). Furthermore, UL37 protein levels peak at late times during infection (3, 300). Therefore, UL37 is considered a γ1, or “leaky-late”, gene (300). The rate of UL37 protein synthesis remains stable between 6-12 h post-infection and the protein appears to be stable, with little, if any, turnover (3). Albright and Jenkins demonstrated that UL37 is phosphorylated in infected cells and the phosphorylation remains stable when analyzed 5 hours after cells were isotopically labeled at 6 and 9 hpi (3). UL37 is phosphorylated when expressed from a vaccinia virus vector, suggesting that a cellular kinase is responsible for the phosphorylation (3).

UL37 is present in the nucleus and cytoplasm of the cell, but is more abundant in the cytoplasm (200, 202, 293, 344). Immunofluorescence studies suggest that localization of UL37 to the nucleus does not depend on the presence of other HSV-1 proteins (293, 344). Recent studies utilizing confocal microscopy and recombinant viruses revealed that in the infected cell UL37 localizes to vesicles of the Golgi complex, in a manner that is dependent on the presence of the UL36 tegument protein, but not capsids (62). UL37 contains a leucine rich region (LRR), encompassing amino acids 263-273, that functions as a nuclear export signal (NES) (344). Interestingly, mutating the residues of the NES to alanines does not affect HSV-1 virus replication in cell culture (62).

Initial attempts failed to detect UL37 in purified virions (300). Subsequent analysis showed that UL37 is packaged into virions and detergent extraction studies revealed that UL37 is incorporated into the tegument region (202, 293). A similar amount of UL37 is also present in L particles (202). Compared to other tegument proteins, UL37 is considered to be a minor virion component, but the number of
molecules of UL37 that are packaged per virion is not known. However, early studies by
Heine and colleagues identified a protein with an estimated molecular weight of 130 kDa,
designated VP6a (or in later studies VP6.5), that comigrated with ICP8 on SDS-PAGE
gels and was packaged at an estimated 30-40 molecules per virion (119, 126). Schmitz
and colleagues speculated that the virion protein designated VP6a/VP6.5 may be UL37
(293). The amount of UL37 packaged into virions appears to be tightly controlled, as
increasing the cellular protein level of UL37 to very high levels does not increase the
amount of UL37 incorporated into virions or L particles (200).

**DOMAINS AND MOTIFS**

Members of the *alpha*-, *beta*- and *gammaherpesvirinae* contain UL37
homologues. The HSV-1 KOS and PRV UL37 proteins share 28% identical residues,
with conserved residues spanning the length of the proteins and unconserved regions of
approximately 100 amino acids at the amino and carboxy-termini. When analyzed by
GlobPlot 2.3, most of the unconserved amino-terminal 50 amino acids and carboxy-
terminal 100 amino acids are predicted to be disordered. The UL37 homologues of
*alpha*-, *beta*-, and *gammaherpesviruses* contain six fully conserved residues including:
L263, L414, W559, P618, F620 and L702. Members of the *alphaherpesvirinae* contain 5
regions of high conservation; residues 104-117, 262-268, 655-662, 669-676 and 903-907.
The relevance, if any, of the conserved residues and regions is unclear, with the exception
of residue 263 which is located in the conserved NES (344). Although the homologues
share similarity throughout the lengths of the proteins, VZV and PRV mutant viruses
containing deletions of UL37 homologues cannot be complemented by HSV-1 UL37,
suggesting that the UL37 homologues in different viruses have different functions or structures (174, 358). A schematic of reported domains and motifs within UL37 is shown in Figure 2.6. UL37 also contains a leucine rich region at amino acids 263-272, which functions as a nuclear export signal (344). A leucine zipper pattern, a motif present in many transcription factors that bind DNA as dimers, is located within amino acids 203-224 of HSV-1 UL37. Interestingly, UL37 is retained on single-stranded DNA columns in an ICP8 dependent manner (299). An alanine rich region encompassing amino acids 44-80 lies within the unconserved amino terminus of UL37. Throughout UL37 are 17 dileucine motifs (LL or LI) and 3 tyrosine-based motifs (YXXΦ, where Φ represents a large hydrophobic residue, M, I, L, F, V), which may possibly facilitate interactions with clathrin adaptor proteins to promote vesicular trafficking. Finally, UL37 contains a conserved TRAF6 binding domain at residues 1099-1104 that contributes to NF-κB activation during infection (180).

**INTERACTION PARTNERS OF UL37**

UL37 is a viral protein that potentially has a large network of binding partners. A summary of interactions reported for UL37 and UL37 homologues is presented in Table 2.3. In yeast two-hybrid analysis and coimmunoprecipitation assays UL37 binds to several capsid and tegument proteins. HSV-1 UL37 interacts with the capsid proteins VP26 and VP19c in yeast two-hybrid studies (171). Another yeast two-hybrid study indicates that the VZV UL37 homologue, ORF21, interacts with the VZV homologues of the capsid proteins encoded by HSV-1 UL26 (336). Furthermore, the VZV UL37 homologue also interacts with the VZV homologue of HSV-1 UL31, a component of
UL37 contains an arginine rich region (ARR) within amino acids 44-86. A leucine zipper motif pattern (L Zip) was identified at residues 203-224. A leucine rich region encompassing amino acids 263-272 functions as a nuclear export signal (LRR/NES) (344). The TRAF6 binding domain (UL37-TRAF6) is located at amino acids 1099-1104 (180). Tyrosine-based trafficking motifs (Y) in the form of YXXΦ are located at amino acids 222-225, 793-796 and 818-821.
<table>
<thead>
<tr>
<th>Interaction Partner</th>
<th>HSV-1 homologue</th>
<th>Virus</th>
<th>Structural Component</th>
<th>Assay</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>UL86</td>
<td>UL19 (VP5)</td>
<td>HCMV</td>
<td>Capsid</td>
<td>Y2H</td>
<td>Bechtel, 2002</td>
</tr>
<tr>
<td>ORF33</td>
<td>UL26</td>
<td>VZV</td>
<td>Capsid</td>
<td>Y2H</td>
<td>Uetz, 2006</td>
</tr>
<tr>
<td>ORF33.5</td>
<td>UL26.5</td>
<td>VZV</td>
<td>Capsid</td>
<td>Y2H</td>
<td>Uetz, 2006</td>
</tr>
<tr>
<td>UL38 (VP19c)</td>
<td>UL38 (VP19c)</td>
<td>HSV-1</td>
<td>Capsid</td>
<td>Y2H</td>
<td>Lee, 2008</td>
</tr>
<tr>
<td>ORF23</td>
<td>UL35 (VP26)</td>
<td>VZV</td>
<td>Capsid</td>
<td>Y2H</td>
<td>Uetz, 2006</td>
</tr>
<tr>
<td>UL35 (VP26)</td>
<td>UL35 (VP26)</td>
<td>HSV-1</td>
<td>Capsid</td>
<td>Y2H</td>
<td>Lee, 2008</td>
</tr>
<tr>
<td>ORF65</td>
<td>UL35 (VP26)</td>
<td>KSHV</td>
<td>Capsid</td>
<td>Y2H, CoIP</td>
<td>Uetz, 2006</td>
</tr>
<tr>
<td>BFRF3</td>
<td>UL35 (VP26)</td>
<td>EBV</td>
<td>Capsid</td>
<td>CoIP</td>
<td>Fossum, 2009</td>
</tr>
<tr>
<td>ORF42</td>
<td>UL15</td>
<td>VZV</td>
<td>Tegument</td>
<td>Y2H, CoIP</td>
<td>Uetz, 2006</td>
</tr>
<tr>
<td>ORF23</td>
<td>UL21</td>
<td>KSHV</td>
<td>Tegument</td>
<td>Y2H, CoIP</td>
<td>Uetz, 2006</td>
</tr>
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<td>BTRF1</td>
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<td>EBV</td>
<td>Tegument</td>
<td>CoIP</td>
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</tr>
<tr>
<td>ORF21</td>
<td>UL23</td>
<td>KSHV</td>
<td>Tegument</td>
<td>CoIP</td>
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</tr>
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<td>ORF67.5</td>
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<td>KSHV</td>
<td>Tegument</td>
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</tr>
<tr>
<td>BFRF4</td>
<td>UL33</td>
<td>EBV</td>
<td>Tegument</td>
<td>CoIP</td>
<td>Fossum, 2009</td>
</tr>
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<td>UL36</td>
<td>HSV-1</td>
<td>Tegument</td>
<td>Y2H, CoIP</td>
<td>Vittone, 2005</td>
</tr>
<tr>
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<td>UL36</td>
<td>PRV</td>
<td>Tegument</td>
<td>CoIP</td>
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</tr>
<tr>
<td>UL36</td>
<td>UL36</td>
<td>HSV-1</td>
<td>Tegument</td>
<td>Y2H</td>
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<td>Tegument</td>
<td>Y2H, CoIP</td>
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<td>Tegument</td>
<td>Y2H</td>
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</tr>
<tr>
<td>UL48</td>
<td>UL36</td>
<td>HCMV</td>
<td>Tegument</td>
<td>CoIP</td>
<td>Bechtel, 2002</td>
</tr>
<tr>
<td>BPLF1</td>
<td>UL36</td>
<td>EBV</td>
<td>Tegument</td>
<td>Y2H</td>
<td>Fossum, 2009</td>
</tr>
<tr>
<td>UL37</td>
<td>UL37</td>
<td>HSV-1</td>
<td>Tegument</td>
<td>Y2H</td>
<td>Lee, 2008</td>
</tr>
<tr>
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<td>HSV-1</td>
<td>Tegument</td>
<td>Y2H, CoIP</td>
<td>Vittone, 2005</td>
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<tr>
<td>UL46</td>
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<td>HSV-1</td>
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<td>Y2H</td>
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<td>ORF45</td>
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<td>Tegument</td>
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<td>Rozen, 2008</td>
</tr>
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<td>ORF47 (gL)</td>
<td>UL1 (gL)</td>
<td>KSHV</td>
<td>Envelope</td>
<td>Y2H, CoIP</td>
<td>Rozen, 2008</td>
</tr>
<tr>
<td>BALF4</td>
<td>UL27 (gB)</td>
<td>EBV</td>
<td>Envelope</td>
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<td>ORF41</td>
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<td>?</td>
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<td>?</td>
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<td>UL54</td>
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<td>Nonstructural</td>
<td>Y2H</td>
<td>Bechtel, 2002</td>
</tr>
</tbody>
</table>
primary enveloped virions, in yeast two-hybrid analysis (336). UL37 and UL37 homologues also interact with other tegument proteins, including UL21, UL46, and UL36 (81, 145, 171, 285, 336, 338). Furthermore, UL37 homologues interact with several glycoproteins, including gL, gB and gN (81, 285). The HCMV UL37 homologue, encoded by the UL47 gene, coimmunoprecipitated in a complex with homologues of the UL36 and VP5 proteins (25). UL37 appears to interact with ICP8, as UL37 elutes from single and double-stranded DNA binding columns in an ICP8 dependent manner (3, 299, 300). Additionally, UL37 interacts with itself in yeast two-hybrid and coimmunoprecipitation studies (171, 338).

**PHENOTYPES OF MUTANT VIRUSES**

In the past decade many studies were undertaken to elucidate the role that UL37 plays in infected cells. Many of these investigations included the construction and analysis of viruses containing deletions or mutations of the UL37 gene. To date, three different HSV-1 viruses have been created containing deletions of nearly the entire UL37 ORF (63, 174, 278). UL37 is essential for the assembly of HSV-1. The amount of infectious virus released from the UL37 null viruses is reduced by >10^5 (63, 174, 278). In the absence of UL37, capsids are assembled, but cytoplasmic capsids appear to lack electron-dense material characteristic of tegument, capsids aggregate in large clusters within the cytoplasm and secondary envelopment of HSV-1 virions is blocked (63, 174, 278). Desai and colleagues observed an accumulation of aggregates of capsids in the nucleus of infected cells, suggesting that UL37 plays a role in nuclear egress (63). In contrast, Leege and Roberts did not observe a defect in the exit of capsids from the
nucleus of cells infected with UL37 deletion viruses constructed in their laboratories (174, 278).

Although UL37 plays an essential role during assembly of HSV-1, UL37 is not essential for envelopment and release of PRV virions. Two recombinant PRV viruses containing deletions of nearly the entire UL37 ORF have been analyzed (150, 187). Release of infectious virions is reduced in the absence of UL37, with titers of approximately 10^3 less PFU/ml as compared to wild-type (150). UL37 plays an important role in secondary envelopment of capsids during assembly. Similar to HSV-1 UL37 deletion viruses, in cells infected with a PRV UL37 deletion virus, large accumulations of capsids containing little or no tegument contact one another indirectly within the cytoplasm (150). Luxton and colleagues observed a reduction in the egress of capsids from the nucleus, although, Klupp and workers reported no impairment of nuclear egress (150, 187).

The UL37 homologue of VZV, encoded by ORF21, is also essential for virus replication in cell culture (358). Cells transfected with VZV cosmids containing an ORF21 deletion mutant fail to produce infectious virus. However, infectious virus is produced when the same VZV cosmids are transfected into an ORF21 expressing cell line (358). Analysis of thoracic and lumbar dorsal root ganglia from rats one month after infection with the ORF21 deletion virus, VZV Roka21D, indicated the presence of transcripts of a latency associated gene, ORF63, suggesting that ORF21 is not necessary for the establishment of latency (358). Interestingly, HSV-1 UL37 does not complement the ORF21 mutant virus (358). Similarly, Leege and colleagues reported that PRV UL37 can not rescue the HSV-1ΔUL37[86-1035] deletion virus and cells expressing HSV-1
UL37 do not complement the PRV-ΔUL37 mutant virus (174). Although the UL37 homologues play critical roles during assembly of the alphaherpesviruses HSV-1, PRV and VZV, it appears that the proteins play different roles in the distinct viruses.

The HCMV homologue of HSV-1 UL37 is encoded by the UL47 gene and plays an important role at early times during infection. A HCMV virus with a deletion of the central portion of the UL47 ORF, AD_{sub}UL47, replicates to titers 100-fold lower than wild-type virus (25). Virion binding and membrane fusion occur in cells infected with AD_{sub}UL47, however, transcription of viral immediate-early genes is delayed by 8 to 10 h (25). It does not appear that the delay in IE gene expression is due to a transactivation defect (25). Bechtel and colleagues suggest that UL47 is involved in the release of viral DNA from the capsid; therefore, in the absence of UL47 this process is slowed (25). Interestingly, virions produced from cells infected with AD_{sub}UL47 contain reduced amounts of UL48, the HSV-1 UL36 homologue. The authors determined that UL48 protein levels, but not transcript levels, are reduced in the absence of UL47 (25).

**UL37-MEDIATED NF-κB ACTIVATION**

In addition to playing a key role in virus assembly, UL37 also activates NF-κB within infected or transfected cells (180). Liu and colleagues determined that residues 1001-1123 of UL37 are necessary for NF-κB activation. UL37 activates NF-κB signaling by binding to TNF receptor-associated factor 6 (TRAF6) through a TRAF6 binding domain encompassing amino acids 1099-1104 (180). The TRAF6 binding motif within UL37, 1099^PVEDDE^1104, fits the general motif of PxExx(Ar/Ac) and is identical to the TRAF6-binding motif in IRAK-M (180, 349). UL37 homologues of alpha-, beta- and
gammaherpesviruses contain putative TRAF6 binding motifs. It appears that approximately one-half of the NF-κB activation that occurs early during infection is UL37-dependent, with additional NF-κB activation by gD (180). NF-κB signaling enhances replication of HSV-1, and a mutant virus containing an E1101A mutation in UL37 that abrogates NF-κB DNA-binding activity exhibits a 2-fold reduction in viral replication (114, 180, 255, 361).

A ROLE FOR UL37 IN CAPSID TRANSPORT

In addition to its function during virus assembly and NF-κB activation, UL37 also appears to play a role in microtubule-mediated transport of capsids. Immunoelectron microscopy revealed that after fusion with the cell membrane, the PRV proteins UL37, UL36 and US3 remain associated with the incoming capsid until it docks at the nuclear pore (110, 186). PRV UL37 is required for rapid transport of incoming capsids to the nucleus; in the absence of UL37 nuclear translocation is delayed by one hour (156). Dual fluorescently-labeled PRV studies indicated that during egress from neuronal cells UL37GFP is transported in association with fluorescently labeled capsids; independent transport of UL37GFP was not observed (186). UL37 appears to play a role in efficient transport of capsids during virus assembly. Luxton and colleagues reported a reduction in nuclear egress of capsids and less directed transport of cytoplasmic capsids in cells infected with a PRV UL37 deletion virus (187). Further supporting a role for UL37 in capsid translocation, Wolfstein and colleagues reported that removal of the outer tegument of HSV-1 virions to expose the inner tegument proteins, UL36 and UL37, improved capsid movement along microtubules in an in vitro motility assay (355).
THE TEGUMENT PROTEIN ENCODED BY THE UL36 OPEN READING FRAME

EXPRESSION AND LOCALIZATION

UL36 is the largest open reading frame identified in the HSV-1 genome (198). The HSV-1 UL36 gene encodes a 3,164 amino acid protein with a predicted molecular mass of 336 kDa (198). The protein encoded by UL36, also referred to as virion protein 1/2 (VP1/2) and infected cell protein 1/2 (ICP1/2), has an actual molecular weight of approximately 270 kDa, although the discrepancy in molecular weight has not been explained (119, 126). McNabb and Courtney showed that VP1/2 is recognized by antisera generated against amino acids 33-44 or amino acids 3048-3057 of UL36, confirming that the amino- and carboxy-termini of the open reading frame are expressed (206). UL36 is expressed at late times during infection, with peak synthesis occurring between 12 and 16 hpi (126, 207). UL36 is considered to be a true late, or $\gamma_2$, gene, as synthesis of UL36 is absolutely dependent on viral DNA replication (207). McNabb and Courtney also showed that UL36 is phosphorylated on serine residues (207). UL36 is incorporated into the tegument of virus particles (119, 126). In terms of copy number per virion, UL36 is a minor tegument protein, with estimates of fewer than 150 molecules of UL36 packaged in the virion (119). UL36 is tightly associated with capsids, as shown by detergent extraction analysis (103, 313).

Detergent cellular fractionation and immunofluorescence studies indicated that UL36 is present in both the nucleus and cytoplasm of HSV-1-infected cells (207, 223). Similarly, in recent studies Abaitua and O’Hare observed full length UL36 in the nucleus.
and cytoplasm of infected cells by confocal immunofluorescence microscopy and detergent cellular fractionation (1). A transferable nuclear localization signal (NLS) was mapped to residues 426-432 of UL36 (1). The NLS appears to contribute to an essential function that UL36 plays in infection, as suggested by complementation assays where cells transfected with a UL36-encoding plasmid containing a deletion of the NLS region fail to complement infection with a UL36 deletion virus (1). In contrast to HSV-1, in PRV-infected cells PRV UL36 does not localize to the nucleus (145, 172, 221). In the case of PRV UL36, two functional NLS domains localize some PRV UL36 to the nucleus of transfected cells, although localization of PRV UL36 in transfected cells is not exclusive to the nucleus (221). These observations suggest that a PRV viral protein may prevent nuclear localization of UL36 during infection (221). The cellular localization of UL36 and the cellular sites of UL36 incorporation into virions continue to be areas of interest and debate.

The UL36 ORF encodes a large tegument protein that is conserved across the herpesvirus family. Based upon the published sequence of strain 17 of HSV-1, UL36 has traditionally been cited as an ORF encoding 3,164 amino acids, (198). However, in August, 2007, the HSV-1 UL36 amino acid sequence published on the PubMed database (genome accession number NC_001806) was updated to exclude the amino terminal 25 amino acids of the previously published UL36 sequence (Fig. 2.7). Therefore, the second methionine of the previously published sequence became the first amino acid of the newly published UL36 sequence, thus reducing the size of UL36 to 3,139 amino acids. The reason for the change of the published protein sequence of UL36 is not clear. Analysis of the UL36 nucleotide sequence does not give an obvious advantage for
Figure 2.7. Nucleotide and Amino Acid Sequence of the Amino-Terminus of HSV-1 UL36. The nucleotide sequence of the amino-terminus of HSV-1 UL36 (accession number NC 001806) is shown in black. The location of the gene segment on the minus strand of the genomic DNA is denoted in black numbering. Methionine codons are highlighted in red, and the corresponding amino acids are shown below the codons (blue). The originally cited start methionine of UL36 is labeled 1 (green) and a second possible ATG translation initiation site is located 26 codons downstream, and is labeled 26 (green).
7168 gcc taa tca ggg gaa ccg ggg cca tgg
  1
tac ggg ggc atg ggt ggc gga aac aac
  m g g g n n
act aac ccc ggg ggt ccg gtc cat aaa
  t n p g g p v h k

  26

cag gcc ggg tct ctg gcc agc agg gca
  q a g s l a s r a
cat atg atc gcg gcc acc cca ccg cac
  h m i a g t p p h
tcc 71820

s
translation initiation at either methionine codon based on the presence of a Kozak sequence; neither methionine codon contains a Kozak sequence surrounding the ATG codon. However, personal experience sequencing and analyzing viral DNA isolated from HSV-1 KOS virions also suggests that the first 25 codons of UL36 may not be expressed in this strain. The ATG codon of the “original” start methionine (labeled #1 in Fig. 2.7) of HSV-1 strain 17, is a GTG codon in HSV-1 KOS. Thus, the second methionine codon (labeled #26 in Fig. 2.7) of the originally published UL36 amino acid sequence may be the actual start codon of the KOS UL36 protein; however, this proposal has not been verified experimentally. One cannot discount the rare possibility that translation initiation of UL36 may occur with a non-traditional (non-ATG) start codon. For the purpose of this dissertation, the amino acid numbering of the HSV-1 UL36 amino acid sequence will follow the assumption that UL36 contains 3,164 amino acids. Use of this numbering system will coincide with the UL36 amino acid designations of previously published studies of HSV-1 UL36.

