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Herpes simplex virus type 1 glycoprotein gM and the membrane associated protein UL11 are required for virus-induced cell fusion and efficient virus entry

In Joong Kim

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HERPES SIMPLEX VIRUS TYPE 1 GLYCOPROTEIN gM AND THE MEMBRANE
ASSOCIATED PROTEIN UL11 ARE REQUIRED FOR VIRUS-INDUCED CELL
FUSION AND EFFICIENT VIRUS ENTRY

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

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Veterinary Medical Sciences through the
Department of Pathobiological Sciences

by

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December 2013

This dissertation is dedicated to my parents,
YongKwang Kim and InSuk Park.

이 박사학위 논문을 나의 사랑하는
아버지 김용광과 어머니 박인숙에게
바칩니다.

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ABSTRACT

HSV-1 facilitates virus entry into cells and cell-to-cell spread by mediating fusion of the viral envelope with cellular membranes and fusion of adjacent cellular membranes. Although virus strains isolated from herpetic lesions cause limited cell fusion in cell culture, clinical herpetic lesions typically contain large syncytia, underscoring the importance of cell-to-cell fusion in virus spread in infected tissues. Certain mutations in gB, gK, UL20 and other viral genes, drastically enhance virus-induced cell fusion *in vitro* and *in vivo*. Recent work has suggested that gB is the sole fusogenic glycoprotein, which is regulated by interactions with viral glycoproteins gD, gH/gL, gK, membrane protein UL20 and cellular receptors. Recombinant viruses were constructed to abolish either gM or UL11 expression in the presence of strong syncytial mutations in either gB or gK. Virus-induced cell fusion caused by deletion of the carboxyl terminal 28 amino acids of gB, or the dominant syncytial mutation in gK (Ala-to-Val at amino acid 40), was drastically reduced in the absence of gM. Similarly, syncytial mutations in either gB or gK did not cause cell fusion in the absence of UL11. Neither the gM nor UL11 gene deletions substantially affected gB, gC, gD, gE and gH glycoprotein synthesis and expression on infected cell surfaces. Two-way immunoprecipitation experiments revealed that the membrane protein UL20, which is found as a protein complex with gK, interacted with gM, while gM did not interact with other viral glycoproteins. Viruses produced in the absence of gM or UL11 entered into cells slower than their parental wild-type virus. Moreover, the mouse eye model study, revealed that although the UL11-null virus replicated less efficiently in mouse corneas, it infected ganglionic neurons with greater efficiency than either the gM or gE-null viruses. Collectively, these results indicate that gM and UL11 are required for efficient membrane fusion events during virus entry and virus spread *in vivo* as well as *in vitro*, and also that gK plays the

most important role in corneal and ganglionic infection in the mouse eye model followed by gM, gE, and UL11 in a descending order of importance relative to the wild-type virus.

CHAPTER I: INTRODUCTION

Statement of Problem and Hypothesis

Herpes simplex virus type 1 (HSV-1) is an important etiology of human diseases characterized by recurrent mucocutaneous lesions in orofacial and genital regions with latency, which affects approximately 60 to 95 percent of the adult population in the world (Ohana et al., 2000, Brady and Bernstein, 2004). HSV-1 infection in the central nervous system and eyes, has clinical importance, since it can occasionally lead to fatal encephalitis and corneal blindness (Whitley and Roizman, 2001). Particularly, although it less frequently occurs than mucocutaneous infections, ocular infection is one of the prominent causes of blindness in developed countries (Liesegang, 2001, Liesegang et al., 1989).

During the HSV-1 life cycle, virus entry into cells and cell-to-cell spread is facilitated by fusion of the viral envelope with cellular membranes and fusion of adjacent cellular membranes. Although virus strains isolated from herpetic lesions cause limited cell fusion in cell culture, clinical herpetic lesions typically contain large syncytia, underscoring the importance of cell-to-cell fusion in virus spread in infected tissues. On the HSV-1 envelope, there are at least 11 glycoproteins as well as several membrane-associated proteins, which may play important roles in viral entry and virus-induced cell fusion due to the location. Among them, certain mutations in gB, gK, UL20 and other viral genes drastically enhance virus-induced cell fusion *in vitro* and *in vivo*. Recent work has suggested that gB is the sole fusogenic glycoprotein, while it is regulated by interactions with viral glycoproteins gD, gH/gL, gK, membrane protein UL20 and cellular receptors [reviewed in (Connolly et al., 2011)].

Among the rest of membrane-associated proteins, the glycoprotein gM and the membrane-associated protein UL11 are highly conserved in all herpesviruses, which have also been

suggested as a potential role in virus-induced cell fusion and virion egress (Kim et al., 2013, Chouljenko et al., 2012). Glycoprotein gM is a conserved type III integral membrane protein with multiple transmembrane domains, that forms a complex with pUL49.5 (gN) [reviewed in (Roizman et al., 2007)]. Deletion of the gM gene does not abrogate HSV-1 replication, but does affect the ability of the virus to spread (Leege et al., 2009). Expression of HSV-1, pseudorabies virus (PRV) and Kaposi sarcoma associated herpesvirus (KSHV or HHV-8) gM and gN in transfected cells inhibited cell fusion caused by simultaneous expression of gB, gD, gH and gL glycoproteins (Koyano et al., 2003, Klupp et al., 2000b). Also, lack of gM was reported to inhibit virus-induced cell fusion caused by a single amino acid substitution in the carboxyl terminus of gB (A855V; gBsyn) (Balan et al., 1994). UL11 is a 96-amino-acid myristoylated and palmitoylated tegument protein anchored into the cytoplasmic side of cell membranes (Baines et al., 1995, Loomis et al., 2001, MacLean et al., 1989, Baird et al., 2008). UL11 interacts with viral glycoprotein gE and tegument proteins UL16 and UL21 and has been suggested to play a role in recruiting viral proteins to the virion assembly site at the trans-Golgi network (TGN) (Baird et al., 2008, Farnsworth et al., 2007, Loomis et al., 2001, Loomis et al., 2003, Leege et al., 2009, Han et al., 2012). Although absence of UL11 in HSV-1 and PRV revealed only moderate defects in viral replication (Fulmer et al., 2007, Leege et al., 2009, Chouljenko et al., 2012, Kopp et al., 2003), the human cytomegalovirus human herpesvirus (HCMV or HHV-5) UL11 homologue is essential for virus replication (Seo and Britt, 2007, Silva et al., 2003).

Therefore, the overall hypothesis of the investigations in this thesis has been that gM and UL11 are involved in these membrane fusion phenomena. The hypothesis predicts that both gM and UL11 directly or indirectly interact and regulate the HSV-1 membrane fusion machinery during virus-induced cell fusion and virus entry. Hence, it is hypothesized that: (a) lacking of

either gM or UL11 on the virus, will negatively affect the ability of dominant syncytial mutations in either gB or gK to cause extensive virus-induced cell fusion; (b) the mutant viruses lacking of either gM or UL11, will exhibit slower kinetics of entry into Vero cells; and (c) the mutant viruses lacking of either gM or UL11, will less efficiently infect ganglionic neurons and establish latency than wild type virus.

Statement of Research Objectives

The goal of this research was to investigate the role of Herpes simplex virus type 1 glycoprotein M (gM) and membrane-associated protein UL11 in relation to virus-induced cell fusion, efficient virus entry and neuronal transmission. The specific aims of this research were:

- I. To investigate whether the lack of either gM or UL11 affected the ability of dominant syncytial mutations in either gB or gK to extensive cell fusion.
 1. To generate and validate a set of mutant viruses containing either gB- or gK-syncytial mutations in the presence or absence of mutations that prevented expression of either the gM or UL11 gene using the HSV-1(F) genome cloned as a bacterial artificial chromosome (BAC).
 2. To characterize the phenotypes of the set of mutant viruses including plaque morphology, fusogenic capacity, and virus replication.
- II. To investigate the role of gM and UL11 involved in virus-induced membrane fusion phenomena during both virus-induced cell fusion and virus entry:
 1. To assess whether the absence of either gM or UL11 affects the synthesis and cell surface expression of viral glycoproteins.
 2. To determine whether gM and UL11 interact directly with gB or the gK/UL20 complex.

3. To analyze whether a lack of either gM or UL11 affects the kinetics of virus entry into Vero cells.

III. To investigate the *in vivo* effects of lack of either gE, gM, gK or UL11 in viral pathogenesis using the scarified mouse eye HSV-1 infection model:

1. To characterize the virus replication kinetics and the plaque morphology phenotypes of recombinant viruses contacting mutants of interest.
2. To assess whether the lack of either gE, gM, gK or UL11 affects the clinical ocular symptom and viral shedding.
3. To determine whether infectious virions infect ganglionic neurons and establish latency.

Overall, the results obtained from this research indicate that:

1. gM and UL11 are required for virus-induced cell fusion and efficient virus entry.
2. Absence of either gM or UL11 does not affect synthesis and cell surface expression of other viral glycoproteins.
3. gM physically interacts with UL20 in virus-infected cells.
4. The mutant viruses lacking either gE, gM, gK or UL11, cause decreased clinical ocular symptom and viral shedding on the infected mice.
5. All three mutant viruses lacking of either gE, gM, or UL11 are able to infect ganglionic neurons and establish latency, albeit at reduced frequencies in comparison to the parental HSV-1(F) strain.
6. The UL11-null virus replicates less efficiently in Vero cell monolayers and mouse eyes, while it infects ganglionic neurons more efficiently than the gM-null or gE-null viruses.

The work is presented in individual chapters in a manuscript format having a specific title for the central theme of each chapter.

CHAPTER II: LITERATURE REVIEW

Historical Perspective of Herpesviruses

Although the first possible description of herpesvirus infection dates back to the 3rd millennium BC on a Sumerian tablet or 1500 BC on an Ebers Papyrus (Roizman et al., 2007), the virus seems to have existed longer than human beings. Three primary subfamilies of mammalian herpesviruses are estimated to have appeared about 200 million year ago and two types of human herpes simplex viruses (HSV) may have diverged at 8 to 10 million years ago (McGeoch et al., 1985, Gentry et al., 1988), while the earliest fossil of *Homo sapiens* has been dated to circa 200,000 years ago (White et al., 2003).

Since the first notion of herpes, the disease has been recognized as a life-long prevalent disease in our history. Herpes (ἕρπης), which means “creep” in Greek, was first used to describe various lesions that appeared to creep or crawl along the skin by an ancient Greek, Hippocrates (460 to 370 BC) (Roizman et al., 2007). Later in Roman history, an early Roman emperor, Tiberius (42 BC to 37 AD) banned kissing at public events to restrain oral herpes spreading (Shors, 2011). During Tiberius’ reign, Aulus Cornelius Celsus (25 BC to 50 AD) described an actual herpetic lesion and treatment in his book, “*De Medicina*” at 30 AD (Celsus, 1814). He stated the herpetic lesion as initiating from round then diffusing as a serpent to form a belt (Roizman et al., 2007) with a suggested treatment of cauterizing the severe open herpes lesion with a hot iron (Celsus, 1814). A reliable medical description was noted by Herodotus (70? to 130? AD) at 100 AD as “an eruption which appears about the mouth at the crises of simple fevers” and it is named as *herpes labialis* (Evans and Kaslow, 1997, Niimura and Emmet, 1989). About a century later, Claudius Galenus (130 to 201 AD), who was a physician of the emperor Marcus Aurelius, elaborately expanded Herodotus’ concept of herpes to latency; the recurrent

infection on the same anatomic site of the patient (Roizman et al., 2007). Between the 16th and 17th century, Shakespeare possibly mentioned oral herpes in ‘Romeo and Juliet’: “O'er ladies' lips, who straight on kisses dream, Which oft the angry Mab with blisters plagues, Because their breaths with sweetmeats tainted are. Act 1. Scene IV”.

Although Galenus' description of herpes is very similar to HSV infection, the term herpes was widely used to describe various skin ailments until the 17th century (Roizman et al., 2007). The term herpes began to be used to specifically describe the HSV infection in the 18th century. A British Physician, Daniel Turner perspicuously illustrated *herpes labialis* as “a choleric pustule breaking forth of the skin diversity” in his monograph on dermatology, “*De Morbis Cutaneis*” in 1714 (Turner, 1731, Wildy, 1973). About two decades later, Jean Astruc, a physician of King Louie XIV, published a book, *De Morbis Veneris*, after studying venereal diseases in French prostitutes (*Puellae publicae*), in which he first described *herpes genitalis* (Astruc, 1736, Roizman et al., 2007).

The modern concept of herpes was suggested by Thomas Bateman as “a restricted group of localized vesicles with a short, self-limiting course” in 1814 (Bateman, 1814). Paul Unna recorded about recurrences of genital herpes lesion in 1883 as “a vocational disease,” and “one of the most benign of affections both to the patient and her public” (Unna, 1883). Few years later, the first book dedicated to herpes, “*Les Herpes Genitaux*” was published (Diday and Doyon, 1886). In 1896, Unna also made a very important contribution to the histopathology of skin diseases which enabled differential diagnosis of herpes from similar diseases especially syphilis under the microscope (Unna, 1896). In the same year, Fournier described the diagnosis and treatment of genital herpes. He advised, “For recurrent herpes the general treatment of arthritis

(by which he may have meant inflammation and pain) may be necessary. As far as hygiene, forbid alcohol, tobacco, also wine fatigue, and sexual excesses” (Fournier, 1896).

During the late 19th and early 20th century, Vidal and Gruter achieved significant scientific advances in herpes research. In 1873, Vidal demonstrated transmission of herpes from one individual to another via human inoculum (Vidal, 1873). In 1924, Gruter furthered this finding to the rabbit as an animal model (Gruter, 1924), crediting him with the first isolation of HSV in the virology community. In 1930, Andrewes and Carmichael found a surprising characteristic of herpes. Unlikely to other known infectious agent, herpes could recur in people with a significantly high neutralizing antibody level (Andrewes and Carmichael, 1930). This was contradictory to widespread canonical belief at that time.

In 1939, Burnet and Williams shaped the present concept of herpes latency, as “Herpes simplex infections, however, once contracted, seem to persist for life. The virus remains for the most part latent; but under the stimulus of trauma, fever, and so forth it may at any time be called into activity and provoke a visible herpetic lesion” (Burnet, 1939). During the 20th century, the evolution of tissue culture and electron microscopy (EM) techniques revolutionized virology research and facilitated the isolation of other families of herpesviruses. In the 1950’s, human herpes virus type 3 [HHV-3; varicella zoster virus (VZV, chickenpox)] (Weller and Stoddard, 1952) and human herpesvirus-5 [HHV-5; human cytomegalovirus (HCMV)] (Craig et al., 1957, Rowe et al., 1956) were isolated. Subsequently, the cultivation of lymphoblastoid tumor cells and B-lymphocyte enabled the isolation of human herpesvirus 4 [HHV-4; Epstein–Barr virus (EBV)] (Epstein et al., 1964). Late 20th century, T-lymphocyte culture allowed the isolation of other betaherpesviruses; human herpesviruses 6A, 6B and 7 (Frenkel et al., 1990, Lopez et al., 1988, Salahuddin et al., 1986). Most recently, human herpesvirus 8 (HHV-8; Kaposi's sarcoma-

associated herpesvirus; KSHV) was discovered via representational difference analysis (RDA) (Chang et al., 1994).

Taxonomy of Herpesviruses

Since the early 20th century, the advent of various new biological techniques - especially tissue culture and electron microscopy - has explosively increased the number of isolated viruses as well as their detailed information including ultrastructure, constituent of virus particle, physicochemical property, etc. The expanded knowledge on viruses spontaneously led to the desire to classify them according to the similarities. For example, all herpesviruses share the same properties of: (a) a core containing a linear double stranded DNA; (b) an icosahedral (200 faceted) capsid consisting of 12 pentavalent and 150 hexavalent capsomeres; (c) a tegument which amorously surrounds the capsid, and (d) a lipid envelope which contains glycoprotein spikes and membrane-associated proteins (Roizmann et al., 1992). Later the antibody techniques allowed the study of antigenic relationships using specific antibodies that are only cross-reactive in closely related viruses (Davison, 2010). For instance, two distinct serotypes of HSV were identified that were designated as subtypes HSV-1 and HSV-2 in future (Schneweiss, 1962). Subsequently, recent rapid genomic sequencing techniques enabled the genome-based phylogenetic analysis.

Initially, the genus *Herpesvirus* was established at the first International Committee on Taxonomy of Virus (ICTV) in 1971 (International Committee on Nomenclature of Viruses. and Wildy, 1971). The genus included 23 viruses and 4 groups of viruses that was named according to general usages at the time (e.g. herpesvirus of saimari) (Davison, 2010). A formal naming system for herpesviruses was founded in 1973 by Roizman and others (Roizman, 1973). This system was adopted to the second ICTV report in 1976 and the genus was elevated to the family

Herpetoviridae (Fenner, 1976). In 1979, to prevent the possible misleading association of this class name to reptiles and amphibians, the third ICTV report the family renamed to *Herpesviridae* that consisted of 21 viruses, (Matthews, 1979, Davison, 2010). The report also classified the family into three subfamilies; *Alpha-*, *Beta-*, and *Gammaherpesvirinae*, and 5 unnamed genera (Matthews, 1979). Subsequently, the formal nomenclature system was elaborated by the ICTV Herpesvirus Study Group and the family expanded to contain 89 viruses in 1981 (Roizman et al., 1981) . In the early 1990's, the system was then consolidated in the fifth report of ICTV (Francki et al., 1991) and the second report of ICTV Herpesvirus Study Group (Roizmann et al., 1992). At the second millennium, the species concept in *Herpesviridae* was adopted in the seventh ICTV report (Van Regenmortel and K., 2000). Recently, the new order *Herpesvirales* was created on the basis of phylogenic studies with very distant relatives of viruses, which consists of 3 families, 3 subfamilies, 17 genera and 90 species (Table 2-1) (Davison et al., 2009). In specific, the existing family *Herpesviridae* was revised to include mammalian, avian and reptilian viruses and two new families were established. The new family *Alloherpesviridae* encompasses bony fish and amphibian viruses, and another new family *Malacoherpesviridae* contains oyster herpesvirus, OsHV1 (Davison, 2010).

The present definition of a virus species by ICTV was adopted in its seventh report (Van Regenmortel and K., 2000), “a polythetic class of viruses that constitutes a replicating lineage and occupies a particular ecological niche” (Van Regenmortel, 1990). However, a rapidly evolving research environment steadily stimulates careful revision of the classical polythetic rule, as well as reclassification of the viruses (Davison, 2010). Although the previous classification according to their biological similarities is well matched to the genome-based phylogenetic analysis, a number of discrepancies appears between them (Davison, 2010). For

Table 2-1. Herpesvirus classification¹. [Table modified from (Davison, 2010)]

Taxon name	Common name	Acronym	Genome size (kb)
Order <i>Herpesvirales</i>			
Family <i>Herpesviridae</i>			
Subfamily <i>Alphaherpesvirinae</i>			
Genus <i>Iltovirus</i>			
Gallid herpesvirus 1*	Infectious laryngotracheitis virus	GaHV1	148,687
Psittacid herpesvirus 1	Pacheco's disease virus	PsHV1	163,025
Genus <i>Mardivirus</i>			
Columbid herpesvirus 1	Pigeon herpesvirus	CoHV1	
Gallid herpesvirus 2*	Marek's disease virus type 1	GaHV2	177,874
Gallid herpesvirus 3	Marek's disease virus type 2	GaHV3	164,270
Meleagrid herpesvirus 1	Turkey herpesvirus	MeHV1	159,160
Genus <i>Simplexvirus</i>			
Ateline herpesvirus 1	Spider monkey herpesvirus	AtHV1	
Bovine herpesvirus 2	Bovine mammillitis virus	BoHV2	
Cercopithecine herpesvirus 2	Simian agent 8	CeHV2	150,715
Human herpesvirus 1*	Herpes simplex virus type 1	HHV1	152,261
Human herpesvirus 2	Herpes simplex virus type 2	HHV2	154,746
Leporid herpesvirus 4	Leporid herpesvirus 4	LeHV4	
Macacine herpesvirus 1	B virus	McHV1	156,789
Macropodid herpesvirus 1	Parma wallaby herpesvirus	MaHV1	
Macropodid herpesvirus 2	Dorcopsis wallaby herpesvirus	MaHV2	
Papiine herpesvirus 2	Herpesvirus papio 2	PaHV2	156,487
Saimiriine herpesvirus 1	Marmoset herpesvirus	SaHV1	
Genus <i>Varicellovirus</i>			
Bovine herpesvirus 1	Infectious bovine rhinotracheitis virus	BoHV1	135,301
Bovine herpesvirus 5	Bovine encephalitis herpesvirus	BoHV5	137,821
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	124,784
Cervid herpesvirus 1	Red deer herpesvirus	CvHV1	
Cervid herpesvirus 2	Reindeer herpesvirus	CvHV2	
Equid herpesvirus 1	Equine abortion virus	EHV1	150,224
Equid herpesvirus 3	Equine coital exanthema virus	EHV3	
Equid herpesvirus 4	Equine rhinopneumonitis virus	EHV4	145,597
Equid herpesvirus 9	Gazelle herpesvirus	EHV9	148,371
Felid herpesvirus 1	Feline herpesvirus 1	FHV1	
Human herpesvirus 3*	Varicella-zoster virus	HHV3	124,884
Phocid herpesvirus 1	Harbour seal herpesvirus	PhoHV1	
Suid herpesvirus 1	Pseudorabies virus	SuHV1	143,461
Unassigned species in the subfamily			
Chelonid herpesvirus 5	Chelonid fibropapilloma-associated herpesvirus	ChHV5	
Chelonid herpesvirus 6	Lung-eye-trachea disease-associated virus	ChHV6	
Subfamily <i>Betaherpesvirinae</i>			
Genus <i>Cytomegalovirus</i>			
Cercopithecine herpesvirus 5	Simian cytomegalovirus	CeHV5	226,204
Human herpesvirus 5*	Human cytomegalovirus	HHV5	235,646
Macacine herpesvirus 3	Rhesus cytomegalovirus	McHV3	221,454
Panine herpesvirus 2	Chimpanzee cytomegalovirus	PnHV2	241,087

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(Table 2-1 continued)

Taxon name	Common name	Acronym	Genome size (kb)
Unassigned viruses in the genus			
Aotine herpesvirus 1	Herpesvirus aotus type 1	AoHV1	219,474
Aotine herpesvirus 3g	Herpesvirus aotus type 3	AoHV3	
Saimiriine herpesvirus 3	Squirrel monkey cytomegalovirus	SaHV3	[189,956]
Genus <i>Muromegalovirus</i>			
Murid herpesvirus 1*	Murine cytomegalovirus	MuHV1	230,278
Murid herpesvirus 2	Rat cytomegalovirus	MuHV2	230,138
Genus <i>Roseolovirus</i>			
Human herpesvirus 6	Human herpesvirus 6	HHV6	159,322
			162,114
Human herpesvirus 7	Human herpesvirus 7	HHV7	153,080
Genus <i>Proboscivirus</i>			
Elephantid herpesvirus 1	Elephant endotheliotropic herpesvirus	EIHV1	
Unassigned species in the subfamily			
Caviid herpesvirus 2	Guinea pig cytomegalovirus	CavHV2	
Suid herpesvirus 2	Pig cytomegalovirus	SuHV2	
Tupaïid herpesvirus 1	Tupaïid herpesvirus	TuHV1	195,859
Subfamily <i>Gammaherpesvirinae</i>			
Genus <i>Lymphocryptovirus</i>			
Callitrichine herpesvirus 3	Marmoset lymphocryptovirus	CalHV3	149,696
Cercopithecine herpesvirus 14	African green monkey EBV-like virus	CeHV14	
Gorilline herpesvirus 1	Gorilla herpesvirus	GoHV1	
Human herpesvirus 4*	Epstein Barr virus	HHV4	171,823
Macacine herpesvirus 4	Rhesus lymphocryptovirus	McHV4	171,096
Panine herpesvirus 1	Herpesvirus pan	PnHV1	
Papiine herpesvirus 1	Herpesvirus papio	PaHV1	
Pongine herpesvirus 2	Orangutan herpesvirus	PoHV2	
Genus <i>Macavirus</i>			
Alcelaphine herpesvirus 1*	Wildebeest-associated malignant catarrhal fever virus	AlHV1	130,608
Alcelaphine herpesvirus 2	Hartebeest malignant catarrhal fever virus	AlHV2	
Bovine herpesvirus 6	Bovine lymphotropic herpesvirus	BoHV6	
Caprine herpesvirus 2	Caprine herpesvirus 2	CpHV2	
Hippotragine herpesvirus 1	Roan antelope herpesvirus	HiHV1	
Ovine herpesvirus 2	Sheep-associated malignant catarrhal fever virus	OvHV2	135,135
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 <i>Percavirus</i>			
Equid herpesvirus 2*	Equine herpesvirus 2	EHV2	184,427
Equid herpesvirus 5	Equine herpesvirus 5	EHV5	
Mustelid herpesvirus 1	Badger herpesvirus	MusHV1	
Genus <i>Rhadinovirus</i>			
Ateline herpesvirus 2	Herpesvirus ateles strain 810	AtHV2	
Ateline herpesvirus 3	Herpesvirus ateles	AtHV3	108,409
Bovine herpesvirus 4	Bovine herpesvirus 4	BoHV4	108,873
Human herpesvirus 8	Human herpesvirus 8	HHV8	137,969
Macacine herpesvirus 5	Rhesus rhadinovirus	McHV5	133,719
Murid herpesvirus 4	Murine herpesvirus 68	MuHV4	119,450
Saimiriine herpesvirus 2*	Herpesvirus saimiri	SaHV2	112,930

(Table 2-1 continued)

Taxon name	Common name	Acronym	Genome size (kb)
Unassigned viruses in the genus			
Leporid herpesvirus 1	Cottontail rabbit herpesvirus	LeHV1	
Leporid herpesvirus 2	Herpesvirus cuniculi	LeHV2	
Leporid herpesvirus 3	Herpesvirus sylvilagus	LeHV3	
Marmodid herpesvirus 1	Woodchuck herpesvirus	MarHV1	
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	
Unassigned viruses in the family			
Accipitrid herpesvirus 1	Bald eagle herpesvirus	AcHV1	
Anatid herpesvirus 1	Duck enteritis virus	AnHV1	158,091
Boid herpesvirus 1	Boa herpesvirus	BoiHV1	
Callitrichine herpesvirus 2	Marmoset cytomegalovirus	CalHV2	
Caviid herpesvirus 1	Guinea pig herpesvirus	CavHV1	
Caviid herpesvirus 3	Guinea pig herpesvirus 3	CavHV3	
Cebine herpesvirus 1	Capuchin herpesvirus AL-5	CbHV1	
Cebine herpesvirus 2	Capuchin herpesvirus AP-18	CbHV2	
Cercopithecine herpesvirus 3	SA6	CeHV3	
Cercopithecine herpesvirus 4	SA15	CeHV4	
Chelonid herpesvirus 1	Grey patch disease-associated virus	ChHV1	
Chelonid herpesvirus 2	Pacific pond turtle herpesvirus	ChHV2	
Chelonid herpesvirus 3	Painted turtle herpesvirus	ChHV3	
Chelonid herpesvirus 4	Argentine turtle herpesvirus	ChHV4	
Ciconiid herpesvirus 1	Black stork herpesvirus	CiHV1	
Cricetid herpesvirus	Hamster herpesvirus	CrHV1	
Elapid herpesvirus 1	Indian cobra herpesvirus	EpHV1	
Erinaceid herpesvirus 1	European hedgehog herpesvirus	ErHV1	
Falconid herpesvirus 1i	Falcon inclusion body disease virus	FaHV1	
Gruid herpesvirus 1	Crane herpesvirus	GrHV1	
Iguanid herpesvirus 1	Green iguana herpesvirus	IgHV1	
Lacertid herpesvirus	Green lizard herpesvirus	LaHV1	
Macacine herpesvirus 6	Rhesus leukocyte-associated herpesvirus strain 1	McHV6	
Macacine herpesvirus 7	Herpesvirus cyclopis	McHV7	
Murid herpesvirus 3	Mouse thymic herpesvirus	MuHV3	
Murid herpesvirus 5	Field mouse herpesvirus	MuHV5	
Murid herpesvirus 6	Sand rat nuclear inclusion agent	MuHV6	
Murid herpesvirus 7	Wood mouse herpesvirus	MuHV7	120,108
Ovine herpesvirus 1	Sheep pulmonary adenomatosis-associated herpesvirus	OvHV1	
Perdicid herpesvirus 1	Bobwhite quail herpesvirus	PdHV1	
Phalacrocoracid herpesvirus 1	Cormorant herpesvirus	PhHV1	
Procyonid herpesvirus 1	Kinkajou herpesvirus	PrHV1	
Sciurid herpesvirus 1	Ground squirrel cytomegalovirus	ScHV1	
Sciurid herpesvirus 2	Ground squirrel herpesvirus	ScHV2	
Sphenicid herpesvirus 1	Black footed penguin herpesvirus	SpHV1	
Strigid herpesvirus 1i	Owl hepatitis virus	StHV1	
Family <i>Alloherpesviridae</i>			
Genus <i>Batrachovirus</i>			
Ranid herpesvirus 1*	Lucké tumor herpesvirus	RaHV1	220,859
Ranid herpesvirus 2	Frog virus 4	RaHV2	231,801

(Table 2-1 continued)

Taxon name	Common name	Acronym	Genome size (kb)
Genus <i>Cyprinivirus</i>			
Cyprinid herpesvirus 1	Carp pox herpesvirus	CyHV1	295,146
Cyprinid herpesvirus 2	Goldfish haematopoietic necrosis virus	CyHV2	
Cyprinid herpesvirus 3*	Koi herpesvirus	CyHV3	
Genus <i>Ictalurivirus</i>			
Ictalurid herpesvirus 1*	Channel catfish virus	IcHV1	134,226
Ictalurid herpesvirus 2	Black bullhead herpesvirus	IcHV2	
Acipenserid herpesvirus 2	White sturgeon herpesvirus 2	AciHV2	
Genus <i>Salmonivirus</i>			
Salmonid herpesvirus 1*	Herpesvirus salmonis	SalHV1	
Salmonid herpesvirus 2	Oncorhynchus masou herpesvirus	SalHV2	
Salmonid herpesvirus 3	Epizootic epitheliotropic disease virus	SalHV3	
Unassigned viruses in the family			
Acipenserid herpesvirus 1	White sturgeon herpesvirus 1	AciHV1	248,531
Anguillid herpesvirus 1	Eel herpesvirus	AngHV1	
Esocid herpesvirus 1	Northern pike herpesvirus	EsHV1	
Percid herpesvirus 1	Walleye epidermal hyperplasia herpesvirus	PeHV1	
Pleuronectid herpesvirus 1	Turbot herpesvirus	PIHV1	
Family <i>Malacoherpesviridae</i>			
Genus <i>Ostreavirus</i>			
Ostreid herpesvirus 1*	Oyster herpesvirus	OsHV1	207,439

*The representative species of each genus

instance, the Marek's disease virus type 1 (Gallid herpes virus type 2) initially classified as a gammaherpesvirus primarily due to the tropism for lymphocytes like varicella zoster virus (VZV), but analysis on its genome sequence indicated close relationship to alphaherpesviruses (Buckmaster et al., 1988).

