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The Role of Herpes Simplex Virus Type 1 Glycoprotein K in Neuroinvasion and Immunopathogenesis of Herpes Keratitis

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THE ROLE OF HERPES SIMPLEX VIRUS TYPE 1 GLYCOPROTEIN K IN
NEUROINVASION AND IMMUNOPATHOGENESIS OF HERPES KERATITIS

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

in

The Interdepartmental Program in
Veterinary Medical Sciences through the
Department of Pathobiological
Sciences

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May 2015

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This dissertation is dedicated to my dear parents; Adel Abdullatif Said and Khadrah Muhammad Hussein.

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TABLE OF CONTENTS

ACKNOWLEDGMENTS	iv
LIST OF TABLES	viii
LIST OF FIGURES.....	ix
ABSTRACT	xi
CHAPTER I: INTRODUCTION.....	1
Statement of Problem and Hypothesis	1
Statement of Research Objectives	2
CHAPTER II: LITERATURE REVIEW	7
History of Herpesviruses	7
Taxonomy of Herpesviruses.....	9
Structure of the Herpes Virion	16
HSV-1 genome:	18
HSV-1 capsid	22
HSV-1 tegument	25
HSV-1 envelope.....	30
Glycoprotein B.....	32
Glycoprotein K.....	35
Glycoprotein M	38
UL 20 protein (pUL20)	40
Life Cycle of Herpes Simplex Virus Type 1	42
Viral attachment, binding and entry	43
Transport of capsids to the nucleus.....	46
Viral gene expression and DNA replication	48
Capsid assembly and DNA packaging.....	50
Primary envelopment.....	51
Secondary envelopment	52
Egress of enveloped virions	56
Retrograde and Anterograde Transport of Alphaherpesviruses in Neurons	58
Neuronal structure and axoplasmic transport	58
Virus binding, attachment and entry	59
Retrograde transport	60
Deposition of Viral DNA into the nucleus	62
Nuclear egress	62
Married model vs separate model in anterograde transport	62
Evidence for the married model	63
Evidence for the separate model	64
Anterograde transport	64
Herpes Simplex Virus Diseases.....	66
Herpes labialis.....	67
Herpes simplex ocular disease	69

Role of corneal epithelial cells in the pathogenesis of primary herpes keratitis	72
Herpes simplex encephalitis	75
Neonatal infections	77
Herpes simplex virus infection in immunocompromised patients.....	79
Prevention and Treatment of Herpes Simplex Infection.....	80
References	83
 CHAPTER III FUNCTIONAL HIERARCHY OF HERPES SIMPLEX VIRUS TYPE-1 (HSV-1) MEMBRANE PROTEINS IN CORNEAL INFECTION AND VIRUS TRANSMISSION TO GANGLIONIC NEURONS....	
Introduction	109
Materials and Methods	113
Cells and viruses.....	113
Construction and characterization of recombinant viruses.....	113
Plaque morphology and viral yields of wild-type and mutant viruses.	114
Ocular infections	114
Quantitative PCR (qPCR) analysis.	115
Results.....	116
Comparison of the replication characteristics of recombinant viruses lacking gM, gE, UL11, or gK	116
Mouse eye infections	118
Determination of viral DNA in trigeminal ganglia of infected mice	119
Discussion.....	121
References.....	124
 CHAPTER IV: A REPLICATION COMPETENT HSV-1(MCKRAE) WITH A MUTATION IN THE AMINO-TERMINUS OF GLYCOPROTEIN K (GK) IS UNABLE TO INFECT MOUSE TRIGEMINAL GANGLIA AFTER CORNEA INFECTION.....	
Introduction	131
Materials and Methods	134
Cells and viruses.....	134
Construction and characterization of recombinant viruses.....	134
Plaque morphology and replication kinetics of wild-type and mutant viruses ..	135
Animals	136
Quantitative PCR (qPCR) analysis	136
Infectious viral particle recovery	137
Results.....	137
Construction and characterization of recombinant viruses.....	137
Mouse eye infections.	139
TG-associated viral DNA and infectious virions.....	141
Discussion.....	143
References	145
 CHAPTER V: CONCLUDING REMARKS	
Summary	150

Current and Future Research	153
References.....	154
APPENDIX I: ADDITIONAL WORK	156
Introduction	156
Objectives and hypothesis	158
Materials and Methods	158
Viruses.....	158
Toll-like receptor-2 (TLR-2) stimulation.	158
QPCR analysis.....	159
Detection of NF-kB activation by western blot	159
Results.....	159
Toll-like receptor 2 (TLR-2) stimulation	159
QPCR analysis for pro-inflammatory cytokine mRNA transcripts in HEK blue cells	160
Determination of NF-kB activation in HCE cells by western blot	161
QPCR analysis for pro-inflammatory cytokine mRNA transcripts in HCE cells ...	161
Summary	164
References.....	164
APPENDIX II: LETTER OF PERMISSION	166
VITA	174

LIST OF TABLES

Table 2.1: Classification of herpesviruses	12
Table 2.2: C-capsids structural protein composition	22
Table 2.3: Tegument proteins and their function.....	26
Table 3.1: Comparison of viral yields between wild type and recombinant viruses.....	118

LIST OF FIGURES

Figure 2.1: The Structure and morphology of herpesvirus	17
Figure 2.2: Herpesvirus genome	19
Figure 2.3: HSV-1 capsid structure	23
Figure 2.4: The location of C-capsid specific components (CCSCs)	24
Figure 2.5: Functional domains of the UL37 protein	29
Figure 2.6: Three- dimensional tomographic reconstruction of HSV-1 virions after denoising ..	32
Figure 2.7: Schematic diagram of gB	34
Figure2.8: Schematic representation of the secondary structure of gK	35
Figure 2.9: The predicted tertiary structure of gK	38
Figure 2.10: A schematic diagram of the predicted secondary structure of HSV-1 gM	40
Figure 2.11: The predicted secondary structure of the UL20 protein	41
Figure 2.12: Schematic diagram of Alphaherpesviruses life cycle	44
Figure 2.13: A colored electron micrograph of HSV-1 capsid at the nuclear pore complex (NPC)	48
Figure 2.14: Schematic illustration of viral protein interactions during secondary envelopment	55
Figure 2.15: Hematoxylin and eosin stained histological section of herpes labialis	68
Figure 2.16: Epithelial keratitis caused by HSV	70
Figure 2.17: Slit lamp photograph of herpes stromal keratitis	71
Figure 2.18: Herpetic encephalitis	77
Figure 2.19: Hematoxylin and eosin stained histological section of the brain	78
Figure 3.1: Schematic representation of mutant viruses	117
Figure 3.2: Plaque morphology of mutant viruses	118
Figure 3.3: Clinical ocular disease development	119
Figure 3.4: Viral shedding	120
Figure 3.5 Viral DNA genome detection by qPCR	121
Figure 4.1. Schematic representation of mutant viruses	138

Figure 4.2: Plaque morphology of the constructed viruses	139
Figure 4.3: Replication kinetics of parental and mutant viruses	140
Figure 4.4: Clinical ocular disease development and scoring	141
Figure 4.5: Viral DNA genome detection by qPCR and infectious viral particle recovery from TGs.....	142
Figure AI.1: Ocular infection of mice	157
Figure AI.2: NF- κ B activation	160
Figure AI.3. QPCR analysis-HEK blue cells	161
Figure AI.4: QPCR analysis, TLR-2 engagement	162
Figure AI.5: Western blot for NF- κ B activation in HCE cells	163
Figure AI.6: QPCR analysis-HCE cells	163

ABSTRACT

Herpes simplex virus type 1 is a common neurotropic pathogen responsible for a multitude of human diseases ranging from mucocutaneous lesions, keratitis to life-threatening encephalitis. The hallmark of the HSV-1 life cycle is infection of sensory neurons, where the virus establishes a latent infection for the life of the host. Viral envelope glycoproteins play important roles in viral life cycle and virus-host interaction. Viral glycoproteins gK, gM, gE and the membrane protein UL11 have been shown to be important for virus assembly, egress and virus spread. To investigate the relative importance of each of gK, gM, gE and UL11 in infection of ganglionic neurons following ocular inoculation, recombinant viruses were constructed, characterized and used to infect mice via the ocular route. Results showed that gK plays the most important role in infection of ganglionic neurons following ocular inoculation in the mouse model. gK null viruses exhibited major defects in replication and cell to cell spread in tissue culture. To further investigate the role of gK in the pathogenesis of herpes keratitis and to delineate gK domains responsible for replication and neuroinvasion, recombinant viruses lacking the amino-terminus of gK were constructed. Characterization of the constructed viruses revealed that the amino terminus of gK is dispensable for replication in tissue culture; however, it is required for neuroinvasion and cell to cell spread. Moreover, the virus lacking the amino terminus in gK was unable to infect corneal epithelium and establish latency in the trigeminal ganglia after ocular inoculation of mice. In the above mentioned investigation, we have observed that lack of the amino terminus of gK is associated with lack of ocular disease in infected mice. Further investigation of this observation (still in progress) revealed a potential role for gK in the immunopathogenesis of keratitis. The research work presented in this

dissertation is of paramount importance as it identifies gK as an important neurotropic determinant, and it delineates gK domains responsible for neuroinvasion and immunopathogenesis in vivo. This work may lead to the development of replication competent, safe vaccine vectors, and paves the way for the discovery of new therapeutics for herpes keratitis.

CHAPTER I: INTRODUCTION

Statement of Problem and Hypothesis

Herpes simplex virus type 1 causes multiple medical disorders such as oral lesions, keratitis, encephalitis and systemic disease. A characteristic feature of the HSV-1 life cycle is infection of sensory neurons, where the virus establishes a latent infection in the trigeminal ganglia. The infection starts at the mucocutaneous areas (site of exposure) followed by entry into the axonal termini of sensory neurons. The virus recruits cellular transport machinery to translocate in retrograde manner to the body of the neuron. In the neuronal cell body, the virus remains latent for the life of the host. Although not completely understood, exposure to ultra violet (UV) light or immunosuppression leads to bouts of reactivation whereby the virus transports in anterograde manner to the primary site of infection. HSV-1 is the leading cause of infectious blindness in the developed world; there is an estimated 400,000 people suffer from herpes ocular disease in the United States. Recurrent herpes keratitis leads to corneal opacity and vision loss. Also, reactivation of latent virus from the trigeminal ganglia (TG) can cause spread to corneal transplants causing corneal graft failure.

Herpes viruses consist of a dense double stranded DNA core enclosed in an icosahedral capsid. The capsid is surrounded by proteinaceous tegument and an envelope derived from cellular membranes. The envelope contains 12 glycoproteins that function in multiple aspects of viral life cycle, and virus-host interactions. The virus uses a core fusion complex composed of; gB, gH/gL, and gD, and the accessory proteins gK and UL20p for optimal fusion. gB and gH/gL are conserved among all Herpesviruses. Although not conserved, gD has functional homologues

in all herpesviruses. gK is conserved among alphaherpesviruses (neurotropic viruses). The envelope protein gK is involved in virion egress, envelopment, and viral fusion and syncytia formation. Viral glycoproteins gK, gM, gE and the membrane protein UL11 have been shown to be important for virus assembly, egress and virus spread. Research in this laboratory has uncovered an important role for gK in neuroinvasion. However, the recombinant gK null viruses that were constructed previously to study gK function exhibited major defects in replication and cell to cell spread in tissue culture, which are considered unfavorable qualities for vaccine vector development. The hypothesis promulgated in this dissertation is that a functional hierarchy among viral membrane proteins exists with respect to their importance in viral life cycle and virus-host interaction in vivo. We also hypothesize that gK structure can be divided into functional domains each of which confers distinct characteristics on virus properties. The hypothesis predicts that the amino terminus of gK, which contains two N-glycosylation sites, is necessary for viral neurotropism and the fusion process during virion entry as well as for immunopathogenesis.

Statement of Research Objectives

The first goal of this research was to explore the relative functional importance of a subset of viral membrane proteins in their role in ocular pathogenesis and establishment of latency in ganglionic neurons. The second goal was to assess the role of the amino terminus of gK in neuroinvasion and immunopathogenesis of keratitis as well as virus replication and spread.

The specific aims of this research include:

- I. To investigate the relative importance of each of gK, gM, gE and the membrane protein UL11 in infection of ganglionic neurons following ocular inoculation of mice.
 - a. To fully characterize the constructed recombinant viruses that lack the expression of either gK, gM, gE or the membrane protein UL11 in tissue culture by analyzing viral growth kinetics and plaque morphology.
 - b. To assess whether the lack of either gE, gM, gK or UL11 affects the clinical ocular disease development and viral shedding.
 - c. To determine whether gK-null, gE-null, gM-null, and UL11-null viruses infect ganglionic neurons and establish latency.
- II. To delineate the role of the amino terminus of gK in neuro-invasion and pathogenesis of herpes keratitis.
 - a. To construct recombinant herpes simplex virus type one on a McKrae background lacking the amino terminus of gK using a two-step Red recombination Bacterial Artificial Chromosome mutagenesis system.
 - b. To fully characterize the newly constructed viruses in tissue culture by analyzing viral growth kinetics and plaque morphology.
 - c. To assess the role of the amino-terminus of gK in neuroinvasion and ocular pathogenesis in a scarified cornea mouse model (in vivo).

- d. To monitor ocular clinical disease progression and score the severity of ocular disease.
- e. To determine whether viral DNA genomes of the recombinant virus lacking the amino terminus of gK and the wild type virus, can be detected in the trigeminal ganglia.
- f. To determine if infectious virions can be recovered from ganglionic neurons after ocular inoculation of mice.
- g. To further investigate the role of the amino terminus of gK in the pathogenesis of keratitis.

Overall, the results obtained from this work indicate that:

- I. Glycoprotein K plays the most important role among gM, gE, and UL11 in infection of ganglionic neurons and establishment of latency.
- II. The mutant viruses lacking either gE, gM, gK or UL11, did not cause significant clinical ocular symptoms and had reduced viral shedding titers than the wild type virus.
- III. The wild type virus and recombinant HSV-1 viruses lacking either gE, gM, or UL11 were able to infect ganglionic neurons and establish latency. However, gK-null virus was unable to infect axonal termini and establish latency in the body of ganglionic neurons.

- IV. UL11-null virus replicated in mouse eyes and Vero cell monolayers less efficiently than gM-null and gE null mutants; however, it infected ganglionic neurons and established latency more efficiently than either the gM or gE-null viruses.
- V. A recombinant herpes virus type 1 lacking the amino terminus of gK replicates efficiently in tissue culture; however, consistently produced significantly smaller plaques than the parental virus. Therefore the amino terminus of gK is dispensable for replication in tissue culture but important for cell to cell spread in vitro.
- VI. HSV-1 McKrae wild type virus causes severe keratitis, conjunctivitis and blepharitis whereas a virus lacking the amino terminus of gK did not cause significant ocular changes.
- VII. The virus lacking the amino terminus of gK was unable to infect axonal termini and establish latency in the trigeminal ganglia after ocular inoculation. This result suggests that the amino terminus portion of gk is the domain responsible for its function in neuroinvasion.

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

Chapter III: Functional hierarchy of herpes simplex virus type-1 membrane proteins in corneal infection and virus transmission to ganglionic neurons.

Chapter IV: A replication competent HSV-1(McKrae) with a mutation in the amino-terminus of glycoprotein K (gK) is unable to infect mouse trigeminal ganglia after cornea infection.

CHAPTER II: LITERATURE REVIEW

History of Herpesviruses

Herpesviruses are ancient pathogens co-evolved with their hosts for millions of years (Kolb et al., 2013). Due to this long herpesvirus-host relationship, phylogenetic analysis of Herpesviruses have been used recently as a surrogate biomarker to study human population structure and past migration patterns throughout history (Kolb et al., 2013) (McGeoch et al., 2000). The earliest accounts that describe herpes virus infections date back to a Sumerian tablet approximately 3000 BCE and in the Ebers Papyrus circa 1500 BC (Roizman et al., 2007; Roizman and Whitley, 2001). The Greek word “Herpes” or Herpin was used by Hippocrates to describe the skin lesions that creep or crawl. However at that time, herpes was applied to many different skin ailments (Roizman et al., 2007; Wildy, 1973).

In the 18th century, the French physician John Astruc, in his capacity as a physician for King Louis XIV, was the first to describe herpes genitalis by studying skin afflictions of French prostitutes. He published his description of genital herpes in his book *De Morbis Venereis* in 1736 (Roizman et al., 2007). In 1883, Paul Gerson Unna described herpes skin lesions as a “vocational disease” of women and also provided descriptions for the recurrences of genital lesions (Roizman et al., 2007; Roizman and Whitley, 2001). Shortly after that; *Les Herpes Denitiaux*, the first book specifically devoted to herpes, was published by Charles-Paul Diday and Adrien Doyon in 1886.

A decade later, Jean Alfred Fournier wrote about the diagnosis, treatment and prevention of genital herpes infections; his recommendations were to abstain from alcohol, tobacco and “sexual excesses”. Evidence for the contagious nature of herpes infections was provided by the French dermatologist Jean Baptiste Émile Vidal as he showed that vesicular lesions can be transmitted from one patient to another, and from one anatomical location to another in the same individual. Vidal used human volunteers in his experiments. Wilhelm Grüter however, used rabbits as animal models to demonstrate that HSV serially transmitted between rabbits. Grüter is credited by the virology community for the isolation of HSV (Gruter, 1924; Roizman et al., 2007).

HSV has been recognized as an infectious agent in the late 1800s; however, the connection between HSV and recurrent infections was not established until 1930s. Andrews and Carmichael have made the observation that recurrent infections occurred only in adults who have been previously infected with the virus evidenced by the presence of neutralizing antibodies (Roizman et al., 2007; Roizman and Whitley, 2001). Burnet and Williams articulated the modern view on latency in 1939; the virus remains latent after infection, and stimuli such as trauma or fever, provoke reactivation and visible herpetic lesions (Burnet, 1939). In 1971 and over 40 years after Grüter’s original isolation of HSV, Schneweis and Nahmias demonstrated the existence of two separate serotypes of HSV and their association with either orolabial herpes (HSV-1) or genital herpes (HSV-2)(Roizman et al., 2007; Schneweis and Nahmias, 1971). The discovery of Acyclovir in the 1980’s dramatically increased the public awareness for genital

herpes; however, the stigmatization of the condition was overshadowed by the arrival of the human immunodeficiency virus and the public concerns of a global HIV epidemic (Cuatrecasas, 2006; Kimberlin, 2014).

During the first half of the 20th century, advancements in tissue culture techniques and virus isolation methods have enabled us to isolate and identify viruses with relative ease. Varicella-zoster virus (VZV) and human cytomegalovirus (CMV) were isolated in the 1950s, and the developments in the cultivation of B lymphocytes resulted in the discovery of Epstein-Barr virus (EBV) in 1964 (Craig et al., 1957; Epstein et al., 1964; Weller and Stoddard, 1952). Developments in T lymphocytes cell culture techniques allowed for the isolation and identification of Human Herpesviruses 6A, 6B and 7 in the late 1980s (Frenkel et al., 1990; Lopez et al., 1988; Salahuddin et al., 1986). Kaposi's sarcoma-associated herpesvirus (KSHV) or human herpesvirus 8 (HHV-8) was discovered in 1994 using representational difference analysis (RDA). This novel technique –at that time- was utilized to identify sequence differences between two complex genomes (Chang et al., 1994).

The rapid advances in molecular biology and sequencing technologies in the last decade will undoubtedly revolutionize our understanding of the inner working of known viruses and facilitate the discovery of a plethora of new viruses (Hall, 2007).

Taxonomy of Herpesviruses

Taxonomy is the science of systematic classification of biological organisms into Taxa, or groups, based on shared characteristics (Davison, 2010; Fenner, 1976). Biological organisms are classified so that hierarchical level “domain” is the most inclusive taxonomic rank, whereas

virus classification system employs the hierarchical levels of Order, Family, Subfamily, Genus, and Species. Classification of viruses is managed and standardized by the committee of the Virology Division of the International Union of Microbiological Societies known as International Committee on Taxonomy of Viruses (ICTV). ICTV has produced nine reports published in intervals since it was established in 1971. These reports contain developments in virus taxonomy (Davison, 2010). In early days, classification of viruses was based on host range and broad pathogenic and epidemiological features, however, technological advances in molecular biology and tissue culture as well as the growing use of electron microscopy and physicochemical methods enabled more accurate viral identification and classification. The development of specific antibodies enabled the study of antigenic relationships and allowed researchers to differentiate between closely related viruses (Davison, 2010). Advances in nucleic acid sequencing and the broad utility of this approach enhanced our ability to differentiate between closely related species at higher resolution and rationalized taxonomy; which eventually lead to the adoption of sequence-based phylogenetic analysis as the key determinant for classification of living organisms (Davison, 2010).

The genus herpesvirus was established in the first ICTV report in 1971 (International Committee on Nomenclature of Viruses. and Wildy, 1971). The genus consisted of 4 groups and 23 viruses. In the second ICTV report in 1976, Herpesvirus genus was elevated to the family Herpetoviridae. The second report also added *Human herpesvirus 2* as a close relative of *Human herpesvirus 1* to the genus *Herpesvirus*. To avoid the misleading association of this family name with reptiles, ICTV renamed *Herpetoviridae* to *Herpesviridae* in the third report in 1979. The third ICTV report also subdivided the *Herpesviridae* family into three subfamilies;

Alphaherpesvirinae, *Betaherpesvirinae* and *Gammaherpesvirinae* (Matthews, 1979). In the ICTV release in 2008, a new order, *Herpesvirales*, was introduced and the family *Herpesviridae* and the new families *Alloherpesviridae* and *Malacoherpesviridae* were created (Davison, 2010; Davison et al., 2009). *Herpesviridae* includes viruses of mammals, birds and reptiles, and the family *Alloherpesviridae* encompasses viruses that infect fish and frogs, while the family *Malacoherpesviridae* incorporates viruses that infect molluscs (Davison et al., 2009).

Members of *Alphaherpesvirinae* subfamily share several common characteristics; ability to infect neuronal cells and establish latency in cell bodies, variable host range, efficient lytic phase of infection in epithelial cells and other non-neuronal cells, relatively short replication cycle and efficient cell to cell spread. Members of *Betaherpesvirinae* share several common characteristics; establish latency in secretory glands, lymphoreticular cells and kidneys, a slow life cycle, restricted host range and cytomegaly (enlarged infected cells). Members of *Gammaherpesvirinae* have several common features which include; they maintain latency in lymphoid tissue, infect a limited host range, replicate in lymphoblastoid cells in tissue culture, some members has a lytic replication in epithelial cells and fibroblasts (Roizman et al., 2007).

The most recent online version of the official ICTV taxonomy released in 2013 recognizes a total of 2827 viral species, while the order *Herpesvirales* currently is home for 3 families, 3 subfamilies, 19 genera and 102 species (table 2-1).

