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The Role of Viral Glycoproteins and Tegument Proteins in Herpes Simplex Virus Type 1 Cytoplasmic Virion Envelopment

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THE ROLE OF VIRAL GLYCOPROTEINS AND TEGUMENT PROTEINS IN HERPES SIMPLEX VIRUS TYPE 1 CYTOPLASMIC VIRION ENVELOPMENT

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ABSTRACT

Herpes simplex virus type 1 (HSV-1) is a ubiquitous neurotropic alphaherpesvirus transmitted by contact with mucocutaneous surfaces of infected individuals. HSV-1 enters the host by fusion of the viral envelope with the host cell plasma membrane, followed by translocation of the viral capsids to the nucleus where viral DNA is injected into the host cell nucleus to initiate viral replication. To generate infectious virions, newly assembled capsids travel to the cytoplasm and undergo a process called secondary envelopment by budding into cytoplasmic vesicles derived from the trans-Golgi network. Cytoplasmic envelopment is a complex process involving interactions between a multitude of viral membrane and tegument proteins. To investigate the relative importance of a subset of viral membrane and tegument proteins in secondary envelopment, a number of recombinant viruses were constructed in the HSV-1(F) genetic background. A mutant virus unable to express gE, gM and the C-terminus of gD was characterized and compared to additional mutants unable to express both gE and gM or gE and the C-terminus of gD and to mutants lacking expression of just one of these glycoproteins, in addition to mutants lacking expression of both pUL11 and gM, and pUL20 alone. Characterization of all mutant viruses by plaque morphology, viral replication kinetics, electron microscopy and particle-to-PFU ratios revealed a hierarchy of defects in cytoplasmic envelopment and infectious virus production, with deletion of pUL20 having the greatest effect, followed by the deletion of pUL11 and gM. Characterization of additional mutants containing multiple mutations revealed that gE, gM and gD do not function in a redundant manner in cytoplasmic envelopment supporting a preeminent role for the pUL20/gK protein complex in cytoplasmic envelopment and egress. An epitope tag insertion adjacent to the pUL37 Y480

(DC480) exhibited a severe defect in cytoplasmic envelopment similar to gK and pUL20-null viruses. Importantly, this mutant virus was partially complemented when grown on cells expressing pUL20, suggesting an interaction with the pUL20/gK protein complex. This pUL37 interaction with pUL20/gK was verified by co-immunoprecipitation and proximity ligation assays suggesting that it facilitates cytoplasmic virion envelopment.

CHAPTER I: INTRODUCTION

Statement of Problem and Hypothesis

Herpes simplex viruses are responsible for a wide spectrum of human disease ranging from cutaneous eruptions to life-threatening viral encephalitis. Infection occurs by direct contact, leading to viral replication at the site of infection before the virus penetrates sensory neurons and travels to dorsal root ganglia to establish a lifelong latent infection punctuated by periods of recurrence at the site of infection. Viral entry into host cells is mediated by assembly of viral fusion proteins that trigger fusion between the viral envelope and the plasma membrane, thus releasing capsids into the cell. Capsids travel to the nucleus to release their cargo of viral DNA for transcription and initiation of progeny virus production.

Virion assembly and egress is a complex multi-step process whereby newly assembled capsids of herpes simplex virus type-1 (HSV-1) must undergo two envelopment steps on their journey out of the cell. Capsids are assembled in the nucleus and undergo primary envelopment and budding into the perinuclear space before losing the envelope by fusion with the outer nuclear envelope to deliver capsids into the cytoplasm. Cytoplasmic capsids travel to sites of secondary envelopment at trans-Golgi membranes and endosomes enriched with viral glycoproteins and associated outer tegument proteins. Interactions between the capsid, the tegument, and viral envelope proteins at TGN membranes facilitate budding of capsids into TGN vesicles to form enveloped virions which are then transported by exocytosis to the cell periphery for release.

The process of secondary envelopment is poorly understood. Deletions of individual membrane and tegument proteins, with the notable exceptions of the membrane proteins pUL20 and gK and the tegument proteins pUL36 and pUL37, do not lead to significant defects in cytoplasmic envelopment, leading to a proliferation of envelopment models in which they function in a redundant or synergistic manner to promote cytoplasmic envelopment. The hypothesis promulgated in this dissertation is that cytoplasmic envelopment is the result of a coordinated action of protein-protein interactions between the cytoplasmic portions of viral membrane proteins and viral tegument proteins that are brought together in close proximity during envelopment. The hypothesis predicts that the pUL20/gK protein complex and the pUL37 tegument protein, both of which are known to play preeminent roles in cytoplasmic envelopment, physically and functionally interact to facilitate the virion envelopment process.

Statement of Research Objectives

The first goal of this research was to assess the synergistic importance of herpes simplex virus type-1 (HSV-1) membrane glycoproteins gD, gE and gM and the membrane-associated tegument protein pUL11 in cytoplasmic virion envelopment in comparison to the membrane proteins pUL20 and gK, which are known to be essential determinants of cytoplasmic virion envelopment. The second goal of this work was to explore the potential role of the inner tegument protein pUL37 in cytoplasmic virion envelopment and to probe the relationship between pUL37 and the pUL20/gK membrane protein complex in order to shed further light on the network of interactions between viral membrane and tegument proteins involved in cytoplasmic virion envelopment. The specific aims of this research include:

I. To clarify the relative contribution of the viral proteins gD, gE, gM and pUL11 to cytoplasmic envelopment and infectious virus production, either alone or in various combinations, in relation to pUL20.

   a. To construct a recombinant virus in the HSV-1(F) genetic background using a BAC mutagenesis system with mutations abrogating expression of gE, gM and the carboxyl-terminal 29 amino acids of gD simultaneously.

   b. To construct additional recombinant viruses in the HSV-1(F) genetic background using a BAC mutagenesis system with mutations abrogating expression of gM alone, pUL11 alone, gE and gM simultaneously, and gM and pUL11 simultaneously.

   c. To fully characterize the newly constructed viruses in addition to previously constructed viruses with mutations abrogating expression of pUL20 alone, gE alone, the carboxyl terminus of gD alone, and gE and the carboxyl terminus of gD simultaneously by analyzing plaque morphology, viral growth kinetics, ultrastructural morphology, and particle-to-PFU ratios.

II. To elucidate the potential role of a poorly characterized domain of pUL37 in cytoplasmic virion envelopment and to explore the possibility of a physical relationship between pUL37 and the pUL20/gK protein complex.

   a. To construct a recombinant virus in the HSV-1(F) genetic background using a BAC mutagenesis system with a mutant pUL37 containing a 12 amino acid protein C epitope tag inserted immediately following Y480 and to fully characterize it by analyzing plaque morphology, viral growth kinetics, ultrastructural morphology, and particle-to-PFU ratios.

b. To test for the existence of interactions between the inner tegument protein pUL37 and the membrane proteins pUL20 and gK by two-way co-immunoprecipitation and proximity ligation assays.

Overall, the results obtained from this work indicate that:

I. Single mutant viruses unable to express gD, gE, gM or pUL11 do not exhibit significant defects in cytoplasmic virion envelopment or infectious virus production. Double mutants unable to express gM and gE or gE and the carboxyl terminus of gD exhibit mild reduction in average plaque size, infectious virus production, and cytoplasmic virion envelopment in comparison to the single mutants, with the mutant lacking gE and the carboxyl terminus of gD showing marginally greater reduction. The mutant virus with simultaneous deletions of gE, gM and the carboxyl terminus of gD shows mild reduction in average plaque size, infectious virus production, and cytoplasmic virion envelopment in comparison to the aforementioned double mutants, suggesting that gE, gM and the carboxyl terminus of gD do not function in a redundant manner in cytoplasmic envelopment. A double mutant lacking both pUL11 and gM exhibits more substantial defects in cytoplasmic virion envelopent and infectious virus production that are greater than those observed with the aforementioned triple mutant. However, all of these mutants exhibit significantly less severe defects than the pUL20-null virus, which illustrates the important role pUL20 and its interacting partner gK play in cytoplasmic virion envelopment.

II. A recombinant HSV-1 with a mutated pUL37 containing a 12 amino acid protein C tag inserted in-frame following Y480 exhibits drastically impaired cytoplasmic envelopment and

infectious virus production similar to that of a pUL37-null virus when grown on Vero cells, but is complemented for virus replication and spread when grown in FRT cells expressing the UL20 gene while the pUL37-null virus is not, suggesting a direct or indirect interaction with the pUL20/gK protein complex. Co-immunoprecipitation experiments and proximity ligation assays show that pUL37 interacts with both pUL20 and gK in infected cells to facilitate cytoplasmic virion envelopment.

The work presented here is placed into individual chapters in a manuscript format with chapter titles reflecting the central theme of the research contained therein.

CHAPTER II: LITERATURE REVIEW

History of Herpes

Herpesviruses have been co-evolving with their hosts, including humans, for millions of years (172, 201). Herpes simplex viruses in particular are well suited to co-migrate and diversify with their human hosts due to their ability to establish latent and persistent infections in their hosts. This intricate evolutionary linkage is illustrated by phylogenetic analyses of Herpes simplex virus type 1 (HSV-1) and Herpes simplex virus type 2 (HSV-2) that estimate the divergence time for HSV-1 and HSV-2 at approximately 2.2 million years BP, roughly corresponding to the advent of the genus *Homo*. (83, 172). Furthermore, the HSV-1 clade structure closely corresponds with the timing and routes of past global human migrations, with HSV-1 strains first diverging in Africa approximately 50,300±16,700 years BP, the Eurasian strains diverging 32,800±10,900 years BP, and the American KOS strain diverging from the Asian CR38 strain at 15,760±5,300 years BP which closely fits the estimated time period in which humans from Asia began to populate the North American continent (165, 172).

For most of human history, disease has been an enigmatic phenomenon, variously ascribed to causes ranging from imbalances of bodily humors to supernatural factors such as divine retribution. Consequently, modern efforts to identify pathogens such as HSV from historical sources are fraught with difficulty, particularly when the disease in question is not known for having dramatic and deadly clinical outcomes. Many of the lesions mentioned in ancient texts are described poorly and may not have been caused by HSV at all, instead resulting from skin malignancies, concurrent infection with a number of other pathogens, or

even by cutaneous diseases that simply do not exist in modern times. These difficulties are further exacerbated by the inconsistent historical usage of the term “herpes”. It was commonly used to refer to a number of other skin conditions, such as lupus, that are not associated with herpesviruses, and, for a time, was even abolished in favor of “formica”. (265).

The earliest evidence of symptoms possibly linked to infection by a herpes simplex virus shows up as descriptions of genital lesions in a ~3000 BC Sumerian tablet and in the ~1500 BC Ebers Papyrus. The first recorded usage of the Greek word “ἕρπης“, or “herpes”, was by Hippocrates in order to describe lesions that appear to crawl along the skin. The Roman author Celsus, writing in the 1st century BCE, described round lesions that eventually “diffused like a serpent to form a belt”, which arguably fits varicella-zoster virus (VZV) better than HSV. Herodotus is believed to be the first to record a link between cutaneous eruptions and fever, and his work was expanded and elaborated by the prominent Greek physician Galen in the 2nd century AD. Galen’s description of lesions that tended to recur at the same anatomical site is notable as the earliest hint of viral latency and reactivation following HSV infection (97, 265).

Better definitions of herpesviral disease had to wait until the 18th century, coinciding with upheavals and innovations in medical science that would later lead to widespread acceptance of the germ theory of disease and the birth of modern medicine. Daniel Turner described a number of cutaneous diseases in his 1714 treatise *De Morbis Cutaneis*, including “facial herpes” (presumably herpes simplex type-1) and “herpes miliaris” (presumably varicella-zoster virus). The first description of “herpes genitalis” followed in 1736 in *De Morbis Venereis*, written by John Astruc and based on his studies of French prostitutes in his capacity as

physician for King Louis XIV. Accounts of genital herpes proliferated after this point, becoming closely associated with prostitution. In 1886, Charles-Paul Diday and Adrien Doyon published *Les Herpes Genitaux*, the first book specifically dedicated to herpes. A decade later, Jean Alfred Fournier released his treatise on the diagnosis and treatment of genital herpes, recommending abstaining from alcohol, tobacco and “sexual excesses” in order to treat and prevent infections. Notably, Paul Gerson Unna described genital herpes in 1883 as a “vocational disease” of women, albeit a disease that is “one of the most benign of affections both to the patient and her public” (97, 265). This nonchalant attitude towards genital herpes persisted up until the mid-1970’s, as evidenced by the absence of genital herpes from studies of psychological morbidity in clinics dealing with sexually transmitted diseases (199). Public awareness of genital herpes peaked in the early 1980's largely due to the development and marketing of acyclovir as an effective herpes treatment and the 1982 publication of a *TIME* cover story branding it as "The New Scarlet Letter", which likely contributed to a stigmatization of the condition. However, herpes was soon supplanted in the public consciousness by the arrival of the human immunodeficiency virus and the worrying prospect of a global HIV epidemic (70, 164).

In the late 19th century, herpes research began to progress from being merely descriptive to experimentation designed to elucidate the nature of the disease. The French dermatologist Jean Baptiste Émile Vidal in 1873 was the first to show that HSV was infectious by passing the infection from one human volunteer to another. However, credit for the isolation of HSV usually goes to Wilhelm Grüter, who used an animal model in his 1924 paper to show that HSV can be serially transmitted between rabbits. Another key to the understanding of HSV biology was published by Andrews and Carmichael in 1930. They observed that recurrent HSV

infections only occurred in adults who carried neutralizing antibodies, thus igniting a debate that culminated in a 1939 publication by Burnet and Williams that promoted the modern view that HSV infection persists for life in a latent state and may be reactivated at any time to produce new lesions due to trauma, fever or other stimuli (97, 265). The existence of two separate types of HSV and their association with either orolabial herpes (HSV-1) or genital herpes (HSV-2) was only demonstrated in 1971 by Schneweis and Nahmias, over four decades after Grüter's original isolation of HSV (277).

During the latter half of the 20th century, rapid technological advances in molecular biology and the advent of electron microscopy have enabled us to isolate and visualize viruses with relative ease, thus leading to the discovery and classification of a growing number of herpesviruses infecting a wide variety of animals, including humans. Advancements in cell culture techniques during the 1950s led to the discovery and isolation of varicella-zoster virus (VZV) and human cytomegalovirus (CMV) (68, 308). Further cell culture developments led to the cultivation of B lymphocytes, resulting in the discovery of Epstein-Barr virus (EBV) in 1964 (89). Human herpesviruses 6A, 6B and 7 were isolated in the late 1980s after the development of techniques to cultivate T lymphocytes (105, 190, 270). The most recently discovered human herpesvirus is HHV-8, otherwise known as Kaposi's sarcoma-associated herpesvirus (KSHV). KSHV was found in 1994 using a then-novel technique called representational difference analysis (RDA) which can be employed to find sequence differences between two complex genomes (47). The proliferation of relatively cheap high-throughput sequencing technologies will undoubtedly lead to the discovery of a plethora of novel herpesviruses, in addition to

providing valuable insights into the inner workings of familiar viruses due to the ready availability of genomic data (124).

Taxonomy of Herpesviruses

Taxonomy is the systematic classification of organisms into groups, or taxa, which are collectively defined by a consensus group of properties (95). Unlike other organisms that have "domain" as the most inclusive taxonomic rank, virus taxa are limited to order, family, subfamily, genus and species, as defined by the International Committee on Taxonomy of Viruses (ICTV). The ICTV was established in 1971 as a means to standardize the classification of viruses, spurred by the growing numbers of viruses being discovered due to advances in molecular biology. While early virologists had to depend on broad biological properties such as host range and disease manifestation to classify novel viruses, the growing use of electron microscopy and improved cell culture techniques provided detailed information on viral morphology and physiology that enabled more accurate viral classification. The development of specific antibodies further refined virus taxonomy by allowing researchers to distinguish between closely related viruses, and advances in nucleotide sequencing led to an explosion of granular genomic data and the eventual adoption of sequence-based phylogenetic analysis as the key taxonomic determinant for all organisms, including viruses (75).

Herpesviruses are a morphologically distinct group, possessing a linear, double-stranded DNA genome varying from 125 kbp to 290 kbp that is contained within an icosahedral capsid that is surrounded by a proteinaceous matrix called the tegument, which is in turn surrounded by a host-derived lipid envelope containing an assortment of membrane-associated viral

proteins. Despite their morphological similarities, herpesviruses comprise three discrete genetic groups and have subsequently been split by the ICTV into three families that together form the order *Herpesvirales*. The family *Herpesviridae* incorporates viruses of mammals, birds and reptiles, while the family *Alloherpesviridae* contains viruses that infect fish and frogs, and the family *Malacoherpesviridae* is home to viruses that infect molluscs (76).

The first ICTV report in 1971 contained a mere 290 different viruses, and all 23 different herpesviruses recognized at the time were placed in the genus *Herpesvirus* (144). This genus was elevated to family and renamed to *Herpetoviridae* in the second ICTV report, which is also notable for the addition of *Human herpesvirus 2* as a close relative of *Human herpesvirus 1* (96). The family was renamed again to *Herpesviridae* in the third ICTV report, presumably to avoid the implied association of *Herpetoviridae* with reptiles. The third ICTV report also marked the division of the family *Herpesviridae* into the three currently recognized subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae* (198). The modern herpesviral taxonomic structure originated prior to the ninth ICTV report with the creation of a new order, *Herpesvirales*, containing the family *Herpesviridae* and the new families *Alloherpesviridae* and *Malacoherpesviridae* (76). According to the most recent (2013) online version of the official ICTV taxonomy available at ictvonline.org, there are now a total of 2828 recognized viral species, while the order *Herpesvirales* currently consists of 3 families, 3 subfamilies, 19 genera and 102 species (Table 2.1).

Table 2.1: Classification of herpesviruses. Adapted with permission from [*Veterinary Microbiology* 143(1-2): 52-69, Herpesvirus systematics]. Copyright (2010) Elsevier. Table has been updated with data from the 2013 ICTV virus taxonomy release available at ictvonline.org. Asterisks indicate the type species in each genus.

Taxon name	Common name	Acronym
Order *Herpesvirales*		
Family *Alloherpesviridae*		
Genus *Batrachovirus*		
*Ranid herpesvirus 1**	Lucké tumor herpesvirus	RaHV1
Ranid herpesvirus 2	Frog virus 4	RaHV2
Genus *Cyprinivirus*		
Anguillid herpesvirus 1	Eel herpesvirus	AngHV1
Cyprinid herpesvirus 1	Carp pox herpesvirus	CyHV1
Cyprinid herpesvirus 2	Goldfish haematopoietic necrosis virus	CyHV2
*Cyprinid herpesvirus 3**	Koi herpesvirus	CyHV3
Genus *Ictalurivirus*		
*Ictalurid herpesvirus 1**	Channel catfish virus	IcHV1
Ictalurid herpesvirus 2	Black bullhead herpesvirus	IcHV2
Acipenserid herpesvirus 2	White sturgeon herpesvirus 2	AciHV2
Genus *Salmonivirus*		
*Salmonid herpesvirus 1**	Herpesvirus salmonis	SalHV1
Salmonid herpesvirus 2	Oncorhynchus masou herpesvirus	SalHV2
Salmonid herpesvirus 3	Epizootic epitheliotropic disease virus	SalHV3
Family *Herpesviridae*		
Subfamily *Alphaherpesvirinae*		
Genus *Iltovirus*		
*Gallid herpesvirus 1**	Infectious laryngotracheitis virus	GaHV1
Psittacid herpesvirus 1	Pacheco's disease virus	PsHV1
Genus *Mardivirus*		
Anatid herpesvirus 1	Duck enteritis virus	AnHV1
Columbid herpesvirus 1	Pigeon herpesvirus	CoHV1
*Gallid herpesvirus 2**	Marek's disease virus type 1	GaHV2
Gallid herpesvirus 3	Marek's disease virus type 2	GaHV3
Meleagrid herpesvirus 1	Turkey herpesvirus	MeHV1
Genus *Scutavirus*		
*Chelonid herpesvirus 5**	Chelonid fibropapilloma-associated herpesvirus	ChHV5

(Table 2.1 continued)

Taxon name	Common name	Acronym
Genus *Simplexvirus*		
Ateline herpesvirus 1	Spider monkey herpesvirus	AtHV1
Bovine herpesvirus 2	Bovine mammillitis virus	BoHV2
Cercopithecine herpesvirus 2	Simian agent 8	CeHV2
*Human herpesvirus 1**	Herpes simplex virus type 1	HHV1
Human herpesvirus 2	Herpes simplex virus type 2	HHV2
Leporid herpesvirus 4	Leporid herpesvirus 4	LeHV4
Macacine herpesvirus 1	B virus	McHV1
Macropodid herpesvirus 1	Parma wallaby herpesvirus	MaHV1
Macropodid herpesvirus 2	Dorcopsis wallaby herpesvirus	MaHV2
Papiine herpesvirus 2	Herpesvirus papio 2	PaHV2
Saimiriine herpesvirus 1	Marmoset herpesvirus	SaHV1
Genus *Varicellovirus*		
Bovine herpesvirus 1	Infectious bovine rhinotracheitis virus	BoHV1
Bovine herpesvirus 5	Bovine encephalitis herpesvirus	BoHV5
Bubaline herpesvirus 1	Water buffalo herpesvirus	BuHV1
Canid herpesvirus 1	Canine herpesvirus	CaHV1
Caprine herpesvirus 1	Goat herpesvirus	CpHV1
Cercopithecine herpesvirus 9	Simian varicella virus	CeHV9
Cervid herpesvirus 1	Red deer herpesvirus	CvHV1
Cervid herpesvirus 2	Reindeer herpesvirus	CvHV2
Equid herpesvirus 1	Equine abortion virus	EHV1
Equid herpesvirus 3	Equine coital exanthema virus	EHV3
Equid herpesvirus 4	Equine rhinopneumonitis virus	EHV4
Equid herpesvirus 8	Asinine herpesvirus 3	EHV8
Equid herpesvirus 9	Gazelle herpesvirus	EHV9
Felid herpesvirus 1	Feline herpesvirus 1	FHV1
*Human herpesvirus 3**	Varicella-zoster virus	HHV3
Phocid herpesvirus 1	Harbour seal herpesvirus	PhoHV1
Suid herpesvirus 1	Pseudorabies virus	SuHV1
Unassigned species in the subfamily		
Chelonid herpesvirus 6	Lung-eye-trachea disease-associated virus	ChHV6
Subfamily *Betaherpesvirinae*		
Genus *Cytomegalovirus*		
Aotine herpesvirus 1	Herpesvirus aotus type 1	AoHV1

(Table 2.1 continued)

Taxon name	Common name	Acronym
Cebine herpesvirus 1	Capuchin herpesvirus AL-5	CbHV1
Cercopithecine herpesvirus 5	Simian cytomegalovirus	CeHV5
*Human herpesvirus 5**	Human cytomegalovirus	HHV5
Macacine herpesvirus 3	Rhesus cytomegalovirus	McHV3
Panine herpesvirus 2	Chimpanzee cytomegalovirus	PnHV2
Papiine herpesvirus 3	Baboon cytomegalovirus	PaHV3
Saimiriine herpesvirus 4	Squirrel monkey cytomegalovirus	SaHV4
Genus *Muromegalovirus*		
*Murid herpesvirus 1**	Murine cytomegalovirus	MuHV1
Murid herpesvirus 2	Rat cytomegalovirus	MuHV2
Murid herpesvirus 8	English rat cytomegalovirus	MuHV8
Genus *Probiscivirus*		
*Elephantid herpesvirus 1**	Elephant endotheliotropic herpesvirus	ElHV1
Genus *Roseolovirus*		
*Human herpesvirus 6A**	Human herpesvirus 6A	HHV6A
Human herpesvirus 6B	Human herpesvirus 6B	HHV6B
Human herpesvirus 7	Human herpesvirus 7	HHV7
Unassigned species in the subfamily		
Caviid herpesvirus 2	Guinea pig cytomegalovirus	CavHV2
Suid herpesvirus 2	Pig cytomegalovirus	SuHV2
Tupaiid herpesvirus 1	Tupaiid herpesvirus	TuHV1
Subfamily *Gammaherpesvirinae*		
Genus *Lymphocryptovirus*		
Callitrichine herpesvirus 3	Marmoset lymphocryptovirus	CalHV3
Cercopithecine herpesvirus 14	African green monkey EBV-like virus	CeHV14
Gorilline herpesvirus 1	Gorilla herpesvirus	GoHV1
*Human herpesvirus 4**	Epstein–Barr virus	HHV4
Macacine herpesvirus 4	Rhesus lymphocryptovirus	McHV4
Panine herpesvirus 1	Herpesvirus pan	PnHV1
Papiine herpesvirus 1	Herpesvirus papio	PaHV1
Pongine herpesvirus 2	Orangutan herpesvirus	PoHV2
Genus *Macavirus*		
*Alcelaphine herpesvirus 1**	Wildebeest-associated malignant catarrhal fever virus	AlHV1
Alcelaphine herpesvirus 2	Hartebeest malignant catarrhal fever virus	AlHV2
Bovine herpesvirus 6	Bovine lymphotropic herpesvirus	BoHV6

(Table 2.1 continued)

Taxon name	Common name	Acronym
Caprine herpesvirus 2	Caprine herpesvirus 2	CpHV2
Hippotragine herpesvirus 1	Roan antelope herpesvirus	HiHV1
Ovine herpesvirus 2	Sheep-associated malignant catarrhal fever virus	OvHV2
Suid herpesvirus 3	Porcine lymphotropic herpesvirus 1	SuHV3
Suid herpesvirus 4	Porcine lymphotropic herpesvirus 2	SuHV4
Suid herpesvirus 5	Porcine lymphotropic herpesvirus 3	SuHV5
Genus *Percavirus*		
*Equid herpesvirus 2**	Equine herpesvirus 2	EHV2
Equid herpesvirus 5	Equine herpesvirus 5	EHV5
Mustelid herpesvirus 1	Badger herpesvirus	MusHV1
Genus *Rhadinovirus*		
Ateline herpesvirus 2	Herpesvirus ateles strain 810	AtHV2
Ateline herpesvirus 3	Herpesvirus ateles	AtHV3
Bovine herpesvirus 4	Bovine herpesvirus 4	BoHV4
Cricetid herpesvirus 2	Pygmy rice rat herpesvirus	CrHV2
Human herpesvirus 8	Kaposi's sarcoma-associated herpesvirus	HHV8
Macacine herpesvirus 5	Rhesus rhadinovirus	McHV5
Murid herpesvirus 4	Murine herpesvirus 68	MuHV4
Murid herpesvirus 7	Wood mouse herpesvirus	MuHV7
*Saimiriine herpesvirus 2**	Herpesvirus saimiri	SaHV2
Unassigned species in the subfamily		
Equid herpesvirus 7	Asinine herpesvirus 2	EHV7
Phocid herpesvirus 2	Phocid herpesvirus 2	PhoHV2
Saguinine herpesvirus 1	Herpesvirus saguinus	SgHV1
Unassigned species in the family		
Iguanid herpesvirus 2	Iguana herpesvirus	IgHV2
Family *Malacoherpesviridae*		
Genus *Aurivirus*		
*Haliotid herpesvirus 1**	Abalone herpesvirus	AbHV1
Genus *Ostreavirus*		
*Ostreid herpesvirus 1**	Oyster herpesvirus	OsHV1

A formal system for naming herpesviruses was implemented prior to the third ICTV report in 1979 (75). It states that each herpesvirus species must be named after the taxon (family or subfamily) of its primary natural host. This host-derived term is followed by the word herpesvirus, and an Arabic number (76). Furthermore, according to a set of rules established by the ICTV that govern viral nomenclature, a species name must be italicized with the first letter of the name being capitalized (e. g. *Human herpesvirus 1*), while a virus name must be written in lower case (unless a proper noun is part of the virus name) and without italics (e. g. herpes simplex virus type 1) (175). The species name cannot be abbreviated; if an abbreviation is used, it represents the physical virus and not the species (75). Like all taxonomic classes, a species is an abstract concept, and the species demarcation criteria can be drastically different among the various established viral categories depending on the quantity of available information. A virus name, on the other hand, represents a physical entity that can be manipulated and can be further subdivided into strains, variants and isolates. An isolate is the most granular unit, representing experimental material that corresponds to a specific instance of a given virus. Furthermore, multiple isolates of the same variant may also have sequence differences due to the presence of quasispecies, which are a mixture of related genotypes that may be present in an environment of high mutation rate (175).

In 1991, the ICTV adopted the definition of a virus species as "a polythetic class of viruses that constitutes a replicating lineage and occupies a particular ecological niche" (301). Based on this definition, current guidelines state that herpesviruses can be classified as separate species only if “(a) their nucleotide sequences differ in a readily assayable and distinctive manner across the entire genome and (b) they occupy different ecological niches by

virtue of their distinct epidemiology and pathogenesis or their distinct natural hosts" (75). These guidelines are now somewhat obsolete due to the fact that modern classification of all organisms, including viruses, is driven by phylogenetic analysis based on sequence comparison data that allows for evolutionary classification (175). The ICTV *Herpesvirales* Study Group is currently considering updates to the herpesvirus species definition and to the methods used to assign viruses to discrete taxonomic groups in order to emphasize sequence-based phylogeny and also to aid in classifying the rapidly growing number of herpesviruses that have been detected only by polymerase chain reaction (PCR) (75).

Herpesviral Disease

Herpesviruses are a diverse group, encompassing over 100 different viruses infecting a wide variety of hosts, both verterbrate and invertebrate. The structure of herpesviruses is characteristic of the group, consisting of a linear double-stranded DNA genome that varies in size from 125 kbp to 290 kbp and is enclosed in an icosahedral capsid composed of 12 pentavalent and 150 hexavalent capsomeres. The capsid is coated by a matrix of tegument proteins, and is further surrounded by a host-derived lipid envelope that contains a variety of membrane-associated viral proteins and glycoproteins (75). Herpesviruses can be further distinguished by their ability to establish a reversible state termed latency where the viral genome persists in the host cell without lytic gene expression or new virus production (97). In contrast to viruses such as retroviruses that rely on viral genome integration into the host cell's chromosomes for their survival, herpesvirus DNA typically persists in the host cell nucleus as circularized extrachromosomal structures called episomes which replicate using the rolling-

circle mechanism, creating long concatemeric DNA strands that are eventually cleaved to produce single genomes to be packaged into newly synthesized capsids. Chromosomal integration of viral DNA has been documented in several herpesviruses but the majority of these cases involve subgenomic DNA fragments, thereby precluding the possibility of reactivation and infectious virus production from the integrated DNA. Currently, the only herpesviruses known to exhibit integration of full-length viral DNA are Epstein-Barr virus (HHV-4), HHV-6A/6B, and Marek's disease virus (GaHV-2) (220).

Nine different herpesviruses have been routinely isolated from humans and implicated in causing human disease. These human herpesviruses consist of herpes simplex virus 1 (HHV-1), herpes simplex virus 2 (HHV-2), varicella-zoster virus (HHV-3), Epstein-Barr virus (HHV-4), cytomegalovirus (HHV-5), Kaposi's sarcoma-associated herpesvirus (HHV-8), and the cluster of HHV-6A, HHV-6B and HHV-7 commonly referred to as roseola virus (23). In addition, the monkey B virus (CeHV-1) and murine gammaherpesvirus-68 (MuHV-4) are occasionally encountered in humans, particularly in laboratory workers handling infected animals, as zoonoses from macaque monkeys and mice, respectively (138, 140).

Herpes simplex viruses (HHV-1 and HHV-2)

Herpes simplex viruses are transmitted by intimate contact between an individual who is shedding virus and a susceptible host. After coming into contact with the mucosa or abraded skin of the susceptible individual, the virus replicates in epithelial cells at the site of infection. The primary infection often results in the presence of vesicular lesions at the site of inoculation, but can also be asymptomatic. Eventually, the virus invades sensory nerve endings and is

transported to neuronal ganglia where it establishes latency that persists for the life of the host. Latent virus may reactivate and start replicating again at any time, possibly triggered by a number of stimuli such as stress or exposure to ultraviolet light. Reactivation typically results in transport of the virus along the peripheral sensory nerves back to the initial site of infection where it replicates and causes transient outbreaks of vesicular lesions termed recurrent infections (23, 97).

Herpes simplex virus 1 (HSV-1) most commonly infects the oropharyngeal area via kissing or other intimate contact and establishes a latent infection in the host's trigeminal ganglia (97). Oropharyngeal HSV-1 infection in adults typically manifests as cold sores on the lips, also known as herpes simplex labialis. Gingivostomatitis is more common in children younger than five years old, and is characterized by intraoral and pharyngeal lesions, accompanied by fever, sore throat and pharyngeal edema. HSV-1 can also infect the eye, leading to dendritic lesions on the cornea accompanied by conjunctivitis. Termed herpetic keratitis, this is the most common infectious cause of blindness in the U.S. (23). Herpes simplex virus 2 (HSV-2) is usually transmitted by genital contact during sexual activity and establishes latency in the host's sacral ganglia (97). Genital HSV-2 infection is known as genital herpes, and manifests as recurrent vesicular lesions usually involving the vulva, vagina and cervix in women, and the penis in affected men (23). HSV-2 clinical manifestations are reportedly less severe in individuals harboring preexisting antibodies to HSV-1 due to previous infection by HSV-1 (317).

Using data from the National Health and Nutrition Examination Survey (NHANES), researchers looked at HSV-1 and HSV-2 seroprevalence in U.S. individuals between 14 and 49

years of age during the period of 2005-2010, showing seroprevalence of 53.9% for HSV-1 and 15.7% for HSV-2. While HSV-2 seroprevalence stayed relatively constant, HSV-1 seroprevalence exhibited a marked decline, particularly in individuals between 14 and 19 years of age whose HSV-1 seroprevalence declined by over 29% from the period of 1976-1980 to the period of 2005-2010, potentially leaving this population at greater risk of genital HSV-2 infection when they begin to engage in sexual activity due to the protective nature of HSV-1 antibodies (33). European serological surveys covering the period between 1989 and 2000 revealed extensive variation in the seroprevalence of HSV-1 and HSV-2 among different countries, although the probability of infection increased with age in all cases. Age standardized HSV-1 seroprevalence varied from a low of 52% in Finland to a high of 84% in Bulgaria. HSV-2 seroprevalence among individuals older than 12 years of age ranged from a low of 4% in England and Wales to a high of 24% in Bulgaria (248). Worldwide, an estimated 536 million people between the ages of 15 and 49 were living with HSV-2 infections in 2003, with an increased prevalence among women and in developing regions (188).

Although less common, HSV-1 and HSV-2 infections can potentially manifest at any skin site. Herpetic lesions on the fingers are known as herpetic whitlow, while individuals participating in contact sports such as wrestling can develop disseminated cutaneous lesions in a condition called herpes gladiatorum (23). Furthermore, HSV-1 is responsible for nearly 30% of genital herpes infections in the U.S. In fact, the frequency of genital HSV-1 infections has been increasing to the point that HSV-1 is the prevailing cause of genital herpes in select populations of U.S. college students, accounting for up to 78% of all genital isolates, presumably due to the growing ubiquity of oral sex practices (164, 261).

The ability of HSV to invade and replicate in neurons allows the virus to establish latency in neuronal ganglia, but it can also lead to a life-threatening condition called herpes simplex encephalitis where the virus invades the central nervous system, leading to hemorrhagic necrosis of the temporal lobes accompanied by fever, altered consciousness, and often death (23). HSV encephalitis is the most common cause of sporadic fatal encephalitis in the U.S. with an incidence of approximately 1 in 200,000 individuals per year and a mortality rate exceeding 70% in untreated individuals, with only 2.5% of patients able to retain normal neurologic function (97). The causative agent of HSV encephalitis is HSV-1 in virtually all recorded cases (309). However, HSV-2 is the predominant causative agent in a different CNS infection called recurrent aseptic meningitis, also known as Mollaret's meningitis in older litarature (299). Unlike cases of HSV encephalitis, cases of recurrent aseptic meningitis are usually benign and self-limiting, resolving without clinical intervention (278).

Neonatal HSV infection is a potentially serious condition typically caused by vertical transmission of HSV from the mother to the infant, occurring in up to 1 in 3000 deliveries in the U.S. HSV-2 is the causative agent in approximately 70% of cases, commonly due to fetal contact with the infected mother's genital secretions during delivery. Disease manifestation and prognosis for neonatal HSV infection varies greatly, ranging from cutaneous lesions affecting the skin, mouth and eyes (~0% mortality rate), to CNS involvement and encephalitis (~15% mortality rate), and potentially to disseminated infection that involves multiple organ systems (~60% mortality rate) (23).

