INSIGHTS INTO HERPES SIMPLEX VIRUS TYPE 1 ASSEMBLY: PROTEIN INTERACTIONS AND VIRION INCORPORATION DETERMINANTS OF THE TEGUMENT PROTEIN VP22

A Dissertation in
Microbiology and Immunology
by
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Doctor of Philosophy

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ABSTRACT

Herpes simplex virus type 1 (HSV-1) assembly involves a complex sequence of events occurring within numerous cellular compartments that culminates in the formation of virions composed of nearly 40 different viral proteins distributed among three morphologically distinct structures: the nucleocapsid, the host-derived lipid envelope containing virally encoded glycoproteins and other transmembrane proteins, and the tegument, a proteinaceous region located between the nucleocapsid and envelope. Although in recent years the major steps of the herpesvirus assembly pathway have become clearer, the molecular mechanisms utilized by viral structural proteins to target to the site of final envelopment (a trans-Golgi network (TGN)-derived vesicle) and interact to form a stable virion, remain poorly characterized. This dissertation focuses on defining the protein-protein interactions and virion incorporation determinants of one specific HSV-1 tegument protein, VP22, and the functional significance of these activities in facilitating packaging of VP22 into assembling virions.

VP22, one of the most abundant tegument proteins with nearly 2,000 copies present within each virion, associates with cellular membranes and localizes to the TGN where it is packaged into assembling virus particles during final envelopment. Current evidence suggests that viral glycoprotein tails play a role in the recruitment of tegument-coated capsids to the site of final envelopment. Initial efforts to identify binding partners of VP22 and elucidate their roles in facilitating virion incorporation of VP22, focused on potential interactions with the cytoplasmic tail of a viral glycoprotein(s). Using a variety of protein-protein interaction assays, VP22 was demonstrated to bind to the cytoplasmic tail of gE. Truncation mutagenesis suggests that residues 165-270 of VP22 facilitate
interaction with gE. In fact, this region of VP22 is sufficient to bind to gE in the absence of additional viral proteins. Using a transfection/infection-based virion incorporation assay, residues 165-270 of VP22 fused to the green fluorescent protein (GFP) competed efficiently with wild-type VP22 for packaging into assembling virus particles. Interestingly, a domain of the protein similar to the region that facilitates gE binding and virion incorporation has been reported to bind VP16. Thus, the focus of the next series of experiments was to elucidate the role VP16 plays in virion incorporation of VP22.

Deletion mutagenesis was used to identify the minimal domain of VP22 that is required for interaction with VP16. Residues 165-225 of VP22 are both necessary and sufficient to facilitate binding to VP16 in the absence of additional virally encoded proteins. However, in order to attain wild-type levels of binding, residues 165-270, which are also sufficient to facilitate binding to the cytoplasmic tail of gE are required. Membrane flotation experiments suggest that this region of VP22 (residues 165-225) has the ability to associate with cellular membranes; however, this activity is not sufficient to facilitate virion incorporation of VP22 and additional protein-protein interactions appear to be necessary. Primary structural alignment reveals that residues 165-270 of HSV-1 VP22 are highly conserved among VP22 homologues of herpesviruses. Site-directed point mutagenesis targeting dileucine motifs conserved within this region enabled partition of the VP16 and gE binding activities. To ascertain the contribution of these binding activities to VP22 virion incorporation, the point mutants were evaluated in the virion packaging assay, with results indicating that interaction with VP16 is not required for incorporation of VP22 into virus particles.
To determine the contribution of gE binding to VP22 virion incorporation, further mutagenesis of potential protein-protein interaction motifs within VP22 was performed. Targeting residues which resembled a WW domain, a well characterized protein-protein interaction module, enabled the identification of mutants which abrogated interaction with gE but retained VP16 binding activity. Upon assessment of these mutants’ ability to incorporate into virus particles, it appeared that failure to bind to gE did not abrogate virus packaging. This result, combined with the observation that VP16 binding is not required for incorporation of VP22, suggests that gE and VP16 binding act redundantly to facilitate assembly of VP22 into the virus particle.

Curiously, a region of VP22 which binds to both VP16 and gE at efficiencies comparable to full-length VP22, fails to be packaged to wild-type levels, suggesting that additional incorporation determinants exist. Wild-type levels of incorporation were only attained when residues 43-86 of VP22 were present, despite the fact that removal of these residues had no deleterious effects on the binding efficiency of VP22 to either VP16 or gE. An acidic cluster of amino acids within this region of the protein, which is required for localization of VP22 at the TGN, was examined for its role in virion packaging. Alanine mutagenesis of the acidic cluster abrogated both binding activities and virion incorporation, suggesting a gross misfolding event. However, upon deletion of the residues compromising this motif, VP22 packaging was not abrogated but reduced to a degree equivalent to a VP22 construct which binds to both gE and VP16 at wild-type levels. Deletion of the acidic cluster had no adverse effect on VP22’s interaction with either VP16 or gE, suggesting that the decrease in virion incorporation observed is not due to a loss of binding activity. These findings indicate that although protein-protein
interactions play a crucial (and possibly redundant) role in VP22 virion incorporation, an acidic cluster of amino acids must also be present to attain efficient packaging. The acidic cluster motif presumably ensures localization of VP22 at the TGN, where interactions with gE and/or VP16 facilitate packaging of the protein into the tegument region of assembling virions.

Collectively, the studies described within this dissertation elucidate the mechanism by which VP22 is incorporated into virus particles, and reveal that in addition to the role protein-protein interactions play, proper trafficking and localization of VP22 also contribute to efficient virion incorporation.
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<td>α-TIF</td>
<td>α trans-inducing factor</td>
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<td>Å</td>
<td>angstrom</td>
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<td>AC</td>
<td>acidic cluster</td>
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<tr>
<td>AIDS</td>
<td>acquired immunodeficiency syndrome</td>
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<td>AP</td>
<td>adaptor protein</td>
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<td>ARF</td>
<td>ADP-ribosylation factor</td>
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<td>ATP</td>
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<td>β-ME</td>
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<td>BHV</td>
<td>bovine herpesvirus</td>
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<td>bp</td>
<td>base pair</td>
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<tr>
<td>C-terminal</td>
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<td>CCSC</td>
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<td>CPE</td>
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<td>downstream activation sequence</td>
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<td>DMEM</td>
<td>Dulbecco’s modified Eagles medium</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>dsDNA</td>
<td>double-stranded deoxyribonucleic acid</td>
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<td>GGA</td>
<td>Golgi-localizing, $\gamma$-adaptin ear homology domain, ARF-binding protein</td>
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<td>kilobase pair</td>
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<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>mm</td>
<td>millimeter</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
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</table>
mRNA  messenger RNA
MT    microtubule
MVB   multivesicular body
N-terminal amino-terminal
ND10  nuclear domain 10
NLS   nuclear localization signal
nm    nanometer
NPC   nuclear pore complex
NTE   NaCl/Tris/EDTA
°C    degree Celsius
ORF   open reading frame
PACS  phosphofurin acidic cluster sorting protein
PAGE  polyacrylamide gel electrophoresis
PBS   phosphate-buffered saline
PCR   polymerase chain reaction
PEG   polyethylene glycol
PKC   protein kinase C
PKD   protein kinase D
PML   promyelocyte
PMP   peripheral membrane protein
POD   promyelocyte oncogenic domain
PrV   pseudorabies virus
RNA   ribonucleic acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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</thead>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>ST</td>
<td>swine testis</td>
</tr>
<tr>
<td>SU</td>
<td>surface</td>
</tr>
<tr>
<td>TAF</td>
<td>template activating factor</td>
</tr>
<tr>
<td>TAP</td>
<td>transporters associated with antigen processing</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TGN</td>
<td><em>trans</em>-Golgi network</td>
</tr>
<tr>
<td>TIEM</td>
<td>transmission immunoelectron microscopy</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane</td>
</tr>
<tr>
<td>TMP</td>
<td>transmembrane protein</td>
</tr>
<tr>
<td>TNFR</td>
<td>tumor necrosis factor receptor</td>
</tr>
<tr>
<td>UL</td>
<td>unique long</td>
</tr>
<tr>
<td>US</td>
<td>unique short</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>vhs</td>
<td>virion host shutoff</td>
</tr>
<tr>
<td>VLP</td>
<td>virus-like particle</td>
</tr>
<tr>
<td>Vmw</td>
<td>virus-encoding molecular weight polypeptide</td>
</tr>
<tr>
<td>VP</td>
<td>virion polypeptide</td>
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<tr>
<td>VSV</td>
<td>vesicular stomatitis virus</td>
</tr>
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<td>VZV</td>
<td>varicella-zoster virus</td>
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<td>wt/vol</td>
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PREFACE

The overall goal of the research presented within this dissertation is to provide insight into the mechanisms utilized by tegument proteins to facilitate virion incorporation. Specifically, protein-protein interactions and virion incorporation determinants of the HSV-1 tegument protein, VP22, are dissected to elucidate the functional significance of these activities in facilitating packaging of VP22 into assembling virions.

Much of this work was aided by intellectual discussions with my laboratory colleagues. Their contribution is acknowledged by inclusion of their names in the multi-author chapters, however all the experiments presented were conducted by myself.
ACKNOWLEDGEMENTS

The pursuit of a doctoral degree is not a trivial endeavor. The intractability of nature is often confounded by the hurdles one faces in life. Thus, its attainment not only signifies growth as a scientist but perhaps more so, growth as a person. My progression as both owes a great deal to a great many people.

I would like to express my utmost appreciation to Dr. Richard Courtney. His wisdom, guidance, and patience, transformed a naive graduate student into an independent scientist. Dr. Courtney’s support and encouragement through both my scientific successes and failures is matched only by his genuine concern for my personal development. I feel privileged to be able to refer to him as my scientific mentor, and friend.

Furthermore, I extend my gratitude to the members of my committee, Drs. Craig Meyers, David Spector, John Wills, and Kristen Eckert, for generously sharing with me their time and passion for scientific discovery. My thanks also go to the faculty, students, and staff of the Microbiology and Immunology Department who throughout my tenure at Penn State have fostered a welcoming and collaborative environment conducive to a rewarding graduate school experience. I would like to specifically thank the administrative staff and members of Central Lab Resources, whose tireless and often unacknowledged toil makes our lives indeterminately easier.

The Irish poet William Butler Yeats once wrote “Think where man’s glory most begins and ends, and say my glory was I had such friends”. I will be eternally grateful for the people whose friendship has supported me throughout the trials and tribulations of graduate school. To Mike and Michelle of the Courtney lab, the members of “Nerd-Herd
2005”, my fellow “Pop-Up Pilgrims”, and especially to the girl with the rumbling stomach, I offer my sincere and heartfelt thanks.

My greatest debt of gratitude is to my parents, Maura and Colm, and my sisters Anne-Marie and Thérèse. Without their unconditional love, support, and encouragement none of this work would have been possible, or even of merit. Words could never suffice to express my thanks and appreciation for all that they have sacrificed to allow me to reach this goal. According, I dedicate my dissertation to them, with all of my love.

*D’ainneoin fad sli o bhaile bionn sibh liom go deo ar m’intinn agus i mo chroi.*
CHAPTER I

INTRODUCTION
Herpes simplex virus type 1 (HSV-1) virion assembly involves a complex sequence of events occurring within numerous disparate cellular compartments that culminates in the formation of virions composed of nearly 40 different viral proteins distributed among three morphologically distinct structures: the nucleocapsid, the host-derived lipid envelope in which virally encoded glycoproteins are embedded, and the tegument, a proteinaceous region located between the nucleocapsid and envelope (Fig. 1.1). Assembly of virus particles begins within the nucleus, with aggregation of capsid proteins into a stable icosahedron into which the viral genome is packaged (Fig. 1.2) (191,455,456). Nucleocapsids are shuttled to the cytoplasm via a budding/fusion event that occurs across the inner and outer membranes of the nucleus, respectively (Fig. 1.2). Subsequently, unenveloped capsids travel through the cytoplasm until they reach a trans-Golgi network (TGN)-derived vesicle. At this site, nucleocapsids are thought to acquire their final lipid bilayer during a budding event that also results in the acquisition of tegument components and all the viral glycoproteins (Fig. 1.2) (236,256,258,284,456,457,613,644,710,739,741,784). Mature virions subsequently follow the secretory pathway to the cell surface, where they are released into the extracellular milieu (456).

Although the cellular sites for capsid assembly and virion envelopment have become increasingly apparent, the process of tegument acquisition (tegumentation) and mechanisms employed by tegument proteins to ensure virion incorporation, remain poorly defined. Tegumentation of capsids could theoretically occur at various stages in the egress pathway: in the nucleus, at the nuclear membrane, during transit through the
Figure 1.1. Basic Structure and Composition of an HSV-1 Virion. The linear, double-stranded DNA genome (black lines) is enclosed within an icosahedral capsid composed of at least five proteins. The nucleocapsid is surrounded by a host-derived lipid envelope that contains at least twelve virally encoded glycoproteins (yellow spikes) and two non-glycosylated transmembrane proteins. Between the nucleocapsid and envelope lies the tegument, a structured layer of over 20 different proteins (red/green/blue concentric circles). Different populations of tegument proteins are known to exist; inner tegument proteins such as VP1/2 associate directly with the nucleocapsid (red region), whereas outer tegument components (e.g. UL11) associate with the lipid envelope (blue region). In addition, another subset of tegument proteins is acquired as a result of interactions with either inner or outer tegument proteins (green region).
Figure 1.2. Assembly Pathway of Infectious HSV-1 Particles. Genomic DNA is cleaved into unit length monomers and packaged into preassembled, icosahedral capsids within the nucleus. Before nuclear egress, proteins which will ultimately comprise the inner tegument may be added to the nucleocapsid (red). Nucleocapsids are then shuttled to the cytoplasm via a budding-fusion event that occurs across the inner and outer leaflets of the nuclear membrane, respectively. During this process, capsids could theoretically attain an additional portion of tegument (green). Upon exiting the nucleus, nucleocapsids traverse the cytoplasm, perhaps picking up further tegument proteins (dark green), to reach the site of final envelopment, a trans-Golgi network (TGN)-derived vesicle. At this site, an envelopment step results in acquisition of the final subset of tegument proteins (blue) as well as the concurrent addition of a lipid envelope and viral glycoproteins (yellow spikes). Subsequently, enveloped particles travel within vesicles through the secretory pathway to the cell surface, where fusion with the plasma membrane releases virions into the extracellular milieu.
cytoplasm or budding at the TGN. It is likely that a variety of intracellular trafficking motifs, which regulate the subcellular localization of tegument components, in combination with a myriad of protein-protein interactions between capsid proteins, tegument proteins, and the cytoplasmic tails of virally encoded glycoproteins, facilitate selective packaging of virion components into the tegument region of assembling virus particles.

The studies presented within this dissertation are focused on providing insight into the mechanisms utilized by tegument proteins to facilitate virion incorporation. Specifically, the protein-protein interactions and virion incorporation determinants of the HSV-1 tegument protein, VP22, were dissected to elucidate the functional significance of these activities in facilitating packaging of the protein into assembling virions. The bedrock of this dissertation VP22, was chosen namely due to its sheer abundance within the virus particle. There are nearly 2,000 copies of VP22 within the tegument region of each virion (290). Furthermore, VP22 possesses the ability to associate with cellular membranes and target to the TGN independently of other viral components (66). Thus, suggesting that VP22 contains intrinsic determinants that facilitate its localization to the site of final envelopment, where it is incorporated into the tegument (66,469). Despite its abundance, the role of VP22 during HSV-1 assembly and mechanism of its incorporation, remain undefined. However, recent studies of a HSV-1 VP22-null virus suggest that the requirement for the protein in the assembly pathway can be bypassed (at least in tissue culture systems), albeit with altered virion composition (179,182).

The focal point of the following chapters is the identification and characterization of protein-protein interactions and intrinsic trafficking signals of VP22 as they pertain to
virion incorporation. Chapter III describes the identification of a novel binding partner of VP22, glycoprotein E (gE). The domain of VP22 sufficient to bind to the cytoplasmic tail of gE competes efficiently with wild-type VP22 for packaging into assembling virus particles, suggesting that interaction with gE may facilitate incorporation of VP22 into the virion. Chapter IV builds upon these findings and describes another binding activity of VP22 that lies within the gE interaction domain. Thus, virion incorporation of the gE binding domain of VP22 could be due to gE binding and/or the newly mapped activity, interaction with VP16. Using mutagenesis strategies to discern between the two activities, it appears that interaction with VP16 is not required for packaging of VP22 into assembling virus particles. Additional data within this chapter suggest that membrane association of VP22 is not sufficient to facilitate virion incorporation, highlighting the existence of additional incorporation determinants, most likely a variety of protein-protein interactions.

In light of the dispensable nature of VP16 binding, Chapter V examines the contribution of interaction with gE to virion incorporation of VP22. Failure to bind gE does not abrogate virion packaging of VP22, suggesting that gE and VP16 binding may act in a redundant fashion to facilitate inclusion of VP22 into the virus particle. Studies within this chapter also underscore the role intracellular trafficking and subcellular localization of VP22 play in virion incorporation; specifically, the functional significance of a trafficking signal, which is required for efficient virus packaging.

The central theme of this dissertation concerns events that occur during HSV-1 virion assembly. To provide a suitable perspective of the findings’ significance, one must reflect on the current understanding of HSV-1 replication in general and the
assembly pathway specifically. A review of this material is furnished in Chapter II with a brief overview of the major steps in the replication cycle and a thorough analysis of assembly. Given the sheer number of herpesviruses identified to date, the scope of the review is confined to HSV-1, with comparisons to other herpesviruses limited to their pertinence to assembly.
CHAPTER II

REVIEW OF THE LITERATURE
A BRIEF HISTORICAL PERSPECTIVE OF HERPESVIRUSES

The history of herpesvirology can be traced back to ancient Greece where, the Greek physician Hippocrates recorded perhaps the first account of a human HSV infection. He described a novel spreading cutaneous lesion which he named “herpes”, meaning to crawl or creep (45). More than half a millennium later a Roman physician, Herodotus, established the association of mouth ulcers and lip vesicles with fever. This correlation, which has survived to the present day, was elaborated on by Galen who recognized that HSV occurrences develop at the same anatomical site (603,742). Over the following centuries, the term ‘herpes’ was used to describe many pathological skin conditions, although it is likely that many of the lesions were attributable to infection with a variety of other viral, fungal, bacterial, or parasitic infections, or concurrent infection with several pathogens.

More rigorous definitions of herpes emerged in the 17th and 18th Centuries. Thomas Bateman described the nature of recurrent HSV infection “as a restricted group of localized vesicles, with a short, self-limiting course” (34). The first link between HSV and the genital organs appeared with publication of De Morbis Venereis by John Astruc, physician for King Louis XIV, in 1736. At that time, French prostitutes were required to undergo medical surveillance; in studying their afflictions, Astruc became the first to describe herpes genitalis (16). Over the next 50 years descriptions of genital herpes abound. Perhaps the most enlightening description of recurrences was published by Unna in 1883 (706). His writings refer to herpes as being “a vocational disease…one of the most benign of afflications both to the patient and her public” (706).
Technical advances of the late 19th century allowed researchers to move away from mere descriptions of clinical manifestations and disease, and undertake hypothesis-driven experimental herpesvirology. In 1893, transmission of infectious agents to human volunteers was in vogue and Vidal had the dubious distinction of proving that HSV was infectious (717). The infectious nature of the virus was unequivocally demonstrated by experiments performed by Lowenstein and Gruter around 1910, where independently, both investigators established that HSV retrieved from human lesions could produce lesions on the cornea of a rabbit (275, 410). This early experimental period saw the host range of HSV expanded to include a variety of laboratory animals, chick embryos and ultimately, cell culture systems. In the late 1950’s, application of these tissue culture systems would permit the isolation and growth of several other human herpesviruses, including cytomegalovirus (CMV) and varicella-zoster virus (VZV) (651, 738). Experimentation was primarily focused on the humoral immune response to HSV infection and latency. Studies highlighted that HSV remains latent within its host and reactivates under certain stimuli despite the presence of neutralizing antibodies, an occurrence in sharp contrast to the behavior of other known infectious agents at that time (11, 169).

The modern age of experimental herpesvirology was ushered in by a variety of significant technological advances. Techniques such as electron microscopy (EM), DNA sequencing and cloning, and genetic engineering permitted the visualization, characterization and manipulation of HSV (603). Virion morphology, organization and sequence of the DNA genome, elaborate viral gene expression cascades, and the identity and function of many viral gene products, were all elucidated (286, 434, 599, 306, 561, 747).
With a greater understanding of HSV, came advances in therapeutics. The discovery of acyclovir, efficacy of the VZV vaccine and recent studies utilizing herpesviruses as viral vectors for the treatment of neurological diseases, serve to exemplify the power and potential benefit to society afforded by scientific research (74,265,422,528,635,744).

**CLASSIFICATION OF HERPESVIRUSES**

Herpesviruses are highly disseminated in nature with approximately 130 different herpesviruses identified from a variety of animal species (601). To date nine herpesviruses have been isolated from humans; HSV-1, HSV-2, VZV, human cytomegalovirus (HCMV), Epstein-Barr virus (EBV), and human herpesviruses 6A, 6B, 7 and 8 (also known as Kaposi’s sarcoma-associated herpesvirus [KSHV]) (601,602). Although these nine human herpesviruses share the same host, the route of infection, tissue tropisms, and causative disease states are quite distinct. Nevertheless, commonalities exist, which relate these nine human herpesviruses to each other, and ultimately to the other plethora of herpesviruses currently characterized (602).

Herpesviruses are large DNA viruses with distinctive virion architecture. Historically, from the 1960s to 1980s, assignment as a herpesvirus was made on the basis of virion morphology. As illustrated in Figure 1.1, the herpesvirus particle has several distinct components. The genomic DNA is densely packed within an icosahedral \((T = 16)\) capsid, which has 162 surface capsomeres and a diameter of 115–130 nm. Of the capsomeres, 150 are primarily composed of six molecules of one protein species, with 11 others pentamers of the same protein. The final pentameric position is occupied by the portal complex. Enclosing the capsid is an amorphous layer, the tegument, composed of
several protein species. This in turn is bounded by the outermost element, a lipid bilayer envelope with embedded protein molecules (typically glycosylated), giving an overall virion diameter of about 200 nm. The whole structure presents a characteristic appearance in negatively-stained or thin-sectioned electron microscopic images, and was used to define membership of the taxonomic family *Herpesviridae*.

In addition to morphological similarities, herpesviruses share several biological properties (601,602). All herpesviruses encode an array of enzymes implicated in nucleotide metabolism (e.g., thymidine kinase, deoxyuridine triphosphatase, ribonucleotide reductase), DNA synthesis (e.g., DNA polymerase, helicase, primase) and protein modification (e.g., protein kinases such as UL13) (601,602). Furthermore, production of infectious herpesvirus particles results in destruction of the infected cell, termed cytopathic effect (CPE). This destruction is characterized by alterations in host macromolecular metabolism, nuclei structure, and cellular membranes with concurrent rearrangement of the cytoskeleton; changes resulting in rounding of the infected cell and ultimately, cell death.

A key characteristic of all herpesviruses is their ability to establish life-long latency as an episome within the infected host and to periodically reactivate when triggered by certain stimuli (e.g. stress, ultraviolet [UV] radiation), thus facilitating dissemination (594). HSV-1 latency is defined as a reversible non-productive infection of a cell by a replication competent-virus (232). During this time, transcriptional activity is virtually nonexistent, with the exception of the latency-associated transcript (LAT), an 8.3 to 8.5 kbp noncoding RNA that can be spliced to yield a 2.0 kbp stable intron (202,595,666). One proposed function of the LAT is suppression of nearby lytic phase
transcripts ICP0, γ34.5, and ICP4 through antisense mechanisms, thereby promoting the establishment and maintenance of latency (107). In addition to putative LAT-mediated suppression of lytic transcripts during latency, mounting evidence suggests that latent gene expression is also regulated at the chromatin level. The latent viral genome is known to associate with nucleosomes (159). Investigation of chromatin modification demonstrates that during latency, the lytic regions of the virus exist in a hypoacetylated, or transcriptionally nonpermissive state, while the LAT promoter and 5’ exon/enhancer remain hyperacetylated, or transcriptionally permissive (375).

Such morphological and biological criteria, in tandem with observations of host range, duration of the viral replication cycle, cytopathology, and properties of latent infection, were used to segregate herpesviruses into one of three subfamilies, Alpha-, Beta- and Gammaherpesvirinae (Table 2.1) (600). Members of the Alphaherpesvirinae such as HSV-1, HSV-2, VZV, pseudorabies virus (PrV), and Marek’s disease virus (MDV), exhibit a broad host range, have a relatively short reproductive cycle, spread rapidly in cell culture systems efficiently destroying infected cells, and are capable of establishing latency in sensory ganglia. The Betaherpesvirinae, including HCMV, are characterized by a very narrow host range (usually species specific) and a long reproductive cycle. Consequently, infection progresses slowly in cell culture and is made obvious by the characteristic enlargement of infected cells (cytomegla). Members of the Betaherpesvirinae can establish latency in secretory glands, kidneys, and are T-lymphotropic, infecting T lymphocytes as well as monocytes and macrophages. The Gammaherpesvirinae, such as EBV and KSHV, also demonstrate a narrow host range
and are distinguished by their predilection for B lymphocytes, where they are capable of establishing a latent infection.

Since the start of the 1980’s, DNA sequence data for herpesvirus genomes has accumulated at an exponential rate with many complete genome sequences and large numbers of partial sequences presently in existence (439). Sequence comparisons have now become the primary approach for evaluating phylogenetic and taxonomic relationships among herpesviruses, and for identifying newly characterized viruses as members of the \textit{Herpesviridae} and assigning them to subfamilies. It became apparent from this approach that mammalian and avian herpesviruses were descended from a common ancestor and that the three taxonomic subfamilies (\textit{Alpha-}, \textit{Beta-} and \textit{Gammaherpesvirinae}) corresponded to major distinct lineages (439). Mammalian herpesviruses populate all three subfamilies, while all characterized avian herpesviruses are in the \textit{Alphaherpesvirinae}. Recently it has become clear that characterized reptilian herpesviruses also belong to the \textit{Alphaherpesvirinae} (262,293,438). However, a completely different picture has emerged for piscine, amphibian and invertebrate herpesviruses: by analysis of their gene contents, piscine and amphibian herpesviruses form a separate group which appears unrelated to the mammalian/avian/reptilian virus group (439). Furthermore, the single known invertebrate herpesvirus (of bivalve molluscs) appears to belong to a third group, distinct from both of the vertebrate virus groups (142,144).

In order to encompass the above findings, the \textit{Herpesviridae} Study Group of the International Committee on Taxonomy of Viruses has developed proposals to revise
### Table 2.1. Human Viruses of the Family *Herpesviridae*

<table>
<thead>
<tr>
<th>Subfamily</th>
<th>Genus</th>
<th>Designation</th>
<th>Vernacular Name</th>
<th>Site of Latency</th>
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<tbody>
<tr>
<td>α</td>
<td>Simplexvirus</td>
<td>Human herpesvirus 1</td>
<td>Herpes simplex virus 1</td>
<td>Sensory Ganglia</td>
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<td></td>
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<td>Human herpesvirus 2</td>
<td>Herpes simplex virus 2</td>
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<td>Varicellovirus</td>
<td>Human herpesvirus 3</td>
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<td>β</td>
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<td></td>
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<td>Roseola infantum / Sixth disease</td>
<td>Lymphoreticular Cells</td>
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<tr>
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<td>Roseolovirus</td>
<td>Human herpesvirus 6B</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>γ</td>
<td>Lymphocryptovirus</td>
<td>Human herpesvirus 4</td>
<td>Epstein-Barr virus</td>
<td>Lymphoid tissue</td>
</tr>
<tr>
<td></td>
<td>Rhadinovirus</td>
<td>Human herpesvirus 8</td>
<td>Kaposi’s sarcoma-associated herpesvirus</td>
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</table>
higher level taxonomic arrangements for herpesviruses. In summary, the developments proposed are as follows. First, membership of the family *Herpesviridae* will be restricted to viruses which belong to the *Alpha*, *Beta*‐ and *Gammaherpesvirinae* subfamilies on the basis of their gene sequences; these comprise all characterized herpesviruses with mammalian, avian and reptilian hosts. Next, the group of piscine and amphibian herpesviruses will be assigned to a new family, the *Alloherpesviridae*, and the single known invertebrate herpesvirus to another new family, the *Malacoherpesviridae*. Finally, all three families will be grouped in a new higher level taxon, the order *Herpesvirales*.

**VIRION ARCHITECTURE OF HERPESVIRUSES**

As illustrated in Figure 1.1, the herpesvirus particle has several distinct morphological components resulting from the coordinated assembly of over 40 different viral proteins: an icosahedral nucleocapsid harboring the viral DNA, a host‐derived lipid envelope in which virally encoded glycoproteins are embedded, and the tegument, a proteinaceous region located between the nucleocapsid and envelope.

**THE HERPESVIRUS GENOME**

The herpesvirus genome consists of a linear, double‐stranded DNA molecule which characteristically contains regions of unique sequence flanked by direct or inverted repeats. Certain genomes (exemplified by HSV‐1) are particularly complex containing two covalently linked unique regions, designated as unique long (*UL*) and unique short
with one or both flanked by inverted repeats, which promote recombination and inversion of the U_L and U_S regions, producing four equimolar genomic isomers (Fig. 2.1) (286,599,634,719). Genomes of *Herpesviridae* members range in size from 124 kbp (simian varicella virus) from the *Alphaherpesvirinae* to 241 kbp (chimpanzee cytomegalovirus from the *Betaherpesvirinae*) (143,259). The genome of channel catfish virus, the sole sequenced member of the *Alloherpesviridae*, is 134 kbp in size, and viruses in this family that infect carp have the largest known genomes (295 kbp) among the *Herpesvirales* (141,319,723). The single member of the *Malacoherpesviridae* (ostreid herpesvirus 1) has a genome of 207 kbp (144). Nucleotide compositions range widely, from 32 to 75% G+C, even for viruses within the same genus (305). Complete sequencing of the HSV-1 genome reveals a linear, double-stranded DNA molecule of 152 kbp, with a G+C content of 68% (41,173,352,434-436).

Protein-coding regions occupy the great majority of a herpesvirus genome. However, sizeable regions also exist that apparently do not specify proteins. Some of these regions express RNAs that appear not to function via translation. Large non-coding RNAs include the latency-associated transcripts in HSV-1 and a spliced RNA of unknown function in HCMV (377,666). MicroRNAs have been predicted or identified in members of the *Alpha-, Beta- and Gammaherpesvirinae* (78,263,551,552). The functional significance of these transcripts is a subject of intense interest at present.

Historically, viral DNA was considered part of a core structure in the form of a torus within viral capsids (226). However, the current model suggests that in a
Figure 2.1. HSV-1 Genome Organization and Isomerization. The genome of HSV-1 consists of two covalently linked regions, the unique long (UL) and unique short (US), which are flanked by inverted repeats. The UL region (red to yellow) is bracketed by inverted repeats known as ab and b’a’ (red and blue boxes). The US region (indicated by light to dark blue) is surrounded by inverted repeats designated a’c’ and ca (red and green boxes). The HSV-1 genome is capable of recombination and generation of four equimolar isomers. This process is attributed to recombination events mediated by the “a” regions of the genome during DNA replication. For clarity purposes, one isomer is designated the prototype (P), whereas the others represent the outcomes of recombination: inversion of the US region (IS), inversion of the UL region (IL), and inversion of both the US and UL regions (ISL). The arrows denote the relative orientation of the US and UL components.
manner reminiscent of bacteriophages, HSV DNA is packaged in a liquid crystalline arrangement within the capsid (54). This genome configuration, along with large amounts of polyamines (spermine and spermidine) packaged within capsids, neutralize negative charges present on the viral DNA (243,555). Neutralization of the charged DNA facilitates a high degree of nucleic acid condensation, which is necessary for packaging of a large genome within the confined space of a capsid.

**THE HERPESVIRUS NUCLEOCAPSID**

The HSV-1 genomic DNA is housed within an icosahedral shell consisting of five predominant proteins, VP5, VP19c, VP23, VP26 and UL6 (Tables 2.2 and 2.3) (245). The icosahedral \( T = 16 \) capsid, has 162 surface capsomeres with a diameter of approximately 115–130 nm depending upon the virus. Of the 162 capsomeres, 150 are primarily composed of six molecules of the major capsid protein VP5 (the hexons), and 11 are pentamers of the same protein. VP26 binds to VP5 at the tips of each hexon, and hexons connect to each other and to pentons via triplexes consisting of one molecule of VP19c and two of VP23 (55,499,697,698,780). The final pentameric position is occupied by the portal complex, which is comprised of UL6, and has an organization reminiscent of the distinctive 12-fold dodecameric ring arrangement seen in tailed double-stranded DNA bacteriophages (101,335,384,708,709). Similarities between herpesvirus capsids and bacteriophages are not only restricted to the portal complex. Analyses by cryo-electron microscopy and computer-based image reconstruction have extended our knowledge of herpesvirus capsid structures into three dimensions and to a
resolution as fine as 8 Å (778). Comparisons of capsids from members of all three families of the Herpesviridae reveal common structural details that provide a compelling case that the three families may have a common evolutionary origin in regard to their capsid architecture. Furthermore, aspects of capsid structure also suggest an evolutionary link between herpesviruses and tailed DNA bacteriophages (92,665). Improvement in resolution of capsid reconstructions during recent years, for both herpesviruses and bacteriophages, has progressed to the point where comparisons can be made at the level of protein secondary structure between the major capsid protein (VP5) of HSV-1 and analogous bacteriophage proteins. gp5, the capsid shell protein of the lambdoid bacteriophage HK97 is a much smaller protein molecule than VP5 but it has very similar dimensions to a domain of VP5 known as the floor domain (745). When comparison is confined to this domain, the overall shape of the two proteins and their secondary structural elements are strikingly similar (29). Although no herpesvirus capsid structure other than that of HSV-1 is known in sufficient detail to carry out an equivalent analysis, given the general uniformity of their capsid structures it seems likely that the floor domain fold of VP5 will be maintained throughout the Herpesviridae. The possession of a common structure in a key domain of the capsid protein provides strong support for an evolutionary linkage between the Herpesviridae and bacteriophages.

Several other viral proteins (UL15, UL17, UL25, UL28, and UL33) interact with the capsid during DNA packaging and/or readying the capsid to exit the nucleus (23,327,611,668,687). Varying classifications throughout the literature as either capsid proteins, capsid-associated proteins, or inner tegument proteins, have resulted in confusion regarding the specific location of these virion components. In the interests of
clarity and comprehension, throughout this review they will be termed tegument proteins, specifically inner tegument proteins. The capsid components of HSV-1 virions will be restricted to VP5, VP19c, VP23, VP24, VP26, and UL6 (Tables 2.2 and 2.3).

THE HERPESVIRUS ENVELOPE

Herpesvirus particles are bounded by a lipid bilayer with embedded protein molecules, giving the virion an overall diameter of approximately 200 nm. The biochemical composition of the membrane is similar to that of Golgi membranes or the plasma membrane (15,192,193,710,728,747). At least fourteen different virally-encoded integral membrane proteins traverse the envelope, twelve of which are glycosylated (Tables 2.2 and 2.3). These envelope proteins are implicated in a number of processes, including viral entry and assembly as well as immune evasion (83,657).

THE TEGUMENT REGION

Sandwiched between the viral envelope and DNA-containing nucleocapsid lies a proteinaceous layer termed the tegument, which is composed of over twenty individual components with varying stoichiometry. Historically, the tegument has been described as an amorphous layer where the distribution of proteins is largely random; however, recent experimental evidence suggests that the tegument is an ordered, yet flexible structure built through specific protein-protein interactions, which demonstrate
# Table 2.2. HSV-1 Virion Components

<table>
<thead>
<tr>
<th>Virion Localization</th>
<th>Gene Designation (Protein Designation) in HSV-1</th>
<th>Conserved in Alphaherpesvirinae</th>
<th>Conserved in Betaherpesvirinae (Gene Designation in HCMV)</th>
<th>Conserved in Gammaherpesvirinae (Gene Designation in EBV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capsid</td>
<td>UL6</td>
<td>Yes</td>
<td>Yes (UL104)</td>
<td>Yes (BBRF1)</td>
</tr>
<tr>
<td></td>
<td>UL18 (VP23)</td>
<td>Yes</td>
<td>Yes (UL85)</td>
<td>Yes (BDLF1)</td>
</tr>
<tr>
<td></td>
<td>UL3 (VP6)</td>
<td>Yes</td>
<td>Yes (UL86)</td>
<td>Yes (BGLF1)</td>
</tr>
<tr>
<td></td>
<td>UL26 (VP24)</td>
<td>Yes</td>
<td>Yes (UL80)</td>
<td>Yes (BVRF2)</td>
</tr>
<tr>
<td></td>
<td>UL35 (VP26)</td>
<td>Yes</td>
<td>Yes (UL46A)</td>
<td>Yes (BFRF3)</td>
</tr>
<tr>
<td></td>
<td>UL38 (VP19c)</td>
<td>Yes</td>
<td>Yes (UL46)</td>
<td>Yes (BDF1)</td>
</tr>
<tr>
<td>Tegument</td>
<td>UL4</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>UL11</td>
<td>Yes</td>
<td>Yes (UL99)</td>
<td>Yes (BBLF1)</td>
</tr>
<tr>
<td></td>
<td>UL13 (PK)</td>
<td>Yes</td>
<td>Yes (UL97)</td>
<td>Yes (BGLF4)</td>
</tr>
<tr>
<td></td>
<td>UL14</td>
<td>Yes</td>
<td>Yes (UL85)</td>
<td>Yes (BGLF3)</td>
</tr>
<tr>
<td></td>
<td>UL15</td>
<td>Yes</td>
<td>Yes (UL83)</td>
<td>Yes (BGLF3)</td>
</tr>
<tr>
<td></td>
<td>UL16</td>
<td>Yes</td>
<td>Yes (UL84)</td>
<td>Yes (BGLF1)</td>
</tr>
<tr>
<td></td>
<td>UL17</td>
<td>Yes</td>
<td>Yes (UL93)</td>
<td>Yes (BGLF1)</td>
</tr>
<tr>
<td></td>
<td>UL21</td>
<td>Yes</td>
<td>Yes (UL87)</td>
<td>Yes (BcRF1)</td>
</tr>
<tr>
<td></td>
<td>UL26</td>
<td>Yes</td>
<td>Yes (UL77)</td>
<td>Yes (BFRF1)</td>
</tr>
<tr>
<td></td>
<td>UL28</td>
<td>Yes</td>
<td>Yes (UL56)</td>
<td>Yes (BFRF1A)</td>
</tr>
<tr>
<td></td>
<td>UL33</td>
<td>Yes</td>
<td>Yes (UL51)</td>
<td>Yes (BFRF1A)</td>
</tr>
<tr>
<td></td>
<td>UL36 (VP1/2)</td>
<td>Yes</td>
<td>Yes (UL48)</td>
<td>Yes (BPLF1)</td>
</tr>
<tr>
<td></td>
<td>UL37</td>
<td>Yes</td>
<td>Yes (UL47)</td>
<td>Yes (BOLF1)</td>
</tr>
<tr>
<td></td>
<td>UL41 (vhs)</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>UL46 (VP11/12)</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>UL47 (VP13/14)</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>UL48 (VP16)</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>UL49 (VP22)</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>UL51</td>
<td>Yes</td>
<td>Yes (UL71)</td>
<td>Yes (BSRF1)</td>
</tr>
<tr>
<td></td>
<td>UL56</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>US2</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>US3</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>US10</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>US11</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>ICP0</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>ICP5</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

Conservation is assigned based on the presence of sequence and/or positional homology.

The HSV-1 genes that are essential for growth in cell culture are underlined.

vhs, virus host shutoff; PK, protein kinase.
<table>
<thead>
<tr>
<th>Virion Localization</th>
<th>Gene Designation (Protein Designation) in HSV-1</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capsid</td>
<td>UL5</td>
<td>Portal protein. Required for DNA packaging and cleavage</td>
</tr>
<tr>
<td></td>
<td>UL18 (VP23)</td>
<td>Component of intercapsomer triplex. Required for capsid assembly</td>
</tr>
<tr>
<td></td>
<td>UL19 (VP5)</td>
<td>Major capsid protein</td>
</tr>
<tr>
<td></td>
<td>UL26 (VP24)</td>
<td>Viral protease involved in release of the scaffold from nucleocapsids</td>
</tr>
<tr>
<td></td>
<td>UL35 (VP26)</td>
<td>Decorates hexon tips</td>
</tr>
<tr>
<td></td>
<td>UL38 (VP19c)</td>
<td>Component of intercapsomer triplex. Required for capsid assembly</td>
</tr>
<tr>
<td>Tegment</td>
<td>UL4</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>UL11</td>
<td>Role in virion egress and secondary envelopment</td>
</tr>
<tr>
<td></td>
<td>UL13 (PK)</td>
<td>Virion protein kinase</td>
</tr>
<tr>
<td></td>
<td>UL14</td>
<td>Role in cell to cell spread</td>
</tr>
<tr>
<td></td>
<td>UL15</td>
<td>ATPase subunit of terminase. Required for DNA packaging</td>
</tr>
<tr>
<td></td>
<td>UL16</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>UL17</td>
<td>Required for DNA cleavage and capsid localization within the nucleus</td>
</tr>
<tr>
<td></td>
<td>UL21</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>UL25</td>
<td>Required for DNA packaging. Caps the portal packaging is complete</td>
</tr>
<tr>
<td></td>
<td>UL28</td>
<td>Subunit of terminase. Required for DNA packaging</td>
</tr>
<tr>
<td></td>
<td>UL33</td>
<td>Subunit of terminase. Required for DNA packaging</td>
</tr>
<tr>
<td></td>
<td>UL36 (VP1/2)</td>
<td>Influences DNA release from capsids. Role in envelopment and egress</td>
</tr>
<tr>
<td></td>
<td>UL37</td>
<td>Role in virion egress and secondary envelopment</td>
</tr>
<tr>
<td></td>
<td>UL41 (vhs)</td>
<td>Causes nonspecific degradation of mRNA</td>
</tr>
<tr>
<td></td>
<td>UL46 (VP11/12)</td>
<td>Modulates transactivation activity of VP16</td>
</tr>
<tr>
<td></td>
<td>UL47 (VP13/14)</td>
<td>Modulates transactivation activity of VP16</td>
</tr>
<tr>
<td></td>
<td>UL48 (VP16)</td>
<td>Transactivates α gene expression</td>
</tr>
<tr>
<td></td>
<td>UL49 (VP22)</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>UL51</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>UL56</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>US2</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>US3</td>
<td>Protein kinase</td>
</tr>
<tr>
<td></td>
<td>US10</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>US11</td>
<td>Binds to U.34 mRNA and acts as an anti-attenuation factor</td>
</tr>
<tr>
<td></td>
<td>ICP0</td>
<td>Promiscuous transactivator</td>
</tr>
<tr>
<td></td>
<td>ICP4</td>
<td>Required for expression of majority of β and γ genes</td>
</tr>
<tr>
<td>Envelope</td>
<td>UL1 (g1)</td>
<td>Required for fusion of membranes</td>
</tr>
<tr>
<td></td>
<td>UL10 (gM)</td>
<td>Unknown. Interacts with gN</td>
</tr>
<tr>
<td></td>
<td>UL20</td>
<td>Required for viral egress</td>
</tr>
<tr>
<td></td>
<td>UL22 (gH)</td>
<td>Required for fusion of membranes</td>
</tr>
<tr>
<td></td>
<td>UL27 (gB)</td>
<td>Required for fusion of membranes</td>
</tr>
<tr>
<td></td>
<td>UL43</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>UL44 (gC)</td>
<td>Involved in cellular attachment</td>
</tr>
<tr>
<td></td>
<td>UL45</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>UL49A (gN)</td>
<td>Unknown. Interacts with gM</td>
</tr>
<tr>
<td></td>
<td>UL53</td>
<td>Involved in viral egress</td>
</tr>
<tr>
<td></td>
<td>US4</td>
<td>Involved in viral egress</td>
</tr>
<tr>
<td></td>
<td>US5</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>US6</td>
<td>Required for post-attachment entry of virus into cells</td>
</tr>
<tr>
<td></td>
<td>US7</td>
<td>Forms Fc receptor with gE. Required for virus spread in polarized cells</td>
</tr>
<tr>
<td></td>
<td>US8</td>
<td>Forms Fc receptor with gE. Required for virus spread in polarized cells</td>
</tr>
<tr>
<td></td>
<td>US9</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

HSV-1 genes essential for growth in cell culture are underlined.
The major protein functions are denoted. The majority of core genes conserved across the α-, β-, and γ-herpesviruses are involved in fundamental aspects of lytic replication of the virus.

vhs, virion host shutoff; PK, protein kinase.
significant redundancy, at least in cultured cells (72,109,156,158,179,182,183,391,407,456,457,480,514,515,576,718,730,776,778). In a manner equivalent to the matrix of other viruses, the tegument interacts with the capsid on one side and the cytoplasmic tails of envelope glycoproteins on the other to link these structural components during the final envelopment process and secure the integrity of the virus particle (287).

Collectively, the tegument could be considered an instrument of mayhem, delivering a myriad of proteins to the cell, many of whose functions have yet to be elucidated. Post-entry, the majority of tegument proteins dissociate from the incoming nucleocapsid, with a subset destined to prime the cell for synthesis of viral components (36,84,444,575). The virion host shutoff (vhs) protein has been implicated in altering the environment of the cell via host gene expression shut-off, mediated by mRNA degradation (379). VP16 serves to transactivate viral immediate-early gene expression, an activity modulated by two additional tegument components, UL46 and UL47 (36,777). The tegument proteins which remain associated with capsids post-entry, are believed to facilitate transport of the capsid to the nucleus via interactions with cytoskeletal motors (12,176,411,754).

In addition to the viral proteins incorporated into the tegument region during virion assembly, cellular proteins such as Hsp70, Hsp90 and annexin, and the cytoskeletal components actin and tubulin, are also packaged (348,337,781). Furthermore, RNA transcripts, both viral and cellular, have been detected in the tegument of HSV-1 virions (40,60,624). Thus, besides its structural importance in linking the envelope and nucleocapsid, the tegument may function as the trunk of herpesvirus
particles, capable of accommodating cargo for translocation into the host cell. As these cellular proteins and RNA transcripts are abundantly present within infected cells during virion formation, it is largely unclear whether any of them are specifically recruited into herpesvirus particles, or whether they only represent filling material. However, if these tegument cargoes are specifically incorporated, they may also act to prime newly infected cells, perhaps by delivering transcripts encoding vhs or immediate-early gene-transactivators that function early in the replication cycle (60). Alternatively, similar to RNA viruses (e.g. retroviruses), it is possible that packaged RNA functions during assembly by acting as a framework upon which major structural proteins assemble (85). Interestingly, the functions provided by viral or cellular proteins incorporated into the tegument, or packaged RNA transcripts, are not required for replication, as viral DNA alone is infectious (255). However tegument proteins are absolutely critical for assembly of infectious virus particles.

The molecular mechanisms of tegumentation and the process of final envelopment are poorly understood (456,457,602). The complexity and redundancy inherent to the tegumentation process is highlighted by the identification of only a handful of tegument proteins deemed to be essential to the HSV-1 assembly process (36,156,158,480,730). However, this essential classification is attributed to a protein based upon observation of null-mutant viruses within cultured cells; scarcely an accurate representation of the requirement for a specific viral protein during virion assembly within the natural host (36,156,158,480,730).

The mechanism(s) by which HSV-1 tegument proteins are selectively packaged into the assembling virion remains to be elucidated. A variety of intracellular trafficking
motifs to facilitate subcellular localization of tegument components, in combination with a myriad of protein-protein interactions between capsid proteins and tegument proteins, tegument proteins and tegument proteins or between tegument proteins and the cytoplasmic tails of virally encoded glycoproteins, likely facilitate selective packaging of proteins into the tegument region of assembling virions. The focal point of this dissertation is an investigation of the mechanisms utilized by tegument proteins to facilitate virion incorporation. Using the HSV-1 protein VP22 as a model tegument component, protein-protein interactions and virion incorporation determinants are dissected to elucidate the functional significance of these activities in facilitating packaging of VP22 into assembling virions.