(Table 2.1 continued)

Taxon name	Common name	Acronym
Genus <i>Scutavirus</i>		
<i>Chelonid herpesvirus 5*</i>	Chelonid fibropapilloma-associated herpesvirus	ChHV5
Genus <i>Simplexvirus</i>		
<i>Ateline herpesvirus 1</i>	Spider monkey herpesvirus	AtHV1
<i>Bovine herpesvirus 2</i>	Bovine mammillitis virus	BoHV2
<i>Cercopithecine herpesvirus 2</i>	Simian agent 8	CeHV2
<i>Human herpesvirus 1*</i>	Herpes simplex virus type 1	HHV1
<i>Human herpesvirus 2</i>	Herpes simplex virus type 2	HHV2
<i>Leporid herpesvirus 4</i>	Leporid herpesvirus 4	LeHV4
<i>Macacine herpesvirus 1</i>	B virus	McHV1
<i>Macropodid herpesvirus 1</i>	Parma wallaby herpesvirus	MaHV1
<i>Macropodid herpesvirus 2</i>	Dorcopsis wallaby herpesvirus	MaHV2
<i>Papiine herpesvirus 2</i>	Herpesvirus papio 2	PaHV2
<i>Saimiriine herpesvirus 1</i>	Marmoset herpesvirus	SaHV1
Genus <i>Varicellovirus</i>		
<i>Bovine herpesvirus 1</i>	Infectious bovine rhinotracheitis virus	BoHV1
<i>Bovine herpesvirus 5</i>	Bovine encephalitis herpesvirus	BoHV5
<i>Bubaline herpesvirus 1</i>	Water buffalo herpesvirus	BuHV1
<i>Canid herpesvirus 1</i>	Canine herpesvirus	CaHV1
<i>Caprine herpesvirus 1</i>	Goat herpesvirus	CpHV1
<i>Cercopithecine herpesvirus 9</i>	Simian varicella virus	CeHV9
<i>Cervid herpesvirus 1</i>	Red deer herpesvirus	CvHV1
<i>Cervid herpesvirus 2</i>	Reindeer herpesvirus	CvHV2
<i>Equid herpesvirus 1</i>	Equine abortion virus	EHV1
<i>Equid herpesvirus 3</i>	Equine coital exanthema virus	EHV3
<i>Equid herpesvirus 4</i>	Equine rhinopneumonitis virus	EHV4
<i>Equid herpesvirus 8</i>	Asinine herpesvirus 3	EHV8
<i>Equid herpesvirus 9</i>	Gazelle herpesvirus	EHV9
<i>Felid herpesvirus 1</i>	Feline herpesvirus 1	FHV1
<i>Human herpesvirus 3*</i>	Varicella-zoster virus	HHV3
<i>Phocid herpesvirus 1</i>	Harbour seal herpesvirus	PhoHV1
<i>Suid herpesvirus 1</i>	Pseudorabies virus	SuHV1
Unassigned species in the subfamily		
<i>Chelonid herpesvirus 6</i>	Lung-eye-trachea disease-associated virus	ChHV6
Subfamily <i>Betaherpesvirinae</i>		
Genus <i>Cytomegalovirus</i>		
<i>Aotine herpesvirus 1</i>	Herpesvirus aotus type 1	AoHV1
<i>Cebine herpesvirus 1</i>	Capuchin herpesvirus AL-5	CbHV1
<i>Cercopithecine herpesvirus 5</i>	Simian cytomegalovirus	CeHV5

(Table 2.1 continued)

Taxon name	Common name	Acronym
Genus <i>Percavirus</i>		
<i>Equid herpesvirus 2*</i>	Equine herpesvirus 2	EHV2
<i>Equid herpesvirus 5</i>	Equine herpesvirus 5	EHV5
<i>Mustelid herpesvirus 1</i>	Badger herpesvirus	MusHV1
Genus <i>Rhadinovirus</i>		
<i>Ateline herpesvirus 2</i>	Herpesvirus ateles strain 810	AtHV2
<i>Ateline herpesvirus 3</i>	Herpesvirus ateles	AtHV3
<i>Bovine herpesvirus 4</i>	Bovine herpesvirus 4	BoHV4
<i>Cricetid herpesvirus 2</i>	Pygmy rice rat herpesvirus	CrHV2
<i>Human herpesvirus 8</i>	Kaposi's sarcoma-associated herpesvirus	HHV8
<i>Macacine herpesvirus 5</i>	Rhesus rhadinovirus	McHV5
<i>Murid herpesvirus 4</i>	Murine herpesvirus 68	MuHV4
<i>Murid herpesvirus 7</i>	Wood mouse herpesvirus	MuHV7
<i>Saimiriine herpesvirus 2*</i>	Herpesvirus saimiri	SaHV2
Unassigned species in the subfamily		
<i>Equid herpesvirus 7</i>	Asinine herpesvirus 2	EHV7
<i>Phocid herpesvirus 2</i>	Phocid herpesvirus 2	PhoHV2
<i>Saguinine herpesvirus 1</i>	Herpesvirus saguinus	SgHV1
Unassigned species in the family		
<i>Iguanid herpesvirus 2</i>	Iguana herpesvirus	IgHV2
Family <i>Malacoherpesviridae</i>		
Genus <i>Aurivirus</i>		
<i>Haliotid herpesvirus 1*</i>	Abalone herpesvirus	AbHV1
Genus <i>Ostreavirus</i>		
<i>Ostreid herpesvirus 1*</i>	Oyster herpesvirus	OsHV1

A definition of a virus species was first created by van Regenmortel in 1989. He defined a virus species as “a polythetic class of viruses that constitute a replicating lineage and occupy a particular ecological niche”. The ICTV adopted this definition in 1991, and formalized for the first time the concept of virus species in its 7th report. A formal system for naming herpesviruses was founded before 1979 with a number of changes taking place over the following years. Herpesvirus nomenclature system maintained that herpesviruse species is

named after the taxon (family or subfamily) of its primary natural host, however, humanherpesviruses maintained human as a designation instead of hominid. This host-derived term is followed by the word herpesvirus, and an Arabic number (1973; Davison, 2010; Fenner, 1976; Matthews, 1979; Roizman et al., 1981; Roizmann et al., 1992). Herpesviruses have been classified as distinct species if “(a) their nucleotide sequences differ in a readily assayable and distinctive manner across the entire genome and (b) they occupy different ecological niches by virtue of their distinct epidemiology and pathogenesis or their distinct natural hosts”(Davison, 2010).

Currently, as phylogenetic analysis based on sequence comparison data has revolutionized biology, and is being used as the basis of taxonomic classification. The ICTV Herpesvirales Study Group is considering updates in Herpesviruse taxonomy scheme, and to accommodate the need to classify the rapidly growing number of herpesviruses that have been detected only by polymerase chain reaction (PCR). The ICTV makes the distinction between the Virus and Virus species; virus refers to the “real physical entities produced by biological evolution and genetics”, it can be manipulated and can be further subdivided into more granular units such as; strains, variants and isolates. Virus species, on the other hand, refers to the “abstract concepts produced by rational thought and logic”.

Structure of Herpes Virion

A mature herpes virion ranges from 120-300 nanometer (nm) in diameter. It is composed of 4 distinct structural components; an electron dense core of double stranded DNA, a capsid shaped as convex polyhedron, a tegument layer and an envelope (Figure 2.1).

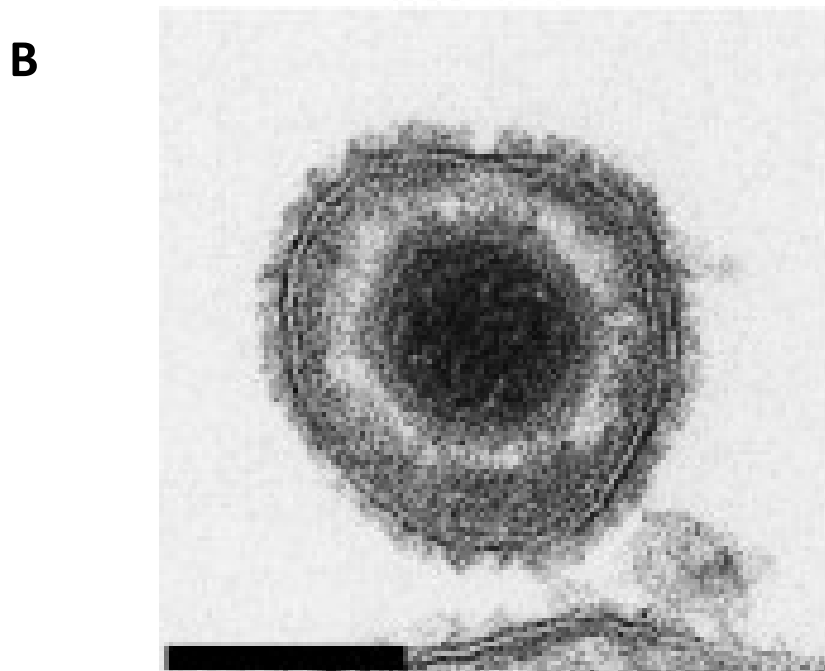
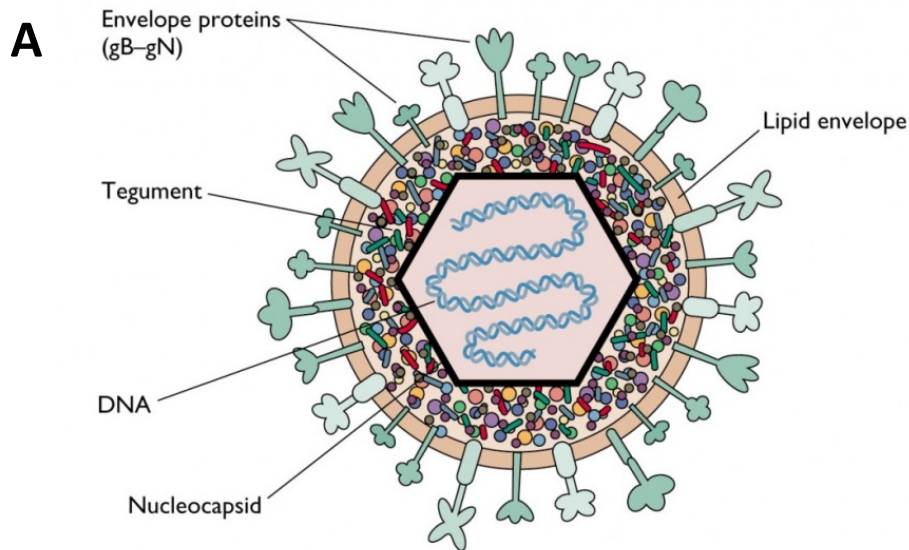


Figure 2.1: The Structure and morphology of herpesvirus. A mature herpes virion ranges from 120-300 nanometer (nm) in diameter. The herpes virion is composed of a dense core containing double stranded DNA genome enclosed in an icosahedral nucleocapsid, the nucleocapsid is surrounded by a proteinaceous material called tegument which is further surrounded by a double-layer membrane called the envelope containing glycoproteins. (A) Schematic diagram for herpesvirus structure. Image from: <http://www.twiv.tv/virus-structure/> (B) electron micrograph of a mature HSV-1 virion, bar = 100 nm (Mettenleiter et al., 2009).

Herpesvirus DNA genome in general ranges from 120 kbp to 250 kbp; Herpes simplex virus genome is 152 kbp long. This DNA core is organized in a toroidal shape or a circle. The DNA is enclosed in a T- 16 symmetrical icosahedral capsid composed of 162 capsomeres. The capsid is surrounded by a proteinaceous material called the tegument which is further surrounded by a double layered envelope derived from cellular membranes. The envelope contains a dozen of glycoproteins and non-glycosylated membrane proteins.

The interactions between the capsid, tegument and envelope proteins help bring the virion particle together during viral assembly and stabilize virion structure. They also facilitate viral protein functions in various steps of viral lifecycle. Interactions between viral envelope proteins and cell membrane receptors facilitate viral binding, attachment and entry in to cells, capsid and tegument proteins also interact with cellular transport proteins to convey the capsid to the nucleus. The interactions between viral proteins and cellular components help perpetuate the HSV-1 life cycle in uninfected cells (Mettenleiter, 2004; Roizman et al., 2007).

HSV-1 genome:

Herpesvirus genome is packaged inside the capsid in the shape of a torus. This toroidal structure (doughnut) is approximately 50 nm high, with an outside diameter of 70 nm and an inside diameter of 18 nm (Nazerian, 1974). Herpes simplex virus type one has a double stranded DNA genome, approximately 152 kbp in length with slight variations between different HSV-1 isolates, and it weighs approximately 100×10^6 Daltons (Kieff et al., 1971). Cytosine + Guanine content in herpesviruses ranges from 31 % -75 % across the genome. HSV-1 genome has a relatively high (68%) cytosine (C) + guanine (G) content. Varicillozooster, another

alphaherpesvirus, for example; has a 46 % genomic G+C content (Baines and Pellett, 2007; Roizman et al., 2007). Herpesvirus genome is arranged into a unique long (UL or U_L) and unique short (US or U_S) segments, flanked by terminal repeats (TR) on each terminus. The UL and US segments are separated by apposed internal repeats (IR) (Figure 2.2) (Roizman et al., 2007).

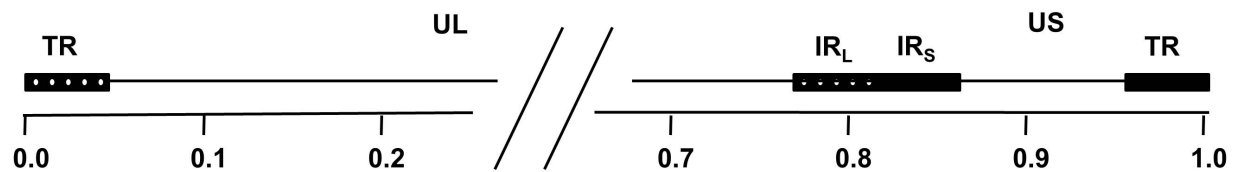


Figure 2.2: Herpesvirus genome: a schematic representation of the prototypical arrangement of Herpesvirus genome. It consists of Unique long (UL), and unique short (US) portions flanked by terminal repeats (TR) and internal repeats (IR). Modified from [mBio 10.1128/mBio.00144-12, A herpes simplex virus 1 (McKrae) mutant lacking the glycoprotein K gene is unable to infect via neuronal axons and egress from neuronal cell bodies] (David et al., 2012).

The repeated sequences flanking the UL segment are approximately 9000 bp in length, whereas, the repeated sequences surrounding the unique short segment are shorter at approximately 6000-7400 bp. The repeats, terminal and internal, are able to invert to one another to form four isomers arranged in varying orientations. These reiterated sequences generally, regulate the expression of several genes (Roizman et al., 2007). ICP0 for example, is present in the repeats flanking the unique long segments and is one of the most conserved genes in the long repeated sequences. ICP4 is one of the most conserved genes in the repeated sequences that flank the US segments. Both genes are immediate-early genes that function in regulation of viral and host cell gene expression and are important for reactivation of latent herpes simplex viruses (Baines and Pellett, 2007; Everett, 2000; Liu et al., 2010).

Most of herpesvirus genes are composed of a 50-200 bp promoter region, followed by a TATA box and an initiation site of 20-25 bases. A 30-300 bases spacer is present between the TATA box and a 5' untranslated leader sequence. This leader sequence is followed by the major open reading frame (ORF) which is followed by 3' untranslated segment, 10-30 bases long, and a polyadenylation sequence. HSV-1 encodes approximately 90 open reading frames (ORF), the majority of which (over 80%) are not spliced. Herpesviruses also produce noncoding RNAs. A well-studied example of these non-coding RNAs is the Latency Associated Transcript (LAT). It is difficult to determine the exact number of encoded proteins in Herpesviruses because some herpesvirus genes do not have a TATA box, possess a second initiation sites and show a disregard of predicted transcription termination sites for several late genes; nonetheless, there are approximately 84 transcriptional units participating in the production of herpes simplex type 1 viral proteins (Davison, 2007; Mavromara-Nazos and Roizman, 1989; McGeoch et al., 2006; Roizman et al., 2007).

The UL region of herpesvirus genome encodes genes that tend to be more conserved among alphaherpesviruses than genes encoded by the US segment. Enzymes that are necessary for viral replication and metabolism are the most conserved and are encoded by the unique long segment; examples include UL5, UL15, UL30 and UL40. (Baines and Pellett, 2007; Roizman et al., 2007).

A number of genes are conserved among all herpesviruses such as gB. Other genes are only shared by alphaherpesviruses such as gK. A few genes unique to HSV-1 and HSV-2 and not shared with other alphaherpesviruses also exist. Important examples are γ 1 34.5 and US11;

they encode a 263 aa and a 161 aa proteins, respectively. US11 is a dsRNA-binding protein; it antagonizes the action of double-stranded RNA-dependent protein kinase (PKR) and inhibits stress-induced translational arrest. γ 1 34.5 has been implicated in the inhibition of autophagy and in the de-phosphorylation of the α subunit of eukaryotic translation initiation factor 2 (eIF-2 α) in order to prevent shutoff of viral protein synthesis by the action of activated PKR (He et al., 1997; Lussignol et al., 2013; Roizman et al., 2007).

Unique to herpes simplex viruses, type 1 and 2, is the Latency associated transcript (LAT), it is a non-coding RNA transcript that accumulates in the nuclei of neurons during latent infection. US4 gene is unique to simplex viruses; it encodes glycoprotein G (gG) which is significantly smaller (~1500 bp smaller) in HSV-1 compared to HSV-2 and has been used in serological assays to differentiate between the two viruses. Other examples of genes unique to simplex virus are; the US5 gene and US8.5 gene they encode glycoprotein J (gJ) which is involved in preventing cytotoxic T-lymphocyte-induced apoptosis of infected cells, and a phosphorylated protein that tends to localize in the nucleoli of infected cells, respectively. The US12 gene encoding ICP47 interferes with MHC-I mediated antigen presentation in infected cells. US 12 is another example of genes exclusively expressed by simplex viruses (Baines and Pellett, 2007; Georgopoulou et al., 1995; Ghiasi et al., 1998; Jerome et al., 1999; Lee et al., 1985; Roizman et al., 2007; York et al., 1994).

A few of viral gene products have been labeled as “non-essential” for viral replication in vitro. These genes were labeled as such as experiments were performed on a single cell type or in a narrow context of a particular condition of an experiment. Later, some of the proteins

labeled as nonessential were proven to be essential in vivo and play important roles in infection of various host cell types, neurotropism, latency, and evasion of host immune responses (Nishiyama, 2004).

HSV-1 capsid

A mature herpesvirus genome is contained within a uniform and symmetric icosahedral capsid which is approximately 15 nm thick, 1250 Angstrom in diameter and weighs 200 Mega Dalton (MDa). The capsid is composed of 162 capsomeres that lie on a T-16 symmetrical icosahedral frame. Five structural proteins form the building blocks of a capsid; VP5 (UL19), VP26 (UL35), VP23 (UL18), VP19C (UL38), and the portal protein pUL6. These five structural proteins and two additional proteins, pUL25 and pUL17, make up the C-capsid specific components in mature DNA-filled capsids (Table 2.2). pUL26/26.5 are part of scaffold capsid proteins that get lost when the DNA is packaged into the capsid. pUL26/26.5 is not present in mature virions (Brown and Newcomb, 2011; Newcomb et al., 1999; Zhou et al., 2000).

Table 2.2: C-capsids structural protein composition. Adapted with modification from (Brown and Newcomb, 2011).

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

The 162 capsomeres are subdivided into 150 hexons, 12 pentons and 320 triplexes (Figure 2.3). The hexons form the edges and faces of the icosahedral capsid; each hexon is composed of 6 copies of VP5 and 6 copies of VP26. Each penton has 5 copies of VP5. Pentons are located at capsid vertices and interact with UL36 tegument protein in mature virions. The capsomeres are linked by the triplexes which are composed of two copies of VP23 and one copy of VP19C arranged as a heterodimer (Brown and Newcomb, 2011; Cardone et al., 2012; Newcomb and Brown, 2010; Schmid et al., 2012; Zhou et al., 2000).

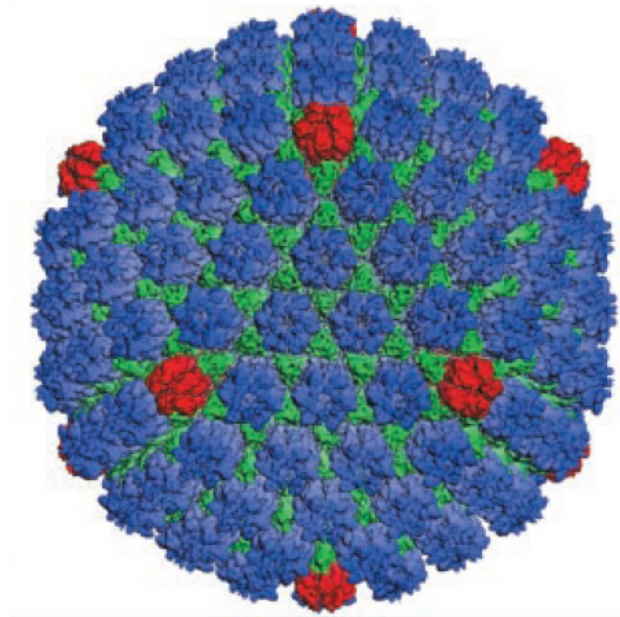


Figure 2.3: HSV-1 capsid structure. HSV-1 capsid with 12 pentons (red), 150 hexons (blue), and 320 triplexes (green). Image from (Zhou et al., 2000).

The capsid has 12 vertices; 11 of which are occupied by pentons. The 12th vertex is occupied by a hollow cylindrical portal structure, 9 nm in length and 16.5 nm in outside diameter with a ~3 nm diameter axial channel. This portal vertex is structurally composed of twelve copies of pUL6 with 12-fold rotational symmetry. The portal vortex is thought to provide the route for packaging and release of viral DNA. It may also play a role in docking of capsid to

the nuclear pore complexes (NPCs). The portal vortex may potentially influence the spatial arrangement of viral tegument and envelope as it has been shown to extend through the tegument layer and possibly come in contact with the envelope (Brown and Newcomb, 2011; Meyring-Wosten et al., 2014; Schmid et al., 2012).

Three morphologically distinct types of capsids (A, B, and C) can be observed by electron microscopy in infected cells. A-capsids are empty as a result of failed DNA packaging process. B-capsids also lack viral DNA, but contain scaffolding proteins. C-capsids are the type of capsids that are ultimately found in mature infectious virions. They contain viral DNA and no scaffolding protein. Also, C-capsids are distinguished by the presence of C-capsid specific components (CCSCs) near capsid vertices (Figure 2.4).

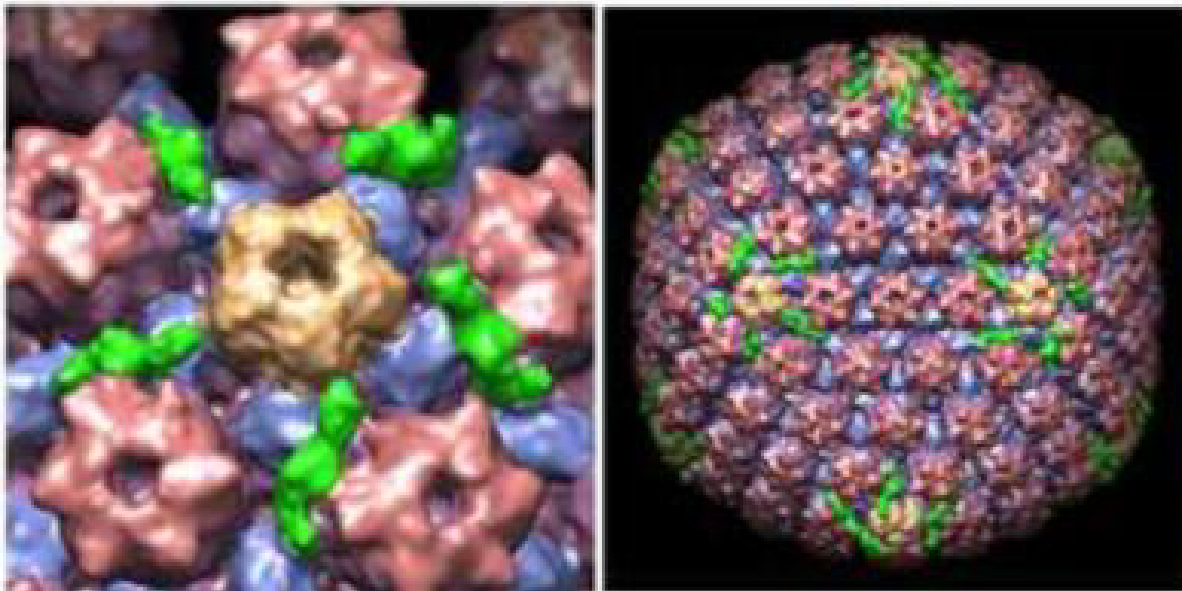


Figure 2.4: The location of C-capsid specific components (CCSCs). CCSCs are highlighted in green. Left panel shows a higher magnification of CCSCs in a single capsid vertex. Right, CCSCs location is shown in the full capsid. Adapted and modified from (Brown and Newcomb, 2011).

CCSCs are a rod shaped structure made up of one copy of pUL25 and one copy of pUL17. It has been shown that CCSCs interface with the tegument by binding to the UL36 protein. CCSCs is thought to fasten neighboring pentons against the internal pressure in DNA-loaded C-capsids (Brown and Newcomb, 2011; Homa and Brown, 1997; Liu and Zhou, 2007).

HSV-1 tegument

The tegument is a proteinaceous matrix and a network layer that bridges the capsid and the envelope. The tegument in mature virions consists of 24 proteins, including pUL7, pUL11, pUL13, pUL14, pUL16, pUL21, pUL23, pUL36 (VP1/2), pUL37, pUL41, pUL46 (VP11/12), pUL47 (VP13/14), pUL48 (VP16), pUL49 (VP22), pUL50, pUL51, pUL55, pUS2, pUS3, pUS10, pUS11, ICP34.5, ICP0 and ICP4 (Table 2.3). Four proteins, pUL46 (VP11/12), pUL47 (VP13/14), pUL48 (VP16) and pUL49 (VP22) are present in over a 1000 copy each and constitute the majority of the tegument volume. By comparison, the tegument proteins pUL36 and pUL37 are present in ~120 copies per virion (Kelly et al., 2009; Newcomb and Brown, 2009). Tegument proteins; pUL36 (VP1/2), pUL37, pUL48 and ICP4 are the only HSV-1 tegument proteins that are essential for viral growth in cell culture (Kelly et al., 2009; Roizman et al., 2007).

Cryo-electron tomography imaging of mature HSV-1 virions shows the tegument as asymmetrically distributed, dense granular layer interspersed with ~40 nm long and ~ 7 nm wide filaments consistent with actin. At early time points in infected cells, the tegument tend to be uniformly distributed around the capsid (Grunewald et al., 2003). As the virion particle

matures over the course of approximately 40 hours, the tegument tends to conglomerate on one side of the capsid, forming a cap-like structure and pushing the capsid off center (Grunewald et al., 2003).

Tegument proteins are subdivided into inner and outer tegument proteins. Inner tegument proteins consist of pUS3, pUL36 and pUL37 which remain associated with the capsid after entry into the host cell. The remaining tegument proteins are categorized as outer tegument proteins which detach from the capsid after entry into host cell. (Mettenleiter, 2006; Newcomb and Brown, 2010).

Table 2.3: Tegument proteins and their function. Adapted and modified from (Kelly et al., 2009).