Previously, establishment of the order *Herpesvirales* was provoked by the PCR-based ongoing discovery of new herpesviruses. Now, the state-of-art sequencing techniques provide reliable genomic sequences and promote genomic-based phylogenic studies. Up to date 51 herpesvirus species or potential species have been sequenced. Furthermore, the next generation sequencings (NGS) could provide the full-gene sequences of target viruses within a few days. In conjunction with bioinformatics, the next generation genomic techniques are overcoming the major weak points of the primary sequence database: (a) accuracy of the sequence and (b) quality of annotation (Davison, 2010). This systemizing information will be able to provide more

accurately annotated reference sequences of the herpesviruses and allow new insight and convenience for comparative research in virology. In this line, reclassification of herpesviruses is an ongoing project and their provisional protein names keep updating based on the genomic-based phylogenic study (Davison, 2010).

Common Biological Properties of Herpesviruses

The members of *Herpesviridae* share four distinct biological properties: 1) The viruses encode a large group of enzymes for their own propagation, which involves in nucleic acid metabolism (e.g. thymidine kinase), DNA synthesis (e.g. DNA helicase/primase) and processing of proteins (e.g. protein kinase); 2) The replication of viral DNAs and assembly of capsids take place in the nucleus. The virion undergoes final processing in the cytoplasm; 3) Production of infectious progeny virus inevitably causes the lysis of the infected cells (lytic infection); 4) The herpesviruses can latently infect in their natural host without producing progeny virus as well as viral products other than small viral RNA transcripts, latency associated transcripts (LAT) [reviewed in (Pellett and Roizman, 2007)].

In specific, each subfamily of *Herpesviridae* shares biological properties: (a) Members of the *Alphaherpesvirinae* commonly show wide host specificity, a relatively short reproductive cycle, rapid spread in tissue cultures, efficient lytic destruction of infected cells, and capability to establish latent infections primarily but not exclusively in sensory ganglia [e.g. Genera *Iltovirus*, *Mardivirus*, *Simplexvirus* (including HSV-1), and *Varicellovirus*]; (b) Members of the *Betaherpesvirinae* subfamily polythetically present narrow host range, long reproduction cycle, slow infection progress in tissue culture, often enlargement of infected cell (cytomegaly), capability to readily establish carrier culture by infecting the virus, and latency in secretory glands, lymphoreticular cells, kidneys or other tissues (e.g. Genera *Cytomegalovirus*,

Muromegalovirus, *Roseolovirus* and *Proboscivirus*); and (c) Members of the *Gammaherpesvirinae* subfamily exhibit that their host-specificity is strictly limited to the family or order to which the natural host belongs in experiments, all replicates in lymphoblastoid cells *in vitro*, some causes lytic infection in some type of epithelial and fibroblastic cells, usually the viruses are specific for either B or T lymphocytes, and latent infection is usually discovered in lymphoid tissue (e.g. *Lymphocryptovirus*, *Macavirus*, *Percavirus* and *Rhadinovirus*) (Davison, 2010, Roizmann et al., 1992, Pellett and Roizman, 2007).

Organization of Herpesvirus Genome

The herpesvirus genome from virion is a linear double stranded DNA but circularizes immediately after releasing from the capsid into the nucleus of infected cell (Pellett and Roizman, 2007). The lengths of genomes vary from 108 to 295 kbp depending on viral species (Table 2-1), which divergence is distinct from the polymorphism in DNA sizes of individual viruses (Davison, 2010). Since the terminal and internal repeated sequences can vary in copy number, the individual viral genome can be different over 10 kbp. The total guanine and cytosine (G/C) content of herpesvirus genomes varies from 31% to 75% (Pellett and Roizman, 2007). In the case of HSV-1, the genome is approximately 152 kbp with a G/C content of 68% (Szpara et al., 2010), which encodes 77 annotated proteins. The genome arranges into two unique regions, Long (UL) and Short (US) according to their relative length (108 kbp and 13 kbp), which are flanked by inverted repeat sequences; two internal repeats (IR) and two terminal repeats (TR) (Figure 2-1). Both regions can invert relative to each other by internal recombination via an element, so-called 'a' sequence, which presents at each terminus as a direct repeat and at the L/S joint in an inverted form. Therefore, the genome from infected cells can present in equimolar four isomers that have different relative orientations of both unique regions (Quinn et al., 2000,

Pellett and Roizman, 2007). The genome also contains variable-number tandem repeats (VNTRs) such as small microsatellite repeats (<100 bp each) and short tandemly reiterated sequences (<500 bp each) (Deback et al., 2009, McGeoch et al., 1988, McGeoch et al., 1986, Szpara et al., 2010). The VNTR presents higher concentration in the terminal repeats which contain a lower percentage of genes than elsewhere in the genome. The VNTR is highly variable and differs not only between the strains but also within the same strain during its replication (Norberg et al., 2004, Umene and Kawana, 2003, Umene et al., 2008, Umene et al., 1984, Szpara et al., 2010).

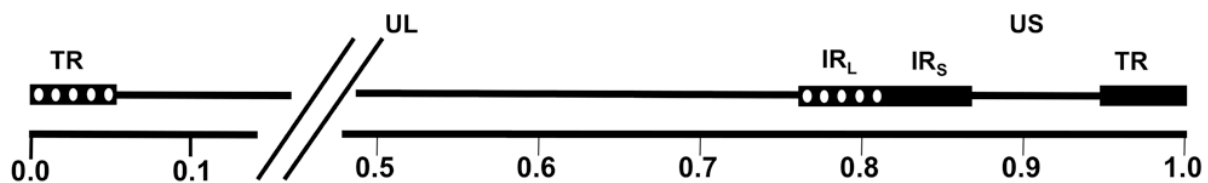


Figure 2-1. Prototypic arrangement of the HSV-1 genome. HSV-1 prototypic genome arrangement is represented with relative positions of the terminal repeats (TR), the unique long (UL) and short (US) segments, and the internal repeats long (IR_L) and short (IR_S). The white dotted or solid regions denote the positions of the related repeats. [Figure from (David, 2012)]

The genome contains three origins of replication: one in UL (*oriL*), and two in US at IR_S and TR_S (*oriS*). Either of them is sufficient for viral replication. Progeny viral genomes are initially replicated as a head-to-tail linked form of concatemeric intermediates. The genome is matured by the terminase, an enzyme complex that mediates the encapsidation and monomeric cleavage of concatemer within the capsid. The cleavage occurs sequence-specifically on two highly conserved DNA sequence motifs, *pac1* and *pac2*, which lie near the ends of the herpesvirus genome, terminal repeats (Brown et al., 2002, Deiss et al., 1986).

Herpesvirus encoding genes typically contain: a promoter/regulatory sequence 50-200 bp upstream of a TATA box; a transcription initiation site 20 to 25 bp downstream of the TATA box; a 5'-nontranslated leader sequence of 30-300 bp; a single major open reading frame (ORF)

with a translation initiation codon; 10 to 30 bp of 3'-nontranslated sequence; and a polyadenylation signal with standard flanking sequences (Pellett and Roizman, 2007). Some exceptions exist such as genes without a TATA box and initiation from a second in-frame methionine (Chou and Roizman, 1986, Markovitz et al., 1999). Due to the high frequency of gene overlaps, the promoter-regulatory sequences of 3' genes are mostly located within the coding domain of 5' genes, except when the gene is arranged in a head-to-head form, while some herpesvirus late genes may have the promoter-regulatory sequence at 3' to the TATA box (Mavromara-Nazos and Roizman, 1989). A few herpesvirus genes have introns which may serve to differentially regulate genes at different stages of the virus life cycle. However the most herpesvirus genes are not spliced. Herpesviruses also produce noncoding RNAs such as latency associated transcript (LAT) of HSV-1. Encoding genes of each species of herpesviruses vary from 70 to 200 (Pellett and Roizman, 2007). The HSV-1 genome contains at least 74 ORFs with a speculation of 84 transcriptional units by 94 putative ORFs (McGeoch et al., 2006, Rajcani et al., 2004).

Architecture of Herpesvirus Virion

The herpesvirus virion consists of three distinct layers: an icosahedral capsid contains an electron-dense core of double stranded viral DNA and is covered by an amorphous proteinaceous tegument and an outer lipid glycoprotein envelope (Figure 2-2). The mature virion appeared as 120 - 260 nm quasispherical shape possibly due to the variable thickness of the tegument. The virion contains approximately 24-71 virally encoded polypeptides: 4-7 capsid proteins, 9-17 in the tegument, and 4-19 in the envelope, with a number whose location is unknown. The herpesvirus particle comprises not only viral proteins but also several host components. Various host proteins are integrated in virion during the maturation process, including cellular structural proteins, enzymes and chaperones. Yet the roles of host-oriented components remain unclear.

The virion additionally contains viral- and cellular-encoded RNAs that can be translated immediately after infection [reviewed in (Pellett and Roizman, 2007)].

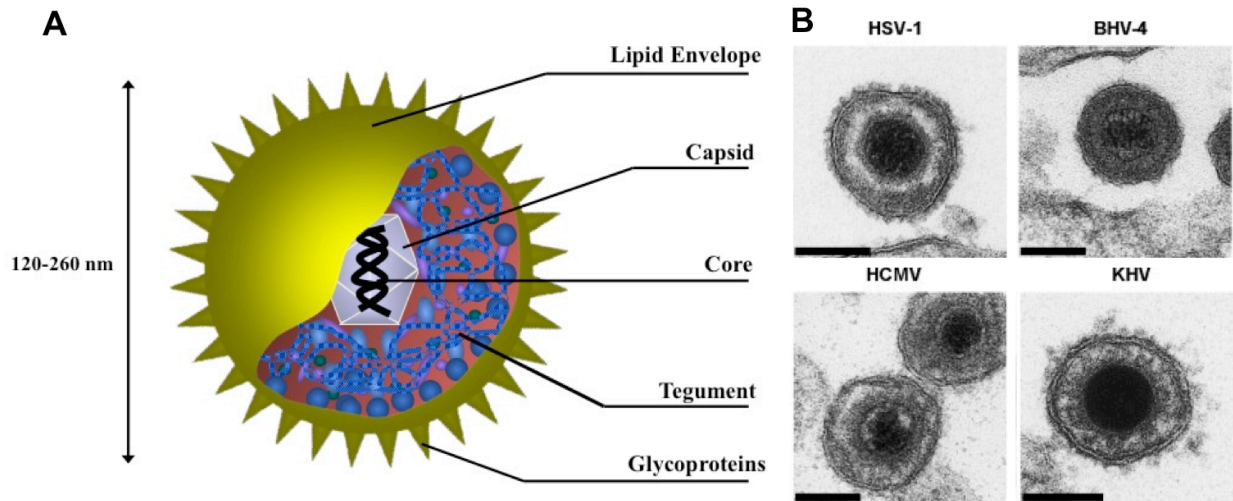


Figure 2-2. Structure and morphology of herpesvirus². The herpesvirus virion is composed of an icosahedral capsid containing the electron-dense core, an amorphous proteinaceous tegument around the capsid, and an outer lipid envelope derived from host cell membranes containing glycoprotein spikes (A). The extracellular virions of herpesviruses shares common morphology: the alphaherpesvirus HSV-1, the betaherpesvirus HCMV, the gammaherpesvirus bovine herpesvirus 4 (BHV-4) and the alloherpesvirus koi herpesvirus (KHV) are shown (B). Bar = 100 nm. [Figure modified from (Melancon, 2004, Mettenleiter et al., 2009)]

Core

The core of a mature virion appears as opaque in the center of a spherical virus under the EM, which is a condensed viral DNA in the shape of a torus. The doughnut structure is 50 nm high, with 18 nm and 70 nm inner and outer diameters, respectively. The arrangement of viral DNA in the torus remains unclear [reviewed in (Pellett and Roizman, 2007)].

Capsid

The capsids in all herpesviruses, including far distantly related fish and oyster herpesviruses, share the same feature (Davison et al., 2005). The icosahedral structure of the capsid is 15 nm

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thick and 125 nm diameter in size and is composed of 162 capsomeres (150 hexons and 12 pentons) with triangulation symmetry of T = 16 (Booy et al., 1991). Cryo-EM studies have elaborated the detailed structure of the HSV nucleocapsid which consists of an outer layer (T = 16 symmetry) and an intermediate layer (T = 4 lattice) (Schrag et al., 1989). In the herpesvirus-infected cells, three main forms of non-enveloped capsids are present, which have the same structure except for the state of the viral DNA core and scaffolding proteins. A-capsids have no core, B-capsids contain the scaffold but no core structure, and C-capsids encapsidate the viral genome but the scaffold is dissociated (Homa and Brown, 1997). The protein components of capsid are listed in Table 2-2 (Baines and Duffy, 2006). Among them, the components of the outer shell is four viral proteins VP5, VP26, VP23 and VP19C (Zhou et al., 2000b). The VP5 is a

Table 2-2. Protein components of HSV-1 capsids³. [Table from (Baines and Duffy, 2006)]

Protein	Gene	Component of	Location in capsid	Approximate copy number per capsid
VP5	UL19	All capsid types	Capsomers	955 (assuming a penton is replaced by portal)
VP19C	UL38	All capsid types	Triplexes	320
VP23	UL18	All capsid types	Triplexes	640
pUL6	UL6	All capsid types	Portal vertex	12
pUL25	UL25	All capsid types	Outside on multiple facets	27-42
pUL17	UL17	A, B and C capsids	Outside on multiple facets	19.2 +/- 5.0 in B capsids
pre-VP22a	UL26.5	Procapsids	Scaffold	> 1000
Protease	UL26	Procapsids	Inside near scaffold	87-147
VP22a	UL26.5	B-capsids	Inside (=cleaved scaffold)	1153
VP21	UL26	B-capsids	Inside (=cleaved protease)	87-147
VP24	UL26	A(?)*, B, and C	Inside (=cleaved protease)	87-147
VP26	UL35	A, B, and C	Hexon tips	952
pUL15	UL15	All capsids types**	Interact with pUL6	1 in B capsids, 12.0 (+/-5.63) in A capsids
pUL28	UL28	Procapsids, B, and C**	Outside with portal	2.4 (+/-1) In B capsids. ≤ 1 in A capsids
pUL33	UL33	Procapsids(?)* A, B, and C(?)*	Outside with pUL15 and pUL28	?

*The presence of VP24 in A capsids and of pUL33 in procapsids and C capsids has not been studied.

**pUL15 and pUL28 are present in C capsids, but in lower amounts compared to the other capsids types.

³ Reprinted from Alpha Herpesviruses: Molecular And Cellular Biology, 175-204, Nucleocapsid Assembly and Envelopment of Herpes, Copyright (2006), with permission from Drs. Baines and Duffy.

primary structural subunit that presents 960 copies per capsid. In detail, there are six copies per hexon ($6 \times 150 = 900$ copies per capsid) and five copies per penton ($5 \times 12 = 60$ copies per capsid) [reviewed in (Pellett and Roizman, 2007)]. Six copies of VP26 are situated on the top of VP5 subunits of each hexon as a ring shape (Zhou et al., 1995). One VP19C and two VP23 proteins form a heterotriplex that acts as a scaffold linking adjacent capsomeres (Spencer et al., 1998). In the capsid, UL6 proteins forms a dodecamer which is a cylinder-like portal to function as a channel for DNA introduction into the capsid during encapsidation as well as DNA release into the nucleus during virus infection (Newcomb et al., 2001). Additionally, the capsid incorporates a protease, VP24 (UL26) that processes the scaffolding during DNA encapsidation (Newcomb et al., 1999, Homa and Brown, 1997).

Tegument

The tegument is a proteinaceous layer between the capsid and envelope with various thicknesses depending on the location of virion within the infected cells (Pellett and Roizman, 2007). The layer appears to be sometimes asymmetrically distributed and fibrous on negative staining EM (Morgan et al., 1959, Morgan et al., 1968, Wildy and Watson, 1962). The tegument of virion appears more symmetrical in perinuclear space and more asymmetrical in cytoplasm (Falke et al., 1959). The extracellular virion sometimes shows asymmetrical tegument but time-dependently the tegument becomes more symmetrically organized (Grunewald et al., 2003, Newcomb and Brown, 2009). The structural analysis of tegument by using multiple approaches including cryo-electron tomography, biochemical assay, viral-gene deletion and yeast two-hybrid screen has suggested that the tegument may be assembled in an orderly shape via defined protein-protein interaction network (Kelly et al., 2009). The tegument proteins interact not only with each other but also with the capsid and envelope proteins (Vittone et al., 2005). This allows

the tegument proteins to form a network layer that bridges the envelope and capsid. The tegument of a mature HSV-1 virion is composed of 24 proteins that play multiple functions in viral replication (Table 2-3) (Kelly et al., 2009, Loret et al., 2008, Mettenleiter, 2004).

Table 2-3. Properties and functions of HSV-1 tegument proteins⁴. [Table from (Kelly et al., 2009)]

Tegument protein ^a	Predicted Mwt (kDa)	Essential (E) or non-essential (NE) in cell culture	Previously described virion protein ^b	Functions
pUL7	33.1	NE	No	Regulates mitochondrial function
pUL11	10.5	NE	Yes	Secondary envelopment
pUL13	57.2	NE	Yes	Protein kinase, tegument dissociation, regulates apoptosis and pUS3, inhibits IFN response
pUL14	23.9	NE	Yes	Nuclear import, regulates apoptosis, nuclear targeting of capsids
pUL16	40.4	NE	Yes	Secondary envelopment
pUL21	57.6	NE	Yes	Secondary envelopment, regulates microtubule assembly
pUL23	41	NE	No	Thymidine kinase, viral DNA replication
pUL36 (VP1/2)	335.9	E	Yes	Capsid transport, secondary envelopment, release of viral DNA, deubiquitinating activity
pUL37	120.6	E	Yes	Secondary envelopment, regulates viral transcription
pUL41	54.9	NE	Yes	Regulates host/viral translation and immune response
pUL46 (VP11/12)	78.2	NE	Yes	Secondary envelopment, regulates pUL48-dependent transcription
pUL47 (VP13/14)	73.8	NE	Yes	Secondary envelopment, regulates pUL48-dependent transcription
pUL48 (VP16)	54.3	E	Yes	Secondary envelopment, regulates viral transcription
pUL49 (VP22)	32.3	NE	Yes	Secondary envelopment, regulates microtubule assembly
pUL50	39.1	NE	No	dUTPase, viral DNA replication
pUL51	25.5	NE	Yes	Unknown
pUL55	20.5	NE	No	Unknown
pUS2	32.5	NE	Yes	Unknown
pUS3	52.8	NE	Yes	Protein kinase, primary deenvelopment, tegument dissociation, regulates actin assembly
pUS10	34.1	NE	Yes	Unknown
pUS11	17.8	NE	Yes	Regulates host translation, capsid transport
ICP34.5	26.2	NE	Yes	Regulates host translation, viral DNA replication and immune response
ICP0	78.5	NE	Yes	Regulates viral transcription
ICP4	132.8	E	Yes	Regulates viral transcription

^a Identified in HSV-1 virions by mass spectroscopy (Loret et al., 2008).

^b Based on Mettenleiter et al. (2006) and Roizman et al. (2007).

⁴ Reprinted from Virus Res 145(2), 173-86, Functional roles of the tegument proteins of herpes simplex virus type 1, Copyright (2009), with permission from Elsevier.

Importantly, their role immediately after infection is modulation of the host cell environment to accommodate for virus replication such as shutting off host cell protein synthesis, inhibiting infection-triggered cell defenses, and stimulating viral gene expression (Pellett and Roizman, 2007). Most of the tegument proteins in *Alphaherpesvirinae* are conserved except pUS2, pUS10, pUS11 and ICP34.5. Moreover, among them, the genes encoding pUL7, pUL11, pUL13, pUL14, pUL16, pUL21, pUL36, pUL37 and pUL51 are conserved throughout the *Herpesviridae* family (Kelly et al., 2009). The most abundant tegument proteins are pUL46 (VP11/12), pUL47 (VP13/14), pUL48 (VP16) and pUL49 (VP22), which present at 1000-2000 copies. For *in vitro* replication, pUL36 (VP1/2), pUL37, pUL48 and ICP4 are essential for the HSV-1 in cell culture [reviewed in (Kelly et al., 2009)]. Non-essential tegument proteins show significant redundancy that can be compensated by increased incorporation of other tegument proteins during the maturation of virus (del Rio et al., 2005, Michael et al., 2006).

Envelope

The envelope is an outer covering lipid bilayer of the virus. Typically it appears as a trilaminar structure that derives from patches of altered cellular membranes (Epstein, 1962, Falke et al., 1959, Morgan et al., 1968, Armstrong et al., 1961). The matured herpesvirus contains at least a dozen different proteins on its envelope, which copy number is varying to exceed 1,000 copies per virion (Roizman et al., 2007, Zhang et al., 2009, Connolly et al., 2011). In detail, 10 glycoproteins have been identified in HSV-1: gB (UL27), gC (UL44), gD (US6), gE (US8), gG (US4), gH (UL22), gI (US7), gK (UL53), gL (UL1), and gM (UL10). The presence of gJ (US5) and gN (UL49.5) were not demonstrated in HSV-1 but in other herpesviruses (Roizman et al., 2007). There are at least two non-glycosylated proteins on the surface of envelope, pUL20 and

pUS9, and possibly pUL24, pUL43, and pUL34. pUL24 and pUL34 are nuclear proteins that present in perinuclear virion but not in matured virion (Roizman et al., 2007).

A recent publication on comprehensive characterization of extracellular HSV-1 virions by mass spectrometry reported the absent of the viral glycoproteins gJ, gK, gN, and pUL43 in the matured HSV virion envelope but gK has been repeatedly identified by western blot analyses (Foster et al., 2001, Klupp et al., 1998, Loret et al., 2008). Additionally, pUL11 is regarded as a membrane-associated protein which anchors to the inner face of the membrane via its two acylation sites and interacts with the cytoplasmic domain of gE (Yeh et al., 2011). Among the envelope glycoproteins, gB, gD, gH and gL have been identified as essential proteins for viral replication by single gene knock-out analyses (Roizman et al., 2007). The envelope proteins play an important role in viral entry by interacting with its cognitive cellular receptors and mediating fusion between the viral envelope and cellular membrane during the entry process (Connolly et al., 2011).

Life Cycle of Herpesvirus

The life cycle of herpesvirus is diagramed in Figure 2-3. As many other enveloped viruses, herpesviruses start their journey of life from the (1) attachment and (2) entry of mature virion into a host cell through direct fusion of their envelope into plasma membrane or endocytosis via either a pH-dependent or -independent manner (Connolly et al., 2011). Followed by entry, (3) the viral capsid is deposited into the cytoplasm and (4) transported to the nucleus via cellular microtubule-associated motor system (Radtke et al., 2010). (5) The capsid then docks on the nuclear pore and releases viral DNA into the nucleus. At the same time, the tegument protein pUL41 (virion host shutoff, VHS), which is dissociated from the infected virus during the entry, shuts off the host protein expression to modulate the cellular environment making it suitable for

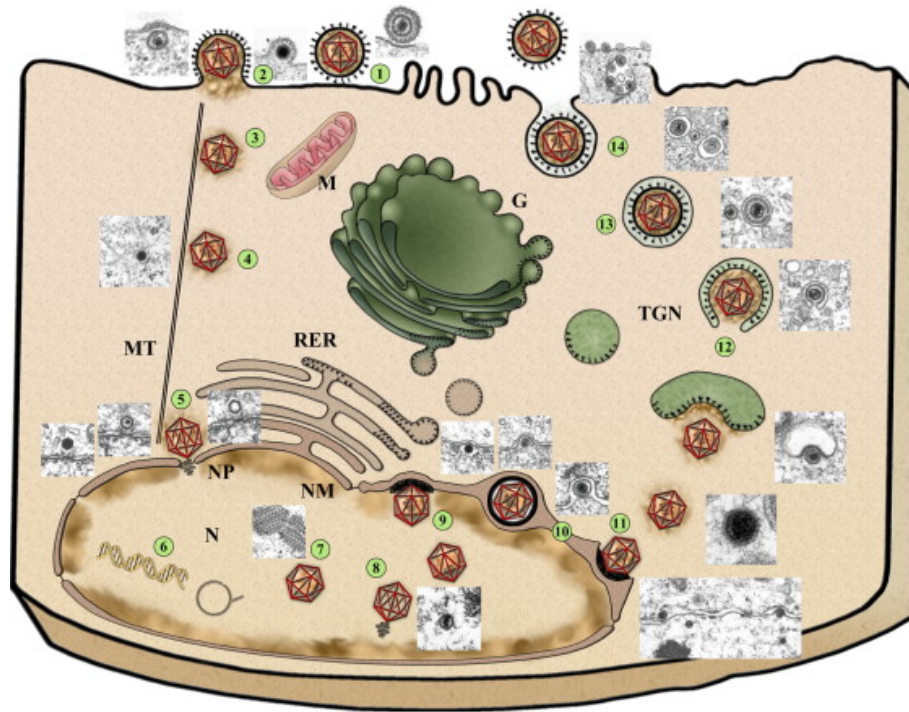


Figure 2-3. Diagram of herpesvirus life cycle⁵. Herpesvirus replication cycle is shown with an electron micrograph of each stage. Cellular structures denote as: N, nucleus; MT, microtubules; NP, nuclear pore; NM, nuclear membrane; TGN, trans-Golgi network (TGN); RER, rough endoplasmic reticulum; M, mitochondrion; G, Golgi apparatus. [Figure from (Mettenleiter et al., 2009)]

the viral replication. (6) Within the nucleus, the viral gene transcription and DNA replication initiates. The viral genes are transcribed in timely cascade fashion that is controlled by both transcriptional and post-transcriptional regulations. The viral DNA is initially replicated in concatemeric form and cleaved into monomers during (8) the encapsidation to (7) a preformed capsid. (9-10) The primary envelope is acquired through the budding into the perinuclear space of the DNA-packaged capsid (nucleocapsid). (11) The primary enveloped virion is subsequently de-enveloped during the egress to the cytoplasm. (12) The de-enveloped virion is coated with tegument proteins in the cytoplasm and then (13) acquires the secondary envelope during budding into a vesicle of trans-Golgi network. (14) The mature virion in the vesicle is

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subsequently exocytosed to extracellular space. The entire process of viral replication takes approximately 18 to 20 hours in the permissive cells [reviewed in (Roizman et al., 2007, Mettenleiter et al., 2009, Mettenleiter et al., 2013)].

Entry via membrane fusion

Herpesvirus enters into cells via different predominant pathways depending on target cell-types: (a) pH-independent fusion of the viral envelope with the host cell membrane (e.g. Vero and Hep-2 cells) (Milne et al., 2005); (b) receptor-mediated endocytosis and subsequent pH-dependent fusion between the viral envelope and the endosomal membrane [e.g. HeLa and Chinese hamster ovary (CHO) cells expressing nectin-1, gD receptor] (Nicola et al., 2003); (c) pH-independent endocytosis (e.g. C10 murine melanoma cells) (Milne et al., 2005).

Unlike the entry pathways of other enveloped viruses, which are usually mediated by single fusion protein, herpesviruses use unique protein machinery for attachment, binding, and membrane fusion (Connolly et al., 2011). During the entry process, viral glycoproteins on the envelope are interacting with each other as well as their cognitive cellular receptors. Throughout the herpesviruses, gB and gH/gL heterodimer are required as a ‘core fusion machinery’ (Spear and Longnecker, 2003, Heldwein and Krummenacher, 2008). Additionally, HSV essentially requires gD and some herpesviruses entail gH/gL-associated proteins such as UL128-131 of cytomegalovirus (CMV) (Ryckman et al., 2006) or gp42 of Epstein-bar virus (EBV) (Li et al., 1997, Wang and Hutt-Fletcher, 1998). Entry of HSV therefore, requires a multipartite fusion machinery comprised of a quartet of viral glycoproteins, gB, gD, and gH/gL along with cognitive cellular receptors (Table 2-4) (Spear, 2004). This fusion complex seems to be formed when HSV contacts to the cell, because the quartet associations alters during the entry (Avitabile et al., 2009).

Table 2-4. Entry related HSV glycoproteins⁶. [Table modified from (Connolly et al., 2011)]

Glycoprotein	Cell receptor	Function
gB	Heparan sulfate DC-SIGN PILR- α MAG NMMHCIIA	Catalyzes membrane fusion
gC	Heparan sulfate	Initial binds cells Facilitate subsequent binding of gD to cells
gD	HVEM Nectin 1 and 2 3-O-sulphated heparin sulphate	Binds cells Triggers fusion
gH/gL	Integrins	Triggers or regulates fusion

Briefly, the entry mechanism into host epithelial cell of HSV can be described in three stages. Initially, the virus attaches to surface of the target cell by binding of gC, and, to a lesser extent gB, to cell-surface glycosaminoglycans (GAGs) moieties of heparan sulfate (HS). Secondly, gD interacts with several cellular receptors along with conformational changes that launch the fusion cascade (Roizman et al., 2007, Gianni et al., 2010). Finally, the gB-mediated membrane fusion between viral envelope and plasma membrane results in release of the tegumented viral capsid into the cytoplasm (Roizman et al., 2007).

