The Pennsylvania State University

The Graduate School

Intercollege Graduate Program in Genetics

INTERACTIONS BETWEEN THE TEGUMENT PROTEINS, UL11 AND UL16, AND THE GLYCOPROTEIN E OF HERPES SIMPLEX VIRUS

A Dissertation in

Genetics

by

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Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

December 2009

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ABSTRACT

The herpesvirus tegument region present between the virion envelope and the nucleocapsid contains more than 20 different virus-encoded proteins. The process of tegument assembly and final envelopment has been unfoldeding over the past few decades. It is thought that a few tegument proteins are added to the capsid in the nucleus, whereas most of them are acquired after entering the cytoplasm or traveling to the site of final envelopment at the *trans*-Golgi network. Overall, the research presented in this dissertation provides insights into the molecular mechanism of protein-protein interactions that may be involved in (or contribute to) assembly and maturation of herpes simplex virus type 1 (HSV-1).

The UL11 (membrane-bound) and UL16 (capsid-associated) tegument proteins are conserved among all herpesviruses, and interaction between the two has been implicated in linking the viral capsid, tegument, and membrane during final envelopment process. Both in transfected and virus-infected cells, a subpopulation of the UL11 protein was found associated with detergent-resistant membranes via modifications with two fatty- acid chains (myristate and palmitate). UL11 can directly interact with UL16 in a manner dependent on the acidic cluster and leucine-isoleucine motifs of UL11. And, *N*-ethylmaleimide-modified UL16 was found to be incapable of binding UL11, suggesting that free cysteines in UL16 somehow play a role in the interaction.

UL16 is stably associated with cytoplasmic capsids isolated from infected cells. In response to initial attachment of virus to the cell surface, an ‘outside-in’ signal is transmitted across the virion membrane, and as a result, UL16 is dissociated from the capsid. The mechanism by which the signal is sent to UL16 remains unclear but seems

likely to be mediated by the glycoproteins on the virion envelope. A GST chimera bearing the cytoplasmic tail of glycoprotein E (gE.CT) was found to be capable of binding UL16 expressed in mammalian or insect cells. To better understand the molecular mechanism of this signaling process, the interaction network emanating from UL16 was investigated. In addition, previously using a GST pull-down assay, it was observed that UL16 interacts with virus-specific proteins from HSV- and PRV-infected cell lysates, providing evidence that UL16 is present in protein complexes.

To characterize native complexes that contain UL16, a recombinant virus was constructed expressing a tagged derivative. Using the combination of tandem affinity purification and mass spectrometry analysis, we identified gE to be present in the complexes isolated from infected cells. The UL16-gE interaction was confirmed in co-immunoprecipitation assays with infected cell lysates. Moreover, mutational analyses of gE.CT have suggested that in infected cells UL16 may interact with gE.CT in both UL11-dependent and -independent manners. Based on all available data, we hypothesize that UL11, UL16, and gE may form a tripartite complex which plays a role in multiple aspects of the virus life cycle, including signaling events during virus attachment, virion maturation, or cell-to-cell spread.

Collectively, our research focused on the protein-protein network has built a foundation for future studies, and also advanced our current knowledge of herpesvirus replication.

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LIST OF ABBREVIATIONS

α-TIF	alpha *trans*-inducing factor
AC	acidic cluster
AD	activating domain
AP	adaptor protein
ATP	adenosine triphosphate
BAC	bacterial artificial chromosome
BD	binding domain
bp	base pair
C-terminal	carboxyl-terminal
CBP	calmodulin binding peptide
CFP	cyan fluorescent protein
CMV	cytomegalovirus
CPE	cytopathic effect
CT	cytoplasmic tail
DMEM	Dulbecco's modified Eagles medium
DNA	deoxyribonucleic acid
dsDNA	double-stranded deoxyribonucleic acid
DRM	detergent-resistant membrane
DTT	dithiothreitol
E	early
EBV	Epstein-Barr virus
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
EHV	equine herpesvirus
EM	electron microscopy
ER	endoplasmic reticulum
ESCRT	endosomal sorting complexes required for transport
ESI	electrospray ionization

FBS	fetal bovine serum
gX	glycoprotein X (X= B, C, D, E, H, I, K, L, etc.)
galK	galactokinase
GFP	green fluorescent protein
GST	glutathione *S*-transferase
GTPase	guanosine triphosphatase
HCMV	human cytomegalovirus
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
HHV	human herpesvirus
His_6	hexahistidine
HS	heparan sulfate
HSV	herpes simplex virus
HVEM	herpesvirus entry mediator
ICP	infected cell protein
IE	immediate-early
IgG	immunoglobulin G
kbp	kilobase pair
kDa	kilodalton
KSHV	Kaposi's sarcoma-associated herpesvirus
L	late
L particle	light particle
LC	liquid chromatography
LI	dileucine motif
m/z	mass-to-charge ratio
M	molar
MALDI	matrix-assisted laser desorption/ionization
MDV	Marek's disease virus
µg	microgram
min	minute
mL	milliliter
µL	microliter

mM	millimolar
mm	millimeter
MOI	multiplicity of infection
mRNA	messenger RNA
MS	mass spectrometry
MS-MS	tandem mass spectrometry
MT	microtubule
MVB	multivesicular body
ND10	nuclear domain 10
NEM	*N*-ethylmaleimide
NLS	nuclear localization signal
nm	nanometer
NPC	nuclear pore complex
°C	degree Celsius
ORF	open reading frame
PACS	phosphofurin acidic cluster sorting protein
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PFU	plaque forming unit
PKC	protein kinase C
PRV	pseudorabies virus
RICH-1	RhoGAP interacting with CIP4 homologues (Rho GTPase-activating protein 17)
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TAP	tandem affinity purification
TEV	tobacco etch virus
TGN	*trans*-Golgi network
TM	transmembrane
TOF	time-of-flight mass analyzer
UAS	upstream activating sequence

U_L	unique long
U_S	unique short
UV	ultraviolet
vhs	virion host shutoff
VP	virion polypeptide
VZV	varicella zoster virus
w/v	weigh/volume
WT	wild type
Y2H	yeast two-hybrid

ACKNOWLEDGEMENTS

First, I would like to thank my advisor, John W. Wills, for his guidance, encouragement, and dedication to this work. His enthusiasm in science has inspired me in many ways. He helped me grow as a scientist and develop ability of independent thinking, which has had a huge impact on me and will keep doing so in my life. Also, many thanks to my committee members, Richard J. Courtney, Rebecca C. Craven, and Sarah K. Bronson as well as two previous members, Anita K. Hopper (currently in Ohio State U.) and John M. Flanagan, for their time, caring, and criticisms to help me progress this project.

I would also like to acknowledge all the previous and current lab members who assisted me and contributed to the research in this dissertation. Their presence in the lab has made the lab an enjoyable environment to work every day. And, Hershey Medical Center core facility personnel and research support team in the Department of Microbiology and Immunology have also provided tremendous help to this work.

Lastly, I would like to thank my parents, S.Y. Yeh and F.Y. Chang, and other family members for encouraging me to pursue doctorate training. I appreciate their endless love and support.

Chapter I

Overview

Herpes simplex virus (HSV) genome encodes nearly 80 proteins, and at least 40 of them are included in the virion along with various host factors (163). The HSV particle is divided into three morphologically distinct structures (Fig. 1.1). In the center is a nucleocapsid which contains the viral double-stranded DNA (dsDNA) genome. Upon infection, HSV delivers the viral genome to the nucleus of infected cells and initiates viral gene expression and DNA replication leading to production of virus progeny. A proteinaceous layer surrounding the nucleocapsid, known as the tegument, is composed of more than 20 virus-encoded proteins which play critical roles in nearly every stage of virus replication. The nucleocapsid and the tegument are enclosed by a lipid bilayer derived from host membranes during virus assembly. The viral envelope is spiked with at least 11 viral glycoproteins that mediate virus entry and membrane fusion.

Due to the complexity of HSV structure, the molecular mechanism of how virus particles are assembled has been a topic of much interest. It is believed that capsid formation and genome encapsidation take place inside the nucleus of infected cells, and the process of tegumentation on newly formed nucleocapsids may begin in the nucleus or during nuclear egress. After entering the cytoplasm, unenveloped nucleocapsids are coated with more tegument proteins and are targeted to *trans*-Golgi network (TGN)-derived vesicles for final envelopment. Vesicles containing mature virions traffic to the cell surface via a secretory pathway and fuse with the plasma membrane, releasing infectious viruses into the extracellular milieu.

The process of tegumentation and final envelopment of nucleocapsids is thought to be accomplished by a complex network of protein-protein interactions between the

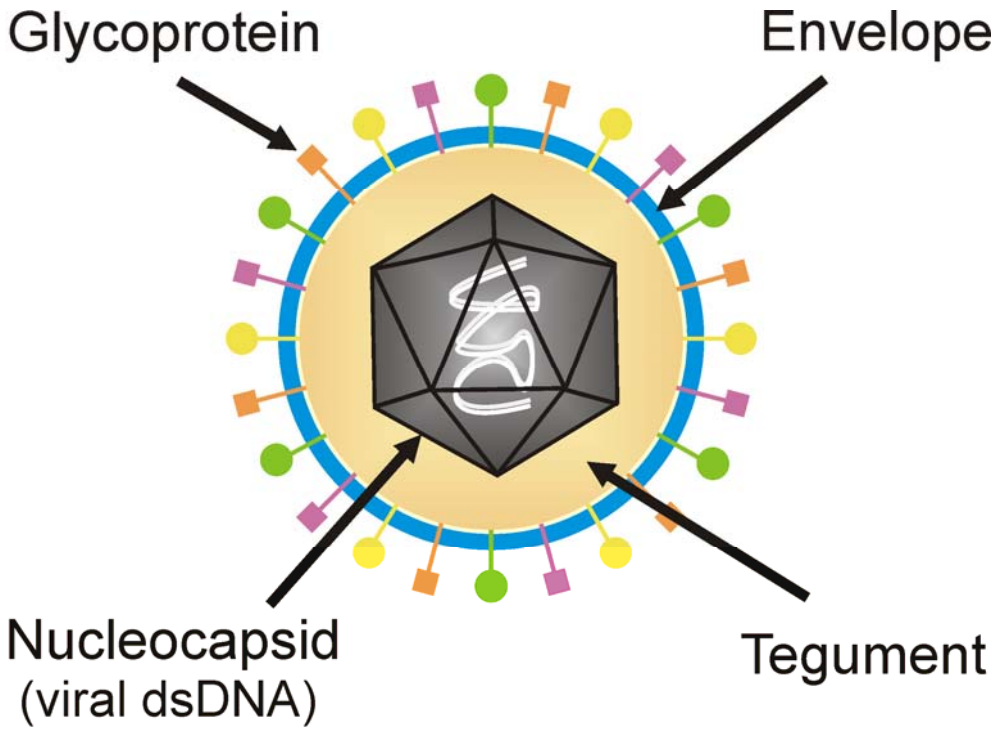


Figure 1.1 HSV virion structure. Three morphologically distinct regions are indicated. A viral envelope (blue) embedded with various glycoproteins (spikes) encloses the tegument (yellow) and the nucleocapsid (icosahedron). The tegument region is filled with proteinaceous materials surrounding the nucleocapsid, which contains the viral double-stranded DNA (dsDNA) genome (white lines).

tegument proteins and the glycoproteins (193). Thus, defining protein-protein interactions that may link the tegument and membrane will be fundamental to expand our current understanding of herpesvirus morphogenesis. The studies described in this dissertation focus on investigating the specific molecular mechanisms between the tegument proteins, UL11 and UL16, and the glycoprotein, gE (Fig. 1.2). A brief overview and a summary of observations are described below.

The UL11 and UL16 tegument proteins are conserved among all herpesviruses, and the interaction between the two is also conserved (161). Because UL11 is associated with the TGN and UL16 is bound to cytoplasmic capsids, the interaction has been proposed to play a role in linking capsids to the TGN for final envelopment (161). Consistent with a role in virus assembly, UL11- and UL16-null mutants have defects in virus production (10,11). However, the precise functions of UL11 and UL16 are still poorly defined. When expressed alone, UL16 is localized throughout a cell. But when coexpressed with UL11, a population of the UL16 molecules can be relocalized to perinuclear compartments reminiscent of the Golgi membranes, suggesting an interaction that is able to take place in the absence of other viral proteins. UL16 recognizes an acidic cluster (AC) and a leucine-isoleucine (LI) motif within UL11, both of which are also responsible for recycling of UL11 from the plasma membrane back to the TGN. Since UL16 interacts with the membrane-trafficking signals of UL11, it seemed possible that there might be host factors involved in the interaction. The studies described in Chapter IV reveal that UL11 is able to directly interact with UL16 in a manner dependent upon the AC and LI motifs, as previously defined. Experiments utilizing a chemical reagent *N*-ethylmaleimide (NEM) also indicate that free cysteine residues present in UL16 might

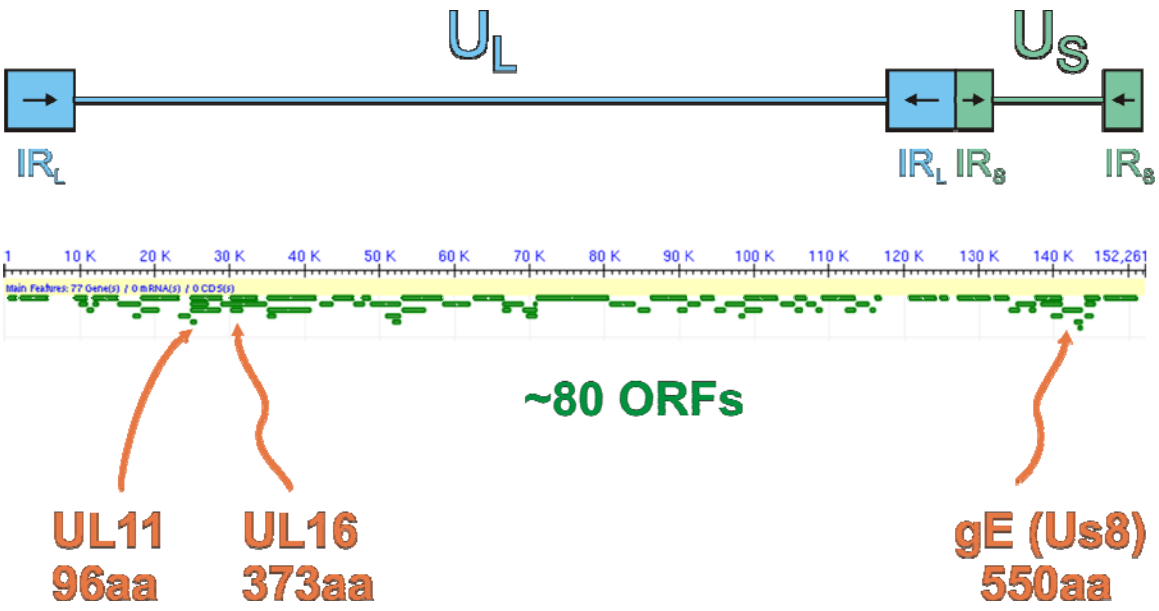


Figure 1.2 Genomic locations of the coding sequences for HSV-1 tegument proteins, UL11 and UL16, and glycoprotein gE. The HSV-1 genome consists of two regions, the unique long (U_L; blue) and unique short (U_S; green) and contains nearly 80 open reading frames (ORFs). The U_L and U_S regions are each flanked with inverted repeats at the ends (IR_L and IR_S, respectively). Genomic locations of the ORFs that encode the tegument proteins, UL11 and UL16, and the glycoprotein E (gE, the product of the U_S8 gene) are indicated.

somehow be involved in the interaction.

Recent studies of the UL16-capsid association have revealed that during virus attachment to its host receptors (heparan sulfate moieties of proteoglycans) a signal is sent across the virion membrane and triggers the release of UL16 from the capsid (191). This 'outside-in' signaling event has led to a speculation that UL16 may interact with the cytoplasmic tails of viral glycoproteins within a virion. Studies presented in Chapter V demonstrate that a GST chimera bearing the cytoplasmic tail of glycoprotein E (gE.CT) is capable of binding UL16 expressed in mammalian or insect cells, indicating that the interaction does not require other viral proteins. In addition, native complexes containing UL16 were isolated using tandem affinity purification technique from HSV-infected cells, and several cellular and viral proteins including gE were identified to be present in these complexes by mass spectrometry analysis. The UL16-gE interaction was confirmed in co-immunoprecipitation assays. Mutagenesis analyses of gE.CT have raised a hypothesis that in infected cells UL16 may interact with gE.CT directly or via UL11. Based on all observations, we speculate that UL11, UL16, and gE may form a tripartite complex which play a role in the signaling event during virus attachment or in other aspects of virus life cycle such as virion maturation or cell-to-cell spread.

The focus of this dissertation was to dissect the specific protein-protein interactions that may be involved in HSV replication. To rationalize and in turn appreciate the significance of the data presented later, it is necessary to understand the major stages of virus life cycle. Therefore, Chapter II will provide a review of the literature pertinent to these aspects.

Chapter II

Literature Review

Herpesviruses and Clinical Diseases

Herpesviruses are well known to be capable of causing overt diseases or remaining silent within their natural hosts for extended periods. Occasionally, latent viruses can be reactivated by certain physiological stimuli, such as stress and UV light, for the lifetime of infected hosts (283). Patients compromised by either immune therapy, underlying disease, organ transplant, or malnutrition are observed to be at increased risk for severe infection and recurrence (251).

The Herpesviridae family is a large group of double-stranded DNA (dsDNA) viruses, which are classified into three subfamilies (alpha, beta, and gamma) based on differences in where and how the viruses replicate and establish latency within natural hosts (250,252). Alphaherpesviruses, including HSV, varicella zoster virus (VZV), and pseudorabies virus (PRV), have a wide host range, establish latency primarily in neurons, and multiply efficiently with a short replication cycle, resulting in rapid disruption of infected cells. Betaherpesviruses, in contrast, have a narrow host range, replicate slowly, and establish latency in various tissues, including secretory glands and lymphocytes. Gammaherpesviruses also have a narrow host range, and are strongly associated with B and T lymphocytes.

To date, night herpesvirus types (HHV1-5, 6a, 6b, 7, and 8) have been isolated from human and are responsible for numerous clinical diseases (250,251). Among these viruses, HSV (-1 and -2, two serotypes) is prevalent throughout the population. Infection with HSV-1 is usually associated with cold sores of the lips and mouth, whereas HSV-2 usually causes sexually-transmitted genital herpes. It has become clear that either strain of HSV can cause either rash, though 75% of genital herpes is caused by HSV-

2. According to the Centers for Disease Control and Prevention, one in five of the general population in the United States has genital herpes while almost three out of four people have a history of cold sores caused by herpesviruses.

Despite replicating in different cell types and causing a wide variety of diseases, herpesviruses share certain features in common. First, all herpesviruses contain genes that encode enzymes for viral DNA synthesis and nucleic acid metabolism. These enzymes include a DNA polymerase, helicase-primase, ribonucleotide reductase, alkaline nuclease, and dUTPase (252). Second, productive infection of herpesviruses leads to death of infected cells, also known as cytopathic effect (CPE). Herpesvirus-induced CPE is usually characterized by alterations in morphology of host organelles, nuclei, and cytoskeleton. Third, they share structural similarities. A linear dsDNA genome is packed within an icosahedral capsid shell, which is surrounded by a proteinaceous layer termed the tegument and a lipid envelope derived from host membranes.

Virion Structure of HSV

HSV particles are generally spherical with the overall diameter of approximately 150 to 200 nm (112,252). Nearly 40 different viral proteins are distributed among three morphologically distinct regions in the virion: the inner nucleocapsid core, tegument, and envelope (Fig. 1.1). Components of each virion region are listed in Table 2. 1.

The nucleocapsid is an icosahedral capsid shell that encloses a linear viral dsDNA genome. Viral DNA is 152 kbp in size with a G+C content of 68% (255). It consists of two unique regions, a unique long (U_L) and a unique short (U_S) region, each terminally flanked with inverted repeats (Fig. 1.2) (181,183,229). Within infected cells,

Table 2.1 HSV virion components

Capsid	Tegument	Envelope
UL6	UL7	UL1 (gL)
UL18 (VP23)	UL11	UL10 (gM)
UL19 (VP5)	UL13 (PK)	UL20
UL26 (VP24)	UL14	UL22 (gH)
UL35 (VP26)	UL16	UL27 (gB)
UL38 (VP19c)	UL17	UL43
	UL21	UL44 (gC)
	UL23	UL45
	UL25	UL49A (gN)
	UL28	UL53 (gK)
	UL36 (VP1/2)	UL56
	UL37	US4 (gG)
	UL41 (vhs)	US6 (gD)
	UL46 (VP11/12)	US7 (gI)
	UL47 (VP13/14)	US8 (gE)
	UL48 (VP16, α-TIF)	US9
	UL49 (VP22)	
	UL51	
	ICP0	
	ICP4	
	US2	
	US3 (PK)	
	US10	

red: proteins conserved among the Herpesviridae family
PK, protein kinase; vhs, virion host shutoff; α-TIF, alpha *trans*-inducing factor

the flanking inverted repeats allow the inversion of U_L and U_S, resulting in the existence of four isomeric forms of DNA molecules (116,249).

The capsid has the form of an icosahedral (T=16) surface lattice, composed of 162 capsomers (150 hexons and 12 pentons) (98,302). The major capsid protein VP5 is the structural subunit of the capsomers, both the hexons and pentons, and accounts for nearly 70% of the mass of the capsid shell (208). The capsomers are connected to one another by triplex structures (nodules) made up by the VP19C and VP23 proteins (212,339). On the distal tips of an individual hexon, one VP26 molecule is bound to each hexon-associated VP5 protein (26,301). The capsid also contains smaller amounts of other proteins including the maturational protease VP24 (a product of U_L26 gene) and the product of U_L6 gene (98).

The tegument, a proteinaceous layer between the envelope and the capsid, is probably the least defined structural element of virus particles in terms of both protein composition and functions. It is thought that the tegument interacts with the capsid on one side and the cytoplasmic tails of glycoproteins or membrane-anchored proteins on the other to secure the integrity of virus particles. However, it is still a puzzle how the tegument attaches to the envelope. Recent studies suggest that the tegument is a flexible, yet ordered structure built upon specific protein-protein interactions. The proteins residing in the tegument region serve several essential functions for virus replication. Upon virus entry, the tegument delivers factors into the cytosol of a host cell to facilitate the initiation of a successful infection, including host gene shut-off and viral gene transactivation. During assembly, the tegument proteins are essential structural components for virion formation.

Surrounding the tegument is a host-derived lipid bilayer embedded with at least 14 virally encoded integral membrane proteins, 12 of which are glycosylated (163,279,281). Using cryo-electron tomography, spikes (or glycoproteins) with varying shapes and lengths can be seen projecting from the envelope (112). These virion envelope proteins perform essential functions for virus entry and immune evasion (36,278).

In addition to virally encoded proteins, several cellular proteins are also present in virions (112,163). For example, HSV packages large amounts of the polyamines, spermine and spermidine (97). The presence of these cationic molecules allows the large viral genome to be packed tightly into capsids by neutralizing the negative charges on the viral DNA (231). Evidence also indicates that HSV incorporates both viral and cellular mRNA molecules (262). However, little is known about whether these molecules have a role upon entry or they serve as structural components for maintaining virion integrity. Alternatively, it is possible that they are merely packaged into virions in a random fashion during virus assembly.

HSV Replication Cycle

To initiate an infection, HSV must first attach to the cell surface and penetrate the plasma membrane. These processes are facilitated by a subset of viral glycoproteins in concert with their cognate host receptors (278). Once within the cell, transcription and replication of the viral genome take place, followed by assembly and release of mature infectious virions. The aim of this section is to provide an overview and key aspects of HSV replication cycle from entry to egress.

Initial Attachment

Binding of HSV to a host cell is mediated by specific interactions between virion envelope glycoproteins and cell surface receptors (Fig. 2.1) (37,282). By selecting for cells that are resistant to HSV infection, heparan sulfate (HS) molecules on cell surface proteoglycans were identified as the primary cellular determinant of virus entry (111,265,330). These cells were defective in HS biosynthesis, and therefore, substantially resistant to virus entry. Two of the viral glycoproteins, gB and gC, are shown to possess the ability to bind heparin-coated columns (119). Studies using mutant viruses lacking these glycoproteins have determined that gC is the primary player during attachment whereas gB plays a secondary role (118,119).

Virion Structural Rearrangement during Attachment

Virus attachment to host cells is well known to trigger intracellular signaling events such as cytoskeleton remodeling, endocytic membrane trafficking, etc. (109,174). The first example that an 'outside-in' signal may be sent across the virion envelope causing an internal change inside a virion has been reported recently (191). This observation suggests that the virion structures (capsid, tegument, and envelope) are interconnected and more dynamic than previously thought. Specifically, in response to initial attachment of virus to cell surface receptors, a capsid-associated tegument protein, UL16, is triggered to be released from the capsid. The signal can also be triggered in a cell-free assay where virions are allowed to bind immobilized heparin beads. In the cell-free assay, a gC-null virus mutant was not capable of transmitting the signal, suggesting gC is required. However, in the cell-binding assay the signaling of the gC-null virus was

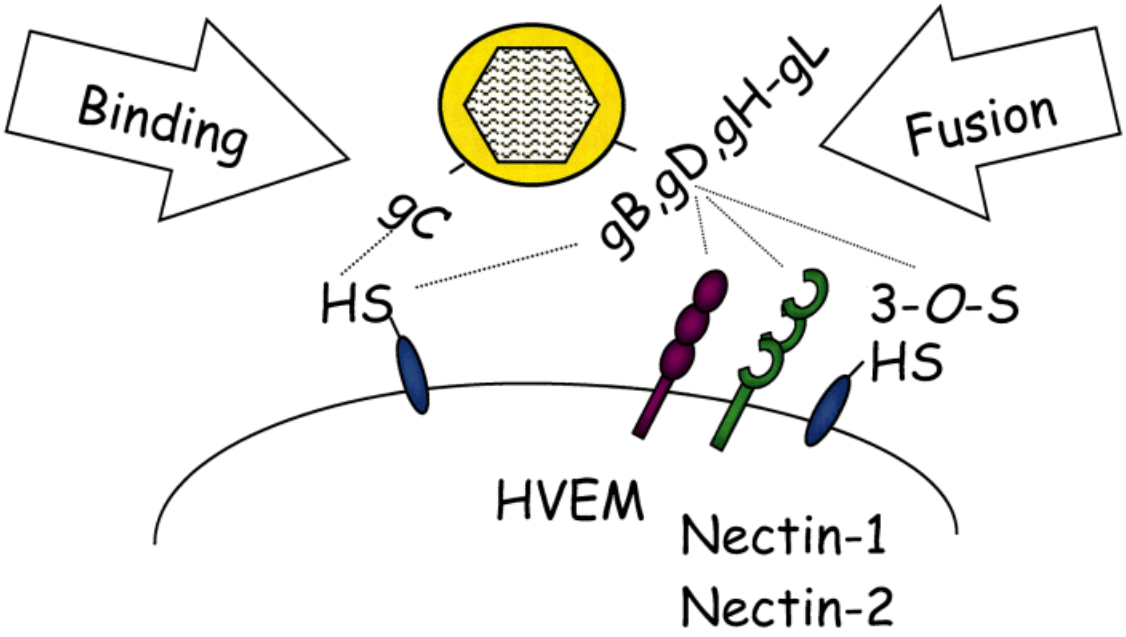


Figure 2.1 Cell surface receptors and viral ligands that participate in HSV entry. The viral envelope contains more than a dozen viral glycoproteins but only five (gB, gC, gD, gH and gL) have been shown to participate in viral entry. Binding of virus to cells can be mediated by the binding of gB or gC to heparan sulfate (HS) chains on cell surface proteoglycans. This facilitates the binding of gD to one of its cell surface receptors. These include HVEM, a member of the TNF receptor family; nectin-1 and nectin-2, two members of the immunoglobulin superfamily; and specific sites in HS generated by certain 3-O-sulfotransferases. Binding of gD to any one of these receptors triggers fusion of the viral envelope with a cell membrane. This membrane fusion requires the action of gB and gH-gL heterodimers as well as gD and a gD receptor.

Image and legend taken from Spear, P.G. 2004. Cellular Microbiology 6 (5): 401-410.
See Appendix A for copyright approval.

found to be unaffected, indicating that the signaling event could be induced in a manner independent of gC when other virus-receptor interactions are available. And, free cysteines in UL16 seem to be critical because treatment of extracellular virions with *N*-ethylmaleimide (NEM) prior to cell binding prevents the UL16 dissociation from the capsid. Despite these findings, the molecular machinery that participates in (or controls) the process and its biological function during virus entry are yet to be determined.

Although initial binding is critical for virus entry, it is not absolutely required. Studies have shown that viruses devoid of both gB and gC are still able to bind to the cell surface, and cells defective in HS biosynthesis are still susceptible to infection, albeit with low efficiencies (15). Further virus penetration requires a fusion event that takes place between the viral envelope and the host membrane.

Membrane Fusion

Depending on cell types, membrane fusion can occur at the plasma membrane as well as endocytic membranes (via either pH-dependent or -independent pathway) (197,214,215,267,326). The entire process requires multiple interactions between cell surface receptors and viral glycoproteins gB, gD, and a gH/gL heterodimer (Fig. 2.1) (216,278). Deletions of any one of these four glycoproteins abolish HSV penetration, despite no effects on virus attachment due to the presence of gC (i.e., binding of virus to heparan sulfate alone is not sufficient for virus entry) (34,60,84,156,254). It is thought that interaction of gD with its cognate host receptors serves as a trigger to initiate membrane fusion. One hypothesis is that alterations of the gD conformation upon receptor binding enables its interactions with gB and/or the gH/gL heterodimer, which in

turn activates fusogenic activity of gB, gH, and gL (44,88,149,280,285). Three categories of host receptors have been found to bind gD and mediate entry: HVEM, a member of the TNF receptor family; nectin-1 and nectin-2, two members of the immunoglobulin superfamily; and specific sites in HS generated by certain 3-O-sulfotransferases (94,199,243,266,320). The ability to use various cell surface receptors for entry allows HSV to infect a wide spectrum of tissues either in cultures or in laboratory animals.

Virus Uncoating

Immediately after fusing with the host cell membrane, HSV deposits its content into the cytosol. The bulk of the tegument is shed and left behind, resulting in the presence of an electron-dense mass associated with the cytoplasmic surface of the plasma membrane (178). The process of tegument dissociation from the incoming nucleocapsids likely occurs as a result of phosphorylation by cellular and viral kinases, as it was shown certain tegument proteins are phosphorylated upon entry, and phosphorylation can promote tegument dissociation from purified capsids in vitro (201). However, it is still unclear whether phosphorylation induces tegument dissociation in vivo.

Due to the large size of viral capsids and high viscosity of the cytoplasm (with protein density approaching 300 mg/ml), capsids must be actively transported through the cytoplasm to the nuclear pores where the viral genome is released into the nucleus (276). Such movement is particularly critical in neuronal cells, where capsids must travel long distances along neuronal axons. Electron microscopic studies have shown that capsids are closely associated with cytoplasmic filaments which have typical morphology and localization of the microtubules (MTs) (67,277). The external surface of cytoplasmic

capsids also displays some appendages, the morphology of which appears to have the dimensions and the shape of the MT minus-end-directed motor dynein, providing further evidence for capsid movement along the MTs (68,166). Although studies searching for viral dynein receptor have suggested several tegument proteins might interact with subunits of cytoplasmic dynein (69,165,175,328,333), it remains to be determined whether these interactions are actually involved in directing capsid transport during entry.