**CLINICAL IMPORTANCE OF HERPESVIRUSES**

To date, nine herpesviruses which result in a variety of diseases have been isolated from humans; HSV-1, HSV-2, VZV, HCMV, EBV, and human herpesviruses 6A, 6B, 7 and 8 (also known as KSHV), with an additional non human herpesvirus from the family cercopithecine, herpes B virus (herpesvirus simiae), also an important human pathogen (127,334,601,602).

**HSV-1/HSV-2**

HSV infections occur worldwide with no seasonal distribution. Viral spread occurs through direct contact with virus-containing secretions. The prevalence of HSV-1 infection increases gradually from childhood, with over 90% of adults possessing
antibodies to HSV-1 by the fifth decade of life. In populations of low socioeconomic status, most people acquire HSV-1 infection before the third decade of life (127,487). In contrast, the seroprevalence of HSV-2 remains low until adolescence and the onset of sexual activity. The incidence of antibodies to HSV-2 in the United States is increasing; currently about 22% of the general population is seropositive (209). Importantly, a large percentage of individuals seropositive for HSV-1 and/or HSV-2 are unaware of their status and comprise an important reservoir of infection (209).

Most HSV-1 infections are acquired early in childhood as subclinical or unrecognized infections (661). Oral lesions progress to ulceration (herpes labialis) and generally heal without scarring over 2-3 weeks. Primary infection with HSV-2 classically presents as herpes genitalis, with extensive vesicle formation and fever, in addition to other symptoms. However, the proportion of primary genital infections due to HSV-1 is increasing; from 10% in 1983 to 32% in 1995, reaching up to 50% in certain populations (70,128,591,721). This trend is thought to result from changing sexual practices, including increased oral-genital exposure (591). Approximately 70 to 90% of individuals with HSV-2 antibodies have not been diagnosed with genital herpes (209). Over one half of such people recognize and present with symptoms after education regarding manifestations of HSV disease (219,385). The large reservoir of unidentified carriers of HSV-2 and frequent asymptomatic reactivation of virus from the genital tract have fostered the continued spread of genital herpes throughout the world. HSV-2 infection is an independent risk factor for the acquisition and transmission of infection with human immunodeficiency virus (HIV) type 1 (127). Among co-infected patients, HIV-1 virions can be shed from herpetic lesions of the genital region. However, the most
serious consequence of genital HSV infection is neonatal herpes. Infection usually occurs during vaginal delivery when the infant is exposed to HSV in maternal secretions. Infection may also be acquired through postnatal contact with immediate family members who have symptomatic or asymptomatic HSV infection or through nosocomial transmission within the hospital. The mortality rate for untreated neonates exceeds 70% (334).

HSV is the most common cause of fatal sporadic encephalitis in the United States (524). Without treatment mortality exceeds 70% and even with early intervention, few survivors recover normal neurological function (251). In addition, HSV is the most common viral cause of corneal blindness in the United States (145,546). HSV also infects almost any area of skin; infections of the thorax, ears, face, and hands have been described among wrestlers (334). Transmission of these infections is facilitated by trauma to the skin sustained during wrestling. Several recent outbreaks of this infection have illustrated the importance of prompt diagnosis and therapy, which are required to contain the spread of this infection.

HERPES B VIRUS

Herpes B virus (herpesvirus simiae) is similar to HSV in terms of genome size, morphology and replication cycle. Herpes B virus is the simian counterpart to HSV in old world monkeys, including the rhesus macaque. The seroprevalence of herpes B virus among macaques in the wild is unknown, but among animals housed in outdoor breeding corrals the seroprevalence is approximately 22% before 2.5 years of age rising to more
than 97% among animals 2.5 years or older (729). About 40 cases of herpes B infection in humans have been described in the literature. Humans can be infected via animal bites, mucosal or eye exposure, inoculation of broken skin, needle sticks or potentially via aerosols (96). Herpes B virus disease is severe in humans with a mortality rate of 70% or higher without treatment, with death occurring within 10 days to 6 weeks after exposure. Human to human transmission appears to be extremely rare, as is asymptomatic infection (95). Acyclovir is effective against herpes B virus although the effective dose is 10-fold higher than for HSV (334).

VZV

VZV is a highly cell-associated virus, spread through the body by cell to cell contact. During acute infection cell-free VZV is released into vesicular fluid of skin vesicles facilitating transmission by direct contact. However, VZV also spreads through airborne transmission via respiratory secretions (106). Varicella (chicken pox) and zoster represent different clinical manifestations of VZV infection (743). Varicella occurs most frequently in children, with zoster usually occurring in adults or immunocompromised patients. Varicella results from primary infection with VZV, whereas zoster results from reactivation of latent virus in sensory and cranial nerve root ganglia (235). The following observations indicate that zoster results from reactivation of latent virus rather than reintroduction of the virus into the host: (i) zoster does not exhibit the seasonal prevalence seen with varicella; (ii) zoster does not occur commonly in parents of young children with chicken pox; and (iii) viruses causing the primary infection and subsequent
zoster are the same, as demonstrated by molecular studies of VZV isolates from patients with zoster after either natural chicken pox or varicella vaccination (633). VZV infection may cause severe or fatal disease in individuals who are receiving immunosuppressive therapy or who have abnormalities in cell-mediated immune responses. Disease can be prevented by vaccination, and a live attenuated varicella vaccine derived from the Japanese Oka strain was licensed in the United States for routine use in susceptible healthy people in 1995 (235). Since its introduction, the incidence of varicella has decreased in the United States by 84% (632). Approximately 15% of vaccinated individuals may develop breakthrough varicella caused by wild-type virus months to years after vaccination because the vaccine is not 100% protective (237). However, it is 97% protective against severe disease (713).

**HCMV**

HCMV, formally designated human herpesvirus 5 has a worldwide distribution infecting humans of all ages with no seasonal pattern of transmission. The seroprevalence of CMV increases with age in all populations and ranges from 40 to 100% (298). The virus is acquired early in life by direct personal contact with individuals who are shedding virus, and the prevalence is highest among lower socioeconomic groups, living in crowded conditions. Infections are classified as being acquired before birth (congenital), at the time of delivery (perinatal), or later in life (postnatal). As CMV has been detected in many body fluids, transmission can occur in a variety of ways (300).
CMV infections are common and generally asymptomatic in otherwise healthy children and adults; however, the consequences of disease in newborns and immunocompromised individuals establish this virus as an important human pathogen (574). Fewer than 5% of congenitally infected infants develop symptoms, but those symptomatic individuals may die of complications within the first few months of life. More commonly they survive but are neurologically damaged (580). CMV infections are frequent and occasionally severe in children or adults with suppressed immune systems, either as a result of chemotherapy, human immunodeficiency virus infection, or recipients of solid-organ and bone marrow transplants. Infection in such patients may be due to reactivation of latent virus, or primary infection/reinfection with exogenous virus, which may be introduced by blood transfusions or the grafted organ (573).

Antiviral drugs are frequently used for treatment of CMV infection, with immunoglobulin products containing CMV-specific antibodies also used as prophylactic treatment for transplant recipients. There are currently no licensed vaccines available for prevention of CMV disease. A number of candidate vaccines have been developed and are in various stages of preclinical and clinical evaluation (618).

EBV

Transmission of EBV occurs by salivary or sexual contact, with virtually everyone becoming infected with the virus at some point (131,190,400). Primary infections occur during the first decade of life in areas with crowded living conditions and poor hygiene. In industrialized countries at least 50% of the population becomes
EBV seropositive before puberty. Primary EBV infections are mostly asymptomatic, especially in early childhood. Around 50% of primary infections in young adults are associated with classical infectious mononucleosis (121). This is a self-limiting, lymphoproliferative illness, generally lasting one to four weeks, with protracted tiredness for up to a year not uncommon (533). In immunocompromised patients, the infection often becomes severe and may be lethal.

EBV is associated with a variety of tumors (506,684). EBV genomes have been found in greater than 95% of Burkitt’s lymphomas and nasopharyngeal carcinoma, and in up to 50% of Hodgkin’s disease. Three types of EBV latency have been defined, based on varying expression of the latency-associated proteins (462). EBV latency is dissimilar in different kinds of tumors with Burkitt’s lymphoma having one type while Hodgkin’s disease and nasopharyngeal carcinoma another (462).

There is no vaccine against EBV although a vaccine against glycoprotein 340, which induces neutralizing antibodies, is presently in clinical trials (416,748). Nucleoside analogues inhibit EBV replication, and are used for treatment of infectious mononucleosis. Such treatment results in termination of viral shedding but does not affect the symptoms, presumably since they are mainly immunomediated (10,704).

**HHV-6A/HHV-6B/HHV-7/HHV-8**

Human herpesvirus 6 variants A and B (HHV-6A and HHV-6B), human herpesvirus 7 (HHV-7) and human herpesvirus 8 (HHV-8; Kaposi’s sarcoma-associated herpesvirus) cause diseases that are clinically significant primarily in young children and immunocompromised patients (298).
Collectively, HHV-6A and HHV-6B are highly prevalent, with seroprevalences in many populations exceeding 90% (146). Most individuals become infected by the age of two or three with HHV-6B the main source of the nearly universal HHV-6 infection (772). Transmission typically occurs within a matter of months of the waning of maternal antibody, likely through saliva. Infections with HHV-6 are generally mild or subclinical in immunocompetent individuals, but clinically important disease does occur and can be severe in immunocompromised people (112). Primary HHV-6 infection causes roseola (also known as roseola infantum or sixth disease) in approximately one quarter of children; symptoms include fever, diarrhea, rash and roseola (772). While HHV-6B is the predominant etiologic agent of roseola, rare cases of HHV-6A-associated roseola have been reported (543). HHV-6 reactivation occurs in approximately one-half of bone marrow transplant recipients and one-third of solid organ transplant recipients (146). In this setting HHV-6 has been associated with encephalitis, seizures, and hepatitis. Interestingly HHV-6B is detected more frequently than HHV-6A in transplant patients (507).

HHV-7 seroprevalence in healthy adults in the United States ranges from 60 to 90%. Following waning of maternal antibody, HHV-7 seroprevalence rapidly increases to over 50% by two years of age and then progresses toward the adult level over the remaining years of childhood. The most plausible route of transmission is via saliva (HHV-7 can be cultured from the saliva of approximately 75% of healthy adults), although HHV-7 has also been detected in breast milk, urine and cervical secretions. Approximately 5% of roseola cases are attributed to HHV-7 infection (543).
The global distribution of HHV-8 is uneven. In Africa, HHV-8 seroprevalence exceeds 50%, whereas in most of Europe and the United States, seroprevalence is less than 10% (424). Most HHV-8 transmission is probably via oral fluids, although questions remain as to precisely how this might occur (292). The reasons for the population-based disparities are likely to be due to differences in child-rearing and family practices. In Africa, the virus is frequently acquired early in life, in a maternal linked manner, possibly via a traditional practice such as premastication of food or use of saliva to soothe bites. In the United States, prevalence rises after adolescence (641).

Little is known about primary HHV-8 infection in healthy individuals. For immunocompromised individuals, various illnesses have been associated with primary HHV-8 infection, including Kaposi’s sarcoma (KS). KS has four major epidemiological forms, all of which are associated with HHV-8: (i) African endemic KS, which primarily affects children with or without HIV infection; (ii) Mediterranean or classic KS, which affects older men from specific regions of Mediterranean Europe who have no known immunological dysfunction; (iii) transplant-associated KS, which affects approximately 1% of solid organ transplant recipients in the United States; and (iv) Acquired immunodeficiency syndrome (AIDS)-associated KS, which is the most common AIDS-associated neoplasm and the most aggressive form of the disease (298,543). KS can be disfiguring, disabling and ultimately, life-threatening. HHV-8 has also been implicated in primary effusion lymphoma, a rare but frequently aggressive subset of body-cavity-based lymphomas, with a very poor prognosis. Multicentric Castleman’s disease, a B-cell hyperplasia, occurs frequently in HIV-infected individuals and is also associated with HHV-8 (98,420).
HERPES SIMPLEX VIRUS TYPE 1 REPLICATION

The replication of HSV-1 is a multifaceted process (Fig. 2.2). To initiate infection, HSV-1 must first attach to the surface of a cell and penetrate the plasma membrane, actions facilitated by a subset of viral glycoproteins in concert with their cognate receptors. Once within the confines of the cell, the virus seeks to propagate and persist. To this end, transcription and replication of the viral genome must occur, with resultant assembly and release of nascent virions, thereby perpetuating the process. The aim of this section of the dissertation is to provide an overview of HSV-1 replication; from entry to egress, key aspects of the viral replicative cycle will be presented, with an emphasis on assembly.

CELLULAR ATTACHMENT AND ENTRY OF HSV-1

Generally, enveloped viruses enter cells by fusion with plasma or endocytic membranes, a process that consists of three basic steps: recognition of a cellular receptor by a viral glycoprotein, triggering of fusion, and fusion execution (353). These steps are carried out by virion glycoproteins, in concert with their cognate receptors. The entry and fusion mechanism of HSV-1 consists of a quartet of viral envelope proteins: gB, gD, gH, and gL, and a variety of cellular receptors. The multipartite system recapitulates the multistep process of fusion, intended to prevent premature activation of the viral fusion machinery in the absence of a cellular receptor.

Historically, it was believed that entry of HSV-1 into permissive cells occurred by direct fusion of the virion envelope with the plasma membrane (752). Initial
Figure 2.2. **General Overview of the HSV-1 Replication Cycle.** A fusion event at the plasma membrane facilitates entry of HSV-1 into a target cell. Upon cytosolic penetration, uncoating of the tegument occurs and VP16, a potent transactivator of immediate-early genes, is released. The nucleocapsids travel along microtubules to nuclear pore complexes, where they release their genome into the nucleoplasm; thereby allowing transcription and replication of the HSV-1 genome to occur. Viral gene expression is a tightly regulated cascade, initiated by VP16-mediated transcription of the immediate-early (α) genes. Proteins encoded by the α genes mediate transcription of early (β) genes, whose products facilitate DNA replication and late (γ) gene transcription. The γ genes encode structural proteins that will comprise progeny virions. Virus particle assembly originates within the nucleus, where capsid formation and DNA packaging occur. Nucleocapsids are then shuttled to the cytoplasm via a budding-fusion event that occurs across the inner and outer leaflets of the nuclear membrane, respectively. Upon exiting the nucleus, nucleocapsids traverse the cytoplasm to the site of final envelopment, a trans-Golgi network (TGN)-derived vesicle. At this site, an envelopment step results in acquisition of the majority of tegument proteins with concomitant attainment of a lipid envelope and complement of viral glycoproteins. Subsequently, enveloped particles travel within vesicles through the secretory pathway to the cell surface, where fusion with the plasma membrane releases HSV-1 virions into the extracellular milieu.
biochemical and genetic approaches aimed at addressing the cellular site of HSV-1 entry supported this model. The virion envelope protein gE is transferred to the plasma membrane of infected cells immediately following viral adsorption, presumably as a result of fusion of the viral envelope with the plasma membrane (534). Viral entry via endocytosis confers access to a low pH environment which proves critical for pH-dependent protein conformational changes necessary for viral fusion, penetration and/or uncoating. However, treatment of target cells with agents that inhibit endosome acidification (amantidine or chloroquine) has no effect on HSV-1 infectivity (639,752). Nevertheless, recent experimental observations suggest that at least three diverse pathways are implicated in HSV-1 entry into cell types susceptible to infection: via direct fusion with the plasma membrane, via fusion within an acidic endosome, and via fusion within a neutral endosome. In Vero cells, fusion at the plasma membrane is known to occur in a pH-independent fashion (371). In other cell types, virion entry proceeds via endocytosis, albeit in a variable fashion. For example, in HeLa cells, primary human keratinocytes, and Chinese hamster ovary (CHO)-K1 cells expressing a gD-receptor, fusion with endosomal membranes requires a low pH environment (503,504). In contrast, viral entry in mouse melanoma cells expressing a human gD receptor also involves virion endocytosis, but fusion does not require low pH (467). In each of these diverse pathways, fusion requires gD, gB, the heterodimeric gH/gL, and a gD receptor (505). However, it remains to be elucidated, whether these glycoproteins act in a similar fashion to form a fusion complex, or whether other cellular components are involved in these dissimilar entry pathways.
Cellular Attachment

The initial step in HSV-1 entry into mammalian cells is attachment of the virus to the cell surface. gC and gB interact independently with cell surface glycosaminoglycans (GAGs) to promote initial attachment (Fig. 2.3) (294,386,637,659). The interaction ensures that virions are tethered and concentrated on the cell surface, greatly increasing the efficiency of infection. GAGs, such as heparan sulfate and chondroitin sulfate are long polyanionic carbohydrate chains that decorate cell surface proteoglycans. Numerous biochemical and genetic approaches revealed the primary attachment factor for HSV-1 as heparan sulfate (273,637,700,758); however, chondroitin sulfate can substitute as an attachment factor in the absence of heparan sulfate (32,273).

The glycoprotein–heparan sulfate interactions, although important, are not absolutely essential for viral entry, at least not for infection of cultured cells. A mutant virus harboring a deletion of the gB poly-lysine sequence responsible for interaction with heparan sulfate is still infectious, although virus binding is reduced; a virus lacking the gC glycoprotein is also still infectious (294,386). If both gB and gC are absent, virus binding to cell surface is severely reduced and infectivity is completely abolished; however, this observation could be attributable to the critical role of gB in membrane fusion, rather than abrogation of virion attachment to the cell surface (294). Mutant cell lines deficient in GAG biosynthesis can be productively infected with HSV-1 and a soluble form of gB interacts with the surface of different cell types, independently of heparan sulfate (32,43). These findings suggest that another receptor may mediate interaction of gB with the cell surface. The exact nature of this non-heparan sulfate gB
receptor is still unknown, and its role in attachment and/or fusion remains to be determined.

**Cellular Entry**

Following gC and/or gB interaction with the cell surface, the next stage in viral entry requires interaction of a virion glycoprotein(s) with a cellular entry receptor(s). Mutational analysis of the twelve known HSV-1 glycoproteins revealed that only four are both necessary and sufficient for virus entry. Deletion of gB, gD, gH, or gL, abrogates plaque formation and viral protein expression but not cell adsorption, presumably due to the presence of gC (75,76,212,397,604). Infectivity of each viral mutant is restored by treatment with the membrane fusogenic agent polyethylene glycol (PEG), indicating a defect in virus entry or membrane fusion. Monoclonal antibodies specific for gB, gD, gH, or gL, are sufficient to block viral infectivity without affecting cell surface attachment, further implicating these glycoproteins in the process of virus entry (489,535,545).

During elucidation of the HSV-1 entry machinery, a variety of observations indicated that a gD-specific receptor may act as the entry receptor for HSV-1. Specifically, expression of gD resulted in resistance of normally susceptible cells to HSV-1 infection, presumably through sequestration of a cellular receptor (82,342). In addition, pre-incubation of target cells with soluble gD prevents HSV-1 infection in a dose-dependent manner (338). Taking these data into consideration, combined with the observation that transient expression of gB, gD, gH, and gL in COS cells prompts plasma
membrane fusion and polykaryote formation (703), the following model surfaced:
Initially, HSV-1 virions associate with the surface of the cell through interactions
between gC and heparan sulfate. Subsequently, gD specifically interacts with a cell
surface receptor, an event which triggers fusion of the virion envelope with the plasma
membrane, mediated by gB, gD, and gH/gL (Fig. 2.3).

The identity of the cellular receptor(s) for HSV-1 was facilitated by the use of
CHO and swine testis (ST) cells, which are both non-permissive for HSV-1 infection.
Screening human and murine cDNA libraries for genes that could mediate HSV-1 entry,
both cells types revealed several different HSV-1 receptors that belong to three distinct
protein families (656): (a) Herpesvirus Entry Mediator (HVEM, also named HveA for
herpesvirus entry mediator A), a member of the tumor necrosis factor receptor (TNFR)
family; (b) nectin-1, a member of a family of intercellular adhesion molecules, whose
ectodomain is made of three immunoglobulin-structured domains; and (c) specific 3-O-
sulfated moieties on heparan sulfate which are generated by the enzymatic activity of D-

It is unclear why HSV interacts with multiple alternative receptors. Differential
use of these receptors may account for entry of HSV into such a wide range of cell types
(43,234,656). Nectin-1 and HVEM exhibit distinctive distributions in human tissues.
The distribution of nectin-1 is compatible with a major role of this receptor in the
infection of sensory neurons and muco-epithelia (115,116,234,279,401,426,427,586).
Although a member of a family of intercellular adhesion molecules, nectin-1 is also
present on the apical surface of polarized epithelial cells, facilitating HSV-1 infection in
the absence of lesions that expose the lateral domains of such cells (228,264).
Figure 2.3. Cellular Attachment and Entry of HSV-1. Initial attachment of extracellular HSV-1 to the surface of a target cell is facilitated by interactions between HSV-1 glycoproteins B and C (gB and gC, respectively) and cell surface glycosaminoglycans, such as heparan sulfate and chondroitin sulfate (gray lines), which decorate cell surface proteoglycans. Upon attachment to a target cell, the HSV-1 glycoprotein D (gD) binds to one of three families of cell surface receptors: the tumor necrosis factor receptor (TNFR) family, nectin-1 a member of a family of intercellular adhesion molecules, and specific 3-O-sulfated moieties on heparan sulfate. Ligation of the gD-receptor, triggers fusion of the virion envelope with the plasma membrane, a process mediated by gB, gD, and gH/gL, collectively.
Attachment → gD - Receptor Ligation → Membrane Fusion

**gB/gC** → **gD** → **gB/gD/gH/gL**

Heparan Sulfate
Chondroitin Sulfate

TNF Receptor Family
Nectins
3-O-Sulfated Heparan Sulfate
HVEM is reported to be restricted to T-lymphocytes in human tissues, suggesting a role for this receptor in HSV infection of activated T lymphocytes, or in lymphoid organs, such as the spleen and thymus. However, data from cultured cells, where HVEM is often found suggests that its distribution may be wider. The specific human tissues targeted by HSV via HVEM remain to be established (373).

The most complex and least understood stage of HSV-1 entry into mammalian cells is fusion of the virion envelope with cellular membranes. In the absence of gD, gB, gH, or gL, HSV-1 fails to enter target cells (75,76,79,110,114,374,771). gD does not possess the characteristics of a fusion protein, thus, it is assumed that the fusion machinery involves gB and the heterodimeric gH/gL. For many viruses the fusion process is performed by specific proteins of the viral membrane. To date, two completely unrelated structural classes have been identified, designated type I and type II fusion proteins.

In the type I model, exemplified in orthomyxoviruses, paramyxoviruses, retroviruses, filoviruses, and coronaviruses, the fusion proteins form homotrimers that are proteolytically cleaved into a surface (SU) and transmembrane (TM) subunit, which remains anchored in the viral membrane (73,203,204,733,749,761,765). When activated, these fusion proteins extend to form a rod-like structure, exposing a hydrophobic α-helical fusion peptide at the N-terminus of the TM subunit which penetrates the target cell membrane. Further conformational changes in the TM subunit bring together two heptad repeats (HR-1 and HR-2) located downstream of the fusion peptides, forming a hairpin. Folding back the fusion protein upon itself brings the viral and cellular membranes into close proximity thereby facilitating fusion (126,329,617).
The type II model, found in flaviviruses and alphaviruses, is characterized by a fusion protein which bears an internal fusion peptide (390,471,582). Initially the fusion protein is tightly associated with a second protein as a heterodimer, folded flat on the viral surface. Receptor binding, results in cleavage of the second protein and an irreversible rearrangement of the fusion protein into a trimer which protrudes from the viral envelope. This conformational change exposes a hydrophobic hairpin loop that penetrates the target cell membrane (126,329,353). Subsequently, a fold-back movement of the fusion protein brings the viral and cellular membranes together.

In stark contrast, HSV-1 relies on multiple distinct glycoproteins (gB, gD, and gH/gL) for fusion. The key role in HSV-1 fusion is thought to be played by the glycoproteins gB and gH, which are conserved across the *Herpesviridae*, although which glycoprotein actually performs membrane fusion remains a source of debate (212,249,548).

In organization, gH resembles previously characterized viral fusion glycoproteins: a possible α-helix contained within the ectodomain of gH possesses the characteristics of an internal fusion peptide and two downstream heptad repeats could potentially interact and adopt a coiled coil hairpin conformation (242). Interestingly, mutation or deletion of the gH α-helix abrogates the ability of HSV-1 to enter cells (241). Replacement of this putative α-helix with the heterologous fusion peptide of HIV gp41 or with the fusion protein G of Vesicular Stomatitis Virus (VSV-G) partially restores virus infectivity and gH fusion activity (241). Furthermore, disrupting the HR’s capability to form coiled coils, also affects the ability of gH to function in the infectivity and cell fusion of cultured cells (241,242). Although gH/gL do not form trimers, these mutagenesis data suggest a
function for gH as a fusion protein in viral entry. Nevertheless, the function of gH is dependent upon formation of a heterodimeric complex with gL, which acts as a chaperone for gH, ensuring proper processing and incorporation into the viral envelope (178,316,545). Upon co-expression with gH, gL associates with the viral envelope; however, in its absence gL, which lacks a transmembrane domain, is secreted from cells (178). HSV-1 virions deficient for gL also lack gH, and although they bind to the cell surface, they fail to penetrate the cellular membrane (316,604).

In a similar fashion to gH, glycoprotein B is conserved among the Herpesviridae family (548). The recently solved crystal structure of HSV-1 gB reveals a trimeric ectodomain where each protomer consists of five distinct domains (identified as I–V) (291). The rod-shaped trimer is organized around a central helical core reminiscent of type I fusion proteins. On the other hand HSV-1 gB is not proteolytically cleaved and might contain an internal fusion peptide similar to type II fusion proteins. Studies involving virus-neutralizing monoclonal antibodies directed towards specific sites on the gB ectodomain found that antibodies that affected fusion reacted with residues in the middle region of the ectodomain (291,296). This suggests that domain IV of gB is exposed on the surface of the virion and may be required for viral penetration and cell fusion. Interestingly, the HSV gB ectodomain structure is homologous to VSV-G (593). As VSV-G is a known viral fusion protein, this structural similarity strongly suggests that gB might be the effector of membrane fusion during HSV entry.

On one hand, the trimeric structure of gB, with overall similarity to other viral fusion glycoproteins, argues that gB may be the fusogen. On the other hand, a number of molecular biology and biochemical properties of gH provide evidence that gH/gL exhibit
features typical of class I fusion glycoproteins, in particular, membrane-interacting regions and heptad repeats. How do gB and gH/gL act in concert to promote virus entry? According to the fusion mechanism typical of smaller viruses, only one glycoprotein ultimately serves as the fusogen. The question then arises as to the role of the other glycoprotein. One possibility is that it enables the fusogen to adopt the fusion-active conformation. Alternatively, neither gB nor gH/gL alone may serve as a fusogen, only facilitating fusion when they are in a complex, or act sequentially. In a recent study, hemifusion (the mixing of the lipids of the outer lipid layers of the cell membrane and virion envelope) and complete fusion (operationally defined as lipid exchange between the two membranes) were dissected. The study provided evidence that gH/gL may cause hemifusion in the absence of gB, but not vice versa. The implication is that gH/gL works prior to and in the absence of gB, with both gH/gL and gB required to facilitate complete fusion (672).

Uncoating of HSV-1 and Release of the DNA Genome

Upon entry into the cytosol of a cell, the HSV-1 virion must deliver its genome to the nucleus to initiate viral transcription. As previously described, the HSV-1 genome is surrounded by a proteinaceous shell, consisting of the tegument and nucleocapsid. Delivery of the genome into the nucleus necessitates shedding these proteinaceous layers in a process termed, uncoating. The process of HSV-1 uncoating involves three major steps: dissociation of tegument components, transport of the viral nucleocapsid from the
plasma membrane to a nuclear pore complex (NPC), and release of the viral genome into the nucleoplasm (Fig. 2.4).

Dissociation of the tegument structure is thought to begin immediately upon entry into the cytosol. The current model for tegument uncoating is based on in vitro data, which suggest that the cellular and viral kinases, casein kinase II (CKII) and UL13, phosphorylate certain tegument proteins promoting their dissociation from the nucleocapsid and each other (479). As previously mentioned, the tegument region harbors a variety of proteins and RNA transcripts of both viral and cellular origin (36,40,60,84,444,575,624). Dissociation of the tegument upon entry serves to liberate these tegument components, thereby facilitating their priming activities within the cell. VP16 transactivates viral immediate-early gene expression, an activity modulated by two additional tegument components, UL46 and UL47 (36,777). vhs serves to alter the host cell environment via mRNA degradation-mediated host gene expression shut-off (379). While RNA transcripts may putatively prime the target cell through delivery of transcripts encoding vhs or immediate-early gene-transactivators that function early in the replication cycle (60,61)

Concomitant with virus penetration and tegument dissociation, nucleocapsids traverse the cytoplasm in route to the nucleus. It has been predicted that it would take an HSV-1 capsid with a diameter of 125 nm 231 years to diffuse 10 mm in the axonal cytoplasm, far exceeding the observed time of one hour post-infection, when entering capsids begin to accumulate at the nuclear pore (654,655). Therefore, the nuclear migration of capsids must be an active and organized process. Instead of diffusion, viral particles use the host cytoskeleton for efficient intracellular transport (170,171,649).
Figure 2.4. Uncoating of HSV-1 and Delivery of the Viral Genome to the Nucleus. 

Upon entry into the cytosol of a cell, the HSV-1 virion must deliver its genome to the nucleus to initiate viral transcription. Uncoating of the nucleocapsid is facilitated by the cellular and viral kinases, casein kinase II (CKII) and UL13, which phosphorylate certain tegument proteins promoting their dissociation from the nucleocapsid and each other. Dissociation of the tegument serves to liberate tegument components, including VP16, vhs, and a variety of proteins and RNA transcripts of both viral and cellular origin. Delivery of VP16 into the cytosol provides the virus with the capacity to transactivate viral immediate-early gene expression (complex formation with the cellular protein host cell factor (HCF), results in the nuclear import of VP16). Whereas, alteration of the host cell environment is facilitated by vhs via mRNA degradation-mediated host gene expression shut-off. Inner tegument components such as VP1/2 and UL37 remain associated with the nucleocapsid after tegument dissociation. Transport of the viral nucleocapsid from the plasma membrane to a nuclear pore complex (NPC) is accomplished by retrograde transport along microtubules (green projections), with inner tegument components believed to recruit microtubule motors to cytosolic capsids. Following intracytoplasmic transport, the nucleocapsid docks at the NPC and releases its genome into the nucleoplasm.
Microtubules (MT) are long cytoskeletal filaments with biochemically distinct ends assembled from α/β-tubulin (508). The fast-growing plus-ends of MT usually orient towards the plasma membrane and in neuronal axons towards the nerve terminals. The less-dynamic MT minus-ends are often stabilized by attachment to a MT-organizing centre located close to the nucleus. Cytoplasmic dynein together with its cofactor dynactin facilitate the majority of transport to MT minus-ends, whereas kinesin-1 and a range of kinesin-related motor complexes catalyse transport to MT plus-ends (297,707). Dynactin, which has no enzymatic activity by itself enhances processivity of dynein, and facilitates binding of the motor complex to many different cargoes (148,620,707).

The role of microtubule-based transport and cytoplasmic dynein in translocation of HSV-1 capsids to the nucleus after entry was highlighted by studies using microtubule depolymerizing drugs to block movement of viral particles from the periphery to the nucleus both in vivo and in vitro (372,691,692). In addition, HSV-1 nucleocapsids were demonstrated to co-localize with dynein and/or the microtubule network at early times after internalization or injection (39,655). Dynein was further implicated in the intracellular transport of HSV-1 capsids, as movement of particles along microtubules was disrupted by overexpression of dynamitin, a protein that can disrupt the large dynein complex (172).

How herpesvirus capsids co-opt dynein has yet to be resolved. Several herpesvirus proteins (UL9, UL34, UL46, and VP26) bind to components of the dynein motor complex in yeast two-hybrid or in vitro assays (176,425,764). The relevance of these interactions is not immediately clear as many of these viral proteins (UL9 and UL34) are not incorporated into extracellular virions and therefore cannot be associated
with capsids during transport to the nucleus (363,585). Others (UL46) are absent from capsids undergoing retrograde transport toward the nucleus and are therefore unlikely to participate in this process (257). While VP26, a surface component of the capsid and thus a likely candidate to recruit dynein, has been shown to be dispensable for nucleocapsid transport (12,119,289).

Curiously, viral capsids covered completely with tegument proteins show little MT motility, whereas capsids lacking the outer tegument, but still containing inner tegument proteins, move along MT and bind dynein as well as dynactin (12,176,411,754). VP1/2 and UL37, proteins of the inner tegument, remain associated with capsids as they traverse the cytosol to the nucleus (257,411). During the egress phase of infection VP1/2 but not UL37, is required for transport of capsids along microtubules (412). Whether the microtubule-based transport of viral particles following entry and during the egress phase proceeds by a related mechanism is unknown; however, although yeast two-hybrid assays have so far failed to identify interactions between dynein and VP1/2 or UL37, they are plausible candidates for recruiting MT motors to cytosolic HSV-1 capsids.

Following intracytoplasmic transport, the capsid docks at the NPC and releases its genome into the nucleoplasm. HSV-1 DNA uncoating is thought to resemble genome release of dsDNA bacteriophages such as T4 and λ (195,196,260,473,597,605). The bacteriophage tail recognizes a specific receptor on the host cell surface, triggering DNA exit from the capsid as a single double helix. The genome is released through the portal channel, found at a unique capsid vertex and specialized for function in DNA entry and exit. DNA uncoating in HSV-1 is considered to resemble that of dsDNA bacteriophage
due to similarities in portal structure and location, and arrangement of DNA within the capsid (2,54,97,384,389,497,526,640,696,709).

Various in vitro studies have led to the identification of both cellular and viral proteins involved in the process of genome release. Importin β and Ran-GTP are required, suggesting that attachment of HSV-1 nucleocapsids to the NPC may involve components of the importin β-dependent nuclear import pathway (519). The viral protein VP1/2 also appears to play a role, as a temperature sensitive viral mutant which maps to the U L36 locus, facilitates capsid binding to the NPC, but DNA release fails to occur (36). Recent experimental evidence has correlated cleavage of the portal protein UL6, with DNA loss from the capsid (492). Proteases potentially involved in the cleavage event could include VP24 (the UL26 gene product) which is located inside the capsid, and VP1/2. Given the above mentioned observation regarding the role of VP1/2 in DNA release, it is an attractive candidate.

Other investigators have proposed a model in which binding of nucleocapsids to a NPC prompts a conformational change in capsid structure that results in an opening at a strategic position with concomitant release of the genome (261). In support of this model, a recent study measuring DNA ejection from capsids upon interaction with a solid surface observed HSV-1 genome release from one capsid vertex, presumably the portal, upon binding (492). DNA release did not depend on proteolytic digestion of the capsid, and inclusion of protease inhibitors failed to suppress ejection. Whether attachment to a solid surface perturbs the capsid directly to facilitate DNA release, or alternatively produces a perturbation that is transmitted to the portal to potentiate portal opening and DNA release, remains to be elucidated.
GENE EXPRESSION AND DNA REPLICATION

After nuclear entry of the HSV-1 genome, a productive infection proceeds by transcription of more than 80 genes by cellular RNA polymerase II in a coordinately-regulated, and sequentially-ordered cascade (129,306). Assignment of HSV-1 genes and their respective protein products to one of three classes (α, β, and γ), emphasizes the temporal nature of this expression cascade. Classification is based upon the time of peak synthesis of the gene product following infection, and the requirements for said expression (306). The first genes transcribed, the α or immediate-early (IE) genes, demonstrate maximal expression levels at 2-4 h post-infection without the requirement of de novo viral protein synthesis. In contrast, the β or early (E) genes are expressed 4-8 h post-infection and require a subset of immediate-early gene products, namely transcriptional transactivators for their expression. The majority of β gene products promote viral DNA replication which subsequently enhances expression of the last class of HSV-1 gene products, the γ or late (L) genes, whose expression crests at 8-10 h post-infection. The γ genes can be further subdivided based on their dependence upon genome replication for expression. γ₁ (leaky-late) gene expression is stimulated by DNA replication, whereas γ₂ (true-late) gene expression strictly requires completion of DNA replication. Despite the elegance of this gene expression cascade, the molecular mechanisms governing the switches by which each temporal class of gene products (and DNA replication) regulate expression of subsequent classes are not completely understood, but remain a subject of intense interest and research.
Structure and Localization of the Genome

During HSV-1 entry, the linear double-stranded DNA genome is inserted into the nucleus. The structure of the genome within the nucleus of productively-infected cells remains poorly elucidated. Early studies found that genomic DNA within the nucleus adopts an endless state and resembles circular DNA when examined by pulse-field gel electrophoresis (554). These findings led investigators to propose that the genome circularizes after entering the nucleus. A caveat to these studies stems from the nature of the experimental system. Experiments were performed in cells that fail to initiate a productive infection (as a result of treatment with protein synthesis and DNA replication inhibitors). Recent experimental evidence suggests that the HSV-1 genome remains in a linear or branched state during a productive infection and circularizes during a quiescent or latent infection, perhaps due to the regulatory activity of ICP0 (326).

Following translocation into the nucleus, the HSV-1 genome targets to discrete nuclear matrix-bound domains, known as nuclear domain 10 (ND10) or promyelocyte (PML) oncogenic domains (PODs) (430). ND10s represent accumulations of various proteins, such as Sp100, PML and Daxx, which are implicated in diverse processes such as proliferation, apoptosis, and transcription (180,431,482,760). In addition, accumulation of several (unrelated) overexpressed proteins at ND10 structures has led to the hypothesis that they may function as nuclear depots recruiting and releasing components, such as viral proteins, towards some physiological end (490). Localization of the HSV-1 genome to these nuclear domains appears to be facilitated by the DNA-binding protein Daxx, which either directly or indirectly (through ICP4, ICP8, and/or
ICP27), interacts with immediate-early promoters and initiates immediate-early gene expression (358,430,676).

Interestingly, at later times in the replication cycle the HSV-1 immediate-early protein ICP0 modifies ND10 components resulting in disruption of the nuclear domain (429). Although the functional significance of ND10 localization and subsequent disruption are not known, other herpesviruses such as HCMV also associate with Daxx early during infection, and specifically disrupt ND10s at later timepoints (325). This suggests that ND10s may provide an important function for herpesvirus replication.

**Immediate-Early Gene Expression**

The α or immediate-early genes, whose expression peaks at 2-4 h post-infection, were initially characterized by their expression in the absence of *de novo* viral protein synthesis (306). This observation suggested that an entering virion and/or cellular component(s) were sufficient to recognize and bind to specific sequences in immediate-early promoters and drive their transcription. The use of constructs containing immediate-early gene promoter sequences fused to the coding region of a reporter gene, enabled the identification of the tegument protein VP16 as the primary transactivator of immediate-early gene transcription (35,84). VP16, which is notorious for its motley nomenclature (VP16, UL48, α trans-inducing factor [α-TIF], Vmw65, and ICP25), is a major structural component of the virion that is readily released from the tegument following entry into the cytoplasm (479,479). Cytoplasmic VP16 forms a complex with the cellular protein host cell factor (HCF), resulting in nuclear import of the complex, due
to a nuclear localization signal (NLS) contained within HCF (381,759). Once within the nucleus the VP16-HCF complex interacts with a cellular transcription factor Oct-1, which recognizes and binds directly to TAATGARAT sequences contained within immediate-early promoter regions (Fig. 2.5) (227,238,414,563). Complex formation with both HCF and Oct-1 provides VP16 with an indirect link to immediate-early promoters and enables the acidic, C-terminal transactivation domain within VP16 to potently induce immediate-early gene expression (694). However, VP16 is not required to facilitate immediate-early gene expression; in its absence, immediate-early genes are still expressed (albeit at greatly reduced efficiency), presumably due to the action of cellular transcription factors (e.g. Oct-1 and Sp1) (238,344).

The immediate-early proteins (ICP0, ICP4, ICP22, ICP27, US1.5) function to regulate viral gene expression and modulate basic host cell functions, with the notable exception of ICP47, which plays a role in immune evasion, inhibiting TAP transport of peptides (192,193,732). ICP4 is an essential regulatory protein, facilitating activation of the majority of early and late genes while repressing certain others, including its own. Transcriptional activation is facilitated by indirect binding to DNA via interactions with host transcription factors such as TFIID, while repression occurs through direct binding to DNA (91,152,160,647). ICP27 is involved in the transcriptional activation of β and γ genes. However, it also plays a role in post-transcriptional regulation of gene expression, by inhibiting host mRNA splicing and facilitating shuttling of intronless viral RNA transcripts into the cytoplasm (283,615). Of the non-essential immediate-early proteins, ICP0 is a promiscuous activator of gene expression modulating the cell cycle through ubiquitin-mediated proteolytic degradation, whereas ICP22 and US1.5 positively
and negatively regulate viral gene expression (194,281,349,568). Although absolutely necessary for establishment of a productive infection, the mechanisms by which the immediate-early proteins perform their functions remain poorly understood.

Early Gene Expression

In contrast to the transactivation of immediate-early genes by VP16, the process by which the β or early genes, whose expression peaks at 4-8 h post-infection, are specifically induced remains ill-defined. Observations during HSV-1 infection indicate that production of immediate-early transactivator proteins results in a robust induction of early gene expression, suggesting that specificity exists for early promoters (306,307). However, examination of early gene promoters reveals that they consist of common regulatory elements (e.g. TATA box, Sp1 binding sites, and CAAT elements) that are found in most eukaryotic genes and lack identifiable binding sites for viral proteins (Fig. 2.5) (14,118,149,345,440,529,670). This indiscriminate nature of early promoters is problematic, considering the tightly controlled cascade of HSV-1 gene expression and the absence of early gene expression without immediate-early protein synthesis. The apparent paradox is partially resolved by the finding that immediate-early proteins such as ICP0 and ICP4 interact with various components of the cellular transcription and translation machinery (91,349,380,650). Thus, it is probable that immediate-early transactivators activate early gene expression by an indirect mechanism perhaps through sequestration of transcription factors, stabilization of the transcriptional complex
Figure 2.5. Schematic Representation of HSV-1 Promoter Architecture. The general arrangement of cis-acting regulatory elements of HSV-1 promoters is shown. Immediate-early promoters contain a TATA box, Sp1 binding sites, and a TAATGARAT element, which is recognized by a complex formed by the viral transactivator VP16 and the cellular proteins, host cell factor (HCF), and Oct-1. In addition, some immediate-early promoters contain binding sites for the virally-encoded transactivator, ICP4. Early gene promoters consist of common eukaryotic cis-acting regulatory elements such as Sp1 binding sites and CAAT elements, upstream of the TATA box. Leaky-late promoters contain elements characteristic of both early and true-late promoters. Similar to early promoters, leaky-late genes contain binding sites for various viral and cellular transcription factors (ICP4, Sp1, and YY1) in the region upstream of their TATA boxes. However, like true-late promoters, they also contain two cis-acting elements at or near the transcriptional start site. These elements (initiator element and downstream activation sequence [DAS]) resemble sequences found within eukaryotic promoters and thus serve as binding sites for various cellular and viral transcription factors. True late promoters contain no cis-acting elements upstream of the TATA box. Instead, they contain a TATA box and the two aforementioned cis-acting elements at or near the transcriptional start site.
Immediate-Early Promoters
IE or α

Early Promoters
E or β

Leaky Late Promoters
L or γ₁

True Late Promoters
L or γ₂
and/or alleviation of transcriptional restriction. Although feasible, how altered cellular
machinery exhibits specificity for early gene promoters within this potential model,
remains ill-defined.

Interestingly, all thirteen of the known early proteins play a role in replication of
the 152 kbp DNA genome, a process that will be discussed in detail in a forthcoming
section (602). Of these proteins, a group of seven (ICP8, UL5, UL8, UL9, UL30, UL42
and UL52) are collectively necessary and sufficient to facilitate DNA replication
(89,90,147,247,248,340,421,434,435,757,782). The other six polypeptides, which are
dispensable for growth in cell culture systems, possess functions for nucleotide
metabolism and repair/modification of synthesized genomes (177,208,248,484,736).

**DNA Replication**

Expression of seven HSV-1 early gene products is necessary and sufficient to
induce HSV-1 DNA replication. The process begins when a dimer of UL9 binds to one
of three redundant origins of replication contained within the viral genome
(205,206,321,417,521,557,667,737). UL9, also known as the origin binding protein,
possesses ATPase and helicase activities which facilitate localized unwinding of the
DNA double helix (417). Through direct interaction with UL9, the single-stranded-
binding protein ICP8 is recruited to the origin where it stabilizes the individual DNA
strands, thereby increasing the efficiency of DNA unwinding mediated by UL9 (47-
50,571). A helicase-primase complex consisting of the UL5 (helicase), UL8, and UL52
(primase) proteins, is assembled at the unwound origin, due to specific interactions
between UL8 and UL9, where it mediates the synthesis of an oligonucleotide primer 6-13 base pairs in length (168,359,402,445,677). Primer synthesis subsequently mediates recruitment of the DNA polymerase complex to the origin. This complex consists of the DNA polymerase enzyme (UL30) and a processivity factor (UL42) which binds directly to DNA and functions to tether the polymerase enzyme to the primer-DNA duplex (229,253,254). Together with ICP8 and the helicase-primase, the DNA polymerase complex catalyzes synthesis of nascent DNA strands by a theta mechanism initially, subsequently switching to a rolling-circle mode by an unknown mechanism (231). The latter mode produces head-to-tail concatamers that are cleaved and packaged into capsids during viral assembly (631,774).

In addition to the core replication machinery described above, HSV-1 encodes five other enzymes that contribute to the DNA replication process (50). Three of these virally encoded enzymes (thymidine kinase, ribonucleotide reductase, and deoxyuridine triphosphatase) enhance efficiency of DNA replication by increasing the cellular deoxyribonucleotide pools. The remaining two enzymes (alkaline nuclease and uracil N-glycosylase) act upon newly-synthesized DNA genomes, cleaving concatamers to permit genome encapsidation and enhancing fidelity of DNA replication by repairing deaminated cytosines, respectively. None of these enzymes are required for growth in cell culture; however, most appear to be necessary for viral replication in the nervous system, which suggests that resting neuronal cells lack the functions provided by these viral enzymes (117,320,569,570).
Considering the enzymatic nature of many HSV-1 early gene products, they represent attractive targets for antiviral therapeutics. The broad substrate specificity of thymidine kinase is the basis for acyclic guanosine analogs, such as acyclovir and gancyclovir. Acyclovir is exclusively phosphorylated by HSV-1 thymidine kinase, but not the host kinase, resulting in production of acyclovir triphosphate. Consequently, acyclovir triphosphate, a substrate for HSV-1 DNA polymerase, instigates DNA polymerase inactivation, chain termination, and viral replication inhibition (577).

**Late Gene Expression**

Late genes, whose expression crests at 8-10 h post-infection, can roughly be divided into two groups based on whether DNA replication stimulates their expression (γ₁ or leaky-late) or is a strict requirement for it (γ₂ or true-late). The difference in expression kinetics between γ₁ and γ₂ appears to be attributable to specific sequence elements within the promoter regions of the respective genes (Fig. 2.5). Unlike the regulatory regions of immediate-early and early genes, true late promoters contain no cis-acting elements far upstream of the TATA box (303,341,630). Instead, they generally contain a TATA box and two cis-acting elements at or near the transcriptional start site (277,278,663). These elements (initiator element and downstream activation sequence [DAS]) strongly resemble sequences found within eukaryotic promoters and thus serve as binding sites for various cellular and viral transcription factors (354,550,753).
Interestingly, leaky-late promoters contain elements that are characteristic of both early and true-late promoters (720). For instance, similar to early promoters, leaky-late genes such as U_L48 (VP16) and U_L19 (VP5) contain binding sites for various viral and cellular transcription factors (ICP4, Sp1, and YY1) in the region upstream of their TATA boxes (308,310,466). However, like true-late promoters, they also contain initiator elements and DAS-like sequences at or near the transcriptional start site (309,396). Comparisons of γ₁ and γ₂ promoter architecture, led to the development of a model in which elements upstream of the TATA box induce limited gene expression before the onset of DNA replication, whereas elements downstream of the TATA box elicit high expression after DNA replication. However, the contribution of DNA replication to late gene expression remains elusive.