**DOMAINS AND MOTIFS**

Several domains involved in UL36 protein interactions and functions have been identified within the large tegument protein (Fig. 2.8). The amino terminus of UL36 corresponds to the 55 kDa UL36 cleavage product involved in the release of the viral genome from the capsid of parental virions (136). The first 450-500 amino acids of UL36 contain ubiquitin protease activity, and a critical conserved cysteine is located in the active site of the enzyme at residue 65 (139). UL36 coimmunoprecipitates with VP5 (207) and a potential interaction domain, residues 1712-1751 of UL36,
Figure 2.8. Schematic Representation of the Domains Identified in UL36.

The active site cysteine of the ubiquitin protease is residue 65 (DeUb) (139). Residues 124-511 of UL36 are involved in binding VP16 (UL36-VP16) (216). A functional nuclear localization signal was identified at residues 426-432 (1). After the capsid transits to the nucleus and docks at the nuclear pore complex, a 55 kDa peptide of UL36 is cleaved, resulting in release of the viral genome into the nucleus (55kDa Cleavage). Residues 512-767 of UL36 facilitate interaction with UL37 (338). The proposed VP5 binding site is located at residues 1712-1751 (17). 35 tandem proline-glutamine repeats encompass amino acids 2911-2980. The extreme carboxy-terminus (amino acids 3104-3164) binds UL25 (54).
was proposed based on its similarity to the sequence of VP26 involved in binding VP5 (17). Residues 124-511 of UL36 are involved in binding the abundant tegument protein VP16 (216). The region of UL36 necessary for binding UL37 encompasses amino acids 512-767 (338), with key residues at F593 and E596 (216). Mutational studies of PRV showed that a 709 amino acid region of the carboxy terminus of PRV UL36, amino acids 2087-2795, is dispensable for the essential function of PRV UL36, this is a region of UL36 that is unconserved across the alphaherpesviruses (35). The extreme carboxy terminus, amino acids 3104-3164, binds the capsid-associated protein, UL25 (54). This region of UL36 localizes to assemblons in infected cells and is essential for productive PRV infection (172).

Analysis of the UL36 amino acid sequence yields several protein motif patterns. UL36 contains a functional NLS within residues 426-432 (1). A putative leucine zipper, a motif often important in the formation of homo- and heterodimers, is located at amino acids 632-653, and another at amino acids 1070-1091 (167, 206). The carboxy-terminus of UL36 contains 35 tandem proline-glutamine repeats spanning residues 2911-2980, of which the functional significance, if any, is unknown. Throughout the UL36 ORF are 35 dileucine motifs (LL or LI) and 17 tyrosine-based motifs (YXXΦ, where Φ represents a large hydrophobic residue, M, I, L, F, V), which possibly facilitate interactions with clathrin adaptor proteins to promote vesicular trafficking. UL36 also contains several late domain motifs. A PPXY late domain motif (in the form of PPTY) is located at residues 2864-2867, two P[T/S]AP late domain motifs consist of amino acids 387-390 (PSAP) and 2825-2828 (PTAP), a YPXL motif encompasses amino acids 218-221
(YPYL) and a PPLP motif is located at residues 2021-2024 (258). Finally, amino acids 2211-2219 and 2430-2443 are potential ATP binding sites (206).

**INTERACTION PARTNERS OF UL36**

As one might expect for a protein of such enormous size, and as indicated by the above-mentioned discussion of interaction domains, UL36 participates in a multitude of interactions with other viral proteins. A summary of the reported interactions of UL36 homologues with viral proteins is shown in Table 2.4; a discussion of noteworthy interactions occurs below. UL36 has been suggested as a key protein responsible for linking the viral capsid to proteins of the outer tegument and envelope (213, 285). This scenario seems plausible, since VP1/2 has been reported to interact with capsid proteins, tegument proteins and envelope glycoproteins. Zhu speculated that the KSHV UL36 homologue, ORF64, binds to the capsid at the amino-terminus and is associated with the viral envelope at the carboxy-terminus (369). However, the orientation of UL36 in the alphaherpesviruses appears to be the opposite (54). HSV-1 UL36 and the UL36 KSHV homologue, ORF64, interact with the major capsid protein, VP5, of HSV-1 and ORF25 of KSHV, respectively, in coimmunoprecipitation assays (207, 285). The VZV and KSHV UL36 homologues also interact with the capsid triplex proteins, encoded by ORF41 of VZV (336) and ORF62 and ORF26 of KSHV, respectively (285). Furthermore, yeast two-hybrid analysis has suggested an interaction of the VZV and EBV UL36 homologues with the UL26 homologues, ORF33 and BDRF1, respectively, which encode the capsid scaffold proteins (81, 336). HSV-1 UL36 interacts with the capsid-associated UL25 protein in coimmunoprecipitation assays (54). In addition, UL36
<table>
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homologues have been reported to interact with several tegument proteins including: UL37 (25, 145, 171, 216, 285, 336, 338), VP16 (171, 338), UL16, UL17, UL21, and UL48 (81, 285). Furthermore, the UL36 homologues of EBV and KSHV have been reported to self-interact (44, 81, 285). UL36 homologues have also been reported to bind gB, gM, gH and gL of KSHV (285), gM of EBV (81) and gE and gN of VZV (336). Finally, UL36 homologues interact with several nonstructural proteins, including the VZV and EBV homologues of UL31, a protein necessary for egress of capsids from the nucleus (81, 274, 336).

PHENOTYPES OF MUTANT VIRUSES

UL36 is an essential tegument protein, executing necessary functions at both early and late times of infection. Desai created a mutant HSV-1 virus containing a deletion of the sequence encoding residues 362-1555 of UL36, resulting in a frameshift mutation that created a stop codon 42 amino acids beyond the deletion; thus most of the 3,164 amino acid protein is not expressed (64). Infection with the resulting virus, KΔUL36, resulted in an accumulation of clusters of capsids in the cytoplasm that lacked tegument and an envelope (64). In a recent study, Roberts and colleagues constructed a mutant virus with a complete deletion of the UL36 ORF, ARΔUL36 (278). Again, UL36 appeared to play an essential role during virus assembly. Viral titers from ARΔUL36 infected cells were reduced 10^5-fold less than wild-type or revertant virus (278). Capsids accumulated throughout the cytoplasm of ARΔUL36 infected cells (278). The authors speculated that the difference in cytoplasmic capsid distribution between KΔUL36 and ARΔUL36 is due to the incomplete nature of the UL36 deletion of KΔUL36, based upon their observation
that the amino-terminal encoded truncated UL36 mutant protein of KΔUL36 is present on cytoplasmic virions (278). Based on HSV-1 recombinant viruses, UL36 clearly plays an essential role at late times of virus assembly.

Similar to HSV-1, PRV UL36 is also necessary for proper tegumentation and secondary envelopment of PRV particles. Infection with PRV-ΔUL36F, a mutant PRV that encodes only the first 20 amino acids of UL36, produces capsids that are dispersed throughout the cytoplasm, occasionally in aggregate clusters, and are not enveloped or released from the cell (91). A second PRV UL36 deletion virus, ΔUL36, contains a deletion of nucleotides 4-8910 of the 9255 nucleotide UL36 ORF and incorporates a GFP fluorescent tag on the VP26 capsid protein (187). In PRV ΔUL36, deletion of UL36 once again results in a lethal effect, however, capsid egress from the nucleus is significantly less than wild-type viruses and results in an accumulation of capsids within the nucleus (187). The reason for the difference in the cellular site where capsids accumulate in the two different UL36 deletion viruses is unclear. However, the phenotypes of both PRV UL36 deletion viruses clearly depict an essential role for UL36 in viral assembly.

THE ROLE OF UL36 IN VIRAL DNA RELEASE

In addition to playing a key role in virus assembly, UL36 associated with incoming virions is required for the release of the viral genome into the nucleus. Early studies of infections with an HSV-1 mutant virus, tsB7, containing a temperature-sensitive mutation within the UL36 ORF, reported an accumulation of capsids at nuclear pores and a block in the release of DNA from the capsids (21). Recent work from the Roizman laboratory has shown that after the capsid docks at the nuclear pore UL36 is
cleaved by a serine-cysteine protease to allow the release of the viral DNA into the nucleus (136). The proteolytic cleavage event produces a 50-60 kDa amino-terminal cleavage product of UL36 that is detected only after the capsid attaches to the nuclear pore (136). The specific cleavage site within UL36 and the protease responsible for the cleavage have not yet been identified.

**UL36 IS A DEUBIQUITINATING ENZYME**

In addition to its roles in viral DNA release and virus assembly, UL36 also functions as a deubiquitinating enzyme in infected cells (139). Kattenhorn and colleagues used mass spectrometry to identify a 47 kDa ubiquitin specific cysteine protease present at late times during HSV-1 infection (after 12 hpi) as a product of the UL36 ORF (139). The proteolytic peptide appears to correspond to the amino-terminal 500 amino acids of UL36, however, the exact identity of the carboxy-terminus of the protease fragment has yet to be identified (139). The deubiquitinating activity of the UL36 homologues is conserved among the *Alpha-, Beta- and Gammaherpesviruses* (105, 112, 131, 139, 291, 341). The active site of the deubiquitinating enzyme is a conserved cysteine at residue 65 of HSV-1, residue 61 of EBV, residue 23 of MCMV (139), residue 26 of PRV (36, 170), and residue 29 of KSHV (105). The crystal structure of the cysteine protease domain of the MCMV UL36 homologue revealed that the herpesvirus-encoded ubiquitin ligase is distinct from other classes of deubiquitinating enzymes in the arrangement of active site residues and the presence of a unique ubiquitin binding β hairpin (292). Recently a viral substrate for the UL36 ubiquitin specific protease has been identified. The EBV UL36 homologue, BPLF1, deubiquitinates the large subunit
(RR1) of the viral ribonucleotide reductase (RR) and results in a decrease in RR activity in cotransfected cells (352).

Studies of recombinant viruses lacking UL36 deubiquitinating activity have provided some insight into the importance of the protease function during infection. Smith and colleagues reported that deletion of nucleotides encoding residues 6-225 of a PRV UL36 mutant virus, Δ6-225, lacks the ubiquitin protease domain and replicates poorly in vitro, with titers 3 logs less than the parental virus (172). They observed normal retrograde transport of virions but a 50% reduction in the frequency of transport of progeny virus particles along axons (172). In similar studies, Mettenleiter and coworkers constructed a mutant virus, PRV-UL36Δ22-248, that encodes a PRV UL36 protein lacking residues 22-248 (34). PRV-UL36Δ22-248 replicates in vitro to titers 15-fold less than wild-type virus, and produces plaque sizes comparable to wild-type virus (34). In an intranasal infection model, PRV-UL36Δ22-248-infected mice survive longer than mice infected with wild-type virus, consistent with an impairment of neuroinvasion of PRV-UL36Δ22-248 (34).

Further studies resulted in the construction of PRV viruses containing point mutations of the cysteine active site residue. Analysis of the point mutant viruses provided further evidence that the deubiquitinating activity of UL36 contributes to neuroinvasion. Mettenleiter’s group constructed a PRV mutant virus, PRV-UL36(C26S), containing a cysteine to serine substitution at the active site residue 26 (36). The authors reported a 20 to 30-fold reduction in viral titers in vitro and noted the presence of large accumulations of unenveloped capsids in the cytoplasm of cells infected with the mutant virus when visualized by electron microscopy (36). In an intranasal infection model,
mice infected with PRV-UL36(C26S) survived twice as long as mice infected with wild-type virus, suggesting a delay in neuroinvasion by viruses lacking UL36 deubiquitinating activity (36). In a similar study, Smith and colleagues created a PRV recombinant virus, C26A, that expresses UL36 containing a cysteine to alanine mutation at the active site residue 26 (170). C26A replicates well in vitro in neuronal and non-neuronal cells, and in vivo the virus was capable of anterograde and retrograde transport in neurons (170). However, using a variety of in vivo infection models, Smith and colleagues elucidated the specific step of pathogenesis in which PRV UL36 deubiquitinating mutants are defective and determined that the C26A mutant virus failed to spread from infected tissues into the mammalian nervous system (170).

Gibson and coworkers constructed a mutant virus, C24I, with an isoleucine mutation of the active site cysteine of the UL36 homologue of HCMV, UL48 (341). They reported that the point mutation slows virus replication but the active site cysteine is not required for virion production in vitro (341). Kim and colleagues also blocked the deubiquitinating activity of HCMV UL48 by creating a mutant virus containing a C24S mutation in the U1.48 ORF (141). They reported that mutation of the active site cysteine of UL48 results in a 10-fold reduction of virions released from cells infected at a low MOI (0.1) (141). The active site cysteine of the UL36 homologue of MHV-68, ORF64, was changed to an alanine (C33A) in a mutant virus constructed by Gredmark-Russ and colleagues (113). Their results showed that loss of ORF64 deubiquitinating activity decreases viral titers in vitro by 10-fold and decreases plaque size by 20% (113). Although deubiquitinating activity is not essential for in vitro viral growth, the ubiquitin specific protease may play a role in vivo, as suggested by the ability of mice infected with
the mutant virus to clear infection more quickly and harbor significantly lower viral loads (113). Studies were also designed to examine the role that UL36 ubiquitin protease activity plays in the tumorigenic potential of Marek’s disease virus (MDV). A MDV mutant virus, vC98A-1, containing a cysteine to alanine mutation at the active site residue 98, showed a minor reduction in titers and plaque size in vitro as compared to the revertant virus (131). They also reported that MDV-transformed lymphoblastoid cells express UL36 RNA, suggesting that UL36 may play a role during the latent/tumor phase of infection (131). Chickens infected with vC98A-1 showed a 3-4 log reduction in viremia levels and a dramatic reduction in oncogenic transformation and tumor metastasis (131).

A ROLE FOR UL36 IN CAPSID TRANSPORT

UL36 appears to play a role in microtubule based transport of capsids at both early and late times of infection. At early times of infection UL36 remains associated with the capsid as it translocates through the cytoplasm and localizes to the nuclear rim (110, 186, 224). The inner tegument proteins, which remain associated with the capsid until it reaches the nucleus, likely facilitate microtubule-based transport of the capsid to the nucleus. An in vitro model was used to examine the role that the essential UL36 protein plays in the initial stages of infection. In this in vitro model, cells were fused to form syncytia and then infected with a UL36 deletion virus at a very low MOI and examined by immunofluorescence to evaluate spread of virions to neighboring nuclei. In the syncytia model, cells infected with a UL36 deletion virus fail to spread infection to other nuclei within the syncytia, suggesting an essential role for UL36 in the initiation of
infection (278). Recent work by Copeland and colleagues showed that cells preloaded with antibodies against UL36 and then infected with HSV-1 show a 51% reduction of nuclear capsid attachment, suggesting a role for UL36 in transport of the incoming capsids to the nucleus and/or attachment to the nuclear pore (55).

UL36 also appears to play a role in microtubule-mediated migration of the capsid through the cytoplasm at later stages of infection. Removal of the outer tegument proteins, to expose the inner tegument proteins including UL36, increases motility of capsids in an in vitro microtubule motility assay (355). In an in vitro microchamber assay, membrane-associated viral particles produced from a UL36 deletion virus show decreased microtubular motility and binding as compared to wild type virus particles (296). Correlative motion analysis of primary sensory neurons infected with a PRV mutant virus expressing RFP-tagged capsids and GFP-tagged UL36 showed that at late times during infection UL36 is always transported in axons associated with capsids (186). Time-lapse microscopy of living cells infected with a fluorescently tagged UL36 deletion virus showed that in the absence of UL36, capsid transport in the cytoplasm is random and non-processive, in contrast to directed movement of capsids observed in the cytoplasm of cells infected with the parental virus (187).

Evidence suggests that UL36 homologues bind proteins that interact directly with molecular motor proteins. The HCMV UL36 homologue, UL48, binds to the ribosome receptor, p180, in transfected or infected cells (249). p180 interacts with the kinesin microtubule motor protein, KIF5B (65). Ogawa-Goto speculated that HCMV particles bind to p180 through UL48 located in the tegument, and that p180 facilitates cytoplasmic capsid transport through its interaction with the kinesin motor proteins (249).
Furthermore, the KSHV UL36 homologue, ORF64, binds to the tegument protein ORF45 in yeast two-hybrid and coimmunoprecipitation assays (285). ORF45 interacts with the kinesin microtubule motor protein, KIF3A (289). The interactions of ORF64 with ORF45 and ORF45 with KIF3A represent another possible mechanism for microtubule-mediated transport via UL36 homologues (289).

THE INTERACTION OF THE UL36 AND UL37 TEGUMEN

PROTEINS

UL36 and UL37 physically interact (145). The interaction of UL36 and UL37 is conserved among the herpesvirus family and has been reported for HSV-1 (171, 216, 338), PRV (145), VZV (336), KSHV (285) and HCMV (25). Vittone and colleagues have determined that the region containing amino acids 512-767 of HSV-1 UL36 is necessary for binding UL37 (338). Mijatov extended these studies to identify residues F593 and E596 of UL36 as essential for interaction with UL37 (216). Furthermore, Fuchs and others reported that deletion of the UL37 binding domain of UL36 does not completely abolish PRV replication, suggesting that the essential function(s) of UL36 is not dependent on binding UL37 (91).

The role that the interaction between UL36 and UL37 may play in the packaging of these proteins into virions has not been fully elucidated. The UL36 homologue of HCMV appears to require the UL37 homologue for protein stability (25). A similar observation was seen with HSV-1 (Fig. 4.1). Desai and colleagues reported that the localization of UL37 in the Golgi complex is dependent on UL36, therefore, it is likely that some UL37 is incorporated into virions during secondary envelopment in a UL36-
dependent manner (62). Based upon the work of Roberts and colleagues, UL36 and UL37 appear to rely on one another for incorporation into L particles (278). Interestingly, the incorporation of UL37 and UL36 into L particles may also be dependent on VP16, as L particles produced from a PRV VP16 deletion mutant, PRV-ΔUL48, do not contain these proteins (87). This observation coincides with the reported interaction of VP16 and UL36 in yeast two-hybrid screens (171, 338). In PRV, immunoelectron microscopy was used to detect UL36 associated with cytoplasmic capsids of a UL37 deletion virus, suggesting that UL36 may be incorporated prior to final envelopment in a UL37-independent manner (145). Krautwald and colleagues also reported that PRV UL36 is incorporated into virions in the absence of PRV UL37 (156). The relationship of the UL36-UL37 interaction to their incorporation into virions, and the cellular sites where these proteins are incorporated continue to be topics of ongoing investigation.

The studies described in this dissertation were initiated with the goal of identifying tegument proteins that may associate with capsids in the nucleus. Based upon its tight interaction with capsids and coimmunoprecipitation with the major capsid protein VP5 (103, 207, 313), we hypothesized that UL36 associates with capsids in the nucleus to possibly facilitate subsequent incorporation of tegument proteins. As described in Chapter III, we found that UL36 and its binding partner, UL37, associate with nuclear capsids. Interestingly, UL36 appears to selectively associate with a subset of capsids, DNA-filled C capsids. UL36 and UL37 are essential for tegumentation and envelopment of HSV-1 virions (63, 278). Our studies show that UL36 and UL37 associate with capsids isolated from the nuclear fraction. In summary, this data suggests
that UL36 and UL37 are among the initial tegument proteins to be incorporated into the
virus particle and may serve as a platform to facilitate the incorporation of other tegument proteins.

The interaction of UL36 and UL37 homologues is conserved across the
herpesvirus family (25, 81, 171, 285, 336, 338). The domain and residues of UL36 that
are necessary for interaction with UL37 have been identified (216, 338). However, the
region of UL37 involved in binding UL36 was unknown. To fill this gap of knowledge,
we utilized coimmunoprecipitation assays to identify the region of UL37 involved in
binding UL36. The results shown in Chapter IV show that the carboxy-terminal half of
UL37, amino acids 568-1123, mediates binding to UL36. This region is also involved in
self-association of UL37, and a second self-association domain lies within the amino-
terminal 300 amino acids of UL37. Furthermore, the carboxy-terminal half of UL37
enables the release of infectious virus particles in a trans-complementation assay,
suggesting this region of UL37 plays an important role in assembly.