It was demonstrated that by 1 h postinfection the nucleocapsids reach the nucleus and attach to the nuclear pore complexes (NPCs), indicating that the MT-mediated capsid transport is rapid and efficient (222). At 4 h postinfection, virtually all capsids are localized at the nuclear rim, where the accumulation of several hundred capsids could be seen. In the presence of MT-depolymerizing agents, such as nocodazole, colchicine and vinblastine, the accumulation of capsids at the nucleus was significantly reduced, further implicating the involvement of MTs in capsid trafficking (148,166). The mechanism for capsids docking at the nuclear pores remains poorly defined. Addition of a specific antibody against a NPC protein importin β and removal of the capsid-associated tegument proteins both have been shown to interfere NPC-capsid binding, suggesting that physical interaction of the NPC components with certain tegument proteins may play a role in this process (222,322).

Almost all of the capsids observed at the nuclear pores lack the electron-dense core, indicative of the release of viral DNA, and the amount of empty capsids also increase over time. Two tegument proteins, VP1/2 (UL36) and UL25, have been implicated in uncoating of the viral genome because they remain strongly associated with capsids during transport to the nuclear pores (17,107,110,234). These speculations were

based on the characterization of temperature-sensitive mutants which showed a phenotype of the accumulation of DNA-filled capsids at the nuclear membrane and a significantly reduced amount of viral genome in the nucleus at the restrictive temperature early in infection. Under the permissive condition, the viral genome is released into the nucleus followed by viral gene expression and DNA replication.

Viral Gene Expression and DNA Replication

After being released into the nucleus, the viral genome is found preferentially adjacent to discrete nuclear matrix-bound domains known as ND10s (176). ND10s are distinct nuclear domains containing proteins (such as PML, Sp100, and Daxx) which are associated with various cellular activities including transcription, apoptosis, and proliferation (177,332,342). It is proposed that HSV gene transcription and DNA replication begin at these nuclear regions, supported by the observations that deposition of viral DNA at ND10s takes places even in the absence of gene expression and that viral RNA transcripts are also detected at locations juxtaposed to ND10s (164,306).

A characteristic of the HSV replication cycle is that its gene expression is in a finely regulated and sequentially ordered cascade (120,252). The viral genes are transcribed by host cell RNA polymerase II in three waves (α, β, γ). The first group of genes being transcribed is designated as α or immediate-early genes.

Immediate-Early Gene Expression

Immediate-early (IE) genes are transcribed and expressed maximally at 2 to 4 h postinfection, and their expression does not require *de novo* viral protein synthesis (120).

VP16, which is encoded by the viral U_L48 gene, was identified as a transcriptional transactivator of IE gene expression (therefore designated as alpha *trans*-inducing factor or α-TIF) (16,39). The virion-associated VP16 is released into the cytoplasm from the tegument after virus entry and shuttled into the nucleus by specifically recognizing a nuclear localization signal (NLS) contained in the cellular protein HCF (150,331). The VP16-HCF complex then interacts with host nuclear transcription factors (Oct-1 and Sp1) and potently induces IE gene expression (96,299). In the absence of VP16, IE gene expression, albeit significantly reduced, does not diminish completely, which is likely attributed to the action of host transcription factors (96,131).

In general, IE proteins function in regulating viral gene expression as well as modulating cellular processes such as cell cycle and RNA transport. These proteins include ICP0, ICP4, ICP22, ICP27 and US1.5. The ICP4 protein binds DNA either directly or indirectly via interaction with cellular transcription factors, leading to activation of almost all early and late gene expression and repression of certain genes including its own (41,55,169,271). ICP0, ICP22, and US1.5 not only regulate viral gene expression but also affect the cell cycle through binding various cyclin proteins (73,135,236). Production of IE proteins is subsequently followed by a robust induction of early gene expression.

Early Gene Expression

Expression of β or early (E) genes peaks at 4 to 8 h postinfection and is dependent upon IE transactivator proteins (discussed above). E gene products function in stimulating viral DNA synthesis, which sequentially enhances expression of the last

group of viral genes (121,252). Of the 13 known E proteins, seven are essential and sufficient for DNA replication (40,53,101,130,173,182,329,340). The other six proteins, though dispensable for virus growth in cell culture, perform functions in quality control of newly synthesized viral genomes, by preventing the incorporation of uracil and repairing viral genome damage (70,81,102,203,316). The mechanism of viral DNA replication is described next.

Viral DNA Replication

HSV encodes its own enzymes that promote viral DNA replication to take place. The process is initiated by the DNA origin-binding protein, UL9, which dimerizes with itself and binds to one of three redundant origins of replication contained in the viral genome, promoting unwinding of the double helix (79,172,223,284,317). The viral protein ICP8 via its interaction with UL9 is recruited to the origin, where it stabilizes the resultant single-stranded DNA and also enhances the rate and extent of UL9 unwinding activity (24). Viral helicase-primase complex (consisting of UL5, UL8, and UL52) is then directed to the unwound origin by UL9 to mediate primer synthesis, which in turn causes dissociation of UL9 from the origin as well as recruitment of DNA polymerase complex to the site (66,139,186,290). The DNA polymerase complex composed of the DNA polymerase (UL30) and an accessory protein (UL42) binds to the primer-DNA duplex and catalyzes synthesis of new viral DNA (90,104). Continued DNA synthesis through a rolling circle mechanism produces head-to-tail genomic concatomers (92,252). The concatomeric DNA molecules are later cleaved into monomers and packaged into

preformed capsids during assembly, a process that requires products of the last group of viral genes.

In addition, HSV encodes enzymes that enhance DNA replication and efficiency (ribonucleotide reductase, thymidine kinase, and deoxyuridine triphosphatase) as well as those for repair of genome damage (alkaline nuclease and uracil N-glycosylase) (252). These proteins seem to be critical for proper viral replication in non-dividing neuronal cells, likely due to the low amounts of certain DNA precursors in neurons (45,124,238,239).

Late Gene Expression

γ or late (L) gene expression is triggered by viral DNA synthesis and peaks at 8 to 10 h postinfection (252). They can be roughly divided into two groups: leaky-late and true-late genes. While viral DNA synthesis stimulates leaky-late gene expression, it is strictly required for true-late gene expression. The differences are likely due to specific elements present in the promoter sequences of the respective genes (137,230,327). However, it is still unclear how DNA replication modulates expression of these L genes.

Proteins encoded by L genes include almost all components that assemble into mature virions, and therefore, possess functions in nearly every step of virus assembly (such as capsid formation, viral genome encapsidation, and virus envelopment). In addition, the products of L genes are also required for early stages of replication cycle, including entry and viral gene expression. The following section will present in detail the current knowledge of how newly synthesized viral DNA and proteins are incorporated into virus progeny particles.

HSV Assembly and Egress

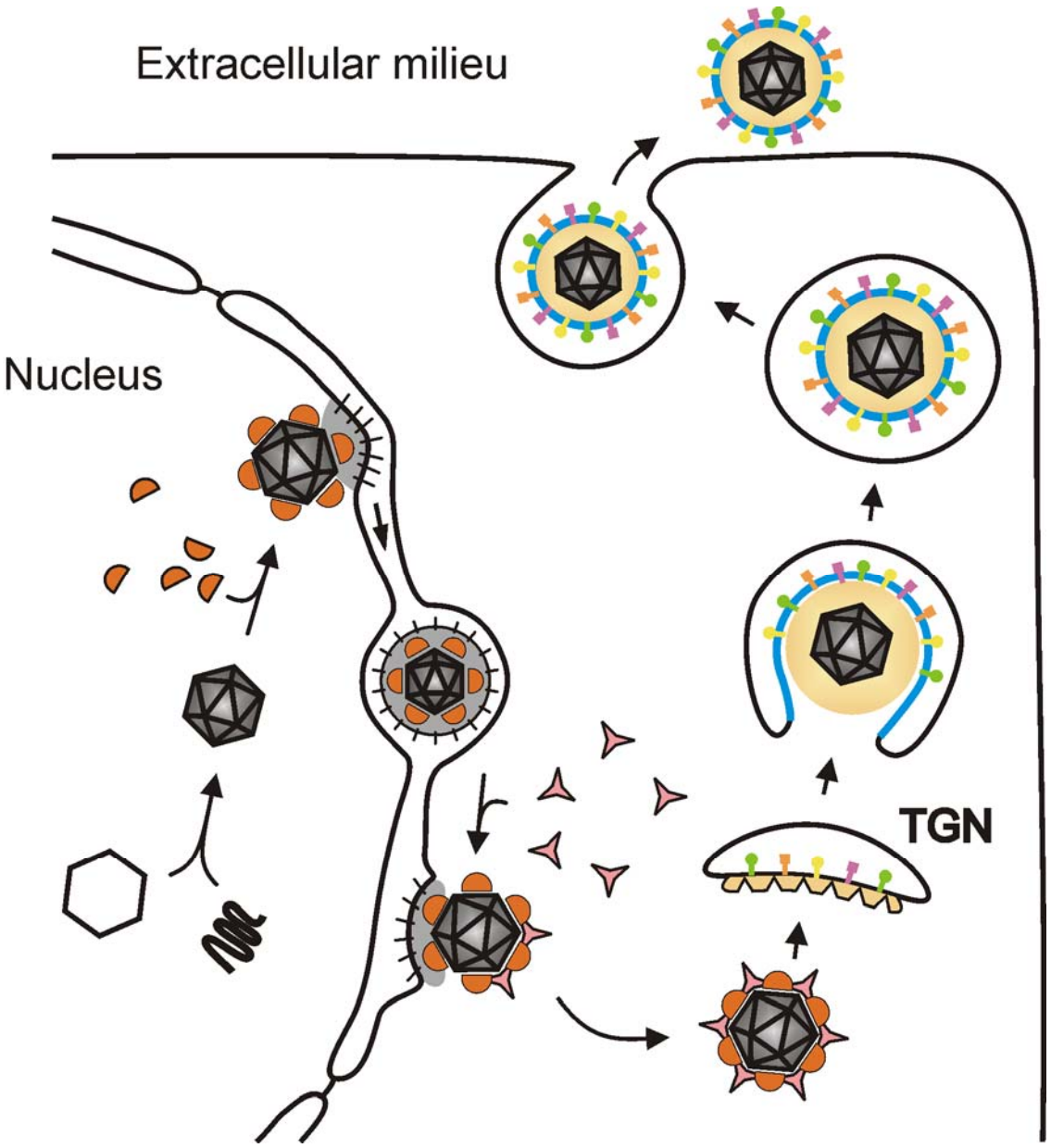
Production of a viral particle within infected cells is accomplished by a series of complex assembly events that involve nearly 40 viral proteins. The whole process begins with the formation of nucleocapsids in the nucleus (Fig. 2.2). Then, nucleocapsids are translocated to the cytoplasm, where they acquire a tegument structure and a host-derived lipid bilayer as the virion envelope. After being released out of cells, mature virions are capable of infecting new cells to continue reproduction. In the following sections, the major steps in the process will be discussed in detail.

Capsid Formation and Viral Genome Encapsidation

In vitro-capsid assembly systems which use insect cells infected with the panel of recombinant baculoviruses have revealed the minimum set of HSV genes required for formation of icosahedral capsids (209,210,291,295). Specifically, capsid-like particles can be observed under electron microscope when five virally encoded capsid proteins (VP5, VP19C, VP23, protease [UL26], and scaffold [pre-VP22a]; see Table 2.1) are allowed to form complexes and assemble into stable structures. Though one may argue that these in vitro reconstitution studies may not reflect exactly how capsid assembly takes place in vivo, these studies have provided fundamental and valuable information for further investigations.

Within infected cells, the first step of capsid assembly involves relocation of viral capsid proteins into the nucleus from the cytoplasm where they are synthesized during the late stage of gene expression. Proteins including VP19C and pre-VP22a possess nuclear localization signals (NLSs) which can function to direct not only themselves but

Figure 2.2 HSV assembly and egress pathway. The viral DNA genome is packaged into preassembled icosahedral capsids in the nucleus. As nucleocapsids are transported towards the inner nuclear membrane, they likely acquire a subset of tegument proteins (orange). Nucleocapsids are subsequently shuttled out of the nucleus by a budding-fusion at the nuclear membrane. Nucleocapsids in the cytoplasm pick up more tegument proteins (pink) as they are transported to *trans*-Golgi network (TGN)-derived vesicles for final envelopment. At this site, nucleocapsids acquire the complete set of tegument proteins (light yellow) as well as the virion envelope (blue) with glycoproteins (spikes). Enveloped, mature virions are then transported within cytoplasmic vesicles to the cell surface where they are deposited into the extracellular milieu.


Extracellular milieu
Nucleus
TGN

also their associating protein VP5 into the nucleus (213,246). Similarly, VP23 localizes to the nucleus only in the presence of VP19C, and the minor capsid protein VP26 is recruited via a complex of pre-VP22a and VP5 (56,61).

Accumulation of capsid proteins in the nucleus leads to production of immature, spherical procapsids. It is thought that this process has already begun when the scaffold protein pre-VP22a interacts with the major capsid protein VP5 in the cytoplasm (122,136). After transport into the nucleus, pre-VP22a proteins self-assemble, and meanwhile, enhance weak interaction between VP5 molecules (227), leading to formation of capsomers (pentons and hexons) and eventually higher-ordered capsomer structures that contain a closed external shell surrounding a thick scaffolding core of pre-VP22a (212,233). Triplex complexes (one copy of VP19C and two copies of VP23) are added to capsomers concomitantly with VP5 and pre-VP22a. They are required for connecting adjacent capsomers to one another and likely function in stabilization of procapsid structures (212). In the absence of pre-VP22a or triplex proteins, capsids can not be produced (210,291,295).

Procapsids are unstable, spherical capsid intermediates which must be transformed into stable icosahedral structures similar to that contained in mature virions (209,300). This maturation step is mediated by a virally encoded protease UL26, which is also present inside procapsids (58). Due to overlapping open reading frames, UL26 contains the entire pre-VP22a sequence and therefore can take part in, but is not essential for, the formation of procapsids (158,294). UL26 protease cleaves itself into VP21 and VP24 and also removes the last 25 amino acids from pre-VP22a (158,235,315). Removal of the 25-amino-acid-long fragment from the C terminus of pre-VP22a abolishes

interaction between pre-VP22a and VP5, leading to dissociation of the scaffolding core from procapsids. This dissociation is accompanied or followed by angularization of immature procapsids to produce more stable, tightly packed polyhedral capsids without a dramatic increase in shell diameter (300). The hexon-associated VP26 protein is also incorporated during or following capsid maturation (26).

Transition from procapsids to polyhedral capsids is an absolute requirement for subsequent encapsidation of viral genome (91,248). Viral DNA is packaged into polyhedral capsids in a process that relies on functions of several viral gene products. UL17 and UL32 are both required for capsid transport to nuclear sites where viral DNA synthesis takes place (152,292). The portal protein UL6 forms a dodecameric ring at one of the capsid vertices (151,211), and recruits a terminase complex (consisting of UL15, UL28, and UL33) that functions in binding, cleaving, and packaging viral genome (2,9,20,293,335). Insertion of viral genome into capsids through the portal is thought to trigger the exit of the scaffold through channels in the capsid structures (180). After entering the capsids, viral genome binds to the capsid-associated UL25 protein which functions to prevent genome from leaking out during the closure of the portal (188,221). Following theses processes, nucleocapsids undergo further structural rearrangements and become more tightly packed between capsomers. The resulting mature, DNA-filled capsids are often referred to as C capsids. Two other types of capsids, A (empty) and B (scaffold-containing) capsids, can also be observed in the nuclei of infected cells; however, both types of capsids are believed to be dead-end products which are incapable of incorporating viral DNA genome (98,303).

Several other viral proteins have also been suggested to play a role in DNA cleavage and packaging. For instance, deletion of a PRV gene that is homologous to HSV U_L21 results in a defect in DNA encapsidation (54,311). VP1/2 (UL36) has been shown to bind the packaging sequence of the viral genome (43), and UL16 has also been implicated in the process due to its ability to bind single-stranded DNA in vitro (224). However, questions still remain unanswered whether or how these proteins are involved in viral genome encapsidation.

Nuclear Egress

Following the packaging of viral genome into preassembled capsids, intranuclear nucleocapsids must leave the nucleus to proceed with further maturation. It has been suggested that nucleocapsids may be driven to the nuclear periphery (at least in part) by myosin motors trafficking along actin filaments, and capsid-associated viral proteins may be involved in the process (82). However, the mechanistic roles of these proteins in intranuclear capsid transport still remain poorly understood.

Due to the fact that these 125-nm-diameter capsids are too large to pass through nuclear pores (287), they must exit the nucleus by budding at the inner nuclear membrane. At the nuclear periphery is an intermediate filament network composed of lamins and lamin-associated proteins. This formidable boundary, known as the nuclear lamina or matrix, is roughly 20 to 80 nm in width and functions in maintaining architecture of the nucleus. It also provides a barrier for large molecules to enter or exit freely through the nuclear membrane (100). To conquer (break down) this obstacle, HSV has developed a strategy which is dependent upon expression and complex formation of the UL31 and

UL34 viral proteins. When either of the two proteins is deleted, capsids are defective in budding at the inner nuclear membrane and are trapped in the nucleus (87,253). Similar defects are also observed in cells infected with CMV and PRV mutants lacking genes homologous to HSV U_L31 or U_L34, indicative of a highly conserved function among all herpesviruses (87,142,204,257). The proper localization of the UL31-UL34 complex along the nuclear rim was shown to depend upon US3 and its kinase activity (142,256). It is proposed that the three proteins together mediate recruitment of the cellular protein kinase C (PKC) to the nuclear rim, where PKC induces phosphorylation of lamins and subsequent dissociation of the nuclear lamina (225,242,263). However, whether these events directly attribute to nuclear egress of capsids is yet to be further investigated.

After nucleocapsids bud into the inner nuclear membrane, enveloped virus-like particles are present in the perinuclear space, and they were shown to contain proteins including UL31, UL34, and US3 (7,87,106,242). It has been debated regarding the fate of these perinuclear virions. An earlier model suggested that perinuclear virions maintain their integrity (with envelope and tegument) and leave the cell through the secretory pathway (38,128). However, evidence has begun mounting and indicates a second model which is now widely accepted. The currently preferred model of HSV egress proposes that perinuclear virions fuse with the outer nuclear membrane, resulting in the release of naked capsids into the cytoplasm. To complete the morphogenesis pathway, naked cytoplasmic capsids need to undergo another budding/envelopment process at vesicles derived from TGN membranes (95,105,269,319). Two different budding events were shown to be distinct not only in the subcellular localizations but also in the requirements

of the viral proteins. The molecular mechanism of final envelopment will be discussed later in detail.

How the envelope of perinuclear virions fuses with the outer nuclear membrane is obscure. It is thought that US3, a component of perinuclear virions, may play a role in this fusion event because the phenotype of a US3 deletion virus showed significant accumulation of enveloped virions in the perinuclear space (142,242). However, there was only 10-fold decrease in virus growth, indicating that US3 activity is not absolutely essential but could be beneficial if present. Whether other viral proteins or cellular proteins play a role in this primary budding-fusion event is still unclear. Several other viral proteins such as UL11, UL20, VP16, and gK have also been suggested in the process (11,12,86,125,206). However, these speculations are largely based on subcellular localization of proteins examined by immunostaining of infected cells or loss-of-function studies of mutant viruses. The studies using mutant viruses all share a big limitation in elucidating the mechanistic roles of viral proteins because it is noted that besides having defects in nuclear egress, these mutant viruses also fail to continue onto final envelopment and egress through the cytoplasm. Therefore, it still remains to be determined whether these proteins actually play a role in nuclear egress.

Upon exiting the nucleus, nucleocapsids traverse the cytoplasm to reach the TGN-derived vesicles, the site of final envelopment. At this site, an envelopment step results in acquisition of the final subset of tegument proteins as well as the concurrent addition of a lipid envelope and viral glycoproteins.

Tegumentation and Final Envelopment

The molecular mechanisms responsible for tegumentation and final envelopment have begun to be understood over the past several decades. A large number of studies were conducted and have helped broaden our knowledge of how these final steps may be accomplished. It is now thought that tegumentation occurs at various stages throughout the capsid egress pathway. Several studies have suggested that tegumentation may begin in the nucleus.

Studies using cryo-electron microscopy image reconstruction have demonstrated that the innermost layer of tegument exhibits icosahedral symmetry around the capsid (338,339). This inner-part material was suggested to be consisted of VP1/2 (UL36) due to its nuclear localization during infection and its interaction with the major capsid protein VP5 (189). Recently, it was reported that VP1/2 and its binding partner UL37 are both present on capsids isolated from the nuclear fraction of infected cells, providing critical evidence for tegumentation of capsids prior to nuclear egress (33). Moreover, it was also proposed that VP1/2 may be recruited to the capsid via interaction with capsid-associated UL25, which performs critical functions in viral genome encapsidation (discussed previously) (47,303).

VP1/2 and UL37 proteins are conserved among all herpesviruses, and so is the interaction between the two (141,196). When either VP1/2 or UL37 is deleted, a large amount of unenveloped capsids accumulate in the cytoplasm, and no virus particle can be produced (57,59). And, in the absence of UL37, VP1/2 is still found on capsids, suggesting that VP1/2 is added to capsids prior to recruitment of UL37. All together, these results reveal that addition of VP1/2 and UL37 to intranuclear capsids is an

essential step for virion maturation, likely serving as attachment sites for other tegument proteins. For instance, VP16 has been proposed to associate with intranuclear capsids via interaction with VP1/2 (310). Using immunoelectron microscopy, VP16 was detected in virus particles in the perinuclear space, suggesting that VP16 is acquired prior to primary envelopment of virus at the inner nuclear membrane (206). However, it is still unclear whether VP16 has any specific functions in nuclear egress. In the perinuclear virions, there also exist other viral proteins including UL31, UL34, and US3, all of which play a role in facilitating nuclear egress (discussed previously). But, only VP16 and US3 stay associated with cytoplasmic capsids and are present in final mature virions (106,206). This observation again has given support to the deenvelopment-reenvelopment model of HSV egress.

After leaving the nucleus, nucleocapsids travel to the site of final envelopment where they acquire the full complement of tegument proteins and the viral envelope which contains various viral membrane proteins and glycoproteins. Researchers have been debating for some time about the identity of cytoplasmic structures where final envelopment takes place. Based on a large number of biochemical and genetic analyses, it is currently accepted that HSV acquires its final envelope by budding into TGN-derived vesicles (194,195). Under electron microscopy, large amounts of unenveloped capsids can be observed as they appear to bud into cytoplasmic vesicles reminiscent of the TGN (108,155). And, viral glycoproteins that are present in the virion envelope are also found to accumulate in the TGN (108,205,286,304). The TGN accumulation of glycoproteins could even occur without the presence of capsids. Biochemical analysis of the extracellular virion envelope also reveals high concentrations of lipids that enrich the

Golgi apparatus, such as sphingomyelin and phosphatidylserine (308). Moreover, using a cell fractionation assay it was demonstrated that HSV associates with fractions containing the TGN and endosomes, providing further evidence for final envelopment at these vesicles (115). Genetic studies also support this model. Recombinant viruses that were engineered to express Golgi-targeted gD exhibited wild-type levels of infectivity and gD incorporation, whereas viruses containing endoplasmic reticulum (ER)-retained gD or gH showed a significant defect in specific infectivity as well as reduced levels of either glycoprotein in secreted virion (31,269,321). Collectively, these studies all point to TGN-derived vesicles as the site of virus final envelopment.

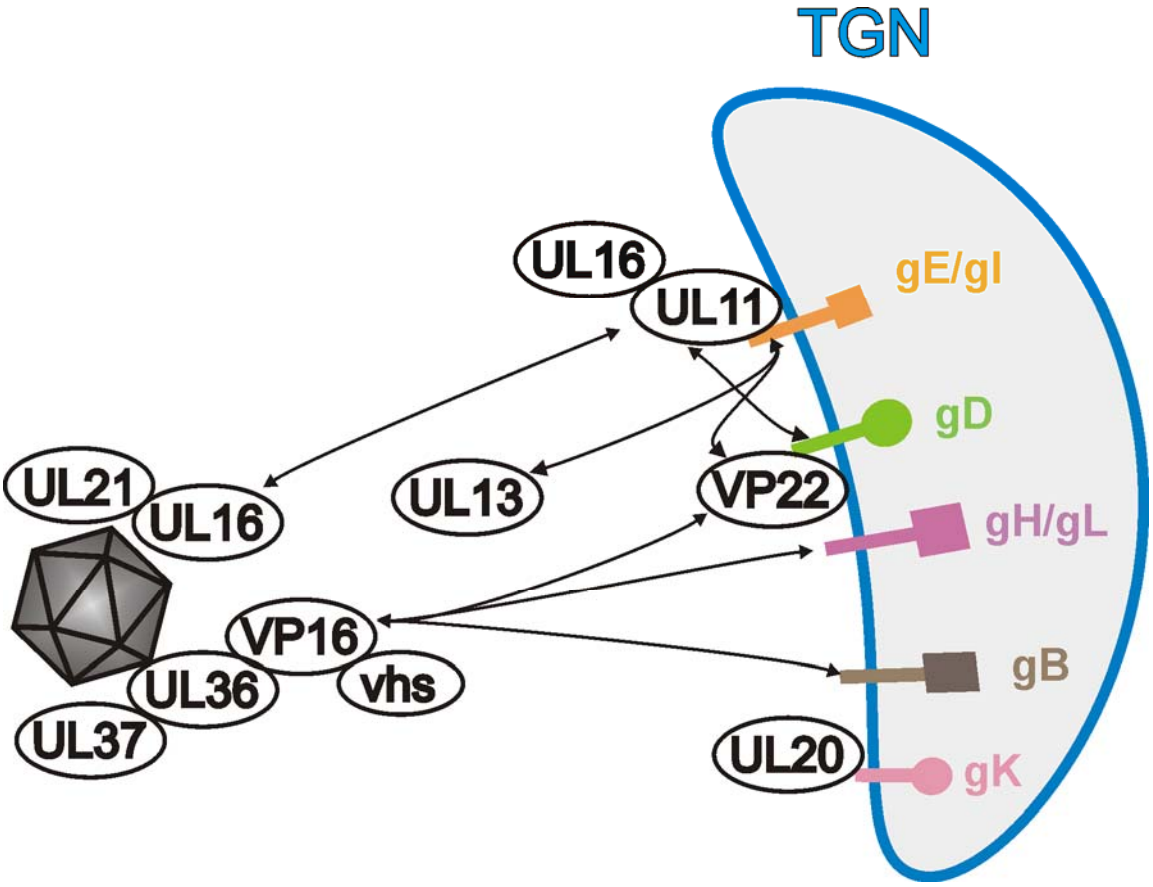
Trafficking of cytoplasmic capsids from the nucleus to the TGN appears to rely on the host cytoskeleton since microtubule (MT)-depolymerizing chemicals were shown to delay capsid transport in the cytoplasm (166,272). While capsid transport during entry may depend on MT-minus-end transport motors (dyneins and dynactin), the plus-end transport motors (kinesins) were suggested to be involved in virus egress, presumably via interactions with capsid-associated tegument proteins (68,298,328). Although several candidates have been implicated, it is still not clear how viral proteins recognize kinesin motors and mediate the capsid movement along microtubules (62,146,165,272).

As nucleocapsids travel through the cytoplasm, tegumentation continues. And, the majority of the tegument is added to nucleocapsids while they bud into TGN-derived vesicles to acquire their lipid bilayer envelope. Recent studies indicate that the process is accomplished via an intricate network of protein-protein interactions that display a significant level of redundancy, at least in cell culture (193). It is thought that the tegument proteins function in bridging capsids (on one side) and the virion membrane

proteins (on the other side), thereby facilitating the final budding process (Fig. 2.3). As described above, the inner tegument proteins, VP1/2 and UL37, associate with capsids in the nucleus, and the absence of either protein shows defects in virion maturation, indicating that subsequent steps of tegumentation cannot be completed (57,59,143). A dramatic defect was also observed in the absence of VP16 which makes up a major part of the virus tegument and has been shown to interact with UL36 (17,310). The VP16-null mutant displayed a large amount of unenveloped cytoplasmic capsids in infected cells, indicative of a blockage at a late stage of virus assembly (i.e., tegumentation and final envelopment) (202). Interestingly, VP16 has been demonstrated to interact with other tegument components, vhs (UL41) and membrane-associated VP22 (UL49) (219,270). Mutant forms of vhs that fail to interact with VP16 were not incorporated into the virion (240). However, virus egress still proceeds in the absence of VP22 and several other tegument proteins including UL13, US3, UL41, UL46, and UL47 (193), representing a significant level of redundancy, at least in cell cultures.

The interactions between tegument-tegument and tegument-envelope proteins at TGN-derived vesicles appear to be capable of initiating the final budding process. It is evident that these interactions can take place independent of the presence of capsids and result in the formation of extracellular light (L) particles (185,247), indicating budding of the tegument can be initiated without capsid egress. Many protein-protein interactions have been implicated in the process. Studies have shown that VP16 may interact with the cytoplasmic tails of gB and gH (341), and VP22 interacts with the gE tail (218). Simultaneous deletions of genes encoding HSV-1 gD/gE/gI or PRV gE/gI/gM also resulted in accumulation of unenveloped capsids within the

Figure 2.3 Potential protein-protein interactions between viral tegument and membrane proteins during final envelopment. HSV final envelopment is thought to be driven by a complex network of protein-protein interactions between various tegument components located on the capsid (icosahedron) and membrane proteins and glycoproteins residing on the TGN membrane.


TGN
UL16
UL11
gE/gI
gD
UL21
UL16
UL13
VP22
gH/gL
VP16
gB
UL36
vhs
UL37
UL20
gK

cytoplasm (29,74). An interaction between the membrane-associated UL11 protein and the capsid-associated UL16 protein has also been suggested to play an important role in the process, which will be discussed later in detail.

In addition, recent studies have reported that inhibition of crucial components of endosomal sorting complexes required for transport (ESCRTs) results in a decrease of released virus (35,52). These components include vascular protein sorting 4 (Vps4) and Vps24, both of which are critical for ESCRT function/recycling and multivesticular body (MVB) luminal vesicle formation (6). Inhibition of either protein appears to lead to incomplete secondary envelopment, which was indicated by the presence of partially enveloped capsids in the cytoplasm. However, it is still unclear whether virus egress actually requires or utilizes this MVB-ESCRT pathway.

Due to the redundancy found in the network of protein-protein interactions during tegumentation and final envelopment, studies that attempt to unfold crucial capsid-tegument-envelope connections have been greatly hampered. Though many of the interactions described above appear to somehow play a role, their specific functions and involvement in virus assembly have not completely been proven.

Release of Mature Viruses

As a result of the final envelopment, mature enveloped virions are enclosed in cytoplasmic secretory vesicles. These vesicles are then transported to the plasma membrane where two membranes fuse, leading to the released of mature virions into the extracellular milieu. The process was suggested to require UL20 and gK based on the studies of virus mutants. Deletion of either gK or UL20 causes accumulation of

enveloped virions within large cytoplasmic vesicles, implicating that these proteins may play a role in directing vesicles to the plasma membrane or facilitating the fusion event between vesicles and the plasma membrane (192).

In polarized epithelial cells, HSV is not only released into the apical surface but also spreads laterally across cell junctions. Current understanding of the molecular mechanism for HSV cell-to-cell spread is discussed next.