A greater risk for severe HSV infections is borne by individuals with impaired immune function, which may occur due to malnutrition, immunosuppressive therapy for organ transplantation, or underlying disease such as cancer or acquired immunodeficiency syndrome (AIDS). HSV infection in these patients can manifest in a variety of ways, ranging from disseminated cutaneous lesions to esophagitis, pneumonitis, colitis, or devastating systemic infections. Recurrent HSV is also more common in immunocompromised patients, particularly in individuals infected with human immunodeficiency virus (HIV) whose disease has progressed to AIDS (97). The genital ulcerations caused by HSV have also been linked to an increased risk of HIV transmission. In a 2002 meta-analysis, researchers estimated that among individuals seropositive for HSV-2, 52% of sexually transmitted HIV infections are attributable to HSV-2 infection, suggesting that widespread treatment and management of HSV-2 could be an effective strategy for HIV prevention (305).

HSV-1 is a ubiquitous, neurotropic virus, and these qualities also made it an obvious pathogen to examine as a possible aetiological agent in the development of Alzheimer's disease. Indeed, research has shown that infection with HSV-1 may predispose individuals to develop Alzheimer's disease, although it is not the sole causative agent. HSV-1 is a particularly strong risk factor for Alzheimer's disease when it is present in the central nervous system of individuals possessing the type 4 allele of the apolipoprotein E gene (APOE-epsilon4) (84). Further studies have found HSV-1 DNA in amyloid plaques within the brains of human Alzheimer's disease patients, and showed that beta-amyloid tends to accumulate in HSV-1 infected mouse brains and cell cultures (315). Elevated HSV-1 antibody titers are also significantly more frequent in Alzheimer's disease patients and are positively correlated with

grey matter volumes (195). Others have suggested that HSV-1 infection may accelerate neurodegeneration in Alzheimer's disease patients by an autoimmune response caused by viral mimicry of key Alzheimer's disease-related proteins (44).

Varicella-zoster virus (HHV-3)

Varicella-zoster virus (VZV) is the alphaherpesvirus responsible for chickenpox and shingles and is present in up to 95% of adult humans. VZV is highly contagious and can be spread by contact with airborne droplets launched by coughing or sneezing in addition to physical contact with rash secretions. Initial viral replication occurs in the oropharynx, followed by spread to dorsal root and cranial nerve ganglia where the virus establishes latency. Primary VZV infection in young children manifests as a disseminated vesicular rash that typically lasts from three to five days and is commonly referred to as chickenpox (23, 115). Herpes zoster, commonly known as shingles, is the recurrent form of VZV infection that occurs after reactivation of latent virus. Herpes zoster is characterized by a painful vesicular rash localized to an area of skin supplied by a single spinal nerve called a dermatome. The rash lasts two to four weeks and is often accompanied by postherpetic neuralgia. Herpes zoster usually affects adults, and occurs with greater frequency and severity in immunocompromised individuals (288).

Cytomegalovirus (HHV-5)

Cytomegalovirus (CMV) is a betaherpesvirus that preferentially replicates and persists in human salivary glands, kidneys and peripheral blood leukocytes. CMV infection is typically spread by saliva, urine, or breast milk and is symptomatic in only around 10% of cases (23). Infected individuals may experience an infectious mononucleosis-like syndrome characterized

by fever, pharyngitis and lymphadenopathy (31). Cytomegalovirus retinitis causes retinal necrosis and primarily affects immunosuppressed individuals. CMV retinitis is the most common cause of vision loss in AIDS patients (28). Congenital CMV infection can also occur, particularly if a woman develops primary CMV infection during gestation. Approximately 1% of live births in the U.S. are affected by congenital CMV (23).

Kaposi's sarcoma-associated herpesvirus (HHV-8)

Kaposi's sarcoma-associated herpesvirus (KSHV) is an oncogenic human gammaherpesvirus that was discovered in 1994 by using a then-novel technique called representational difference analysis to compare genomic DNA between Kaposi's sarcoma-affected tissue and unaffected tissue (47). KSHV is spread by close contact and infects a variety of human cell types, including epithelial cells and lymphocytes, with a tendency to establish latency in B lymphocytes (48). Clinical disease caused by KHSV such as the eponymous Kaposi's sarcoma, a cutaneous tumor, typically occurs only in AIDS patients and other immunocompromised individuals (9). KSHV can also cause a hyperproliferation of B lymphocytes, leading to primary effusion lymphomas or multicentric Castleman's disease, which is characterized by the development of lymphatic tumors at multiple sites, possibly due to expression of virally encoded interleukin-6 (vIL-6) (10, 46, 48).

Human herpesvirus 7 (HHV-7)

Human herpesvirus 7 and its relatives human herpesvirus 6A and human herpesvirus 6B are members of the betaherpesviral genus *Roseolovirus* and are closely associated with the childhood disease roseola, which is characterized by fever and an accompanying facial rash

(23). Human herpesvirus 6B is believed to be the primary causative agent of roseola, but infection with HHV-7 may help reactivate latent HHV-6B, leading to roseola symptoms. HHV-7 infects and goes latent in CD4+ T lymphocytes and is ubiquitous in the population, particularly among children (155). Over 95% of adults are seropositive for HHV-7, with over 75% of children seropositive by age 6 (45, 58).

Human herpesvirus 6 (HHV-6A and HHV-6B)

Human herpesvirus 6A and human herpesvirus 6B share 95% sequence homology and were only recognized as distinct species in 2012 (3, 125). HHV-6 is a betaherpesvirus relative of cytomegalovirus and is highly prevalent in the population, infecting more than 90% of young children. Clinical disease is primarily due to infection with HHV-6B, manifesting as the disease exanthem subitum, also known as roseola infantum (11, 155). After primary infection, HHV-6 preferentially replicates in activated CD4+ T lymphocytes and persists in a latent state in monocytes for the life of the host (98). Several studies have shown evidence of complete HHV-6 integration into host genomes, with estimates of integrated HHV-6 prevalence in the world human population approaching 1%. HHV-6 is also able to integrate into the genomes of human germ line cells and thus transmit itself from parent to offspring, leading to individuals carrying chromosomally-intergrated HHV-6 in every nucleated cell in the body at chromosomal sites identical to their parents. HHV-6 appears to preferentially incorporate itself into the telomeric regions of human chromosomes, possibly with the aid of a recently identified protein (encoded by the U94 gene) that shares partial homology with a parvovirus protein (REP68/78) implicated in chromosomal integration. This genomic integration is thought to occur by chromosomal

incorporation of the viral concatemeric DNA formed after rolling-circle replication of the episome, rather than by incorporating the episomal DNA itself or the initial linear viral DNA genome. Notably, HHV-6 is one of only three herpesviruses, along with Epstein-Barr virus and Marek's disease virus, that are known to undergo chromosomal integration of full-length viral DNA (11, 220).

Epstein-Barr virus (HHV-4)

Epstein-Barr virus (EBV) is an oncogenic human gammaherpesvirus that preferentially infects B lymphocytes and epithelial cells. EBV infection is associated with infectious mononucleosis and with tumors such as Hodgkin's lymphoma, Burkitt's lymphoma and various nasopharyngeal carcinomas. EBV chromosomal integration was primarily observed in activated B lymphocytes, presumably due to increased permissiveness of replicating DNA to viral integration, but integration in epithelial cells has also been documented. The chromosomal regions where EBV preferentially integrates are typically associated with gene-poor heterochromatin regions containing multiple repeats (141, 220).

Marek's disease virus (GaHV-2)

Marek's disease virus (MDV) is an oncogenic avian alphaherpesvirus that targets T lymphocytes, leading to the development of lymphoid tumors in chickens and turkeys. Similarly to HHV-6, MDV preferentially integrates into telomeric regions, presumably aided by an RNA telomerase subunit that is thought to aid in genomic integration by forming telomeric elongations at the ends of the MDV genome. Chromosomally integrated MDV has long been

recognized in MDV-associated lymphoma cell lines, which suggests a possible correlation between oncogenicity and chromosomal integration in MDV (78, 220, 263).

In addition to the possible link between chromosomal integration and tumor formation in MDV, several integration sites during EBV infection have been shown to overlap with cellular genes, including tumor suppressor genes and proto-oncogenes, raising the possibility of tumorigenesis occurring through changes in cellular gene expression caused by viral genome integration. HHV-6 chromosomal integration can also be of great physiological significance due to the documented ubiquity of integration and vertical transmission of integrated HHV-6 in humans. Individuals carrying integrated HHV-6 in every cell due to vertical transmission can be misdiagnosed as having active HHV-6 infections due to the presence of HHV-6 DNA in blood samples. Chromosomally-integrated HHV-6 could also be spread from such individuals via tissue and cord blood transplants, possibly leading to complications. Furthermore, telomeric integration of viral genomes as occurring in HHV-6 and MDV could destabilize the telomeres and lead to defects such as telomere truncations, premature chromosomal senescence, or the disruption of telomeric regions associated with various rare syndromes such as chromosome 9q subtelomere deletion syndrome (220).

Prevention and Treatment of Herpes Simplex Virus Infection

Apart from obvious preventative measures such as maintaining good hygiene, avoiding contact with infected individuals and using condoms or other barrier methods during sexual activity, the existence and use of an effective vaccine would be the ideal method of preventing HSV infection. Unfortunately, several strategies for HSV vaccine construction have been tested

with limited success, including inactivated virus vaccines, genetically attenuated live virus vaccines, recombinant subunit vaccines (typically expressing gB or gD), genetic immunization using DNA plasmids expressing HSV proteins, and the use of replicating nonpathogenic vectors to express HSV antigens. More recently, encouraging results were published regarding the use of epitope-based lipopeptide vaccines (72). Several vaccine candidates are currently undergoing clinical testing. ACAM529 is a vaccine under development by Sanofi Pasteur that is undergoing phase I clinical trials. ACAM529 is a replication-defective vaccine constructed by deleting the UL5 and UL29 genes of HSV-2, and has been shown to induce a strong immune and protective response in vivo (225). ImmunoVEX-HSV2, developed by BioVex, is a live-attenuated vaccine constructed by deleting a number of HSV-2 genes responsible for evading the host's immune response, specifically the genes responsible for blocking interferon (IFN) responses and for downregulating major histocompatibility complex (MHC) presentation and dendritic cell activation. ImmunoVEX-HSV2 showed promising results in phase I clinical trials, but its current fate is uncertain following Amgen's acquisition of BioVex in 2011 (60, 152, 196).

Acyclovir is the current mainstay of therapeutic anti-HSV treatments, and is particularly effective against HSV-1, HSV-2, VZV and EBV (158). Developed in the late 1970's, acyclovir quickly became a blockbuster drug for Burroughs Wellcome Co., largely due to effective marketing practices and broader public awareness of HSV infection (70, 164, 238). Acyclovir is a nucleoside analogue that mimics deoxyguanosine. In infected cells, acyclovir is selectively phosphorylated by viral thymidine kinase at significantly higher efficiency than by mammalian thymidine kinase, forming acyclovir monophosphate which is subsequently converted to acyclovir diphosphate and acyclovir triphosphate by various cellular enzymes. Acyclovir

triphosphate inhibits HSV DNA polymerase by acting as a substrate and being incorporated into the growing DNA chain instead of deoxyguanosine triphosphate (dGTP), terminating strand elongation and trapping the DNA polymerase when it is unable to add a new deoxynucleoside triphosphate due to lack of a 3' hydroxyl moiety on acyclovir. Penciclovir and ganciclovir (particularly effective against CMV) have a mode of action highly analogous to that of acyclovir, except they are not obligate chain terminators of DNA synthesis due to the presence of a 3' hydroxyl-like moiety. Valacyclovir, Famciclovir and Valganciclovir are prodrugs of acyclovir, penciclovir and ganciclovir, respectively, which have been structurally modified to improve oral bioavailability. These drugs have largely replaced older nucleoside analogues such as vidarabine, idoxuridine and trifluridine due to greater selectivity and lower patient toxicity (97).

Other common drugs that are active against a broad range of herpes viruses and act by preventing transcription by the viral DNA polymerase are cidofovir and foscarnet. Cidofovir is a deoxynucleotide analogue of deoxycytidine monophosphate (dCMP). Inside infected cells, cidofovir is metabolized by cellular enzymes to a diphosphorylated form that inhibits viral DNA polymerase by incorporating into DNA, slowing elongation and eventually resulting in chain termination when two cidofovir residues are incorporated in a row (97, 173). Foscarnet is a non-nucleoside inhibitor of viral DNA polymerase. It is a pyrophosphate analogue that inhibits viral DNA polymerase directly by competitively inhibiting the release of normal pyrophosphate that is required for the DNA polymerase to continue strand elongation. Foscarnet is nephrotoxic and typically used as a last line of defense against viruses exhibiting resistance to the abovementioned nucleoside analogues (97). Fomivirsen is a relatively new drug targeted at treating CMV patients using a different mode of action. It is an antisense drug consisting of a

synthetic 21-member phosphorothioate oligonucleotide that binds to complementary messenger RNA of a major immediate early region protein of CMV, thus blocking translation of the targeted messenger RNA (111). Another drug called docosanol has proven to be effective against both HSV-1 and HSV-2 when applied topically. It consists of a 22-carbon saturated alcohol that is believed to work by inhibiting fusion of the HSV viral envelope with the host cell plasma membrane, preventing viral entry into target cells (157).

Numerous experimental drugs such as viral helicase-primase inhibitors have shown great promise as anti-HSV drugs in a number of studies (166). Imiquimod, a toll-like receptor agonist that causes localized immune system upregulation when applied topically, has documented anti-HSV activity (133). Withaferin A, a compound derived from the plant *Withania somnifera* is also reported to have anti-HSV activity via inhibition of viral DNA polymerase (120). Less conventionally, treatment with narrowband laser light at 1072-nm has been shown to reduce the healing time of HSV-1 cold sores (126). Furthermore, extracts from the plants *Aloe vera*, *Swertia chirata* and *Melissa officinalis*, as well as a vast amount of other plants or plant-derived compounds used in traditional medicine have shown promising anti-HSV activity in preliminary studies (162, 200, 302, 304). Numerous alternative medicines and dietary supplements have also been advocated for the treatment or prevention of HSV-associated disease, but the antiviral activity of most of these compounds (such as zinc, L-lysine, etc.) is yet to be established by rigorous peer-reviewed studies (249).

Structure of the Herpes Virion

A mature HSV-1 virion is composed of four distinct elements. The core consists of the ~152 kb linear double-stranded DNA genome arranged as a toroidal structure and enclosed within a T-16 symmetrical icosahedral capsid composed of 162 capsomeres. The capsid is coated with a layer of viral proteins called the tegument, and is in turn enclosed by a lipid envelope originating from host cellular membranes that is enriched with viral glycoproteins and other membrane-associated proteins. A variety of interactions between the viral proteins that make up the capsid, tegument and envelope are responsible for shepherding the viral particle through the complex assembly process within the host cell, and for maintaining the structural integrity of mature virions. Further interactions between viral envelope proteins and receptors on the cell membranes of host cells are responsible for fusion of the viral envelope with the targeted cell's plasma membrane, thus perpetuating the HSV-1 life cycle by enabling entry of virus particles into uninfected cells (97, 214).

HSV-1 genome

The HSV-1 genome is comprised of two unique regions designated as the unique long (UL) and unique short (US) that are flanked by repeating sequences designated as internal repeats (IR) or terminal repeats (TR) based on their relative position within the HSV-1 genome (Figure 2.1). The repeats surrounding the unique long segment are approximately 9000 bp in length, while the repeats flanking the unique short segment are shorter at approximately 6000-7400 bp. The entire HSV-1 genome is around 152 kbp in length with the exact number varying slightly between different HSV-1 isolates, and is characterized by a relatively high G+C content

of ~68%, a trait shared with the closely related HSV-2. By comparison, the genomic G+C content of varicella-zoster virus, a fellow alphaherpesvirus, is a mere ~46% (19).

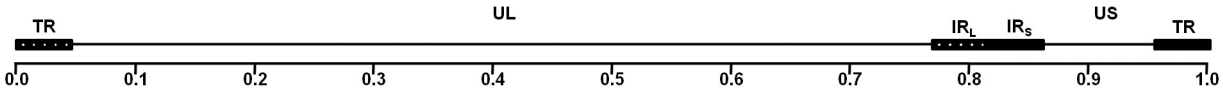

Figure 2.1: Arrangement of the HSV-1 genome. UL: unique long region, US: unique short region, TR: terminal repeats, IR: internal repeats.

The number of open reading frames (ORFs) within the HSV-1 genome is approximately 90, and most ORFs have a polyadenylation site at the 3' end and are flanked at the 5' end by a transcription initiation site with an upstream TATA box and a promoter even further upstream (Figure 2.2). Over 80% of HSV genes do not undergo splicing and many genes share polyadenylation sites. The exact number of encoded proteins is difficult to determine because not all RNAs are ultimately translated to proteins. Other complications include the presence of antisense ORFs, the occasional lack of TATA boxes, and the disregard of predicted transcription termination sites for several genes late in infection resulting in oversized run-on transcripts (74, 97).

Generally speaking, the genes encoded in the unique short segment tend to be less conserved among other alphaherpesviruses than the genes encoded in the unique long segment. Genes that encode enzymes responsible for DNA replication and metabolism show the highest relative conservation and include UL5, UL15, UL30 and UL40. The repeating regions that surround these segments also regulate the expression of several genes, but the repeats are less conserved than the UL and US segments themselves. The most conserved part of the long

repeated region flanking the UL segment encodes the ICP0 gene, while the most conserved area of the short repeated region flanking the US segment is the ICP4 gene, both of which are immediate-early genes that regulate viral and host cell gene expression and are important for reactivation of latent HSV infection (19, 90, 185).

A number of genes also exist that are unique to the two simplexviruses HSV-1 and HSV-2 and are not found in other alphaherpesviruses. A notable example is γ1 34.5, which encodes a 263 aa protein that is a critical determinant of HSV neurovirulence and has been implicated in autophagy inhibition and in the dephosphorylation of the α subunit of eukaryotic translation initiation factor 2 (eIF-2α) in order to prevent shutoff of viral protein synthesis by the action of activated double-stranded RNA-dependent protein kinase (PKR), which are abilities that γ1 34.5 shares to some extent with the similarly simplexvirus-restricted gene product encoded by US11 (128, 191). Another example is the latency associated transcript (LAT), which accumulates in the nuclei of sensory neurons that harbor latent HSV infections. Other genes unique to simplexviruses include the US4 gene encoding glycoprotein G (gG) which is ~1500 bp smaller in HSV-1 compared to HSV-2 and has been used in serologic assays to differentiate between the two viruses, the US5 gene encoding glycoprotein J (gJ) which is involved in preventing cytotoxic T-lymphocyte-induced apoptosis of infected cells, the US8.5 gene which encodes a phosphorylated protein that tends to localize in the nucleoli of infected cells, and the US12 gene encoding ICP47 which interferes with MHC-I mediated antigen presentation in infected cells (19, 112, 113, 148, 179, 323).

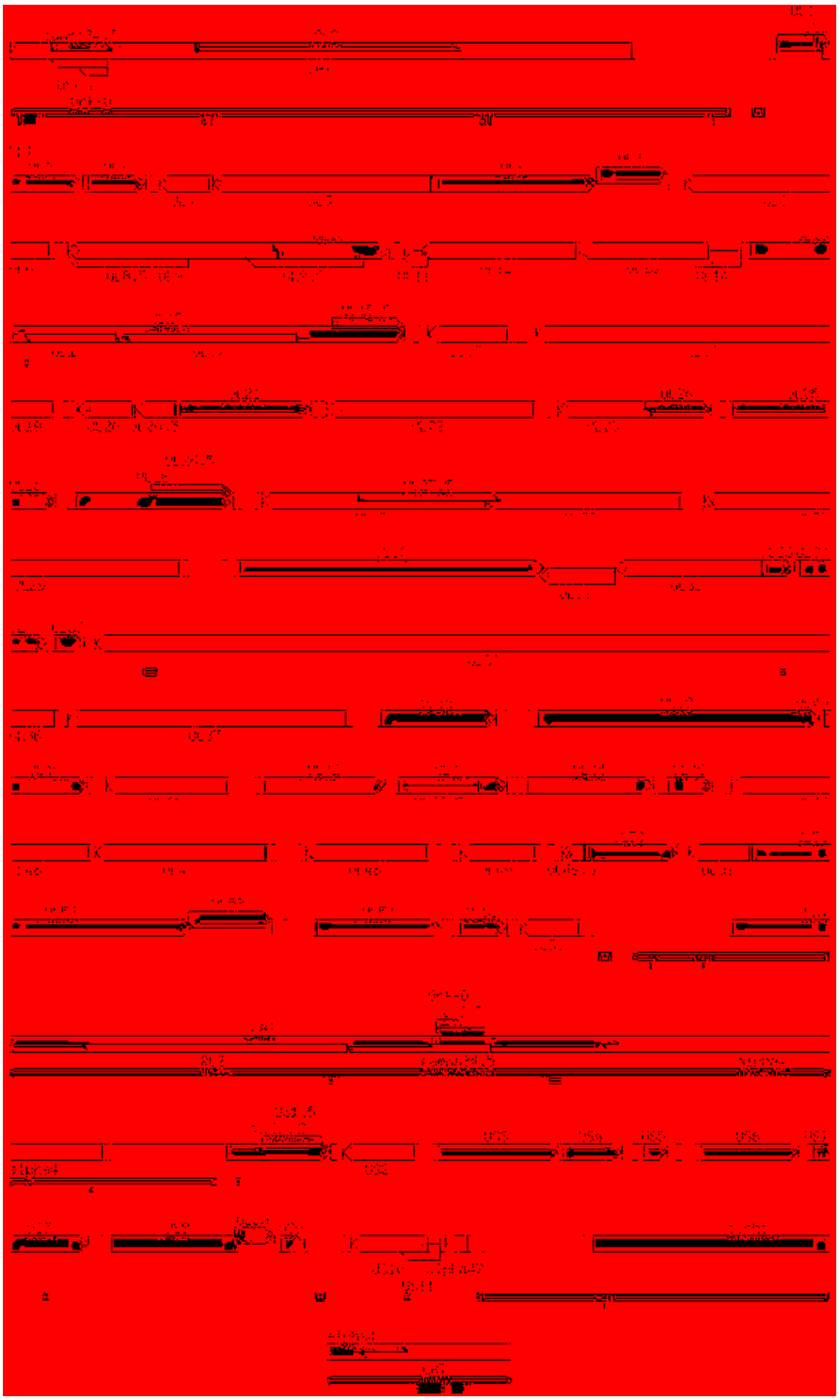

Figure 2.2: Genetic map of HSV-1, showing gene arrangement and orientation. Plus strand genes are colored blue and represented by arrows pointing to the right, minus strand genes are colored teal and represented by arrows pointing to the left, intergenic regions are colored grey, transfer RNA is colored red, ribosomal RNA is colored yellow, and repeats are colored black. Available from the Los Alamos National Laboratory: Human Oral Genomic and Metagenomic Resource at www.oralgen.org.

HSV-encoded proteins often perform multiple functions and exhibit a complex web of protein-protein interactions, making them difficult to characterize. While a number of proteins have been labeled in the literature as "nonessential" for viral replication *in vitro*, this label only applies within the narrow context of a particular study, typically performed on a single cell line. Many of these "nonessential" proteins are essential *in vivo* and play important roles in infecting a variety of host cell types, neurotropism, latency, and evasion of host immune responses (237). Furthermore, most studies are performed using highly passaged viral strains that have adapted to life in laboratory tissue culture. Low-passage clinical isolates typically have more aggressive and neurovirulent phenotypes than laboratory strains and these differences are reflected in their genomes, with numerous amino acid differences detected using high-throughput sequencing (193, 293).

HSV-1 capsid

HSV-1 has a highly uniform and symmetric icosahedral capsid 1250 Å in diameter with a total molecular mass of the capsid shell of approximately 200 MDa. Capsids are composed of five structural proteins: the 149 kD major capsid protein VP5 (UL19), the 12 kD protein VP26 (UL35), the 34 kD protein VP23 (UL18), the 50 kD protein VP19C (UL38), and the portal protein pUL6. Two additional proteins, pUL25 and pUL17, make up the C-capsid specific components in mature DNA-filled capsids (Table 2.2) (35, 324).

Table 2.2: Protein composition of C-capsids. Adapted with permission from [*Current Opinion in Virology* 1(2): 142-149, Herpesvirus capsid assembly: insights from structural analysis]. Copyright (2011) Elsevier.

Gene	Protein	Amino acids	Copies/capsid	Location in capsid
UL19	VP5	1374	955	Hexons; pentons
UL38	VP19C	465	320	Triplexes
UL18	VP23	318	640	Triplexes
UL35	VP26	112	900	Tip of hexon
UL6	pUL6	676	12	Unique vertex
UL17	pUL17	703	60	Near vertices
UL25	pUL25	546	60	Near vertices

Capsids have 162 capsomeres subdivided into 150 hexons, 12 pentons and 320 triplexes that lie on a T-16 symmetrical icosahedral frame (Figure 2.3). Each penton has 5 copies of VP5, while each hexon has 6 copies of VP5 and 6 copies of VP26 (324). The hexons form the edges and faces of the icosahedral capsid (276). The triplexes serve as links between adjacent capsomeres and are composed of two copies of VP23 and one copy of VP19C arranged as a heterotrimer (324). Pentons are located at capsid vertices and are in contact with the surrounding tegument layer in mature virions, specifically interacting with the tegument protein UL36 which forms filamentous structures extending from the pentons (43, 231). Pentons are found on 11 of the 12 capsid vertices, with the 12th vertex occupied by a portal structure similar to ones found in tailed bacteriophages (276). This portal vertex is a hollow cylindrical structure 9 nm in length and 16.5 nm in outside diameter with a ~3 nm diameter axial channel and is composed of 12 copies of pUL6, exhibiting 12-fold rotational symmetry (35). The portal presumably provides the route for the release and packaging of viral DNA, and therefore may also be important for successful docking of the viral capsid at nuclear pore

complexes (NPCs), which are responsible for mediating transport between the cytosol and the nucleus (218). The distal end of the portal vertex has been shown to extend through the tegument layer and possibly lie in contact with the envelope, suggesting that the location of the portal vertex may have an influence on the spatial arrangement of viral tegument and envelope that surrounds the capsid (276).

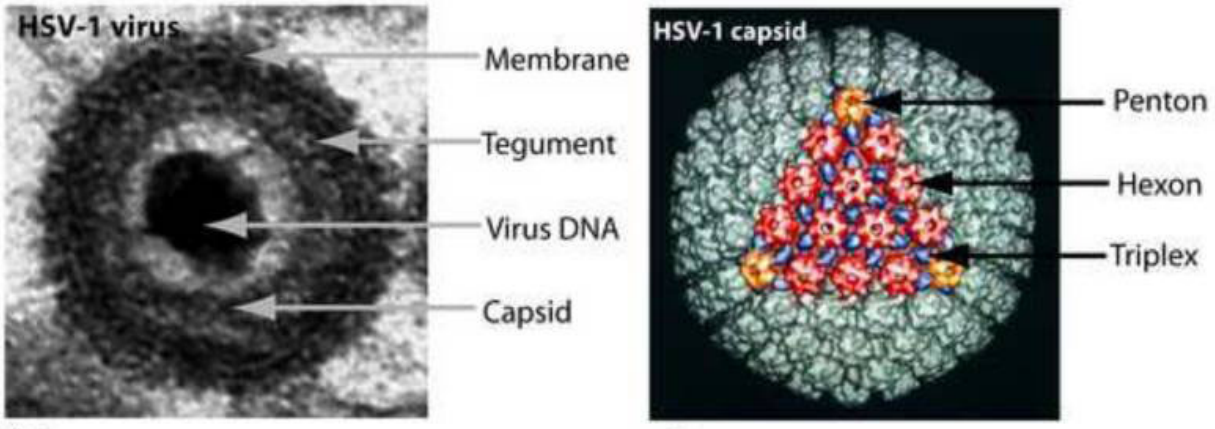

Figure 2.3: Virion and capsid structure. Adapted with permission from [*Current Opinion in Virology* 1(2): 142-149, Herpesvirus capsid assembly: insights from structural analysis]. Copyright (2011) Elsevier.

Three structurally similar but morphologically distinct types of capsids exist in infected cells. The three capsid types differ in the contents of the capsid cavity: A-capsids are the abortive result of failed DNA packaging and are empty, B-capsids contain scaffolding protein but no viral DNA and are likely also dead end byproducts of the capsid assembly process, while C-capsids contain viral DNA and no scaffolding protein and are the capsids ultimately found in mature infectious virions (137, 184). Another distinguishing feature of C-capsids is the presence of rod-shaped structures called C-capsid specific components (CCSCs) near capsid vertices that have been shown to interface with the tegument by binding to the UL36 protein. CCSCs are

attached to viral capsids in the nuclei of infected cells upon completion of DNA packaging, and each CCSC is made up of one copy of pUL25 and one copy of pUL17. Five CCSCs extend radially outward from each capsid vertex, presumably helping to brace neighboring pentons against internal capsid pressure in DNA-loaded C-capsids (Figure 2.4) (35). This pressure occurs because the confined HSV-1 genome is subjected to bending stress in addition to repulsive hydration and electrostatic forces. The internal pressure of HSV-1 capsids is approximately 18 atmospheres, which is roughly comparable to the ~25 atmosphere internal pressure in bacteriophage λ. This high internal capsid pressure likely contributes to successful ejection of the HSV genome into the nucleus upon docking with the nuclear pore complex (25).

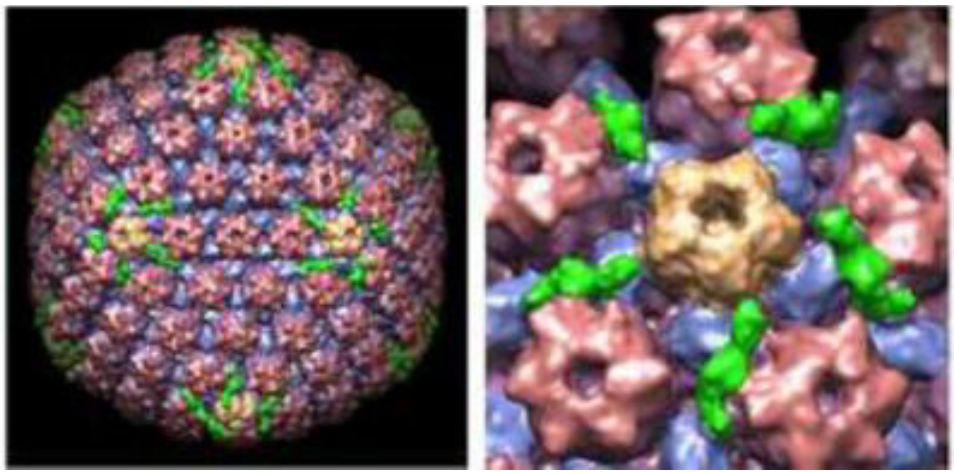

Figure 2.4: Location of CCSCs on the capsid. CCSCs are colored green and visualized on the entire capsid at left and magnified at right. Adapted with permission from [*Current Opinion in Virology* 1(2): 142-149, Herpesvirus capsid assembly: insights from structural analysis]. Copyright (2011) Elsevier.

HSV-1 tegument

The tegument is a proteinaceous matrix that surrounds the capsid and links the capsid to the viral envelope. The tegument plays a variety of roles in addition to being a structural component of the virion. Tegument components are involved in regulation of gene expression, immune modulation, capsid transport within the cell, and both primary and secondary envelopment steps in the viral life cycle (97, 160).

The tegument of HSV-1 is currently believed to consist of 24 proteins, including pUL7, pUL11, pUL13, pUL14, pUL16, pUL21, pUL23, pUL36 (VP1/2), pUL37, pUL41, pUL46 (VP11/12), pUL47 (VP13/14), pUL48 (VP16), pUL49 (VP22), pUL50, pUL51, pUL55, pUS2, pUS3, pUS10, pUS11, ICP34.5, ICP0 and ICP4 (Table 2.3). The most abundant of these proteins are pUL46 (VP11/12), pUL47 (VP13/14), pUL48 (VP16) and pUL49 (VP22). These four proteins are present at more than 1000 copies per virion and together make up the majority of tegument volume (160). By contrast, the tegument proteins pUL36 and pUL37 are present in ~120 copies per virion (232). The only tegument proteins shown to be essential for growth in cell culture are pUL36 (VP1/2), pUL37, pUL48 (VP16) and ICP4 (160). In fact, a mutant PRV lacking all of the hyper-abundant tegument proteins pUL46, pUL47, pUL48 and pUL49 is able to survive in vitro, suggesting a certain amount of redundancy in the structural and functional contributions of most tegument proteins (107).

Table 2.3: Properties of HSV-1 tegument proteins. Adapted with permission from [*Virus Research* 145(2): 173-186, Functional roles of the tegument proteins of herpes simplex virus type 1]. Copyright (2009) Elsevier.

Tegument protein	**Predicted Mwt (kDa)**	**Essential (E) or nonessential (NE) in cell culture**	**Functions**
pUL7	33.1	NE	Regulates mitochondrial function
pUL11	10.5	NE	Secondary envelopment
pUL13	57.2	NE	Protein kinase, tegument dissociation, regulates apoptosis and pUS3, inhibits IFN response
pUL14	23.9	NE	Nuclear import, regulates apoptosis, nuclear targeting of capsids
pUL16	40.4	NE	Secondary envelopment
pUL21	57.6	NE	Secondary envelopment, regulates microtubule assembly
pUL23	41.0	NE	Thymidine kinase, viral DNA replication
pUL36 (VP1/2)	335.9	E	Capsid transport, secondary envelopment, release of viral DNA, deubiquitinating activity
pUL37	120.6	E	Secondary envelopment, regulates viral transcription
pUL41	54.9	NE	Regulates host/viral translation and immune response
pUL46 (VP11/12)	78.2	NE	Secondary envelopment, regulates pUL48-dependent transcription
pUL47 (VP13/14)	73.8	NE	Secondary envelopment, regulates pUL48-dependent transcription
pUL48 (VP16)	54.3	E	Secondary envelopment, regulates viral transcription
pUL49 (VP22)	32.3	NE	Secondary envelopment, regulates microtubule assembly
pUL50	39.1	NE	dUTPase, viral DNA replication
pUL51	25.5	NE	Unknown
pUL55	20.5	NE	Unknown
pUS2	32.5	NE	Unknown
pUS3	52.8	NE	Protein kinase, primary deenvelopment, tegument dissociation, regulates actin assembly
pUS10	34.1	NE	Unknown
pUS11	17.8	NE	Regulates host translation, capsid transport
ICP34.5	26.2	NE	Regulates host translation, viral DNA replication and immune response
ICP0	78.5	NE	Regulates viral transcription
ICP4	132.8	E	Regulates viral transcription

Mounting evidence suggests that the tegument is not an amorphous mass as described in older literature. Cryo-electron tomography of mature HSV-1 virions revealed the tegument to be asymmetrical with a tendency to congregate on one side of the capsid, forming a cap-like structure and resulting in the capsid being pushed off-center and closer to the envelope on the side opposite the bulk of the tegument "cap" (Figure 2.5B). The tegument appears as a dense particulate layer interspersed with filaments ~7 nm in width and up to ~40 nm in length consistent with actin (122). Actin filaments have been detected in several viruses that undergo membrane budding during egress, including in the alphaherpesvirus pseudorabies virus (PRV), suggesting the same may be true in HSV-1 (122, 284, 314). The tegument contains ~40% of the total protein mass of the virion and occupies the majority of internal space within the envelope, accounting for two-thirds of the volume with the capsid making up the remaining one-third (122, 184).

Intriguingly, tegument in cell-associated virus is uniformly distributed around the capsid. The asymmetric distribution of the tegument appears to be the end result of a maturation process that occurs over the course of approximately 40 hours. During this process, the tegument becomes more asymmetric and gains increasing resistance to removal by Triton X-100. This suggests that the tegument becomes more tightly associated with the capsid in mature virions, possibly as an adaptation to preserve structural integrity and infectivity during prolonged exposure to the extracellular environment (232).