Tegument protein	Predicted Mwt (kDa)	Functions
pUL7	33.1	Regulates mitochondrial function
pUL11	10.5	Secondary envelopment
pUL13	57.2	Protein kinase, tegument dissociation, regulates apoptosis and pUS3, inhibits IFN response
pUL14	23.9	Nuclear import, regulates apoptosis, nuclear targeting of capsids
pUL16	40.4	Secondary envelopment
pUL21	57.6	Secondary envelopment, regulates microtubule assembly
pUL23	41.0	Thymidine kinase, viral DNA replication
pUL36 (VP1/2)	335.9	Capsid transport, secondary envelopment, release of viral DNA, deubiquitinating activity
pUL37	120.6	Secondary envelopment, regulates viral transcription
pUL41	54.9	Regulates host/viral translation and immune response
pUL46 (VP11/12)	78.2	Secondary envelopment, regulates pUL48-dependent transcription
pUL47 (VP13/14)	73.8	Secondary envelopment, regulates pUL48-dependent transcription

(Table 2.3 continued)

Tegument protein	Predicted Mwt (kDa)	Functions
pUL50	39.1	dUTPase, viral DNA replication
pUL51	25.5	Unknown
pUL55	20.5	Unknown
pUS2	32.5	Unknown
pUS3	52.8	Protein kinase, primary deenvelopment, tegument dissociation, regulates actin assembly
pUS10	34.1	Unknown
pUS11	17.8	Regulates host translation, capsid transport
ICP34.5	26.2	Regulates host translation, viral DNA replication and immune response
ICP0	78.5	Regulates viral transcription
ICP4	132.8	Regulates viral transcription

Tegument proteins play important roles not only as structural but also as functional proteins (Table 2.3) (Kelly et al., 2009). UL36 protein (pUL36 or VP1/2) is a 3164 amino acid protein encoded by the open reading frame UL36 (Roizman et al., 2007). The inner tegument protein pUL36 have been shown to interact with capsid proteins VP5 and pUL25 and acts as the link between the capsid and the outer tegument proteins (Bucks et al., 2011; Collier et al., 2007). Also, pUL36 directly interacts with another inner tegument protein pUL37, which directly interacts with the envelope proteins gK and pUL20, possibly providing a scaffold for the rest of the tegument (Jambunathan et al., 2014; Mijatov et al., 2007; Newcomb and Brown, 2010). The amino terminus of pUL36 contains a 420 aa ubiquitin-specific cysteine protease; this domain with its active site at Cys65 deubiquitinates viral proteins to avoid degradation by the proteasomal pathway (Kattenhorn et al., 2005). The amino terminus of VP1/2 also contains a highly conserved functional nuclear localization signal (Abaitua and O'Hare, 2008). The ubiquitin-specific cysteine protease domain of UL36 has been shown to down regulate IFN-Beta production by deubiquitinating TNF receptor-associated factor 3 (TRAF3) (Wang et al., 2013).

pUL36 along with other inner tegument proteins (pUL37 and pUS3) also recruits the motor molecule dynein and its cofactor dynactin during retrograde transport (Radtke et al., 2010). UL36 protein plays a role in DNA packaging and release from the capsid.

The HSV-1 UL37 gene encodes a 120.6 kD, 1123 aa protein that encompasses a variety of functional domains (Figure 2.5). UL37 protein is essential for viral assembly, secondary envelopment in the cytoplasm and regulation of viral transcription (Bucks et al., 2011; Kelly et al., 2009; Mettenleiter, 2002). The amino terminus of UL37 contains a self association domain (in the absence of UL36), and leucine-rich nuclear export signal (NES)(Bucks et al., 2011; Watanabe et al., 2000). The function of the central domain of UL37 is yet unclear. The carboxy terminus of UL37 contains a motif that activates NF- κ B signaling by binding TNF receptor-associated factor 6 (TRAF6). Modulation of NF- κ B activity possibly affects the transcriptional activation of viral intermediate early genes such as ICP0 (Amici et al., 2006; Bucks et al., 2011; Liu et al., 2008). The domain spanning residues 578-899 of the carboxyl terminus of UL37 have also been shown to interact with a cytoskeletal cross-linker called dystonin/BPAG1 which is involved in microtubule stabilization and transport (Pasdeloup et al., 2013). The pUL36 and pUL37 are highly conserved among all Herpesviruses which highlight their importance as structural as well as functional viral proteins (Mettenleiter, 2002).

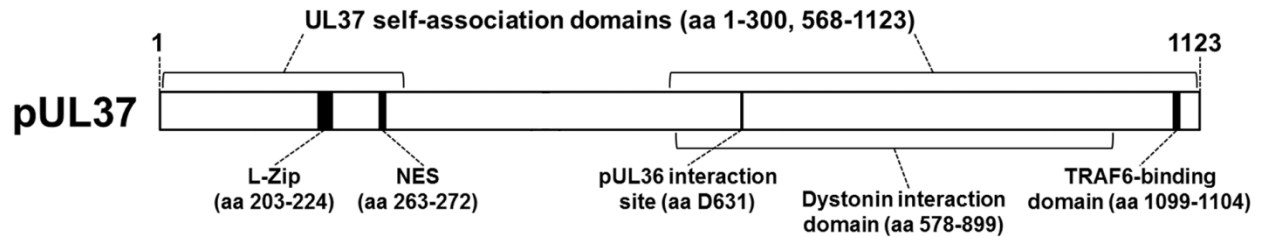


Figure 2.5: Functional domains of the UL37 protein. Adapted with modification from (Jambunathan et al., 2014).

US3 and UL13 proteins are versatile viral serine/threonine protein kinases. They contribute along with cellular kinases, to the phosphorylation of other tegument proteins prior to their dissociation from the capsid during entry (Asai et al., 2007; Kato et al., 2005; Kelly et al., 2009). US3 has an anti-apoptotic activity through phosphorylation of pro-apoptotic Bcl-2 family members. By contrast, pUL13 has a pro-apoptotic activity (Benetti and Roizman, 2007; Hagglund et al., 2002; Jerome et al., 1999; Kelly et al., 2009).

The UL41 gene encodes the 54.9 kD outer tegument protein known as virion host shutoff (vhs) protein. Vhs dissociates from the virion capsid during entry and functions as an endoribonuclease RNase A to degrade mRNA resulting in shutting down the host cell gene expression as well as viral protein synthesis. Viral mRNAs though, are rescued by VP16; at a later stage of infection pUL48 (VP16) interacts with pUL41 to inhibit pUL41 activity and facilitate its incorporation into virion particles. (Lam et al., 1996; Taddeo and Roizman, 2006).

VP16, also referred to as (α -transinducing factor (α -TIF), is an outer tegument protein that is required for initiation of transcription of viral alpha genes such as ICP0 and ICP4 in a lytic infection (Campbell et al., 1984; Kelly et al., 2009; Roizman et al., 2007).

ICP0 protein is encoded by the RL2 gene from the reiterated sequences region. ICP0 protein is a 775- aa promiscuous transactivator of genes (Kelly et al., 2009; Roizman et al., 2007). It contains a RING finger domain and ubiquitin ligase E3 domain (Roizman et al., 2007). The expression of ICP0 protein directly depends on pUL48 as discussed above. The efficiency of ICP0 functions are enhanced by its interaction with ICP4. The two major functions of ICP0 are; to facilitate the transition from α to β viral-gene expression and to block the inhibition of viral gene expression by host cell interferons through its ubiquitin ligase activity (Gu and Roizman, 2007; Harle et al., 2002).

The tegument is an important structural and functional component of the virion particle. It accounts for approximately 40% of the total virion protein mass and two-thirds of the virion particle volume (Grunewald et al., 2003; Liu and Zhou, 2007).

HSV-1 envelope

The envelope is a host-derived lipid bilayer outer covering of the virus. Herpes Simplex Virus envelope contains at least 12 different glycoproteins and several other non-glycosylated membrane-associated viral proteins. The glycoproteins include pUL1 (gL), pUL10 (gM), pUL22 (gH), pUL27 (gB), pUL44 (gC), pUL49.5 (gN), pUL53 (gK), pUS4 (gG), pUS5 (gJ), pUS6 (gD), pUS7 (gI) and pUS8 (gE). The non-glycosylated membrane proteins include pUL20, pUL24, pUL43, pUL45, pUL56 56 and pUS9 (Campadelli-Fiume and Menotti, 2007; Roizman et al., 2007). Although pUL34 is not present in the envelope of extracellular virions, it mediates primary envelopment in concert with pUL31 (Roizman et al., 2007).

Cryo-electron tomography of HSV envelope reveals that the envelope is approximately 5 nm thick with 600-750 non-randomly clustered glycoprotein spikes of highly variable length, spacing and morphology embedded in the viral envelope (Figure 2.6) (Grunewald et al., 2003).

In these tomography images, the glycoprotein spikes appears to be clustered in the areas where the tegument is dense, in support of the documented experimental evidence of the interaction between HSV-1 tegument proteins and envelope glycoproteins (Grunewald et al., 2003). It has been shown, using mutational analysis and biochemical approaches that the cytoplasmic portions of gB, gD and gH bind to the outer tegument protein VP16 (pUL48) (Gross et al., 2003; Kamen et al., 2005; Zhu and Courtney, 1994). Also, the interactions between the cytoplasmic tails of gD and gE and the tegument proteins pUL11 and pUL49 (VP22) have been demonstrated by co-immunoprecipitation experiments (Chi et al., 2005; Farnsworth et al., 2007a; O'Regan et al., 2007). In PRV, UL49 physically interacts with the cytoplasmic domains of the membrane protein US9 and the carboxyl terminus of gE/gI and gM (Fuchs et al., 2002a; Lee et al., 2008). Most recently, the interaction between the membrane proteins gK and pUL20 and pUL37 have been reported using co-immunoprecipitation experiments and proximity ligation assays (Jambunathan et al., 2014).

Viral membrane proteins play important roles in viral life cycle and in the interaction between the virus and the host. Various glycoproteins participate in virion attachment and entry through interacting with host cell receptors. Membrane proteins also play crucial roles during egress and envelopment as well as infectious virus production via interactions with the tegument (Roizman et al., 2007).

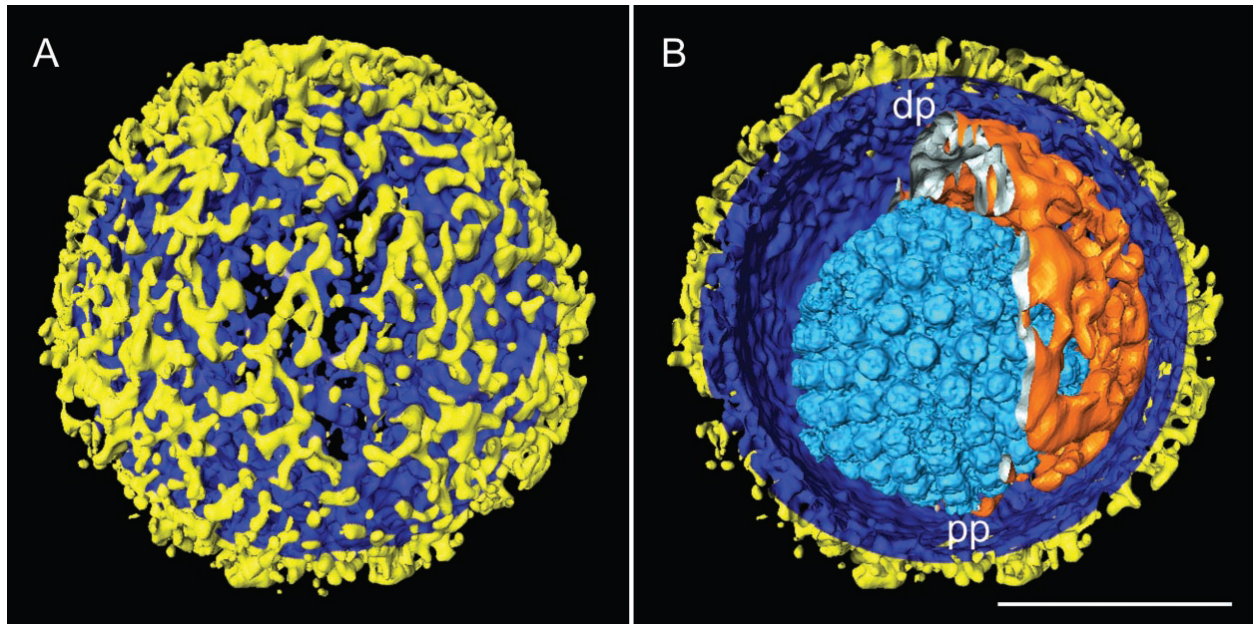


Figure 2.6: Three- dimensional tomographic reconstruction of HSV-1 virions after denoising. (A) Glycoprotein spikes in yellow are protruding from the membrane colored in blue. (B) Virion interior, the capsid is shown in light blue and the tegument “cap” (orange) inside the envelope (blue and yellow). pp, proximal pole; dp, distal pole. Scale bar, 100 nm. Adapted from (Grunewald et al., 2003).

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Glycoprotein B. Glycoprotein B is a 904 amino acid glycosylated transmembrane glycoprotein encoded by the gene UL27. gB is the most conserved component of the fusion machinery of herpes viruses. The extracellular portion (ectodomain) of gB is the largest domain composed of 696 aa with multiple N-glycosylation sites. The crystal structure of the gB ectodomain reflects features similar to both class I and class II fusion proteins with a striking

homology to glycoprotein G from vesicular stomatitis virus (VSV G). gB possesses an alpha-helical coil-coil core that relates it to class I fusion proteins and an extended beta hairpin with hydrophobic tips that relates gB to class II fusion proteins. The gB fusogenic activity is accomplished through a large scale conformational change of "open" and "closed" or folding and unfolding rearrangements of the trimetric gB complex (Figure 2.7)(Heldwein et al., 2006).

gB is one of four glycoproteins known as the fusion complex (gD, gH, gL, and gB). The interactions between gD and the gH/gL heterodimer with gB presumably trigger conformational rearrangements in gB to initiate fusion. It has also been shown that gB binds to cellular receptors such as the myelin-associated glycoprotein (MAG) paired immunoglobulin-like type-2 receptor alpha (PILR-alpha), and non-muscle myosin heavy chain IIA (NMHC-IIA) which may also assist in triggering the gB conformational changes necessary for fusion (Chowdhury et al., 2012; Connolly et al., 2011) (Arii et al., 2010), (Sato et al., 2008; Suenaga et al., 2010). Moreover, accessory proteins such as gK and UL20 may also be required for optimal fusion process. It has been reported that the direct interaction of gB with gK and UL20 modulates gB-mediated cell fusion (Chouljenko et al., 2010; Foster et al., 2004; Melancon et al., 2005).

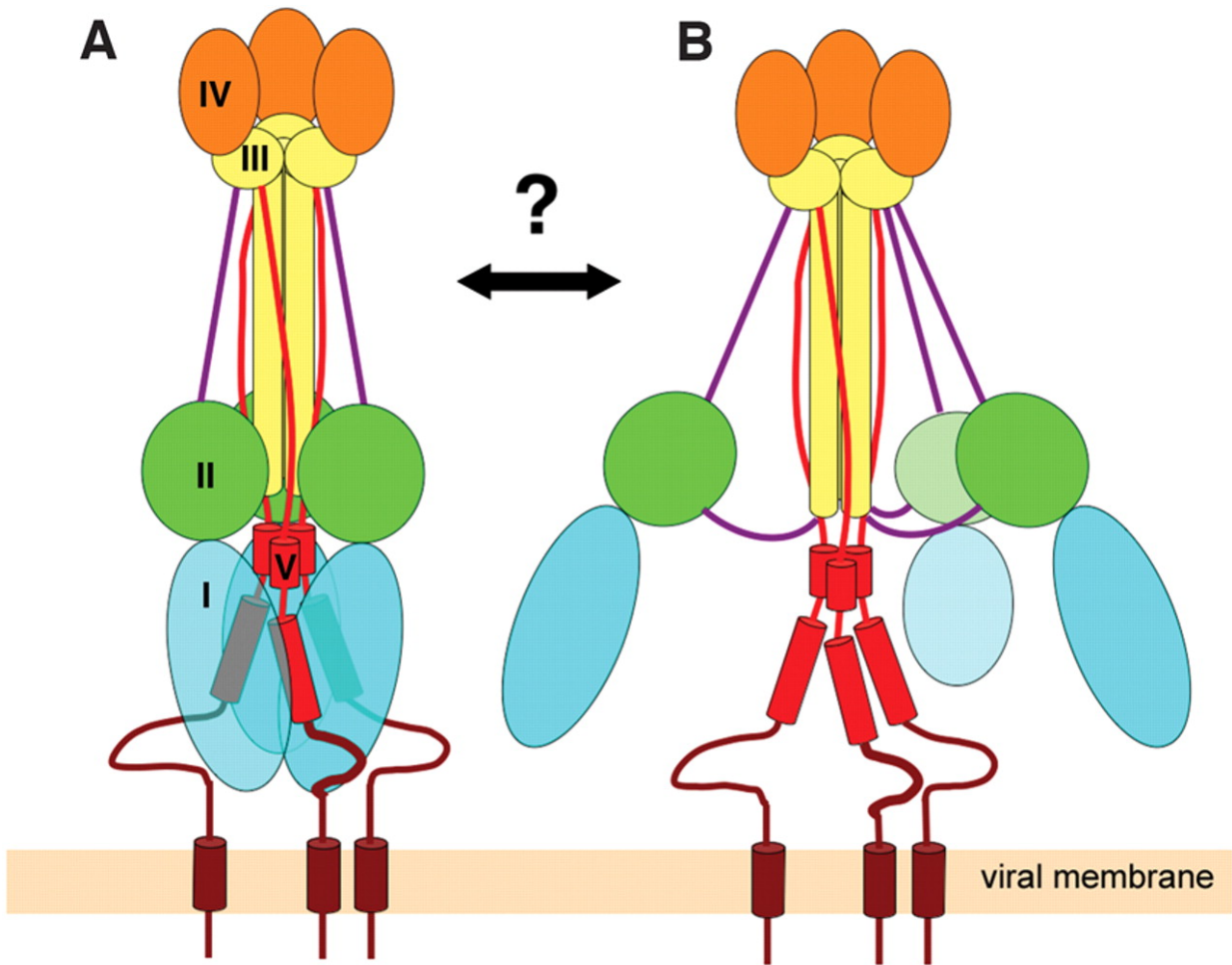


Figure 2.7: Schematic diagram of gB, showing conformational refolding between a “closed” (A) and “open” (B) state in an umbrellalike fashion. gB Domains are shown schematically in different colors. Adapted from (Heldwein et al., 2006).

In addition to its role in virus induced cell fusion during entry, gB has been shown to bind to Toll Like Receptor 2 /Toll-like Receptor 6 heterodimer leading to MyD 88 mediated activation of NF-kB (Cai et al., 2013).

Glycoprotein K. gK is a 338 aa glycosylated HSV-1 envelope protein that is encoded by UL53 gene. gK is highly conserved among all alphaherpesviruses. The secondary structure of gK is composed of an amino terminus and a carboxyl terminus both located extracellularly, four membrane spanning domains and small cytoplasmic domains. The amino terminus of gK contains a 30 aa signal sequence and two N-linked glycosylation sites at residues 48 and 58 (Figure 2.8; gK secondary structure)(Foster et al., 2003; Ramaswamy and Holland, 1992).

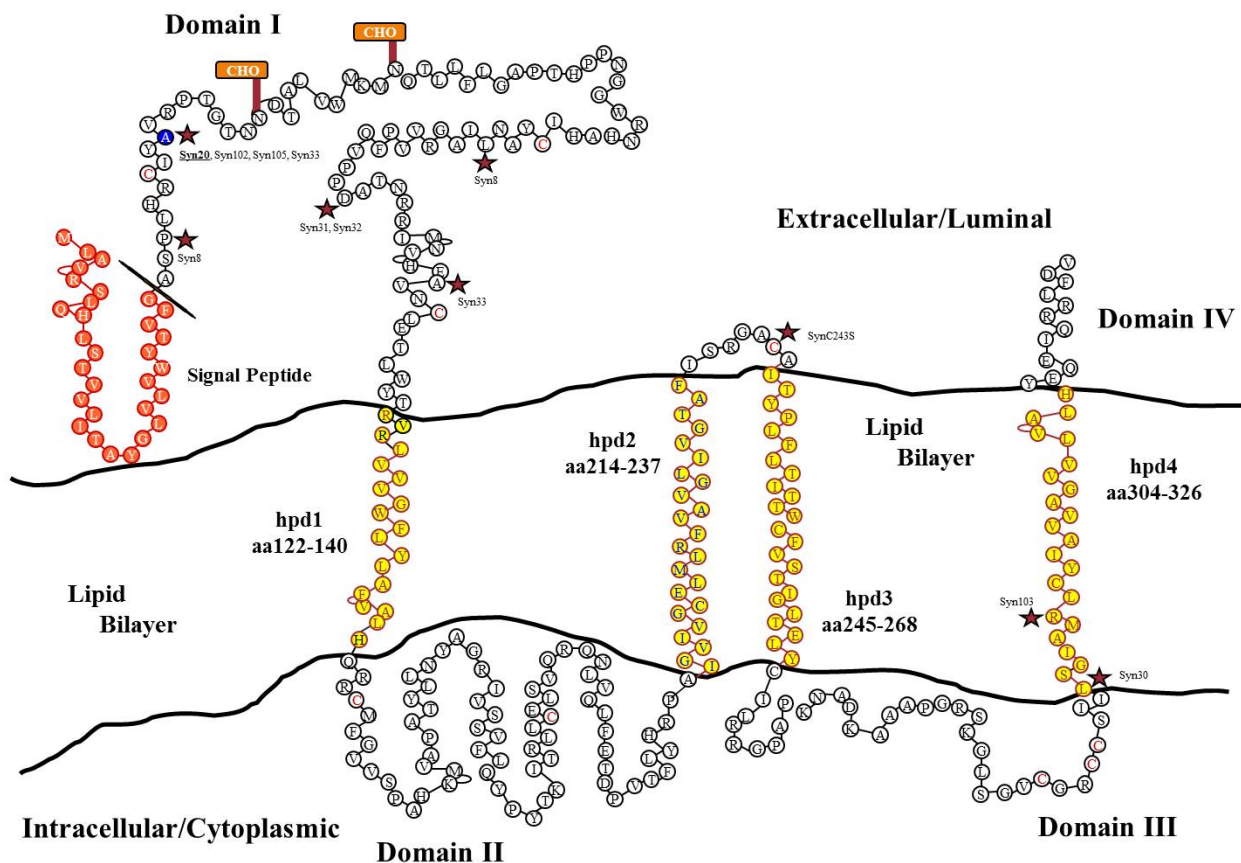


Figure 2.8: Schematic representation of the secondary structure of gK. The 30 amino acid signal peptide is shown in red. The four transmembrane domains are shown in yellow.

Several single amino acid mutations in gK, primarily in the amino terminus, have been shown to cause syncytia formation in tissue culture (Pogue-Geile et al., 1984; Ruyechan et al., 1979). gK forms a heterodimer with the non-glycosylated membrane protein UL20. The interaction between gK and UL20 is important for the transport of the protein complex (gK-UL20 heterodimer) through the Golgi network (Foster et al., 2008; Melancon et al., 2007). gK plays a critical role in the cytoplasmic virion envelopment and infectious virus production. gK deletion mutants accumulate in the cytoplasm as un-enveloped virions and are unable to egress from infected cells (Foster and Kousoulas, 1999; Hutchinson et al., 1992; Hutchinson and Johnson, 1995; Jayachandra et al., 1997; Ramaswamy and Holland, 1992). The role of gK in virus-induced cell fusion was studied, using a variety of deletion, truncation and syncytial mutants of gK. Work in Dr. Kousoulas' laboratory demonstrated that the amino terminus of gK physically interacts with the amino terminus of gB and modulates gB mediated virus induced cell fusion (Chouljenko et al., 2009; Chouljenko et al., 2010). Also, cleavage of the amino terminus of HSV-1 gK significantly reduced virus entry (Jambunathan et al., 2011). The amino terminus of gK is also required for efficient entry into Chinese Hamster Ovary cells (CHO cells) expressing the gB receptor; paired immunoglobulin-like type-2 receptor alpha (PILR α)(Chowdhury et al., 2013). gK overexpression was correlated with the accumulation of large numbers of virion particles in the perinuclear space (Hutchinson and Johnson, 1995). Mutant viruses lacking the expression of both gB and gK or gB and UL20 accumulated capsids in the cytoplasm suggesting that pUL20, gK and gB do not function redundantly in the de-envelopment process at the outer nuclear membrane (Melancon et al., 2005).

In addition to gK roles in; virus-induced cell fusion, envelopment and virion egress, it plays important roles in neuroinvasion and ocular pathogenesis (David et al., 2008; David et al., 2012). Further experiments to delineate gK domains that are important for neuroinvasion have demonstrated that the amino terminus of gK, however dispensable for replication in tissue culture, is important for neuroinvasion and the pathogenesis of keratitis (David et al., 2012; Kim et al., 2014; Saied et al., 2014). It has been shown that the signal peptide of gK contains a T cell epitope, however, since the signal peptide is cleaved, its significance in immunomodulation is yet to be determined (Osorio et al., 2007).

I collaborated with Misagh Naderi and Dr. Michal Brylinski at the Computational Systems Biology group, Louisiana State University to generate a predicted tertiary (folded) structure for gK (Figure 2.9) and UL20 protein. gK was modeled based on validated templates. The predicted structure showed that the transmembrane domains are alpha helices, the amino terminus of gK is composed of beta sheets and short alpha helices, and domain II folds into two connected alpha helices. The predicted folding of domain II into alpha helices suggests that this domain may have an important function, which is open for investigation. The gK tertiary structure prediction is still in progress as we are still addressing some important factors that, if considered in the model, may alter the predicted tertiary structure of gK.



Figure 2.9: The predicted tertiary structure of gK. The amino terminus in blue (dark and light), the last transmembrane domain and the carboxyl terminus are in red. Domain III is predicted to have two connected alpha helices.

Glycoprotein M. Glycoprotein M (gM) is a 473 aa, highly conserved type III integral membrane protein encoded by UL10 gene. gM forms a functional complex with pUL49.5 (gN). The structure of gM (Figure 2.10) is composed of amino terminus cytoplasmic domain that contains 2 initiation codons, 8 membrane spanning domains, 2 N-glycosylation sites in extracellular domains, and a carboxyl terminal cytoplasmic domain that contains 4 tyrosine-based endocytosis motifs (YXX Φ ; YDEV426, YAKI438, YDTV454 and YSTV470)(Baines and

Roizman, 1993; Crump et al., 2004; Roizman et al., 2007). Deletion UL10 gene does not abolish HSV-1 replication, but reduces the ability of the virus to spread from cell to cell in tissue culture (Browne et al., 2004; Chouljenko et al., 2012; Leege et al., 2009; MacLean et al., 1993). Glycoprotein M mutant viruses showed alterations in several viral membrane proteins localization patterns in the trans-Golgi network (TGN); which implies that gM may function in retention or retrieval of viral glycoproteins in or from the trans-Golgi network (TGN)(Crump et al., 2004; Klupp et al., 2000; Leege et al., 2009).