Cell-to-Cell Spread

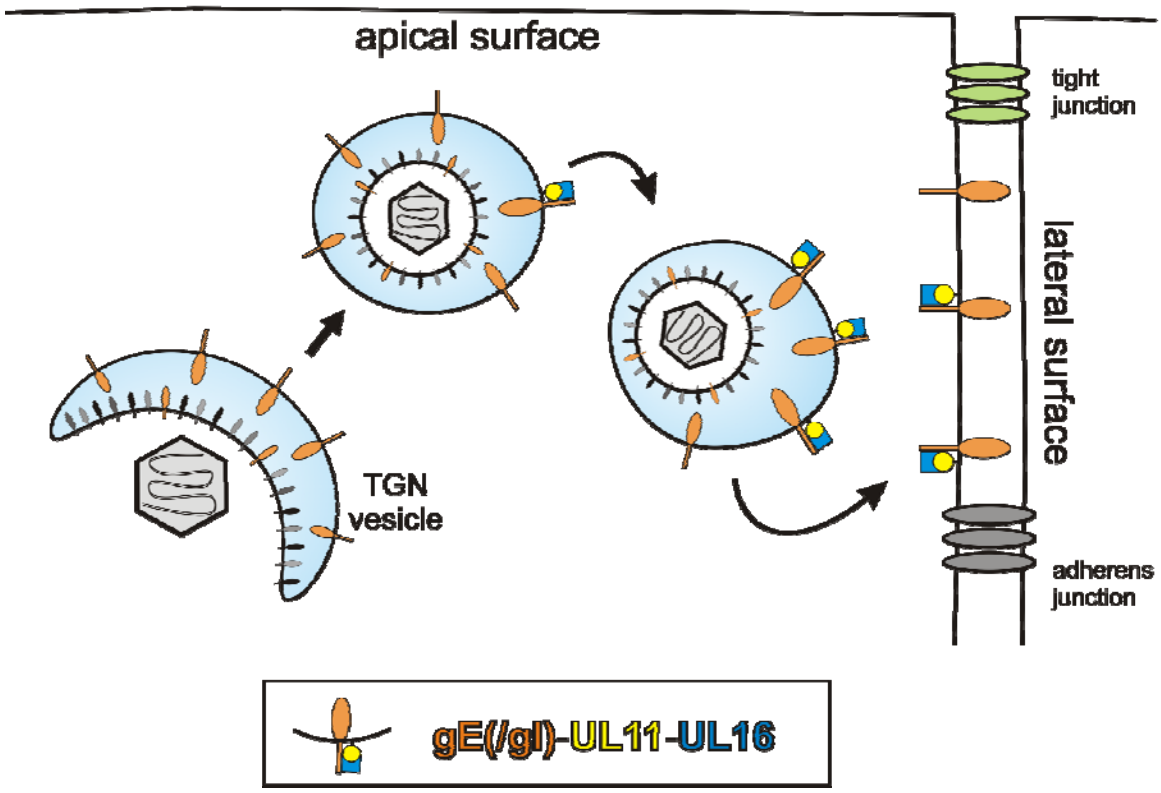
Cell-associated HSV can move basolaterally from an infected cell to its adjacent cells via cell-to-cell spread. The recruitment of viral receptors at the intercellular contact interface may promote efficient receptor engagement during virus entry. This specialized spreading mode shields viruses from exposure to the host immune system and contributes to rapid and efficient virus dissemination (258). And, it is important for virus pathogenesis in animals (14,64,307).

HSV cell-to-cell spread occurs not only in polarized epithelial cells but also between neurons and other cells in the nervous system. Main mechanisms of cell-to-cell spread include cell-cell fusion and movements across tight junctions of polarized epithelial cells or across neuronal synapse. All of these processes require the coordination of gB, gD, and gH/gL, four glycoproteins that are essential for entry of extracellular viruses and membrane fusion at the cell surface (described previously). Cell-cell fusion can result in syncytium formation or might only be restricted to localized fusion events that allow transport of viral materials while maintaining structural integrity of individual cells (258). Studies using the cell-cell fusion assay indicated that fusion can be induced

by expressing a combination of gB, gD, and gH/gL without other viral proteins, clearly demonstrating the importance of their interactions in mediating the fusion mechanism (44,285). In addition, a heterodimeric complex of viral glycoproteins gE and gI (gE/gI) is also critical for efficient cell-to-cell spread (Fig. 2.4). gE- or gI-null mutants of alphaherpesviruses, including HSV, PRV, and VZV, all displayed greatly reduced virus spread in cultured epithelial and neuronal tissues as well as in animals (22,46,63,64,297). It has been hypothesized that gE/gI functions in sorting vesicles containing virions to the basolateral cell surface, where membrane fusion occurs to release virions into the intercellular space. Subsequent entry of these virions into a neighboring uninfected cell requires the interaction of gD with its host receptor nectins to activate membrane fusion machinery composed of gB and gH/gL. An interaction between gE and an unknown host receptor has also been implicated, but the identity of the gE receptor is still under investigation (65). The specific role of gE/gI in HSV cell-cell spread will be discussed later.

Several reports have also suggested that UL11 may function in cell-to-cell spread. Deletion of HSV UL11 and its homologs led to defects in infectious virus production and small plaques on cultured cells (11,145,260). The small plaque size may be attributed to reduced production of extracellular viruses, or a failure in targeting vesicles containing virions to basolateral membranes (i.e., cell-to-cell spread), or the combination of both. Further support was provided by the observation that UL11 contains signals that modulate its intracellular trafficking between the TGN and plasma membrane (160). Also, it has recently been shown that UL11 interacts in some manner with the cytoplasmic tails of gE and gD, both of which are required for efficient cell-to-cell spread (75).

Figure 2.4 HSV cell-to-cell spread. In polarized cells, mature virions contained in TGN-derived vesicles can be targeted to the lateral surface where they infect adjacent cells. The process requires actions of a heterodimeric complex composed of gE/gI (orange lollipop), which is thought to facilitate basolateral sorting of TGN vesicles towards the cell surface. UL11 (yellow circle) interacts with the cytoplasmic tail of gE, and has been implicated in virus cell-to-cell spread. It is speculated that UL11 (along with its binding partner, UL16 [blue square]) could also be involved in this lateral transport of TGN vesicles. At late times postinfection, gE/gI accumulates on the lateral surface where it interacts with an unknown cellular receptor. At the cell surface, vesicles fuse with the plasma membrane and release virions into the intercellular space.


apical surface
tight junction
lateral surface
adherens junction
TGN vesicle
gE(/gI)-UL11-UL16

Interestingly, deletion of a UL11 binding partner, UL16, also displayed small plaques on cultured cells (data not shown), and UL16 specifically recognizes the trafficking sequences within UL11 (161,334). So if UL11 does play a role in cell-to-cell spread, UL16 could also be involved. Moreover, Chapter V also provided evidence that UL16 can interact with the cytoplasmic tail of gE, further implicating UL16 in cell-to-cell spread.

To define the specific roles of UL11, UL16, and gE in virus life cycle, it is essential to characterize the molecular basis of how these proteins interact. A brief introduction of several broadly used approaches for studying and identifying protein-protein interactions will be presented next and followed by an overview of what is currently known about the functions of these three proteins in virus replication.

Analysis of Protein-Protein Interactions

Most of the time the majority of proteins inside a cell are involved in complex formation with various macromolecules, for example, DNAs, RNAs, lipids, or other proteins. Through interactions with different partners in different subcellular localizations at a particular time, protein functions are highly regulated. Determination of the structure and composition of large complexes that serve as molecular machines has advanced our knowledge on many essential biological activities such as transcription, translation, replication, cell motility, molecular transport, etc. (49,126,179,200,323). Viruses, including herpesviruses, are pathogens that reproduce in a host organism by hijacking cellular machines and manipulating host environment. Thus, their life cycle is a complex process mediated through numerous specific interactions among viral genome, viral

proteins, and host factors in a finely modulated fashion. Investigating how these elements interact with each other is fundamental for a better understanding of virus morphogenesis.

As the main goal of this dissertation, characterization of the molecular basis of specific protein-protein interactions that may take place during HSV infection will help expand our knowledge of herpesvirus biology. The following section will present several biochemical methods that are widely used to probe and study protein-protein complexes. The methods described below include two-hybrid systems, co-immunoprecipitation, and affinity purification. Because each technique has its own advantages and disadvantages, it is often necessary and critical to prove and double-check the results using various combinations of these techniques and others. A brief overview of mass spectrometry analysis will also be presented, which is often combined with co-immunoprecipitation and affinity purification for acquiring identities of unknown members of protein complexes.

Two-Hybrid Systems

Two-hybrid systems are powerful tools for studying macromolecular interactions via high-throughput screening and selection. Results obtained from these methods often provide a first, critical hint for identification of binding partners. In conjunction with mutagenesis strategies these methods are useful for analyzing details of specific molecular interactions. A well-known example is the yeast two-hybrid (Y2H) system assay, which has been widely used to study and probe novel protein-protein interactions since it was established in 1989 (78). The initial Y2H was designed using the GAL4 transcriptional activator of the yeast *Saccharomyces cerevisiae*. GAL4 activates

transcription of genes required for yeast growth on galactose, which forms the principle of selection. For the screening purpose, GAL4 is split into two separate parts, the DNA binding domain (DBD) and activating domain (AD). Two domains are modular and can function in close proximity without being directly connected. DBD, which is the domain responsible for binding to an upstream activating sequence (UAS), is fused to a known protein as the bait. AD, which is responsible for activating downstream reporter genes, is fused to the prey. The prey can either be a known protein or a library of known or unknown proteins. Plasmids encoding these fusion proteins are transfected into a cell. If the bait and prey proteins interact, transcription of the reporter gene can then take place, rendering a detectable or selectable change in the cell phenotype. To date, the same principle has been adapted to develop many alternative methods to detect protein-protein interactions (32,132,144,288,312).

Although the Y2H screens are used by many researchers, it is important to keep in mind these methods have several caveats. The main issue is the possibility of a high number of false positive and/or false negative identifications. This could be due to overexpression levels of proteins, misfolding of proteins in chimeric forms, missing proper protein modifications when expressed in the yeast, or forced mislocalization of cytosolic proteins to the yeast nucleus. Moreover, some proteins might interact when they are coexpressed in a two-hybrid system, but in reality they are never present in a cell at the same time. It is also noted that certain proteins as the bait can cause self-activation and induce transcription of downstream reporter genes even with no contact with the prey. Any of these reasons alone can lead to false results. Because of a high error rate, the overall confidence of a two-hybrid assay is rather low. It is necessary to confirm

interactions using alternative high confidence assays, for example co-immunoprecipitation, which is discussed next.

Co-Immunoprecipitation

Co-immunoprecipitation (co-IP) is routinely used to address whether proteins of interest form a complex within a cell (in vivo). It is also a useful tool for probing novel binding partners of a known protein. Co-IP, meaning immunoprecipitation of protein complexes, is performed by selecting an antibody specifically targeting a known protein that is likely to be involved in a large complex. By targeting a particular protein with an antibody, it becomes possible to isolate and concentrate the intact protein complex from a sample and subsequently identify unknown members of the complex.

Since immunoprecipitation is a protein isolation method based on specific biochemical interactions between antigen and antibody, the ability of an antibody to precipitate its target protein is mainly dependent upon whether the epitope of the target is exposed. Under some circumstances, an antibody might fail to detect the target. For instance, when a protein is engaged in complexes, it is possible that the epitope could be hidden and therefore not accessible. Or instead, the epitope would not be exposed until the protein physically complexes with specific binding partner(s) and undergoes a conformational change. It is also possible that addition of an antibody may compete for binding to the target and/or block critical motifs required for interaction with its partners, thereby leading to disruption/inhibition of complex formation. These issues might be overcome by using antibodies against different epitopes in the protein of interest. Repeating experiments by targeting different members of the protein complex also allows

careful verification of involvement of each protein in the complex. For proteins that traffic dynamically between various subcellular localizations or serve in many biological activities, it is very likely that they may form transient interactions with different binding partners at different times for various functions. Thus, in a co-IP experiment proteins that are collected along with a known protein may not exist in a single complex, but may instead represent multiple (sub)complexes. These possibilities would need to be further investigated by analyzing details of individual interactions.

Another caveat of co-IP is that the high-capacity advantage of the sepharose/agarose beads could become a high-capacity disadvantage. This often occurs when the beads are not completely saturated with antibodies, resulting in partially antibody-coated beads which have free spots to bind nonspecifically to anything that sticks. Therefore, co-IP often requires initial optimization by carefully calculating the quantity of available antibody and binding capacity of the beads. And, a preclear step could also be carried out to lower the amount of nonspecific bindings by incubating samples with some beads before the actual immunoprecipitation procedure.

Another major technical hurdle with immunoprecipitation is that not every antibody is available and it is sometimes difficult to generate an antibody which only specifically recognizes its target. To overcome these issues, researchers alternatively engineer a ‘tag’ onto either the N or C terminus of the protein of interest. The tagged protein can then be immunoprecipitated with an antibody raised against the tag. The advantage is that the same tag and antibody can be used repeatedly to study different proteins. Commonly used tags are the green fluorescent protein (GFP), FLAG tag,

glutathione-*S*-transferase (GST), polyhistidine (His_6) tag, etc. GST and His_6 tags can also be isolated using affinity purification, which is discussed in the following section.

Affinity Purification

Affinity purification (or chromatography) is a powerful technique that allows simple and rapid purification of proteins expressed in bacteria, insect cells, and mammalian cells. The approach is achieved by using a particular moiety of a protein or a foreign amino acid sequence that is fused to a known protein as a 'tag'. The tag sequence can be inserted at either 5' or 3' terminus of the gene of interest in an expression vector. A linker sequence and a cleavage site for specific proteases or chemical agents are also often engineered between the tag and the protein. Introduction of linker sequences helps promote independent folding of two entities whereas use of cleavage sites allows liberation of the tag from the protein. Once fusion proteins are constructed and expressed in a cell, the purification process by affinity resin/column is generally straightforward. Examples are the hexahistidine (His_6) and glutathione-*S*-transferase (GST) tags, both of which were extensively used throughout the studies presented in this dissertation.

The His_6 tag offers a fast, one-step purification of recombinant protein by means of immobilized metal ion (e.g., nickel or cobalt) affinity chromatography (5,42,232,274). For example, the nickel ion (Ni^{2+}) is held by chelation with reactive groups that are covalently bound to a solid support (agarose or sepharose). The most commonly used chelators are nitrilotriacetic acid and iminodiacetic acid, which have four and three sites available for interaction with a Ni^{2+} ion, respectively (42,275). The coordination sites left on Ni^{2+} will be free to interact with functional groups of recombinant proteins (i.e., a

stretch of polyhistidine residues). After binding, the resin can be washed with imidazole (up to 20 mM) to remove nonspecific binding proteins, followed by an elution with 150 to 300 mM imidazole. The purity and amount of protein can then be analyzed by SDS-PAGE gels, immunoblotting, and other protein quantitation methods. A secondary chromatography, for example a sizing column, can also be used to eliminate impurities. One advantage of using a small tag like His_6 (roughly 1 kDa in molecular size) is that the tag is unlikely to interfere with the folding of proteins in a significant way. However, this technique is sometimes finicky and restricted to certain conditions due to the presence of the metal ion. For example, reducing conditions cannot be used. EDTA or many other types of chelating agents or some ionic detergents should also be avoided.

Another commonly used tag in the recombinant fusion system is the GST protein. GSTs are ubiquitous enzymes found in many species ranging from prokaryotes to human (4,23). Because of the ability to catalyze the conjugation of reduced glutathione to a variety of electrophilic xenobiotic compounds (such as carcinogens, drug metabolites, etc.), GSTs are considered to be important detoxification enzymes for an organism (4). Although GST, roughly 26 kDa in size, is quite big compared to the His_6 or FLAG tag, it is chosen as a fusion moiety due to several reasons. GST-fusion proteins can be purified without denaturation using tripeptide glutathione-coated sepharose beads, offering simple one-step purification. The beads can be washed to remove contaminating proteins. If necessary, bead-bound GST fusions can also be released into solution by adding reduced glutathione without affecting protein functions and activities. Moreover, the GST molecule is modular so it is not likely to disrupt folding of the tagged protein. Many commercially available sources of GST-based vectors also contain a cleavage site, which

enables efficient removal of GST. Due to these properties, GST fusions are widely used for antibody generation, protein-protein interaction studies, and many biochemical analyses. For studying protein-protein interactions, the GST pull-down assay is performed by first incubating GST fusions with cell lysates or by expressing GST fusions in cells. Then, any proteins that are associated with GST fusions can be concentrated on the beads and subsequently analyzed by SDS-PAGE gels, immunoblotting, or mass spectrometry. Despite these advantages, the GST fusion system has its limitations. GST is well known to form homodimers, and therefore, two different GST fusion proteins cannot be used in the same reaction/assay. Dimerization of GST fusion proteins on glutathione beads could also lead to low efficiency of elution of bound proteins with reduced glutathione or incomplete protease cleavage of the recombinant proteins.

Tandem affinity purification (TAP), which makes use of two subsequent affinity purification steps, has been recently established and shown to be more specific and sensitive for studying large networks of protein-protein interactions (93,245). The initially designed TAP tag combines two affinity tags [protein A and calmodulin binding peptide (CBP)], between which is a specific cleavage site of tobacco etch virus (TEV) protease. Insertion of the TEV cleavage site allows the Protein A tag to be removed after the first purification step (IgG beads), and the resulting CBP-tagged protein can then be harvested on the second column (calmodulin resin). This method has been shown to efficiently promote an enrichment of native multi-protein complexes containing the tagged protein. In conjunction with mass spectrometry analysis, the composition of these multi-protein complexes can be identified in an extensive fashion. One advantage of this method is that native protein complexes can be obtained without prior knowledge of their

constituents. It also largely reduces the chance for contaminating proteins to be isolated, yielding a higher purity. However, there is still a possibility that a TAP tag might interfere with folding of the tagged protein and affect its interaction with binding partners. Or, the tag may not be sufficiently exposed to the affinity resins, thereby generating ineffective results. These issues might be overcome by choosing smaller affinity tags that compose the TAP tag (48,93,237). In addition, it is important to keep in mind that the TEV protease might cleave certain proteins that are in the complex, though the frequency is rarely low because of the high specificity of the enzyme. To date, the TAP method has become a powerful tool for systematically studying the proteomics of an organism in a large scale (19,83,93,133,237,245). Following the affinity purification, acquiring identities of unknown members that may be functionally linked in large complexes can be accomplished using mass spectrometry analysis.

Mass Spectrometry Analysis and Protein Identification

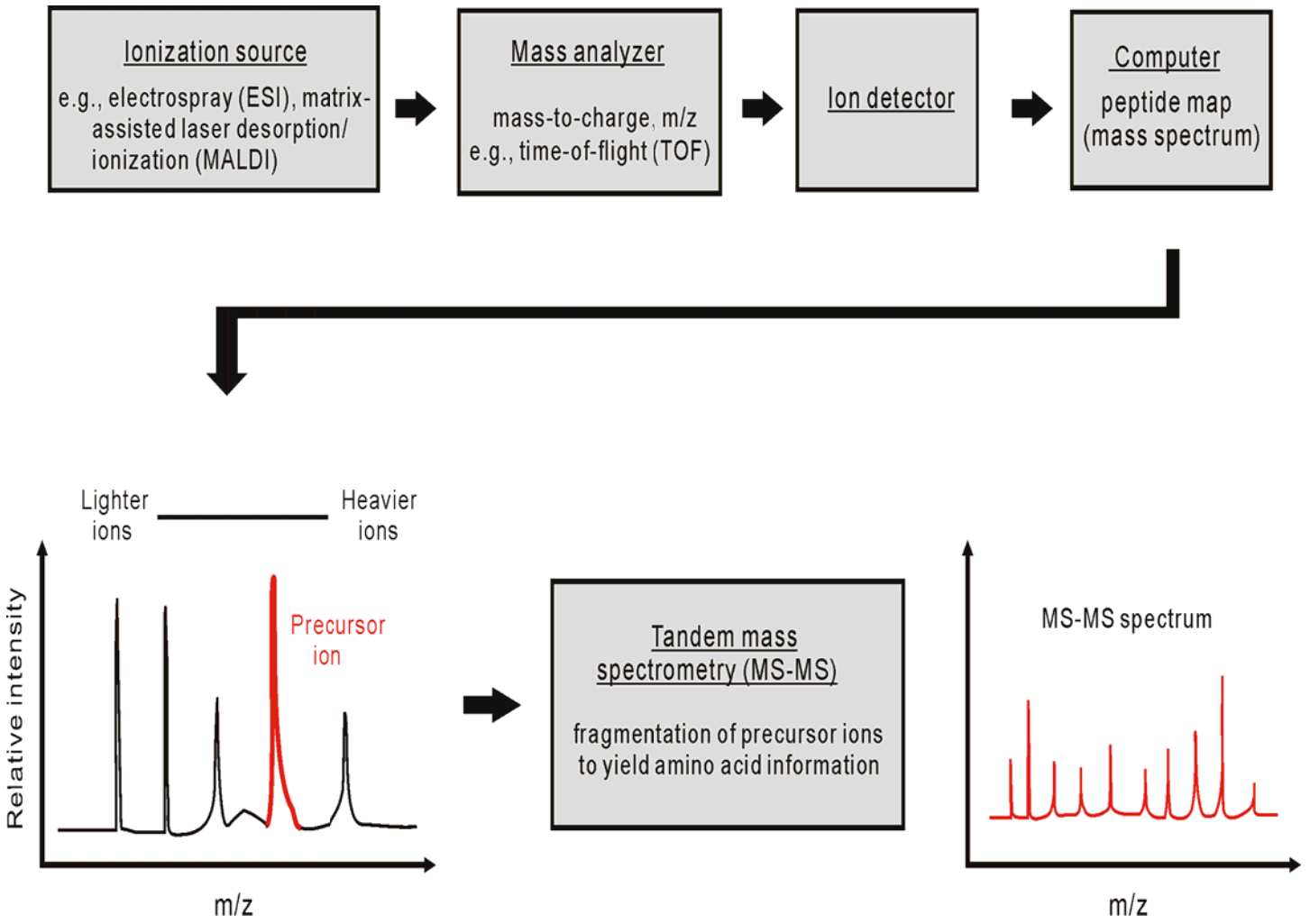
Mass spectrometry (MS) is an analytical tool used for determining the molecular mass of a sample ranging from small compounds (e.g., organic chemicals) to large biomolecules (e.g., oligonucleotides, peptides, and proteins). Because of its versatility, MS is widely adopted in academia and industry for various purposes, such as drug discovery and screening, food quality control, and biomolecule identifications. This section will provide a brief introduction of MS and discuss its application for protein analysis.

Mass spectrometers normally consist of three parts, including an ionization source, a mass analyzer, and an ion detector (Fig. 2.5) (80,157,217). Specifically, a test sample is

introduced into the ionization source of the instrument since ions are easier to manipulate than neutral molecules. There are many ionization methods available. For instance, electrospray ionization (ESI) generates ions from macromolecules in liquid medium without their fragmentation, and matrix-assisted laser desorption/ionization (MALDI) uses a laser beam for ionization of macromolecules without breaking chemical bonds. Each method has its own advantages and disadvantages, and is usually chosen depending upon the type of sample and the mass spectrometer available. The resulting ions travel through the mass analyzer where they are separated according to their mass-to-charge (m/z) ratios. Once the separated ions are detected, the signals are sent to a recorder where the m/z ratios along with their relative abundance are stored and presented in the format of a m/z spectrum (or mass spectrum). High vacuum is applied throughout the mass spectrometer to allow ions a reasonable chance of travelling from one end of the instrument to the other without any hindrance from air molecules.

For protein analysis, samples are often first separated on SDS-PAGE gels, and then, desired protein bands are excised, digested with trypsin into peptide fragments, and extracted from the gel pieces. Alternatively, protein samples can be directly digested with trypsin without being analyzed by gel electrophoresis. The tryptic peptides can then be introduced into the mass spectrometer for determination of their molecular mass (198,217). Specific peptides (precursor ions, as indicated in Fig. 2.5) inside the mass spectrometer can be selected for further tandem mass spectrometry analysis (MS-MS), by which precursor ions are fragmented and analyzed to generate their structural information.

Figure 2.5 Basic composition of a mass spectrometer and tandem mass spectrometry analysis. The test sample is first introduced into the ionization source of the instrument. Generated ions travel through the mass analyzer where they are separated according to their mass-to-charge (m/z) ratios. Once the separated ions are detected, the signals are sent to a recorder (computer) where the m/z ratios along with their relative abundance are stored and presented in the format of a m/z spectrum (or mass spectrum). Specific precursor ions inside the mass spectrometer can be selected for further tandem mass spectrometry analysis (MS-MS), by which precursor ions are fragmented and analyzed to yield their structural information.


Ionization source
e.g., electrospray (ESI), matrix-assisted laser desorption/ ionization (MALDI)
Mass analyzer
mass-to-charge, m/z
e.g., time-of-flight (TOF)
Ion detector
Computer
peptide map
(mass spectrum)
Lighter ions
Heavier ions
Relative intensity
Precursor ion
m/z
Tandem mass spectrometry (MS-MS)
fragmentation of precursor ions to yield amino acid information
MS-MS spectrum
m/z

Acquired amino acid sequences are subsequently used to identify the corresponding proteins by searching against databases containing updated protein sequence information (like GenBank, Swiss-Prot, etc.).

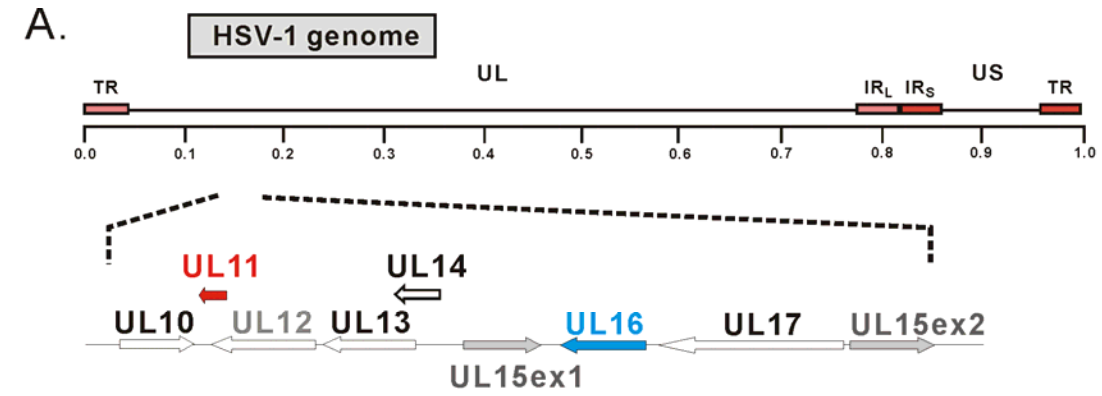
Differences in the mass spectrometer type and its setup as well as biochemical properties and complexity of protein samples can be various sources of variability in MS analysis, which can lead to lack of reproducibility and limitations in sensitivity to detect/select peptides with low abundance (21,217). And, mis-identifications (false negatives), environmental contaminants and database matching are also common sources of problems (21), which may be solved by establishing improved search engines and databases.

Nevertheless, due to its ability of analyzing protein complexes in a large scale, MS-based proteomics has become a powerful tool for studies of protein-protein interaction networks. In Chapter V, making use of MS in conjunction with tandem affinity purification has allowed efficient characterization of the UL16-associated complexes and provided the first evidence that UL16 forms a physical complex with gE in infected cells. Interestingly, UL11, a UL16 binding partner, has also been shown to interact with the cytoplasmic tail of gE in some manner (75). The following sections will provide an overview of the known/potential roles of these three proteins in virus life cycle.

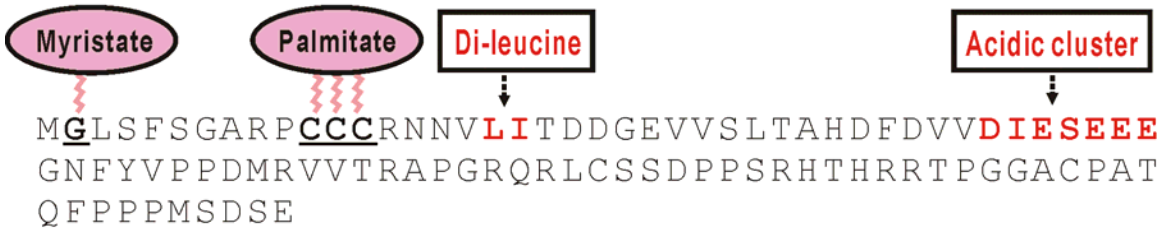
UL11 and UL16, and their Possible Roles during Virus Replication

Of over 80 proteins encoded by HSV, more than 20 are incorporated into the tegument region. Questions remain as to how these proteins are distributed within the

Figure 2.6 HSV-1 UL11 and UL16 tegument proteins. (A) The U_L11 and U_L16 coding sequences are transcribed from the minus strand, as indicated by the arrows. Flanking open reading frames are also indicated. (B) The UL11 amino acid sequence. Four motifs have been characterized within the N-terminal region: two acylation signals (purple), myristylation of the glycine residue and palmitylation of at least one nearby cysteine residue; and two motifs 'di-leucine' and 'acidic cluster' (red) that are critical for protein trafficking and interaction with UL16. (C) The UL16 amino acid sequence. The protein contains 20 cysteine residues (green), six of which are highly conserved among all herpesviruses (red).



B. UL11 (96aa)



C. UL16 (373aa)

MAQLGPRRPLAPPGPPGTLPRPDSRAGARGTRDRVDDL
GTDVDSIARIVNSVFVWRVVRADERLKIFRCLTVLTEP
LCQVALPNPDPGRALFCEIFLYLTRPKALRLPPNTFFA
LFFFNRERRYCAIVHLRSVTHPLTPLLCTLTFARIRAA
TPPEETPDPTTEQLAEEPVVGELDGAYLVPAKTPPEPG
ACCALGPGAWWHLPSGQIYCWAMDSDLGSLCPPGSRAR
HLGWLLARITNHPGGCESCAPPPHIDSANALWLSSVVT
ESCPCVAPCLWAKMAQCTLAVQGDASLCPLLFGHPVDT
VTLLQAPRRPCITDRLQEVVGGRCGADNIPPTSAGWRL
CVFSSYISRLFATSCPTVARAVARASSSDPE

virion as well as the role of individual components during virus replication cycle. As the major focus of this dissertation, two associating tegument proteins, UL11 and UL16, and their possible functions are discussed below.

The HSV U_L11 gene encodes a small, 96-amino-acid tegument protein (Fig. 2.6A and B). There are approximately 800 copies per virion (162). The UL11 protein localizes to cytoplasmic faces of both the nuclear and Golgi membranes in infected cells (8). When expressed alone, UL11 is predominantly associated with the TGN, which is a highly conserved property shared among UL11 homologs of all herpesviruses (160,260,268). It has also been shown that UL11 can direct a foreign protein in a chimeric form to the Golgi instead of the plasma membrane (27).

Deletion of UL11 from HSV, PRV, and HCMV resulted in a phenotype of accumulation of unenveloped nucleocapsids in the cytoplasm of infected cells (11,145,268). Based on these observations, UL11 has been proposed to play a critical role in virus final envelopment at the TGN. However, it is unclear whether UL11 is directly involved in initiating budding or functions to recruit proteins that are required for the budding process. HSV UL11 associates with membranes via N-terminal modifications with two fatty-acid chains (myristate and palmitate), and the protein also contains an acidic cluster (AC) and a di-leucine (LI) motif, both of which are responsible for its TGN accumulation (160). Mutagenesis analyses that lead to defects in myristylation and palmitylation result in incapability of membrane association whereas deletions of the acidic cluster and LI motifs fail in the retrieval of UL11 from the plasma membrane back to the Golgi. Moreover, it was recently shown that fatty-acid modifications of UL11 are important for association with detergent-resistant membranes (DRMs), and the AC and

LI motifs also regulate levels of DRM association (13). Because HSV assembly has been suggested to utilize DRMs, it is thought that the tegument proteins that localize to DRMs might play a critical role in virion maturation (13,153).