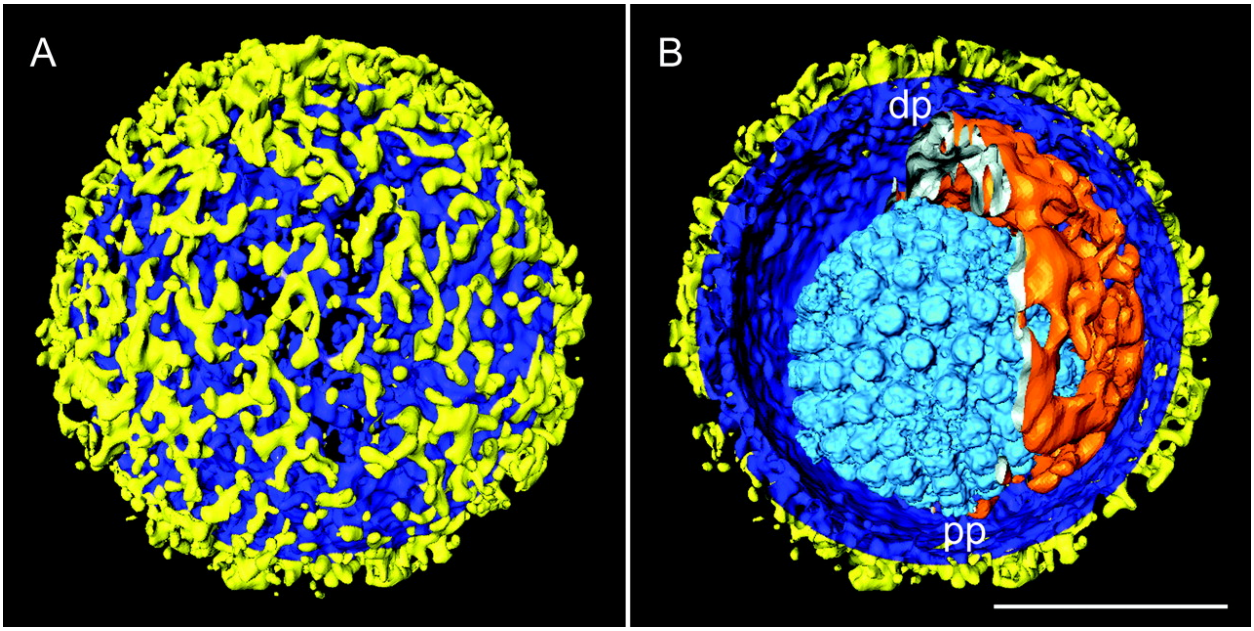

Figure 2.5: Three-dimensional rendering of an HSV-1 virion from cryo-electron tomography. (A) External view, showing distribution of glycoprotein spikes colored in yellow protruding from the membrane colored in blue. (B) Cutaway view, showing the capsid in light blue and the tegument in orange. pp: proximal pole, dp: distal pole, scale bar: 100 nm. Adapted with permission from [*Science* 302(5649): 1396-1398, Three-dimensional structure of herpes simplex virus from cryo-electron tomography]. Copyright (2003) American Association for the Advancement of Science.

Most tegument is accumulated on the capsid in the cytoplasm, particularly during secondary envelopment when partly tegumented capsids bud into tegument-coated regions of trans-Golgi derived vesicles enriched with viral membrane proteins (97, 232). Interactions between tegument proteins and membrane-associated proteins are crucial for secondary envelopment. However, interactions between the capsid and the tegument are not strictly required for the successful assembly and structural integrity of the tegument layer or for subsequent envelopment in the cytoplasm, as evidenced by the existence of "light particles" or L-particles which are composed of enveloped tegument proteins without a capsid (205, 303).

Tegument proteins can be categorized into the inner tegument proteins consisting of pUS3, pUL36 and pUL37 which remain associated with the capsid after entry into the host cell, and the outer tegument proteins which detach from the capsid after entry in order to prime the cell for infection (216, 231). The inner tegument protein pUL36 binds directly to the capsid via documented interactions with the capsid proteins VP5 and pUL25 and serves as the bridge between the capsid and the outer tegument (37, 61). In turn, it has been proposed that the outer tegument protein pUL48 functions as an adaptor that links the capsid-associated inner tegument to the membrane-associated outer tegument (216). Yeast two-hybrid assays have shown that pUL48 physically interacts with pUL36 and with the outer tegument proteins pUL41, pUL46, pUL47 and pUL49 (232, 303). Moreover, virions lacking pUL48 are dispersed throughout the cytoplasm and are unable to localize to TGN membranes or undergo secondary envelopment (221). Interactions between pUL48 and the capsid proteins pUL19 (VP5), pUL38 (VP19C) and pUL35 (VP26) have also been detected, but these interactions are not believed to be biologically relevant because pUL48 is unable to bind to capsids of mutant HSV-1 lacking pUL36 (171). In addition to directly binding to the outer tegument, pUL36 indirectly interacts with the viral envelope via its binding partner pUL37 which has been shown to directly interact with the envelope proteins gK and pUL20 (145, 219). The presence of pUL36 and pUL37 is critical for the successful construction and organization of the tegument as well as for the cytoplasmic envelopment and maturation of HSV-1. The importance of pUL36 and pUL37 is further reinforced by their status as the most highly conserved tegument proteins across all herpesvirus subfamilies (215).

UL36 protein. Weighing in at 335.9 kD, the 3164 aa protein pUL36 (VP1/2) is nearly three times more massive than any other tegument protein (160). pUL36 is phosphorylated on serine residues and is expressed late during infection, only becoming detectable approximately 8 hours post infection (207, 208). pUL36 is present at 100-150 copies per virion and is an important structural component of the tegument (231). Highly conserved among all herpesviruses, pUL36 plays important roles both at the start of infection and during tegumentation and secondary envelopment in the cytoplasm (81, 208, 260).

pUL36 is recruited to cytoplasmic capsids that later colocalize with TGN membranes and membrane proteins at sites of secondary envelopment (273). Although other tegument proteins such as pUL16, pUL46 (VP11/12) and pUL48 (VP16) have been shown to reversibly bind to capsids, pUL36 is thought to be the key link between the capsid and the tegument (171, 210, 216, 226). pUL36 forms filamentous structures attached to capsid pentons that extend outward through the tegument, possibly serving as a flexible scaffold for the direct or indirect binding of other tegument proteins (43, 231). Attachment of pUL36 to the capsid is accomplished by binding to the major capsid protein VP5 and by a noncovalent interaction between the carboxyl terminus of pUL36 and the C-capsid specific component protein pUL25 (37, 61, 232). pUL36 contains two binding sites for the capsid protein pUL25, one located in the region encompassing aa 2430-2893, and the other located in the C-terminal 167 aa portion. The C-terminal 167 residues of pUL36 are needed to retain pUL36 on incoming capsids during entry, but not required for successful targeting of cytoplasmic capsids to sites of viral assembly at TGN membranes as determined by colocalization experiments. However, HSV-1 with a C-terminal truncation of 735 aa from pUL36 encompassing both putative pUL25 binding sites was unable

to recruit pUL36 to capsids in the cytoplasm and did not colocalize with membrane proteins, suggesting that the pUL25 binding site spanning residues 2430-2893 is sufficient for recruitment of pUL36 onto capsids in the cytoplasm and for targeting capsids to glycoprotein-enriched cytoplasmic TGN membranes as determined by immunofluorescence (273). Characterization of an HSV-1 mutant virus lacking the entire pUL36 protein did not show any significant defects in primary envelopment, but secondary envelopment was severely impaired, with numerous unenveloped DNA-filled capsids accumulating in the cytoplasm (81). Further experiments demonstrated that the presence of pUL36 is crucial for successful virus assembly and localization to sites of cytoplasmic envelopment both in vitro and in vivo (160, 273).

pUL36 forms the inner tegument layer along with its binding partner pUL37 (303). The interaction between pUL36 and pUL37 has been confirmed in PRV in addition to HSV-1, and is mediated by residues F593 and E596 of pUL36 (168, 219). The outer tegument is thought to be connected to the inner tegument primarily via interactions between the amino terminus of pUL36 and pUL48 (216, 273). During entry, pUL36 releases the outer tegument from the incoming capsid while remaining tightly bound to the capsid along with the other inner tegument proteins pUL37 and pUS3 (231, 273). In turn, pUL48 has been shown to interact with a variety of other tegument proteins using yeast two-hybrid assays, including pUL41, pUL46, pUL47 and pUL49 (232, 303). The presence of pUL36 is required for the successful incorporation of pUL37 and pUL48 onto capsids (171). Both pUL36 and pUL37 are also present in L-particles, presumably due to interactions with each other and with outer tegument proteins such as pUL48, showing that interactions between pUL36 and the capsid are not required for the incorporation of pUL36 into tegument (292).

The presence of pUL36 and pUL37 is critical for the assembly and release of infectious virions. Viruses unable to express either one of these genes cannot form enveloped virions due to the block in cytoplasmic envelopment and accumulate unenveloped capsids in the cytoplasm. In order to study the role these proteins play during the earliest stages of infection, researchers fused adjacent cells to form syncytia and allow direct capsid transfer to uninfected cells, entirely bypassing the typical entry steps at the plasma membrane. Mutant virus lacking pUL37 was able to infect other nuclei within the syncytia while virus lacking pUL36 was not, thus showing that pUL36 plays an important role at the start of infection in addition to its role in virion assembly while pUL37 is not required for initiation of infection (260). Impairment of pUL36 function during infection by the use of a temperature-sensitive pUL36 mutant (tsB7) results in the abrogation of DNA release from capsids and the accumulation of capsids at nuclear pores (24). After the capsid docks to the nuclear pore complex, cleavage of pUL36 is necessary for the successful release of viral DNA into the host cell nucleus, suggesting that pUL36 may serve to plug the portal vertex after viral DNA is packaged into the capsid (153).

The amino terminal region of pUL36 contains a functional nuclear localization signal and pUL36 has been detected in both the nucleus and cytoplasm of infected cells (1, 208). However, analysis of purified nuclear capsids of HSV-1 has yielded mixed results, with one study detecting and one failing to detect any associated pUL36 (38, 297). Additional experiments with PRV virions isolated from the perinuclear space following primary envelopment failed to recognize any bound pUL36 using immunogold analysis (168). Nuclear pUL36 may be involved in cleavage and packaging of viral DNA and in late gene expression, as determined by experiments with a temperature-sensitive pUL36 mutant virus (2, 24, 51).

The amino terminus of pUL36 also encodes a 420 aa ubiquitin-specific cysteine protease with the active site located at Cys65. This domain may be involved in the deubiquitination of viral proteins to avoid proteasomal degradation, and possibly in cytoplasmic envelopment, due to ubiquitination being implicated in membrane trafficking and multivesicular body formation (156). The ubiquitin-specific protease domain of pUL36 has also been shown to function in evasion of interferon-mediated innate immunity by deubiquitinating TRAF3, resulting in downregulation of beta interferon (IFN-β) production (306). The deuibiquitinating activity of pUL36 is highly conserved, as evidenced by the presence of equivalent deuibiquitinating activity in pUL36 homologues encoded by the betaherpesvirus murine cytomegalovirus (MCMV) and the gammaherpesvirus Epstein-Barr virus (EBV), both of which retain the conserved catalytic cystein residue at the active site despite having less than 15% sequence identity with HSV-1. Portions of pUL36 corresponding to N-terminal 1-285 aa of MCMV and 1-205 aa of EBV also displayed deubiquitinating activity (274). Abolishing the deubiquitinating activity of pUL36 by mutating the catalytic cysteine residue at the active site to a serine in PRV did not abrogate binding to pUL37 but resulted in ~25 fold viral titer reduction and ~40% plaque size reduction compared to wild type virus, with increased accumulation of unenveloped capsids in the cytoplasm and reduced neurovirulence in mice (30). Another study has provided evidence for autocatalytic activity in the deubiquitinating domain of pUL36, suggesting that it can act on itself to influence the stability of the pUL36 protein. (29).

Viral capsids depend on the action of microtubule motors for both retrograde and anterograde transport within the host cell. Retrograde transport toward the cell body is mediated by the inbound motor dynein and its cofactor dynactin, while anterograde transport

toward the cell periphery is mediated by the outbound motor kinesin. Using differentially tegumented capsids in a mammalian cell-free system designed to replicate an intact microtubule network with the associated motors, researchers have shown that capsids with exposed inner tegument proteins such as pUS3, pUL36 and pUL37 were able to recruit dynein and its cofactor dynactin, as well as both kinesin-1 and kinesin-2. In contrast, capsids lacking either just the inner tegument or all tegument were not able to recruit microtubule motors (253). Another study using a microchamber system to reconstitute viral trafficking along microtubules in vitro showed that pUL36 is required for successful anterograde capsid transport along microtubules (279).

UL37 protein. The UL37 gene of HSV-1 encodes a large 1123 aa (120.6 kD) highly conserved tegument protein that is essential for viral growth in cell culture and is crucial for viral assembly and secondary envelopment in the cytoplasm (37, 160, 215). The UL37 protein is expressed late in the infection cycle and is stably phosphorylated soon after translation of the UL37 gene. Phosphorylation of pUL37 is likely performed by a cellular kinase and is not dependent on the presence of any known HSV-1 binding partner because pUL37 expressed by a recombinant vaccinia virus is also phosphorylated (5). The quantity of pUL37 loaded onto mature virions is tightly regulated, since overexpression of pUL37 does not increase the amount of pUL37 detected in virions (203).

The UL37 protein encodes a variety of functional domains (Figure 2.6). Co-immunoprecipitation experiments revealed that pUL37 domains spanning residues 1-300 and 568-1123 are involved in self-association in the absence of its binding partner pUL36 (37). The

amino terminus contains an alanine-rich region (ARR) spanning residues 44-80, a leucine zipper motif covering residues 203-224 and a leucine-rich nuclear export signal (NES) encompassing residues 263-272 (37, 307). The carboxyl terminus contains a domain spanning residues 1099-1104 involved in binding TNF receptor-associated factor 6 (TRAF6) to activate NF-kappaB signaling (37, 187). The C-terminal 578-899 aa of pUL37 can interact with a spectraplakin protein called dystonin/BPAG1 known as a cytoskeletal cross-linker that is involved in microtubule stabilization and transport. Viral replication and cytoplasmic capsid mobility during egress from infected cells is impaired in dystonin-depleted cells, suggesting that pUL37 may play a role in capsid trafficking along microtubules (246). The C-terminus of pUL37 is also responsible for binding to pUL36 (37, 161). Scanning alanine mutagenesis of pUL37 revealed that residue D631 of pUL37 mediates binding to pUL36. Knocking out this residue resulted in significantly decreased ability of the virus to replicate, with mutant viral titers approximately 2 logs lower than those of wild type virus (161). Trans-complementation experiments using a plasmid encoding the C-terminal portion of pUL37 spanning residues 568-1123 that includes the putative pUL36 interaction site showed that the plasmid is sufficient to partially rescue a virus unable to express pUL37 (37).

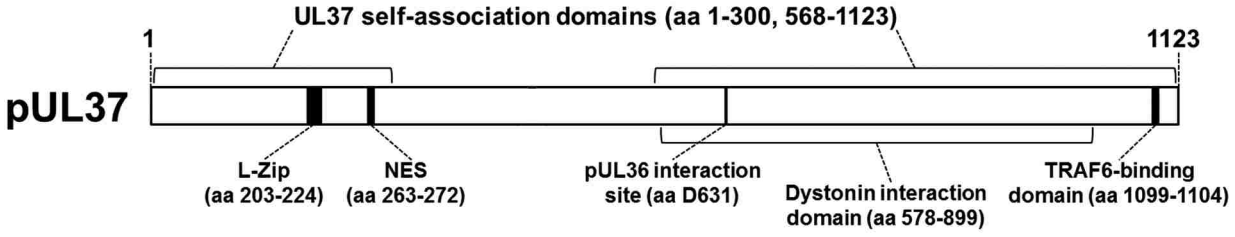

Figure 2.6: Functional domains of the UL37 protein.

The function of the central portion of pUL37 spanning aa 301-567 is not well defined. A mutant HSV-1 with a 12 aa protein C epitope tag inserted in-frame after residue 480 of pUL37 exhibited a severe defect in cytoplasmic envelopment, but was partially complemented for replication and spread when grown on cells expressing pUL20. Two-way interactions between pUL37 and the membrane proteins pUL20 and gK were confirmed using co-immunoprecipitation and proximity ligation assays, thus providing the first direct link between the inner tegument and the envelope, although the exact locations of the relevant binding sites remain unknown (145). pUL20 and gK are known to be among the most important mediators of secondary envelopment, and their interactions with pUL37 may serve to facilitate the process of cytoplasmic virion envelopment (52, 145).

pUL37 forms a complex with pUL36, and this interaction is conserved across all three subfamilies of the Herpesviridae. Interactions between proteins homologous to pUL36 and pUL37 have been documented in the alphaherpesviruses HSV-1, PRV and VZV, in the betaherpesvirus HCMV and in the gammaherpesvirus KSHV (26, 168, 181, 219, 268, 300, 303). The presence of pUL36 is necessary for incorporation of pUL37 onto capsids (171). pUL37 is likely added to capsids after pUL36, since pUL36 is still detected on both HSV-1 and PRV capsids in mutants lacking pUL37 (80, 170). Along with pUL36, pUL37 may be involved in the organization of tegument structure. pUL37 attaches to capsid-bound pUL36 at the vertices, together forming thin flexible strands ranging from 15 to 70 nm in length that extend throughout the tegument, possibly providing a scaffold for the rest of the tegument (231). Deletion of either pUL36 or pUL37 prevents the acquisition of appreciable amounts of

tegument in the cytoplasm and blocks cytoplasmic envelopment in HSV-1, resulting in cytoplasmic accumulation of unenveloped capsids (80, 81, 260).

The role of the interaction between pUL36 and pUL37 in secondary envelopment is still unclear. A PRV mutant virus engineered to remove the pUL36 domain responsible for interacting with pUL37 exhibits impaired cytoplasmic envelopment (109). The lack of a complete block in secondary envelopment after disruption of pUL36-pUL37 binding may be due to decreased importance of pUL37 for secondary envelopment in PRV. Complete deletion of the PRV version of pUL37 disrupts cytoplasmic envelopment to a lesser extent than pUL37 deletion in HSV-1 (170, 260). The phenotype of HSV-1 pUL37-null mutants is characterized by unenveloped capsids distributed throughout the cytoplasm that do not colocalize with TGN vesicles, suggesting that pUL37 may play a role in directing capsids to secondary envelopment sites (245). Trafficking of pUL37 to putative sites of secondary envelopment at TGN membranes in HSV-1 infected cells is dependent on the presence of pUL36, and occurs even in the absence of capsids (79).

Interaction between pUL37 and the capsid proteins pUL35 and pUL38 has been documented by yeast two-hybrid screens in HSV-1 (181). However, pUL37 is only recruited to capsids in the presence of pUL36, implying that direct interactions between the capsid and pUL37 are not an important determinant for the accretion of pUL37 onto capsids (271). Both pUL36 and pUL37 are also present in L-particles, further indicating that interactions with capsid proteins are not strictly required for their incorporation into tegument (204, 292). Successful incorporation of either pUL36 or pUL37 into L-particles is dependent on the presence of the

other, reflecting their close association (260). Integration of pUL36 and pUL37 into L-particles is probably mediated by interactions with outer tegument proteins (106). Interactions between pUL36 and pUL48, as well as between pUL37 and pUL46, have been documented by yeast two-hybrid screening (181, 303). Surprisingly, the presence of pUL36 or pUL37 is not required to form L-particles, suggesting that they are not needed for outer tegument assembly or for the membrane invagination process during budding at sites of secondary envelopment (109, 170, 260). However, L-particles lack capsids and are not infectious, with a tegument structure dissimilar to that of normal virions. pUL36 and pUL37 likely influence tegument organization and act as a link between the capsid and the outer tegument and envelope, thus performing a crucial role during secondary envelopment (145, 170, 251, 260).

Although early work with a pUL37 deletion mutant hinted at a role for pUL37 in capsid egress from the nucleus, follow-up research suggested that the observed overabundance of capsids in nuclear fractions was due to large clusters of cytoplasmic capsids precipitating with the nuclei (80, 260). pUL37 has been detected on purified intranuclear capsids along with pUL36, but neither one is required for translocation of capsids from the nucleus to the cytoplasm in both HSV-1 and PRV (38, 109, 183, 260). Transport of pUL37 to the nucleus in infected cells is possibly due to cytoplasmic interaction with the DNA-binding protein ICP8 (280, 281). Modulation of NF-kappaB signaling by binding to TRAF6 and interaction with known DNA-binding proteins such as ICP8 suggest a potential role for pUL37 in transcriptional regulation of immediate early genes (6, 187, 280).

In vitro experiments have shown that inner tegument proteins such as pUL37 and its binding partner pUL36 are required for the successful recruitment of microtubule motors such as kinesin, dynein, and the dynein cofactor dynactin onto capsids and for the transport of viral capsids along microtubules (253, 273). pUL37 is added to capsids in the cytoplasm along with pUL36, and the presence of both is required for efficient transport of viral capsids to sites of secondary envelopment at the TGN (271). pUL36 and pUL37 remain bound to capsids during infection and entry into host cells and promote nucleus-directed capsid mobility along microtubules, likely by binding to dynein (4, 63, 313). However, pUL37 deletion mutants in both HSV-1 and PRV show that it is not strictly required for localization of incoming capsids to the nucleus (174, 260).

The recently published crystal structure of the amino terminal half of pUL37 in the HSV-related alphaherpesvirus PRV provides a compelling structural argument for a role of pUL37 in viral trafficking. The N-terminus of PRV pUL37 encompassing residues 1 to 479 is an elongated structure rich in alpha helices that is structurally similar to members of the complexes associated with tethering containing helical rods (CATCHR) family of eukaryotic cellular multisubunit tethering complexes (MTCs) involved in vesicular transport (Figure 2.7). This similarity is presumably due to convergent evolution because sequence identity between the pUL37 N-terminus and MTC subunits is less than 10%. Structural stability of the pUL37 N-terminus in PRV is maintained by the internally located conserved residue W477, as demonstrated by the construction of a shorter N-terminal construct spanning residues 1-476.

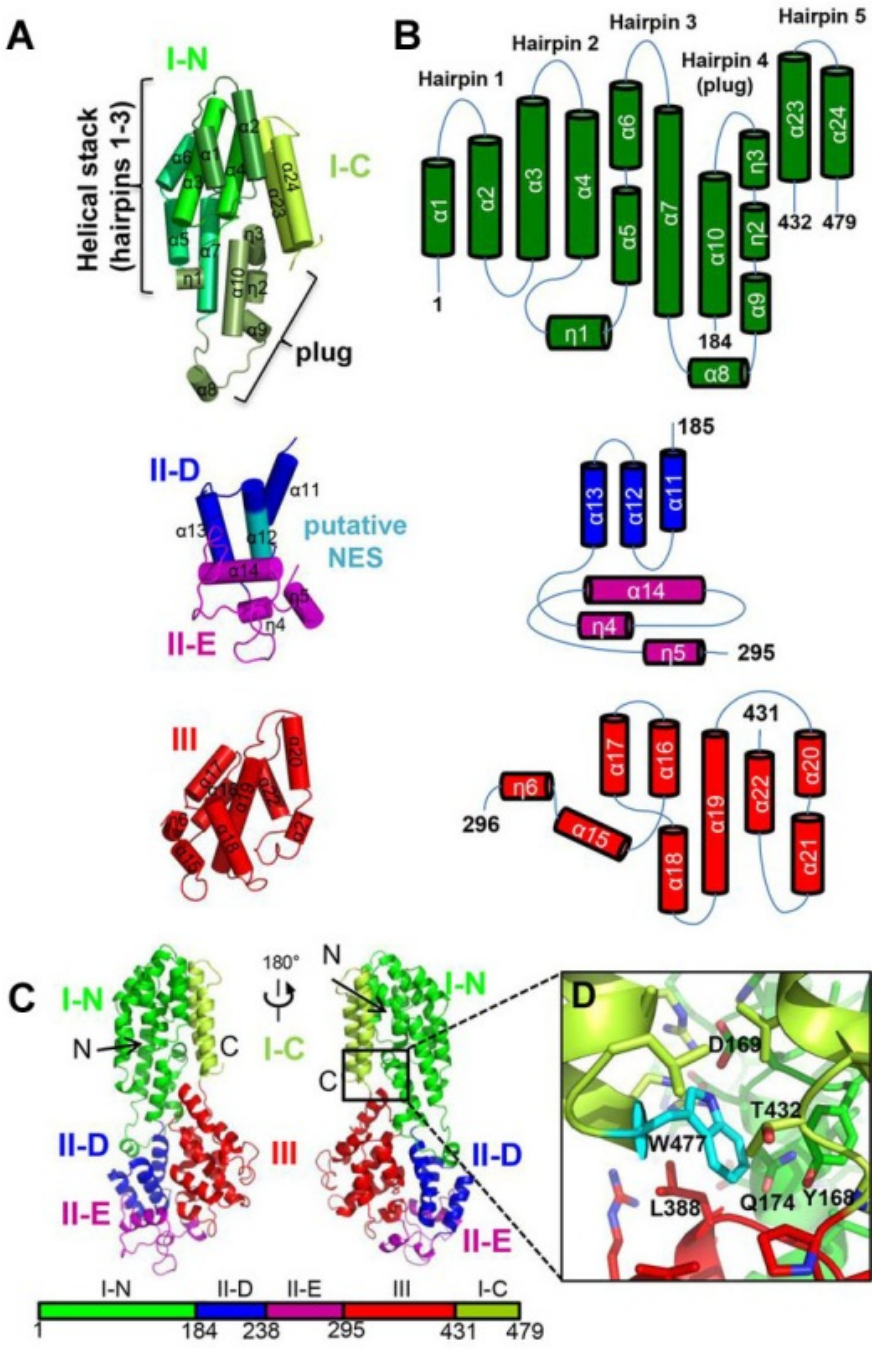

Figure 2.7: Structure and organization of the PRV N-terminal 1-479 aa of pUL37. (A) Secondary structure of individual domains. Domain I components are shown in different shades of green. Domain II subdomains are shown in blue and purple. The nuclear export signal (NES) portion of domain II is shown in teal. Domain III is shown in red. (B) Organization of helices within each domain. (C) Crystal structure of the entire PRV pUL37 N-terminus encompassing residues 1-479. (D) Magnified view of the region surrounding residue W477. Adapted with permission from [*Journal of Virology* 88(10): 5462-5473, Crystal structure of the herpesvirus inner tegument protein UL37 supports its essential role in control of viral trafficking]. Copyright (2014) American Society for Microbiology.

Mutagenesis of conserved surface-exposed regions selected by evolutionary trace analysis (ETA) in conjunction with the 3D structural data revealed a cluster of residues important for spread between infected cells. Mutation of the domain III residues Q324, D362, R365, H421, H425 to alanine impaired cell-to-cell spread without reducing the rate of viral production, suggesting a role for pUL37 in transporting mature virions to cell junctions for infection of adjacent cells (251).

The multifunctional UL37 protein also undoubtedly plays a variety of as yet undiscovered roles in viral replication by interacting with a plethora of host cell proteins. Yeast two-hybrid screening of human brain cDNA libraries revealed potential interaction between pUL37 and a number of host cell proteins, including the Iκβ kinase complex associated protein (IKAP), the 20S proteasome subunit beta type 5 (PSMB5), the autophagy regulator Beclin1 (BECN1), the serine threonine kinase thousand and one kinase 3 (TAOK3), the NIF3L1 binding protein THO complex 7 homolog (THOC7), the nuclear RNA helicase DEAD box protein 5 (DDX5), the molecular chaperonin containing TCP1 subunit 3 (CCT3), the mitochondrial protein asparaginyl-tRNA synthetase 2 (NARS2), and the transcriptional repressors GTPase activating Rap/RanGAP domain-like 1 (GARNL1) and zinc finger protein 350 (ZNF350) (159).

HSV-1 envelope

The envelope contains at least 12 glycoproteins in addition to several other membrane-associated viral proteins. The glycoproteins include pUL1 (gL), pUL10 (gM), pUL22 (gH), pUL27 (gB), pUL44 (gC), pUL49.5 (gN), pUL53 (gK), pUS4 (gG), pUS5 (gJ), pUS6 (gD), pUS7 (gI) and pUS8 (gE). The non-glycosylated membrane proteins include pUL20, pUL24, pUL43, pUL45, pUL56

and pUS9 (42, 264). While pUL34 is also technically a membrane-associated protein, it is not present on the envelope of extracellular virions. Instead, pUL34 localizes to the inner nuclear membrane and acts in concert with pUL31 to mediate primary envelopment (264). The various envelope proteins play important roles in both virus entry and egress from infected cells. During entry, membrane proteins are involved in binding to cellular receptors and in the initiation and regulation of membrane fusion, while during egress they play crucial roles during envelopment and infectious virus production via interactions with the tegument (97).

Unlike the capsid, both the tegument and the envelope are pleiomorphic structures. The envelope is a host-derived lipid bilayer ~5 nm thick with an average diameter of ~186 nm that increases to ~225 nm when the protruding viral glycoprotein spikes are included. Cryo-electron tomography of mature enveloped virions reveals 600-750 nonrandomly distributed glycoprotein spikes of highly variable morphology embedded in the viral envelope (Figure 2.5A). The tegument appears to be in contact with the internal portions of adjacent envelope glycoprotein spikes and may serve to anchor the glycoproteins, as evidenced by increased spike cluster density in the membrane overlying the thickened "cap" portion of the asymmetrical tegument (122). Accordingly, a number of direct interactions between envelope glycoproteins and outer tegument proteins have been documented in HSV-1. The tegument protein pUL48 (VP16) binds to the cytoplasmic tails of gB, gD and gH (119, 154, 325). Co-immunoprecipitation experiments indicate that the tegument proteins pUL11 and pUL49 (VP22) interact with the cytoplasmic portions of gD and gE (49, 93, 239). The PRV version of pUL49 (VP22) has been shown to interact with the carboxyl terminal domains of gE/gI and gM (108). Interaction between pUL49 and the cytoplasmic portion of the membrane protein pUS9 has also been

detected (181). Most recently, the membrane proteins gK and pUL20 have been reported to bind to pUL37, marking the first documented interaction between envelope proteins and the inner tegument (145).

Glycoprotein B. Glycoprotein B (gB) is a 904 residue glycosylated transmembrane protein encoded by the UL27 gene of HSV-1. gB is the most conserved component of the viral entry and fusion machinery composed of gB, gD and the gH/gL heterodimer. A crystal structure of the gB ectodomain revealed a trimeric structure strikingly similar to vesicular stomatitis virus glycoprotein Gr, a known fusion protein. The gB structure reflects aspects of both class I and class II fusion proteins and suggests that fusogenic activity of gB is mediated by a reversible conformational change between an "open" and "closed" orientation of the trimeric gB complex (Figure 2.8) (130). This conformational rearrangement to initiate fusion is presumably mediated by interactions with gD and the gH/gL heterodimer, but may also be assisted by the documented direct binding of gB to cellular receptors such as the paired immunoglobulin-like type-2 receptor alpha (PILR-alpha), myelin-associated glycoprotein (MAG) and non-muscle myosin heavy chain IIA (NMHC-IIA) (12, 56, 62, 272, 291). gB-mediated fusion is further influenced by direct interactions with the membrane proteins pUL20 and gK (54, 102, 213). Recently, deletion of gM and the membrane-associated tegument protein pUL11 has been shown to affect membrane fusion events, presumably due to interactions with members of the viral fusion complex. Indeed, interaction between gM and pUL20 was detected by two-way co-immunoprecipitation experiments (163).

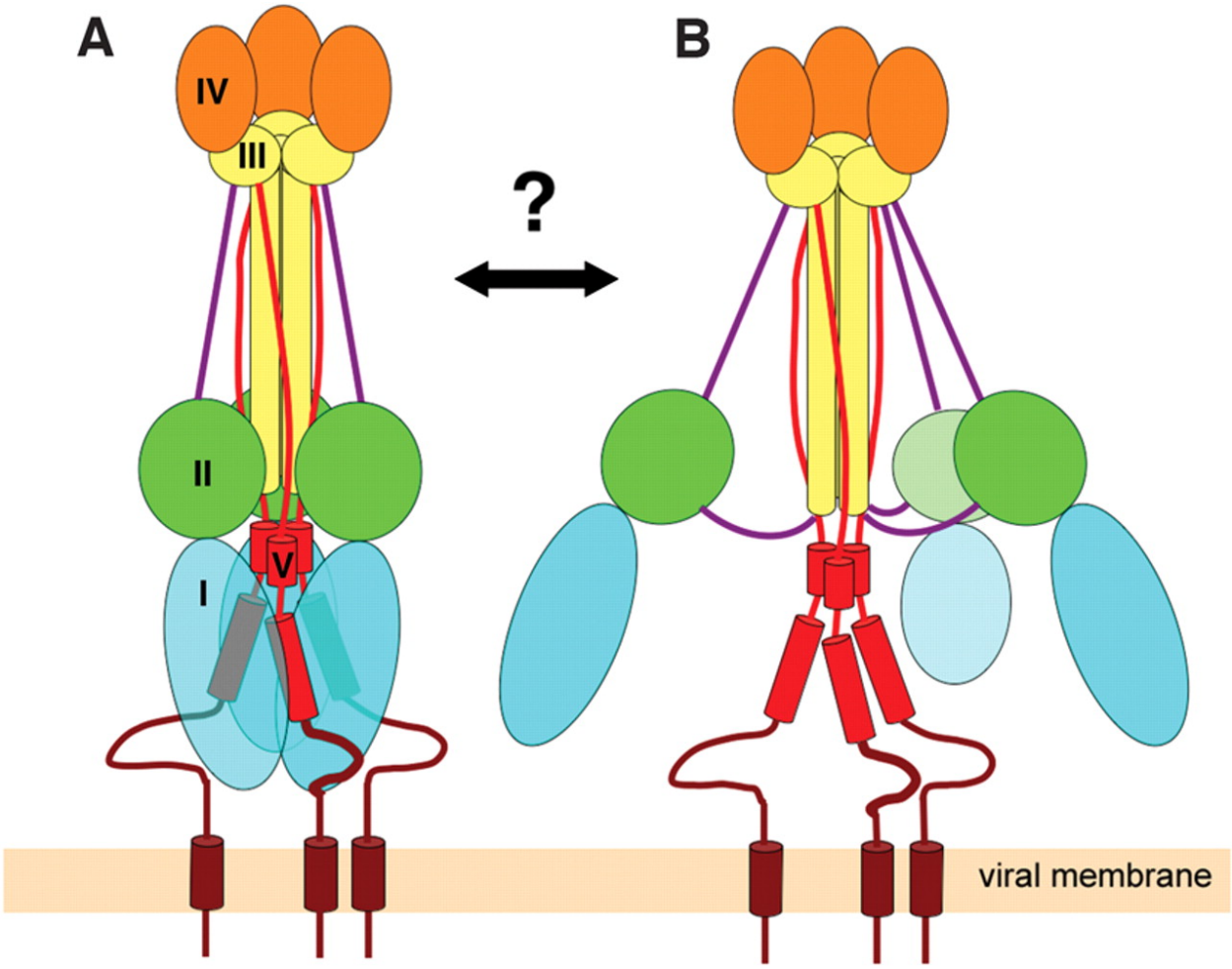

Figure 2.8: Schematic model of gB illustrating predicted conformational change between a "closed" (A) and "open" (B) state. Domains of gB are shown in different colors. Adapted with permission from [*Science* 313(5784): 217-220, Crystal structure of glycoprotein B from herpes simplex virus 1]. Copyright (2006) American Association for the Advancement of Science.

Glycoprotein K. Glycoprotein K is a 338 residue glycosylated transmembrane protein encoded by the UL53 gene of HSV-1 and is conserved in all alphaherpesviruses. gK contains four hydrophobic transmembrane domains, a 30 residue signal sequence and two N-linked glycosylation sites at residues 48 and 58 (Figure 2.9) (254). The presence of gK is critically important for successful cytoplasmic virion envelopment and infectious virus production. gK deletion mutants are unable to egress and accumulate in the cytoplasm as unenveloped virions

(101, 142, 143, 147, 254). gK exists as a complex with the membrane protein pUL20 in infected cells. Studies indicate that transport of gK to the plasma membrane and to sites of secondary envelopment at TGN membranes is mediated by the presence of pUL20 (100, 103, 212).