Late gene expression results in the synthesis of proteins which function in nearly every stage of viral replication. In addition to playing roles in assembly-related processes such as capsid assembly, genome cleavage/packaging, and nucleocapsid envelopment, late proteins facilitate virus entry and subsequent gene expression. This diversity of function is due to the fact that virtually all mature virion components are late proteins. Despite their overall importance in viral replication, collectively late proteins remain the most poorly characterized temporal class of HSV-1 gene products.
HSV-1 VIRION ASSEMBLY AND EGRESS

Following DNA replication and subsequent induction of late gene expression, newly-synthesized virion components accumulate, initiating the HSV-1 assembly process. Virion assembly is perhaps the most complex step of HSV-1 replication, requiring the coordination, among various cellular organelles, of over forty different structural proteins, at least ten nonstructural proteins, the 152 kbp genome, and a variety of cellular factors. In ensuing sections, the major steps in the HSV-1 assembly process will be reviewed in order of their occurrence: nucleocapsid assembly, encapsidation of the viral genome, nuclear egress, tegumentation, final envelopment, and virion release (Fig. 1.2). Emphasis will be placed on discussion of the molecular events associated with tegumentation and final envelopment, specifically trafficking of virion proteins to the site of final envelopment and the myriad of protein-protein interactions that facilitate packaging of virion components into assembling virus particles, a major focus of this dissertation.

Capsid Assembly

Capsid assembly is perhaps one of the most dissected aspects of the herpesvirus replication cycle. Ultrastructural studies of infected cell complexes and structures, the development of \textit{in vitro} baculovirus-based assembly systems, and genetic analysis of capsid components have all contributed to our current understanding of this aspect of herpesvirology (665). Assembly and packaging of the nucleocapsid of HSV-1 is a
complex, multi-step process involving the coordinated activity of some fifteen viral proteins (22). Mechanistically, this pathway has much in common with capsid assembly of tailed bacteriophages, suggesting common albeit distant evolutionary origins (29,31,664). In both HSV-1 and double-stranded DNA bacteriophage assembly, a capsid preformed through interactions of the major capsid protein with a scaffold protein not found in the mature virion, is subsequently filled with DNA. Not only do HSV-1 and bacteriophage capsids bear structural similarities, the mechanism by which viral genomic DNA is incorporated into the capsid also parallels that of bacteriophages (29,778). Packaging of HSV-1 DNA into the capsid structure is facilitated by a portal complex whose 12-fold dodecameric ring arrangement of subunits is similar in structure and dimension to portals found in capsids of DNA bacteriophages (101,102,335,384,709). Like bacteriophage portals, the HSV-1 portal functions as a channel through which viral DNA enters the preformed capsid (302,476,477). The mechanism for introduction of DNA into the HSV-1 capsid is also thought to resemble the mechanism found in bacteriophages, where the portal and terminase subunits [UL15 and UL28 in HSV-1 (1,24,740)] form a packaging machine (93,225).

It is well established that final nucleocapsid assembly occurs within the nucleus; however, the initial stages of assembly have their roots in the cytoplasm. Following synthesis in the cytoplasm, the structural components of the capsid (VP5, UL6, VP19C, VP23 and VP26), and non-structural proteins and their precursors (UL26, UL26.5), are translocated to the nucleus. Transport is facilitated by either an intrinsic nuclear localization signal (NLS), or by association with NLS-containing proteins. VP19C, a triplex protein, the UL6 portal protein, and abundant scaffold protein UL26.5 (preVP22a)
localize to the nucleus in the absence of additional viral proteins (538,539). Whereas the major capsid protein VP5, the triplex protein VP23, and VP26, which binds to the hexon tips, require a chaperon in order to enter the nucleus. In the case of VP5, nuclear recruitment is facilitated by UL26.5 or VP19C (502,588). VP23 is translocated into the nucleus with VP19C, and VP26 is imported by a complex of VP5 and UL26.5 (154,588). Similar protein-protein interactions facilitating nuclear import of NLS-deficient proteins have been observed with CMV capsid proteins as well HSV-1 proteins involved in DNA cleavage/packaging (369,553,756).

Interaction of capsid proteins in the cytoplasm to facilitate nuclear import appears to have an additional role; preventing assembly of aberrant structures. Interaction of VP5 and UL26.5 serves to prevent VP5 from forming anomalous capsid structures composed of VP5, VP19C, and VP23. Cells infected with HSV-1 contain few if any aberrant structures; however, when a null virus lacking the UL26 and UL26.5 genes infects cells, only aberrant structures are found (157). In addition, as protease processing of the scaffold protein can occur in the absence of capsid structures, interaction of VP5 with the scaffold protein may serve to prevent cleavage until after capsid assembly takes place (685).

Within the nucleus, capsids are first assembled as precursor particles or procapsids, an immature, spherical precursor to the icosahedral capsid, into which DNA is packaged (Fig. 2.6). The surface shell of procapsids is composed of hexamers and pentamers of the major capsid protein VP5, encoded by the U119 gene. Capsomers are coordinated by triplexes of VP23 and VP19C, with the portal, a
Figure 2.6. Basic Structure and Composition of an HSV-1 Procapsid. The procapsid surface shell is made up of hexamers (light blue subunits) and pentamers (dark blue subunits) of VP5 (UL19), coordinated by triplexes (green ovals), which are heterotrimers of VP19C (UL38) and VP23 (UL18). The inner shell is composed of the scaffold proteins UL26.5 and UL26. UL26 is an extended version of UL26.5, with the viral protease VP24 and a linker fused to the N-terminus. A single dodecameric ring of UL6 is present at one of the 12 five-fold vertices, termed the portal vertex.
dodecameric ring of UL6 through which the viral DNA will ultimately enter the preformed capsid, at one of the fivefold vertices, the others occupied by the pentons (497,499). In addition to its role in DNA encapsidation (to be addressed later), the portal plays a crucial role in procapsid assembly (494,498). Capsid assembly must take place in such a way that a portal is incorporated into one of the 12 capsid vertices and excluded from all other locations, including the remaining 11 vertices. To ensure that capsids house only one portal complex, it is believed that the portal complex can act as an initiation factor for capsid assembly (Fig. 2.7) (494).

Procapsid assembly depends upon the presence of scaffold proteins UL26 and UL26.5 which share amino acid sequence at their C-termini due to an overlapping reading frame (157,403-405,685). Interaction of scaffold proteins with VP5 is thought to drive capsid formation by bringing the major capsid molecules together through a mechanism of scaffold self-interaction (542,566,567). The regions of UL26.5 and UL26 required for interaction with VP5, as well as the regions required for scaffold intermolecular self-interaction have been identified and shown to be critical for capsid formation (496,549,685,686). Procapsids can assemble in the absence of UL26, however a protease activity housed within this protein is required for maturation of the procapsid (230,564,565). The components brought together by scaffold self-interaction are termed partial procapsids, contain the major capsid protein, scaffold and triplex proteins, and extend in small increments to create the closed structure (686).

In addition to its self-association to form the dodecameric ring, UL6 also interacts with the UL26.5 scaffold protein, an interaction that is crucial for incorporation of a portal into assembling capsids (491,643). If a portal is present during the initial steps of
Figure 2.7. HSV-1 Procapsid Formation in the Absence and Presence of the Portal.

The diagram illustrates the observation the procapsids are assembled normally in the absence of the portal (A). If the portal is present, however, it is suggested to be involved in initiation of procapsid assembly and to be donated by way of a complex with the scaffold protein (B).
procapsid formation, the scaffold acts to present the portal complex to the growing capsid shell and it becomes incorporated into early capsid precursors (one portal complex per capsid) (494). If the portal is not present during this assembly initiation phase, it fails to be incorporated, even if added a later time to the assembly process, prior to procapsid completion (Fig. 2.7) (494). Interestingly, HSV-1 capsid formation can occur in the absence of the portal, suggesting that the partial procapsid structures can initiate capsid formation, independently of UL6 (496,678,686,735).

Shortly after assembly, procapsids undergo a major transition termed maturation, involving profound structural and compositional changes (Fig. 2.8) (495,695,699). UL26, which houses the viral protease, cleaves itself generating the proteins VP21 and VP24 (protease) (404,495,500,567,590,731). Subsequently, VP24 cleaves the product of the UL26.5 gene at a site 25 amino acids from the C-terminus of the protein, abolishing interaction between the scaffold protein and VP5, and releasing the scaffold from the capsid interior (163,164,230,404,565). The capsid undergoes an energy-independent change in shape, switching from a spherical form to the mature icosahedral symmetry, with concomitant remodeling of the hexamers (274,295). A continuous inner floor layer is formed and sites are created around the hexamer outer tips to which the small protein VP26 binds (55,500,779). Cleavage of the UL26 and UL26.5 proteins is not required for procapsid assembly, but the cleavage event is essential for capsid maturation and subsequent DNA encapsidation (230,565,592).

Because procapsids mature in vivo shortly after assembly, they are rarely captured in ultrastructural studies (699). However, three types of capsids are observed in, and may
**Figure 2.8. Maturation Pathway for HSV-1 Procapsids.** During procapsid maturation, the viral protease (VP24) is released by autoproteolysis of UL26, and processes the C-termini of the internal scaffold proteins at the interface between the inner and outer shells, facilitating profound structural and compositional changes. The shape of the capsid switches from round to polyhedral as hexamers (light blue subunits) and pentamers (dark blue subunits) of VP5 are remodeled, forming a continuous inner floor layer and creating sites on hexon outer tips to which the small protein VP26 (UL35) binds. Three types of capsids with mature shells are observed in the nuclei of infected cells: DNA-containing C-capsids and two capsid forms that lack DNA, B-capsids, which retain the scaffolding protein, and A-capsids, which do not. The capsids are represented schematically in cross-section.
be isolated from, the nuclei of infected cells: DNA-containing C-capsids, B-capsids, which retain scaffold protein and lack DNA, and A-capsids, which lack both DNA and scaffold proteins (244,493,547,636). All three have mature shells, however the latter two capsids types appear to be aberrant byproducts, probably resulting from failure to complete (A-capsids), or properly initiate (B-capsids) DNA packaging.

Encapsidation of the Viral Genome

Encapsidation of the HSV-1 DNA genome involves cleavage of progeny DNA concatamers into unit monomers and packaging of a monomer through a portal complex in the capsid shell. In a generalized model of cleavage and packaging of genomic DNA of double-stranded DNA bacteriophages, the terminase enzyme binds to specialized sites in the concatameric DNA and subsequently cleaves the duplex DNA to generate one mature genome end. The terminase with attached concatameric DNA then binds to the portal complex, a dodecameric ring structure located at one vertex in the capsid which forms a channel. Interaction of the portal with the terminase-genome complex generates the packaging motor, fueled by ATP hydrolysis, which pumps the DNA against growing mechanical forces into the capsid resulting in removal of the internal scaffold structure. To keep the tightly condensed DNA in the head, the portal channel has to be properly sealed, preventing DNA leakage. This can either be achieved by a conformational change of the portal protein, or by addition of completion proteins (94).

In HSV-1, cleavage of viral concatameric DNA into unit-length genomes and stable packaging into preformed capsids requires the products of the U₇₆, U₇₁₅, U₇₁₇,
UL25, UL28, UL32, and UL33 genes, which are conserved in all subfamilies of Herpesviridae (4,20,21,105,302,383,433,539,611,612,679-681). Mutants deficient in these genes show characteristic phenotypes, with an intranuclear accumulation of concatameric viral DNA and concomitant absence of C-capsids (668). The absence of UL25 is less prohibitive (668); cleavage and packaging take place, but the DNA does not appear to be stably encapsidated, as fewer C-capsids are seen in the nuclei of infected cells (447).

HSV-1 UL32 is thought to be involved in efficient localization of capsids to replication compartments, directing preassembled capsids to the site of DNA packaging (383). A similar function has been suggested for the HSV-1 UL17 protein (611,680). Subsequently UL6, which comprises the portal complex, recruits the products of the UL15 and UL28 genes which are believed to form subunits of the terminase (21,560,768). The HSV-1 UL33 protein forms a complex with the UL15 and UL28 gene products, constituting a third component of the terminase (37,38). This enzymatic complex is believed to recognize and cleave at specific sites on the replicating concatamer of viral DNA and translocate DNA into the capsid (37,38). In support of this hypothesis, the UL28 protein and its herpesvirus homologues preferentially bind pac1 sequences within the a sequence of the genome (1,51,616). Furthermore, UL15 is capable of binding ATP and is hypothesized to function as a molecular motor responsible for driving DNA into the capsid (24,768). The phenotype of a UL25-negative HSV-1 mutant (described above) suggests that UL25 may function as a head completion protein or plug which seals the portal channel after DNA packaging (447). Interestingly, the HSV-1 UL25 protein has recently been reported to interact with the capsid shell and viral DNA (447,516). In
addition, UL25, in combination with UL17, has also been implicated in the egress of capsids from the nucleus (mentioned in greater detail below) (699).

Entry of the DNA genome into the capsid is proposed to induce a decrease in pH within the capsid, contributing to the dissociation and subsequent exit of scaffold proteins through channels in the capsid (432). Following encapsidation of the DNA, the nucleocapsid is thought to adopt a novel conformation, resulting in an increased association of capsomeres and closure of capsid pores (302).

As previously mentioned the mechanism of assembly and structure of herpesvirus capsids parallel those of bacteriophages (29,31,664). Furthermore, the process by which DNA is encapsidated is reminiscent of bacteriophages, suggesting common, albeit distant evolutionary origins (1,24,29,93,101,102,225,335,384,708,709,740,778). In support of this theory, the arrangement of the dsDNA in the capsid appears very similar in both herpesviruses and bacteriophages. It has been described as a liquid crystalline state in which concentric rings with 2.4 to 2.6 nm striations can be visualized by electron microscopy (54,778). In contrast to bacteriophage T7, HSV-1 DNA does not appear to possess a central protein plug or spindle inside the capsid around which the genome is arranged, and it has been suggested that the herpesvirus genome is packed as an extended, predominantly linear DNA molecule (596,778). It was calculated that the closely packed DNA genomes of HSV-1, HCMV, and rhesus monkey rhadinovirus would occupy approximately 90 to 92% of the total available space, suggesting that genomes are packed as naked DNA without associated histone-like basic proteins (769).
Nuclear Egress

Prior to nucleocapsid egress, significant changes in nuclear architecture are required. As previously described, capsid assembly and genome packaging precede nuclear shuttling of nucleocapsids. Viral gene expression, DNA replication and genome packaging are proposed to occur in replication compartments adjacent to nuclear matrix-associated ND10 structures (430). As a result, nucleocapsids are thought to be tethered to ND10 structures via interactions with tegument and non-structural proteins, forming a large complex termed an assemblon (727). Thus, capsids must dissociate from ND10-based assemblon structures in order to become available for nuclear shuttling. Coincidently, most alphaherpesviruses studied to date encode a protein (e.g. HSV-1 ICP0) that disrupts ND10 structures by selectively targeting components of ND10 domains (e.g. PML and Sp100) for degradation at late times during infection, when assembly is favored (368,429,537). However, it must be noted that ICP0 is not required for the production of infectious virus, thus while an attractive model, the relationship between ND10 disruption and nuclear egress is purely correlative (281).

Given their size of approximately 125 nm, HSV-1 nucleocapsids appear to be excluded from access to the cytoplasm by way of nuclear pore complexes, which exhibit an upper transport threshold of 28 nm (530-532,671). Thus, assembled nucleocapsids are believed to egress the nucleus via a budding event across the nuclear membrane. Following assembly, nucleocapsids move towards and contact the inner nuclear membrane, in an actin-dependent fashion (211). To achieve intimate contact with the inner nuclear membrane, further alterations to nuclear architecture must occur. The
nuclear lamina, a meshwork of intermediate filaments that underlie the membrane providing structural support, must be softened and at least partially dissolved to permit changes in the shape and rigidity of the nuclear envelope (271,626). This process requires two virally encoded proteins, UL31 and UL34, both of which are structurally and functionally conserved across *Herpesviridae* (224,363,364,583-585). UL34 and its homologues are predicted type II transmembrane proteins that complex with UL31 (224,250,382,409,583). Complex formation is important for correct localization of both binding partners at the nuclear membrane; a prerequisite for primary envelopment, as in the absence of either protein, nuclear egress is drastically impaired.

UL31 and UL34 bind to nuclear lamins A/C or B and are required for recruitment of cellular protein kinase C (PKC), which phosphorylates intranuclear lamins A/C and/or B (250,486,536,583,584). This results in local dissolution of the lamin network and underlying chromatin layer, thus enabling nucleocapsids to access the inner nuclear membrane (583,642). Viral protein kinases also appear to play a role in this process. Modification of the lamin B receptor occurs after HSV-1 infection, resulting in redistribution of lamins A/C and B (626). The UL13 kinase of HCMV is directed to the lamin B receptor within the nuclear membrane by interaction with the cellular protein p32 (423). US3, an HSV-1 kinase, also assists in positioning of the UL31/UL34 complex, as well as localization of PKC to the nuclear rim (585,642). Although UL34 is a substrate for HSV-1 US3 kinase, US3 is not necessary for UL34 phosphorylation, indicating that other viral and/or cellular kinases may also phosphorylate UL34 (364,608).
In wild-type virus infections, there is a preference for primary envelopment of DNA-containing nucleocapsids (C-capsids), over envelopment of immature capsid forms that lack viral DNA (742). A recent study has highlighted the role that UL25 and UL17 play in the mechanism of preferential envelopment (699). Despite their presence on all forms of intranuclear capsids, cryo-electron microscopy and image reconstruction suggests that UL25 and UL17 appear to form a heterodimeric C-capsid-specific component (CCSC). The authors speculate that pressure imposed on the nucleocapsid inner surface by packaged DNA elicits structural changes on the outer surface, facilitating CCSC binding. However, thus far, no capsid or capsid-associated protein has been identified that physically interacts with components of the inner nuclear membrane (and presumably the UL31/UL34 complex) to drive primary envelopment.

The fate and origin of perinuclear particles are a source of controversy and debate within the herpesvirus field. Initial egress models suggested that perinuclear particles retain their envelope (and tegument), with virion glycoproteins undergoing modification during release from the cell, as the virus particle moves through the secretory pathway (139,623). Recent studies have revisited and elaborated upon this lumenal egress pathway, proposing that egress of herpesvirus capsids from the nucleus and formation of enveloped virions occur by two alternate pathways (392,746). The first route is similar to that described above, and involves primary envelopment of intranuclear capsids at the inner nuclear membrane followed by transport of these primary virions through the endoplasmic reticulum (ER) and secretory Golgi pathway to the cell surface. The second, and suggested predominant route of egress, involves dilation of nuclear pores resulting in direct access of capsids to the cytoplasm, which subsequently undergo
envelopment in the Golgi compartment. The majority of enveloped virions observed in the perinuclear space are suggested, not to arise from nucleocapsid budding at the inner nuclear membrane, but rather as a result of capsids released from the nucleus through dilated nuclear pores, budding from the cytoplasm into the outer membrane/ER (392,746). Egress would subsequently be facilitated by trafficking through the ER and Golgi pathway to the extracellular milieu (392,746). These studies are based on a limited number of morphological analyses without any biochemical or genetic support, and fail to account for a wealth of biochemical, genetic and morphological data (392,746). Specifically, primary enveloped particles contain UL31 and UL34 (or their various homologues) which are not present in any of the hitherto-analyzed mature herpesvirus virions (224,584). Furthermore, important constituents of mature virions, such as VP22 and VP13/14, are absent from primary virions (488). The lipid composition of the mature virus envelope displays striking differences from that of the nuclear membrane (710) and viral envelope proteins engineered to be retained in the ER fail to become part of the mature virion (741). Thus the integrity of the primary enveloped particle cannot be maintained during maturation, and a de-envelopment step appears to be required. In addition, there are no other reports of the accumulation of nucleocapsids at nuclear pores during herpesvirus infection and the integrity of nuclear pores appears to be maintained until late in herpesvirus infection (282). Contrastingly, nucleocapsids are routinely found accruing along the inner nuclear membrane and enveloped virions within the perinuclear space are an accepted feature of herpesvirus infections, regardless of virus type or host cell (256,456-459). Finally, viral mutants that impair envelopment at the inner nuclear membrane have been identified, and the resulting reduction in perinuclear virions is
associated with reduced infectious yield, implying a key role for the perinuclear virion (224,363,365,584,585).

A competing model, the envelopment-deenvelopment-reenvelopment pathway, suggests that after budding into the perinuclear space, enveloped particles fuse with the outer nuclear membrane, releasing naked nucleocapsids into the cytoplasm, where they undergo a secondary envelopment event at a cytoplasmic vesicle to acquire their final lipid bilayer (662). This reenvelopment event would also result in the acquisition of tegument and viral glycoproteins with mature virions subsequently following the secretory pathway to the cell surface, for release into the extracellular milieu (662).

While not every aspect of the envelopment-deenvelopment-reenvelopment model has been proven (the mechanism of fusion during nuclear egress and identity of viral and/or cellular factors that facilitate this process have yet to be elucidated), it explains the relevant observations outlined above, which any alternative model has to account for. Thus, due to overwhelming evidence, the reenvelopment pathway has now garnered general acceptance.

During egress from the nucleus, it is plausible that nucleocapsids may acquire a portion of their tegument complement, either prior to nuclear egress or during budding at the inner nuclear membrane. Recent studies from Bucks et al., (2007) suggest that VP1/2 and UL37 are added to nucleocapsids prior to their egress from the nucleus (72). VP1/2 would appear to be an ideal candidate for intranuclear tegumentation as it localizes to the nucleus during infection and binds to the major capsid protein, VP5 (448-451,778). Furthermore, a recent study has identified UL25, which associates tightly with capsids and plays a critical role in DNA encapsidation, as a binding partner of VP1/2 (123).
Interestingly, a C-terminal portion of VP1/2 which is indispensable for viral replication, facilitates interaction with UL25; suggesting that the essential function of the VP1/2 carboxyl terminus may be to anchor the VP1/2 tegument protein to capsids (56,123). VP1/2 also interacts with UL37, possibly recruiting this protein to intranuclear capsids (361,464,705,718). In the absence of UL37, VP1/2 is still detected on capsids, suggesting that VP1/2 is added to assembled capsids prior to addition of UL37 (364,365). Nevertheless, interaction of VP1/2 and UL37 with intranuclear capsids remains controversial, with other groups suggesting that at most minimal copies of VP1/2 are added to intranuclear capsids; the remaining copies acquired during a cytoplasmic tegumentation event (699). The vhs polypeptide has also been shown to associate with intranuclear B and C-capsids. However, an additional population of the protein is incorporated into virions in the cytoplasm or during final envelopment (576).

Biochemical studies of virions in the perinuclear space are hampered by inefficient separation from other intracellular particles. However, using transmission immunoelectron microscopy (TIEM), these primary enveloped virions were demonstrated to be strikingly different from mature virus particles in terms of morphology and protein content (256,469). This finding implicates different populations of tegument proteins in nuclear egress and final envelopment, and further strengthens the hypothesis that HSV-1 assembly proceeds via the envelopment-deenvelopment-reenvelopment pathway, rather than the lumenal pathway. Perinuclear particles contain UL31 and UL34 (224,583,584), which are absent from mature virions, demonstrating a distinct difference in composition between primary and mature virions. In contrast, major tegument proteins of mature virions are either absent from perinuclear particles
(such as VP13/14 and VP22), or present in different quantities (VP16) (488). To date, only the US3 protein kinase has been shown to be present in primary and mature virions to a similar extent.

With regard to viral envelope glycoproteins, although several of them have been detected at the inner nuclear membrane, it is disputed whether they are part of the perinuclear virion (28,333,460,469,693). Current evidence suggests that gB, gC, gD, and gM become incorporated into the primary envelope upon budding through the inner nuclear membrane (28,333,693). However, presumably upon fusion with the outer nuclear membrane, any viral glycoproteins found in perinuclear particles would be lost concurrently with the primary envelope, and subsequently reacquired during a final envelopment event at a cytoplasmic vesicle.

In order to continue along the maturation pathway, nucleocapsids must traverse into the cytoplasm. Fusion of the primary envelope with the outer nuclear membrane/ER occurs independently of the four conserved herpesvirus glycoproteins (gB, gD, gH, or gL) required for fusion of the mature virion envelope with the plasma membrane during entry. However, an HSV-1 mutant lacking both gB and gH fails to cross the nuclear envelope (perinuclear particles accumulate in the perinuclear space or in membrane vesicles that bulge into the nucleoplasm) (200). As the fusion process associated with HSV-1 entry requires the cooperative action of gB, gD, gH, and gL, these observations suggest that the two types of fusion (egress versus entry) are dissimilar processes. A defect in deenvelopment, resulting in accumulation of primary enveloped virions has also been observed in HSV-1 mutants that lack US3 kinase activity, suggesting that
phosphorylation of a component of primary-enveloped virions by US3, a component of perinuclear particles, may trigger deenvelopment (364, 585, 622).

**Site of Final Envelopment**

Upon release into the cytoplasm, nucleocapsids traffic to a cytoplasmic vesicle where they undergo a secondary envelopment event resulting in the acquisition of a lipid bilayer and final complement of tegument and viral glycoproteins. The identity of this cytoplasmic budding site, a key component of the envelopment-deenvelopment-reenvelopment model, has long been disputed. Such disparate cytoplasmic structures as the ER-Golgi intermediate compartment, post-Golgi vacuoles, tegusomes, aggresomes, and early and late endosomes have been proposed as the location of reenvelopment (71, 218, 284, 304, 513, 598, 613, 614).

Current evidence points to TGN-derived vesicles as the site of cytoplasmic budding. Initially, electron microscopy identified large numbers of unenveloped capsids which appeared to bud into tegument-coated vesicles that were reminiscent of TGN membranes (236, 256, 258, 343, 366, 662). The egress model of HSV-1 infers that the cellular compartment used for reenvelopment contributes not only an envelope to HSV-1 but also a complement of viral glycoproteins. Accumulation at the TGN of the viral glycoproteins present in the virion envelope, was observed in immunofluorescence studies; as one would predict if capsids acquire their envelope at this site (5-7, 62, 132, 216, 266, 330, 446, 469, 614, 689, 702, 784, 785). Moreover, this accumulation occurred independently of capsid egress (702). These microscopy studies are
complemented by biochemical analyses of the phospholipid composition of the virion envelope which detect high concentrations of sphingomyelin and phosphatidylserine, lipids that enrich the Golgi apparatus, in the HSV-1 envelope (710). In addition, detailed cellular fractionation studies of infected cells, demonstrated that HSV-1 co-purifies with the TGN and/or endosomes (284). These biochemical data coupled with detailed ultrastructural studies indicate a Golgi-derived compartment as the site of HSV-1 final envelopment, although a role for endosomes cannot formally be excluded.

**Cytoplasmic Trafficking of HSV-1 Nucleocapsids during Egress**

Similar to the events of entry and uncoating, HSV-1 nucleocapsids appear to traffic to the site of secondary envelopment by hijacking the cytoskeletal network (544). Treatment with nocodazole, a microtubule depolymerizing agent, (but not cytochalasin D which disrupts actin filaments), abrogates cytoplasmic transport of nucleocapsids during egress (412,468). The molecular motor kinesin-1 and a range of kinesin-related motor complexes catalyse transport away from the nucleus (297,707). Interestingly, movement of viral nucleocapsids along microtubules is abrogated by overexpression of dynamitin, a protein that can disrupt the large dynein complex, a cofactor of kinesin-2 (172).

The viral proteins involved in recruitment of cytoplasmic nucleocapsids to the microtubule network are poorly-defined. When a fragment of the kinesin-1 heavy chain (the stalk/tail region thought to bind organelles) was used as bait to identify putative viral binding partners, the major capsid protein VP5, and tegument proteins VP16, VP22, and US11 were detected (162). A recent *in vitro* study demonstrated that viral capsids
completely covered with tegument proteins show little microtubule motility, whereas capsids with the outer tegument removed by exposure to high salt concentrations, but still containing inner tegument proteins, move along microtubules efficiently (12,176,411,754). Biochemical analysis of the protein composition of these inner tegument-coated capsids confirmed the presence of VP1/2, UL37, and VP16; VP22 was also detected, albeit in low abundance. Notably, US11 despite its ability to bind to the heavy chain of kinesin-1 was not required for MT mobility in vitro (754).

The tegument proteins VP1/2, UL37, VP16, US3, but not VP22, are found associated with cytoplasmic nucleocapsids (220,257,469). Furthermore, in the related alphaherpesvirus PrV, VP1/2 is required for microtubule-dependent transport in the cytoplasm, with UL37 (which requires VP1/2 for capsid-association) necessary for wild-type levels of transport, but not essential for the process (223,361,412). These findings suggest that VP1/2, may bind a host motor complex either directly or indirectly, possibly through another tegument protein, thereby facilitating transport of viral nucleocapsids to the site of final envelopment. In support of this model, yeast two-hybrid analysis has identified VP16 as a potential binding partner of VP1/2 (718). In addition, the HCMV VP1/2 homologue binds to a cellular protein p180, which in turn binds kinesin (161,517,718).

A caveat to elucidation of HSV-1 nucleocapsid transport during egress stems from the nature of the experimental system used. The experiments described above were performed in vitro or in Vero cells (African Green Monkey kidney epithelial cells). Yet, HSV-1 is capable of infecting neurons as well as epithelial cells. Initial infection of sensory neurons is followed by retrograde trafficking of capsids to nerve cell bodies in
ganglia where latency is established. Periodic reactivation and virus replication produce virions that are transported in the anterograde direction in axons leading to reinfection of epithelial tissues. It is presently controversial how alphaherpesviruses are transported in the anterograde direction in neuronal axons, with two different models proposed (Fig. 2.9) (13,87,99,100,150,301,411,468,544,609,652,653). In the first model, denoted the separate model, unenveloped capsids are transported on axonal microtubules separately from vesicles containing viral glycoproteins. In this model, assembly of enveloped virions occurs at axon termini, as capsids bud into membrane vesicles containing viral glycoproteins (544). Alternatively, the married model suggests that capsids acquire an envelope and glycoproteins in neuronal cell bodies and subsequently travel through axons as enveloped virions (13,99,100). Presumably, on reaching axon termini, the outer membranes surrounding these virions fuse with the plasma membrane delivering virions to the extracellular milieu. Studies focused on differentiating between the two models highlight the divergence between nucleocapsid transport in neuronal versus epithelial cells.

As with nucleocapsid transport in epithelial cells, anterograde transport of cytoplasmic nucleocapsids also appears to be microtubule-dependent in cultured neural cells; however, the requirements for transport are markedly different (162,372,468,544). In neurons US9 is required for anterograde axonal transport of capsids, but not for transport of viral glycoproteins; however, in epithelial cells, no defect is observed in the absence of US9 (387). Thus, one must be vigilant in interpreting results across these diverse experimental systems.
Figure 2.9. Proposed Models for Assembly and Egress of HSV-1 from Neuronal Cells. (A) The separate model. Unenveloped capsids are transported on axonal microtubules (green) separately from vesicles containing viral glycoproteins. Inner tegument proteins (red and green concentric circles) are likely associated with capsid surfaces, while other tegument proteins (blue concentric circles) associate with glycoproteins. In this model, assembly of enveloped virions occurs at axon termini, by budding of capsids into membrane vesicles containing viral glycoproteins. Fusion of the vesicle with the plasma membrane releases virions onto the surface of the neuron that can subsequently infect adjacent epithelial cells. (B) The married model. Capsids acquire an envelope containing glycoproteins and their complement of tegument proteins in neuronal cell bodies and travel as enveloped virions within vesicles on axonal microtubules. Upon reaching axon termini, the vesicle membranes surrounding these virions fuse with the plasma membrane delivering virions to the extracellular milieu that can subsequently infect adjacent epithelial cells.
Cytoplasmic Tegumentation

It has become clear that cytoplasmic tegumentation can initiate at two distinct sites, the viral nucleocapsid and the TGN-derived cytoplasmic vesicle which will ultimately contribute the future virion envelope. These two subassemblies combine during the process of final envelopment to produce a mature virion. Although appearing naked by conventional electron microscopy, biochemical and immunolabeling studies indicate that cytoplasmic nucleocapsids carry the US3, UL16, UL37, VP1/2, and VP16 proteins (220,452,469,488). VP1/2 and UL37 have been suggested to be added to viral nucleocapsids prior to their egress from the nucleus, whereas immunolabeling studies have detected VP16 and US3 in perinuclear particles (72,488,585,622). However, whether these proteins are in fact added during nuclear egress and remain associated with the translocated capsid, or are reassembled early after nuclear egress is unclear.

Electron cryomicroscopy studies have suggested that the inner tegument demonstrates icosahedral symmetry at the penton vertices of the capsid (274,778). Detergent treatment of virions removes the envelope and solubilizes some tegument proteins but leaves others (notably VP1/2) in an insoluble, capsid/tegument fraction, while more vigorous treatment results in the loss of virtually all envelope and tegument proteins except for VP1/2 (658,716). As tegument attached at the pentons is also resistant to removal by detergent, it seems highly likely that the detergent-insoluble VP1/2 is located at or near these positions (716). Thus, VP1/2 represents a strong candidate for the icosahedrally ordered inner tegument component. The identification of VP1/2 as a tegument component added to the viral nucleocapsid early during the assembly process
correlates with its classification as an inner tegument protein (72,220,469). As VP1/2 remains associated with viral nucleocapsids upon entry, and has been implicated in microtubule-dependent cytoplasmic transport during both entry and egress, the location of VP1/2 within the virion also compliments its functionality, at least in relation to nucleocapsid transport (257,411).

VP1/2, which is conserved across the *Herpesviridae*, also plays a critical role in the assembly of all herpesviruses examined (158,648,767). An HSV-1 VP1/2-null mutant results in the accrual of numerous cytosolic C-capsids that lack a tegument layer and fail to undergo secondary envelopment (158). A PrV VP1/2-null virus, demonstrates a more severe phenotype than that of the HSV-1 mutant, with viral nucleocapsids typically restricted to the nucleus (412). Although, the reason for these disparate phenotypes is not immediately clear, it must be noted that the mutant UL36 allele introduced into HSV-1 left ~62% of the coding sequence intact, which could have resulted in production of a truncated protein with partial activity (158). VP1/2 is anchored to the capsid through interaction with UL25, with a C-terminal portion of VP1/2 which facilitates interaction with UL25, essential for viral replication (56,123). Interestingly, UL25 is also required for nuclear egress and has been implicated in the formation of a C-capsid specific component which results in the preferential egress of DNA-filled capsids from the nucleus (699,362). Collectively, these findings suggest that association of VP1/2 with intranuclear capsids may facilitate their egress from the nucleus, either directly or indirectly through the recruitment of additional cellular or viral factors.
Regarding viral binding partners of VP1/2, two protein constituents of cytoplasmic capsids (UL37 and VP16), interact with the UL36 gene product (220,361,464,469,705,718). While the majority of VP16 is released from capsids upon entry, UL37 remains associated with viral nucleocapsids until they dock at the nuclear pore in a similar fashion to VP1/2 (257). One could infer from this observation that UL37 may form a second layer of inner tegument. As UL37 is present on intranuclear capsids, interaction with VP1/2 may recruit UL37 to capsids prior to nuclear egress. Indeed the PrV homologue of VP1/2 is required for UL37 capid association. Interestingly, deletion of UL37 abrogates virus maturation, with a distinct accumulation of unenveloped nucleocapsids observed in the cytoplasm (156). Nucleocapsids were predominantly detected at the nuclear periphery, suggesting that nuclear egress can occur, albeit inefficiently, but subsequent steps of tegumentation and envelopment are blocked (156). Microtubule-dependent transport of viral nucleocapsids occurs independently of UL37, implying that the UL37-null phenotype is not simply due to failure to transport capsids to the correct cellular compartment for final envelopment (412). These findings suggest that although UL37 may be recruited to capsids within the nucleus, its essential role in the assembly pathway functions after nuclear egress, downstream of a critical function of VP1/2.

Of the tegument proteins identified as components of cytoplasmic nucleocapsids, VP16 is the only other gene product deemed essential for tegumentation and secondary envelopment (480,730). VP16, although present on perinuclear particles, is efficiently removed from extracellular virions with detergent treatment and high salt concentrations (488,658,716). In addition, upon viral entry VP16 dissociates from the capsid structure
to perform one of its functions; transactivation of immediate-early gene expression (36,36,84,444,575,777). Collectively these observations imply that VP16 is not a component of the inner tegument. Furthermore, the levels of VP16 on perinuclear particles are lower than those in extracellular virions, and TIEM studies have demonstrated that additional VP16 is added to capsids during secondary envelopment (469,488). Consistent with its differential localization within the virion in relation to the inner tegument, the phenotype of a VP16-null viral mutant is quite distinct from that observed upon deletion of either VP1/2 or UL37. Tegumented nucleocapsids are detected within the cytoplasm; however, they are unable to undergo secondary envelopment (480,730). Thus it appears that VP16 is required for one or more steps in the virus egress pathway, downstream of the essential activities of VP1/2 and UL37.

In addition to providing the virion membrane and complement of viral glycoproteins, secondary envelopment at TGN-derived cytoplasmic vesicles also results in the acquisition of the majority of tegument components. Tegment proteins such as VP22, UL11, and VP13/14 appear to be added primarily during this step in the assembly pathway (469). Furthermore, additional copies of protein species present on cytoplasmic nucleocapsids also appear to be acquired during this envelopment event (155,220,469,488,576,585,622,687). This observation suggests that proteins such as UL17, UL37, VP1/2, VP16, and vhs may have multiple roles at different stages of the assembly pathway. Virion incorporation of tegument and glycoprotein components during secondary envelopment requires the targeting of such constituents to membranes of the TGN. While the nucleocapsid traffics to the TGN as a multiprotein complex, other structural components likely target to the Golgi as monomers. Before attempting to
understand how HSV-1 proteins accumulate at the site of final envelopment, the biology of the Golgi apparatus should be considered.

The Golgi apparatus represents the central junction for membrane trafficking. Consisting of four compartments, the cis, medial, and trans-cisterna, and the trans-Golgi network (TGN), the Golgi apparatus and its resident proteins are components of the cellular secretory pathway. The responsibilities of this pathway include synthesizing lipids, processing glycoproteins, cycling cellular proteins between the Golgi and ER, and sorting proteins to and from the plasma membrane through the TGN and endosomal/lysosomal pathway (201,246). Considering the large flow of proteins and lipids through the Golgi, proteins have adopted several different mechanisms to localize there (Fig. 2.10). Retention of proteins at the Golgi apparatus can be explained through two competing models: the kin-recognition model and the bilayer thickness model (485). The kin-recognition model suggests that Golgi resident transmembrane proteins (TMPs) oligomerize to form structures too large to enter transport vesicles. The bilayer-thickness model proposes that distinct lipid domains partition Golgi resident proteins from transient ones. Both models implicate the length and composition of the transmembrane domain as the retention determinant. Regardless of which model is correct, the transmembrane domain of several cellular glycotransferases, the coronavirus E1 protein, and the rubella virus E2 protein appears responsible for Golgi retention (124,299,673).

The kin-recognition and bilayer thickness models are applicable for retention of only TMPs at the Golgi. Many peripheral membrane proteins (PMPs) localize to the Golgi in the absence of a transmembrane domain. Localization of PMPs to the Golgi could be explained by post-translational modification with fatty acids, such as addition of

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myristyl (14C), farnesyl (15C), palmityl (16C), or geranylgeranyl (20C) groups (579,773). The hydrophobic nature of fatty acids is thought to retain modified proteins at the site where the modification occurs; thus, the compartment where the fatty acyl transferase is located (755). For instance, the vaccinia virus p37 protein requires modification by palmitylation in order to localize to membranes of the TGN, suggesting that the appropriate fatty acyl transferase is located at the TGN (269).

PMPs and TMPs also utilize an assortment of vesicular trafficking signals that facilitate retention at the Golgi. Trafficking or sorting signals are usually short amino acid motifs that can be classified into three general categories: tyrosine-based motifs (YXXΦ), dileucine motifs, and acidic cluster motifs. These motifs recruit members of the clathrin adaptor family [adaptor protein-1 (AP-1), AP-2, AP-3, Phosphofurin acidic cluster sorting protein-1 (PACS-1), and Golgi-localized, γ-adaptin ear homology domain, ARF-binding protein (GGAs)] to a membrane, which subsequently facilitate clathrin coat formation and vesicular trafficking (53,356,357,724). These motifs may traffic proteins to and from the Golgi apparatus. For example, the cellular endoprotease furin exits the TGN as a result of AP-1 recognition of an YKGL motif (682). Upon reaching the plasma membrane, furin is endocytosed through detection of a dileucine motif by AP-2 (670). Once internalized to an endosomal compartment, phosphorylation of an acidic cluster motif within furin recruits PACS-1, which returns furin to the TGN (724).

Finally, a PMP or TMP can indirectly localize to the Golgi apparatus through association with a protein that resides in the Golgi using one of the four above-mentioned mechanisms. Viral proteins, such as the coronavirus M protein and the bunyavirus
Figure 2.10. Localization of Proteins to the Golgi Apparatus. Considering the large flow of proteins and lipids through the Golgi apparatus, resident proteins are thought to have adopted one of several mechanisms to facilitate their localization. (A) The bilayer thickness model proposes that distinct lipid domains partition Golgi resident proteins from transient ones. (B) The kin-recognition model suggests that Golgi resident transmembrane proteins (TMPs) oligomerize to form structures too large to enter transport vesicles. (C) Many peripheral membrane proteins (PMPs) localize to the Golgi in the absence of a transmembrane domain. Localization to the Golgi may be attributable to post-translational modification with fatty acids. The hydrophobic nature of fatty acids is thought to retain modified proteins at the site where the modification occurs; thus, the compartment where the fatty acyl transferase is located. (D) Proteins also utilize an assortment of vesicular trafficking signals that facilitate retention at the Golgi. Trafficking or sorting signals are usually short amino acid motifs that can be classified into three general categories: tyrosine-based motifs (YXXΦ), dileucine motifs, and acidic cluster motifs. These motifs recruit members of the clathrin adaptor family [adaptor protein-1 (AP-1), AP-2, AP-3, Phosphofurin acidic cluster sorting protein-1 (PACS-1)], to a membrane, which subsequently facilitate clathrin coat formation and vesicular trafficking (dashed circles). Finally a protein can indirectly localize to the Golgi apparatus through association with a protein that resides in the Golgi using one of the four above-mentioned mechanisms.
Extracellular milieu

PACS-1

AP-1

AP-2

TGN

A

B

C

D
glycoprotein 2 rely on interactions with Golgi resident proteins to facilitate their intracellular localization (9,108,399).

As required by the proposed HSV-1 envelopment model, all virion structural components must descend upon the TGN. Although only a small subset of HSV-1 proteins have been examined for targeting to the cellular site of final envelopment, studies reveal that herpesvirus proteins utilize many of the mechanisms outlined in Figure 2.10 to localize to the TGN. The glycoproteins gB, gE, gI, and US9 contain YXXΦ, dileucine, and acidic cluster motifs within their cytoplasmic tails that enable these proteins to traffic between the plasma membrane and TGN (63,6,7,64,65,133,267,304,331,446,522,523,613,689,701,785).

In the case of gE, endocytosis from the plasma membrane and subsequent TGN localization is facilitated by a tyrosine-based motif and an acidic stretch of amino acids in the cytoplasmic tail of the glycoprotein, thus ensuring incorporation of gE into the envelope of nascent virions (6,7). Specifically, the tetrapeptide of the tyrosine-based motif is recognized by the µ-2 subunit of AP-2. Formation of a clathrin-coated vesicle is facilitated by AP-2 which acts as an adaptor between the membrane protein (gE) and clathrin (52,518). Recycling of gE to the plasma membrane post-endocytosis has been demonstrated, but there is also a pathway for gE to recycle to the TGN, the site of final tegumentation and envelopment (6,522). The acidic cluster of amino acids present in the cytoplasmic tail mediates TGN trafficking (6,267,724). Phosphorylation of serine and threonine residues within this acidic cluster motif mediates interaction with PACS-1, which directs gE from endosomes to the TGN (134,207,627,725). Differential phosphorylation of residues adjacent to the gE acidic cluster by viral kinases or the
cellular casein kinase II, targets endocytosed gE to either the cell surface or the TGN, thereby increasing cell-to-cell spread or virus assembly (350,351,474).

Given the high degree of conservation of the different C-terminal sorting signals in the alphaherpesvirus glycoproteins, it would seem likely that these molecules traffic inside the cell following identical signal-mediated transport processes. Yet this assumption is not true. HSV-1 gI, cannot be internalized from the plasma membrane on its own and requires interaction with gE for efficient endocytosis and targeting to the TGN. Whereas gB requires an intact dileucine motif, not an acidic cluster motif like gE, for postendocytic trafficking to the TGN from endosomes (6,267,724).

The HSV-1 glycoproteins gK and UL20 when expressed singly, localize at the ER; however, when coexpressed they enable each other to traffic and localize to the TGN (18,19,213,217,317,726). They are not detectable at the plasma membrane and, at least for gK, this may be due to endocytosis (215,216). Interestingly, when UL20 and gK are expressed with the glycoproteins essential for fusion, there is a decrease in cell surface expression of gD, gB, and gH/gL, suggesting that viral gene products may also facilitate glycoprotein endocytosis (18,19). HSV-1 gM displays interesting analogies to gK and UL20. Although deemed nonessential in the alphaherpesviruses, gM is conserved throughout the Herpesviridae. In gM-expressing cells, gD and gH/gL relocalize from the plasma membrane to the TGN (132). This raises the possibility that gM may act to reduce cell-cell fusion by decreasing cell surface expression of the HSV-1 fusion glycoproteins. However, the gM-mediated decrease in cell surface expression is not restricted to the fusion proteins, and may actually function to maintain sufficient concentrations of envelope proteins at the site of secondary envelopment (132).
Tegument proteins which form the outer layer of tegument are either membrane-associated themselves or need to associate with membrane bound proteins. The HCMV tegument protein UL99 utilizes myristylation and an acidic cluster motif to localize to the TGN (346). Whereas, the HSV-1 UL11 tegument protein requires both myristylation and palmitylation for efficient Golgi targeting, and an acidic cluster motif to facilitate retrieval from the plasma membrane to the Golgi (57,406). Palmitylation of HSV-1 UL51, and prenylation of PrV US2, have been implicated in membrane association of these tegument proteins (113,512).

The tegument components VP22 and vhs associate with cellular membranes in the absence of additional viral proteins (66,388,483). Yet, primary sequence analysis does not reveal an amino-terminal signal sequence, membrane-spanning domain, or any known motifs for fatty acylation or isoprenylation. Interestingly, both VP22 and vhs can be removed from membranes by treatment with salt in the absence of infection (66,388,483). However, in the presence of infection, vhs can not be removed by similar treatment (483). These results suggest that in the absence of infection membrane binding is mostly due to electrostatic interactions, whereas during infection there are salt resistant mechanisms that stabilize membrane binding. It is plausible that in the absence of infection, VP22 and vhs may bind to a cellular protein through electrostatic interactions; however, upon infection a virally-encoded or virally-induced protein may act as a membrane binding partner which stabilizes the interaction. This would not be without precedent as many tegument proteins that are added to the nucleocapsid during secondary envelopment rely on protein-protein interactions to ensure their localization to membranes of the TGN (109,183,270,280,360,407,646,718,776,784). In fact VP16, a
binding partner of vhs interacts with the cytoplasmic tails of a variety of viral glycoproteins, and recent studies have identified gD as a binding partner for VP22 (109,270,646,784). Extensive protein-protein interaction networks incorporating glycoproteins, tegument proteins, and nucleocapsid components have, and continue to be elucidated.