Because the carboxy-terminal half of UL37 mediates binding to UL36 and self-
association, we set out to determine if UL37 can interact with UL36 and UL37
simultaneously, resulting in a UL37-UL37-UL36 complex. The final observations
presented in Chapter IV provide evidence that UL37 does not self-associate when UL36
is abundant. Therefore, self-association of UL37 appears to be regulated by UL36. A
discussion of the potential roles of UL37 self-association and regulation by UL36 is
presented in Chapter V.
CHAPTER III

HERPES SIMPLEX VIRUS TYPE 1 TEGUMENT PROTEINS UL36 AND UL37 ARE ASSOCIATED WITH INTRANUCLEAR CAPSIDS

Adapted from:
ABSTRACT

The assembly of the tegument of herpes simplex virus type 1 (HSV-1) is a complex process that involves a number of events at various sites within virus-infected cells. Our studies focused on determining whether tegument proteins, UL36 and UL37, are added to capsids located within the nucleus. Capsids were isolated from the nuclear fraction of HSV-1-infected cells and purified by rate-zonal centrifugation to separate B capsids (containing the scaffold proteins and no viral DNA) and C capsids (containing DNA and no scaffold proteins). Western blot analyses of these capsids indicated that UL36 associated primarily with C capsids and UL37 associated with B and C capsids. The above results demonstrate that at least two of the tegument proteins of HSV-1 are associated with capsids isolated from the nuclear fraction, and these capsid-tegument protein interactions may represent initial events of the tegumentation process.
INTRODUCTION

Virions of herpes simplex virus type 1 (HSV-1) have three morphologically distinct structures: an icosahedral capsid that encloses the genome, a proteinaceous tegument layer surrounding the capsid, and a host-derived lipid envelope containing viral glycoproteins and other membrane proteins (281). Capsid assembly occurs in the nucleus yielding procapsids, as well as A, B, and C capsids, which can be separated in density gradients (103, 238, 261). Procapsids contain the major capsid protein, VP5, triplex proteins VP19C and VP23, the viral protease VP24, and the scaffold proteins VP22a and VP21 (234, 281). Scaffold proteins are released from the capsid as the viral genome enters through the UL6 portal complex to form the mature C capsid that becomes the nucleocapsid of the mature virion (240). A capsids do not contain scaffold proteins or viral genome and B capsids lack the viral genome but retain scaffold proteins. A and B capsids are considered abortive packaging products (261, 281).

Mature capsids exit the nucleus through an envelopment-deenvelopment process. They acquire a temporary envelope by budding into the inner nuclear membrane and subsequently lose this envelope upon fusion with the outer nuclear membrane and translocation into the cytoplasm (reviewed in 212). The UL31 and UL34 gene products rearrange the nuclear lamina and are necessary for efficient egress of capsids from the nucleus (48, 272, 273, 283 and reviewed in 212).

Although the events of capsid assembly and DNA packaging have been extensively studied, the molecular mechanisms associated with the assembly of the tegument have been described in limited detail. The addition of tegument onto capsids could occur within the nucleus, as the capsid traverses the inner and outer nuclear
membranes, within the cytoplasm, and finally as the capsid acquires its envelope from vesicles derived from the trans-Golgi network (212, 281). Immunoelectron microscopy studies have shown the association of the tegument protein VP16 with primary enveloped virions located within the perinuclear space (232).

Studies described within this report were focused on determining whether specific tegument proteins are associated with capsids isolated from the nuclear fraction of HSV-1-infected cells. The two tegument proteins included within these studies are UL36 and UL37. The UL36 gene encodes a 270 kDa protein encoded also known as VP1/2 (119, 126, 191, 206, 225, 313). Previous studies have suggested that approximately 150 molecules of UL36 are present in each virion and that UL36 is tightly associated with capsids (103, 207, 313). UL36 is localized to both the cytoplasm and nucleus of virus-infected cells (207, 223). UL36 is an essential viral protein with apparent functions during early and late times after infection. The phenotype of a mutant virus (tsB7) containing a temperature-sensitive mutation in the UL36 gene suggests that UL36 is involved in the release of the viral genome from capsids during the initial events of virus infection (21). Immunoelectron microscopy studies have suggested that UL36 and UL37 remain associated with the capsid until it docks at the nuclear pore (110). Another mutant virus, KΔUL36, encoding only the amino terminal 361 amino acids residues of the UL36 protein, results in an accumulation of DNA-containing capsids in the cytoplasm that lack the major tegument proteins and an envelope (64). Furthermore, the pseudorabies virus UL36 homologue is necessary for microtubule-based capsid transport in the cytoplasm (187). In addition, recent studies have shown that the cleaved amino
terminal 500 amino acids of UL36 has deubiquitinating activity that is conserved among homologues of the herpesvirus family (139, 291).

The 120 kDa protein encoded by the UL37 gene is phosphorylated (3, 202, 293) and is found in both the cytoplasm and nucleus of virus-infected cells (293). A null mutant of UL37 (K△UL37) results in an accumulation of unenveloped capsids forming aggregates along the inner nuclear membrane and in the cytoplasm suggesting that UL37 plays an essential role in the virion assembly process (63). The UL37, as well as the UL36 gene product, are conserved among all subfamilies of Herpesviridae (212). The pseudorabies virus homologues of the HSV-1 UL36 and UL37 proteins physically interact (145). Similar findings were recently reported for HSV-1 UL36 and UL37 (338).

The accumulation of capsids within or near the nucleus for the UL37-null mutant K△UL37 suggested the possibility that this tegument protein and its binding partner, UL36, are added to capsids in the nucleus. In addition, the strong association of UL36 with capsids suggested that UL36 may be one of the innermost tegument proteins of the virus. Using cell fractionation and density gradient analyses, we found that UL36 and UL37 are associated with capsids purified from the nucleus. Moreover, it appears that UL36 associates predominantly with C capsids as compared to B capsids. These results suggest that the addition of UL36 and UL37 may represent an initial tegumentation event in the assembly of HSV-1 virions.
RESULTS

Purity of isolated nuclear fractions. Throughout these studies, capsids from the nuclear fraction of virus-infected cells were used; therefore, it was essential to demonstrate that the nuclear fractions were free of components from the cytoplasmic fraction. The nuclear and cytoplasmic fractions from virus-infected cells were harvested at 15 hpi and analyzed by Western blotting for two marker proteins of the cytoplasm and nucleus, calnexin and lamin B1, respectively (Fig. 3.1). The blot was probed simultaneously with antibodies to calnexin and lamin B1. Repeated analyses showed that nuclear fractions were free of detectable levels of the endoplasmic reticulum protein, calnexin, and the cytoplasmic fraction did not contain detectable levels of lamin B1.

Purity of intranuclear capsids. Since the focus of this study is on capsids isolated from the nuclear fraction, it was important to demonstrate that this capsid preparation was free of capsids associated with the cytoplasmic fraction. Vero cells were infected with HSV-1 KOS, harvested at 15 hpi and the cells were resuspended in 1.0% NP40 lysis buffer. This cell preparation was then mixed with the cytoplasmic fraction from HSV-1 K26GFP virus-infected cells harvested at 15 hpi. All capsids within the K26GFP cytoplasmic fraction contained the VP26-GFP chimeric protein. Our goal was to determine if capsids obtained from the purified nuclear fraction of the KOS infected cells were cross-contaminated with the VP26-GFP capsids from the K26GFP cytoplasmic fraction. After a 30-minute incubation on ice in 1.0% NP40 lysis buffer, the nuclei were purified following the protocol described in the Methods. Capsids from the purified nuclear fraction were resolved by rate-zonal centrifugation as described in the Methods. Fractions from the density gradient were analyzed by SDS-PAGE and Western blotting...
Figure 3.1. Analysis of the Purity of the Nuclear Fraction Obtained from HSV-1-Infected Cells. Approximately $2 \times 10^6$ Vero cells were infected with HSV-1 at a MOI of 10 pfu/cell. At 15 h after infection, the cells were harvested and separated into the cytoplasmic and nuclear fractions as described in the Methods. The proteins of the nuclear and cytoplasmic fractions were resolved by SDS-PAGE followed by Western blot analysis simultaneously using antibodies to the nuclear marker lamin B1 (70 kDa) and the endoplasmic reticulum marker calnexin (90 kDa) to assay the same nitrocellulose blot.
Figure 3.2. Analysis of the Purity of the Nuclear Capsid Preparation. (A) Approximately 1.5 x 10^8 HSV-1 KOS infected Vero cells were harvested at 15 hpi, resuspended in 1% NP40 lysis buffer and then mixed with the cytoplasmic fraction from approximately 1.3 x 10^8 Vero cells infected with HSV-1 K26GFP virus. This mixture was incubated on ice for 30 minutes and the nuclear fraction was then purified as described in the Methods. Capsids from the nuclear fraction were purified and separated on a 20-50% linear sucrose gradient following rate- zonal centrifugation. 0.5 ml fractions were collected from the bottom of the tube. The proteins within each fraction were precipitated with 10% TCA, separated by SDS-PAGE followed by Western blot analysis. The blot was probed sequentially with antibodies to GFP and the major capsid protein VP5. K26GFP lysate (far right) shown in all panels served as a positive control for the Western blots. (B) Western blot analysis of capsids isolated from the cytoplasmic fraction from approximately 2.6 x 10^7 K26GFP infected Vero cells purified and analyzed as described above. K26GFP lysate is shown in the far right lanes as a control.
A Nuclear capsids isolated from the nuclear fraction of KOS infected cells mixed with the K26GFP cytoplasmic fraction

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B Cytoplasmic capsids isolated from K26GFP cytoplasmic lysate

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with antisera to GFP and VP5 (Fig. 3.2). As shown in Fig. 3.2A, VP5 was detected in fractions 11-15 of the gradient, indicating the location of capsids within the gradient. However, no GFP-containing capsids were detected in the gradient fractions indicating that the cytoplasmic GFP-containing capsids were effectively removed during the purification of the nuclear fraction. As a positive control, the VP26-GFP capsids within the cytoplasmic fraction of K26GFP infected cells were also resolved on another gradient and GFP-containing capsids were readily detected in fractions 11-15 by Western blotting (Fig. 3.2B). The amount of the cytoplasmic fraction used to obtain the VP26-GFP capsids loaded onto the gradient in Fig. 3.2B was approximately 1/5 the amount of lysate that was mixed with the KOS infected cells prior to the purification of the nuclear fraction. In both preparations, the major capsid protein VP5 was detected in fractions 11-15. As will be presented below, it is within these fractions that most of the UL36 or UL37 is detected with capsids isolated from the nuclear fraction. The results from this experiment suggest that the nuclear isolation protocol used in this study yields a population of nuclear capsids that is not contaminated with capsids from the cytoplasmic fraction.

**Tegument proteins UL36 and UL37 associate with capsids isolated from the nuclear fraction.** At 15 h after infection, HSV-1-infected Vero cells were fractionated into cytoplasmic and nuclear fractions. Capsids from the nuclear fraction were loaded onto a 20-50% (wt/wt) linear sucrose gradient and resolved by rate-zonal centrifugation. Fractions were collected, precipitated with 10% trichloroacetic acid, and analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting. Blots were reacted with five different antibodies (Fig. 3.3). To identify where capsids were located within the
Figure 3.3. Rate-Zonal Centrifugation of HSV-1 Capsids Isolated from the Nuclear Fraction and Analyzed for the Presence of Tegument Proteins. Capsids were obtained from the nuclear fraction of HSV-1-infected Vero cells harvested at 15 h after infection and separated on a 20-50% linear sucrose gradient following rate-zonal centrifugation. 0.5 ml fractions were collected from the bottom of the tube. The proteins within each fraction were precipitated with 10% TCA, separated by SDS-PAGE followed by Western blot analysis using primary antibodies to specific tegument proteins followed by the addition of the secondary anti-rabbit or anti-mouse antibody conjugated to horseradish peroxidase. Western blot analysis was done using antibodies to capsid proteins VP5 (the major capsid polypeptide) and VP21/VP22a (scaffold proteins), tegument proteins UL36 and UL37, and the DNA binding protein UL42.
gradient, an antibody specific for the major capsid polypeptide, VP5, was used. Western blotting with anti-VP5 serum showed that most capsids were in fractions 11-15. Antibodies specific for UL36 and UL37 showed that these two tegument proteins were also present in the capsid-containing fractions. Scaffold proteins VP21 and VP22a are associated with B capsids and not with DNA-containing C capsids (281). When the blot was reacted with an antibody specific for scaffold proteins, the major reactivity was seen in fraction 12 with fractions 11 and 13 also showing the presence of some scaffold protein. In contrast, most of the UL36 reactivity was detected in fraction 14 that contains high amounts of VP5 but little to no scaffold proteins. When the blot was reacted with a monoclonal antibody specific for the HSV-1 nonstructural DNA-binding protein UL42 (97), no reactivity was detected in fractions containing VP5. The results presented above suggest that tegument proteins UL36 and UL37 are associated with capsids isolated from the nuclear fraction of virus-infected cells.

As a control for the specific binding of selected tegument proteins to capsids isolated from the nucleus, two additional tegument proteins were assayed, VP22 and UL11. VP22 is one of the most abundant tegument proteins of purified HSV-1 virions (119). Depending on the time after infection, VP22 is localized within the cytoplasm as well as the nucleus of virus-infected cells (223, 264). UL11 is a myristoylated tegument protein that is localized primarily to the Golgi region (37, 189). Capsids from the nuclear fraction were isolated, purified and assayed by Western blotting as described above. Antibody to VP5 was used to identify the location of the capsids resolved by rate-zonal centrifugation. In addition, antibody to the tegument protein UL37 was included as a positive control. As shown in Fig. 3.4, density gradient fractions demonstrating reactivity
Figure 3.4. Rate-Zonal Centrifugation of HSV-1 Capsids Isolated from the Nuclear Fraction and Analyzed for the Presence of Tegument Proteins VP22 and UL11. At 15 h after infection the capsids from the nuclear fraction were obtained, separated by rate-zonal centrifugation as described in the legend to Fig. 3.3. The same blot was probed sequentially using antibodies specific for the major capsid protein VP5 and tegument proteins VP22, UL11, and UL37 (positive control).
with antisera to VP5 and UL37 were similar to that shown previously in Fig. 3.3. However, when the blot was reacted with antisera specific for either VP22 or UL11, no reactivity was detected. Western blot analysis of purified extracellular virions showed strong reactivity with antisera to VP22 and UL11 (data not shown). These results further support the specificity of the detection of tegument proteins UL36 and UL37 with capsids isolated from the nuclear fraction of HSV-1-infected cells.

**The detection of UL36 and UL37 in density gradients is capsid dependent.**

To ascertain whether the co-sedimentation of UL36 and UL37 with VP5 was due to their association with capsids, the analysis was done in the absence of capsid formation. A mutant virus (K23Z) defective in the synthesis of the VP23 triplex protein was kindly provided by Dr. Prashant Desai. When this mutant infects Vero cells, no capsids form due to the absence of the Ul18 gene, which encodes the VP23 triplex protein (60). Vero cells and the VP23 complementing cell line (C32) were infected with the K23Z virus and harvested at 15 h after infection. Prior to cell fractionation, a sample of the total cell lysate from K23Z virus-infected Vero and complementing cells (C32) was collected and assayed by SDS-PAGE and Western blotting for the presence of VP5, VP16 and UL37 (Fig. 3.5). In both cases, expression of VP5, VP16 and UL37 was readily detected by immunoblotting. These same cell lysates were fractionated, and capsids obtained from the nuclear fraction were resolved on a density gradient, precipitated with TCA, and analyzed by SDS-PAGE and Western blotting (Fig. 3.6). As expected, capsids from the complementing C32 cells exhibited VP5, UL36, and UL37 in similar fractions as seen in other gradients presented above. Most of the detectable UL36 was in fraction 13, the same fraction where there was a strong reactivity to anti-VP5. The detection of UL37
Figure 3.5. SDS-PAGE Analysis of the Cell Lysates of Vero (VP23 Non-Complementing) and C32 (VP23 Complementing) Cells Infected with the HSV-1 VP23-Negative Mutant Virus, K23Z. The two cell lines were infected with the K23Z virus at a MOI of 10 pfu per cell and harvested at 15 h after infection. The whole cell lysates were resolved by SDS-PAGE followed by Western blot analysis. The same blot was probed sequentially with antibodies to VP5, VP16, and UL37.
Figure 3.6.  Rate-Zonal Centrifugation of Capsids Isolated from Cells Infected with the VP23-Negative Mutant (K23Z) and Analysis for the Presence of Tegument Proteins UL36 and UL37.  Vero (non-complementing cell line) and C32 (complementing cell line expressing VP23) cells were infected with the VP23-negative mutant virus, K23Z, at a MOI of 10 pfu/cell.  At 15 h after infection the capsids from the nuclear fraction were obtained, separated by rate-zonal centrifugation, analyzed by SDS-PAGE followed by Western blot analysis as described in the legend to Fig. 3.3. The same blot was probed sequentially for the presence of VP5 and tegument proteins UL36 and UL37.
was also seen in similar fractions as described for other gradients. In contrast, neither VP5, UL36, nor UL37 was detectable in the region of the gradient where capsids normally sediment when the nuclear fraction from the nonpermissive Vero cells was analyzed. Although VP5, UL36 and UL37 are present in the nucleus in the absence of capsid formation, they are not readily detected in the linear sucrose gradient in the absence of capsid formation. When not associated with capsids, these proteins do not effectively pellet through the 35% (wt/wt) sucrose cushion purification step that precedes the linear sucrose gradient analysis. In some experiments, small but detectable amounts of UL37 were observed in fractions toward the bottom of the gradient suggesting the formation of non-capsid associated complexes. Overall, these results further support that UL36 and UL37 sediment in a capsid-dependent manner due to their association with intranuclear capsids.

**UL36 associates primarily with C capsids.** The results shown in Figure 3.3 suggest that the sucrose gradient resolved capsids into two populations. Based on the reactivity of the fractions with the anti-scaffold antibody, it appeared that the slower sedimenting capsid population contained a preponderance of B capsids. However, UL36 was predominantly found with the faster sedimenting capsid population, a fraction that showed no detectable reactivity with antiserum specific for scaffold proteins and presumably contains mostly C capsids. To confirm that the faster migrating band of capsids was indeed composed of DNA-containing C capsids, Vero cells were infected with wild-type HSV-1 and labeled with $^3$H-thymidine from 3-15 h after infection. These cells were harvested, fractionated into cytoplasmic and nuclear fractions, and capsids isolated from the nuclear fraction were resolved by rate-zonal centrifugation as described
above. Each fraction was analyzed for $^3$H-thymidine, precipitated with TCA, and analyzed by SDS-PAGE and western blotting (Fig. 3.7). A peak of $^3$H-thymidine counts was detected in fraction 13 and immunoblotting showed the presence of strong reactivity for the anti-VP5 and anti-UL36 sera within this fraction. In contrast, when these same fractions were reacted with the anti-scaffold antibody, essentially all reactivity was detected in fraction 11. These data further confirm that density gradients were able to resolve populations of B capsids and C capsids, and that the majority of the detectable UL36 is associated with C capsids.
Figure 3.7. Rate-Zonal Centrifugation of $^3$H-Thymidine-Labeled Capsids and Analysis for the Presence of the Tegument Protein UL36.

Capsids were obtained from the nuclear fraction of HSV-1-infected Vero cells that were labeled with 10 µCi per ml of $^3$H-thymidine from 3 to 15 h after infection. The capsids were separated by rate-zonal centrifugation as described in the legend to Fig. 3.3. Prior to TCA precipitation of the proteins within each of the fractions, 50 µl was removed and $^3$H-thymidine cpm in each fraction were determined by liquid scintillation analysis (panel A). Following TCA precipitation, the same fractions were resolved by SDS-PAGE followed by Western blot analysis (panel B). The same blot was sequentially probed with antibodies to the tegument protein UL36 and capsid proteins VP5 and VP21/VP22a (scaffold proteins) as described in the legend to Fig. 3.3.


**DISCUSSION**

To our knowledge, the results within this report are the first to show that specific tegument proteins are added to capsids prior to their exit from the nucleus. This is consistent with the tight interaction of UL36 with virion capsids (103, 207, 313). In fact, Zhou and colleagues suggested that UL36 may be an inner tegument protein associated with pentons of the capsid based on the interpretation of computer generated models from cryoelectron micrographs of HSV-1 virus particles (366). Recent observations by Desai using a UL36 mutant virus, KΔUL36, showed that capsids synthesized in cells lacking the expression of UL36 were located within the cytoplasm; however, they lacked any of the major tegument proteins and did not progress to the envelopment stage of virus assembly (64). The rationale for analysis of the presence of UL37 on capsids within the nuclear fraction was supported by several observations. First, it was recently shown that the UL36 and UL37 gene products of pseudorabies virus, as well as HSV-1, physically interact (145, 338). Second, other studies have shown that viruses not expressing UL37 were not enveloped and formed aggregates in the nucleus and cytoplasm (63). From the above observations, it appears that UL36 and UL37 may associate with capsids before subsequent tegumentation and envelopment events can occur. Thus it is possible that these two proteins are components of the “inner” tegument region and may serve as a platform for the addition of other tegument proteins to the capsid.