The syncytial phenotype caused by mutation in the carboxyl terminus of gB [A855V (gBsyn) and gB Δ 28] was inhibited by deletion of gM in these syncytia-forming viruses. Also, recombinant viruses lacking gM had slower entry kinetics in Vero cells (Balan et al., 1994; Davis-Poynter et al., 1994; Kim et al., 2013). Work in this laboratory showed that deletion of gM and the membrane protein UL11 affected membrane fusion events. Two-way co-immunoprecipitation experiments have detected an interaction between gM and UL20 (Kim et al., 2013).

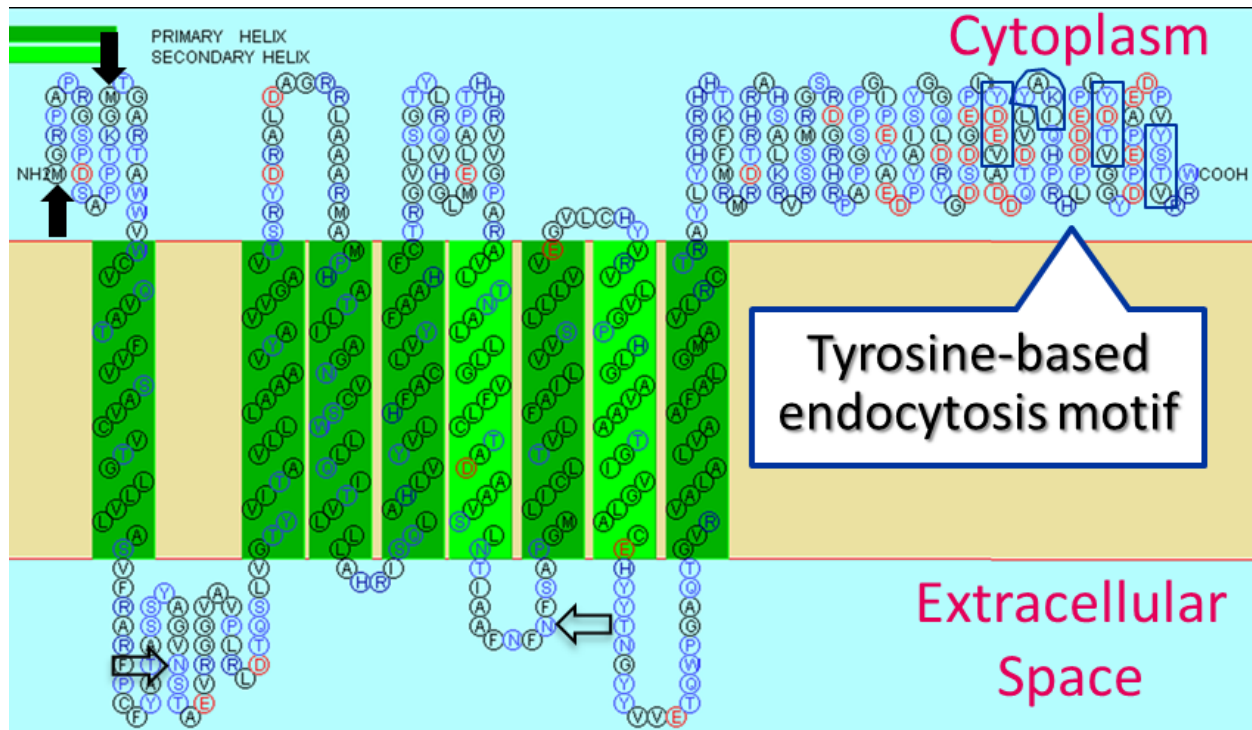


Figure 2.10: A schematic diagram of the predicted secondary structure of HSV-1 gM. The yellow box indicates plasma membrane. Two initiation methionines (empty arrows) and two potential N-glycosylation sites (black solid arrows), C-terminal tyrosine-based endocytosis motifs are indicated by blue boxes. The dark and light green boxes indicate alpha helices. [Structure built by SOSUI prediction (Hirokawa et al., 1998)].

UL 20 protein (pUL20). UL20 protein is 222 aa non-glycosylated membrane protein that is conserved in all alphaherpesviruses. The predicted secondary structure of pUL20 is composed of five domains; the amino terminus (Domain I) and the carboxyl terminus (Domain V) are located within the cytoplasm, 4 transmembrane domains and Domains II and IV located extracellularly (Figure 2.11).

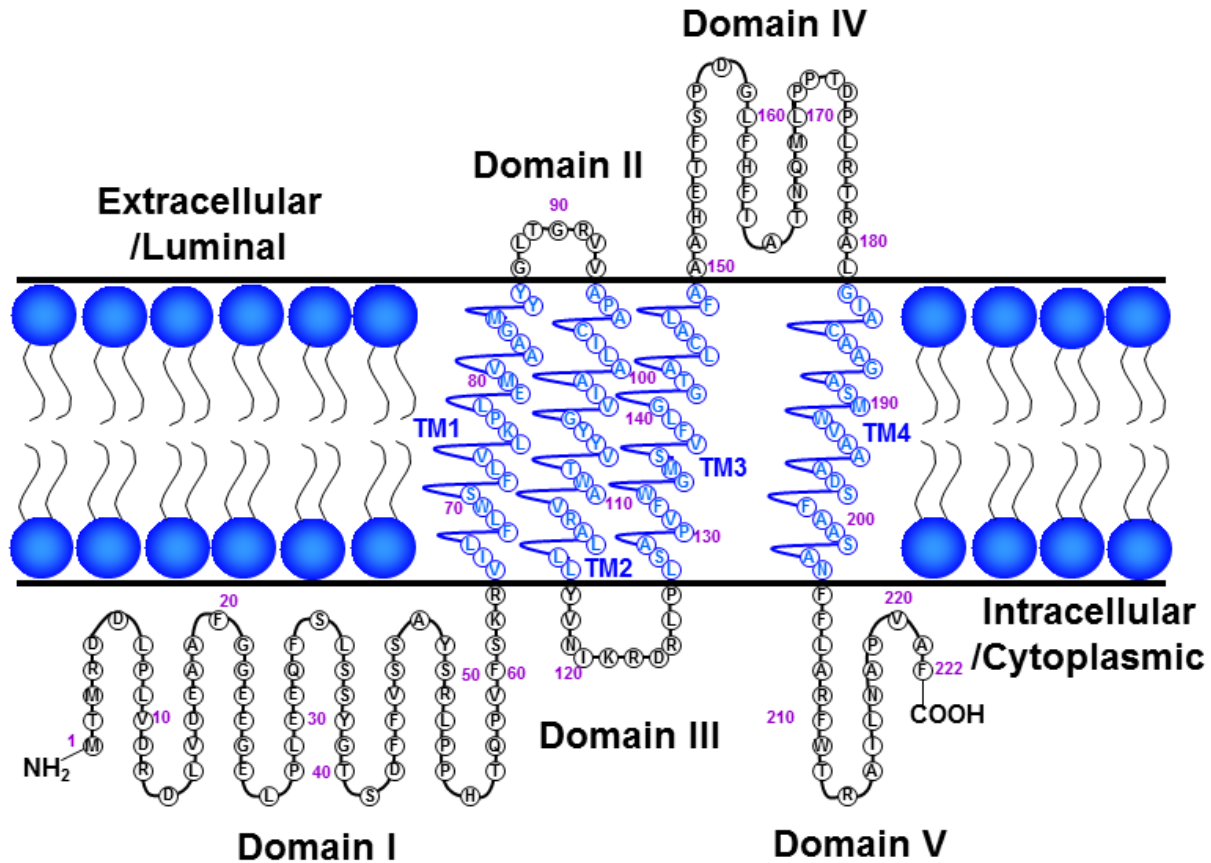


Figure 2.11: The predicted secondary structure of the UL20 protein. The protein is composed of four transmembrane domains and five predicted regions are shown. Adapted with permission from PhD dissertation titled “The Role of Viral Glycoproteins and Tegument Proteins in Herpes Simplex Virus Type 1 Cytoplasmic Virion Envelopment” by Dmitry V. Chouljenko, Louisiana State University, School of Veterinary Medicine, Department of Pathobiological Sciences, August 2014.

pUL20 forms a heterodimer with gK. The interaction between gK and UL20 is important for the transport of the protein complex (gK-UL20 heterodimer) through the golgi network (Foster et al., 2008; Melancon et al., 2007). This gK/pUL20 complex functions in virion envelopment, egress, and virus-induced cell fusion. pUL20 functional domains play independent roles in virus-induced cell fusion and cytoplasmic virion envelopment; mutations in tyrosine residues in the amino terminus of pUL20 compromised infectious virus production

and virus induced cell fusion, however, allowed efficient intracellular transport and TGN localization of UL20p and gK (Melancon et al., 2004; Melancon et al., 2007). Most recently, work in this laboratory showed that phenylalanine residues at the carboxyl terminus of pUL20 play crucial roles in viral cytoplasmic envelopment and infectious virion production. Interestingly, replacement of phenylalanine-to-alanine at amino acid position 210 in the carboxyl terminus of pUL20 caused a gain-of-function phenotype (Charles et al., 2014).

Life Cycle of Herpes Simplex Virus Type 1

Infection with HSV-1 starts at the mucocutaneous membranes where the virus replicates and then infects axonal endings and transports in retrograde manner to the body of the neuron in the trigeminal ganglia to establish latent infection. Exposure to UV light or stressors leads to reactivation of the virus whereby virions transport in anterograde manner to the primary site of infection for lytic replication and viral shedding.

Infection of epithelial cells at the primary site of infection is a multistep involving virus entry, transport of capsids to the nucleus, viral gene expression and DNA replication, capsid assembly, primary envelopment, secondary envelopment, and egress and maturation of infectious virions. During entry, viral glycoproteins interact with cell surface receptors for the attachment, binding and fusion between viral envelope and the cell membrane. After entry, the virus sheds the envelope at the surface and the capsid and the associated inner tegument proteins travel to the nucleus, dock at the nuclear pore complex and deposit viral DNA to the nucleus leading to the start of another viral replication cycle. In the nucleus of neurons,

however, the virus establishes latency for the life of the host. During latency, viral DNA associates with nucleosomes in a circular episome form, and the Latency Associated Transcript (LAT) is produced. Assembly of capsids and packaging of DNA take place in the nucleus.

In a process called primary envelopment, nucleocapsids travel to the cytoplasm by moving across the nuclear membrane. In the cytoplasm, tegument proteins are assembled on the nucleocapsid. The interaction between tegument proteins and viral envelope protein-enriched TGN-derived vesicles facilitates acquiring the envelope in a process known as secondary envelopment. Enveloped virions enclosed inside the vesicles transport to plasma membrane to release virions into the extracellular environment (Figure 2.12) (Bastian et al., 1972; Roizman et al., 2007; Vittone et al., 2005).

Viral attachment, binding and entry

Mature infectious virion particles initiate the infection by virion attachment to the cellular receptors. This attachment step is mediated by binding of the amino terminus glycoprotein C (gC) to cell surface receptors such as heparan sulfate and chondroitin sulfate glycosaminoglycans (GAGs)(Campadelli-Fiume and Menotti, 2007; Herold et al., 1991; Roizman et al., 2007; Tal-Singer et al., 1995; WuDunn and Spear, 1989). It has also been shown that gB participates in the attachment step by binding to GAGs on cell surfaces(Laquerre et al., 1998). The ability of gB to mediate the attachment may explain the observation that deletion of gC does not impair viral attachment(Griffiths et al., 1998). Interestingly, deletion of heparan

sulfate and chondroitin sulfate glycosaminoglycans (GAGs) significantly reduces viral infectivity but does not completely abolish infection from taking place (Gruenheid et al., 1993). The attachment step is reversible and does not require energy (Campadelli-Fiume and Menotti, 2007).

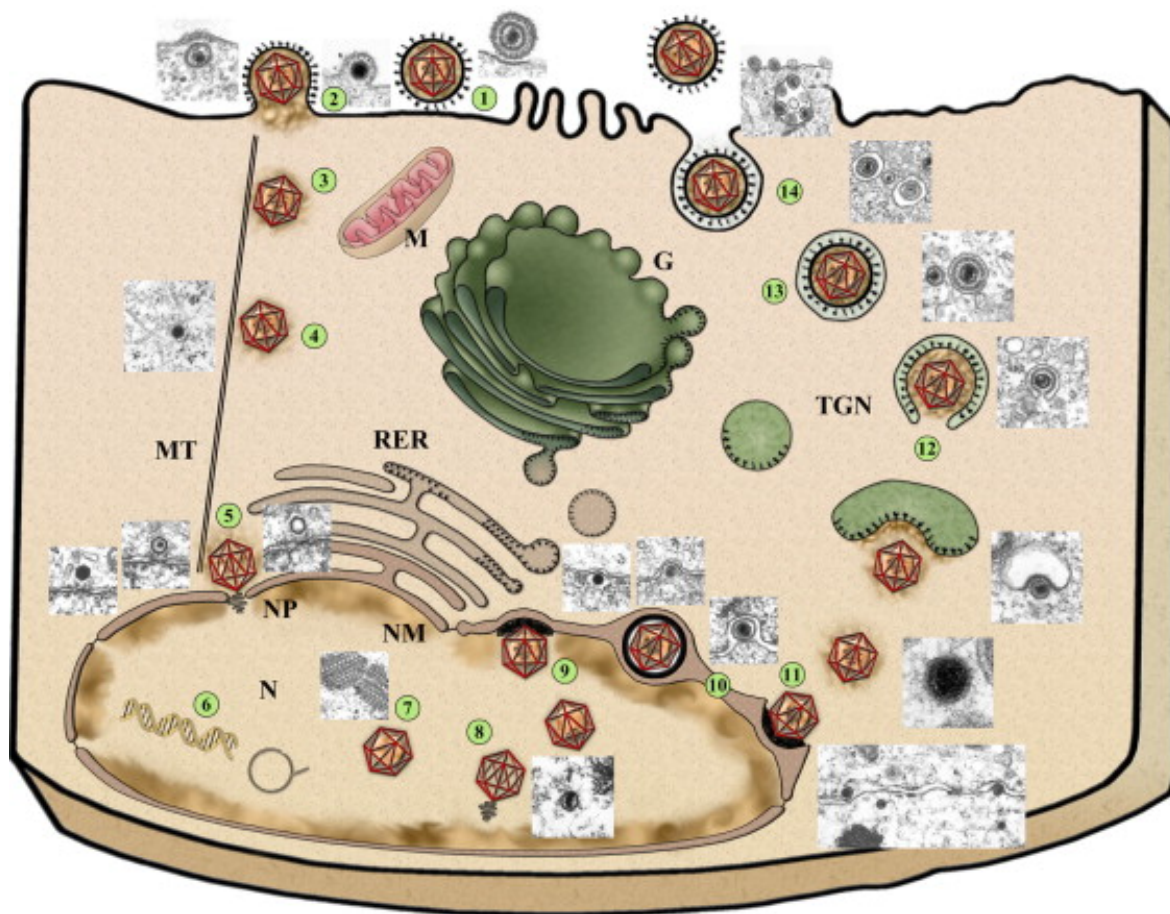


Figure 2.12: Schematic diagram of Alphaherpesviruses life cycle. Electron micrographs of the respective stages are shown. (1) Attachment. (2) Penetration. (3) Transport of the capsid to the nucleus (N). (4) Interaction of nucleocapsid with microtubules (MT). (5) Docking at the nuclear pore (NP). (6) Release of viral genome into the nucleus. Here, transcription of viral genes and genome replication takes place. (7) Assembly of Capsids. (8) Encapsidation of viral genome into preformed capsids. (9) Newly formed nucleocapsids leave the nucleus by budding at the Inner Nuclear Membrane (INM). (10) Virions located in the perinuclear space fuse (11) with the outer nuclear membrane. (12) Intracytosolic capsids bud into vesicles of the Trans-Golgi Network (TGN) in a step called secondary envelopment. After transport to the cell surface (13), (14) vesicle and plasma fuse to release mature virions to the extracellular space. Adapted from (Mettenleiter et al., 2009).

Typically, herpes simplex viruses enter cells via fusion of the viral envelope with plasma membranes; however, it can enter a wide range of cells via either pH independent or pH-dependent endocytosis (Milne et al., 2005; Nicola et al., 2003). The entry step is mainly mediated by the interaction between the ectodomain of Glycoprotein D (gD) with cellular receptors. The transmembrane and cytoplasmic domains of gD do not seem to play a role in viral entry, however, they serve to anchor gD to the viral envelope (Browne et al., 2003).

Cellular receptors for gD include members of tumor necrosis factor receptor family (TNF) such as herpes virus entry mediator A (HVEM or HveA). This family of cellular receptors is expressed on many mammalian epithelial cells, fibroblasts as well as cells of the immune system such as B and T lymphocytes and monocytes, however, neurons do not express this family of receptors (Kwon et al., 1997; Spear, 2004). Members of immunoglobulin superfamily also serve as receptors for gD; examples include nectin-1, nectin-2, paired immunoglobulin-like type 2 receptor alpha (PILR-2 alpha) and 3-O sulfated heparan sulfates. Nectin 1 alpha and beta are expressed on neuronal cells as well as epithelial cells fibroblasts and hematopoietic cells (Cocchi et al., 1998a; Cocchi et al., 1998b; Geraghty et al., 1998). Binding of gD to its cognate receptor triggers conformational changes in gB, gH, and gL which leads to the fusion of viral envelope with cell membrane (Campadelli-Fiume and Menotti, 2007; Cocchi et al., 2004; Roizman et al., 2007). 3-O-sulfated heparan sulfate is expressed in neurons as well as a variety of other tissues and serves as a receptor for the ectodomain of gD (Campadelli-Fiume and Menotti, 2007).

Fusion activity in HSV is executed by a multipartite fusion complex composed of gB and the gH/gL heterodimer. The presence of this fusion machinery is absolutely required for fusion to proceed (Cai et al., 1988; Campadelli-Fiume and Menotti, 2007; Forrester et al., 1992; Roop et al., 1993). gL is necessary for incorporation of gH in the virion particle and its absence abrogates entry (Roop et al., 1993). It has been shown that gB binds to a variety of cellular receptors including the paired immunoglobulin-like type-2 receptor alpha (PILR- α), myelin-associated glycoprotein (MAG) and non-muscle myosin heavy chain IIA (NMHC-IIA), however, the importance of this binding in the fusion process is yet to be determined (Arii et al., 2010; Chowdhury et al., 2012; Satoh et al., 2008; Suenaga et al., 2010).

The fusogenic activity of gB is regulated by accessory proteins. Work in this laboratory has shown that gK and UL20 directly interact with gB and modulate its function in virus induced cell fusion. gM may indirectly participate in optimal gB fusogenic activity through interaction with pUL20 (Chouljenko et al., 2010; Foster et al., 2004; Kim et al., 2013; Melancon et al., 2005).

Transport of capsids to the nucleus

Upon fusion, the viral envelope is shed at the cell membrane and the nucleocapsids and tegument proteins are deposited in the cytosol. The outer tegument proteins such as pUL41, pUL47 and pUL48 (VP16) then dissociate from the nucleocapsid and the associated inner tegument proteins (Liu et al., 2010; Mettenleiter, 2006). pUL41, also known as the Virion Host Shutoff protein (VHS) inhibits the production of host proteins by inhibiting host mRNA synthesis and processing, and by speeding mRNA degradation. VHS targets both host and viral mRNAs, however, at late stages of infection viral mRNAs are rescued by VP16; which interacts with

pUL41 to inhibit its activity and to facilitate its incorporation into virion particles (Everly et al., 2002; Lam et al., 1996; Roizman et al., 2007; Taddeo and Roizman, 2006). VP16 (pUL48), also known as alpha trans-inducing factor (α TIF) travels to the nucleus, forms a complex with the cellular protein Oct-1 and initiates the transcription of viral immediate early genes (Newcomb and Brown, 2009). Nucleocapsids and the associated inner tegument proteins are propelled along microtubules to the nucleus (retrograde transport) via the cellular motor molecules complex called Dynein. Anterograde transport from the nucleus to the plasma membrane is mediated by the Kinesin motor molecule (Diefenbach et al., 2008; Garner, 2003; Sodeik et al., 1997). Inner tegument proteins pUL36, pUL37 and US3 remain in the capsid and their presence is required to recruit cellular transport motor molecules (Sodeik et al., 1997). After transport, capsids dock onto the nuclear pore complex and the viral DNA is extruded from the capsid into the nucleus (Figure 2-13). pUL36 plays an important role in DNA deposition into the nucleus through its interaction with the nuclear pore complex proteins (Batterson et al., 1983; Batterson and Roizman, 1983; Ojala et al., 2000; Sodeik et al., 1997). pUL36 acts as a plug to keep viral DNA packaged in the capsid. Cleavage of pUL36 is necessary to release viral DNA into the cell nucleus (Jovasevic et al., 2008). pUL25 is also involved in the release of viral genome into the nucleus; pUL25 interacts with nucleoporins CAN/Nup214 and hCG1. pUL25 also binds to the portal protein pUL6 and pUL36 (Jovasevic et al., 2008).

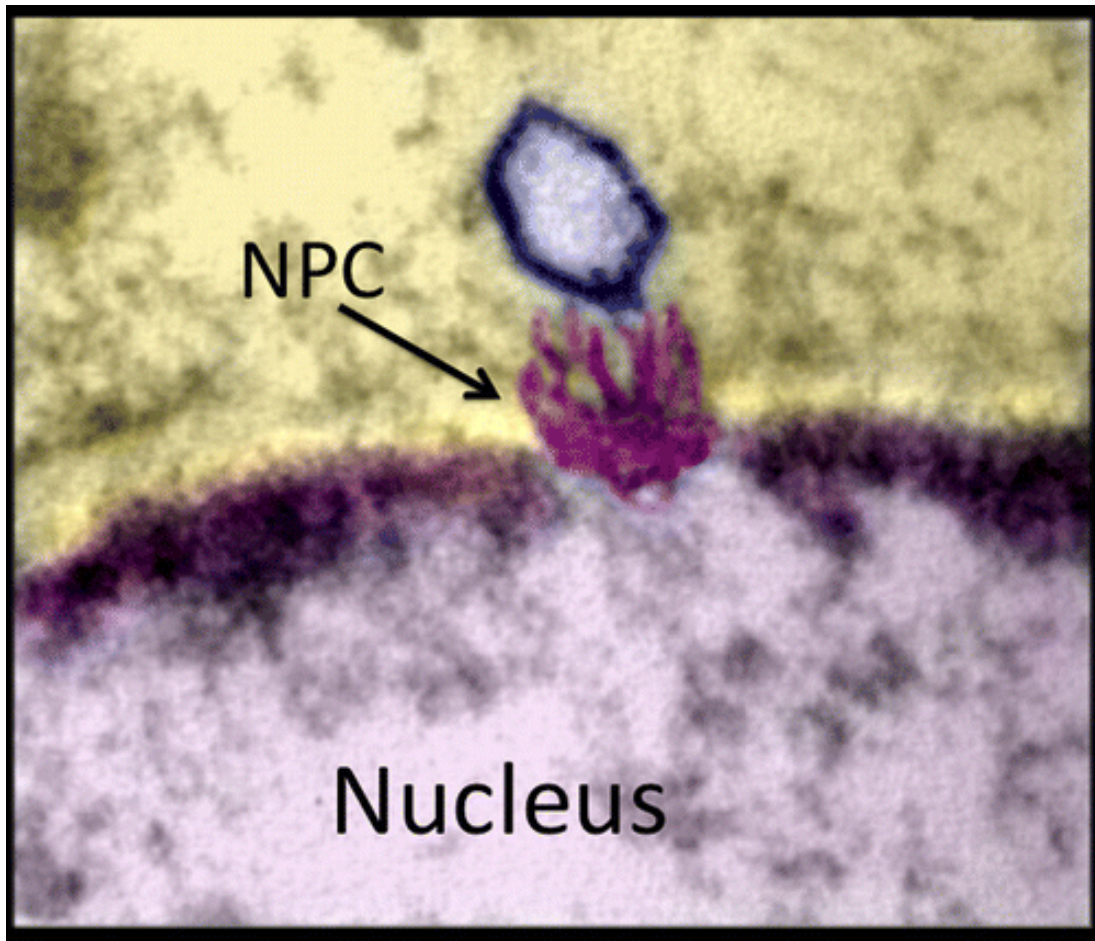


Figure 2.13: A colored electron micrograph of HSV-1 capsid at the nuclear pore complex (NPC). The figure shows an empty capsid shortly after deposition of viral DNA through the NPC. Adapted from (Bauer et al., 2013).