Studies using co-immunoprecipitation, GST pull-down assays, and a yeast two hybrid assay have identified the UL16 tegument protein as a binding partner of UL11 (161,310). The HSV U_L16 gene locates within the U_L15 intron on the anti-sense strand and encodes a 373-amino-acid tegument protein (Fig. 2.6A and C) (10). Like UL11, UL16 is conserved among all herpesviruses. The protein contains 20 cysteine residues, six of which are highly conserved (Fig. 2.6C). And, nearly 10 of these cysteines were found to be free and can be modified by *N*-ethylmaleimide (NEM) (190). UL16 has been shown to be associated with cytoplasmic capsids in infected cells (190,207), and therefore, its interaction with UL11 has been implicated in linking capsids to the TGN (190). Previous studies have reported that a UL16-null HSV mutant displayed defects in virus production, with virus titers at 3 to 10-fold lower than those of the wild-type virus (10). And, the virus also appears to produce smaller plaques on Vero cells (data not shown). Moreover, it has recently been reported that a mutant gammaherpesvirus lacking the gene homologous to HSV U_L16 displayed accumulation of partially tegumented capsids in the cytoplasm and also showed defects in release of infectious virions, providing further evidence that UL16 may play a role in virion morphogenesis and egress (113).

Previously, it was shown that UL11 is able to interact with UL16 expressed in transfected cells, revealing that no other viral proteins are required for the interaction (161). And, when expressed alone UL16 diffuses throughout the cell, but when coexpressed with UL11, it can be relocalized to perinuclear compartments reminiscent of the Golgi membranes (161). The interaction is highly specific and dependent upon the AC and LI motifs within

UL11. While foreign ACs from other proteins are able to substitute for that of UL11 in trafficking and TGN localization, they disrupt the interaction with UL16, suggesting a high specificity in the recognition (161,162). It is intriguing that UL16 recognizes the trafficking signals of UL11 (i.e., the AC and LI motifs), raising a possibility that UL16 might be a viral homolog of a clathrin adaptor subunit and function in complex with particular cellular proteins to regulate subcellular localization of UL11. Therefore, it seemed possible that host proteins might be involved in the interaction. Experiments described in Chapter IV were initiated to investigate whether UL11 directly or indirectly interacts with UL16 and how these two proteins interact with each other.

Moreover, studies also showed that UL16 interacts with UL21, another conserved capsid-associated tegument protein (140). UL21 has been shown to associate with microtubules, implicative of a role in transport of cytoplasmic capsid during virus egress (289). Based on all available observations, the tripartite UL11-UL16-UL21 complex has been proposed to play a role in recruiting nucleocapsids to the TGN during final envelopment (161,190,207); however, this hypothesis has never been proven.

In addition to a role in virus egress, UL16 was also found to be involved in a signaling event during virus attachment (described previously). However, the molecular machinery involved in the process is unclear. In Chapter V, evidence was provided that UL16 forms a complex with the glycoprotein gE, suggesting that gE might also be involved in this signaling event. Though the role of gE during virus attachment still remains to be determined, it has been strongly implicated in virus egress and cell-to-cell spread.

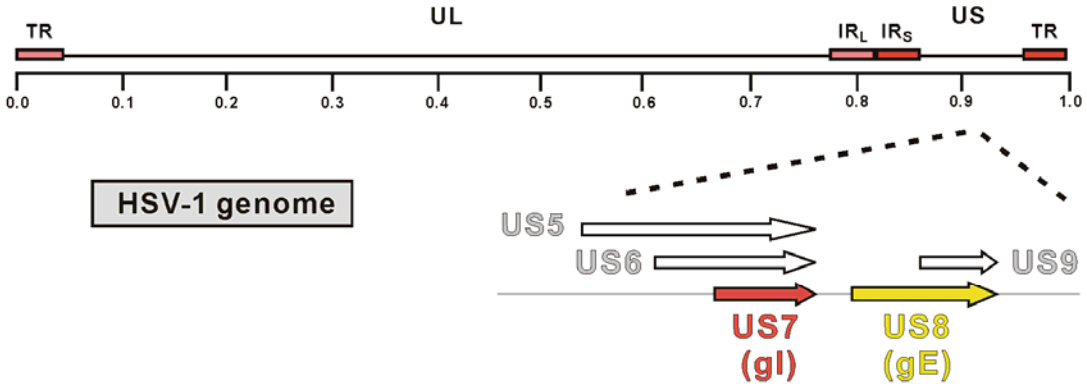
Viral Glycoprotein gE

HSV-1 gE, encoded by the U_S8 gene, is a 550-amino-acid type I membrane glycoprotein composed of an ectodomain, a transmembrane (TM) domain, and a cytoplasmic tail (CT) (Fig. 2.7A and B). A 25-amino-acid-long signal peptide which is responsible for ER targeting is initially synthesized at the N terminus and subsequently cleaved by host signal peptidase on the ER membrane. As the gE polypeptide chain continues to be synthesized, it is translocated into the ER lumen and further modified by oligosaccharyltransferases, which is an ER-residing enzyme complex that catalyzes the transfer of oligosaccharides from the glycosyl donor dolichol to nascent proteins (154). This cotranslational modification, known as N-glycosylation, takes place at asparagine (N) residues in the ectodomain of gE. Once the gE protein is correctly folded, it moves from the ER to the Golgi where N-glycan chains continue to be trimmed and elongated. And, depending on protein accessibility to saccharide-modifying proteins in the Golgi, glycosylation process may not always go to completion (117). Therefore, there could be heterogeneity in the type and length of the glycan side chains at each glycosylation site, which in turn results in several glycosylated variants differing in mass as observed in SDS-PAGE gels (71,129).

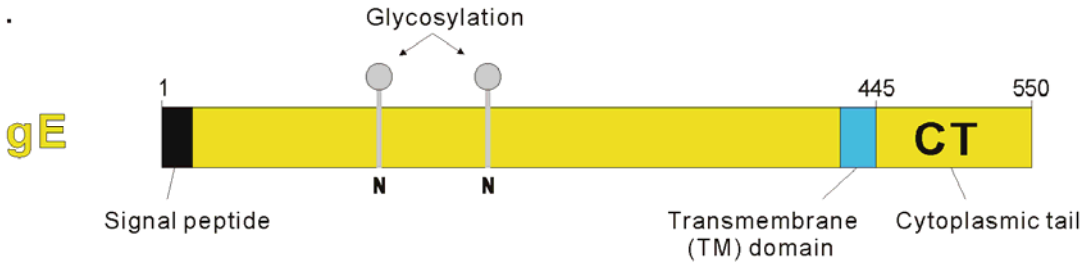
gE is highly conserved in alphaherpesviruses, including HSV, PRV, and VZV, and all homologs are known to form a physical complex with another viral glycoprotein gI. Like gE, gI (encoded by U_S7 gene) is also a type I membrane glycoprotein consisting of an ectodomain, a TM domain, and a CT (Fig. 2.7A and C). The heterodimeric gE/gI complex, built upon interaction between two ectodomains, has been shown to play an essential role in virus cell-to-cell spread (Fig. 2.4) and additionally function as an IgG Fc receptor (18,63,64,71). Mutant viruses lacking gE or gI displayed defects in cell-to-cell

Figure 2.7 HSV-1 glycoproteins gE and gI. (A) The U_s7 and U_s8 coding sequences are transcribed from the plus strand, as indicated by the arrows. Flanking open reading frames are also indicated. (B and C) Schematic representations of gE and gI and amino acid sequence of each cytoplasmic tail (CT). The residue numbers for the CT domains are indicated. Both gE and gI are type I membrane glycoproteins with an N-terminal signal peptide and a transmembrane (TM) domain near the C terminus. The potential sites for addition of N-linked glycans are marked by grey lollipops. gE.CT has two YXXΦ motifs (underlined) and a cluster of acidic residues (red) flanked by three serines for phosphorylation (B). gI.CT has a di-leucine motif (underlined) at the very end of the tail (C).

A.



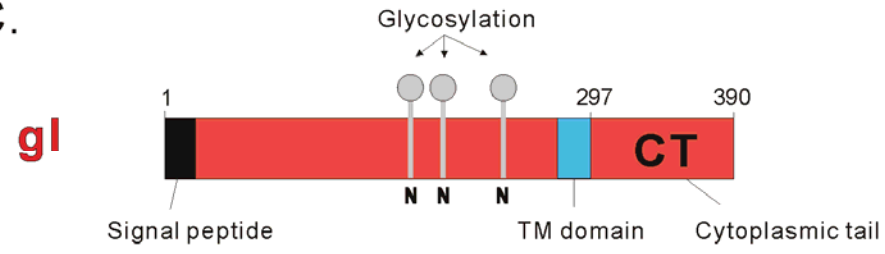
B.



445

...RRRAWRAVKSRASGKGPT**YIRV**ADSEL**YADWSSDSEGERD**
QVPWLAPPERPDSPSTNGSGFEILSPTAPSVYPRSDGHQSR
RQLTTFGSGRPDRRYSQASDSSVFW

C.



297

...RCQRRYRRSRRPIYNPQIPTGISCAVNEAAMARLGAELKS
HPSTPPKSRRRSSRTPMPSLTAIAEESEPAGAAGLPTPPVDP
TTSTPTPP**LL**V

spread in both cultured cells and animals (22,46,63,64,297). It has been shown that in cultured polarized epithelial cells gE/gI accumulates at the TGN at early times of infection and later traffics to the basolateral surfaces, suggesting that gE/gI may function in targeting virions to cell junctions (65). The ectodomains of gE/gI are also necessary for cell-to-cell spread. It was hypothesized that at late times of infection, gE/gI accumulates and are tethered at cell junctions where its ectodomain binds to a yet-to-be-determined host receptor, thereby mediating virus movement across epithelial cell junctions (325). Thus, gE/gI appears to play at least two critical roles that promote virus cell-to-cell spread: basolateral targeting and receptor engagement at cell junctions. Studies indicated that though the ectodomain of gE/gI is sufficient for accumulation at cell junctions, viruses lacking the cytoplasmic tails behave much like gE- or gI-null mutants. They all produced smaller plaques and showed defects in cell-to-cell spread, further supporting the indispensible function of the gE/gI tails (187,296).

HSV-1 gE.CT is 106-amino-acid-long and contains several intracellular trafficking motifs which are also found in PRV and VZV homologs. There are two tyrosine-based motifs (YXXΦ, where Y standing for tyrosine, X for any amino acid, and Φ for a bulky, hydrophobic amino acid)) and a stretch of acidic amino acids containing serine residues that are phosphorylated (Fig. 2.7B) (3). gE.CT YXXΦ motifs were suggested to interact with cellular clathrin adaptors AP-1 (μ1 subunit), which may promote virion incorporation into clathrin-coated transport vesicles, and the acidic cluster may be involved in TGN accumulation via interaction with phosphofurin acidic cluster sorting protein-1 (PACS-1) (3,51,77,129,187). gI.CT also contains a dileucine (LL) motif that may serve functions in protein endocytosis, TGN accumulation, and localization to

the basolateral surfaces of polarized cells through interactions with AP-1 (β1 subunit) (Fig. 2.7C). Based on these observations, gE/gI CTs were suggested to function in sorting vesicles containing virions to basolateral surface, facilitating cell-to-cell spread.

Furthermore, it is intriguing that when coexpressed in cells gE/gI predominantly localizes to the TGN whereas a virus infection results in accumulation at cell junctions at later times (129,187). These observations suggest that another viral factor(s) may bind to gE/gI and facilitate its trafficking to cell junctions. One possibility is the UL11 tegument protein, which has been implicated in the process and also has been shown to interact with gE.CT (discussed previously). And, deletion of a UL11 binding partner, UL16, also displayed small plaques on cultured cells (data not shown). So if UL11 does play a role in cell-to-cell spread, UL16 could also be involved. In Chapter V, evidence was presented that UL16 is capable of interacting with gE.CT, further implicating a potential role of UL16 in cell-to-cell spread.

To address the specific functions of UL11, UL16, and gE in virus replication cycle, it is fundamental to characterize the molecular basis of how these proteins interact with each other. Thus, studies described in Chapter IV and Chapter V were initiated to analyze and define these molecular interactions.

Chapter III

Materials and Methods

Cells and Virus Propagation

Cells and Viruses

Vero (African monkey kidney) and A7 (human melanoma) cells were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 5% fetal bovine serum (FBS), penicillin (65 μg/ml), and streptomycin (131 μg/ml) at 37°C in a humidified CO_2 incubator. The KOS strain of HSV type 1 (HSV-1) (273) was used for these studies, along with several recombinants described later. For experiments with pseudorabies virus (PRV), the Becker strain was used. In all cases, infected cells were grown in DMEM supplemented with 2% FBS, 25 mM HEPES buffer, glutamine (0.3 μg/ml), penicillin, and streptomycin.

Plaque Assay

Virus stocks were diluted in 10-fold steps with phosphate-buffered saline (PBS) containing 1% FBS, and incubated with confluent Vero cells in duplicate 60 mm-plates for 1 h at 37°C. After 1 h, unbound viruses were removed by one wash with PBS-FBS, and cells overlaid with 0.5% (w/v) methylcellulose were incubated for additional 2 to 3 days. Cells were subsequently stained with crystal violet, and plaques were counted.

Single-Step Virus Growth

60 mm-plates of Vero cells (3 X 10^6/plate) were infected with specified viruses at a multiplicity of infection (MOI) of 5 at 37°C. After 1 h incubation, cells were washed with PBS-FBS and then overlaid with 4 ml of DMEM containing 2% FBS. At indicated times postinfection, medium and cells from each plate were harvested and treated as

follows. Medium was cleared of cells by centrifugation at 3,000 rpm for 5 min, and frozen at -80°C. Cells were scraped into PBS, washed three times with PBS, and freeze-thawed three times to release cell-associated viruses. Each sample was titered on Vero cells using plaque assay describe above.

Antibodies

A His_6-specific mouse monoclonal antibody was purchased from Novagen (product number 70796-3). The UL11-, UL16-, and GFP-specific antibodies produced in rabbits (Cocalico Biologicals, Inc.) were raised against purified GST-UL11, GST-UL16, and His_6-GFP antigens, and have been described previously (13,161,190). The αHis_6-GFP-specific serum (diluted 1:3,000) recognizes GFP, CFP, and also the His_6 tag, which was fused to the N terminus of GFP and UL16 for purification from *Escherichia coli* (see Chapter IV-Results). The rabbit polyclonal antibody against VP5 was kindly provided by Richard J. Courtney (Pennsylvania State University). The polyclonal gE antibody (UP1725), kindly provided by Harvey M. Friedman (University of Pennsylvania), was produced in rabbits, using baculovirus-expressed gE amino acids 24 to 409 as the antigen.

Recombinant viruses

KOS.UL11-GFP

A recombinant virus expressing UL11-GFP was created by homologous recombination and has been described previously (13). Sequences from upstream and downstream of the U_L11 gene (360 bp each) were PCR amplified from the KOS genome and ligated to the 5' and 3' termini of U_L11-*gfp*, respectively. The composite DNA

fragment was inserted into pSP72 vector (Promega) using BamHI and HpaI sites, and then cotransfected into A7 cells with the genome of the KOS strain of HSV-1. Recombinant virus was selected by five rounds of plaque purification by fluorescence microscopy. The resulting virus was confirmed by a combination of PCR analyses using primers that flank the U_L11-*gfp* coding sequence (yielding a larger product than untagged UL11) and the failure to express the wild-type, untagged UL11, as determined by immunoblotting and radiolabeling-immunoprecipitating for UL11. The recombinant was analyzed for specific infectivity and plaque size, as well as localization and kinetics of UL11-GFP expression. All characteristics examined were undiminished compared with the parental virus (data not shown).

KOS.UL16-CFP

A derivative of HSV that expresses UL16-CFP was created by homologous recombination. Briefly, the *gfp* gene of plasmid pCMV.UL16-GFP (161) was replaced with the *cfp* gene using flanking BamHI and BsrGI sites. Next, a fragment containing 300 bp downstream of U_L16 was PCR amplified from the KOS genome with a forward primer containing a BsrGI site and a reverse primer containing a NotI site, and this fragment was inserted at the 3' end of the U_L16-*cfp* coding sequence. The resulting plasmid was linearized with EcoRI and transfected into A7 cells, along with purified KOS DNA. Plaques produced by the recombinant virus were identified by fluorescence microscopy, and these were picked for six rounds of purification. Confirmation that the desired virus was obtained was provided by PCR analyses using primers that flank the U_L16-*cfp* coding sequence (yielding a larger product than untagged U_L16) and the failure

to express wild-type UL16 (as determined both by immunoblotting and radiolabeling-immunoprecipitation assays [data not shown]). Moreover, the recombinant was found to be identical to the wild type with regard to specific infectivity and plaque size, as well as subcellular localization and kinetics of UL16-CFP expression (data not shown).

KOS.UL16-TAP

To construct a recombinant virus that expresses UL16 fused with a TAP (tandem affinity purification) tag (Fig. 3.1A), a bacterial artificial chromosome (BAC) containing the HSV-1 KOS strain genome was used [KOS BAC, generously provided by David A. Leib (99)]. Recombinant HSV-1 clones were made using a *galK* positive/negative selection-based recombineering strategy, as described previously (314). Briefly, a *galK* expression cassette was inserted at the 3' terminus of U_L16 open reading frame in the KOS BAC. Next, the *galK* cassette was replaced with a DNA fragment that encodes a TAP tag sequence (pCAGGS TAP tag C cassette, a gift from Dr. de la Torre, Scripps Research Institute). Starting from the position closest to UL16, the TAP tag is composed of a calmodulin binding peptide (CBP), a tobacco etch virus (TEV) protease cleavage site, and two IgG binding domains of Protein A (Fig. 3.1A). The resulting BAC was then transfected into A7 cells by means of the calcium phosphate precipitation method. After 5 to 6 days, transfected cells were harvested when showing cytopathic effects, and used to infect new Vero cells to produce a viral stock. Confirmation that the desired virus was obtained was provided by PCR analyses using primers that flank the U_L16-TAP coding sequence (yielding a larger product than untagged UL16) and the failure to express wild-type UL16 (as determined by immunoblotting). Kinetics of UL16-TAP expression was

found to be identical to the wild-type KOS strain. The recombinant was also found to be indistinguishable from the wild type with regard to plaque size and specific infectivity (see Chapter V-Results).

BV.UL16-GFP

To create a recombinant baculovirus that expresses UL16-GFP, the chimeric gene was first PCR amplified from pCMV.UL16-GFP (161) using a forward primer containing a BspHI site and a reverse primer containing a NotI site. This fragment was cloned into the pTriEx-1.1 vector (Novagen), which was used to produce the recombinant baculovirus by homologous recombination in insect cells via the BacVector-3000 transfection kit (Novagen). *Spodoptera frugiperda* cells (Sf21) were maintained in Grace's insect medium supplemented with Yeastolate (Mediatech), lactalbumin hydrolysate (Mediatech), penicillin, streptomycin, and 10% FBS in a humidified incubator at 28°C without CO_2. Plaques produced by recombinant baculoviruses were identified by fluorescence microscopy, and these were picked for several rounds of purification. Virus stocks were amplified by infecting suspension cultures of Sf21 cells at a MOI of 0.2. Virions were purified from culture medium at 5 to 7 days postinfection and concentrated, and titers were determined as described previously (28,220) (work performed by D.G. Meckes, Jr.).

Mammalian Expression Vectors

For expression of UL16-GFP derivatives in mammalian cells, deletion mutations and alleles for single amino acid substitutions were created in pCMV.UL16-GFP (161)

by either PCR technology or QuikChange site-directed mutagenesis (Stratagene). Codons for cysteines were individually replaced with ones for serine (-AGC-). A plasmid expressing full-length gE (pCMV3-gE) (71) was a kind gift from Harvey M. Friedman (University of Pennsylvania).

Construction and Purification of *E. coli*-Expressed Proteins

GST-Fusion Constructs

Plasmids encoding wild-type GST-UL11 and mutants lacking the LI, AC, or residues 51 to 96 were described previously (161). To construct a GST-UL11 mutant that lacks the three consecutive cysteines located near the N terminus, the U_L11.CCC- allele was PCR amplified from pCMV.UL11-GFP.CCC- (160) and cloned into pGEX-4T-3 (GE Healthcare). The resulting plasmid was subsequently used to make GST-UL11.4C-, which does not contain any cysteines in UL11. This was accomplished by changing the codon for the remaining cysteine in UL11.CCC- (residue 83) to that for alanine (-GCA-) using QuikChange site-directed mutagenesis according to the manufacturer's protocol. The wild-type U_L16 gene was also cloned into pGEX-4T-3 to generate a plasmid that expresses GST-UL16. A plasmid encoding the cytoplasmic tail of gE fused to GST (GST-gE.CT) was a kind gift from David C. Johnson (The Oregon Health and Science University) and has been described previously (218). For expression of GST-gE.CT derivatives, an acidic cluster (DEED/As) and deletion mutants were created by QuikChange site-directed mutagenesis. Codons for four acidic amino acids (two aspartates and two glutamates within the sequence SS**D**S**E**G**E**R**D**QV) in gE.CT were substituted with ones for alanine using a forward primer (5'-GAC TTG AGC TCG **GCC**

AGC **GCG** GGA **GCA** CGC **GCC** CAG GTC CCG TG-3') and a reverse primer containing the reverse-complementary sequence. Plasmids encoding the cytoplasmic tail of gB and gD fused to GST (GST-gB.CT or -gD.CT) were provided by Richard J. Courtney (Pennsylvania State University). All GST-fusion proteins were purified from *E. coli* on glutathione beads using the standard methods described by the manufacturer (GE Healthcare).

His_6-Tagged Proteins

To construct plasmids that encode His_6-UL11 (wild-type and LI mutant versions) or His_6-UL16, the alleles were cloned into pET-28 (Novagen). The resulting plasmids were transformed into *E. coli* BL21 (DE3) cells (Novagen), and 0.1 mM isopropyl-β-D-thiogalactopyranoside was added to the cultures to induce protein expression. Proteins were purified by using the His-Bind kit (Novagen). Briefly, approximately 1 g of bacteria were pelleted and resuspended in 10 ml of PBS containing protease inhibitors (Sigma, P8340). The bacteria were lysed by sonication and treatment with 1% Triton X-100 for 30 min at 4°C. After the removal of cell debris and insoluble material by centrifugation at 14,000 x g for 10 min, the lysates were incubated with nickel beads for 30 min. The beads were washed according to the His-bind protocol, and proteins were eluted in 300 mM imidazole and dialyzed overnight against 20 mM Tris-HCl (pH 7.9). Proteins were quantified in standard bicinchoninic acid assays or by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining. On average, the yields of His_6-UL11 and His_6-UL16 proteins from 1 g of bacteria were 0.2 mg and 0.1 mg, respectively.

GST Pull-Down Assay

GST pull-down assays were performed as described previously (161). To analyze the interaction of UL11 with UL16 mutants, pCMV.UL16-GFP derivatives were transfected into A7 cells by means of the calcium phosphate precipitation method. At 20 h posttransfection, the cells were harvested in NP-40 lysis buffer (0.5% NP-40, 150 mM NaCl, 50 mM Tris-HCl [pH 8.0]), and glutathione bead-bound GST-UL11 was added. Proteins bound to the beads were separated by SDS-PAGE, transferred to nitrocellulose membranes, and analyzed by enhanced chemiluminescence-based immunoblotting (GE Healthcare). The same protocol was used to analyze the interaction of GST-UL11 with UL16 produced in Vero cells infected with HSV (wild-type KOS or KOS.UL16-CFP) or insect cells infected with BV.UL16-GFP (all produced in 60-mm plates at a MOI of 1). To detect proteins that were pulled down with GST-UL16 from HSV- or PRV-infected cells, the cells were first labeled with [^{35}S]methionine, as described previously (161). To look for host proteins that might bind to GST-UL11 in complex with UL16-GFP produced in insect cells, SDS-PAGE gels were subjected to zinc staining following the manufacturer's instructions (Bio-Rad). To analyze the interaction of GST-gE.CT chimeras with UL16 derived from various sources, the same procedure was performed using glutathione bead-bound GST-gE.CT chimeras.

NEM Treatment

To covalently modify free cysteines in UL16, NEM was used at a final concentration of 10 mM (25). For in vitro binding assays, His_6-UL16 proteins made in bacteria were purified on nickel beads, as described above. Before they were eluted from

beads, His_6-UL16 proteins were treated with NEM for 30 min at room temperature. The beads were washed thoroughly, and the proteins were eluted and dialyzed. For baculovirus- or HSV-infected cells, NEM was added to cells that had been resuspended in PBS, and after incubations of 30 min at room temperature, the cells were washed three times with PBS before NP-40 lysis. To examine the effect of NEM on the UL11-UL16 complex, GST pull-down assays were done as before. Then, glutathione bead-bound GST constructs and the associated proteins were treated with NEM for 30 min at room temperature before being analyzed on SDS-PAGE gels.

Co-Immunoprecipitation from Transfected or Infected Cells

Confluent monolayers of Vero or A7 cells were infected with a MOI of 5 with HSV (wild-type KOS or KOS.UL16-CFP) or transfected with specified mammalian expression vectors. At 20 h postinfection, cells were scraped into PBS, pelleted by centrifugation at 1,000 x g for 5 min, and resuspended into NP-40 lysis buffer with protease inhibitors (Sigma, P8340). Infected cells were left on ice for 10 min, and nuclei were removed by centrifugation for 2 min at 18,000 x g. Lysates were precleared overnight with protein A agarose beads (Roche) at 4°C with rocking, and subsequently incubated with UL16-, GFP-, or gE-specific antibodies. Following 5-h incubation at 4°C, lysates were incubated with protein A beads for 2 h with rocking. The beads were washed thoroughly with NP-40 lysis buffer and resuspended in sample buffer. Bound proteins were separated in 10% SDS-PAGE gels and analyzed by immunoblotting with UL16-, GFP-, VP5-, or gE-specific antibodies (diluted 1:3,000). To avoid background resulting from the antibodies used in the immunoprecipitation, True-Blot HRP- conjugated

secondary antibody (eBioscience) that recognizes only native Fc epitopes was used at a dilution of 1:7,500.

Analysis of Virion Protein Incorporation

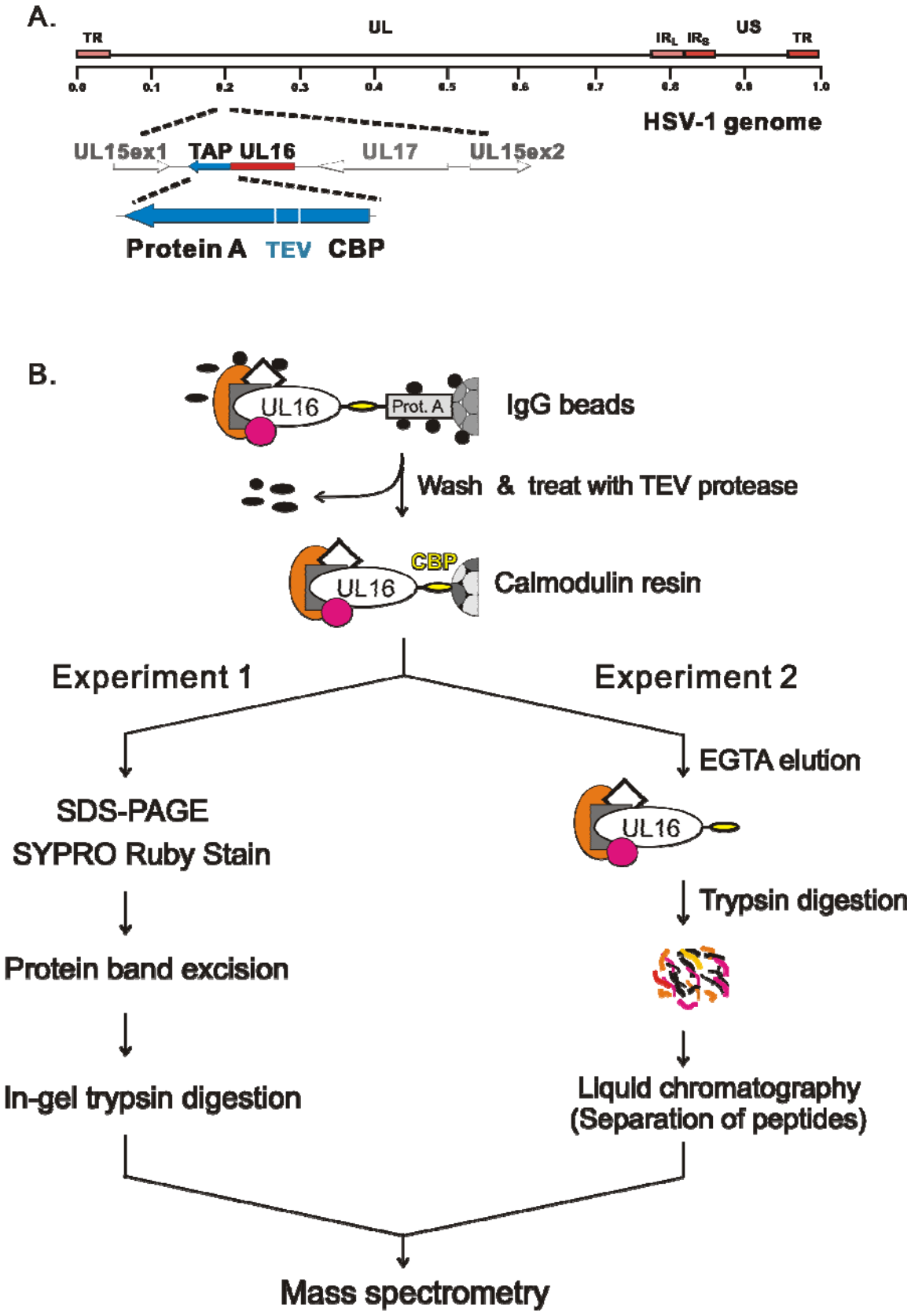
Confluent monolayers of Vero cells were infected at a MOI of 5. At 20 h postinfection, medium was collected and cell debris was removed by centrifugation for 10 min at 1,000 x g. Then, extracellular virions were pelleted by centrifugation for 1 h at 83,500 x g in a SW41 rotor through a 30% (w/v) sucrose cushion (1.5 ml). Virion proteins were separated by 10% SDS-PAGE gels and analyzed by immunoblotting using VP5-, UL16-, or GFP-specific antibodies.

Tandem Affinity Purification/Mass Spectrometry Analysis

Tandem Affinity Purification (TAP)

Vero cells infected with KOS.UL16-TAP at a MOI of 5 were lysed in NP-40 buffer at 20 h postinfection. As depicted in Fig. 3.1B, cell lysates were first incubated with IgG Sepharose beads (GE Healthcare) overnight at 4°C. The IgG beads were then washed with TEV protease cleavage buffer (0.1% NP-40, 150 mM NaCl, 1 mM dithiothreitol [DTT], 10 mM Tris-HCl [pH 8.0]), and incubated with TEV protease (generously provided by John M. Flanagan, Pennsylvania State University) at room temperature for 4 h. Sample were kept at 4°C overnight to allow complete digestion. The cleavage product UL16-CBP and its associated proteins were harvested using calmodulin resin (GE Healthcare) in calmodulin binding buffer (0.1% NP-40, 150 mM NaCl, 10 mM β-mercaptoethanol, 10 mM Tris-HCl [pH 8.0], 1 mM Mg acetate, 1 mM imidazole, 2

Figure 3.1 Experimental design of tandem affinity purification (TAP). (A) The diagram depicts insertion of the coding sequence of the TAP tag (blue) at the 3' end of the U_L16 open reading frame (red). Starting from the position closest to UL16, the TAP tag composes of a calmodulin binding peptide (CBP), a tobacco etch virus (TEV) protease cleavage site, and Protein A. (B) Protein complexes containing UL16 were pulled down using the IgG beads (via Protein A moiety) and treated with the TEV protease. After cleavage, the resulting CBP-tagged UL16 and associating proteins were collected using calmodulin resin. Two different approaches were then used to acquire final samples for mass spectrometry analysis. In Experiment 1, proteins bound to the beads were resolved in SDS-PAGE gels and visualized using SYPRO Ruby Protein Gel Stain. The detected protein bands were excised, digested with trypsin, and analyzed by mass spectrometry. In Experiment 2, proteins were eluted from the beads using EGTA and digested with trypsin. The resulting peptide mixture was then analyzed using liquid chromatography followed by mass spectrometry.