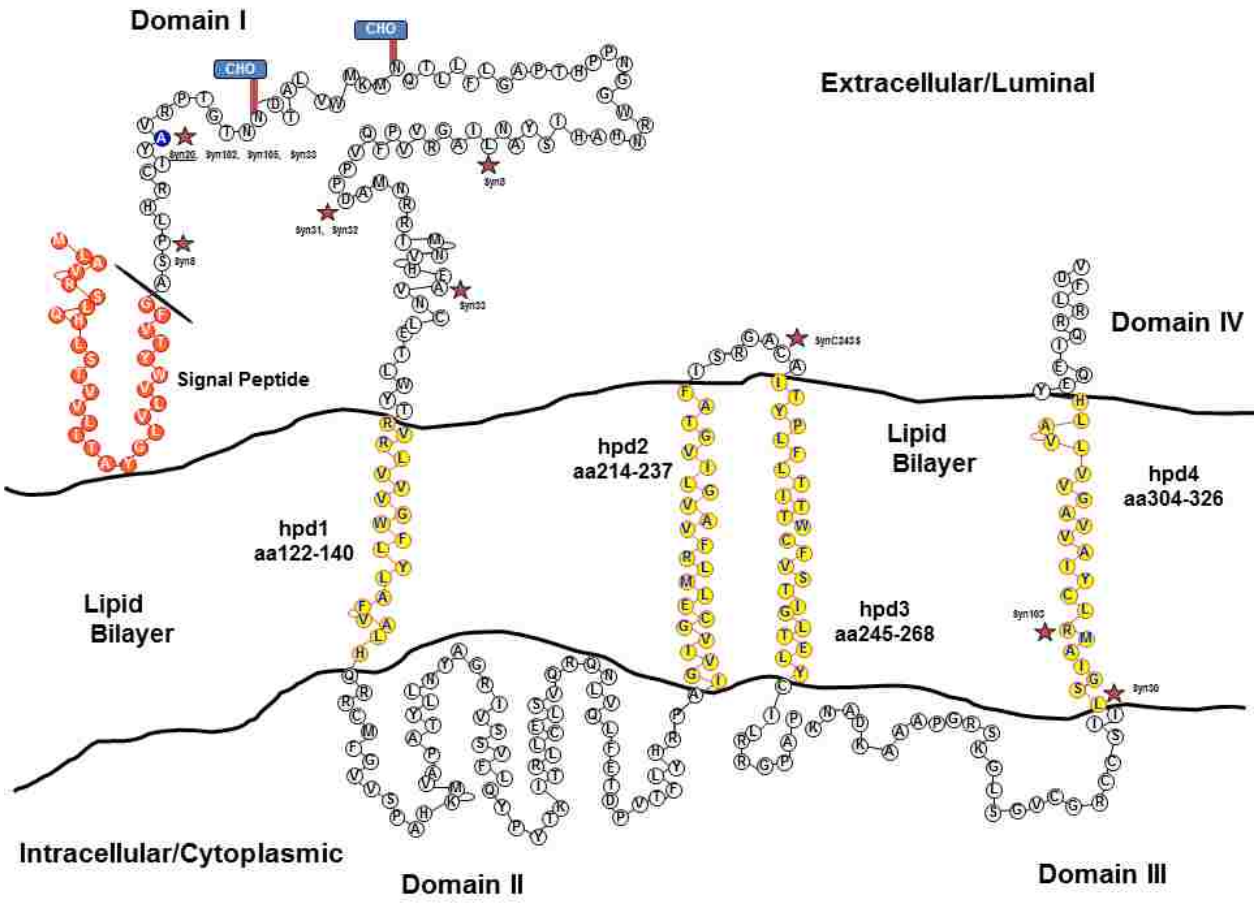

Figure 2.9: Schematic illustration of glycoprotein K. The four transmembrane regions, the 30 aa N-terminal signal peptide, the two N-linked glycosylation sites, and the locations of known syncytial mutations are shown.

Studies using a variety of deletion, truncation and syncytial mutants of gK have illustrated the importance of gK in mediating virus-induced cell fusion (16, 17, 53, 142, 213). A number of gK mutations, primarily within the amino terminus of gK, have been identified that

promote formation of syncytia in infected cell monolayers (252, 269). The recent detection of a direct interaction between the gK amino terminus and the extracellular portion of the key fusion protein gB suggests that the prevalence of syncytial mutations in the amino terminus of gK is not coincidental (54).

Overexpression of gK was correlated with an increase in the number of enveloped virions in the perinuclear space, suggesting a role in gB-mediated de-envelopment at the outer nuclear membrane (143). However, simultaneous deletion of both gB and gK as well as both gB and pUL20 did not prevent capsids from accumulating in the cytoplasm, indicating that pUL20, gB and gK do not function in a redundant manner for de-envelopment (213).

Virus lacking either the entire gK or merely the amino terminus of gK enters cells at a slower rate than wild-type virus (104, 146). The amino terminus of gK appears to be required for efficient virus entry into cells via the gB-associated paired immunoglobulin-like type-2 receptor alpha (PILRα) (55). Virus lacking gK is unable to enter neurons via their axonal termini, illustrating the further importance of gK as a determinant of neurovirulence (73, 222). gK may also play a role in immunomodulation due to the presence of a T cell epitope within its amino terminal signal domain (241).

UL20 protein. The UL20 protein is a 222 residue nonglycosylated hydrophobic transmembrane protein that is conserved in all alphaherpesviruses (Figure 2.10) (194). pUL20 is an important mediator of virus-induced cell fusion and cytoplasmic virion envelopment, with pUL20 deletion mutants characterized by accumulation of unenveloped virions in the cytoplasm

(17, 21, 102, 211). Virus-induced cell fusion was also prevented in recombinant viruses unable to express pUL20 that contained syncytial mutations in gB or gK (102).

pUL20 forms a complex with gK, and expression of pUL20 is important for intracellular transport of gK to the cell surface and to TGN membranes (100, 212). Both pUL20 and gK directly interact with gB and function as regulators of gB-mediated fusion (54, 102, 149, 213). Interaction between pUL20 and gM has also been reported (163). The pUL20 domains involved in virus-induced cell fusion and cytoplasmic virion envelopment are functionally independent, as evidenced by pUL20 mutants which abrogate cytoplasmic envelopment without inhibiting virus-induced cell fusion or transport of pUL20 and gK to the cell surface and to TGN membranes (211, 212).

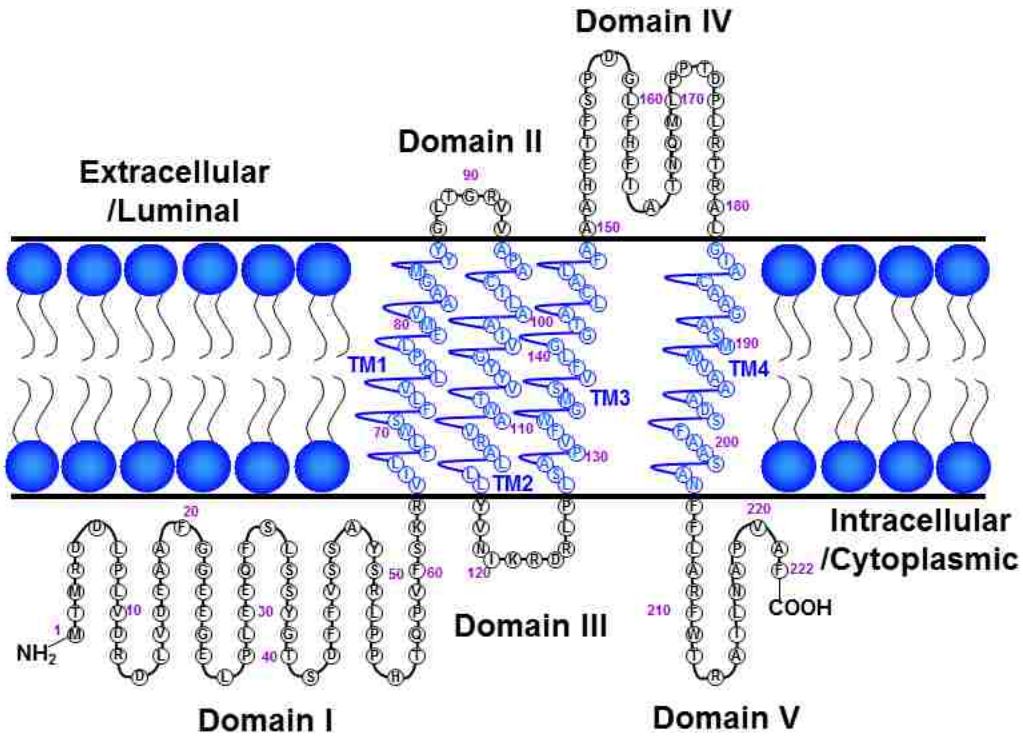

Figure 2.10: Schematic illustration of the UL20 protein. The four transmembrane regions and five predicted domains are shown.

Life Cycle of Herpes Simplex Virus

HSV infects the host at mucocutaneous surfaces and replicates before invading local sensory nerves and propagating via neurons to sensory ganglia, specifically the trigeminal ganglia for ocular and oral herpes and sacral ganglia for genital herpes, where it establishes lifelong latency. In order to initiate infection, the DNA-containing capsid is deposited in the cytoplasm of a targeted cell either by fusion of the viral envelope with the plasma membrane or by endocytosis followed by fusion of the viral envelope with the membrane of the endocytic vesicle. This process is mediated by the action of a subset of viral envelope glycoproteins that bind to a variety of cellular receptors. The deenveloped virion is then transported to nuclear pore complexes to release viral DNA into the nucleus, leading to either the start of another viral replication cycle or the establishment of latency. New capsids are assembled in the nucleus and loaded with viral DNA. Capsids travel to the cytoplasm by moving across the nuclear membrane in a process called primary envelopment. In the cytoplasm, capsids acquire tegument and undergo secondary envelopment by budding into TGN-derived vesicles enriched with viral membrane proteins in a process that is thought to be largely mediated by interactions between tegument proteins and the cytoplasmic tails of TGN-embedded viral glycoproteins. Enveloped virions are then transported inside the vesicles to the plasma membrane for release into the extracellular environment (97, 303).

Virus entry

In order to initiate infection, virus must first attach to the host cell membrane. HSV attachment is mediated primarily by binding of the envelope glycoprotein gC to

glycosaminoglycans (GAGs) on the cell surface, although gB has also been implicated in attachment (42, 131, 132). The dominant cell surface receptors involved in viral attachment are the widely expressed heparan sulfate and chondroitin sulfate GAGs, which are beleived to bind the amino terminal portion of gC (295). Surprisingly, deletion of gC does not impair viral attachment in every tested HSV-1 strain, suggesting that other viral proteins such as gB are involved (118). Deletion of heparan sulfate and chondroitin sulfate is significantly more deleterious to viral infectivity, but does not prevent infection from happening (121). Viral attachment is reversible and functions separately from fusion, as evidenced by the retention of unfused viral envelopes and subsequent infectivity in detached virus(42).

Entry of HSV into cells typically occurs by fusion of the viral envelope to the host cell plasma membrane, although attached virus may also enter via endocytosis and fusion of the viral envelope to the surrounding endocytic vesicle (235). Entry is primarily mediated by binding of the gD ectodomain to cellular receptors, initiating a conformational change which triggers fusion by activating the gB, gH/gL fusion complex (59). In addition, virus entry is facilitated by binding of gB to cellular receptors including the paired immunoglobulin-like type-2 receptor alpha (PILR-α), myelin-associated glycoprotein (MAG) and non-muscle myosin heavy chain IIA (NMHC-IIA) (12, 56, 272, 291).

The transmembrane and cytoplasmic domains of gD serve to anchor gD in the viral envelope and do not appear to play a role in entry (36). gD binds to three families of cellular entry receptors: nectins, tumor necrosis factor (TNF) receptors, and modified heparan sulfate receptors (42). Nectins are a group of immunoglobulin-type intercellular adhesion molecules

that can dimerize with other nectins and are commonly found at cadherin-based cell junctions (294). Nectins are widely expressed in human tissues and are divided into four subtypes (41). Nectin 1 is utilized for HSV-1 entry, while HSV-2 is able to exploit both nectin 1 and nectin 2 (42). TNF receptors are involved in signal transduction pathways regulating cell differentiation, proliferation and apoptosis. gD binds to a TNF receptor dubbed herpesvirus entry mediator (HVEM) that is primarily expressed on cells of the immune system such as B and T lymphocytes and monocytes, as well as in epithelial cells and a number of other tissues, excluding neurons (176, 287). 3-O-sulfated heparan sulfate is the third type of gD receptor and is expressed in neurons in addition to a variety of other tissues (42). Variations in the distribution of cellular gD receptors across cell types likely affect HSV tissue tropism, and the preferential distribution of HVEM on immune system components that are not commonly targeted ty HSV infection suggests a possible role for the HVEM-gD interaction in immune evasion (42, 177).

Fusion activity in HSV is executed by a highly conserved multipartite system of viral glycoproteins composed of gB and the gH/gL heterodimer (42). The crystal structure of gB revealed a trimeric protein that bears a close resemblance to the known fusion protein vesicular stomatitis virus G protein, while gH exhibits properties similar to those of class 1 fusion glycoproteins (116, 130). Both gB and the gH/gL heterodimer are required for fusion to proceed (39, 99, 267). Absence of gL is prevents incorporation of gH into viral particles and abrogates entry into cells (267). It has recently been suggested that gB is the sole fusogenic protein, with gD and gH/gL merely serving to trigger a conformational change in gB to initiate fusion (62).

Presence of gD in addition to gB and gH/gL is necessary and sufficient for successful entry into host cells (42, 236). Transfection of plasmids expressing gD, gB, gH and gL into cells leads to reconstitution of a functional fusogenic complex in the presence of a suitable gD receptor, suggesting that no additional viral proteins are strictly required for membrane fusion (114, 298).

The fusogenic action of gB is regulated by direct or indirect interactions with other viral glycoproteins. Studies utilizing HSV-1 mutants with a tendency to fuse adjacent infected cells to create multinucleated giant cells called syncytia have indicated that pUL20, gK, gM and pUL11 perform regulatory roles in virus-induced fusion. Additionally, both pUL20 and gK have been shown to directly interact with gB, while gM may in turn interact with pUL20 (54, 102, 163, 213).

Capsid transport to the nucleus

After fusion with the host cell membrane, tegumented viral capsids are deposited into the cytoplasm. Capsids are propelled to the nucleus along microtubules by dynein motors. Experiments designed to replicate viral transport along the microtubule network in vitro have shown that the presence of inner tegument proteins such as pUS3, pUL36 and pUL37 on the capsid is required to recruit the microtubule motors responsible for both retrograde (dynein and dynactin) and anterograde (kinesin) transport (253). However, microtubule interactions do not completely account for intracellular capsid mobility since disruption of the microtubule network by nocodazole treatment does not completely abrogate HSV-1 infection (286).

As capsids travel towards the nucleus to deposit viral DNA through nuclear pore complexes (NPCs), outer tegument proteins such as pUL41, pUL47 and pUL48 (VP16) disassociate from the capsids to prime the host cell for viral gene expression and production of progeny virus (184, 216). The UL41 gene encodes the 58 kD virion host shutoff (vhs) protein. vhs dissassociates from the tegument during infection and travels to the nucleus where it degrades mRNA, shutting down gene expression and protein synthesis in the host cell (91). vhs also degrades viral mRNA and may contribute to the switch to late gene expression by degrading transcripts of immediate-early and early genes (240). Downregulation of vhs activity in the later stages of infection may be accomplished by the documented interaction of vhs with the tegument protein pUL48 (275, 283). pUL48, also known as the alpha trans-inducing factor (αTIF), is major structural component of the tegument. pUL48 travels to the nucleus after infection and forms a complex with the cellular protein Oct-1 to initiate the viral gene expression cascade by starting the transcription of immediate-early genes (232). pUL47 also localizes to the nucleus after infection and may be involved in regulating pUL48-mediated expression of immediate-early genes (303).

Upon capsid docking to the nuclear pore complex (NPC) (Figure 2.11), cleavage of the attached inner tegument protein pUL36 is necessary to release viral DNA into the nucleus, suggesting that pUL36 acts as a sort of plug to retain the packaged viral DNA (153). The highly pressurized viral DNA is released from the capsid portal as a single linear strand, which is quickly condensed upon entering the nucleus by host intranuclear proteins into DNase-insensitive rod-like structures (25, 218).

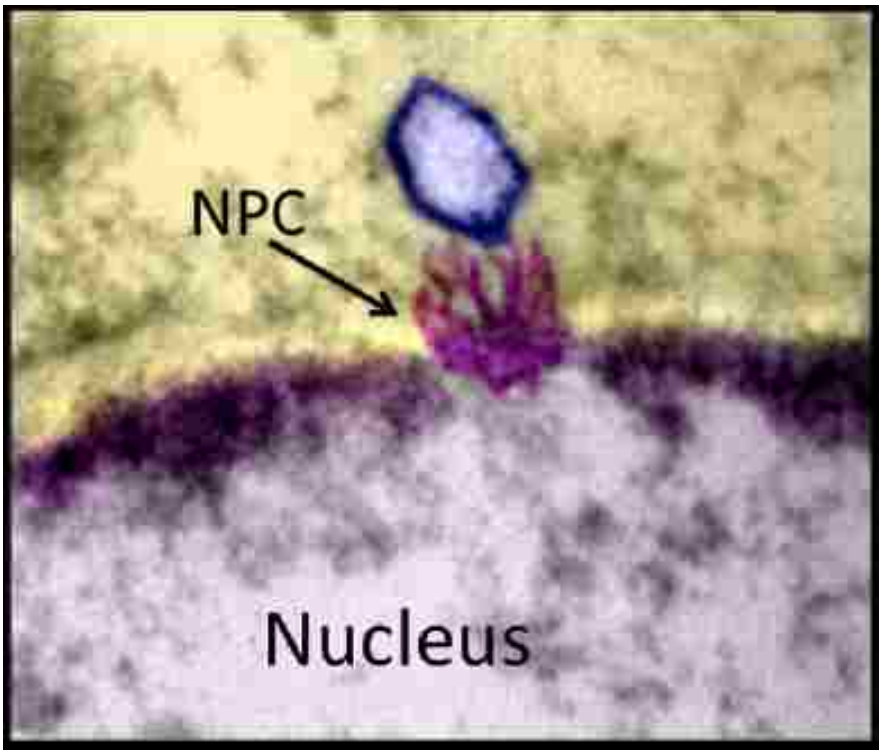

Figure 2.11: Artificially colored electron micrograph of empty HSV-1 capsid shortly after releasing viral DNA through the nuclear pore complex (NPC). Adapted with permission from [*Journal of the American Chemical Society* 135(30): 11216-11221, Herpes virus genome, the pressure is on]. Copyright (2013) American Chemical Society.

Viral gene expression and DNA replication

Freshly injected linear viral DNA rapidly circularizes due to the actions of the DNA repair protein XRCC4 and DNA ligase IV and serves as a template for subsequent replication (227). Immediate early (IE or alpha) genes encoding ICP0, ICP4, ICP22, ICP27, ICP47 and pUS1.5 are expressed very soon after infection, prior to replication of viral DNA. alpha gene expression is stimulated by pUL48 (αTIF), and the alpha gene products themselves modulate further gene expression, stimulating expression of early (beta) genes which encode components of the viral DNA replication machinery (97). The origin-binding protein pUL9 initiates replication by loading

the viral replisome complex onto origins of replication in the viral genome. The replisome consists of a virally encoded DNA polymerase composed of pUL30 and pUL42, a helicase-primase composed of pUL5, pUL8 and pUL52, and the single-stranded DNA-binding protein pUL29 (ICP8) (228). Viral DNA replication proceeds using a rolling circle mechanism, and newly synthesized viral DNA is used as the template for transcription of late (gamma) genes primarily encoding structural proteins that are utilized for the formation of progeny virus. Viral gene transcription is handled by the host RNA polymerase II, and viral proteins are synthesized in the cytoplasm (66, 228).

In latently infected neurons, viral DNA persists in the nucleus as a circular episome and normal lytic gene expression does not occur. Instead, the virus expresses latency-associated transcripts (LATs) which silence viral gene expression and have been implicated in protecting neurons from apoptosis (250). LAT transcript levels decrease upon viral reactivation, triggering alpha, beta and gamma gene expression and allowing viral replication to proceed (97).

Capsid assembly

HSV-1 capsids are assembled in the nuclei of infected cells in large nuclear inclusion bodies that form approximately four hours after initial infection and persist for the duration of infection (Figure 2.12) (35). At the start of assembly, VP5 forms complexes with the scaffolding proteins VP21 and VP22a which are cleaved from the overlapping UL26 and UL26.5 gene products (97). These complexes congregate to form 125 nm spherical procapsids, with the portal vertex being incorporated during initiation of procapsid assembly (35, 233). DNA transfer

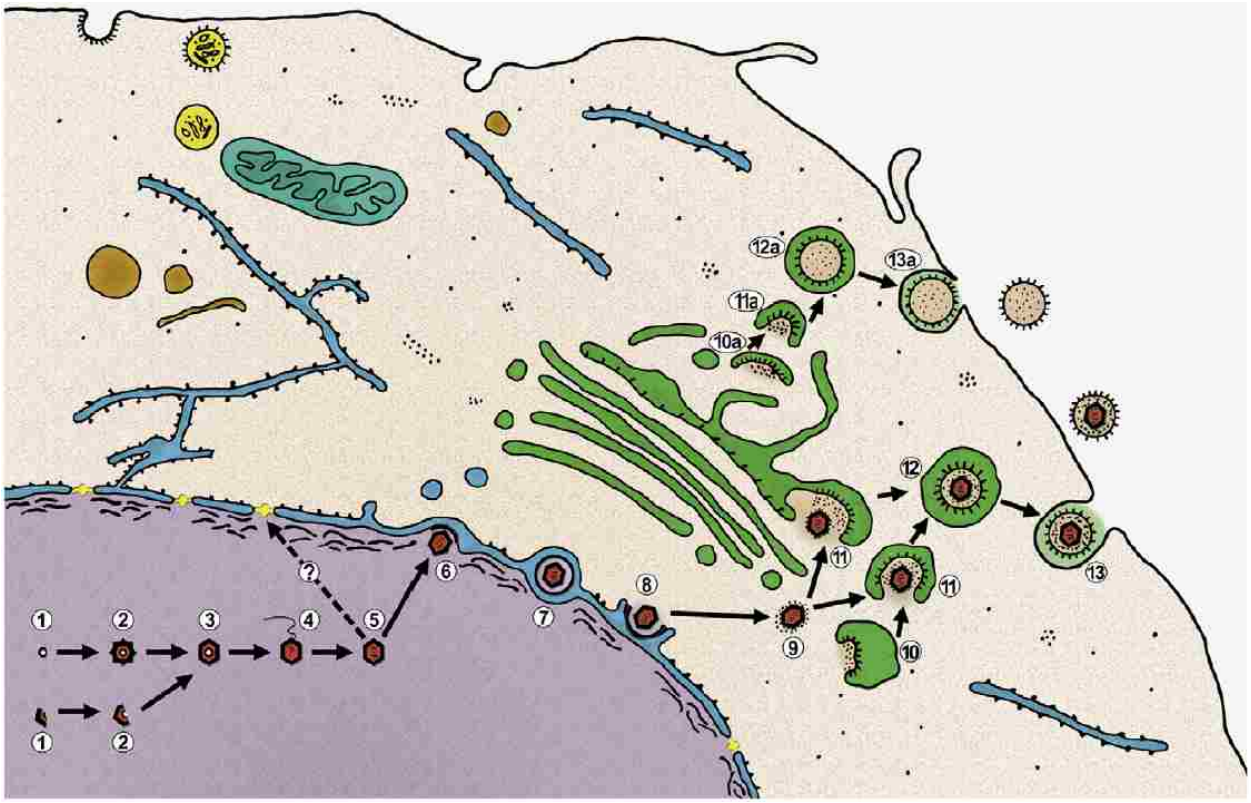

Figure 2.12: Diagram of HSV assembly and egress. (1-3) Intranuclear capsids assemble in the nucleus. (4) Capsids are packaged with viral DNA. (5) Capsids travel to the inner nuclear membrane. (6) Capsid budding into the inner nuclear membrane. (7) Enveloped capsid in the perinuclear space. (8) Fusion of envelope with the outer nuclear membrane and de-envelopment. (9) Tegument proteins assemble on the capsid. (10) Viral membrane proteins and additional tegument proteins gather at sites of secondary envelopment. (11) Capsids bud into TGN-derived membranes to acquire envelope. (12) Enveloped virion fully enclosed in cytoplasmic vesicle. (13) Mature virions are transported to the plasma membrane for release. (10a-13a) Formation of L-particles consisting of enveloped tegument proteins without a capsid. Adapted with permission from [*Current Opinion in Microbiology* 9(4): 423-429, Herpesvirus assembly: a tale of two membranes]. Copyright (2006) Elsevier.

into the capsid through the portal vertex is mediated by interactions between the portal and the tripartite terminase complex consisting of the viral proteins pUL15, pUL28 and pUL33 which is involved in cleaving concatemeric DNA into monomers during packaging. The pUL15, pUL28 and pUL33 protein complex initially forms in the cytoplasm and is eventually translocated to the

nucleus, aided by the presence of a nuclear localization signal (NLS) in pUL15 (97, 318). During DNA packaging, the capsid angularizes to its mature polyhedral shape as the scaffolding protein exits the still-leaky procapsid and is cleaved away by the viral maturational protease VP24 encoded by the UL26 gene (35). Following angularization, the minor structural capsid protein VP26 is recruited to the maturing capsids in an ATP-dependent manner (50). The tegument protein VP1-2 (pUL36) encoded by the UL36 gene likely serves to plug the portal after DNA packaging, as evidenced by research showing that cleavage of pUL36 after the capsid attaches to the nuclear pore complex is required to release viral DNA into the nucleus during infection (153).

Primary envelopment

In order to leave the nucleus, newly constructed DNA-filled capsids bud into the inner nuclear membrane, forming enveloped particles in the perinuclear space that subsequently undergo de-envelopment at the outer nuclear membrane to release the capsid into the cytoplasm (Figure 2.12). Although the nucleus contains pores that allow for the passage of certain proteins, HSV-1 capsids generally do not use this route for egress from the nucleus due to the relatively large 125 nm diameter of the capsid. Further evidence for the envelopment and de-envelopment pathway for nuclear egress is provided by observations indicating that nuclear pores are not disrupted during capsid translocation to the cytoplasm (134, 149).

Primary envelopment at the inner nuclear membrane requires the action of a nuclear envelopment complex (NEC) composed of pUL31 and pUL34 (258, 266). pUL34 is a type II integral membrane protein that is anchored within the inner nuclear membrane (282). pUL31 is

a phosphoprotein that has been shown to associate with the C-capsid specific components (CCSCs) at capsid vertices. pUL31 preferentially binds to the pUL25 component of CCSCs, but is also able to interact with the pUL17 CCSC component in the absence of pUL25. Interaction of the NEC with CCSCs may drive the preferential selection of DNA-containing capsids for primary envelopment (319).

The capsid-associated pUL31 is thought to bind to pUL34 at the inner nuclear membrane to initiate budding into the perinuclear space (259). Interactions with the capsid are further supported by the detection of both pUL31 and pUL34 within enveloped perinuclear virions, although they are not found within mature virions (110, 259). Surprisingly, the presence of capsids is not strictly required to initiate budding into the perinuclear space due to the fact that transient expression of only pUL31 and pUL34 of PRV is sufficient to form perinuclear vesicles (169).

Before capsids can even reach the inner nuclear membrane, they must first penetrate the nuclear lamina. The lamina is a dense filamentous layer composed of lamins A, B and C that coats the inner nuclear membrane (77). The NEC mediates localized disassembly of the lamina by recruiting protein kinase Cα and protein kinase Cδ to phosphorylate lamin B, while the capsid-associated kinase pUS3 phosphorylates lamin A and lamin C (178, 223, 244). The NEC also disrupts the lamina by binding to lamin A and lamin C, possibly due to competitive inhibition of interactions between the lamins (224, 257).

Tegument proteins are not necessary for primary envelopment, although perinuclear virions are already coated with a thin layer of tegument composed of pUL11, pUL36, pUL37,

pUL41, pUL48, pUL49 and pUS3 (18, 215, 217, 229, 243, 255, 259). The remainder of the tegument is added before and during secondary envelopment in the cytoplasm (149). Viral glycoproteins including gB, gD, gH, gL and gM have been detected in perinuclear virions as well as in the inner and outer nuclear membranes (22, 94, 289). gD and gM are actively recruited by the NEC to the inner nuclear membrane and gD has even been shown to bind to pUL34, but the role of viral glycoproteins in primary envelopment is unclear (310).

Perinuclear virions are released into the cytoplasm by fusion of the perinuclear envelope with the outer nuclear membrane in a process called de-envelopment. This fusion is presumably mediated by gB and the gH/gL heterodimer, which are also involved in fusion during entry (129). Accordingly, HSV mutants lacking gB and gH show enveloped virions stuck within the perinuclear space, unable to undergo de-envelopment and enter the cytoplasm (94). gB is a key HSV-1 fusion protein whose action is tightly regulated to avoid runaway fusion of intracellular membranes (130, 149). pUL20 and gK are able to physically interact with gB and act as regulators of gB-mediated fusion (54, 102, 143, 149). Furthermore, phosphorylation of gB by pUS3 has been shown to promote de-envelopment at the outer nuclear membrane (312, 316).

Secondary envelopment

Cytoplasmic capsids begin to acquire more tegument proteins such as pUL36 and pUL37 during their journey to sites of secondary envelopment at trans-Golgi network (TGN) derived vesicles and endosomes (Figure 2.12) (271). Additional tegument is bound to the cytoplasmic surface of TGN membranes and is added to the capsids during secondary envelopment (34,

149). The inner tegument proteins pUL36 and pUL37 mediate the transport of viral capsids to TGN membranes via interactions with the microtubule-based transport machinery (192, 271). pUL36 and pUL37 interact with viral capsids and with each other to form filaments attached to capsid vertices, and their presence is necessary for acquisition of the outer tegument and for cytoplasmic envelopment (80, 81, 231, 260).

The process of secondary envelopment in the cytoplasm involves a complex web of interactions between the capsid, the tegument and the cytoplasmic tails of envelope glycoproteins embedded in TGN membranes (Figure 2.13) (149, 216). Capsids coated with the inner tegument proteins pUL36 and pUL37 are connected to the outer tegument via the documented interactions between pUL36 and pUL48 and between pUL37 and pUL46 (181, 303). pUL48 acts as a sort of hub within the outer tegument and interacts with the tegument proteins pUL41, pUL46, pUL47 and pUL49 (88, 232, 283, 303). Another interaction between outer tegument proteins is the binding of pUL21 to pUL16, verified in both HSV-1 and in PRV (127, 167). In turn, pUL16 binds to the membrane-associated tegument protein pUL11 (189, 321). pUL11 interacts with the membrane proteins gD and gE and has been proposed as a bridge between the tegumented capsid and the envelope during secondary envelopment (93, 209, 321). Indeed, mutant viruses lacking expression of pUL11 exhibit mild impairment of cytoplasmic envelopment and infectious virus production, but not nearly to the same extent as mutants lacking pUL20 or gK (20, 52, 213).

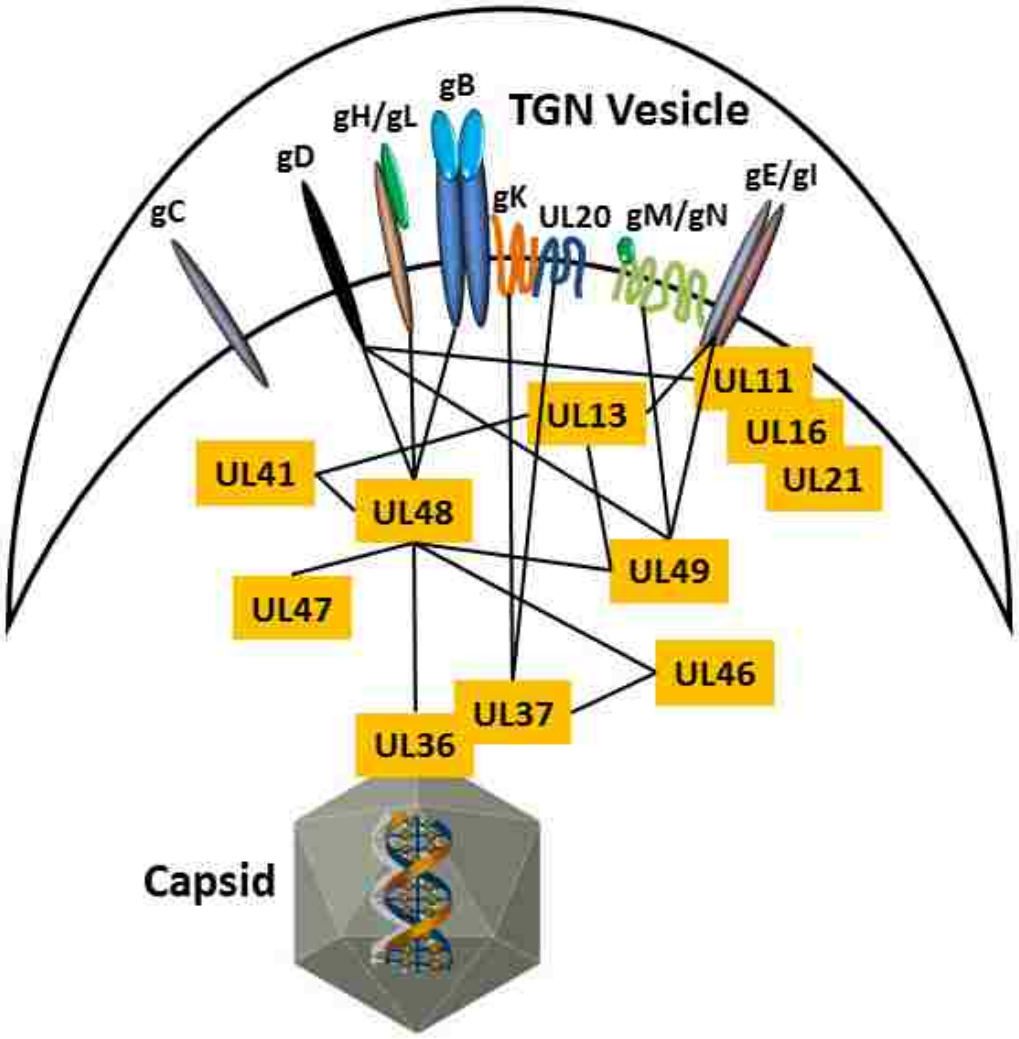

Figure 2.13: Diagram of viral protein interactions during secondary envelopment. Yellow rectangles denote tegument proteins.

Viral glycoproteins such as gB, gD, gE/gI are sorted to TGN membranes due to modification with mannose-6-phosphate (M6P) and to the presence of encoded sorting domains, including di-leucine motifs, tyrosine motifs and acidic clusters, that are similar to those found on host TGN proteins (27, 123, 206). Interactions with these targeted glycoproteins enable viral proteins that lack such domains to still be recruited to TGN membranes for

incorporation into mature virions (69). The cytoplasmic domains of viral membrane proteins embedded in TGN vesicles interact with tegument proteins to anchor them onto the membrane, and both the membrane proteins and the membrane-bound tegument proteins also interact with the incoming partially tegumented capsids to promote budding and subsequent envelopment (149, 217).

In addition to the interaction between pUL11 and glycoproteins D and E, a variety of other links between membrane glycoproteins and tegument proteins have been identified (93). The tegument-associated protein kinase pUL13 has been shown to phosphorylate both gE and pUL49, in addition to modulating the function of pUL41 (67, 234, 242). Pulldowns and chemical cross-linking experiments have shown that the outer tegument protein pUL48 (VP16) is able to bind to the cytoplasmic tails of gB, gD and gH (119, 154, 325). The tegument proteins pUL11 and pUL49 (VP22) interact with the cytoplasmic portions of gD and gE (49, 93, 239, 290). The PRV version of pUL49 can interact with the carboxyl terminal domains of gE/gI and gM (108). Interaction between pUL49 and the cytoplasmic portion of the membrane protein pUS9 has been detected via yeast two-hybrid screening (181). The absence of pUL49 also has a negative impact on the incorporation of gD, gE/gI and ICP0 into virions, although incorporation of the ICP0 binding partner ICP4 is unaffected (86, 87, 320). Interactions between the membrane and the inner tegument or capsids have also been reported. The membrane proteins pUL20 and gK have been shown to bind to the inner tegument protein pUL37 via co-immunoprecipitation and proximity ligation assays, which may provide an explanation for the relative importance of pUL20 and gK in facilitating cytoplasmic virion envelopment. (52, 101, 145). Furthermore, the cytoplasmic tail of gD is reportedly able to directly bind capsids (49).

Single glycoprotein deletions in HSV-1, with the notable exception of gK, do not have a significant effect on secondary envelopment (52, 101, 149). It has been suggested that gD, gE and gM are essential mediators of secondary envelopment in the cytoplasm and act in a redundant manner, which would explain why single deletions of these glycoproteins do not significantly perturb cytoplasmic envelopment (92, 149, 217). Early studies indicated that HSV-1 mutants lacking both gD and the gE/gI heterodimer showed nearly complete abrogation of cytoplasmic envelopment (92). A similar phenotype was observed for PRV mutants lacking gM and gE/gI (32). However, follow-up analysis of HSV-1 mutants lacking gE, gM, or the cytoplasmic portion of gD, either alone or in combination, did not show significantly impaired cytoplasmic envelopment and infectious virus production even in the absence of all three simultaneously, suggesting that these proteins are not essential and do not function in a redundant manner for cytoplasmic envelopment (52, 180). A recombinant HSV-1 lacking both gM and pUL11 did exhibit impaired secondary envelopment, but not to the same extent as mutants unable to synthesize gK or the non-glycosylated membrane protein pUL20, thus illustrating the preeminent role pUL20 and gK play in the secondary envelopment process (52, 101, 182). It is theoretically possible that lack of pUL20 or gK does not actually prevent cytoplasmic envelopment from occurring. pUL20 and gK are known fusion modulators, and their absence may cause the newly enveloped virions to immediately undergo de-envelopment back into the cytoplasm due to fusion of the virion envelope with the surrounding TGN vesicle. However, this possibility is yet to be thoroughly investigated (54, 102, 143, 149, 212, 213).