The UL36 and UL37 gene products are conserved among all members of the herpesvirus subfamilies (212), which may indicate their key role in the assembly process. In addition, the studies of Desai (63, 64) suggest that these two proteins may be important for targeting the capsid to the proper maturation pathway for the addition of other
tegument proteins and envelopment. Whether the addition of UL36 and UL37 to assembled capsids only occurs within the nucleus cannot be determined from the studies described within this report and is an aspect currently being addressed. Yeast two-hybrid analyses have shown the interaction of UL36 with the tegument proteins VP16 and UL37, and also demonstrated the self-association of UL37 (338). Further understanding of these interactions may help to elucidate the composition of the “inner tegument” region. Interestingly, the Kaposi’s sarcoma-associated herpesvirus homologue of UL36, ORF64, is proposed to bind to the capsid at its N-terminus and attach to the viral envelope at its C-terminus (369).

An unexpected finding from our studies was that UL36 is predominantly associated with C capsids as compared to B capsids. Although major differences in conformation and protein content of B vs. C capsids have not been suggested, subtle differences have been reported (23, 106, 248, 298, 327, 366). For example, studies have shown that three- to four-fold greater amounts of UL25 are associated with C capsids as compared to B capsids, suggesting that UL25 may function to seal the capsid portal after DNA packaging (248, 298). Cryoelectron microscopic observations suggest that the channel within pentons of B capsids appears open, in contrast the pentagonal channel in C capsids appears closed (366). The selective binding of UL36 to C capsids suggests that either the conformation or the selective presence of other capsid or tegument proteins enhances the opportunity for the binding of UL36. Currently, our results do not demonstrate if the binding of UL37 is preferential to C or B capsids. Studies are currently in progress to determine if there is a sequential order for the binding of UL36 and UL37 to capsids.
MATERIALS AND METHODS

Cells and viruses. African green monkey kidney (Vero) cells (ATCC CCL-81) were grown in Dulbecco’s modified Eagles medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 2.25% sodium bicarbonate, 25mM HEPES buffer, glutamine (300 µg/ml), penicillin (100 µg/ml) and streptomycin (131 µg/ml) as described previously (246, 247). Cells were infected in the same medium except the serum concentration was reduced to 2.0 %. HSV-1 KOS strain was used as the wild-type virus (310). An HSV-1 mutant virus (designated K23Z) with a defect in the synthesis of one of the capsid triplex proteins, VP23, and the complementing cell line (designated C32) that expresses this protein, were generously provided by Prashant Desai (Johns Hopkins University) (60, 263). The C32 complementing cell line was grown as described above, with the addition of 1.0 mg/ml G418 (263).

Antibodies. Rabbit polyclonal antibodies to UL36 (207), VP5 (McNabb, unpublished data), VP22 (183) and UL11 (183) have been described previously. Rabbit polyclonal antibody to GFP was generated against His-GFP antigen. Rabbit polyclonal antibody to VP16 was purchased from Clontech. Mouse monoclonal antibodies were used to detect the HSV-1 DNA-binding protein encoded by the UL42 gene (194) and the UL26 gene products (scaffold proteins) (antisera designation MCA406) (238). A rabbit polyclonal antibody (designated 780) to the UL37 gene product was generously provided by Frank Jenkins (University of Pittsburgh) (293). Goat polyclonal antibodies to calnexin and lamin B1 were used to assess the purity of nuclear fractions.

Capsid isolation. Approximately 1.5 x 10^8 Vero cells grown in 18 100 mm plates were infected with HSV-1 at a multiplicity of infection (MOI) of 10 pfu/cell. At
15 h after infection, cells were harvested by scraping, pelleted using low speed centrifugation (225 x g, 10 minutes at 4°C) and washed in phosphate-buffered saline (PBS). Cells were disrupted in NP40 lysis buffer (approximately 9 x 10^6 cells/ml) [0.15 M NaCl, 0.01 M Tris-HCl (pH 7.2), 0.002 M MgCl₂, 1.0% NP-40 (IGEPAL CA-630), 0.005 M DL-dithiothreitol, and 0.01% Protease Inhibitor Cocktail (Sigma)] (103, 208). The nuclei were pelleted using low speed centrifugation (225 x g, 7 minutes at 4°C), and the supernatant was considered the cytoplasmic fraction. The nuclear pellet was washed in NP40 lysis buffer and transferred to a clean conical tube to reduce contaminating cytoplasmic debris that might be stuck to the sides of the tubes. Capsids from the nuclear fraction were isolated as previously described with slight modifications (297). The nuclear pellet was resuspended in NP40 lysis buffer, lysed by three cycles of freeze/thaw and then sonicated for three one-minute pulses. Cell debris was cleared by centrifugation at 8,000 rpm for 30 minutes at 4°C in an SW41 rotor. The supernatant was treated with 100 U DNase at 37°C for 10 minutes and the capsids were pelleted through a 35% (wt/wt) sucrose cushion [prepared in TNE (500 mM NaCl, 1 mM EDTA, 20 mM Tris pH 7.6)] in a SW41 rotor centrifuged at 24,500 rpm for 1 hour at 4°C. The pellet was resuspended in TNE and sonicated three times at 10 seconds each. The sample was layered onto a 20-50% (wt/wt) linear sucrose gradient (prepared in TNE) and centrifuged at 24,500 rpm for 1 h at 4°C in an SW41 rotor. After centrifugation, 0.5 ml fractions were collected from the bottom of the tube. Trichloroacetic acid (TCA) was added to each fraction for a final concentration of 10% TCA and incubated at 4°C for a minimum of one hour. Precipitated proteins were pelleted by centrifugation (14,000 x g for 15 minutes at room temperature), washed in 100% ethanol, dried by centrifugation in a heat-
vac for 5 minutes, resuspended in 2X sample buffer [119 mM Tris-HCl pH 6.8, 19% glycerol, 0.05% bromophenol blue, 3.8% SDS, 9.5% βME, 0.5 M urea], boiled for 5 minutes, and electrophoretically separated on a SDS-polyacrylamide gel (PAGE). After transfer to nitrocellulose membranes, proteins were detected by Western blot analysis with primary antibodies to specific HSV-1 proteins, secondary antibodies conjugated to horseradish peroxidase (Sigma), ECL reagents (Amersham Biosciences, Piscataway, NJ), chemiluminescence autoradiography and Kodak BioMax XAR film. For sequential probing of the same nitrocellulose blot, membranes were stripped in 60 mM Tris-HCl pH 8.0, 2% SDS and 0.75% βME at 56°C for 37 minutes to remove bound antibodies.

**Radiolabeling viral DNA.** To radiolabel the viral DNA associated with C capsids, approximately 1.5 x 10^8 Vero cells grown in 18 100 mm plates were infected with HSV-1 at a MOI of 10 pfu/cell. At 3 h after infection, [methyl-^3H]thymidine (specific activity of 79 Ci/mmol, 1.0 mCi/ml) was added to 5 plates at a concentration of 10 μCi per ml. At 15 h after infection, infected cells were harvested and intranuclear capsids were purified on a linear sucrose gradient as described above. From each of the collected fractions, 50 μl was removed and assayed for ^3H-thymidine activity by liquid scintillation analysis. The remainder of the 0.5 ml fractions was TCA precipitated, resolved on an SDS-PAGE gel and Western blotted for the presence of viral proteins.
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CHAPTER IV

IDENTIFICATION OF INTERACTION DOMAINS IN THE HERPES SIMPLEX VIRUS TYPE 1 TEGUMENT PROTEIN UL37
ABSTRACT

Herpes simplex virus type 1 (HSV-1) UL37 is a 1,123 amino acid tegument protein that self-associates and binds to the tegument protein UL36 (VP1/2). Studies were undertaken to identify regions of UL37 involved in these protein-protein interactions. Residues within the carboxy-terminal half of UL37, amino acids 568-1123, are important for interaction with UL36 in coimmunoprecipitation assays. Coimmunoprecipitation assays also revealed that amino acids 1-300 and 568-1123 of UL37 are capable of self-association. UL37 appears to self-associate only under conditions when UL36 is not present or is present in low amounts, suggesting UL36 and UL37 may compete for binding. Transfection-infection experiments were performed to identify domains of UL37 that complement the UL37 deletion virus, KΔUL37. The carboxy terminal region of UL37 (residues 568-1123) partially rescues the defective infection. These results suggest the C-terminus of UL37 may contribute to its essential functional role within the virus-infected cell.
INTRODUCTION

Herpesvirus virions are composed of three morphologically distinct structures: the capsid, the tegument and the envelope (reviewed in 282). The 150 kbp linear DNA genome is enclosed within the icosahedral capsid. The virion is bounded by a host-derived lipid envelope that contains approximately 12 virus-encoded glycoproteins (reviewed in 282). The proteinaceous region between the capsid and envelope is termed the tegument. More than 25 different virus-encoded proteins, as well as trace amounts of host proteins, make up the tegument region (reviewed in 140, 213).

During herpesvirus replication, capsids are assembled and the viral genome is packaged in the nucleus of the infected cell. The capsid exits the nucleus through an envelopment-deenvelopment process as it acquires an envelope by budding into the inner nuclear membrane and enters the perinuclear space. The envelope subsequently fuses with the outer nuclear membrane and the capsid is released into the cytoplasm (reviewed in 213). It has also been reported that capsids may escape the nucleus by exiting through damaged nuclear pores (177, 353). Detectable quantities of tegument proteins UL36 (VP1/2) and UL37 (41), as well as vhs (269), have been reported on capsids isolated from the nucleus. Other tegument proteins including VP16, VP22 and US3 have been shown to associate with capsids within the perinuclear space (109, 232, 252, 274) suggesting the earliest events in tegumentation occur within these sites. After exiting the nucleus the cytoplasmic capsid travels through the secretory pathway. It has been suggested that some tegument proteins, such as UL16, interact with the capsid as it travels in the cytoplasm on its journey toward the trans-Golgi network (TGN) (209). The capsid obtains a lipid envelope by budding into vesicles derived from the TGN. Many, if not all,
tegument proteins are associated with TGN-derived vesicles through membrane binding domains or through interactions with membrane bound glycoproteins and/or membrane bound tegument proteins. The TGN-membrane-associated tegument proteins and glycoproteins are incorporated into the virion during final envelopment (213).

The morphogenesis and structure of the tegument appear to be complex, with many details only recently being uncovered. With the exception of the innermost layers, the tegument region is described as asymmetrical in nature (117, 366). Recent evidence suggests the tegument region is not a static structure, but instead undergoes a time-dependent maturation process after release from the infected cell (235). The maturation of the virion results in changes in tegument structure including loss of protein symmetry surrounding the capsid and increased resistance to detergent extraction (235). There is also evidence that structural changes in the tegument occur upon binding of the virion to the host cellular receptor heparan (210). Virion binding to the heparan receptor causes the disassociation of at least one tegument protein, UL16, from the capsid (210).

A myriad of protein-protein interactions among tegument proteins have been described. Protein-protein interactions likely play important roles in facilitating the incorporation of tegument proteins into the assembling virion. Two proteins of interest in this study are the tegument proteins encoded by the U_L36 and U_L37 genes. UL36, also known as VP1/2, binds UL37 (145). UL36 is the largest known herpesvirus-encoded protein and the HSV-1-encoded UL36 protein is approximately 270 kDa (119, 126, 313). HSV-1 UL36 interacts with the capsid proteins VP5 and UL25 (54, 207, 254), and is bound tightly to the capsid (103, 313). UL36, and its homologues in all herpesvirus subfamilies, functions as a deubiquitinating enzyme (139, 291). The conserved UL36
tegument protein is important for productive infection, playing roles at early, as well as late times during infection. During translocation of incoming capsids to the nucleus, UL36 associated with the incoming capsid is cleaved, resulting in changes to the capsid that allow the viral genome to be released into the nucleus (136). Studies of HSV-1 and PRV UL36 deletion viruses indicate that in the absence of UL36, DNA filled capsids accumulate in the cytoplasm (in the case of HSV-1) or in the nucleus (in the case of PRV), are not associated with appreciable amounts of tegument and do not undergo secondary envelopment (64, 91, 187, 278).

UL37 encodes a conserved 120 kDa phosphorylated tegument protein (3, 202, 293, 300). UL37 is distributed throughout the cell and contains a nuclear export signal (200, 202, 293, 344). UL37 functions to activate NF-κB signaling by binding TRAF6 (180). The amount of UL37 packaged into the virion is tightly controlled, as increasing expression of the protein does not increase the abundance of UL37 in virions (200).

During virus assembly UL37 may have a function in the cytoplasm as well as the nucleus. In cells infected with a mutant virus containing a deletion of HSV-1 UL37, KΔUL37, DNA-filled capsids accumulate in the nucleus (63); however, in cells infected with another HSV-1 UL37 deletion virus, ΔUL37[86-1035], an accumulation of nuclear capsids was not observed (174). Roberts and colleagues constructed a third HSV-1 UL37 deletion virus, FRΔUL37. Infection with FRΔUL37 did not result in an accumulation of capsids in the nucleus but yielded an accumulation of large aggregates of cytoplasmic capsids, similar to capsid aggregates also observed in KΔUL37-infected cells (63, 278). None of the HSV-1 UL37 deletion viruses yield productive infections (63, 174, 278). In contrast, PRV UL37 is not strictly essential, with titers approximately 100-1,000-fold
lower than wild type and no accumulation of capsids in the nucleus (150, 187). Secondary envelopment is inhibited with HSV-1 and PRV UL37 deletion viruses, resulting in an accumulation of capsids in the cytoplasm (63, 150, 174, 187, 278).

Coimmunoprecipitation and yeast two-hybrid interaction studies have revealed that HSV-1 UL37 may interact with several other viral proteins. In a yeast two-hybrid analysis, UL37 interacts with the capsid proteins VP26 and VP19c (171). UL37 also participates in interactions with other tegument proteins, including UL46 and UL36 (145, 171, 336, 338). Furthermore, UL37 interacts with itself, as indicated by yeast two-hybrid analysis and coimmunoprecipitation studies (171, 338).

Based on its tight association with the capsid, it has been suggested that UL36 is one of the first tegument proteins to associate with the capsid, and acts as a foundation to bind other tegument proteins to the capsid (213). Studies revealing the association of UL36 and UL37 with capsids isolated from the nuclear fraction also support this hypothesis (41). The interaction of UL36 with the capsid may aid or enhance the incorporation of UL36 binding partners, such as UL37 or VP16 (171, 213, 338). UL36 and UL37 physically interact, but the functional importance of the interaction is perhaps only beginning to be understood (145). Desai and colleagues reported that the localization of UL37 in the Golgi complex is dependent on UL36 (62). Furthermore, based upon the work of Roberts and colleagues, UL36 and UL37 appear to rely on one another for incorporation into L particles (278). Interestingly, the incorporation of UL37 and UL36 into L particles may be dependent on VP16, as L particles produced from a PRV VP16 deletion mutant, PRV-ΔUL48, does not contain these proteins (87).
Consistent with this finding, UL36 has been reported to interact with VP16 in yeast two-hybrid screens (171, 338).

Vittone and colleagues have determined that the region containing amino acids 512-767 of HSV-1 UL36 is necessary for binding UL37 (338). Mijatov extended these studies to identify residues F593 and E596 of UL36 as essential for the interaction (216). Furthermore, Fuchs and others reported that deletion of the UL37 binding domain of UL36 does not abolish PRV replication, suggesting that the essential function(s) of UL36 is not dependent on binding UL37 (91). The region of UL37 that participates in its interaction with UL36 as well as the region(s) of UL37 that participates in self-association have not been identified and are the focus of our report. These studies are important for several reasons. First, the interaction of UL36 and UL37 is conserved among the alpha-, beta-, and gammaherpesviruses (25, 145, 171, 285, 336, 338). Furthermore, UL36 plays an essential role in the assembly of HSV-1 and PRV (64, 91, 187, 278). In addition, UL37 plays an essential role for assembly of HSV-1 and varicella zoster virus (VZV) and a critical role in the assembly of pseudorabies virus (PRV) (63, 150, 187, 278, 358). A greater understanding of the interaction between UL36 and UL37 will help elucidate the role(s) that the UL36-UL37 interaction plays during assembly of herpesvirus virions. We have identified a region within the carboxy-terminal half of UL37 to be necessary for binding to UL36. Furthermore, we have determined that both carboxy and amino-terminal regions of UL37 are capable of self-association, and can interact with one another. In a trans-complementation assay, when plasmids encoding UL37 truncation mutants were transfected into cells followed by infection with a UL37
deletion virus, a mutant protein encompassing the carboxy-terminal half of UL37 was found to complement a UL37 deletion virus.
RESULTS

Expression of UL36.512-767HA is dependent on UL37. Vittone and colleagues identified amino acids 512-767 of HSV-1 UL36 as necessary for interaction with UL37 (338). Plasmids encoding residues 512-767 of UL36 and a hemagglutinin (HA) or GFP tag were generated for use in the current study. In studies of CMV, Bechtel and colleagues reported that expression of the UL36 homologue, UL48 (also known as HMWP), is dependent on the presence of the UL37 homologue, encoded by UL47 (also known as HMWP-binding protein) (25). In CMV, the decrease in UL48 protein is not accompanied by a decrease of UL48 mRNA, suggesting that UL47 is required for the stability of UL48 (25). Based on these previous findings, cotransfection experiments were performed to determine if UL37 is necessary to stabilize HSV-1 UL36.512-767HA or UL36.512-767GFP. A plasmid encoding UL36.512-767HA was cotransfected into Vero cells with increasing amounts of plasmids encoding GFP or UL37GFP. 24 h after transfection the cells were lysed in NP40 lysis buffer and analyzed by SDS-PAGE and Western blotting with rabbit anti-HA or anti-GFP antisera to verify protein expression. In a dose-dependent manner, UL36.512-767HA was detected in cells cotransfected with UL37GFP (Fig. 4.1A). However, UL36.512-767HA was not detected when cotransfected with GFP. These results suggest UL37 stabilizes UL36. As similar results were observed with UL36 and UL37 homologues of CMV (25), this phenomenon may be conserved across the herpesvirus family. In contrast, when a GFP-expressing plasmid, UL36.512-767GFP, was cotransfected into cells with increasing amounts of UL46HA or UL37HA, protein expression of UL36.512-767GFP was readily detected in all samples (Fig. 4.1B). This suggests that the GFP fusion protein stabilizes residues 512-767 of
Figure 4.1. Expression of UL36.512-767HA and UL36.512-767GFP in Transfected Cells. (A) UL36.512-767HA expression when cotransfected with increasing amounts of GFP or UL37GFP. Vero cells were cotransfected with 4µg of UL36.512-767HA and increasing amounts of GFP or UL37GFP plasmid DNA. Cells were lysed in NP40 lysis buffer and a portion of the lysate was analyzed for protein expression by Western blotting. Rabbit anti-HA (top panel, αHA) and anti-GFP antibodies (bottom panel, αGFP) were used to detect protein expression. (B) UL36.512-767GFP expression when cotransfected with increasing amounts of UL37HA or UL46HA. Vero cells were cotransfected with 4µg of UL36.512-767GFP and increasing amounts of UL46HA or UL37HA plasmid DNA. Cells were lysed in NP40 lysis buffer and a portion of the lysate was analyzed for protein expression by Western blotting as described above.
UL36 in the UL36.512-767GFP construct such that it is not dependent on UL37 for protein expression. Therefore, the more stable GFP-tagged UL36.512-767 mutant protein was utilized in subsequent assays to investigate the domain(s) of UL37 necessary for interaction with UL36.

**UL36.512-767GFP interacts with UL37 in HSV-1-infected cells.** To determine if the UL36.512-767GFP interacts with virus-expressed UL37, cells were transfected with UL36.512-767GFP and subsequently infected with an HSV-1 mutant virus defective in capsid assembly, K23Z, or a virus lacking expression of UL36, KΔUL36. Cells were lysed in NP40 lysis buffer, the nuclear fraction was discarded and goat anti-GFP antisera was used to immunoprecipitate GFP. Immune complexes were captured by incubating the lysates with Protein G-agarose beads. SDS-PAGE and Western blotting were performed to verify protein expression (Fig. 4.2A) and reveal the interaction of UL36.512-767GFP with UL37 in HSV-1-infected cells (Fig. 4.2B). Virus-expressed UL37 coimmunoprecipitated with UL36.512-767GFP in K23Z-infected cells and KΔUL36-infected cells, indicating that the interaction of UL36.512-767GFP and UL37 does not require capsid assembly or expression of full-length UL36, respectively.