Viral gene expression and DNA replication

After deposition of viral genome in the nucleus, viral DNA circularizes forming endless genomes. It has been shown that cellular DNA ligase IV and the DNA repair protein XRCC4 are required for the formation of circular viral genomes during productive infection (Muylaert and Elias, 2007; Strang and Stow, 2005). Production of viral proteins can be classified based on the temporal gene expression into; immediate early genes or alpha gene expression, early genes or

Beta gene expression, and late genes or Gamma gene expression (Roizman et al., 2007). As discussed before, VP16 (alpha-TIF) dissociates from the tegument once in the cytoplasm, binds to host C1 and the complex then enters the nucleus and binds to Oct-1(Katan et al., 1990; Kristie and Sharp, 1990). This tripartite complex initiates the transcription of viral immediate early genes (alpha genes) (Newcomb and Brown, 2009). Alpha genes; ICP0, ICP4, ICP22, ICP27, ICP47 and pUS1.5 are expressed immediately after infection and their products peak at 2-4 hours post infection. In general, alpha gene products are required for the activation of the expression of beta genes. ICP0 stimulates expression of all classes of viral genes (Everett, 2000). ICP4 is required for transcription of early and late gene promoters(Everett, 2000). Expression of beta genes peaks at 4-8 hours after infection. Beta gene products function in viral DNA replication process and gamma gene expression. Examples of beta gene products include; ribonucleotide reductase, thymidine kinase, viral DNA polymerase (UL30), DNA binding proteins (UL42 and UL29 or ICP8), ORI binding protein (UL9), and the helicase/primase complex (UL5, 8, and 52)(Muylaert et al., 2011; Roizman and Campadelli-Fiume, 2007). Gamma genes include structural viral proteins, tegument and glycoproteins and their gene expression peaks after viral DNA replication process has started. Gamma genes are divided into gamma 1 and gamma 2 genes based on their temporal expression. Gamma 1 gene expression starts earlier sometimes as early as alpha genes (known as leaky genes), and gamma 2 genes are expressed only after DNA replication has begun. An example of gamma 2 genes include gK (Roizman and Campadelli-Fiume, 2007). During viral replication, the origin-binding protein pUL9 facilitates loading of the viral replisome complex on viral genome origins of replication. Viral replisome complex is composed of the viral DNA polymerase (pUL30 and pUL42), a helicase-primase (pUL5, pUL8 and

pUL52), and the single-stranded DNA-binding protein pUL29 (ICP8)(Muylaert et al., 2011). Viral DNA replication uses a rolling circle mechanism and the newly formed viral genomes are produced. Host RNA polymerase II handles viral RNA transcription using the newly synthesized viral DNA as a template (Costanzo et al., 1977; Muylaert et al., 2011). In neurons, the virus establishes a latent infection where the productive lytic sequence of gene expression does not occur. Instead, viral genome remains in the nucleus in an episomal form and the non coding RNA latency associated transcript (LAT) accumulates in the nuclei of neurons. LAT silences other viral genes and protects neurons from apoptosis (Perng et al., 2000).

Capsid assembly and DNA packaging

Viral proteins are synthesized in the cytoplasm of infected cells. The structural components of the capsid are then imported to the nucleus for assembly. The overlapping UL26 and UL26.5 gene products are cleaved to produce the scaffolding proteins VP21 and VP22a. UL26.5 also produces VP24 which is a protease that cleaves viral capsid scaffold proteins. Two copies of VP19C and one copy of VP23 form a complex in the cytoplasm and transport together into the nucleus. Within the viral nuclear inclusion bodies, the major capsid protein VP5 binds to the scaffolding proteins VP21 and VP22a forming 125 nm spherical pro-capsids as hexones, pentons and triplexes are assembled(Brown and Newcomb, 2011; Nicholson et al., 1994; Rixon et al., 1996; Roizman et al., 2007). DNA packaging into the capsid occurs through the portal (composed of 12 copies of pUL6). The viral terminase complex composed of pUL15, pUL28 and pUL33 aids in viral DNA packaging by cleaving the viral DNA concatamers into monomers. Maturation of capsid takes place as VP24 (protease) cleaves the scaffolds and the capsid starts

to assume mature polyhedral shape(Brown and Newcomb, 2011). As the capsid angularizes, VP26 assembles on the maturing capsid in an energy dependent manner(Chi and Wilson, 2000). After that, pUL36 is recruited at the portal to serve as plug keeping the viral DNA securely contained within the capsid(Jovasevic et al., 2008).

Primary envelopment

The relatively small nuclear pore (~39 nm) does not allow the passage of the 125 nm diameter of the viral capsid to the cytoplasm. The relatively large capsids bud into the inner nuclear membrane forming enveloped capsids in the peri-nuclear space, then the enveloped capsids undergo de-envelopment at the outer nuclear membrane to exit into the cytoplasm. This model is known as the envelopment and de-envelopment pathway (Hofemeister and O'Hare, 2008; Johnson and Baines, 2011).

Acquisition of an envelope at the inner nuclear membrane is mediate by pUL31 and pUL34. pUL34 is a virus-encoded membrane anchored protein present at the inner nuclear lamina and forms a heterodimer with pUL31(Shiba et al., 2000). The pUL34/pUL31 complex, also known as the nuclear egress complex (NEC), recruits viral and cellular kinases to phosphorylate and soften the nuclear lamina (Mettenleiter et al., 2013; Reynolds et al., 2001; Roller et al., 2000). The NEC recruits cellular kinases such as protein kinase C alpha and C delta to phosphorylate lamin B, whereas, lamin A and lamin C are phosphorylated by the viral kinases pUS3 and pUL13. Phosphorylation of lamins disrupts and dissolves the nuclear lamina allowing the interaction of nucleocapsids with the inner nuclear membrane (Mettenleiter et al., 2013; Mou et al., 2007; Park and Baines, 2006). C-capsids (DNA-containing capsids) are preferentially

selected for the primary envelopment process. UL31 protein favorably binds to pUL25 component of the C-capsid specific (CCSCs) at capsid vertices. It can also interact with the pUL17 CCSC component in the absence of pUL25 (Yang et al., 2014). Both pUL31 and pUL34 have been detected in virions present in the perinuclear space. Interestingly however, mature virions do not contain pUL31 and pUL34 (Fuchs et al., 2002b).

Nucleocapsids with a primary envelope are already coated with a thin layer of tegument proteins, and the rest of tegument proteins will be added before or during secondary envelopment (Mettenleiter, 2002; Mettenleiter et al., 2009; Padula et al., 2009). In a process called de-envelopment, the enveloped nucleocapsids in the perinuclear space fuse with the outer nuclear membrane to exit to the cytoplasm. The core fusion machinery gB and gH/gL which is involved in virion fusion during entry also mediates the de-envelopment process. Deletion of gB and gH leads to accumulation of enveloped virions in the peri-nuclear space (Farnsworth et al., 2007b; Wright et al., 2009). Furthermore, phosphorylation of gB by the viral kinase pUS3 promotes the fusion of enveloped nucleocapsids with the outer nuclear membrane (Wisner et al., 2009).

Secondary envelopment

Nucleocapsids released into the cytoplasm acquire more tegument as they travel to the site of secondary envelopment at trans-Golgi network (TGN) derived vesicles and endosomes. Already covered with a thin layer of tegument, capsids get coated with more pUL36 and pUL37 tegument proteins. Nucleocapsids are propelled along microtubules to the sites of secondary envelopment at the TGN as a result of the interaction between pUL36 and pUL37 tegument

proteins and the cellular motor molecule Kinesin. Also, the presence of pUL36 and pUL37 is required for the successful incorporation of other tegument proteins (Luxton et al., 2006; Sandbaumhuter et al., 2013).

Additional tegument proteins are added to the capsid during the secondary envelopment; tegument proteins bind to the cytoplasmic surface of TGN membranes and as the capsids bud into TGN derived vesicles they acquire envelopes (Johnson and Baines, 2011).

After expressed in the cytoplasm, viral envelope proteins are sorted to the TGN membranes where the carboxyl termini facing towards the cytoplasm. Viral glycoproteins are subjected to modifications by mannose-6-phosphate (M6P), and they contain a variety of sorting domains, including di-leucine motifs, tyrosine motifs and acidic clusters, similar to those found on host TGN proteins. The above mentioned modifications and the presence of sorting domains facilitate sorting of viral glycoproteins such as gB, gD, gE/gI to TGN (Beitia Ortiz de Zarate et al., 2004; Gu et al., 2001; McMillan and Johnson, 2001).

A complex network of interactions between the capsid and tegument proteins, and the carboxyl termini of envelope glycoproteins facilitate secondary envelopment (Figure 2.14).

The inner tegument protein pUL36 have been shown to interact with capsid proteins VP5 and pUL25 and acts as the link between the capsid and the outer tegument proteins (Bucks et al., 2011; Coller et al., 2007). Also, pUL36 and pUL37 interact with pUL48 and pUL46, respectively. pUL48, an outer tegument protein, interacts with the tegument proteins pUL41, pUL46, pUL47 and pUL49 (Elliott et al., 1995; Newcomb and Brown, 2009; Smibert et al., 1994; Vittone et al., 2005). pUL21 and pUL16 form a complex; this interaction has been documented

in HSV-1 and PRV (Klupp et al., 2005). pUL16 also interacts with the membrane-associated tegument protein UL11 (Loomis et al., 2003; Yeh et al., 2008). pUL11 in turn, interacts with gD and gE serving as a bridge between the tegument covered capsid and the envelope to facilitate secondary envelopment (Farnsworth et al., 2007a; Meckes et al., 2010; Yeh et al., 2008). The cytoplasmic domains of gE and gD and gM interact with pUL49 (VP22) which presence is important for the incorporation of gD, gE/gI and ICP0 into virions (Stylianou et al., 2009) (Chi et al., 2005; Farnsworth et al., 2007a; O'Regan et al., 2007). Interestingly, absence of pUL49 (VP22) does not affect the incorporation of ICP4, the binding partner of ICP0, in virion particles (Duffy et al., 2006; Elliott et al., 2005; Yao and Schaffer, 1994). Recruitment of VP22 into virion particles is also dependent on its interaction with the cytoplasmic tail of gE (Stylianou et al., 2009). The interaction between pUL48 and the cytoplasmic portions of gB, gD and gH has been documented using chemical cross-linking assays and protein pull-down experiments (Foster and Kousoulas, 1999; Kamen et al., 2005; Zhu and Courtney, 1994). In addition to phosphorylation of nuclear lamina, pUL13 phosphorylates both gE and pUL49 and modulates pUL41 function (Coulter et al., 1993; Ng et al., 1998; Overton et al., 1994).

Another complex network of interactions between viral membrane and the inner tegument has been reported. Proximity ligation assay and co-immunoprecipitation experiments showed that the inner tegument protein UL37 binds to the membrane proteins pUL20 and gK highlighting (Jambunathan et al., 2014). Also, the cytoplasmic portion of gD has been shown to bind directly to the capsid (Chi et al., 2005).

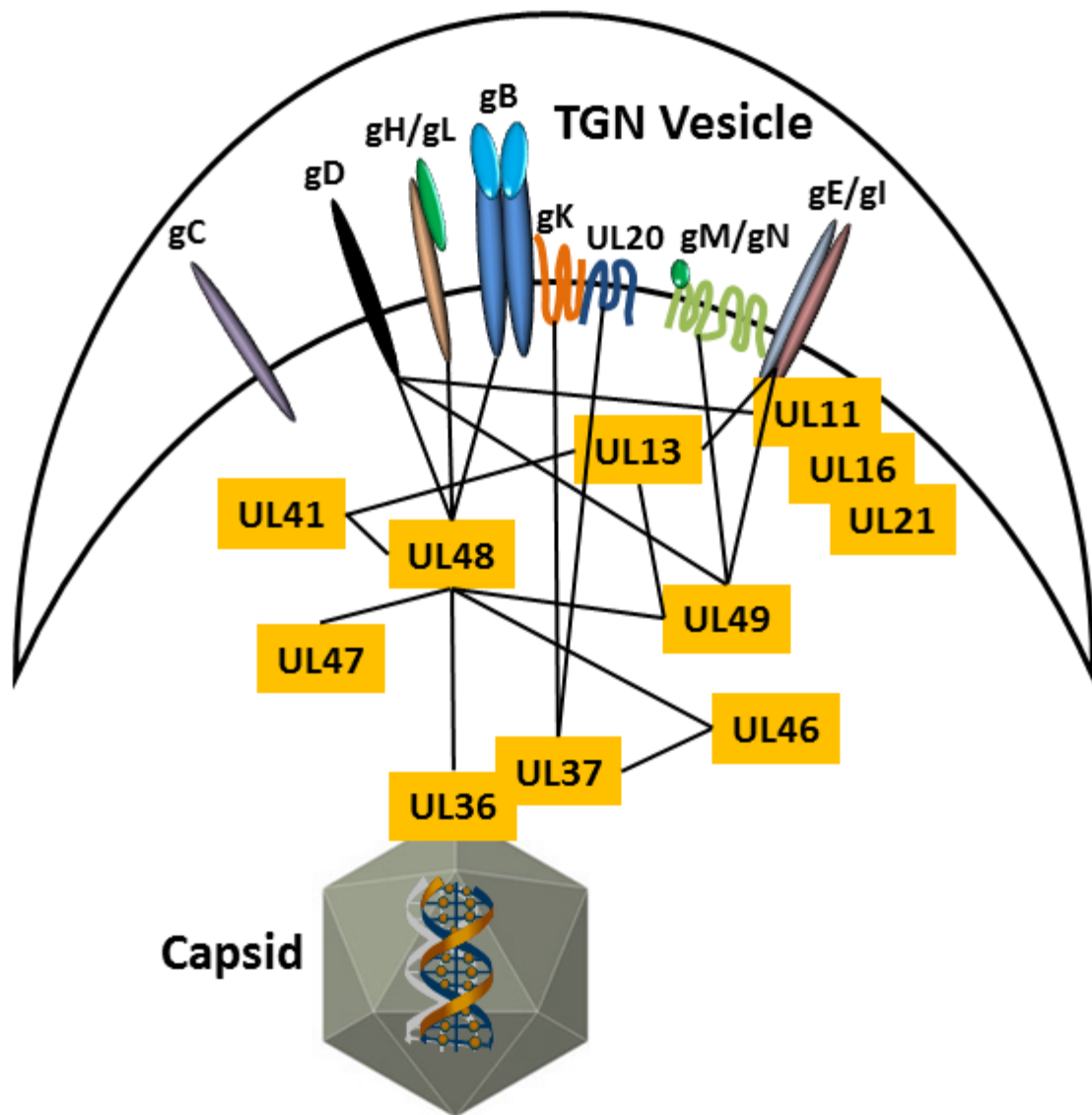


Figure 2.14: Schematic illustration of viral protein interactions during secondary envelopment. Yellow rectangles denote tegument proteins. Adapted with permission from PhD dissertation titled “The Role of Viral Glycoproteins and Tegument Proteins in Herpes Simplex Virus Type 1 Cytoplasmic Virion Envelopment” by Dmitry V. Chouljenko, Louisiana State University, School of Veterinary Medicine, Department of Pathobiological Sciences, August 2014.

gK and the non-glycosylated membrane protein UL20 play important roles in cytoplasmic envelopment and egress (Foster and Kousoulas, 1999; Jambunathan et al., 2014). And as discussed above, the gD (carboxyl terminus), gE, gM, and the membrane-associated protein UL11 play important roles in cytoplasmic virion envelopment and egress from infected

cells. The relative importance of gD, gE, gM, gK, pUL20 and pUL11 in virion secondary envelopment and egress have been studied. Lack of gK and UL20 causes the most severe perturbances in virion envelopment and egress (Chouljenko et al., 2012; Foster and Kousoulas, 1999; Jambunathan et al., 2014).

Nucleocapsids obtain envelopes as they bud into vesicles derived from TGN. These vesicles are enriched with viral envelope glycoproteins. This budding process is topologically similar to the formation of intraluminal vesicles in multivesicular bodies in cells. Formation of intraluminal vesicles in multivesicular bodies is mediated by the endosomal sorting complexes required for transport (ESCRTs) and the ATPase VPS4 complexes. Experiments using ESCRT-dominant-negative cells, as well as RNA interference experiments revealed that ESCRT proteins are likely involved in virion envelopment in both HSV-1 and HCMV. It has been shown that gK possesses a putative ESCRT-binding motif which possibly participates in the recruitment of ESCRT protein to facilitate membrane curvature during the budding process. Interestingly, ESCRT-III proteins and VPS4A/B have been shown to be incorporated into mature HSV-1 virions (Pawliczek and Crump, 2009).

Egress of enveloped virions

After secondary envelopment, enveloped virions enclosed within TGN derived vesicles are transported to the plasma membrane and released to the extracellular space via exocytic pathways (Johnson and Baines, 2011; Mettenleiter, 2002; Mettenleiter et al., 2009). The transport process is assisted by myosin Va and the cellular serine-threonine protein kinase D. Myosin Va translocate enveloped virion laden cellular vesicles along cortical actin filaments

(Roberts and Baines, 2010). The cellular serine-threonine protein kinase D is involved in scission of TGN vesicles. It also regulates their transport to the basolateral cell membrane (Remillard-Labrosse et al., 2009). In polarized epithelial cells, TGN-derived vesicles containing enveloped virions are sorted to cell-cell junctions where they fuse with the plasma membrane and release virions (Mettenleiter, 2002). Sorting of virus containing TGN vesicles to cell junctions is presumably mediated by gE/gI membrane protein complex and the inner tegument protein pUL37 (Johnson et al., 2001; Pitts et al., 2014). Placing virions at the cell to cell junction facilitates cell to cell spread and protects the virus from exposure to host immune response elements such as antibodies and complement (Johnson and Baines, 2011). Syncytial cells are large multinucleated cells formed by the fusion of cell membranes of multiple cells after infection in vivo. Plasma membranes of infected cells are enriched with viral glycoproteins. Viral entry fusion machinery expressed on infected cell membranes mediate the fusion of adjacent cells creating syncytia. Syncytia formation is particularly beneficial to the virus as it helps the virus hide from immune surveillance (Roizman et al., 2007). The fusion of TGN-derived vesicles containing enveloped virions is unlikely mediated by viral glycoprotein embedded in the vesicles membranes. Viral glycoproteins embedded in TGN-derived vesicles are oriented so that the carboxyl termini are to the cytoplasm. The amino termini of these glycoproteins are located within the vesicles and do not come in contact with the cell plasma membrane to mediate fusion (Johnson and Baines, 2011).

Retrograde and Anterograde Transport of Alphaherpesviruses in Neurons

Neuronal structure and axoplasmic transport

Neurons are highly polarized cells with unique structure; they are composed of cell bodies which contain the nucleus, and highly active RNA transcription machinery known as Nissl substance. Neurons have dendritic extensions and an axon that specializes in initiation; propagation and transmission of electrical impulses. The axon has an efficient transport system for long distance cargo transport to move molecules and organelles between the body of the body of the neuron and axonal termini. Microtubules are key structural elements in the axon. Microtubules are composed of heterodimeric subunits of alpha and beta tubulins that assemble in a linear head to tail configuration to form protofilaments. Then, 13 protofilaments form a hollow conduit, approximately 24 nm in diameter. Microtubules assemble from Microtubule Organizing Center (MTOC) with distinct polarity; in the axon they are oriented so that an active + end is towards the axonal termini, whereas, a stable – end is oriented towards the MTOC. In dendrites, microtubules have mixed polarity. The end microtubule at the proximal axon contains filamentous actin, microtubule end binding (EB) proteins EB1 and EB3. Ankyrin G acts as a scaffold to anchor these structural components together. The proximal axon and its complex elements regulate the transport of cellular components to the axonal compartment (Kramer and Enquist, 2013), (Hedstrom et al., 2008; Leterrier et al., 2011; Song et al., 2009).

Transport within axons is mediated by two classes of motor molecules kinesin and dynein. Both use ATP hydrolysis to drive the transport. Dynein is a 1.2 megadalton that contains heavy chains, intermediate chains, light intermediate chains and light chains. Dynein forms a

dynactin complex with multiple subunits to bind various molecules. Dynein motor molecule mediates microtubule-dependent retrograde transport (towards the – end). Kinesin superfamily (KIF) is markedly more diverse. KIFs are classified into 3 broad categories; N-kinesins M-kinesins, C-kinesins depending on the position of the motor domain; near the amino terminus, near the middle, or near the carboxyl terminus, respectively. KIFs mediate microtubule dependent transport towards the + end (anterograde transport) (Diefenbach et al., 2008; Goldstein and Yang, 2000; Hirokawa et al., 2009; King, 2000; Schroer, 2004).

Virus binding, attachment and entry

Mature infectious virion particles initiate the infection of neurons virion attachment to the cellular receptors. The envelope glycoprotein gC and gB attach to glycosaminoglycane chains of heparan sulfate proteoglycans, this initial attachment does not require energy (Kramer and Enquist, 2013; Laquerre et al., 1998; WuDunn and Spear, 1989). After that, gD binds to a wide variety of receptors on cell membrane. Cellular receptors for gD include members of tumor necrosis factor receptor family (TNF) such as herpes virus entry mediator A (HVEM or HveA). Members of immunoglobulin superfamily also serve as receptors for gD; examples include nectin-1, nectin-2, paired immunoglobulin-like type 2 receptor alpha (PILR-2 alpha) and 3-O sulfated heparan sulfates. Nectin-1 is present on the sensory neuronal endings. VZV encodes gE that may have gD like functions during binding, a proposed receptor for VZV gE

is the insulin degrading enzyme. Binding of gD to its cognate receptor triggers conformational changes in gB, gH, and gL which leads to the fusion of viral envelope with cell membrane. (Heldwein and Krummenacher, 2008; Kramer and Enquist, 2013; Li et al., 2007; Roizman et al., 2007).

During binding and fusion, viral tegument proteins are reorganized to become asymmetrically distributed so that the dense aggregation faces away from the cell (Meckes and Wills, 2008; Smith, 2012). HSV-1 enters neuronal cells via a pH-independent fusion of the viral envelope with neuronal plasma 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).

Viral tegument proteins are classified into inner and outer tegument proteins. Inner tegument proteins are: pUL14, pUL16, pUL21, pUL36, pUL37, pUS3, and ICP0 (Kramer and Enquist, 2013; Radtke et al., 2010). Outer tegument proteins are; pUL46, pUL47, VP16, and VP22 (Luxton et al., 2005; Smith, 2012). Tegument proteins dissociate from the capsid upon entry into the cytosol. However, a subset of inner tegument proteins (UL36, UL37 and US3) remain attached to the capsid. These three inner tegument proteins participate in the retrograde axonal transport of capsid to the nucleus (Copeland et al., 2009; Kramer and Enquist, 2013; Luxton et al., 2005; Smith, 2012).

Retrograde transport

After virus-cell membrane fusion and deposition of capsid and the attached inner tegument proteins in the cytosol, the capsid is transported in retrograde manner to the

nucleus. It has been shown recently that alphaherpesviruses induce local protein synthesis in axons for efficient retrograde transport. Proteins involved in cytoskeletal reorganization, cell signaling and metabolism are among the infection induced proteins (Koyuncu et al., 2013).

Retrograde transport depends on intact microtubules and the motor complex; Dynein and the dynein co-factor dynactin. Alphaherpesvirus capsids transport to the nucleus at a rate of 1 $\mu\text{m/s}$ which is consistent with the fast retrograde axonal transport. As expected capsid transport was abolished when cells were pretreated with microtubule depolymerizing agent such as Nocodazol and colchisin. This indicates that the virus exploits and piggy back on preexisting axonal transport mechanisms. Multiple capsid and tegument proteins have been proposed to be involved in the recruitment of dynein motor molecule for retrograde transport. pUL34 and pUL9 have been shown to bind to dynein 1 intermediate chain (DYNC11)1a, and LC8 respectively, in vitro (Diefenbach et al., 2008; Kramer and Enquist, 2013; Ye et al., 2000). However, neither is incorporated in mature virion particles. Moreover, deletion of pUL34 did not prevent infection with HSV-1 (Reynolds et al., 2002; Roller et al., 2000). UL35 encodes VP26; a structural capsid protein. Studies have demonstrated that VP26 interacts with the dynein light chain (Tctex-1), both in yeast two-hybrid system and in vitro (Diefenbach et al., 2008; Douglas et al., 2004). Inner tegument proteins UL36, UL37 and US3 have emerged as candidates for virus-motor molecule interaction. Deletion of UL37 and US3 did not cause dramatic disruption in viral retrograde transport. However, UL36 null mutants were largely unable to reach the nucleus (Kramer and Enquist, 2013; Luxton et al., 2006). US9 null viruses do not exhibit defects in the retrograde transport (Brideau et al., 2000a).

Deposition of Viral DNA into the nucleus

After capsids reach the minus end at the MTOC, they translocate to the nuclear pore complex where viral DNA is released into the nuclear pore. Proteolytic cleavage of UL36 after docking to the NPC induces conformational changes that lead to the release of viral DNA into the nucleus (Jovasevic et al., 2008). pUL25 capsid protein have been shown to interact with nuclear pore complex components (Nup 214 and hCG1). It is possible that this pUL25 plays a role in DNA release into the nucleus (Smith, 2012). Once inside the nucleus the linear viral DNA circularizes and serves as a template for replication (Roizman et al., 2007).

Nuclear egress

A heterodimeric complex composed of pUL34 and pUL 31 (NEC) mediates the primary envelope-mediated nuclear egress of the nucleocapsid into the perinuclear space. This complex recruits viral kinases (US3 and pUL13) and cellular protein kinase C to locally phosphorylate and soften the nuclear lamina. (Leach and Roller, 2010; Mettenleiter et al., 2013). The initial budding through the inner nuclear membrane is followed by fusion and de-envelopment event at the outer nuclear membrane. pUS3 is involved in the de-envelopment and translocation of nucleocapsid from the periuclear through the outer nuclear membrane (Mettenleiter et al., 2013; Mou et al., 2009).

Married model vs separate model in anterograde transport

The mechanism of anterograde transport of alpha herpesvirus in neuronal cells is highly controversial, to a large extent because of disparate results obtained with pseudorabies virus

(PRV) and HSV-1. Two principal mechanisms of axonal transport have been proposed: (i) independent axonal transport of viral glycoproteins and capsids and assembly of the enveloped virions in the plasma membrane (separate model) and (ii) transport of fully enveloped virions that have formed via budding of capsids into TGN-derived membranes (married model) reviewed in (Diefenbach et al., 2008; Kratchmarov et al., 2012).