Impaired virion egress was recently noted using an HSV-1 double mutant lacking gB and gD, leaving open the possibility that gB may cooperate with gD to facilitate cytoplasmic envelopment (40, 151). Glycoprotein B is primarily responsible for fusion of the viral envelope to the host cell membrane during entry (130). gB-mediated fusion is modulated by interactions between gB and other membrane proteins, including gD, gH, gK and pUL20 (13-15, 54, 62, 114). pUL20 forms a complex with gK and has also been shown to interact with gM (100, 163). Deletion of one or more viral proteins may lead to defects in secondary envelopment by directly or indirectly affecting the stability and function of other viral proteins due to disruption of the complex tangle of interactions among tegument proteins and viral glycoproteins (52).

Host cell proteins are likely also involved in secondary envelopment. ESCRT proteins in particular may facilitate membrane curvature during the budding process. This is supported by experiments with dominant-negative forms of ESCRT component proteins that show abrogation of secondary envelopment in both HSV-1 and HCMV (247, 296). Viral proteins likely interact with ESCRT components to recruit ESCRT machinery to sites of cytoplasmic envelopment at TGN membranes. Sequence-based screening of HSV-1 proteins for potential ESCRT-binding partners revealed a putative ESCRT-binding motif in gK that is similar to retroviral motifs called late domains which promote ESCRT recruitment (247).

Egress to extracellular spaces

After undergoing secondary envelopment by budding into cytoplasmic membranes derived from the trans-Golgi network (TGN) and endosomes, enveloped virions enclosed within cellular vesicles are translocated to the plasma membrane for release by co-opting the cell's

exocytic machinery (Figure 2.12) (149, 215, 217). These doubly-enveloped virions are morphologically identical to extracellular virions, but show significant differences from enveloped virions found in the perinuclear space (117). Transport of TGN-derived vesicles containing enveloped virions to the plasma membrane is assisted by the action of cellular serine-threonine protein kinase D, which is involved in fission of cargo-laden TGN vesicles and regulation of their transport towards the basolateral plasma membrane (256). HSV is also known to utilize myosin Va to translocate enveloped virion-containing vesicles to the plasma membrane along cortical actin filaments (262). Upon reaching the plasma membrane, TGN-derived transport vesicles fuse with the plasma membrane to release their enveloped virion cargo into the extracellular space. This fusion is unlikely to be mediated by the viral fusion machinery utilized during entry because the viral glycoproteins embedded in the transport vesicles are incorrectly oriented. As tegumented capsids bud into TGN-derived vesicles to acquire an envelope, they are in contact with the cytoplasmic portions of viral glycoproteins embedded in TGN membranes. Therefore, the portions of viral glycoproteins that were located inside the TGN-derived vesicles are oriented to the outside surface of the final enveloped virion, with the formerly cytoplasmic portions facing inside the virion (149).

In polarized epithelial cells, progeny virions are often sorted to cell-cell junctions instead of simply being released into the extracellular space. This sorting is presumably mediated by viral proteins attached to or incorporated into the TGN-derived vesicles containing mature enveloped virions (149). Studies have implicated the gE/gI membrane protein complex and the inner tegument protein pUL37 in mediating transport of progeny virions to cell-cell junctions (150, 251). Trafficking to cell-cell junctions provides important benefits for the virus. Placing

virions in close proximity to adjacent cells facilitates the spread of infection. Minimizing exposure to the extracellular space also allows virions to avoid elements of the host immune response such as antibodies and components of the complement cascade (149). Immune system exposure is further reduced by the propensity of HSV to cause fusion between cell membranes of adjacent cells in vivo. The plasma membranes of infected cells are enriched with viral glycoproteins, including members of the fusion complex involved in fusion of the viral envelope with the plasma membrane during entry. Viral entry fusion machinery is thus repurposed to create large multinucleated cells called syncytia (97, 135). These factors help explain why titers of neutralizing antibodies in HSV-infected patients frequently do not correlate with disease severity or frequency of recurrence (64, 65).

HSV-1 egress in neurons: "married" vs. "separate" models

HSV-1 shares with other alphaherpesviruses the ability to invade neurons and establish a life-long latent infection. After replicating at the initial site of infection, HSV-1 enters sensory neurons via their axon termini by fusion of the viral envelope with the neuronal plasma membrane. The viral capsid, along with associated inner tegument proteins, is then transported in a retrograde fashion from the periphery of the neuron to the cell body where viral DNA is deposited in the neuronal nucleus for replication or establishment of latency. This retrograde transport is mediated by dynein motors associated with the neuron's internal microtubule network (7, 82). In neurons, the minus-ends of microtubules are anchored in the neuronal cell body while the growing plus-ends are located at the axon termini. Dynein, along with its cofactor dynactin, are large protein complexes involved in the ATP-dependent movement of

bound cargo towards microtubular minus-ends. In HSV-1, the essential inner tegument protein pUL36 and its binding partner pUL37 remain associated with the capsid after entry and have been hypothesized to play a role in retrograde transport by interacting with the dynein/dynactin complex, possibly in a redundant fashion with other viral capsid or inner tegument proteins. The outer capsid protein VP26, as well as the viral proteins pUL34 and pUL9, has been shown to bind to dynein in vitro, but recent studies with VP26-null mutant virus showed no defect in dynein-mediated retrograde capsid transport. The pUL34 and pUL9 proteins are not present in mature virions and are not involved in retrograde transport (71, 82). Also, *in vivo* models have shown the membrane protein gE to be important for retrograde spread in HSV-1 but not in PrV (202).

Upon reactivation from latency in neurons, HSV-1 assembly and egress directly from the neuronal cell body proceeds in much the same way as in non-neuronal cells. However, neurons are highly polarized cells and a subset of newly constructed HSV-1 particles undergoes anterograde transport driven by microtubule-dependent kinesin motors whereby the viral particles travel along the axon from the neuronal cell body toward the plus-ends of microtubules at the axon terminus, leading either to reinfection of the innervated region and virus shedding or to further invasion of the central nervous system by infection of other neurons (82, 230). Two competing models exist for the anterograde transport of HSV-1 that occurs during viral egress from infected neurons. In the “married” model, the secondary envelopment step (where the tegumented viral capsid acquires its final envelope by budding into cytoplasmic vesicles enriched with viral glycoproteins and other membrane-associated proteins) occurs within neuronal cell bodies and the resulting mature enveloped virions are

transported within secretory vesicles toward the axon termini for release. In the "separate" model, unenveloped viral capsids coated with inner tegument proteins are transported along the axons separately from axonal vesicles containing the viral glycoproteins and other membrane-associated proteins, with the secondary envelopment step and subsequent viral release by exocytosis occurring at the axon termini (82, 149, 230).

Kinesins are a large group of molecular motor proteins that typically use ATP hydrolysis to move cargo toward the plus-ends of microtubules. One or more different types of kinesin may be involved in viral transport, possibly based on changes in the composition of viral cargo (82). The prototypical kinesin-1 is a tetramer consisting of two identical heavy chains and light chains with a "tail" region that binds cargo and two "heads" with ATPase activity that bind and "walk" along microtubules by stepping past one another hand-over-hand (322). The tail region is composed of both heavy and light chains, both of which are capable of interacting with cargo either directly or via adaptor proteins. The "separate" and "married" models for HSV-1 anterograde transport have direct implications for elucidating the mechanism and binding partners of kinesin in viral anterograde transport. If the "separate" model is valid, kinesin's cargo would consist of tegumented capsids and kinesin could be expected to interact with some of the exposed capsid or inner tegument proteins (82). In fact, interaction between the carboxyl terminus of the HSV-1 tegument protein pUS11 and a kinesin-1 cargo-binding domain has been documented, but HSV-1 virions lacking pUS11 were still capable of binding kinesin-1, suggesting a level of redundancy in kinesin-virion interactions (82, 85). The major tegument protein pUL36 has also been proposed to play a role in kinesin binding and anterograde transport in both HSV-

1 and PrV. A recent study showed decreased microtubule binding and motility in an in vitro microchamber assay using a mutant HSV-1 expressing a partially deleted pUL36 protein (279).

On the other hand, if the “married” model is valid and enveloped virions are transported within secretory vesicles containing embedded viral glycoproteins then kinesin could be expected to interact with either cellular receptors or viral glycoprotein cytoplasmic tails located on the outer vesicle. Such an interaction has been documented between kinesin and the cellular receptor amyloid precursor protein, which associates with vesicles containing enveloped HSV-1 during neuronal egress (82, 85). Direct interaction has also been documented between kinesin and the HSV-2 membrane-associated protein pUL56 (82). Furthermore, while studies in various alphaherpesviruses such as PrV and BHV-5 have indicated that the viral envelope proteins gE/gI and pUS9 are critical for anterograde neuronal transport, in HSV-1 only gE/gI are essential for anterograde spread while pUS9 is dispensable (57, 202). It has also been postulated that the possible interactions between kinesin and tegument proteins such as pUS11 and pUL36 may be amenable to interpretation under the “married” model by considering the transport of partially enveloped virions where some of the tegument proteins are exposed and may bind to kinesin. Alternatively, there is some evidence that pUL36 may bind to the surface of the cytoplasmic organelles which enclose the enveloped virions, allowing for direct interactions between pUL36 and kinesin (279). However, the fact that HSV-1 lacking gE/gI cannot undergo anterograde neuronal transport means that any putative interactions between kinesin and pUL36 or pUS11 are insufficient to rescue this critical function in the absence of gE/gI.

In contrast to pseudorabies virus (PrV), where the “married” model for anterograde neuronal transport has seen wide acceptance after numerous studies utilizing electron microscopy and live-cell imaging of fluorescently labeled PrV, the picture for HSV-1 has been less clear (82, 186, 197). Earlier studies with HSV-1 lent support to the “separate” model by observing unenveloped nucleocapsids in the axons of infected neurons by electron microscopy and a lack of colocalization between fluorescently labeled capsids and envelope proteins in neuronal axons while also observing some colocalization of labeled capsid, tegument and envelope proteins in neuronal growth cones at axon termini, presumably due to secondary envelopment at axon termini (82, 136, 285). However, many of these studies suffered from flaws, such as the assumption that the motionless unenveloped virus particles observed in the axon after fixation for electron microscopy or conventional fluorescent microscopy were undergoing anterograde transport to the axon terminus instead of retrograde transport to the neuronal cell body after secondary infection, a problem often exacerbated by the high MOIs (up to 1000 PFU/neuron) used in the experiments (311). EM experiments are further complicated by the significantly lower quantities of virus found in the axons of neurons infected with HSV-1 compared to neurons infected with PrV (230).

More recent experiments provide solid evidence for the “married” model for HSV-1 anterograde neuronal transport. In a high-resolution electron microscopy study of explanted primary neurons from rat superior cervical ganglia, approximately 75% of observed intraaxonal HSV-1 virus particles were enveloped, supporting the “married” model (230). Another EM study using both rat and mouse primary neurons from superior cervical ganglia in Campenot neuronal growth chambers also reported a majority of enveloped HSV-1 particles in neuronal axons

(139). Also, a recent study that used live-cell imaging to evaluate anterograde transport in chick embryo and rat dorsal root ganglion-derived neurons by a recombinant HSV-1 simultaneously expressing both an RFP-tagged capsid protein (VP26) and a GFP-tagged membrane protein (gB) showed that up to 70% of virus particles were enveloped during anterograde axonal transport (8). Furthermore, a similar recent live-cell imaging study using a recombinant HSV-1 expressing YFP-tagged VP26 and RFP-tagged gB in human SK-N-SH neuroblastoma cells and rat superior cervical ganglion neurons in microfluidic chambers showed clear evidence of both enveloped and unenveloped viral capsids in anterograde transport. Caveats include the smaller proportion of enveloped particles in the axons of human neurons compared to rat neurons, which may be explained in part by differences between the human and rat anterograde transport machinery, and the possibility that adding bulky fluorescent proteins to the HSV-1 capsid and envelope could impact the ratios of enveloped and unenveloped particles in axons. Thus, it is possible to find varying degrees of evidence for both the “separate” and “married” models for HSV-1 anterograde transport, depending on the type of neuron used for the experiment and the imaging method employed (311). Although there is little doubt now that anterograde transport of fully enveloped virions can occur in HSV-1, it is also true that some portion of exiting HSV-1 virions are transported as tegumented capsids, suggesting the two mechanisms could coexist in HSV-1 to a greater extent than in PrV.

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CHAPTER III: FUNCTIONAL HIERARCHY OF HERPES SIMPLEX VIRUS 1 VIRAL GLYCOPROTEINS IN CYTOPLASMIC VIRION ENVELOPMENT AND EGRESS*

Introduction

Herpes simplex virus 1 (HSV-1) virion assembly begins in the nucleus with the construction of viral capsids, which acquire certain tegument proteins and then bud through the inner nuclear membrane, forming enveloped virions within the perinuclear space (primary envelopment). Enveloped virions fuse with the outer nuclear membranes, allowing capsid deposition in the cytoplasm of cells (10, 45, 47); reviewed in reference (42). In the cytoplasm, viral capsids are coated with tegument proteins and acquire final viral envelopes by budding into glycoprotein-enriched regions of the *trans*-Golgi network (TGN) membranes (secondary envelopment) (17, 51, 54, 56). This final virion morphogenesis step delivers fully enveloped virions into cytoplasmic vesicles, which are ultimately transported out of the cell (33). Secondary envelopment of cytoplasmic capsids is facilitated by interactions between tegument proteins and the cytoplasmic domains of viral glycoproteins and other membrane proteins anchored in TGN-derived membranes (17, 51, 54, 56); reviewed in references (32, 42).

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Deletion or forced retention of either gD or gH within the endoplasmic reticulum does not cause drastic defects in cytoplasmic virion envelopment and egress, although both glycoproteins are essential for viral infectivity (7, 19, 38, 55). Similarly, gB is not required for cytoplasmic envelopment and egress, inasmuch as gB-null viruses acquire envelopes and can be rendered infectious after treatment with the fusogen polyethylene glycol (8, 41). However, partial deletion of the carboxyl terminus of gB was reported to cause substantial reductions in cytoplasmic virion envelopment and egress (9), suggesting that truncated gB may cause a dominant negative effect. Recently, a gB-gD double mutant but not a gD-null virus exhibited substantial defects in late stages of virus egress, indicating that gB may cooperate with gD in facilitating virion envelopment (34). Single or simultaneous deletion of HSV-1 gE and gM genes did not cause any appreciable defects in cytoplasmic virion envelopment and infectious virus production (6). Similarly, lack of either gD or gE expression caused a mild (2- to 3-fold) reduction in enveloped virions. However, simultaneous deletion of HSV-1 gD and gE or gD, gE, and gI genes caused drastic accumulation of unenveloped capsids in the cytoplasm. Because neither of these gene deletions alone caused similar defects, it was concluded that gD and gE function in a redundant manner in cytoplasmic virion egress (16). Deletion of the UL11 gene produced mild defects in cytoplasmic virion egress (27), while deletion of either the gK or UL20 gene or specific mutations within these two genes caused drastic inhibition of cytoplasmic virion envelopment (23, 31, 41). Also, it was reported that simultaneous deletion of HSV-1 UL11 and gM caused drastic inhibition of cytoplasmic virion envelopment (37). Together, these results suggest that there are multiple cooperative relationships among viral glycoproteins and membrane proteins facilitating cytoplasmic virion envelopment.

We have reported that gK and UL20 have distinct functions in virus-induced cell fusion and cytoplasmic virion envelopment. These functions are genetically separable, since mutations in UL20 that drastically inhibit virion envelopment do not affect virus-induced cell fusion (24, 40). UL20 and gK function in virus-induced cell fusion by physically binding to gB and gH in infected cell surfaces (12, 13). Their roles in cytoplasmic envelopment and egress are not defined, but it is likely that they interact with other viral glycoproteins and tegument proteins to facilitate virion envelopment. Infectious virus production is directly dependent on the ability of viruses to assemble in the cytoplasm mature virions containing fully glycosylated glycoproteins and spread from infected to uninfected cells. Moreover, there are no known glycoprotein mutations that inhibit virion egress without affecting cytoplasmic envelopment. In the study described in this report, we determined particle-to-PFU ratios, relative plaque size (indicative of relative virus spread), and kinetics of virus growth and conducted ultrastructural visualization of glycoprotein-deficient mutant and wild-type virions in the same HSV-1(F) genetic background to gain an understanding of the relative contribution of individual viral glycoproteins in infectious virion morphogenesis and egress. The results show that UL20 and gK are the most important viral determinants for cytoplasmic virion envelopment, egress, and infectious virus production in comparison to gM, gD with deletion of the carboxyl-terminal 29 amino acids (gDΔct), gE, and UL11 alone or in various combinations.

Materials and Methods

Cells and antibodies

African green monkey kidney (Vero) cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, NY), supplemented with 10% fetal calf serum and antibiotics. Antibodies used include anti-HSV-1 gE monoclonal antibody (Virusys, Sykesville, MD) and Alexa Fluor 488-conjugated goat anti-mouse IgG monoclonal antibody (Invitrogen-Molecular Probes, Carlsbad, CA) for the indirect immunofluorescence assays (IFAs), as well as anti-HSV-1 gD, anti-HSV-1 gB, anti-HSV-1 gC monoclonal antibodies (Virusys, Sykesville, MD), rabbit anti-gM antibody (a gift from Joel Baines, Cornell University, Ithaca, NY), and rabbit anti-UL11 antibody (a gift from John Wills, Pennsylvania State University, Hershey, PA) for the Western immunoblot assays.

Construction of HSV-1 mutant viruses

Mutagenesis was accomplished in *Escherichia coli* using the markerless two-step RED recombination mutagenesis system and synthetic oligonucleotides (36, 53) (Table 3.1) implemented on the bacterial artificial chromosome (BAC) plasmid pYEbac102 carrying the HSV-1(F) genome (52) (a kind gift from Y. Kawaguchi, University of Tokyo, Tokyo, Japan). Construction of the HSV-1 mutants gDΔct (US6), ΔgE (US8), ΔUL20, ΔgM1, ΔgE+gDΔct, and ΔgE+ΔgM1 was described previously (36). The ΔgM2 recombinant virus was constructed by altering two potential initiation codon sites (from ATG to CTG and ATG to ATT, respectively) located 57 bp apart at the beginning of the UL10 open reading frame (ORF) (5) (Figure 3.1, Table 3.1). The ΔUL11 virus was constructed by changing the initiation codon from ATG to CTG.

Table 3.1: RED recombination primers.

	Primer Name	Primer Sequence	Purpose
I	5' gE-EnPac	ATTGGCCTCCCATTTACCCGAGATCGGCTGCTATCCCCGGGACCCTCGT	AgL (start codon point mutation)
II	5' gM-EnPac	CGCGGGGCGGTGGTGGGGTTTCTTAGGATGACGACGATAAGTAGGG	
III	5' gD-366Del	AACACAACACCCGAGAACAAACCCACCACCGCCCCGCGATCCAGGTCCC	gDΔct (deletion from amino acid 366 creating stop codon)
IV	5' gD-366Del	GCGGATACCAGCCGATCTTCGGGTACAACCAATTAACCAATTCTGATTAG	
V	5' gV1-EnPac	CTCCGCGTTAGCGATACGCTCGACGTGTACTGTTCGCACTCGTCGTCCTAG	ΔgM1 (start codon point mutation)
VI	5' gV1-EnPac	TGCGAGCGCCCGGCCCCCAGGATTACAAGGATGACGACGATAAG	
VII	5' gV2-EnPac	TTCGTGGGGCGCCGCGGAGTCGGCAGATCCTCTGGCGCCCGGGCGTCCCA	ΔgM2 (start codon and downstream site point mutations)
VIII	5' gV2-EnPac	GTGGGGACGACGAGTCCGAACCAACCAATTAACCAATTCTGATTAG	
IX	5' UL11-EnPac	CGCCCGGCCCCCAGAGGATCTCCGGACTCCTCGCGCCCCCACGAAAGCCAT	ΔUL11 (start codon point mutation)
X	5' UL11-EnPac	ATACCGCGCGCCGCGGACGCCGTGATTACAAGGATGACGACGATAAG	
		ACGTCGCCACCTGCACACCCGAGACCCACCACCGCCGTCCGCGCCCCGGTAA	
		TGCCTTTCGTCGGGGGGCGCCAACCAATTAACCAATTCTGATTAG	
		GCCATCCAGCGAAGCAGCCGCCTGGCATTCGACGACACGTCGCCGAGCT	
		GTGGGCCTCTCGTTCTCCGGGATTACAAGGATGACGACGATAAG	
		ACGTTGTTTCGGCAGCAGCAGGGCCGGCGCCCCGGAGAACGAGAGGCCCA	
		GAGCTCGGCGAGCGTGTCGTCCAACCAATTAACCAATTCTGATTAG	

❖ For RED recombination primers, the regions homologous to HSV-1 DNA are in standard upper case, the underlined sequences denote sites of mutation, the underscores represent deletion and the regions which bind to the marker gene are in solid italics

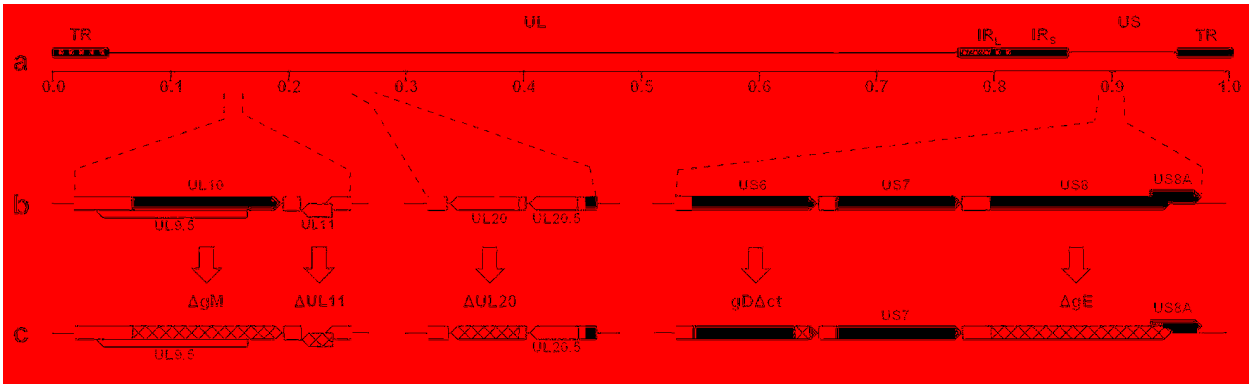

Figure 3.1: Genomic map of mutated genes. (a) Prototypic arrangement of the HSV-1 genome with the unique long (UL) and unique short (US) regions flanked by the terminal repeat (TR) and internal repeat (IR; L, long; S, short) regions; (b) expanded genomic regions of the UL10, UL11, UL20, US6, and US8 ORFs; (c) effect of ATG mutagenesis on gM, gE, UL11, and UL20 gene expression (hatched regions), as well truncation of gD after gDΔct mutagenesis (hatched gD region).

The ΔgE+gDΔct recombinant virus was used as the backbone for construction of the ΔgE+gDΔct+ΔgM2 triple mutant by altering the two potential initiation codon sites in gM, as described above for ΔgM2 virus. The ΔgM2+ΔUL11 double mutant was constructed by altering the initiation codon of UL11 from ATG to CTG in the ΔgM2 virus.

Confirmation of the targeted mutations, recovery of infectious virus and marker-rescue experiments

HSV-1 BAC DNAs were purified from 50 ml of overnight BAC cultures with a Qiagen large-construct kit (Qiagen, Valencia, CA). Using PCR test primers designed to lie outside the target mutation site(s), all mutated DNA regions were sequenced to verify the presence of the desired mutations in BACs. Similarly, viruses recovered from infected Vero cells were sequenced to confirm the presence of the desired mutations. Viruses were recovered from cells transfected with BACs as we have described previously (36). To determine whether the ΔUL11+ΔgM2 mutant virus contained any other genomic mutations, rescue experiments were performed with approximately 1-kbp DNA fragments spanning the UL11 and gM initiation codon mutations. Vero cells were transfected with the DNA fragments, and 24 h later, transfected cells were infected with the ΔUL11+ΔgM2 virus. Virus stocks were prepared 24 h postinfection (hpi) and plated at limiting dilution on Vero cells. Approximately 10 to 15% of viral plaques appeared to be similar to wild-type virus (data not shown). DNA sequencing of wild-type-like viral plaques confirmed the absence of the UL11 and gM mutations.

Plaque morphology of mutant viruses and relative plaque area measurements

Visual analysis of plaque morphology of mutant viruses was performed as we have previously described (24, 27, 36, 41). Plaque area measurements and data analysis were

essentially as described previously (36), except that photographs of viral plaques analyzed were taken at 50x magnification and 30 randomly selected plaques were imaged for each of the mutant and wild-type viruses under consideration.

One-step viral growth kinetics

Analysis of one-step growth kinetics was performed as we have described previously (20, 25). Briefly, nearly confluent Vero cell monolayers were infected with each virus at 4°C for 1 h at multiplicities of infection (MOIs) of 0.2 and 3. Thereafter, plates were incubated at 37°C in 5% CO_2 and virus was allowed to penetrate for 1 h at 37°C. Any remaining extracellular virus was inactivated by low-pH treatment (pH 3.0), and cells were incubated at 37°C in 5% CO_2. Supernatants and cell pellets were separated at different times postinfection and stored at -80°C.

SDS-PAGE, Western immunoblotting, and indirect immunofluorescence assay

Subconfluent Vero cell monolayers were infected with the indicated virus at an MOI of 3. At 24 hpi, cells were collected by low-speed centrifugation, washed with phosphate-buffered saline (PBS), and processed as described previously (21, 36). The indirect immunofluorescence assay for the detection of gE expression was performed as described previously (36).

Electron microscopy

The ultrastructural morphology of virions within infected cells was examined by transmission electron microscopy essentially as described previously (22, 23, 31, 36, 41). All infected cells processed for electron microscopy were prepared at 16 h postinfection and visualized by transmission electron microscopy.

Preparation of cytoplasmic and extracellular virions

Cytoplasmic virions were separated by glycerol shock treatment essentially as previously described by Sarmiento and Batterson (46), with the following modifications. Extracellular virions were prepared from supernatants of infected cells. Specifically, viruses were adsorbed on wells of a 12-well plate of nearly confluent Vero cell monolayers at 4°C for 1 h at an MOI of 1. Afterwards, plates were incubated at 37°C in 5% CO_2 for 1 h to allow the virus to penetrate into cells. Any remaining extracellular virus was inactivated by low-pH treatment (pH 3.0), and cells were incubated at 37°C in 5% CO_2 for 18 h and supernatants were collected. Infected cells were washed once with ice-cold PBS (pH 7.4), and the remaining extracellular virus was inactivated by low-pH treatment (pH 3.0). After removing the low-pH PBS, 900 µl of PBS (pH 7.4) was added to each well. To minimize nuclear disruption, the glycerol concentration was gradually increased by adding 150 µl of 90% glycerol (prewarmed to 37°C) to each well, followed by mixing and incubation at 37°C for 5 min, which was repeated three times to obtain a final concentration of 30% glycerol. The glycerol-treated samples were centrifuged at 1200x *g* for 10 min at 4°C, and supernatants were discarded. The cell pellet was suspended in 250 µl of ice-cold lysis buffer (0.01 M Tris-HCl, pH 7.4, 1.0 mM MgCl2, and 1.0 mM CaCl) by gentle mixing. The cell suspension was incubated on ice for 5 min and centrifuged at 700 x *g* for 10 min at 4°C to separate the nuclei and cellular debris from the cytoplasmic fraction. The supernatant (cytoplasmic fraction) was carefully transferred to new 1.5-ml tubes and used for TaqMan real-time PCR as described previously (49, 50).

Q-PCR

Quantitative PCR (Q-PCR) was utilized to derive the number of viral genomes within cytoplasmic and extracellular samples that remained protected after DNAase treatment, as described previously for Kaposi's sarcoma-associated herpesvirus (KSHV) (49, 50). Specifically, the primers and probe (6-carboxytetramethylrhodamine [TAMRA]) for the real-time PCR were designed to detect HSV-1 US6 (gD). Cytoplasmic and extracellular fractions were collected 18 hpi, and 100 µl of each suspension was used for the extraction of viral DNA. The cytoplasmic and extracellular suspensions were treated with Turbo DNase I (Ambion, Inc.) for 1 h at 37°C. Viral DNA was extracted using a DNeasy blood and tissue kit (Qiagen, Inc.) as per the manufacturer's instructions. Equal volumes of viral DNA were used for TaqMan PCR analysis. Purified HSV-1 bacterial artificial chromosome (YE102) DNA was used to generate the standard curve. Samples were also tested, and genome numbers were determined using validated standards provided by the Path-HSV1-genesig real-time PCR detection kit for human herpesvirus 1 (herpes simplex virus 1) (PrimerDesign, Ltd., South Hampton, United Kingdom).

Results

Construction and molecular analysis of recombinant viruses

We showed previously that recombinant viruses carrying a deletion of the carboxyl terminus of gD (gDΔct) and the entire gE gene or gE and gM were able to efficiently acquire cytoplasmic envelopes (36). To compare the relative roles of UL20, gM, gE, gD, and UL11 in cytoplasmic envelopment and virion egress, we generated additional mutant viruses lacking expression of one or more of these genes (Figure 3.1). All mutant viruses were produced using the two-step RED recombination mutagenesis system (53) implemented on the pYEbac102

bacterial artificial chromosome carrying the entire HSV-1(F) genome (52), as described in Materials and Methods. Further characterization of the gE and gM double-null virus produced earlier (36) suggested that a second initiation codon located 19 amino acids downstream from the first initiation codon can be utilized to produce a truncated gM (data not shown). Therefore, to ensure a complete lack of gM expression, the mutant virus ΔgM2 was created by altering both initiation codons, and both versions of the mutated gM genes were used to sequentially construct the desired set of mutations. The gM and UL11 defects were readily rescued by DNA fragments overlapping the corresponding mutations, as evidenced by the appearance of more than 10% viral plaques that were similar to wild-type plaques. Moreover, DNA sequencing of selected rescued viruses revealed the presence of wild-type gM and UL11 sequences (data not shown). The triple mutant virus gDΔct+ΔgM2+ΔgE was generated from the previously characterized gDΔct+ΔgE mutant virus (36). Overall, these results suggest that there were no spurious nucleotide changes elsewhere in the viral genomes of viruses containing the gDΔct, gE, gM, and UL11 mutations.

Recovery and plaque morphologies of infectious viruses produced by HSV-1 BAC DNAs

To generate virus stocks from the mutant BAC genomic constructs, individual BAC DNAs were transfected into Vero cells and initial virus stocks were recovered and characterized as detailed in Materials and Methods. The plaque morphologies of all mutant and wild-type viruses were examined in Vero cells. As expected, the HSV-1(F) wild-type virus produced the largest plaques, while the ΔUL20 mutant produced the smallest plaques, consisting of less than 5 cells (Figure 3.2A). Deletion of UL11 and gM (ΔUL11+ ΔgM2 virus) caused the production of viral plaques that were 4- to 5-fold larger than those of ΔUL20 virus. Deletion of either gE, the

carboxyl terminus of gD (gDΔct), or gM (ΔgM2) alone did not drastically reduce the average plaque size, while simultaneous deletion of gD (gDΔct), gM (ΔgM2), and gE (ΔgE) reduced average plaque sizes to the same extent as deletion of UL11 alone (Figure 3.2A). To better assess the virus plaque sizes produced by individual mutant viruses, randomly chosen viral plaques were measured and statistically analyzed as described in Materials and Methods. This analysis confirmed that the most drastic reduction in average viral plaque sizes was produced by ΔUL20, followed by the ΔUL11+ΔgM2, ΔUL11+ΔgM1, ΔUL11, and gDΔct+ΔgM2+ΔgE viruses (Figure 3.2B).

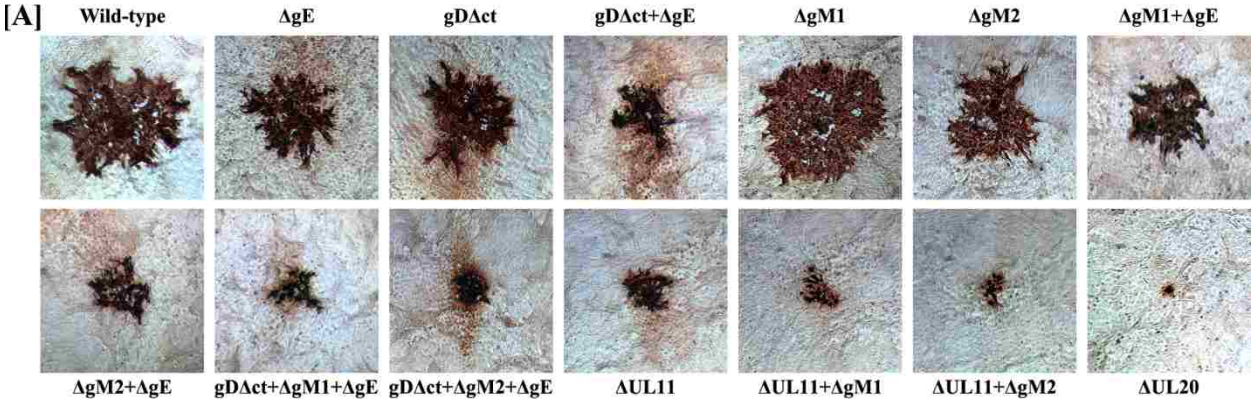

Figure 3.2A: Plaque phenotypes of wild-type and mutant viruses. Confluent Vero cell monolayers were infected with each virus at an MOI of 0.001, and viral plaques were visualized at 48 hpi by immunohistochemistry as described in Materials and Methods.

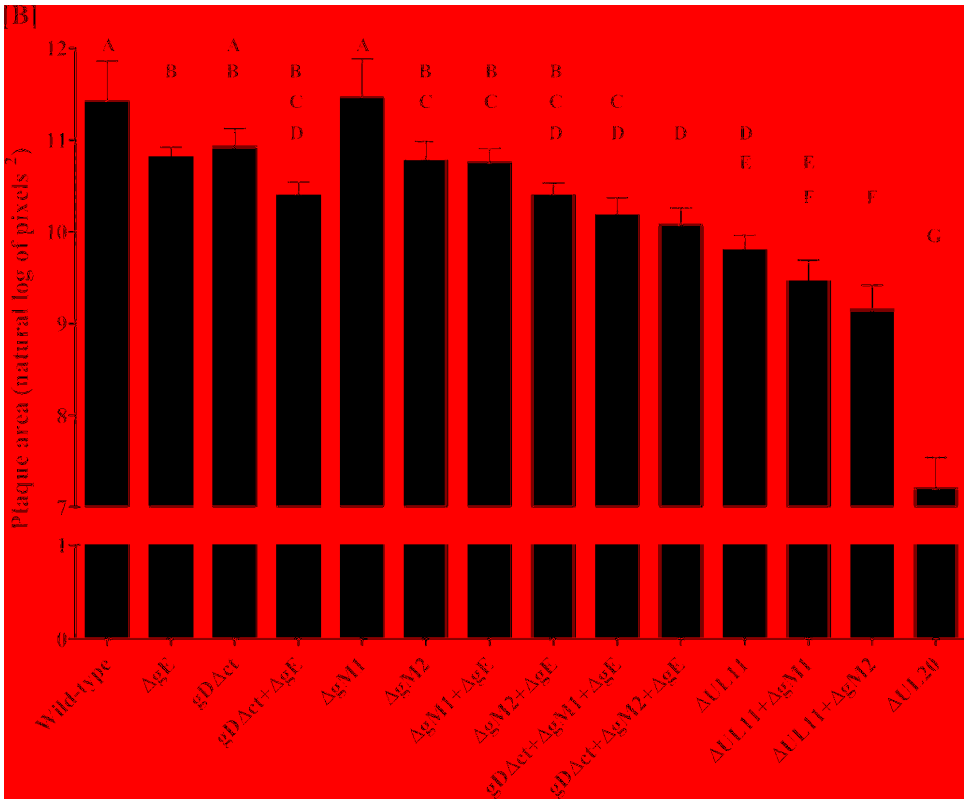

Figure 3.2B: Plaque area analysis. Confluent Vero cell monolayers were infected with each virus at an MOI of 0.001, and viral plaques were visualized at 48 hpi by immunohistochemistry as described in Materials and Methods. Thirty different viral plaques were randomly selected, imaged, measured, and statistically analyzed as described in Materials and Methods. Natural log-transformed data depicted as bar graphs for each virus are shown as geometric means with 95% confidence intervals. Tukey's test was performed after one-way analysis of variance to examine pairwise differences between the means for each of the 14 viruses. Viruses that were significantly different from each other ($P \leq 0.05$) are labeled with different capital letters (A, B, C, D, E, F, and G).