Initial attachment is mainly mediated by gC, although other glycoproteins also have receptor binding properties (Herold et al., 1991, Shukla and Spear, 2001). The N-terminal major binding site of gC is comprised of amino acid residues 129-RR-130 and 142-IRCRFRNSTRMEFRLQIW R-160 as well as another region around G247 (Trybala et al., 1994, Mardberg et al., 2001). Albeit gC is dispensable for either entry or viral replication, its presence on the viral envelope can increase binding efficiency approximately 10 times (Herold et al., 1991, Laquerre et al., 1998, Shukla and Spear, 2001). During infection of dendritic cells, the C-type lectin dendritic

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cell-specific ICAM3-grabbing non-integrin (DC-SIGN; also known as CD209) also can serve as a binding partner to gB and gC and facilitate attachment (Ma et al., 2008).

Membrane fusion during HSV entry is triggered by gD binding to relevant host cell receptors. gD, the receptor binding glycoprotein and the major determinant of HSV tropism, interacts with at least three classes of receptors: immunoglobulin super family [e.g. nectin-1 (HveC) and nectin-2 (HveB)]; tumor necrosis factor superfamily [e.g. herpesvirus entry mediator (HVEM; HveA)]; and 3-O-sulfated heparan sulfate in some cells (Figure 2-4) (Montgomery et al., 1996, Geraghty et al., 1998, Shukla et al., 1999, Campadelli-Fiume et al., 2000, Spear et al., 2000, Yoon et al., 2003, Satoh et al., 2008). In detail, nectin-1 or nectin-2, cell adhesion molecules belongs to an immunoglobulin superfamily; HVEM is a member of the tumor necrosis factor receptor family; and specific modification in heparan sulfate (3-O-S HS) catalyzed by

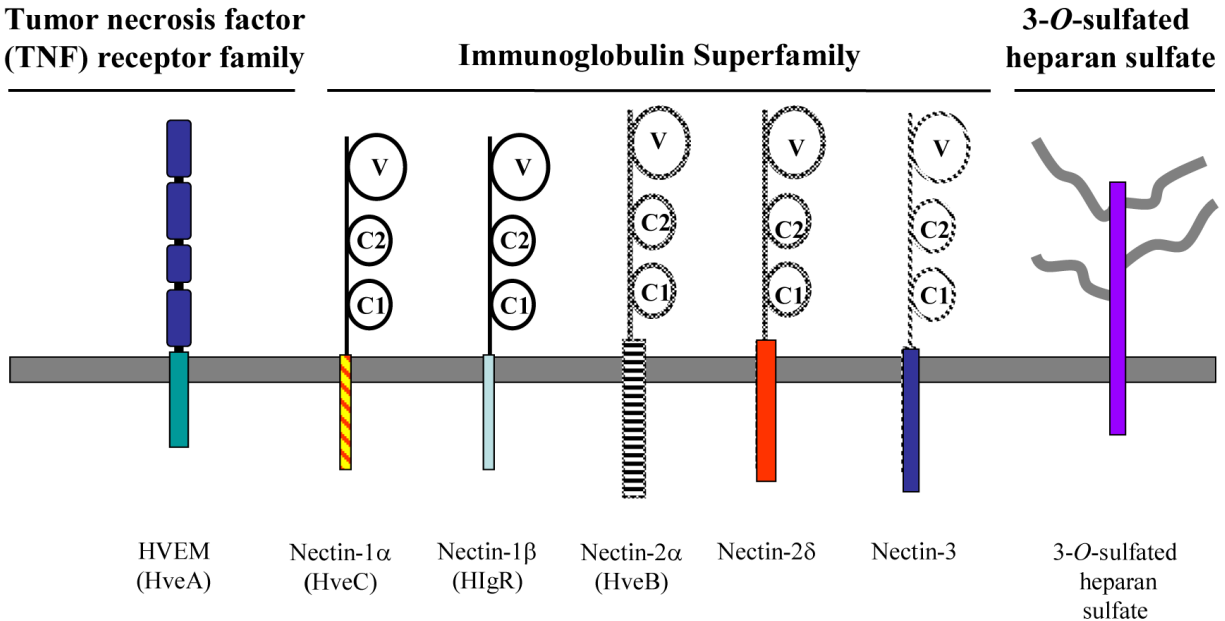


Figure 2-4. Cellular receptors for HSV-1 entry. The three classes of cell surface receptors for HSV entry are schematized: the tumor necrosis factor (TNF) receptor family consisting of HVEM; the immunoglobulin superfamily consisting of the nectins; and 3-O-sulfated heparin sulfate. [Figure from (Melancon, 2004)]

particular isoforms of 3-O-sulfotransferase (Spear et al., 2006). Since gD can bind to heparan sulfate, a ubiquitous cell surface component, HSV may have the broad host ranges, at least for viral entry to cells. Interestingly, HSV-1 and -2 gDs share 82% identity in amino acid sequence (Spear et al., 2006). The difference gives rise to receptor preference; HVEM and nectin-1 are active as entry receptors for both HSV-1 and HSV-2, whereas 3-O-H HS and nectin-2 preferentially mediates entry of HSV-1 and HSV-2, respectively (Spear et al., 2006). Among the receptors, however it is not clear which one is more important *in vivo* (Connolly et al., 2011). In mouse models of both genital herpes and herpes simplex encephalitis showed preference in nectin-1 for a receptor (Kopp et al., 2009, Taylor et al., 2007, Wu et al., 2009). By using the specific selectivity, the modification of HSV gD to accept heterologous ligand surprisingly, can redirect the tropism so that the engineered virus selectively infects the targeted cells such as tumor cells (Kamiyama et al., 2006, Menotti et al., 2008, Menotti et al., 2009, Zhou and Roizman, 2006). The soluble ectodomain of gD, which comprises binding and profusion domains, was indicated to be responsible for initiating the fusion cascade (Cocchi et al., 2004). Later, structural analyses solved the binding domains of gD. Specifically, HVEM interacts with an N-terminal loop of gD spanning residues 7-15 and 24-32 (Carfi et al., 2001, Connolly et al., 2003, Connolly et al., 2002), 3-O-H HS requires gD residues 7-32 for fusion trigger (Yoon et al., 2003), and nectin-1 and nectin-2 interact with N-terminal residues – particularly tyrosine 38 – as well as other residues within the core of gD (Connolly et al., 2005, Manoj et al., 2004). The binding of gD to receptor causes the conformational change of gD (Fusco et al., 2005, Krummenacher et al., 2005) and the C-terminal region residues 260–310 of gD, termed as ‘pro-fusion domain’, triggers fusion (Cocchi et al., 2004). Briefly, N-terminal receptor-binding site that is flexible in the absence of receptor binding is autoinhibited by C-terminus of itself, but the

C-terminus is dislodged after receptor binding to enable the receptor binding sites (Fusco et al., 2005, Krummenacher et al., 2005). This change and/or the exposed residues trigger gB and gH/gL to effect virus-cell and cell-cell fusion (Atanasiu et al., 2007). Recently, it is proposed that the receptor-activated gD induced conformational change of gH/gL, particularly to the N-terminus of gH and the C terminus of gL (Atanasiu et al., 2013). A key feature of this fusion model is that the glycoprotein quartet complexes are not preformed, but, rather they assemble at the onset of or at fusion execution (Avitabile et al., 2009). Recently it is demonstrated that gD can recruit gB and gH/gL independently of one another, which is contrary to their stepwise recruitment to gD, as predicted by the hemifusion model (Avitabile et al., 2009, Gianni et al., 2010). Furthermore, it is revealed that gB and gH/gL interact with each other independent of gD (Avitabile et al., 2009). Therefore, how gD is related to the formation of fusion complex remains unclear. Collectively, the major role of gD is a sensor and trigger of fusion; it senses that virions approach a receptor-positive cell and signals to the downstream glycoproteins gB and gH/gL to initiate fusion between the virion envelope and the cell membrane (Gianni et al., 2010, Avitabile et al., 2009).

To be functional, gH and gL must form a non-covalent complex on cell surface and in the virion envelope (Avitabile et al., 2009). Previously gH/gL heterodimer has been suggested to have a fusogenic capability due to heptad repeats in gH, which is a common feature of type I fusion protein (Subramanian and Geraghty, 2007). However, the crystal structure of gH/gL ectodomain revealed that the candidate fusogenic peptide region is actually buried, and does not resemble any of known fusion proteins; therefore, gH/gL does not function as a fusion protein but a regulator of gB in fusion machinery (Chowdary et al., 2010). Although it is not essential for either entry or fusion, gH/gL has a binding property to the cell membrane, which may mediate

direct virion entry (Galdiero et al., 1997). The RGD motif on gH is responsible to binding of cellular $\alpha V\beta 3$ integrin (Parry et al., 2005). On the other hand, a recent report showed that gH/gL can bind to the cell surface independently from $\alpha V\beta 3$ integrin, which implicates the presence of another cognitive binding partner (Gianni et al., 2010). Structurally, while proximal to viral membrane domains of gH is conserved, N-terminal domains of gH/gL showed heterogeneity among herpesviruses [reviewed in (Connolly et al., 2011)]. Therefore, the membrane-distal portion of the heterodimer is suggested that may interact with other virus-specific or cell-specific proteins (Connolly et al., 2011). Mapping of multiple neutralizing antibody binding domains in gH/gL may reflect that the complex has several interactive partners for function (Connolly et al., 2011). The membrane-distal and -proximal domains of the gH/gL ectodomain are also revealed to promote fusion (Galdiero et al., 1997, Jackson et al., 2010).

As mentioned earlier, although gB can bind to several host cell receptors to trigger viral entry *in vitro*, including paired immunoglobulin-like type 2 receptor- α (PILR- α) (Satoh et al., 2008), non-muscle myosin heavy chain IIA (NMMHCIIA; myosin9) (Arii et al., 2010), and myelin-associated glycoprotein (MAG) (Suenaga et al., 2010), the interactions between gB and those receptors are not demonstrated *in vivo*. In the dominant model, therefore it is believed that gD serves as a receptor-binding glycoprotein and gB acts as a sole fusion protein (Connolly et al., 2011). gB is a class III viral fusion protein that has hydrophobic fusion loops, as predicted from its domain structure that contains five distinct domains, and is similar to that of the trimeric postfusion form of glycoprotein G of vesicular stomatitis virus (VSV) (Heldwein et al., 2006). Presumably, gB directly participates in fusion by bringing the viral and the host cell membranes together (Chowdary and Heldwein, 2010).

Structurally, HSV-1 gB is a membrane-anchored protein that is composed of 904 amino acids including a secretory signal (residues 1 to 30), a large ectodomain (residues 31 to 773), a transmembrane anchor (residues 774 to 795), and an intraviral/cytoplasmic domain (residues 796 to 904) (Bzik et al., 1986, Bzik et al., 1984, Pellett et al., 1985, Chowdary and Heldwein, 2010, Rasile et al., 1993). As with other fusion proteins, the core of gB is composed of an alpha-helical coiled coil (Heldwein et al., 2006). The fusion loop is located in domain I, which is close to the transmembrane region (Hannah et al., 2009). Long linker regions that lead into and out of domain I and domain II are predicted to cause large-scale rotation to mediate conformational change from pre- to post-fusion structures (Connolly et al., 2011). The cytoplasmic domain of gB functions as an important negative regulator of cell membrane fusion, as it interacts stably with anionic lipid membranes with a concomitant increase in helical content (Chowdary and Heldwein, 2010). In the cytoplasmic domain, the residues within the region from position 801 to 851 are important for trimerization of gB (Chowdary and Heldwein, 2010). Those two specific regions in the cytoplasmic domain are also suggested to be critical for membrane interactions: residues 852 to 868 and 877 to 904 (Chowdary and Heldwein, 2010). The residues 877 to 904 may be required for stable interaction with anionic liposomes while residues 852 to 868 may additionally contribute to this interaction (Chowdary and Heldwein, 2010). Collectively, although gB has some binding properties to the host cell receptor, it mainly functions as a fusion protein by using its fusion loop to destabilize the host cell membrane. The fusogenicity of gB is activated after structural transformation that is induced by other viral proteins gH/gL.

Overall, gD is associated with gH/gL and gB through residues 260-310 in the profusion domain, and residues 240-260 and also 304-305, respectively. gH/gL is also associated with gB but it is uncertain whether the interaction requires gD-receptor binding. The currently dominant

fusion model is as follows (Figure 2-5): (a) binding gD to its receptor induces conformational change of gD; (b) The transformed gD activates gH/gL; (c) consequently activated gH/gL triggers gB to cause fusion (Atanasiu et al., 2010, Atanasiu et al., 2013).

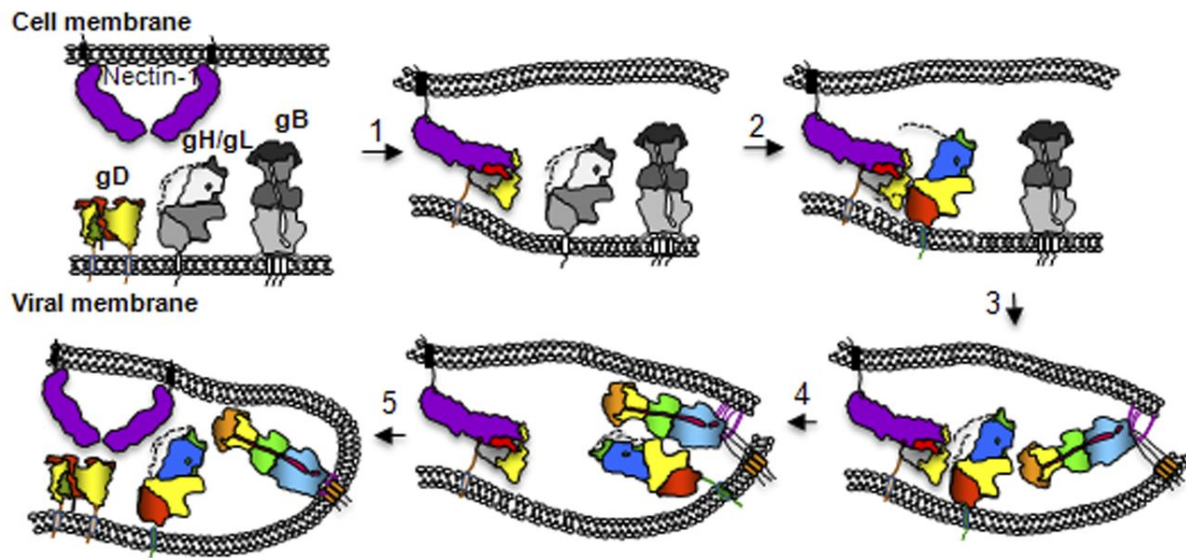


Figure 2-5. Schematic representation of fusion process. [Figure from (Eisenberg et al., 2012)]

Although extensive membrane fusion can be induced by co-expressing glycoproteins gB, gD and gH/gL in cell lines (Turner et al., 1998, Muggeridge, 2000), the virus-induced fusion requires other viral proteins including gK, pUL20, gM, gE, gI, pUL11, pUL16, pUL21 and possibly more (Davis-Poynter et al., 1994, Melancon et al., 2005, Melancon et al., 2007, Chouljenko et al., 2009, Han et al., 2012, Kim et al., 2013). Our laboratory has shown that HSV-1 gK and pUL20 functionally and physically interact and that these interactions are absolutely necessary for their coordinate intracellular transport, cell-surface expression, and membrane fusion functions in the HSV-1 life cycle (Foster et al., 2001). Furthermore, we have shown that a peptide comprised of the amino terminal 82 amino acids of gK (gKa) expressed *in trans* complemented gB-mediated cell fusion and could physically interact with gB and gH in infected

cells (Chouljenko et al., 2009). These results suggest that gB-mediated virus-induced cell fusion is regulated via direct interactions with gK and pUL20 (Chouljenko et al., 2009, Chouljenko et al., 2010). Moreover, our laboratory has shown that HSV-1 recombinant viruses, gK Δ 31-68 and gK Δ 31-117 exhibited slower kinetics of entry into Vero and CHO cells expressing different HSV-1 cellular receptors than their parental wild-type virus (Jambunathan et al., 2011, Chowdhury et al., 2013). Also, our laboratory has found that gK-null virions did not infect neuronal axons (David et al., 2008, David et al., 2012). These findings implicate the importance of gK/pUL20 during the virus entry. In this thesis, we demonstrated that both gM and pUL11 is required for virus-induced cell fusion. Moreover, mutant viruses lacking either gM or pUL11 exhibited slower kinetics of entry into Vero cells than the parental virus. This suggests that gM and pUL11 are involved in membrane fusion phenomena during both virus-induced cell fusion and virus entry (Kim et al., 2013). Furthermore, UL11 is known to physically interact with gE, pUL16 and pUL21 which are also required for virus-induced cell fusion (Balan et al., 1994, Chatterjee et al., 1989, Davis-Poynter et al., 1994, Han et al., 2012). Overall, the virus requires complicated fusion machinery composed in multiple viral proteins to induce the efficient viral entry.

Host protein shutoff

The host cell is equipped with a potent surveillance system to prevent and eliminate an intruder. During the infection, therefore, the virus immediately encounters this innate immune response by the host cell (Shu et al., 2013). To avoid this response, HSV also has its own strategy. At almost same instance of invasion into the host cell, the virus readily starts to control the host cell environment to accommodate viral replication by using prepackaged viral proteins and mRNA (Roizman et al., 2007). During the acute phase of lytic infection, virion host shutoff

endoribonuclease (VHS-RNase, pUL41) mainly plays this critical role (Strom and Frenkel, 1987). The VHS-RNase is released into the cell as a part of tegument during the viral entry and immediately begins to degrade mRNAs in polyribosomes (Ejercito et al., 1968, Kwong and Frenkel, 1987, Read and Frenkel, 1983, Strom and Frenkel, 1987). Specifically, the VHS-RNase binds to eIF4H of eIF4F complex and decaps the mRNA (Du et al., 2011, Oroskar and Read, 1989). Subsequently, 5'-exonuclease *Xrn1* degrades the decapped mRNA (Sokoloski et al., 2009, Kumar and Glaunsinger, 2010). For many years it was enigmatic how the viral proteins are produced, since the VHS degrades both viral and host mRNAs non-specifically. Initially it was hypothesized that the outpacing production of viral mRNA, rather than its degradation, may lead to synthesis of viral proteins, and at the end the accumulated proteins negatively control the VHS activity (Roizman et al., 2007). However, a recent report suggested that the VHS-RNase actually degrades mRNA in a highly selective manner and under the control of at least four viral proteins: VP16 (UL48) (Lam et al., 1996); VP22 (UL49) (Taddeo et al., 2007); VP13/14 (UL47) (Shu et al., 2013) and ICP27 (Poon and Roizman, 1997). Therefore, the RNase activity is modulated to target the stable host mRNAs and the viral immediate early (IE) mRNAs while sparing other viral mRNAs (Taddeo et al., 2013, Shu et al., 2013). Among those, two late proteins VP16 and VP22 quench the RNase activity at the late stage of viral replication (Lam et al., 1996, Taddeo et al., 2007). Other partners, ICP27 and pUL47, are an IE protein and a tegument protein, respectively. Two proteins work together to attenuate the VHS-RNase activity possibly by intervening in the mRNA-decapping process of the RNase (Shu et al., 2013).

Capsid transport to the host cell nucleus

Followed by fusion of the HSV-1 envelope with a host cell membrane, the viral capsid along with several capsid-associated tegument proteins [e.g. VP1/2 and VP16 (α -TIF)] is released

(Antinone et al., 2006, Jovasevic et al., 2008). To accomplish the viral replication, the capsid needs to be conveyed to the host cell nucleus then the viral genome is released and replicated in the nucleus. In the cytosol, passive diffusion of a macromolecule larger than 500 kDa is almost impossible due to a highly viscous environment with the enriched proteins (up to 300 mg/ml), thus the cells employ an active transport system to efficiently convey the macromolecules (Sodeik, 2000). The transport system is comprised of microtubules that have structural polarity with a highly dynamic “plus” end and a reactively stable “minus” end (Yang et al., 2006). On the microtubule (MT), two-headed motor proteins, kinesin and dynein mediate the transport of intracellular cargoes toward the plus-end and minus-end, respectively (Vale, 2003, Vallee et al., 2004). In the cell, the plus-end extends into the cytoplasm and the minus-end anchors to the microtubule-organizing centers (MTOC) (e.g. centrosomes) (Job et al., 2003). Therefore, the transport can be divided into two directions: (a) anterograde direction (MTOC to plasma membrane, plus-end-direction) and (b) retrograde direction (plasma membrane to MTOC, minus-end-direction). HSV-1 snatches this cellular microtubule-associated motor system to convey its capsid toward the nuclear pore complex (NPC) of the host cell nucleus (Ojala et al., 2000, Radtke et al., 2010). The accumulated capsids subsequently dock onto NPC and are destabilized then release the DNA into the nucleus (Ojala et al., 2000, Sodeik et al., 1997). The entire process from the capsid-penetration to DNA-release has been observed to occur within one hour (Sodeik et al., 1997).

Multiple proteins participate in the process of the capsid-NPC binding and viral DNA release. Specifically, inner HSV1 tegument proteins VP1/2 (UL36) and pUL37 are required for recruiting microtubule motors onto viral capsids, capsid transport along microtubules, and binding to nuclear pores (Ojala et al., 2000, Radtke et al., 2010, Shanda and Wilson, 2008,

Wolfstein et al., 2006). The pUL36 is believed to function with binding to microtubule motors and nuclear pores (Batterson et al., 1983, Luxton et al., 2006, Radtke et al., 2010, Roberts et al., 2009, Wolfstein et al., 2006). pUL36 is associated on capsid until arrival at the nuclear envelope (Antinone and Smith, 2010, Copeland et al., 2009, Granzow et al., 2005, Luxton et al., 2005, Morrison et al., 1998). pUL36 has several binding domains to other tegument and capsid proteins. Its N-terminal domain interact with tegument proteins VP16 and pUL37 and C-terminal binding sites with a capsid protein pUL25 (Coller et al., 2007, Ko et al., 2010, Klupp et al., 2002, Mettenleiter, 2002, Roberts et al., 2009, Vittone et al., 2005). The pUL25, which is involved in the release of incoming genomes at the nuclear pores, interacts with nucleoporins CAN/Nup214 and hCG1, and also binds to a portal protein pUL6 and a tegument protein pUL36 (Batterson et al., 1983, Cardone et al., 2007, Newcomb et al., 2007, Jovasevic et al., 2008, Roberts et al., 2009).

Gene expression in infected cells

Within the nucleus, DNA ligase IV/XRCC4 immediately circularizes the released linear viral DNA through ligating head to tail, and the circular viral DNA serves as a template for replication and transcription (Duyk et al., 1985, Garber et al., 1993, Lilley et al., 2005, Muylaert and Elias, 2007, Shirata et al., 2005, Strang and Stow, 2005). Host RNA polymerase II is responsible for transcribing the viral genes and the transcribed mRNAs are subsequently translocated to cytoplasm for viral protein synthesis (Alwine et al., 1974, Costanzo et al., 1977). During the lytic infection, HSV-1 produces at least 84 different proteins, in a highly coordinated cascade fashion (Figure 2-6) (Roizman et al., 2007), which can be divided into five groups based on the peak time of expression: α (immediate early), β_1 (early early), β_2 (early late), γ_1 (late), and γ_2 (late late) (Honess and Roizman, 1975, Roizman et al., 1975). The α proteins appears in 2-4

hours post-infection and stimulate expression of β genes (Roizman et al., 2007). Approximately 4-8 hours post-infection, the expression of β genes approaches peak rate, which are involved in the replication of viral DNA and nucleotide metabolism (Roizman et al., 2007). At the late phase of viral gene expression, the synthesis of viral DNA enhances expression of the γ genes, which predominantly encode structural proteins (Roizman et al., 2007). Despite the gene classification, all groups of viral genes are expressed in a continuous cascade fashion under a complex feedback system (Figure 2-6), rather than clearly demarcated stages (Roizman et al., 2007).

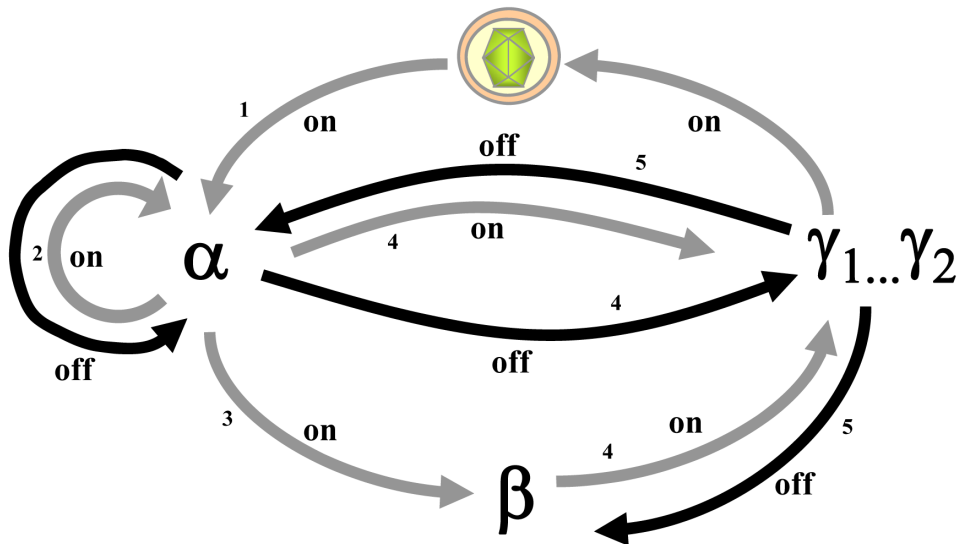


Figure 2-6. Schematic map of the regulation of HSV gene expression. 1) α -TIF, from the tegument activates initial transcription of α genes. 2) Autoregulation of gene expression. 3) Activation of β gene expression by α genes. 4) Activation of γ gene expression by α and β genes. 5) γ genes stop expression of α and β genes late in infection. [Figure from (Roizman et al., 2007)]

Recent studies gradually unveiled the detailed cascade of HSV-1 gene expression. Upon entry of the viral genome into the nucleus, the repressive histones and cellular repressors immediately bind to the viral DNA and all viral genes are repressed (Figure 2-7. B) (Roizman et al., 2011). During the early stage of virus infection, the viral DNA is associated with nuclear domain 10 (ND10) bodies that attract a large variety of histones, histone-modifying enzymes,

coactivators, corepressors and more (Knipe and Cliffe, 2008, Kutluay and Triezenberg, 2009, Kwiatkowski et al., 2009). The major component of ND10 is promyelocytic leukemia protein (PML) and seemingly it is involved in the antiviral activity in association with interferons (IFN) (Everett and Chelbi-Alix, 2007). The ND10 body increases its size and number by exposure to IFNs or histone deacetylase (HDAC) inhibitors as well as intrusion of exogenous DNA into cells (Maul et al., 2000, Negorev and Maul, 2001).

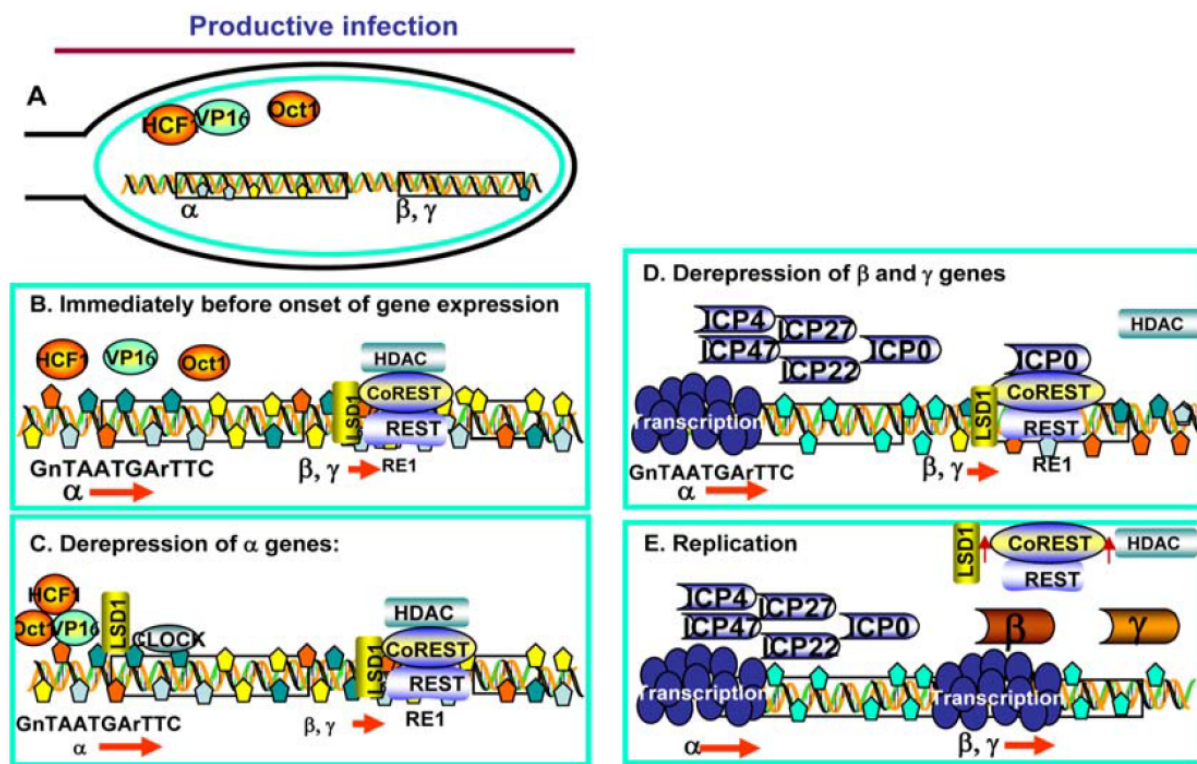


Figure 2-7. A model for HSV gene-expression cascade during the lytic infection. [Figure from (Zhou et al., 2013)]

The depression of gene expression initiates from α genes and follows to β and γ genes (Roizman, 2011). A viral tegument protein VP16, which was dissociated from the tegument of infected virus during the entry, initiates the α gene expression (Figure 2-7: A) (Post et al., 1981, Ishov and Maul, 1996, Maul et al., 1996). After coupled with host cell factor 1 (HCF1) in

cytoplasm, VP16 translocates to the nucleus, and recruits host cell octamer binding protein 1 (Oct1), and then the complex binds to the response element (GnTAATGArTTC) in the α gene promoters (Figure 2-7: B and C). For the purpose of remodeling chromatin, the complex recruits lysine-specific demethylase 1 (LSD1) and the CLOCK histone acetyl transferase to commence the transcription of six α genes (Figure 2-7: D and E): infected-cell proteins (ICP) 0, -4, -22, -27, -47, and U_S1.5 (Katan et al., 1990, Kristie and Sharp, 1990, La Boissiere et al., 1999, Liang et al., 2009, Mackem and Roizman, 1982, Roizman et al., 2011, Roizman, 2011, Roizman et al., 2007). Concurrently, ND10 bodies become disassembled by ICP0 (Gu and Roizman, 2003). Among the six α genes, ICP4, -0, -27, -22, and U_S1.5 are responsible for stimulating expression of β genes in some types of cells (Roizman et al., 2007).