Evidence for the married model. Live cell imaging of double labeled viruses in proximal and distal regions of axons after infection of primary neuronal cultures of dorsal root ganglia (DRG) of embryonic rats revealed that there is a co-localization of capsid and membrane protein signals during anterograde experiments. Antinone et al 2006 have constructed recombinant PRV viruses with GFP labeled gD and mRFP labeled Vp26 (Antinone and Smith, 2006). The two fluorescent signals co-localized in 86% of particles traveling in anterograde direction. However, 14 % of mRFP signal had no GFP signal associated with it. This lack of co-localization can be attributed to limitations in detectability. Taylor et al 2012 had similar dual signal using GFP-US9 labeled PRV virus (Antinone and Smith, 2006; Taylor et al., 2012). Imaging of dual-fluorescent HSV-1 recombinant viruses were shown to support the married model. HSV virus expressing mRFP-VP26 and GFP-gB was reported to have 70% co-localization of both signals in chicken and rat DRG(Antinone et al., 2010). Recent high resolution electron microscopic imaging of neurons infected with PRV or HSV-1 showed that the majority (approximately 70%) of virions travel in anterograde manner are enveloped (Negatsch et al., 2010).

Evidence for the separate model. Immunofluorescent antibody (IFA) technique was used to determine the assembly state of virion particles in anterograde set up experiments. Fluorescent antibodies against capsid proteins and envelope proteins have shown minimal co-localization supporting the separate model (Snyder et al., 2006). Cell imaging using cryo-electron tomography on hippocampal axons revealed that axons predominantly contained naked capsids (Ibiricu et al., 2011).

It is clear that the support for the separate model tends to depend on negative evidence. Limitations of detection in IFA techniques would significantly confound the final interpretation. Also, hippocampal neurons tend to develop long and arborized dendrites, and the cryo-electron microscopy pictures were obtained without identification of the exact sections of axons. Transport in dendrites has not been characterized before. Therefore, the assembly status of the virion traveling in dendrites in anterograde manner may not be the same as in axons (Kratchmarov et al., 2012).

Anterograde transport

Once in the cytosol, nucleocapsids obtain inner tegument proteins including UL36, UL37 and US3. UL 36 is essential for secondary envelopment and egress events. UL36 also interacts with motor proteins to transport the capsid to the site of secondary envelopment. The nucleocapsids and associated tegument composition of incoming virions is similar to the newly formed. Which pauses the question; how are capsids differentially directed to the nucleus upon entry or to the site of secondary envelopment during egress?. Post translational modifications or other subtle but important differences in tegument component may govern the differential

targeting of capsids to the nucleus during entry, and newly formed capsids to the site of secondary envelopment (Kramer and Enquist, 2013; Smith, 2012).

UL36 and US11 are viral protein candidates for recruitment of kinesin motor molecules during anterograde transport. Both proteins have been shown to interact with kinesin in biochemical assays. However, UL36 and US11 are components of virion tegument and they are unlikely to participate in the anterograde transport of enveloped virions. (Kramer and Enquist, 2013).

US9, gE and gI play important roles in sorting enveloped virions into the axonal compartment. HSV-1 gE is a type 1 membrane glycoprotein of 552 amino acids. gE is expressed as a heterodimer with gI; the complex facilitates cell to cell spread by sorting newly formed virion particles to cell junctions and lateral cell surfaces (Collins and Johnson, 2003).

The cytoplasmic tail of PRV gE (last 123 amino acids) contains tyrosine based internalization motif (specifically amino acids 478-481). This motif is important in gE/gI constitutive endocytosis from the plasma membrane. gE-null or gE cytoplasmic tail deleted viruses have a small plaque phenotype in non-neuronal- cell type, and defective for anterograde spread in neurons in vivo (Brideau et al., 2000b; McGraw et al., 2009). gE/gI complex also stabilizes the interaction of US9 with KIF1A (kinesin motor protein important in anterograde transport) (Kratchmarov et al., 2013a).

US9 is a type II membrane anchored protein. US9-null viruses produce similar plaque-size as wild type in epithelial cell tissue culture. However, mutants lacking US9 are defective in anterograde transmission (Chowdhury et al., 2011; Lyman et al., 2007; Taylor et al., 2012). US9

was also found to interact with KIF1A(Kramer et al., 2012). US9 has an acidic cluster of 10 amino acids, within the cluster Y49 and Y50 are known to be required for US9-KIF1A interaction and to be important for productive anterograde spread in vivo (Brideau et al., 2000b; Kramer et al., 2012; Kratchmarov et al., 2013b). Moreover, phosphorylation of two serine residues S51& S53 in the acidic cluster of US9 was shown to be essential for anterograde spread in vivo (Brideau et al., 2000b; Kratchmarov et al., 2013b). Although not essential, phosphorylation of the two serine residues S51 & S53 enhances the US9-KIF1A based transport (Kratchmarov et al., 2013b). The S51 & S53 serine residues in US9 are conserved in BHV-1, HSV-1, HSV-2, VZV, EHV-1, BHV-1, BHV-5 (Chowdhury et al., 2011) and PRV.

Herpes Simplex Virus Diseases

Herpes simplex virus infection causes a wide range of clinical manifestations ranging from vesicles on the mucocutaneous membranes to fatal encephalitis (Roizman et al., 2007). Being an enveloped virus, HSV-1 is susceptible to desiccation, UV radiation, and lipid solvents. Therefore, transmission of the virus is by the direct and intimate contact with individuals shedding the virus. After infection the virus is shed from the site of infection for 7-10 days (Amir et al., 1997). Serum neutralizing antibodies can be detected as early as 4-7 days post infection and peak at 3 weeks post infection (Buddingh et al., 1953). The seroprevalence for Herpes simplex virus in USA and Europe is estimated to be 70-85% (Fatahzadeh and Schwartz, 2007; Looker and Garnett, 2005; Nahmias et al., 1990). Seroprevalence to HSV-1 is more common than to HSV-2(Nahmias et al., 1990). HSV-1 usually is an oral infection whereas HSV-2 infects genitalia. Increasing reports have showed that both viruses have been detected at alternate

sites; however, HSV-1 infections of the genitalia tend to be less severe with lower frequency of recurrence (Corey et al., 1983; Kalinyak et al., 1977). Approximately 30% of genital herpes infections in the U.S. are caused by HSV-1. However, in specific populations such as college students in the US, HSV-1 accounts for up to 78% of genital isolates (Kimberlin, 2014; Roberts et al., 2003). A global prevalence study showed that the total number of people aged 15-49 years who are infected with HSV-2 in 2003 is 536 million (Looker et al., 2008).

Herpes labialis

Herpes labialis or cold sore is infection of the mucocutaneous areas of the lips and mouth. It is the most common manifestation of herpes simplex infection (Roizman et al., 2007; Whitley and Miller, 2001). The virus infects and replicates in epithelial cells of the oropharynx. This primary infection may result in vesicular lesions or bullae at the site of inoculation, but the primary infection can also be asymptomatic. Histologically; the bulla will appear as an intraepidermal vesicle containing serofibrinous fluid and swollen, acantholytic keratinocytes, some of which has eosinophilic intranuclear inclusion bodies (Figure 2.15).

Vesicles may rupture leading to erosion and ulceration, and occasionally the herpes infection is complicated by secondary bacterial infection. The virus eventually infects sensory nerve endings and transports to the body of the neuron in the trigeminal ganglia where it establishes persistent (latent) infection for the life of the host. Triggered by a number of stimuli such as stress and UV light, latent virus may reactivate resulting in the transport of the virus to the initial site of infection where it replicates and causes outbreaks of recurrent vesicular lesions (Baron et al., 1996; Roizman et al., 2007).

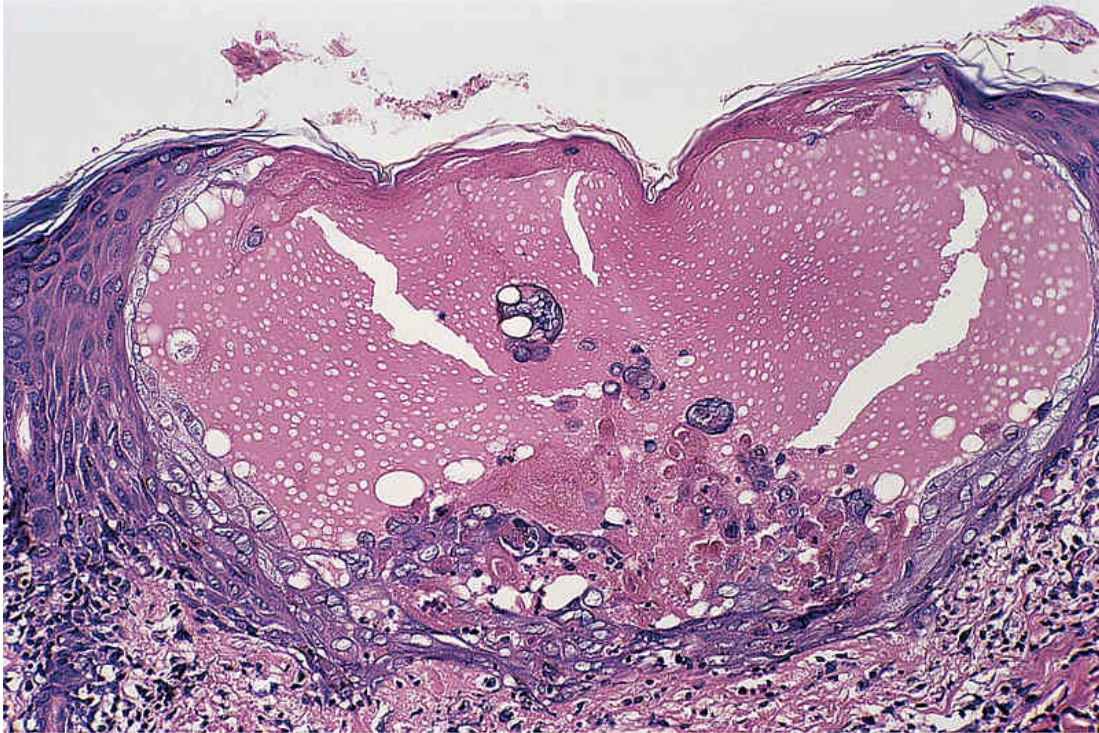


Figure 2.15: Hematoxylin and eosin stained histological section of herpes labialis showing an intraepidermal vesicle containing serofibrinous fluid and swollen, acantholytic keratinocytes, some of which has eosinophilic intranuclear inclusion bodies. The dermis is infiltrated with inflammatory cells. Adapted from Weedon: Skin Pathology 3rd edition (2010).

The frequency and the severity of the recurrent infection varies between individuals, approximately 30% of seropositive individuals experience recurrences with 40% of them have a recurrence once a year (Wald and Corey, 2007). Recurrent disease is often presented with tingling or itching, and pain for nearly 6 hours. These symptoms are then followed by the typical cold sore vesicle formation within 24-48 hours (Spruance, 1984; Spruance et al., 1977) (Spruance and Crumpacker, 1982). The vesicular lesion usually resolves on average after seven days (Segal et al., 1974; Ship et al., 1977). Recurrent infection and viral shedding can also be

asymptomatic (Fatahzadeh and Schwartz, 2007; Koelle and Wald, 2000). Intraoral and pharyngeal lesions accompanied by fever are known as Gingivostomatitis are more common in children younger than five years old (Roizman et al., 2007).

Herpes simplex ocular disease

Herpes simplex ocular disease is one of the serious consequences of HSV infection. Ocular herpes virus infection is the leading cause of infectious blindness in the developed world. Approximately 400,000 people in the United States have suffered herpes simplex ocular disease with 58,000 total episodes per year. A recent study has estimated the average annual incidence of new cases to be 11.8 per 100,000 population. The prevalence is approximately 149 cases per 100,000 population (Liesegang, 2001; Liesegang et al., 1989; Young et al., 2010).

Primary ocular infection can cause ocular disease, but frequent episodes of reactivation lead to more devastating consequences. Herpes simplex virus infected eyes manifest as conjunctivitis, blepharitis, ocular discharge and keratitis (Darougar et al., 1985; Rowe et al., 2013; Zhu and Zhu, 2014). The majority of herpes ocular infections are caused by HSV-1 rather than HSV-2 (Roizman et al., 2007). Herpes virus infection of the cornea can be divided into; epithelial keratitis which accounts for 50%-80% of all ocular herpes infection, and stromal keratitis (Labetoulle et al., 2005; Wilhelmus et al., 1981).

In epithelial keratitis, the virus replicates in corneal epithelial cells and causes epithelial cell destruction. It manifests initially as small vesicles that coalesce and ultimately rupture leading to what is known as dendritic ulcers. Dendritic ulcers in the cornea appear as central ulcerations with raised borders, and terminal end bulb (Figure 2.16). Inflammatory cell

infiltrates include polymorphonuclear cells, lymphocytes and plasma cell, occasional syncytial cell formation and eosinophilic intranuclear inclusions (Maudgal and Missotten, 1978; Metcalf and Reichert, 1979).

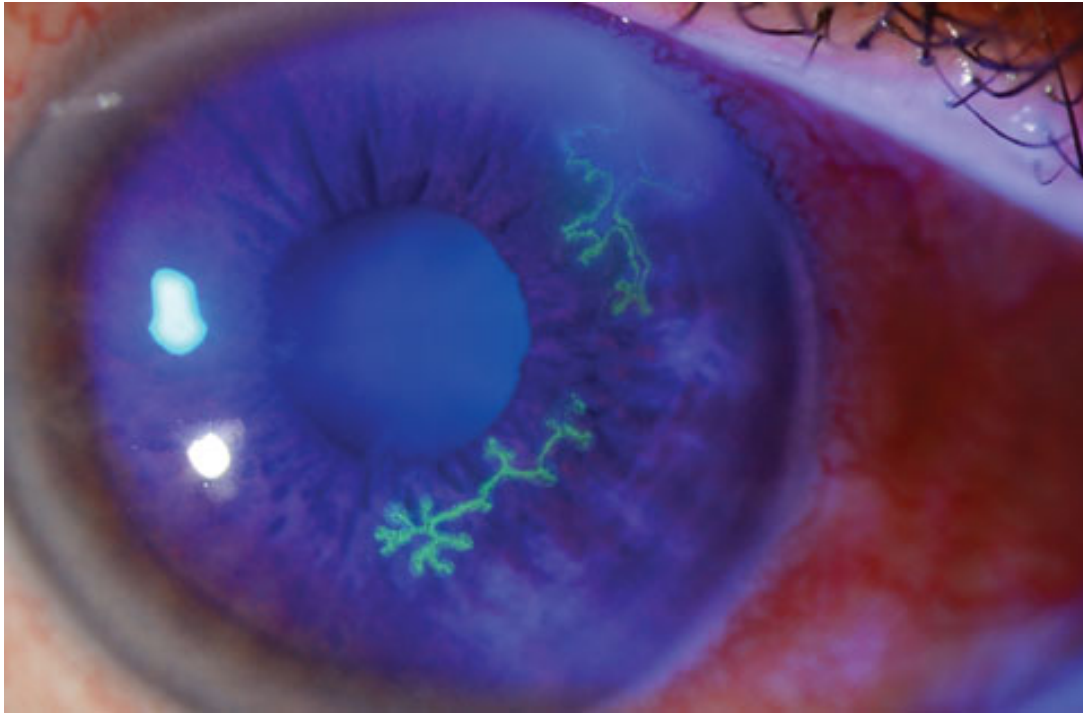


Figure 2.16: Epithelial keratitis caused by HSV-1; Dendritic ulcer in the corneal epithelium shown here with fluorescein staining. The image was taken from <http://medilinks.blogspot.com/2012/01/photos-for-herpes-simplex-keratitis-hsk.html>

Stromal keratitis is characterized by stromal edema, neovascularization, necrosis and inflammatory infiltrates. Repeated bouts of reactivation lead to corneal stromal scarring. Even after clearance of inflammatory cells, new blood vessel growth in the cornea and the destruction of the structural integrity of the stroma cause marked opacity and vision impairment (Figure 2.17) (Zhu and Zhu, 2014) (Rowe et al., 2013) (Gimenez et al., 2013).



Figure 2.17: Slit lamp photograph of Herpes simplex stromal keratitis. Note stromal opacity and neovascularization of the cornea (Rowe et al., 2013).

The infection can spread to deeper eye structures and cause infection and inflammation of the anterior chamber, iris and retina (David et al., 2008; Holland et al., 1987; Liesegang, 2001). However, the acute retinal necrosis, iridocyclitis and panuveitis are rare in immunocompetent individuals (Zhu and Zhu, 2014). Severe stromal destruction and corneal opacity may be treated with corneal transplants, however, reactivation of latent virus from the trigeminal ganglia can cause graft failure (Cockerham, 2001; Cockerham et al., 1997; De Kesel et al., 2001; Tullo, 2003).

One of the possible consequences of herpes ocular infection is neurotrophic keratopathy; a loss of sensation in the cornea due to degeneration of sensory nerves (Hamrah et al., 2012; Liesegang, 1999). Loss of sensation in the cornea may also lead to reduction in tear film production (dry eye), which increases the chances for corneal abrasions and ulcerations, inflammation, secondary bacterial infection and increased frequency of herpetic ocular recurrences (Tullo, 2003) (Keijser et al., 2002).

Role of corneal epithelial cells in the pathogenesis of primary herpes keratitis. The annual incidence of new cases of herpes keratitis is 11.8 per 100,000 population (Liesegang et al., 1989; Young et al., 2010). Infection starts by the entry and replication of the virus in epithelial cells at site of exposure, followed by entry into sensory neurons via their axonal termini. The virus recruits cellular transport machinery to translocate in retrograde manner to the body of the neuron. In the neuronal cell body, the virus remains latent for the life of the host. Exposure to UV light or immunosuppression leads to bouts of reactivation whereby the virus transports in anterograde manner to the primary site (Roizman et al., 2007).

The innate immune system is the first line of defense against pathogens. Cells of the innate immune system are equipped with Pattern Recognition Receptors (PRRs) which serve as sensors for conserved pathogen associated molecular patterns (PAMPs) displayed on the invading pathogens (Boehme and Compton, 2004). Toll like Receptors (TLRs) are a class of PRRs that are expressed predominantly on cells that are likely to first encounter the pathogen. Professional antigen presenting cells and phagocytic cells express the highest levels of TLRs (Akira et al., 2006; Medzhitov, 2007). In general, binding of PAMPs to TLRs leads to the

production of pro-inflammatory cytokines, and interferons, and up regulation of co-stimulatory molecules(Yamamoto and Takeda, 2010).

Corneal epithelial cells are one of the most important active participants in herpes Simplex keratitis. They are the first cell type to encounter the virus upon primary ocular infection with HSV. Corneal epithelial cells not only take part in pathogen recognition via the expression of a wide range of PRR including TLR-2, TLR-7 and TLR-9 (Johnson et al., 2005; Kumar et al., 2006; Li et al., 2006) but also secrete β -defensins, cytokines and chemotactic factors upon microbial invasion(Akpek and Gottsch, 2003).

TLR2 plays a key role in the recognition of a wide range of microbial antigens from bacteria, fungi, parasites and viruses reviewed in (Kawai and Akira, 2010). TLR2 generally forms heterodimers with TLR1 or TLR6. Moreover, TLR2 has the ability to act together with other cell surface receptors to assist PAMP recognition. These include CD36 and CD14 (Hoebe et al., 2005; Jiang et al., 2005). Generally, binding of PAMPs to TLR-2 leads to the downstream signaling through MyD88 dependent pathway which activates NF- κ B and promotes the production of pro-inflammatory cytokines such as IL-1, IL-6, IL-8, and IL-12 (Kawai and Akira, 2010). It has been demonstrated that TLR-2 recognizes structural components of viruses (Bieback et al., 2002; Cooper et al., 2005). Members of herpes virus family bind to and activate TLR-2 MyD88 dependent pathways (Ariza et al., 2009; Boehme et al., 2006; Wang et al., 2005). HSV-1 gB has been shown to interact with TLR-2 which leads to pro-inflammatory cytokine production via MyD88 dependent signaling. This binding and downstream cascade activation did not require

CD14 (Cai et al., 2013; Leoni et al., 2012; Sato et al., 2006). HSV-1 gB recognition occurs predominantly by TLR-2/TLR6 heterodimer and to a lesser extent by TLR-2/TLR heterodimer (Cai et al., 2013).

Following a primary infection with HSV-1, the cornea is infiltrated with a diverse set of inflammatory cells composed of; neutrophils (the predominant cell type), macrophages, natural killer cells (NK), T cells, and corneal dendritic cells (Miller et al., 1996; Stuart and Keadle, 2012). The development of primary herpes keratitis is believed to be a result of interaction of virus with the innate immune system rather than a direct viral cytopathic effect on corneal cells (Stuart and Keadle, 2012).

During primary keratitis, the infection induces the production of IL-6, which stimulates the production of neutrophils chemotactic factors (CCL3 and CXCL2). CCL3 and CXCL2 along with CCL1, CCL4, CCL5 CXCL10 attract monocytes, NK cells and DCs (Stuart and Keadle, 2012). Also, the levels of pro-inflammatory cytokines IL-1 alpha and IL-6 were increased upon primary herpes corneal infection. This increase was followed by an overlapping set of cytokine production (IFN-gamma, IL-12, IL-17, IL-4 and IL-10). This cytokine profile is indicative of the involvement of CD4⁺ T cells; Th1 and Th17 in the immune response (Biswas et al., 2004; Niemialtowski and Rouse, 1992; Stuart and Keadle, 2012). CD8⁺ cells play a minor role in the disease (Stuart and Keadle, 2012).

TLR-2 pathway activation is responsible for the detrimental inflammatory cytokine production that culminates in immunopathology (Sarangi et al., 2007; Wang et al., 2012). On the other hand, resistance to acute HSV-1 infection is mediated by p204 / IFN-inducible protein 16 (IFI-16). CD Conrady et al suggested that recognition of HSV-1 by the innate cytoplasmic sensor (IFI-16) is the principal initiator of IFN production in the cornea. IFI-16 initiates IFN production through stimulator of interferon genes (STING) dependent manner (Conrady et al., 2012).

Recently, the interaction between DC and NK in the development of HSK has been under increased scrutiny. Frank et al have studied the role of dendritic cells as well as the interaction between DC and NK in the cornea during primary HSV-1 keratitis (Frank et al., 2012). He showed that DCs are necessary for optimal clearance of HSV-1 from the cornea. However, they assume this function by directing local NK cells which in turn, serve as the proximal mediator of viral clearance from the cornea (Frank et al., 2012).

Herpes simplex encephalitis

Herpes simplex virus is the most common cause of viral encephalitis worldwide (Aryee and Thwaites, 2015). Herpetic encephalitis is rare in immunocompetent individuals. In immunocompromised adult patients; primary or recurrent infection can result in encephalitis (Fatahzadeh and Schwartz, 2007). Of the 7.3 million hospitalizations for encephalitis in the US between 1979-1988, Herpes simplex virus accounts for 10% of the cases (Stahl et al., 2011a; Stahl et al., 2011b). Herpes simplex encephalitis has an incidence of 1 in 250,000–500,000 individuals (Baringer, 2008; Whitley, 2006). If left untreated, herpes simplex encephalitis has a

70% fatality rate (Wald and Corey, 2007). Only 2.5 % of survivors returned to normal function, the remaining patients may sustain some degree of dysfunction (Whitley, 2006). Ninety percent of herpetic encephalitis cases in adults are caused by HSV-1, whereas, HSV-2 is a more common cause of encephalitis in neonates (Ito et al., 2000). The virus gains access to the central nervous system (CNS) in primary infection through the olfactory and trigeminal nerves. Reactivation of a latent virus can lead to transport of virions to the CNS causing encephalitis (Whitley, 2006). The classical clinical presentation of herpetic encephalitis is headache, fever, neurologic deficits and altered mental state. However, these clinical signs are not pathognomonic (Whitley, 2006).

At early stages of herpetic encephalitis, the brains of affected adults show bilateral, asymmetrical acute inflammation, congestion, and/or hemorrhage, most prominently in the temporal lobes. The meninges overlaying the affected areas appear congested or cloudy. After two weeks, the changes progress to liquefactive necrosis (Figure 2.18). Histologically, there is congestion of meninges and the cortex and subcortical white matter at early stages; however, at late stages (after two weeks), hemorrhagic necrosis, perivascular cuffing and glial nodules become evident. Inflammation consists of mononuclear infiltrates, gliosis and satellitosis and neuronophagia. Found in only 50% of patients, intranuclear inclusion bodies are most often visible in the first week of infection. The intranuclear inclusion bodies (Cowdry type A inclusions) are characterized by an eosinophilic homogeneous appearance surrounded by a clear zone with margined chromatin (Figure 2.19) (Booss and Kim, 1984; Kapur et al., 1994; Stahl et al., 2011a; Whitley, 2006). Cases of aseptic meninges are rare and the changes seen are mononuclear inflammatory cell infiltrates in the meninges and a slight increase in mononuclear

cell counts in the cerebrospinal fluid (CSF) (Dudgeon, 1969; Koskiniemi et al., 1980; Skoldenberg et al., 1984). Diagnosis of herpetic encephalitis relies on the presence of clinical signs, MRI imaging, PCR on brain biopsies and CSF, histopathological findings, serological tests (CSF) and viral isolation (Cinque et al., 1996; Whitley et al., 1989).