**Identification of the region of UL37 involved in binding UL36.** A series of hemagglutinin (HA) tagged UL37 truncation mutants was constructed and used in coimmunoprecipitation assays to identify the region of UL37 involved in binding UL36 (Fig. 4.3A). N-terminal and C-terminal truncation mutants of UL37, containing a hemagglutinin (HA) tag at the carboxy-terminus, were cotransfected into Vero cells with UL36.512-767GFP. Cells were lysed in NP40 lysis buffer, the nuclear fraction was discarded and anti-HA antisera was used to immunoprecipitate the UL37HA mutant
Figure 4.2. Coimmunoprecipitation of UL37 with UL36.512-767GFP in HSV-1-Infected Cells. Plasmids encoding GFP or UL36.512-767GFP were transfected into cells, infected with the designated viruses 24 h post-transfection and harvested 24 h post-infection. (A) Cells were lysed in NP40 lysis buffer, and protein expression was analyzed by Western blotting using polyclonal rabbit anti-UL37 antisera (top panel, αUL37) and polyclonal rabbit anti-GFP antisera (bottom panel, αGFP). (B) Coimmunoprecipitation of UL37 with UL36.512-767GFP in infected cells. Transfected cell lysates were incubated with goat anti-GFP antibody and then bound to protein-G agarose beads. Immunoprecipitated material was analyzed on a SDS-PAGE gel and transferred to nitrocellulose. Proteins were detected by Western blotting using polyclonal rabbit anti-UL37 antisera (top panel, αUL37) and polyclonal rabbit anti-GFP antisera (bottom panel, αGFP).
Figure 4.3. Coimmunoprecipitation of UL36.512-767GFP with N-Terminal and C-Terminal UL37HA Truncation Mutants. (A) A schematic representation of HA tagged full length, N-terminal and C-terminal UL37 truncation mutants. (B) Expression of UL37HA truncation mutants and UL36.512-767GFP. Vero cells were cotransfected with UL36.512-767GFP and the indicated UL37HA constructs, harvested at 24 h post-transfection and lysed in NP40 lysis buffer. Each cytoplasmic lysate was analyzed by Western blotting using rabbit HA antibody (αHA, top panel) or goat GFP antibody (αGFP, bottom panel). (C) Coimmunoprecipitation of UL36.512-767GFP with UL37 HA constructs. Cotransfected cytoplasmic lysates were incubated with an anti-HA rabbit antibody and then bound to protein-G agarose beads. Immunoprecipitated material was separated on an SDS-PAGE gel and transferred to nitrocellulose. Proteins were detected by Western blotting using a goat anti-HA antibody (αHA, top panel) or goat anti-GFP antibody (αGFP, bottom panel).
proteins. Immune complexes were captured by incubating the lysates with Protein G-agarose beads. SDS-PAGE and Western blotting were performed to verify expression of the cotransfected plasmids (Fig. 4.3B) and identify the UL37HA mutants capable of coimmunoprecipitating UL36.512-767GFP (Fig. 4.3C). As expected, full-length UL37HA coimmunoprecipitated UL36.512-767GFP. UL37 mutants encoding amino acids 1-300 or 1-567 of UL37 did not coimmunoprecipitate UL36. Extension of the amino-terminal mutants to include residues 1-809 restored binding to UL36. Furthermore, mutants UL37.568-1123HA and UL37.301-1123HA were also capable of binding UL36.512-767.GFP. These results suggest that residues 568-809 of UL37 are important for interaction with UL36.

To further address the role that amino acids 568-809 of UL37 play in the interaction with UL36, a UL37 mutant containing an internal deletion of these amino acids, UL37.Δ568-809HA, was constructed (Fig. 4.4A). Vero cells were cotransfected with UL37HA constructs and UL36.512-767GFP, subsequently lysed for coimmunoprecipitation with anti-HA antisera, and protein interactions visualized by SDS-PAGE and Western blotting. Expression of the transfected plasmids in cell lysates is shown in Fig. 4.4B. Immunoprecipitated material is presented in Fig. 4.4C. As also seen in Figure 4.3, mutants UL37.1-809HA, UL37.568-1123HA and UL37.301-1123HA coimmunoprecipitated UL36.512-767GFP. When analyzed in coimmunoprecipitation assays, deletion of amino acids 568-809 within full-length UL37 decreased binding to UL36 to nearly background levels (Fig. 4.4C). This result suggests that amino acids 568-809 of UL37 are functionally important for the interaction of UL36 and UL37, or alternatively this region plays an integral role in the structure of UL37.
Figure 4.4. Analysis of the Ability of UL36.512-767GFP to Interact with a UL37HA Mutant Lacking Residues 568-809. (A) A schematic representation of the UL37HA truncation mutants. UL37HA mutants were cotransfected into Vero cells with UL36.512-767GFP. Immunoprecipitation and Western blot analysis was performed as described in the legend to Fig. 4.3. (B) Expression of the GFP and HA fusion proteins in whole cell lysates was analyzed by Western blotting with rabbit anti-HA (αHA, top panel) and rabbit anti-GFP (αGFP, bottom panel) antibodies. (C) The ability of the UL37HA mutants to bind UL36.512-767GFP was analyzed by Western blotting membranes of the coimmunoprecipitated material with goat anti-GFP antibody (αGFP, bottom panel). Rabbit anti-HA antibody and rabbit TrueBlot HRP-conjugated anti-rabbit secondary antibody was used to verify immunoprecipitated UL37HA constructs (αHA, top panel).
that is necessary for the interaction.

Results shown in Figures 4.3 and 4.4 suggest that amino acids 568-809 of UL37 are necessary for the interaction with UL36. Experiments were performed to determine if these amino acids are sufficient to enable UL36 binding. HA tagged UL37 mutants containing amino acids 568-809, or amino acids extending outward from that region were generated (Fig. 4.5A). Plasmids encoding the UL37HA mutants and UL36.512-767GFP were cotransfected into cells and analyzed by coimmunoprecipitation and Western blotting. Several of the internal UL37 mutants reproducibly exhibited relatively low protein expression as compared to the full length protein (Fig. 4.5B). When the internal UL37 mutants were immunoprecipitated with anti-HA antisera, Western blotting with anti-GFP antisera revealed that UL36.512-767GFP failed to coimmunoprecipitate with any of the internal UL37HA mutant proteins (Fig. 4.5C). These results suggest that although amino acids 568-809 of UL37 are necessary for interaction with UL36, they are not sufficient for this interaction. The failure of the UL37HA mutants to bind UL36 suggests that amino acids outside the region of 568-809 are involved in binding to UL36 and/or maintaining the proper conformation of UL37 necessary for the interaction with UL36.

As shown in Figures 4.3 and 4.4, the C-terminal half of UL37, containing residues 568-1123, is sufficient for binding UL36.512-767GFP. In an effort to define a smaller region of UL37 that is sufficient for UL36 binding, additional HA tagged plasmids encoding internal regions of UL37 were constructed (Fig. 4.6A). UL37HA mutants and UL36.512-767GFP were cotransfected into cells to be used in coimmunoprecipitation
Figure 4.5. Coimmunoprecipitation of UL36.512-767GFP with Internal UL37HA Mutants. (A) A schematic representation of the internal UL37HA truncation mutants. Plasmids encoding internal domains of UL37HA were cotransfected into Vero cells with UL36.512-767GFP. Immunoprecipitation and Western blot analysis was performed as described in the legend to Fig. 4.3. (B) Expression of the GFP and HA fusion proteins in whole cell lysates was analyzed by Western blotting with rabbit anti-HA (αHA, top panel) and rabbit anti-GFP (αGFP, bottom panel) antibodies. (C) The ability of the internal UL37HA mutants to bind UL36.512-767GFP was analyzed by Western blotting membranes of the coimmunoprecipitated material with goat anti-GFP antibody (αGFP, bottom panel). Rabbit anti-HA antibody and rabbit TrueBlot HRP-conjugated anti-rabbit secondary antibody was used to verify immunoprecipitated UL37HA constructs (αHA, top panel).
assays. Protein expression (Fig. 4.6B) and protein interactions (Fig. 4.6C) were visualized by SDS-PAGE and Western blotting. The internal UL37HA mutants reproducibly displayed multiple protein bands, suggestive of protein degradation. Of note, the GFP Western blot of cell lysates (Fig. 4.6B, bottom panel) depicts multiple protein bands in the UL37.489-1021 sample due to incomplete stripping of anti-HA antibody from the membrane before the GFP Western blot was performed. Coimmunoprecipitation with anti-HA antibody showed that UL37 mutants encoding amino acids 391-1021 or 743-1123 do not reproducibly bind UL36.512-767GFP above background levels. However, UL37 mutants containing amino acids 489-1021, 568-1021, or 624-1123 coimmunoprecipitated UL36.512-767GFP; although binding is markedly lower than binding with full length UL37. The smallest mutant of UL37 that facilitates an interaction with UL36 is UL37.568-1021HA. Together, these results suggest that amino acids 624-1021 would also be sufficient for interaction with UL36. To address this issue a UL37 mutant expressing amino acids 624-1021 was constructed and analyzed in coimmunoprecipitation assays as described above (Fig. 4.7A). Surprisingly, UL37.624-1021HA was not able to interact with UL36, although protein expression of this mutant was considerably less than that of full length UL37 (Fig.4.7B and C). Collectively, these coimmunoprecipitation studies indicate that residues 568-809 of UL37 are necessary for interaction with UL36. The carboxy-terminal half of UL37 is sufficient for interaction with UL36, but further truncation of this region results in little, if any, binding to UL36. These results suggest that UL37 is conformationally-sensitive to truncations within the carboxy-terminus.
Figure 4.6. Coimmunoprecipitation of UL36.512-767GFP with Additional Internal UL37HA Mutants.  (A) A schematic representation of the internal UL37 truncation mutants. Plasmids encoding internal domains of UL37HA were cotransfected into Vero cells with UL36.512-767GFP. Immunoprecipitation and Western blot analysis was performed as described in the legend to Fig. 4.3. (B) Expression of the GFP and HA fusion proteins in whole cell lysates was analyzed by Western blotting using rabbit anti-HA (αHA, top panel) and rabbit anti-GFP (αGFP, bottom panel) antibodies. (C) The ability of the internal UL37HA mutants to bind UL36.512-767GFP was analyzed by Western blotting the immunoprecipitated material with goat anti-GFP antibody. Rabbit anti-HA antibody and rabbit TrueBlot HRP-conjugated anti-rabbit secondary antibody was used to verify immunoprecipitated UL37HA constructs.
A

UL37

UL37.391-1021

UL37.489-1021

UL37.568-1021

UL37.624-1123

UL37.743-1123

B

Cell Lysates

UL36.512-767GFP

C

IP: αHA

UL36.512-767GFP

αHA

αGFP
Figure 4.7. Coimmunoprecipitation of UL36.512-767GFP with UL37.624-1021HA. (A) A schematic representation of the UL37HA truncation mutants. Plasmids encoding the UL37HA truncation mutants were cotransfected into Vero cells with UL36.512-767GFP. Immunoprecipitation and Western blot analysis was performed as described in the legend to Fig. 4.3. (B) Expression of the GFP and HA fusion proteins in whole cell lysates was analyzed by Western blotting using rabbit anti-HA (αHA, top panel) and rabbit anti-GFP (αGFP, bottom panel) antibodies. (C) The ability of the UL37HA mutants to bind UL36.512-767GFP was analyzed by Western blotting the immunoprecipitated material with goat anti-GFP antibody. Rabbit anti-HA antibody and rabbit TrueBlot HRP-conjugated anti-rabbit secondary antibody was used to verify immunoprecipitated UL37HA constructs.
Identification of domains involved in self-association of UL37. UL37 has been shown by yeast two-hybrid and coimmunoprecipitation assays to self associate (171, 338). Coimmunoprecipitation assays were performed to investigate if UL37 is capable of self-association in the presence of other viral proteins. Because UL37HA and virus-expressed, untagged UL37 are approximately the same molecular weight and are difficult to distinguish on an SDS-PAGE gel, a cotransfection assay was used to investigate UL37 self-association. Plasmids encoding UL37HA and UL37GFP were cotransfected into cells and infected with viruses defective for capsid assembly (K23Z), UL36 expression (KΔUL36) or UL37 expression (KΔUL37). Cells were lysed in NP40 lysis buffer and protein interactions were assayed by coimmunoprecipitation with rabbit anti-HA antisera. Western blotting with goat antibodies to GFP and HA were used to verify protein expression (Fig. 4.8A). Anti-GFP immunoblots showed that UL37GFP coimmunoprecipitated with UL37HA in HSV-1 infected cells in a manner that is not dependent on the formation of capsids or UL36 expression (Fig. 4.8B).

To ascertain the region(s) of UL37 involved in self-association of the protein, GFP-tagged UL37 mutants were generated (Fig. 4.9A). A plasmid encoding full length UL37HA was cotransfected into cells with plasmids encoding UL37GFP mutants. Protein interactions were assayed by coimmunoprecipitation with anti-GFP antisera. Western blotting with rabbit antibodies to GFP and HA were used to verify protein expression (Fig. 4.9B). Anti-HA immunoblots show that mutants encoding either N-terminal or C-terminal regions of UL37 were capable of interacting with full length UL37HA (Fig. 4.9C). Mutants containing amino acids 1-809, 1-300, 301-1123 or 589-
Figure 4.8. Coimmunoprecipitation of UL37HA and UL37GFP in HSV-1-Infected Cells. Plasmids encoding UL37HA and UL37GFP were transfected into cells and subsequently infected with HSV-1 viruses K23Z, KΔUL36 or KΔUL37 which are defective in capsid formation, UL36 expression and UL37 expression, respectively. Cells were infected at 24 h post-transfection and subsequently harvested 24 h post-infection. (A) Cells were lysed in NP40 lysis buffer, and protein expression was analyzed by Western blotting using goat anti-GFP (top panels, αGFP) and anti-HA (bottom panels, αHA) antibodies. (B) Coimmunoprecipitation of UL37GFP with UL37HA in infected cells. Transfected cell lysates were incubated with rabbit anti-HA antibody and then bound to protein-G agarose beads. Immunoprecipitated material was analyzed on a SDS-PAGE gel and transferred to nitrocellulose. Proteins were detected by Western blotting using goat anti-GFP (top panels, αGFP) and anti-HA antibodies (bottom panels, αHA).
1123 were able to coimmunoprecipitate full length UL37HA (Fig. 4.9C). A construct encoding amino acids 1-567 repeatedly did not interact with UL37HA in binding assays; this is an intriguing observation because a smaller mutant encoding amino acids 1-300 does bind full length UL37HA. This finding suggests that removal of the C-terminal half of UL37GFP may cause the protein to misfold, thus blocking its ability to bind UL37HA. However, further truncation of the UL37GFP mutant from the C-terminus, resulting in UL37.1-300GFP, restored binding to UL37HA, possibly by exposing an amino-terminal UL37 self-association domain that was presumably masked or misfolded in UL37.1-567GFP.

UL37 appears to contain two domains involved in interaction with full length UL37, residues 1-300 and 568-1123. Cells were cotransfected with HA and GFP tagged UL37 constructs and coimmunoprecipitation assays were used to determine if the amino and carboxy-terminal interaction domains bind to one another and if the mutant UL37 domains self interact (Fig. 4.10A). In UL37 binding assays, UL37.1-567GFP, which does not bind full length UL37, was used as a negative control. Immunoprecipitation with anti-GFP antisera and Western blotting with anti-HA antibody revealed that UL37.1-300HA and UL37.568-1123GFP interact in cotransfected cells (Fig. 4.10C). Binding assays also showed that UL37.1-300GFP is capable of binding UL37.1-300HA and that UL37.568-1123GFP is capable of interacting with UL37.568-1123HA (Figs. 4.10C and E, respectively). Fig. 4.10G shows that HA and GFP-tagged mutants containing amino acids 1-567 are not capable of self-association, further suggesting that this mutant protein lacks proper structure that enables self-interaction. These results suggest that UL37 contains two domains involved in self-association of the protein.
Figure 4.9. Coimmunoprecipitation of UL37HA with GFP tagged N-Terminal and C-Terminal UL37 Mutants. (A) A schematic representation of full length, N-terminal and C-terminal UL37 truncation mutants fused to GFP. (B) Expression of the UL37 constructs. Vero cells cotransfected with the indicated constructs were harvested approximately 24 h post-transfection and lysed in NP40 lysis buffer. A fraction of the cytoplasmic lysates was analyzed using rabbit antibodies to GFP (αGFP, top panel) and HA (αHA, bottom panel). (C) Coimmunoprecipitation of UL37GFP constructs with UL37HA. Transfected cell lysates were incubated with goat anti-GFP antibody and then bound to protein-G agarose beads. Immunoprecipitated material was analyzed on a SDS-PAGE gel and transferred to nitrocellulose. Proteins were detected by Western blotting using rabbit antibodies to GFP (αGFP, top panel) and HA (αHA, bottom panel).
Figure 4.10. Interactions of Amino- and Carboxy-Terminal UL37 Self-Association Domains Analyzed by Coimmunoprecipitation-Western Blot Analysis.  (A) A schematic representation of the UL37 N-terminal and C-terminal HA and GFP fusion constructs used in the coimmunoprecipitation assays.  (B,D,F) Expression of cotransfected UL37HA and UL37 GFP mutants.  Vero cells were cotransfected with the indicated plasmids, harvested at approximately 24 h post-transfection and lysed in NP40 lysis buffer.  A portion of each cytoplasmic lysate was analyzed for protein expression using rabbit antibodies to HA (αHA, top panels) and GFP (αGFP, bottom panels).  (C,E,G) Coimmunoprecipitation of UL37 HA-tagged and UL37 GFP-tagged mutant proteins.  Coimmunoprecipitations were performed and analyzed as described in the legend to Fig. 4.9.  Western blotting with rabbit anti-HA (αHA, top panels) and anti-GFP (αGFP, bottom panels) antibodies was used to determine if the UL37HA mutants coimmunoprecipitated with the GFP fusion constructs.
A

B Cytoplasmic Lysates
UL37.1-300HA

D Cytoplasmic Lysates
UL37.568-1123HA

F Cytoplasmic Lysates
UL37.1-567HA

C IP αGFP
UL37.1-300HA

E IP αGFP
UL37.568-1123HA

G IP αGFP
UL37.1-567HA
Mutants encoding either of the interaction domains are capable of self-association with full length UL37 and the two interaction domains can be coimmunoprecipitated in a complex. Furthermore, HA-tagged mutants encoding residues 1-300 or 568-1123 can self-interact with GFP-tagged mutants encoding residues 1-300 or 568-1123, respectively.

Results from UL36-UL37 binding assays indicated that amino acids 568-809 of UL37 are necessary for the interaction with UL36 (Fig. 4.3 and 4.4). To determine if these residues are also necessary for self-association of UL37, we generated a UL37.Δ568-809GFP mutant to be used in coimmunoprecipitation studies. Plasmids encoding UL37.Δ568-809GFP and UL37HA were cotransfected into cells, lysed and analyzed by coimmunoprecipitation with anti-GFP antisera. Immunoblots with anti-HA antisera indicate that residues 568-809 of UL37 are not sufficient for self-association of UL37. UL37.Δ568-809GFP coimmunoprecipitated UL37GFP, indicating that amino acids 568-809 of UL37 are not necessary for the self-interaction (Fig. 4.11C). These results suggest that this region, amino acids 568-809, is integral for the interaction with UL36 but not for self-association of UL37.