A.
TR
UL
IRL IRS
US
TR
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
HSV-1 genome
UL15ex1 TAP UL16 UL17 UL15ex2
Protein A TEV CBP
B.
UL16
Prot. A
IgG beads
Wash & treat with TEV protease
CBP
UL16
Calmodulin resin
Experiment 1
Experiment 2
EGTA elution
SDS-PAGE
SYPRO Ruby Stain
UL16
Trypsin digestion
Protein band excision
In-gel trypsin digestion
Liquid chromatography
(Separation of peptides)
Mass spectrometry

mM $CaCl_2$). Collected proteins from two experiments were analyzed with different approaches as follows. **Experiment 1**: Proteins bound to the beads were eluted in sample buffer, resolved in SDS-PAGE gels, and visualized using SYPRO Ruby Protein Gel Stain following the manufacturer's instructions (Invitrogen). The detected protein bands were excised and analyzed by mass spectrometry (see below, In-Gel Trypsin Digestion/MS-MS). **Experiment 2**: Proteins bound to the beads were eluted from the beads using ethylene glycol tetraacetic acid (EGTA) and digested with sequencing grade modified trypsin (10 ng/µl; Promega, V5111) for 4 h at 4°C. The resulting peptide fragments were concentrated in a speed-vac, rehydrate-dried three times with 250 µl pure water, and resuspended in 100 µl millipore water. The final solution was then analyzed using liquid chromatography combined with matrix-assisted laser desorption/ionization mass spectrometry (see below, LC-MALDI/MS-MS).

In-Gel Trypsin Digestion/MS-MS (For samples in Experiment 1)

The following procedure was performed by Michael Ward and John O. Semmes at Eastern Virginia Medical School. Protein gel bands excised from SDS-PAGE gels were cut into 1 to 2 mm cubes, washed three times with 500 µl Ultra-pure water, and incubated in 100% acetonitrile for 45 min. Samples were reduced with 50 mM DTT at 56°C for 45 min and alkylated with 55 mM iodoacetamide for 1 h at room temperature. The material was dried in a speed-vac, rehydrated in a 12.5 ng/µl sequencing grade modified trypsin solution (Promega, Madison, WI), and incubated in an ice bath for 40 to 45 min. The excess trypsin solution was removed and replaced with 40 to 50 µl of 50 mM ammonium bicarbonate, 10% acetonitrile (pH 8.0). The mixture was then incubated

overnight at 37°C. Elastase digests were performed as described for trypsin at an enzyme concentration of 15 ng/µl without acetonitrile in the reaction buffer. Peptides were extracted twice with 25 µl 50% acetonitrile, 5% formic acid and dried in a speed-vac. Digests were resuspended in 20 µl buffer A (5% acetonitrile, 0.1% formic acid, 0.005% heptafluorobutyric acid [HFBA]), and 10 µl were loaded onto a 12-cm x 0.075-mm fused silica capillary column packed with 5 µM diameter C18 beads (The Nest Group, Southboro, MA) using a N2 pressure vessel at 1100 psi. Peptides were eluted over 80 min, by applying a 0 to 80% linear gradient of buffer B (95% acetonitrile, 0.1% formic acid, 0.005% HFBA) at a flow rate of 200 µl/min with a pre-column flow splitter, resulting in a final flow rate of ~200 µl/min directly into the source. In some cases, the gradient was extended to 150 min to acquire more MS-MS spectra. A LTQ™ Linear Ion Trap (ThermoFinnigan, San Jose, CA) was run in an automated collection mode with an instrument method composed of a single segment and five data-dependent scan events with a full MS scan followed by four MS-MS scans of the highest intensity ions. Normalized collision energy was set at 35, activation Q was 0.250 with minimum full scan signal intensity at 1 x 105 with no minimum MS2 intensity specified. Dynamic exclusion was turned on utilizing a three minute repeat count of 2 with the mass width set at 1.0 m/z. Sequence analysis was performed with MASCOT (Matrix Sciences, London GB) using an indexed viral and Human subset database of the non-redundant protein database from National Center for Biotechnology Information (NCBI) web site (http://www.ncbi.nlm.nih.gov/).

LC-MALDI/MS-MS (For samples in Experiment 2)

The following procedure was performed by Anne Stanley and Bruce Stanley (Pennsylvania State University). The tryptic peptides of EGTA-eluted samples were separated on a reverse phase nanoflow C18 HPLC column, and eluted at a flow rate of 2.5 µl/min in a gradient (95% A [0-8 min], 5% B [0-8 min], 40% B [8.1-40 min], 80% B [41-44 min], 5% B [44-49 min]). Buffer A was 2% acetonitrile, 0.1% trifluoroacetic acid, and buffer B was 98% acetonitrile, 0.1% trifluoroacetic acid. The eluted fractions combined with MALDI matrix solution (7 mg/ml recrystallized α-cyano-hydroxycinnamic acid [CHCA], 2 mg/ml ammonium phosphate, 0.1% trifluoroacetic acid, 80% acetonitrile) were automatically spotted onto a stainless steel MALDI target plate every six seconds (0.6 µl per spot), for a total of 370 spots. MS Spectra are then acquired from each sample spot using the newly updated default calibration, using 400 laser shots per spot, laser intensity 3200 (which can change somewhat with laser age and tuning). A plate-wide interpretation was then automatically performed, choosing the highest peak of each observed m/z value for subsequent MS-MS analysis. Up to 2500 laser shots at laser power 4200 were accumulated for each MS-MS spectrum. Sequence analysis was performed using the Paragon and ProGroup algorithm as implemented in the ProteinPilot 2.0 software (MDS Sciex/Applied Biosystems).

Chapter IV

Analysis of Interaction between the UL11 and UL16 Tegument Proteins of Herpes Simplex Virus

Adapted from Yeh, P.C., D.G. Meckes, Jr., and J.W. Wills. 2008.

J. Virol. 82 (21): 10693-10700.

ABSTRACT

The UL11 and UL16 tegument proteins of herpes simplex virus are conserved throughout the herpesvirus family. Previous studies have shown that these proteins interact, perhaps to link UL16-bound nucleocapsids to UL11, which resides on the cytoplasmic face of the *trans*-Golgi network, where maturation budding occurs. Little is known about the interaction except that it requires the leucine-isoleucine (LI) and acidic cluster motifs in UL11 and that no other viral proteins are involved. In particular, the important question of whether these two proteins bind to each other directly has not been addressed. Accordingly, UL11 and UL16 were expressed in bacteria, and the purified proteins were found to retain the ability to interact in a manner that was dependent upon the LI and acidic cluster. In an attempt to map the UL11-binding site contained in UL16, a large number of deletion mutants were constructed. The first 40 (nonconserved) amino acids were found to be dispensable, but all the other constructs failed to bind UL11 or had poor expression in transfected cells, suggesting that UL16 is very sensitive to alterations and probably lacks a multidomain structure. As an alternative strategy for identifying residues that are important for the interaction, the cysteines of UL16 were investigated, because many of these are highly conserved. Approximately half of the 20 cysteines in UL16 have been shown to be covalently modified by *N*-ethylmaleimide, and this treatment was found to block the interaction with UL11. Moreover, individual serine replacements of six of the most conserved cysteine residues were made, and four of these disrupted the interaction with UL11 without affecting protein stability. However, the UL11-UL16 interaction does not involve the formation of interspecies disulfide bonds,

because binding occurred even when all the cysteines in UL11 were eliminated. Thus, UL16 directly interacts with UL11 and does so in a manner that requires free cysteines.

INTRODUCTION

Herpesviruses have three morphological structures: the icosahedral capsid, which contains the viral DNA; the tegument, a proteinaceous compartment surrounding the capsid; and the lipid envelope embedded with virus-encoded glycoproteins. While the formation of the capsid has been studied in depth (209,241,248,264), the assembly of tegument proteins and final envelopment remain poorly understood. It is thought that some tegument proteins are added to the capsid in the nucleus (33), whereas others are acquired after entering the cytoplasm or traveling to the site of final envelopment at the *trans*-Golgi network (TGN) (76,193,194). Several molecular interactions have been implicated in linking the virus capsid, tegument, and membrane during the envelopment process. Examples include VP22 (tegument)-gD (membrane), VP16 (tegument)-gH (membrane), and UL11 (membrane)-UL16 (tegument/capsid) (193,194). The focus of this report is the interaction of the UL11 and UL16 tegument proteins of herpes simplex virus (HSV).

UL11, a 96-amino-acid protein, is conserved among all herpesviruses and thought to play a role in the virus budding process at the TGN during nucleocapsid envelopment (11,145,171,268). UL11-null mutants are defective in virus replication and exhibit increased numbers of unenveloped capsids in the nucleus and cytoplasm (11,30,145,171,268). UL11 is a myristylated protein that accumulates on the cytoplasmic faces of nuclear and Golgi apparatus-derived membranes in infected cells and localizes primarily to the TGN when expressed alone (8,160,170,260). In addition, UL11 has multiple sequences that are required for its proper membrane localization, including cysteines for palmitylation, a leucine-isoleucine (LI) motif, and an acidic cluster (AC)

(13,160). These sequences also regulate the amount of UL11 that is associated with detergent-resistant membranes (13).

UL16 is a 373-amino-acid protein that is also conserved among all herpesviruses (224,324). It has been identified as a binding partner of UL11 by co-immunoprecipitation, glutathione *S*-transferase (GST) pull-down, and yeast two-hybrid assays, and the interaction requires the LI and AC motifs of UL11 (161,310). ACs from other proteins are able to substitute for that of UL11 in trafficking assays, but they disrupt the interaction with UL16, suggesting a high specificity in the recognition (161,162). More recently, we reported that UL16 dynamically interacts with the capsid (190). In particular, UL16 is stably associated with capsids in the cytoplasm but not those in extracellular virions. Free cysteines appear to play a critical role in this maturation event, because the addition of *N*-ethylmaleimide (NEM; a chemical that reacts with free cysteines) stabilizes UL16 on the capsids of extracellular virions (190).

We initially proposed that the interaction between UL11 (on the membrane) and UL16 (on the capsid) might provide a bridging function that contributes to the budding process at the TGN (161), but this hypothesis has never been proved. And, while it is clear that no other viral proteins are needed for binding, the crucial question of whether UL11 directly interacts with UL16 has never been addressed. It seemed possible that a cellular protein might be involved, because UL16 recognizes the same information (i.e., the LI and AC motifs) that is used by cellular machinery to traffic UL11 out of detergent-resistant membranes in the absence of other viral proteins (13,160). That is, UL16 might be a viral homolog of a clathrin adaptor subunit, and if so, it might need to form a complex with particular cellular proteins to function (51,138). The goals of the

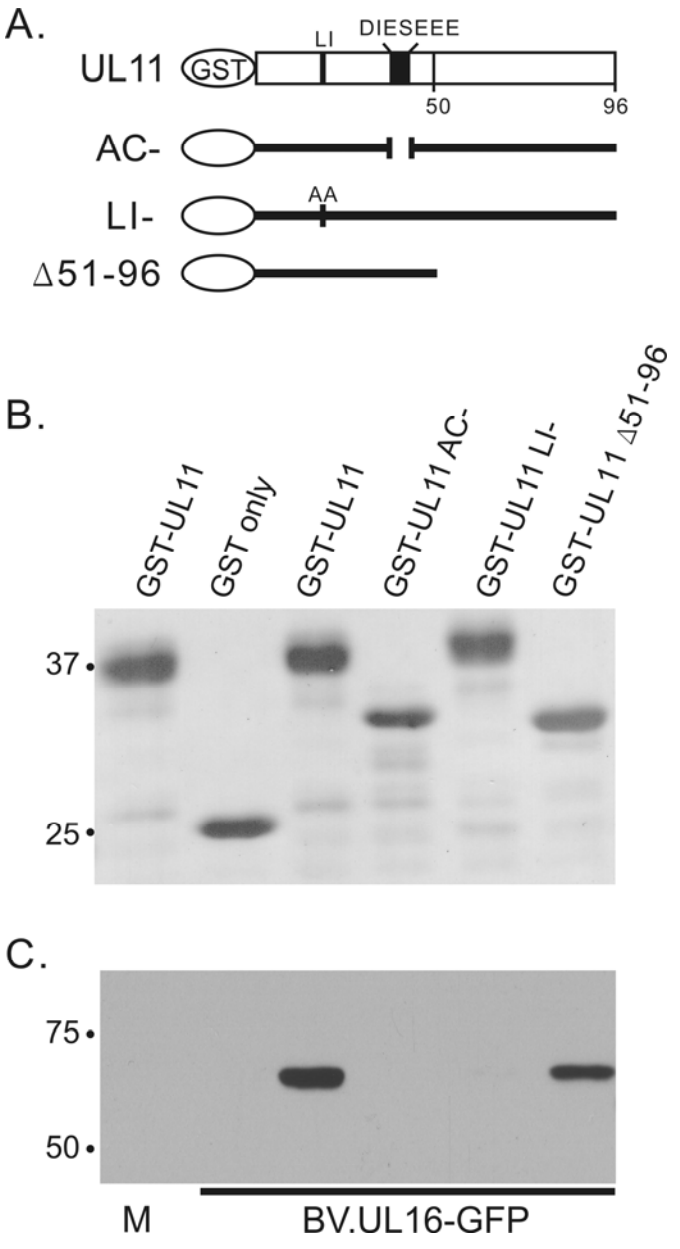
experiments described here were to address first whether the interaction is direct or indirect, and second, which part of UL16 is required for UL11 binding. The results prove that the interaction is indeed direct and suggest that the binding mechanism requires several (but not all) of the conserved cysteines within UL16.

RESULTS

Search for host proteins that bind UL11 in complex with UL16. It is quite clear that the interaction between UL11 and UL16 does not require any other viral proteins (161,310); however, it seemed possible that host proteins might be involved in the interaction because UL16 recognizes the membrane-trafficking signals of UL11 (i.e., UL16 might be a viral homolog of a clathrin adaptor subunit and function in complex with particular cellular proteins). To look for additional binding partners, a recombinant baculovirus was constructed that expresses UL16-GFP at high levels. To determine whether this source of UL16-GFP was still capable of binding specifically to GST-UL11, Sf21 cells were infected with BV.UL16-GFP, and NP-40 lysates were prepared 20 h postinfection. Equivalent amounts of glutathione bead-bound GST or GST-UL11 fusion proteins (Fig. 4.1A and B) were added to lysate samples, and bound proteins were analyzed by SDS-PAGE and immunoblotting for GFP. Like its mammalian-expressed counterpart (161), baculovirus-expressed UL16-GFP was pulled down by wild-type GST-UL11 and mutant Δ51-96 but not by the AC- or LI- mutants (Fig. 4.1C). These results show that no mammalian-specific host factor is necessary for the UL11-UL16 interaction. To look for host factors that might be in the complex, bound proteins were resolved in SDS-PAGE gels and subjected to zinc staining, but no distinct difference was observed between mock- and baculovirus-infected cells (data not shown). This negative result is consistent with the hypothesis that UL11 and UL16 may directly interact.

Binding of purified UL11 and UL16. To further address whether the UL11-UL16 interaction is direct or indirect, these two proteins were made and purified from

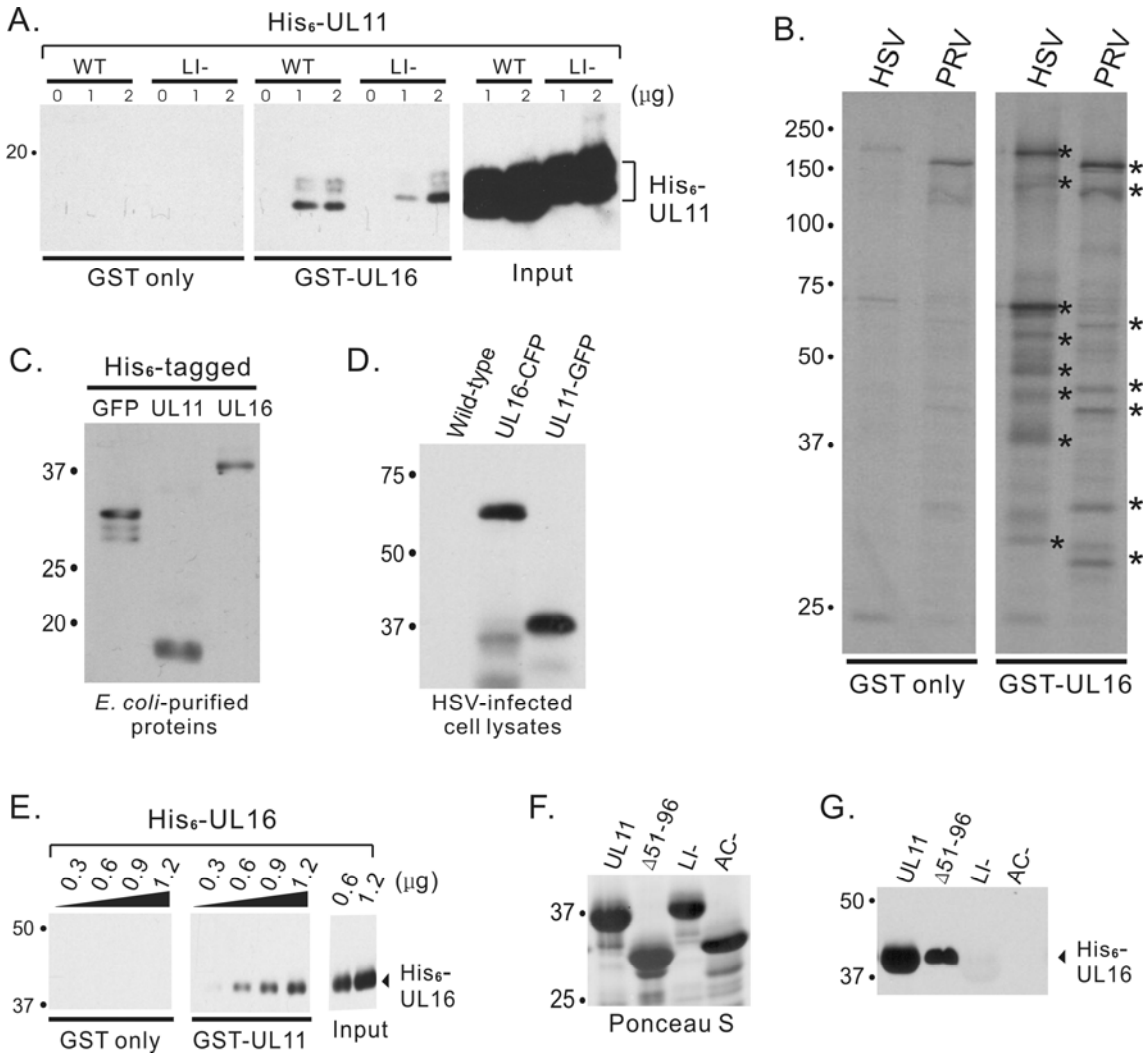
Figure 4.1 Baculovirus-expressed UL16-GFP interacts with UL11 via the acidic cluster and LI motifs. (A) Diagrams of GST-UL11 constructs. Approximately equal amounts of these constructs as estimated from a Ponceau S-stained gel (B) were mixed with lysates from uninfected (M, mock) or BV.UL16-GFP-infected Sf21 insect cells. The UL11 AC- mutant has the approximately same mobility as the Δ51-96 mutant, which has been observed previously (161,162). (C) Proteins bound to GST constructs were loaded onto SDS-PAGE gels and analyzed by immunoblotting for GFP. When analyzed using densitometry, approximately 40% of the amount of UL16-GFP protein in the lysates (input) was pulled down by GST-UL11.


A.
UL11
GST
LI
DIESEEE
50
96
AC-
LI-
AA
Δ51-96
B.
GST-UL11
GST only
GST-UL11
GST-UL11 AC-
GST-UL11 LI-
GST-UL11 Δ51-96
37
25
C.
75
50
M
BV.UL16-GFP

bacteria to completely eliminate eukaryotic host factors. In the first attempt, His_6-tagged UL11 was expressed in *E. coli*, purified on nickel beads, eluted, and dialyzed. A mutant that lacks the LI motif was also produced to use as a negative control for binding specificity. Various amounts of these His_6-UL11 proteins were mixed with glutathione bead-bound GST-UL16 in NP-40 lysis buffer for 2 h at room temperature, conditions identical to those used for the baculovirus experiments. Although no His_6-UL11 was pulled down by GST alone, the GST-UL16 construct pulled down only a small amount (less than 1%) of the input His_6-UL11, as detected with a His_6-specific monoclonal antibody, and very little difference was seen using the LI mutant (Fig. 4.2A). Because UL16 has many cysteines (190), it seemed likely that GST-UL16 from *E. coli* might be misfolded to some degree. If so, the misfolding must be specific for the UL11 interaction because GST-UL16 was capable of pulling down virus-specific proteins from HSV- and PRV-infected cell lysates (Fig. 4.2B), while no prominent proteins were pulled down by GST-UL16 from uninfected cell lysates (data not shown).

In a second attempt to look for direct interactions, the GST and His_6 tags were switched. The hope was that His_6-UL16 might be better able to fold if it was not linked to GST, which is well known to form a dimer. The switch of tags produced a minor problem in that the His_6-specific monoclonal antibody (Novagen; used in Fig. 4.2A) unexpectedly cross reacted with purified GST-UL11 (data not shown). Fortunately, this problem could be eliminated by using a polyclonal rabbit antibody against His_6-GFP. This antibody reacts with His_6-GFP, His_6-UL11, and His_6-UL16 (Fig. 4.2C), but it does not react with UL11 or UL16 (Fig. 4.2D, lane 1) unless they are tagged with CFP or GFP (Fig. 4.2D, lanes 2 and 3, respectively). Using this antiserum in the interaction assay, purified GST-

Figure 4.2 Direct interaction assays with UL11 and UL16. (A) The indicated amounts of His_6-UL11 proteins were incubated with purified GST only or GST-UL16. Bound proteins were separated by SDS-PAGE and detected by immunoblotting using a monoclonal antibody specific for the His_6 tag. WT, wild type. (B) To examine the abilities of purified GST or GST-UL16 to pull down virus-specific proteins, they were incubated with radiolabeled HSV- or PRV-infected cell lysates. Proteins bound were separated by SDS-PAGE, and radiolabeled proteins were detected by autoradiography. Examples of virus-specific proteins pulled down by GST-UL16 were indicated with asterisks. (C and D) Immunoblot analyses were used to show the specificity of rabbit anti-His_6-GFP serum for various bacteria-expressed His_6-tagged proteins (C) and viral proteins made by wild-type and recombinant HSV strains (D). (E) The indicated amounts of His_6-UL16 proteins were incubated with purified GST only or GST-UL11. Bound proteins were separated by SDS-PAGE and detected by immunoblotting. (F and G) Aliquots (1.8 μg) of His_6-UL16 protein were incubated with approximately equal amounts of GST-UL11 or mutants (Δ51-96 or LI- or AC- mutants) as estimated from a Ponceau S-stained gel (F), and bound proteins were analyzed by immunoblotting (G).

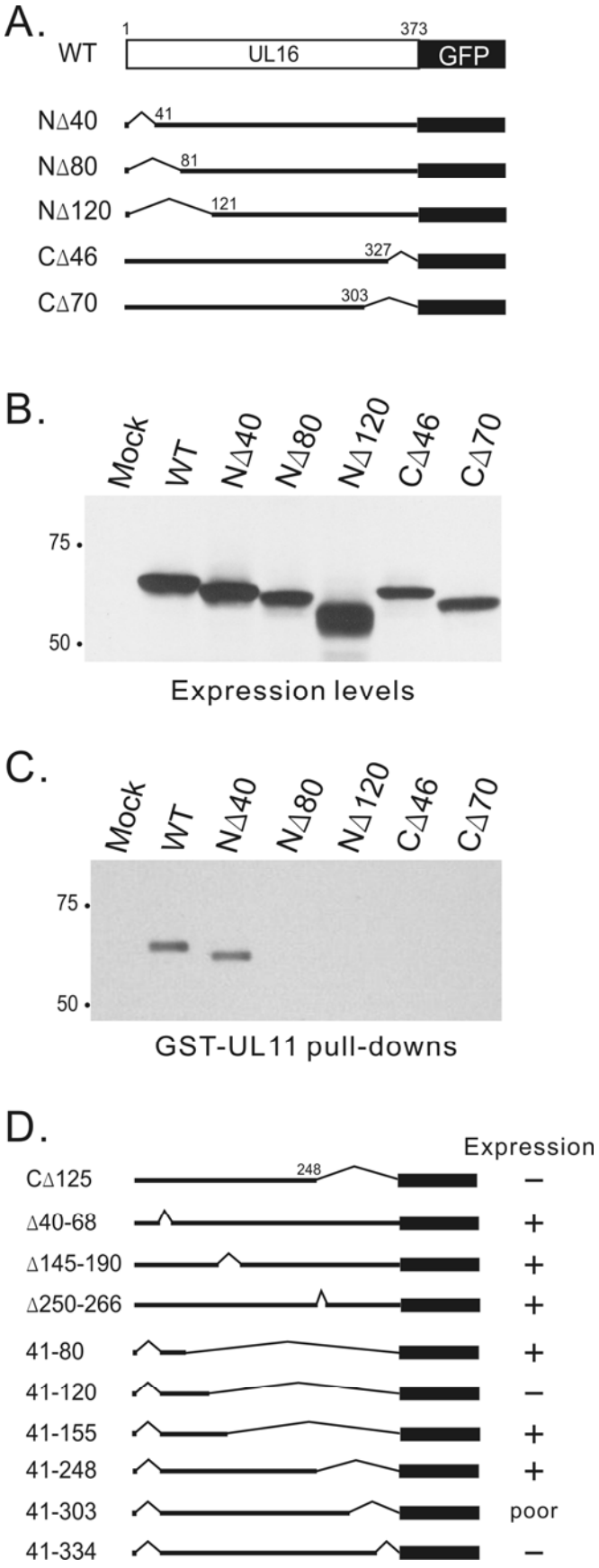

A.
His6-UL11
WT
LI-
WT
LI-
WT
LI-
0 1 2 0 1 2 0 1 2 0 1 2 1 2 1 2
(μg)
20
His6-UL11
GST only
GST-UL16
Input
B.
HSV
PRV
HSV
PRV
250
150
100
75
50
37
25
GST only
GST-UL16
C.
His6-tagged
GFP UL11 UL16
37
25
20
E. coli-purified proteins
D.
Wild-type
UL16-CFP
UL11-GFP
75
50
37
HSV-infected cell lysates
E.
His6-UL16
0.3 0.6 0.9 1.2 0.3 0.6 0.9 1.2 0.6 1.2 (μg)
50
37
His6-UL16
GST only
GST-UL11
Input
F.
UL11
Δ51-96
LI-
AC-
37
25
Ponceau S
G.
UL11
Δ51-96
LI-
AC-
50
37
His6-UL16

UL11 was readily able to pull down purified His_6-UL16 in a dose-dependent manner (Fig. 4.2E). Importantly, the Δ51-96 mutant could also bind to His_6-UL16, but the LI and AC mutants could not (Fig. 4.2G), even though approximately equal amounts of each of the GST-fusion proteins were used (Fig. 4.2F). These data prove that UL11 directly interacts with UL16 and that both the acidic cluster and LI motifs are essential for the interaction.

Deletion analysis of UL16. In an attempt to map the UL11-binding site contained in UL16, several N-terminal and C-terminal deletion mutants were constructed in the context of the eukaryotic expression vector, pCMV.UL16-GFP (Fig. 4.3A), and these were transfected into A7 cells. All mutants expressed well (Fig. 4.3B); however, in the GST-UL11 pull-down experiment, only one mutant, NΔ40, was found to retain the ability to bind to UL11 (Fig. 4.3C). Because the N-terminal 40 amino acids reside in the least conserved region among UL16 homologs (not shown), it is not surprising that they were found dispensable for UL11 binding. Numerous other truncation and internal deletion mutants were constructed (Fig. 4.3D), but many of these had no or poor expression (summarized in Fig. 4.3D), and all those that did express were unable to bind to UL11 (data not shown). These results suggest that UL16 is very sensitive to mutations, at least with regard to the requirements for UL11 binding.

Sensitivity of the interaction to NEM treatment. As an alternative strategy for identifying residues that are important for the interaction, the cysteines of UL16 were investigated because many of these are highly conserved (324). NEM is a very small (125-Da) membrane-permeable molecule that has long been used to chemically modify

Figure 4.3 The first 40 amino acids of UL16 are not required for UL11 binding. (A) Diagrams of UL16 wild-type (WT) and N- and C-terminal truncation mutants, which were all constructed as GFP-fusion proteins. (B) Expression levels of indicated constructs in transiently transfected A7 cells. At 20 h posttransfection, cells were harvested in sample buffer, loaded onto SDS-PAGE gels, and analyzed by immunoblotting for GFP. (C) Transfected cells were lysed and incubated with purified GST-UL11 proteins. Bound proteins were analyzed by SDS-PAGE and immunoblotting for GFP. (D) Summary of expression levels of other UL16 mutants.