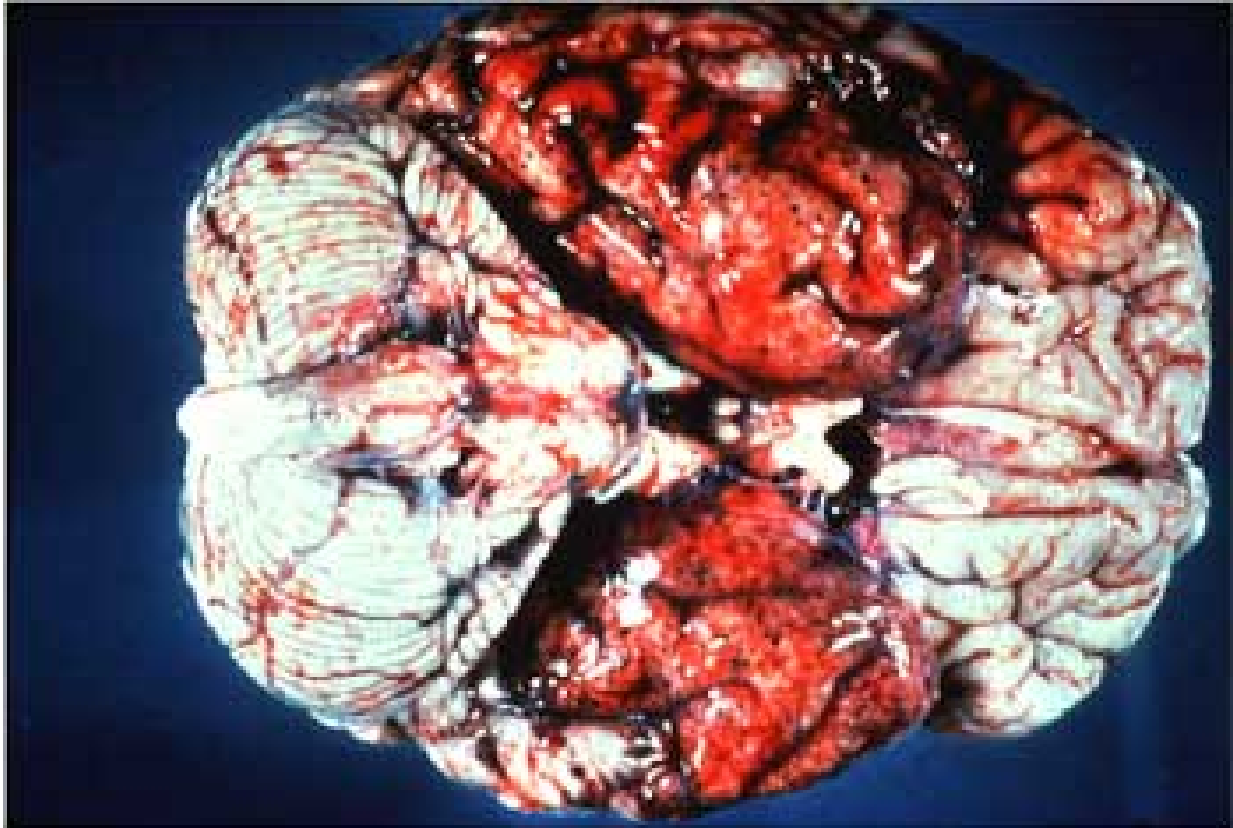


Figure 2.18: Herpetic encephalitis. Bilateral asymmetrical necrohemorrhagic encephalitis, temporal and frontal lobes. Adapted from laboratory medicine, Yale school of medicine website; <http://labmed.yale.edu/education/cme/casestudies/7/10.aspx>. The website credits University of Iowa College of Medicine Virtual Hospital, Dr. Gary Baumbach, M.D., Department of Pathology, for the picture.

Neonatal infections

Infection of neonates with HSV can occur in utero (intrauterine), intrapartum and postpartum. Intrapartum infection constitute the most common mode of infection (75-80% of

the cases), whereas, postpartum infection accounts for approximately 10% of the cases (Brown et al., 2003). The majority of intrapartum neonatal infections are caused by HSV-2, however, HSV-1 infection may also occur (Roizman et al., 2007). Intrauterine infections are very rare, but they often have detrimental consequences such as disseminated cutaneous lesions and multiple developmental abnormalities including: lissencephaly, microphthalmia and microcephaly to mention a few (Hutto et al., 1987; Roizman et al., 2007).



Figure 2.19: Hematoxylin and eosin stained histological section of the brain. Intranuclear inclusion bodies (Cowdry type A inclusions) are characterized by an eosinophilic homogeneous appearance surrounded by a clear zone with margined chromatin. Adapted from laboratory medicine, Yale school of medicine website; <http://labmed.yale.edu/education/cme/casestudies/7/11.aspx>. The website credits Dr. Peter Anderson, University of Alabama at Birmingham, Department of Pathology, for the image.

There are estimated 1500 cases of neonatal infections with HSV per year in the US whereby the infection rate is 1 in 3000 births. Risk of transmission dramatically decreases with caesarean section delivery, or if the mother seroconverts before delivery (Brown et al., 2003;

Johnston et al., 2008). Neonatal infection with HSV can result in a wide range of clinical presentations including disseminated disease, central nervous system disease, or cutaneous disease. Forty five percent of neonatal infections involve the mouth, eye and skin. Thirty percent of the cases are central nervous system disease, and the disseminated disease accounts for the remaining 25% of the cases (Kimberlin, 2001; Kimberlin et al., 2001; Pinninti and Kimberlin, 2013a, b; Whitley, 1988). Although disseminated HSV disease has a higher mortality rate (29%) than CNS disease (4%), the frequency of disabilities is much lower. 69% of neonatal CNS disease cases suffer from disability and retarded brain development 1 year after the disease compared to 17% of disseminated disease cases (Pinninti and Kimberlin, 2013b).

Symptoms of disseminated disease and CNS infection in neonates are not specific. The signs include; fever, anorexia, respiratory distress and lethargy. Disseminated disease in neonates involves the spread of the virus to multiple visceral organ systems (lungs, liver, eyes, skin, and brain) causing severe complications such as pneumonia, hepatitis, encephalitis and coagulopathy. Central nervous system infection in neonates is similar to herpetic encephalitis in adults (Thompson and Whitley, 2011).

Herpes simplex virus infection in immunocompromised patients

Immunocompromised individuals are at a greater risk of severe, more common, and greater spread of primary or recurrent HSV infections. Impaired immune system function may occur due to metabolic disturbances and malnutrition, immunosuppressive therapy for cancer treatment or organ transplant, or acquired immunodeficiency syndrome (AIDS). The clinical manifestations of HSV infection in such patients ranges from vesicular dermatitis to esophagitis,

pneumonitis, colitis, and devastating systemic infections (Fatahzadeh and Schwartz, 2007; Roizman et al., 2007). More incidents of reactivation (60-95%) have been observed in immunosuppressed individuals (Arduino and Porter, 2008). It is noteworthy to mention that HSV-2 infection increases the risk of acquiring HIV by 2-4 folds. Also HSV-2-seropositive individuals have a 5-fold greater risk of acquiring HIV compared with HSV-2-negative individuals (Corey et al., 2004; Wald and Link, 2002).

Prevention and Treatment of Herpes Simplex Infection

Elimination of contact with seropositive individuals, the use of therapeutics and vaccines represent the main strategies to prevent and treat herpes virus infection. Preventative measures such as maintaining good hygiene, avoiding contact with infected individuals and using barrier methods during sexual activity such condoms would be ideal to eliminate contact with seropositive individuals. Unfortunately, this preventive measure is difficult to implement due to asymptomatic shedding. Seventy percent of HSV-1 infected persons shed the virus from the oral cavity at least once a month with many of the shedding incidents are without symptoms (Miller and Danaher, 2008).

Anti-herpes virus therapeutic agents have been effective in reducing the duration of primary and recurrent infection as well as the symptoms. Acyclovir is effective against HSV-1, HSV-2, VZV and EBV (Kaye, 2009). It inhibits viral DNA polymerase activity and viral DNA synthesis. Acyclovir is a nucleoside analogue that mimics deoxyguanosine but lacks the 3' hydroxyl moiety. In infected cells, viral thymidine kinase monophosphorylates acyclovir. Subsequently, cellular kinases convert acyclovir monophosphate to acyclovir triphosphate by

adding 2 additional phosphates. Acyclovir is incorporated in the growing DNA strand instead of deoxyguanosine triphosphate (dGTP). As a result, DNA polymerase is inhibited and DNA strand elongation is terminated (Coen, 2007; Wilson et al., 2009). Viral specificity of acyclovir is reflected in the fact that; 1) acyclovir is selectively phosphorylated by viral TK kinase at a higher efficiency than mammalian thymidine kinase, 2) acyclovir inhibits cellular DNA polymerase weakly compared to viral DNA polymerase (Kleymann et al., 2002) (Crumpacker and Schaffer, 2002; Wilson et al., 2009). The emergence of acyclovir resistant HSV stains (approximately 6% of HSV isolates from HIV infected patients are acyclovir resistant), and the limited oral bioavailability of acyclovir pose significant problems. Therefore, alternatives to acyclovir have been researched in recent years. Foscarnet is a pyrophosphate analog that has been used as an alternative to acyclovir. Foscarnet inhibits viral DNA polymerase by preventing the release of pyrophosphate; a process required for DNA strand elongation. Foscarnet is used as the last resort because it is nephrotoxic and requires intravenous mode of administration. Valacyclovir, Famciclovir and Valganciclovir are nucleoside analogues with similar mode of action to acyclovir, but with improved oral bioavailability (Coen, 2007). New classes of therapeutics such as aminothiazolylphenyl-containing drugs and thiazole urea derivatives have been experimentally shown to be promising drug alternatives for the treatment of HSV infections. These classes of drugs target helicase–primase complex rather than DNA polymerase. The new drugs increase the affinity of the primase and helicase subunits to DNA strands leading to the inhibition of viral DNA synthesis (Crumpacker and Schaffer, 2002; Kleymann et al., 2002) (Crute et al., 2002).

Many experimental drugs such as glycosylation inhibitors and plant extracts such as *Aloe vera*, *Swertia chirata* and *Melissa officinalis*, and Toll like Receptor agonists have been examined for their efficacy and toxicity to be used as anti-herpesvirus therapeutics. Some of those compounds have shown promising preliminary results.

The goal of development of vaccine therapeutics to HSV is to augment immune responses against it and to suppress recurrences. HSV vaccine strategies include the construction of genetically attenuated live virus vaccines, killed or inactivated virus vaccines, recombinant subunit vaccines, DNA vaccines as well as immunizations with replication competent and replication incompetent vectors. An example of a recently constructed genetically modified attenuated live virus vaccine is ACAM529. ACAM529 is an anti-HSV-2 vaccine that has been optimized for commercial manufacturing (Sanofi Pasteur) and is currently under phase I clinical trials. This vaccine has shown both strong immunogenicity and high efficacy in vivo (Mundle et al., 2013). Promising results have been observed with HSV-2 live-attenuated ICP0 deletion virus (ICP10DPK) which showed prevention of recurrent disease in 37% and 43.5% of vaccinated individuals in two separate trials (Casanova et al., 2002). Lipidon H and lipidon G is a whole heat-inactivated virus from HSV-1 and HSV-2, respectively. This vaccine was shown to reduce incidents of recurrences; however, the results were inconclusive due to lack of use of proper controls (Weitgasser, 1977). An experimental HSV-2 subunit vaccine (GEN-003/MM-2) composed of HSV-2 gD2 and a truncated form of ICP4 prepared with Matrix M-2 (MM-2) adjuvant was able to elicit humoral immune responses, CD4(+) and CD8(+) T cells resulting in decreased recurrent disease symptoms and reduced the frequency of recurrent viral shedding in Guiana pigs (Skoberne et al., 2013). Lipopeptide vaccines represent a

new strategy in vaccine development; the preparations are made for mucosal topical ocular and intravaginal application. The principle is predicated on the recent findings that HSV protective epitopes are recognized by T cells from symptomatic individuals versus asymptomatic (Dasgupta et al., 2009). Recent work in this laboratory has shown that a single vaccination using a recombinant virus with a mutation in gK and UL20 conferred 100% protection against lethal challenge with HSV-1 and HSV-2 wild type viruses (Stanfield et al., 2014) . This vaccine is currently being tested by the National Institute of Allergy and Infectious Diseases (NIAID), Vaccine and Treatment Evaluation Units (VTEUs) in Cincinnati.

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CHAPTER III: FUNCTIONAL HIERARCHY OF HERPES SIMPLEX VIRUS TYPE-1 (HSV-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 (Cockerham, 2001; Cockerham et al., 1997; De Kesel et al., 2001; Liesegang, 2001; Liesegang et al., 1989; Tullo, 2003). Typically, primary infection of epithelial cells exposed on orofacial mucocutaneous tissues leads to invasion of embedded axonal termini embedded within 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).

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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 (Diefenbach et al., 2008; Johnson and Baines, 2011; Mettenleiter et al., 2009). 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; Satoh et al., 2008; Shukla et al., 1999; Spear et al., 2000; Spear and Longnecker, 2003) 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) (Goldgeier et al., 2002; Roizman et al., 2007). 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. (Muggeridge, 2000; Turner et al., 1998). 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 (Chouljenko et al., 2009; Chouljenko et al., 2010; Foster et al., 2001).

Glycoproteins gM and gE and the membrane-associated UL11 protein have been also implicated in virus-induced cell fusion and virion egress (Chouljenko et al., 2012; Kim et al., 2013). 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 (Klupp et al., 2000; Koyano et al., 2003). 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 retrograde spread defect *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; Baird et al., 2008; Loomis et al., 2001; MacLean et al., 1989). 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; Han et al., 2012; Leege et al., 2009; Loomis et al., 2001; Loomis et al., 2003). Although absence of UL11 in HSV-1 and PRV revealed only moderate defects in viral replication (Chouljenko et al., 2012; Fulmer et al., 2007; Kopp et al., 2003; Leege et al., 2009), the HCMV UL11 homologue is essential for virus replication (Seo and Britt, 2007; Silva et al., 2003).

Recently, we showed that lack of either the gK or 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 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 mouse corneas, it infected ganglionic neurons with greater efficiency than the gM and 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; Lee et al., 2009; Tanaka et al., 2003; Tischer et al., 2006). All viruses were grown on Vero cells and equal PFU were used for all viruses in the ocular infection experiments. The gK-null virus was tittered on the VK302 complementing cell line.

Construction and characterization of recombinant viruses

The recombinant viruses were constructed in previous studies (Chouljenko et al., 2012; Kim et al., 2013; Melancon et al., 2005). Briefly, mutagenesis was performed in *E. coli* using

two-step red mutagenesis implemented on HSV-1(F) strain 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). 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 viruses 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 viral yields 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 a horseradish peroxidase conjugated goat-anti-rabbit antibody (Dako Carpinteria, CA). The reactions were developed using NovaRed substrate (VectorLabs, Burlingame, CA, USA). Viral yields 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 by standard plaque assay. Plaques were stained by immunohistochemistry and visualized using dissecting microscope.

Ocular infections

Balb/c mice were used for all experiments and were handled in accordance with the ARVO statement for the use of animals in ophthalmic and vision research and Louisiana State University-School of Veterinary Medicine IACUC regulations. Corneal scarification and ocular inoculation of mice were performed as described previously (David et al., 2008; Saied et al., 2014). 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 a mock-infected. After anesthesia, 25-26 G needle was used to scarify mice corneas in a grid like pattern (4 cross hatches per eye). 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. Clinical ocular changes were monitored daily for keratitis, blepharoconjunctivitis and ocular discharge. Mice eyes were rinsed at 24, 48 and 72 hours post infection and the number of infectious virions within the rinsing solution was determined by plaque assay.

Quantitative PCR (qPCR) analysis

Trigeminal ganglia (TG) were collected 30 days post infection (dpi) for qPCR analysis and stored at -80 °C. After thawing, Qiagen DNeasy blood and tissue kit (Qiagen, Valencia, CA) was used to extract total DNA from the TG per manufactures instructions. QPCR was performed as initially described previously for Kaposi's sarcoma herpesvirus (KSHV) (Subramanian et al., 2008; Subramanian et al., 2010) and utilized for HSV-1 DNA detection in neuronal cell cultures (Kim et al., 2013). Briefly, the primers and probe (6-carboxytetramethylrhodamine (TAMRA) for the real-time PCR were designed to detect HSV-1 US6 (gD). The amount of DNA extracted from

each trigeminal ganglion was measured using Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE). Total DNA concentration from each ganglion was approximately 80 ± 5 mg per ganglion. Equal volumes of viral DNA were used for qPCR analysis. Purified plasmid containing the gD gene was 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 (Figure 1) 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 1) (see “Materials and methods” section). 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 2). 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 2). 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 (Table 3.1).

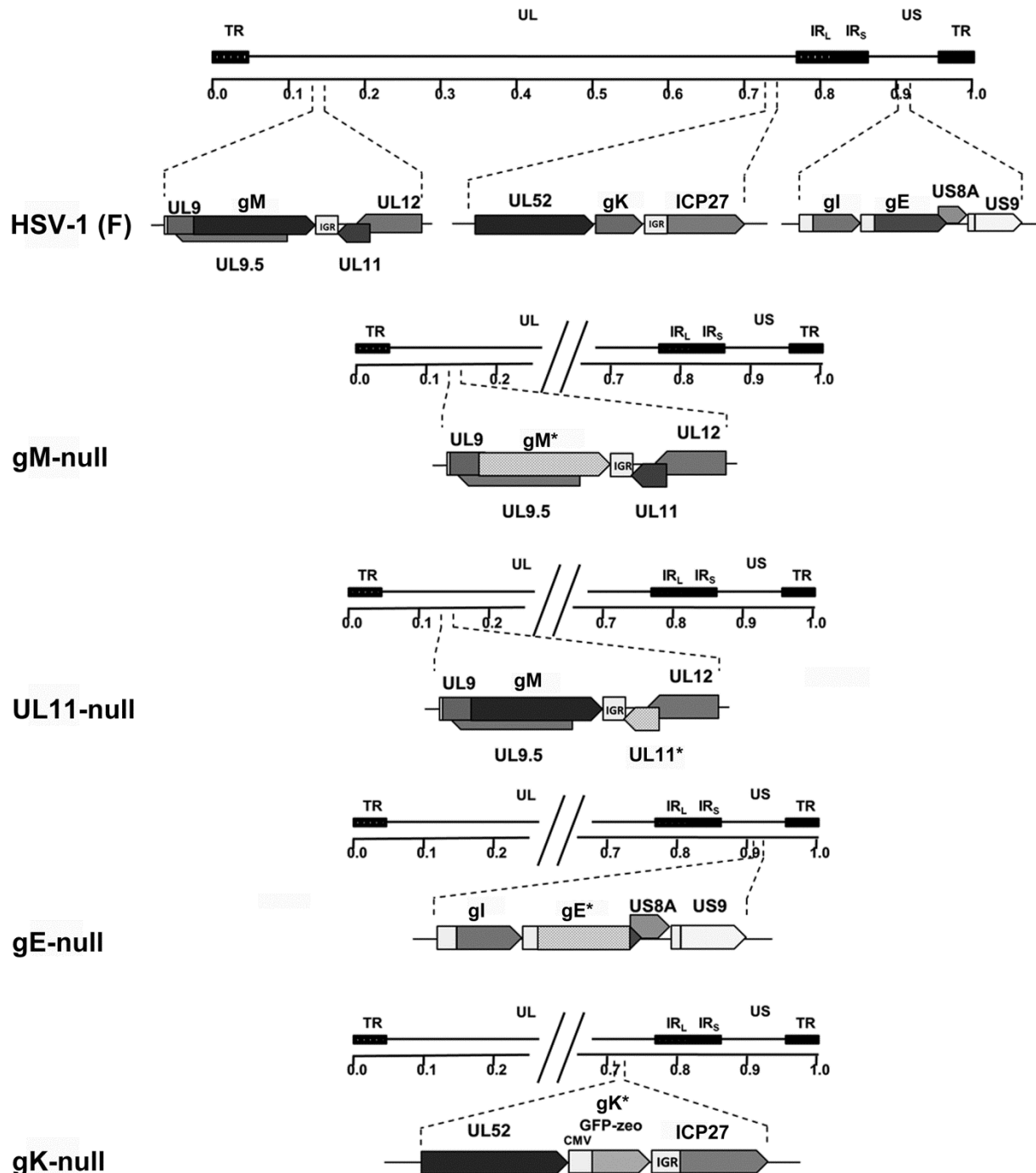


Figure 3.1: Schematic representation of mutant viruses. The top line represents the prototypic arrangement of the HSV-1 genome with the unique long (UL) and unique short (US) regions flanked by the terminal repeat (TR) and internal repeat (IR) regions. IGR denotes the intergenic region. The relevant 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).

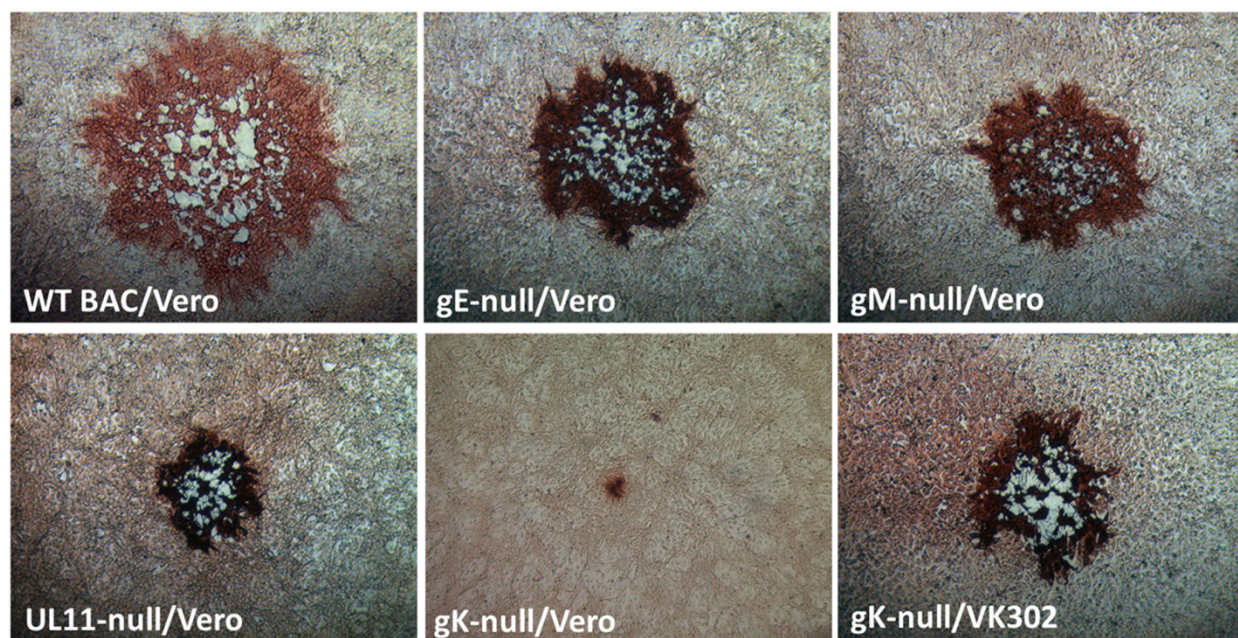


Figure 3.2: Plaque morphology of mutant viruses. Confluent monolayers of Vero or VK302 cells were infected at an approximate MOI of 0.001 and representative plaques were identified as indicated by immunohistochemistry staining. Viral plaques were photographed at the same magnification. VK302 is a cell line expressing gK upon infection.

Table 3.1: Comparison of viral yields between wild type and recombinant viruses:

Virus\HPI	0 HPI	24 HPI	48 HPI
WT BAC	$8.67 \times 10^1 \pm 6.67 \times 10^1$	$4.87 \times 10^7 \pm 8.19 \times 10^6$	$6 \times 10^7 \pm 2 \times 10^7$
gE-null	$3.33 \times 10^1 \pm 1.76 \times 10^1$	$4.93 \times 10^7 \pm 4.67 \times 10^6$	$8.667 \times 10^7 \pm 1.764 \times 10^7$
gM-null	$3.33 \times 10^1 \pm 1.33 \times 10^1$	$9.33 \times 10^6 \pm 3.33 \times 10^6$	$1.13 \times 10^7 \pm 1.76 \times 10^6$
UL11-null	$3.33 \times 10^1 \pm 6.67$	$8.07 \times 10^6 \pm 8.82 \times 10^5$	$1.13 \times 10^7 \pm 4.05 \times 10^6$
gK-null	$6.67 \times 10^1 \pm 4.80 \times 10^1$	$1.13 \times 10^4 \pm 3.52 \times 10^3$	$2.47 \times 10^4 \pm 1.27 \times 10^4$

Table 3.1 shows viral yields in plaque forming units (PFU)/ml. Hours post infection (HPI). Triplicate samples were titrated and the standard error of mean at each time point was calculated.

Mouse eye infections

Mouse 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 3.3). Viral shedding was determined daily 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 3.4).

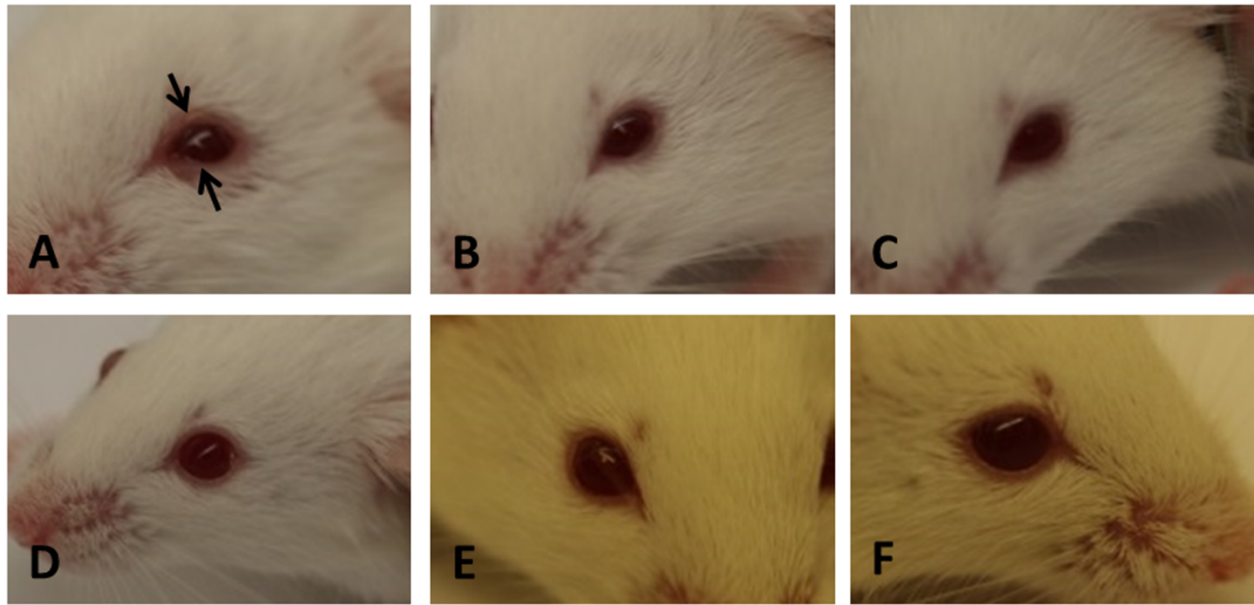


Figure 3.3: Clinical ocular disease development. Representative pictures of infected eyes are shown. Mice eyes were monitored for the development of blepharoconjunctivitis, hyperemia, keratitis and ocular discharge (A) WT BAC group, notice the blepharitis; thickening of upper and lower eyelids (arrows). (B) gM-null group. (C) gE-null group. (D) UL11-null group. (E) gK-null group (F) Mock group.