The ensuing expression of β and γ genes are regulated by the sum total effect of all transcriptional factors (Roizman et al., 2007). In the depression cascade of β and γ genes, at least in low multiplicity of infection, an α protein ICP0 functions a key role through interacting and blocking the multiple cellular repressors, especially the repressor complex HDAC/CoREST/LSD1/REST (HCLR) (Andres et al., 1999, Chong et al., 1995, Schoenherr and Anderson, 1995, Humphrey et al., 2001, You et al., 2001). The HCLR complex is composed of HDAC, corepressor element-1 silencing transcription factor (CoREST), LSD1, and RE1-silencing transcription factor (REST). The primary function of the HCLR complex is repression of neuronal genes in non-neuronal cells (Ballas et al., 2005, Griffith et al., 2001, Koenigsberger et al., 2000, Shimojo and Hersh, 2004). A newly synthesized α protein, ICP0 interacts with CoREST of HCLR complex, and displaces the HDAC from CoREST. Consequently, this dissociation enables the expression of β and γ genes (Figure 2-7: C and D) (Gu and Roizman,

2007). Slightly later in infection, a fraction of LSD1, CoREST, and HDAC-1 is translocated to the cytoplasm after onset of viral DNA synthesis (Gu et al., 2005).

Overall, the viral genome is repressed immediately on entry into the nucleus and depression of viral genes is implemented in two stages: first the expression of α genes is initiated by VP16 and then the ensuing expression of β and γ genes are provoked by newly synthesized α proteins, especially ICP0 (Roizman et al., 2011).

Viral DNA replication

HSV-1 DNA replication is initiated after the expression of β genes, which includes viral replication proteins. The viral genome replication of HSV essentially requires seven replication proteins: a DNA polymerase (Pol; pUL30), a DNA polymerase accessory protein (pUL42), the origin-binding protein (OBP; pUL9), a single-stranded DNA binding protein (ICP8; pUL29) and a helicase-primase complex (pUL5, pUL8 and pUL52) (Purifoy et al., 1977, Conley et al., 1981, Challberg, 1986, Wu et al., 1988). Additionally, six non-essential proteins are associated with the DNA replication: alkaline endo-exonuclease (pUL12); uracil N-glycosylase (pUL2); deoxyuridine triphosphatase (pUL50); thymidine kinase (pUL23); ribonucleotide reductase (pUL39 and pUL40) [reviewed in (Weller and Coen, 2012)].

Due to the circularized structure of viral DNA in the nucleus (Strang and Stow, 2005, Lilley et al., 2005, Shirata et al., 2005, Muylaert and Elias, 2007), progeny viral genomes are replicated in a head-to-tail linked form of concatemeric intermediates by the rolling circle replication (Jacob et al., 1979). The replication starts from one of three origins: one being located in UL (*oriL*) and the other two situated in US at IR_S and TR_S (*oriS*). Initially, a dimer of UL9 binds to an origin and unwinds the DNA and then ICP8 binds to ssDNA (Macao et al., 2004). On this site, other replication proteins are recruited and a replisome is organized (Muylaert et al., 2011). The

replication of viral DNA is initiated as a theta structure, and then continued to a rolling circle form by the replisome (Skaliter et al., 1996).

Capsid assembly and DNA packaging

At the late stage of infection, the γ genes are transcribed and the transcribed γ -gene mRNAs are translocated to cytoplasm. The components of capsid proteins are synthesized in the cytoplasm and translocated back to the nucleus where the capsid is assembled (Roizman et al., 2007). In the nucleus, the capsid assembly may initiate with formation of the portal ring, which is composed of 12 copies of pUL6 (Newcomb et al., 2001, Newcomb et al., 2003). Since the pUL6 molecule contains interacting domains with the scaffolding proteins (pUL26.5), the portal ring is believed to contribute as an initiation site for self-assembly of the scaffolding protein sphere (Huffman et al., 2008, Yang and Baines, 2008). The sphere is composed of dual scaffolding systems (Figure 2-8): (1) internal scaffold shell is comprised of laterally packed dimers of elongated protein, pUL26.5 (preVP22a), and additionally, maturational protease (VP24) is coupled to the pUL26.5 on the inner face of the internal shell (Newcomb et al., 2000); (2) the external scaffold is composed of triplex proteins, consisting of two copies of VP19C (UL38) and one VP23 (UL18). On the scaffolding sphere, pUL19 (VP5) is coordinately arranged by contacting with the triplexes without direct contact each other (Trus et al., 1995). Consequently, the procapsid surface shell is composed of one dodecamer of pUL6, 150 hexamers and 11 pentamers of VP5, and 320 triplexes of VP19C and VP23 [reviewed in (Cardone et al., 2012)].

Once the procapsid assembly is complete, the protease VP24 is activated and initiates the maturation of the capsid. The protease first detaches itself from the scaffolding protein and undergoes cleaving the C-terminal 25 amino acid residues of scaffolding proteins, which results

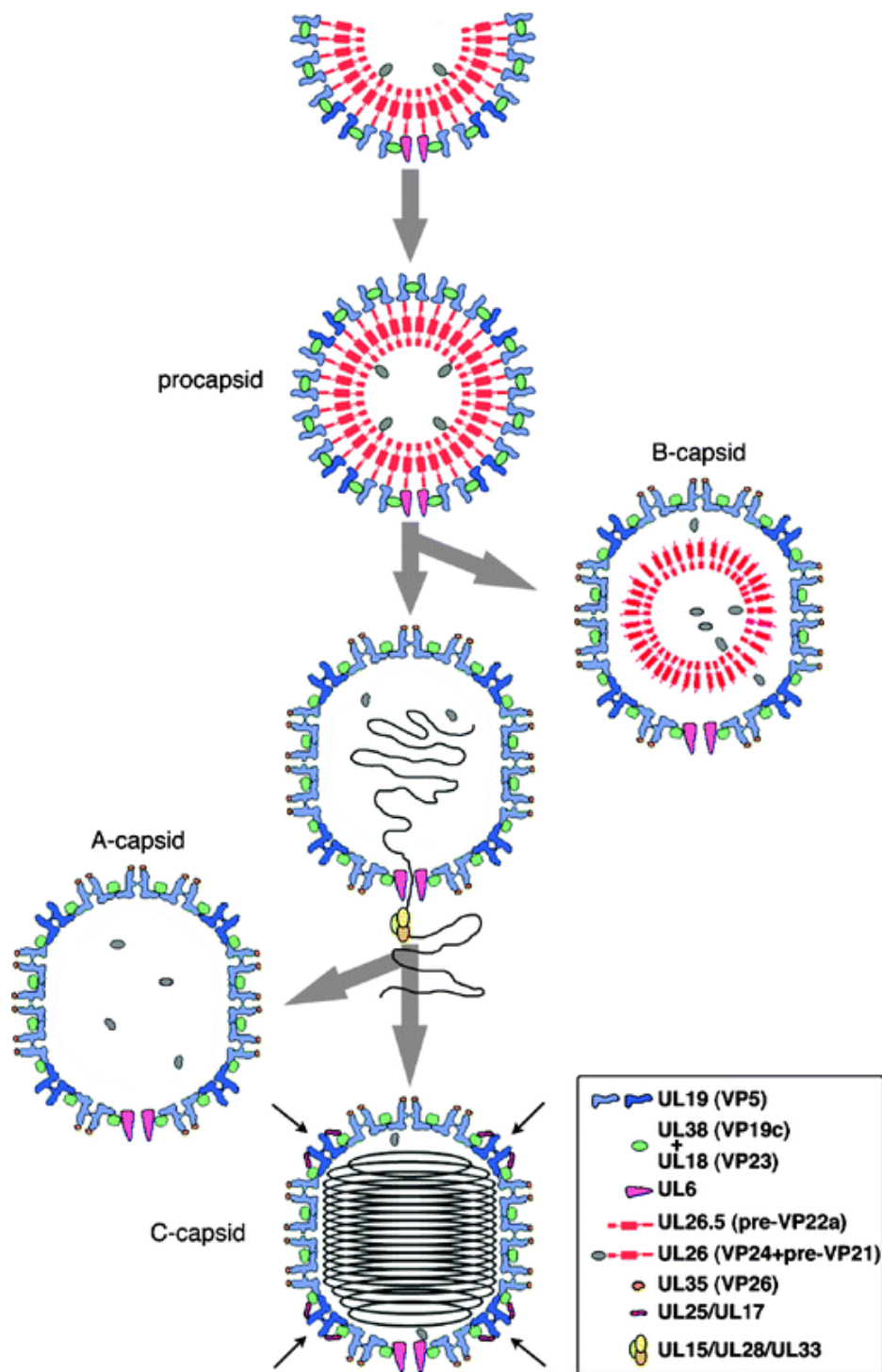


Figure 2-8. Assembly and maturation of HSV-1 nucleocapsid⁷. [Figure from (Cardone et al., 2012)]

⁷ Springer and the original publisher, Adv Exp Med Biol, 726, 423-39, Procapsid assembly, maturation, nuclear exit: dynamic steps in the production of infectious herpesvirions. Cardone et al., Figure 1, copyright (2012), is given to the publication in which the material was originally published, by adding; with kind permission from Springer Science and Business Media"

in dissociation of outer and internal shell. The dissociation of scaffolding proteins allows the neighboring capsomers to directly contact each other via their innermost domains. Thus, the spherical shape of outer shell transforms to icosahedral structure (Heymann et al., 2003, Trus et al., 1996). The viral DNA packaging follows the structural transition and promotes expulsion of the dissociated scaffolding proteins from the capsid except the protease VP24 (Newcomb et al., 1996, Trus et al., 1996). The DNA packaging is initiated by a multipartite enzyme complex, terminase that is comprised of at least pUL15, pUL28 and pUL33 (Beard et al., 2002). Initially, the terminase associates with portal protein, and subsequently mediates the encapsidation and monomeric cleavage of the concatemeric viral DNA within the capsid (Yang et al., 2007). The cleavage occurs at the sequence-specific motifs, *pac1* and *pac2*, which are located near the ends of the herpesvirus genome (Brown et al., 2002, Deiss et al., 1986).

As a result of the process in capsid maturation, fully matured capsids as well as two types of abortive capsids are accumulated in the nucleus due to the miscarriage in DNA packaging (Figure 2-8): (1) B capsid, which appears at a rate of 20-30%, is due to failure of initiating the DNA packaging, thus the B capsid still contains the internal scaffolding shell but no core structure; (2) A capsid, which occurs infrequently, appears when the scaffold proteins are expelled but the DNA encapsidation is unsuccessful; (3) and finally, the C capsid appears as the fully matured form of capsid which is most abundantly produced (Homa and Brown, 1997, Baines, 2011).

Interestingly, the herpesviruses and bacteriophages share high similarity in process of nucleocapsid maturation. Therefore, this may indicate their common ancestry (Agirrezabala et al., 2005, Baker et al., 2005, Hendrix, 2005, Johnson and Chiu, 2007, Rixon, 2008, Steven et al., 2005).

Virion egress

Once the nucleocapsid is assembled, the virion continues maturation by acquiring the tegument proteins and final envelopment through virion egress from the nucleus to extracellular space to become an infectious virion. During the virion egress, virion encounters various physical barriers. Representatively, the nuclear lamina obstructs direct access of nucleocapsid to inner nuclear membrane (INM), which is a dense mesh-network on the inner side of nuclear membrane (NE) (Aebi et al., 1986). The size of capsid (c.a. 125 nm), which is too large to pass across the nuclear pore (< 39 nm) (Pante and Kann, 2002), is another obstacle for transportation of the capsid across NE [reviewed in (Mettenleiter et al., 2013)].

Therefore, the predominant model explains the virion egress as multiple steps of “envelopment-deenvelopment-reenvelopment” (Skepper et al., 2001). Initially, the virions may approach and contact to INM via nuclear actin filaments with dissolving the nuclear lamina (Forest et al., 2005). The capsid then begins to escape from the nucleus by budding into perinuclear space on the INM and consequently acquires primary envelope (envelopment). Subsequently, the capsid is released to cytosol by fusing the primary envelope with the outer nuclear membrane (ONM) and becomes naked (deenvelopment). Within the cytoplasm, especially in the trans-Golgi network (TGN), final tegumentation and envelopment occur on the deenveloped virion (reenvelopment) [reviewed in (Baines, 2007, Johnson and Baines, 2011, Mettenleiter et al., 2009, Mettenleiter et al., 2013, Roizman et al., 2007)].

During the nuclear egress steps, a heterodimer of pUL31 and pUL34 (nuclear egress complex; NEC) plays an important role for the primary envelopment (Klupp et al., 2000a, Yamauchi et al., 2001, Fuchs et al., 2002b, Leelawong et al., 2011, Yang and Baines, 2011, Roller et al., 2010, Roller et al., 2011, Ryckman and Roller, 2004, Shiba et al., 2000).

Structurally, pUL34 is an NE-anchored type II protein and provides a binding domain for pUL31 (Reynolds et al., 2001, Shiba et al., 2000). NEC recruits viral and cellular kinases that phosphorylate the nuclear lamins (Muranyi et al., 2002). The nuclear lamina is therefore, disrupted by NEC-dependent manner (Muranyi et al., 2002, Marschall et al., 2011). Viral kinases, pUS3 and pUL13 target lamin A and C, whereas a cellular kinase, protein kinase C (PKC) prefers to phosphorylate lamin B (Leach and Roller, 2010, Mou et al., 2007). In this line, pUL31, pUL34 and pUS3 seem to play together. However, deletion of pUL31, pUL34, or pUS3 showed different phenotypes. While impairing either pUL31 or pUL34 arrested capsids in nucleoplasm (Roller et al., 2000, Reynolds et al., 2001, Wagenaar et al., 1995, Chang et al., 1997, Ott et al., 2011), deletion of US3 induced accumulation of virions in perinuclear space (Klupp et al., 2001, Reynolds et al., 2002, Wagenaar et al., 1995). Also, the primary enveloped virion in the perinuclear space contains all three proteins, whereas only pUS3 remains in the tegument layer of the cytoplasmic or extracellular virion (Granzow et al., 2004, Leelawong et al., 2011, Loret et al., 2008, Padula et al., 2009, Reynolds et al., 2002, Yang and Baines, 2011). These results indicated that the complex of pUL31 and pUL34 plays a role during the primary envelopment and is readily discharged from the capsid during the deenvelopment.

The preferential selection of mature capsids for the nuclear egress seems to be related with a heterodimer of pUL17 and pUL25 [C-capsid-specific complex (CCSC)], which binds to vertex-proximal sites on C-capsids (Cardone et al., 2012). As mentioned earlier, the viral DNA encapsidation induces a subtle conformational change in capsid, which enhances the affinity to CCSC (Trus et al., 2007). Since the CCSC interacts with pUL31 (Yang and Baines, 2011), augmented binding of CCSC on the capsid may contribute to recruitment of other protein(s) involved in the primary envelopment (Cardone et al., 2012). Overall, the CCSC associated with

capsids couples with pUL31, and pUL31 on the capsid complexes with NM-integrated pUL34, and then the capsid buds into the perinuclear space.

The primary enveloped virus particles in the perinuclear space subsequently undergo deenvelopment via fusing the primary envelope and ONM. Consequently, the capsid is released into the cytosol and naked again. HSV-induced membrane fusion also occurs during the virus entry (Mettenleiter et al., 2013). Although the herpesvirus entry essentially requires a quartet of viral glycoproteins gB, gD and gH/gL for the fusion (Heldwein and Krummenacher, 2008), the requirement of those viral glycoproteins during the deenvelopment is enigmatic. Unlike the viral entry, deletion either of gB or gH only produced minor defects in deenvelopment, whereas lacking both proteins blocked the nuclear egress and caused accumulation of primary enveloped virion in the perinuclear space (Farnsworth et al., 2007). In this regards, the mechanism between virus entry and deenvelopment may be different from each other. Interestingly, abrogation of pUS3, which phosphorylates pUL31 (Mou et al., 2009) as well as cytoplasmic domain of gB (Wisner et al., 2009), showed the same phenotype as deletion of both gB and gH (Reynolds et al., 2002, Ryckman and Roller, 2004). Since the alteration of fusion loop in gB with deletion of gH displayed the same defects as above (Wright et al., 2009), it is more likely that the fusion during the deenvelopment is mediated by gB after phosphorylated by pUS3 (Costanzo et al., 1977). However, unlike HSV, Pseudorabiesvirus (PRV) mutants lacking either gB and gH, or gB and gD did not affect in nuclear egress (Klupp et al., 2008), which suggests there may be an alternative mechanism for deenvelopment (Costanzo et al., 1977).

During the virion egress from the nucleus to extracellular spaces, another major event in virion maturation is tegumentation. While the processes in capsid assembly and genome packaging are well appreciated, yet the tegumentation process remains undetailed (Henaff et al.,

2013). Initially, the tegument was thought to be an amorphous structure; however, the addition of tegument proteins to capsids seems to be an ordered process of adding “layers” of protein during virus egress, thus the layers are able to be provisionally divided into inner and outer layers (Mettenleiter, 2006, Mettenleiter et al., 2006). The inner tegument layer is a highly ordered filamentous structure composed in VP1-2 (UL36) and pUL37, which directly bind to the penton vertices of capsid (Chen et al., 1999, Newcomb and Brown, 2010, Zhou et al., 1999). Unlike the inner layer, the outer layer seems to be less ordered in structure and may not directly contact the capsid (Katan et al., 1990, Zhou et al., 1999). Furthermore, a recent study suggested that the initial tegument layer on the nuclear C-capsid may be composed of up to 5 tegument proteins: pUS3, pUL36, pUL37, ICP0, and ICP4; subsequently, the virion loses some of those in egress steps; and the virion acquires other tegument proteins during reenvelopment (Henaff et al., 2013). Thus, the tegument of mature extracellular virion becomes more complicated, consisting of at least 23 different viral proteins through extensive and redundant protein-protein interactions (Loret et al., 2008).

The tegument-coated nucleocapsids in the cytoplasm then become a mature virion by acquiring a final viral envelope via budding into TGN (reenvelopment) (Farnsworth and Johnson, 2006, Sugimoto et al., 2008, Turcotte et al., 2005, Wisner and Johnson, 2004). This capsid budding process is thought to be facilitated by interactions between tegument proteins on cytoplasmic capsids and the carboxyl termini of viral glycoproteins embedded in TGN membrane (Farnsworth and Johnson, 2006, Sugimoto et al., 2008, Turcotte et al., 2005, Wisner and Johnson, 2004). For efficient interactions between the tegument proteins and membrane glycoproteins, the membrane proteins are concentrated at the virion budding site. Some membrane proteins contain sorting sequences that direct the glycoproteins to TGN, such as

tyrosine and di-leucine motifs, acid clusters, and phosphorylated amino acids, or oligosaccharides (Gu et al., 2001). Another group of viral proteins are transported to the TGN via interaction with the viral proteins containing the sorting motifs (Wisner and Johnson, 2004). These interactions between the tegument proteins and membrane proteins are thought to occur in redundant fashion, since viruses lacking single membrane proteins (gB, gD, gC, gE, gI, gH or gL) do not show drastic impairment in reenvelopment, except gK or UL20 [reviewed in (Johnson and Baines, 2011)]. Although mutant viruses lacking two or more viral proteins show synergistic defects in the cytoplasmic reenvelopment, deletion of either the gK or UL20 gene produced significantly greater defects in virion envelopment and overall virus replication than deletion of either the carboxyl terminus of gD, UL11, gM or gE alone or in various combinations (Chouljenko et al., 2012). Recently, it was shown that VP22 forms a protein complex with gM and gE (Maringer et al., 2012); gB and gM physically interacts with gK/UL20 heterodimer (Chouljenko et al., 2009, Chouljenko et al., 2010, Kim et al., 2013); and gE forms a protein complex with UL11, UL16 and UL21 (Yeh et al., 2011, Han et al., 2011). Overall, during the reenvelopment, there occur redundant and multiple numerous interactions between the membrane-anchored viral proteins and capsid-bound tegument proteins, and these interactions seem to facilitate the budding process of cytoplasmic virion into the TGN [reviewed in (Johnson and Baines, 2011)].

Via the budding process, the fully mature enveloped virion is delivered in a transport vesicle and released into the extracellular space via fusion of the transport vesicle with the plasma membrane [(Johnson and Huber, 2002), reviewed in (Kristie and Sharp, 1990, Mettenleiter et al., 2009)]. This release process is presumably mediated by cellular exocytic pathway. The exocytosed viral particles tend to attach on the outer cell membrane rather than freely release in the extracellular space [reviewed in (Johnson and Baines, 2011)]. During the late phase of infection, the

TGN recycling loop is disrupted by HSV and this may promote the transport of progeny viruses toward plasma membrane [(Wisner and Johnson, 2004, Campadelli et al., 1993), reviewed in (Johnson and Baines, 2011)]. In the polarized cells, such as epithelial cells and neuronal cells, HSV can direct the progeny virus to the basolateral surface rather than apical surface to promote cell-to-cell spread (Johnson et al., 2001). This sorting process is mediated by the HSV gE/gI heterodimer, therefore mutating gE, gI, or the cytoplasmic domain of gE impairs basolateral sorting (Johnson et al., 2001). Along with virus induced cell fusion, this cell-to-cell spreading is very important for HSV infection because these aspects are thought to facilitate transmission to adjacent cells. In this manner HSV may spread without exposure to the host humoral immune system, particularly neutralizing antibodies [reviewed in (Roizman et al., 2007)] .

Latent infection

Although latency is the most interesting characteristic of herpesviruses, the exact mechanism of latency remains unclear. According to the recent reviews (Roizman, 2011, Roizman et al., 2011, Zhou et al., 2013), detailed features of the latency and reactivation of viruses are assumed as follows (Figure 2-9): After establishing the primary infection of herpesvirus at mucosal tissue, the virus replicates explosively then causes the cell-to-cell spread known as the lytic cycle. Once the viruses are transmitted to nerve endings that innervate the infected tissue, viruses travel to the nuclei of sensory ganglionic neurons (somata), in a retrograde manner, where latent infection occurs. During the first 2 weeks of infection, the virus can keep replicating and the progeny viruses can be detected by the end of the 3rd week; however, the viral replication stops. Whereas viral DNA can be readily detected, infectious virion is disappeared in this stage [reviewed in (Roizman, 2011)]. Interestingly, in latency-destined neurons VP16/HCF1 complex is not translocated into the nucleus (Figure 2-9: A) (Kristie et al., 1999, La Boissiere et al., 1999)

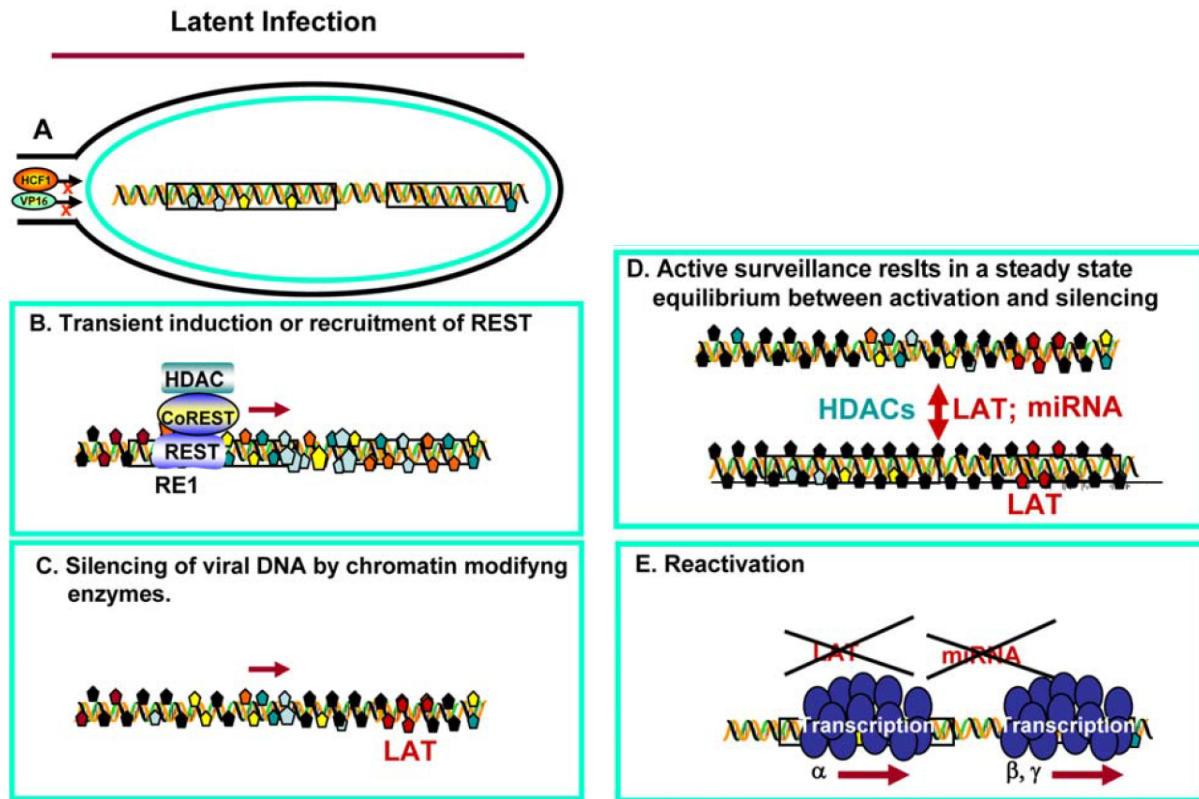


Figure 2-9. A model for HSV gene-expression during the latent infection. [Figure from (Zhou et al., 2013)]

and the REST is induced or recruited from satellite cells; therefore, alpha gene expression is not ensuing (Figure 2-9: B). At this phase, the viral DNA is held in the nucleus of the neuronal cell in a circular episome form that is closely associated with nucleosomes (Deshmane and Fraser, 1989). Since the latency-associated transcripts (LAT) promoter and 5' exon are associated with acetylated histones, or active chromatin, and lytic-gene promoters are associated with heterochromatin forms of histones during the latent infection, the lytic gene expression is almost silenced but the LATs are expressed (Figure 2-9: C) (Knipe and Cliffe, 2008, Kwiatkowski et al., 2009, Neumann et al., 2007a, Wang et al., 2005b). As a result, LATs and viral miRNAs are accumulated in the latently infected neurons and they survey the neurons. Recent studies suggest that in the latency-destined neuron, HSV hijacks the HDAC-1 (histone deacetylase)- or HDAC-

2/CoREST/LSD1/REST repressor complex to silence the viral DNA itself (Figure 2-9: D) (Ballas and Mandel, 2005, Tapia-Ramirez et al., 1997, Gopalakrishnan, 2009). Although LATs seem to have a potential role to establish the latent infection based on the earlier findings, it may not be true, since the LAT-null mutant still can establish latency (Javier et al., 1988, Wang et al., 2001). On the other hand, LATs function to prevent neurons from apoptosis, protecting themselves from being destroyed by immune response (Ahmed et al., 2002, Sawtell, 1997, Thompson and Sawtell, 2001, Thompson and Sawtell, 2000). Although the exact mechanism remains unknown, CD8 + T cells also seem to play a role in regulating infection in nervous tissue to suppress the alpha gene expression in latent infection (Liu et al., 1996).

Reactivation of the virus usually occurs when the neuronal, or primary infected tissue, is stressed by UV radiation, heat, trauma, other local stress as well as systemic stress or immunocompromise to the host (Figure 2-9: E) [reviewed in (Roizman et al., 2011)]. During the reactivation from latency, suppression of HDACs plays a pivotal role (Danaher et al., 2005, Neumann et al., 2007b). Once the stress occurs, transcription of all viral genes is begun, and LAT and miRNAs become degraded (Du et al., 2011). The produced progeny viruses can travel in an anterograde direction and establish peripheral infection on its enervating sites. The ensuing recurrent infection process is very similar to primary infection. The progeny viruses can also spread in a retrograde direction (toward second or third neurons) more proximal to the central nervous system.

Glycoprotein M (UL10)

Glycoprotein M (gM) is a 473 aa type III integral membrane glycoprotein encoded by UL10, which is conserved throughout the entire family, *Herpesviridae* and forms a complex with pUL49.5 (gN) [reviewed in (Roizman et al., 2007)]. Structurally, gM was predicted to contain 2

initiation sites in N-terminal cytoplasmic domain, 8 transmembrane domains, 2 N-glycosylation sites in extracellular domains, and 4 tyrosine-based endocytosis motifs (YXXΦ; YDEV₄₂₆, YAKI₄₃₈, YDTV₄₅₄ and YSTV₄₇₀) in the C-terminal cytoplasmic domain (Figure 2-10) [(Crump et al., 2004, Baines and Roizman, 1993) and reviewed in (Roizman et al., 2007)]. Due to the glycosylation, gM is initially expressed as a 47-kDa precursor, modified to a 50-kDa molecule with high-mannose-type oligosaccharides, and ultimately matured into a 53- to 63-kDa protein (Baines and Roizman, 1993). Deletion of the gM gene does not abrogate HSV-1 replication, but inhibits the ability of the virus to spread (Browne et al., 2004, Chouljenko et al., 2012, MacLean et al., 1993, Leege et al., 2009).