**Stoichiometry of UL37 Self-Association.** UL37 interacts with other molecules of UL37, however, the stoichiometry of the interaction is unknown. To address this issue cells were transfected with UL37HA or UL37.1-567HA, as a negative control for self-association. 24 h post-transfection cells were lysed in NP40 lysis buffer, nuclei and cell debris discarded and the cytoplasmic supernatant was loaded onto the top of 5-20% (w/v) sucrose gradients. Centrifugation at 35,000 rpm for 37.5 h at 4°C caused sedimentation of proteins. Spencer and colleagues used a similar approach to characterize the
**Figure 4.11. Coimmunoprecipitation of UL37HA and UL37Δ568-809GFP.** (A) A schematic representation of the UL37GFP constructs cotransfected with full length UL37HA construct. (B) Expression of UL37HA. Vero cells cotransfected with the indicated constructs were harvested at approximately 24 h post-transfection and lysed in NP40 lysis buffer. A portion of the cytoplasmic lysates were analyzed for protein expression using a rabbit anti-HA antibody. (C) Coimmunoprecipitation of UL37Δ568-809GFP with UL37HA. Coimmunoprecipitations were performed and analyzed as described in the legend to Fig. 4.9. Coimmunoprecipitated proteins were detected by Western blotting with rabbit anti-GFP (αGFP, bottom panel) and rabbit anti-HA (αHA, top panel) antibodies.
A

UL37

UL37

UL37.Δ568-809

UL37.Δ568-809

B

Cytoplasmic Lysates

UL37HA

GFP

UL37GFP

UL37.Δ568-809

UL37.Δ568-809

αHA

C

IP αGFP

UL37HA

GFP

UL37GFP

UL37.Δ568-809

UL37.Δ568-809

αHA

αGFP

kDa

150

100

75

50

37

25
multimerization of HSV-1 capsid triplex proteins (315). Bovine serum albumin was loaded onto a separate gradient and analyzed as sedimentation standard. Fractions were collected from the top of the gradient, protein was TCA precipitated, and analyzed by SDS-PAGE and Western blotting with anti-HA antibody. UL37HA sedimented midway down the gradient, with peaks of protein in fractions 6, 8 and 9 (Fig. 4.12A). UL37 is also present in fractions 5, 7, and 10. The approximate molecular weights and sedimentation values (S) of the proteins that sedimented in the fractions were calculated using the Martin and Ames equation and BSA as a standard (193). The molecular weight of UL37 in the peak fractions 6, 8 and 9 was determined to be 94, 148 and 181 kDa, respectively. The molecular weight of UL37 is approximately 120 kDa. Therefore, the sedimentation analysis suggests that UL37 forms dimeric structures, but larger complexes of self-associated UL37 were not detected. The calculated molecular weight of protein found in fractions 9 and 10 (181 kDa and 215 kDa respectively) is less than the anticipated molecular weight of a dimer of UL37HA (240 kDa). It is possible that proteases may cleave UL37HA at the amino terminus, resulting in truncated UL37 proteins that self-associate with the C-terminal self-association domain. UL37.1-567HA, which does not self-associate in coimmunoprecipitation assays, was also analyzed as a negative control (Fig. 4.12A). UL37.1-567HA peaked in fractions 4 and 5, with calculated molecular weights of proteins in these fractions of 49 and 68 kDa respectively (Fig. 4.12B). These results are consistent with those of the coimmunoprecipitation assays suggesting that UL37.1-567HA does not self-associate. Results from this assay suggest that in transfected cells UL37 does not self-associate into protein complexes containing more than two molecules of UL37. These results also suggest that dimers of UL37 may
**Figure 4.12. Stoichiometry of UL37 Self-Association.** (A) Vero cells were cotransfected with UL37HA or UL37.1-567HA. 24 h post-transfection cells were lysed in NP40 lysis buffer, nuclei and cell debris discarded and the cytoplasmic supernatant was loaded onto the top of 5-20% (w/v) sucrose gradients in SW41 tubes and centrifuged at 35,000 rpm for 37.5 h at 4°C. Fractions were collected from the top of the gradients, protein was TCA precipitated, and analyzed by SDS-PAGE and Western blotting with anti-HA antibody. (B) Bovine serum albumin was loaded onto a separate gradient and analyzed as a control. The approximate molecular weights and sedimentation values (S) of proteins in the fractions were calculated using the Martin and Ames equation and BSA as a standard (193).
## Sedimentation Analysis

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<th>Fraction</th>
<th>Distance from meniscus (mm)</th>
<th>S</th>
<th>Approximate molecular weight (kDa)</th>
</tr>
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<td>25</td>
<td>4.7</td>
<td>68</td>
</tr>
<tr>
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<td>7</td>
<td>37</td>
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</table>
have a unique conformation that causes UL37 to sediment slower than expected in sucrose gradients.

**Do UL37 and UL36 compete for binding to UL37?** UL37 contains two self-association domains, amino acids 1-300 and 568-1123. The UL36 binding domain of UL37 overlaps the C-terminal UL37 self-association domain. In the infected cell UL37 and UL36 are present in the cytoplasm and nucleus at approximately the same times. These observations raise the question whether UL36 and UL37 compete for binding to UL37. Coimmunoprecipitation studies were used to determine if UL37 is capable of binding UL37 and UL36 simultaneously or if one binding reaction inhibits the other. Cells were cotransfected with UL37GFP, UL37HA and increasing amounts of UL36.512-767GFP plasmids. Cells were lysed, proteins were coimmunoprecipitated with anti-HA antisera and immune complexes were visualized by SDS-PAGE and Western blotting. Western blotting of cytoplasmic lysates with anti-GFP antibody verified protein expression of both GFP-expressing plasmids (Fig. 4.13A). Western blotting of the immunoprecipitated material with anti-GFP antisera was used to determine if UL37GFP and/or UL36.512-767GFP coimmunoprecipitated with UL37HA. When 0.5 µg of plasmid encoding UL36.512-767GFP was cotransfected into cells with UL37HA and UL37 GFP, anti-GFP Western blots revealed that UL37HA interacts with UL37GFP (Fig. 4.13B). However, as the expression of UL36.512-767GFP protein increased, the amount of UL37GFP that coimmunoprecipitated with UL37HA decreased. The self-interaction of UL37HA and UL37GFP appeared to occur only when UL36.512-767GFP is not present in high quantities. Based on these results it appears that UL37GFP and
Figure 4.13. Coimmunoprecipitation Analysis of UL37HA when Coexpressed with UL37GFP and UL36.512-767GFP. (A) Vero cells were cotransfected with the indicated amounts UL36.512-767GFP, UL37HA and UL37GFP plasmids. Approximately 24 h post-transfection cells were harvested, lysed in NP40 lysis buffer and a portion of the cytoplasmic lysate was analyzed for protein expression by Western blotting using goat anti-GFP antibodies. Arrows indicate the positions of UL37GFP and UL36.512-767GFP. (B) Coimmunoprecipitation of UL37GFP and/or UL36.512-767GFP with UL37HA. Coimmunoprecipitation with anti-HA antisera was performed as described in the legend to Fig. 4.3. Immunoprecipitated material was analyzed by Western blot analysis using a goat anti-HA antibody (αHA, top panel) and goat anti-GFP antibody (αGFP, bottom panel). Arrows indicate the positions of UL37GFP and UL36.512-767GFP in the bottom panel.
A  Cytoplasmic Lysates

B  IP: αHA
UL36.512-767GFP compete for binding to UL37HA. This result suggests that UL37HA does not bind UL37GFP and UL36.512-767GFP simultaneously.

**Identification of domains of UL37 that complement the UL37 deletion virus, KΔUL37.** UL37 is necessary for assembly of HSV-1 virions; in the absence of UL37 proper tegumentation and secondary envelopment do not occur (63, 278). A trans-complementation assay was designed to determine the regions of UL37 that rescue the replication deficient KΔUL37 virus to result in the production of infectious virions. Cells were transfected with the indicated mutant UL37HA plasmids and 24 h later infected with KΔUL37. UL46HA was transfected as a negative control. 48 h after infection, cells and media were harvested and subjected to three rounds of freeze/thaw, cellular debris was pelleted and infectious virions were quantified by plaque assay on BD45 cells (Vero cells that stably express UL37). Western blotting of the transfected-infected cell lysates was performed to verify expression of each of the transfected plasmids (data not shown). The amounts of infectious virions released from cells transfected with UL37HA mutants were determined and are represented as the percent of PFU/ml released from cells transfected with full length UL37HA (Fig. 4.14). Plaque assays revealed that a mutant protein encoding the C-terminal half of UL37, residues 568-1123, rescued the KΔUL37 virus to approximately 12% of the levels released when a plasmid encoding full length UL37HA was transfected (Fig. 4.14). The other tested UL37HA mutants yielded viral titers comparable to the UL46HA control sample. These results indicate that the carboxy-terminal half of UL37 confers partial complementation to the replication defective KΔUL37 virus. This result suggests that this region of UL37, which is involved
in binding UL36 and self-association, may play an essential role in the function of UL37
during infection.
Figure 4.14. Transfection-Infection Experiments to Assess the Ability of UL37HA Mutants to Complement the KΔUL37 Virus. Cells were transfected with the indicated UL37HA constructs, and approximately 24 h later infected with KΔUL37 virus with an MOI of 1. Approximately 48 h after infection the cells and media were harvested and lysed by three cycles of freeze/thaw. Cellular debris was pelleted and the supernatant was tittered on UL37 expressing cells (BD45) to quantitate the amount of infectious virus released. Titrations for each UL37 construct represent the average of a minimum of three independent experiments, error bars represent the calculated standard error of measurement, (SEM). For each independent experiment the titers were compared by determining the percent of virus released as compared to UL37HA (%PFU/ml for the UL37HA sample equals 100% for each experiment).
Rescue of UL37 Deletion Virus with Transfected UL37 Constructs

%PFU/ml as compared to full length UL37HA

UL46HA  UL23HA  1-567HA  1-300HA  301-1123HA  568-1123HA  568-320HA  568-1021HA  624-1123HA  743-1123HA  624-1021HA  delta568-320HA
DISCUSSION

The studies described within this report were designed to identify domains of the UL37 tegument protein involved in interaction with UL36 and self-association. To our knowledge, these are the first studies identifying domains of UL37 involved in these interactions. These studies will help elucidate the role of the interaction between UL36 and UL37 and the role that self-association of UL37 play during viral assembly. A schematic summary of domains and epitopes of UL37 identified by this report and others is presented in Figure 4.15.

Several observations throughout these studies suggested that the conformation of UL37 is sensitive to mutation. First, UL37.1-567HA was the only mutant that did not interact with full length UL37. Although UL37.1-567HA contains the amino-terminal self-association sequence (amino acids 1-300); UL37.1-567HA did not interact with either of the self-association domains (regions 1-300 and 568-1123). These results suggest that, due to truncation, the structural conformation of UL37.1-567HA is altered in such a way that inhibits the self-interaction of UL37. Results from mapping the domain of UL37 involved in UL36 binding also suggest that the conformation of UL37 is sensitive to truncations. The carboxy-terminal half of UL37, encompassing amino acids 568-1123, is sufficient for interaction with UL36. However, deletion of 102 amino acids from the unconserved C-terminus of UL37 yields a mutant, UL37.568-1021HA, that binds UL36 at low levels. Furthermore, UL37.624-1123HA showed low levels of binding to UL36 (Fig. 4.6). Collectively, these results suggested that a smaller construct encoding amino acids 624-1021 should also bind UL36. However, when a plasmid encoding this region was constructed and analyzed in coimmunoprecipitation
Figure 4.15. Schematic Representation of Identified Domains and Epitopes of UL37. Amino acids 1-300 function as a UL37 multimerization domain (N UL37-UL37 dom), an arginine rich region encompasses residues 44-80 (ARR), a leucine zipper motif spans residues 203-224 (L Zip), and a leucine rich region at amino acids 263-272 functions as a nuclear export signal (LRR/NES) (344). The C-terminal half of UL37, amino acids 568-1123, functions as a UL37 self-association domain (C UL37-UL37 dom), is involved in binding UL36 (UL36-UL37 dom) and this region partially complements the KΔUL37 virus in transfected cells (Resc. KΔUL37). UL37 activates NFκ-B through binding TRAF6 at residues 1099-1104 (UL37-TRAF6 dom) (180). Throughout UL37 are 17 dileucine motifs (LL or LI) and 3 tyrosine-based motifs (YXXΦ, where Φ represents a large hydrophobic residue) which may facilitate interactions with clathrin adaptor proteins to promote vesicular trafficking (31, 142, 143, 340). Conserved residues span the length of UL37, with the exception of approximately 100 unconserved amino acids at the amino and carboxy-termini.
assays, UL37.624-1021HA failed to bind UL36 (Fig. 4.7), suggesting that amino acids surrounding this region within the carboxy-terminus are important in maintaining the conformation of UL37 needed to bind UL36. Interestingly, Luxton and colleagues reported that a GFP tag on the amino-terminus of PRV UL37 was deleterious for viral growth, perhaps because GFP at this position disrupts the conformation of UL37 (186).

The UL37 domains involved in interaction with UL36 and self-association overlap in the carboxy-terminal region of UL37. We examined if UL37 binds to molecules of UL36 and UL37 at the same time, forming a complex. From cells cotransfected with UL37HA, UL37GFP and UL36.512-767GFP, we observed that as the amount of UL36.512-767GFP protein increased, the amount of UL37GFP that coimmunoprecipitated with UL37HA decreased (Fig. 4.13). Thus, it appears that UL36.512-767GFP competes with UL37GFP for binding to UL37HA. It is important to note that in this coimmunoprecipitation analysis a fragment of UL36 inhibits self-association of UL37; however, it is unknown whether full length UL36 also inhibits UL37 self-association.

The role that self-association of UL37 may play during infection is unclear. When UL37HA and UL37GFP are cotransfected into cells that are subsequently infected with HSV-1, the self-association of UL37HA and UL37GFP is readily detected in coimmunoprecipitation assays (Fig. 4.8). Although UL37 is overexpressed through transient transfection in this experimental design, it appears that UL37 does self-associate within the infected cell. Fuchs and colleagues reported that deletion of the domain of UL36 necessary for binding UL37 does not abrogate PRV virus production, but reduces viral titers by 50-fold and produces capsids within the cytoplasm in large ordered
structures (91). Therefore, it appears that in PRV, UL36 is not required to interact with UL37 in order to execute its essential function. UL37 plays a critical role for assembly of HSV-1, but is not strictly essential for PRV replication. The function and/or necessity of the UL36-UL37 interaction may differ between HSV-1 and PRV. It is not yet known whether UL37 needs the UL36-UL37 interaction to perform its essential function during HSV-1 infection. Similarly, it is unknown if the self-interaction of UL37 plays a vital role in virus replication. Exactly how the competition between UL36 and UL37 to bind UL37 may impact virus assembly is unclear.

The amount of UL37 packaged into virions is tightly controlled (200). Furthermore, relatively few copies of VP1/2 are packaged into virions; an estimated 100-150 copies per virion (119). Based upon the incorporation of UL36 and UL37, it is possible that the protein levels of UL37 and UL36 within the infected cell are tightly controlled. UL36 protein levels have been reported to be dependent on the presence of UL37 (Fig. 4.1 and reference 25). Proper regulation of the protein levels of UL36 and UL37 in the infected cell will also impact competition between UL36 and UL37 for binding with other molecules of UL37. We speculate that the self-association of UL37 and the interaction of UL37 with UL36 play roles at different sites of the infected cell. Although UL37 and UL36 are binding partners, they may not always be abundant at the same locations in the cell, thus allowing UL37 to self associate in some locations and bind UL36 in others.

To identify the regions of UL37 that perform necessary functions for assembly of infectious particles, plasmids encoding regions of UL37 were transfected into cells that were subsequently infected with the UL37 deletion virus, KΔUL37. Cells and media
were harvested and analyzed by plaque assays to identify the regions of UL37 that rescue the replication defective virus. Surprisingly, the only region of UL37 that provided partial complementation was the region encoding amino acids 568-1123. The domain involved in UL36 binding and a self-association domain are located within this region. However, other mutants that bind to UL36, such as UL37.301-1123HA and UL37.1-809HA did not rescue the mutant virus. Similarly, other UL37 mutants that self-associate did not complement KΔUL37. These results are difficult to interpret. It is possible that some UL37 mutant proteins may perform a dominant-negative function in the infected cell, interacting with binding partners, but in a manner that disrupts virus assembly. Similarly, it is also unclear if the failure of some UL37 mutant proteins to rescue KΔUL37 may be attributed to aberrant conformational structures that inhibit UL37 from performing essential function(s) during infection. Several mutants that bind UL36 and/or self-associate also did not rescue the UL37 deletion virus. These results suggest that UL37 performs a function, in addition to self-association and UL36 binding, that maps to residues 568-1123 and is necessary for productive infection. The specific essential function(s) that UL37 plays during virus assembly remains to be determined.
MATERIALS AND METHODS

**Cells and Viruses.** Vero cells (ATCC CCL-81) were grown in Dulbecco’s modified Eagles Medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 2.25% sodium bicarbonate, 25mM HEPES buffer, glutamine (300 μg/ml), penicillin (100 μg/ml) and streptomycin (131 μg/ml). Infected cells were grown in DMEM with a reduced FBS concentration of 2%. HSV-1 KOS strain was used as the wild type strain (310). A UL37 deletion virus, KΔUL37, and Vero cell line that stably expresses UL37 (BD45), were kind gifts from Prashant Desai (63). BD45 cells were grown in DMEM with 5% FBS.

**Antibodies.** Rabbit anti-HA antisera used for Western blotting and immunoprecipitation was purchased from Sigma. Goat anti-HA antibody used for Western blotting was purchased from Santa Cruz. Rabbit polyclonal anti-GFP antibody used for Western blotting was kindly provided by John Wills, Penn State College of Medicine. Goat polyclonal anti-GFP antibody used for Western blotting and immunoprecipitation was purchased from Rockland. Rabbit TrueBlot HRP-conjugated anti-rabbit antibody was purchased from eBioscience.

**Cloning.** The UL37 gene was amplified from the HSV-1 KOS genome by PCR amplification using Platinum Pfx polymerase (Invitrogen). A forward primer containing a Bg/II site 50 bp upstream of the start codon of UL37 and a reverse primer containing an EcoRI site in place of the stop codon of UL37 were used for amplification. The DNA product was digested with EcoRI and Bg/II and ligated into the vector pEGFP-n2 (Clontech) also digested with the same restriction enzymes. Two separate clones produced from separate PCR reactions were sequenced and compared. Amino acid
sequences of the UL37 codons were identical and one plasmid was selected and designated pUL37GFP.

The pUL37HA plasmid was generated by excising the UL37 fragment from the pUL37GFP plasmid, described above, using EcoRI and BglII. This fragment was ligated into a hemagglutinin (HA) containing plasmid, pUL46HA, (231) that was digested with the same restriction enzymes. The parental vector of pUL46HA was the pEGFP-n2 GFP vector that was previously modified to replace the GFP sequence with the HA sequence (184). The newly insert of the newly constructed plasmid, pUL37HA, was sequenced to ensure that UL37 was in frame with HA.

UL37 C-terminal GFP and HA truncation mutants were constructed by using the forward primer used to make pUL37GFP and a reverse primer that contains an EcoRI site immediately downstream of the truncation site. The PCR products were digested with BglII and EcoRI and ligated into pUL37GFP or pUL46HA digested with the same restriction enzymes. The resulting inserts were sequenced to ensure that they contained the specified UL37 truncation sequences and were in frame with GFP or HA.

To construct the UL37HA N-terminal truncation mutants the UL37HA plasmid was modified to contain a HindIII restriction enzyme site immediately upstream of the start codon of UL37. The QuickChange II XL Site-Directed Mutagenesis kit (Stratagene) was used according to the manufacturer’s instructions, to insert the HindIII site into pUL37HA to generate pUL37HA-HindIII. pUL37HA-HindIII contains 50 bp of genomic sequence upstream of the UL37 start site, followed by a HindIII restriction site immediately upstream of the first UL37 amino acid. UL37HA N-terminal truncation mutants were amplified with forward primers containing a HindIII site and start codon
immediately upstream of the first codon of the specified truncation mutant. The reverse primer used was the same reverse primer utilized to create pUL37HA and contains an EcoRI restriction site. The above mentioned primers were used to amplify the mutant UL37 inserts, which were then digested with HindIII and EcoRI and ligated into pUL37HA-HindIII previously digested with the same enzymes. The resulting N-terminal UL37HA truncation mutants contained 50 bp of genomic upstream sequence, a start codon, the truncated amino acid sequence and the HA tag. The UL37HA truncation mutant plasmids were sequenced to verify the correct mutant UL37 coding regions. The UL37GFP N-terminal truncation mutants were constructed by excising the UL37 mutant fragments from the respective UL37HA plasmids using EcoRI and BglII and then ligating the sequences into pUL37GFP previously digested with the same enzymes.

Plasmids containing internal sequences of UL37 were made by amplifying the UL37 sequence with forward primers containing a HindIII site and start codon immediately before the first UL37 codon of the mutant protein. Reverse primers contained an EcoRI site that was immediately downstream of the last UL37 codon of the mutant protein. The inserts were amplified by PCR, digested with HindIII and EcoRI and ligated into pUL37.568-1123HA previously digested with the same enzymes. The resulting plasmids contain 50 bp of HSV-1 sequence upstream of the UL37 start codon, followed by a methionine start codon, the internal UL37 sequence and the HA tag.

UL37Δ568-809.HA and UL37Δ568-809.GFP were generated using the QuickChange II XL Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer’s instructions. PCR primers complementary to nucleotides encoding amino acids 563-567 and 810-814 were used to generate a PCR product from UL37HA and
UL37GFP templates, looping out codons 568-809 of UL37. Clones were screened by restriction enzyme digestion and sequenced to verify the removal of codons 568-809.

The UL36.512-767 gene fragment was amplified from HSV-1 KOS genomic DNA by PCR amplification using Platinum Pfx polymerase (Invitrogen). A forward primer containing a BglII site and an ATG start sequence immediately before codon 512 and a reverse primer containing a MfeI site directly after codon 767 was used to amplify codons 512-767 of UL36. The amplified DNA was digested with BglII and MfeI and ligated into the vector pEGFP-n2 (Clontech) digested with BglII and EcoRI. The resulting plasmid, pUL36.512-767GFP, was sequenced to verify the correct amino acid sequence and that the gene fragment was in frame with GFP.