Replication characteristics of HSV-1 mutants

To examine the effect of the various engineered mutations on virus replication, Vero cells were infected at an MOI of either 0.2 or 3.0 with either the wild-type or each mutant virus, and viral titers were obtained at 24 and 48 hpi (Table 3.2). At an MOI of 0.2, ΔUL20 replicated approximately 3 log units less efficiently at both 24 and 48 hpi than the HSV-1(F) virus. All other mutant viruses achieved titers that were approximately 1 log unit or higher than the ΔUL20

virus titer at 24 hpi and 48 hpi (Table 3.2). Similar results were obtained when an MOI of 3.0 was used, with the exception that all mutant viruses other than ΔUL20 virus replicated more efficiently at the high MOI than at the low MOI, with viral titers approaching those produced by the HSV-1(F) wild-type virus at 48 hpi (Table 3.2).

Table 3.2: Comparison of wild-type and mutant virus replication. Viral titers were determined at different times after infection of Vero cells at an MOI of 0.2 (A) or MOI of 3.0 (B). The experiment was performed a second time and the titers obtained were averaged, with the standard deviation calculated for each time point. Not done (ND).

A

Mutant viruses	0 hpi	24 hpi	48 hpi
Wild-type	$6.00x10^{1} \pm 28.3$	$1.67x10^{7} \pm 9.02x10^{5}$	$1.63x10^{7} \pm 6.11x10^{5}$
ΔgE	$4.00x10^{1} \pm 0.00$	$8.67x10^{6} \pm 1.16x10^{6}$	$2.31x10^{7} \pm 1.35x10^{6}$
gDΔct	$2.00x10^{1} \pm 0.00$	$2.88x10^{6} \pm 1.68x10^{5}$	$1.56x10^{7} \pm 8.72x10^{5}$
gDΔct+ΔgE	$3.00x10^{1} \pm 14.1$	$5.67x10^{5} \pm 4.37x10^{4}$	$6.53x10^{6} \pm 1.12x10^{6}$
ΔgM2	$2.00x10^{1} \pm 0.00$	$4.27x10^{6} \pm 7.69x10^{5}$	$4.80x10^{6} \pm 6.11x10^{5}$
ΔgM2+ΔgE	$1.00x10^{1} \pm 14.1$	$4.07x10^{6} \pm 5.21x10^{5}$	$9.20x10^{6} \pm 5.03x10^{5}$
gDΔct+ΔgM2+ΔgE	$3.00x10^{1} \pm 14.1$	$5.73x10^{5} \pm 5.93x10^{4}$	$2.55x10^{6} \pm 1.55x105$
ΔUL11	$1.00x10^{1} \pm 14.1$	$4.53x10^{5} \pm 4.16x10^{4}$	$1.14x10^{7} \pm 1.44x10^{6}$
ΔUL11+ΔgM2	$2.00x10^{1} \pm 0.00$	$1.13x10^{5} \pm 1.62x10^{4}$	$1.64x10^{6} \pm 8.00x10^{4}$
ΔUL20	$4.00x10^{1} \pm 28.3$	$3.81x10^{4} \pm 6.22x10^{2}$	$6.13x10^{4} \pm 2.31x10^{3}$

B

Mutant viruses	0 hpi	24 hpi	48 hpi
Wild-type	$4.60x10^{2} \pm 84.8$	$1.03x10^{7} \pm 8.97x10^{5}$	$7.61x10^{6} \pm 2.39x10^{6}$
ΔgE	$2.50x10^{2} \pm 42.4$	$1.27x10^{7} \pm 1.43x10^{6}$	$8.55x10^{6} \pm 3.76x10^{6}$
gDΔct	$2.40x10^{2} \pm 28.3$	$3.99x10^{6} \pm 3.15x10^{5}$	$4.40x10^{6} \pm 5.77x10^{5}$
gDΔct+ΔgE	$1.80x10^{2} \pm 56.6$	$3.40x10^{5} \pm 2.31x10^{4}$	$3.60x10^{6} \pm 6.43x10^{5}$
ΔgM2	$1.60x10^{2} \pm 56.6$	$4.73x10^{6} \pm 5.21x10^{5}$	$4.73x10^{6} \pm 7.33x10^{5}$
ΔgM2+ΔgE	$7.00x10^{1} \pm 14.1$	$7.13x10^{6} \pm 1.10x10^{6}$	$7.13x10^{6} \pm 8.51x10^{5}$
gDΔct+ΔgM2+ΔgE	$1.50x10^{2} \pm 14.1$	$3.17x10^{6} \pm 1.89x10^{5}$	$2.43x10^{6} \pm 1.30x10^{5}$
ΔUL11	$3.80x10^{2} \pm 28.3$	$1.99x10^{7} \pm 4.81x10^{5}$	$2.41x10^{7} \pm 1.33x10^{5}$
ΔUL11+ΔgM2	ND	ND	ND
ΔUL20	$2.70x10^{2} \pm 14.1$	$1.34x10^{5} \pm 2.34x10^{3}$	$1.61x10^{5} \pm 3.51x10^{3}$

Protein expression profiles of viral mutants

To confirm that the engineered gene mutations resulted in lack of expression of the relevant protein and to investigate whether deletion of one or more viral glycoproteins affected the synthesis of other viral glycoproteins, all mutant viruses were tested for the expression of gB, gC, gD, gE, gM, and UL11. As expected, all mutant viruses containing the ΔgM2 mutation failed to express gM (Figure 3.3). The ΔgM1 virus expressed a truncated gM glycoprotein migrating in a manner consistent with the deletion of 19 amino acids from the amino terminus of gM, as previously reported by others (5) (data not shown). The ΔUL11 mutation caused a lack of UL11 expression for all recombinant viruses containing this mutation. The gDΔct deletion caused the appearance of a truncated gD migrating with the expected apparent molecular mass, as we have reported previously (36). Mutant viruses unable to produce gM and gE or gM, gE, and gDΔct (DME2) appeared to express substantially smaller amounts of gB than all other viruses tested, while all viruses synthesized equivalent levels of gC. In addition, the gDΔct+gE or

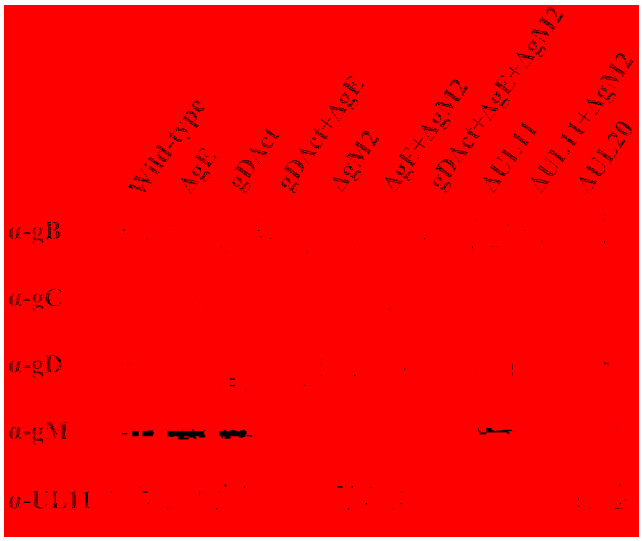

Figure 3.3: Western immunoblot analysis of glycoproteins specified by mutant viruses. α-gB, α-gC, α-gD, α-gM, and α-UL11 denote antibodies specific for each protein.

gM+gE+gDΔct viruses showed substantial reductions in the relative production of the UL11 protein (Figure 3.3). The expression of gE was tested by indirect immunofluorescence, since the available antibody did not react strongly enough in immunoblots. IFA results showed that gE was not expressed in cells infected with viruses specifying the ΔgE mutation, while gE expression was unaffected by any of the other gene deletions (Figure 3.4).

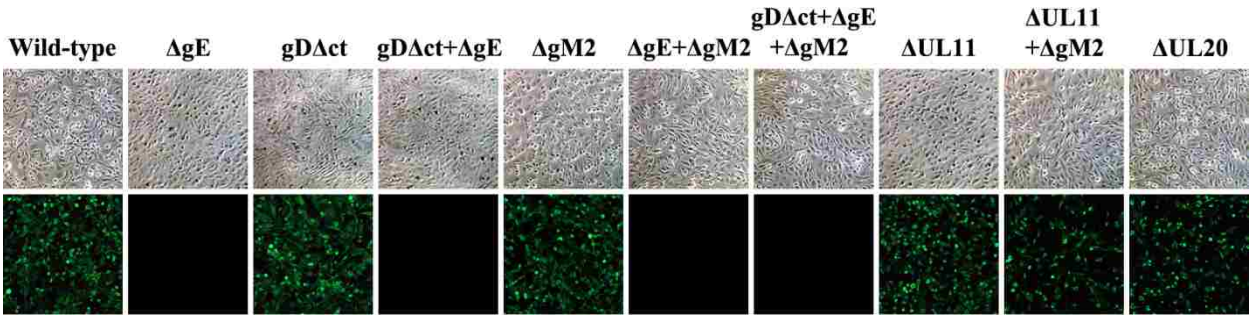

Figure 3.4: Immunofluorescence detection of gE expression. Vero cells were infected at an MOI of 1 with the viruses indicated, and gE expression was detected by indirect immunofluorescence at 24 hpi. (Top row) Phase contrast micrographs of infected cells treated with anti-gE Mab; (bottom row) fluorescent micrographs of the same infected cells. All mutant viruses containing the ATG-to-CTG mutations in gE (ΔgE) failed to react with anti-gE antibody, while viruses expressing the wild-type gE reacted with anti-gE antibody.

Ultrastructural characterization of wild-type and mutant viruses

The ultrastructural phenotypes of all viruses relative to the wild-type parental virus were investigated at 16 hpi utilizing transmission electron microscopy and visually examining more than 50 individual virus-infected Vero cells. As expected, the wild-type virus did not exhibit any apparent defects in cytoplasmic virion envelopment or egress, as evidenced by the presence of fully enveloped virions intracellularly and extracellularly (Figure 3.5). Ultrastructural visualization of Vero cells infected with the different mutant viruses revealed a diverse range of cytoplasmic defects in virion envelopment. The most pronounced effects were produced by the

ΔUL20 and ΔUL11+ΔgM2 viruses, which produced numerous unenveloped capsids. In contrast, the DME2 virus produced fully enveloped virions that were excreted out of infected cells (Figure 3.5).

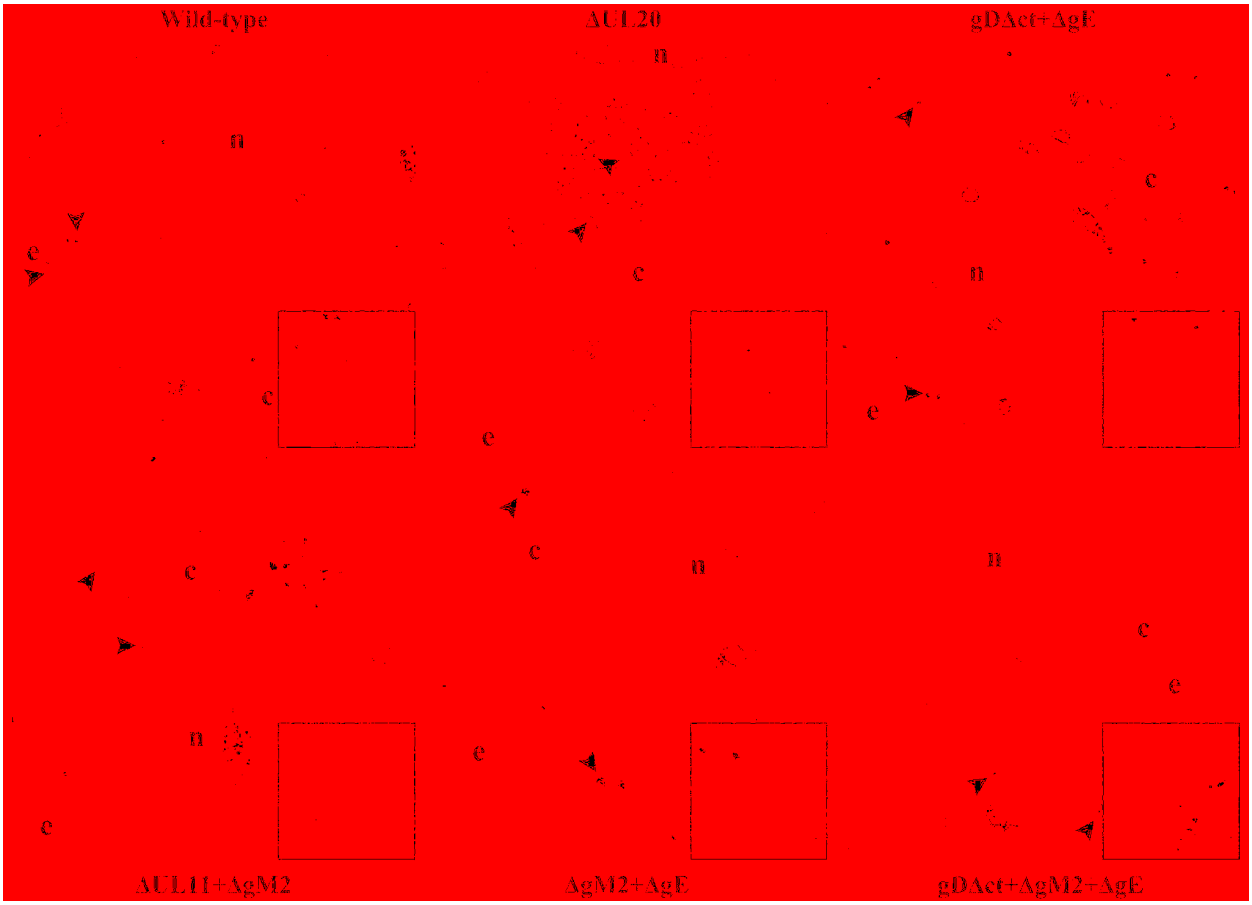

Figure 3.5: Ultrastructural morphology of wild-type and mutant viruses. Electron micrographs of Vero cells infected with different viruses at an MOI of 3 and processed for electron microscopy at 16 hpi are shown. Enlarged sections of each micrograph are included as insets in each panel. The nucleus (n), cytoplasm (c), and extracellular space (e) are marked. Representative virions are marked with black arrowheads.

Quantification of relative efficiency of infectious virus production and egress from infected cells

The number of virus particles (enveloped and unenveloped capsids) can be indirectly estimated by determining the number of viral genomes obtained after DNase I treatment (see Materials and Methods). The ΔUL20 virus infection resulted in the least efficient production of

infectious virus within cells, followed by infection with the ΔUL11+ΔgM2 virus. All other viruses produced infectious virions with intermediate efficiencies ranging between those of virus strains HSV-1(F), ΔUL20, and ΔUL11+ΔgM2 (Table 3.3). As expected, particle/PFU ratios in supernatants of infected cells were much lower than those obtained from the cytoplasmic fraction of infected cells for all viruses, with the exception of the ΔUL20 virus, which produced high numbers of noninfectious virion particles in the supernatant.

Table 3.3: Determination of viral particle-to-PFU ratios. *Takes into account data from Q-PCR, replication kinetics and plaque size quantification. †Cells infected with ΔUL20 HSV-1 display a more apoptotic phenotype than cells infected with wild-type virus, leading to release of more viral genomes into the supernatant.

Virus	Cytoplasm (Particles/PFU)	Supernatant (Particles/PFU)	Overall defect*
Wild-type	**109**	**6**	**+**
ΔgE	**425**	**4**	**++**
gDΔct	**381**	**10**	**++**
gDΔct+ΔgE	**855**	**218**	**+++**
ΔgM2	**2355**	**4**	**++**
ΔgM2+ΔgE	**417**	**9**	**++**
gDΔct+ΔgM2+ΔgE	**1779**	**12**	**+++**
ΔUL11	**590**	**60**	**+++**
ΔUL11+ΔgM2	**3460**	**70**	**++++**
ΔUL20	**8509**	**1702†**	**+++++**

Discussion

Mature virions acquire their viral envelopes by budding into cytoplasmic membranes originating from the TGN. Multiple interactions between the cytoplasmic portions of viral glycoproteins and tegument proteins facilitate cytoplasmic virion envelopment [reviewed in references (32, 42)] (Figure 3.6). The fact that lack of expression of UL11, gD, gE, or gM alone does not drastically affect cytoplasmic virion envelopment has led to the hypothesis that the cytoplasmic portions of glycoproteins gD, gM, and gE and membrane-associated protein UL11

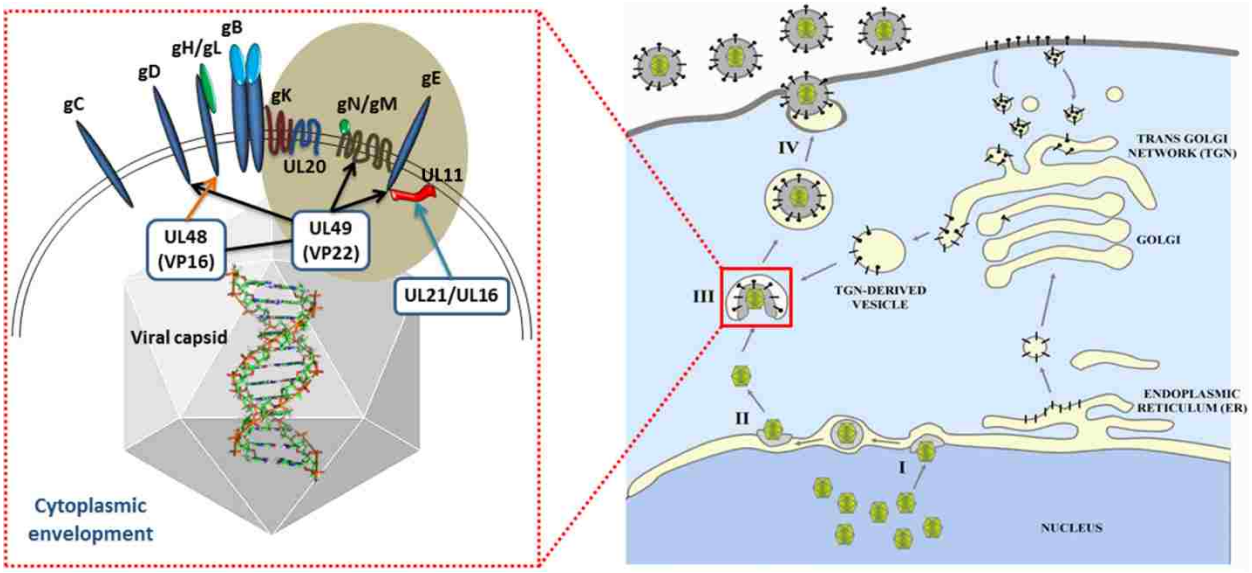

Figure 3.6: Diagrammatic description of glycoprotein-tegument protein interactions during cytoplasmic virion envelopment at TGN-derived vesicles. (Right) The different steps in virion egress from infected cells: I, budding of nuclear capsids into perinuclear spaces; II, deenvelopment at the outer nuclear membrane and release of capsids into cytoplasm; III, secondary envelopment at TGN membranes; IV, extracellular transport of enveloped virions. Glycoproteins are shown as black bars being synthesized in the endoplasmic reticulum and transported via vesicles to the Golgi apparatus and expressed on infected cell surfaces. (Left) Interactions of tegumented capsids with the carboxyl termini of glycoproteins and membrane proteins: UL49 (VP22) is shown to interact with gD, gM, and gE; UL48 (VP16) interacts with gH and VP22; UL21 and UL16 interact with UL11 [reviewed in references (34, 42)]. UL20, UL11, gK, gM, and gE are shown within a brown sphere to highlight their potential cooperative relationships in infectious virion morphogenesis.

function in a redundant manner to facilitate virion envelopment (16, 32, 37, 42). To directly test this hypothesis and compare the relative importance of viral glycoproteins and membrane proteins in virion envelopment, we generated a set of mutant viruses carrying one or multiple mutations in viral glycoproteins gD, gE, and gM and in UL11 and compared them to viruses lacking expression of UL20. The results presented herein show that lack of expression of the UL20 (or gK) gene causes the most severe inhibition of cytoplasmic virion envelopment in comparison to that for all other viruses lacking expression of one or more of the gD, gE, gM, and UL11 genes.

Previously, we reported that a recombinant virus lacking expression of gE (ΔgE) and expressing a truncated version of gD (gDΔct) did not exhibit any major defect in cytoplasmic virion envelopment (28). These results suggest that gE and gD do not appear to function in a redundant manner to facilitate cytoplasmic virion envelopment, despite their known interactions with tegument proteins and the membrane-associated protein UL11 (18, 30, 32, 36, 43, 48, 57) (Figure 3.6). Deletion of the gM gene resulted in a reduction in the average size of viral plaques produced (5) and an increase in the accumulation of unenveloped capsids in the cytoplasm of HSV-1 infected cells (6, 39). Similar results have been reported for the alphaherpesviruses pseudorabies virus (PRV) and equine herpesvirus 1 (EHV-1) (15, 44). These defects in cytoplasmic virion envelopment are associated, at least in part, with the interactions between gM and the major tegument protein UL49 demonstrated for PRV (26) and HSV-1 (48) (Figure 3.6). We show here that simultaneous deletion of the carboxyl terminus of gD and the entire gM and gE genes causes mild reductions in average plaque size, cytoplasmic virion envelopment, and infectious virus production in comparison to those for the virus carrying the

gDΔct+ΔgE combined mutations, suggesting that gM does not play a significant redundant role with gD and gE in these events. Direct comparison with the ΔUL20 virus reveals that deletion of the UL20 gene is substantially more deleterious to infectious virus production than the combined effect of the gDΔct, ΔgM2, and ΔgE mutations.

Lack of gM expression caused decreased synthesis of gB, while deletion of the carboxyl-terminal 29 amino acids of gD (gDΔct) caused decreased synthesis of UL11. It was recently shown that the carboxyl terminus of gE interacts with UL11, causing their coincorporation into virion particles (48). gD and gH have been shown to bind to gB and modulate its fusogenicity (1-4, 14, 28). We have shown that gK and UL20 interact with both gB and gH (13). We have recently found that UL20 interacts with gM (V. N. Chouljenko and K. G. Kousoulas, unpublished data), suggesting that that the gK-UL20 and gM-gN complexes interact (Figure 3.6). Virion tegument protein VP22 (UL49) interacts with cytoplasmic domains of gD, gE, and gM, as well as its tegument partner, VP16 (UL48) (11, 18, 26). Moreover, VP16 binds to the carboxyl terminus of gH (29). Collectively, these results suggest that there are multiple interactions among viral glycoproteins and tegument proteins and that deletion of one or more viral proteins may indirectly affect the functions and stability of other interacting proteins.

Lack of expression of both the UL11 and gM genes inhibited cytoplasmic virion envelopment in Vero cells to a lesser extent than in other cells, while a similar deletion in PRV caused drastic inhibition of infectious virus production (35, 37). Therefore, it was of interest to generate a similar mutant virus in the HSV-1(F) genetic background to directly compare it with other mutant viruses. Simultaneous deletion of the HSV-1(F) UL11 and gM genes caused a substantial reduction in cytoplasmic virion envelopment and infectious virus production which

was greater than that observed in the case of the gDΔct+ΔgM2+ΔgE triple mutant virus. However, the ΔUL11 and ΔgM2 defects were markedly less severe than the ΔUL20 defect, suggesting that UL20 and its interacting partner, gK (21, 24), play more important roles than gD, gM, and gE alone or in combination. Recently, it was reported that gB may function with gD in a redundant manner to facilitate cytoplasmic virion envelopment (34). Therefore, lack of gK or UL20 may affect the binding ability of gB, gD, and gH to bind to tegument proteins. Alternatively, gK and UL20 may directly bind to tegument proteins, facilitating cytoplasmic virion envelopment. Additional experiments are needed to discern the functions of gK and UL20 in cytoplasmic virion envelopment, egress, and infectious virus production.

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CHAPTER IV: HERPES SIMPLEX VIRUS 1 PROTEIN UL37 INTERACTS WITH VIRAL GLYCOPROTEIN K AND MEMBRANE PROTEIN UL20 AND FUNCTIONS IN CYTOPLASMIC VIRION ENVELOPMENT*

Introduction

The herpes simplex virus 1 (HSV-1) double-stranded DNA genome is enclosed in an icosahedral capsid and embedded within a proteinaceous tegument containing multiple viral proteins, all of which is packaged within an envelope decorated with multiple membrane proteins and glycoproteins (8, 21, 36, 49, 55). HSV-1 encodes at least 26 tegument proteins and 11 glycoproteins as well as several nonglycosylated membrane-associated proteins. The final steps in virion envelopment occur in the cytoplasm of infected cells and involve multiple interactions among viral proteins, including interaction between cytoplasmic portions of viral glycoproteins and tegument proteins bound to cytoplasmic capsids, as well as tegument-tegument protein interactions (7, 8, 41, 51, 54).

HSV-1 UL37 is an approximately 120-kDa phosphorylated tegument protein expressed in both mature virions and light particles (1). The UL37 protein localizes to both the nucleus and cytoplasm in infected cells. The UL37 cytoplasmic localization is considered to be due to the presence of a nuclear export signal (NES) sequence within the UL37 protein that mediates

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transport of the UL37 protein to the cytoplasm in the absence of any other viral proteins (10, 37, 49, 56). The UL37 protein is capable of self-association and interacts via its carboxyl terminus with the UL36 major tegument protein, and this interaction is necessary and sufficient for transport of the UL36-UL37 protein complex to the *trans*-Golgi network (TGN), where virion envelopment is thought to occur (6, 10, 27, 30).

Deletion of the UL37 gene specified by either HSV-1 or pseudorabies virus (PRV) inhibits cytoplasmic virion envelopment, causing the accumulation of unenveloped capsids in the cytoplasm of infected cells (11, 33, 46). However, while the UL37 gene is absolutely essential for HSV-1 replication, deletion of the PRV UL37 gene allows the resultant mutant virus to replicate, albeit 100-fold less efficiently than the parental wild-type virus, thus demonstrating that certain PRV UL37 functions may be shared with other PRV proteins (11, 29). The pUL36 in the UL37-UL36 protein complex is thought to recruit the microtubule motors dynein, kinesin-1, and kinsein-2 onto capsids in the cytosol (47). These interactions are further supported by *in vitro* experiments in which capsids complexed with inner tegument proteins were able to associate with the microtubule motors, while the capsids lacking the tegument proteins did not (44). Thus, the UL37 protein plays important roles in capsid trafficking during virion entry, in a retrograde manner, i.e., towards the nucleus, and in conjunction with dynein motors and the microtubular network, while also playing an important role in capsid transport to the TGN (2, 12, 35, 42, 45, 57).

The UL36-UL37 protein complex is part of a multiprotein complex within the virion particle and within infected cells. Specifically, the UL36 protein interacts with the outer tegument protein pUL48 (55) and the minor capsid protein UL25 (43). Additionally, the UL37

protein interacts with the UL46 and the UL35 proteins (32). Also, it has been reported that UL37 is phosphorylated by cellular enzymes and that it interacts in the cytoplasm with ICP8, the major HSV-1 DNA binding protein, and is transported to the nucleus of infected cells (1). The HSV-1 UL37 protein contains a TRAF6 (tumor necrosis factor receptor-associated factor 6) binding domain that binds to TRAF6 and activates NF-kB expression, required for efficient viral replication, in a Toll-like receptor 2 (TLR2)-independent manner (34). These results suggest that the UL37 protein specifies additional functions in viral replication beyond its role in virion entry and cytoplasmic virion envelopment.

HSV-1 gK is a multimembrane-spanning viral glycoprotein encoded by the UL53 gene. It is expressed on virions, exists as a functional complex with the membrane-associated UL20 viral protein, and plays a role in both virion entry and cytoplasmic virion envelopment (15, 18, 19). The functions of the gK-UL20 protein complex in virion entry and cytoplasmic virion envelopment can be segregated genetically and physically from each other (9, 14-16, 23, 39). Functions of gK-UL20 in virion entry involve direct interactions of the amino termini of both gK and UL20 with the fusogenic viral glycoprotein gB as well as with the membrane fusion regulator glycoprotein gH (9). These glycoproteins regulate fusion of the viral envelope with cellular membranes during virus entry, as well as virus-induced cell-to-cell fusion, i.e., formation of multinucleated cells (syncytia) which allow the virus to spread from cell to cell (4, 5, 13, 40).

Here we demonstrate for the first time that the UL37 protein physically interacts with both gK and UL20 proteins in infected cells. Moreover, overexpression of the UL20 protein in FRT cells complements viral replication of the UL37 mutant DC480 virus, which is unable to

acquire cytoplasmic envelopes, suggesting that the gK-UL20 interactions with UL37 facilitate cytoplasmic virion envelopment.

Materials and Methods

Cell lines

African green monkey kidney (Vero) cells were obtained from the American Type Culture Collection (Rockville, MD). The Vero-based UL37-complementing cell line BD45 was a gift from Prashant Desai (Johns Hopkins University, Baltimore, MD). The construction and use of the UL20-expressing FRT/UL20 cell line were detailed previously (39). The gK-transformed cell line VK302 was a gift from David C. Johnson (Oregon Health Sciences University, Portland, OR). All cells were maintained in Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal calf serum and antibiotics.

Construction of HSV-1 mutant viruses

Mutagenesis was accomplished in *Escherichia coli* by using the markerless two-step Red recombination mutagenesis system and synthetic oligonucleotides (31, 53) (DC480 Forward, GGGTCGAACGTGTTTGGTCTGGCGCGGGAATACGGGTACTATGCCAACTACGATTACAAGGATGACGA CGATAAG; and DC480 Reverse, CCGCCCGTGCGTGTGCTCGCTGGCGCCCTGGACCCGCCTGAAAGTT TTTACCTTGCCATCGATCAGTCGCGGATCCACCTGGTCTCGTAGTTGGCATAGTACCCGTACAACCAATT AACCAATTCTGATTAG) implemented on the bacterial artificial chromosome (BAC) plasmid pYEbac102 carrying the HSV-1(F) genome (52) (a gift from Y. Kawaguchi, University of Tokyo, Japan). Construction of the HSV-1 mutant virus ΔUL20 (UL20-null) was described previously (31). The DC480 recombinant virus was constructed by inserting a 12-amino-acid epitope tag (EDQVDPRLIDGK) for protein C (protC tag) into the HSV-1 UL37 gene immediately after the

codon for amino acid 480 (Figure 4.1). The recombinant mutant virus YE102-VC1 was modified to express gK and UL20 genes containing V5 and 3XFLAG antigenic epitopes, respectively, and was described previously (23). Construction of the HSV-1 UL37-null mutant virus (generously provided by Prashant Desai, Johns Hopkins University, Baltimore, MD) was described previously (11).

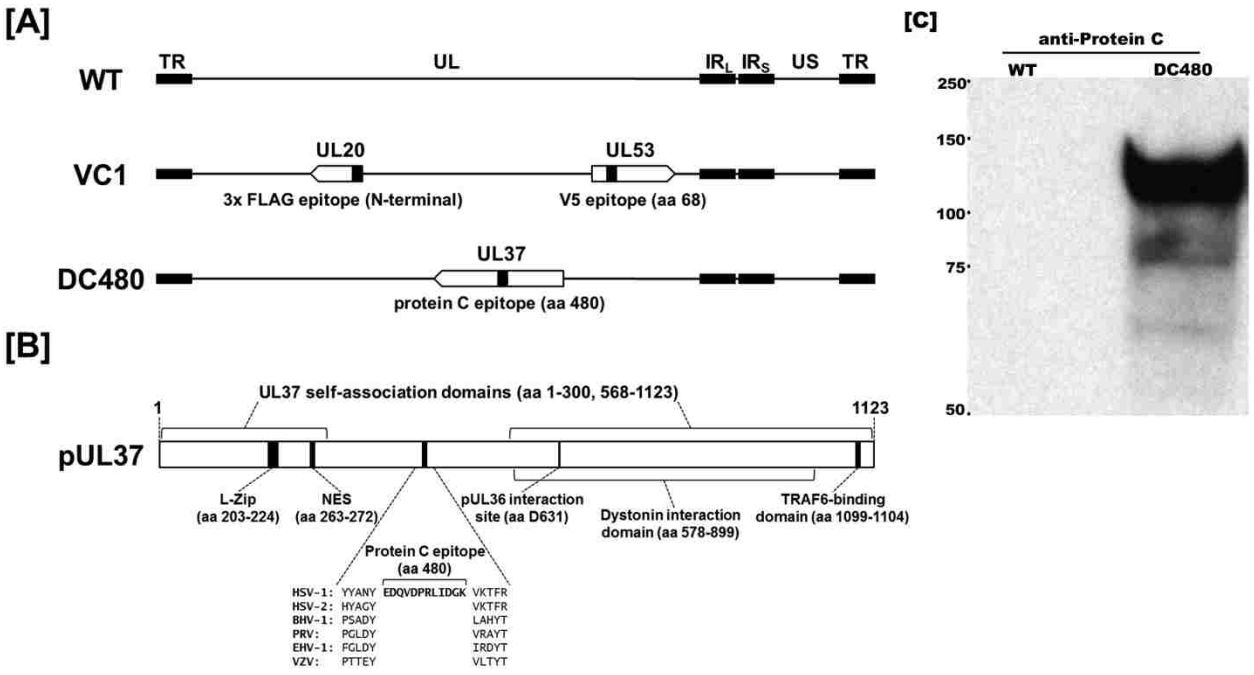

Figure 4.1: Schematics of recombinant viruses and the UL37 gene. (A) Schematic arrangements of the wild-type (WT) virus and mutant viruses VC1 and DC480, with the unique long (UL) and unique short (US) regions flanked by the terminal repeat (TR) and internal repeat (IR) regions. The VC1 virus expresses UL20 and gK tagged with 3XFLAG and V5 epitope tags, respectively. The DC480 virus contains a 12-amino-acid (aa) protein C epitope tag inserted in frame immediately following amino acid 480. (B) Schematic of the UL37 protein showing its functional domains. (C) Western immunoblot of cell extracts obtained from Vero cells infected with the DC480 virus, using anti-protC antibody. Lane 1, wild-type virus-infected cellular extracts; lane 2, DC480 virus-infected cellular extracts.

Confirmation of targeted mutations, recovery of infectious virus, and plaque morphology analysis

HSV-1 BAC DNAs were purified from 50-ml overnight BAC cultures by use of a Qiagen large-construct kit (Qiagen, Valencia, CA). All mutated DNA regions were sequenced to verify the presence of the desired mutations in BACs. Similarly, viruses recovered from infected Vero cells were sequenced to confirm the presence of the desired mutations. Viruses were recovered from cells transfected with BACs as described previously (31). Visual analysis of the plaque morphology of mutant viruses was performed as previously described (20, 31, 39, 40).

Viral growth kinetics

Analysis of viral growth kinetics was performed as we described previously (14, 19). Briefly, nearly confluent Vero cell monolayers were infected with each virus at 4°C for 1 h at a multiplicity of infection (MOI) of 0.2. Thereafter, plates were incubated at 37°C and 5% CO_2, and virus was allowed to penetrate for 1 h at 37°C. Any remaining extracellular virus was inactivated by low-pH treatment (pH 3.0), and cells were incubated at 37°C and 5% CO_2.

Electron microscopy

The ultrastructural morphology of virions within infected cells was examined by transmission electron microscopy essentially as described previously (16, 17, 25, 31, 40). All infected cells processed for electron microscopy were prepared at 18 h postinfection (hpi) and visualized on a JEOL transmission electron microscope.

Quantitative PCR (qPCR) analysis of cytoplasmic virions

Cytoplasmic virions were separated by glycerol shock treatment essentially as originally described by Sarmiento and Batterson (48) and later modified by our laboratory (8). Real-time PCR was carried out on viral DNA from the cytoplasmic fractions of Vero cells.