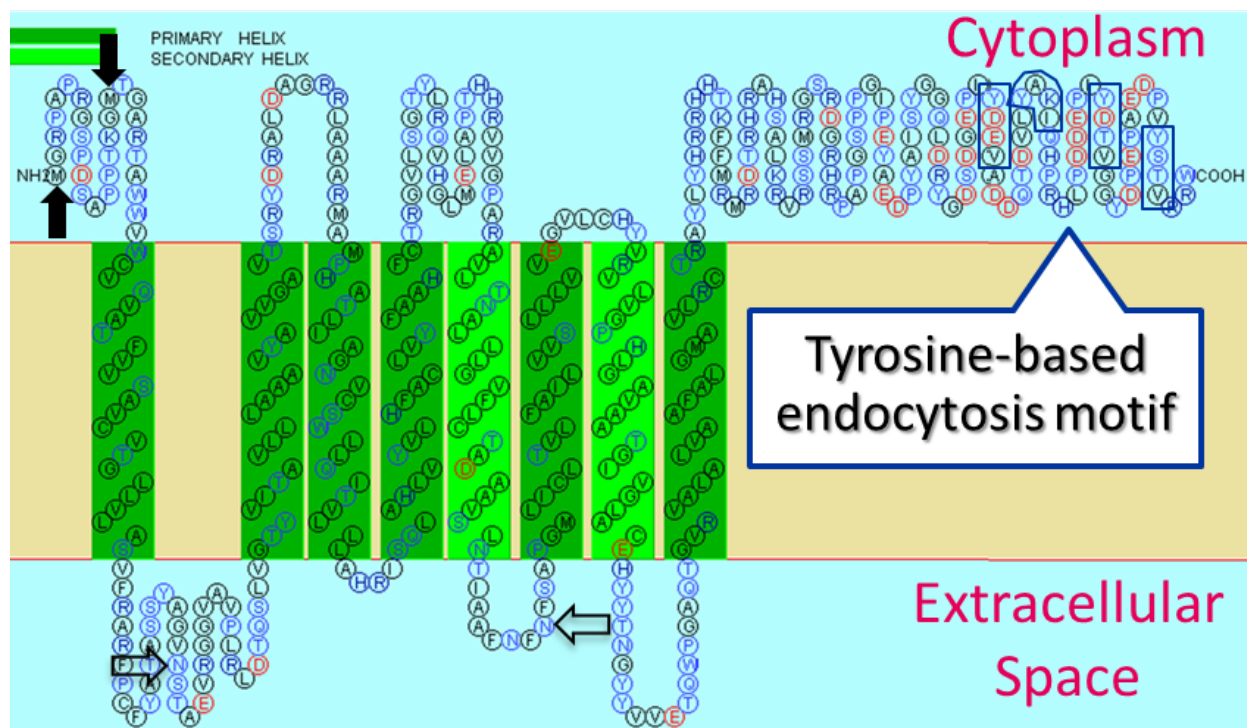


Figure 2-10. A predicted structure of glycoprotein M (gM) of HSV-1. The yellow-filled two redlined box indicates plasma membrane. The black solid and empty arrows indicate two initiation methionines and two potential N-glycosylation sites, respectively. C-terminal tyrosine-based endocytosis motifs are indicated by blue boxes. The dark and light green boxes indicate primary and secondary helix respectively. [Structure built by SOSUI prediction (Hirokawa et al., 1998)].

The alteration of gM expression causes relocalization of several membrane proteins from the cell surface to the TGN (Crump et al., 2004, Klupp et al., 2000b), which implies that gM may function to retain viral glycoproteins at the trans-Golgi network (TGN), or retrieve them from the plasma membrane to the TGN (Leege et al., 2009). Expression of HSV-1, pseudorabies virus (PRV) and Kaposi's sarcoma associated herpesvirus (KSHV or HHV-8) gM and gN in transfected cells, inhibited cell fusion caused by simultaneous expression of glycoproteins gB, gD, gH and gL suggesting that gM/gN may modulate membrane fusion (Koyano et al., 2003, Klupp et al., 2000b). Also, lack of gM was reported to inhibit virus-induced cell fusion caused by alteration of the carboxyl terminus of gB [A855V (gBsyn) and gB Δ 28] (Balan et al., 1994, Davis-Poynter et al., 1994, Kim et al., 2013). Moreover, mutant viruses lacking gM exhibited slower kinetics of entry into Vero cells than the parental wild type virus (Kim et al., 2013). *In vivo*, a mouse model experiment with gM-null HSV-1, revealed that the virus growth at the periphery and spread to and/or growth in the nervous system were impaired, although the mutant virus could establish latency (MacLean et al., 1993). Intranasal infection of a gM-null PRV to piglets also produced ca.100-fold reduced level of excreting viruses from the nasal mucosa, and transiently elevated body temperature comparing to wild-type or revertant virus (Dijkstra et al., 1997). Collectively, these results indicate that gM is required for efficient virus entry, replication and spread *in vitro* as well as *in vivo*.

Tegument protein UL11

UL11 is a 96-amino-acid myristoylated and palmitoylated tegument protein anchored into the cytoplasmic side of cell membranes and contains acidic cluster and di-leucine motif (Figure 2-11) (Baines et al., 1995, Loomis et al., 2001, MacLean et al., 1989, Baird et al., 2008). The two acylation sites of UL11 are important for efficient targeting and binding to plasma membrane,

and virion packaging of UL11; whereas, the acidic cluster and di-leucine motif are related with TGN localization of UL11 (Loomis et al., 2001, Loomis et al., 2006). UL11 has been suggested to play a role in recruiting viral proteins to the virion assembly site at the TGN (Farnsworth et al., 2007, Leege et al., 2009, Han et al., 2012). UL11 is also known to interact with UL16 and gE through its N-terminal (Baird et al., 2008, Loomis et al., 2003, Loomis et al., 2001) and C-terminal domains (Yeh et al., 2011), respectively. Although absence of UL11 in HSV-1 and PRV revealed only moderate defects in viral replication with accumulation of unenveloped capsids within the cytoplasm (Fulmer et al., 2007, Leege et al., 2009, Chouljenko et al., 2012, Kopp et al., 2003), the HCMV UL11 homologue is essential for virus replication (Seo and Britt, 2007, Silva et al., 2003). HSV-1 UL11 was shown to form a protein complex with gE, UL16 and UL21 that may be required for efficient virus spread (Han et al., 2012). Moreover, recent evidences suggested that UL11 is required for membrane fusion phenomena during both virus-induced cell fusion and virus entry (Kim et al., 2013).

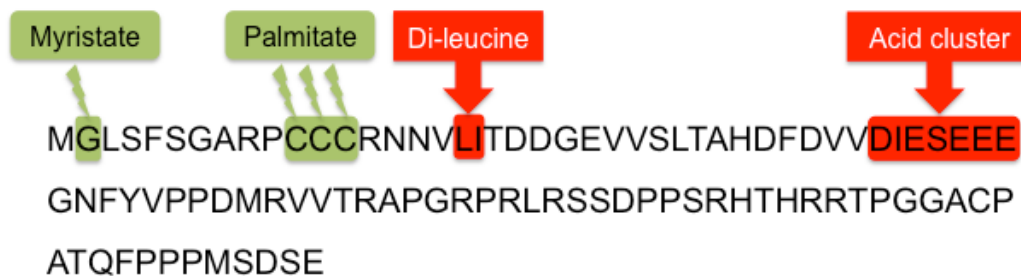


Figure 2-11. Structure and amino acid sequence of UL11. Four motifs of UL11 are indicated: myristylation of glycine, palmitation of cysteine, di-leucine motif and acid cluster.

Roles of other non-essential envelope proteins

HSV-1 encodes at least 11 glycoproteins as well as several membrane-associated proteins, which play important roles in viral entry and virus-induced cell fusion. Among those proteins, gB, gD, gH and gL have been identified as essential core proteins for HSV-1 replication in a cell

culture system by single gene knock-out analyses [reviewed in (Roizman et al., 2007)]. On the contrary, although other proteins are dispensable for virus replication in cell culture system, deletion of either of these proteins results in alteration of viral pathogenesis *in vivo*.

gC (UL27) is known to induce initial binding of herpesvirus to 3-O-sulfated heparan sulfate on the host cell by using its N-terminal 120 aa binding site, which can increase binding by approximately 10-fold to host cells (Roizman et al., 2007, Moffat et al., 1998). *In vivo*, gC confers immune evasion by blocking complement activation to HSV infection. gC has two domains that can modulate the complement activation by decoy complement components. One domain binds to C3 and C3 fragments C3b, iC3b, and C3c, and another domain binds to properdin that mediates the decay of the C3 convertase and interferes in its interaction with C3b (Chang et al., 2005, Moffat et al., 1998, Roizman et al., 2007). gC also can prevent C5 from binding to C3b, a required interaction to generate the C5-convertase and membrane attack complex (Chang et al., 2005). Therefore, infection with gC-null HSV or VZV to model animals, revealed less clinical severity and viral reproduction due to rapid virus neutralization (Chang et al., 2005, Moffat et al., 1998). On the other hand, in mouse and guinea pig models, gC and gD combined subunit vaccine showed enhanced protection from recurrence of HSV-2 than gD single subunit vaccine through blocking immune evasion domains (Awasthi et al., 2011). Furthermore, passive immunization of gC-specific IgG to intact mice conferred protection from HSV-1 infection but not to C3 knock-out mice. These results indicate a close relationship of gC to the complement activation cascade *in vivo* (Awasthi et al., 2009).

gE (US8) is expressed as a heterodimer with gI (US7) and functions to facilitate cell-to-cell spread by sorting newly formed virion particles to cell junctions and lateral cell surfaces (Collins and Johnson, 2003). Unlike PRV, HSV-1 gE-null mutants exhibited a defect in retrograde

transport *in vivo* but not *in vitro* (McGraw et al., 2009). *In vivo*, intranasal inoculated gE-null PrV or retina infection of gE-null HSV-1 could not spread to second-order neurons of the trigeminal, sympathetic, or parasympathetic route in murine models (Masse et al., 1999, Wang et al., 2005a). Although gE-null BHV could still establish latent infection at sensory ganglia, it also produced a lesser number of latent DNA than gE-positive parental virus (Mars et al., 2000). In the murine flank model, gE-null HSV-1 caused reduced size of skin lesions at the inoculation site (Wang et al., 2005a). Additionally, the gE/gI complex has a high affinity to Fc portion of IgG that possibly provides an immune evasion by interrupting antibody mediated cellular toxicity to herpesvirus infected cells (Li et al., 2007). Taken together, gE/gI complex provides immune evasion activity and neurovirulence by promoting spread at the axon terminals of infected neurons and interrupting cell-mediated immune response *in vivo* (Enquist et al., 2002).

US9 protein (pUS9) has been reported as an important protein for neuronal spread and neurovirulence (Brideau et al., 2000, Butchi et al., 2007, Chowdhury et al., 2002, Polcicova et al., 2005, Negatsch et al., 2010). pUS9 localizes to the TGN, and functions in anterograde transport of newly formed virion particles in neurons by targeting other envelope proteins (Brideau et al., 2000; Lyman et al., 2007; (Lyman et al., 2007, Tomishima and Enquist, 2001, Butchi et al., 2007, Snyder et al., 2008, Brideau et al., 2000, Negatsch et al., 2010). PRV US9-null mutants showed an apparent defect in anterograde spread in neural circuits, due to impaired transport of essential viral membrane proteins to axon terminals, which facilitates spread to the connected neuron, but PRV gE/gI-null mutants did not show the defect (Enquist et al., 2002). Although the function of pUS9 is mainly related to the neuronal system, it may have indirect functions in cytoplasmic egression (Negatsch et al., 2010).

Homologues of gG (US4), which function remains unclear, were identified in HSV-1, EHV-1 and -4, BHV-1 and -5, but not in VZV (Bryant et al., 2003). The gG-null HSV-2 did not exhibit phenotypic abnormalities, defect in nuclear egress, or noticeable attenuation of viral pathogenesis *in vivo* (Atkinson et al., 1978). In tissue culture, since BHV-1 gG-null virus showed smaller plaque than that of the wild type, its role is possibly related to a direct cell-to-cell transmission mechanism (Nakamichi et al., 2000). Controversially, disruption of PRV gG gene by insertion showed no difference between wild type and mutant viruses. However, this result seems not to be caused by gG disruption but by serine/threonine kinase activity of pUS3. Since DNA insertions in gG possibly have polar effects to reduce the production of US3 protein, this impacts the spread of viral infection (Sullivan and Smith, 1987). Also, gG of EHV-1, BHV-1 and BHV-5 showed a strong affinity to CC, CXC and C chemokines, but not CX3CL1, which suggest that its potential role in immune evasion is by interfering with the interaction of chemokines with its cognitive receptors and GAGs (Bryant et al., 2003).

Although gJ (US5) is embedded on the membrane, its relationship to binding or entry to the host cell remains unclear, because HSV-1 gJ-null mutant did not cause any remarkable difference in virus growth and phenotype *in vivo* and *in vitro* (Balan et al., 1994). On the other hand, gJ has been suggested to have anti-apoptotic activity through inhibiting cytotoxic T lymphocyte (CTL) killing mechanism (Zhou et al., 2000a, Aubert et al., 2008, Aubert et al., 2006). In detail, gJ may inhibit apoptosis by depleting cellular ATP stores by disrupting activity of subunit 6 in F₀F₁ ATP synthase. gJ was identified to induce reactive oxygen species (ROS) formation, but the relationship between ROS production and apoptosis, mediated by HSV infection is unclear (Aubert et al., 2008).

gK (UL53), which has been identified as a modulator of gB in our lab, is a 338 aa extreme hydrophobic protein containing two N-link mannose glycosylation sites and 4 membrane spanning domains (Debroy et al., 1985, Pertel and Spear, 1996, Hutchinson et al., 1992a, Ramaswamy and Holland, 1992). In specific, the N-terminal amino acid 31-68 of gK directly interacts with cytoplasmic domain of gB and modulated gB-mediated virus-induced membrane fusion and virion egress (Melancon et al., 2005, Chouljenko et al., 2009). This interaction was confirmed by precise cleavage of N-terminal 68 amino acids on the purified virion by single putative tobacco etch virus (TEV) protease, since the cleavage caused a similar level of reduction in virus entry to that of the gK-null HSV-1 (Jambunathan et al., 2011). Also, HSV-1 gK functionally and physically interact with UL20 and these interactions are absolutely necessary for their coordinate intracellular transport, cell-surface expression and membrane fusion functions in the HSV-1 life cycle (Foster et al., 2001, Foster et al., 2004, Jambunathan et al., 2011, Foster et al., 2008). The partner protein, pUL20 is a 222 aa membrane protein that is assumed to have four membrane spanning domains with cytoplasmically located amino and carboxyl termini (Melancon et al., 2004). Since two proteins are closely related in function, lacking either UL20 or gK expression induces the most severe defects in cytoplasmic envelopment, egress, and infectious virus production (Chouljenko et al., 2012). Moreover, since certain mutations in the UL20 gene (Baines et al., 1991, McLean et al., 1990, Melancon et al., 2004) and the UL53 gene coding for gK (Bond and Person, 1984, Debroy et al., 1985, Hutchinson et al., 1992b, Pogue-Geile et al., 1984, Pogue-Geile and Spear, 1987, Ruyechan et al., 1979) also drastically enhance virus-induced cell-fusion (syncytial or *syn* mutations), it reflects their important roles in the gB-mediated virus-induced cell fusion. Our lab has shown that gK-null HSV-1(McKrae) exhibited severely reduced capability to infect corneal cells and

neurons without the direct exposure of neuronal endings by extensive cornea scarification in a mouse model (David et al., 2012). On the other hand, interestingly, gK immunized mice showed significantly severe corneal scarring after ocular HSV-1 infection than naïve mice (Ghiasi et al., 1995, Ghiasi et al., 1994). Moreover, a recombinant HSV-1 harboring two additional copies of gK showed a more increased pathogenic severity than wild-type virus in mice (Mott et al., 2007). The mutant virus was identified that caused accelerated CD8⁺ T-cell (CTL) response that induced significantly severe corneal scarring than wild type virus did, although the reproduction levels of both viruses are similar (Mott et al., 2007). Subsequent study identified an immunodominant T-cell stimulatory region in signal sequence of gK, and furthered that ITAYGLVL is a highly conserved potential CD8⁺ T-cell epitope (Mott et al., 2009).

gN (UL49.5) has shown that the gene is dispensable without remarkable changes in HSV-1, EHV-1 and PRV but essential for MDV in cell culture (Adams et al., 1998, Tischer et al., 2002). Interestingly, gN of genus Varicellovirus including BHV-1, EHV-1, EHV-4, and PRV has TAP-inhibiting activity, which can interfere with the MHC class I mediated antigen presentation, whereas the UL49.5 proteins of VZV, HSV-1 and HSV-2 do not show the similar function (Verweij et al., 2011).

UL43 protein (pUL43) is predicted as a transmembrane protein that is conserved throughout the alpha- and gammaherpesviruses but lacking in betaherpesviruses (Klupp et al., 2005). The absence of pUL43 in HSV-1 did not affect viral replication in cell culture or in mouse model including neuronal and transneuronal spread (MacLean et al., 1991). EBV pUL43 homologue, BMRF2, contains RGD motifs that are absent in PRV and HSV-1. The motifs were suggested to interact with cellular integrin, and play a role in infection of polarized epithelial cells (Tugizov et al., 2003). Although PRV pUL43 was not essentially required for virus growth in cell culture

(Powers et al., 1994), coexpression of PRV pUL43 inhibited fusion induced by a transient expression-fusion assay (Klupp et al., 2005). However, this phenomenon needs further investigation to determine the reason.

The homologues of HSV-1 UL45, a type II membrane protein, have been identified in HSV-2, MDV, ILTV, EHV-1 and EHV-4 but not in VZV, BHV-1 and PRV [reviewed in (Oettler et al., 2001)]. UL45 protein (pUL45) may facilitate virus egress and mediate fusion events during HSV infection since a syncytial variant of HSV (HSV-gB Y854K) requires pUL45 for cell-to-cell fusion (Haanes et al., 1994). However, HSV-1 pUL45 was dispensable for growth on Vero and HeLa cells, but a deletion mutant produced smaller plaque size and altered morphology (Visalli and Brandt, 1991). Unlike HSV-1, lacking of pUL45 on EHV-1 produced unnoticeable differences in plaque size and morphology (Oettler et al., 2001). *In vivo*, UL45 protein may be required for efficient growth in the central nervous system at low dose infections, since the UL45-null virus grew more poorly than wild type virus in a mouse infection model (Visalli and Brandt, 2002). However, a recent study showed that the presence of pUL45 did not affect the conformational change of gB and virus entry (Dollery et al., 2010). The role of pUL45, therefore still remains unclear.

Taken together, although the non-essential envelope proteins are dispensable in the cell culture system, they affect the pathogenesis of herpesvirus by influencing in immune response, cell-to-cell spreading, neurotransmission, and viral replication in orchestrated work with other viral products. Particularly, gC, gE/gI, gG, gJ, and gN suppress immune response and gK elevates CTL response in herpesvirus infection. gE/gI, US9, and UL45 together promote the neuro-invasiveness of herpesviruses. Moreover, as mentioned earlier in this chapter, the viral proteins closely interact with each other and form a complicated network (Figure 2-12). A recent

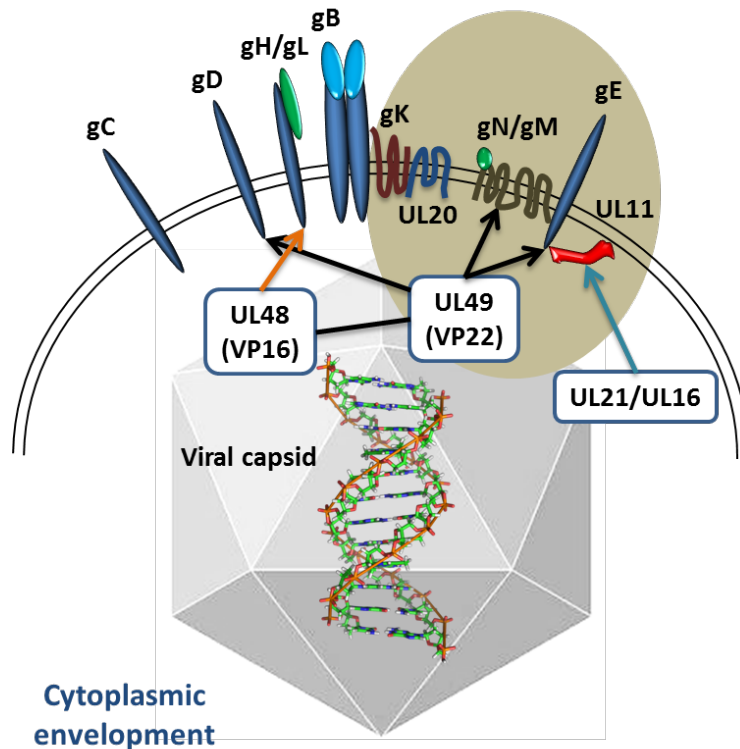


Figure 2-12. Interactions of tegumented capsids with the carboxyl termini of glycoproteins and membrane proteins. UL49 (VP22) is shown to interact with gD, gM and gE; UL48 (VP16) interacts with gH and VP22; UL21 and UL16 interact with UL11. UL20, UL11, gK, gM and gE are shown within a brown sphere to highlight their potential cooperative relationships in infectious virion morphogenesis. [Figure modified from (Chouljenko et al., 2012)]

study showed that the carboxyl terminus of gE interacts with UL11 inducing their co-packing into virion particles (Stylianou et al., 2009). gD and gH are known to directly interact with gB to modulate its fusogenicity (Atanasiu et al., 2007, Avitabile et al., 2007, Avitabile et al., 2009, Avitabile et al., 2003, Gianni et al., 2009, Connolly et al., 2011). Recently gK/UL20 is shown to interact with both gB and gM (Chouljenko et al., 2010, Kim et al., 2013). Furthermore, it was shown that VP22 (UL49) forms a protein complex via interacting with cytoplasmic domain of gD, gE, gM as well as its tegument partner VP16 (UL48) (Maringer et al., 2012, Chi et al., 2005, Farnsworth et al., 2007, Fuchs et al., 2002a). VP16 is also known to interact with the carboxyl terminus of gH (Gross et al., 2003). Overall, these results suggest that the viral glycoproteins and

tegument proteins interact with each other in multiple ways and deleting one or more of them may indirectly affect the functions and stability of other interacting partner proteins (Chouljenko et al., 2012).

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CHAPTER III: HERPES SIMPLEX VIRUS TYPE 1 GLYCOPROTEIN gM AND THE MEMBRANE ASSOCIATED PROTEIN UL11 ARE REQUIRED FOR VIRUS-INDUCED CELL FUSION AND EFFICIENT VIRUS ENTRY⁸

Introduction

Herpes simplex virus type 1 (HSV-1) encodes at least 11 glycoproteins as well as several membrane-associated proteins, which play important roles in viral entry and virus-induced cell fusion. Virus-induced cell-fusion is apparent in herpetic lesions and is thought to facilitate virion transmission to adjacent cells without exposure to the host humoral immune system, particularly neutralizing antibody [reviewed in reference (Roizman et al., 2007)]. Certain mutations in the UL20 gene (Baines et al., 1991, McLean et al., 1990, Melancon et al., 2004), the UL24 gene (Jacobson et al., 1998, Sanders et al., 1982), the UL27 gene encoding glycoprotein gB (Bzik et al., 1984, Pellett et al., 1985), and the UL53 gene coding for gK (Bond and Person, 1984, Debroy et al., 1985, Hutchinson et al., 1992, Pogue-Geile et al., 1984, Pogue-Geile and Spear, 1987, Ruyechan et al., 1979) drastically enhance virus-induced cell-fusion (syncytial or *syn* mutations). Recently, it has been suggested that gB is the sole fusogenic glycoprotein, while glycoproteins gD and gH/gL are required to activate gB's fusogenicity in conjunction with specific cellular receptors (Connolly et al., 2011). In this membrane fusion model, binding of gD to its cognate receptors including nectin-1, herpes virus entry mediator (HVEM), and other receptors (Montgomery et al., 1996, Geraghty et al., 1998, Shukla et al., 1999, Campadelli-Fiume et al., 2000, Spear et al., 2000, Spear and Longnecker, 2003, Satoh et al., 2008) is thought to trigger sequential conformational changes in gH/gL and gB causing fusion of the viral envelope with cellular membranes during virus entry, as well as fusion among cellular membranes (Hannah et

⁸ This chapter previously appeared as Kim et al., 2013. Herpes Simplex Virus 1 glycoprotein M and the membrane-associated protein UL11 are required for virus-induced cell fusion and efficient virus entry. *J Virol*, 87, 8029-37. It is reprinted by permission of American Society for Microbiology.

al., 2007, Heldwein et al., 2006). Extensive membrane fusion can be induced by co-expressing glycoproteins gB, gD and gH/gL in cell lines (Turner et al., 1998, Muggeridge, 2000) suggesting that these glycoproteins are sufficient for membrane fusion. However, virus-induced cell fusion is regulated by a number of other viral proteins, since wild type viruses cause limited amount of fusion (Kousoulas et al., 1978), and lack of either glycoprotein gK or the membrane protein UL20 severely inhibits membrane fusion (Melancon et al., 2004, Melancon et al., 2005).

We have shown that HSV-1 gK and UL20 functionally and physically interact and that these interactions are absolutely necessary for their coordinate intracellular transport, cell-surface expression and membrane fusion functions in the HSV-1 life cycle (Foster et al., 2001b). Furthermore, we have shown that a peptide comprised of the amino terminal 82 amino acids of gK (gKa) expressed *in trans* complemented gB-mediated cell fusion and could physically interact with gB and gH in infected cells (Chouljenko et al., 2009). These results suggest that gB-mediated virus-induced cell fusion is regulated via direct interactions with gK and UL20 (Chouljenko et al., 2009, Chouljenko et al., 2010).

Glycoprotein gM is a conserved type III integral membrane protein with multiple transmembrane domains, that forms a complex with pUL49.5 (gN) [reviewed in (Roizman et al., 2007)]. Deletion of the gM gene does not abrogate HSV-1 replication, but inhibits the ability of the virus to spread (Leege et al., 2009). gM expression causes relocalization of several membrane proteins from the cell surface to the TGN (Crump et al., 2004, Klupp et al., 2000). Thus, gM may function to retain viral glycoproteins at the trans-Golgi network (TGN), or retrieve them from the plasma membrane to the TGN (Leege et al., 2009). Expression of HSV-1, pseudorabies virus (PRV) and Kaposi's sarcoma associated herpesvirus (KSHV or HHV-8) gM and gN in transfected cells inhibited cell fusion caused by simultaneous expression of

glycoproteins gB, gD, gH and gL suggesting that gM/gN may modulate membrane fusion (Koyano et al., 2003, Klupp et al., 2000). Also, lack of gM was reported to inhibit virus-induced cell fusion caused by a single amino acid substitution in the carboxyl terminus of gB (A855V; gBsyn) (Davis-Poynter et al., 1994, Balan et al., 1994).

UL11 is a 96-amino-acid myristoylated and palmitoylated tegument protein anchored into the cytoplasmic side of cell membranes (Leege et al., 2009, Yeh et al., 2008). UL11 has been suggested to play a role in recruiting viral proteins to the virion assembly site at the TGN (Leege et al., 2009). UL11 is known to interact with UL16 and gE through its N-terminal (Baird et al., 2008, Loomis et al., 2003, Loomis et al., 2001) and C-terminal domains (Yeh et al., 2011) respectively. Although absence of UL11 in HSV and PRV revealed only moderate defects in viral replication, the human cytomegalovirus human herpesvirus (HCMV or HHV-5) UL11 homologue is essential for virus replication (Leege et al., 2009). HSV-1 UL11 was recently shown to form a protein complex with gE, UL16 and UL21 that may be required for efficient virus spread (Han et al., 2012).

Recently, we utilized mutant viruses lacking one or more viral genes to show that the deletion of either the gK or UL20 gene produced significantly greater defects in virion envelopment and overall virus replication than deletion of either the carboxyl terminus of gD, UL11, gM or gE alone or in various combinations (Chouljenko et al., 2012). Herein, we investigated whether the lack of either gM or UL11 affected the ability of dominant syncytial mutations in either gB or gK to cause extensive virus-induced cell fusion. We found that both gM and UL11 are required for virus-induced cell fusion. Moreover, mutant viruses lacking either gM or UL11 exhibited slower kinetics of entry into Vero cells than the parental virus suggesting that gM and UL11 are involved in membrane fusion phenomena during both virus-induced cell fusion and virus entry.

Materials and Methods

Cells, antibodies and plasmids

African green monkey kidney (Vero) cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies-Gibco; Carlsbad, CA), supplemented with 10% fetal calf serum (FCS; Life Technologies-Gibco; Carlsbad, CA) and Primocin antibiotic (Invivogen; San Diego, CA). Antibodies used include anti-HSV-1 gB, gC, gD, gE and ICP5 (VP5) monoclonal antibodies (mAb) (Virusys; Sykesville, MD), Alexa Fluor 488-conjugated goat anti-mouse IgG (Life Technologies-Molecular Probes; Carlsbad, CA), anti-HSV-1 gH mAb (Abcam; Cambridge, MA), anti-FLAG mAb (Sigma; St. Louis, MO), rabbit anti-HSV-1 gM polyclonal antibody (pAb) (a gift from Dr. Joel Baines, Cornell University, Ithaca, NY, USA), and rabbit anti-HSV-1 UL11 pAb (a gift from Dr. John Wills, Pennsylvania State University, Hershey, PA, USA). For transient complementation experiments, HSV-1(F) UL10 (gM) and UL11 genes were cloned into pCDNA3.1 plasmid (Life Technologies-Invitrogen; Carlsbad, CA) and named pCgM and pCUL11, respectively.