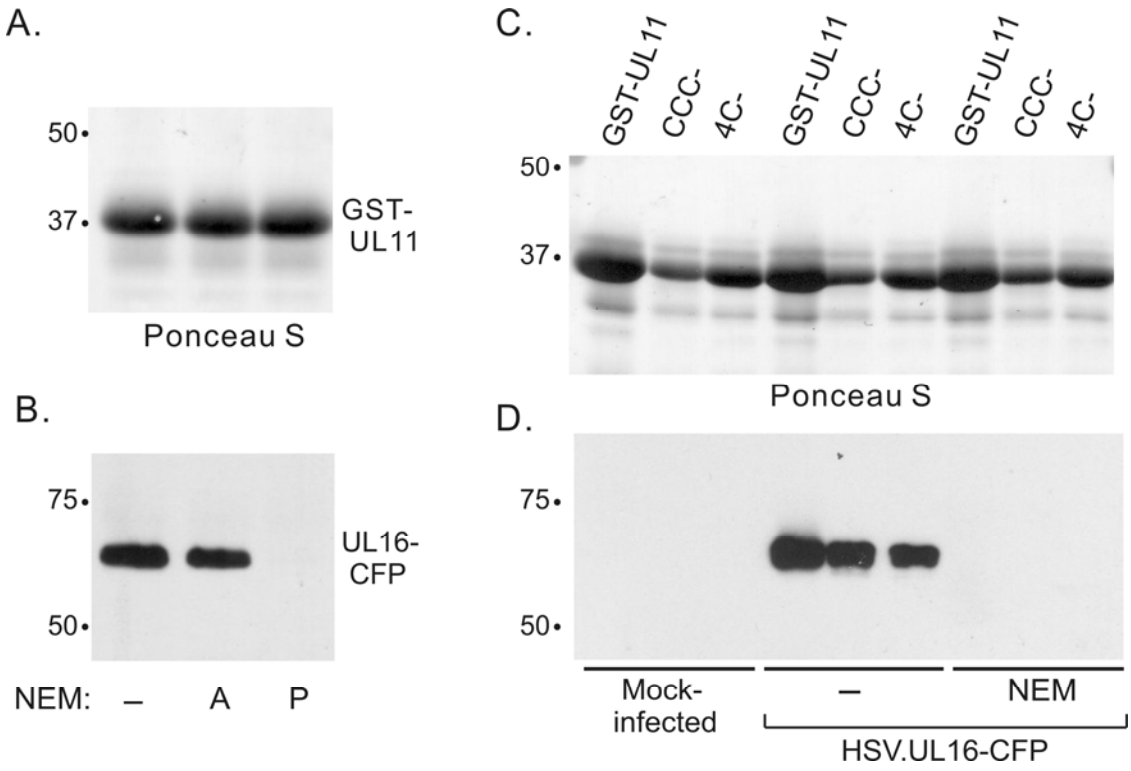

A.
1
373
WT
UL16
GFP
NΔ40
41
NΔ80
81
NΔ120
121
CΔ46
327
CΔ70
303
B.
Mock
WT
NΔ40
NΔ80
NΔ120
CΔ46
CΔ70
75
50
Expression levels
C.
Mock
WT
NΔ40
NΔ80
NΔ120
CΔ46
CΔ70
75
50
GST-UL11 pull-downs
D.
Expression
CΔ125
248
–
Δ40-68
+
Δ145-190
+
Δ250-266
+
41-80
+
41-120
–
41-155
+
41-248
+
41-303
poor
41-334
–

free cysteines (50). Recent studies have shown that about half of the 20 cysteines in UL16 can be modified by NEM, and this treatment stabilizes the interaction of the protein with the extracellular capsid (190). To examine the possibility that free cysteines might also be important for UL11 binding, UL16 was treated with NEM and then tested for binding.

Vero cells were infected with a recombinant HSV that expresses UL16-CFP. At 20 h postinfection, infected cells were treated with NEM for 30 min at room temperature, washed, and then lysed in NP-40 buffer to test for binding in GST-UL11 pull-down assays (Fig. 4.4A and B). A blockage of the UL11-UL16 interaction was observed (Fig. 4.4B, lanes 1 and 3). Identical results were obtained using wild-type HSV (data not shown). The same blockage was also observed when baculovirus-expressed UL16-GFP was treated with NEM in the GST-UL11 pull-down assay (data not shown). In these assays, the cells were washed thoroughly to remove excess NEM before being lysed, but it was important to consider the possibility that residual NEM might disrupt the interaction. To address this, UL11 and UL16 proteins were allowed to bind prior to the addition of NEM, and the complex was found to be stable (Fig. 4.4B, lane 2). Hence, it is clear that modification of UL16 with NEM blocks UL11 binding.

The NEM results raised the possibility that free cysteines in UL11 and UL16 might form irrelevant disulfide bonds when mixed together. To examine this, cysteine mutants of UL11 were constructed. Of the four cysteines in UL11, three are located near the N terminus (residues 11 to 13), and the fourth is located in the dispensable second half of the molecule (residue 83). UL11 mutants lacking three (CCC-) or all four (4C-) of the cysteines were expressed as GST fusion proteins and purified from bacteria. Both

Figure 4.4 UL11 cannot interact with NEM-modified UL16 from infected cell lysates. (A and B) Vero cells were infected with HSV.UL16-CFP at an MOI of 1. At 20 h postinfection, cells were scraped into PBS. One set of cells were treated with NEM for 30 min before detergent lysis. Cell lysates were incubated with approximately equal amounts of glutathione bead-bound GST-UL11 as estimated from a Ponceau S-stained gel (A). Bound proteins were analyzed by SDS-PAGE and immunoblotting (B). For lanes 2 and 3, the addition of NEM was either after (A) or prior (P) to the 2-h incubation. (C and D) GST-UL11 fusion proteins as detected in a Ponceau S-stained gel (C) were mixed with either untreated or NEM-treated HSV.UL16-CFP-infected cell lysates, and bound proteins were analyzed by SDS-PAGE and immunoblotting (D).


A.
50
37
GST-
UL11
Ponceau S
B.
75
50
UL16-
CFP
NEM: – A P
C.
GST-UL11
CCC-
4C-
GST-UL11
CCC-
4C-
GST-UL11
CCC-
4C-
50
37
Ponceau S
D.
75
50
Mock-
infected
–
NEM
HSV.UL16-CFP

mutants retained the ability to interact with UL16-CFP produced in infected cells (Fig. 4.4C and D). Identical results were obtained using untagged UL16 expressed by wild-type HSV (data not shown). Moreover, pretreatment of the infected cells with NEM blocked the interaction (Fig. 4.4C and D, lanes 7 to 9).

The UL11 mutants were also examined for their ability to interact with His_6-UL16 purified from bacteria as described in Fig. 4.2, and as expected, direct binding was observed (Fig. 4.5B and C). When the His_6-UL16 protein was treated with NEM prior to its elution from the nickel beads, a large increase in its mass was observed (Fig. 4.5A), indicating the presence of multiple free cysteines. As predicted, the modified protein lost its ability to bind UL11 (Fig. 4.5B and C). Collectively, these results demonstrate that interspecies disulfide bonds are not required for a stable interaction.

Cysteine-substitution mutants of UL16. It would be a daunting task to analyze all 20 of the cysteines in UL16; however, a small subset of these are highly conserved (Fig. 4.6A). To determine whether any of these are important for the interaction with UL11, six were individually replaced with serine in the context of UL16-GFP. Plasmids encoding the resulting mutants, designated C1S to C6S, were transfected into A7 cells, and each was found to be expressed as well as wild-type UL16-GFP (Fig. 4.6B). Mutants C1S and C2S retained the ability to interact with GST-UL11, but the other four were completely inactive for binding (Fig. 4.6C). These results further support the hypothesis that free cysteines (perhaps conserved ones) in UL16 play a critical role in the interaction with UL11 (discussed in Chapter VI).

Figure 4.5 NEM modification of UL16 inhibited the in vitro UL11-UL16 interaction. (A) His_6-UL16 proteins purified from bacteria were treated with or without NEM and analyzed by immunoblotting. (B and C) Aliquots (1.2 μg) of untreated or NEM-treated His_6-UL16 proteins were incubated with approximately equal amounts of GST-UL11 fusion proteins as estimated from a Ponceau S-stained gel (B), and bound proteins were analyzed by immunoblotting (C).

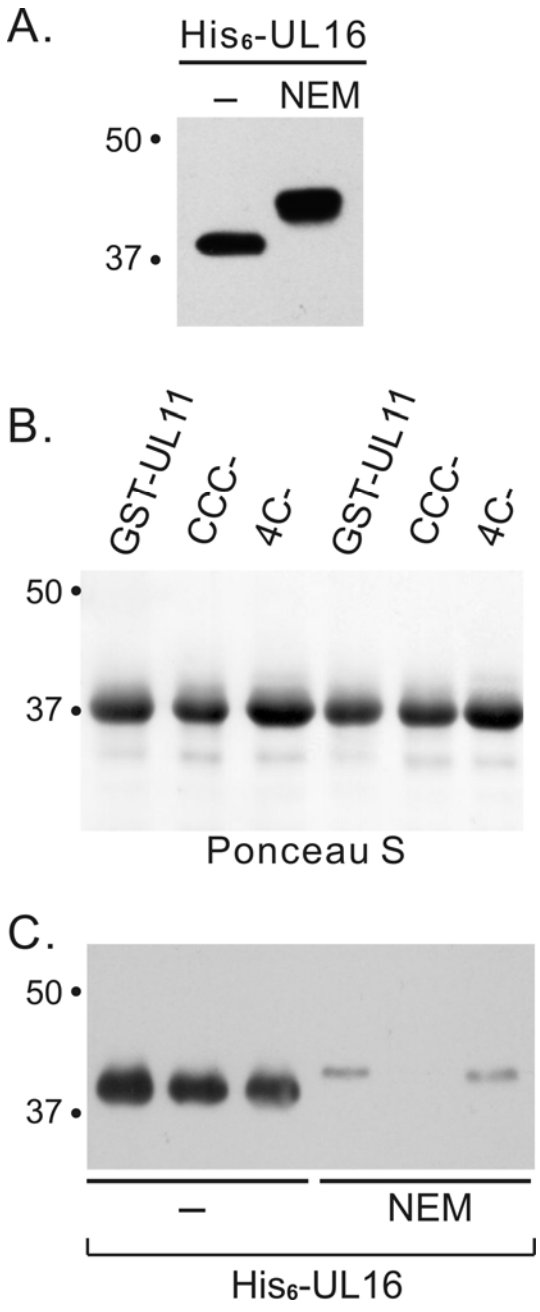
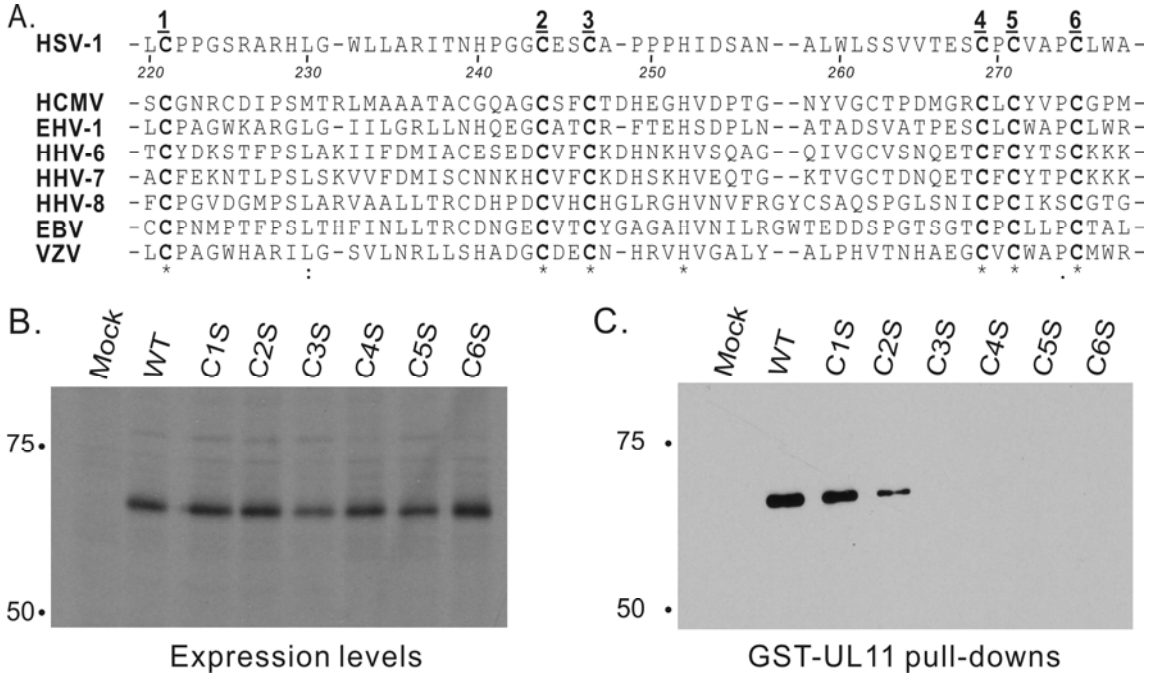

A.
His6-UL16
–
NEM
50
37
B.
GST-UL11
CCC-
4C-
GST-UL11
CCC-
4C-
50
37
Ponceau S
C.
50
37
–
NEM
His6-UL16

Figure 4.6 Conserved cysteines within UL16 are important for UL11 binding. (A) Alignment of regions in UL16 homologs containing six highly conserved cysteine residues. The residue numbers in this region of HSV-1 are indicated. HCMV, human cytomegalovirus; EHV, equine herpesvirus; HHV, human herpesvirus; EBV, Epstein-Barr virus; VZV, varicella zoster virus. (B) Expression levels of HSV-1 UL16-GFP wild type (WT) and six cysteine-to-serine substitution mutants (C1S to C6S). Codons for the six cysteines indicated in panel A were individually replaced with codons for serine and constructed into the GFP expression vector. The alleles were transfected into A7 cells, and protein expression was examined by immunoblotting for GFP. (C) GST-UL11 pull-down assays. Transfected cells were lysed with detergent and incubated with glutathione bead-bound GST-UL11. Bound proteins were analyzed by SDS-PAGE and immunoblotting for GFP.


A.
1 2 3 4 5 6
HSV-1 -LCPPGSRARHLG-WLLARITNHPGGCESCA-PPPHIDSAN--ALWLSSVVTESCPCVAPCLWA-
220 230 240 250 260 270
HCMV -SCGNRCDIPSMTRLMAAATACGQAGCSFCTDHEGHVDPTG--NYVGCTPDMGRCLCYVPCGPM-
EHV-1 -LCPAGWKARGLG-IILGRLLNHQEGCATCR-FTEHSDPLN--ATADSVATPESCLCWAPCLWR-
HHV-6 -TCYDKSTFPSLAKIIFDMIACESEDCVFCKDHNKHVSQAG--QIVGCVSNQETCFCYTSCKKK-
HHV-7 -ACFEKNTLPSLSKVVFDMISCNNKHCVFCKDHSKHVEQTG--KTVGCTDNQETCFCYTPCKKK-
HHV-8 -FCPGVDGMPSLARVAALLTRCDHPDCVHCHGLRGHVNVFRGYCSAQSPGLSNICPCIKSCGTG-
EBV -CCPNMPTFPSLTHFINLLTRCDNGECVTCYGAGAHVNILRGWTEDDSPGTSGTCPCLLPCTAL-
VZV -LCPAGWHARILG-SVLNRLLSHADGCDECN-HRVHVGALY--ALPHVTNHAEGCVCWAPCMWR-
B.
Mock WT C1S C2S C3S C4S C5S C6S
75
50
Expression levels
C.
Mock WT C1S C2S C3S C4S C5S C6S
75
50
GST-UL11 pull-downs

Chapter V

Interaction between the UL16 Tegument Protein and the Cytoplasmic Tail of Glycoprotein E of Herpes Simplex Virus

ABSTRACT

Previous studies of UL16 and its capsid association have demonstrated that binding of HSV to its initial attachment receptors on the host cell surface induces a rapid structural rearrangement within the tegument, representing the first evidence that an 'outside-in' signal can be sent across the viral membrane prior to virus entry. Specifically, interaction of virus with heparan sulfate molecules triggers the release of UL16 from the capsid. The mechanism by which the signal is sent to UL16 remains unclear but seems likely to be mediated by virion envelope glycoproteins. Here, we showed that a GST chimera bearing the cytoplasmic tail of glycoprotein E (gE.CT) is capable of binding UL16 expressed in mammalian or insect cells. And, gE.CT was also found to interact with a UL16 binding partner, UL11, consistent with a previous finding. To better understand the molecular mechanism responsible for this signaling process, the interaction network emanating from UL16 was investigated. Previously using a GST pull-down assay, it was observed that UL16 interacts with virus-specific proteins from HSV- and PRV-infected cell lysates, providing evidence that UL16 is present in protein complexes. To characterize native UL16 complexes in a natural infection, we constructed a recombinant HSV expressing a tagged derivative. Using the combination of tandem affinity purification and mass spectrometry, we identified gE as a component of UL16 complexes isolated from infected cells. The UL16-gE interaction was confirmed using co-immunoprecipitation assays. And, mutational analyses revealed that an acidic cluster of gE.CT is not required for UL16 binding, and raised a possibility that in infected cells UL16 may interact with gE.CT in both UL11-dependent and -independent manners. We hypothesize that UL11, UL16, and gE may form a tripartite complex which is involved in

the multiple aspects of virus life cycle, including signaling events during virus attachment, or virion maturation, or even cell-to-cell spread.

INTRODUCTION

Herpes simplex virus (HSV) has three morphologically distinct structures: the double-stranded DNA-containing icosahedral capsid, the lipid bilayer envelope embedded with approximately 11 glycoproteins, and the tegument between the two containing more than 20 viral proteins. The initial virus attachment to a host cell requires glycoproteins gC or gB binding to heparan sulfate (HS) moieties present on cell surface proteoglycans, and further virus penetration depends on sophisticated coordination between four glycoproteins: gD, gB, and a heterodimer gH/gL (278). The initial attachment facilitates gD interaction with a gD receptor, and then the receptor binding-induced conformational changes of gD activate fusion machinery composed of gB and gH/gL.

Our recent studies of a capsid-associated tegument protein, UL16, have revealed that the three virion structures communicate. Specifically, UL16 is stably associated with cytoplasmic capsids isolated from infected cells (190). In response to virus attachment to HS on the host cell surface, an 'outside-in' signal is transmitted across the virion membrane, and as a result, triggers UL16 dissociation from the capsid (191). The signal can also be triggered in a cell-free assay where virions are allowed to bind immobilized heparin beads. In this cell-free assay, a gC-null virus mutant was not capable of transmitting the signal, indicating that gC is required. Free cysteines present in UL16 also appear to be critical because treatment of extracellular virions with *N*-ethylmaleimide (NEM) prior to cell binding can prevent the release of UL16 from the capsid. Despite these observations, the molecular machinery that participates in this signaling process is yet to be determined.

Since the signaling event occurs during virus attachment (to HS), it was thought that the signal might be sent through gC or gB to UL16 (i.e., UL16 might interact with the cytoplasmic tail [CT] of gC or gB within a virion). gB.CT (109-amino-acid) seemed more likely to be involved because gC.CT contains only 14 amino acids in length. Moreover, although the signaling of the gC-null mutant is abolished in the heparin bead-binding assay, the mutant virus can still trigger the release of UL16 from the capsid in the cell-binding assay, suggesting possible involvement of other molecules besides gC and a more complex mechanism. Interestingly, it was recently reported that gC and gE on the virion envelope block access of neutralizing antibodies to gB and gD, implicating that these four glycoproteins may exist in complexes (123). Due to dynamic interactions that could occur during virus entry between the virion envelope and the host cell membrane, it is possible that one or more of these four glycoproteins are capable of triggering the signaling event, even in the absence of gC.

Furthermore, it was recently shown that UL11, a binding partner of UL16, is associated in some manner with the tails of gE and gD (75). Therefore, it is possible that the signal can be sent through gE or gD. However, there are no studies suggesting UL16 can interact with gE or gD. Here, we showed that gE.CT is capable of binding UL16 expressed in mammalian or insect cells, indicating that no other viral proteins are required for the interaction. Consistent with the previous report, gE.CT was found to interact with UL11, and the interaction appears to be direct and likely mediated by the second half of UL11 (the function of which has never been described) and the N-terminal region of gE.CT (work performed by J. Han). Previously using a GST pull-down assay, UL16 was found to interact with virus-specific proteins from HSV- and PRV-infected

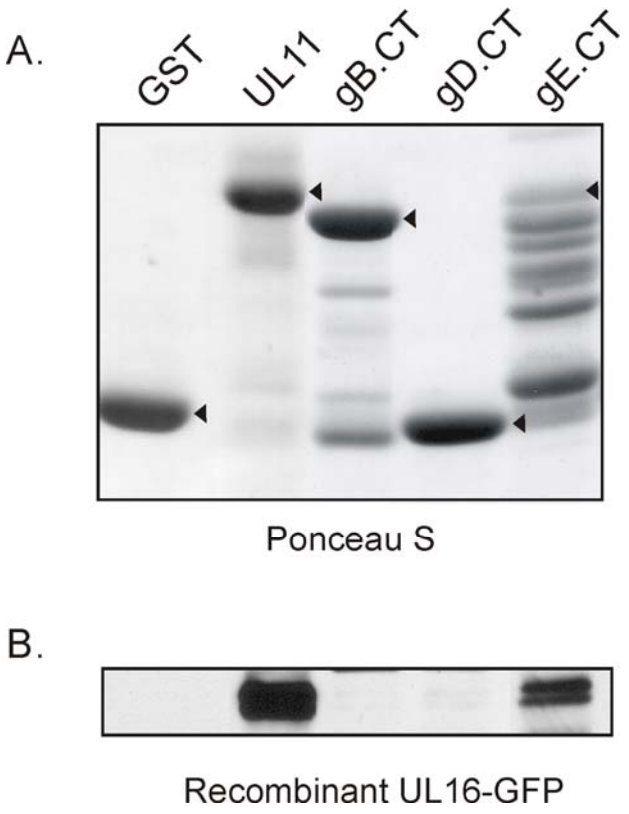
cell lysates, providing evidence that UL16 is present in protein complexes (334). Using the combination of tandem affinity purification (TAP) and mass spectrometry (MS) as well as co-immunoprecipitation assays, it was confirmed that UL16 forms a complex with gE within infected cells. And, mutational analyses indicated that the interaction does not require an acidic cluster of gE.CT, although its sequence resembles that of UL11 which is essential for UL16 interaction (161,334). It was also found that UL16 appears to be capable of binding gE.CT directly or via UL11 binding, the latter of which is blocked by NEM modification of free cysteines present in UL16 (334). Based on all available data, we hypothesize that UL11, UL16, and gE may form a tripartite complex which is involved in the multiple aspects of virus life cycle, including the signaling event during virus attachment, or virion maturation, or even cell-to-cell spread.

RESULTS

Interaction between UL16 and the cytoplasmic tail of gE. Based on the observation that UL16 is dissociated from the capsid upon virus binding to its host receptor heparan sulfate (HS) molecules (191), it seemed possible that virion envelope glycoproteins may be involved in this signaling event (i.e., the 'outside-in' signal is transmitted through interactions between UL16 and the cytoplasmic tail [CT] of viral glycoproteins within a virion). Two glycoproteins, gB and gC, are known to bind HS during virus attachment (118,119), and since gC.CT contains only 14 amino acid residues, gB.CT (109-amino-acid) seemed more likely to interact with UL16. In addition, UL11, a binding partner of UL16, was previously reported to bind the tails of gD and gE in some manner (75); however, it has never been determined whether UL16 is capable of binding these tails.

To address whether UL16 interacts with the tail of gB or gD or gE, the tails were constructed as GST fusions and tested for UL16 binding. A recombinant baculovirus that expresses high levels of UL16-GFP was used to infect Sf21 cells, and NP-40 cell lysates were prepared 20 h postinfection. Glutathione bead-bound GST or GST fusions (Fig. 5.1A) were then incubated with lysates, and bound proteins were analyzed by SDS-PAGE and immunoblotting for GFP. UL11 was used as a positive control because it was previously shown to readily pull down the recombinant UL16-GFP (Fig. 5.1, lane 2) (334). The results showed that gE.CT, but not gB.CT, is capable of pulling down UL16 from infected insect cell lysates (Fig. 5.1B, lanes 3 and 5), suggesting that the UL16-gE.CT interaction does not require any other viral or mammalian proteins. Due to the

Figure 5.1 Interaction of UL16 with gE.CT. (A and B) Approximately equal amounts of glutathione bead-bound GST constructs as indicated by arrowheads on a Ponceau S-stained gel (A) were mixed with lysates from Sf21 insect cells infected with a recombinant baculovirus expressing UL16-GFP. Proteins bound to GST constructs were loaded onto SDS-PAGE gels and analyzed by immunoblotting for GFP (B). The GST-UL11 construct was used as a positive control.

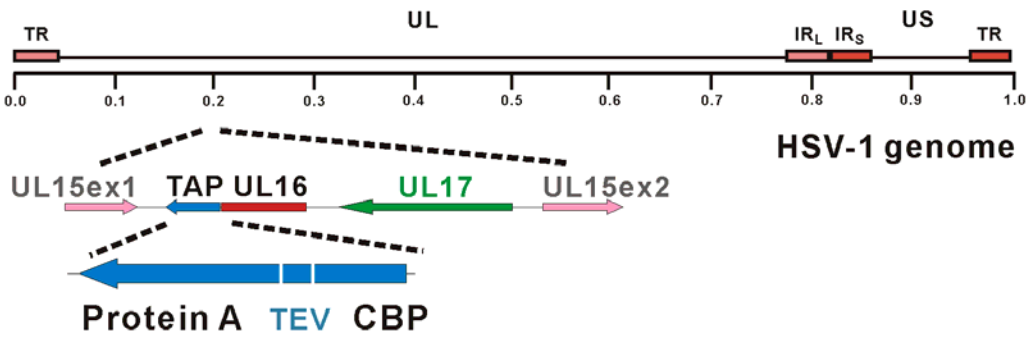

A.
GST
UL11
gB.CT
gD.CT
gE.CT
Ponceau S
B.
Recombinant UL16-GFP

mobility of GST-gD.CT similar to that of GST alone (Fig. 5.1A, lanes 1 and 4), it still remains to be determined whether gD.CT could interact with UL16.

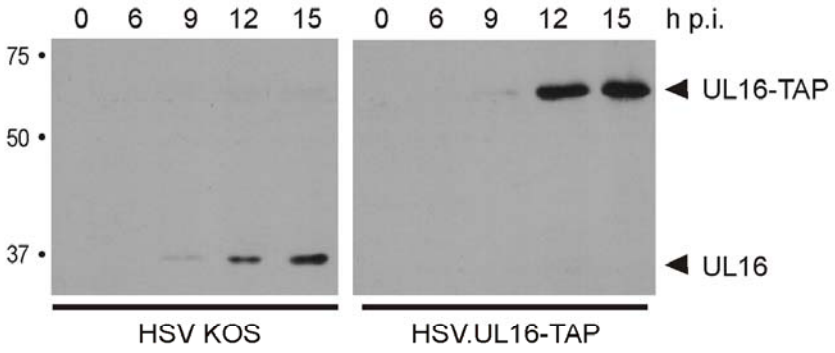
Complex formation of UL16 and gE. Previous studies have demonstrated that a GST-UL16 fusion pulls down virus-specific proteins from HSV- and PRV-infected cell lysates, suggesting that UL16 may be involved in protein complexes during an infection (334). To characterize native UL16-associated complexes, a recombinant HSV that expresses UL16-TAP was constructed (Fig. 5.2A) using the bacterial artificial chromosome (BAC) technology (see Chapter III). The recombinant was found to be indistinguishable from the wild-type KOS strain with regard to kinetics of UL16 expression as well as single-step virus growth (Fig. 5.2B and C). Vero cells infected with the recombinant were lysed in NP-40 buffer at 20 h postinfection. Subsequent purification steps were performed as depicted in Fig. 3.1. Briefly, IgG beads were incubated with NP-40 lysates overnight at 4°C, treated with tobacco etch virus (TEV) protease at room temperature for 4 h, and kept at 4°C overnight to allow complete digestion. Although it is possible that the TEV protease might cleave certain proteins present in the complex, the frequency is rarely low because of the high specificity of the enzyme. And, there appears to be no TEV cleavage site contained in any of HSV-encoded proteins (according to the protein sequences published in NCBI website, data not shown). Following the cleavage, samples containing UL16 and associated proteins were collected on calmodulin resin. Then, bound proteins were obtained using two different approaches and analyzed by mass spectrometry in separate institutes. Table 5.1 summarized the results from these two experiments, both of which have identified gE to

Figure 5.2 Construction of the HSV.UL16-TAP recombinant. (A) The diagram depicts insertion of the coding sequence of the TAP tag (blue) at the 3' end of the U_L16 open reading frame (red). Starting from the position closest to UL16, the TAP tag composes of a calmodulin binding peptide (CBP), a tobacco etch virus (TEV) protease cleavage site, and Protein A. (B and C) Comparison of the wild-type KOS strain and HSV.UL16-TAP with regard to kinetics of UL16 expression at indicated times postinfection (B) and single-step virus growth (C).

A.


TR
UL
IRL IRS
US
TR
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
HSV-1 genome
UL15ex1 TAP UL16 UL17 UL15ex2
Protein A TEV CBP

B.


0 6 9 12 15 0 6 9 12 15 h p.i.
75
50
37
UL16-TAP
UL16
HSV KOS
HSV.UL16-TAP

C.

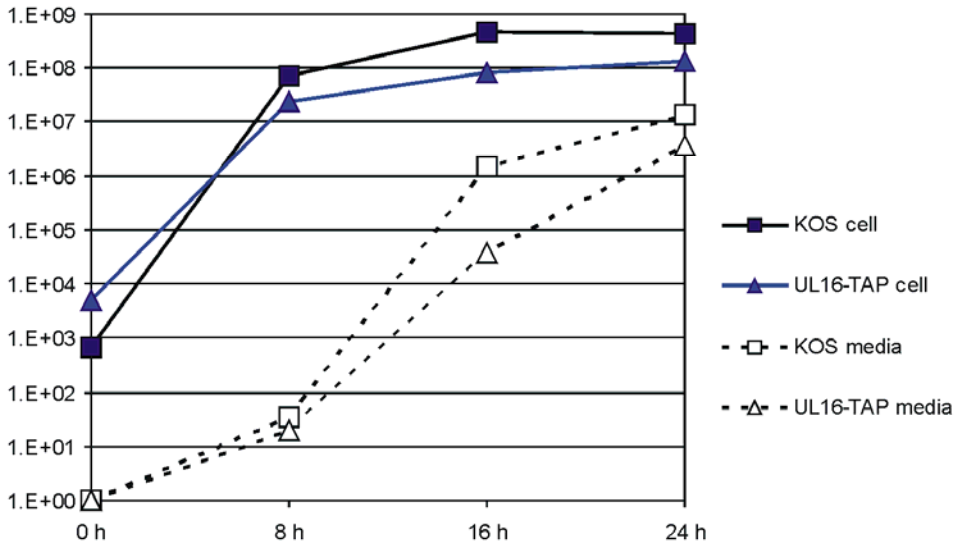

1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
0 h
8 h
16 h
24 h
KOS cell
UL16-TAP cell
KOS media
UL16-TAP media

Table 5.1 Proteins identified by tandem affinity purification (TAP).

Experiment 1

Species	Gene (protein)	gi#	Peptides from MS-MS
HSV	UL19 (VP5)	137571	ASEAYFLGQLQVAR HAMAPATIAAVR GRPVDGVLVTTAPIK QTLDELESAPQTTR LIVETGSAVSTATAASDVQFK
	UL33	136883	DTIPDCALR
	UL39	132605	LLEVMDSLDAK DDAEGLSDPRPR DGETLRPNTLLLK ADGTLPASTLVR
	US2	137124	ILASEPLGTPVVVRPANLR
	US8 (gE)	44844054	LPPPPAPPR VSVGEDVSLLPAPGPTGR TDFVWQER NAVVEQPLPQR APVPLAMAYAPPAPSATGGLR ETDSGLYTLSVGDIKDPAR
Homo Sapiens	UBX domain-containing protein 5	146325810	IQEIVVETPTLAAER
	Filaggrin-2	74755309	SGQSSYGQHSSGSSQSSGYGQHGSR
	CLIP-associating protein 1	74723323	IMATSGVVAVR
	Stomatin-like protein 2	60415944	ILEPGLNILIPVLDR
Homo Sapiens	Myosin, heavy polypeptide 9, non-muscle	119580501	QLLQANPILEAFGNAK ANLQIDQINTDLNLER
Likely contam-inants?	Keratins (various types)		

Experiment 2

Gene (protein)	gi#	Peptides from MS-MS
UL21	9629401	VGPAGVSPAPPPNNTDSSSLVPGAQDSAPPGPTLR DVPVGSDVQADSTALLR LSPFPALVR TSPGVLISGLR
UL49 (VP22)	136927	ANELVNPDVVQDVDAATATR TDEDLNELLGITTIR MAAVQLWDMSR LHFSTAPPNPDAPWTPR SAASRPTERPRAPAR STAPPNPDAPWTPR
US8 (gE)	44844054	LPPPPAPPR VSVGEDVSLLPAPGPTGR TDFVWQER AMAYAPPAPSATGGLR QLTTFGSGRPDRR SDGHQSRRQLTTFGSGR
PS1TP5-binding protein 1 (HCG15971)	93211215	SYELPDGQVITIGNER VAPEEHPVLLTEAPLNPK DNGSGMCKAGFAGDDAPR AVFPSIVGR
Rho GTPase-activating protein 17 (Rich1)	119576183	SSGTNFQGLPSK IDTLKEEMDEAGNK IVTDSNSRVSEPHR PKDPVSAAVPAPGR
Myosin, heavy polypeptide 9, non-muscle	119580501	QLLQANPILEAFGNAK ANLQIDQINTDLNLER
Keratins (various types)		

gi#: sequence identification number in GenBank.