**Coimmunoprecipitation.** Vero cells grown to approximately 90-95% confluency in 60 mm or 100 mm plates were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Approximately 20-24 h post-transfection cells were washed twice with phosphate-buffered saline (PBS) and harvested by scraping. Cells were pelleted by centrifugation at 1000 x g for 10 min at 4°C and resuspended in 1% NP40 lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2mM MgCl, 1% NP40, 0.1% Sigma protease inhibitor cocktail). Cells were then incubated on ice for 30 min and nuclei were pelleted by centrifugation at 1000 x g for 10 min at 4°C. The cytoplasmic fraction was clarified further by centrifugation at 14,000 rpm for 5 min at 4°C in a microfuge. The cytoplasmic lysate was precleared for approximately 1 h with protein G-agarose beads (Roche) that were washed three times in lysis buffer. Protein G-agarose beads were pelleted at 14,000 rpm for 5 min at 4°C in a microfuge. Immunoprecipitation antibody was added to the precleared samples and rocked at 4°C for
a minimum of 1 h. Protein G-agarose beads were added to the samples and rocked overnight at 4°C. Immune complexes were washed three times in lysis buffer. 2x sample buffer (3.6% SDS, 18% βME, 114 mM Tris pH 6.8, 0.05% bromophenol blue and 18% glycerol) was added to the beads and the immunoprecipitated material was separated by SDS-PAGE, transferred to nitrocellulose membranes and analyzed by Western blotting using the antibodies mentioned above, ECL reagents, and autoradiography using Kodak BioMax XAR film. For sequential probing of the same nitrocellulose membrane, blots were submerged in stripping buffer (60 mM Tris-HCl pH 8.0, 2% SDS and 0.75% βME) at 56°C for approximately 35 minutes to remove bound antibodies.

**Assay to determine stoichiometry of UL37 self-association.** Vero cells grown in 60 mm plates to approximately 90-95% confluency were transfected with the indicated plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Approximately 20-24 h post-transfection cells were washed two times in PBS, harvested by scraping and resuspended in 1% NP40 lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2mM MgCl₂, 1% NP40, 0.1% Sigma protease inhibitor cocktail). Cells were incubated on ice for 30 min and nuclei were pelleted by centrifugation at 1000 x g for 10 min at 4°C. The cytoplasmic fraction was clarified further by centrifugation at 14,000 rpm for 5 min at 4°C in a microfuge, pelleted material was discarded. Approximately 1/3 of the cytoplasmic lysate was layered on top of linear 5-20% (w/v) sucrose gradients (dissolved in 1% NP40 lysis buffer without protease inhibitors) that were prepared using a BioComp Gradient Master. The gradients were centrifuged for 37.5 h at 35,000 rpm at 4°C in Beckman SW41 tubes. After centrifugation approximately 0.75 ml fractions were collected from the top of the gradient using a
Brandel fraction collector. 25 μg bovine serum albumin (BSA) was added to each fraction to increase protein concentration before the addition of 30% trichloroacetic acid (TCA) to each fraction for a final concentration of 10%. Proteins in the fractions were TCA precipitated at 4°C overnight, pelleted via microcentrifugation, resuspended in 2x sample buffer and separated by SDS-PAGE. Protein sedimentation was analyzed by Western blotting using rabbit anti-HA antisera, ECL reagents, and autoradiography using Kodak BioMax XAR film.

The calculation of molecular weights and sedimentation coefficients (S) were determined based on comparison with the sedimentation of BSA using the method of Martin and Ames \[ \left( \frac{\text{MW}_{\text{unknown}}}{\text{MW}_{\text{standard}}} \right)^{2/3} = \frac{\text{distance traveled by unknown}}{\text{distance traveled by standard}} \] (193). The sedimentation coefficient was also determined using the Martin and Ames method \[ \left( \frac{S_{\text{unknown}}}{S_{\text{standard}}} \right) = \frac{\text{distance traveled by unknown}}{\text{distance traveled by standard}} \]. SDS-PAGE and Coomassie Blue staining was used to determine the position of BSA within the sucrose gradients.

**Trans-Complementation Assay.** Vero cells grown in 60 mm plates to approximately 90-95% confluency were transfected with the indicated plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Approximately 24 h after transfection cells were infected with KAUL37 HSV-1 at an MOI of 1. Infected cells were grown in 3.5 ml of DMEM containing 2% FBS. 48 h after infection media and cells were harvested by scraping. Cells were lysed by three cycles of freeze thaw and cell debris was pelleted by centrifugation at 750 x g for 10 min at 4°C. Cellular debris was analyzed by SDS-PAGE and Western blotting to verify expression of the transfected plasmids. Media supernatant was stored at -80°C before analysis by
plaque assay. Infectious virions in the media supernatant were calculated by plaque titration assays on BD45 cells. Titrations were performed in duplicate. Each plasmid was analyzed for trans-complementation a minimum of three times and results of the independent titrations were averaged and presented as percent PFU/ml as compared to full length UL37HA.
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CHAPTER V

DISCUSSION
The assembly of herpesvirus particles is a complex process that begins in the nucleus, continues in the cytoplasm and culminates with envelopment of virus particles at vesicles derived from the TGN. Over 25 virus-encoded tegument proteins are packaged into virions in a complex process. Most tegument proteins localize to multiple locations in the infected cell and interact with several tegument proteins and/or glycoproteins. Therefore, in many cases the specific cellular locations of tegument incorporation and the mechanisms by which proteins associate with assembling virus particles remain elusive. The studies presented in this dissertation provide the first evidence that UL36 and UL37 associate with capsids in the nucleus. This work is among the initial reports of tegumentation occurring within the nucleus. This novel finding, however, has generated several more questions regarding the details of virion incorporation of UL36 and UL37. UL37 interacts with UL36 and also self-associates. Subsequent experiments presented in this dissertation identified and characterized domains of UL37 involved in these interactions. Presented below is a discussion of the insights these data have provided, the limitations of these data, questions surrounding the incorporation of UL36 and UL37 that remain to be answered and speculative models/roles for these proteins during assembly.

UL36 AND UL37 ASSOCIATION WITH CAPSIDS AND VIRION INCORPORATION

The work presented in Chapter III reveals the association of UL36 and UL37 with capsids isolated from the nucleus of HSV-1-infected cells. Extensive analysis was performed to verify the purity of the nuclear fraction including: biochemical analysis for a cytoplasmic marker protein, analysis of nuclear capsids for the presence of TGN-
associated tegument proteins and intentional contamination with (and subsequent removal) of labeled cytoplasmic material. These control experiments repeatedly yielded convincing results showing that the nuclear fraction was free of cytoplasmic material. Capsids isolated from detergent treated nuclear fractions contained detectable amounts of UL36 and UL37. Interestingly, UL36 appeared to associate only with C capsids and was not detected on A capsids or B capsids. These novel studies were among the first to suggest that tegumentation begins within the nucleus, and includes the incorporation of two essential tegument proteins, UL36 and UL37.

CONTRASTING STUDIES REGARDING THE ASSOCIATION OF UL36 AND UL37 WITH INTRANUCLEAR CAPSIDS

The experiments contained within this dissertation provide solid evidence for the association of UL36 and UL37 with capsids isolated from the nuclear fraction. There are several remaining questions and contrasting reports regarding the association of UL36 and UL37 with nuclear capsids. Several laboratories have analyzed capsids from the nucleus of HSV-1 or PRV-1-infected cells using a variety of methods and did not detect these proteins in association with intranuclear capsids. Several studies failed to detect PRV UL36 and/or UL37 on perinuclear capsids when analyzed by immunoelectron microscopy (109, 145, 221). It is possible that the sites of incorporation of UL36 and UL37 differ among PRV and HSV-1, and PRV UL36 may not be added to the virion before the capsid exits the nucleus. Numerous contradictory findings regarding the cellular localization and incorporation of PRV and HSV-1 UL36 have been reported. Several groups have reported, in contrast to HSV-1 UL36 (1, 207, 224), that PRV UL36
does not localize to the nucleus of PRV-infected cells (145, 172, 221). Furthermore, it is also possible that UL36 antisera used in the aforementioned immunoelectron microscopy studies was not suitable to detect small quantities of UL36 bound to capsids due to poor avidity of the antisera and/or the UL36-capsid interaction masking immunogenic UL36 epitopes.

In addition, two studies isolated HSV-1 capsids from nuclei of cells lysed in Triton X-100. Wolfstein and colleagues did not detect UL37 or UL36 on nuclear B or C capsids by Western blotting (355). Trus and coworkers analyzed the protein content of nuclear A and C capsids by Coomassie blue staining and did not observe stained protein bands corresponding to the molecular weights of UL36 or UL37 (334). The contradictory findings between these results and those presented in this dissertation are not easily explained. It is possible that the nuclear capsids isolated by Trus and colleagues contained UL36 but at a level that was not detectable by Coomassie blue staining. In my experience it is difficult to visualize capsid-associated and virion-associated UL36 by Coomassie blue staining or isotopic labeling. Trus and colleagues isolated highly purified capsids by using two sucrose gradient purification steps. Following the first gradient purification step, the capsid fractions were resuspended in TNE buffer and pelleted without a sucrose cushion. Although unlikely, it is possible that high speed pelleting of capsids without a sucrose cushion may cause proteins to dissociate from capsids. Finally, perhaps the most significant difference in the isolation procedures of the contrasting studies is the use of lysis buffer containing 1% Triton X-100 detergent as compared to 1% NP40 (Igepal CA-630) used to lyse cells in the studies described in this dissertation. Triton X-100 and NP40 are non-denaturing nonionic
detergents with similar structure and are considered by many investigators to be interchangeable in biological applications. However, according to the chemical manufacturer Sigma-Aldrich, Triton X-100 is slightly more hydrophilic than NP40, and the detergents should not be considered functionally interchangeable in most applications. It is possible that the more hydrophilic detergent Triton X-100 removed some or all UL36 from nuclear capsids, whereas lysis buffers containing NP40 caused less (if any) UL36 to dissociate from capsids. It is important to note that the initial studies reporting the tight association of UL36 with capsids were done using 1% NP40 lysis buffer and resulted in little, if any, UL36 release from capsids (103, 207, 313). Careful examination of Wolfstein’s studies suggests that Triton X-100 treatment of HSV-1 virions did indeed cause the release of UL36 from capsids, as indicated by the presence of UL36 in supernatant-containing-fractions following the centrifugation step to pellet capsids (355). Direct comparison of the protein content of nuclear capsids isolated with NP40 and Triton X-100 has not been reported.

Other published studies provide evidence that supports the model of UL36 incorporation within the nucleus. Abaitua and coworkers identified a functional and conserved NLS in HSV-1 UL36 (1). This group also showed that full length UL36 colocalizes with VP26 and VP5 within capsid assemblons of the nucleus (1). Furthermore, they reported that the UL36 NLS is required to functionally complement a UL36-deletion virus (1). Additionally, work by Coller and colleagues also suggests that UL36 binds to capsids within the nucleus. They reported that the C-terminal domain of PRV UL36 (the capsid binding domain, cbd) binds UL25 in the nucleus and localizes to capsid assemblons in infected cells where it becomes packaged into virions (54). When
viewed by fluorescence microscopy, the UL36 cbd is associated with capsids located within the nucleus (54). The incorporation of the PRV UL36 cbd is an example of nuclear tegumentation and further demonstrates the ability of UL36 to bind capsids within the nucleus.

EGRESS OF CAPSIDS FROM THE NUCLEUS

In our studies capsids were purified from the nuclear fraction of detergent-treated cells. Detergent treatment of nuclei removes the outer nuclear membrane, which presumably releases perinuclear capsids that have undergone primary envelopment. Therefore, tegument proteins associated with capsids isolated from detergent-treated nuclei must interact with the capsid prior to budding into the inner nuclear membrane. In other words, UL36 and UL37 associate with capsids prior to primary envelopment. This raises the question: Do UL36 and UL37 remain associated with capsids after they undergo primary envelopment and are subsequently released into the cytoplasm? UL31 and UL34, localized to the inner nuclear membrane, are incorporated into primary enveloped virions and are released during fusion of the primary envelope with the outer nuclear membrane. It is possible that UL36 and UL37 also dissociate from the capsid as the primary envelope is lost. Circumstantial evidence, however, suggests that this is not the case.

The populations of UL36 and UL37 that associate with nuclear capsids likely remain associated with capsids during assembly and are found in mature virions. Several observations support this hypothesis. The first line of evidence to support this claim is the ability of UL36 to remain tightly associated with capsids during detergent and urea
treatment of virions when other tegument proteins are released (103, 313). UL36 also interacts with the major capsid protein VP5 and the capsid-bound tegument protein UL25 (54, 207, 254). UL37, in addition to binding UL36, interacts with capsid proteins VP26 and VP19c in yeast two-hybrid assays (171). Collectively, these observations suggest that UL36 binds the capsid tightly through direct contact with capsid proteins and capsid-bound proteins, therefore the removal of UL36 upon deenvelopment seems unlikely. Although the VZV and EBV homologues of UL36 and UL37 interact with UL31 homologues in yeast two-hybrid analysis (Table 2.4) (81, 336), there is no evidence that HSV-1 UL36 or UL37 interacts with UL31 or UL34 to possibly facilitate removal of the tegument proteins from the capsid during deenvelopment at the ONM.

The second factor that suggests that nuclear populations of UL36 and UL37 are not removed from capsids during nuclear egress is the fact that UL36 and UL37 play significant roles in microtubule-based cytoplasmic trafficking of capsids (187, 355). Therefore, efficient capsid transport would require the incorporation of UL36 and/or UL37 before capsids exit the nucleus or directly thereafter. It seems unlikely and inefficient for capsids to associate with UL36 and UL37 in the nucleus, lose these proteins upon nuclear exit, and then quickly bind UL36 and UL37 upon capsid release into the cytoplasm. For these reasons, it appears that the populations of UL36 and UL37 that are bound to nuclear capsids remain associated during nuclear egress to facilitate capsid transport during viral egress. To thoroughly test whether UL36 and UL37 remain capsid-associated during nuclear egress, the protein content of capsids needs to be analyzed immediately after release of capsids from the perinuclear space. An in vitro
nuclear egress assay has been described and may be useful in the future to address this issue (271).

UL36 ASSOCIATES SPECIFICALLY WITH C CAPSIDS

UL36 appears to selectively associate with C capsids, but not A capsids or B capsids. Interestingly, initial studies by Gibson and Roizman characterizing different capsid forms also reported that UL36 was bound to C capsids but not A and B capsids (103). Major differences in the surface protein components of A, B and C capsids have not been reported. However, subtle differences in the amounts of capsid-bound UL25 have been reported (237, 248, 326, 334). A capsids and B capsids appear to contain about 15-40 copies per capsid, and C capsids appear to contain about 50-80 copies per capsid (334). The interaction of UL36 and UL25 likely facilitates the association of UL36 with the capsid and the greater amount of UL25 on C capsids possibly contributes to the selective binding of UL36 to C capsids. vhs has been reported to associate with capsids within the nucleus; however, vhs binds nuclear B and C capsids (269). Therefore, the presence of vhs likely does not affect the selectivity of UL36-capsid binding. There are also subtle differences in the capsid surface structures of B capsids and C capsids. Zhou and colleagues reported that the penton channels of C capsids are fully closed, however penton channels of B capsids are constricted by VP5 protrusions, but are not fully closed (366). Because UL36 is suggested to bind directly to the major capsid protein VP5 and capsid-bound UL25, subtle differences in surface structure of A, B and C capsids may inhibit or facilitate UL36 binding. This dissertation work did not include the isolation and analysis of procapsids. Since major differences exist in the
structure and composition of procapsids as compared to angular capsids (33, 238, 332, 368), it is unlikely that UL36 and UL37 bind procapsids, although this proposal has not been tested experimentally.

**A MODEL OF UL36 AND UL37 TEGUMENTATION IN THE NUCLEUS**

UL36 and UL37 are interaction partners and associate with nuclear capsids. The question naturally arises: Do UL36 and UL37 associate with capsids as a complex? Or, do UL36 and UL37 bind capsids in a sequential fashion? The ability of UL37 to bind B capsids (which appear to lack UL36) suggests that detectable amounts of UL37 can bind capsids independently of UL36 (Fig. 3.3). Preliminary results analyzing the protein content of nuclear capsids produced by an HSV-1 UL36 deletion virus suggested that UL37 was partially dependent on the presence of UL36 for capsid association, although the interaction of UL37 with nuclear capsids was not abolished in the absence of UL36 (data not shown). However, recent work by Ko and colleagues showed that capsids isolated from whole cell lysates of cells infected with an HSV-1 UL36 deletion virus lacked UL37 and VP16 proteins, suggesting that UL37 and VP16 require UL36 for virion incorporation (151). Small amounts of UL37 appear to associate with B capsids; however, B capsids do not mature into infectious particles and are considered to be the result of failure to properly initiate DNA packaging. Therefore, it is unclear if the association of UL37 with B capsids is relevant to the proper maturation pathway of infectious virions.

Since UL36 binds the major capsid protein, VP5, and capsid-associated UL25, and is bound tightly to the capsid, it has been suggested that UL36 is the first tegument
protein to associate with capsids (140, 212). Furthermore, cryoelectron microscopy studies by Zhou and colleagues showed that a large tegument protein, presumably UL36, is symmetrically located on the capsid around pentameric vertices (366). Preliminary experiments performed in our laboratory showed that in the absence of UL37, the amount of UL36 associated with nuclear capsids appeared to be dramatically decreased (data not shown). However, interpretation of these results was complicated by the fact that expression of UL36 was significantly decreased in the absence of UL37, a phenomenon that has been described by others (25). In contrast, a recent study by Ko and colleagues reported that UL36 was detectable on capsids isolated from whole cell lysates of cells infected with a HSV-1 UL37 deletion virus (151). This observation suggests that UL36 is capable of binding capsids independently of UL37. In PRV, immunoelectron microscopy showed that UL36 is associated with cytoplasmic capsids produced by a UL37 deletion virus, further supporting the idea that UL36 is incorporated prior to final envelopment in a UL37-independent manner (145).

Although UL36 may bind capsids in the absence of UL37, it is probable that in wild-type infection UL36 interacts with UL37 before binding to capsids because UL37 enhances protein stability of UL36 (Fig. 4.1 and 25). UL36 and UL37 likely interact in the cytoplasm, translocate to the nucleus via the conserved UL36 NLS (1) and bind to pentons of DNA-filled capsids (Fig. 5.1). It is important to note, however, that other viral proteins are not required for UL37 localization in the nucleus (293). The UL36 NLS is necessary for a plasmid encoding UL36 to rescue an HSV-1 UL36 deletion virus in trans-complementation assays (1), suggesting that the NLS of UL36 plays an essential role during virus assembly. It is likely that UL36 localization in the nucleus and association
**Figure 5.1. Model of UL36 and UL37 Association with Capsids in the Nucleus.** UL36 and UL37 associate with intranuclear capsids. In HSV-1 infected cells it is unclear if UL36 and UL37 form a complex before binding C capsids or if they bind capsids independently of one another. (A) In HSV-1-infected cells UL36 and UL37 likely interact in the cytoplasm, translocate to the nucleus and bind pentameric capsomers. (B) In the absence of UL37 expression, UL36 may localize to the nucleus and bind capsids independently of UL37. UL36 protein stability appears to be dependent on UL37 expression, therefore the relevance of this scenario is uncertain. (C) UL37, but not UL36, is associated with nuclear B capsids. B capsids do not mature into infectious virions. Therefore it is unclear if the association of UL37 with B capsids is relevant to the proper maturation pathway of infectious virions.
with nuclear capsids is required for subsequent tegumentation and assembly of HSV-1 virions. Based on previous studies, it appears that UL36 may facilitate the association of UL37 with DNA filled C capsids (151). Presumably the large tegument protein, UL36, can bind UL37 and UL25 simultaneously, as UL36 utilizes amino acids at the extreme carboxy-terminus to bind UL25 and residues at the amino-terminus to interact with UL37. The binding of UL25 to C capsids is thought to act as a signal for egress of DNA-filled capsids from the nucleus (147, 334). The association of UL36 and UL37 with nuclear capsids, although not essential for nuclear egress, may play a role in facilitating exit from the nucleus. In fact, Desai and colleagues observed an accumulation of capsids aggregated in the nucleus of cells infected with the HSV-1 UL37-deletion virus, KΔUL37 (63). Furthermore, Luxton and coworkers also reported a reduction in nuclear egress in cells infected with a PRV UL37 deletion virus and a PRV UL36 deletion virus (187).

**POTENTIAL INCORPORATION OF UL36 AND UL37 IN THE CYTOPLASM**

Several observations suggest that UL36 and UL37 are not only incorporated into virions within the nucleus, but significant amounts may also be incorporated as the virus acquires an envelope by budding into vesicles derived from the TGN. First, UL37 localizes to the Golgi complex via its interaction with UL36 (62). Additionally, membrane flotation analysis of the cytoplasmic fraction of cells infected with an HSV-1 mutant unable to assemble capsids, K23Z, have shown that UL36 and UL37 are found on cytoplasmic membrane fractions of HSV-1 infected cells, suggesting that UL36 and UL37 may associate with membranes involved in final envelopment of virions (data not shown). Finally, UL36 and UL37 are found in L particles, which lack capsids but contain
tegument and envelope proteins and are produced by budding events at the site of final envelopment (278, 318). This data suggests that populations of UL36 and UL37 are incorporated at TGN membranes during final envelopment.

It is likely that a large proportion of UL36 and UL37 is incorporated in the cytoplasm during final envelopment. This speculation is based upon several factors. First, detection of UL36 and UL37 on capsids isolated from the nucleus is more easily observed with protein detection assays of relatively high sensitivity and/or specificity, suggesting that the total content of UL36 and UL37 found in virions is not incorporated in the nucleus. Second, a large proportion of UL36 in the cytoplasm of infected cells is associated with membrane fractions.