Final Envelopment

A highly ordered network of protein-protein interactions drives the process of secondary envelopment, with concomitant tegument acquisition. As described above, tegumentation occurs at two distinct sites, the viral nucleocapsid and the TGN-derived cytoplasmic vesicle, which contributes the future virion envelope. These two subassemblies must ultimately interact to promote secondary envelopment.

A variety of identified protein-protein interactions suggest possible bridging mechanisms between the two subassemblies (Fig. 2.11). For example, as previously mentioned VP22 associates with membranes and binds to the cytoplasmic tail of gD (109). PrV VP22 also interacts with the cytoplasmic tails of gE and gM (221). VP16, which is found on cytoplasmic nucleocapsids, is a binding partner of VP22 (183). Thus, VP22 may act as a bridging protein, with both VP16 and a glycoprotein tail binding simultaneously, to facilitate budding of VP16-coated capsids into glycoprotein/tegument protein layered vesicles. However, recent studies of a HSV-1 VP22-null virus suggests that the requirement for VP22 in the assembly pathway can be bypassed, at least in cultured cells (179,182). Thus, this may be only one of a plethora of protein-protein
interaction networks that work redundantly to facilitate the final stages of envelopment. With that in mind, UL11 has been shown to target to, and bind membranes of, the TGN and interact with UL16, which is believed to associate with cytoplasmic nucleocapsids (57,406,407,527,452,718). However, in a similar fashion to VP22, both UL11 and UL16 are deemed non-essential for HSV-1 replication in cultured cells (25,26). In addition, identification of UL37 and VP1/2 at the TGN, proteins known to intimately associated with nucleocapsids, suggests another mechanism by which nucleocapsids may interact with cytosolic membranes to facilitate final tegumentation and envelopment (155,658,716,718).

Similarly, VP16 interacts with gH, gB, gD, and the tegument protein vhs (which are all found on cytoplasmic membranes) and the inner tegument protein VP1/2 (388,483,646,776,270,783). However, unlike VP22 and UL11, VP16 is indispensable for secondary envelopment (480,730). The dramatic defects seen in the assembly pathway with a HSV-1 VP16-null mutant suggest that one or more of the protein-protein interactions involving VP16 are required for assembly and egress of HSV-1 (480,730).

During the HSV-1 assembly pathway, cytoplasmic tails of viral glycoproteins recruit a subset of tegument proteins to membranes of the TGN (270,783,109). In a manner analogous to the functional redundancy displayed by many of their tegument binding partners, deletion of individual glycoproteins has little effect on assembly (58,59,197,221). However, simultaneous deletion of gM and the gE/gI heterodimer in PrV results in the formation of capsid-bound tegument aggregates in the cytoplasm (58,59,221). In HSV-1, simultaneous deletion of gD and gE results in accumulation of unenveloped capsids in the cytoplasm that are embedded in tegument-like material (197).
Figure 2.11. Protein-Protein Interactions that May Facilitate Final Envelopment of HSV-1. As tegumentation occurs at two distinct sites, the viral nucleocapsid and the cytoplasmic face of trans-Golgi network (TGN)-derived vesicles, these two subassemblies must ultimately interact with each other to promote secondary envelopment. A highly ordered network of protein-protein interactions drives the process of final envelopment; however, only a small subset of protein-protein interactions (designated by solid lines or overlapping proteins) among virion components are known. Dashed black lines indicate proposed physical interactions implied by chemical cross-linking studies. Dashed red lines are physical interactions identified in the related virus pseudorabies virus (PrV).
The phenotypes of both mutant viruses indicate that the block to secondary envelopment does not preclude tegumentation of cytoplasmic nucleocapsids. These observations suggest that cytosolic tails of viral glycoproteins act as a platform or scaffold upon which tegument proteins accumulate. Ultimately, glycoprotein-tegument protein enrichment at the TGN will act as a foundation, with additional protein-protein interactions providing a link between the nucleocapsid and the TGN-derived vesicle, thereby promoting final tegumentation and envelopment.

As outlined above, gM, which is conserved across the *Herpesviridae*, is thought to play a role in either retaining envelope glycoproteins at the TGN, or retrieving them from the cell surface, thereby maintaining sufficient concentrations of viral glycoproteins at the site of secondary envelopment (132). Similarly, the tegument protein UL11 is able to direct heterologous proteins to the Golgi. For example, a hybrid protein in which the HSV-1 UL11 polypeptide was fused to the Gag protein of HIV-1 is directed to the Golgi region; thereby enabling Gag budding to occur at Golgi vesicles rather than at the plasma membrane (57). Despite their suggested roles in glycoprotein and tegument protein localization at the TGN, both gM and UL11 can be deleted individually with minimal effects on virus assembly. However, deletion of both proteins yields a dramatic phenotype, with a large intracytoplasmic accumulation of capsids embedded in tegument (58,59,367). It is hypothesized that in the absence of both gM and UL11, neither an enrichment of glycoproteins at the future budding site nor targeting of tegument proteins to this site occurs, resulting in abrogation of secondary envelopment. While emphasizing the importance of the glycoprotein-tegument protein platform in the assembly process, one must note that the mutant viruses characterized were from PrV (58,59,367).
Although deletion of gM and gE in PrV abrogates secondary envelopment, a similar deletion in HSV-1 demonstrates no significant impairment (69,367). However, simultaneous deletion of gD and gE in HSV-1 does abrogate final envelopment (197). Furthermore, VP22 is a binding partner of gM in PrV and of gD in HSV-1. Thus, it is plausible that although the requirements for secondary envelopment are different within herpesvirus subfamilies, PrV gM and HSV-1 gD may be functionally equivalent.

Interestingly, nucleocapsids are not required to initiate budding at TGN-derived vesicles. Particles commonly referred to as light (L) particles because of their lower density in Ficoll gradients, are produced by many herpesviruses during their normal replicative cycle in both cell culture and within the natural host (674,8,324,442,443,589). L particles lack a nucleocapsid, yet contain both tegument proteins and envelope glycoproteins, and are formed independently of normal virus maturation (442,443,589,674). These particles contain clues as to the identity of the minimal budding machinery of HSV-1. In other words, the minimal complement of viral and cellular proteins that will result in the aggregation of virion proteins on the cytoplasmic surface of a membrane followed by induction of membrane curvature and subsequent pinching off of the particle. The composition of L particles is similar, but not identical, to infectious virions; containing at least five additional phosphoproteins not normally present within virions (442,443,589,674). Despite these compositional differences, L particles can facilitate a wild-type HSV-1 infection by delivering to uninfected cells tegument proteins such as VP16 and vhs that function to initiate a productive infection (138,442). Interestingly, the presence of UL37 and VP1/2 in L particles strengthens the hypothesis that additional copies of such inner tegument proteins as are added to
nucleocapsids during secondary envelopment. However, compositional analysis has also highlighted the complexity of the task to identify the virion components that comprise the minimal budding machinery, as L-particles contain over 30 proteins. Nevertheless, L particle production is blocked, at least in PrV, in the absence of gE and gM, or UL11 and gM (58,367). This observation implies that an interaction between membrane proteins and tegument proteins, as seen in secondary envelopment, may be required to facilitate particle formation.

Given the complexity of the virus, elucidation of the budding machinery of HSV-1 is undoubtedly a formidable task. However, as viruses are not arbitrary entities and are based on order and conserved biological properties, examination of the mechanisms that facilitate budding of other enveloped viruses may provide insight to the multifaceted envelopment of HSV-1. Taking such an approach, a recent report which drew parallels from the assembly process of enveloped RNA viruses, has identified a requirement for a cellular factor in the cytoplasmic envelopment of HSV-1 (135).

Although relatively simple in terms of structure and composition, parallels can easily be drawn between the architecture of RNA viruses and that of HSV-1. Both possess a proteinaceous core protecting their nucleic acid genome and a host-derived lipid envelope containing transmembrane proteins. Of particular interest, many RNA viruses such as retroviruses, rhabdoviruses, filoviruses and orthomyxoviruses, possess a proteinaceous layer between the nucleocapsid and the envelope created by multimerization of a matrix (M) or matrix-like protein. The tegument proteins of herpesviruses appear positionally and possibly functionally equivalent to the matrix proteins of RNA viruses. Positionally, tegument proteins link the nucleocapsid to the
envelope, and the same is true for matrix proteins. However, it is intriguing to consider the functional analogy. In a similar fashion to the production of L particles, the retroviral Gag polyprotein, the rhabdoviral M protein, filoviral VP40, and orthomyxoviral M1 protein are sufficient to produce enveloped, extracellular, virus-like particles in the absence of other viral components (240,393,688).

A simplistic model for M protein-mediated assembly involves accumulation and interaction of matrix proteins on a target membrane. The collective interactions of M proteins with the membrane and each other induce curvature of the membrane, followed by a pinching-off step and release of an extracellular particle (Fig. 2.12) (233).

Extensive analysis of the retroviral Gag polyprotein has led to the identification of three amino acid domains that are cooperatively required for budding. The membrane-binding (M) domain targets Gag to the plasma membrane and mediates its attachment to the membrane (715,780). Once at the plasma membrane, Gag proteins indirectly associate with one another through an interaction (I) domain, which is an RNA-binding motif that preferentially recognizes genomic RNA (57,85,252,734). Assembly and aggregation of Gag multimers on the plasma membrane is sufficient to cause curvature of the membrane. However a third domain, the late (L) domain, is essential for release of the budding particle (311,760,763,770). Through one of three currently identified motifs, PT/SAP motifs, YPXL motifs and PPPY motifs, the L domain recruits the endosomal sorting complexes required for transport (ESCRTs) in order to complete the pinching-off step (46,153,478). ESCRTs comprise the cellular machinery that facilitates budding into multivesicular bodies (MVBs) and consists of the multiprotein complexes
Figure 2.12. Budding Mechanism of Enveloped Viruses. The envelopment process of many RNA viruses requires targeting of virion components to the cellular site of envelopment. Aggregation of these components on the membrane can be attributed to their interactions with the membrane and each other. This aggregation induces membrane curvature, which proceeds until a spherical bud is formed. Membrane fission and release of a particle occurs as a result of a pinching-off event that requires fusion of the membrane stalk.
Pinching-off

Formation of a spherical bud

Aggregation and induction of membrane curvature

Targeting and interaction of components to the designated membrane
ESCRT-1, -2, and -3 together with several additional proteins (313). This machinery is normally responsible for the sorting of proteins into MVB luminal vesicles that are destined for degradation in lysosomes (272,645); a budding process that topologically bears great similarity to the budding of enveloped virions either at the plasma membrane or into the lumen of TGN-derived vesicles (Fig. 2.13). From the initial observation with retroviral Gag, it is now well accepted that many families of enveloped RNA viruses (including the Filoviridae, Rhabdoviridae, Arenaviridae, and Paramyxoviridae) require ESCRT proteins for viral budding (46,153,478,541). Such common usage of the ESCRT proteins by completely unrelated virus families demonstrates the importance of this cellular membrane budding machinery for enveloped viral pathogens.

The first demonstration of an involvement of ESCRT proteins in assembly of HSV-1 has recently been obtained by two independent groups (80,135). Replication of HSV-1 was inhibited by expression of dominant-negative Vps4, an ATPase that disassembles and thereby recycles the ESCRT machinery. The block in viral replication appears to occur very late in the assembly pathway. Electron microscopy studies indicate that envelopment of HSV-1 can initiate in the presence of dominant negative Vps4, but cannot proceed to completion. These partially-enveloped virions are otherwise morphologically similar to those observed in normal cells. This finding suggests that all the viral proteins necessary for the interaction of capsids with membranes, which drive envelopment to a late stage, must be present on these structures, yet envelopment cannot be completed.

As outlined above, enveloped RNA viruses recruit the ESCRT machinery to sites of viral budding through interaction of viral L domain motifs with ESCRT proteins. The
Figure 2.13.  **Topological Similarity Between Protein Sorting into Multivesicular Bodies and Budding of Enveloped Virions.** Cell surface receptors can be endocytosed upon binding their ligand. The vesicle is released from the plasma membrane into the cytoplasm by a membrane fission event. The endocytic vesicle can subsequently travel to and fuse with an endosome. The receptor is degraded by budding into the endosome thus forming a multivesicular body (MVB). Release of the invaginated vesicle also requires membrane fission. The topology of MVB formation is similar to budding of enveloped virions either at the plasma membrane or into the lumen of *trans*-Golgi network (TGN)-derived vesicles. Current evidence suggests that the machinery used by many enveloped virus to facilitate their release may be similar to that used during MVB formation.
majority of these RNA viruses express proteins that contain one or two L domain motifs that are necessary and sufficient for recruitment of ESCRT proteins.

The situation with herpesviruses is likely to be more complex. All three classes of L domain consensus motifs are present in several HSV-1 structural proteins including the major capsid protein (VP5), the tegument protein VP16, the very large tegument protein VP1/2, the cytoplasmic domain of gE, and at least two additional tegument proteins (UL51 and UL56). The frequency of putative L domain motif occurrence in HSV-1 virion components may signify the importance of engaging ESCRT proteins for the assembly of infectious virions and may also reflect the functional redundancy of different proteins in herpesvirus assembly (778). While such redundancy of function may allow for the loss of one or more L domain motifs without abrogating virion production, it also complicates analysis of the role of such motifs by mutagenesis.

Release into the Extracellular Milieu

The net result of the secondary envelopment process is an enveloped herpesvirus virion within a secretory vesicle. The vesicle is transported to the plasma membrane, where vesicle and plasma membranes fuse and mature virions are released from the infected cell. Although this process mimics the release of secretory molecules from the cell, viral proteins are apparently involved, although their function in this particular step of viral replication remains enigmatic.

In HSV-1 and PrV, the UL20 and gK proteins have been implicated in this process (25,214,222,318,332). Deletion of gK and the integral membrane protein UL20,
results in accumulation of enveloped particles within large cytoplasmic vesicles (25,27,214,216,222,332). These observations suggest that UL20 and gK play a role in translocation of infectious virions from the cytoplasm to extracellular spaces.

Curiously, pH neutralization through treatment with chloroquine and bafilomycin A1 (which specifically blocks endosomal acidification) abolishes HSV-1 infectivity, with no concomitant reduction in the numbers of enveloped particles found within neutralized organelles (284). This seemingly important role for acidic pH in acquisition or perhaps maintenance of infectivity during HSV-1 egress raises the intriguing possibility that the requirement for an acidic pH may reflect some acid-dependent processing event critical for virion maturation.

Maturation events occurring within the Golgi complex and secretory vesicles are not uncommon (415,511,587,610). Structural changes in the West Nile virus capsid have been observed as virus particles travel through the low-pH environment of the TGN. Neutralization of organelle pH with ammonium chloride results in accumulation of immature particles within the cell (572,775). In regard to HSV-1, a recent study from Meckes et al., (2007) demonstrated a dynamic interaction between UL16 and capsids, with a binding and release mechanism that is pH regulated (452). Thus, one could speculate that herpesviruses utilize the acidic pH of secretory vesicles to induce changes in the tegument necessary for infectivity.

In terms of the contribution of cellular components to the pathway by which virion containing TGN-derived vesicles leave the cell, studies have shown that protein kinase D (PKD) is an important regulator for transport of cargo destined for the cell surface (239,328,398). Cytosolic PKD is recruited to the TGN in a diacyl glycerol
(DAG) dependent fashion (33). DAG depletion effectively prevents the transport of many molecules to the plasma membrane (33). Furthermore, a PKD mutation which abrogates kinase activity causes accumulation of cargo filled tubules at the TGN, consistent with a role for PKD in fission (398). Given the important role of PKD in the transport of cargo from the TGN to the cell surface, it could potentially be involved in HSV-1 egress. Interestingly, PKD inactivation causes retention of HSV-1 at the TGN. This suggests a role for PKD in viral egress, and further highlights another host mechanism hijacked by HSV-1 to facilitate its replication (463).

In addition to release into the extracellular space, HSV-1 is also capable of spreading laterally in vivo and in vitro. Cell-to-cell spread facilitates pathogenesis because it enables virions to exit infected cells and enter uninfected neighboring cells without being exposed to the host humoral immune response (30,88,165). HSV-1 cell-to-cell spread in cultured epithelial cells involves a process by which progeny virions are targeted specifically to epithelial cell junctions (339). By virtue of being sorted to epithelial cell junctions there is preferential movement of virus between cells rather than into extracellular fluids.

Similar to virion penetration, cell-to-cell spread requires gB, gD, and gH/gL (77,212,397,604); however, in contrast to these envelope glycoproteins, the HSV-1 glycoproteins gE and gI, which function primarily as a heterodimer, promote cell-to-cell spread without any obvious role in the entry of extracellular particles (76,165,166,212,397,604). HSV-1 gE- or gI-null mutants display markedly reduced spread between cultured epithelial and neuronal cells and in epithelial and neuronal tissues (165-167,556).
HSV-1 gE/gI appears to participate in at least two processes that promote cell-to-cell spread. The extracellular domains of gE/gI are necessary for spread (125,339). gE/gI accumulates at cell junctions late in infection, apparently tethered there in a manner similar to that of various cell adhesion molecules, and when expressed in trans, gE/gI can interfere with cell-to-cell spread (125,446,750). It is hypothesized that the extracellular domain of gE binds to receptors that are selectively expressed at cell junctions, promoting virus movement across epithelial cell junctions (198). However, the cytoplasmic domain of gE is involved in a second process that promotes cell-to-cell spread. Mutants lacking the gE cytoplasmic domain behave much like an HSV-1 gE-null mutant, with dramatically reduced spread between cultured epithelial cells and within the corneal epithelium (556,750).

The gE cytoplasmic domain promotes extensive accumulation of gE/gI at the TGN in HSV-1-infected cells at early times of infection and when gE/gI is expressed by transfection or with viral vectors (7,167,446,750). The TGN serves as the intracellular compartment where secondary envelopment of HSV occurs, and as described above, HSV gE/gI and gD serve essential but redundant functions during acquisition of the virion envelope in the cytoplasm (140,197,644,710,741,751). Thus, gE/gI accumulates extensively at the TGN during virus particle assembly and, in conjunction with gD, promotes secondary envelopment there. Furthermore, not only does the gE cytoplasmic tail facilitate cell-to-cell spread through assembly of virus particles, the gE cytoplasmic domain is required for the specific sorting of enveloped virions formed in the TGN to epithelial cell junctions (339). When gE, or the gE cytoplasmic domain, is deleted, virions traffic to apical surfaces rather than to lateral cell junctions. These observations
support a working model in which gE/gI heterodimers present in membrane vesicles affect intracellular sorting decisions, perhaps by coupling to cell sorting machinery, resulting in transport of virions specifically to lateral cell surfaces (Fig. 2.14) (339).

In support of this model, upon relocalization of HSV-1 virions from sites of envelopment at the TGN to lateral cell surfaces, gE/gI despite containing TGN sorting motifs, is redistributed from the TGN to epithelial cell junctions (446,750,751). This process appears to involve global rearrangement or redistribution of the TGN because gB and host TGN proteins (TGN46 and carboxypeptidase D) also move to the plasma membrane (751). Redistribution is specific to lateral but not apical surfaces, and does not require assembly of enveloped virions in the TGN, suggesting that there are viral proteins that function to redistribute enveloped virus particles from the TGN to cell junctions.

In polarized epithelial cells, the TGN and endosomes are the major compartments in which membrane and secreted proteins are sorted to either basolateral or apical cell surfaces (86,428,454). Cellular proteins are sequestered into subdomains of the TGN where vesicles form that are directed to basolateral or apical cell surfaces. The cytoplasmic domains of cargo proteins interact with cytosolic adaptor molecules, e.g., the clathrin adaptors AP-1, AP-3, and PACS-1, that promote coating of vesicles and direct transport (210,724). Clathrin adaptors recognize tyrosine (YXXΦ, where Φ is a large hydrophobic amino acid) and dileucine motifs in the cytoplasmic domains of membrane proteins, among other signals.

The HSV-1 gE cytoplasmic domain is 106 amino acids in length and contains several cell sorting motifs, which are also found in PRV and VZV homologues (312,378,428,437,522,689). The HSV gE cytoplasmic domain contains two tyrosine
motifs (YXXΦ) as well as an acidic cluster of amino acids, components of which are phosphorylated. Tyrosine motifs bind the μ1 and μ2 components of AP-1 and AP-2 clathrin adaptors to promote incorporation into clathrin-coated transport vesicles (198). The HSV gI cytoplasmic domain also contains a dileucine motif at the C-terminus that likely binds the β1 component of AP-1 clathrin adaptors. The gE cytoplasmic domain interacts with the acidic cluster-binding protein PACS-1 that directs molecules to the TGN (198,276,724). However, a recent study investigating determinants within the gE cytoplasmic tail that are required to promote gE/gI trafficking to cell junctions and cell-to-cell spread, mapped the activity to a region of the cytoplasmic tail which contains no obvious cell sorting motifs (198). Furthermore, gE/gI remains at the TGN compartment when other HSV proteins are not present. Thus, it is intriguing to consider that a viral protein may bind to this region and facilitate trafficking to cell junctions to promote cell-to-cell spread. Alternatively, after accumulation in the TGN to promote secondary envelopment gE/gI may interact with other HSV-1 proteins in a process that unmasks cryptic sorting sequences in the gE cytoplasmic domain that promote delivery to cell junctions.

In summary, herpesviruses have evolved an elaborate pathway for egress from the host cell and for assembly of a highly complex particle. Two envelopment processes occur in diverse subcellular compartments involving different viral and cellular proteins. Whereas intranuclear events, such as capsid formation and genome packaging, resemble similar steps in dsDNA bacteriophages, final maturation occurs in the cytoplasm after translocation of capsids through the nuclear membrane. Given the extensive similarities
Figure 2.14. Function of Glycoprotein E and I Heterodimers in Cell-to-Cell Spread. Heterodimers of glycoprotein E (gE) and I (gI) present in membrane vesicles that enclose newly enveloped virions affect intracellular sorting decisions, perhaps by coupling to cell sorting machinery, and thereby transport virions specifically to lateral cell surfaces (A). As gE/gI heterodimers remain at the trans-Golgi network (TGN) compartment when other HSV-1 proteins are not present, a viral protein may bind to the cytoplasmic tail of gE and facilitate trafficking to cell junctions to promote cell-to-cell spread (B). Alternatively, gE/gI may interact with other HSV-1 proteins in a process that unmasks cryptic sorting sequences in the gE cytoplasmic domain that promote delivery to cell junctions (B). Virions that reach cell junctions move into narrow spaces between cells and interact with glycoprotein D (gD) and gE/gI receptors that promote HSV-1 entry into adjacent cells.
of herpesviruses to dsDNA bacteriophages, it is intriguing to speculate that ancestors of herpesviruses were parasites of the prokaryotic precursors of the eukaryotic cell nucleus, and that they left their previous hosts using the primary budding/fusion mechanism with the subsequent need to acquire a second budding/fusion system after the original host became enveloped as an endosymbiont.

**VP22**

It is likely that the myriad of protein-protein interactions between capsid proteins and tegument proteins, tegument proteins and tegument proteins and between tegument proteins and the cytoplasmic tails of virally encoded glycoproteins not only drive the HSV-1 assembly process but facilitate selective packaging of tegument components into assembling virions. In an effort to provide insight into the specific mechanisms utilized by tegument proteins to facilitate virion incorporation, the thrust of this dissertation focuses on dissection of the protein-protein interactions and virion incorporation determinants of the HSV-1 tegument protein, VP22, and elucidation of the functional significance of these activities in facilitating packaging of VP22 into assembling virions.

VP22, encoded by the U1.49 gene of HSV-1, contains 301 amino acids and is one of the most abundant tegument proteins with an estimated 2000 copies per virion (189,290,391). Despite its abundance, the role of VP22 during HSV-1 assembly and mechanism of incorporation, remain undefined. Extensive studies of the subcellular localization of VP22 have yielded controversial results in which the protein is capable of both nuclear and cytoplasmic accumulation; however, these conflicting reports correlate with differing experimental methods (184,185,187,188,370,558). In our experience,
VP22 possesses the ability to associate with cellular membranes and target to the TGN independently of other viral components (66). This finding suggests that VP22 contains intrinsic determinants that facilitate localization to the site of final envelopment, where it becomes incorporated into the tegument. The aforementioned nuclear accumulation of VP22 during infection may correspond to a recently reported interaction of VP22 with cellular template-activating factor I (TAF-I), a chromatin remodeling protein that promotes ordered transfer of histones to naked DNA (470,520,712). The interaction with TAF-I prevents nucleosome deposition on DNA. Thus, VP22 is hypothesized to prevent rapid recruitment of repressive histones to input DNA by inhibiting TAF-I activity (712).

In addition to interactions with TAF-I, studies suggest that VP22 also possesses the ability to multimerize, with dimerization domains mapping to both N-terminal and C-terminal portions of the protein (481,718). In addition to forming multimeric structures, VP22 also interacts with VP16, a protein essential for secondary envelopment and egress (182,183,480,730). Furthermore, ultrastructural studies indicate that during viral assembly, a portion of the VP16 virion complement is acquired by nucleocapsids prior to final envelopment (469,488). Thus, interaction with VP16-coated cytoplasmic nucleocapsids may facilitate incorporation of TGN-localized VP22 into assembling virus particles.

Although little is known about the molecular details of final envelopment, it is likely that protein-protein interactions between tegument proteins and the cytoplasmic tails of envelope glycoproteins facilitate the budding process. VP22 binds to the cytoplasmic tail of gD and may facilitate interaction of viral nucleocapsids with glycoproteins lining up on the membranes of TGN-derived vesicles, perhaps through its
interaction with VP16 (109,514). Interestingly, VP22 is a major component of L particles and could potentially be a component of the HSV-1 minimal budding machinery (674). Simultaneous deletion of gD and gE results in accumulation of unenveloped capsids in the cytoplasm that are embedded in tegument-like material, a similar phenotype to that seen with a VP16-null virus (197,480,730). In PrV the cytoplasmic tails of the envelope glycoproteins gE and gM bind to VP22 in a yeast two-hybrid study (221). Interestingly, simultaneous deletion of PRV gM and the gE/gI heterodimer also results in the formation of capsid-bound tegument aggregates in the cytoplasm (58,59,221).

Primary structural alignment of VP22 reveals a core region encompassing amino acids 165-270, that it is highly conserved among VP22 homologues of alphaherpesviruses (Chapter IV Fig. 4.13A). This region of the protein houses a variety of motifs that may facilitate interaction of VP22 with its binding partners. Two tryptophan residues at positions 189 and 221, and phenylalanine residues at amino acids 196 and 201 resemble a WW domain, a well characterized protein-protein interaction motif. WW domains are typically 35 to 40 amino acids in length, characterized by two tryptophan residues that bookend aromatic residues with a proline residue located C-terminally of the second tryptophan (323). Interestingly, WW domain ligands include proline-rich peptide motifs and phosphorylated serine/threonine-proline sites, examples of which are found in the cytoplasmic tails of a variety of viral glycoproteins and within the region of VP16 which facilitates interaction with VP22 (285). Two conserved dileucine motifs, which function as binding sites for a variety of proteins are also present within this conserved domain of VP22 (288,407).
Studies of two HSV-1 VP22-null viruses indicate that the requirement for VP22 during secondary envelopment can be bypassed at least in tissue culture systems, although the protein may play an important role in viral egress (179,182). VP22-null viruses demonstrate a variety of cell-specific replication defects with altered virion composition (179,182,559). In the absence of VP22, decreased amounts of both gE and gB are packaged into assembling virus particles, with virion levels of gD, a binding partner of VP22, also reduced (179,182,559). Incorporation of VP22 into assembling virus particles occurs independently of the phosphorylation status of the protein (562). However, studies suggest that efficient incorporation of viral glycoproteins, specifically gD, may require the nonphosphorylated form of VP22 (562). In addition to the defects observed with glycoprotein virion incorporation, the immediate early proteins ICP0 and ICP4 were also packaged at reduced levels (179,182). In the absence of VP22, ICP0, which normally colocalizes with VP22 at characteristic cytoplasmic domains within the cell reminiscent of the TGN, remains diffuse within the cytoplasm with resultant inefficient virion packaging (182). ICP4, a binding partner of ICP0 is also detected at reduced levels in virions (182,762). The exact relationship between these three proteins is unclear; however, it is intriguing to note that the VZV UL49 homologue interacts with its ICP4 homologue, IE62 (660). This observation suggests that HSV-1 ICP4 may facilitate VP22-dependent recruitment of ICP0 to cytoplasmic domains.

Although dispensable for viral replication in cultured Vero cells, VP22 appears to be required for efficient viral spread both in vitro and in vivo (179,182,559). Plaque sizes are dramatically reduced (~95%) when Vero cell monolayers are infected with a VP22-null virus, with the defect suggested to be primarily due to decreased viral release (179).
Furthermore, VP22 is required for efficient viral spread during infection of the mouse cornea (179). VP22 may promote extracellular virus release through a direct or indirect mode of action. Alteration of the cytoskeleton to promote efficient transport of virion-containing vesicles to the plasma membrane may be one direct mechanism by which VP22 facilitates viral spread.

As VP22 is conserved across the alphaherpesviruses, one may garner further insight into the role that VP22 plays in viral spread by elucidation of its function in related viruses. Although VP22 is dispensable for replication of both PRV and bovine herpesvirus (BHV), VP22 is an essential protein of MDV and VZV (151,175,394,395,690). MDV and VZV are highly cell-associated herpesviruses both in vitro and in vivo and spread directly from cell-to-cell, with little to no infectious virus released (120,175,268). Interestingly, both gE and gI, which form a noncovalently linked complex in infected cells and virions, are essential for the growth of both viruses, at least in certain cell types, while they are dispensable in all other alphaherpesviruses tested (30,122,355,465,523,621,766,786). Given this finding, combined with the role of gE/gI in promoting cell-to-cell spread and interaction of VP22 with gE in the related herpesvirus PrV, it is intriguing to speculate that highly cell-associated alphaherpesviruses may utilize the UL49-gE/gI pathway to spread from cell-to-cell. However, in herpesviruses that produce cell-free virions the loss of the UL49 homologue is less crucial for virus propagation (44,76,165,166,212,224,397,418,419,472,604,621).

In support of this model, the absence of VP22 affects HSV-1 growth in polarized epithelial cells [Madin-Darby bovine kidney cells (MDBK)] where cell-to-cell spread is the primary mode of virus transmission (182). The process of cell-to-cell spread enables
virions to exit infected cells and enter uninfected neighboring cells without being exposed
to the host humoral immune response, perhaps explaining why an HSV-1 VP22-null
virus fails to spread during infection of the mouse cornea (30,88,165,179). Furthermore,
residues 37 to 187 of MDV VP22, which are sufficient to facilitate cell-to-cell spread of
VP22 and are homologous to the conserved core of HSV-1 VP22, complement a VP22-
deficient MDV virus when expressed in trans, suggesting an important role for this VP22
property in MDV-1 infection (175).

As discussed previously, the microtubule network plays a crucial role in the
transport of viral nucleocapsids in cultured neurons, both during entry and egress
(301,468,544). However, in nonneuronal cells drugs which affect the microtubule
network show little significant inhibition of virus replication, suggesting that while
microtubules may be important for neuronal transport, additional transport mechanisms
may be used in nonneuronal cells (372). Consistent with this hypothesis, in Vero cells
addition of nocodazole or taxol from 4 h post-infection onwards has little effect on virion
release (17). Furthermore, proposals that transport of virus particles may occur on
microtubules do not exclude actin from participating in transport; an accepted concept is
that long-range movement of vesicles occurs on microtubules and short-range movement
occurs on actin filaments. Nerve ends contain few microtubules, and synaptic vesicles
are thought to travel on actin cables at the periphery, beyond the reach of microtubules
(68).

Given the apparent defect in cell-to-cell spread observed with VP22-null viruses,
it is interesting to note that VP22 interacts with two components of the cellular
cytoskeleton, the microtubule network and actin filaments. Cytoskeletal exploitation
during virus infection of host cells is commonplace, with some viruses disrupting one or more elements of the cytoskeleton (174,336,347,453,722). A more infrequent occurrence is induction of cytoskeletal polymerization and/or stabilization, in many cases to facilitate virus egress (103,104,136,137). VP22 exhibits the properties of a classical cellular microtubule-associated protein (MAP), binding to microtubules and reorganizing them into thick bundles which are highly resistant to depolymerizing agents such as nocodazole (185). Microtubules contained in these bundles are modified by acetylation, a marker for microtubule stability. VP22 colocalizes with microtubules in both transfected and infected cells although VP22-induced microtubule stabilization in transfected cells is more efficient than that observed in HSV-1-infected cells (185). Presumably VP22 has multiple roles during the replicative cycle. Therefore, while the majority of the VP22 population in transfected cells is available for assembly into microtubule bundles, it is likely that a fine balance exists between the different activities of VP22 in an infected cell. VP22-induced microtubule stabilization may facilitate capsid trafficking, particularly in neurons, where the viral capsid has a considerable distance to travel from the periphery of the cell to the nucleus. It may also be involved in virus egress from the cell, assisting transport of naked capsids to the site of final envelopment or of virion-containing vesicles to the cell boundary.

VP22 interacts with actin filaments through an association with the actin-associated motor protein nonmuscle myosin IIA, which is recruited to the actin cytoskeleton and has been reported to be involved in numerous dynamic cellular processes, including vesicle secretion (629,711). Inhibition of the ATPase activity of
nonmuscle myosin IIA with the myosin-specific inhibitor butanedione monoxime, fails to severely inhibit entry of infectious virus, but does impair virus release (711).

HSV-1-infected cells frequently form pronounced plasma membrane protrusions which establish intimate contact with adjacent cells (711). This behavior is not a passive effect of virus-induced CPE but an active process induced by infection, and may be relevant for cell-to-cell spread. Interestingly, GFP-VP22-containing particles are detected with nonmuscle myosin IIA-containing filaments which traverse these protrusions (711).

Although controversial, some researchers have suggested that VP22 is capable of intercellular spread via a mechanism potentially involving actin microfilaments (3,42,184,606). Given the ability of VP22 to also interact with microtubules, it is intriguing to speculate that a connection between the two activities may provide an elegant mechanism for coordinated delivery of VP22 into surrounding cells during virus infection (185). Furthermore, the ability of VP22 to elicit cell-to-cell spread not only of itself but of proteins and peptides fused to VP22, strengthens the hypothesis that a potential role of this abundant tegument protein in the replication cycle may be to facilitate cell-to-cell spread of HSV-1 virions (184).

While the role(s) of VP22 during viral replication is gradually elucidated, the process by which VP22 is selectively packaged into the assembling virion remains poorly understood. Virion incorporation of VP22 and all tegument components presumably relies upon a variety of intracellular trafficking motifs. In combination with a myriad of protein-protein interactions, these motifs direct the protein to a compartment of the cell where virion incorporation can occur. Analysis of the protein composition of HSV-1 L particles, suggests that tegument components vhs, VP16, and VP1/2, which are believed
to be added to capsids within the nucleus, are also recruited into the tegument region during secondary envelopment at the TGN (72,469,483,488,576,674). In contrast, TIEM studies suggest that VP22 is only recruited into the tegument region during final envelopment (469). VP22 associates with cellular membranes and targets to the TGN independently of other viral components (66). Although VP22 contains intrinsic determinants that facilitate its localization to the site of final envelopment, studies in PrV suggest that localization to the correct site of virion incorporation is not sufficient to facilitate virus packaging (66,469). Deletion of two binding partners of PrV VP22, gE and gM, results in particles lacking the tegument protein, with deletion of gE alone resulting in decreased levels of VP22 in virions (461,58). Furthermore, the Bartha strain of PrV which lacks the glycoproteins gI and gE, fails to package VP22, suggesting that binding to the cytoplasmic tail of a viral glycoprotein may be an important determinant in virion incorporation of VP22 (413). Studies of the HSV-1 tegument protein UL11, also suggest that localization to the correct site of virion incorporation is not sufficient to facilitate virus packaging (408). UL11 contains sites for myristylation and palmitylation, which facilitate membrane-binding and targeting to the Golgi apparatus and are essential for efficient virion packaging of the protein (57,406,408). However, an acidic cluster motif, which is not needed for Golgi apparatus-specific targeting but is involved in recycling the protein from the plasma membrane, is also deemed essential (406,408). Thus it appears that UL11 requires to traffic through a specific pathway to achieve efficient virion incorporation, perhaps enabling the formation of essential protein-protein interactions or posttranslational modifications.
VP22 is extensively phosphorylated, with the phosphorylation state of the protein implicated in both gD and microtubule binding (188,562). While the majority of VP22 in infected cells is highly phosphorylated, the non-phosphorylated form of VP22 is the predominant species in the virion. Although phosphorylation appears to play a role in the virion incorporation of BHV VP22 (perhaps through the inability of hypophosphorylated VP22 to bind to an interacting partner or localize to the correct cellular site), the phosphorylation state has no effect on the relative packaging of HSV-1 VP22 (562,578).

Interestingly, studies suggest that the herpesvirus tegument may be flexible regarding both protein composition and stoichiometry (776,777). Overexpression of VP22 within infected cells results in a 3-fold increase in its incorporation with little effect on other tegument components (391). This observation is consistent with the hypothesis that the incorporation of a tegument protein is partly determined by local protein concentration. In contrast, the amount of HSV-1 UL37 in virions is strictly controlled despite a 20-fold increase of UL37 in infected cells (441). Thus, apparently different tegument proteins utilize different mechanisms of virion incorporation.

Numerous studies have deleted a tegument component and evaluated the subsequent effects on virion protein composition (26,150,179,182,461,525,628,776). However, as tegument proteins have been shown to be involved in numerous protein-protein interactions, one must be cautious when interpreting the direct impact of a single deletion on the specific composition of the viral particle.
CHAPTER III

A CONSERVED REGION OF THE HERPES SIMPLEX VIRUS TYPE 1 TEGUMENT PROTEIN VP22 FACILITATES INTERACTION WITH THE CYTOPLASMIC TAIL OF GLYCOPROTEIN E (gE)

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ABSTRACT

Herpes simplex virus type 1 (HSV-1) virions, contain a proteinaceous layer termed the tegument that lies between the nucleocapsid and viral envelope. Current evidence suggests that viral glycoprotein tails play a role in the recruitment of tegument-coated capsids to the site of final envelopment; vesicles derived from the trans-Golgi network (TGN). We have identified an interaction between VP22, an abundant tegument protein and the cytoplasmic tail of glycoprotein E (gE). This interaction was identified by coimmunoprecipitation studies and confirmed by a glutathione-S-transferase (GST) pulldown from infected cell lysates. Truncation mutagenesis suggests that residues 165-270 of VP22 facilitate interaction with the cytoplasmic tail of gE. In fact, this region of VP22 is sufficient to bind to gE in the absence of additional viral proteins. Using a transfection/infection-based virion incorporation assay, residues 165-270 of VP22 fused to the green fluorescent protein (GFP) competed efficiently with wild-type VP22 for packaging into assembling virus particles.
INTRODUCTION

Herpes simplex virus type 1 (HSV-1) assembly involves a complex sequence of events occurring within numerous cellular compartments that culminates in the formation of virions composed of nearly 40 different viral proteins distributed among three morphologically distinct structures: the nucleocapsid, the host-derived lipid envelope in which virally encoded glycoproteins are embedded, and the tegument, a proteinaceous region located between the nucleocapsid and envelope (601). It is well established that capsid assembly and the subsequent packaging of the viral genome occur in the nucleus (191,456-459). As with other herpesviruses, the most widely accepted model for HSV-1 assembly and egress suggests that nucleocapsids are shuttled to the cytoplasm via a budding/fusion event that occurs across the inner and outer membranes of the nucleus, respectively. Current evidence suggests that nucleocapsids then travel through the cytoplasm until they reach trans-Golgi network (TGN)-derived vesicles. At this site, nucleocapsids are thought to acquire their final envelope during a budding event that also results in the acquisition of viral glycoproteins (236,256,258,284,456,457,613,644,710,739,741,784). Mature virions subsequently follow the secretory pathway to the cell surface, where they are released into the extracellular milieu (456,457).

In contrast to nucleocapsid assembly, the molecular mechanisms responsible for the addition of tegument proteins to the nucleocapsid and the process of final envelopment itself, are poorly defined (456,457,601). The addition of tegument proteins to the nucleocapsid may occur at various stages during the egress pathway: in the nucleus, at the nuclear membrane, in the cytoplasm or during budding at the TGN.
Elucidating how the process of tegumentation occurs is important as evidence indicates that tegument proteins, possibly in concert with certain viral glycoproteins, contain all of the functions required for budding at the TGN (442,443,589,674). Our studies have focused on the role the protein-protein interactions of one specific HSV-1 tegument protein, VP22, plays in facilitating the process of tegumentation and final envelopment.

VP22, encoded by the Ul49 gene of HSV-1, contains 301 amino acids and is one of the most abundant tegument proteins with an estimated 2000 copies per virion (189,290,391). Despite its abundance, little is known regarding the role of VP22 during HSV-1 infection. Two VP22-null viruses have recently been described and demonstrate a variety of cell-specific replication defects with altered virion composition (179,182,559). Previous studies have shown that VP22 associates with membranes and localizes to acidic compartments of the cell including the TGN (66). VP22 also interacts with another abundant tegument protein VP16, which is essential for secondary envelopment and egress (182,183,480,730). Recent transmission immunoelectron microscopy (TIEM) studies suggest that during viral assembly, detectable amounts of VP16 are added to the capsid in the nucleus, with additional VP16 added as the nucleocapsid moves through the cytoplasm, prior to final envelopment (469,488). In contrast, VP22 is incorporated into virions during final envelopment as nucleocapsids bud into TGN-derived vesicles (469). Although little is known about the molecular details of this final envelopment step, it is likely that protein-protein interactions between tegument proteins and the cytoplasmic tails of envelope glycoproteins may occur during the budding process. In fact, VP22 binds to the cytoplasmic tail of glycoprotein D (gD) and may facilitate the interaction of the viral nucleocapsids with glycoproteins lining up on the membranes of TGN-derived
vesicles (109). In pseudorabies virus (PrV) the cytoplasmic tails of the envelope glycoproteins gE and gM bind to VP22 in a yeast two-hybrid study (221). Consistent with this observation, simultaneous deletion of gM and the gE/gI heterodimer results in reduced amounts of VP22 in the mature PrV particle, and in the formation of capsid-bound tegument aggregates in the cytoplasm (58,59,221). In HSV-1, simultaneous deletion of gD and gE results in accumulation of unenveloped capsids in the cytoplasm that are embedded in tegument-like material (197). These studies not only highlight the important role certain viral glycoproteins play in the assembly process but also the considerable amount of redundancy present, as deletion of gD, gM or gE alone had little effect on assembly (58,59,197,221).

In the current study, an interaction of VP22 with the cytoplasmic tail of gE was identified. Deletion mutagenesis was utilized to identify the minimal domain of VP22 that facilitates this interaction. These studies revealed a central region that is both necessary and sufficient to facilitate interaction with the gE tail. Furthermore, this domain of VP22 fused to the green fluorescent protein (GFP) competed efficiently with wild-type VP22 for packaging into assembling virus particles, suggesting that interaction with the cytoplasmic tail of gE may facilitate incorporation of VP22 into the virion.

This report expands upon the network of protein-protein interactions that facilitates the process of final tegumentation and envelopment, and further elucidates the mechanism by which VP22 is incorporated into virus particles.
RESULTS

Identification of an interaction between VP22 and the cytoplasmic tail of gE.

Given the apparent role gE plays in the assembly process, combined with the findings in PrV that VP22 binds to the cytoplasmic tail of gE, we examined the interaction of HSV-1 gE with VP22 (58,59,197,221).

We initially used a glutathione-S-transferase (GST) pulldown assay to examine interaction of the cytoplasmic tail of gE with VP22. Plasmids expressing the cytoplasmic tail of gE fused to the C-terminus of the GST protein (GST-gECT), or GST alone were expressed in *Escherichia coli* cells and the GST fusion proteins were purified on glutathione-Sepharose beads. Approximately equal amounts of the GST fusion proteins were used in the pulldown assay as determined by Coomassie-blue staining (Fig. 3.1A). The full-length GST-gECT fusion protein is indicated by an arrowhead. Various faster migrating bands were also purified from the bacterial lysates, which react with both GST and gE specific antisera, suggesting that these bands are breakdown products of the GST-gECT fusion protein. As a source of VP22 for these studies we used a mutant (166v) of the HSV-1 strain 17(+) (a kind gift from Peter O’Hare, Marie Curie Research Institute, United Kingdom), which expresses a 65 kilodalton (kDa), GFP-tagged form of VP22 (GFP-VP22) rather than the native VP22. This virus was chosen for our initial studies rather than a wild-type virus due to cross reactivity of our VP22 antibody with GST. Cells were infected with the GFP-VP22-expressing HSV-1 virus, lysed in NP-40 lysis buffer and incubated with equivalent amounts of purified GST fusion proteins (GST alone or GST-gECT) bound to glutathione-Sepharose beads. After a 3-h incubation,
Figure 3.1. Binding of the gE Cytoplasmic Tail to VP22. (A) Coomassie-blue stained gel of the cytoplasmic tail of gE fused to the C-terminus of the GST protein (GST-gECT) and GST alone. GST-gECT or GST were expressed in Escherichia coli cells and purified on glutathione-Sepharose beads. The arrowhead denotes the full-length GST-gECT fusion protein. (B) GST pulldown from HSV-1-infected cell lysates using GST-gECT. A7 cells were either mock infected or infected with a GFP-VP22-expressing HSV-1 mutant (166v) or the wild-type parental strain 17(+). At 10 h post-infection, cells were lysed in NP-40 lysis buffer (see Materials and methods). A fraction of each cell lysate was analyzed by Western blotting for viral protein expression. The remaining infected cell lysates were precleared with glutathione-Sepharose 4B beads and the samples were then incubated with approximately equal amounts of purified GST fusion proteins on glutathione-Sepharose beads. Beads were washed extensively with lysis buffer and bound proteins were separated by SDS-PAGE and transferred to nitrocellulose. GFP-VP22 was detected by Western blot analysis using a rabbit polyclonal antibody specific for GFP. As a control for specificity, the blots were stripped and reprobed with a rabbit polyclonal antibody raised against the major capsid protein VP5. The blots were also probed for the presence of the major tegument protein VP16 using a rabbit monospecific polyclonal antibody. The plus and minus designations indicate whether the cells were infected or mock infected, respectively.
A.

B.

Cell Lysate  

GST-ECT  

GST-ECT  

GST-ECT  

HSV:  

-  

+  

+  

166v Infection  

VP22  

VP5  

VP16  

VP22  

VP5  

VP16  

17(+) Infection  

144
beads were washed extensively with lysis buffer, bound material was separated by sodium dodecyl sulfate (SDS)-10% polyacrylamide gel electrophoresis (PAGE) and then analyzed on Western blots (Fig. 3.1B). Unlike GST alone, the GST-gECT fusion protein pulled-down a 65 kDa protein (the expected size of GFP-VP22) that was recognized by the anti-GFP antiserum. This band was not detected in the mock-infected sample or in cells infected with the parental 17(+) strain of HSV-1 (Fig. 3.1B). The specificity of the interaction between the cytoplasmic tail of gE and VP22 was highlighted by reprobing the blot with antibodies raised against the major capsid protein VP5 (Fig. 3.1B). Curiously, when the blot was reprobed with antibodies specific for the abundant tegument protein VP16, the cytoplasmic tail of gE appeared to bind to VP16 (Fig. 3.1B). As VP22 is known to bind to VP16, this result raised the possibility that VP22 may act as a bridge facilitating interaction of VP16 with gE. Alternatively, although an interaction between VP16 and gE has yet to be described, VP16 may facilitate binding of VP22 to gE.