Specifically, the primers and probe (6-carboxytetramethylrhodamine [TAMRA]) for the real-time PCR were designed to detect HSV-1 US6 (gD) (gD forward primer, ACGTACCTGCGGCTCGTGA AGA; probe, 6-carboxyfluorescein [FAM]-GCCAAGGGCTCCTGTAAGTACGCCCT-TAMRA; and gD reverse primer, TCACCCCCTGCTGGTAGGCC). The cytoplasmic suspensions were treated with Turbo DNase I (Ambion, Inc.) for 1 h at 37°C. Viral DNA was extracted using a DNeasy blood and tissue kit (Qiagen, Inc.) per the manufacturer's instructions. Equal volumes of viral DNA were used for TaqMan PCR analysis. Purified HSV-1 bacterial artificial chromosome (YE102) DNA was used to generate the standard curve (8).

Immunoprecipitation and immunoblot assays

Confluent Vero cells in T75 flasks were infected with the double-tagged recombinant virus YE102-VC1 (gK-V5 and UL20-FLAG) or the UL37-null virus at an MOI of 2. At 24 hpi, the infected cells were lysed with NP-40 cell lysis buffer (Life Technologies) supplemented with protease inhibitor tablets (Roche). The samples were centrifuged at 13,000 rpm for 10 min at 4°C. The supernatants were then used for immunoprecipitation. The proteins from virus-infected cells were immunoprecipitated using protein G magnetic Dynabeads according to the manufacturer's instructions (Invitrogen). Briefly, the beads were bound to their respective antibodies and left on a nutator for 10 min, followed by the addition of cell lysates. The lysate-bead mixture was kept on the nutator for 10 min at room temperature and subsequently washed three times with phosphate-buffered saline (PBS). The protein was eluted from the magnetic beads in 40 µl of elution buffer and used for immunoblot assays. Sample buffer containing 5% β-mercaptoethanol was added to the protein and heated at 55°C for 15 min. Proteins were resolved in a 4 to 20% SDS-PAGE gel and immobilized on nitrocellulose

membranes. For Western immunoblots, subconfluent Vero cell monolayers were infected with the indicated virus at an MOI of 3. At 24 hpi, cells were collected by low-speed centrifugation, washed with PBS, and processed as described previously (15, 31). Immunoblot assays were carried out using monoclonal mouse anti-protein C antibody (Abcam, Cambridge, England), monoclonal mouse anti-VP5 antibody (Abcam, Inc., Cambridge, MA), monoclonal mouse anti-FLAG antibody (Sigma-Aldrich, Inc., St. Louis, MO), monoclonal mouse anti-V5 antibody (Invitrogen), horseradish peroxidase (HRP)-conjugated goat anti-mouse antibodies against the light-chain (Fab) and heavy chain (Fc) (Abcam, Inc., Cambridge, MA), polyclonal rabbit anti-UL37 antibody (a gift from Frank J. Jenkins, University of Pittsburgh Cancer Institute), and HRP-conjugated goat anti-rabbit antibody (Abcam, Inc., Cambridge, MA).

***In situ* PLA of protein interactions in virus-infected cells**

Vero cells were grown on 8-well chamber slides (Nunc Lab-Tek II chamber slide system) and infected with F strain virus (untagged) or the tagged virus VC1 (gk-V5 and UL20-FLAG) at an MOI of 10. At 18 h postinfection, the cells were fixed with ice-cold methanol for 10 min at -20°C. After three washes with PBS, the samples were blocked for 2 h at 37°C with Duolink blocking buffer in a humidity chamber. Primary antibodies that were raised in two different species were diluted in antibody diluting buffer, added to the samples, and incubated overnight at 4°C. Mouse anti-V5 antibody (Invitrogen) and rabbit anti-FLAG antibody (Sigma-Aldrich, Inc., St. Louis, MO) were used for gK and UL20 detection, respectively (positive control). Mouse anti-FLAG and rabbit anti-UL37 antibodies were used for the HSV-1(F)-infected (virus with no epitope tags) and VC1-infected cells to detect UL20-UL37 interaction. Mouse anti-V5 and rabbit anti-UL37 antibodies were used for HSV-1(F)- and VC1-infected cells to detect gK-UL37

interaction. Mouse anti-ICP8 (Abcam) and rabbit anti-FLAG was used to detect ICP8-UL20 interaction. Unbound primary antibodies were removed by washing with 1X Tris-buffered saline (TBS)-Tween 20 (0.05%) three times for 5 min each. Duolink Anti-Rabbit Plus and Anti-Mouse Minus *in situ* proximity ligation assay (PLA) probes were added to the samples (1:5 dilution) and incubated at 37°C for 1 h. After the incubation step, washes were done with Duolink wash buffer A, twice for five min each. The ligation stock was diluted 1:5 in high-purity water and added to the wells (40 to 80 µl), and the slides were incubated at 37°C for 30 min. The slides were washed with buffer A three times for five min each, amplification solution (40 to 50 µl) was added, and the slides were incubated for 1.5 h at 37°C. The slides were then washed with wash buffer B twice for 10 min each, and once with 0.01% buffer B. The slides were mounted with mounting medium (Duolink II), stored at -20°C, and protected from light until confocal images were taken. The confocal images were taken using a 60X objective on an Olympus Fluoview FV10i confocal laser scanning microscope.

Results

Construction and characterization of UL37 mutant viruses

The recombinant virus DC480 was constructed by insertion of a protein C (protC) epitope tag immediately after the UL37 gene region encoding amino acid 480 (Figure 4.1A). The protC epitope tag was inserted in frame immediately after a conserved tyrosine residue located within the central portion of UL37, distal to known functional domains of UL37 (Figure 4.1B). Expression of the UL37-protC-tagged protein was confirmed by Western immunoblot analysis with anti-protC antibody. The UL37-protC construct was detected as a protein species

migrating with an apparent electrophoretic mobility of approximately 120 to 130 kDa (Figure 4.1C), in agreement with previous published reports (10, 38, 49).

Plaque morphology and replication kinetics of the DC480 recombinant virus

The DC480 virus produced very small viral plaques on Vero and VK302 cells that were similar in appearance to those produced by the UL20-null virus and comprised, on average, 5 to 10 cells, but it formed nearly wild-type-like viral plaques on BD45 cells expressing the UL37 gene (Figure 4.2). The UL37-null virus produced very small viral plaques on Vero, VK302, and FRT cells but formed wild-type-like plaques on BD45 cells. The UL20-null virus formed wild-type-like viral plaques on the FRT cell line expressing the UL20 gene, as expected. Interestingly, the

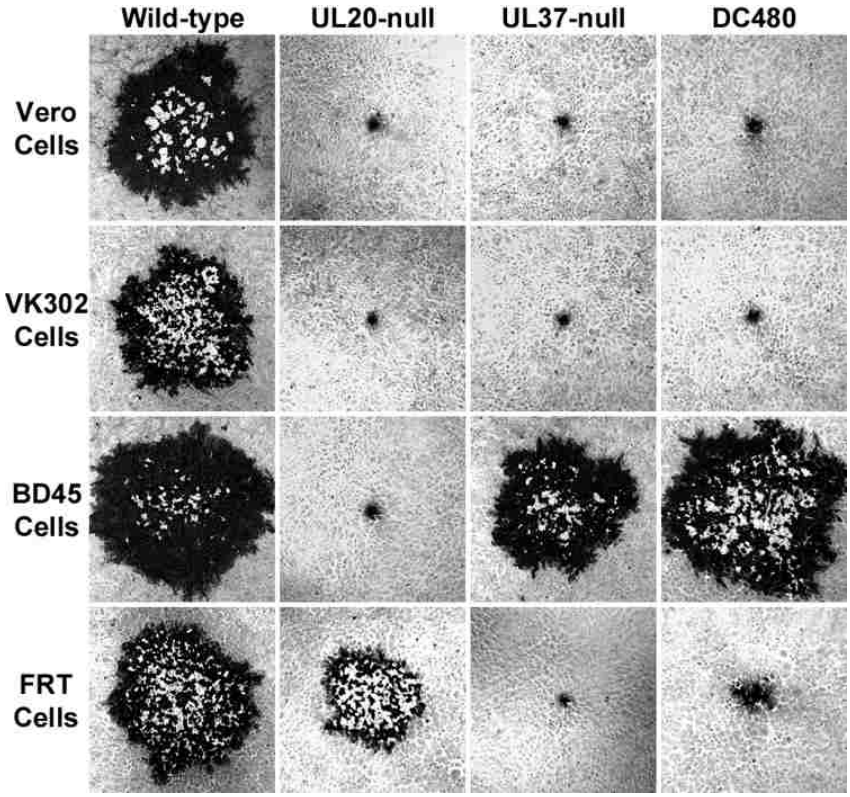

Figure 4.2: Representative plaque morphologies of wild-type and mutant viruses. Vero, BD45 (UL37 expressing), FRT (UL20 expressing), and VK302 (gK expressing) cell monolayers were infected at an MOI of 0.001, and viral plaques were visualized by immunohistochemistry using polyclonal rabbit anti-HSV-1 antibody at 48 hpi.

DC480 virus formed plaques on FRT cells that were approximately 5 to 10 times larger than the plaques produced by the same virus on Vero or VK302 cells (Figure 4.2). We reported previously that a lack of UL20 gene expression inhibits infectious virus production (17, 39). The DC480 virus replicated substantially less efficiently than the UL20-null virus in Vero cells, producing infectious virion titers that were nearly 4 log lower than those of the wild-type virus at late times postinfection, while the UL20-null titers were approximately 2 ½ log lower than those of the wild-type virus (Figure 4.3A). In contrast, the DC480 virus replicated nearly 20-fold more efficiently in FRT cells than in Vero cells, while the wild-type virus replicated equally efficiently in both FRT and Vero cells (Figure 4.3B). The replication efficiency of the UL37-null virus on FRT cells was approximately 20-fold lower than that of the DC480 virus. As expected, the UL20-null virus was able to replicate in FRT cells substantially better than in Vero cells, although final titers remained lower than those of the wild-type virus (Figure 4.3A and B).

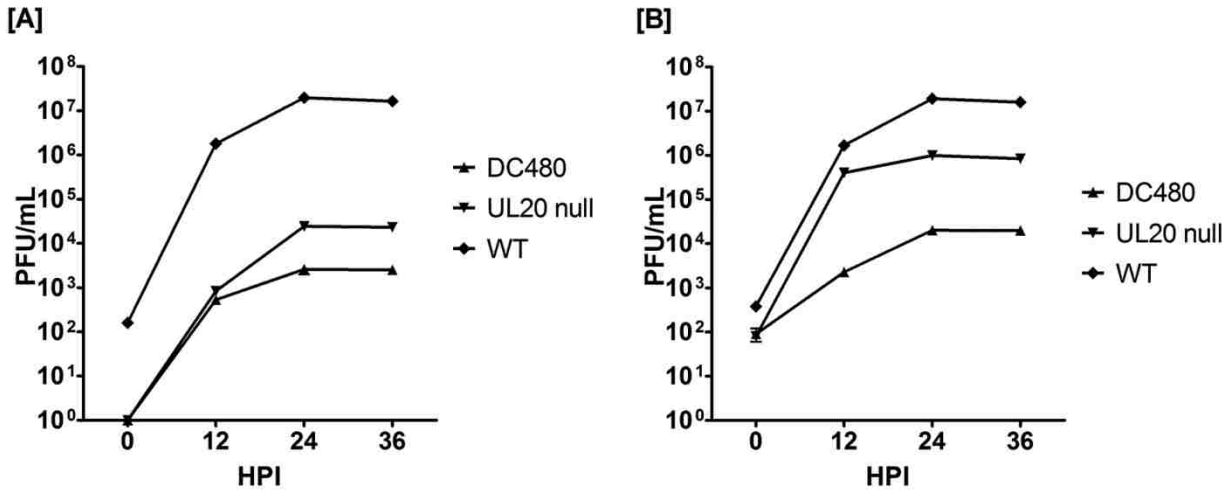

Figure 4.3: Replication kinetics of wild-type and mutant viruses. Confluent Vero (A) or FRT (B) cell monolayers were infected with each virus shown at an MOI of 0.2. Viral titers were obtained by plaque assay on the appropriate cell lines. The titers obtained were averaged, and the standard error of the mean was calculated for each time point.

Evaluation of envelopment efficiencies of the DC480 virus

Ultrastructural visualization of infected cells revealed that the DC480 virus exhibited drastic defects in virion envelopment, as evidenced by the accumulation of unenveloped capsids in the cytoplasm of infected Vero cells and the absence of enveloped virions extracellularly, as previously reported for the UL37-null virus (11, 42), in comparison to wild-type virus infection. In contrast, the DC480 virions appeared to be egressing efficiently out of BD45 cells (Figure 4.4).

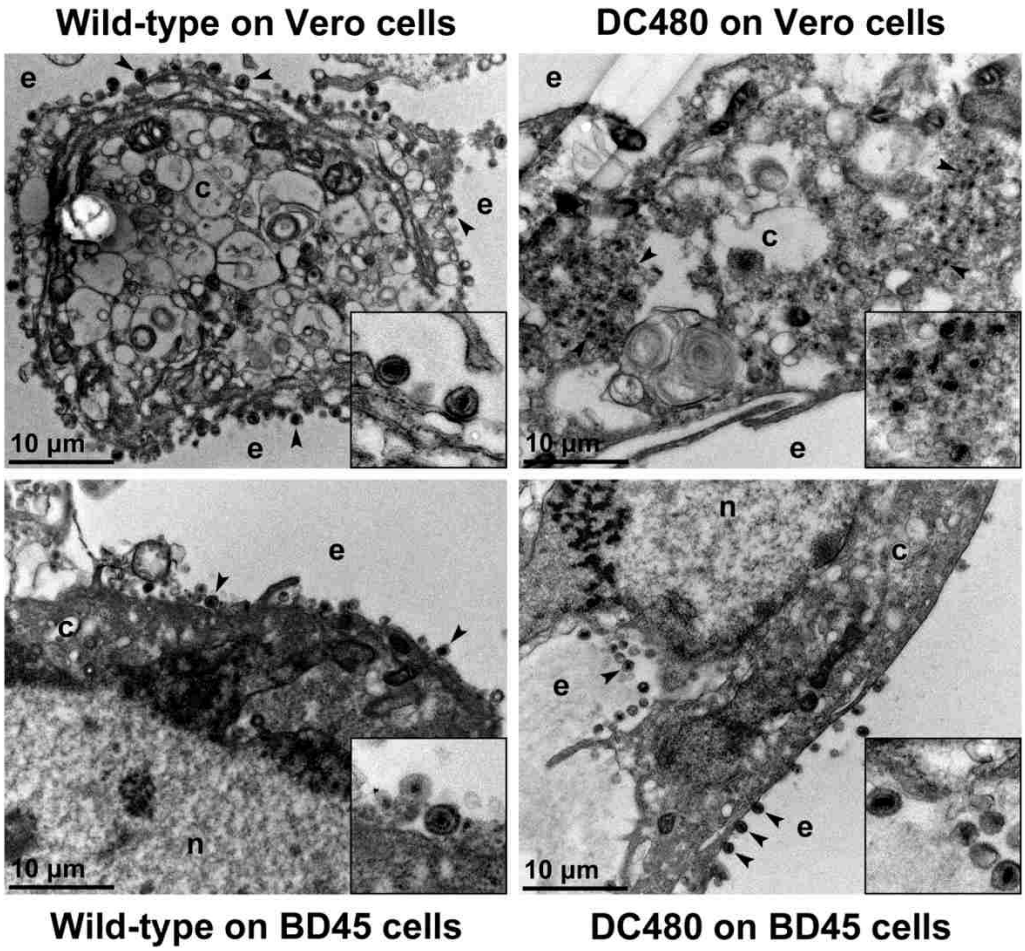

Figure 4.4: Ultrastructural morphology of wild-type and mutant viruses. Electron micrographs of Vero or BD45 cells infected at an MOI of 3 with wild-type or DC480 virus and processed for electron microscopy at 18 hpi are shown. Enlarged sections of the micrographs are included as insets. The nucleus (n), cytoplasm (c), and extracellular space (e) are marked. Representative virions are marked with black arrowheads.

We previously described the use of qPCR for determination of the relative efficiency of viral cytoplasmic envelopment (8). In this assay, the total number of viral genomes found within DNase-resistant viral capsids is determined via qPCR and compared to the number of infectious virions obtained by plaque assay. The wild-type virus was efficiently enveloped and formed into infectious virions in both Vero and BD45 cells at an MOI of 1. In contrast, the viral envelopment and infectious virion production efficiency of the DC480 virus were inhibited >700-fold in Vero cells in comparison to BD45 cells. Ratios of the numbers of encapsidated DC480 viral genomes and PFU were only approximately 2-fold lower than that of the wild-type virus on BD45 cells (Figure 4.5).

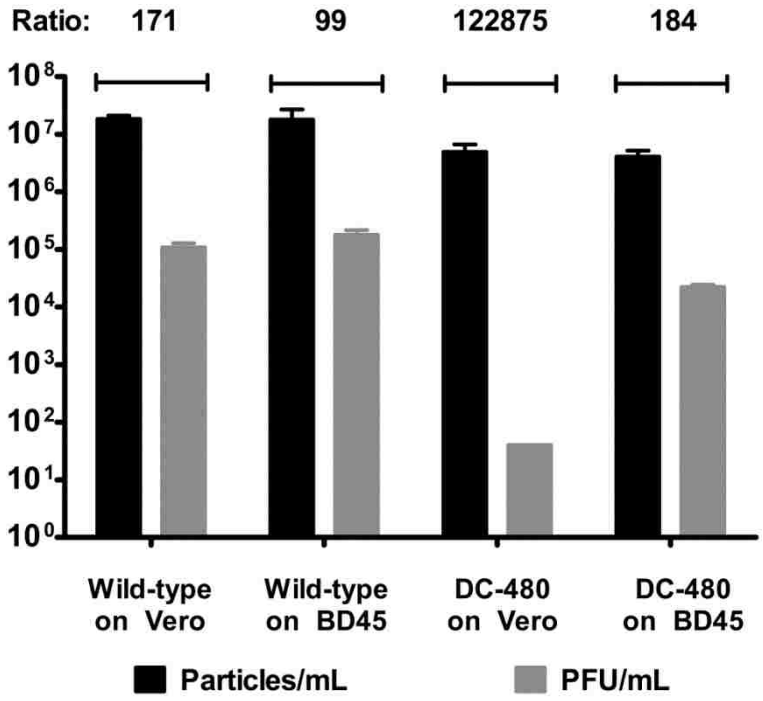

Figure 4.5: Determination of relative efficiencies of virion envelopment of wild-type and mutant viruses in Vero or BD45 cells. The total number of viral genomes in the cytoplasm of infected Vero or BD45 cells at 24 hpi that were protected from DNase I treatment was obtained by qPCR. The total number of intracellular infectious virions was obtained by plaque assay. Ratios reflecting relative efficiencies of envelopment and infectious virion production were obtained by dividing the average number of capsid-protected viral genomes by the number of PFU. Error bars represent standard errors of the means.

Determination of UL37 interactions with gK and UL20

To evaluate whether the UL37 protein interacts physically with either gK or the UL20 membrane protein, coimmunoprecipitation experiments were performed using the VC1 virus (Figure 4.1A), which expresses gK tagged with a V5 epitope inserted in frame within the amino terminus of gK and UL20 tagged with a 3XFLAG epitope inserted in frame after amino acid 4 of the UL20 protein (23). Detection of the UL37 protein was achieved by using a rabbit anti-UL37 polyclonal antibody (49). The UL37 protein was detected as migrating with an apparent molecular mass of 130 kDa (Figure 4.6A, arrow). Immunoprecipitation of cellular extracts obtained from wild-type-infected cells by use of the UL37 antibody and subsequent immunoblot detection by the UL37 antibody showed a UL37-specific protein species migrating with an apparent molecular mass of 130 kDa (Figure 4.6A). However, this 130-kDa protein species was absent in the lysate and the immunoprecipitates from the UL37-null virus (Figure 4.6A). Immunoblot probing of immunoprecipitates of the UL20 protein with the anti-3XFLAG antibody or immunoprecipitates of gK with the anti-V5 antibody readily detected the presence of the UL37 protein migrating at approximately 130 kDa (Figure 4.6A). Immunoblot probing of infected lysate extracts with the anti-UL20 (anti-3XFLAG) antibody revealed the presence of UL20 migrating with an apparent molecular mass of approximately 28 kDa (Figure 4.6B), as we reported previously (9). The UL20 protein was also readily detected in UL20, gK, and UL37 immunoprecipitates (Figure 4.6B). The UL20 protein failed to immunoprecipitate VP5, while VP5 was readily detected in the lysate, migrating with an apparent molecular mass of 150 kDa (Figure 4.6C) when probed with anti-VP5 antibody. Immunoblot probing with the anti-gK (anti-V5) antibody readily detected gK in VC1 virus-infected cell extracts (Figure 4.6D) as well as gK,

UL20, and UL37 immunoprecipitates. Although the apparent molecular mass of gK is approximately 38 kDa (19, 22), larger gK-related protein species were detected in UL20 and UL37 immunoprecipitates, presumably due to gK-UL20-UL37 protein complexes that were not fully resolved to individual protein species (Figure 4.6D).

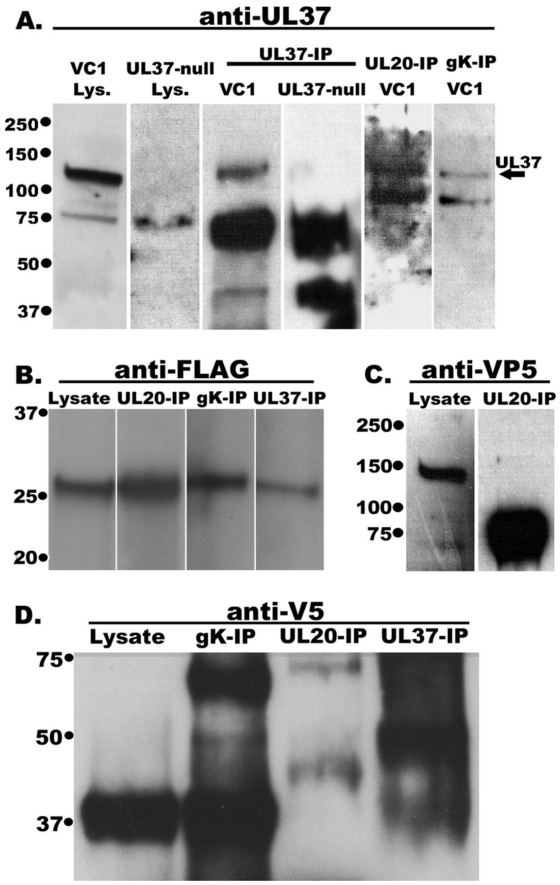

Figure 4.6: Western immunoblots of infected cell lysates and anti-UL37, anti-gK, and anti-UL20 immunoprecipitates. Vero cells were infected with the double-tagged virus YE102-VC1, expressing gK tagged with the V5 epitope tag and UL20 tagged with the 3XFLAG epitope, or with UL37-null virus. Infected cell lysates were obtained at 24 hpi, and immunoprecipitates were obtained using anti-gK (anti-V5; gK-IP), anti-UL20 (anti-FLAG; UL20-IP), and anti-UL37 (UL37-IP) antibodies in conjunction with magnetic beads. Immunoprecipitates were electrophoretically separated by SDS-PAGE and transferred to nitrocellulose membranes. The blots were probed with rabbit anti-UL37 antibody and HRP-conjugated secondary anti-rabbit antibody (A), mouse anti-FLAG antibody and HRP-conjugated goat anti-mouse antibody against heavy chain (Fc) (B), mouse anti-VP5 antibody and HRP-conjugated goat anti-mouse antibody (C), or mouse anti-V5 antibody and HRP-conjugated goat anti-mouse antibody against light chain (Fab) (D). In panels B, C, and D, lanes labeled “lysate” denote cellular extracts of VC1, and the other lanes represent immunoprecipitated samples from VC1-infected cells.

Determination of UL37 interactions with gK and UL20 by PLA

To further validate the observed interactions of the UL37 protein with gK and the UL20 membrane protein, we utilized PLA. In this assay, primary antibodies against UL37, gK (anti-V5,) and UL20 (anti-3XFLAG) are first bound to their respective antigens. Species-specific secondary antibodies, called PLA probes, each having a unique short DNA primer covalently attached, are used to bind to the primary antibodies. When the PLA probes are in close proximity, the DNA primers can interact through two other circle-forming oligonucleotides that are added later. Enzymatic ligation of these two fluorescently labeled oligonucleotides followed by polymerase-dependent rolling circle amplification results in the generation of intense fluorescence, which is visualized as distinct bright spots by use of a fluorescence microscope. This assay can determine even transient or weak intracellular interactions (24, 50). PLA readily detected the known gK-UL20 interactions (Figure 4.7F). Potential interactions were also detected between UL20 and UL37 (Figure 4.7B) and between gK and UL37 (Figure 4.7E), while wild-type virus-infected cells did not exhibit fluorescence signals (Figure 4.7A and D). The fluorescence signals were also absent in our ICP8-UL20 control, suggesting the absence of potential interaction between these proteins (Figure 4.7C).

Discussion

HSV-1 cytoplasmic virion envelopment involves multiple interactions among viral glycoproteins and tegument proteins. The UL37 protein has been shown to be transported, in complex with the UL36 protein and in a capsid-independent manner, to the TGN, where it functions in cytoplasmic virion envelopment. Here we describe the phenotypic and replication

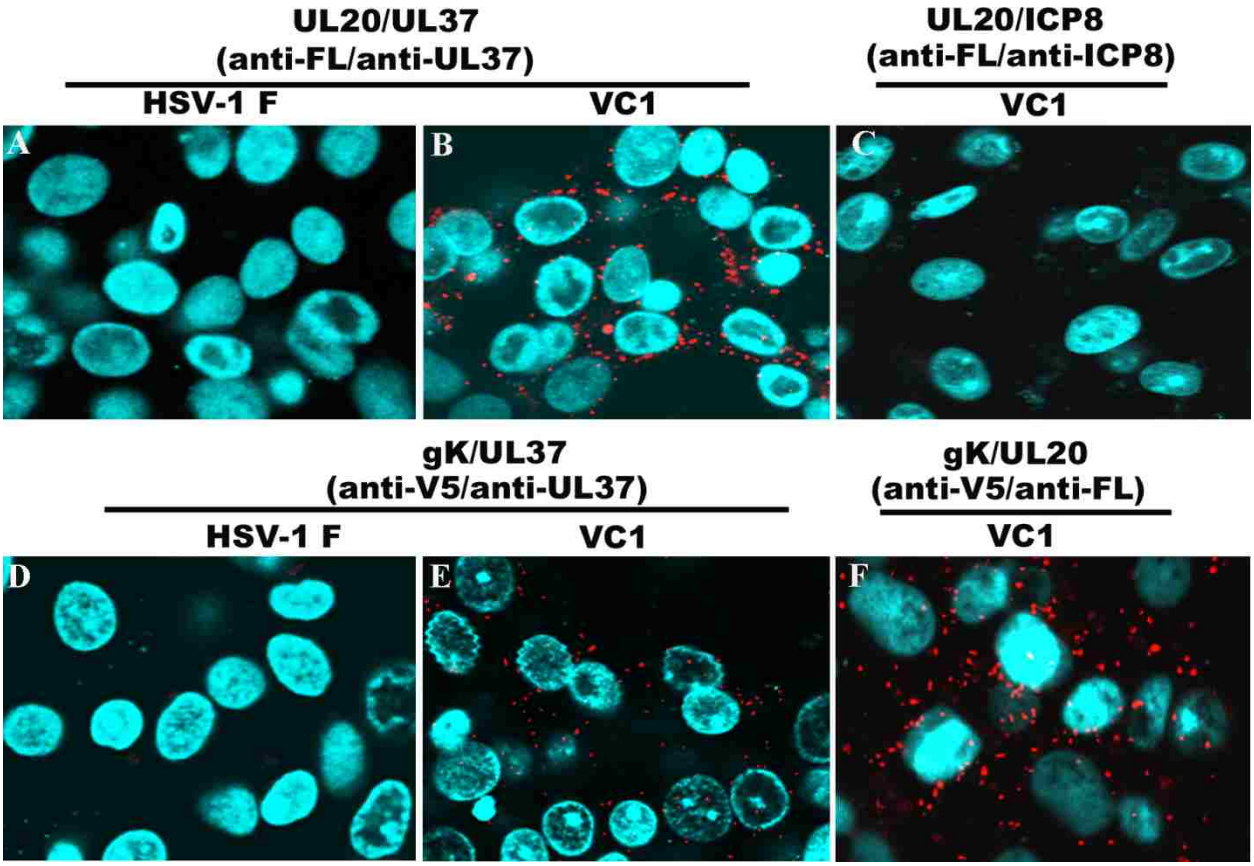

Figure 4.7: PLA to determine UL37 interactions with gK and UL20. PLA was performed using anti-UL37 (rabbit), anti-UL20 (anti-3xFLAG mouse antibody or anti-3XFLAG rabbit antibody), anti-ICP8 (mouse), and anti-gK (anti-V5 mouse antibody) antibodies in conjunction with appropriate secondary antibodies and oligonucleotides as described in Materials and Methods. Fluorescence images of cells infected with wild-type or VC1 virus were recorded using an Olympus confocal microscope at 18 hpi. (A and B) HSV-1(F)- and HSV-1(VC1)-infected Vero cells treated with anti-FLAG (anti-UL20) and anti-UL37 antibodies, respectively. (C) HSV-1(VC1)-infected Vero cells treated with anti-FLAG (anti-UL20) and anti-ICP8 antibodies. (D and E) HSV-1(F)- and HSV-1(VC1)-infected Vero cells treated with anti-V5 (anti-gK) and anti-UL37 antibodies, respectively. (F) HSV-1(VC1)-infected Vero cells treated with anti-V5 (anti-gK) and anti-FLAG (anti-UL20) antibodies.

properties of the UL37 mutant virus DC480, which contains a 12-amino-acid epitope tag inserted in frame at approximately the middle portion of the UL37 protein. The DC480 virus exhibits drastic defects in cytoplasmic virion envelopment, similar to those of mutant viruses lacking either the gK or UL20 gene. Partial complementation of the UL37 mutant virus in UL20-

expressing FRT cells, along with coimmunoprecipitation experiments, revealed that the UL37 protein interacts with gK and UL20, suggesting that these interactions facilitate cytoplasmic virion envelopment.

Functional domains of the UL37 protein

The UL37 protein contains multiple domains that appear to have distinct functions during virus replication. To facilitate detection of the UL37 protein, we inserted a protC epitope tag within the central portion of the UL37 protein, distal to the amino acid regions that are known to be involved in UL37 self-association, to bind to the UL36 protein, or to contain other known functional domains (Figure 4.1). The mutant DC480 expressed a full-length UL37 protein that was readily detectable by Western immunoblots using anti-protC antibody. However, this epitope insertion resulted in a severe cytoplasmic envelopment defect similar to that of the UL37-null virus, as evidenced by ultrastructural examination of infected cells showing accumulation of unenveloped capsids in the cytoplasm. This apparent defect in cytoplasmic virion envelopment in the DC480 virus was further supported by highly elevated DNase-resistant particle-to-PFU ratios in comparison to those for wild-type virus infections. Alignment of a large subset of known UL37 amino acid sequences revealed the presence of highly conserved residues among the alphaherpesviruses, as noted previously (28). The protC epitope tag insertion was specifically targeted to be immediately adjacent to a highly conserved tyrosine (Y) residue at amino acid position 480. UL37 is also reported to be phosphorylated in infected cells (1). Reversible phosphorylation can result in conformational changes that can significantly alter the functions of proteins, as evidenced by the regulation of the p53 tumor suppressor protein via phosphorylation (3). Tyrosine sulfation is also a widespread posttranslational

modification of eukaryotic proteins that modulate protein-protein interactions (26). Thus, it is possible that the protC epitope insertion alters Y-specific posttranslational modifications of the UL37 protein or alters protein-protein interactions that directly involve the amino acid region immediately adjacent to the conserved Y residue. Alternatively, disruption of this UL37 domain by the protC epitope insertion may change the overall conformation of the protein, thus affecting other functional domains of the UL37 protein. It has been shown that the UL37 protein is phosphorylated by cellular enzymes and that it interacts in the cytoplasm with the major DNA binding protein, ICP8. UL37 interactions with ICP8 enable transport of the UL37 protein to the nuclei of infected cells away from the cytoplasm; therefore, the UL37-ICP8 interactions are not likely to play a role in cytoplasmic virion envelopment (1).

As expected, both the DC480 and the UL37-null viruses were efficiently complemented for virus replication and spread on the BD45 cells transformed with an HSV-1 gene fragment containing the UL37 gene (11). Surprisingly, the DC480 virus was also complemented for virus replication and virus spread in FRT cells expressing the UL20 gene under the control of its native promoter, while the UL37-null virus was not. The observed DC480 virus complementation for virus growth on UL20-expressing FRT cells strongly suggests direct or indirect physical interactions between the UL37 protein and the UL20-gK protein complex. Coimmunoprecipitation experiments using the available anti-UL37 polyclonal antibody and either gK or UL20, tagged with a V5 or 3xFLAG epitope tag, respectively, showed that the UL37 protein interacted with both gK and UL20 in infected cells, thus providing additional evidence for physical interactions between UL37 and the gK-UL20 protein complex. It has been reported previously that the UL36-UL37 complex migrates to TGN membranes in the absence of capsids

(10). There was no VP5 protein detected in UL20 immunoprecipitates, suggesting that the observed interactions between UL20 and gK involved a UL37 protein that was not attached to viral capsids. The UL37-UL36 protein complex may interact with gK-UL20 after its intracellular transport to TGN membranes. Alternatively, the UL36-UL37 protein complex may interact with gK-UL20 in the endoplasmic reticulum and be cotransported to the TGN sites of virion envelopment. Additional experiments are required to distinguish between these two possibilities.

We have shown that the gK-UL20 protein complex plays critical roles in cytoplasmic virion envelopment and egress from infected cells. Our current results as well as previous reports (6, 10, 27, 30) clearly indicate that the highly conserved UL37 protein is a crucial determinant for cytoplasmic virion envelopment. Thus, the facts that these two protein complexes (gK-UL20 and UL36-UL37) interact within infected cells and at TGN sites and are involved in cytoplasmic virion envelopment are not surprising. Similar interactions must occur within the virion particle, suggesting that during virion entry these interactions must be regulated to allow the capsid to enter into the cytoplasm, while virion envelopes and their glycoprotein content remain fused with cellular plasma membranes. Interactions of viral glycoproteins, including gK and its partner protein, UL20, with cellular receptors may trigger inhibition of the UL36-UL37 interactions with the gK-UL20 protein complex. Additional work is required to investigate these possibilities.

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CHAPTER V: CONCLUDING REMARKS

Summary

Herpes simplex virus type-1 (HSV-1) virion assembly is a complex multi-step process. HSV-1 DNA is packaged into capsids in the nucleus, forming mature nucleocapsids which associate with a subset of tegument proteins such as pUL36, pUL37, pUL41, pUL48, pUL49 and pUS3 and then undergo primary envelopment by budding at the inner nuclear membrane into the perinuclear space, acquiring a temporary envelope studded with viral proteins (11-13). This envelope is lost after the particle fuses with the outer nuclear membrane in a process that may involve the fusion machinery implicated in viral entry which consists of gB, the heterodimer gH/gL, and the gB-associated fusion regulators gK/UL20. Other mechanisms involving different viral or host proteins that are not typically used during entry may also be involved in de-envelopment at the outer nuclear membrane because mutants lacking gB, gH/gL and gK/UL20 in various combinations do not exhibit a complete block in nuclear egress (2, 9). After de-envelopment, the nucleocapsid is deposited into the cytosol where additional inner tegument proteins assemble at the capsid, while the outer tegument proteins along with the glycoproteins and other membrane-associated viral proteins gather at the secondary envelopment sites in cytoplasmic vesicles derived from the trans-Golgi network. In secondary envelopment, tegumented capsids interact with the cytoplasmic domains of viral membrane proteins embedded in TGN-derived vesicles, leading to acquisition of the final viral envelope by budding into the vesicles (9). The enveloped virions are then transported in membrane vesicles (which also contain embedded viral glycoproteins) to the plasma membrane for release by

exocytosis, presumably assisted by microtubule-associated kinesin motors that ferry cargo towards the plus-ends of microtubules in the cell periphery (3).