The mechanism by which UL36 is membrane-associated has not been determined (Fig. 5.2). UL36 may utilize a variety of mechanisms to facilitate membrane binding including (but not limited to): intrinsic hydrophobic domains, trafficking signals and interactions with other membrane-bound viral proteins. UL36 may associate with membranes through its interaction with membrane-associated VP16 (81, 171, 338). L particles produced by a PRV VP16 deletion virus appear to lack UL36 and UL37, suggesting that VP16 plays an integral role in incorporation of these proteins at the TGN (87). Furthermore, UL36 homologues have been shown to bind glycoproteins in yeast two-hybrid and coimmunoprecipitation assays (81, 285, 336). This data suggests that UL36 and/or UL36 homologues may associate with membranes through interaction with cytoplasmic tails of glycoproteins localized to TGN membranes. However, it is unclear if UL36 homologues of the simplex viruses also interact with viral glycoproteins.
**Figure 5.2. Model of UL36 and UL37 Association with TGN Membranes.** UL36 and UL37 localize to TGN-derived membranes to facilitate incorporation into infectious virions and L particles. Localization of UL37 to TGN-derived membranes is dependent on expression of UL36 (62). The association of UL36 with TGN-derived membranes may be mediated by its interaction with VP16. It is unknown if UL36 associates with membranes independently of other viral proteins, however, UL36 contains several putative motifs that may facilitate trafficking to and interaction with membranes.
It is unknown if UL36 contains intrinsic signals that mediate membrane binding in the absence of other viral proteins. The UL36 amino acid sequence contains several motifs that possibly facilitate trafficking to and interaction with membranes. Throughout the UL36 ORF are 35 dileucine motifs (LL or LI) and 17 tyrosine-based motifs (YXXΦ, where Φ represents a large hydrophobic residue, M, I, L, F, V), which may facilitate interactions with clathrin adaptor proteins to promote vesicular trafficking. Furthermore, the HSV-1 UL36 amino acid sequence does not contain predicted transmembrane helices. However, residues in the C-terminus of KSHV UL36 were suggested to mediate interaction with the viral envelope (369). Curiously, when KSHV virions were treated with trypsin, UL36 appeared to be cleaved at the C-terminus, suggesting that UL36 contains a transmembrane spanning domain and that a portion of UL36 extends to the exterior of the virus envelope (369). The implications of this observation are unclear since similar results have not been reported in other herpesviruses and further characterization of this observation in KSHV has not been reported. As UL36 is a protein of enormous size, it contains several regions with hydrophobic amino acids that may also facilitate hydrophobic interactions with membranes. In addition, acidic cluster motifs are located at residues 399-404 (EEDDDD) and 3049-3054 (DDEDSD). However, mutational analyses have not been performed to verify if these motifs actually play a role in membrane association and these sequences do not appear to be conserved.

Although the mechanism that UL36 utilizes for membrane association has not been elucidated, it is clear that UL37 relies upon UL36 to localize to the Golgi complex (62). Roberts and colleagues reported that UL37 is not detected in L particles in a HSV-1 UL36 deletion virus (278). These observations suggest that the interaction of UL36 and
UL37 plays a role in the incorporation of UL37 in both the nuclear and cytosolic compartments. It also appears that UL37 is required for incorporation of UL36 at the TGN, as L particles produced by a HSV-1 UL37 deletion virus contain only trace amounts, if any, UL36 (278). The mechanisms regarding incorporation of UL36 and UL37 in PRV may differ from HSV-1. PRV UL36 appears to be present in PRV virus particles produced by a UL37 deletion virus (156). Further work characterizing the membrane association of UL36 and UL37 may help elucidate the interactions that facilitate their incorporation during secondary envelopment. In conclusion, current evidence suggests that the interaction of HSV-1 UL36 and UL37 is important for the incorporation of these proteins in the nucleus, in addition to incorporation at vesicles derived from the TGN.

PUTATIVE ROLES OF UL37 DOMAINS AND PROTEIN INTERACTIONS

UL37 is a conserved tegument protein that is essential for production of HSV-1 virions. Deletion of UL37 from the HSV-1 viral genome blocks assembly at late times of infection with an accumulation of unenveloped DNA-filled capsids in the cytoplasm (63, 174, 278). This phenotype is reminiscent of that seen in cells infected with HSV-1 virus mutants lacking expression of UL36, the UL37 binding partner (64, 278). Vittone and colleagues showed that amino acids 512-767 of UL36 are sufficient to bind UL37 (338). Mijatov and coworkers extended these studies by identifying F593 and E596 as key residues of UL36 that mediate the UL36-UL37 interaction. In contrast, no information existed regarding the domains of UL37 that are necessary for interaction with UL36, self-
association of UL37 or required for virus assembly. The studies described in Chapter IV of this dissertation were initiated to identify domains of UL37 required for interaction with UL36. Coimmunoprecipitation studies showed that amino acids 568-809 of UL37 are necessary and the carboxy-terminal half of UL37 is sufficient for binding UL36. Further mutational analysis of the C-terminus of UL37 suggested that the conformation of UL37 is sensitive to mutations. To determine if UL37 utilizes the same residues for self-association and UL36 binding, we performed coimmunoprecipitation studies to locate regions involved in UL37 self-interaction. Interestingly, UL37 contains two domains sufficient for self-association, including the region involved in UL36 binding, 568-1123, and residues 1-300. Because the C-terminus of UL37 serves dual purposes of UL36 binding and self-association, we were interested if UL37 can bind UL36 and self-associate simultaneously. Experimental evidence suggests that as the amount of UL36 increases, the level of UL37 self-association decreases; therefore, it appears that self-association of UL37 occurs only when UL36 is not in abundance. Furthermore, a trans-complementation assay was utilized to determine the regions of UL37 that rescue the UL37 deletion virus, KΔUL37 in trans. Several UL37 mutants capable of UL36 binding and/or UL37 self-association were assayed in trans-complementation assays, but the only UL37 mutant capable of rescuing the UL37 deletion virus to result in the production of infectious virions was UL37.568-1123HA. Conclusively, these results suggest that the carboxy terminus of UL37 functions in self-association, UL36 binding and virus assembly. The following sections will discuss the implications and limitations of these results and address experimental possibilities to extend upon these insights.
LOCALIZATION OF UL37 MUTANTS

UL37 plays an essential role in virus assembly. The conserved tegument protein is present in the nucleus and cytoplasm of infected and transfected cells (200, 202, 293, 344). UL37 contains a nuclear export signal (NES) encompassing amino acids 263-273 (344). Interestingly, mutating the residues of the NES to alanines did not affect HSV-1 virus replication in cell culture (62). In the absence of UL37, unenveloped capsids accumulate in the cytoplasm of PRV or HSV-1-infected cells (63, 150, 174, 187, 278) with some studies reporting accumulation of capsids in the nucleus (63, 187). Based upon these observations, UL37 may have functional roles in the nucleus and cytoplasm of infected cells.

A limitation to the studies presented in Chapter IV of this dissertation is the lack of localization data for the UL37 mutants. Cellular fractionation and Western blotting have shown that all of the UL37 mutant proteins utilized in these studies localize, to some extent, in the cytoplasm of infected cells. However, the proportions of the total protein present in the cytoplasm, and the specific sites of cytoplasmic localization are unknown. Immunofluorescence analysis of the UL37 mutants may provide insights regarding UL37 protein interactions and virus assembly. As mentioned previously, the conformation of UL37 appears to be sensitive to mutations. It is possible that mutations disrupt the folding and/or cellular localization of UL37, resulting in failure to bind UL36. One mutant, UL37.568-1123HA, complemented the UL37 deletion virus in trans-complementation assays, resulting in the production of infectious virions (Fig. 4.14). However, another UL37 mutant, UL37.301-1123HA, that encodes residues encompassing this region, binds UL36 and self-associates, surprisingly did not rescue the
defective infection in these assays. A comparison of the localization of UL37HA, UL37.301-1123HA and UL37.568-1123HA may reveal differences in protein trafficking that may explain the failure of UL37.301-1123HA to rescue the UL37 deletion virus. Such analysis may also shed light on the specific cellular compartment(s) where UL37 facilitates virus assembly.

Furthermore, localization studies of internal UL37 mutants that failed to bind UL36 may provide insights about the UL36-UL37 interaction. Initial coimmunoprecipitation studies suggested that amino acids 568-809 of UL37 were necessary for interaction with UL36. Although this region is required for UL36 binding, UL37.568-809HA was not sufficient to bind UL36 (Fig. 4.5). In addition, several larger internal UL37 mutants showed little or no interaction with UL36 (Figs. 4.5 and 4.6). Comparison of localization patterns of internal UL37 mutants may explain the inability of some mutants to bind UL36. It is possible that some UL37 mutants fail to bind UL36 due to altered localization within the cell. Comparison of different localization patterns of several mutants may also reveal domains of UL37 necessary for trafficking to specific cellular compartments.

IDENTIFICATION OF DOMAINS THAT FACILITATE INCORPORATION OF UL37

In the studies described in this dissertation, we determined a domain of UL37 that is necessary for UL36 binding. Furthermore, we identified regions of UL37 involved in self-association. However, the domain(s) of UL37 that mediates incorporation into HSV-1 virions was not identified. Future studies of UL37 should include determining if the
regions of UL37 required for UL36 interaction and self-association are also necessary for incorporation of UL37 into virions.

Investigations to identify incorporation domains of UL37 should include analyzing extracellular virus particles released from UL37 trans-complementation assays for the incorporation of transfected UL37 mutants. UL37.568-1123HA was the only UL37 mutant analyzed that resulted in the production of infectious virions. The ability of UL37.568-1123HA to rescue the defective infection suggests it was incorporated into extracellular particles in the trans-complementation assay. Perhaps the best method to perform incorporation studies would be to construct recombinant HSV-1 viruses that express mutant UL37 proteins (in the context of a UL37 deletion virus). This system would enable analysis of tegument incorporation when UL37 mutants are expressed by normal viral kinetics. It would be interesting to determine which UL37 mutants are incorporated into extracellular particles. UL37 mutants may be incorporated into virus particles that are not infectious. In such a scenario, deciphering why the virus particles are not infectious could provide novel insights regarding UL37 function and/or interactions. Furthermore, it would be interesting to determine if interaction with UL36 is a requirement for UL37 incorporation into extracellular virus particles. To address this question, the incorporation of a UL37 mutant containing a deletion of the UL36 binding domain should be analyzed. Current evidence suggests that interaction with UL36 facilitates UL37 incorporation in the nucleus and cytoplasm of HSV-1 infected cells (62, 278). However, it is possible that interaction of UL37 with other viral proteins also facilitates UL37 packaging into virions.
FURTHER CHARACTERIZATION OF DOMAINS OF UL37

The studies of Chapter IV identified UL37 residues as 568-1123 as sufficient for interaction with UL36. Further truncation of this region of UL37 dramatically reduced interaction with UL36. Future studies involving characterization of the UL36 binding domain should involve site-directed mutagenesis of highly conserved residues of UL37. UL37 contains six residues that are conserved across the herpesvirus family. Four of the six conserved amino acids lie within the region of UL37 necessary for UL36 binding, amino acids 568-809. This high degree of conservation suggests that these UL37 residues play important roles during infection. The conserved residues include: L263, L414, W559, P618, F620 and L702. Substitution of the conserved UL36 binding domain residues with the small amino acid alanine, or replacing these residues with an amino acid of similar size and/or charge may abrogate the ability of UL37 to bind UL36. Conversely, mutation of these amino acids may have no affect on UL36-UL37 binding, but may impact another function of UL37. Amino acid substitutions could be constructed in UL37-expressing plasmids to be used in coimmunoprecipitation and trans-complementation assays. However, generation of mutant viruses containing UL37 acid substitutions would enable a more comprehensive investigation of possible effects resulting from UL37 amino acid changes.

PUTATIVE FUNCTIONS OF UL37 SELF-ASSOCIATION AND A POTENTIAL ROLE FOR UL36 INHIBITION OF UL37 SELF-ASSOCIATION

UL37 self-associates and interacts with the large tegument protein UL36. Coimmunoprecipitation studies have suggested that a molecule of UL37 binds either
another molecule of UL37 or binds UL36 (Fig. 4.13). UL37 does not appear to bind both UL36 and UL37 at the same time. This result suggests that protein complexes consisting of several multimerized UL37 molecules bound to UL36 do not exist in HSV-1-infected cells. The roles of UL37 self-association during infection and the inhibition of UL37 self-association by UL36 are not clear. Based upon current evidence, a discussion of putative functions of these protein-protein interactions is presented below.

The interaction of UL37 with UL36 appears to be important for UL37 capsid-association and incorporation into L particles (151, 278). Abundant levels of UL36 inhibit self-association of UL37. Therefore, the UL36-UL37 interaction appears to play an important role in UL37 incorporation, and it is unlikely that the self-association of UL37 facilitates UL37 incorporation into virions. This presents the question: How might UL37 self-association play a role in HSV-1-infected cells?

The self-association of UL37 may facilitate UL37-mediated NF-κB activation. UL37 activates NF-κB signaling in HSV-1-infected cells by binding to the TRAF6 adaptor protein (180). Prior to discussing how self-association of UL37 may impact NF-κB signaling, it is important to understand the role of TRAF6 in the NF-κB signaling cascade (reviewed in 308). TRAF6 is a ubiquitin ligase that plays a pivotal role in NF-κB activation by the IL-1 receptor (IL-1R) and Toll-like receptor (TLR) (59). Activation of IL-1R or TLR leads to recruitment of MyD88, IRAK1, IRAK4 and TRAF6 (308). The formation of the protein complex is thought to facilitate oligomerization of TRAF6 (50). TRAF6 oligomerization activates its ubiquitin ligase activity which results in polyubiquitination of TRAF6. The formation of lysine 63 (K63)-linked polyubiquitin chains recruits the TAK1/TAB2 and IKK/NEMO complexes (Fig. 5.3) (308).
Recruitment of the IKK kinase complex causes autophosphorylation and activation of TAK1. TAK1 phosphorylates IKKβ, resulting in IKK activation. IKK phosphorylates IκB and targets it for proteasomal degradation through K48-linked polyubiquitination (49, 290). Degradation of IκB unmasks the NLS of the p50/p65 NF-κB dimer, facilitating its entry into the nucleus and activation of gene transcription.

In HSV-1-infected cells UL37 is bound to TRAF6 in a complex with TAB1, TAB2 and TAK1 (180). During infection it is possible that self-association of UL37 serves to promote oligomerization of TRAF6 through the UL37-TRAF6 interaction, thus activating the NF-κB pathway without ligand binding to IL-1R or TLR. In this model, dimers of UL37 bind two molecules of TRAF6 (Fig. 5.3). The interaction of UL37 with TRAF6 may facilitate TRAF6 oligomerization and therefore activate TRAF6 ubiquitin ligase activity resulting in the formation of K63 (lysine 63)-linked polyubiquitin chains, recruitment of the kinase complexes, degradation of IκB and NF-κB activation.

In the NF-κB activation pathway, IKK phosphorylates IκB and targets the NF-κB inhibitor for degradation through K48-linked polyubiquitination. The degradation of IκB allows NF-κB to enter the nucleus to activate transcription of target genes. Interestingly, HSV-1 UL36, and its homologues in all herpesvirus subfamilies, are ubiquitin proteases that specifically deubiquitinate K48 linkages (139). It is possible that during infection UL36 regulates activation of the NF-κB pathway by cleaving K48-linked ubiquitin chains from IκB. In this scenario, UL36 inhibits proteasomal degradation of IκB by cleaving ubiquitin tags, therefore preventing the release of IκB from NF-κB and inhibiting the translocation of NF-κB to the nucleus. Therefore, the deubiquitinating activity of UL36 may inhibit NF-κB activation at the final step in the activation pathway.
Figure 5.3. **Putative Function of UL37 Self-Association and a Potential Role for UL36 Inhibition of UL37 Self-Association.** Amino acids 1099-1104 of UL37 mediate interaction with TRAF6. Dimers of UL37 may bind and induce oligomerization TRAF6. Oligomerization of TRAF6 activates its ubiquitin ligase activity which causes a cascade of events resulting in the activation of NF-κB. UL36 functions as a K48-ubiquitin protease (139). UL36 may cleave K48-linked ubiquitin from IκBα to prevent the degradation of IκBα and thereby inhibit NF-κB activation. UL36 appears to inhibit self-association of UL37. UL36-mediated inhibition of UL37 self-association prevents virus-induced TRAF6 activation, therefore decreasing NF-κB activation. In this model UL37 self-association functions to activate NF-κB signaling and UL36 functions to regulate UL37-mediated NF-κB activation.
During infection NF-κB signaling is activated through TLR2 and non-TLR2 pathways as part of the host innate immune response (160, 161, 255). NF-κB signaling appears to benefit virus infection, as HSV-1 grows best in cells with NF-κB activity (114, 255). Evidence suggests that NF-κB is recruited to activate transcription of the IE genes in infected cells (5). During infection UL37 may activate NF-κB signaling to enhance viral IE gene transcription. UL36 may then down-regulate UL37-mediated NF-κB activation to inhibit NF-κB-activated deleterious effects on the cell, such as apoptosis or recognition by the immune system, which may hinder virus replication.

In summary, when UL36 is abundant, the interaction of UL36 and UL37 likely facilitates their incorporation into virus particles. Furthermore, UL36 may also inhibit NF-κB signaling by preventing the degradation of IκB as described above. In contrast, when UL36 protein levels are relatively low, UL37 may bind UL36, and excess molecules of UL37 may self-associate to activate NF-κB through TRAF6 binding. Therefore, based on results described in this dissertation and known functions of UL36 and UL37, this model suggests that UL36 inhibition of UL37 self-association serves as a mechanism to regulate UL37-induced activation of NF-κB.
REFERENCES


involved in the establishment of murine gammaherpesvirus 68 infection. J. Virol. 83:10644-10652.


132. Jayachandra, S., A. Baghian, and K. G. Kousoulas. 1997. Herpes simplex virus type 1 glycoprotein K is not essential for infectious virus production in actively replicating cells but is required for efficient envelopment and translocation of
infectious virions from the cytoplasm to the extracellular space.  J. Virol. 71:5012-5024.


simplex virus type 1 is the presence of the major tegument protein pUL36 (VP1/2). J. Virol.


230. Murphy, M. A. Packaging Determinants of the Herpes Simplex Virus Type 1 Tegument Protein pUL46. 2009. The Pennsylvania State University. Dissertation


Bachenheimer. 1998. Herpes simplex type 1 induction of persistent NF-kappa B
nuclear translocation increases the efficiency of virus replication. Virology
247:212-222.


characterization of herpes simplex virus type 1 mutants defective in the UL6

requires a functional ESCRT-III complex but is independent of TSG101 and


of a truncated form of the herpes simplex virus glycoprotein gH-gL complex. J.

Biochemical studies of the maturation of herpesvirus nucleocapsid species.
Virology. 74.

262. Pereira, L. 1994. Function of glycoprotein B homologues of the family

263. Person, S. and P. Desai. 1998. Capsids are formed in a mutant virus blocked at
the maturation site of the UL26 and UL26.5 open reading frames of herpes
simplex virus type 1 but are not formed in a null mutant of UL38 (VP19C).

nucleus during synchronized herpes simplex virus type 1 infection. J. Virol.
73:6769-6781.


269. Read, G. S. and M. Patterson. 2007. Packaging of the virion host shutoff (Vhs) protein of herpes simplex virus: two forms of the Vhs polypeptide are associated with intranuclear B and C capsids, but only one is associated with enveloped virions. J. Virol. 81:1148-1161.


274. Reynolds, A. E., E. G. Wills, R. J. Roller, B. J. Ryckman, and J. D. Baines. 2002. Ultrastructural localization of the herpes simplex virus type 1 UL31,
UL34, and US3 proteins suggests specific roles in primary envelopment and egress of nucleocapsids. J. Virol. 76:8939-8952.


327. Thurlow, J. K., F. J. Rixon, M. Murphy, P. Targett-Adams, M. Hughes, and V. G. Preston. 2005. The herpes simplex virus type 1 DNA packaging protein UL17 is a virion protein that is present in both the capsid and the tegument compartments. J. Virol. 79:150-158.


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O’Regan, K.J., M.A. Murphy, M.A. Bucks, J.W. Wills and R.J. Courtney. 2007. Incorporation of the Herpes Simplex Virus Type 1 tegument protein VP22 into the virus particle is independent of interaction with VP16. Virology 369 (2):263-280

Bucks, M.A., K.J. O’Regan, M.A. Murphy, J.W. Wills and R.J. Courtney. 2007. Herpes Simplex Virus Type 1 tegument proteins VP1/2 and UL37 are associated with intranuclear capsids. Virology 361 (2):316-324

O’Regan, K.J., M.A. Bucks, M.A. Murphy, J.W. Wills and R.J. Courtney. 2007. A conserved region of the Herpes Simplex Virus Type 1 tegument protein VP22 facilitates interaction with the cytoplasmic tail of glycoprotein E (gE). Virology 358 (1):192-200