Construction of HSV-1 mutant viruses

Mutagenesis was accomplished in *Escherichia coli* using the markerless two-step RED recombination mutagenesis system using synthetic oligonucleotides (Lee et al., 2009, Tischer et al., 2006) implemented on the bacterial artificial chromosome (BAC) plasmid pYEbac102 carrying the HSV-1(F) genome (Tanaka et al., 2003) (a kind gift from Dr. Kawaguchi, University of Tokyo, Japan). Construction of the HSV-1 mutants Δ gM2 (Δ gM) and Δ UL11 was described previously (Chouljenko et al., 2012). Briefly, the Δ gM 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) (Baines and Roizman, 1993) (Figure 3-1). The Δ UL11 virus was constructed by changing the initiation codon from ATG to CTG. The gB Δ 28 recombinant virus was produced by introducing a stop codon causing truncation of gB by 28 amino acids. The gKsyn20 recombinant virus was constructed by introducing a point mutation (ala-to-val) at gK amino acid position 40. The Δ gM and Δ UL11 viruses were used as the backbone for construction of the double mutants, gB Δ 28/ Δ gM, gKsyn20/ Δ gM, gB Δ 28/ Δ UL11 and gKsyn20/ Δ UL11 by introducing designated mutation, respectively (Figure. 3-1).

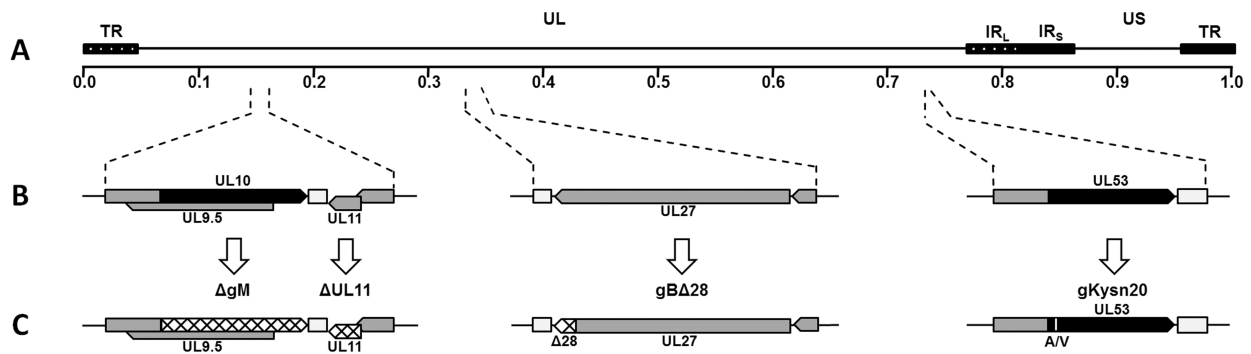


Figure 3-1. Schematic representation of mutant viruses. A: Represents the prototypic arrangement of the HSV-1(F) YE102 genome with the unique long (UL) and unique short (US) regions flanked by the terminal repeat (TR) and internal repeat (IR) regions. B: Relative genomic positions and gene arrangements of targeted genes encoding viral glycoproteins gB (UL27), gM (UL10) and gK (UL53) and membrane protein UL11. C: Schematic representation of engineered mutations. Mutant viruses containing the Δ gM and Δ UL11 mutations were produced by changing the initiation codons of each gene (hatched regions). Mutant viruses containing gB Δ 28 were produced by introduction of a stop codon causing truncation of gB by 28 amino acids (hatched region in gB). Mutant viruses containing gKsyn20 were produced by a point mutation (ala-to-val) at gK amino acid position 40 (white vertical line in gK).

Confirmation of the targeted mutations and recovery of infectious viruses

HSV-1 BAC DNAs were purified from 50 ml of overnight BAC cultures with the Qiagen large-construct kit (Qiagen; Valencia, CA). Using PCR test primers designed to lie outside of the

target mutation site(s), all mutated DNA regions were sequenced to verify the presence of the desired mutations in BACs. Viruses were recovered from cells transfected with BAC plasmids as we have described previously (Lee et al., 2009), and mutations were confirmed by DNA sequencing. To further validate the recombinant viruses, the entire genomes of recovered viruses from BACs were sequenced via the Ion Torrent Next Generation Sequencing (Life Technologies-Invitrogen; Carlsbad, CA). Briefly, total genomic DNA (gDNA) was extracted from the virus-infected Vero cells using the PureLink Genomic DNA Mini Kit (Life Technologies-Invitrogen; Carlsbad, CA). High-quality fragment libraries of each virus were prepared from the extracted total gDNAs using the Ion Xpress Plus Fragment Library Kit (Life Technologies-Invitrogen; Carlsbad, CA). The fragment libraries were subsequently applied to Ion 316 chips and were analyzed on the Ion Personal Genome Machine System (Life Technologies-Invitrogen; Carlsbad, CA).

Plaque morphology of mutant viruses on Vero cells and transiently complementing cells

Visual analysis of plaque morphology of mutant viruses was performed as we have previously described (Foster et al., 2004, Fulmer et al., 2007, Lee et al., 2009, Melancon et al., 2005). Confluent monolayers of Vero cells were transfected with 1 μ g of gM or UL11-expressing plasmid (one well of a 12-well tissue culture plate) using Lipofectamin 2000 transfection reagent (Life Technologies-Invitrogen; Carlsbad, CA). At 24 hours post-transfection, cells were infected at a multiplicity of infection (MOI) of 0.0001 with each mutant virus lacking gM or UL11. At 48 hours post-infection (hpi), the infected cells were fixed with ice-cold methanol. Plaques were visualized after immunohistochemical staining with polyclonal anti-HSV rabbit sera as described earlier (Chouljenko et al., 2009). More than 50 independent viral plaques were examined to assess relative levels of syncytia formation and select individual plaques as representative plaques for each experiment.

One-step viral growth kinetics

Analysis of one-step growth kinetics was performed as we have described previously (Foster et al., 2003, Foster et al., 2001b). Briefly, nearly confluent Vero cell monolayers in 12 well plates were infected with each virus in triplicate at either a low MOI (0.2) or high MOI (3.0) for 1 hour at 4°C. Thereafter, plates were incubated at 37°C/5% CO₂ and virus was allowed to penetrate for 1 hour at 37°C. Any remaining extracellular virus was inactivated by low-pH treatment (pH 3.0) for 10-15 sec, and the plates were incubated at 37°C/5% CO₂. At 12 and 24 hpi, virus stocks were prepared and viral titers were calculated by end-point titration on Vero cells. Viral plaques were counted after immunohistochemical staining with rabbit anti-HSV-1 pAb, as previously described (Chouljenko et al., 2009).

SDS-PAGE and Western immunoblot assay

Western immunoblot analysis was carried out essentially same as described earlier (Chouljenko et al., 2009). Briefly, confluent Vero cell monolayers were infected with the indicated virus at an MOI of 3. At twenty-four hpi, cells were collected by low speed centrifugation, washed twice with ice-cold PBS, and lysed with NP-40 lysis buffer (Life Technologies-Novex; Carlsbad, CA). The collected samples were mixed with SDS-PAGE sample buffer (BioRad; Hercules, CA) at 1:1 ratio and were electrophoretically separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tris-HEPES-SDS gradient 4-20% gels, Thermo scientific; Waltham, MA). Following electrophoresis, four identical gels were transferred to nitrocellulose membrane under a constant current. Membranes were blocked in Tris-buffered saline containing 0.1% Tween 20 (TBST) plus 5% nonfat milk for 1 hour at room temperature and were probed with primary mAbs for over-night at 4°C. Goat anti-mouse secondary antibody conjugated with HRP and ECL substrate (GE Healthcare; Little Chalfont, United Kingdom) were used for detection purposes.

Quantification of cell-to-cell fusion

Luciferase-based cell-to-cell fusion assay was performed essentially same as described earlier (Chouljenko et al., 2009). Briefly, subconfluent monolayer of Vero cells in 6-well plate was transfected with 2 µg of plasmid containing either the T7 polymerase gene under the CMV promoter (effector cells) or the T7-dependent luciferase gene (target cells) by using Lipofectamine 2000 transfection reagent respectively. As positive and negative controls, cells were transfected with both of the plasmids simultaneously and with pCAGGS empty vector plasmid, respectively. After 12 hours from each transfection the two different cell populations, effector and target cells or negative cells and target cells, were detached and were reseeded to a new 12-well plate at 1:1 ratio for experimental groups and negative control groups, respectively. At 24 hours post-transfection cells were infected with wild type and mutant viruses at an MOI of 0.2, respectively. At 12 and 24 hpi cells were washed twice with ice-cold PBS and lysed with passive lysis buffer (Promega; Madison, WI). The lysates were clarified by centrifugation at 10,000 ×g for 5 min at 4°C and were reacted with luciferase substrate (Promega; Madison, WI). The intensity of luciferase activity was measured by a TD-20/20 luminometer (Turner Designs; Sunnyvale, CA) with 5 sec delay and 10 sec read.

Analysis of membrane-associated proteins

Biotinylation of cell surface proteins was used to identify membrane associated proteins of Vero cells infected with each designated viruses. Briefly, surface proteins of Vero cells infected with the designated viruses at MOI of 3 were biotinylated at 12 hpi, and isolated with a Pierce Cell Surface Protein Isolation Kit (Thermo scientific; Waltham, MA). The isolated proteins, whole lysates and flow through were analyzed by western blots with specific antibodies to gB, gC, gD, gH, gM, UL11 and VP5.

Two-way immunoprecipitation experiments

Immunoprecipitation of proteins were essentially same as earlier described (Jambunathan et al., 2011). Vero cell monolayers were infected with HSV-1(F) or HSV-1(F)-YE102-VC1 (having UL20 protein tagged with the 3XFLAG epitope and gK tagged with V5 epitope) (Jambunathan et al., 2011). Cellular extracts were prepared at 24 hpi and the designated proteins were precipitated by protein G magnetic Dynabeads (Life Technologies-Novex; Carlsbad, CA) bound to the corresponding antibodies according to the manufacturer's instructions. The presence of gB, gM, and UL20 was visualized by western immunoblots using anti-gB, anti-gM, and anti-Flag (UL20) antibodies before or after immunoprecipitation with the same set of antibodies.

Virus penetration kinetics assay

The kinetics of virus penetration was measured as described previously (Jambunathan et al., 2011, Foster et al., 2001b). Briefly, subconfluent monolayers of Vero cells in 12-well tissue culture plates were infected at 4°C for 1 hour with approximately 250 PFU of wild type HSV-1(F), Δ gM and Δ UL11 viruses. Subsequently, infected cell cultures were incubated at 34°C to allow virus penetration. Immediately thereafter (0) and at 30, 60, 120, and 180 min, the virus inoculum was removed, and washed once with PBS (pH 7.4), and then remaining extracellular virus was inactivated by treatment with low-pH PBS (pH 3.0). The cells were washed once with PBS and DMEM without serum sequentially, and then overlaid with 1% methylcellulose in DMEM supplemented with 2% FCS. The cells were fixed with ice-cold methanol at 48 hpi and virus plaques were counted after immunohistochemical staining (Chouljenko et al., 2009). Mean values and standard deviations of three independent experiments were calculated. To determine the entry kinetics, linear regression slopes during the exponential growth period, from 0 to 120 min, were calculated.

Results

Construction of recombinant viruses

Deletion of the carboxyl terminal 28 amino acids of gB ($\Delta 28$), or an ala-to-val mutation (syn20) in the amino-terminus of gK cause extensive virus-induced cell fusion (Baghian et al., 1993, Bond and Person, 1984, Foster et al., 2003). To investigate the role of gM and UL11 in virus-induced cell fusion, we constructed a set of mutant viruses containing these syncytial mutations in the presence or absence of mutations that prevented expression of either gM or UL11 genes using the HSV-1(F) genome cloned as a BAC (see Materials and Methods). The set of mutant viruses that were constructed included: 1) gB $\Delta 28$; 2) gB $\Delta 28/\Delta$ gM; 3) gB $\Delta 28/\Delta$ UL11; 4) gKsyn20; 5) gKsyn20/ Δ gM; 6) gKsyn20/ Δ UL11 (Figures 3-1 and -2).

Validation of recombinant viruses

Individual mutant viruses were recovered by transfecting DNA of each constructed mutant BAC into Vero cells and the entire genomes of the parental HSV-1(F) virus and all mutant viruses were sequenced (see Materials and Methods). Viral genomes matched very well to the published HSV-1(F) genomic sequence (GenBank accession number GU734771.1) with the exception of 37 nucleotide changes resulting in 13 amino acid changes between the published HSV-1(F) and our HSV-1(F) strain, which is derived from the pYE102bac (Table 3-1). Comparison of the HSV-1(F)-pYE102bac with each mutant virus revealed the presence of each engineered mutation, while no other nucleotide changes were observed.

Characterization of mutant viruses

The plaque morphology of each recombinant virus was characterized as described in Materials and Methods. As expected, the recombinant viruses gB $\Delta 28$ and gKsyn20 caused extensive cell fusion (Figure 3-2: A). In contrast, lack of either gM or UL11 expression in the

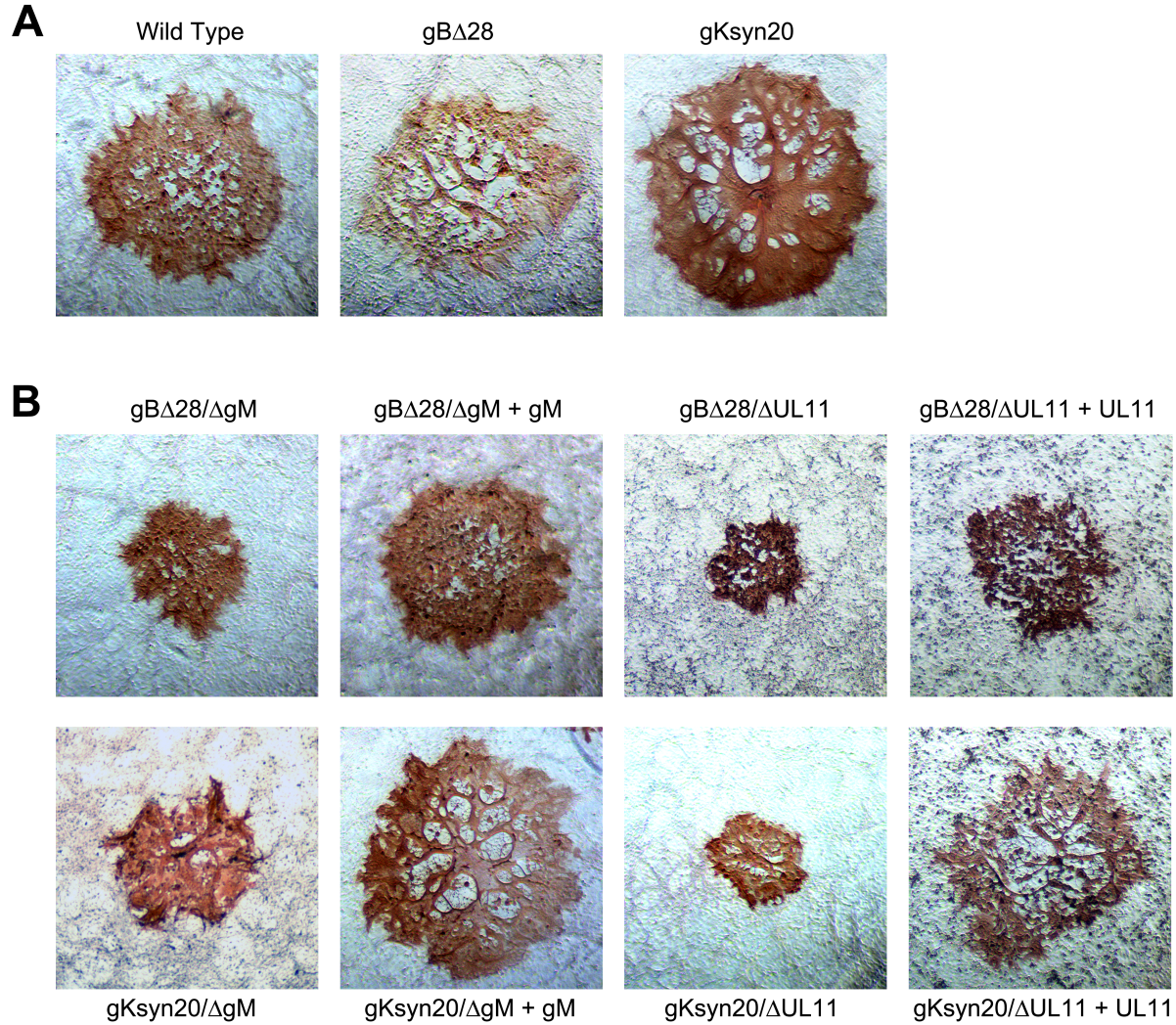


Figure 3-2. Plaque morphology of mutant viruses in comparison to HSV-1(F) YE102. Confluent monolayers of Vero cells were infected with wild type and mutant viruses at an MOI of 0.0001 and immunohistochemically stained at 48 hpi using polyclonal anti-HSV rabbit sera as described in Materials and Methods. A: Representative viral plaques of HSV-1 (F) YE102 wild type virus and syncytial mutant viruses gB Δ 28 and gKsyn20 on Vero cells. B) Representative viral plaques of syncytial mutant viruses lacking gM or UL11 in the absence or presence of transient complementation with either gM or UL11 expressing plasmids.

presence of either gB Δ 28 or gKsyn20 mutations caused significantly reduced levels of cell-to-cell fusion and decreased plaque sizes (Figure 3-2: B). To test whether these plaque changes were due primarily to lack of either gM or UL11 gene expression, complementation assays were performed in which Vero cells were transfected with plasmids expressing either gM or UL11 and

Table 3-1. Summary of differences between the published HSV-1(F) and the HSV-1(F) from pYE102bac^a.

Gene name ^b	nt position	aa difference	aa position	Gene description
UL1	9,604			gL
UL6	15,564			Capsid portal protein
UL8	18,753			Helicase-primase subunit
	19,678			Helicase-primase subunit
UL13	26,904	C → Y	507	Tegument serine/threonine protein kinase
NC	34,226			Tegument serine/threonine protein kinase
	35,711			
UL19	40,404	A → T	15	Major capsid protein
UL21	43,555			Tegument protein
NC	47,555			
UL24	47,695			Nuclear protein
	47,910	A → V	86	Nuclear protein
	47,911			Nuclear protein
UL27	53,638			gB
UL34	69,681	S → N	45	Nuclear egress membrane protein
UL37	81,439			Tegument protein
	82,015			Tegument protein
	82,806			Tegument protein
	82,815			Tegument protein
UL38	84,529	C → R	45	Capsid triplex subunit 1
UL39	87,759	A → V	424	Ribonucleotide reductase subunit 1
	88,762			Ribonucleotide reductase subunit 1
UL48	103,969			Transactivating tegument protein VP16
	104,340	T → A	212	Transactivating tegument protein VP16
UL50	107,306	P → T	134	Deoxyuridine triphosphatase
UL55	115,491	M → I	35	Nuclear protein UL55
UL56	116,406	A → T	137	Membrane protein UL56
NC	132,421			
	134,690			
	137,938			
US7	139,976			gI

^ant, nucleotide; aa, amino acid.

^bNC, noncoding region.

subsequently infected with the mutant viruses. These experiments revealed that more than 50% of viral plaques (not shown) were rescued to either gB Δ 28 or gKsyn20 plaque morphologies (Figure 3-2: B). To quantify the fusogenic capacity of mutant viruses, we utilized a luciferase-based assay to measure cell-to-cell fusion (see Materials and Methods). The gKsyn20 mutation caused much higher virus-induced cell fusion than the gB Δ 28 mutation, most likely because this virus spread more rapidly than the gB Δ 28 virus. Moreover, the amount of gKsyn20-caused cell fusion was much higher than that obtained by transfecting both luciferase gene and T7 polymerase in the same cell population (positive control). Deletion of either gM or UL11 completely abrogated gKsyn20-mediated cell fusion (Figure 3-3: A). Similarly, deletion of either

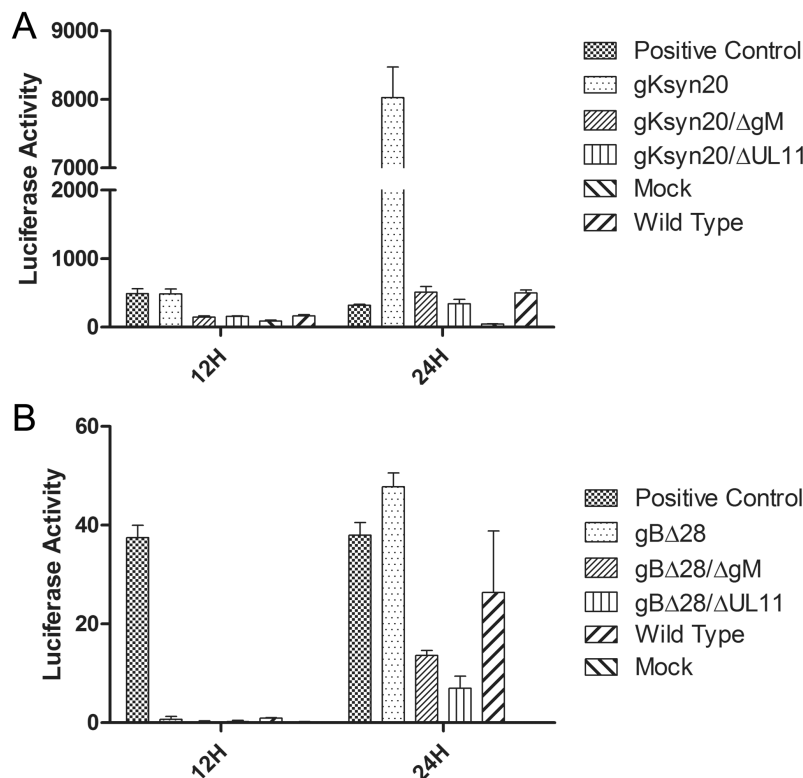


Figure 3-3. Fusion activity of wild type and mutant viruses. Fusion activity of each virus was quantified by luciferase-based assay (see Materials and Methods). The extent of fusion was assessed at 12 and 24 hpi for the wild type virus and all mutant viruses containing the gKsyn20 mutation (A) or the gB Δ 28 mutation (B).

gM or UL11 severely inhibited gB Δ 28-induced cell fusion exhibiting cell fusion levels lower than those produced by the wild type virus (Figure 3-3: B). To assess the relative effect of each mutation alone or in combination on virus replication, viral titers were obtained at 24 or 48 hpi after infection of Vero cells at an MOI of either 0.2 or 3.0. All viruses appeared to replicate with approximately equal efficiencies to that of the parental wild type virus irrespective of the presence of the engineered mutations (Figure 3-4).

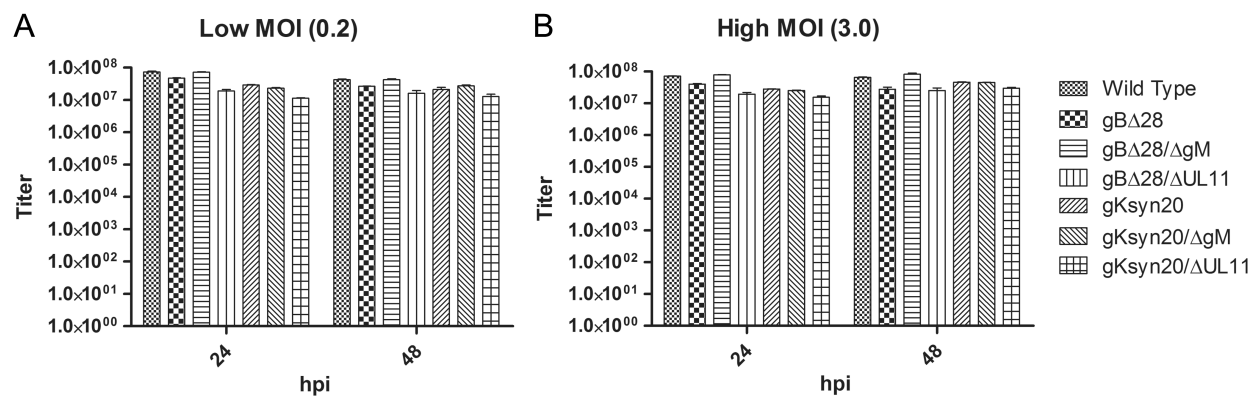


Figure 3-4. Replication kinetics of the wild type and mutant viruses. Confluent Vero cell monolayers were infected with each virus in triplicate at either a low MOI (0.2) (A) or high MOI (3.0) (B) and viral titers were obtained by plaque assay on Vero cells at 24 and 48 hpi. Error bars represent standard deviations.

Effect of mutations on the synthesis and cell-surface expression of viral glycoproteins

The effect of lack of either gM or UL11 expression on the synthesis of viral glycoproteins, gB, gC, gD, gM and membrane-associated protein UL11 were assessed using western immunoblots of whole cell lysates. Lack of either gM or UL11 expression was confirmed via the inability of anti-gM or anti-UL11 antibodies to detect the presence of either protein in their respective mutant viruses. Overall, neither lack of gM or UL11 expression drastically affected the synthesis of gB, gC and gD. Furthermore, lack of gM did not affect the synthesis of UL11, and lack of UL11 did not affect gM levels (Figure 3-5).

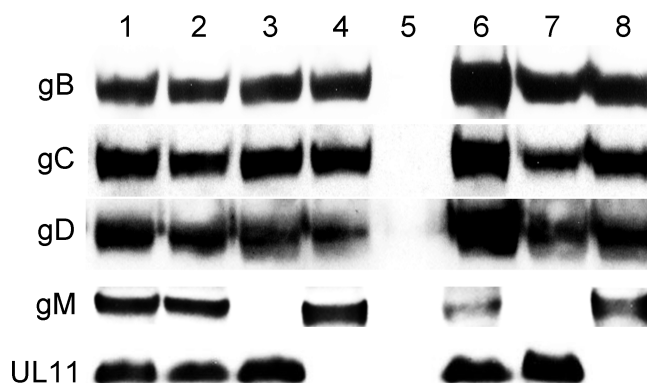


Figure 3-5. Characterization of glycoprotein expression by wild type and mutant viruses. The overall synthesis of each viral protein was assessed by western immunoblotting. Each lane represents infected cellular extracts from cells infected with either wild type or mutant viruses; 1) wild type, 2) gB Δ 28, 3) gB Δ 28/ Δ gM, 4) gB Δ 28/ Δ UL11, 5) mock infection, 6) gKsyn20, 7) gKsyn20/ Δ gM, and 8) gKsyn20/ Δ UL11. gB, gC, gD, gM and UL11 denote antibodies specific for each protein.

Surface-glycoprotein expression profiles of Vero cells infected with the designated viruses were analyzed by cell-surface biotinylation experiments (Figure 3-6). Viral glycoproteins expressed on infected cell surfaces were biotinylated under live conditions. Biotinylated proteins were subsequently isolated by streptavidin immunoprecipitation and analyzed by western immunoblots (see Materials and Methods). The HSV-1 capsid protein VP5 was included in the immunoblots as a negative control, since it is not expected to be expressed in infected cellular plasma membranes. Lack of either gM or UL11 did not affect overall expression of viral glycoproteins gB, gC, gD and gH on HSV-1(F)-infected cell surfaces (Figure 3-6: A). Similar results were obtained for the gB Δ 28 virus (Figure 3-6: B), except in the case of gKsyn20 infected cells, most likely due to rapid loss of fused cells (syncytia) (Figure 3-6: C).