To ascertain whether interaction between VP22 and gE occurs in the context of an infected cell, immunoprecipitation assays were performed. Cells were infected with the GFP-VP22-expressing HSV-1 mutant 166v, and lysed in NP-40 lysis buffer. A fraction of each cell lysate was analyzed by Western blotting for expression of the viral proteins GFP-VP22, gE, VP5 and VP16 (Fig. 3.2A). The remainder of the infected cell lysate was incubated with anti-gE antibodies (a kind gift from Dr. Harvey Friedman, University of Pennsylvania, Philadelphia) and the resulting antibody-antigen complexes were collected with protein A-agarose beads. After extensive washes, immunoprecipitating proteins were separated by SDS-PAGE and transferred to nitrocellulose. Coimmunoprecipitated
Figure 3.2. Coimmunoprecipitation of VP22 and gE. (A) Expression of viral proteins. A7 cells were either mock infected or infected with a GFP-VP22-expressing HSV-1 mutant (166v). At 10 h post-infection, cells were lysed in NP-40 lysis buffer (see Materials and methods). A fraction of each cell lysate was analyzed by Western blotting for viral protein expression. (B) Coimmunoprecipitation of GFP-VP22 and gE. The remaining infected cell lysates were precleared overnight at 4 °C with protein A-agarose beads. The lysates were then incubated with a rabbit polyclonal antibody raised against gE and the resulting antibody-antigen complexes were collected with protein A-agarose beads. After extensive washes with lysis buffer, proteins that immunoprecipitated with anti-gE antibody were separated by SDS-PAGE and transferred to nitrocellulose. Coimmunoprecipitated GFP-VP22 was detected by immunoblot using a goat polyclonal antibody specific for GFP. As a control for specificity, the blots were stripped and reprobed with a rabbit polyclonal antibody raised against the major capsid protein VP5. The blots were also probed for the presence of the major tegument protein VP16 using a rabbit monospecific polyclonal antibody. The plus and minus designations indicate whether the cells were infected or mock infected, respectively.
GFP-VP22 was detected by an immunoblot using a goat polyclonal antibody raised against the GFP protein. GFP-VP22 coimmunoprecipitated with gE, but was not precipitated with protein A-agarose beads alone, or an irrelevant rabbit polyclonal antibody to a hemagglutinin epitope (αHA) (Fig. 3.2B). As previously observed with the GST pulldown assay, in the presence of VP22, VP16 appears to bind to gE suggesting the existence of a gE/VP22/VP16 complex (Fig. 3.2B). The specificity of the interaction was again highlighted by the absence of any co-immunoprecipitating VP5. Collectively, the results of these experiments suggest that in the context of a viral infection, VP22 interacts with the cytoplasmic tail of gE, perhaps in a VP16 dependent fashion.

**Mapping the domain of VP22 that facilitates interaction with gE.** To map the domain of VP22 responsible for its interaction with gE, N-terminal and C-terminal truncation mutants of VP22 were tagged with GFP creating the fusion proteins represented in Fig. 3.3A and Fig. 3.4A, respectively. All deletions were designed to avoid major disruptions in protein folding that could result from truncating the protein in the middle of a hydrophobic region (66). To evaluate the ability of VP22 mutants to interact with the cytoplasmic tail of gE, a GST pulldown assay was performed. A7 cells were transfected with the indicated constructs. 20 h later the transfected monolayers were lysed with NP-40 lysis buffer and a fraction of each cell lysate was analyzed for expression of the VP22-GFP fusion proteins by Western blotting using a rabbit polyclonal antibody specific for GFP (Fig. 3.3B and Fig. 3.4B). The remaining cell lysates were incubated with equivalent amounts of purified GST fusion proteins (either the cytoplasmic tail of gE fused to GST or GST alone) bound to glutathione-Sepharose beads. After a 3-h incubation, beads were washed extensively with lysis
Figure 3.3. Characterization of the Ability of VP22 N-Terminal Truncation Mutants to Bind to the Cytoplasmic Tail of gE in a GST Pulldown Assay. (A) N-terminal truncation mutants of VP22 fused to GFP. A schematic representation of full-length and N-terminal truncated forms of VP22 fused to the N-terminus of the GFP protein. (B) Expression of VP22 truncation mutants in transfected cells. A7 cells were transfected with the indicated constructs and at 20 h post-transfection, the transfected monolayers were lysed with NP-40 lysis buffer. A fraction of each cell lysate was analyzed for expression of the VP22-GFP fusion proteins by Western blotting using a rabbit polyclonal antibody specific for GFP. (C) GST pulldown from transfected cell lysates using GST-gECT. The GST fusion proteins were expressed in *Escherichia coli* cells, purified on glutathione-Sepharose beads, and approximately equal amounts of each were added to the remainder of the transfected cell lysates. Beads were washed extensively with lysis buffer and bound proteins were separated by SDS-PAGE and transferred to nitrocellulose. Western blot analysis was performed using a rabbit polyclonal antibody raised against the GFP protein. The positions of molecular mass markers (in kilodaltons) are indicated on the left.
Figure 3.4. Characterization of the Ability of VP22 C-Terminal Truncation Mutants to Bind to the Cytoplasmic Tail of gE in a GST Pulldown Assay. (A) C-terminal truncations of VP22 fused to GFP. A schematic representation of full-length and C-terminal truncated forms of VP22 fused to the N-terminus of the GFP protein. Expression of VP22 truncation mutants in transfected cells (B) and their ability to bind to the cytoplasmic tail of gE (C) were analyzed as described in the legend to Fig. 3.3. The positions of molecular mass markers (in kilodaltons) are indicated on the left.
buffer, and bound material was separated by SDS-PAGE and then analyzed on Western blots. The GST-gECT fusion protein pulled-down VP22-GFP from the transfected-cell lysate, whereas the GST alone control did not, indicating that the interaction between VP22 and gE can occur in the absence of other viral proteins and is not dependent upon VP16 (Fig. 3.3C). Deletion of the N-terminal 43, 86, 120 or even 164 amino acids of VP22 failed to abrogate gE binding. In contrast, when the N-terminal 225 or 270 residues of VP22 were deleted, binding was abolished. These results suggest that the region of VP22 required to facilitate interaction with gE lies within the C-terminal 137 amino acids. Analysis of the C-terminal truncations of VP22 showed that restoration of the N-terminal 43, 86, 120, 164 or 225 amino acids of VP22 was not sufficient to promote interaction of VP22 with gE (Fig. 3.4C). However, upon restoration of the first 270 amino acids (VP22.1-270), binding to the cytoplasmic tail of gE was detected (Fig. 3.4C). Collectively, the results of these experiments indicate that amino acids 165-270 of VP22 facilitate interaction with the cytoplasmic tail of gE.

**Residues 165-270 of VP22 are sufficient to facilitate binding to gE.** Primary structural alignment of this region (amino acids 165-270) of VP22 reveals that it is highly conserved among VP22 homologues of alphaherpesviruses (Chapter IV Fig. 4.13A). To examine whether residues 165-270 of VP22 are sufficient to facilitate binding to the cytoplasmic tail of gE, this segment of VP22 was fused to the N-terminus of GFP (VP22.165-270). In addition, a VP22-GFP construct was made in which these amino acids are absent [VP22.Δ165-270] (Fig. 3.5A). Both mutants were evaluated for their ability to bind to the cytoplasmic tail of gE in a GST pulldown assay. Each of the mutants was expressed at levels similar to VP22-GFP (Fig. 3.5B).
Figure 3.5. Identification of the Minimal Domain of VP22 that Facilitates Interaction with the Cytoplasmic Tail of gE. (A) gE interaction domain mutants of VP22 fused to GFP. Schematic representation of VP22, deletion of the predicted gE interaction domain of VP22, and the interaction domain alone fused to the N-terminus of the GFP protein. Expression of VP22 mutants in transfected cells (B) and their ability to bind to the cytoplasmic tail of gE (C) were analyzed as described in the legend to Fig. 3.3. The positions of molecular mass markers (in kilodaltons) are indicated on the left.
Upon deletion of amino acids 165-270, VP22 binding to gE was abrogated (Fig. 3.5C). In contrast residues 165-270 of VP22, when fused to GFP, did bind to the cytoplasmic tail of gE, whereas GFP alone did not. These data suggest that amino acids 165-270 of VP22 comprise a minimal domain of the protein that is both necessary and sufficient to facilitate its interaction with gE in the absence of additional viral proteins.

**VP16 binding to gE is facilitated by VP22.** As our results suggest that VP16 is not required to facilitate interaction of VP22 with gE, we were curious to determine if VP22 acts as a bridging protein, linking VP16 to the cytoplasmic tail of gE. To test this hypothesis, A7 cells were infected with a VP22-null virus (UL49−) (a kind gift from Dr. Joel Baines, Cornell University, Ithaca) or the wild-type parental strain F. The cells were subsequently lysed in NP-40 lysis buffer and viral protein expression and binding to the cytoplasmic tail of gE were analyzed as described above. Although high levels of VP16 expression were observed with both a VP22-null virus infection and that of the parental F strain, the GST-gECT fusion protein only pulled-down VP16 in the presence of VP22 (Fig. 3.6). The specificity of binding was highlighted by reprobing the blot with antibodies raised against the major capsid protein VP5 (Fig. 3.6). Unfortunately, due to cross reactivity of our VP22 antibody with GST, we were unable to detect VP22 pulled-down by GST-gECT. These results indicate that VP22 facilitates binding of VP16 to gE, perhaps with through the formation of a tripartite protein complex.

**Virion incorporation of the region of VP22 that binds to gE.** Recent studies have shown a correlation between the ability of VP22 to bind VP16 and its packaging into virus particles (182). Given that the domain of VP22 we
Figure 3.6. Characterization of the Ability of VP16 to Bind to the Cytoplasmic Tail of gE in the Absence of VP22.

A7 cells were either mock infected or infected with a VP22-null virus (U_{L49}^-) or the wild-type parental strain F. At 10 h post-infection, cells were lysed in NP-40 lysis buffer and viral protein expression and binding to the cytoplasmic tail of gE were analyzed by Western blotting as described in the legend to Fig. 3.1. The major tegument protein VP16 was detected using a rabbit monospecific polyclonal antibody and VP22 was detected using a rabbit polyclonal antibody raised against GST-VP22. As a control for specificity, the blots were stripped and reprobed with a rabbit polyclonal antibody raised against the major capsid protein VP5. The plus and minus designations indicate whether the cells were infected or mock infected, respectively.
have identified as sufficient to facilitate binding to the cytoplasmic tail of gE overlaps with the domain shown to bind VP16 [(280); Chapter IV], it was of interest to ascertain if the gE interaction domain of VP22 was packaged into assembling virus particles. To examine this possibility, we utilized a transfection/infection-based packaging assay in which our VP22-GFP mutant proteins compete with virally expressed wild-type VP22 for incorporation into assembling virions. Vero cells were transfected with plasmids encoding various GFP fusion proteins and subsequently infected with HSV-1 or mock-infected. At 18 h post-infection, extracellular virions were harvested and then pelleted through a sucrose cushion. The pelleted virions were analyzed by Western blotting using GFP-specific antisera to detect the VP22-GFP fusion proteins. To confirm that approximately equal amounts of virus were loaded with each sample, Western blots were stripped and reprobed for the abundant tegument protein VP16.

Analysis of the expression of the various VP22-GFP constructs within transfected/infected cells showed that each was expressed and migrated at the expected molecular weight (Fig. 3.7A). With regard to packaging, wild-type VP22-GFP was incorporated into virions, whereas GFP alone was undetectable, despite high expression in transfected/infected cells, indicating that GFP itself does not have a significant effect on packaging (Fig. 3.7B). Furthermore, none of the VP22-GFP mutant proteins were detected in media from transfected/mock-infected cells, indicating that incorporation is in fact a virus-specific event and not due to aggregates that can pellet through the sucrose cushion (data not shown). Residues 165-270 of VP22, which are sufficient for binding to the cytoplasmic tail of gE, were packaged into assembling virions, albeit at slightly reduced levels when compared to wild-type VP22 (Fig. 3.7B). Upon deletion of the gE
**Figure 3.7. Virion Incorporation of the VP22 Domain that Facilitates Interaction with gE.** Vero cells were transfected with the indicated VP22-GFP constructs, and 20 h later, they were infected with HSV-1. After an additional 18-h incubation, cell lysates were prepared (A) and virions were collected from the media by centrifugation through a 30% sucrose cushion (B). Cell lysates and extracellular virus were separated by SDS-PAGE and transferred to nitrocellulose. Western blot analysis was performed using a rabbit polyclonal antibody specific for GFP. As a loading control, the blot was stripped and reprobed with a rabbit monospecific polyclonal antibody raised against the HSV-1 tegument protein VP16.
interaction domain (VP22Δ165-270), the resulting mutant failed to be incorporated to any significant level (Fig. 3.7B). The results of these experiments suggest that residues 165-270 of VP22 may harbor determinants which facilitate incorporation of the protein into assembling virus particles.
DISCUSSION

Recent studies have not only highlighted the important role viral glycoproteins play in the herpesvirus assembly pathway, but also the considerable amount of redundancy in the process itself (58,59,197,221). Deletion of individual glycoproteins (gD, gM or gE) has little effect on assembly; however, simultaneous deletion of two or more glycoproteins such as HSV-1 gD and gE/gI or PrV gM and gE/gI can have dramatic effects on assembly (58,59,197,221). Current evidence suggests that during the HSV-1 assembly pathway, viral glycoprotein tails may play an important role in the recruitment of tegument-coated capsids to the site of final envelopment (109,197). The cytoplasmic tails of HSV-1 glycoproteins interact with various tegument proteins including VP22 (109,270,501,783). Furthermore, deletion of the cytoplasmic tails of two PrV glycoproteins (gE and gM), which interact with VP22, resulted in reduced amounts of VP22 packaged in the mature particle. These results suggest that glycoprotein-tegument protein interactions may facilitate the incorporation of certain tegument proteins into the assembling virion (221).

The goal of the present study was to determine if the HSV-1 tegument protein VP22 interacts with the cytoplasmic tail of gE. Using GST pulldown assays and coimmunoprecipitation studies, we have identified an interaction of VP22 with the cytoplasmic tail of gE that can occur in the absence of additional viral proteins. Deletion mutagenesis identified a region of VP22 encompassing amino acids 165-270 that is both necessary and sufficient to facilitate interaction with gE. Primary structural alignment reveals that the gE interaction domain of HSV-1 VP22 is highly conserved among VP22 homologues of herpesviruses (Chapter IV Fig. 4.13A). Interestingly, this region of the
protein is also capable of binding VP16 in a manner independent of other viral polypeptides [(515); Chapter IV].

When VP16 is deleted, cytoplasmic capsids fail to become enveloped indicating that VP16 plays a key role in the tegumentation and egress of HSV-1 (480,730). TIEM studies suggest that VP16 is added to the nucleocapsid prior to final envelopment whereas the membrane-associated VP22 is incorporated as nucleocapsids bud into TGN-derived vesicles (469). VP22 can relocalize the normally diffuse VP16 to a perinuclear compartment that resembles the TGN (183) and our studies suggest that VP16 binds to gE in a VP22-dependent manner. As VP22 binds both VP16 and the cytoplasmic tail of gE, it may act as a bridge, with both VP16 and gE binding simultaneously. Alternatively, VP16 and gE binding to VP22 may be mutually exclusive. In such a scenario, VP22 may still act a bridge between VP16 and gE, as a recent report suggested that VP22 may possess the ability to multimerize (481,718). This VP16-VP22-gE linkage may facilitate the budding of cytoplasmic nucleocapsids into TGN-derived vesicles.

VP16 has been crosslinked to the cytoplasmic tails of gB, gH, and gD (783) and recently has been shown to bind to the cytoplasmic tail of gH (270). Thus, the interaction of VP22 with VP16 may be only one of numerous interactions that work redundantly to facilitate the final stages of envelopment. In fact, the recent descriptions of a HSV-1 VP22-null virus highlights the redundancy inherent to the process, as the requirement for VP22 in the assembly pathway can be bypassed, albeit with an altered virion composition (179,182). In light of our findings, it is interesting to note that virions generated with the VP22-null mutant contained reduced amounts of gE as well as gD (179). Other tegument proteins may also play an important role in the final stages of assembly and egress.
UL11 for example, has been shown to target to, and bind membranes of the TGN (57,406) and interact with UL16 (407), which is believed to associate with capsids (527). Nevertheless, the dramatic defects seen in the assembly pathway with the HSV-1 VP16-null mutant suggests that one or more of the protein-protein interactions involving VP16 may be required for the assembly and egress of HSV-1 virions (480,730).

The process by which HSV-1 tegument proteins are selectively packaged into the assembling virion remains poorly understood. Previous studies suggest that the herpesvirus tegument may be flexible regarding both protein composition and stoichiometry (776,777). Overexpression of VP22 within infected cells results in a 3-fold increase in its incorporation with little effect on other tegument components (391). While the majority of VP22 in infected cells is highly phosphorylated, the non-phosphorylated form of VP22 is the predominant species in the virion; however, studies have shown that VP22 phosphorylation has no effect on the relative packaging of VP22 (562). Recent studies have suggested that two determinants are involved in the packaging of VP22, a domain comprising the C-terminal 89 amino acids and an internal VP16 interaction domain (which overlaps with the VP22 gE interaction domain) (280). Using a transfection/infection-based incorporation assay in which a GFP-tagged VP22 expressed in \textit{trans} competes with virally encoded VP22 for packaging into virions, we demonstrated that a region of VP22, corresponding to the gE interaction domain (residues 165-270), was packaged into assembling virus particles, although at reduced levels relative to wild-type VP22. This region facilitates VP16 binding [(515); Chapter IV] but lacks the C-terminal packaging determinant identified in other studies, which may explain the reduced packaging (280). The C-terminal determinant harbors no currently
identified activities of VP22; however, it may facilitate gD binding, whereby VP22 binding to VP16, gE, and gD would facilitate wild-type levels of incorporation. A VP22 virion packaging signal has been described in the C-terminus of VP22 which may facilitate gD binding (personal communication from Dr. John Blaho, Mount Sinai School of Medicine, New York). This signal is absent from the region encompassing amino acids 165-270 of VP22; however, it is contained in VP22.Δ165-270 and thus may account for the low levels of incorporation seen with this mutant, perhaps due to gD binding.

Further mutagenesis will allow the careful dissection of the VP16/gE/gD binding domains of VP22 and may shed light on the minimal requirement for virion incorporation of VP22.
MATERIALS AND METHODS

Cells and viruses. Vero (ATCC CCL-81) and A7 (human melanoma) cells, a gift from Gary Thomas (The Oregon Health and Science University, Portland) were grown in Dulbecco’s modified Eagles medium (DMEM) (GIBCO) supplemented with 10% fetal bovine serum (FBS), 2.25% sodium bicarbonate, 25 mM HEPES buffer, glutamine (300 µg/ml), penicillin (100 µg/ml), and streptomycin (131 µg/ml). Infected cells were grown in DMEM supplemented with 2% FBS, 25 mM HEPES buffer, glutamine (300 µg/ml), penicillin (100 µg/ml), and streptomycin (131 µg/ml). The viruses used in this study were HSV-1 strains KOS (650), F (179) and 17(+) (67), and the mutant viruses U149 (a VP22-null virus) a kind gift from Joel Baines (Cornell University, Ithaca, New York) (179) and 166v (a GFP-VP22-expressing mutant) a kind gift from Peter O’Hare (Marie Curie Research Institute, United Kingdom) (186).

Construction of VP22-GFP chimeras. Plasmids encoding VP22 fused to GFP and the N-terminal and C-terminal truncations of VP22 fused to GFP were described previously (66).

To make pVP22.Δ165-270.GFP, the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) was used according to the manufacturer’s instructions. Using pVP22.GFP as a template (66), mutagenic primers were designed complementary to 15-bp immediately upstream of codon 165 and 15-bp immediately downstream of codon 270, essentially looping out the sequence encoding amino acids 165-270 of VP22 in pVP22.GFP; thereby creating pVP22.Δ165-270-GFP.

pVP22.165-270-GFP was constructed by using pVP22.165-301-GFP as a source of the U149 gene fragment (66). DNA was amplified by using a forward primer...
containing a *Bgl*II site 92-bp upstream of the start codon of VP22 and a reverse primer containing a *Hind*III site immediately downstream of codon 270 for VP22. This product was digested with *Bgl*II and *Hind*III and ligated into the vector pEGFP-N2 (Clontech) digested with the same restriction enzymes, to produce pVP22.165-270-GFP. All constructs were sequenced to confirm the identity of VP22 and to ensure that the gene encoding VP22 (or the mutated forms) was in frame with the gene encoding the GFP protein.

**Construction and purification of GST fusion proteins.** The vector encoding the cytoplasmic tail of gE fused to GST (GST-gECT) was a kind gift from David Johnson (The Oregon Health and Science University, Portland, Oregon).

To express and purify the cytoplasmic tail of gE fused to GST and GST alone, plasmids encoding the respective constructs were transformed into BL21 competent cells (Stratagene) according to the manufacturer’s instructions and overnight cultures were prepared. Approximately 10 ml of these cultures were used to inoculate fresh 100 ml cultures and grown at 37 °C to $A_{600}=0.4$. To induce expression, 100 µL of 1 M IPTG (GIBCO) was added and the cultures were grown at 30 °C for 3-4 h, followed by centrifugation at 10,000 x $g$ for 10 min at 4 °C. The bacterial pellet was resuspended in 10 ml of lysis buffer (100 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1 mM CHAPS, 400 mM NaCl, 1% Triton X-100, Complete Mini protease inhibitors [Roche]). The suspension was sonicated and clarified by centrifugation at 27,000 x $g$ for 10 min at 4 °C. A volume of 133 µl of glutathione-Sepharose 4B beads (Pharmacia) that had been washed twice with phosphate-buffered saline (PBS) was added to the supernatant and incubated overnight at 4 °C. Beads were washed thoroughly with PBS and the yield of each
purified GST fusion protein was determined by SDS-PAGE and subsequent staining with Coomassie blue to detect the recombinant protein.

**GST pulldown assay.** GST fusion proteins were purified from bacterial cultures, as described above. Confluent monolayers of A7 cells grown in 60-mm plates were infected with HSV-1 strains 17(+), F, a GFP-VP22-expressing mutant (166v) of the HSV-1 strain 17(+) or a VP22-null virus (U L49-) at a multiplicity of infection (MOI) of 10. At 10 h post-infection, cells were washed twice with PBS and lysed in 1.2 ml of NP-40 lysis buffer (1% NP-40, 200 mM NaCl, 50 mM Tris-HCl [pH 7.4], 2 mM MgCl2) containing Complete Mini protease inhibitors. A sample was removed for analysis of viral protein expression. Infected cell lysates were precleared overnight at 4 °C with glutathione-Sepharose 4B beads that had been previously washed twice with PBS, and the samples were then incubated with approximately equal amounts of purified GST fusion proteins on glutathione-Sepharose beads (as determined by Coomassie blue-stained gel) for 3 h at 4 °C. Beads were washed three times with NP-40 lysis buffer and once with 10 mM Tris-HCl (pH 7.4). GFP-VP22 or VP16 bound to GST constructs was detected by Western blotting using a rabbit polyclonal antibody raised against the GFP protein (Santa Cruz) or a rabbit monospecific polyclonal antibody raised against a C-terminal peptide of VP16 (Clontech), followed by a goat anti-rabbit antibody conjugated to horseradish peroxidase (Sigma), ECL reagents (Pharmacia), and autoradiography on Kodak BioMax XAR film. As a control for specificity, the blots were reprobed with a rabbit polyclonal antibody raised against the major capsid protein VP5. To further control for the quantities of GST fusion proteins used in the pulldown, nitrocellulose membranes were stripped (60 mM Tris-HCl [pH 8.0], 2% SDS, 0.75% β-
mercaptoethanol [β-ME] for 45 min at 55 °C) and reprobed with a goat polyclonal antibody raised against the GST protein (Rockland).

To analyze the VP22-gE interaction within transfected cells, a similar GST pulldown assay was used. Confluent monolayers of A7 or Vero cells grown in 100-mm plates were transfected with the indicated constructs by using the calcium phosphate method as described previously (130) or Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. At 20 h post-transfection, monolayers were washed twice with PBS and scraped into 10 ml of cold PBS. A 1 ml sample of the cell suspension was removed for analysis of expression of the VP22-GFP fusion proteins, and the remaining cells were pelleted by centrifugation (1000 x g for 5 min at 4 °C). The 9 ml samples were lysed in NP-40 lysis buffer. Transfected-cell lysates were precleared overnight at 4 °C with glutathione-Sepharose 4B beads that had been previously washed twice with PBS, and the samples were then incubated with approximately equal amounts of purified GST fusion proteins on glutathione-Sepharose beads (as determined by Coomassie blue-stained gel) for 3 h at 4 °C. Beads were washed three times with NP-40 lysis buffer and once with 10 mM Tris-HCl (pH 7.4). VP22-GFP fusion proteins bound to GST constructs were detected by Western blotting using a rabbit polyclonal antibody raised against the GFP protein (Santa Cruz), a goat anti-rabbit antibody conjugated to horseradish peroxidase (Sigma), ECL reagents (Pharmacia), and autoradiography on Kodak BioMax XAR film. To further control for the quantities of GST fusion proteins used in the pulldown, nitrocellulose membranes were stripped as described above and reprobed with a goat polyclonal antibody raised against the GST protein (Rockland). To detect expression of the VP22-GFP fusion proteins in transfected cells, proteins in the 1
ml cell suspension sample were analyzed by Western blotting using a rabbit polyclonal antibody against GFP, a goat anti-rabbit antibody conjugated to horseradish peroxidase, ECL reagents, and autoradiography on Kodak BioMax XAR film.

**Immunoprecipitation-Western assay.** Confluent monolayers of A7 cells grown in 60-mm plates were infected with a GFP-VP22-expressing mutant (166v) of the HSV-1 strain 17(+) at a MOI of 10. At 10 h post-infection, cells were washed twice with PBS and lysed in 1.2 ml of NP-40 lysis buffer as described above. A sample was removed for analysis of viral protein expression. Infected-cell lysates were precleared overnight at 4 °C with protein A-agarose beads (Roche) that had been washed twice in lysis buffer. The lysates were then incubated with a rabbit polyclonal antibody raised against gE (a kind gift from Harvey Friedman, University of Pennsylvania, Philadelphia), protein A-agarose beads alone, or an irrelevant rabbit polyclonal antibody for 1 h at 4 °C, and immune complexes were collected with protein A-agarose beads that had been washed twice with lysis buffer. Beads were washed three times with NP-40 lysis buffer and once with 10 mM Tris-HCl (pH 7.4). Coimmunoprecipitated GFP-VP22 or VP16 was detected by Western blotting using a goat polyclonal antibody raised against the GFP protein (Rockland) or a rabbit monospecific polyclonal antibody raised against a C-terminal peptide of VP16 (Clontech), followed by the appropriate species of secondary antibody conjugated to horseradish peroxidase, ECL reagents, and chemiluminescence autoradiography on Kodak BioMax XAR film.

As a control for specificity, the blots were stripped as described above and reprobed with a rabbit polyclonal antibody raised against the major capsid protein VP5.
**Virion incorporation assay.** Confluent monolayers of Vero cells grown in 100-mm plates were transfected with the indicated constructs using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. At 20 h post-transfection, cells were infected with HSV-1 KOS strain at a MOI of 10 or mock infected. At 18 h post-infection (38 h post-transfection), the medium was removed by pipetting and centrifuged at 1000 x g for 10 min at 4 °C to remove cellular debris. The supernatant was retained and extracellular virions were purified by centrifugation (115,000 x g for 1 h in a Beckman SW41 rotor) through a 30% (wt/vol) sucrose cushion (1.7 ml) in NTE buffer (100 mM NaCl, 10 mM Tris, 1 mM EDTA, [pH 7.4]). Pelleted virions and infected cells were disrupted in 1X sample buffer (50 mM Tris-HCl [pH 6.8], 10% glycerol, 2% SDS, 5% β-ME). GFP-tagged proteins were detected by Western blotting using a rabbit polyclonal antibody raised against the GFP protein (Santa Cruz), a goat anti-rabbit antibody conjugated to horseradish peroxidase, ECL reagents, and chemiluminescence autoradiography on Kodak BioMax XAR film. As a loading control, nitrocellulose membranes were stripped as described above and reprobed with a rabbit monospecific polyclonal antibody raised against a C-terminal peptide of the HSV-1 tegument protein VP16 (Clontech).
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CHAPTER IV

INCORPORATION OF THE HERPES SIMPLEX VIRUS TYPE 1 TEGUMENT PROTEIN VP22 INTO THE VIRUS PARTICLE IS INDEPENDENT OF INTERACTION WITH VP16

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ABSTRACT

Herpes simplex virus type 1 (HSV-1) virions contain a proteinaceous layer termed the tegument that lies between the nucleocapsid and viral envelope. The mechanisms underlying tegumentation remain largely undefined for all herpesviruses. Using glutathione S-transferase (GST) pulldowns and coimmunoprecipitation studies, we have identified a domain of the tegument protein VP22 that facilitates interaction with VP16. This region of VP22 (residues 165-225) overlaps the glycoprotein E (gE) binding domain of VP22 (residues 165-270), which is sufficient to mediate VP22 packaging into assembling virus particles. To ascertain the contribution of VP16 and gE binding activities of VP22 to its virion incorporation, a transfection/infection based virion incorporation assay, using point mutants that discern between the two binding activities, was utilized. Our results suggest that interaction with VP16 is not required for incorporation of VP22 into virus particles and that binding to the cytoplasmic tail of gE is sufficient to facilitate packaging.
INTRODUCTION

Herpesviruses share common virion morphology; icosahedral capsids containing the viral genome are surrounded by an amorphous layer of at least 20 proteins known as the tegument, which is in turn enclosed by a lipid bilayer composed of eleven or more virally encoded glycoproteins (601). While it is well established that capsid assembly and packaging of the viral genome occur in the nucleus, the compartment(s) in which the tegument and envelope are acquired is less well-defined (191,456,457). As with other herpesviruses, the current model for herpes simplex virus type 1 (HSV-1) assembly and egress suggests that nucleocapsids are shuttled to the cytoplasm via a budding/fusion event that occurs across the inner and outer membranes of the nucleus, respectively. The unenveloped nucleocapsids then travel through the cytoplasm until they reach a trans-Golgi network (TGN)-derived vesicle. While at this site, nucleocapsids are thought to acquire their final lipid bilayer during a budding event that also results in the acquisition of tegument and viral glycoproteins (236,256,258,284,456,457,613,644,710,739,741,784). The mature virions subsequently follow the secretory pathway to the cell surface, where they are released into the extracellular milieu (456).

In contrast to nucleocapsid assembly, the molecular mechanisms of tegumentation and the process of final envelopment are poorly understood (456,457,601). Tegumentation of nucleocapsids can theoretically occur at various stages in the egress pathway: in the nucleus, at the nuclear membrane, in the cytoplasm or during budding at the TGN. Recent studies have demonstrated that a subset of tegument proteins are added to the capsid prior to nuclear egress; however, the mechanism behind the addition of the
majority of the tegument components remains elusive (72,488). Understanding how the process of tegumentation occurs is important as evidence indicates that tegument proteins, possibly in concert with certain viral glycoproteins, contain all of the functions required for budding at the TGN (442,443,589,674). Our studies have focused on defining the protein-protein interactions of one specific HSV-1 tegument protein, VP22, and the role these interactions play in facilitating incorporation of VP22 into assembling virions.

Encoded by the UL49 gene of HSV-1, VP22 is a highly phosphorylated, 301-amino-acid protein which is one of the most abundant tegument components, with an estimated 2000 copies of the protein packaged per virion (189,290,391). Despite its abundance, the role of VP22 during HSV-1 assembly and the mechanism of its incorporation, remain undefined. VP22 interacts with VP16, another abundant tegument protein, which is essential for secondary envelopment and egress (182,183,480,730). Transmission immunoelectron microscopy (TIEM) studies suggest that during viral assembly, detectable amounts of VP16 are added to the capsid in the nucleus, with additional VP16 added as the nucleocapsid moves through the cytoplasm, prior to final envelopment (469,488). In contrast, VP22 is packaged into virions during final envelopment as nucleocapsids bud into TGN-derived vesicles (469). Consistent with this observation, previous studies have shown that VP22 associates with membranes and localizes to acidic compartments of the cell including the TGN (66).

Although little is known about the molecular details of final tegumentation and envelopment, it is likely that protein-protein interactions between tegument proteins or between tegument proteins and the cytoplasmic tails of virally encoded glycoproteins
facilitate the process and may result in the incorporation of viral proteins into the assembling particle. Two VP22-null viruses have recently been described and demonstrate a variety of cell-specific replication defects with altered virion composition including decreased packaging of both glycoprotein D (gD) and glycoprotein E (gE) (179,182,559). VP22 binds to the cytoplasmic tails of both gD and gE, and may facilitate interaction of viral nucleocapsids with glycoproteins lining up on the membranes of TGN-derived vesicles, perhaps through its interaction with VP16 (109,514). In HSV-1, simultaneous deletion of gD and gE results in accumulation of unenveloped capsids in the cytoplasm that are embedded in tegument-like material, a similar phenotype to that seen with a VP16-null virus (197,480,730). In pseudorabies virus (PrV) the cytoplasmic tails of the envelope glycoproteins gE and gM bind to VP22 in a yeast two-hybrid study (221). Interestingly, simultaneous deletion of gM and the gE/gI heterodimer results in reduced amounts of VP22 in the mature PrV particle, and in the formation of capsid-bound tegument aggregates in the cytoplasm (58,59,221). Furthermore, the Bartha strain of PrV, which lacks the glycoproteins gI and gE, fails to package VP22 (413).

Previous studies from our laboratory demonstrated that the gE binding domain of VP22 (residues 165–270) competes efficiently with wild-type VP22 for packaging into assembling virus particles [(514); Chapter III]. Interestingly, a recent study suggested that a similar domain of VP22 may facilitate binding to VP16, and reported a correlation between the ability of VP22 to bind VP16 and its incorporation into virus particles (280). The focus of the current study was to elucidate the role these protein-protein interactions play in the virion packaging of VP22.
Deletion mutagenesis was used to identify the minimal domain of VP22 that is required for interaction with VP16. The experiments presented in this report extend the findings of Hafezi et al., (2005) and reveal a central region that is both necessary and sufficient to facilitate interaction with VP16. Membrane flotation experiments suggest that this region of VP22 tagged with the green fluorescent protein (GFP) has the ability to associate with cellular membranes; however, this activity is not sufficient to facilitate virion incorporation of VP22 and additional protein-protein interactions appear to be required. Using site-directed point mutagenesis to discern between the VP16 and gE binding activities of VP22, virion incorporation studies suggest that VP16 binding is not required for incorporation of VP22 into assembling virus particles. This report extends our knowledge of the network of protein-protein interactions that facilitates the process of final tegumentation and envelopment and further defines the mechanism by which VP22 is incorporated into virus particles.
RESULTS

Mapping the domain of VP22 that facilitates interaction with VP16. To ascertain the domain of VP22 that facilitates interaction with VP16, a series of N-terminal and C-terminal truncation mutants was made in the context of a GST-VP22 fusion protein (Fig. 4.1A). All deletions were designed to avoid major disruptions in protein folding that could result from truncating the protein in the middle of a hydrophobic region. The truncation mutants were evaluated in a glutathione S-transferase (GST) pulldown assay for their ability to bind to VP16. HSV-1-infected cell lysates were incubated with equivalent amounts of purified GST fusion proteins bound to glutathione-Sepharose beads. After a 3-h incubation, beads were washed extensively with lysis buffer, and bound material was separated by sodium dodecyl sulfate (SDS)-10% polyacrylamide gel electrophoresis (PAGE) and then analyzed on Western blots. Unlike GST alone, the GST-VP22 fusion protein reacted with a 65 kDa protein that was recognized by anti-VP16 antiserum (Fig. 4.1B). Upon deletion of the N-terminal 43, 86, 120 or even 164 amino acids of VP22, VP16 binding was still detectable; however, VP22.87-301 bound to VP16 weakly, a reproducible observation that may suggest problems in protein folding with this truncation mutant. When the N-terminal 225 or 270 residues of VP22 were deleted, binding was abolished. These results indicate that the region of VP22 that facilitates interaction with VP16 lies within the C-terminal 137 amino acids.

To further map the VP16 interaction domain of VP22, we also characterized the ability of the C-terminal truncation mutants to bind VP16 from HSV-1-infected cell lysates (Fig. 4.1C). The first 43, 86, 120 or 164 amino acids of VP22 were not sufficient
Figure 4.1. Characterization of the Ability of VP22 Truncation Mutants to Bind VP16 in a GST Pulldown Assay. (A) N-terminal and C-terminal truncations of VP22 fused to GST. A schematic representation of full-length, N-terminal and C-terminal truncated forms of VP22 fused to the C-terminus of the GST protein. (B) and (C) GST pulldown from HSV-1-infected cell lysates using GST-VP22 and N-terminal and C-terminal truncation mutants respectively. The GST fusion proteins were expressed in *Escherichia coli* cells, purified on glutathione-Sepharose beads, and approximately equal amounts of each were added to NP-40 lysates of Vero cells that had been infected with HSV-1. Beads were washed extensively with lysis buffer and bound proteins were separated by SDS-PAGE and transferred to nitrocellulose. Western blot analysis was performed using a rabbit monospecific polyclonal antibody raised against VP16. (D) VP16 interaction domain mutants of VP22 fused to GST. Schematic representation of VP22, deletion of amino acids 87-120 of VP22 or the predicted VP16 interaction domain of VP22, and the interaction domain alone fused to the C-terminus of the GST protein. (E) GST pulldown from HSV-1-infected cell lysates using VP16 interaction domain mutants of VP22. Purified fusion proteins were tested for their ability to bind VP16 from HSV-1 infected cells, as described in the legend to Fig. 1B and 1C. The positions of molecular mass markers (in kilodaltons) are indicated on the left.
to facilitate binding of VP22 to VP16; however, constructs containing the first 225 or 270 residues were able to bind. Collectively, these experiments indicate that amino acids 165-225 of VP22 facilitate interaction with VP16.

**Residues 165-225 of VP22 are sufficient to pulldown VP16.** To examine whether residues 165-225 of VP22 are sufficient to facilitate interaction with VP16, this segment of VP22 was fused to the C-terminus of GST. In addition, a GST-VP22 construct was made in which these amino acids are absent (Fig. 4.1D). Both mutants were evaluated for their ability to interact with VP16 from HSV-1-infected cell lysates. As predicted, VP16 failed to bind to the fusion protein in which amino acids 165-225 were deleted (VP22.Δ165-225) (Fig. 4.1E). Binding was detected when amino acids 87-120 were deleted (Fig. 4.1E), indicating that the VP22-VP16 interaction is not sensitive to all internal deletions. Furthermore, amino acids 165-225, when fused to GST, were sufficient to pulldown VP16 from infected-cell lysates (Fig. 4.1E), although not to the level of wild-type VP22. These data suggest that amino acids 165-225 of VP22 comprise a minimal domain of the protein that is both necessary and sufficient to facilitate its interaction with VP16.

**Coimmunoprecipitation of GFP-tagged VP22 truncation mutants with VP16.** Immunoprecipitation assays from transfected/infected cell lysates were performed to ascertain if a similar interaction of VP22 and VP16 occurred within the context of an infected cell. N-terminal and C-terminal truncation mutants of VP22 were tagged with GFP creating the fusion proteins represented in Fig. 4.2A. Vero cells were transfected with plasmids encoding GFP or the truncated forms of VP22 fused to GFP. At 20 h post-transfection, cells were infected with HSV-1 at a MOI of 10. After an additional period
of 10 h, the infected cells were lysed with NP-40 lysis buffer. A fraction of each cell lysate was analyzed by Western blotting to verify that the GFP-tagged truncation mutants were expressed in transfected/infected cells (Fig. 4.2B and Fig. 4.2D). The remaining lysates were then incubated with goat anti-GFP antibodies followed by protein G-agarose beads. Immunoprecipitated material was separated by SDS-PAGE and analyzed by Western blotting using rabbit anti-VP16 antibodies to assay for immunoprecipitation of VP16 with the VP22 truncation mutants. When the N-terminal truncations were analyzed for their ability to interact with VP16, results similar to those found with the GST pulldown assay were observed. Deletion of the first 43, 86, 120 or 164 amino acids had no effect on the ability of VP16 to coimmunoprecipitate with VP22 (Fig. 4.2C), whereas deletion of the N-terminal 225 or 270 residues of VP22 abrogated VP16 binding. Thus, these results confirm that the region of VP22 required to facilitate interaction with VP16 resides within the C-terminal 137 amino acids.

Analysis of the C-terminal truncations of VP22 showed that restoration of the N-terminal 43, 86, 120 or 164 amino acids of VP22 was not sufficient to promote interaction of VP22 and VP16 when assayed by coimmunoprecipitation from transfected/infected cells (Fig. 4.2E). In contrast to the data from the GST pulldown assay, restoration of the first 225 amino acids did not allow VP16 to bind VP22 in transfected/infected cells (Fig. 4.2E). The variance seen between the results of the two assays may be attributable to the location of the protein fusion. The GFP tag fused to the C-terminus of the VP22 truncation mutant may contribute some steric hindrance, which affects interaction between VP22.1-225 and VP16. VP16 did coimmunoprecipitate with VP22.1-270 in which the first 270 amino acids of VP22 are fused to GFP (Fig. 4.2E).
Figure 4.2. Coimmunoprecipitation of N-Terminal and C-Terminal VP22 Truncation Mutants with VP16. (A) N-terminal and C-terminal truncation mutants of VP22 fused to GFP. A schematic representation of full-length, N-terminal and C-terminal truncated forms of VP22 fused to the N-terminus of the GFP protein. (B) and (D) Expression of VP22 N-terminal and C-terminal truncation mutants respectively, in transfected/infected cells. Vero cells expressing GFP, VP22-GFP constructs or mock transfected cells (Mock) were infected with HSV-1, and lysed 10 h post-infection with NP-40 lysis buffer. A fraction of each cell lysate was analyzed by Western blotting using a goat polyclonal antibody specific for GFP. (C) and (E) Coimmunoprecipitation of VP16 with N-terminal and C-terminal truncation mutants of VP22, respectively. The remainder of the transfected/infected cell lysate was incubated with a goat polyclonal antibody against GFP and the resulting antibody-antigen complexes were collected with protein G-agarose beads. After extensive washes with lysis buffer, material that immunoprecipitated with anti-GFP antibody was separated by SDS-PAGE and transferred to nitrocellulose. Coimmunoprecipitated VP16 was detected by immunoblot using a rabbit monospecific polyclonal antibody raised against a C-terminal peptide of VP16. The positions of molecular mass markers (in kilodaltons) are indicated on the left.
Collectively, the immunoprecipitation assay in transfected/infected cells suggests that amino acids 165-270 of VP22 facilitate interaction with VP16.

To clarify whether residues 165-225 or 165-270 are indeed sufficient to facilitate interaction with VP16, these two peptides and VP22-derivatives lacking each of these segments were fused to GFP for analysis in the coimmunoprecipitation assay (Fig. 4.3A). Western blot analysis with anti-GFP antibodies indicated that the VP22 interaction domain mutants were expressed in transfected/infected cells and migrated with the expected molecular masses (Fig. 4.3B). Analysis of the mutants for their ability to interact with VP16 in the immunoprecipitation-Western assay demonstrated that upon deletion of either domain (165-225 or 165-270) from VP22, VP16 failed to coimmunoprecipitate (Fig. 4.3C). In contrast, amino acids 165-270 fused to GFP did coimmunoprecipitate VP16 at levels similar to wild-type VP22. When amino acids 165-225 of VP22 fused to GFP were expressed in the transfected/infected cell system, VP16 was reproducibly detected, although at reduced levels when compared to the wild-type VP22 construct or VP22.165-270 GFP. Overall, these findings suggest that residues 165-225 of VP22, whether fused to GST or GFP, are unable to facilitate optimal binding with VP16 as compared to the wild-type VP22 construct.

**The VP16 interaction domain of VP22 can bind to VP16 in the absence of additional viral proteins.** The GST pulldown experiments and coimmunoprecipitation studies were performed with infected cell lysates and transfected/infected cell lysates respectively. Thus, in addition to our mutant constructs, virally encoded wild-type VP22 is present in both experimental systems. Recent studies have suggested that VP22 may possess the ability to multimerize (481,718). Therefore wild-type VP22 could
Figure 4.3. Immunoprecipitation-Western Analysis of the VP22 Domain that Facilitates Interaction with VP16. (A) VP16 interaction domain mutants of VP22 fused to GFP. Schematic representation of VP22, deletion of the VP16 interaction domain of VP22 (as indicated by immunoprecipitation-Western), and the interaction domain alone fused to the N-terminus of the GFP protein. Also represented, deletion of the VP16 interaction domain of VP22 (as indicated by GST pulldown), and this interaction domain alone fused to GFP. Expression of VP22 truncation mutants in transfected/infected cells (B) and their ability to coimmunoprecipitate with VP16 (C) were analyzed as described in the legend to Fig. 4.2.
theoretically act as a bridge between VP16 and VP22 truncation mutants under study.

To examine whether the VP22-GFP mutants used in our VP16 interaction studies are capable of multimerizing with a wild-type VP22 construct, we utilized an immunoprecipitation-Western assay. Vero cells were cotransfected with a hemagglutinin (HA) tagged wild-type VP22 construct (VP22-HA) and the VP22-GFP mutant constructs represented in Fig. 4.3A. At 24 h post-transfection, cells were lysed with NP-40 lysis buffer and a fraction of each cell lysate was analyzed by Western blotting to verify that VP22-HA and the GFP-tagged mutants were expressed in transfected cells (Fig. 4.4A). The remaining lysates were then incubated with goat anti-GFP antibodies followed by protein G-agarose beads. Immunoprecipitated material was separated by SDS-PAGE and analyzed by Western blotting using rabbit anti-HA antibodies to assay for immunoprecipitation of VP22-HA with GFP-tagged VP22 mutants (Fig. 4.4B). Analysis of the mutants for their ability to interact with VP22-HA demonstrated that although VP22-GFP is capable of multimerization with HA-tagged VP22 (Fig. 4.4B), deletion of residues 165-225 or 165-270 from VP22 abrogated interaction (Fig. 4.4B). When amino acids 165-225 of VP22 fused to GFP were assayed in the system, VP22-HA failed to be detected, suggesting that this region of VP22 which binds to VP16 weakly, is unable to multimerize. In contrast, VP22.165-270 was able to interact with the HA-tagged VP22 construct (Fig. 4.4B). These experiments suggest that residues 165-270 of VP22 are capable of multimerizing with a wild-type VP22 construct.

In light of these findings, we examined the interaction of VP22 and VP16 in the absence of infection. Thus, removing the possibility that virally encoded VP22 may act
Figure 4.4. Characterization of the Ability of VP16 Binding Domains of VP22 to Interact with the Wild-Type Protein. (A) Expression of VP22-HA and the various VP22-GFP mutant constructs represented in Fig. 4.3A in cotransfected Vero cells. Vero cells expressing VP22-HA and either GFP, VP22-GFP constructs, or a mock transfected control (Mock) were lysed with NP-40 lysis buffer. A fraction of each cell lysate was analyzed by Western blotting using a goat polyclonal antibody specific for GFP and a rabbit polyclonal antibody raised against the HA epitope. (B) Coimmunoprecipitation of VP22-HA with VP22-GFP mutant constructs. The remainder of the cotransfected cell lysate was incubated with a goat polyclonal antibody against GFP and the resulting antibody-antigen complexes were collected with protein G-agarose beads. After extensive washes with lysis buffer, material that immunoprecipitated with anti-GFP antibody was separated by SDS-PAGE and transferred to nitrocellulose. Coimmunoprecipitated VP22-HA was detected by immunoblot using a rabbit monospecific polyclonal antibody raised against the HA epitope. The positions of molecular mass markers (in kilodaltons) are indicated on the left.
as a bridge between residues 165-270 of VP22 and VP16. This methodology enables us to confirm that we have indeed identified the minimal domain of VP22 responsible for interaction with VP16. Vero cells were transfected with plasmids encoding GFP alone or the various mutants of the VP16 interaction domain of VP22. At 20 h post-transfection, cells were lysed with NP-40 lysis buffer and a fraction of each cell lysate was analyzed by Western blotting to verify that the GFP-tagged constructs were expressed (Fig. 4.5A). The remaining lysates were then incubated with either GST or GST-VP16 bound to glutathione-Sepharose beads. After a 3-h incubation, beads were washed extensively with lysis buffer and bound material was separated by SDS-PAGE and then analyzed by Western blotting. VP22 fused to GFP was efficiently pulled-down from transfected cell lysates, indicating that the interaction can occur in the absence of additional viral proteins (Fig. 4.5B and Fig. 4.5C). Deletion of either domain (165-225 or 165-270) from VP22 reduced binding to GST-VP16 to background levels. When residues 165-270 of VP22 were assayed in this system, this region bound to GST-VP16 at enhanced levels (Fig. 4.5B and Fig. 4.5C). In contrast, residues 165-225 although capable of binding to VP16, did so at a reduced efficiency (approximately 30% of wild-type VP22). Collectively, these results suggest that residues 165-225 of VP22 are sufficient to facilitate binding to VP16 in the absence of additional virally encoded proteins; however, in order to attain wild-type levels of binding, residues 165-270 of VP22 are required.