Experiment 1: performed by Dr. John O. Semmes and Michael Ward at EVMS. Experiment 2: PSU mass spectrometry core facility.

be present in a complex with UL16. Other proteins listed in Table 5.1 also represent possible binding partners of UL16. Their functions and potential involvement with UL16 will be discussed later (see Chapter VI).

To validate the UL16-gE interaction detected by the TAP pull-down, co-immunoprecipitation assays were performed using Vero cells infected with a recombinant HSV expressing UL16-CFP (334). As a loading control, the expression levels of gE and VP5 (the major capsid protein) in cell lysates were monitored by immunoblotting (Fig. 5.3A). gE and its associated proteins were immunoprecipitated from infected cell lysates with the polyclonal gE antiserum. Then, immunoprecipitated proteins were separated by SDS-PAGE and subjected to immunoblotting. The results confirmed that UL16 forms a complex with gE within infected cells (Fig. 5.3B). Since interaction of UL16 with gE.CT was found to not require other viral proteins (Fig. 5.1B), it was thought that UL16 might be able to interact with the full length of gE when they are coexpressed in a cell in the absence of other viral proteins. In an attempt to address this question, a cotransfection assay was performed. However, the interaction cannot be detected even though UL16 and gE both expressed well in these cells (data not shown), suggesting that an unknown factor may be critical for the interaction, such as proper protein modification(s) or protein conformations, or the presence of other viral protein(s).

Deletion analyses of gE.CT. Because the N terminus of gE.CT is highly conserved among all gE homologs, it seemed possible that the region may contain the UL16-binding site. Therefore, C-terminal truncation mutants were constructed as GST chimeras (Fig. 5.4A). An acidic cluster mutant of gE.CT (DEED/As) was also created

Figure 5.3 Co-immunoprecipitation of UL16 and gE from infected cell lysates. (A) Vero cells were infected with HSV.UL16-CFP at a MOI of 10. At 20 h postinfection, uninfected (M, mock) or infected cells were harvested and washed with PBS. As a loading control, the expression levels of gE and VP5 in infected cell lysates were monitored by immunoblotting using specific rabbit polyclonal antibodies. (B) Infected cells were lysed with detergent and subjected to immunoprecipitation (IP) using rabbit polyclonal antibodies α-gE or -GFP, and precipitated proteins were analyzed by SDS-PAGE and immunoblotting.

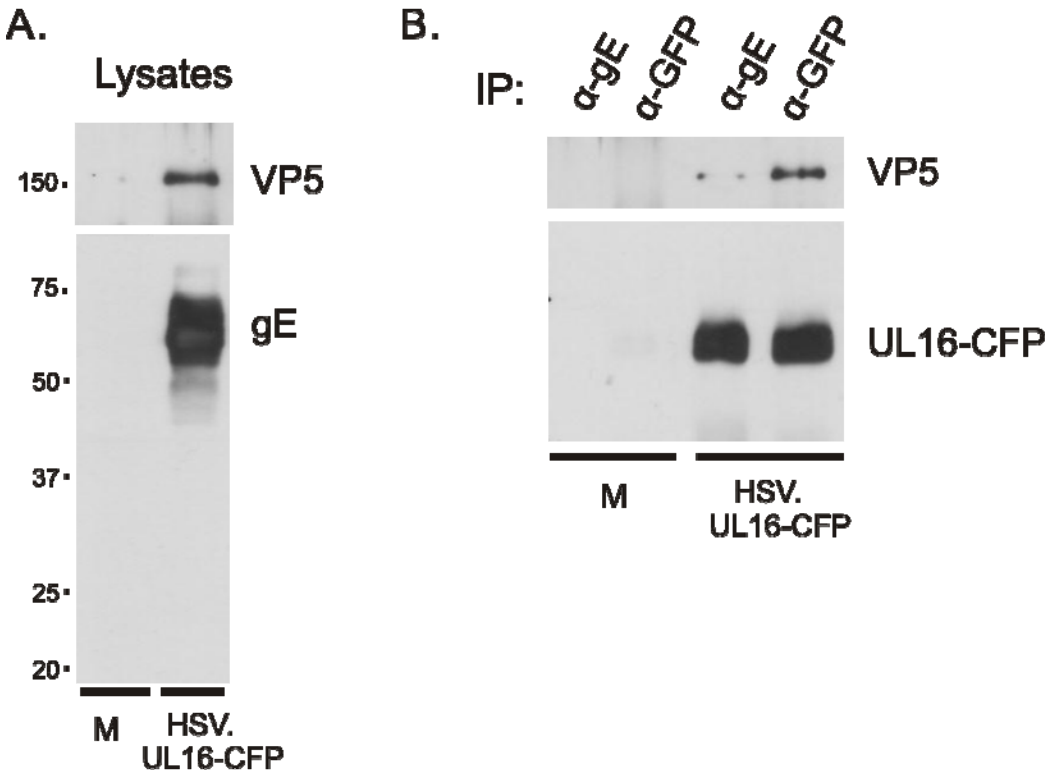
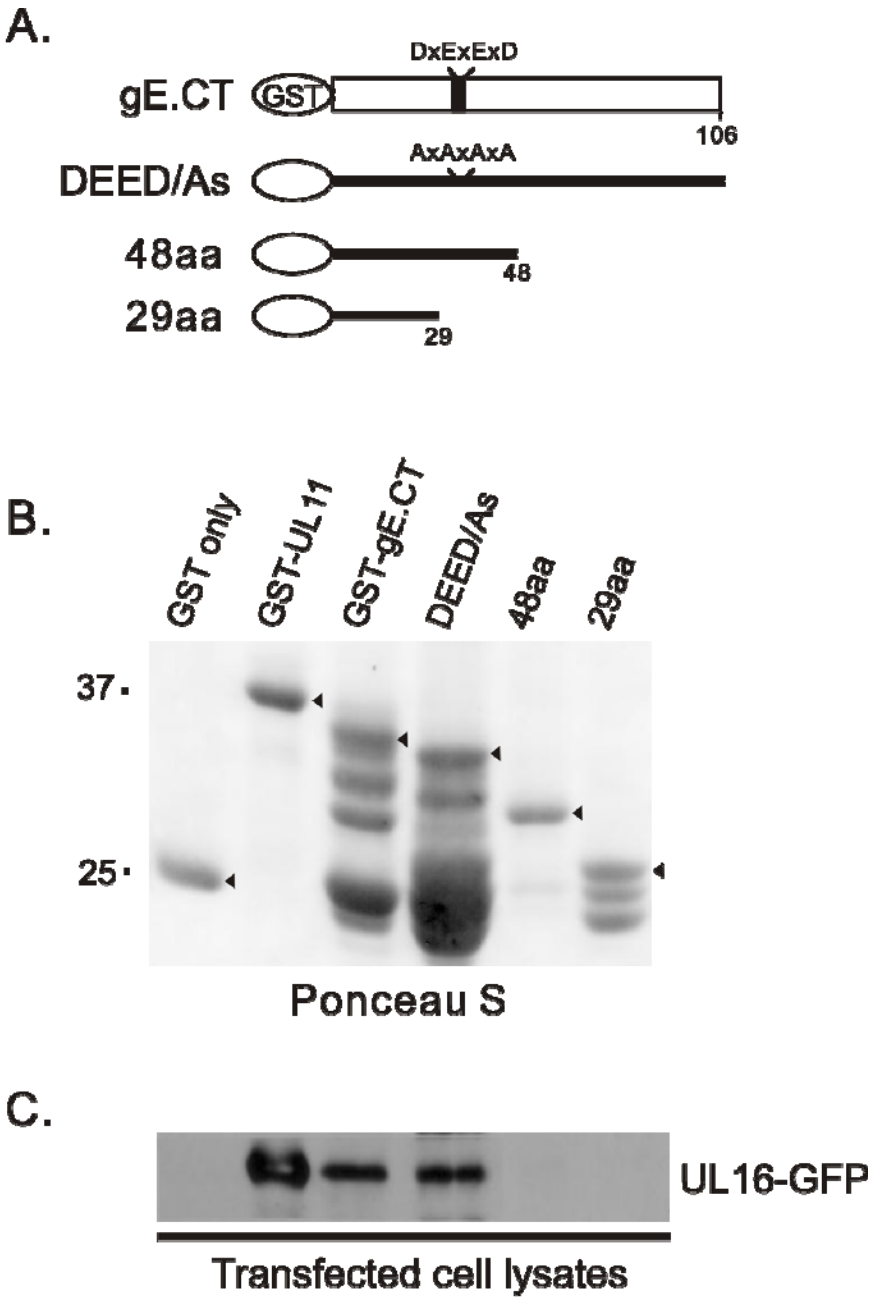

A.
Lysates
150
75
50
37
25
20
VP5
gE
M
HSV.
UL16-CFP
B.
IP:
α-gE
α-GFP
α-gE
α-GFP
VP5
UL16-CFP
M
HSV.
UL16-CFP

Figure 5.4 Interactions of gE.CT mutants with UL16 expressed in mammalian cells. (A) Diagrams of GST-gE.CT wild-type and mutant constructs. DEED/As, codons for four acidic amino acids (D, aspartate; E, glutamate) were all replaced with codons for alanine. (B and C) Approximately equal amounts of glutathione bead-bound GST constructs as indicated by arrowheads on a Ponceau S-stained gel (B) were mixed with lysates from UL16-GFP-transfected A7 cells. Proteins bound to GST constructs were loaded onto SDS-PAGE gels and analyzed by immunoblotting for GFP (C).


A.
DxExExD
gE.CT
GST
106
AxAxAxA
DEED/As
48aa
48
29aa
29
B.
GST only
GST-UL11
GST-gE.CT
DEED/As
48aa
29aa
37
25
Ponceau S
C.
UL16-GFP
Transfected cell lysates

because this stretch of acidic amino acids resembles the acidic cluster of UL11, which is required for UL16 interaction (161,334). A7 cells were transfected with an eukaryotic expression vector, pCMV.UL16-GFP. After 24 h posttransfection, glutathione bead-bound GST fusion proteins were added to NP-40 lysates. The results indicated that the acidic cluster in gE.CT is not required for the interaction (Fig. 5.4B and C, lane 4). Identical results were obtained when HSV.UL16-CFP-infected Vero cell lysates were used (Fig. 5.5A, lane 5).

When the C-terminal region of gE.CT was deleted, the binding with UL16 was lost from transfected cell lysates (Fig. 5.4B and C, lanes 5 and 6), suggesting that the C terminus of gE.CT may be critical for binding. Unexpectedly, the interactions of these mutant constructs with UL16 from infected cell lysates remained intact and were inhibited by NEM treatment (Fig. 5.5A, lanes 6 and 7 in both panels), whereas NEM had no effects on interaction of UL16 with the full length or the acidic cluster mutant of gE.CT (Fig. 5.5A, lanes 4 and 5). Further support that NEM does not disrupt the UL16-gE.CT interaction was from the observation that either untreated or NEM-treated baculovirus-expressed UL16-GFP both interacted with gE.CT (Fig. 5.5B, lanes 5 and 7). These results suggested that free cysteines present in UL16 do not seem to play a role in the UL16-gE.CT interaction, though they are required for the UL11-UL16 interaction (Fig. 5.5B, lanes 4 and 6) as described previously (334). Furthermore, parallel studies of UL11 and its interaction with gE.CT have revealed that the interaction seems to be direct and likely mediated by the second half of UL11 (the function of which has never been described) and the N-terminal region of gE.CT (work performed by J. Han). Collectively, these results led to a hypothesis that in infected cells UL16 may interact with the N-

terminal region of gE.CT via UL11 binding (NEM-sensitive) or associate with the C-terminal region of gE.CT in a fashion that is independent of UL11 (NEM-resistant) (Fig. 5.6).

In an attempt to address whether the C terminus of gE.CT is sufficient for UL16 interaction, two truncation mutants lacking the N-terminal region (29 or 48 amino acids) were constructed and tested for binding; however, it was found that both mutants failed to bind baculovirus-expressed UL16 (data not shown). One concern was that these mutants might not have proper conformations with regard to the UL16 binding. Alternatively, other mutants with smaller deletions or point mutations will be required for further defining the UL16-binding site contained in gE.CT.

Figure 5.5 Interaction of UL16 with the full length of gE.CT was not affected by NEM treatment. (A) Vero cells were infected with HSV.UL16-CFP at a MOI of 10. At 20 h postinfection, cells were scraped into PBS. One set of cells were treated with NEM for 30 min before detergent lysis (bottom panel). Either untreated or NEM-treated HSV.UL16-CFP-infected cell lysates were incubated with approximately equal amounts of GST constructs (data not shown). Bound proteins were then analyzed by SDS-PAGE and immunoblotting. GST-UL11a and b, two separate preparations of GST-UL11 from *E. coli*. (B) Sf21 insect cells infected with a recombinant baculovirus expressing UL16-GFP were either untreated or treated with NEM prior to detergent lysis. Cell lysates were incubated with approximately equal amounts of GST constructs (data not shown). Proteins bound to GST constructs were loaded onto SDS-PAGE gels and analyzed by immunoblotting for GFP.

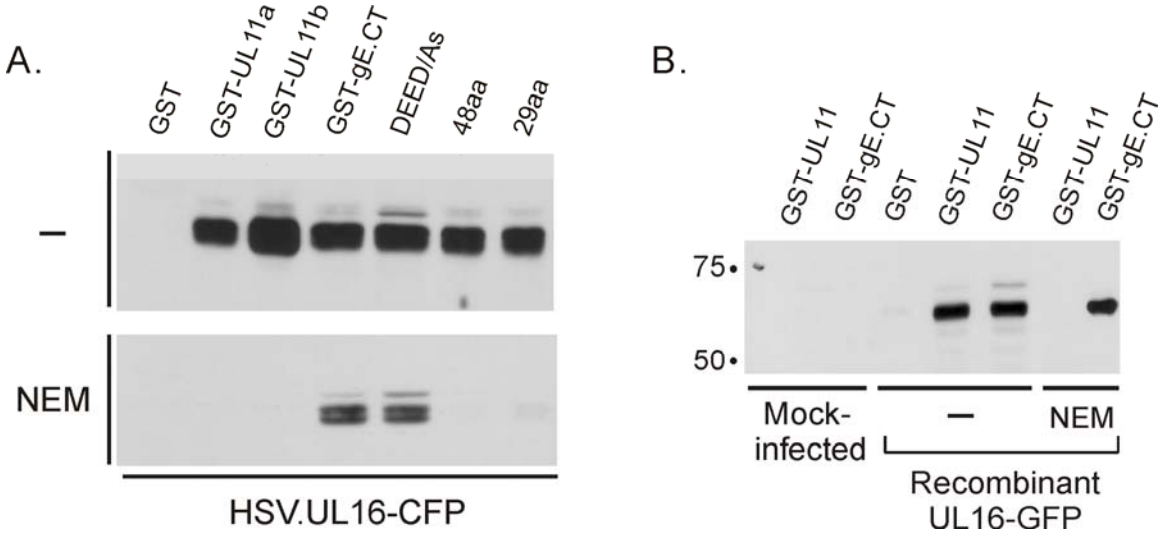
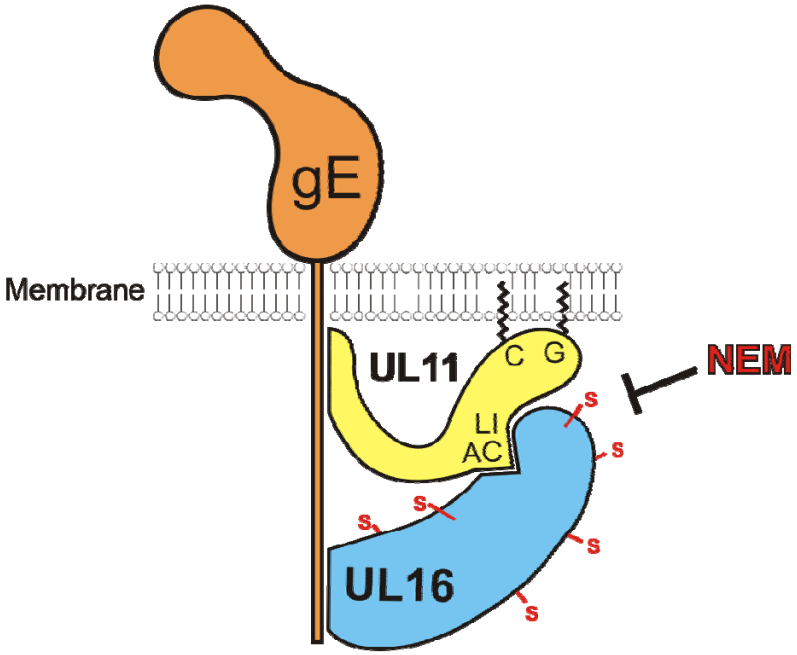
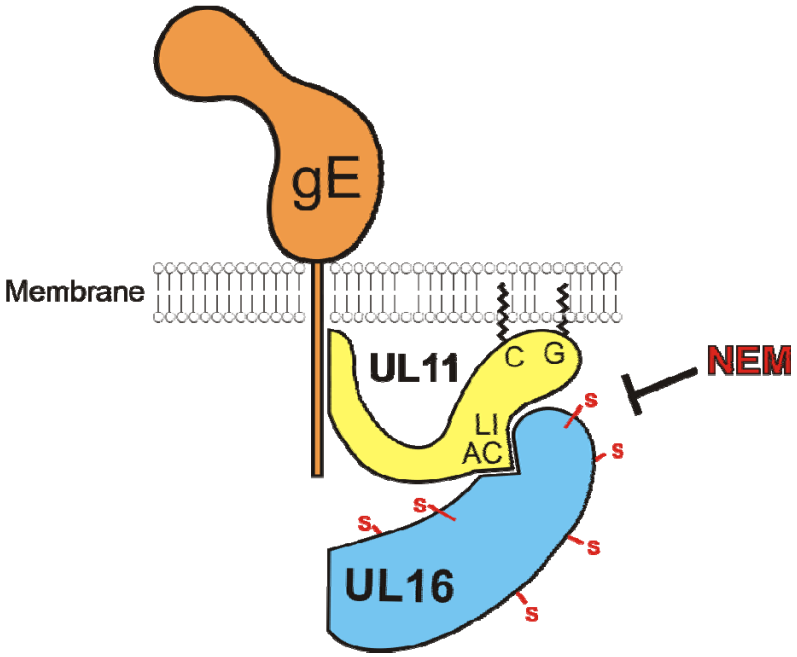

A.
GST
GST-UL11a
GST-UL11b
GST-gE.CT
DEED/As
48aa
29aa
–
NEM
HSV.UL16-CFP
B.
GST-UL11
GST-gE.CT
GST
GST-UL11
GST-gE.CT
GST-UL11
GST-gE.CT
75•
50•
Mock-infected
–
NEM
Recombinant UL16-GFP

Figure 5.6 Model for the UL16-gE interaction. gE is a type I transmembrane glycoprotein and UL11 is associated with the membrane via its N-terminal modifications of two fatty-acid chains (myristate and palmitate). The UL11-UL16 direct interaction is mediated by the acidic cluster (AC) and leucine-isoleucine (LI) motifs of UL11 as well as free cysteine residues within UL16, which can be blocked by NEM modification. (A) UL16 can interact not only with the N-terminal region of gE.CT via UL11 binding (NEM-sensitive) but also with the C-terminal region of gE.CT in a fashion that is independent of UL11 (NEM-resistant). (B) In the absence of the C-terminal region of gE.CT, UL16 has to interact with UL11 to stay associated with gE.CT. NEM modification blocks the UL11-UL16 and in turn the UL16-gE interactions.

A.


gE
Membrane
UL11
C G
LI
AC
NEM
S
S
S
S
S
UL16
S

B.


gE
Membrane
UL11
C G
LI
AC
NEM
S
S
S
S
S
UL16
S

Chapter VI

Discussion

We previously hypothesized that the interaction between UL11 and UL16 may provide a bridging function that contributes to the budding process at the TGN. In this study, we have more precisely defined this molecular interaction as well as others that may be involved in HSV morphogenesis. It was presented for the first time that UL16 is capable of interacting with the cytoplasmic tail of glycoprotein E (gE.CT) in both a UL11-dependent or -independent manner. The specific role of this UL11-UL16-gE tripartite complex in virus replication remains to be determined; however, our findings provide insights into the possible functions of these proteins. This chapter will be focused on the potential implications of these findings and discuss how the UL11-UL16-gE interactions may play a role in herpesvirus biology.

Potential Roles during Virus Attachment

Little is known about the virion structure. It has three morphologically distinct layers (capsid, tegument, and envelope) and contains nearly 40 virally encoded proteins in total. The details of how these proteins build a virion and specific locations of individual proteins within a virion are poorly understood. Studies of UL16 and its capsid association suggest a communication activity between the three layers of proteins. In response to HSV initial attachment to its cell surface receptors, a signal is sent across the virion envelope, and triggers a rapid and efficient structural rearrangement within the tegument, specifically, the release of UL16 from the capsid (191). The molecular mechanism controlling this signaling event is not known.

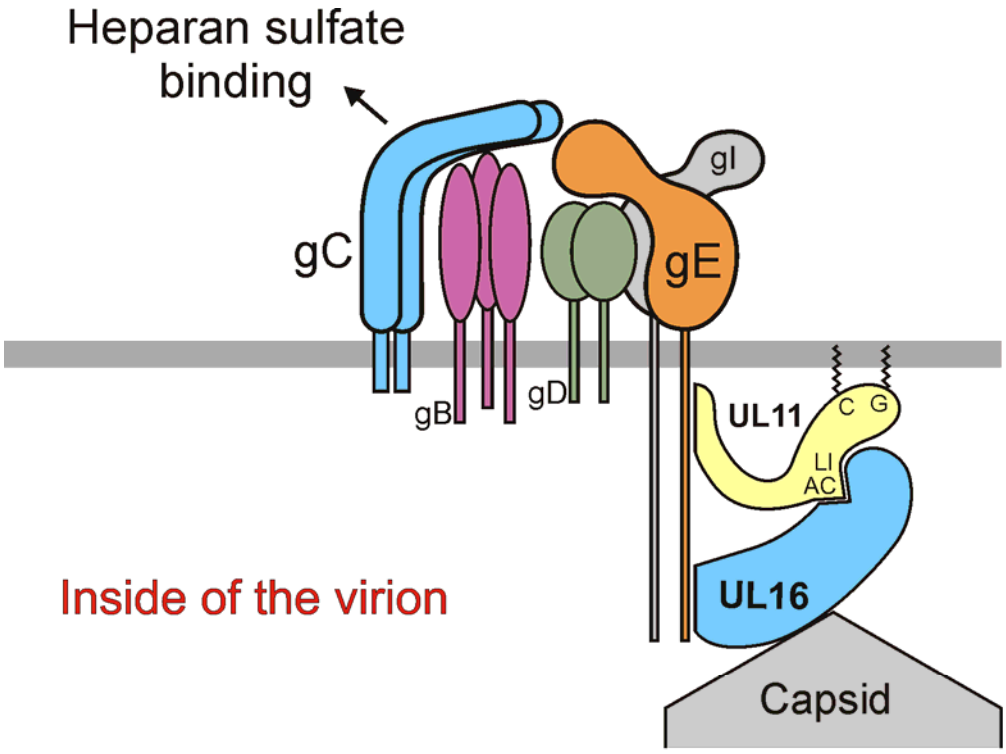
Evidence provided in Chapter V that UL16 is able to interact with gE.CT has provoked a speculation: the signal may be sent via gE.CT to UL16 during virus attachment. On the virion envelope, gC and gE(gI) appear to be in a complex with gB and

gD, blocking access by neutralizing antibodies (123), and inside the virion, UL11, UL16, and gE.CT may form a tripartite complex (Fig. 6.1). Membrane association of UL11 is mediated by its N-terminal myristylation and palmitylation signals (160). UL16 specifically recognizes the acidic cluster (AC) and leucine-isoleucine (LI) motifs within UL11, and also interacts with gE.CT either directly or through UL11 binding. And, our recent studies also indicate that UL11 interacts with gE.CT through its C terminus (work performed by J. Han). As for gI, though it forms a heterodimer with gE within infected cells, it is not known whether gI.CT is also involved in interaction with UL11 or UL16.

In this proposed model, binding of gC to heparan sulfate leads to clustering of glycoproteins, which then changes the UL11-UL16-gE.CT tripartite interactions and in turn loosens the interaction of UL16 with the capsid. In the absence of gC, binding of gB or gD to its receptor on the cell surface might be sufficient to induce structural changes of gE, subsequently promoting UL16 dissociation from the capsid. This could explain why the signal could still be observed with a gC-null mutant in a cell-binding assay as previously described (191).

By introducing mutations that abrogate interactions between the tripartite complex, it may become possible to test whether these proteins are indeed involved in this signaling event during virus attachment. Notably, other protein-protein interactions that may exist inside the virion could also be involved. For instance, VP16 may interact with the cytoplasmic tails of gB and gH (341), VP22 interacts with the gE tail (218), and VP16 and VP22 may also interact with each other (114,219). Therefore, the signaling event could be more complex than portrayed here (Fig. 6.1). It is unclear whether there are other changes within the virion during this structural rearrangement. But, as more is

Figure 6.1 Model for the UL16-capsid release mechanism during virus attachment. On the virion envelope, gC and gE(gI) are in a complex with gB and gD, and inside the virion, UL11, UL16, and gE.CT form a tripartite complex. Membrane association of UL11 is mediated by its N-terminal myristylation and palmitylation signals. UL16 specifically recognizes the acidic cluster (AC) and leucine-isoleucine (LI) motifs within UL11, and also interacts with gE.CT directly or via UL11 binding. Upon virus attachment to its initial cellular receptor (i.e., gC binds heparin sulfate [HS]), clustering of one or more viral glycoproteins may induce conformational changes of gE, which subsequently transmits a signal to UL16, thereby triggering its dissociation from the capsid.


Heparan sulfate
binding
gI
gC
gE
gB
gD
UL11
C G
LI
AC
Inside of the virion
UL16
Capsid

learned about the interactions that may take place within the virion, we may likely begin to uncover the molecular machinery required for (or controlling) this signaling event and ultimately understand its purposes for virus entry.

Implications for Virion Maturation

The process of HSV tegumentation and final envelopment to virion maturation is currently believed to be mediated by interactions between tegument proteins and the cytoplasmic tails of glycoproteins (Fig. 2.3). In this dissertation, we have precisely defined a previously recognized interaction between two tegument proteins, UL11 and UL16, and also presented for the first time that UL11, UL16, and gE may form a tripartite complex. Studies in Chapter IV demonstrate that UL11 and UL16 are capable of interacting directly and in a manner that is dependent upon the AC and LI motifs of UL11. Chapter V shows UL16 can interact with gE.CT, likely in both UL11-dependent and -independent manners. Interestingly, UL11 has also been reported to interact with gD.CT (75). And, UL11- and gD/gE-null viruses are all severely defective in final envelopment and virion release, suggesting important roles played by these proteins during virus egress (11,74). An attempt to test whether UL16 can also interact with gD.CT was not successful due to a technical issue (Fig. 5.1); however, this possibility should be further investigated in future studies.

To evaluate the potential contributions of these interactions to virus egress, mutations must be introduced into the virus. Simultaneous deletions of UL11, UL16, and gE may severely block budding and display defects similar to those observed with the gD-/gE- mutant virus. And, it is not clear whether these conserved proteins are part of

HSV budding machinery. It will be particularly interesting to test whether coexpression of UL11, UL16, and gE/gD in the absence of other viral proteins is sufficient to drive vesicle formation. As more is learned about how and where these proteins interact, we may gain a better understanding of their specific roles during virion maturation.

It was previously shown that the interaction of UL16 with capsids changes during egress (190). In particular, UL16 is stably associated with cytoplasmic capsids isolated from NP-40-treated cell lysates, but this interaction is very different in extracellular virions, where disruption with NP-40 under identical conditions releases UL16 from the capsid. More recently, we showed that the natural trigger for the release of UL16 from the capsid occurred upon the attachment of virus to the host cell (191). Free cysteines appear to play a critical role in this release mechanism because the addition of *N*-ethylmaleimide (NEM) stabilizes UL16 on the capsids of extracellular virions (i.e., it is no longer released from the capsid by NP-40 treatment). Stabilization is also observed when extracellular virions are exposed to low pH (5.0 to 5.5), conditions that protonate free cysteines, making them less reactive (190). However, there are likely to be many proteins in the virion that have free cysteines, and hence, the change in the UL16-capsid association properties seen with NEM treatments could be complex, and are experimental rather than physiological.

In the experiments described in Chapter IV, NEM modification of purified UL16 was found to directly block the interaction with purified UL11, a finding that was further supported by the inability of some (but not all) UL16 cysteine substitution mutants to bind UL11. Based on these observations, we hypothesize that the interaction of UL16 with the capsid may be destabilized as a result of binding to UL11 during budding. In

other words, when the capsid traverses the cytoplasm and arrives at the TGN, UL16 may engage UL11 and undergo a conformational change, making it sensitive to release by NP-40. Although these ideas are highly speculative, our findings represent the initial glimpses of a molecular mechanism that is present in the tegument.

Possible roles for cysteines in UL16

There are 20 cysteines in UL16, and sequence alignments revealed a cluster in the second half of the protein that is highly conserved (Fig. 4.6A) (324). The substitution mutants described here reveal that some, but not all, of these residues are important for UL11 binding. While it is possible that these cysteines directly contact UL11, there are several other (perhaps more likely) possibilities for what they do. Some cysteines may be needed merely for the proper folding of the protein, much like any other amino acid in UL16. Other cysteines might serve more complex structural roles, either as participants in disulfide bonds or as components of metal-binding domains (e.g., zinc fingers). It is also possible that UL16 is an enzyme that utilizes one or more free cysteines in its active site. For example, protein disulfide isomerases, which catalyze the formation and breakage of disulfide bonds, generally have a C-X-X-C motif in their active site, and this motif is also found among the conserved residues of UL16 (Fig. 4.6A). Further complicating the study of this protein is the possibility that all of these roles for cysteines will be found in UL16. Moreover, the role of any particular cysteine may change during virion budding and egress (e.g., particular disulfide bonds may be formed, broken, and rearranged along the way). Hence, much caution is needed when interpreting the results of any cysteine mutants, especially within the far more complicated context of recombinant viruses.

Interestingly, NEM does not abrogate the interaction of UL16 with gE.CT, suggesting that free cysteines present in UL16 do not play a role and that gE.CT might recognize other residues/domains contained in UL16. We have learned that UL16 is highly sensitive to deletions throughout its length, at least with regard to UL11 binding (Chapter IV). This means elucidation of the residues in UL16 that actually contact UL11 will be difficult; however, these UL16 deletion mutants may become handy for investigating the gE-binding site contained in UL16.