UL20 protein physically interacts with gM in virus-infected cells

To determine whether gM and UL11 interact directly with gB or the gK/UL20 complex, we performed two-way immunoprecipitation experiments. The results revealed that the gM interacted with UL20 but not gB, since gM immunoprecipitates contained UL20 (Figure 3-7:

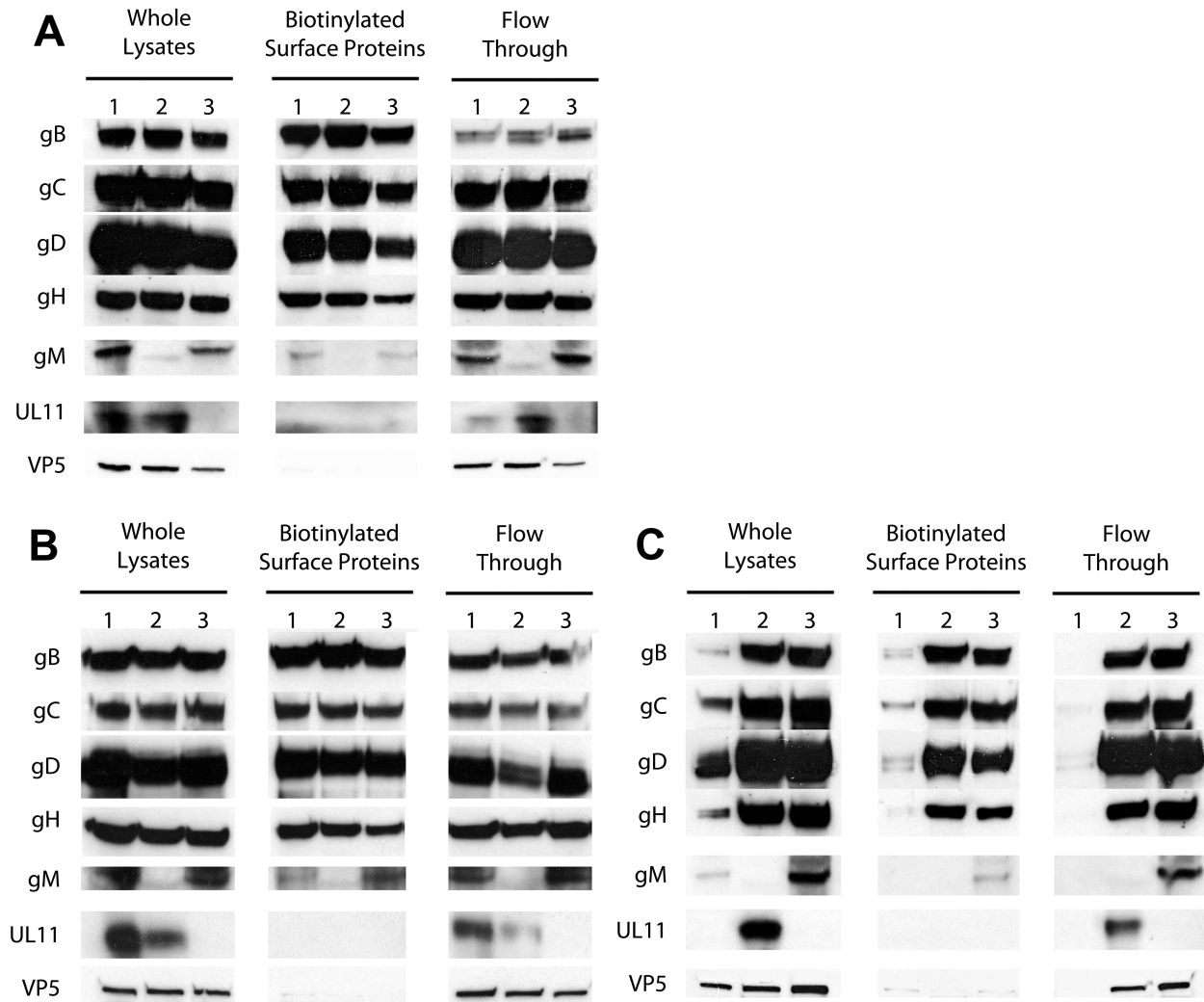


Figure 3-6. Cell-surface expression of viral glycoproteins on infected cell-surfaces by wild type and mutant viruses. At 12 hpi, whole lysates were prepared and membrane-associated proteins were isolated (see Materials and Methods). Flow through-labeled lanes represent cytoplasmic proteins. Each lane represents wild type or mutant virus infected cell extracts: (A) 1. wild type, 2. Δ gM and 3. Δ UL11; (B) 1. gB Δ 28, 2. gB Δ 28/ Δ gM and 3. gB Δ 28/ Δ UL11; (C) 1. gKsyn20, 2. gKsyn20/ Δ gM and 3. gKsyn20/ Δ UL11. gB, gC, gD, gH, gM, UL11 and VP5 denote antibodies specific for each protein.

lane 2), but not gB (Figure 3-7: Lane 8), and UL20 immunoprecipitates contained gM (Figure 3-7: lane 4). Immunoprecipitations with gB, gD, or gH failed to reveal any interactions with gM (not shown). Similar immunoprecipitations with UL11 failed to reveal any interactions with gB, gC, gD, gH, gM, gK or UL20 (not shown).

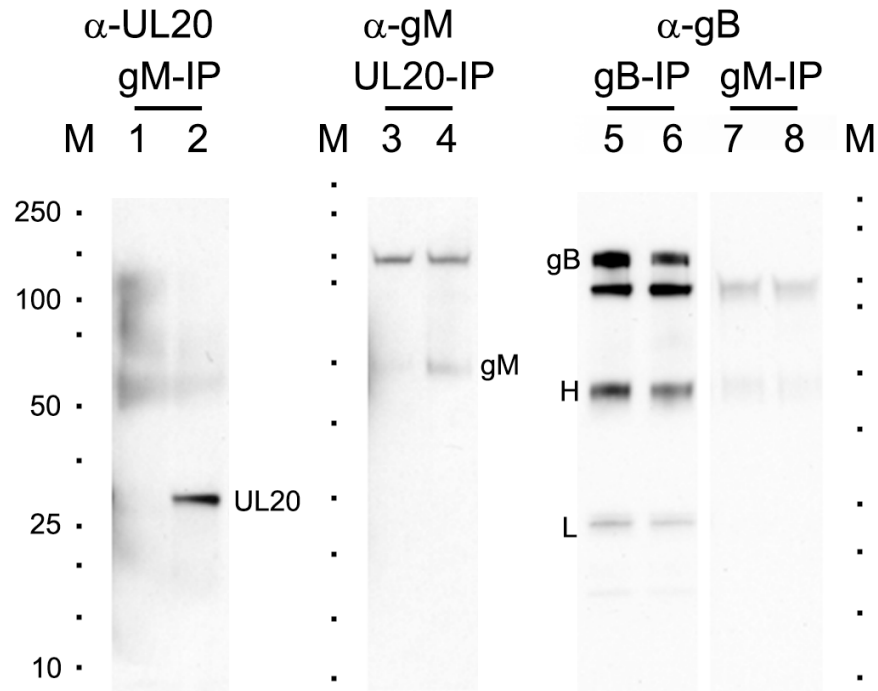


Figure 3-7. UL20 protein interacts with gM in virus-infected cells. Vero cell monolayers were infected with HSV-1(F) or HSV-1(F)-YE102-VC1 (having UL20 protein tagged with the 3XFLAG epitope and gK tagged with V5 epitope). Cellular extracts were prepared at 24 hpi and the presence of gB, gM or UL20 was detected using western immunoblots after immunoprecipitation (IP) with designated antibodies. Lane 1, 3, 5 and 7: antigen from HSV-1 (F) infected cells; lane 2, 4, 6 and 8: antigen from HSV-1(F)-YE102-VC1 infected cells; lane M demarcates positions of molecular mass markers (10 to 250 kDa; Bio-Rad). Anti-gB (α -gB), anti-gM (α -gM) and anti-FLAG (α -UL20) denote probed antibodies and gB-IP, gM-IP and UL20-IP (FLAG-IP) represent antibodies used for immunoprecipitation. gB, H, L, gM and UL20 denote gB, heavy chain, light chain, gM and UL20 proteins respectively.

HSV-1 gM and UL11 are required for wild-type-like virus entry

Virus-induced cell fusion and fusion of the viral envelope with cellular membranes during virus entry are thought to occur via similar mechanisms involving viral glycoproteins. Therefore, we tested whether lack of either gM or UL11 affected the kinetics of virus entry into Vero cells. Lack of either gM or UL11 caused slower entry of both viruses into Vero cells in comparison to wild-type virus (Figure 3-8). Similarly, lack of either gM or UL11 caused slower virus entry in the presence of either the gB Δ 28 or gKsyn20 mutations (not shown).

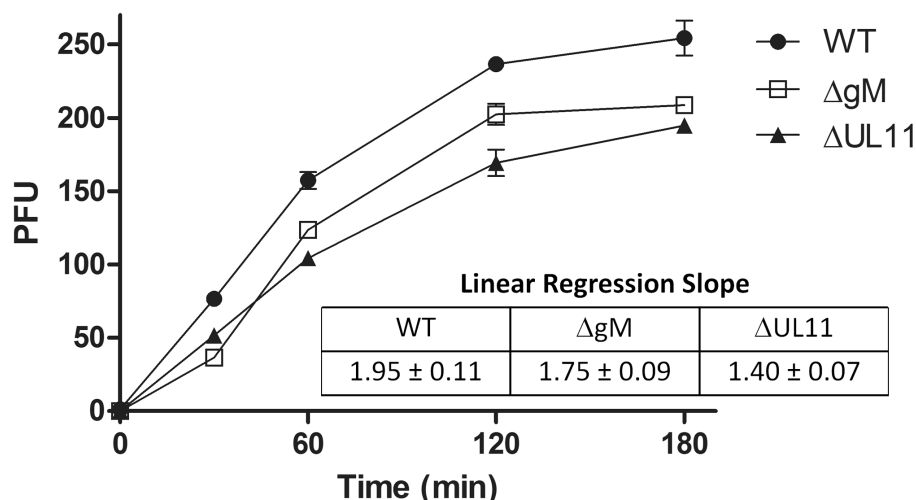


Figure 3-8. Entry kinetics of HSV-1(F), Δ gM and Δ UL11 viruses into Vero cells. Vero cell monolayers were infected with either HSV-1(F), Δ gM, or Δ UL11 viruses at 250 PFU per tissue culture well. The kinetics of virus entry at 34°C was measured (see Materials and Methods). Mean values and standard deviations of three independent experiments are shown. The relative efficiency of entry was calculated by obtaining the linear regression slope during the exponential viral growth period (0-120 min). The mean value and the standard deviation of each slope are shown.

Discussion

Herpes viral proteins and glycoproteins have been shown to extensively interact within the virion particle and in infected cells. Interactions among viral glycoproteins gB, gD, gH, gL, gK and the membrane protein UL20 are known to be involved in regulation of membrane fusion phenomena occurring during virus entry and virus-induced cell fusion. The purpose of this investigation was to assess whether glycoprotein gM and the membrane-associated protein UL11, both of which are highly conserved in all herpesviruses are involved in these membrane fusion phenomena. Herein, we show that the absence of either gM or UL11 expression abrogated virus-induced cell fusion caused by strong syncytial mutations in either gB or gK, and that gM interacted with the gK/UL20 protein complex. Moreover, virions produced in the absence of gM or UL11 entered slower into cells than their parental wild type virus. These results suggest that

both gM and UL11 directly or indirectly interact and regulate the HSV-1 membrane fusion machinery during virus-induced cell fusion and virus entry.

Glycoprotein gM is a highly hydrophobic glycoprotein predicted to span membranes eight times. It is highly conserved in all alphaherpesviruses suggesting that it plays essential role in herpes infections. Previous work indicated that insertional inactivation of the gM gene inhibited cell fusion caused by a syncytial mutation in gB (gBsyn; ala-to-val at gB amino acid 855) (Davis-Poynter et al., 1994, Balan et al., 1994). This particular syncytial mutation causes significantly lower amounts of virus-induced cell fusion than the gB Δ 28 mutation, the latter being the strongest gB syncytial mutation causing extensive cell fusion of most cell types in tissue culture (Silverman et al., 2012, Foster et al., 2001a). Similarly, the gKsyn20 mutation (ala-to-val at gK amino acid 40) causes extensive virus-induced cell fusion in most cell types (Chouljenko et al., 2009). Our results show that lack of gM abrogated virus-induced cell fusion caused by either gKsyn20 or gB Δ 28 mutations in agreement with previous findings (Davis-Poynter et al., 1994, Balan et al., 1994). Glycoprotein gM has been suggested to function in either retaining viral glycoproteins to TGN membranes, or retrieving them from plasma membranes to TGN (Leege et al., 2009). In contrast to these published findings, deletion of gM did not appear to appreciably impact the overall level of other viral glycoproteins, nor their relative levels expressed on cell surfaces. Transient expression of HSV-1, PRV and KSHV gM appeared to inhibit cell fusion caused by simultaneous expression of gB, gD, gH and gL suggesting potential interactions between gM and one or more of these viral glycoproteins (Koyano et al., 2003, Klupp et al., 2000). However, extensive two-way co-immunoprecipitation experiments failed to indicate interactions between gM and either gB or gD suggesting that gM may indirectly interact with these proteins to regulate cell fusion. Additional protein-protein

interaction experiments revealed that gM interacts with the UL20 protein, the interacting partner of gK. We have shown that the gK/UL20 protein complex interacts with gB (Chouljenko et al., 2010), therefore, it is likely that lack of gM affects the ability of gK/UL20 complex to bind and regulate the fusogenic properties of gB. In this regard, gM is a new member of the HSV-1 fusion machinery composed of gB, gH, gD, gL, gK, UL20 and now gM. Recently, it was shown that VP22 forms a protein complex with gM and gE that may affect the functions of these proteins (Maringer et al., 2012), thus gE may also participate in this glycoprotein network on infected cell surfaces and virion envelopes.

The UL11 protein was of particular interest to these investigations due to its high conservation among all herpesviruses and its ability to interact with the carboxyl terminus of glycoprotein gE and multiple tegument proteins (Yeh et al., 2011). We show that UL11 is expressed on infected cell surfaces at very low levels, if at all, in agreement with the prediction that UL11 is attached to cytoplasmic sides of plasma membranes via its myristoylated and palmitoylated residues and its interaction with the cytoplasmic tail of gE (Leege et al., 2009, Yeh et al., 2008, Baird et al., 2008, Loomis et al., 2003, Loomis et al., 2001, Yeh et al., 2011). Surprisingly, lack of UL11 severely inhibited both gB Δ 28 and gKsyn20-caused virus-induced cell fusion. The gB Δ 28 results confirm recent findings that UL11 is required for gBsyn (A855V), but significantly extend these findings, since deletion of the gB carboxyl terminus causes extensive cell fusion in all cell types, unlike the gBsyn (A855V) mutation (Silverman et al., 2012). Moreover, lack of UL11 inhibited fusion caused by the gKsyn20 mutation, which is a dominant syncytial mutation causing fusion of all cell types tested.

Lack of gE (Balan et al., 1994, Davis-Poynter et al., 1994) and UL11 (Han et al., 2012) were reported to prevent gBsyn-mediated virus-induced cell fusion. Immunofluorescence and flow

cytometry experiments failed to detect substantially lower amounts of gE in cells infected with either gM-null or UL11-null viruses (not shown). This suggests that the observed inhibition of virus-induced cell fusion in the absence of UL11 is not due to lack of gE cell-surface expression. In contrast to our findings, it was recently shown that deletion of UL11 appeared to reduce gE cell-surface expression in Vero but not HaCaT cells by fluorescence microscopy (Han et al., 2012). It is possible that different results are obtained due to the use of HSV-1 (KOS) (Han et al., 2012) versus HSV-1(F) (this study). Additional studies will be needed to resolve whether lack of UL11 substantially affects gE expression and function on infected cell surfaces.

Virus-induced cell fusion and fusion of the viral envelope with cellular membranes during virus entry are thought to occur via similar mechanisms involving viral glycoprotein gB and cellular receptors (Connolly et al., 2011). We have shown that gK and UL20 are expressed in virion envelopes and are involved in virus entry, since deletion of gK or a small segment of gK that interacts with gB cause virions to enter slower than their parental wild type virus into cells and alter their ability to utilize HSV-1 specific receptors (Foster et al., 2001b, Jambunathan et al., 2011, Chowdhury et al., 2013). Similarly, virions produced in the absence of either gM or UL11 exhibited slower kinetics of entry into Vero cells than their parental virus. gM is known to be expressed in virion envelopes (Baines and Roizman, 1993). Also, UL11 is a structural component of virions (Loret et al., 2008) and interacts with gE and other tegument proteins in infected cells (Han et al., 2012). Recently, it was shown that VP22 bridges a complex between gE and gM (Maringer et al., 2012). Therefore, it is conceivable that UL11 binding to gE can indirectly affect both gE and gM functions causing the observed inhibition of both virus-induced cell fusion and the slower kinetics of the UL11-null virus in comparison to its parental wild-type virus. Alternatively, it is possible that absence of gM or UL11 affect the incorporation of other

viral glycoproteins in virion envelopes causing the observed inhibition in virus entry. Additional investigations beyond the scope of this work are needed to address this issue in the future.

Collectively, these results suggest that both gM and UL11 are required for virus-induced cell fusion and efficient virus entry, most likely, because they directly or indirectly interact with the viral fusion machinery. Glycoprotein gM directly interacts with the gK/UL20 protein complex. We did not detect any UL11 interactions with either gB, gC, gD, gH, gM, or gK/UL20. It is possible that such interactions are weak or of transient nature that cannot be detected via co-immunoprecipitation experiments. UL11 interacts with gE, and VP22 interacts with both gE and gM (Maringer et al., 2012) suggesting that gE also participates in indirect interactions with the fusion machinery. Additional experiments are needed to ascertain the intracellular localization of UL11 and its potential functions in virus-induced cell fusion. Overall, it is apparent that multiple protein-protein interactions occur among viral glycoproteins and tegument proteins that regulate membrane fusion phenomena in HSV-1 infections.

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CHAPTER IV: FUNCTIONAL HIERARCHY OF HERPES SIMPLEX VIRUS TYPE-1 MEMBRANE PROTEINS IN CORNEAL INFECTION AND VIRUS TRANSMISSION TO GANGLIONIC NEURONS

Introduction

Ocular herpes simplex virus (HSV) infection is one of the most common infectious causes of blindness in developed countries. Moreover, frequent reactivation of latent virus can cause recurrent herpes keratitis and corneal graft failure (Liesegang et al., 1989, Liesegang, 2001, Cockerham, 2001, Cockerham et al., 1997, De Kesel et al., 2001, Tullo, 2003). Typically, primary infection of epithelial cells exposed on orofacial mucocutaneous tissues leads to invasion of axonal termini embedded with the cornea via fusion of the viral envelope with neuronal synaptic membranes. Subsequently, capsids are transported in a retrograde manner to neuronal cell somata where viral genomes can establish life-long latency (Antinone and Smith, 2010). Infrequently and particularly in newborn infections, neuronal infection can cause acute encephalitis and significant morbidity and mortality (Fatahzadeh and Schwartz, 2007). Immunosuppression as well as other external stimuli can cause reactivation of the virus from the latent state and re-infection of peripheral tissues including corneas and orofacial surfaces (Smith, 2012).

Virion entry into all cells including neurons involves viral glycoproteins gD, gB, gH, gL, and gC (Connolly et al., 2011). HSV-1 enters neuronal cells via a pH-independent fusion of the viral envelope with neuronal axonal membranes (Nicola et al., 2005, Qie et al., 1999), while it can enter a wide range of non-neuronal cells via either pH-independent or pH-dependent endocytosis (Milne et al., 2005). Retrograde transport of capsids to neuronal somata after infection of axonal termini is mediated by the dynein motor in conjunction with the neuronal microtubular network (Mettenleiter et al., 2009, Johnson and Baines, 2011, Diefenbach et al., 2008). Glycoprotein gB is thought to be the sole fusogenic glycoprotein, while glycoproteins gD

and gH/gL are required to activate gB's fusogenicity in conjunction with specific cellular receptors (Connolly et al., 2011). Binding of gD to its cognate receptors including nectin-1, HVEM, and other receptors (Campadelli-Fiume et al., 2000, Geraghty et al., 1998, Montgomery et al., 1996, Shukla et al., 1999, Spear et al., 2000, Spear and Longnecker, 2003, Satoh et al., 2008) triggers sequential conformational changes in gH/gL and gB causing fusion of the viral envelope with cellular membranes during virus entry, as well as during virus-induced cell-to-cell fusion (Hannah et al., 2007, Heldwein et al., 2006).

Wild-type virus strains typically cause limited but measurable amount of cell-to-cell fusion in cell culture (Kousoulas et al., 1978). However, most viral lesions in human and animal tissues are characterized by the presence of multinucleated cells (syncytia). Virus-induced cell-fusion is an important mechanism for virion transmission to adjacent cells without exposure to the host humoral immune system, particularly neutralizing antibody [reviewed in reference (Roizman et al., 2007)]. Extensive membrane fusion can be induced by co-expressing glycoproteins gB, gD and gH/gL in cell lines indicating that gB, gD, and gH/gL are essential components for both virus entry and virus-induced cell fusion (Turner et al., 1998, Muggeridge, 2000). Additional viral genes encode proteins that have been shown to be involved in virus-induced cell fusion without necessarily drastically affecting virus entry kinetics. These genes include: the UL20 gene (Baines et al., 1991, McLean et al., 1990, Melancon et al., 2004), the UL24 gene (Jacobson et al., 1998, Sanders et al., 1982), and the UL53 gene coding for gK (Bond and Person, 1984, Debroy et al., 1985, Hutchinson et al., 1992, Pogue-Geile et al., 1984, Pogue-Geile and Spear, 1987, Ruyechan et al., 1979). Also, certain mutations within the UL27 gene encoding glycoprotein gB drastically enhance virus-induced cell-fusion presumably because they destabilize the virus-encoded fusion machinery (Bzik et al., 1984, Pellett et al., 1985). We have shown that lack of

either glycoprotein gK or the membrane protein UL20 severely inhibits membrane fusion (Melancon et al., 2004, Melancon et al., 2005). Moreover, we have shown that HSV-1 gK and UL20 functionally and physically interact and that these interactions are absolutely necessary for their coordinate intracellular transport, cell-surface expression and membrane fusion functions in the HSV-1 life cycle (Foster et al., 2001, Chouljenko et al., 2009, Chouljenko et al., 2010).

Glycoproteins gM and gE and the membrane-associated UL11 protein have been also implicated in virus-induced cell fusion and virion egress (Kim et al., 2013, Chouljenko et al., 2012). Glycoprotein gM is a conserved type III integral membrane protein with multiple transmembrane domains, that forms a complex with pUL49.5 (gN) [reviewed in Roizman et al. (2007)]. Deletion of the gM gene does not abrogate HSV-1 replication, but does affect the ability of the virus to spread (Leege et al., 2009). Expression of HSV-1, pseudorabies (PRV) and Kaposi sarcoma associated herpesvirus (KSHV or HHV-8) gM and gN in transfected cells inhibited cell fusion caused by simultaneous expression of gB, gD, gH and gL glycoproteins (Koyano et al., 2003, Klupp et al., 2000). Also, lack of gM was reported to inhibit virus-induced cell fusion caused by a single amino acid substitution in the carboxyl terminus of gB (A855V; gBsyn) (Balan et al., 1994). HSV-1 glycoprotein gE is expressed as a heterodimer with gI and functions to facilitate cell-to-cell spread by sorting newly formed virion particles to cell junctions and lateral cell surfaces (Collins and Johnson, 2003). Unlike PRV, HSV-1 gE-null viruses exhibited a defect in retrograde transport *in vivo* but not *in vitro* (McGraw et al., 2009). UL11 is a 96-amino-acid myristoylated and palmitoylated tegument protein anchored into the cytoplasmic side of cell membranes (Baines et al., 1995, Loomis et al., 2001, MacLean et al., 1989, Baird et al., 2008). UL11 interacts with viral glycoprotein gE and tegument proteins UL16 and UL21 and has been suggested to play a role in recruiting viral proteins to the virion assembly

site at the trans-Golgi network (TGN) (Baird et al., 2008, Farnsworth et al., 2007, Loomis et al., 2001, Loomis et al., 2003, Leege et al., 2009, Han et al., 2012). Although absence of UL11 in HSV-1 and PRV revealed only moderate defects in viral replication (Fulmer et al., 2007, Leege et al., 2009, Chouljenko et al., 2012, Kopp et al., 2003), the HCMV UL11 homologue is essential for virus replication (Seo and Britt, 2007, Silva et al., 2003).

Recently, we showed that the lack of either the gK or the UL20 gene expression produced significantly greater defects in virion envelopment and overall virus replication than deletion of either the carboxyl terminus of gD, or lack of either the UL11, gM or gE gene expression alone or in various combinations (Chouljenko et al., 2012). Herein, we investigated the *in vivo* effects of lack of either gE, gM, gK or UL11 using the scarified mouse eye HSV-1 infection model. The gK-null virus was unable to efficiently infect neurons and establish latency, in contrast to all other mutant viruses. Surprisingly, although the UL11-null virus replicated less efficiently in mouse corneas, it infected ganglionic neurons with greater efficiency than either the gM or gE-null viruses. These results suggest that in the scarified mouse eye model system, replication in the eye is not directly associated with ability to infect neuronal endings and establish latency in ganglionic neurons. Moreover, gK serves more important functions in neuronal infection than either gM, gE or the UL11 protein.

Materials and Methods

Cells and viruses

African green monkey kidney (Vero) cells were obtained from the American Type Culture Collection (Rockville, MD) and grown and propagated in Dulbecco's modified Eagle medium (DMEM) supplemented with 5-10% fetal bovine serum (FBS) and antibiotics. The gK-complementing VK302 cell line (generously provided by David Johnson, Oregon Health

Sciences University, Portland, OR) was maintained in DMEM supplemented with 10% FBS and antibiotics. The HSV-1(F) cloned as a bacterial artificial chromosome was a gift from Dr.

Kawaguchi (Chouljenko et al., 2012, Tanaka et al., 2003, Lee et al., 2009, Tischer et al., 2006).

Construction and characterization of recombinant viruses

The recombinant viruses were constructed previously (Chouljenko et al., 2012, Melancon et al., 2005, Kim et al., 2013). Briefly, mutagenesis was performed in *E. coli* using the two-step red mutagenesis implemented on the HSV-1(F) genome cloned into the bacterial artificial chromosome plasmid vector pYEBac102, as described previously (Kim et al., 2013). The gM-null virus was constructed by altering two potential initiation codon sites (from ATG to CTG and from ATG to ATT, respectively). The UL11-null and gE-null viruses were constructed by changing their respective initiation codons from ATG to CTG, as described in detail previously (Kim et al., 2013). The gK-null virus was constructed by replacing gK with a GFP-zeo gene cassette (Melancon et al., 2005). All recombinant viral genomes were sequenced using next generation sequencing (NGS) to confirm the introduction of the intended mutations and the absence of collateral unwanted nucleotide changes, as described earlier (Kim et al., 2013).

Plaque morphology and replication kinetics of wild-type and mutant viruses

Confluent monolayers of Vero and VK302 cells were infected with WT BAC, Δ gM, Δ gE, Δ gK, Δ UL11 viruses at MOI of 0.001. Cells were fixed by methanol at 48 hpi. After fixation, immunohistochemistry was performed using horseradish peroxidase conjugated goat-anti-rabbit antibody (Dako Carpinteria, CA). The reactions were developed using NovaRed substrate (VectorLabs, Burlingame, CA, USA). Replication kinetics were obtained essentially as described previously (Saied et al., 2013). Briefly, confluent monolayers of Vero cells were infected with WT BAC, Δ gM, Δ gE, Δ gK, Δ UL11 viruses at MOI of 0.1. Infections were halted

by freezing at 0, 24, and 48 hpi. Plates were prepared in triplicates and viral titers were determined for each time point. Plaques were stained by immunohistochemistry and visualized using a dissecting microscope.

Ocular infections

Corneal scarification and ocular inoculation of mice were performed as described previously (Subramanian et al., 2008, Subramanian et al., 2010). Briefly; female Balb/c mice were divided into 6 groups; each group was inoculated with either WT BAC, Δ gM, Δ gE, Δ gK, Δ UL11 viruses, or mock-infected. After anesthesia, a 25-26 G needle was used to scarify mice corneas in a grid-like pattern. Infections were performed using 10^5 PFU for each virus applied to each eye by a micropipette. After inoculation, the eyelids were gently held shut for a few seconds. The clinical ocular changes were monitored daily for keratitis, blepharoconjunctivitis and ocular discharge. Mice eyes were rinsed at 24, 48 and 72 hpi and the number of infectious virions within the rinsing solution was determined by plaque assay.

Quantitative PCR (qPCR) analysis

Trigeminal ganglia were collected 30 days post infection (dpi) for qPCR analysis and stored at -80°C. After thawing, the Qiagen DNeasy blood and tissue kit (Qiagen, Valencia, CA) was used to extract total DNA from the trigeminal ganglia per manufactures instructions. QPCR was performed as initially described previously for Kaposi's sarcoma herpesvirus (KSHV) (David et al., 2012) and utilized later for HSV-1 DNA detection in neuronal cell cultures (Kim et al., 2013). Briefly, the primers and probe (6-carboxytetramethyl-rhodamine (TAMRA) for the real-time PCR were designed to detect HSV-1 US6 (gD). Equal volumes of viral DNA were used for TaqMan PCR 196 analysis. Purified plasmid containing the gD gene was initially used to generate the standard curve.

Results

Comparison of the replication characteristics of recombinant viruses lacking gM, gE, UL11, or gK

The gM-null, UL11-null and gE-null viruses were constructed previously via mutagenesis of their respective initiation codons utilizing the cloned HSV-1(F) genome into a bacterial artificial chromosome (WT BAC), while the gK-null was constructed via gene replacement mutagenesis (Figure 4-1) (see Materials and Methods). The gK-null virus formed very small plaques on Vero cells, while it was efficiently complemented and formed much larger plaques on the VK302 cell lines expressing gK (Figure 4-2: A). The gE and gM-null viruses produced viral plaques that on average were approximately 50% smaller, while the UL11-null virus produced viral plaques that were approximately 75% smaller than the wild-type virus (Figure 4-2: A). The replication kinetics of the gM and UL11-null viruses were similar yielding final titers that were approximately one log less than the wild-type virus at 48 hpi. The gE-null virus replicated with similar kinetics to the wild-type virus, while the gK-null virus replicated very inefficiently yielding final titers that were at least three logs less than the wild-type virus at 48 hpi (Figure 4-2: B).

Mouse eye infections

Mice corneas were scarified and infected with 10^5 PFU in each eye. Clinical ocular changes were monitored daily for the development of blepharoconjunctivitis, keratitis and ocular discharge. Mice infected with the wild-type virus exhibited mild-clinical symptoms at 7 dpi, while none of the mice infected with the mutant viruses exhibited any significant ocular changes (Figure 4-3). Viral shedding was determined up to 72 hpi daily. The wild-type virus produced the highest viral titers in eyewashes, followed by the gM-null, gE-null, UL11-null and gK-null (Figure 4-4).