The secondary envelopment process involves an intricate web of protein-protein interactions between capsid, tegument, and viral envelope proteins that is not fully understood. It has been hypothesized that the HSV-1 membrane glycoproteins gD, gE and gM play a crucial role in this process by acting in a redundant manner to promote secondary envelopment via interactions with VP22 or pUL11 (9). To explore the relative importance of these membrane proteins in secondary envelopment, we constructed recombinant mutant viruses unable to express pUL11, gE, gM, or the carboxyl terminus of gD in a number of combinations, and compared them to a mutant unable to express pUL20. Mutant viruses were constructed using a markerless RED recombination system implemented on a bacterial artificial chromosome (BAC), allowing us to abrogate expression of targeted genes without introducing extraneous sequences or affecting nearby genes. To characterize the mutant viruses, we used plaque morphology to assess virus spread, viral growth kinetics to measure infectious virus production, ultrastructural morphology to examine virus intracellular localization and ability to egress, and particle-to-PFU ratios to indirectly quantify cytoplasmic virion envelopment. A triple mutant virus lacking expression of gE, gM and the carboxyl terminus of gD showed minimal reduction in secondary envelopment and infectious virus production compared to single mutants lacking pUL11, gE, gM or C-terminal gD and to wild-type virus. Double mutants unable to express both gE and gM, or gE and C-terminal gD were likewise mildly affected, but a pUL11/gM double mutant showed more significant replication defects. None of these mutants approached the extent of egress defects exhibited by the pUL20 mutant virus, thus reinforcing

the view that the membrane proteins gK and UL20 are the key determinants of viral secondary envelopment (1).

An additional mutant virus was constructed with a protein C epitope tag inserted in-frame immediately adjacent to Y480 (DC480) near the center of the multifunctional inner tegument protein pUL37. Surprisingly, this 12 amino acid insertion was sufficient to block infectious virus production on Vero cells, displaying a phenotype similar to that of pUL37-null, pUL20-null or gK-null viruses. The DC480 mutant virus also showed partial complementation for virus replication and spread when grown on cells engineered to express pUL20, suggesting a potential interaction between the two proteins. We have provided compelling evidence for such an interaction between pUL37 and pUL20, and also between pUL37 and gK by two-way co-immunoprecipitation and proximity ligation assays (7).

pUL20 and gK act as fusion modulators and have been shown to interact with each other and with the major HSV-1 fusion protein gB (2, 4, 5, 10). In addition to their role in fusion, pUL20 and gK are known as important mediators of secondary envelopment, and their interactions with pUL37 may serve to facilitate the process of cytoplasmic virion envelopment (1, 6-8).

In conclusion, this work has made contributions to understanding the relative importance of a number of HSV-1 membrane and tegument proteins in cytoplasmic virion envelopment and egress. It has also leveraged the emergence of powerful new technologies such as proximity ligation assays to shed light on the complex tangle of protein-protein interactions that underpins viral secondary envelopment.

Current and Future Research

The work presented in the preceding two chapters raises interesting questions about the identity of specific domains of pUL37 that are involved in binding to pUL20 and gK, and vice versa, and work to determine the precise binding domains is ongoing. The close proximity of the protein C epitope insert in the DC480 mutant to two highly conserved tyrosine residues, and the fact that pUL37 is phosphorylated, also invites investigation into the role of posttranslational modifications on pUL37 structure and function. Additional research involving alanine-scanning mutagenesis of conserved pUL37 residues will investigate the role these and other conserved residues play in posttranslational modification of pUL37 or in binding to the pUL20/gK protein complex to facilitate cytoplasmic virion envelopment. The recently published crystal structure of the amino terminal half of PRV pUL37 will further inform selection of conserved residues for mutagenesis by allowing targeted modification of externally located residues.

In order to investigate the possibility that groups of other viral glycoproteins act in a redundant manner to promote secondary envelopment, additional multi-protein deletion mutants could be created. Furthermore, to rule out spurious mutations that might be introduced into the viral genome during mutagenesis, whole-genome sequencing of HSV-1 mutants can be performed. This is facilitated by the increasing prevalence and decreasing cost of high-throughput sequencing. It is also possible that some of the mutant viral phenotypes exhibiting partial defects in cytoplasmic envelopment presented in Chapter 2 were due to as yet undiscovered direct or indirect interactions with the pUL20 or gK protein, leading to

disruption of pUL20 or gK function in secondary envelopment. Alternatively, deleting enough proteins will undoubtedly perturb the multitude of protein-protein interactions occurring between the tegument and envelope during secondary envelopment, thus affecting the stability or function of other viral proteins and creating defects even if the deleted proteins are typically not involved in mediating envelopment. It is even possible that pUL20 and gK do not actually prevent cytoplasmic envelopment from occurring. Instead, their absence may cause the newly enveloped virions to immediately undergo de-envelopment back into the cytoplasm due to fusion of the virion envelope with the surrounding TGN vesicle. This possibility creates a ripe target for future investigation.

Vesicle formation in multivesicular bodies is mediated by endosomal sorting complexes required for transport (ESCRT) components and associated proteins such as the ATPase VPS4. Functional VPS4 was shown to be required for cytoplasmic virion envelopment in HSV-1, presumably by facilitating ESCRT-mediated membrane deformation during cytoplasmic envelopment and/or "pinching off" the viral envelope as the virus buds into TGN-derived vesicles. Viral proteins likely interact with ESCRT components to recruit ESCRT machinery to sites of cytoplasmic envelopment. gK is a prime candidate for such interactions due to the presence of a putative ESCRT-binding motif in gK similar to retroviral motifs called late domains which promote ESCRT recruitment (14). The presence of a putative ESCRT-binding motif in gK provides a potential mechanism by which gK and its binding partner pUL20 may influence cytoplasmic virion envelopment and merits further investigation.

Many aspects of the HSV-1 secondary envelopment interactome remain undefined. Obtaining a complete understanding of viral assembly will undoubtedly lead to more potent antiviral targets and will also lay the groundwork for the construction of fully synthetic viral particles that will put a biological twist on the concept of nanoscale robots.

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APPENDIX I: ADDITIONAL WORK

Introduction

The ubiquitous herpes simplex virus type-1 (HSV-1) is a prototypical member of the Alphaherpesvirinae (7). HSV-1 possesses a ~152 kb double-stranded DNA genome enclosed within an icosahedral capsid composed of 12 pentavalent and 150 hexavalent capsomeres (39). The capsid is coated with a layer of viral proteins called the tegument, and is in turn enclosed by a lipid envelope originating from host cellular membranes that is enriched with viral glycoproteins and other membrane-associated proteins. A variety of interactions between the viral proteins that make up the capsid, tegument and envelope are responsible for maintaining the structural integrity of mature virions and for shepherding the viral particle through the complex cytoplasmic envelopment process at trans-Golgi network-derived vesicles and endosomes (16, 28).

The UL37 gene of HSV-1 encodes a large 1123 aa (120.6 kD) highly conserved tegument protein that is essential for viral growth in cell culture and is crucial for viral assembly and secondary envelopment in the cytoplasm (4, 18, 29). The quantity of pUL37 loaded onto mature virions is tightly regulated, since overexpression of pUL37 does not increase the amount of pUL37 detected in virions (26).

pUL37 forms a complex with pUL36, and this interaction is conserved across all three subfamilies of the Herpesviridae. Interactions between proteins homologous to pUL36 and pUL37 have been documented in the alphaherpesviruses HSV-1, PRV and VZV, in the

betaherpesvirus HCMV and in the gammaherpesvirus KSHV (3, 21, 24, 30, 35-37). The presence of pUL36 is necessary for incorporation of pUL37 onto capsids (23). pUL37 is likely added to capsids after pUL36, since pUL36 is still detected on both HSV-1 and PRV capsids in mutants lacking pUL37 (8, 22). Along with pUL36, pUL37 may be involved in the organization of tegument structure. pUL37 attaches to capsid-bound pUL36 at the vertices, together forming thin flexible strands ranging from 15 to 70 nm in length that extend throughout the tegument, possibly providing a scaffold for the rest of the tegument (31). Deletion of either pUL36 or pUL37 prevents the acquisition of appreciable amounts of tegument in the cytoplasm and blocks cytoplasmic envelopment in HSV-1, resulting in cytoplasmic accumulation of unenveloped capsids (8, 9, 33).

The UL37 protein encodes a variety of functional domains. Co-immunoprecipitation experiments revealed that pUL37 domains spanning residues 1-300 and 568-1123 are involved in self-association in the absence of its binding partner pUL36 (4). The amino terminus contains an alanine-rich region (ARR) spanning residues 44-80, a leucine zipper motif covering residues 203-224 and a leucine-rich nuclear export signal (NES) encompassing residues 263-272 (4, 38). The carboxyl terminus contains a domain spanning residues 1099-1104 involved in binding TNF receptor-associated factor 6 (TRAF6) to activate NF-kappaB signaling (4, 25). The C-terminal 578-899 aa of pUL37 can interact with a spectraplakin protein called dystonin/BPAG1 known as a cytoskeletal cross-linker that is involved in microtubule stabilization and transport. Viral replication and cytoplasmic capsid mobility during egress from infected cells is impaired in dystonin-depleted cells, suggesting that pUL37 may play a role in capsid trafficking along

microtubules (32). The C-terminus of pUL37 is also responsible for binding to pUL36 (4, 19). Scanning alanine mutagenesis of pUL37 revealed that residue D631 of pUL37 mediates binding to pUL36. Knocking out this residue resulted in significantly decreased ability of the virus to replicate, with mutant viral titers approximately 2 logs lower than those of wild type virus (19). Trans-complementation experiments using a plasmid encoding the C-terminal portion of pUL37 spanning residues 568-1123 that includes the putative pUL36 interaction site showed that the plasmid is sufficient to partially rescue a virus unable to express pUL37 (4).

The function of the central portion of pUL37 spanning aa 301-567 is not well defined. A mutant HSV-1 with a 12 aa protein C epitope tag inserted in-frame immediately after residue Y480 of pUL37 exhibited a severe defect in cytoplasmic envelopment, but was partially complemented for replication and spread when grown on cells expressing pUL20 (14). Moving the protein C epitope tag 100 nucleotides upstream of Y480 abrogated pUL20-mediated complementation, resulting in a pUL37-null phenotype (data not shown). The protein C epitope tag was inserted proximal to two highly conserved tyrosine residues Y474 and Y480. Tyrosine sulfation is a common form of posttranslational modification, and insertion of the protein C epitope may have altered posttranslational modification of the nearby tyrosine residues (17). Alternatively, the protein C epitope may directly disrupt protein-protein interactions mediated by adjacent residues, or even trigger a conformational change in the pUL37 protein that could affect binding to other proteins (14).

The UL37 protein is expressed late in the infection cycle and is stably phosphorylated soon after translation of the UL37 gene. Phosphorylation of pUL37 is likely performed by a cellular kinase and is not dependent on the presence of any known HSV-1 binding partner because pUL37 expressed by a recombinant vaccinia virus is also phosphorylated (1). Phosphorylation is a widespread form of posttranslational modification that can affect a multitude of protein functions, including modulation of protein-protein interactions and control of intracellular trafficking (1, 12). Phosphorylation of proteins such as the p53 tumor suppressor has been shown to mediate conformational changes that can affect protein function and regulation (2). Many viral proteins are also phosphorylated, by either viral or cellular kinases. The HSV-1 tegument includes at least three components that are protein kinases, encoded by the UL13, UL23 and US3 genes (18, 29).

Two-way interactions between pUL37 and the membrane proteins pUL20 and gK were confirmed using co-immunoprecipitation and proximity ligation assays, thus providing the first direct link between the inner tegument and the envelope, although the exact locations of the relevant binding sites remain unknown (14). pUL20 and gK act as modulators of virus-induced fusion and interact with each other in addition to binding the major HSV-1 fusion protein gB, while pUL20 has also recently been shown to interact with gM (6, 10, 11, 20, 27). In addition to their role in fusion, pUL20 and gK are known to be among the most important mediators of secondary envelopment, and their interactions with pUL37 may serve to facilitate the process of cytoplasmic virion envelopment (5, 13-15).

Results

Both pUL37 and the pUL20/gK complex are conserved within the alphaherpesvirinae subfamily, reflecting their important roles in the viral life cycle (34). Alignments of a number of alphaherpesviral UL37 homologs show the presence of highly conserved residues scattered throughout the pUL37 ORF (19). We have performed additional alignments of UL37 homologs from 19 different alphaherpesviruses, illustrating the highly conserved nature of a number of residues including Y474 and Y480 (Figure A1.1). In order to discern the roles these conserved residues may play in mediating interactions between pUL37 and pUL20/gK to promote cytoplasmic virion envelopment, we have implemented a set of 13 single and double amino acid changes of highly conserved pUL37 residues in transient expression vectors using alanine scanning site-directed mutagenesis. Here we demonstrate that a subset of these pUL37 mutants is unable to complement infection with the DC480 mutant virus carrying a protein C tag at pUL37 Y480 (Figure A1.2). The ability of these constructs to successfully complement infection with pUL37-null virus will be characterized in future experiments. These constructs will also be assessed for alterations in patterns of cellular localization in the context of concurrent infection with pUL37-null, as well as for their ability to bind pUL20 and gK via co-immunoprecipitation. Changes in posttranslational modifications that may occur as a result of the mutations in pUL37, particularly alterations in the phosphorylation profile, will be detected via antibodies to phosphoserine and phosphotyrosine and by radiolabeling with P32 (Figure A1.3).

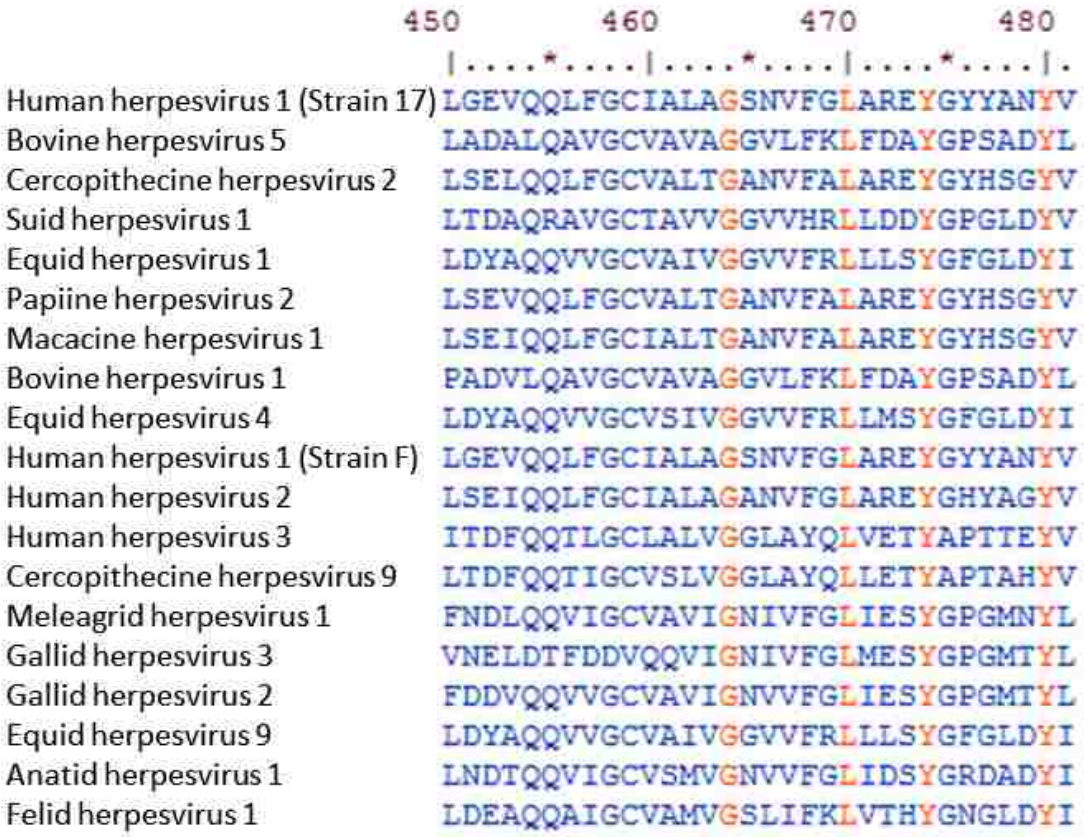


Figure A1.1: Partial alignment of UL37 homologs from 19 alphaherpesviruses. Y474 and Y480 are the only tyrosine residues conserved in the UL37 gene.

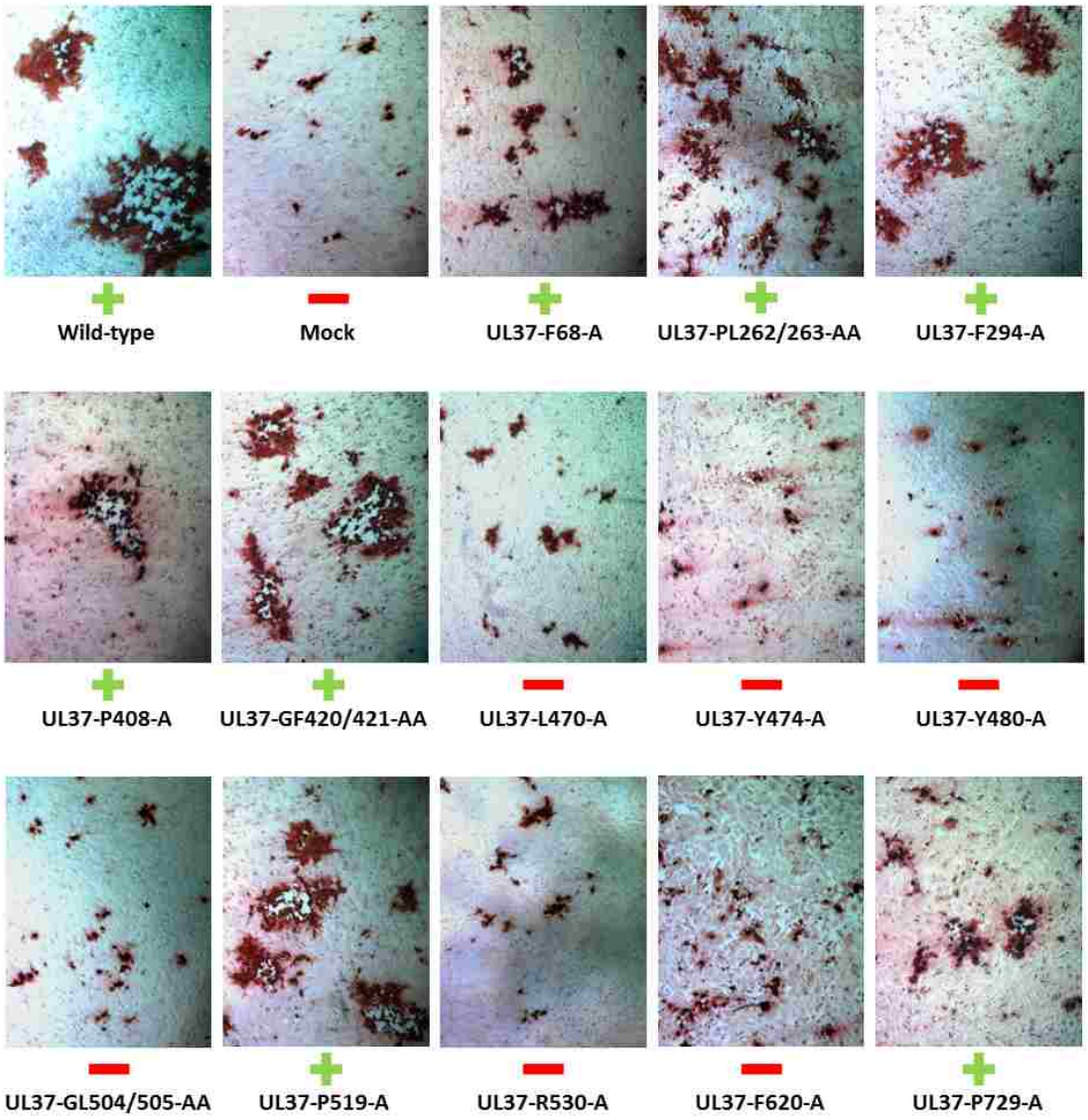
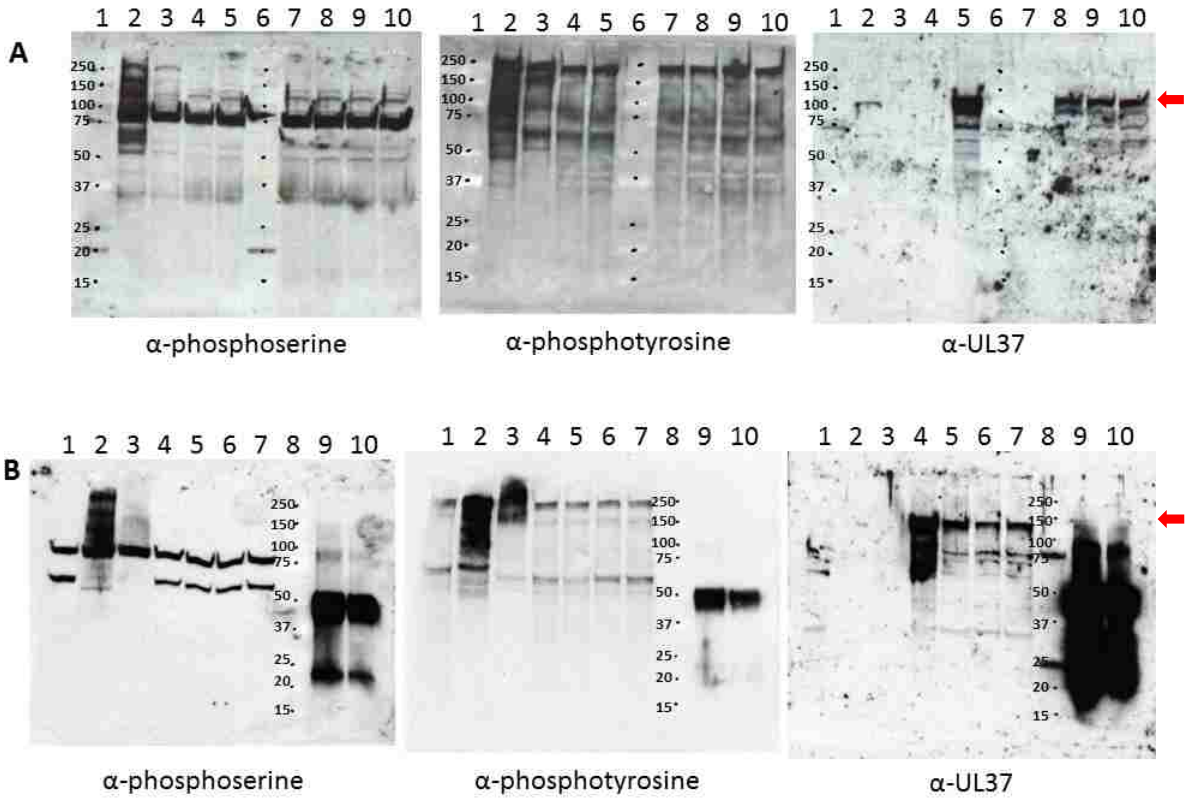


Figure A1.2: Trans-complementation assay. A trans-complementation assay was performed on Vero cells to assess a set of 13 pUL37 constructs for their ability to complement infection with the DC480 mutant virus which contains a protein C epitope insertion adjacent to Y480 in pUL37. Replication of the DC480 virus on Vero cells has previously been reported as highly impaired and similar to that of pUL37-null and pUL20-null, with plaques mostly composed of single infected cells (14). pUL37 constructs UL37-F68-A, UL37-PL262/263-AA, UL37-F294-A, UL37-P408-A, UL37-GF420/421-AA, UL37-P519-A and UL37-P729-A with the noted residues altered to alanine were able to complement the DC480 virus at least in part, while constructs UL37-L470-A, UL37-Y474-A, UL37-Y480-A, UL37-GL504/505-AA, UL37-R530-A and UL37-F620-A with the noted residues altered to alanine were not able to complement infection with the DC480 mutant, raising the possibility of these residues being important for virion assembly and/or egress. Full or partial complementation is marked with a plus sign. Lack of complementation is denoted by a minus sign. Mock signifies infection with the DC480 mutant virus followed by mock transfection.



Row A		Row B	
Lane 1	Ladder	**Lane 1**	Wild-type pUL20
Lane 2	Calyculin/okadaic acid treated cell lysate	**Lane 2**	Calyculin/okadaic acid treated cell lysate
Lane 3	EGF-treated cell lysate	**Lane 3**	EGF-treated cell lysate
Lane 4	UL37 in reverse orientation	**Lane 4**	Wild-type pUL37
Lane 5	Wild-type pUL37	**Lane 5**	pUL37-620F
Lane 6	Ladder	**Lane 6**	pUL37-480Y
Lane 7	Wild-type pUL20	**Lane 7**	pUL37-474Y
Lane 8	pUL37-620F	**Lane 8**	Ladder
Lane 9	pUL37-480Y	**Lane 9**	Wild-type pUL37 pulldown
Lane 10	pUL37-474Y	**Lane 10**	pUL37-null pulldown

Figure A1.3: Phosphorylation assay. (Row A) Transfection with the specified constructs only. Lanes 2 and 3 are positive controls for phosphorylation, while lanes 4 and 7 are negative controls. (Row B) Transfection with the specified constructs followed by infection with DC480 mutant virus. Lane 1 is negative control, lanes 2 and 3 are positive controls for phosphorylation, and lanes 9 and 10 are from a suboptimal pulldown. Red arrow is pointing at the 120 kD pUL37 band. Expression of pUL37 is readily detectable using anti-pUL37 antibody, but successful detection of pUL37 phosphorylation using antibodies to phosphoserine or phosphotyrosine is yet to be accomplished.

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APPENDIX II: LETTER OF PERMISSION

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1. Chouljenko DV, Kim IJ, Chouljenko VN, Subramanian R, Walker JD, Kousoulas KG. **Functional hierarchy of herpes simplex virus 1 viral glycoproteins in cytoplasmic virion envelopment and egress**. J. Virol. April 2012 vol. 86 no. 8, 4262-4270. doi: 10.1128/JVI.06766-11.

2. Jambunathan N, Chouljenko D, Desai P, Charles AS, Subramanian R, Chouljenko VN, Kousoulas KG. **Herpes simplex virus 1 protein UL37 interacts with viral glycoprotein gK and membrane protein UL20 and functions in cytoplasmic virion envelopment**. J. Virol. June 2014 vol. 88 no. 11, 5927-5935. doi: 10.1128/JVI.00278-14.

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Sincerely,
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Functional Hierarchy of Herpes Simplex Virus 1 Viral Glycoproteins in Cytoplasmic Virion Envelopment and Egress

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Herpes simplex virus 1 (HSV-1) viral glycoproteins gD (carboxyl terminus), gE, gK, and gM, the membrane protein UL20, and membrane-associated protein UL11 play important roles in cytoplasmic virion envelopment and egress from infected cells. We showed previously that a recombinant virus carrying a deletion of the carboxyl-terminal 29 amino acids of gD (gDΔct) and the entire gE gene (ΔgE) did not exhibit substantial defects in cytoplasmic virion envelopment and egress (H. C. Lee et al., J. Virol. 83:6115–6124, 2009). The recombinant virus ΔgM2, engineered not to express gM, produced a 3- to 4-fold decrease in viral titers and a 50% reduction in average plaque sizes in comparison to the HSV-1(F) parental virus. The recombinant virus containing all three mutations, gDΔct-ΔgM2-ΔgE, replicated approximately 1 log unit less efficiently than the HSV-1(F) parental virus and produced viral plaques which were on average one-third the size of those of HSV-1(F). The recombinant virus ΔUL11-ΔgM2, engineered not to express either UL11 or gM, replicated more than 1 log unit less efficiently and produced significantly smaller plaques than UL11-null or gM-null viruses alone, in agreement with the results of Leege et al. (T. Leege et al., J. Virol. 83:896-907, 2009). Analyses of particle-to-PFU ratios, relative plaque size, and kinetics of virus growth and ultrastructural visualization of glycoprotein-deficient mutant and wild-type virions indicate that gDΔct, gE, and gM function in a cooperative but not redundant manner in infectious virion morphogenesis. Overall, comparisons of single, double, and triple mutant viruses generated in the same HSV-1(F) genetic background indicated that lack of either UL20 or gK expression caused the most severe defects in cytoplasmic envelopment, egress, and infectious virus production, followed by the double deletion of UL11 and gM.

Herpes simplex virus 1 (HSV-1) virion assembly begins in the nucleus with the construction of viral capsids, which acquire certain tegument proteins and then bud through the inner nuclear membrane, forming enveloped virions within the perinuclear space (primary envelopment). Enveloped virions fuse with the outer nuclear membranes, allowing capsid deposition in the cytoplasm of cells (10, 45, 47; reviewed in reference 42). In the cytoplasm, viral capsids are coated with tegument proteins and acquire final viral envelopes by budding into glycoprotein-enriched regions of the *trans*-Golgi network (TGN) membranes (secondary envelopment) (17, 51, 54, 56). This final virion morphogenesis step delivers fully enveloped virions into cytoplasmic vesicles, which are ultimately transported out of the cell (33). Secondary envelopment of cytoplasmic capsids is facilitated by interactions between tegument proteins and the cytoplasmic domains of viral glycoproteins and other membrane proteins anchored in TGN-derived membranes (17, 51, 54, 56; reviewed in references 32 and 42).

Deletion or forced retention of either gD or gH within the endoplasmic reticulum does not cause drastic defects in cytoplasmic virion envelopment and egress, although both glycoproteins are essential for viral infectivity (7, 19, 38, 55). Similarly, gB is not required for cytoplasmic envelopment and egress, inasmuch as gB-null viruses acquire envelopes and can be rendered infectious after treatment with the fusogen polyethylene glycol (8, 41). However, partial deletion of the carboxyl terminus of gB was reported to cause substantial reductions in cytoplasmic virion envelopment and egress (9), suggesting that truncated gB may cause a dominant negative effect. Recently, a gB-gD double mutant but not a gD-null virus exhibited substantial defects in late stages of virus egress, indicating that gB may cooperate with gD in facilitating virion envelopment (34). Single or simultaneous deletion of HSV-1 gE and gM genes did not cause any appreciable defects in cytoplasmic virion envelopment or infectious virus production (6). Similarly, lack of either gD or gE expression caused a mild (2- to 3-fold) reduction in enveloped virions. However, simultaneous deletion of HSV-1 gD and gE or gD, gE, and gI genes caused drastic accumulation of unenveloped capsids in the cytoplasm. Because neither of these gene deletions alone caused similar defects, it was concluded that gD and gE function in a redundant manner in cytoplasmic virion egress (16). Deletion of the UL11 gene produced mild defects in cytoplasmic virion egress (27), while deletion of either the gK or UL20 gene or specific mutations within these two genes caused drastic inhibition of cytoplasmic virion envelopment (23, 31, 41). Also, it was reported that simultaneous deletion of HSV-1 UL11 and gM caused drastic inhibition of cytoplasmic virion envelopment (37). Together, these results suggest that there are multiple cooperative relationships among viral glycoproteins and membrane proteins facilitating cytoplasmic virion envelopment.

We have reported that gK and UL20 have distinct functions in virus-induced cell fusion and cytoplasmic virion envelopment. These functions are genetically separable, since mutations in UL20 that drastically inhibit virion envelopment do not affect virus-induced cell fusion (24, 40). UL20 and gK function in virus-in-

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Herpes Simplex Virus 1 Protein UL37 Interacts with Viral Glycoprotein gK and Membrane Protein UL20 and Functions in Cytoplasmic Virion Envelopment

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ABSTRACT

We have shown that glycoprotein K (gK) and its interacting partner, the UL20 protein, play crucial roles in virion envelopment. Specifically, virions lacking either gK or UL20 fail to acquire an envelope, thus causing accumulation of capsids in the cytoplasm of infected cells. The herpes simplex virus 1 (HSV-1) UL37 protein has also been implicated in cytoplasmic virion envelopment. To further investigate the role of UL37 in virion envelopment, the recombinant virus DC480 was constructed by insertion of a 12-amino-acid protein C (protC) epitope tag within the UL37 amino acid sequence immediately after amino acid 480. The DC480 mutant virus expressed full-size UL37 as detected by the anti-protC antibody in Western immunoblots, accumulated unenveloped capsids in the cytoplasm of infected cells, and produced very small plaques on African green monkey kidney (Vero) cells that were similar in size to those produced by the UL20-null and UL37-null viruses. The DC480 virus replicated nearly 4 log less efficiently than the parental wild-type virus when grown on Vero cells. However, DC480 mutant virus titers increased nearly 20-fold when the virus was grown on FRT cells engineered to express the UL20 gene in comparison to the titers on Vero cells, while the UL37-null virus replicated approximately 20-fold less efficiently than the DC480 virus on FRT cells. Coimmunoprecipitation experiments and proximity ligation assays showed that gK and UL20 interact with the UL37 protein in infected cells. Collectively, these results indicate that UL37 interacts with the gK-UL20 protein complex to facilitate cytoplasmic virion envelopment.

IMPORTANCE

Herpes simplex viruses acquire their final envelopes by budding into cytoplasmic membranes derived from the *trans*-Golgi network (TGN). The tegument proteins UL36 and UL37 are known to be transported to the TGN sites of virus envelopment and to function in virion envelopment, since mutants lacking UL37 accumulate capsids in the cytoplasm that are unable to bud into TGN membranes. Viral glycoprotein K (gK) also functions in cytoplasmic envelopment, in a protein complex with the membrane-associated protein UL20 (UL20mp). This work shows for the first time that the UL37 protein functionally interacts with gK and UL20 to facilitate cytoplasmic virion envelopment. This work may lead to the design of specific drugs that can interrupt UL37 interactions with the gK-UL20 protein complex, providing new ways to combat herpesviral infections.

The herpes simplex virus 1 (HSV-1) double-stranded DNA genome is enclosed in an icosahedral capsid and embedded within a proteinaceous tegument containing multiple viral proteins, all of which is packaged within an envelope decorated with multiple membrane proteins and glycoproteins (1–5). HSV-1 encodes at least 26 tegument proteins and 11 glycoproteins as well as several nonglycosylated membrane-associated proteins. The final steps in virion envelopment occur in the cytoplasm of infected cells and involve multiple interactions among viral proteins, including interaction between cytoplasmic portions of viral glycoproteins and tegument proteins bound to cytoplasmic capsids, as well as tegument-tegument protein interactions (1, 6–9).

HSV-1 UL37 is an approximately 120-kDa phosphorylated tegument protein expressed in both mature virions and light particles (10). The UL37 protein localizes to both the nucleus and cytoplasm in infected cells. The UL37 cytoplasmic localization is considered to be due to the presence of a nuclear export signal (NES) sequence within the UL37 protein that mediates transport of the UL37 protein to the cytoplasm in the absence of any other viral proteins (3, 11–13). The UL37 protein is capable of self-association and interacts via its carboxyl terminus with the UL36 major tegument protein, and this interaction is necessary and sufficient for transport of the UL36-UL37 protein complex to the *trans*-Golgi network (TGN), where virion envelopment is thought to occur (11, 14–16).

Deletion of the UL37 gene specified by either HSV-1 or pseudorabies virus (PRV) inhibits cytoplasmic virion envelopment, causing the accumulation of unenveloped capsids in the cytoplasm of infected cells (17–19). However, while the UL37 gene is

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To Whom It Concern,

This letter is to confirm that Dmitry V. Chouljenko has permission from the American Society for Microbiology (ASM) to use to any material from the following articles, including the entire article, in his dissertation.

1. Chouljenko DV, Kim IJ, Chouljenko VN, Subramanian R, Walker JD, Kousoulas KG. Functional hierarchy of herpes simplex virus 1 viral glycoproteins in cytoplasmic virion envelopment and egress. J. Virol. April 2012 vol. 86 no. 8, 4262-4270. doi: 10.1128/JVI.06766-11.

2. Jambunathan N, Chouljenko D, Desai P, Charles AS, Subramanian R, Chouljenko VN, Kousoulas KG. Herpes simplex virus 1 protein UL37 interacts with viral glycoprotein gK and membrane protein UL20 and functions in cytoplasmic virion envelopment. J. Virol. June 2014 vol. 88 no. 11, 5927-5935. doi: 10.1128/JVI.00278-14.

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Dmitry Vladimirovich Chouljenko was born in Kiev, Ukraine and followed his parents to the United States at the age of 9. Dmitry graduated from Baton Rouge Magnet High School, and went on to pursue a college degree at Louisiana State University in Baton Rouge. He graduated with a Bachelor of Science degree in biological sciences, with a minor in entomology and a concentration in marine biology. Soon afterwards, he was accepted into the Louisiana State University graduate program and began work on a Doctor of Philosophy degree, focusing on herpes simplex virus research under the mentorship of Dr. Konstantin G. Kousoulas. After graduation in August 2014, Dmitry intends to further his education by pursuing post-doctoral research that leverages molecular biology and bioinformatics to gain insight into the biology and management of infectious diseases.