Potential Roles in Cell-to-Cell Spread

In addition to virion maturation, UL11 and gE have also been implicated in transport of vesicles containing virions to the basolateral cell surface for virus cell-to-cell spread (Fig. 2.4) (11,14,63,145,260). This specialized spreading mode facilitates rapid and efficient virus dissemination. Our findings that UL16 interacts with not only UL11 but also the cytoplasmic tail of gE support the hypothesis that UL16 may as well play a role in this process. Interestingly, deletion of UL16 produces small plaques on Vero cells compared to wild-type virus, a similar phenotype displayed by the UL11- and gE-null mutants ((11,63), and data not shown). Production of small plaques is unlikely to be explained by a merely 10-fold decrease in virus release. Thus, it will be of interest to further investigate the role of UL16 in cell-to-cell spread in cultured polarized cells or even animal models. Furthermore, it was exciting that characterization of native UL16-associated complexes in infected cells has identified RICH-1, which is a Rho GTPase-activating protein that plays a role in maintenance of cell junctions (Table 5.1) (167). Future studies will be required to investigate the specificity of the interaction and

determine whether RICH-1 is involved in virus cell-to-cell spread. The potential function of RICH-1 in maintaining integrity of cell junctions will be discussed later.

Other Possible Binding Partners of UL16

Using the combination of tandem affinity purification (TAP) and mass spectrometry (MS), numerous HSV and cellular proteins were identified to be present in native UL16 complexes isolated from infected cells (Table 5.1). It was noted that most of these proteins were only identified in one experiment but not in the other, which could be due to some technical issues or various differences in methods that were used to collect and analyze two separate samples (Chapter III). This may also explain why UL11, a known UL16 binding partner, was not found in either experiment. There were various types of keratins, which are very likely to be contaminants. Keratin contamination is a common and significant problem in mass spectrometry analysis because keratin is a major component in skin, hair, dust, etc. Gloves should always be worn and all care should be taken while handling samples. It is important to keep in mind that any efforts to reduce the chance of keratin contamination would improve identification of the proteins of interest by the mass spectrometer.

Experiments presented in Chapter V were mainly focused on confirming and characterizing the interaction between UL16 and gE. Questions still remain to be addressed whether the rest of the proteins listed in Table 5.1 are actually in a complex with UL16 during an infection. The following section will discuss their known/potential functions and the possibility of their involvement with UL16 (if any).

HSV-Encoded Proteins

UL19 (VP5). The U_L19 gene encodes the major capsid protein VP5 which is the structure subunit of the capsomers, both the hexons and pentons. It was previously shown that UL16 is stably associated with cytoplasmic capsids isolated from NP-40-treated cell lysates (190); however, the mechanism of how UL16 becomes associated with the capsid still remains undefined. The identification of VP5 by MS may be due to a few capsids that were purified concurrently with UL16.

UL21. UL21 is a 65-kDa tegument protein and has been shown to associate with microtubules, implicative of a role in transport of cytoplasmic capsid during virus egress (289). Our studies using co-immunoprecipitation and GST pull-down assays showed that HSV-1 UL21 and UL16 form a complex in infected cells (work performed by D.G. Meckes, Jr.). The interaction appears to be conserved because it has been demonstrated in another alphaherpesvirus, pseudorabies virus (PRV) (140). Thus, we have expected UL21 to be identified in our experiments. Deletion analysis indicates that the first half of UL21 is not required for UL16 binding (see Appendix B).

UL33. The UL33 protein (19-kDa) together with UL15 and UL28 forms a terminase complex that functions in binding, cleaving, and packaging viral DNA, and is essential for the assembly of DNA-filled capsids (2,20). A HSV-1 UL33 deletion mutant failed to process and encapsidate viral DNA into preassembled capsids, resulting in accumulation of immature capsids and no production of infectious viruses (226). UL16 has also been suggested to play a role in DNA packaging (207). Interestingly, a yeast two-hybrid analysis has also found that the KSHV homolog of UL16 interacts with the

UL33 homolog (305). Therefore, it will be of interest to further investigate this observation.

UL39. The U_L39 gene encodes the large subunit of ribonucleotide reductase, ICP6 (136-kDa). The protein forms a tight complex with a small subunit encoded by U_L40 to function in catalyzing the biosynthesis of deoxyribonucleotides from the corresponding ribonucleotides, which provides the precursors for viral DNA synthesis (85). A HSV-1 mutant from which 90% of ICP6 was deleted was extremely impaired in its ability to induce lesions in animals, and neither infectious nor latent virus could be recovered from the ganglia of these animals (124). These findings suggest that this enzyme plays a critical role in virulence and latency. It was also shown that inhibition of ICP6 leads to formation of small plaques on cultured cells (337). Intriguingly, HSV-2 UL16 has been suggested to play a role in DNA synthesis because it binds to single-stranded DNA in vitro (224). Since the specific role of UL16 in the nucleus is still poorly defined (discussed later), it may be interesting to test whether an interaction indeed takes place between HSV-1 UL16 and UL39.

UL49 (VP22). VP22, encoded by the U_L49 gene, is 40-kDa and the most abundant tegument proteins with an estimated 2000 copies per virion (72). The role of VP22 during virus assembly and mechanism of incorporation have been implicated in many reports (75,218,219). A GST pull-down assay was performed to test whether UL16 interacts with VP22 expressed in transfected cells; the result showed no binding between the two (data not shown). Interestingly, VP22 has been demonstrated to interact with the cytoplasmic tail of gE (218), and therefore, there is a possibility that gE might function in

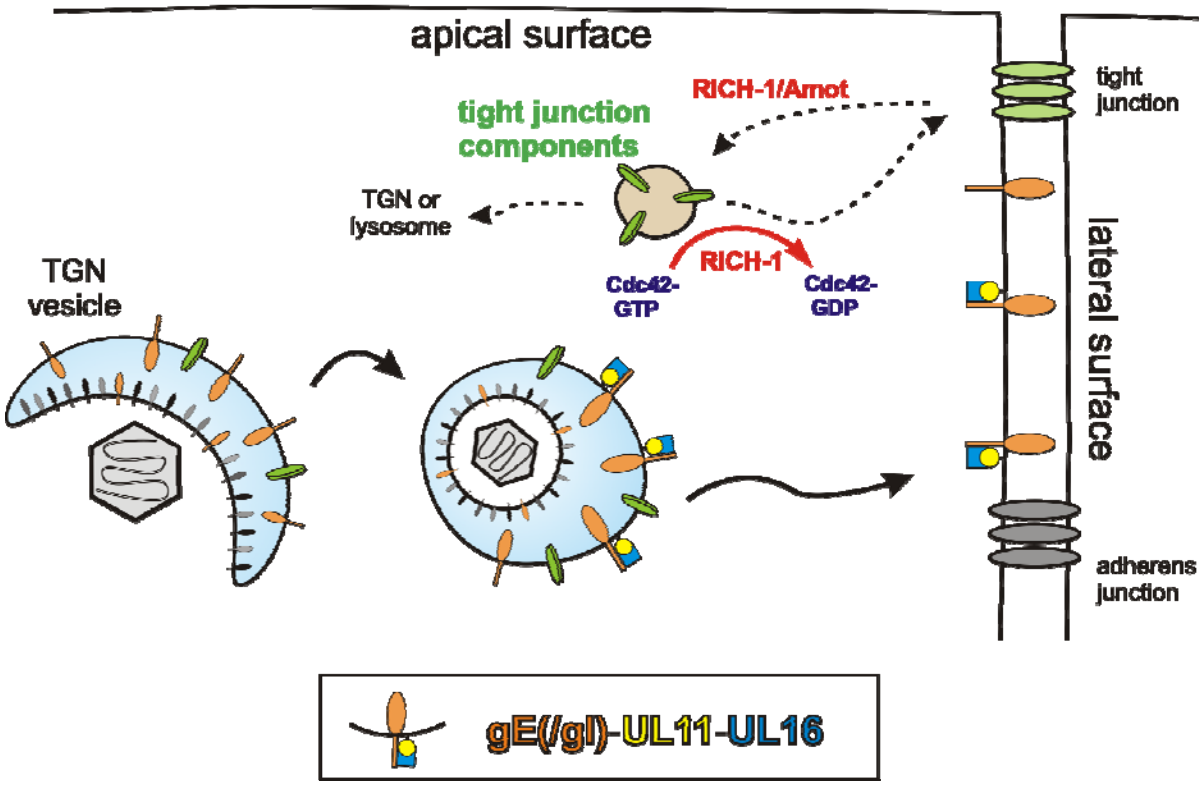
bridging the UL16-VP22 interaction. Whether VP22 is present in a complex with gE and UL16 (i.e., three proteins form a tripartite complex) is required to be further investigated.

US2. US2 is a 32-kDa virion protein which has been shown to be dispensable for virus growth in cell culture or animals (127,159). Due to the high level of redundancy, the specific role of US2 in virus replication cycle is still unclear. However, it was shown that HSV-2 US2 interacts and colocalizes with cytoskeratin 18, displaying a filamentous or dot-like cytoplasmic pattern (103). Interestingly, a UL16 binding partner, UL21, was also shown to be associated with the host cytoskeleton. Therefore, it could be interesting to see whether US2 is associated with cytoplasmic capsids and whether it has a role in capsid trafficking in the cytoplasm. If it does, the capsid-associated UL16 protein might be able to form some sort of complex with US2 during infection.

Cellular Proteins

Rho GTPase-activating protein 17 (or RICH-1 [RhoGAP interacting with CIP4 homologues]) is a 95-kDa protein that was identified as a binding partner of CIP4 (Cdc42-interacting protein 4), and was shown to be a specific GAP for Cdc42 and Rac1 (two members of Rho GTPase family) (244). The role of RICH-1 in polarized epithelial cells has recently been described. It was found that RICH-1 forms a complex with the scaffolding protein angiomotin (Amot), and RICH-1/Amot functions in maintaining integrity of tight junctions by regulating activity of Cdc42 GTPase as well as intracellular trafficking of specific apical-polarity components of tight junctions (Fig. 6.2) (167,318). It was particularly exciting that RICH-1 was identified as a possible binding partner of UL16 because we have speculated that the UL11-UL16-gE tripartite complex may play a

Figure 6.2 Model for the role of RICH-1 in HSV cell-to-cell spread. To maintain the integrity of tight junctions (green) in polarized cells, RICH-1 forms a complex with the scaffolding protein Amot, and triggers the endocytosis of tight junction components. Once the tight junction proteins are present in endocytic vesicles, their fate is determined by the GTP status of Cdc42. RICH-1 dissociates from Amot to serve as a GTPase-activating protein on Cdc42, which would result in recycling of the tight junction proteins back to the plasma membrane. However, if Cdc42 is not deactivated, the tight junction proteins will be transported to the TGN or to the lysosome for degradation. In infected polarized cells, mature HSV virions contained in TGN-derived vesicles can be targeted to the lateral surface where they infect adjacent cells. The process requires actions of a heterodimeric complex composed of gE/gI (orange lollipop), which is thought to facilitate basolateral sorting of TGN vesicles towards the cell surface. UL11 (yellow circle) interacts with the cytoplasmic tail of gE, and has been implicated in virus cell-to-cell spread. It is speculated that UL11 (along with its binding partner, UL16 [blue square]) could also be involved in this lateral transport of TGN vesicles. Since RICH-1 was identified as a potential binding partner of UL16, it might as well serve a role in HSV cell-to-cell spread.


apical surface
tight junction components
RICH-1/Arnot
TGN or lysosome
RICH-1
Cdc42-GTP
Cdc42-GDP
TGN vesicle
tight junction
lateral surface
adherens junction
gE(/gI)-UL11-UL16

role in virus cell-to-cell spread across cell junctions (Fig. 6.2). Future studies will be necessary to address the specificity of interaction between UL16 and RICH-1 as well as to investigate whether RICH-1 is involved in HSV cell-to-cell spread.

Filaggrin-2 (FLG-2) is a histidine- and glutamine-rich protein of approximately 248 kDa. Due to its Ca^{2+}-binding property, FLG-2 may be nonspecifically pulled down during the second purification step of the TAP procedure, in which excess of Ca^{2+} is provided to allow efficient interaction of calmodulin with calmodulin binding peptide (Fig. 3.1). The protein shares common structural features of the S100 fused-type protein (SFTP) family, which consists of essential components that function in epidermal differentiation and maintain epithelial homeostasis and barrier (259). S100 family represents the largest family within the superfamily of proteins carrying the EF-hand motif, which is a helix-loop-helix structural domain that binds a Ca^{2+} ion.

Host cytoskeleton-associated proteins

Following replication, transport of newly synthesized HSV particles to the cell periphery is likely to make use of one or more components of the cellular cytoskeletal system and their associated motor proteins. The microtubule plus-end transport motors (kinesins) have also been suggested to be involved in virus egress, presumably via interactions with the capsid-associated tegument proteins (68,298,328). Interestingly, a UL16 binding partner, UL21, has also been shown to associate with microtubules, implicative of a role in transport of cytoplasmic capsid during virus egress (289). Here, we have identified five cellular proteins that appear to be associated with the host cytoskeleton or play some sort of function in the system. Therefore, it could be

interesting to see whether these proteins play a role in capsid trafficking in the cytoplasm. If they do, the capsid-associated UL16 protein might be able to form some sort of complex with them during an infection.

UBX domain-containing protein 5 (UBXD5) is a 57-kDa protein, a.k.a. UBX domain protein 11 (UBXN11), Socius (SOC), or colorectal tumor-associated antigen (COA-1). The 'Ubiquitin regulatory X' (UBX) domain-containing proteins serve as the major regulators of Cdc48/p97, which is the highly conserved ATPase functioning in homotypic membrane fusion and misfolded protein degradation (261). Expression of the protein (COA-1) has also been related to colorectal cancer patients with metastatic disease (168). The rat homolog, Socius, has been implicated in reorganization of the actin cytoskeleton via interactions with Rnd1, a member of the Rho GTPase family (134).

PS1TP5-binding protein 1, also known as HCG15971, is a 42-kDa, ubiquitous actin-like protein. It is involved in the formation of actin filaments which are major components of the host cytoskeleton. Interactions with motor proteins (the myosin superfamily) play crucial roles in muscular contraction, various types of cell motility, and movement of organelles and vesicles inside the cell.

CLIP-associating protein 1 (Cytoplasmic linker-associated protein 1, or CLASP1) is a 169-kDa protein localized to microtubule (MT) plus-ends. CLIPs and CLASPs, as plus-end tracking proteins, regulate the dynamic properties of MT (1,89). CLIPs promote MT growth and regulate motor proteins (dynein-dynactin) localization, and CLASPs stabilize specific subsets of MTs in response to signaling cues. CLIPs and CLASPs interact and cooperate to direct the MT network, thereby regulating cellular asymmetry.

These proteins are also critical for mitotic fidelity by regulating spindle and kinetochore function (228).

Stomatin-like protein 2 (SLP-2) is a 38-kDa protein and a novel and unusual member of the stomatin superfamily that interacts with the peripheral erythrocyte cytoskeleton. The protein has been suggested to link stomatin or other integral membrane proteins to the peripheral cytoskeleton, thereby playing a role in regulating ion channel conductances or the organization of sphingolipid and cholesterol-rich lipid rafts (313). SLP-2 also appears to be involved in regulating cell growth and cell adhesion in human esophageal squamous cell carcinoma, indicating a role in tumorigenesis (336).

Myosin heavy chain 9 (or myosin-9, nonmuscle myosin IIA) is a 226-kDa protein which plays several important cellular functions such as cytokinesis, cell and shape, cell-cell adhesion, etc. Due to the capability of binding calmodulin, it was speculated that the protein may be pulled down by calmodulin resin during the TAP purification (Fig. 3.1), and therefore, likely to be a contaminant. Myosins are actin-binding motors that utilize ATP to provide energy for movement (147). The heavy chain of these motors bears a calmodulin-binding domain, and its association with two myosin light chains regulates the function of the intact myosin molecule in response to Ca^{2+} binding. It has been suggested that myosin-9 may play a role in HSV egress, based on the observation that a myosin inhibitor reduced the release of virus into the extracellular milieu while having much less effect on the yield of cell-associated virus (309). However, the defects observed in these experiments might be due to global disruption of the actin cytoskeleton. Whether the myosin-9 protein is directly or indirectly involved in trafficking of virus particles virus still remains unclear.

Potential Functions of UL16 in the Nucleus

Because UL16 has been shown to be capable of binding DNA in vitro and colocalize with capsid proteins (VP5 and VP22a) in the nucleus, it was suggested to participate in viral DNA synthesis or encapsidation (207,224). In a recent report, UL16 was detected at high levels in the nucleus of HSV-infected cells, and although UL16 was not found on intranuclear capsids purified from sucrose density gradients (190), it did not rule out the possibility that UL16 might be associated with unstable, immature procapsids. Despite these observations, the function of UL16 in the nucleus still remains undefined. When expressed alone, UL16-GFP fusion localizes diffusely throughout transfected Vero cells (190), and since the protein is too large to diffuse through the nuclear membrane, it must either have its own nuclear localization signal (NLS) or associate with a cellular protein that contains one. Sequence alignment of the UL16 homologs has implicated several basic residues (i.e., lysines and arginines) in the molecule that might serve as a NLS (324); but, it has not been proven whether any of the basic residues indeed compose a NLS. Mutagenesis analysis (with specific point mutations) will be necessary to address this question.

Intriguingly, when UL16-GFP-transfected cells were subsequently infected with HSV (in a transfection-infection assay), UL16-GFP was found to accumulate in the nucleus early times postinfection, preceding expression of the virally encoded UL16 (approximately 8 h postinfection), and this nuclear localization persisted to 18 h postinfection (190). This finding reveals that nuclear retention of the UL16 molecule may be mediated by associations with viral factors that function in the nucleus at an early stage during HSV infection, such as DNA replication or cleavage/packaging (224).

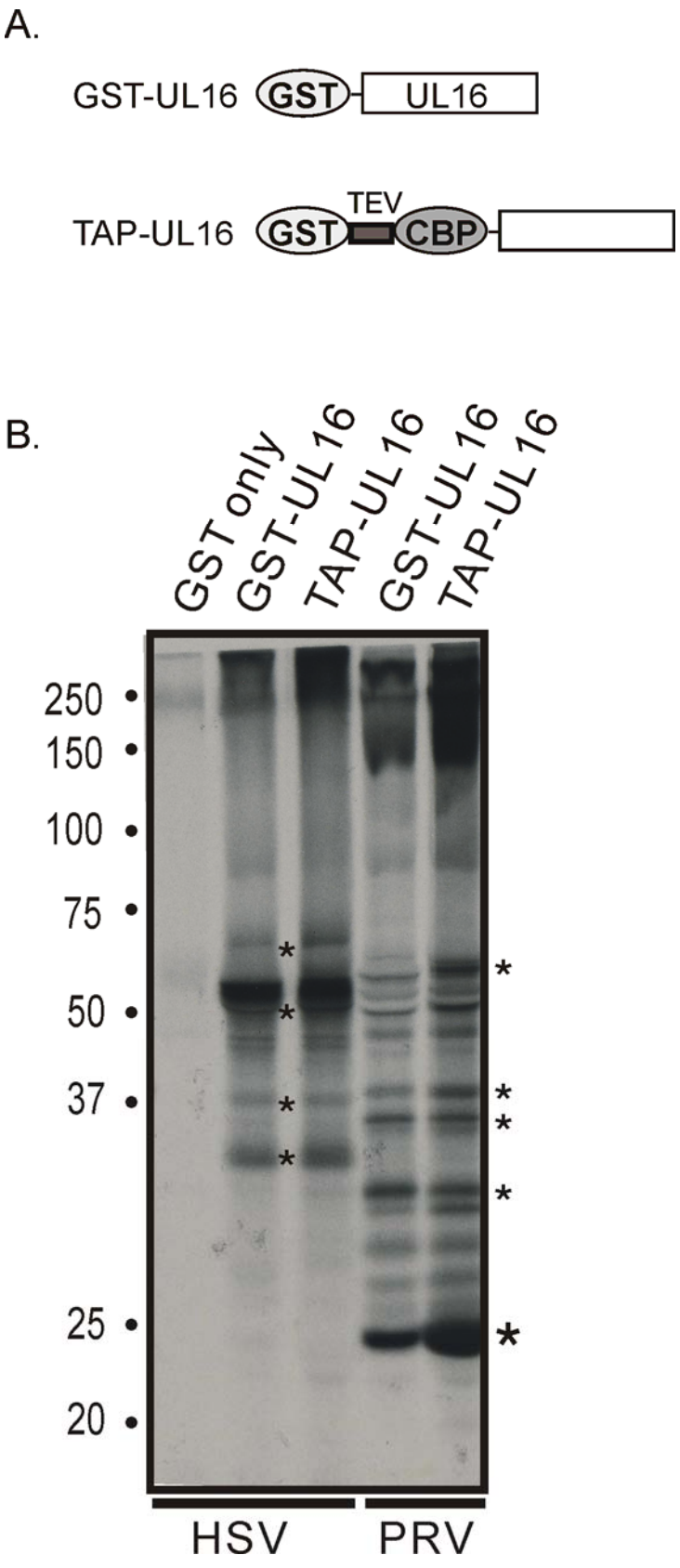
Further support was provided by the finding in a yeast two-hybrid analysis that the KSHV homolog of UL16 was found to interact with the UL33 homolog, an essential component of the DNA packaging machinery (305). Intriguingly, the TAP experiments described in Chapter V also identified UL33 and UL39 to be present in UL16-associated complexes isolated from the cytoplasm of infected cells. Whether UL16 interacts with UL33 or UL39 in the cytoplasm or in the nucleus should be investigated in future studies. In addition, two UL16 binding partners, UL11 and UL21, have also been found in the nucleus. UL11 is associated with nuclear periphery whereas UL21 is associated with intranuclear capsids in infected cells (8,54). Like UL16, their actual functions in the nucleus are still poorly understood. And, it is also unclear whether UL16 interacts with UL11 and UL21 in the nucleus.

In an attempt to explore the UL16-interaction network in the nucleus, pull-down experiments were performed using *E. coli*-expressed chimeras: GST-UL16 and GST-TEV-CBP-UL16 (or TAP-UL16) (Fig. 6.3A). Vero cells were infected with either HSV or PRV, and 5 h postinfection cells were radiolabeled for additional 2 h. Then, nuclear fractions were isolated from infected cells as described previously (33,190) and incubated with UL16 constructs purified from bacteria. It was found that both UL16 chimeras were able to pull down virus-specific proteins from nuclear fractions of HSV- or PRV-infected cells (Fig. 6.3B). The TAP procedure was then carried out in an attempt to isolate cleaner protein complexes, but was hampered by some technical difficulties. In a second attempt, nuclear fractions were isolated from HSV.UL16-TAP-infected Vero cells. The TAP procedure was performed as described in Chapter III. However, no proteins could be successfully isolated (data not shown). This could be due to several reasons, for example,

insufficient starting materials, or instability of UL16-associated complexes, or a highly transient, dynamic association of UL16 with its partners. And as mentioned above, there might be a possibility that UL16 is associated with unstable, immature procapsids, which might also result in difficulties for purification. It will be interesting to investigate whether UL16 is present on procapsids using virus mutants that are defective in capsid maturation. For instance, viruses with deletions in the gene encoding for protease UL26 accumulate immature procapsid structures in the nucleus (91,233). It might also be interesting to test whether UL16 can bind to any capsid-like structures that are produced by in vitro-capsid assembly systems using the panel of recombinant baculoviruses that express HSV capsid proteins (209,210,291).

Experiments described in this dissertation are mainly focused on analyzing interactions between UL16, UL11 and gE in the cytoplasm of infected cells, and our findings have shed lights into the potential roles of these proteins in various stages of virus life cycle. Thus, future studies that are designed to explore/characterize the nuclear UL16-interaction network will be expected to provide more insights into not only the specific role of UL16 in the nucleus but also overall HSV biology.

Figure 6.3 UL16 interacts with virus-specific proteins from nuclear fractions of HSV- or PRV-infected cells. (A) Diagrams of the UL16 constructs. (B) To examine the abilities of purified GST-UL16 or TAP-UL16 to pull down virus-specific proteins from the nuclei of infected cells, Vero cells were infected with HSV or PRV. 5 h postinfection, cells were radiolabeled with [^{35}S]methionine for additional 2 h. Then, nuclear fractions isolated from infected cells were incubated with the indicated constructs. Bound proteins were separated by SDS-PAGE, and radiolabeled proteins were detected by autoradiography. Examples of virus-specific proteins pulled down by GST-UL16 and TAP-UL16 were indicated with asterisks.


A.
GST-UL16
GST
UL16
TAP-UL16
TEV
GST
CBP
B.
GST only
GST-UL16
TAP-UL16
GST-UL16
TAP-UL16
250
150
100
75
50
37
25
20
HSV
PRV

Appendix A

Permission for Use of Figure 2.1

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Email: katie.wade@wiley.com

*Figure 1 of Spear P.G. 2004. Cellular Microbiology 6 (5): 401-410.

Appendix B

The First Half of the UL21 Tegument Protein is Not Required for UL16 Binding

Previously using GST pull-down and co-immunoprecipitation assays, UL11 was found to associate with not only the capsid-associated UL16 tegument protein but also an unknown 65-kDa protein from HSV-infected cell lysates (161). The 65-kDa protein was excised from the SDS-PAGE gel, digested with trypsin, and subsequently identified by mass spectrometry as another tegument protein UL21 (work performed by A.L. Harper).

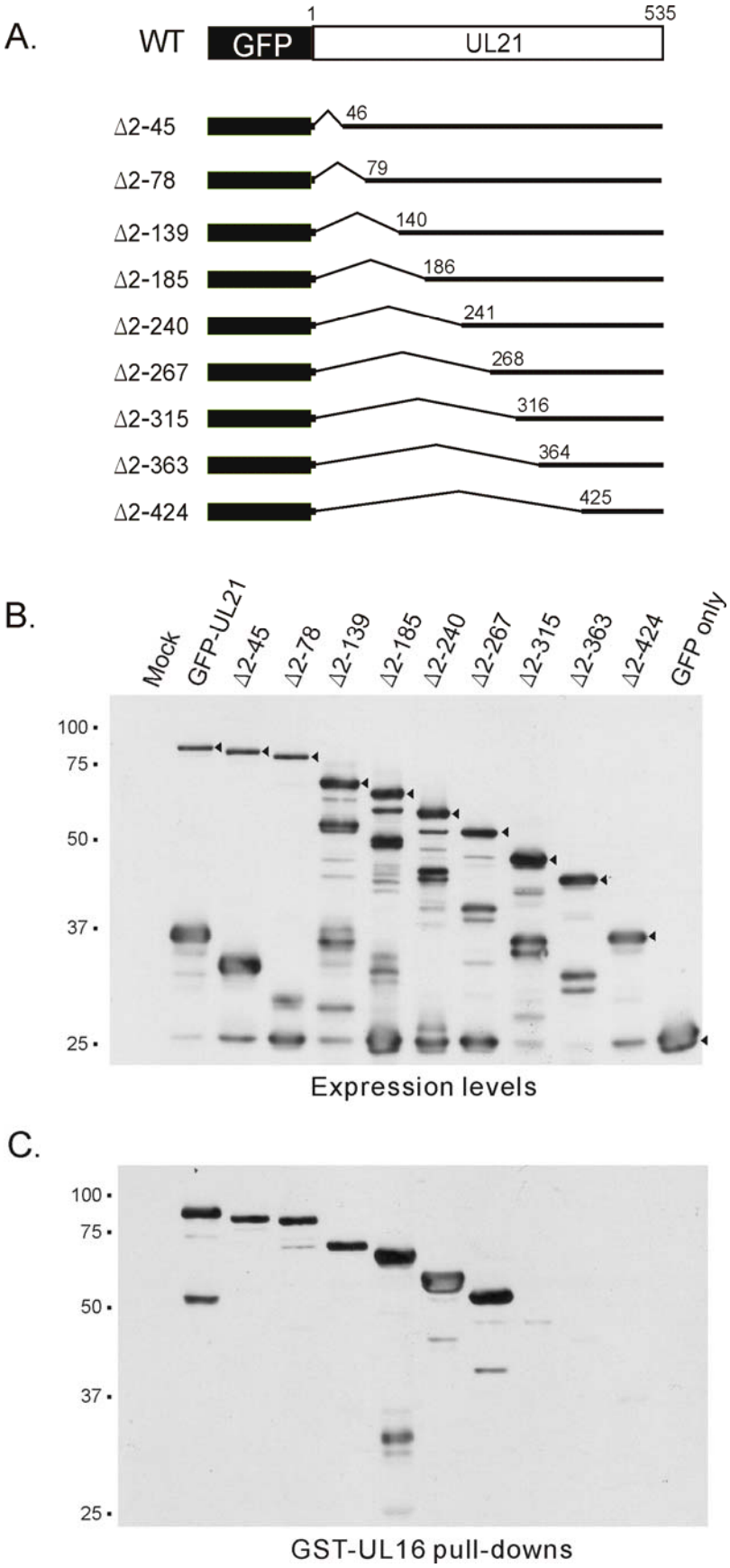
UL21 is conserved among all herpesviruses and has been shown to associate with microtubules, implicative of a role in transport of cytoplasmic capsid during virus egress (289). Using co-immunoprecipitation and GST pull-down experiments, it was confirmed that HSV UL21 and UL16 form a complex in infected cells (work performed by D.G. Meckes, Jr.). The UL21-UL16 interaction has also been observed in another alphaherpesvirus, pseudorabies virus (PRV) (140). Together with the UL11-UL16 interaction, the tripartite UL11-UL16-UL21 complex is suggested to function in connecting and moving the capsid along the microtubules to the TGN membrane for final envelopment.

When UL21 is expressed in cells, it can be readily pulled down by GST-UL16, suggesting that UL21 and UL16 are able to interact with each other in the absence of other viral proteins. Moreover, purified proteins from *E. coli* are able to interact with each other in vitro, indicative of a direct interaction mechanism (work performed by J.A. Marsh).

Deletion analysis of UL21. In an effort to map the UL16-binding site contained in UL21, several N-terminal deletion mutants were constructed (by A.L. Harper) in the context of the eukaryotic expression vector, pCMV.GFP-UL21 (Fig. B.1A), and these

were transfected into Vero cells using Lipofectamine 2000 (Invitrogen). All mutants expressed well compared to wild type (Fig. B.1B). In the GST-UL16 pull-down experiment, the mutants with deletions in residues 2 to 267 were found to retain the ability to bind UL16, and the binding was lost with the mutants missing regions in the second half of UL21 (residues 268 to 535) (Fig. B.1C). These results indicate that the first half (residues 2 to 267) of UL21 is not required for UL16 binding.

Figure B.1 The first half (residues 2 to 267) of UL21 is not required for UL16 binding. (A) Diagrams of UL21 wild-type (WT) and N-terminal truncation mutants, which were all constructed as GFP-fusion proteins. (B) Expression levels of indicated constructs in transiently transfected Vero cells (arrowheads). At 20 h posttransfection, cells were harvested in sample buffer, loaded onto SDS-PAGE gels, and analyzed by immunoblotting for GFP. (C) Transfected cells were lysed by NP-40 and incubated with equal amounts of purified GST-UL16 proteins (data not shown). Bound proteins were analyzed by SDS-PAGE and immunoblotting for GFP.


A.
WT
GFP
UL21
1
535
Δ2-45
46
Δ2-78
79
Δ2-139
140
Δ2-185
186
Δ2-240
241
Δ2-267
268
Δ2-315
316
Δ2-363
364
Δ2-424
425
B.
Mock
GFP-UL21
Δ2-45
Δ2-78
Δ2-139
Δ2-185
Δ2-240
Δ2-267
Δ2-315
Δ2-363
Δ2-424
GFP only
100
75
50
37
25
Expression levels
C.
100
75
50
37
25
GST-UL16 pull-downs

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