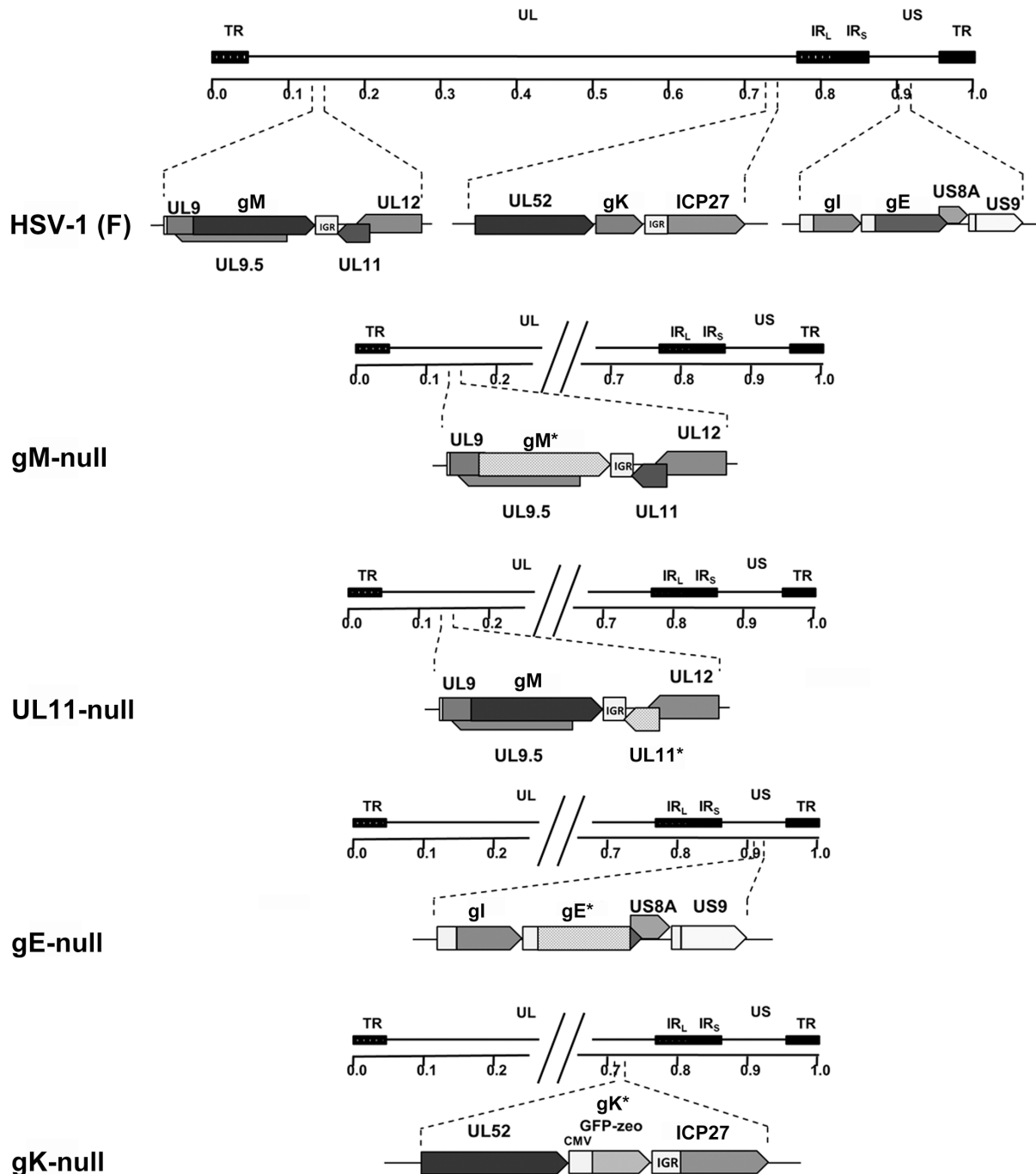


Figure 4-1. Schematic representation of mutant viruses. Schematic representation of mutant viruses. The top line represents the prototypic arrangement of the HSV-1 genome with the unique long (UL) and unique short (US) regions flanked by the terminal repeat (TR) and internal repeat (IR) regions. The relevant genome regions of the parental wild-type HSV-1(F) genome cloned as a bac (WT BAC) and the gM, gE, and UL11-null viral genomes are shown. * Denotes mutagenesis of initiation codons that prevent gene expression. The gK-null virus was constructed by replacing the UL53 gene that encodes gK with a GFP-Zeo gene cassette under the control of the cytomegalovirus IE promoter (CMV) (Melancon et al., 2005).

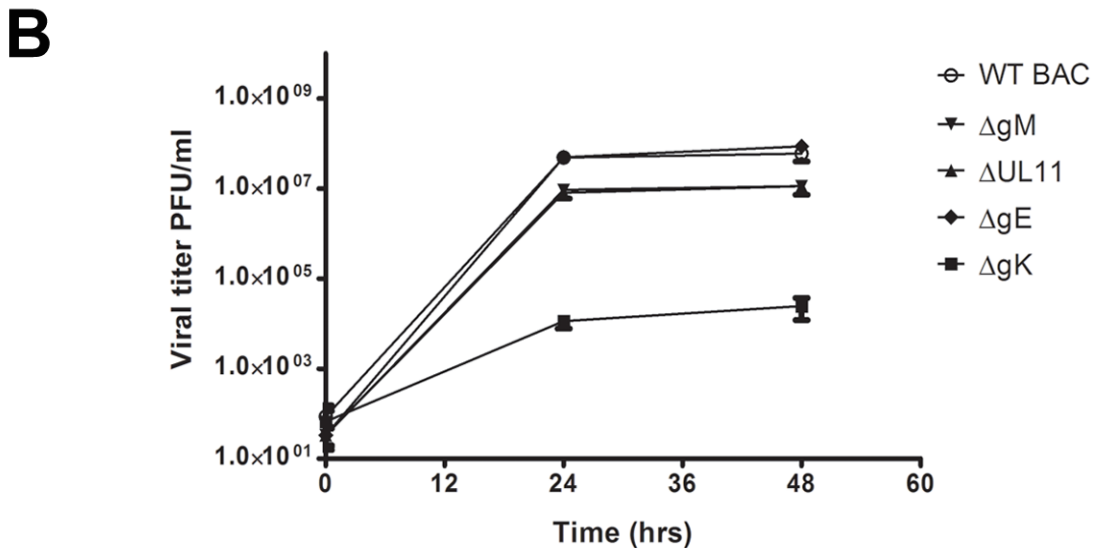
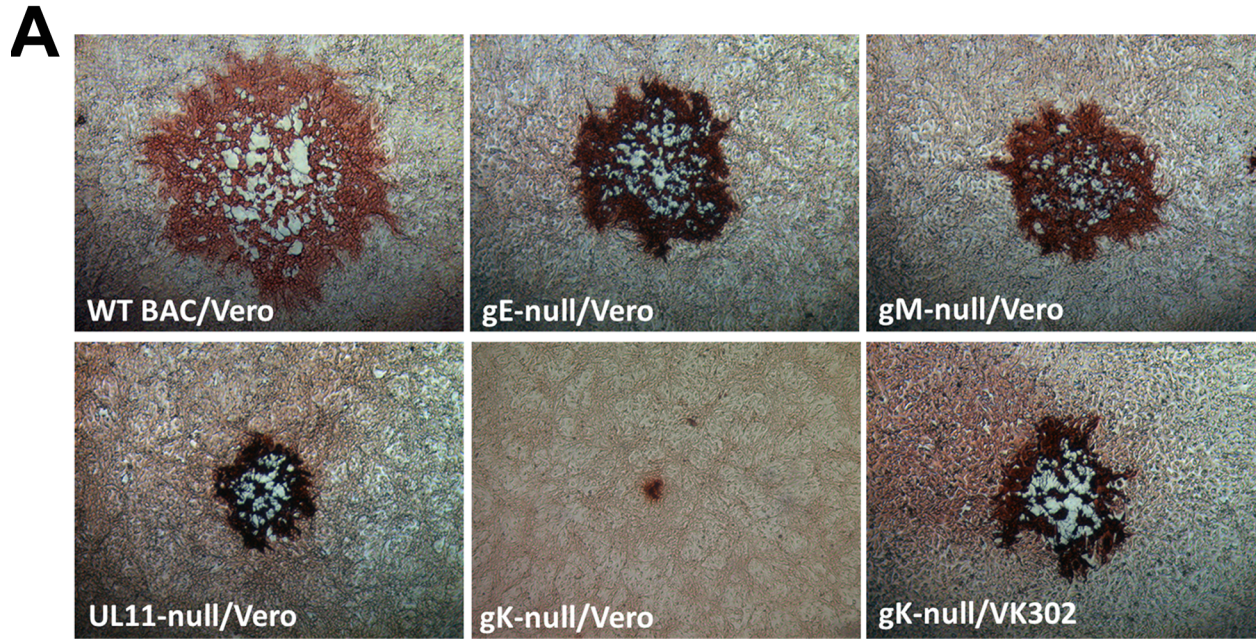


Figure 4-2. Plaque morphology and replication kinetics of parental and recombinant viruses. Plaque morphology and replication kinetics of parental and constructed recombinant viruses. (A) Confluent monolayers of Vero or VK302 cells were infected at an approximate MOI of 0.001 and representative plaques were identified as indicated by immunohistochemistry staining. VK302 is a cell line expressing gK upon infection. (B) Confluent monolayers of Vero cells were infected at an approximate MOI of 0.1 with WT BAC, gM-null, UL11-null, gE-null, and gK-null viruses. Viral titers were obtained by the standard titration plaque assay on Vero at the indicated times. PFU; Plaque Forming Unit.

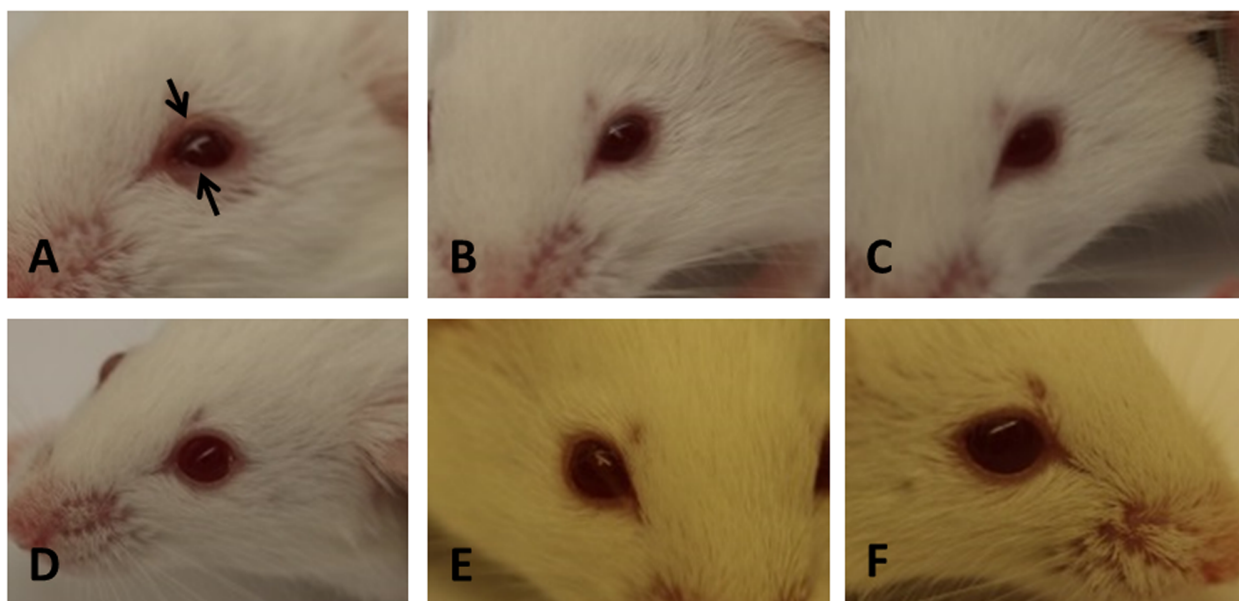


Figure 4-3. Clinical ocular disease development. Clinical ocular disease development: Representative pictures of infected eyes are shown. Mice eyes were monitored for the development of blepharoconjunctivitis, hyperemia, keratitis and ocular discharge (A) WT BAC group, the observed mild blepharitis characterized by thickening of the upper and lower eyelids is indicated by arrows. (B) gM-null-infected mice. (C) gE-null-infected mice. (D) UL11-null-infected. (E) gK-null-infected mice. (F) Mock-infected mice.

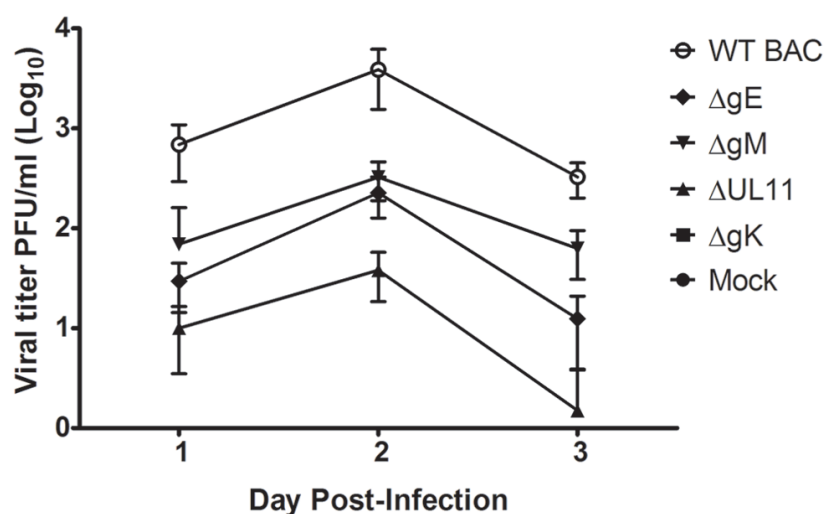


Figure 4-4. Viral shedding. Viral titers from eye rinses obtained at 24, 48 and 72 hpi. Viral titers were obtained by the standard plaque assay.

Determination of viral DNA in trigeminal ganglia of infected mice

The trigeminal ganglia (TG) were collected from mice at 30 dpi and were tested for the presence of viral DNA genomes by qPCR, as we have described previously (David et al., 2012, Saied et al., 2013). On average, approximately 112 viral DNA genomes were detected in TG of mice infected with the wild-type virus. Viral genomes were detected in the TGs of mouse infected with all mutant viruses except the gK-null-infected mice. Specifically, 2-of-10 mice infected with the gM-null virus contained on average 6 viral genomes, 4-of-10 mice infected with the gE-null virus contained on average 6 viral genomes, and 6-of-10 UL11-null-infected mice contained on average 24 viral genomes (Figure 4-5).

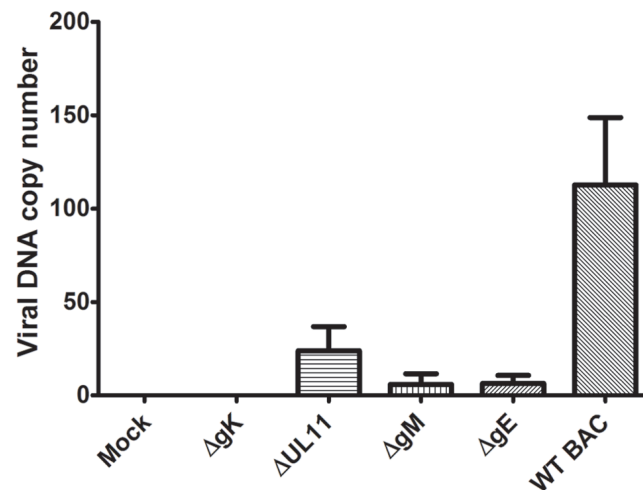


Figure 4-5. Viral DNA genome detection by qPCR. Equal volumes of viral DNA were used for TaqMan PCR. Purified plasmid containing the gD gene was initially used to generate the standard curve.

Discussion

We have demonstrated previously that HSV-1 gK plays a pivotal role in neuronal infection. Specifically, lack of gK prevents infection of trigeminal ganglia after infection of scarified mouse eye corneas, and inhibits retrograde and anterograde translocation of virions via neuronal

axons (David et al., 2008, David et al., 2012). The purpose of the present investigations was to compare and contrast the ability of mutant viruses lacking gK, gM, gE, or UL11 to productively infect mouse corneas and ganglionic neurons *in vivo* and establish latency in the trigeminal ganglia. Our results confirm previous findings that lack of gK causes severe reduction in the ability of the virus to infect corneal cells and neurons despite the direct exposure of neuronal endings to virus after extensive cornea scarification. In contrast, viruses lacking either gM, gE or UL11 infected mouse corneas and ganglionic neurons and established latent infections. Importantly, although the UL11-null virus replicated inefficiently in mouse eyes, it infected ganglionic neurons and established latency more efficiently than either the gM or gE-null viruses.

The wild-type HSV-1(F) virus produced only mild ocular changes after infection of mouse eyes characterized by mild blepharoconjunctivitis only at late times post infection (7 days). These results are significantly different to our previous findings that the HSV-1(McKrae) strain produce drastic ocular changes marked by severe blepharitis, hyperemia, keratitis and high ocular discharge 7-8 days post infection (Saied et al., 2013). The HSV-1(F) infection results are consistent with previous findings that this viral strain was relatively apathogenic after intracranial infection of mouse brains (Sedarati and Stevens, 1987). Moreover, the HSV-1(F) strain utilized in the present investigations was recovered from the bac-cloned HSV-1(F) genome (YEbac102) (Tanaka et al., 2003). Thus, it is possible that incorporation of the bac plasmid within the viral genome contributed to the observed lack of ocular immunopathogenicity. The genomic sequences of the HSV-1(F), KOS and McKrae strains have been obtained (Macdonald et al., 2012b, Macdonald et al., 2012a, Szpara et al., 2010). Alignment of the HSV-1(F) and McKrae genes encoding viral glycoproteins reveals multiple single amino acid changes (Chowdhury et al., 2012). Additional changes are predicted in many other genes between

McKrae and F strains (not shown). One or more of these predicted amino acid changes may be responsible for the observed ocular pathogenic differences between these two viral strains.

Mouse corneas contain over 50,000 neuronal axonal termini per mm^2 . Therefore, cross-hatching scarification exposes neuronal endings to direct viral infection. In this regard, cornea scarification provides a model for assessing the ability of viruses to directly infect ganglionic neurons through their axonal termini. Using this model, we showed previously that lack of gK drastically impaired the ability of the virus to infect ganglionic neurons and establish latency. In this paper, we confirm these findings and compare them to the ability of gM-, gE-, and UL11-null viruses to infect ganglionic neurons. All three mutant viruses were able to infect ganglionic neurons, albeit at reduced frequencies in comparison to the parental HSV-1(F) strain. These results suggest that gM, gE and UL11 serve potentially secondary roles in corneal ganglionic neuronal infections in comparison to gK. Surprisingly, although the UL11-null virus replicated less efficiently in Vero cell monolayers and mouse eyes, it infected ganglionic neurons more efficiently than the gM-null or gE-null viruses. This result supports our working hypothesis that ocular viral replication may contribute, but not serve the most important role in infection of ganglionic neurons in the scarified mouse eye infection model system.

Recently, we showed that gM interacts with the gK/UL20 protein complex and that UL11 is required for virus-induced cell fusion caused by syncytial mutations in gB or gK, respectively (Kim et al., 2013). Thus, the absence of either gM or UL11 from the virion particle may perturb gB and gK/UL20 fusogenic functions preventing efficient entry into neuronal axons embedded within scarified mouse corneas. Additional work is needed to directly demonstrate potential defects in axonal cytoplasmic entry for these viruses. These experiments are currently in progress.

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CHAPTER V: CONCLUDING REMARKS

Summary

Herpes simplex virus type 1 (HSV-1) encodes at least 11 glycoproteins as well as several membrane-associated proteins. Earlier works have shown that those proteins play important roles in viral entry and virus-induced cell fusion. Specifically, certain mutations in the UL20 gene, UL27 gene encoding glycoprotein gB, the UL53 gene coding for gK and other viral genes drastically enhance virus-induced cell-fusion. Later, it has been suggested that gB is the sole fusogenic glycoprotein, while glycoproteins gD and gH/gL are required to activate gB's fusogenicity in conjunction with specific cellular receptors (Connolly et al., 2011). Although extensive membrane fusion can be induced by co-expressing those glycoproteins gB, gD and gH/gL in cell lines (Turner et al., 1998, Muggeridge, 2000), virus-induced cell fusion is regulated by a number of other viral proteins. Our lab has shown that wild type viruses cause limited amount of fusion (Kousoulas et al., 1978), and lack of either glycoprotein gK or the membrane protein UL20 severely inhibits virus-induced membrane fusion (Melancon et al., 2004, Melancon et al., 2005). Also, recently we have shown that the deletion of either the gK or UL20 gene produced significantly greater defects in virion envelopment and overall virus replication than deletion of either the carboxyl terminus of gD (gDct), UL11, gM or gE alone or in various combinations (Chouljenko et al., 2012). Moreover, lacking either gB, gH/gL, gD, gE, gI, gM or UL11 was reported to inhibit virus-induced cell fusion caused by a single amino acid substitution in the carboxyl terminus of gB (A855V; gBsyn) (Davis-Poynter et al., 1994, Balan et al., 1994, Han et al., 2012). These earlier findings suggest that the virus-induced membrane fusion is coordinately regulated by multiple membrane associated proteins.

Upon this main hypothesis, in chapter 2, we investigated whether the lack of either gM or UL11 affected the ability of dominant syncytial mutations in either gB or gK to cause extensive virus-induced cell fusion. We found that both gM and UL11 are required for virus-induced cell fusion. Moreover, mutant viruses lacking either gM or UL11 exhibited slower kinetics of entry into Vero cells than the parental wild type virus suggesting that gM and UL11 are involved in membrane fusion phenomena during both virus-induced cell fusion and virus entry. These results suggest that both gM and UL11 directly or indirectly interact and regulate the HSV-1 membrane fusion machinery during the virus-induced cell fusion and virus entry.

Our initial hypothesis was that gM and UL11 may affect the synthesis or membrane expression of viral proteins, since gM and UL11 were previously suggested to play a role in directing or recruiting proteins to TGN (Leege et al., 2009, Farnsworth et al., 2007, Han et al., 2012). Herein, we investigated the effect of lacking either gM or UL11 expression on the synthesis of viral glycoproteins, gB, gC, gD, gM and UL11 using western immunoblots of whole cell lysates. Neither lack of gM or UL11 expression drastically affected the synthesis of gB, gC and gD. Furthermore, lack of gM did not affect the synthesis of UL11, and lack of UL11 did not affect gM levels. Subsequently, we furthered our investigation to the surface-glycoprotein expression profiles of Vero cells infected with the set of viruses by using the cell-surface biotinylation experiments. Briefly, viral glycoproteins expressed on infected cell surfaces were biotinylated under live conditions. Biotinylated proteins were then isolated by streptavidin immunoprecipitation and analyzed by western immunoblots. Lack of either gM or UL11 did not affect overall expression of viral glycoproteins gB, gC, gD and gH on HSV-1(F)-infected cell surfaces. Similar results were obtained for the gB Δ 28 mutant and gKsyn20 mutant viruses infected cells. These finding suggest that gM and UL11 may interact directly with gB or the

gK/UL20 complex to cause the observed phenomena. We performed extensive two-way immunoprecipitation experiments to determine the direct interaction between gM or UL11, and other viral proteins. The results revealed that gM physically interacted with UL20 in the virus-infected cells. Immunoprecipitations with gB, gD, or gH failed to reveal any interactions with gM. Similar immunoprecipitations with UL11 failed to reveal any interactions with gB, gC, gD, gH, gM, gK or UL20.

As mentioned earlier, lack of gE (Balan et al., 1994, Davis-Poynter et al., 1994) and UL11 (Han et al., 2012) were reported to prevent gB-mediated virus-induced cell fusion. Also, it was recently shown that deletion of UL11 appeared to reduce gE cell-surface expression in Vero but not HaCaT cells by fluorescence microscopy (Han et al., 2012). However, we failed to detect substantially lower amounts of gE on the membrane of Vero cells infected with either gM-null or UL11-null virus, using immunofluorescence and flow cytometry experiments. This suggests that the observed inhibition of virus-induced cell fusion in the absence of UL11 is not due to lack of gE cell-surface expression. Additional studies will be needed to resolve the role of UL11.

Subsequently, we expanded our investigations to *in vivo* study, using a scarified mouse eye model. HSV-1 infection in central nervous system and eyes have clinical importance, since it can occasionally lead to fatal encephalitis and corneal blindness (Whitley and Roizman, 2001). Although it occurs less frequently than mucocutaneous infections, ocular infection is one of the prominent causes of blindness in developed countries (Liesegang, 2001, Liesegang et al., 1989). After initial infection to the mouse eye, HSV-1 replicates in corneal epithelium, and transmits to neuronal cell bodies via epithelial-neuronal interfaces by retrograde manner, and then establishes latency (Cunningham et al., 2006, Diefenbach et al., 2008).

Herein, we investigated the *in vivo* effects of lacking either gE, gM, gK or UL11 in viral pathogenesis as described in the chapter 3. Briefly, after infecting the set of viruses including gE-null, gM-null, gK-null, and UL11-null viruses with their parental wild type virus, clinical ocular symptom and viral shedding were assessed. The mice infected with wild type virus exhibited mild-clinical symptoms at 7 dpi, while no mice infected with the mutant viruses exhibited any remarkable ocular changes. Also, the wild type virus shed the highest viral titer in eyewashes, followed by the gM-null gE-null, UL11-null, and gK-null. Interestingly, retrieved wild type virus from infected eyes caused extensive cell-to-cell fusion on Vero cell culture, but gM-, gE-, UL11-, and gK-null viruses did not.

To investigate the neuroinvasiveness of each virus, trigeminal ganglia from the virus-infected mice were harvested at 30 dpi and tested for the presence of viral DNA genomes by qPCR. The average viral genome copy numbers from trigeminal ganglia was approximated 112 copies from wild type virus infected group, while there were approximately 6 copies from either gM-null or gE-null virus infected group, and 24 copies from UL11-null virus infected group. The viral genome was detected from all wild type infected mice (10/10), albeit the mutant virus genomes were detectable in significantly less heads of mice: specifically, 0/10 in gK-null, 2/10 in gM-null, 4/10 in gE-null, and 6/10 in UL11-null groups. Overall, All three mutant viruses were able to infect ganglionic neurons, albeit at reduced frequencies in comparison to the parental HSV-1(F) strain. These results suggest that gM, gE and UL11 serve potentially secondary roles in corneal ganglionic neuronal infections in comparison to gK. Surprisingly, although the UL11-null virus replicated less efficiently in Vero cell monolayers and mouse eyes, it infected ganglionic neurons more efficiently than the gM-null or gE-null viruses. This result supports our

working hypothesis that ocular viral replication may contribute, but not serve the most important role in infection of ganglionic neurons in the scarified mouse eye infection model system.

Collectively, these results suggest that the both gM and UL11 are required for virus-induced cell fusion and efficient virus entry, most likely, because they directly or indirectly interact with the viral fusion machinery. Specifically, we found that gM directly interact with the gK/UL20 protein complex, although we did not detect any UL11 interactions with either gB, gC, gD, gH, gM or gK/UL20, possibly due to weak interactions or transient nature. Overall, it is apparent that multiple protein-protein interactions occur among the viral glycoproteins and tegument proteins that regulate membrane fusion phenomena in HSV-1 infections *in vivo* as well as *in vitro*. Also, the level of ocular viral replication is not closely correlated with infecting ganglionic neurons.

Current and Future Work

UL11 has been shown to interact with gE, UL16, and UL21 and lacking either of those proteins inhibited gBsyn-mediated cell fusion (Han et al., 2012). Recently, it was shown that VP22 bridges a complex between gE and gM (Maringer et al., 2012). Therefore, it is conceivable that UL11 binding to gE can indirectly affect both gE and gM functions causing the observed inhibition of both virus-induced cell fusion and the slower kinetics of the UL11-null virus in comparison to its parental wild-type virus. Alternatively, it is possible that absence of gM or UL11 affect the incorporation of other viral glycoproteins in virion envelopes causing the observed inhibition in virus entry. Another interesting potential pathway is interaction among UL11, gE and gH. Although it is not demonstrated in HSV-1, VZV gE has been shown to interact with gH and temper the cell-to-cell fusion by regulating cell surface expression of gH via co-endocytosis of gH-gE complex (Pasieka et al., 2004). Because those two proteins are highly conserved and share similar properties in all herpesviruses, it is plausible that HSV-1 could share

the same properties. This thesis did not conclude the exact properties of UL11 in the virus-induced fusion event. Additional investigations beyond the scope of this work are needed to address this issue in the future.

We showed in chapter 3 that gE, gM and UL11 as well as most importantly gK/UL20, are required for efficient corneal ganglionic neuronal infection. Surprisingly, although the UL11-null virus replicated less efficiently in Vero cells and mouse corneal surface, it infected ganglionic neurons more efficiently than the gM-null or gE-null viruses. This result supports our working hypothesis that ocular viral replication may contribute, but not serve the most important role in infection of ganglionic neurons in the scarified mouse eye infection model system. In chapter 2, we showed that gM interacts with the gK/UL20 protein complex and that UL11 is required for virus-induced cell fusion caused by syncytial mutations in gB or gK, respectively (Kim et al., 2013). Thus, the absence of either gM or UL11 from the virion particle may perturb gB and gK/UL20 fusogenic functions preventing efficient entry into neuronal axons embedded within scarified mouse corneas. Therefore, our future investigation will focused on direct demonstration of potential defects in axonal cytoplasmic entry for these viruses. These experiments are currently in progress.

Latency and neuronal infection as well as virulence of herpesviruses are prevalent, long-term dilemma in vaccine study. Therefore, the exact feature of fusion event and properties of relevant proteins will allow another insight in development of vaccine and viral therapeutics.

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In Joong Kim was born in 1976 to InSuk Park and YongKwang Kim in Daejeon, Republic of Korea. Three and five years later, In Joong had his brother and sister, Younjung and Bangshil (later Nahye), respectively. In Joong spent most of his childhood at Doma-dong, Daejeon, while receiving elementary education from Yuchon elementary school. The family moved to Seoul, Korea, and he graduated from Munchang elementary school in 1988. At Seoul, In Joong graduated from Seoun middle school and Banpo high school, in 1991 and 1994, respectively. In Joong, then proceeded to pursue his degree in veterinary medicine at Konkuk University and achieved his degree and license in 2000. He furthered his education in veterinary microbiology and infectious disease at Konkuk University and graduated the master's program in 2002. At the same time, he began his profession as a veterinary officer at National Veterinary Quarantine and Research Service (NVRQS). After the graduation, In Joong began to serve as a research scientist at NVRQS by 2009. After 10 years of dedication in the field of public health and research in veterinary infectious diseases, In Joong decided to pursue his doctoral degree in research of herpes simplex virus and joined the laboratory of Dr. Konstantin Gus Kousoulas at the School of Veterinary Medicine, Louisiana State University in 2009. Upon graduation, In Joong continues his profession in veterinary medicine as an anatomical pathology resident at the College of Veterinary Medicine, Kansas State University, where he hopes to broaden his research and clinical bases. Hence, the contribution to academia as a translational researcher is his long-term goal after acquiring the Diploma of American College of Veterinary Pathology.