Residues 165-225 of VP22 fail to bind to the cytoplasmic tail of gE. Previous studies have shown that residues 165-270 of VP22, which facilitate wild-type levels of binding to VP16, are also sufficient to facilitate binding to the cytoplasmic tail of gE [(514); Chapter III]. However, the ability of the VP16 interaction domain of VP22
Figure 4.5. Analysis of the VP22 Domains that Facilitate Interaction with VP16 in a GST Pulldown Assay. (A) Expression of the VP22 truncation mutants represented in Fig. 4.3A, in transfected cells. Vero cells were transfected with the indicated constructs. At 20 h post-transfection, the monolayers were lysed with NP-40 lysis buffer. A fraction of each cell lysate was analyzed for expression of VP22-GFP fusion proteins by Western blotting using a rabbit polyclonal antibody specific for GFP. (B) GST pulldown from transfected cell lysates using GST-VP16. The GST fusion proteins were expressed in Escherichia coli cells, purified on glutathione-Sepharose beads, and approximately equal amounts of each were added to the remainder of the transfected cell lysates. Beads were washed extensively with lysis buffer and bound proteins were separated by SDS-PAGE and transferred to nitrocellulose. Western blot analysis was performed using a rabbit polyclonal antibody raised against the GFP protein. The positions of molecular mass markers (in kilodaltons) are indicated on the left. (C) Binding efficiency of VP22 truncation mutants to VP16. Using densitometry, binding efficiency was quantitated by dividing the amount of VP22-GFP protein detected in the pulldown assay (normalized for the amount of GST-VP16 present) by the amount in the cell lysate (normalized for the amount of actin present). In each experiment, the wild-type VP22-GFP construct was set at 100% binding efficiency. Error bars represent standard deviations for four replicate experiments.
A.

B.

C.

% of wild-type VP22

GFP  VP22  Δ165-225  165-225  Δ165-270  165-270
encompassing amino acids 165-225 to bind to gE, has not been evaluated. To examine this activity, we exploited a modified version of the GST pulldown assay described above, using the cytoplasmic tail of gE fused to the C-terminus of GST (GST-gECT) as bait rather than VP16. Vero cells were transfected with plasmids encoding GFP alone or the various mutants of the VP16 interaction domain of VP22 and a fraction of each cell lysate was analyzed by Western blotting to verify that the GFP-tagged constructs were expressed (Fig. 4.6A). The remaining lysates were analyzed in the GST pulldown assay for their ability to bind to GST-gECT, with bound material separated by SDS-PAGE and then analyzed by Western blotting (Fig. 4.6B).

As we observed in a previous study, both VP22 fused to GFP and VP22.165-270 bound efficiently to the cytoplasmic tail of gE [(514); Chapter III], with the latter construct binding to levels approximately 85% of wild-type (Fig. 4.6B and Fig. 4.6C). In contrast, deletion of either VP16 binding domain (165-225 or 165-270) from VP22 resulted in abrogation of binding to GST-gECT (Fig. 4.6B and Fig. 4.6C). Similarly, VP22.165-225, although capable of binding to VP16, failed to bind to the cytoplasmic tail of gE at levels greater than background (Fig. 4.6B and Fig. 4.6C). These results suggest that although capable of facilitating binding to VP16, residues 165-225 of VP22 fail to bind to the cytoplasmic tail of gE.

**Membrane association of the VP16 interaction domain of VP22.** VP22 is known to partition with the cellular membrane fraction in the absence of additional HSV-1 proteins (66). We were curious to determine if the membrane association activity mapped to the same region of VP22 that facilitates VP16 and gE binding, and whether membrane association is sufficient to facilitate virion incorporation or if additional
Figure 4.6. Characterization of the Ability of VP22 Domains that Facilitate Interaction with VP16 to Bind to the Cytoplasmic Tail of gE in a GST Pulldown Assay. (A) Expression of the VP22 truncation mutants represented in Fig. 4.3A, in transfected cells. Vero cells were transfected with the indicated constructs and 20 h post-transfection, the monolayers were lysed with NP-40 lysis buffer. A fraction of each cell lysate was analyzed for expression of VP22-GFP fusion proteins by Western blotting using a rabbit polyclonal antibody specific for GFP. (B) GST pulldown from transfected cell lysates using GST-gECT. The GST fusion proteins were expressed in *Escherichia coli* cells, purified on glutathione-Sepharose beads, and approximately equal amounts of each were added to the remainder of the transfected cell lysates. Beads were washed extensively with lysis buffer and bound proteins were separated by SDS-PAGE and transferred to nitrocellulose. Western blot analysis was performed using a rabbit polyclonal antibody raised against the GFP protein. The positions of molecular mass markers (in kilodaltons) are indicated on the left. (C) Binding efficiency of VP22 mutants to the cytoplasmic tail of gE. Using densitometry, binding efficiency was quantititated by dividing the amount of VP22-GFP protein detected in the pulldown assay (normalized for the amount of GST-gECT present) by the amount in the cell lysate (normalized for the amount of actin present). In each experiment, the wild-type VP22-GFP construct was set at 100% binding efficiency. Error bars represent standard deviations for four replicate experiments.
protein-protein interactions are required. To examine the membrane association of the interaction domains of VP22, we utilized a membrane flotation assay. Vero cells were transfected with plasmids encoding GFP alone or the various mutants of the VP16 interaction domain of VP22. At 24 h post-transfection, monolayers were harvested and resuspended in hypotonic buffer. After mechanical lysis and clarification, the resulting supernatants were added to 85% (wt/vol) sucrose to yield a final sucrose concentration of 72%. A discontinuous gradient was formed by overlaying this mixture with 65% and 10% sucrose. Due to the buoyant density of membranes, ultracentrifugation results in flotation of membrane-associated proteins to the interface between the 65% and 10% sucrose layers. Twelve fractions were collected from the bottom of the tube and proteins were separated by SDS-PAGE and analyzed by Western blotting. GFP failed to associate with membranes in this assay and was found predominantly in fractions 1 through 3 where one would expect non-membrane associated proteins to migrate (Fig. 4.7). In contrast, VP22-GFP was detected in fractions 10 and 11 (corresponding to the 65%-10% interface) where membrane associated proteins are found (Fig. 4.7). Deletion of either domain (165-225 or 165-270) from VP22 abrogated membrane association with both mutant proteins detected solely in fractions 1 through 3. However, when residues 165-270 of VP22 were assayed in this system, this region associated with membranes at levels similar to that seen with the full-length construct (Fig. 4.7). Interestingly, when VP22.165-225 was examined in the flotation assay, it exhibited a higher level of membrane association when compared to the wild-type VP22 construct (compare protein levels in fraction 10 versus fraction 1 through 3, Fig. 4.7). Collectively, these results suggest that the membrane association activity of VP22 does overlap with both VP16 and
Figure 4.7. Membrane Flotation of VP22 Domains that Facilitate Interaction with VP16 and gE. Vero cells were transfected with the VP22 mutant constructs represented in Fig. 4.3A. At 24 h post-transfection, the monolayers were scraped into PBS, washed and resuspended in hypotonic buffer. Swollen cells were disrupted by passage through a 25 gauge needle and nuclei were removed by low-speed centrifugation. The resulting supernatants were added to 85% (wt/vol) sucrose in NTE buffer to yield a final sucrose concentration of 72%. A discontinuous gradient was formed by overlaying this mixture with 65% sucrose followed by 10% sucrose. After centrifugation at 100,000 x g for 18 h at 4 °C in a Beckman SW41 rotor, twelve fractions were collected from the bottom of the tube. After TCA precipitation of each fraction, proteins were separated by SDS-PAGE and transferred to nitrocellulose. GFP-tagged proteins were detected by Western blotting using a goat polyclonal antibody raised against the GFP protein. Fractions 1-3 contain non-membrane associated proteins, whereas fractions 10 and 11 correspond to the 65-10% sucrose interface and contain cellular membranes.
gE binding domains of VP22, with the highest level of membrane association observed with residues 165-225 of VP22, which are unable to bind to gE (Fig 4.6 B and Fig. 4.6C) and facilitate only moderate levels of VP16 binding (30% of wild-type VP22).

**Role of VP16 binding and membrane association in packaging of VP22 into assembling virions.** Recent studies have reported a correlation between incorporation of VP22 into virus particles and VP16 binding (280). Thus, it was of interest to ascertain whether the domain of VP22 that facilitates interaction with VP16 was packaged into assembling virus particles. To examine this possibility, we utilized a transfection/infection-based packaging assay. Vero cells were transfected with plasmids encoding various VP22-GFP fusion proteins and subsequently infected with either wild-type HSV-1 (Fig. 4.9), or a VP22-null virus, UL49− (a kind gift from Dr. Joel Baines, Cornell University, Ithaca) (Fig. 4.8) to eliminate any contribution that VP22 multimerization may play in the incorporation of VP22 truncation mutants under study. At 18 h post-infection, extracellular virions were harvested and pelleted through a sucrose cushion. The pelleted virions were analyzed by Western blotting using GFP-specific antisera to detect VP22-GFP fusion proteins. To confirm that approximately equal amounts of virus were loaded with each sample, Western blots were stripped and reprobed for either the major tegument protein VP16 (wild-type infection) or the major capsid protein VP5 (UL49− infection).

Expression analysis of the various VP22-GFP constructs within transfected/infected cells showed that each fusion protein was expressed and migrated at the expected molecular weight (Fig. 4.8A and Fig. 4.9A). With regard to packaging with the VP22-null mutant, wild-type VP22-GFP was incorporated into virus particles,
whereas GFP alone was virtually undetectable, despite high expression in transfected/infected cells, indicating that GFP itself does not have a significant effect on packaging (Fig. 4.8B and Fig. 4.8C). Furthermore, none of the VP22-GFP mutant proteins were detected in media from transfected/mock-infected cells, and many transfected constructs failed to be packaged upon infection indicating that incorporation is in fact a specific event and not due to aggregates that can pellet through the sucrose cushion (Fig. 4.8B, Fig. 4.8C and data not shown). We found that deletion of residues 165-225 of VP22 reduced packaging to background levels, however upon deletion of the 165-270 interaction domain, the resulting mutant was packaged at extremely low (approximately 8% of wild-type VP22) but reproducible levels (Fig. 4.8B and 4.8C). A VP22 virion packaging signal has been described in the C-terminus of VP22 which may facilitate gD binding (personal communication from Dr. John Blaho, Mount Sinai School of Medicine, New York). This signal is contained in VP22.Δ165-270 and may account for the low levels of incorporation observed with this mutant. Curiously, VP22.Δ165-225 which also contains the putative virion packaging signal, failed to be packaged at similar levels to VP22.Δ165-270, an observation that may be attributable to differential folding between the two mutants.

VP22.165-225, which binds to VP16 albeit at reduced levels compared to wild-type VP22 and associates with cellular membranes, failed to be packaged into virions at detectable levels. However, residues 165-270 of VP22, which are sufficient for optimal binding to VP16 and also associate with cellular membranes and bind to the cytoplasmic tail of gE, did facilitate virion incorporation to levels approaching 54% of the wild-type
Figure 4.8. Virion Incorporation of the VP22 Domains that Facilitate Interaction with VP16 in the Absence of Virally Encoded VP22. Vero cells were transfected with the indicated VP22-GFP constructs, and 20 h later, they were infected with a VP22-null virus (U<sub>L49</sub>). After an additional 18-h incubation, cell lysates were prepared (A) and virions were collected from the media by centrifugation through a 30% sucrose cushion (B). Cell lysates and extracellular virus were separated by SDS-PAGE and transferred to nitrocellulose. Western blot analysis was performed using a rabbit polyclonal antibody specific for GFP. As a loading control, the blot was stripped and reprobed with a rabbit polyclonal antibody raised against the HSV-1 major capsid protein VP5. The positions of molecular mass markers (in kilodaltons) are indicated on the left. (C) Packaging efficiency. Using densitometry, packaging efficiency was quantitated by dividing the amount of VP22-GFP protein detected in extracellular virus particles (normalized for VP5) by the amount in the cell lysate (normalized for VP5). In each experiment, the wild-type VP22-GFP construct was set at 100% packaging efficiency. Error bars represent standard deviations for four replicate experiments.
Figure 4.9. Virion Incorporation of the VP22 Domains that Facilitate Interaction with VP16 in the Presence of a Wild-Type HSV-1 Infection. Expression of the indicated VP22-GFP constructs in transfected cells which were subsequently infected with HSV-1 KOS (A) and their incorporation into virus particles (B) were analyzed as described in the legend to Fig. 4.8. The positions of molecular mass markers (in kilodaltons) are indicated on the left. (C) Packaging efficiency was calculated as described in the legend to Fig. 4.8C, using the major tegument protein VP16 as a loading control rather than VP5. Error bars represent standard deviations for four replicate experiments.
VP22-GFP construct (Fig. 4.8B and Fig. 4.8C). Interestingly, when these experiments were repeated with wild-type HSV-1 rather than a VP22-null virus, despite the mutant constructs differential binding to wild-type VP22 (Fig. 4.4B), the relative levels of incorporation remained the same (Fig. 4.9B and Fig. 4.9C). This observation indicates that full-length VP22 does not facilitate incorporation of truncated VP22 constructs into the virus particle. These results suggest that the ability to associate with cellular membranes is not sufficient to facilitate virion incorporation and that additional protein-protein interactions (VP16 binding, at least to wild-type levels, and gE binding but not VP22 multimerization) may be an important determinant for incorporation of VP22 into virions.

**Truncation mutagenesis of the domain of VP22 that facilitates binding to both VP16 and gE.** Previous studies have demonstrated that the gE binding domain of VP22 also maps to residues 165–270 [(514); Chapter III]. Residues 165-225 of VP22, which are unable to bind to the cytoplasmic tail of gE (Fig. 4.6B and Fig. 4.6C), failed to be packaged into assembling virus particles despite their ability to bind to VP16. Thus, conceivably both binding activities may be required to facilitate VP22’s incorporation.

In an attempt to identify and possibly separate the two discrete activities contained within this domain, and to define the contribution of VP16 and gE binding to VP22’s incorporation, a series of N-terminal and C-terminal truncations of the 165-270 domain of VP22 were created (Fig. 4.10A). The truncation mutants were evaluated for their ability to bind to VP16 in immunoprecipitation-Western assays from transfected/infected cell lysates as described above. Despite high levels of expression of the truncation mutants in transfected/infected cells (Fig. 4.10B), VP16 only
Figure 4.10. Immunoprecipitation-Western Analysis of Truncation Mutants of the gE Binding Domain of VP22. (A) Truncation mutants of the gE binding domain of VP22 fused to GFP. Schematic representation of VP22, deletion of the gE interaction domain of VP22, the interaction domain alone, and N-terminal and C-terminal truncations of the gE interaction domain fused to the N-terminus of the GFP protein. Expression of truncation mutants of the gE binding domain of VP22 in transfected/infected cells (B) and their ability to coimmunoprecipitate with VP16 (C) were analyzed as described in the legend to Fig. 4.2.
coimmunoprecipitated with the full-length VP22-GFP fusion protein (Fig. 4.10C). The C-terminal truncations VP22.165-255 and VP22.165-240 both contain the VP16 binding domain of VP22 (residues 165-225) that facilitated moderate binding to VP16 (~30% of wild-type levels). Curiously, no detectable VP16 binding was found with either of these mutants (Fig. 4.10B).

To determine whether truncation of the 165-270 domain of VP22 would also have a deleterious effect on VP22’s interaction with gE, we employed a similar strategy to that described above, using a purified GST-gECT fusion protein to pulldown GFP-tagged truncation mutants from transfected cells. The transfected lysates demonstrated high levels of expression of the indicated GFP-tagged constructs (Fig. 4.11A); however, as seen with VP16 binding, even the smallest truncation abrogated interaction with gE (Fig 4.11B and Fig. 4.11C). Perhaps unsurprisingly, given the loss of both VP16 and gE binding, the truncation mutants of VP22.165-270 also failed to be packaged into assembling virus particles despite high expression levels (Fig. 4.12). Collectively these experiments suggest that the region of VP22 that facilitates VP16 and gE binding (residues 165-270) may be sensitive to truncation. Even minor deletions of either N-terminal or C-terminal residues abrogated both binding activities and virion incorporation, perhaps indicating a role for the structure of this domain in its functionality.

**VP22 dileucine motif mutants fail to bind to VP16.** Primary structural alignment reveals that residues 165-270 of HSV-1 VP22 are highly conserved among VP22 homologues of herpesviruses (Fig. 4.13A). In a further attempt to uncouple the two binding activities, a more subtle approach of targeting conserved residues for site-
Figure 4.11. Characterization of the Ability of Truncation Mutants of the gE Binding Domain of VP22 to Interact with the Cytoplasmic Tail of gE in a GST Pulldown Assay. Expression of truncation mutants of the gE binding domain of VP22 represented in Fig. 4.10A in transfected cells (A) their ability to bind to the cytoplasmic tail of gE (B) and binding efficiency (C) were analyzed as described in the legend to Fig. 4.6. The positions of molecular mass markers (in kilodaltons) are indicated on the left. Error bars represent standard deviations for four replicate experiments.
Figure 4.12. Virion Incorporation of Truncation Mutants of the gE Binding Domain of VP22 in the Presence of a Wild-Type HSV-1 Infection. Expression of truncation mutants of the gE binding domain of VP22 in transfected cells which were subsequently infected with HSV-1 KOS (A) and their incorporation into virus particles (B) were analyzed as described in the legend to Fig. 4.9. The positions of molecular mass markers (in kilodaltons) are indicated on the left. (C) Packaging efficiency was calculated as described in the legend to Fig. 4.9C. Error bars represent standard deviations for four replicate experiments.
directed point mutagenesis was utilized. Two conserved “dileucine motifs” were the initial targets for mutagenesis. These residues were chosen because dileucine motifs function as binding sites for a variety of proteins (288,407). The residues in question were mutated to alanines in the context of full-length VP22 fused to the N-terminus of GFP, creating the constructs LL (-), LI (-) and LL (-)/ LI (-) (Fig. 4.13B).

Each of these mutants was examined for their ability to bind to VP16 in both the presence and absence of infection using the previously described coimmunoprecipitation and GST-VP16 pulldown assays, respectively. Initial localization studies demonstrated that the dileucine motif mutants display a subcellular localization reminiscent of wild-type VP22 in transfected/infected cells (Fig. 4.13C), suggesting that failure of any mutant to bind VP16/gE or to be incorporated into virus particles is not due to a gross mislocalization within the cell.

Expression analysis of the various dileucine motif mutants within transfected/infected (Fig. 4.14A) and transfected only cells (Fig. 4.14C) showed that each was expressed at levels similar to wild-type VP22, and migrated at the expected molecular weight. With regards to VP16 binding, VP16 coimmunoprecipitated efficiently with wild-type VP22, but mutation of either dileucine motif found in VP22 abrogated binding (Fig. 4.14B). A similar result was observed in the absence of infection; VP22 fused to GFP was efficiently pulled-down from transfected cell lysates, whereas LL (-), LI (-) or LL (-)/ LI (-) failed to bind to GST-VP16 (Fig. 4.14D and Fig. 4.14E). These results suggest that mutation of either VP22 dileucine motif to alanine residues disrupts interaction with VP16.
Figure 4.13. Analysis of the VP22 Sequence. (A) Sequence alignment of VP22 homologues. The alignment of alphaherpesvirus VP22 homologues is shown, which includes that of herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), pseudorabies virus (PRV), bovine herpesvirus-1 (BHV-1), equine herpesvirus-4 (EHV-4), varicella-zoster virus-Dumas (VZV-D), and Marek’s disease virus-Georgia (MDV-GA). Blue text indicates identity and green text denotes similarity. The conserved “dileucine” motifs are boxed. (B) Dileucine motif mutants of VP22 fused to GFP. Schematic representation of wild-type VP22, and VP22 amino acid substitution mutants used in this study, fused to the N-terminus of the GFP protein. (C) Localization of VP22 dileucine motif mutants. Vero cells were transfected with the indicated VP22-GFP constructs, and 20 h later, they were infected with HSV-1. At 18 h post-infection, (38 h post-transfection), cells were fixed with paraformaldehyde and examined by confocal microscopy with the appropriate wavelength to excite GFP and DAPI.
A.

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B.

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- 235/236  251/252  301

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GFP  VP22  VP22.LL (-)  VP22.LI (-)  VP22.LL (-) / Li (-)
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C.

- GFP
- VP22
Figure 4.14. Characterization of the Ability of VP22 Dileucine Motif Mutants to Bind VP16. Expression of VP22 dileucine motif mutants in transfected/infected cells (A) and their ability to coimmunoprecipitate with VP16 (B) were analyzed as described in the legend to Fig. 4.2. Expression of VP22 dileucine motif mutants in transfected cells (C), their ability to bind to GST-VP16 (D), and binding efficiency (E) were analyzed as described in the legend to Fig. 4.5. The positions of molecular mass markers (in kilodaltons) are indicated on the left. Error bars represent standard deviations for four replicate experiments.
The cytoplasmic tail of gE binds to the VP22 dileucine motif mutant LI (-).

Since mutation of VP22’s dileucine motifs abrogated VP16 binding, their ability to bind to the cytoplasmic tail of gE was examined. Again, a purified GST-gECT fusion protein was used to pulldown the VP22 dileucine motifs mutants from transfected cells. Briefly, Vero cells were transfected with plasmids encoding GFP or the VP22 dileucine motif mutants and a fraction of each cell lysate was analyzed by Western blotting to verify that the GFP-tagged constructs were expressed (Fig. 4.15A). The remaining lysates were analyzed in the GST pulldown assay for their ability to bind to GST-gECT, with bound material separated by SDS-PAGE and then analyzed by Western blotting (Fig. 4.15B).

As we observed previously, VP22 fused to GFP bound efficiently to the cytoplasmic tail of gE (514). In contrast, LL (-) and LL (-)/ LI (-) both failed to bind to GST-gECT (Fig. 4.15B and Fig. 4.15C). Interestingly, mutation of the second dileucine motif in VP22 (LI) did not abrogate interaction with gE, although binding was noticeably reduced (approximately 33% of wild-type levels) when compared to the VP22-GFP construct (Fig. 4.15B and Fig. 4.15C).

Collectively, our analysis of the VP22 dileucine motif mutants suggests that VP16 and gE binding activities that map to residues 165-270 of VP22 can be separated by mutation of the second dileucine motif to alanines. This mutation results in a construct that fails to bind to VP16 but does retain the ability to interact with the cytoplasmic tail of gE, albeit at reduced levels when compared to wild-type VP22.

**Virion incorporation of VP22 dileucine motif mutants.** With the identification of a VP22 mutant that fails to bind VP16, we set out to determine the contribution of VP16 binding to VP22’s incorporation into virions. We utilized the
**Figure 4.15. Characterization of the Ability of VP22 Dileucine Motif Mutants to Bind to the Cytoplasmic Tail of gE in a GST Pulldown Assay.** Expression of VP22 dileucine motif mutants in transfected cells (A), their ability to bind to the cytoplasmic tail of gE (B), and binding efficiency (C) were analyzed as described in the legend to Fig. 4.6. The positions of molecular mass markers (in kilodaltons) are indicated on the left. Error bars represent standard deviations for four replicate experiments.
transfection/infection-based packaging assay described above using either a VP22-null virus (U\textsubscript{L49}−) (Fig. 4.16) or wild-type HSV-1 (Fig. 4.17). Expression analysis of the various VP22-GFP constructs within transfected/infected cells showed that the dileucine motif mutants were expressed at levels similar to wild type VP22 and migrated at the expected molecular weight (Fig. 4.16A and Fig. 4.17A). With regard to packaging, similar results were seen with both the VP22-null virus and wild-type HSV-1. Wild-type VP22-GFP was incorporated into virus particles, whereas GFP alone was undetectable, despite high expression in transfected/infected cells (Fig. 4.16B and Fig. 4.16C [U\textsubscript{L49}−]) and (Fig. 4.17B and Fig. 4.17C [HSV-1]). Mutation of the LL dileucine motif of VP22 alone [VP22.LL (−)], or in combination with the LI motif [VP22.LL (−) / LI (−)] resulted in constructs that failed to be incorporated to any significant level (Fig. 4.16B and Fig. 4.16C [U\textsubscript{L49}−]) and (Fig. 4.17B and Fig. 4.17C [HSV-1]). However, the VP22.LI (−) construct (which interacts with gE but fails to bind to VP16), was incorporated into virus particles albeit at levels below wild-type VP22 (approximately 37% with U\textsubscript{L49}− and 33% with HSV-1). This reduced level of packaging may be a result of the impaired ability of this mutant to bind to the cytoplasmic tail of gE (Fig. 4.15B and Fig. 4.15C). Collectively, these results suggest that VP16 binding is not required for incorporation of VP22 into assembling virus particles, and that the determinant contained within residues 165-270 that facilitates VP22 incorporation, may in fact be gE binding.
Figure 4.16. Virion Incorporation of VP22 Dileucine Motif Mutants in the Absence of Virally Encoded VP22. Expression of VP22 dileucine motif mutants in transfected/infected cells (A) and their incorporation into virus particles (B) were analyzed as described in the legend to Fig. 4.8. The positions of molecular mass markers (in kilodaltons) are indicated on the left. (C) Packaging efficiency was calculated as described in the legend to Fig. 4.8C. Error bars represent standard deviations for four replicate experiments.
Figure 4.17. Virion Incorporation of VP22 Dileucine Motif Mutants in the Presence of a Wild-Type HSV-1 Infection. Expression of the indicated VP22-GFP constructs in transfected cells which were subsequently infected with HSV-1 KOS (A) and their incorporation into virus particles (B) were analyzed as described in the legend to Fig. 4.9. The positions of molecular mass markers (in kilodaltons) are indicated on the left. (C) Packaging efficiency was calculated as described in the legend to Fig. 4.9C. Error bars represent standard deviations for four replicate experiments.
DISCUSSION

The pathway of herpesvirus tegument assembly is poorly defined. It is not clear in which compartments(s) of the cell the virus acquires its tegument proteins, and the mechanism by which tegument proteins are selectively packaged into the assembling virion remains poorly understood. It is likely that a myriad of protein-protein interactions between capsid proteins and tegument proteins, tegument proteins and tegument proteins or between tegument proteins and the cytoplasmic tails of virally encoded glycoproteins facilitate the process. The HSV-1 tegument protein VP22 is packaged into virions during final envelopment as nucleocapsids bud into TGN-derived vesicles (469). This observation suggests that interaction between VP22 and viral proteins on the cytoplasmic face of the TGN vesicle, perhaps in concert with binding to tegument proteins located on the surface of the approaching capsid, ensures the accrual of VP22 in the tegument of the assembling particle. In support of this model, VP22 is known to associate with membranes and bind to the cytoplasmic tails of both gD and gE (66,109,514). PrV VP22 harbors a region homologous to the gE binding domain of HSV-1 VP22 that facilitates interaction of VP22 with the cytoplasmic tails of gE and gM, and VP22 fails to be incorporated into virions which do not express both glycoproteins (221). VP22 also binds to VP16, an abundant tegument protein that has been crosslinked to the cytoplasmic tails of gB, gD and gH (783) and has been shown to bind to the cytoplasmic tail of gH (270). In addition, VP16 is added to the nucleocapsid prior to final envelopment and its presence on the surface of capsids undergoing secondary envelopment may facilitate VP22’s incorporation into the tegument (469).
In a previous study we demonstrated that the gE binding domain of VP22 (residues 165-270) can compete efficiently with wild-type VP22 for packaging into assembling virions [(514); Chapter III]. A recent study suggested that a similar domain of VP22 may facilitate binding to VP16, and reported a correlation between VP22’s incorporation into virus particles and VP16 binding (280). The goal of the present study was to clarify the role VP16 binding plays in the incorporation of VP22 and further elucidate the mechanism by which this tegument protein is packaged.

Using a variety of N-terminal and C-terminal truncation mutants of VP22 in both GST pulldown assays and coimmunoprecipitation studies, we have identified the minimal domain of VP22 that facilitates interaction with VP16. A region encompassing amino acids 165-225 is both necessary and sufficient to bind to VP16 when expressed in the absence of additional viral proteins, however such binding is only 30% of that seen with the wild-type construct. Upon extension of this domain to include residues 165-270, VP16 binding was enhanced to levels greater than those observed with wild-type VP22. This region of VP22 is highly conserved across the herpesviruses and conservation of sequence may indicate a conservation of structure. Thus, the enhanced binding to VP16 may be due to a more overt exposure of the binding interface than wild-type VP22 presents. Our mapping studies confirm and extend those from a previous study which indicated that the VP16 binding domain of VP22 may be located between residues 160 and 212 (280).

To elucidate the role VP16 binding may play in packaging of VP22, we proceeded to evaluate the two domains (165-225 and 165-270) in our transfection/infection-based virion incorporation assay. The domain that facilitates
binding to both VP16 and the cytoplasmic tail of gE (165-270) was packaged at levels approaching wild-type VP22 (~54%). This region of VP22 binds to VP16 at levels greater than the wild-type construct, but binding to gE occurs at levels approximately 80% of wild-type. Thus the reduced level of incorporation demonstrated by this mutant may be due to the decreased efficiency of gE binding. Residues 165-225 (which bind to VP16 but not gE) were not packaged to any significant level suggesting, in agreement with previous studies, that VP16 binding may not be sufficient to facilitate VP22’s incorporation (280,514).

Both interaction domains of VP22 associate with cellular membranes, with VP22.165-225 exhibiting a higher level of membrane association than either residues 165-270 of VP22 or the wild-type construct. In light of the incorporation data, these results suggest that the ability to associate with cellular membranes is not sufficient to facilitate virion incorporation and that additional protein-protein interactions (VP16 binding, at least to wild-type levels, and gE binding) may be important determinants for incorporation of VP22 into virions.

When the virion incorporation experiments were repeated using wild-type HSV-1 rather than a VP22-null virus to infect, despite the mutant constructs differential binding to wild-type VP22, the relative levels of incorporation remained the same. Thus it would appear that full-length VP22 does not facilitate incorporation of truncated VP22 constructs into the virus particle, perhaps signifying that multimerization does not play a key role in the incorporation of VP22 into the tegument region of assembling virus particles.
Curiously, upon deletion of residues 165-270 of VP22, the resulting mutant was packaged into virus particles at extremely low (approximately 8% of wild-type VP22) but reproducible levels. It has been suggested that the C-terminus of VP22 may contain a packaging determinant of VP22 (personal communication from Dr. John Blaho, Mount Sinai School of Medicine, New York) (280). Such a packaging signal may represent the domain of VP22 that facilitates interaction with the cytoplasmic tail of gD (109). Perhaps this signal is contained within VP22Δ165-270 and may account for the low levels of incorporation seen with this mutant.

Previous studies on the virion packaging determinants of VP22 indicated that only VP22 constructs capable of binding to VP16 were packaged to significant levels (280). Using site-directed point mutagenesis of potential protein-protein interaction motifs, we were able to separate the VP16 and gE binding activities within VP22. Despite abrogation of VP16 binding with the construct VP22.LI (-), packaging into assembling virus particles still occurred, albeit at reduced levels. It is unclear whether the observed reduction is a result of the inability of the LI (-) mutant to bind to VP16 or is due to the decreased gE binding efficiency that occurs upon mutation of this motif. Nevertheless these results indicate that VP16 binding is not necessary for incorporation of VP22.

While the correlation between sub-optimal packaging and inefficient binding to the cytoplasmic tail of gE suggests an important role for gE binding in the incorporation of VP22, one cannot rule out the contribution that other activities of VP22 may play in the process. VP22 associates directly with cellular membranes and is also believed to possess the ability to multimerize (66,481). We have demonstrated that amino acids 165 to 270 of VP22 associate with cellular membranes in the absence of additional viral
proteins and based upon mapping of the multimerization domains of VP22 by Mouzakitis et al., (2005), this region (165-270) should possess the ability to multimerize. Furthermore, our results indicate that VP22.165-270 can bind to an HA-tagged wild-type VP22 construct in the absence of additional viral proteins. Thus, to attain levels of incorporation greater than those observed with VP22.165-270, it may be necessary to define a domain of VP22 that can bind to both gE and VP16 at wild-type levels.

These two binding activities may in fact act in a redundant fashion to facilitate VP22’s incorporation, perhaps in conjunction with the unidentified activity located in the C-terminal region of the protein (gD binding?). Functional redundancy, at least for virus growth in cell culture, is a common theme in herpesvirology. In HSV-1, deletion of gD or gE has little effect on assembly, however simultaneous deletion of these glycoproteins results in the accumulation of unenveloped capsids in the cytoplasm (197). Similarly, concurrent deletion of PrV’s gE and gM causes the formation of capsid-bound tegument aggregates in the cytoplasm, whereas deletion of either glycoprotein individually has little effect (58,59,221). As VP22 binds to both gD and gE in HSV-1, and to gE and gM in PrV, in addition to its VP16 binding, VP22 may act as a bridging protein, with both VP16 and a glycoprotein tail binding simultaneously (109,221,514). These interactions may facilitate budding of VP16-coated capsids into glycoprotein/tegument protein layered vesicles. However, recent studies of a HSV-1 VP22-null virus suggests that the requirement for VP22 in the assembly pathway can be bypassed (179,182). Thus, this may be only one of a plethora of protein-protein interaction networks that work redundantly to facilitate the final stages of envelopment. Perhaps the incorporation determinants of VP22 are but another example of the redundancy inherent to the process.
Identification of a domain harboring the ability to direct molecules into the tegument could represent a useful vector in the field of drug discovery and may allow the targeting of factors with the ability to disrupt HSV-1 assembly. Studies are in progress to assess the role of gE in VP22’s incorporation and to identify the minimal activities of VP22 required to achieve wild-type levels of incorporation.
MATERIALS AND METHODS

Cells and viruses. Vero cells (ATCC CCL-81) were grown in Dulbecco’s modified Eagles medium (DMEM) (GIBCO) supplemented with 10% fetal bovine serum (FBS), 2.25% sodium bicarbonate, 25 mM HEPES buffer, glutamine (300 \( \mu \text{g/ml} \)), penicillin (100 \( \mu \text{g/ml} \)), and streptomycin (131 \( \mu \text{g/ml} \)). Infected cells were grown in DMEM supplemented with 2% FBS, 25 mM HEPES buffer, glutamine (300 \( \mu \text{g/ml} \)), penicillin (100 \( \mu \text{g/ml} \)), and streptomycin (131 \( \mu \text{g/ml} \)). The viruses used in this study were the KOS strain (650) and a VP22-null virus (U_L49), a kind gift from Joel Baines (Cornell University, Ithaca, New York) (179).

Construction of VP22-GFP and VP22-HA chimeras. Plasmids encoding VP22 fused to GFP, the N-terminal and C-terminal truncations of VP22 fused to GFP and the internal deletions of VP22 (pVP22.\( \Delta \)165-225-GFP, pVP22.\( \Delta \)165-270-GFP, pVP22.165-225-GFP and pVP22.165-270-GFP) were described previously (66,514).

To make pVP22.184-270-GFP, the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) was used according to the manufacturer’s instructions. Using pVP22.165-270-GFP as a template (66,514), mutagenic primers were designed complementary to 15-bp immediately upstream of codon 165 and 15-bp immediately downstream of codon 183, essentially looping out the sequence encoding amino acids 165-183 of VP22 in pVP22.165-270-GFP; thereby creating pVP22.184-270-GFP. A similar strategy was used to create pVP22.204-270-GFP, pVP22.165-255-GFP and pVP22.165-240-GFP.

To make pVP22.LL(-)-GFP, the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) was used according to the manufacturer’s instructions. Using pVP22-GFP
as a template (Brignati et al., 2003), mutagenic primers were designed which change the nucleotide sequence at codons 235 and 236 of VP22 to encode for alanine residues rather than two leucines; thereby creating pVP22.LL(-)-GFP. A similar strategy was used to create pVP22.LI(-)-GFP and pVP22.LL(-)/LI(-)-GFP.

To make pVP22-HA, the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) was used according to the manufacturer’s instructions. Using pVP22-GFP as a template, mutagenic primers were designed which inserted a sequence encoding the HA tag (YPYDVPDYA) followed by a stop codon immediately after the nucleotide sequence of codon 301 of VP22.

All constructs were sequenced to confirm the identity of VP22 and to ensure that the gene encoding VP22 (or the mutated forms) was in frame with the gene encoding the GFP protein or the sequence that encodes the HA tag.

**Construction and purification of GST fusion proteins.** The vector encoding the cytoplasmic tail of gE fused to GST (GST-gECT) was a kind gift from David Johnson (The Oregon Health and Science University, Portland, Oregon).

The U₁₄₈ gene was PCR amplified by using Platinum Taq polymerase (Invitrogen) from the HSV-1 KOS viral genome. To construct the vector encoding GST-VP16, PCR was performed with a forward primer containing a EcoRI site inserted immediately upstream of the U₁₄₈ start codon and a reverse primer that contains a NotI site immediately downstream of the U₁₄₈ stop codon. Following amplification, the VP16-encoding DNA was digested with EcoRI and NotI and ligated into pGEX-4T-3 (Amersham Biosciences) digested with the same restriction enzymes, to produce pGST-VP16.
The UL49 gene was PCR amplified by using Platinum Taq polymerase (Invitrogen) from the plasmid pVP22-GFP. To construct the vector encoding GST-VP22, PCR was performed with a forward primer containing a BamHI site inserted immediately upstream of the UL49 start codon and a reverse primer that contains a mutation to reinsert the UL49 stop codon and a NotI site immediately downstream of the regenerated stop codon. Following amplification, the VP22-encoding DNA was digested with BamHI and NotI and ligated into pGEX-4T-3 (Amersham Biosciences) digested with the same restriction enzymes, to produce pGST-VP22.

GST-VP22 N-terminal truncation mutants were made in similar fashion. A forward primer containing an EcoRI site upstream of the site of truncation and the same reverse primer used in the construction of pGST-VP22 were used to PCR amplify the truncated UL49 gene. This product was digested with EcoRI and NotI and ligated into the pGEX-4T-3 vector digested with the same restriction enzymes, to produce pGST-VP22.AA-301, where “AA” designates the first amino acid of VP22 encoded by the mutant.

GST-VP22 C-terminal truncation mutants were constructed by using a forward primer containing an EcoRI site upstream of the UL49 start codon and a reverse primer containing a stop codon followed by a NotI site immediately downstream of the site of truncation. This product was digested with EcoRI and NotI and ligated into the pGEX-4T-3 vector digested with the same restriction enzymes, to produce pGST-VP22.1-AA, where “AA” designates the last amino acid encoded by the mutant.

To construct pGST-VP22.Δ87-120, the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) was used according to the manufacturer’s instructions.
Using pGST-VP22 as a template, mutagenic primers were designed complementary to 15-bp immediately upstream of codon 87 and 15-bp immediately downstream of codon 120, essentially looping out the sequence encoding amino acids 87-120 of VP22 in pGST-VP22; thereby creating pGST-VP22Δ87-120.

To construct pGST-VP22Δ165-225, pVP22Δ165-225-GFP was used as the source of the UL49 allele (514). The forward primer used to create the GST-VP22 C-terminal truncation mutants (containing an EcoRI site upstream of the UL49 start codon), and the reverse primer used in the construction of pGST-VP22 (containing a mutation to reinsert the UL49 stop codon and also a NotI site immediately downstream of the regenerated stop codon) were used to PCR amplify the internally-deleted gene. This product was digested with EcoRI and NotI and ligated into the multiple cloning site (MCS) of pGEX-4T-3 to produce pGST-VP22Δ165-225.

pGST-VP22.165-225 was constructed using pVP22-GFP as the template. The UL49 gene fragment was PCR amplified using a forward primer containing an EcoRI site immediately upstream of the codon for amino acid 165 of VP22 and a reverse primer containing a stop codon followed by a NotI site immediately downstream of the codon for residue 225 of VP22. This product was digested with EcoRI and NotI and ligated into the pGEX-4T-3 vector digested with the same restriction enzymes, to produce pGST-VP22.165-225. All constructs were sequenced to confirm the identity of VP16 and VP22 (or various mutant forms) and to ensure that the start codons were in frame with the gene encoding the GST protein.

To express and purify the various GST fusion proteins, plasmids encoding the respective constructs were transformed into BL21 competent cells (Stratagene) according
to the manufacturer’s instructions and overnight cultures were grown. Approximately 10 ml of these cultures were used to inoculate fresh 100 ml cultures and grown at 37 °C to 

\[ A_{600} = 0.4 \]. To induce expression, 100 µL of 1 M IPTG (GIBCO) was added and the cultures were grown at 30 °C for 3-4 h, followed by centrifugation at 10,000 x g for 10 min at 4 °C. The bacterial pellet was resuspended in 10 ml of lysis buffer (100 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1 mM CHAPS, 400 mM NaCl, 1% Triton X-100, Complete Mini protease inhibitors [Roche]). The suspension was sonicated and clarified by centrifugation at 27,000 x g for 10 min at 4 °C. A volume of 133 µl of glutathione-Sepharose 4B beads (Pharmacia) that had been washed twice with phosphate-buffered saline (PBS) was added to the supernatant and incubated overnight at 4 °C. Beads were washed thoroughly with PBS and the yield of each purified GST fusion protein was determined by SDS-PAGE and subsequent staining with Coomassie blue to detect the recombinant protein.

**GST pulldown assay.** GST fusion proteins were purified from bacterial cultures, as described above. Confluent monolayers of Vero cells grown in 60-mm plates were either infected or mock infected with the HSV-1 KOS strain at a multiplicity of infection (MOI) of 10. At 10 h post-infection, cells were washed twice with PBS and lysed with NP-40 lysis buffer (0.5% NP-40, 150 mM NaCl, 50 mM Tris-HCl [pH 8.0]) containing Complete Mini protease inhibitors. Infected cell lysates were precleared overnight at 4 °C with glutathione-Sepharose 4B beads that had been previously washed twice with PBS, and the samples were then incubated with approximately equal amounts of purified GST-fusion proteins on glutathione-Sepharose beads (as determined by Coomassie blue-stained gel) for 3 h at 4 °C. Beads were washed three times with NP-40 lysis buffer and
once with 10 mM Tris-HCl (pH 7.4). VP16 bound to GST constructs was detected by Western blotting using a rabbit monospecific polyclonal antibody raised against the HSV-1 tegument protein VP16 (Clontech), a goat anti-rabbit antibody conjugated to horseradish peroxidase (Sigma), ECL reagents (Pharmacia), and autoradiography on Kodak BioMax XAR film. To further control for the quantities of GST-fusion proteins used in the pulldown, nitrocellulose membranes were stripped (60 mM Tris-HCl [pH 8.0], 2% SDS, 0.75% β-mercaptoethanol [β-ME] for 45 min at 55 °C) and reprobed with a goat polyclonal antibody raised against the GST protein (Rockland).

To analyze the VP22-gE and VP22-VP16 interaction within transfected cells, a similar GST pulldown assay was used. Confluent monolayers of Vero cells grown in 100-mm plates were transfected with the indicated constructs by using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. At 20 h post-transfection, monolayers were washed twice with PBS and scraped into 10 ml of cold PBS. A 1 ml sample of the cell suspension was removed for analysis of expression of the VP22-GFP fusion proteins, and the remaining cells were pelleted by centrifugation (1000 x g for 5 min at 4 °C). The 9 ml samples were lysed in NP-40 lysis buffer and processed as described above. VP22-GFP fusion proteins bound to GST constructs were detected by Western blotting using a rabbit polyclonal antibody raised against the GFP protein (Santa Cruz), a goat anti-rabbit antibody conjugated to horseradish peroxidase (Sigma), ECL reagents (Pharmacia), and autoradiography on Kodak BioMax XAR film. To further control for the quantities of GST-fusion proteins used in the pulldown, nitrocellulose membranes were stripped as described above and reprobed with a goat polyclonal antibody raised against the GST protein (Rockland). To detect expression of
the VP22-GFP fusion proteins in transfected cells, proteins in the 1 ml cell suspension sample were analyzed by Western blotting using a rabbit polyclonal antibody against GFP, a goat anti-rabbit antibody conjugated to horseradish peroxidase, ECL reagents, and autoradiography on Kodak BioMax XAR film. To ensure equal loading, nitrocellulose membranes were stripped as described above and reprobed with a goat polyclonal antibody raised against actin (Santa Cruz). Using densitometry, binding efficiency was quantitated by dividing the amount of VP22-GFP protein detected in the pulldown assay (normalized for the amount of GST-VP16 or GST-gE present) by the amount in the cell lysate (normalized for the amount of actin present).

Immunoprecipitation-Western assay. Confluent monolayers of Vero cells grown in 100-mm plates were transfected with the indicated constructs by using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. At 20 h post-transfection, the monolayers were infected with HSV-1 KOS strain at a MOI of 10. At 10 h post-infection (30 h post-transfection), cells were washed twice with PBS and scraped into 10 ml of cold PBS. A 1 ml sample of the cell suspension was removed for analysis of expression of the VP22-GFP fusion proteins, and the remaining cells were pelleted by centrifugation (1000 x g for 5 min at 4 °C). The 9 ml samples were lysed in NP-40 lysis buffer containing Complete Mini protease inhibitors as described above and precleared overnight at 4 °C with protein G-agarose beads (Roche) that had been washed twice in lysis buffer. The lysates were then incubated with a goat polyclonal antibody raised against GFP (Rockland) for 1 h at 4 °C, and immune complexes were collected with protein G-agarose beads that had been washed twice with lysis buffer. Beads were washed three times with NP-40 lysis buffer and once with 10 mM Tris-HCl (pH 7.4).
Coimmunoprecipitated VP16 was detected by Western blotting using a rabbit monospecific polyclonal antibody raised against a C-terminal peptide of the HSV-1 tegument protein VP16 (Clontech), a goat anti-rabbit antibody conjugated to horseradish peroxidase, ECL reagents, and chemiluminescence autoradiography on Kodak BioMax XAR film. To detect expression of the VP22-GFP fusion proteins in transfected/infected cells, proteins in the 1 ml cell suspension sample were analyzed by Western blotting using a goat polyclonal antibody against GFP, a rabbit anti-goat antibody conjugated to horseradish peroxidase, ECL reagents, and autoradiography on Kodak BioMax XAR film.

**Multimerization assay.** Confluent monolayers of Vero cells grown in 100-mm plates were co-transfected with VP22-HA and the indicated GFP-tagged constructs by using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. At 24 h post-transfection, cells were washed twice with PBS and scraped into 10 ml of cold PBS. A 1 ml sample of the cell suspension was removed for analysis of expression of the transfected proteins, and the remaining cells were pelleted by centrifugation (1000 x g for 5 min at 4 °C). The 9 ml samples were lysed in NP-40 lysis buffer containing Complete Mini protease inhibitors as described above and precleared overnight at 4 °C with protein G-agarose beads (Roche) that had been washed twice in lysis buffer. The lysates were then incubated with a goat polyclonal antibody raised against GFP (Rockland) for 1 h at 4 °C, and immune complexes were collected with protein G-agarose beads that had been washed twice with lysis buffer. Beads were washed three times with NP-40 lysis buffer and once with 10 mM Tris-HCl (pH 7.4). Coimmunoprecipitated VP22-HA was detected by Western blotting using a rabbit polyclonal antibody raised against the hemagglutinin
epitope (Sigma), a goat anti-rabbit antibody conjugated to horseradish peroxidase, ECL reagents, and chemiluminescence autoradiography on Kodak BioMax XAR film. To detect expression of the HA and GFP-tagged VP22 fusion proteins in co-transfected cells, proteins in the 1 ml cell suspension sample were analyzed by Western blotting using anti-GFP or anti-HA antibodies, the appropriate species of secondary antibody conjugated to horseradish peroxidase, ECL reagents, and autoradiography on Kodak BioMax XAR film.

**Membrane flotation assay.** Confluent monolayers of Vero cells grown in 100-mm plates were transfected with the indicated constructs by using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. At 24 h post-transfection, monolayers were washed twice with PBS and scraped into 10 ml of cold PBS. Cells were pelleted by centrifugation (1000 x g for 5 min at 4 °C) and resuspended in hypotonic buffer (50 mM Tris HCl [pH 7.4] and 3 mM MgCl₂) supplemented with Protease Inhibitor Cocktail [Sigma]. Following incubation on ice for 15 minutes, cells were lysed by 10 passages through a 25 gauge needle. Cell lysates were subsequently centrifuged at 1000 x g for 10 min at 4 °C to remove unbroken cells and nuclei. The resulting supernatants were added to 85% (wt/vol) sucrose in NTE buffer (100 mM NaCl, 10 mM Tris, 1 mM EDTA [pH 7.4]) to yield a final sucrose concentration of 72%. A discontinuous gradient was formed by overlaying this mixture with 65% sucrose followed by 10% sucrose. This sucrose gradient was then centrifuged at 100,000 x g for 18 h at 4 °C in a Beckman SW41 rotor. Twelve 1 ml fractions were collected from the bottom of the tube and trichloroacetic acid (TCA) was added to each fraction to a final concentration of 10%. TCA precipitation was allowed to occur overnight at 4 °C and the
resulting pellets were washed in 100% ethanol, dried and then solubilized in 1X sample buffer (50 mM Tris-HCl [pH 6.8], 10% glycerol, 2% SDS, 5% β-ME). GFP-tagged proteins were detected by Western blotting using a goat polyclonal antibody raised against the GFP protein (Rockland), a rabbit anti-goat antibody conjugated to horseradish peroxidase, ECL reagents, and chemiluminescence autoradiography on Kodak BioMax XAR film.

**Virion incorporation assay.** Confluent monolayers of Vero cells grown in 100-mm plates were transfected with the indicated constructs using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. At 20 h post-transfection, cells were infected with either HSV-1 KOS or a VP22-null virus (U_{L49}^{-}) at a MOI of 10, or mock infected. At 20 h post-infection (38 h post-transfection), the medium was removed by pipetting and centrifuged at 1000 x g for 10 min at 4 °C to remove cellular debris. The supernatant was retained and extracellular virions were purified by centrifugation (115,000 x g for 1 h in a Beckman SW41 rotor) through a 30% (wt/vol) sucrose cushion (1.7 ml) in NTE buffer (100 mM NaCl, 10 mM Tris, 1 mM EDTA, [pH 7.4]). Pelleted virions and infected cells were disrupted in 1X sample buffer (50 mM Tris-HCl [pH 6.8], 10% glycerol, 2% SDS, 5% β-ME). GFP-tagged proteins were detected by Western blotting using a goat polyclonal antibody raised against the GFP protein (Rockland), a rabbit anti-goat antibody conjugated to horseradish peroxidase, ECL reagents, and chemiluminescence autoradiography on Kodak BioMax XAR film. As a loading control, nitrocellulose membranes were stripped as described above and reprobed with either a rabbit polyclonal antibody raised against the major capsid protein VP5 or a rabbit monospecific polyclonal antibody raised against a C-terminal peptide of
the HSV-1 tegument protein VP16 (Clontech). Using densitometry, packaging efficiency was quantitated by dividing the amount of VP22-GFP protein detected in extracellular virus particles by the amount in the cell lysate (both normalized for either VP5 or VP16).

Confocal microscopy. Vero cells were transfected with the indicated constructs using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. At 20 h post-transfection, cells were infected with HSV-1 KOS strain at a MOI of 10 or mock infected. At 18 h post-infection (38 h post-transfection), cells were washed twice in Tris-buffered saline (TBS), fixed with 3% paraformaldehyde in PBS and mounted on slides using SlowFade antifade reagent with DAPI (Molecular Probes). Fluorescence was observed with a Leica TSC SP2 AOBS confocal microscope using the appropriate wavelength to excite GFP and/or DAPI. Images were formatted in Adobe Photoshop 7.0.
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CHAPTER V

VIRION INCORPORATION OF THE HERPES SIMPLEX VIRUS

TYPE 1 TEGUMENT PROTEIN VP22 DOES NOT REQUIRE

INTERACTION WITH GLYCOPROTEIN E (gE) AND IS

FACILITATED BY AN ACIDIC CLUSTER OF AMINO ACIDS
ABSTRACT

Herpes simplex virus type 1 (HSV-1) virions contain a proteinaceous layer termed the tegument that lies between the nucleocapsid and viral envelope. To elucidate determinants that facilitate packaging of VP22 into the tegument, a transfection/infection based virion incorporation assay, using point mutants that discern between binding activities of VP22, was utilized. Failure to bind to gE did not abrogate virion packaging. As VP16 binding is also dispensable for incorporation, gE and VP16 binding may act redundantly to facilitate inclusion of VP22 into the virus particle. Interestingly, a region of VP22 which binds to both VP16 and gE, fails to be packaged to wild-type levels, suggesting that additional incorporation determinants exist. Wild-type levels of incorporation were attained when residues 44-86 of VP22 were restored. Mutational analysis of an acidic cluster of amino acids within this region, suggests that this motif is required to facilitate wild-type levels of VP22 virion incorporation.
INTRODUCTION

Assembly of herpes simplex virus type 1 (HSV-1) involves a intricate sequence of events, which coordinate incorporation of over 40 different viral proteins into one of three morphologically distinct virion structures: the nucleocapsid, the host-derived lipid envelope containing virally encoded glycoproteins, and the tegument, a proteinaceous region located between the nucleocapsid and envelope (601). Although, extensive studies have established that capsid assembly and packaging of the viral genome occur in the nucleus, the compartment(s) in which the tegument and envelope are acquired is less well-defined (191,455,456). The current model for HSV-1 assembly and egress suggests that nucleocapsids are exported to the cytoplasm via a budding/fusion event that occurs across the inner and outer membranes of the nucleus, respectively. Unenveloped nucleocapsids subsequently ferry through the cytoplasm until they reach a trans-Golgi network (TGN)-derived vesicle, where a budding event results in concurrent acquisition of a final lipid bilayer and complement of viral glycoproteins (236,256,258,284,456,457,613,644,710,739,741,784). Ultimately, virion-containing vesicles follow the secretory pathway to the cell surface, where mature virus particles are released into the extracellular milieu (456).

In contrast to nucleocapsid assembly, the molecular mechanisms that facilitate addition of tegument proteins to the nucleocapsid during the assembly pathway and the process of final envelopment itself are poorly understood (456,457,601,602). Tegumentation of nucleocapsids can theoretically occur at various stages in the egress pathway: in the nucleus, at the nuclear membrane, in the cytoplasm or during budding at the TGN. Recent studies have demonstrated that a subset of tegument proteins are added
to the capsid prior to nuclear egress; however, the mechanism(s) by which tegument proteins are selectively packaged into the assembling virion has yet to be defined (72,488). It is likely that a myriad of protein-protein interactions between capsid proteins, tegument proteins, and the cytoplasmic tails of virally encoded glycoproteins facilitate the process. Our studies have focused on defining the protein-protein interaction motifs of one specific HSV-1 tegument protein, VP22, and the roles these play in facilitating virion incorporation.

The HSV-1 U1.49 gene encodes the 301-amino-acid VP22, which is one of the most abundant proteins in the tegument region (189,290,391). Despite its abundance, the role of VP22 during HSV-1 assembly and mechanism of its incorporation remain undefined. Two VP22-null viruses have recently been described and demonstrate a variety of cell-specific replication defects and altered virion composition, including decreased packaging of both glycoprotein D (gD) and glycoprotein E (gE), two known binding partners of VP22 (109,179,182,514,559). VP22 associates with membranes and localizes to acidic compartments of the cell including the TGN (66). In addition to binding to cytoplasmic tails of a subset of viral glycoproteins, presumably at the TGN, VP22 is known to interact with another abundant tegument protein, VP16 (109,182,183,199,514). Transmission immunoelectron microscopy (TIEM) studies suggest that during the viral assembly pathway, detectable amounts of VP16 are added to the capsid in the nucleus, with additional VP16 added as the nucleocapsid moves through the cytoplasm prior to final envelopment (469,488). In contrast, VP22 is packaged into virions during final envelopment, as nucleocapsids bud into TGN-derived vesicles (469). These findings suggest that interaction between VP22 and viral glycoproteins on the
cytoplasmic face of the TGN vesicle, perhaps in concert with binding to tegument proteins located on the surface of the approaching capsid (such as VP16), may ensure accrual of VP22 in the tegument and may even facilitate final envelopment. However, the recent description of a HSV-1 null virus demonstrates that the requirement for VP22 in the assembly pathway can be bypassed. Thus, other protein-protein interaction networks which are capable of facilitating final envelopment must exist, possibly functioning in a redundant manner with those featuring VP22.

It is likely that virion incorporation of VP22 is facilitated by a variety of protein-protein interactions. A VP22 construct deficient for VP16 binding but competent for interaction with the cytoplasmic tail of gE is still packaged into assembling HSV-1 particles (515). Interestingly, simultaneous deletion of pseudorabies virus (PRV) gM and the gE/gI heterodimer results in capsid-bound tegument aggregates in the cytoplasm and reduced amounts of VP22 in the mature PrV particle (58,59,221). Furthermore, the Bartha strain of PrV, which lacks glycoproteins gI and gE, fails to package VP22, suggesting that binding to the cytoplasmic tail of a viral glycoprotein may be an important packaging determinant (413).

To ascertain the contribution of gE binding to virion packaging, site-directed mutagenesis was used to disrupt the VP16 and gE binding activities of VP22. Assessment of these mutants in a virion incorporation assay demonstrated that failure to bind to gE did not abrogate packaging of VP22. Wild-type levels of incorporation were only attained when residues 43-86 of VP22 were present. An acidic cluster of amino acids within this region, which is required for localization of VP22 at the TGN, appears to be an important determinant for virion packaging.
This report further elucidates the mechanism by which VP22 is incorporated into virus particles and reveals that in addition to protein-protein interactions, proper trafficking and localization of VP22 also contribute to efficient virion packaging.
RESULTS

Site-directed point mutagenesis of VP22. The gE binding activity of VP22 has been mapped to a domain encompassing residues 165 to 270, which are highly conserved among VP22 homologues of herpesviruses and also facilitate binding to VP16 [(514); Chapter III]. To ascertain the contribution of gE binding to virion incorporation of VP22, site-directed point mutagenesis was used to target conserved residues within this domain, in an attempt to abrogate gE binding while leaving the ability to bind VP16 intact.

Two tryptophan residues at positions 189 and 221 of VP22 and phenylalanine residues at amino acids 196 and 201 were the initial targets for mutagenesis (Fig. 5.1A). These targets were chosen due to their similarity to a “WW” domain, a well characterized protein-protein interaction motif. “WW” domains are typically 35 to 40 amino acids in length, characterized by two tryptophan residues that bookend aromatic residues with a proline residue located C-terminally of the second tryptophan (323). Interestingly, “WW” domain ligands include proline-rich peptide motifs and phosphorylated serine/threonine-proline sites, examples of which are found in the cytoplasmic tail of gE (285). The tryptophan residue at position 189 and/or 221 was changed conservatively to a phenylalanine, creating the VP22 constructs W189F, W221F and W189F/W221F (Fig. 5.1A). Studies suggest that aromatic residues located between the tryptophans of a “WW” domain confer ligand specificity to this protein-protein interaction motif (322,714). Thus, phenylalanines at positions 196 and 201 of VP22 were mutated to either alanines (F196A and F201A) or more conservatively, to tryptophan residues (F196W and F201W) (Fig. 5.1A). Localization studies demonstrated that all mutant constructs displayed a subcellular localization reminiscent of wild-type VP22 in transfected/infected cells (Fig.
Figure 5.1. **Site-Directed Point Mutagenesis of VP22.** (A) Point mutants of VP22 fused to GFP. Schematic representation of wild-type VP22 and amino acid substitution mutants used in this study fused to the N-terminus of the GFP protein. (B) Localization of VP22 point mutants. Vero cells were transfected with the indicated VP22-GFP constructs, and 20 h later, they were infected with HSV-1. At 18 h post-infection, (38 h post-transfection), cells were fixed with paraformaldehyde and examined by confocal microscopy with the appropriate wavelength to excite GFP.
Thus, failure of any mutant to bind VP16/gE or to be incorporated into the virus particle is not due to a gross mislocalization within the cell.

**Characterization of the ability of VP22 point mutants to bind to the cytoplasmic tail of gE.** To determine whether mutation of the conserved tryptophan or phenylalanine residues within VP22 would abrogate binding to gE, a glutathione S-transferase (GST) pulldown assay was utilized. The cytoplasmic tail of gE fused to the C-terminus of the GST protein (GST-gECT), or GST alone were expressed in *Escherichia coli* cells and subsequently purified. Approximately equal amounts of GST fusion proteins were used in the pulldown assay as determined by Coomassie-blue staining (Fig. 5.2A). The full-length GST-gECT fusion protein is indicated by an arrowhead. Various faster migrating bands were also purified from the bacterial lysates, which react with both GST and gE specific antisera, suggesting that these bands are breakdown products of the GST-gECT fusion protein. Vero cells were transfected with plasmids encoding the green fluorescent protein (GFP) or the various mutants of VP22 fused to the N-terminus of GFP represented in Fig. 5.1A. The transfected monolayers were lysed with NP-40 lysis buffer and a fraction of each cell lysate was analyzed by Western blotting to verify that the GFP-tagged constructs were expressed (Fig. 5.2B and Fig. 5.2C). The remaining lysates were incubated with equivalent amounts of purified GST fusion proteins (either the cytoplasmic tail of gE fused to GST or GST alone) bound to glutathione-Sepharose beads. The beads were subsequently washed extensively with lysis buffer, and bound material was separated by SDS-PAGE and then analyzed on Western blots.
Figure 5.2. Characterization of the Ability of VP22 Point Mutants to Bind to the Cytoplasmic Tail of gE in a GST Pulldown Assay. (A) Coomassie-blue stained gel of the cytoplasmic tail of gE fused to the C-terminus of the GST protein (GST-gECT) and GST alone. GST-gECT or GST were expressed in *Escherichia coli* cells and purified on glutathione-Sepharose beads. The arrowhead denotes the full-length GST-gECT fusion protein. (B) and (C) Expression of VP22 point mutants in transfected cells. Vero cells were transfected with the indicated constructs and 20 h post-transfection, the monolayers were lysed with NP-40 lysis buffer. Expression of VP22-GFP fusion proteins was analyzed by Western blotting using a rabbit polyclonal antibody specific for GFP. (D) and (E) GST pulldown from transfected cell lysates using GST-gECT. Approximately equal amounts of the GST fusion proteins purified in (A) were added to the remainder of the transfected-cell lysates. Beads were washed extensively with lysis buffer and bound proteins were separated by SDS-PAGE and transferred to nitrocellulose. Western blot analysis was performed using a rabbit polyclonal antibody raised against the GFP protein. The positions of molecular mass markers (in kilodaltons) are indicated on the left. (F) Binding efficiency of VP22 point mutants to the cytoplasmic tail of gE. Using densitometry, binding efficiency was quantitated by dividing the amount of VP22-GFP protein detected in the pulldown assay (normalized for the amount of GST-gECT present) by the amount in the cell lysate (normalized for the amount of actin present). In each experiment, the wild-type VP22-GFP construct was set at 100% binding efficiency. Error bars represent standard deviations for four replicate experiments.
Upon analysis of transfected lysates, each of the VP22 point mutants under study demonstrated high levels of expression (Fig. 5.2B and Fig. 5.2C). Curiously, a faster migrating band was detected with the F196A construct which was not present in lysates of other point mutants (Fig. 5.2C lane 5). The band reacted with GFP antiserum suggesting that it is GFP-tagged and thus may represent a VP22 breakdown product that is stabilized by the F196A mutation. With regards to gE binding, VP22-GFP was efficiently pulled-down from transfected-cell lysates by GST-gECT but not by the GST alone control (Fig. 5.2D and Fig. 5.2F). In contrast, W189F or W189F/W221F (Fig. 5.2D and Fig. 5.2F), and F196A, F201A or F201W (Fig. 5.2E and Fig. 5.2F) failed to bind to gE. F196W exhibited levels of binding to GST-gECT greater than those seen with wild-type VP22 (approximately 140%) (Fig. 5.2F), whereas the mutant W221F although still retaining binding activity, did so at a diminished level (54% of wild-type) (Fig. 5.2F). Taken together, these results indicate that a variety of conservative amino acid substitutions (W189F, W189F/W221F, and F201W) are capable of inhibiting VP22’s ability to bind to gE and may be useful tools in the task of deciphering the role gE binding plays in virion incorporation of VP22.

**Interaction of VP22 point mutants with VP16 in a coimmunoprecipitation assay.** With the identification of conservative amino acid substitutions capable of abrogating VP22’s binding with gE, we were anxious to test the effect of these mutations on interaction with VP16. Our hope was to separate the two binding activities within VP22. To this end, immunoprecipitation assays from transfected/infected cell lysates were performed to ascertain if the VP22 point mutants had a deleterious effect upon interaction with VP16. The VP22 constructs represented in Fig. 5.1A were transfected
into Vero cells and at 20 h post-transfection, cells were infected with HSV-1 at a MOI of 10. After an additional period of 10 h, the monolayers were lysed with NP-40 lysis buffer and a fraction of each cell lysate was analyzed by Western blotting to verify that the GFP-tagged truncation mutants were expressed in transfected/infected cells (Fig. 5.3A and Fig. 5.3B). [As noted earlier, a faster migrating band was detected with F196A which was not present in the lysates of other point mutants (Fig. 5.3B lane 4)]. The remaining lysates were then incubated with goat anti-GFP antibodies followed by Protein G-agarose beads. Immunoprecipitated material was separated by SDS-PAGE and analyzed by Western blotting using rabbit anti-VP16 antibodies to assay for immunoprecipitation of VP16 with VP22 point mutants. When the VP22 point mutants were analyzed for their ability to interact with VP16 within transfected/infected cells, VP16 coimmunoprecipitated with W221F, F196A, F196W, F201W, and the wild-type VP22 construct (Fig. 5.3C and Fig. 5.3D). W189F retained the ability to interact with VP16 (albeit poorly), however binding was abrogated with mutants F201A and W189F/W221F.

**Interaction of VP22 point mutants with VP16 in the absence of additional viral proteins.** The coimmunoprecipitation studies were performed with transfected/infected cell lysates. Thus, in addition to our mutant constructs, virally encoded VP22 is present in both experimental systems. Recent studies have suggested that VP22 may possess the ability to multimerize (481,515,718). Therefore, virally expressed VP22 could theoretically act as a bridge between VP16 and the VP22 mutants under study. As several of the VP22 point mutants assayed appeared to retain the ability to bind to VP16, we sought to examine the interaction in the absence of infection in order
Figure 5.3. Coimmunoprecipitation of VP22 Point Mutants with VP16. (A) and (B) Expression of VP22 point mutants represented in Fig. 5.1A in transfected/infected cells. Vero cells expressing GFP, VP22-GFP constructs or mock transfected cells (Mock) were infected with HSV-1, and lysed 10 h post-infection with NP-40 lysis buffer. A fraction of each cell lysate was analyzed by Western blotting using a goat polyclonal antibody specific for GFP. (C) and (D) Coimmunoprecipitation of VP16 with VP22 point mutants. The remainder of the transfected/infected cell lysate was incubated with a goat polyclonal antibody against GFP and resulting antibody-antigen complexes were collected with protein G-agarose beads. After extensive washes with lysis buffer, material that immunoprecipitated with anti-GFP antibody was separated by SDS-PAGE and transferred to nitrocellulose. Coimmunoprecipitated VP16 was detected by immunoblot using a rabbit monospecific polyclonal antibody raised against a C-terminal peptide of VP16. The positions of molecular mass markers (in kilodaltons) are indicated on the left.
to verify that the binding detected was authentic and not facilitated by virally encoded VP22. To attain this goal we exploited a modified version of the GST pulldown assay described above using VP16 fused to the C-terminus of GST (GST-VP16) as bait rather than the cytoplasmic tail of gE. GST-VP16 and GST alone were expressed in *Escherichia coli* cells and subsequently purified, with approximately equal amounts of each used in the pulldown assay as determined by Coomassie-blue staining (Fig. 5.4A). Vero cells were transfected with plasmids encoding GFP alone or the indicated VP22 point mutants and a fraction of each cell lysate was analyzed by Western blotting to verify that the GFP-tagged constructs were expressed (Fig. 5.4B). The remaining lysates were analyzed in the GST pulldown assay to look for binding to GST-VP16, with bound material separated by SDS-PAGE and then analyzed by Western blotting (Fig. 5.4C).

VP22 fused to GFP was efficiently pulled-down from transfected cell lysates, indicating that the interaction can occur in the absence of additional viral proteins (Fig. 5.4C and Fig. 5.4D). In agreement with the results of coimmunoprecipitation studies, W189F/W221F and F201A, point mutants which abrogated interaction with gE, failed to bind to VP16 to levels above background (Fig. 5.4C and Fig. 5.4D). In contrast, W221F and F196W, which bind to gE at levels approaching 54% and 140% of wild-type VP22 respectively, were efficiently pulled-down by GST-VP16 (Fig. 5.4C and Fig. 5.4D). Interestingly, the levels of binding to VP16 observed with these mutants parallel those seen with gE binding. W221F bound to VP16 at approximately 41% of wild-type VP22 levels, with F196W more efficient at 130% (Fig. 5.4D). It is appealing to speculate that the apparent repressive and enhancing effects these mutants display upon VP16 and gE...
Figure 5.4. Analysis of VP22 Point Mutants in a GST Pulldown Assay. (A) Coomassie-blue stained gel of VP16 fused to the C-terminus of the GST protein (GST-VP16) and GST alone. GST-VP16 or GST were expressed in *Escherichia coli* cells and purified on glutathione-Sepharose beads. Expression of VP22 point mutants represented in Fig. 5.1A in transfected Vero cells (B), and their ability to bind to VP16 (C) were analyzed as described in the legend to Fig. 5.2 with GST-VP16 used as bait rather than GST-gECT. (D) Binding efficiency of VP22 point mutants to VP16. Using densitometry, binding efficiency was quantitated by dividing the amount of VP22-GFP protein detected in the pulldown assay (normalized for the amount of GST-VP16 present) by the amount in the cell lysate (normalized for the amount of actin present). In each experiment, the wild-type VP22-GFP construct was set at 100% binding efficiency. Error bars represent standard deviations for four replicate experiments. The ability of each VP22 mutant to bind to gE (as determined in Fig. 5.2) is also represented. A plus indicates that the mutant construct retains binding activity, whereas a minus denotes abrogation of binding.
binding may be due to their impact on the structure of VP22. W221F, potentially masks the gE and VP16 binding sites, with F196W having the contradictory effect, exposing the binding interfaces.

Coimmunoprecipitation experiments predicted that three mutants (W189F, F196A and F201W), which were unable to interact with gE, would retain the ability to bind to VP16. When these mutants were assayed for their ability to bind to GST-VP16, W189F demonstrating a binding efficiency of 77% of wild-type VP22, and F196A and F201W exhibiting levels of 84% and 87%, respectively (Fig. 5.4C and Fig. 5.4D). Interestingly, the faster migrating band that was observed upon expression of F196A in our previous experiments was also detected in this assay and was efficiently pulled-down by the GST-VP16 fusion protein (Fig. 5.4C lane 8), suggesting that this putative breakdown product of VP22 may possess the ability to bind to VP16.

Collectively, our analysis of VP22 point mutants suggests that the VP16 binding activity housed within the conserved central region of VP22, can be separated from the gE binding activity by mutation of a tryptophan residue at position 189 or one of two phenylalanine residues at positions 196 or 201.

**Binding to the cytoplasmic tail of gE is not required for virion incorporation of VP22.** Previously, we have shown that incorporation of VP22 into assembling virus particles is independent of interaction with VP16 [(515); Chapter IV]. With the identification of VP22 constructs that fail to bind to gE yet retain the ability to interact efficiently with VP16, we now possess the tools to examine the role gE binding plays in virion incorporation of VP22. To accomplish this goal we utilized a transfection/infection-based packaging assay. Vero cells were transfected with plasmids
encoding various VP22-GFP fusion proteins and subsequently infected with either a VP22-null virus (U₄₉⁻) (Fig. 5.5) or wild-type HSV-1 (Fig. 5.6). The use of a VP22-null virus (a kind gift from Dr. Joel Baines, Cornell University, Ithaca) eliminates the possibility of multimerization between virally encoded VP22 and the mutant construct under study that may otherwise confound the results of the incorporation assay. At 18 h post-infection, extracellular virions were harvested and subsequently pelleted through a sucrose cushion. Pelleted virions were analyzed by Western blotting using GFP-specific antisera to detect VP22-GFP fusion proteins. To confirm that approximately equal amounts of virus were loaded with each sample, Western blots were stripped and reprobed for the major capsid protein VP5 or the abundant tegument protein VP16.

Analysis of the VP22-GFP constructs within transfected/infected cells showed that they were expressed at levels similar to the wild-type construct and migrated at the expected molecular weight (Fig. 5.5A and Fig. 5.6A). With regard to packaging, similar results were seen with both the VP22-null virus and wild-type HSV-1, suggesting that multimerization with virally encoded VP22 does not facilitate incorporation of the point mutants into the virus particle [(515); Chapter IV]. Specifically, wild-type VP22-GFP was incorporated into virus particles, whereas GFP alone was undetectable, despite high expression in transfected/infected cells indicating that GFP itself does not have a significant effect on packaging (Fig. 5.5B and Fig. 5.5C [U₄₉⁻]) and (Fig. 5.6B and Fig. 5.6C [HSV-1]). Furthermore, none of the VP22-GFP mutant proteins were detected in media from transfected/mock-infected cells (data not shown), and prior utilization of this assay has demonstrated that many constructs fail to be packaged upon infection,
Figure 5.5. Virion Incorporation of VP22 WÆF and FÆA/W Point Mutants in the Absence of Virally Encoded VP22. Vero cells were transfected with the indicated VP22-GFP constructs, and 20 h later, they were infected with a VP22-null virus [UL49]. After an additional 18-h incubation, cell lysates were prepared (A) and virions were collected from the media by centrifugation through a 30% sucrose cushion (B). Cell lysates and extracellular virus were separated by SDS-PAGE and transferred to nitrocellulose. Western blot analysis was performed using a rabbit polyclonal antibody specific for GFP. As a loading control, the blot was stripped and reprobed with a rabbit monospecific polyclonal antibody raised against the HSV-1 major capsid protein VP5. The positions of molecular mass markers (in kilodaltons) are indicated on the left. (C) Packaging efficiency. Using densitometry, packaging efficiency was quantitated by dividing the amount of VP22-GFP protein detected in extracellular virus particles (normalized for VP5) by the amount in the cell lysate (normalized for VP5). In each experiment, the wild-type VP22-GFP construct was set at 100% packaging efficiency. Error bars represent standard deviations for four replicate experiments. The ability of each VP22 mutant to bind to either gE (as determined in Fig. 5.2) or VP16 (as determined in Fig. 5.3 and Fig. 5.4) is also represented. A plus indicates that the mutant construct retains binding activity, whereas a minus denotes abrogation of binding.
Figure 5.6. Virion Incorporation of VP22 W→F and F→A/W Point Mutants in the Presence of a Wild-Type HSV-1 Infection. Expression of the indicated VP22-GFP constructs in transfected/infected cells (A) and their incorporation into virus particles (B) were analyzed as described in the legend to Fig. 5.5. The positions of molecular mass markers (in kilodaltons) are indicated on the left. (C) Packaging efficiency was calculated as described in the legend to Fig. 5.5C, using the tegument protein VP16 as a loading control rather than VP5. Error bars represent standard deviations for four replicate experiments. The ability of each VP22 mutant to bind to either gE (as determined in Fig. 5.2) or VP16 (as determined in Fig. 5.3 and Fig. 5.4) is also represented. A plus indicates that the mutant construct retains binding activity, whereas a minus denotes abrogation of binding.
indicating that incorporation is in fact a specific event and not due to aggregates that can pellet through the sucrose cushion (408,514,515). The VP22 mutant constructs W189F/W221F and F201A, which abrogated binding to both VP16 and gE failed to be packaged into virions (Fig. 5.5B and Fig. 5.5C [UL49–]) and (Fig. 5.6B and Fig. 5.6C [HSV-1]). VP22 mutants such as these, which are deficient for a variety of activities, most likely suffer from gross conformational issues as a result of the mutation itself, rather than the identification of a key residue involved in three singular activities of VP22.

The VP22 mutants W221F and F196W, which retained the ability to interact with both VP16 and gE were packaged into assembling virus particles but with markedly different efficiencies (Fig. 5.5B and Fig. 5.5C [UL49–]) and (Fig. 5.6B and Fig. 5.6C [HSV-1]). W221F was packaged at approximately 41% of wild-type levels with a UL49– infection and 38% with HSV-1 as the infecting virus, a result presumably attributable to the mutant’s impaired ability to bind VP16 (41% of wild-type VP22 levels) and gE (54% of wild-type VP22 levels). In contrast, F196W which demonstrated levels of binding to both VP16 and gE in excess of wild-type (130% and 140% respectively), was packaged into assembling virus particles at approximately 120% of the full-length VP22-GFP construct with a UL49– infection and 115% in the presence of a wild-type infection. This finding suggests that an increase in binding to one or both binding partners of VP22 may enhance incorporation of the protein into assembling virions.

The VP22 mutants of particular interest to this project, W189F, F196A, and F201W (which interact with VP16 but not gE), were incorporated into assembling virus particles albeit at levels below wild-type VP22 (Fig. 5.5B and Fig 5.5C [UL49–]) and (Fig.
The reduced level of virus packaging may be a result of the impaired ability of these mutants to bind to VP16 (W189F ~77%, F196A ~84%, and F201W ~87%), possibly complemented by their failure to bind to gE. Interestingly, the faster migrating band detected upon expression of F196A, which we hypothesize may be a breakdown product of VP22 that the mutation stabilizes, was also incorporated into virus particles. These results suggest that binding to the cytoplasmic tail of gE is not required for incorporation of VP22 into assembling virus particles.

**Extension of the gE binding domain of VP22 to the carboxyl terminus of the protein restores binding to wild-type levels.** Previously, we demonstrated that a domain of VP22 encompassing residues 165-270 facilitates binding to both VP16 and gE [(515); Chapter IV]. Curiously, despite possessing membrane association activity, in addition to high binding efficiencies to both VP16 (399% of wild-type levels) and gE (86% of wild-type levels), this domain of VP22 is only packaged to 54% of the levels seen with a full-length VP22-GFP construct [(515); Chapter IV]. It is attractive to propose that in order to attain wild-type levels of virion incorporation of VP22, the construct under study must be able to bind to both gE and VP16 at wild-type efficiencies. In an effort to determine whether such a VP22 construct would achieve levels of virion packaging equal to a full-length VP22-GFP fusion protein, we attempted to identify a domain of VP22 capable of interacting with both binding partners (VP16 and gE) to wild-type levels. The conserved core of VP22 (amino acids 165-270) facilitates greater than wild-type levels of binding to VP16 most likely through an overt exposure of the VP16 binding interface (515). We hypothesized that extension of this domain either N-terminally or C-terminally may allow for a more accurate structural conformation, with a
resultant normalization of VP22-VP16 and VP22-gE binding to wild-type levels. To this end, a variety of N-terminal and C-terminal extensions of the domain 165-270 of VP22 were tagged with GFP, creating the fusion proteins represented in Fig. 5.7A. Vero cells were transfected with plasmids encoding the VP22 truncation mutants or GFP alone and each construct was evaluated for the ability to bind to a purified GST-gECT fusion protein. Each of the GFP-tagged constructs under study demonstrated high levels of expression and migrated at the expected molecular weight (Fig. 5.7B). VP22 fused to the GFP protein bound efficiently to the cytoplasmic tail of gE (Fig. 5.7C and Fig. 5.7D). Upon deletion of amino acids 165-270, VP22 binding to gE was abrogated (Fig. 5.7C and Fig. 5.7D). In contrast, residues 165-270 bound to the cytoplasmic tail of gE at levels approaching 86% of wild-type VP22 (Fig. 5.7C and Fig. 5.7D). Upon extension of the gE binding domain to encompass the final C-terminal residue of VP22 (VP22.165-301), binding to the cytoplasmic tail of gE increased to levels greater than wild-type (approximately 230%). When residues that lie towards the N-terminus of the protein were restored, binding to gE decreased from the enhanced levels witnessed with VP22.165-301 and returned to levels approaching wild-type VP22 (Fig. 5.7C and Fig. 5.7D). This reduction in binding efficiency to gE from the enhanced levels of VP22.165-301 (and its presumably overt exposure of the gE binding interface), is most likely due to restoration of a conformation approaching that of the native protein.

Collectively, our analysis of the VP22 truncation mutants suggests that the gE binding activity housed within residues 165-270 of VP22 can be enhanced to levels equal to or greater than a wild-type VP22 construct by extension of the domain to the carboxyl and amino termini of the protein.
Figure 5.7. Characterization of the Ability of VP22 Truncation Mutants to Bind to the Cytoplasmic Tail of gE in a GST Pulldown Assay. (A) VP22 derivatives fused to GFP. A schematic representation of full-length and N-terminal truncation mutants of VP22 fused to the N-terminus of the GFP protein. Also represented, deletion of the region of VP22 that facilitates binding to both VP16 and gE and this domain alone fused to GFP. Expression of VP22 derivatives in transfected Vero cells (B), their ability to bind to the cytoplasmic tail of gE (C), and binding efficiency (D) were analyzed as described in the legend to Fig. 5.2. The positions of molecular mass markers (in kilodaltons) are indicated on the left. Error bars represent standard deviations for four replicate experiments.
Analysis of the ability of truncation mutants of VP22 to bind to VP16 in a GST pulldown assay. The VP22 mutant proteins identified above, which restore wild-type levels of binding to gE house the domain (residues 165-270) that facilitates interaction with VP16. To confirm that extension of this domain (165-270) to facilitate wild-type levels of binding to gE did not have a detrimental effect on interaction with VP16, we exploited the GST-VP16 pulldown assay described above. Vero cells were transfected with plasmids encoding the VP22 truncation mutants and each construct demonstrated high levels of expression and migrated at the expected molecular weight (Fig. 5.8A). VP22 fused to GFP was efficiently pulled-down from the transfected cell lysates, whereas deletion of amino acids 165-270 abrogated binding to VP16 (Fig. 5.8B and Fig. 5.8C). In contrast, residues 165-270 of VP22, bound to VP16 at levels greater than wild-type (Fig. 5.8B and Fig. 5.8C). VP22.165-301, which facilitated levels of binding to gE of 230%, was also pulled-down by VP16 at efficiencies greater than wild-type VP22 (255%) (Fig. 5.8B and Fig. 5.8C). As the domain of VP22 under study was extended towards the N-terminus, VP16 binding was restored to wild-type levels (VP22.121-301 ~107%, VP22.87-301 ~102% and VP22.44-301 ~140%). These data suggest that extension of the VP16/gE interaction domain of VP22 to facilitate wild-type levels of gE binding does not diminish VP16 binding to levels below those of a wild-type construct.

A VP22 construct that binds to both VP16 and gE at wild-type levels is impaired for virion packaging. To determine whether VP22 constructs that facilitate wild-type levels of binding to both VP16 and gE are packaged into virions at levels equivalent to VP22-GFP, we utilized the transfection/infection-based packaging
Figure 5.8. Analysis of VP22 Truncation Mutants in a GST Pulldown Assay. Vero cells were transfected with the VP22-GFP constructs represented in Fig. 5.7A. Expression of VP22 constructs in transfected cells (A), their ability to bind to GST-VP16 (B), and binding efficiency (C) were analyzed as described in the legend to Fig. 5.4. The positions of molecular mass markers (in kilodaltons) are indicated on the left. Error bars represent standard deviations for four replicate experiments.
assay described above. A wild-type HSV-1 strain was used to infect the transfected monolayers, allowing any contribution of heretofore unknown VP22 packaging determinants to be ascertained. Western blot analysis of the various VP22-GFP constructs within transfected/infected cells showed that the mutants were expressed at levels similar to wild-type VP22 and migrated at the expected molecular weight (Fig. 5.9A). With regard to packaging, VP22-GFP was incorporated into virus particles, whereas GFP alone was undetectable, despite high expression in the transfected/infected cells (Fig. 5.9B and Fig. 5.9C). As we had seen previously, VP22.165-270 which binds to VP16 and gE, was incorporated to approximately 49% of wild-type levels. In contrast, upon deletion of residues 165-270, VP22 virion packaging occurred at extremely low (approximately 8% of wild-type VP22) but reproducible levels, (Fig. 5.9B and Fig. 5.9C). A virion packaging signal has been described in the C-terminus of VP22 (personal communication from Dr. John Blaho, Mount Sinai School of Medicine, New York). This signal is contained in VP22.Δ165-270 and may account for the low levels of incorporation seen with this mutant.

Surprisingly, VP22.87-301, which displayed wild-type binding to both VP16 and gE, was not packaged at wild-type levels but at an efficiency comparable to VP22.165-270 (Fig. 5.9B and Fig. 5.9C). Similar results were attained with VP22.121-301 and VP22.165-301. However, upon inclusion of residues 44-86, incorporation into virus particles returned to levels resembling VP22-GFP (95%) (Fig. 5.9B and Fig. 5.9C). These results suggest that optimal VP22 incorporation levels are not facilitated by wild-type binding to both VP16 and gE alone, but require residues 44-86 of VP22.
Figure 5.9. Virion Incorporation of VP22 Truncation Mutants. Expression of the indicated VP22-GFP constructs in transfected/infected cells (A) and their incorporation into virus particles (B) were analyzed as described in the legend to Fig. 5.6. The positions of molecular mass markers (in kilodaltons) are indicated on the left. (C) Packaging efficiency was calculated as described in the legend to Fig. 5.6C. Error bars represent standard deviations for four replicate experiments. Comparison of VP22.87-301 to both wild-type VP22 and VP22.44-301 indicates that the difference in incorporation levels observed is statistically significant (P < 0.001). The difference in packaging levels between VP22.44-301 and the full-length protein is not significant (P > 0.05).
Deletion of the acidic cluster of VP22 does not abrogate interaction with VP16 or gE. HSV-1 virion components must traffic to the TGN for incorporation into virus particles. Some virion proteins traffic to the TGN as a multiprotein complex (e.g. nucleocapsids), whereas other structural proteins likely target to the TGN as monomers. Trafficking signals are usually short amino acid motifs within a protein that can be classified into three general categories: tyrosine-based motifs (YXXΦ), dileucine motifs, and acidic cluster motifs (53,356,357,724). A variety of herpesvirus proteins have hijacked these cellular mechanisms to localize to the TGN. The tegument protein UL11 and glycoproteins gB, gE, and gI contain dileucine and acidic cluster motifs that enable these proteins to traffic to the TGN (5,7,406,446,701).

Analysis of the primary structure of amino acids 44-86 of VP22 reveals a cluster of acidic amino acids between residues 61-77, which is necessary for localization of VP22 to the TGN (unpublished results from Michael Brignati). To determine whether the packaging determinant that, in tandem with optimal gE and VP16 binding, facilitates wild-type levels of VP22 incorporation is in fact the acidic cluster motif, the residues comprising the primary acidic cluster (amino acids 71-77) were changed to alanines (AC→Ala) or deleted (ΔAC1) (Fig. 5.10A). A smaller cluster of acidic amino acids is also located at residues 61-64 of VP22. To eliminate the possibility that deletion mutagenesis may enable this secondary motif to functionally substitute for the primary acidic cluster we deleted both, creating the mutant ΔAC1 + ΔAC2 (Fig. 5.10A). To ensure that mutagenesis of the acidic clusters did not have a detrimental effect on the ability of these constructs to bind to gE and VP16, the mutants were assayed by GST pulldown using the cytoplasmic tail of gE or VP16 as bait (Fig. 5.10).
Figure 5.10. Characterization of the Ability of VP22 Acidic Cluster Mutants to Bind to VP16 or the Cytoplasmic Tail of gE in a GST Pulldown Assay. (A) Acidic cluster mutants of VP22. A schematic representation of HA-tagged VP22, mutation of the primary acidic cluster of VP22 to alanine residues, and deletion of the primary acidic cluster or both primary and secondary acidic clusters from VP22. Expression of VP22 acidic cluster mutants in transfected Vero cells (B and E), their ability to bind to the cytoplasmic tail of gE (C) or VP16 (F) and binding efficiency (D and G) were analyzed as described in the legend to Fig. 5.2 and Fig. 5.4, respectively, with Western blot analysis performed with a HA specific antibody rather than one specific for GFP. The positions of molecular mass markers (in kilodaltons) are indicated on the left. Error bars represent standard deviations for four replicate experiments.
Expression analysis of the various acidic cluster mutants within transfected cells showed that each was expressed and migrated at the expected molecular weight (Fig. 5.10B and Fig. 5.10E). With regards to gE binding, VP22-HA was efficiently pulled-down from transfected cell lysates; however, AC\(\rightarrow\)Ala failed to bind, suggesting that the structural conformation of VP22 may be sensitive to alanine mutagenesis (Fig. 5.10C and Fig. 5.10D). In contrast, deletion of one or both of the acidic clusters from VP22 had little impact upon interaction with gE (Fig. 5.10C and Fig. 5.10D). Similar results were seen with GST-VP16, where VP22-HA and the acidic cluster deletion mutants bound efficiently and AC\(\rightarrow\)Ala abrogated interaction with VP16 (Fig. 5.10F and Fig. 5.10G). These results suggest that deletion of the acidic cluster does not have a detrimental effect on binding of VP22 to gE or VP16.

**An acidic cluster is required to facilitate wild-type levels of VP22 virion packaging.** To elucidate the role of the acidic cluster in virion incorporation of VP22, we utilized the packaging assay described above. Western blot analysis of the acidic cluster mutants of VP22 within transfected/infected cells showed that the mutants were expressed at levels similar to wild-type VP22 and migrated at the expected molecular weight (Fig. 5.11A). VP22-HA was packaged into assembling virus particles; however, AC\(\rightarrow\)Ala failed to be incorporated, presumably due to structural effects ensuing from insertion of seven consecutive alanines (Fig. 5.11B and Fig. 5.11C). Upon deletion of the primary acidic cluster (residues 71-77 of VP22), an intermediate phenotype was observed (Fig. 5.11B and Fig. 5.11C). \(\Delta AC^1\) failed to attain wild-type levels of incorporation (77%), yet was more efficiently packaged than VP22.87-301 (51%) (Fig. 5.9C).
This finding suggests that the secondary acidic cluster may partially substitute for the absence of the primary motif. However, upon deletion of both acidic clusters from VP22 (ΔAC₁ + ΔAC₂), packaging was reduced to levels similar to VP22.87-301. Collectively, these results suggest that an acidic cluster of amino acids are required to facilitate efficient incorporation of VP22 into the virion, perhaps ensuring localization of VP22 at the TGN.
Figure 5.11. Virion Incorporation of VP22 Acidic Cluster Mutants. Expression of the indicated VP22-HA constructs in transfected/infected cells (A) and their incorporation into virus particles (B) were analyzed as described in the legend to Fig. 5.6, with Western blot analysis performed with a HA specific antibody rather than one specific for GFP. The positions of molecular mass markers (in kilodaltons) are indicated on the left. (C) Packaging efficiency. Using densitometry, packaging efficiency was quantitated by dividing the amount of VP22-HA protein detected in extracellular virus particles (normalized for VP16) by the amount in the cell lysate (normalized for VP16). Error bars represent standard deviations for four replicate experiments. Comparison of ∆AC¹ and ∆AC¹ + ∆AC² to each other and to wild-type VP22 indicates that the difference in incorporation levels observed is statistically significant (P < 0.001).
A. 

Cell Lysate

B. 

Virus

C. 

% wild-type VP22
DISCUSSION

The HSV-1 tegument protein VP22 is packaged into virions during final envelopment as nucleocapsids bud into TGN-derived vesicles (469). This observation suggests that VP22 traffics to the TGN where interactions with viral glycoproteins, perhaps in concert with binding to tegument proteins located on the surface of cytoplasmic capsids ensures tegumentation of the protein.

In support of this model, VP22 binds to both gD and gE, and the VP22 homologue of the related alphaherpesvirus, PrV, interacts with the cytoplasmic tails of gE and gM (66,109,109,221,514,514,515). Furthermore, VP22 interacts with VP16, whose presence on the surface of capsids undergoing secondary envelopment may facilitate packaging of VP22 into the tegument (183,469,488). A previous study demonstrated that the gE binding domain of VP22 (residues 165-270) which also facilitates binding to VP16, is packaging into assembling virions [(514) and Chapter III]. VP16 binding is not an essential virion incorporation determinant of VP22 [(515) and Chapter IV]. Thus, to further elucidate the mechanism by which VP22 is packaged into assembling virus particles our studies have focused on defining the functional significance of gE binding in tegumentation of VP22.

To abrogate the VP22-gE interaction with minimal disruption to the overlapping VP16 binding activity, residues 165-270 of VP22 were subjected to extensive point mutagenesis. A variety of point mutants which abrogated gE binding, but not that of VP16, were packaged into assembling virus particles, albeit at reduced levels. It is unclear whether the observed reduction is a result of the inability of these mutants to bind to gE or is due to the decreased VP16 binding that occurs upon mutation of VP22.
Nevertheless these results indicate that gE binding is not required for virion incorporation of VP22. As tegumentation of VP22 is also independent of interaction with VP16, both binding activities (gE and VP16) may act in a redundant, or perhaps additive fashion, to facilitate virion packaging of VP22. Functional redundancy, as observed with the virion incorporation determinants of VP22, is a common theme across herpesvirology, at least for virus growth in cell culture (58,59,221).

A VP22 mutant which fails to bind to both VP16 and gE and nonetheless retains the ability to be packaged into the tegument region of assembling virus particles has yet to be identified, suggesting that one of these activities must be functional to facilitate virion incorporation of VP22. However, binding to both VP16 and gE to wild-type levels is not sufficient to facilitate wild-type levels of VP22 incorporation, indicating the existence of other packaging determinants. It has been suggested that the C-terminus of VP22 may contain an incorporation determinant of VP22 (personal communication from Dr. John Blaho, Mount Sinai School of Medicine, New York) (280). However, mutants which house this region, in addition to maintaining gE and VP16 binding activities, are still packaged at reduced levels.

Considering the dynamic nature of the TGN, one would anticipate that a resident protein, such as VP22, would contain sorting sequences that facilitate its vesicular trafficking and retrieval (66). Acidic cluster motifs are well characterized trafficking motifs, shuttling proteins to and from the Golgi apparatus. Many herpesvirus proteins utilize such motifs to localize to the TGN (5,7,406,446,701). An acidic cluster of amino acids appears to be required for virion incorporation of the HSV-1 tegument protein UL11; however, this motif is not sufficient as UL11 constructs which localize to the TGN
and associate with membranes are not packaged into virus particles (408). Optimal VP22 virion incorporation required a region of the protein encompassing residues 44-86, with an acidic cluster of amino acids within this domain facilitating efficient packaging. The acidic cluster motif is necessary for localization of VP22 at the TGN (unpublished results from Michael Brignati), and may facilitate efficient incorporation by ensuring correct subcellular localization, where protein-protein interactions with gE and/or VP16 recruit VP22 into assembling virions. W189F/W221F and F201A are both unable to interact with either VP16 or gE. Although localizing to a subcellular compartment reminiscent of the TGN, and harboring an acidic cluster and residues 165-225 (which facilitate membrane association of VP22), neither VP22 mutant was packaged into virus particles (515). This finding suggests that in a similar fashion to UL11, localization to the putative site of final envelopment is not sufficient to facilitate virus packaging of VP22, and additional determinants are required (presumably protein-protein interactions with VP16 and/or gE).

A logical model would suggest that VP22 must first associate with membranes through the actions of the membrane association domain and then localize to the TGN via the acidic cluster motif. The converse is unlikely to occur because acidic cluster motifs are membrane trafficking motifs and would require membrane association for functionality. After localization to the TGN, protein-protein interactions with VP16 and/or gE ensure retention of VP22 in the assembly pathway with resultant incorporation into assembling virus particles. In absence of an acidic cluster, interactions with VP16 and gE may only recruit a portion of VP22 into the assembly pathway (perhaps through
encounters with VP16-coated capsids, or with the cytoplasmic tail of gE as VP22 traffics through the TGN).

Due to the volume of VP22 constructs assayed in this study it was impractical to examine the various mutant alleles using recombinant viruses. However, our group and others have identified and mapped many protein-protein interactions and virion incorporation determinants using these \textit{in vitro} experimental systems (406, 407, 408, 483, 514, 515). Despite these limitations, our findings further elucidate the molecular mechanisms that result in tegumentation of VP22; specifically the crucial (and possibly redundant) role played by protein-protein interactions and the first description of the functional significance of a VP22 trafficking signal for virion incorporation.
MATERIALS AND METHODS

Cells and viruses. Vero (ATCC CCL-81) and A7 (human melanoma) cells, a gift from Gary Thomas (The Oregon Health and Science University, Portland) were grown in Dulbecco’s modified Eagles medium (DMEM) (GIBCO) supplemented with 10% fetal bovine serum (FBS), 2.25% sodium bicarbonate, 25 mM HEPES buffer, glutamine (300 µg/ml), penicillin (100 µg/ml), and streptomycin (131 µg/ml). Infected cells were grown in DMEM supplemented with 2% FBS, 25 mM HEPES buffer, glutamine (300 µg/ml), penicillin (100 µg/ml), and streptomycin (131 µg/ml). The viruses used in this study were the KOS strain (650) and a VP22-null virus (UL49), a kind gift from Joel Baines (Cornell University, Ithaca, New York) (179).

Construction of VP22-GFP and VP22-HA chimeras. Plasmids encoding VP22 fused to GFP, the N-terminal truncations of VP22 fused to GFP and the internal deletions of VP22 (pVP22.Δ165-270-GFP and pVP22.165-270-GFP) were described previously (66,514).

To make plasmids encoding the various point mutant variants of VP22, the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) was used according to the manufacturer’s instructions. Using pVP22-GFP as a template (Brignati et al., 2003), mutagenic primers were designed which changed the nucleotide sequence of codon 189 of VP22 to encode for a phenylalanine residue rather than a tryptophan; thereby creating pVP22.W189F-GFP. A similar strategy was used to create pVP22.W221F-GFP, pVP22.F196A-GFP, pVP22.F196W-GFP, pVP22.F201A-GFP and pVP22.F201W-GFP. To construct plasmids encoding the double point mutant of VP22, W189F/W221F, the
QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) was used with pVP22.W189F-GFP acting as a template.

The plasmid encoding VP22 fused to the hemagglutinin epitope (pVP22-HA), has been described previously (515). To construct pVP22.AC→Ala-HA, the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) was used according to the manufacturer’s instructions. Using pVP22-HA as a template, mutagenic primers were designed which changed the nucleotide sequence of codons 71-77 of VP22 to encode for alanines rather than the residues of the acidic cluster motif. This QuikChange XL Site-Directed Mutagenesis methodology was also utilized to make pVP22.ΔAC¹-HA. Mutagenic primers were designed complementary to 15-bp immediately upstream of codon 71 and 15-bp immediately downstream of codon 77, essentially looping out the sequence encoding amino acids 71-77 of VP22 in pVP22-HA. Using pVP22.ΔAC¹-HA as a template, a similar approach was used to construct pVP22.ΔAC¹+ΔAC²-HA.

All constructs were sequenced to confirm the identity of VP22 and to ensure that the gene encoding VP22 (or mutated forms) was in frame with the gene encoding the GFP protein or the sequence that encodes the HA tag.

**Construction and purification of GST fusion proteins.** Construction of the plasmid encoding VP16 fused to the C-terminus of the GST protein (GST-VP16) was described previously (515). The vector encoding the cytoplasmic tail of gE fused to GST (GST-gECT) was a kind gift from David Johnson (The Oregon Health and Science University, Portland, Oregon).

To express and purify the various GST fusion proteins, plasmids encoding the respective constructs were transformed into BL21 competent cells (Stratagene) according
to the manufacturer’s instructions and overnight cultures were prepared. Approximately 10 ml of these cultures were used to inoculate fresh 100 ml cultures and grown at 37 °C to $A_{600}=0.4$. To induce expression, 100 µL of 1 M IPTG (GIBCO) was added and the cultures were grown at 30 °C for 3-4 h, followed by centrifugation at 10,000 x g for 10 min at 4 °C. The bacterial pellet was resuspended in 10 ml of lysis buffer (100 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1 mM CHAPS, 400 mM NaCl, 1% Triton X-100, Complete Mini protease inhibitors [Roche]). The suspension was sonicated and clarified by centrifugation at 27,000 x g for 10 min at 4 °C. A volume of 133 µl of glutathione-Sepharose 4B beads (Pharmacia) that had been washed twice with phosphate-buffered saline (PBS) was added to the supernatant and incubated overnight at 4 °C. Beads were washed thoroughly with PBS and the yield of each purified GST fusion protein was determined by SDS-PAGE and subsequent staining with Coomassie blue to detect the recombinant protein.

**GST pulldown assay.** To analyze the VP22-gE and VP22-VP16 interaction within transfected cells, a GST pulldown assay was used. Confluent monolayers of Vero cells grown in 100-mm plates were transfected with the indicated constructs by using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. At 20 h post-transfection, monolayers were washed twice with PBS and scraped into 10 ml of cold PBS. A 1 ml sample of the cell suspension was removed for analysis of expression of the VP22-GFP or VP22-HA fusion proteins, and the remaining cells were pelleted by centrifugation (1000 x g for 5 min at 4 °C). The 9 ml samples were lysed in NP-40 lysis buffer. Transfected-cell lysates were precleared overnight at 4 °C with glutathione-Sepharose 4B beads that had been previously washed twice with PBS, and the samples
were then incubated with approximately equal amounts of purified GST fusion proteins on glutathione-Sepharose beads (as determined by Coomassie blue-stained gel) for 3 h at 4 °C. Beads were washed three times with NP-40 lysis buffer and once with 10 mM Tris-HCl (pH 7.4). GFP or HA-tagged VP22 fusion proteins bound to GST constructs were detected by Western blotting using a rabbit polyclonal antibody raised against the GFP protein (Santa Cruz) or a rabbit polyclonal antibody specific for the hemagglutinin epitope (Sigma), a goat anti-rabbit antibody conjugated to horseradish peroxidase (Sigma), ECL reagents (Pharmacia), and autoradiography on Kodak BioMax XAR film.

To further control for the quantities of GST fusion proteins used in the pulldown, nitrocellulose membranes were stripped (60 mM Tris-HCl [pH 8.0], 2% SDS, 0.75% β-mercaptoethanol [β-ME] for 45 min at 55 °C) and reprobed with a goat polyclonal antibody raised against the GST protein (Rockland). To detect expression of the VP22-GFP or VP22-HA fusion proteins in transfected cells, proteins in the 1 ml cell suspension sample were analyzed by Western blotting using a rabbit polyclonal antibody against GFP or the hemagglutinin epitope, a goat anti-rabbit antibody conjugated to horseradish peroxidase, ECL reagents, and autoradiography on Kodak BioMax XAR film. To ensure equal loading, nitrocellulose membranes were stripped as described above and reprobed with a goat polyclonal antibody raised against actin (Santa Cruz). Using densitometry, binding efficiency was quantitated by dividing the amount of VP22-GFP or VP22-HA fusion protein detected in the pulldown assay (normalized for the amount of GST-VP16 or GST-gECT present) by the amount in the cell lysate (normalized for the amount of actin present).
**Immunoprecipitation-Western assay.** Confluent monolayers of Vero cells grown in 100-mm plates were transfected with the indicated constructs by using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. At 20 h post-transfection, the monolayers were infected with HSV-1 KOS strain at a MOI of 10. At 10 h post-infection (30 h post-transfection), cells were washed twice with PBS and scraped into 10 ml of cold PBS. A 1 ml sample of the cell suspension was removed for analysis of expression of the VP22-GFP fusion proteins, and the remaining cells were pelleted by centrifugation (1000 x g for 5 min at 4 °C). The 9 ml samples were lysed in NP-40 lysis buffer containing Complete Mini protease inhibitors as described above and precleared overnight at 4 °C with protein G-agarose beads (Roche) that had been washed twice in lysis buffer. The lysates were then incubated with a goat polyclonal antibody raised against GFP (Rockland) for 1 h at 4 °C, and immune complexes were collected with protein G-agarose beads that had been washed twice with lysis buffer. Beads were washed three times with NP-40 lysis buffer and once with 10 mM Tris-HCl (pH 7.4). Coimmunoprecipitated VP16 was detected by Western blotting using a rabbit monospecific polyclonal antibody raised against a C-terminal peptide of the HSV-1 tegument protein VP16 (Clontech), a goat anti-rabbit antibody conjugated to horseradish peroxidase, ECL reagents, and chemiluminescence autoradiography on Kodak BioMax XAR film. To detect expression of the VP22-GFP fusion proteins in transfected/infected cells, proteins in the 1 ml cell suspension sample were analyzed by Western blotting using a goat polyclonal antibody against GFP, a rabbit anti-goat antibody conjugated to horseradish peroxidase, ECL reagents, and autoradiography on Kodak BioMax XAR film.
**Virion incorporation assay.** Confluent monolayers of Vero cells grown in 100-mm plates were transfected with the indicated constructs using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. At 20 h post-transfection, cells were infected with either HSV-1 KOS or a VP22-null virus (U149) at a MOI of 10, or mock infected. At 18 h post-infection (38 h post-transfection), the medium was removed by pipetting and centrifuged at 1000 x g for 10 min at 4 °C to remove cellular debris. The supernatant was retained and extracellular virions were purified by centrifugation (115,000 x g for 1 h in a Beckman SW41 rotor) through a 30% (wt/vol) sucrose cushion (1.7 ml) in NTE buffer (100 mM NaCl, 10 mM Tris, 1 mM EDTA, [pH 7.4]). Pelleted virions and infected cells were disrupted in 1X sample buffer (50 mM Tris-HCl [pH 6.8], 10% glycerol, 2% SDS, 5% β-ME). GFP or HA-tagged proteins were detected by Western blotting using a goat polyclonal antibody raised against the GFP protein (Rockland) or a rabbit polyclonal antibody specific for the hemagglutinin epitope (Sigma), followed by the appropriate secondary antibody conjugated to horseradish peroxidase, ECL reagents, and chemiluminescence autoradiography on Kodak BioMax XAR film. As a loading control, nitrocellulose membranes were stripped as described above and reprobed with either a rabbit polyclonal antibody raised against the major capsid protein VP5 or a rabbit monospecific polyclonal antibody raised against a C-terminal peptide of the HSV-1 tegument protein VP16 (Clontech). Using densitometry, packaging efficiency was quantitated by dividing the amount of VP22-GFP or VP22-HA protein detected in extracellular virus particles by the amount in the cell lysate (both normalized for either VP5 or VP16).
Confocal microscopy. Vero cells were transfected with the indicated constructs using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. At 20 h post-transfection, cells were infected with HSV-1 KOS strain at a MOI of 10 or mock infected. At 18 h post-infection (38 h post-transfection), cells were washed twice in Tris-buffered saline (TBS), fixed with 3% paraformaldehyde in PBS and mounted on slides using SlowFade antifade reagent with DAPI (Molecular Probes). Intrinsic GFP fluorescence was observed with a Leica TSC SP2 AOBS confocal microscope using the 488 nm laser line. Images were formatted in Adobe Photoshop 7.0.

Statistical analysis. For all statistical considerations, the data were normalized to the control prior to analysis. Statistical calculations were performed using GraphPad Prism version 5 software. One way analysis of variance (ANOVA) was used with Bonferroni post test analysis. Statistical significance was set to at least $p$-value < 0.05.
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CHAPTER VI

DISCUSSION
VIRION INCORPORATION OF VP22

Herpes simplex virus type 1 (HSV-1) assembly involves a complex sequence of events that culminates in the formation of virions composed of nearly 40 different viral proteins. Although recent advances have elucidated major steps of the herpesvirus assembly pathway, the molecular mechanisms utilized by viral structural proteins to target to the site of final envelopment and interact to form a stable virion remain poorly characterized. The studies presented within this dissertation provide insight into the mechanisms utilized by tegument proteins to facilitate virion incorporation. Specifically, the protein-protein interactions and intrinsic virion incorporation determinants of the HSV-1 tegument protein VP22 are dissected to elucidate the functional significance of these activities in facilitating packaging of VP22 into assembling virions.

Our initial endeavors identified an interaction between VP22 and the cytoplasmic tail of the viral glycoprotein gE (Fig. 3.1). As viral glycoprotein tails accumulate at the cytosolic face of TGN-derived vesicles [the proposed site of secondary envelopment and subcellular localization of VP22 (702)] such an interaction appeared an excellent candidate for potentially mediating virion incorporation of VP22. Primary structural alignment reveals that the domain that facilitates gE binding is highly conserved among VP22 homologues of herpesviruses (Fig. 4.13A). Furthermore, the VP22 homologue of a related herpesvirus PrV, also binds to the cytoplasmic tail of gE, with the activity mapping to a similar domain (221). Thus conservation of sequence within this domain may indicate conservation of function. Interestingly, this region of VP22 is sufficient to mediate virion incorporation, suggesting that interaction with the cytoplasmic tail of gE may facilitate packaging of VP22 into the virus particle (Fig. 3.7). However, additional
interaction studies identified that this region of VP22 also interacts with VP16 (Fig. 4.5). Thus, virion incorporation of VP22 may be facilitated by a variety of activities.

Attempts to separate the gE and VP16 binding activities within this conserved domain of VP22 highlighted the exquisite sensitivity of this protein to mutation. Initial studies had suggested that amino acids 165-225 of VP22 facilitate weak binding to VP16 (Fig. 4.1). Upon extension of this domain to encompass residue 270, VP16 binding was increased to greatly enhanced levels compared to the binding efficiency of the wild-type protein (Fig. 4.5). While no structural data exist regarding VP22, one could hypothesize that such an increase in binding efficiency may result from a more overt exposure of the VP16 binding interface, perhaps suggesting a role for the structure of this conserved domain in the functionality it harbors. This hypothesis was strengthened as minor deletions of N-terminal or C-terminal residues of the conserved domain, leaving amino acids 165-225 of VP22 intact, abrogated VP16 binding (Fig. 4.10). Furthermore, point mutagenesis had dramatic effects on both binding activities. This was most likely due to resultant global effects on protein folding, rather than identification of residues critical to the activities of VP22 assayed. In addition, alanine mutagenesis of an N-terminal portion of the protein, outside the conserved central domain, abrogated both binding activities (Fig. 5.10). Collectively, these data suggest that structurally VP22 as a whole may be sensitive to mutagenesis.

Nevertheless our mutagenesis strategy isolated a variety of VP22 mutants which were deficient in one or both binding activities (data summarized in Figure 6.1). Analysis of these constructs in a virion packaging assay suggested that neither gE binding
Figure 6.1. Summary of the Data Regarding the Importance of Binding Activities to Virion Incorporation of VP22. The percentages are relative to the binding or packaging efficiency of a wild-type VP22 construct which was set at 100%. The percentages garnered from virion incorporation studies reflect the use of a wild-type virus in the assay (a) or a VP22-null virus (b). ND represents binding efficiencies that were not determined.
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<td>F201W</td>
<td>87%</td>
<td>4%</td>
<td>30%</td>
<td>32%</td>
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nor interaction with VP16 is essential for virion incorporation of VP22 (Fig. 4.16 and Fig. 5.5). In the absence of gE or VP16 binding, virion incorporation is decreased; however, we were unable to determine whether the observed reduction is a result of the inability to bind to VP16 (or gE) or due to deleterious effects of the mutation on the efficiency of gE (or VP16) binding (Fig. 6.1). Nonetheless, our results suggest that in a fashion analogous to many other aspects of herpesvirology, VP16 and gE binding may act in a redundant, or perhaps additive fashion, to facilitate incorporation of VP22 into assembling virus particles.

VP22 is packaged into virions during final envelopment as nucleocapsids bud into TGN-derived vesicles (469). This observation suggests that interaction between VP22 and viral proteins on the cytoplasmic face of the TGN vesicle, perhaps in concert with binding to tegument proteins located on the surface of the approaching capsid, may ensure accrual of VP22 in the tegument of the assembling particle. The cytoplasmic tail of gE may act as a scaffold for recruitment of VP22 to the cytosolic face of TGN-derived vesicles. VP16 is added to the nucleocapsid prior to final envelopment and its presence on the surface of capsids undergoing secondary envelopment may also contribute to packaging of VP22 into the tegument (469). In addition, VP16 can associate with infected cell membranes through interactions with a variety of glycoprotein tails (270,783). Thus, membrane bound VP16 may also play a role in virion incorporation of VP22. To examine whether these two viral proteins work redundantly to facilitate incorporation of VP22, the VP22 mutant [VP22.LI (-)] which abrogates VP16 binding with minimal disruption to the gE binding activity, could be analyzed in a
transfection/infection-based virion packaging assay in a gE-null background. (The reciprocal experiment would not be possible as VP16-null viral mutants fail to undergo secondary envelopment). If VP16 and gE are the only binding partners that facilitate VP22 virion incorporation then one would expect packaging to occur at background levels. Alternatively, packaging studies could be undertaken with a VP22 construct that harbors both point mutations which individually abrogated interaction with VP16 or gE. However, in light of the sensitivity of VP22 to mutation and the deleterious effect that each mutation had on the efficiency of the residual binding activity, a dual mutation may result in gross disruption of protein structure. Such an outcome may perturb other heretofore unidentified activities within VP22 that contribute to its virion incorporation.

In addition to binding to gE and VP16, VP22 has been reported to interact with the cytoplasmic tail of gD, an interaction which may link VP22 to the site of final envelopment (109). Whether gD binding plays a role in virion incorporation of VP22 remains to be elucidated. However, it is curious to note that a VP22 construct (VP22.87-301), which binds to both VP16 and gE at wild-type levels, is not incorporated into virus particles at optimal efficiency (Fig. 5.9). Wild-type levels of incorporation were attained when amino acids 44-301 of VP22 were examined (Fig. 5.9). This result suggests that additional incorporation determinants for VP22 exist, presumably located within residues 44-86. The domain of VP22 which interacts with gD has not been characterized; however, a conserved acidic cluster of amino acids is found within this region of VP22 encompassing residues 61-77. Previous studies from our group have identified the acidic cluster of VP22 as necessary for localization of the protein to the TGN. Acidic clusters are well characterized trafficking motifs, shuttling proteins to and from the Golgi
apparatus. Considering the dynamic nature of the TGN, one would anticipate that a resident protein such as VP22 would contain sorting sequences that facilitate its vesicular trafficking and retrieval (66). Many herpesvirus proteins utilize such motifs to localize to the TGN (5,7,406,446,701). An acidic cluster of amino acids appears to be required for virion incorporation of the HSV-1 tegument protein UL11; however, this motif is not sufficient, as UL11 constructs which localize to the TGN and associate with membranes are not packaged into virus particles (408).

Examination of the contribution of the acidic cluster of VP22 to virion incorporation demonstrated that the motif is required to facilitate wild-type levels of incorporation of VP22 into the virion (Fig. 5.11). Furthermore, upon deletion of the primary acidic cluster from VP22, a secondary group of acidic amino acids, encompassing residues 61-64, could in some measure, functionally substitute. Deletion of the acidic cluster motif had no detrimental effect on the gE and VP16 binding activities of VP22 (Fig. 5.10); however, the effect on gD binding was not determined. Although one cannot rule out the possible contribution of gD binding, or other unidentified activities which may map to this region of VP22, the results suggest that the acidic cluster facilitates efficient incorporation of VP22 into the virion. Presumably, the motif facilitates localization at the TGN, where protein-protein interactions with gE and/or VP16 result in incorporation of VP22 into the tegument region of assembling virions.

In light of these findings a logical model for virion incorporation of VP22 suggests that the protein associates with membranes, with specific localization to the TGN, facilitated by an acidic cluster of amino acids. At the TGN, protein-protein
interactions with gE and perhaps membrane associated VP16 ensure retention of VP22 in the assembly pathway. Upon secondary envelopment of VP16-coated capsids at this cellular site, VP22 is packaged into assembling virus particles. In absence of an acidic cluster, interactions with VP16 and/or gE may only recruit a portion of VP22 into the assembly pathway (perhaps through encounters with VP16-coated capsids, or with the cytoplasmic tail of gE as VP22 traffics through the TGN) with the remainder continually trafficking between vesicles, unable to be retrieved to the TGN.

Previous studies have shown that the herpesvirus tegument is flexible regarding both protein composition and stoichiometry (776,777). Overexpression of VP22 within infected cells results in a 3-fold increase in its incorporation (391); an observation that is consistent with the hypothesis that virion incorporation of a tegument protein is partly determined by local protein concentration. Throughout our studies we noted that the quantity of VP22 packaged in our virion incorporation assay was variable, with levels of incorporation correlating with VP16 and gE binding efficiencies. Interestingly, a VP22 mutant (F196W) which binds to gE and VP16 at levels greater than wild-type resulted in increased incorporation of VP22 when compared to the full-length VP22-GFP construct, despite similar levels of expression within the cell (Fig. 5.5). Although coarsely measured, it is fascinating to speculate that this particular mutation may result in a VP22 molecule that has a higher affinity than other virion components for binding sites on gE and/or VP16, possibly out competing them for incorporation into the virion. It would be interesting to determine whether virion packaging of tegument components known to bind VP16 and/or gE (e.g. vhs or UL11) is reduced upon expression of this VP22 point mutant rather than the native protein (199,619,646). Conversely, as a VP22-null virus
packages decreased amounts of both gD and gE (179,182), would expression of F196W result in enhanced incorporation of gD, gE, and other VP22 binding partners? However, as tegument proteins have been shown to be involved in numerous protein-protein interactions, one must be cautious when interpreting the direct impact of a single protein on the specific composition of the viral particle. The complexity inherent to the mechanisms of tegumentation and envelopment also extends to comprehension of experimental results pertaining to the process.

**MEMBRANE ASSOCIATION OF VP22**

VP22 possesses the ability to associate with cellular membranes and target to the TGN independently of other viral components (66). This suggests that VP22 contains intrinsic determinants that facilitate localization to the site of final envelopment, where it becomes incorporated into the tegument. Membrane association of VP22 maps to a region of the protein that encompasses amino acids 120-225 (66). Studies contained within this report further defined this domain to residues 165-225 (Fig. 4.7 and Fig. 6.2). Although expression of this region results in a subcellular localization reminiscent of wild-type VP22 (Fig. 6.3B), the construct is not packaged into assembling virus particles, implying that membrane association is not sufficient to facilitate virion incorporation (Fig. 4.8). One could argue that this construct lacks an acidic cluster and thus, although membrane associated, fails to localize to the TGN. However, VP22.165-270 also lacks an acidic cluster and yet is packaged into virus particles (Fig. 4.8).
Figure 6.2. Functional Domains of VP22. A schematic representation of the various domains within VP22 identified to date. The amino acid residues that comprise the acidic cluster of VP22, and the conserved residues that were subject to mutagenesis during our studies, are highlighted in red.
Membrane association domain
Minimal VP16 binding domain
Domain that facilitates efficient VP16 binding
gE binding domain
Virion incorporation domain
Dimerization domains
Nucleic acid binding domain
Furthermore, many of the point mutations of VP22 resulted in constructs that fail to be incorporated into virus particles, despite demonstrating a punctuate fluorescence indicative of membrane association (Fig. 5.1 and Fig. 5.5). Other HSV-1 tegument proteins such as UL11 display a similar phenotype, with localization and membrane association at the TGN insufficient to facilitate virion incorporation (408). Presumably, additional activities are required for virus packaging; perhaps specific protein-protein interactions or posttranslational modifications are important determinants for incorporation of VP22 into virions.

The mechanism of VP22 membrane association is unknown. However, as membrane association occurs in the absence of viral proteins, VP22 may bind to membranes either directly by interacting with phospholipids, or indirectly by binding to a cellular membrane-associated protein. VP22 contains a high number of basic residues and is susceptible to extraction from membranes under high electrostatic conditions (66). Thus, one could hypothesize that positively charged basic amino acid residues of VP22 interact with acidic phospholipids of cellular membranes. Intriguingly, of all the constructs tested, VP22.165-225 demonstrates the highest level of membrane association by gradient analysis and also exhibits the highest net positive charge (number of basic residues minus acid amino acids) (Fig. 6.3). Furthermore, amino acids 241-261 of VP22 demonstrate sequence similarity to a protein kinase C (PKC) homology-2 (C2) domain (Fig. 6.4). C2 domains are Ca^{2+}-dependent membrane binding domains that cooperatively increase the affinity of basic residues for membranes (314,315). Although a crystal structure of VP22 has yet to be attained, circular dichroism performed on amino
Figure 6.3. Potential Role of Protein Charge in Membrane Association of VP22.

(A) Primary sequence of VP22 with basic residues colored blue and acidic residues denoted in red. (B) Localization and net charge of various membrane-associated domains of VP22. Vero cells were transfected with the indicated VP22-GFP constructs, and 20 h later, they were infected with HSV-1. At 18 h post-infection, (38 h post-transfection), cells were fixed with paraformaldehyde and examined by confocal microscopy with the appropriate wavelength to excite GFP. Net charge was calculated by subtracting the number of acidic residues within a domain from the number of basic residues.
A.

MTSRRSVKSG PREVPREDYE DLYYTPSSGM ASPDSPPDTST RRGALQTRSR
QREGEVRFVQY DESDYALYGG SSSEDDEHPE VPRTRRPVSSG AVLSGPGPAR
APPPPAGSGG AGRTPTTAPR APTRTQKVATK APAAAPAETT RGRKSAQPES
AALPDAPAST APTRSKTPAQ GLARKLHFST APPNPAPWT PRVAGFNKRV
FCAAVGRLLA MHRMAAVQL WDMSRPRTDE DLNELLGITT IRVTVCEGKN
LIQRANELVN PDVVDVDVA TATRGRSAAS RPTERPRAFAR SASSRPPRPV
E

B.

Net Charge
(Basic Residues minus Acidic Residues)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Charge</th>
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<tr>
<td>VP22</td>
<td>+14</td>
</tr>
<tr>
<td>VP22.165-225</td>
<td>+8</td>
</tr>
<tr>
<td>VP22.165-270</td>
<td>+3</td>
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Figure 6.4. Putative Protein Kinase C Homology-2 Domain in VP22. The alignment of amino acids 241-261 of VP22 with a consensus protein kinase C (PKC) homology-2 (C2) domain is shown. Blue text indicates identity, and green text denotes similarity.
HSV-1 VP22

Consensus C2
acids 159-301 of VP22 indicates that the protein is capable of binding divalent cations (376).

If VP22 does interact with cellular membranes through its basic residues, this would not be the first description of such a viral protein. The membrane-binding domain of Rous sarcoma virus Gag protein consists of 11 basic residues within an 86 amino acid domain (81). Basic residues are also a significant component of the membrane-binding capacity of the VSV matrix protein, HIV-1 Gag, and Ebola virus VP40 (111,181,233,607,780).

Alternatively, VP22 may bind to a membrane-anchored protein to facilitate membrane association. This membrane bound protein would be cellular in origin as flotation analysis was performed in transfected cells. Curiously, in a fashion analogous to the membrane associated tegument protein vhs (483), membrane association of VP22 increases to approximately 100% upon infection with a VP16-null virus (personal communication from Michelle Bucks, Penn State College of Medicine, Hershey, Pennsylvania). Thus, perhaps the cellular factor that facilitates membrane association in the absence of infection is homologous to a viral or virally-induced protein. The interaction of VP22 with various glycoprotein tails could be informative regarding the identity of the cellular protein. First, the gE binding domain of HSV-1 VP22 maps to residues 165-270 which overlap the membrane association domain. Secondly, the putative gE/gM binding domain of PrV VP22 maps to a similar region. These findings suggest that glycoprotein cytoplasmic tail structure may be conserved. Alignment of the glycoprotein tails followed by database mining for cellular homologues may shed light on potential cellular binding partners of VP22. If the cellular protein is homologous to the
cytoplasmic tail of gE, one might predict that a VP22 construct in which the gE binding activity is disrupted would demonstrate decreased membrane association in transfected cells. Conversely, overexpression of the cellular binding partner of VP22 may increase membrane association of the protein to levels resembling those observed during infection. As a final point, if glycoprotein tails are the exclusive virally-induced means of facilitating membrane association of VP22, would a VP22 construct deficient for glycoprotein binding exhibit reduced membrane association? Moreover, would VP22 levels of membrane association upon infection with a gE/gD-null virus mimic those observed in transfected cells?

PUTATIVE FUNCTIONS OF VP22 AT EARLY TIMES DURING INFECTION

Collectively, the tegument could be considered the toolbox of the virion, delivering a myriad of proteins to the cell, many of whose functions have yet to be elucidated. Post-entry, the majority of tegument proteins dissociate from the incoming nucleocapsid, with a subset destined to prime the cell for synthesis of viral components (36,84,444,575). VP22 is one of the most abundant components of the tegument, and the complement packaged into assembling virions may play a variety of roles in newly infected cells.

Some groups have noted a nuclear accumulation of VP22 during infection (479,558). Following HSV-1 infection the incoming genome is in a linear non-nucleosomal state, with IE gene activation stimulated by the incoming structural protein VP16 (36). During latency the viral genome is in a largely transcriptionally repressed circular configuration associated with non-acetylated histones. VP22 interacts with
cellular template-activating factor I (TAF-I), a chromatin remodeling protein that promotes ordered transfer of histones to naked DNA (470,520,712). The interaction with TAF-I prevents nucleosome deposition on DNA. Thus, VP22 is hypothesized to prevent rapid recruitment of repressive histones to input DNA by inhibiting the activity of TAF-I (712).

In addition to the viral proteins contained with virions, RNA transcripts of both viral and cellular origin have been detected in the tegument of HSV-1 virions (40,60,624). While only a fraction of viral open reading frame (ORF) transcripts are represented, suggesting possible selectivity, the cellular RNAs epitomize the abundant RNAs within infected cells during virion formation (624). Thus it is largely unclear whether any of the cellular transcripts are specifically recruited into herpesvirus particles, or whether they only represent filling material. Although the role of virion RNA is unknown, the prevailing hypothesis posits that packaged RNAs prime a newly infected cell to create an environment for efficient initiation of infection. Interestingly, VP22 is one of three tegument proteins that can bind RNA. The other proteins are UL47 and the product of the U511 gene; however, US11 is not required for the packaging of RNA into virus particles (624,625). Given the abundance of VP22 within the tegument region it may facilitate packaging of RNA into virions. Furthermore, recombinant VP22 consisting of only residues 159-301 can bind short oligonucleotides, inducing the assembly of spherical particles, termed Vectosomes (510). VP22 has been shown to mediate translocation of RNase sensitive genetic information from one cell to another where it is subsequently expressed (509). Thus, Vectosomes may allow VP22 to bind RNA in the cell where the protein is made and carry the RNA to adjacent uninfected cells.
where it is expressed. Although the VP22 domain that binds to RNA has yet to be elucidated, it is of interest to note that amino acids 198-262 demonstrate sequence similarity to a nucleic acid binding domain known as a helicase and RNase D C-terminal (HRDC) domain (Fig. 6.5).

Alternatively, similar to RNA viruses (e.g. retroviruses), it is possible that packaged RNA functions during assembly by acting as a framework upon which the major structural proteins assemble (85). If indeed RNA is a structural component of the tegument, it would be of interest to examine whether RNA is present within L particles. The tegument architecture of L particles is not dependent upon the presence of a nucleocapsid, which may indirectly provide a substructure (442,674). Removal of the envelope and subsequent application of exogenous RNase may highlight possible contributions of RNA to the tegument structure.

Given the ability of VP22 to bind RNA, it is interesting to note that a recent study from Taddeo et al., (2007) demonstrated that accumulation of the vhs protein requires both VP16 and VP22 (625,675). As the steady-state level of vhs mRNA is not dependent on the presence of VP16 or VP22, the authors propose that in the absence of VP16 and VP22, vhs functions to sequester mRNA in compartments inaccessible to the cellular translational machinery (675). VP22 only binds to vhs in the presence of VP16, an interaction that is believed to rescue vhs mRNA (perhaps through the ability of VP22 to bind RNA) and make it available to the machinery of the cell for translation (675). It is intriguing to speculate that VP22 may also direct vhs mRNA to adjacent uninfected cells, another possible mechanism by which HSV-1 alters the cellular environment to facilitate efficient initiation of infection.
Figure 6.5. Potential Helicase and RNase D C-Terminal Domain in VP22. The alignment of amino acids 198-262 of VP22 with a consensus helicase and RNase D C-terminal (HRDC) domain is shown. Blue text indicates identity, and green text denotes similarity.
HSV-1 VP22: KRVFCAAVGLAAHARMMAAVQ------------LWDM--SRPRTDE
Consensus HRDC: RERQLRLRALREWDEIAREEDVSPYVVLPDATLDIAEKLPRTVA

HSV-1 VP22: DLNELLGITTIRTVCEG--KNLQRANELVNPD
Consensus HRDC: ELLAIDGVGERKRRYGKEILAVIRAARDGPSEA
POTENTIAL ROLES OF VP22 DURING ASSEMBLY AND EGRESS

One of the final steps in the HSV-1 assembly pathway is envelopment of the viral nucleocapsid at a cytoplasmic vesicle, resulting in acquisition of a virion envelope and full complement of tegument and viral glycoproteins. Viral glycoprotein tails play a critical role in this envelopment step as evidenced by a gE/gD-null mutant in HSV-1 and a gE/gM-null virus of PrV, which form unenveloped capsids in the cytoplasm embedded in tegument-like material (58). Presumably glycoprotein tails act as a scaffold upon which tegument proteins aggregate to bridge the budding nucleocapsid and membrane, facilitating secondary envelopment. The cytoplasmic tail of VZV gpI (equivalent to HSV-1 gE) is required to localize tegument proteins to the cis-face of TGN cisternae (725). In the absence of the cytoplasmic tail, tegument proteins fail to accumulate on one face of the TGN and instead form a uniform coat surrounding the vesicle, with resultant disruption to final tegumentation and envelopment (725).

In a reciprocal fashion, VP22 may be one of a number of tegument proteins that act redundantly to ensure enrichment of glycoprotein tails at the TGN (179,182). Curiously, virus particles assembled in the absence of VP22 show reduced virion packaging of the glycoprotein binding partners of VP22 (179,182). Thus, interaction of VP22 with gE and gD, as it relates to virion incorporation, appears to be mutually beneficial, facilitating efficient incorporation of both binding partners. VP22-dependent aggregation of gE and gD at the site of final envelopment may occur through retention of the glycoproteins at one face of the TGN. Alternatively, the acidic cluster of VP22 may facilitate retrieval of glycoproteins from other cellular sites to the TGN, thereby ensuring their aggregation at the site of final envelopment and subsequent virion incorporation.
In addition to the essential role of certain viral proteins, cellular factors are also required to facilitate secondary envelopment of HSV-1, specifically the ESCRT complex which mediates the pinching-off step (135). A motif known as an L domain is believed to recruit the ESCRT complex to the site of viral budding. Putative L domains are present in several HSV-1 structural proteins, perhaps signifying the importance of engaging ESCRT proteins for the assembly of infectious virions. As outlined above, simultaneous deletion of gE and gD from HSV-1 blocks secondary envelopment; however, deletion of either glycoprotein alone has little effect (197). Curiously, gE is one of the structural proteins that possess a potential L domain whereas gD does not. Although, VP22 harbors sequences which are similar (STAP, PDAP, and PTTAP) to the consensus sequence of an L domain (PTAP), it is unlikely to be able to recruit the ESCRT complex. However, VP22 may recruit an L domain containing protein to the TGN, and anchor it at the membrane through its ability to bind to the cytoplasmic tail of gE or gD. Thus, in the absence of gE or gD, ESCRT complexes would still be recruited, either directly through the putative L domain within gE or indirectly through VP22-dependent recruitment. In the absence of both glycoprotein tails, secondary envelopment is unable to proceed as both methods of ESCRT recruitment are abrogated. It would be relatively straightforward to test the basic tenets of this hypothesis. Namely, that deletion of both gE and gD abrogates VP22 association with membranes of the TGN and that a gE/VP22-null mutant would be unable to complete secondary envelopment.

During the assembly process, tegumentation occurs at two distinct sites; on nucleocapsids (in the nucleus and during transit though the cytoplasm) and at the cytoplasmic face of TGN vesicles. Biochemical analyses suggest that certain tegument
components, including vhs, VP16, VP1/2, and UL37, are acquired at both locales (488,576,699). VP22 localizes to the site of final envelopment and is a constituent of a myriad of protein-protein interaction networks involving gD, gE, VP16, vhs, ICP0, and ICP4 (109,183,514,515). Thus, in addition to interacting with glycoproteins at the site of final envelopment, VP22 may recruit other tegument proteins to the TGN. To date, the consequences of VP22 deletion on recruitment of proteins into the tegument region has been restricted to analysis of virus particles produced in the absence of VP22. However, tegument proteins, which are normally incorporated into virions at both tegumentation sites, may be present in VP22-null virus particles due to their interaction with nucleocapsids; and thus inhibit detection of subtle differences which occur at the membrane of VP22-null virus infected cells. Therefore, to elucidate the role VP22 plays in recruiting or retaining virion components at the site of final envelopment, one should examine the protein composition of infected-cell membranes from a VP22-null infection.

The process of cell-to-cell spread enables HSV-1 virions to exit infected cells and enter uninfected neighboring cells without being exposed to the host humoral immune response. In polarized epithelial cells, cell-to-cell spread is the primary mode of virus transmission, a process mediated by the gE/gI heterodimer (750). The presence of the heterodimer in the membrane of virion harboring vesicles affects intracellular sorting decisions resulting in transport of virions specifically to lateral cell surfaces (339). Interestingly, plaque sizes are dramatically reduced (~95%) when Vero cell monolayers are infected with a HSV-1 VP22-null virus, suggesting a possible role for VP22 in viral spread (179). Furthermore, VP22 is an essential protein of MDV and VZV (151,175,394,395,690). MDV and VZV are highly cell-associated herpesviruses both in
vitro and in vivo and spread directly from cell-to-cell, with little to no infectious virus released (120,175,268). The cytoplasmic tail of gE houses a variety of trafficking signals which were initially assumed to mediate transport of virion containing vesicles to lateral surfaces of the cell (198). However, a recent study of determinants within the cytoplasmic tail of gE that are required to promote cell-to-cell spread mapped the activity to a region which contains no obvious cell sorting motifs (198). Furthermore, gE/gI remains at the TGN compartment when other HSV proteins are not present. Thus, a viral protein may bind to the cytoplasmic tail of gE unmasking cryptic sorting sequences that promote delivery to cell junctions. Alternatively, the gE/gI heterodimer may interact with other HSV-1 proteins that contain intrinsic trafficking sequences, which direct cell-to-cell spread.

Interestingly, the region of the gE that facilitates cell-to-cell spread also binds to VP22 (198). Although, VP22 binding may simply expose a concealed trafficking signal within the cytoplasmic tail of gE, intriguingly VP22 itself houses a variety of putative trafficking motifs (Fig. 6.6). HSV-1 VP22 possesses two dileucine motifs, an acidic cluster, and a tyrosine-based motif (YEDL), which are present in both VZV and MDV (Fig. 6.7). However, the functional significance of these motifs for trafficking of VP22 has yet to be elucidated. Thus, it is unclear which motifs may function in directing virions to the basolateral surfaces of the cell. Nevertheless, upon deletion of the acidic cluster from VP22, the protein is detected at puncta reminiscent of endosomes which are distal from the nucleus (unpublished results from Michael Brignati). Hence, the acidic cluster is proposed to retrieve VP22 from endosomes to the TGN,
Figure 6.6. Putative Role for the Interaction Between VP22 and gE in Cell-to-Cell Spread. After secondary envelopment at a *trans*-Golgi network (TGN)-derived vesicle, virions are directed to basolateral surfaces of the cell rather than the apical surface. The cytoplasmic tail of glycoprotein E (gE) has been implicated in mediating trafficking of these virion containing vesicles to the cell periphery. In one model, denoted (A), VP22 (blue protein) binds to the cytoplasmic tail of gE (red membrane bound protein) with resultant exposure of a cryptic trafficking signal within gE. This putative trafficking motif subsequently directs transport of the vesicle to the cell periphery (pathway denoted by a red arrow). An alternative model (B) posits that VP22 promotes cell-to-cell spread by binding indirectly to virion containing vesicles through the cytoplasmic tail of gE, with subsequent direction to basolateral surfaces of the cell facilitated by intrinsic trafficking motifs within VP22. This pathway is denoted by the blue arrow.
Interaction of VP22 with gE unmask a cryptic sorting sequence.

VP22 binds to gE and uses an intrinsic trafficking motif to direct vesicles to basolateral surfaces.
Figure 6.7. Conservation of Putative Trafficking Motifs across VP22 Homologues. The sequence alignment of VP22 homologues from HSV-1, MDV and VZV is shown. The residues highlighted in red comprise potential acidic cluster motifs, those in green represent tyrosine-based motifs, whereas the blue residues may function as dileucine motifs.
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<th>HSV-1</th>
<th>VZV-D</th>
<th>MDV-GA</th>
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<td>MTSRRSVKSGPREVPREDYEYLDYYTPSSCMASPDSPDPDTSSRAKOQTRAPRGEVRVFQYDESDYALYGGSSSEDDE</td>
<td>-MASSGDRLCRSN---------AVRRKTTPSYSQYRTARSSVVGPPDDSDSGLGYITTVGADSPSP</td>
<td>-MGDSERRKSERRSLGYPAYDDLSPARRPSTRTQRNLNQDDLKHGSFDTDHPTQKHAKASAVSEDESSTTRGGFT</td>
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<tr>
<td>HSV-1</td>
<td>HFEVPRTRRFVGAVLSAPGPARAPPPAGSSEGAG---RTPTTAPRAPARTQTVATKAFAPAAEETTRGRKSAQPESAALP</td>
<td>VYADLYFEHKNTPRVHQPNDSSGSEDDEDFIDDEVVAIFELRHVELVEDAYVENPLSVKPSFSTKNAAVPKLE</td>
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<tr>
<td>HSV-1</td>
<td>DAPASTAPRSTPKPAOQLRKLHDSAPPNDAPWTPRVAFTQFKVFAVGLAMARMAAVQLWDMSRPRTEDDL</td>
<td>DAPK-RAAPGAGAIAAG---RPIFSTAPKTATSSWCQPTPSYKRFCEAVRVRVAMQAQKAACAAWNSNPPRNNEEL</td>
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<tr>
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<tr>
<td>HSV-1</td>
<td>E----------</td>
<td>QTGRTINRSRASRASRTDTRK</td>
<td></td>
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<tr>
<td>HSV-1</td>
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<tr>
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<td>MDV-GA</td>
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in a manner analogous to the role of the acidic cluster of gE or furin (724). Plasma membrane targeting may be facilitated by the tyrosine-based motif, which performs a similar function in furin, with the dileucine motifs responsible for subsequent endocytosis (670,682). Thus, VP22 may promote cell-to-cell spread by binding indirectly to virion containing vesicles through the cytoplasmic tail of gE, with subsequent direction to basolateral surfaces of the cell facilitated by a tyrosine-based motif (Fig. 6.6). After virion release, VP22 (and perhaps gE), are endocytosed and return to the TGN through the actions of a dileucine motif and acidic cluster. This model is eminently testable. A HSV-1 viral mutant, which expresses a VP22 protein unable to bind to gE, would elucidate the role of the interaction in cell-to-cell spread. While sequential mutation of the putative trafficking motifs within VP22 combined with phenotype analysis and localization studies of VP22 within the infected cell, would also test the hypothesis.

Perhaps the best-characterized exploitation of the host cell cytoskeleton to facilitate virus release is that of vaccinia virus, which has been shown to induce actin polymerization directly behind its virus particle as a means of propelling the virus through the cell (136,137). The virus protein(s) responsible for this activity has not yet been identified, but it has been shown that disruption of the actin cytoskeleton in infected cells inhibits virus release, indicating that actin is essential to the virus replicative cycle (540). In addition to a potential role in directing virions to basolateral surfaces of the cell, VP22 may facilitate cell-to-cell spread through modification of the cytoskeleton of infected cells.

VP22 exhibits the properties of a classical cellular microtubule-associated protein, binding to microtubules and reorganizing them into thick bundles which are highly
resistant to depolymerizing agents such as nocodazole (185). Microtubules contained in these bundles are modified by acetylation, a marker for microtubule stability. VP22 colocalizes with microtubules in both transfected and infected cells, although VP22-induced microtubule stabilization in transfected cells is more efficient than that observed in HSV-1-infected cells (185). Presumably VP22 has multiple roles during the replicative cycle. Therefore, while the majority of the VP22 population in transfected cells is available for assembly into microtubule bundles, it is likely that a fine balance exists between the different activities of VP22 in an infected cell. Studies suggest that underphosphorylated VP22 binds to microtubules (562). Hence, differential phosphorylation may dictate the portion of VP22 distributed between the activities of microtubule stabilization and virion assembly. Interestingly, the amount of gD packaged into virions is reduced when a constitutively hypophosphorylated VP22 is expressed during infection (562).

VP22-induced microtubule stabilization may facilitate capsid trafficking, particularly in neurons, where the viral capsid has a considerable distance to travel from the nucleus to the periphery of the cell. Intriguingly, VP22 binds to the heavy chain of the molecular motor kinesin-1, which transports cargo away from the nucleus to the cell periphery (162,707). Furthermore, VP22 recruits VP16 to microtubules, and thus may facilitate transport of VP16-coated capsids to the site of final envelopment. The interaction of VP22 with microtubules may also be involved in virus egress from the cell, assisting transport of virion-containing vesicles to the cell boundary.

VP22 also interacts with actin filaments through an association with actin-associated nonmuscle myosin IIA; a motor protein which is recruited to the actin
cytoskeleton and has been reported to be involved in numerous dynamic cellular processes, including vesicle secretion (629,711). Inhibition of the ATPase activity of nonmuscle myosin IIA with the myosin-specific inhibitor butanedione monoxime, fails to severely inhibit entry of HSV-1, but does impair virus release (711). HSV-1-infected cells frequently form pronounced plasma membrane protrusions which establish intimate contact with adjacent cells (711). Interestingly, GFP-VP22-containing particles are detected with nonmuscle myosin IIA-containing filaments which traverse these protrusions (711).

The actin cytoskeleton has been implicated in facilitating transport over short intracellular distances. An accepted concept is that long-range movement of vesicles occurs on microtubules and short-range movement occurs on actin filaments. Nerve ends contain few microtubules, and synaptic vesicles are thought to travel on actin cables at the periphery, beyond the reach of microtubules (68). Thus, VP22 may mediate cell-to-cell spread through coordinated sequestration of both microtubule and actin cytoskeletal components.
SUMMARY

Although the major steps of herpesvirus assembly have recently been elucidated, the molecular mechanisms utilized by viral proteins to target to the site of final envelopment and interact to form stable virions, remain poorly characterized. The studies presented within this dissertation have focused on providing insight into the mechanisms utilized by tegument proteins to facilitate virion incorporation. Specifically, the protein-protein interactions and virion incorporation determinants of the HSV-1 tegument protein, VP22, were dissected to elucidate the functional significance of these activities in facilitating packaging of VP22 into assembling virions.

Despite the abundance of VP22 within virions, little is known regarding its role during HSV-1 infection. Curiously, the protein-protein interactions and trafficking motifs characterized within these pages are also implicated in many of the putative activities of VP22. Thus, our studies have implications beyond the molecular mechanisms that mediate virion incorporation of VP22, and may act as a foundation to facilitate elucidation of the activities of VP22 during HSV-1 infection.
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