Determination of viral DNA in trigeminal ganglia of infected mice

The TG harboring latent viral DNA 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 11,200 viral DNA genomes were detected per single 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 600 viral genomes, 4-of-10 mice infected with the gE-null virus contained on average 600 viral genomes, and 6-of-10 UL11-null-infected mice contained on average 2400 viral genomes. The numbers of viral genomes recovered from the wild-type infections were significantly different in comparison to all mutant viruses by one-way ANOVA test (Figure 3.5).

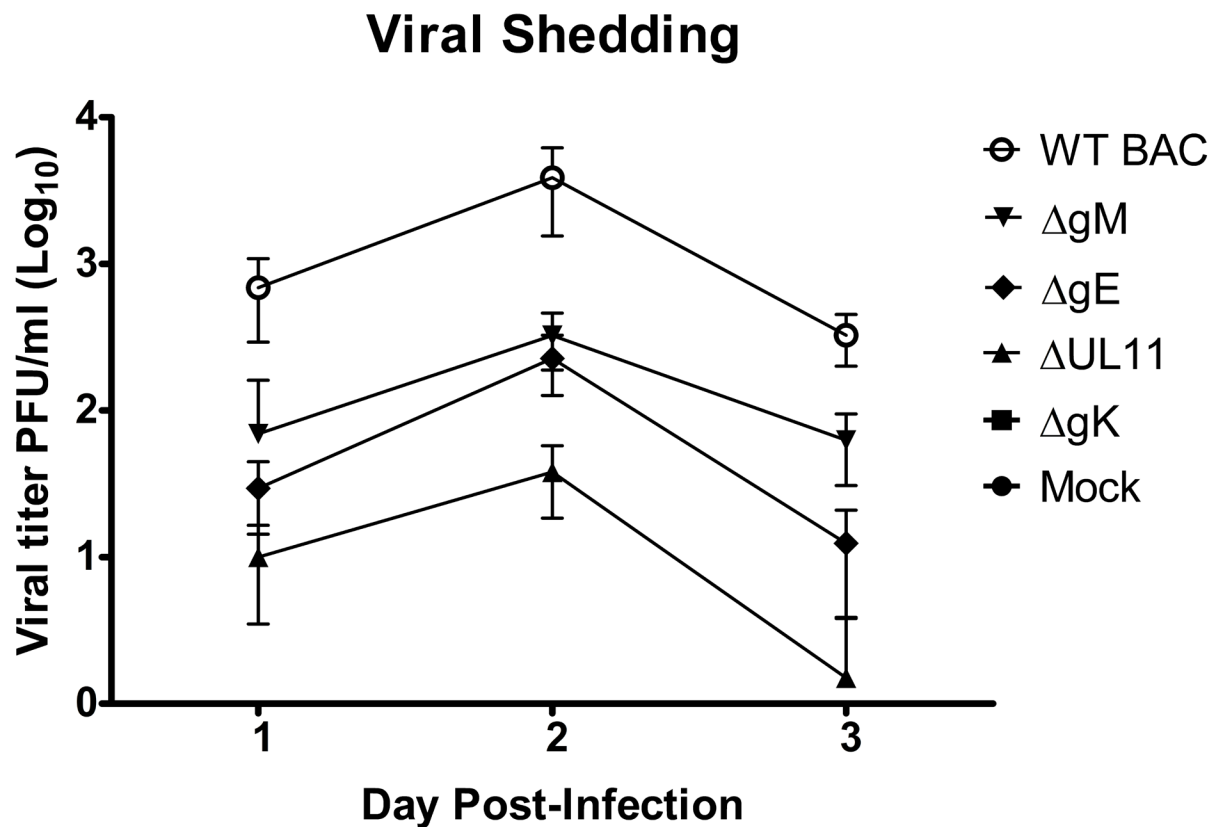


Figure 3.4: Viral shedding. Viral titers were obtained from eye rinses obtained at 24, 48 and 72 hpi. ΔgK and Mock values are zeros.

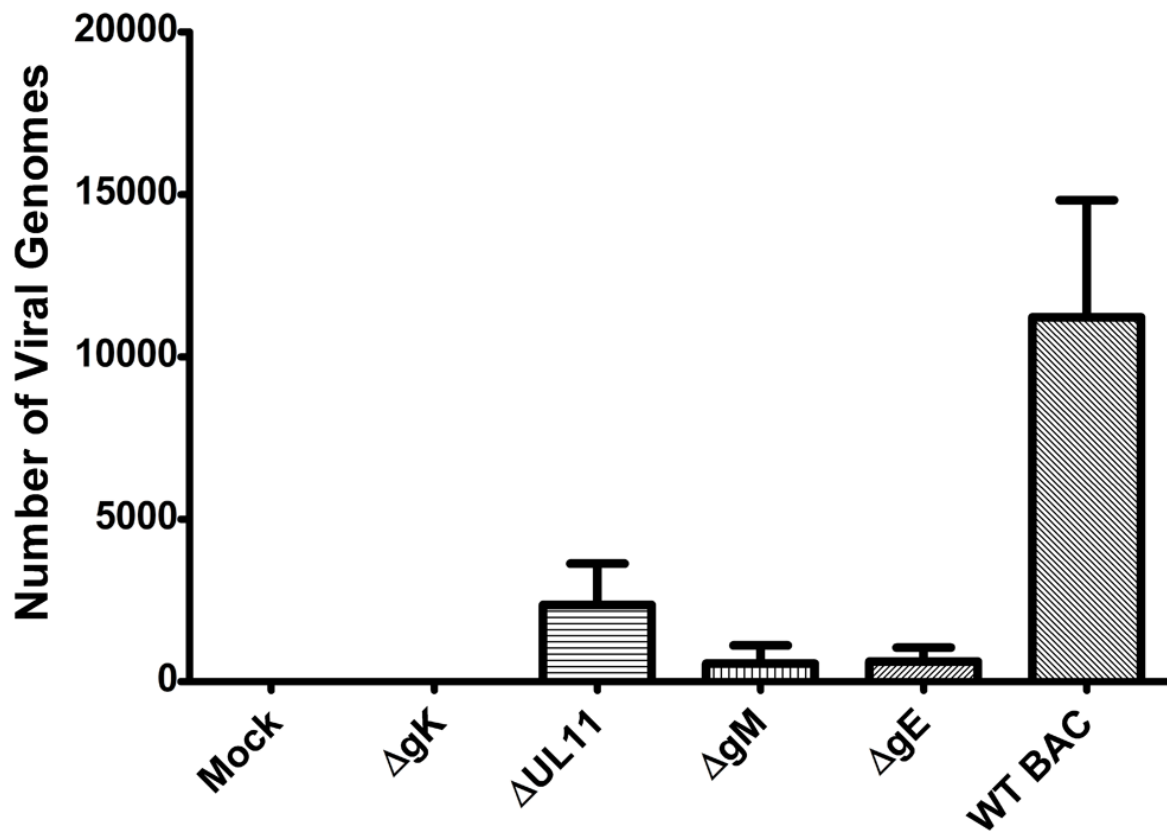


Figure 3.5: Viral DNA genome detection by qPCR. Equal volumes of viral DNA were used for TaqMan PCR. Purified plasmid containing the gD gene was used to generate the standard curve.

Discussion

We have demonstrated previously that HSV-1 gK plays a pivotal role in neuronal infection. Specifically, lack of gK prevents infection of TG 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 infected mouse corneas and ganglionic neurons *in vivo* and establish latency in the TG. 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 after cornea scarification. In contrast, viruses lacking either gM, gE or UL11 infected mouse corneas 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., 2014). 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 (pYE102). 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., 2012a, b; 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 when McKrae and F strains are compared (not shown). One or more of these predicted amino acid changes may be responsible for the observed ocular pathogenetic differences between these two viral strains.

Mouse corneas contain over 50,000 neuronal axonal termini per mm². 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 article, 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 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 IV: A REPLICATION COMPETENT HSV-1(MCKRAE) WITH A MUTATION IN THE AMINO-TERMINUS OF GLYCOPROTEIN K (GK) IS UNABLE TO INFECT MOUSE TRIGEMINAL GANGLIA AFTER CORNEA INFECTION*

Introduction

HSV-1 causes multiple medical disorders such as cold sore, keratitis, encephalitis and systemic disease (Whitley et al., 1998). The hallmark of the HSV-1 life cycle is infection of sensory neurons, where the virus establishes a latent infection for the life of the host (Roizman et al., 2007; Whitley et al., 1998). HSV-1 primary infection starts at the mucocutaneous surface and then the virus invades the axonal termini to undergo retrograde transport to neuronal cell body (Antinone and Smith, 2010). Upon, reactivation of the virus from the latent state, the virus transports in anterograde manner to the periphery (Smith, 2012).

Ocular HSV is one of the most common infectious causes of blindness in developed countries with estimates of the incidence of herpes ocular disease episodes ranging from 4.1 to 20.7 cases per 100,000 in developed countries. In the United States, approximately 500,000 people suffer from herpes simplex ocular disease (Liesegang, 2001; Liesegang et al., 1989). A nationwide prospective study in France conducted in 2002 estimated the incidence of herpes keratitis to be 25.8 cases per 100,000 persons annually (Labetoulle et al., 2005). Recurrent herpes keratitis leads to corneal opacity and vision loss. Also, reactivation of latent virus from the trigeminal ganglia (TG) can cause spread to corneal transplants causing corneal graft failure (Cockerham, 2001; Cockerham et al., 1997; De Kesel et al., 2001; Tullo, 2003).

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HSV-1 enters neuronal cells via a pH-independent fusion of the viral envelope with neuronal plasma 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). Virus entry into all cells involves the coordinated functions of the glycoproteins gD, gB, gH, gL, and gC (Connolly et al., 2011). Fusion of the viral envelope with cellular including neuronal membranes causes deposition of the viral capsid into the cytoplasm, which is subsequently transported to the cell nucleus. Retrograde transport of capsids to neuronal somata after infection of axonal termini is highly dependent on the cellular microtubule network and is most likely mediated via direct interactions by one or more tegument and capsid proteins with the dynein motor and the dynein cofactor dynactin (Diefenbach et al., 2008; Johnson and Baines, 2011; Mettenleiter et al., 2009).

The UL20 and UL53 (gK) genes are highly conserved in all alpha herpesviruses and encode proteins of 222 and 338 amino acids, respectively, each with four membrane spanning domains (Debroy et al., 1985; Foster et al., 2003; MacLean et al., 1991; Melancon et al., 2004; Ramaswamy and Holland, 1992). The amino and carboxyl termini of gK are located extracellularly, while the amino and carboxyl termini of UL20 are located intracellularly (Foster et al., 2003; Melancon et al., 2004). gK is post-translationally modified by N-linked carbohydrate addition at the amino-terminus of gK, while the UL20 protein (UL20p) is not glycosylated (Debroy et al., 1985; Hutchinson et al., 1992; Ramaswamy and Holland, 1992). HSV-1 gK is a structural component of virions and it interacts with UL20p on virion and cellular membranes. HSV-1 gK and UL20 interaction is required for their coordinated intracellular transport, cell surface expression, and functions in virus-induced cell fusion, virus entry, virion

envelopment and egress from infected cells (Foster et al., 2003; Foster et al., 2008; Foster and Kousoulas, 1999; Foster et al., 2004a; Foster et al., 2001a; Foster et al., 2004b; Hutchinson et al., 1995; Jambunathan et al., 2011; Jayachandra et al., 1997). The amino terminus of gK interacts with gB and gH and can complement gB-mediated cell fusion indicating that the gK/UL20 protein complex can modulate virus entry and virus-induced cell fusion via modulating gB and gH membrane fusion functions (Chouljenko et al., 2009; Chouljenko et al., 2010).

Virions that lack gK enter into African monkey kidney cells (Vero), albeit with lower efficiency than the wild-type virus (Foster et al., 2001b; Jambunathan et al., 2011). Deletion of amino acids 31-68 within the amino terminus of gK inhibits virus-induced cell-to-cell fusion and virus entry without drastically inhibiting virion envelopment and egress. Specifically, a recombinant virus lacking gK amino acids 31-68 replicated fairly efficiently, while gK deletions from amino acids 31-47 and 31-117 exhibited major defects in infectious virus production (Chouljenko et al., 2009). However, deletion of gK amino acids 31-68 inhibited virus-induced cell fusion caused by syncytial mutations in gK and entry into PILR-alpha expressing Chinese hamster ovary cells (Chouljenko et al., 2009; Chowdhury et al., 2013). We have shown that gK is essential for neuronal infection and virulence (David et al., 2008). Specifically, we have reported that gK-null virus was unable to infect axonal termini and egress from neuronal cell bodies (David et al., 2012).

To further investigate the role of gK in neuroinvasiveness, we engineered the gK 31-68 amino acid deletion in the HSV-1(McKrae) genome cloned as a bacterial artificial chromosome (McK). In vivo experiments utilizing the mouse eye model system revealed that this virus was

unable to efficiently infect mouse TG after ocular infection of scarified mouse eyes. These results indicate that the amino terminus of gK plays pivotal role in corneal infection, neuroinvasiveness and acute herpes keratitis.

Materials and Methods:

Cells and viruses

African green monkey kidney (Vero) cells were obtained from the American Type Culture Collection (Rockville, MD) and grown 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 clinical ocular isolate and the neuroinvasive strain of HSV-1 (McKrae) was a gift from J. M. Hill (Louisiana State University Health Sciences Center, New Orleans, LA).

Construction and characterization of recombinant viruses

The McKrae strain of HSV-1 was originally cloned into a bacterial artificial chromosome (BAC) vector. The construction methodology for mutant viruses for this experiment is essentially similar to the techniques that were previously described (Chowdhury et al., 2013; Tanaka et al., 2003). Briefly, the BAC plasmid PBeloBAC 11 (NEB) was inserted between the UL3 and UL4 viral genes. An excisable EGFP cassette bracketed by flippase recognition target (FRT) sequences was cloned into the BAC plasmid vector. Vero cells were transfected with the plasmid vector and infected with HSV-1 (McKrae) virus. Fluorescent plaques were selected and

the presence of BAC plasmid was confirmed by PCR-assisted DNA sequencing (Chowdhury et al., 2013). The recombinant virus McK Δ gK31-68 was constructed by deleting 38 amino acids (gK amino acids 31- 68) from the amino terminus of gK as described previously for the HSV-1 (F) genome cloned into BAC plasmid pYEbac102 (Tischer et al., 2006). During the construction of this virus the EGFP gene is spontaneously removed via FRT-mediated recombination. A rescued virus was also produced by transfection of a gene segment spanning the gK gene followed by infection with a gK mutant virus. All viruses were sequenced using the Ion Personal Genome Machine (PGM) sequencer (Life Technologies-Invitrogen, Carlsbad, CA) to confirm the Δ gK31-68 deletion and the absence of any other nucleotide changes within coding sequences.

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

For plaque size and morphology, confluent monolayers of Vero and VK302 cells were infected with McK, McKR (rescued virus), or McK Δ gK31-68 viruses at MOI of 0.001. Cells were fixed by methanol at 48 hpi. After fixation, immunohistochemistry was performed using a horseradish peroxidase conjugated goat-anti-rabbit polyclonal antibody (Dako Carpinteria, CA). The reactions were developed using NovaRed substrate (VectorLabs, Burlingame, CA, USA). Replication kinetics were determined as described previously (David et al., 2008; David et al., 2012). Briefly, confluent monolayers of Vero cells were infected with McK, McKR, or McK Δ gK31-68 viruses at MOI of 1. Infections were stopped by freezing at 0, 6, 12, 18 and 24 hours post infection (hpi). Plates were prepared in triplicates and viral titers were determined for each time point by standard plaque assay. Plaques were stained by immunohistochemistry and visualized using a dissecting microscope.

Animals

Female BALB/c mice (6-to-10-week-old) were divided into four groups; each group was infected with McK, McKR, McKΔgK31-68 viruses, or mock-infected. All animals were handled in accordance with the ARVO statement for the use of animals in ophthalmic and vision research and Louisiana State University-School of Veterinary Medicine IACUC regulations. Animals were anesthetized, and the corneas were scarified in a grid pattern using a 25-26 G needle as described previously (David et al., 2008). Each eye was inoculated with 10^5 plaque forming units (pfu) applied using a micropipette. After inoculation, the eyelids were gently held shut for a few seconds. The clinical ocular disease development and progression were monitored and scored daily. The scoring criteria included the degree of blepharoconjunctivitis, hyperemia, keratitis and ocular discharge. The ocular disease was scored on a scale of 0-3. Zero represented a normal eye with no clinical symptoms, and 3 represented severe clinical presentation of the ocular changes.

Quantitative PCR analysis

TG were collected 30 days post infection (dpi) for quantitative PCR (qPCR) analysis and stored at -80° C. After thawing, trigeminal ganglia were processed using the Qiagen DNeasy blood and tissue kit (Qiagen, Valencia, CA) to isolate total DNA per the manufacturer's instructions. qPCR was performed as initially described previously for Kaposi's sarcoma herpesvirus (KSHV) (Subramanian et al., 2008; Subramanian et al., 2010) and subsequently utilized for HSV-1 DNA detection in neuronal cell cultures (David et al., 2012). Briefly, the primers and probe (6-carboxytetramethylrhodamine (TAMRA) for the real-time PCR were

designed to detect the HSV-1 US6 (gD). Equal amounts of total DNA were used for TaqMan PCR analysis. A baseline cutoff value of 0 was set at approximately 50 genomes, since samples containing less than 50 genomes exhibited high variability among the triplicate samples. Purified plasmid containing the gD gene was used to generate standard curves as described previously (David et al., 2012).

Infectious viral particle recovery

TG explants were collected from mice 30 dpi and were incubated at 37° C and 5% CO₂ immersed in DMEM media with no FBS for 48 hrs. Then, ganglia explants were macerated and co-cultured with Vero cells for another 48 hrs. Virus stocks were prepared and the number of infectious virions was determined by standard plaque assay.

Results

Construction and characterization of recombinant viruses

Construction and characterization of the McK virus was described previously (Chowdhury et al., 2013). This virus has a bac containing a EGFP gene cassette inserted within the intergenic region between the UL3 and UL4 genes (Figure 4.1A). The McK genome was utilized to engineer the McKΔgK31-68 (Figure 4.1B) virus using the two-step red-mediated recombination (Tischer et al., 2006). Subsequently, a rescued virus (McKR) was constructed (Figure 4.1C). Lack of spurious mutations and confirmation of the engineered mutations was obtained by complete genomic sequencing of all engineered viruses (see Materials and Methods section).

The wild-type (McKrae), McK and the McKR viruses produced on average similar plaque sizes on Vero cells (Figure 4.2 A, B, C). However, the McKΔgK31-68 virus consistently produced plaques that were on average approximately half-the-size of the McK and the McKR viruses (Figure 4.2D). The McKΔgK31-68 smaller plaque size phenotype was reversed on the gK expressing cells VK302 on which all viruses produced on average similar size plaques (Figure 4.2 I, J, K, L). As expected, only the McK virus exhibited EGFP fluorescence (Figure 4.2 F, N). The McK, McKΔgK31-68 and McKR viruses replicated with similar kinetics in Vero cells at an MOI of 1 with the exception that the McKΔgK31-68 appeared to have a delay in infectious virus production at 6 hpi in comparison to the McK and McKR viruses that did not negatively affect infectious virus production at later times post infection (Figure 4.3).

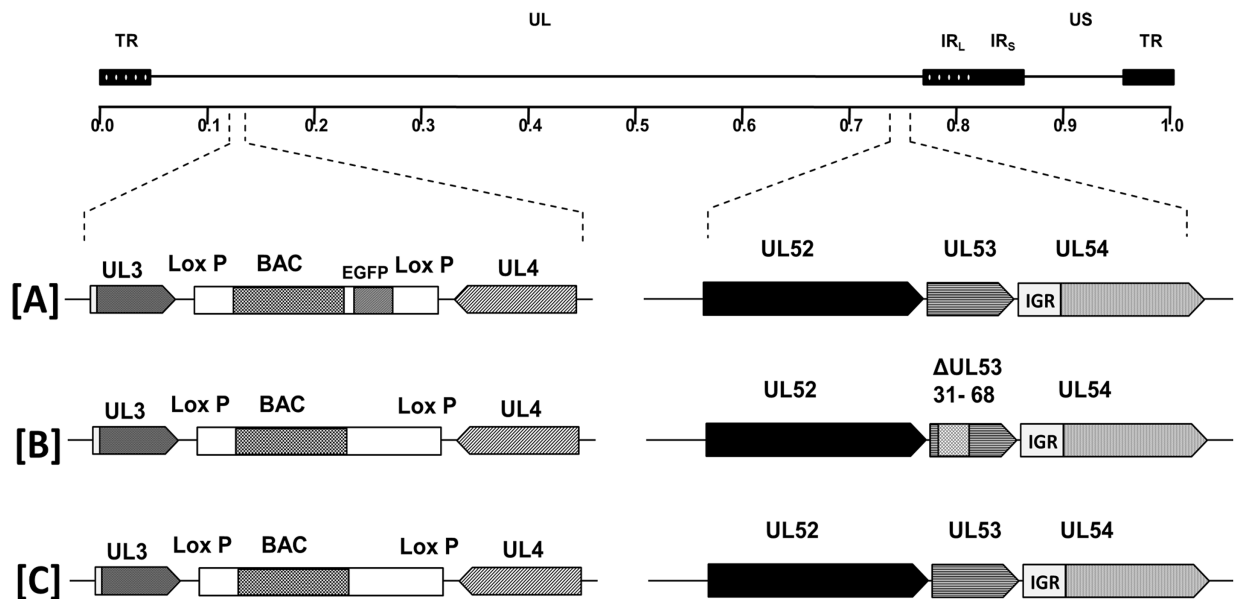


Figure 4.1: Schematic representation of mutant viruses. The top line represents the prototypic arrangement of the HSV-1 genome with the unique long (UL) and unique short (US) regions flanked by the terminal repeat (TR) and internal repeat (IR) regions. (A) McK. (B) McKΔgK31-68. (C) McKR.

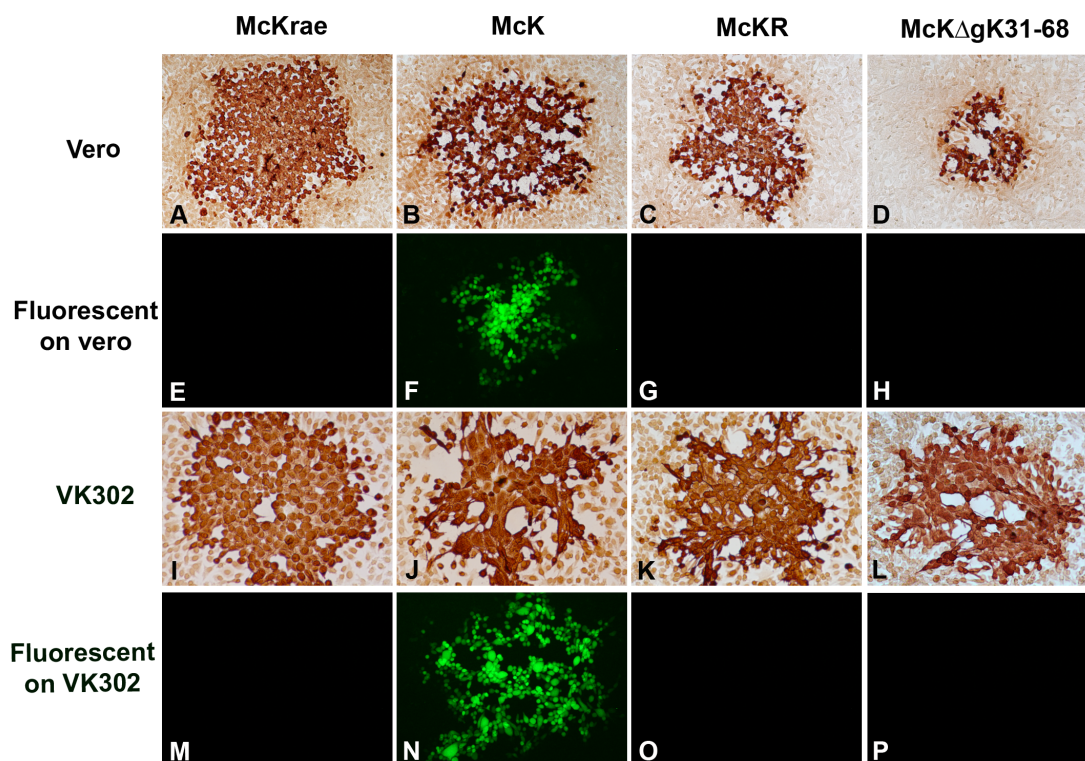


Figure 4.2: Plaque morphology of the constructed viruses. Confluent monolayers of Vero cells were infected at an MOI of 0.001 and representative plaques were identified and visualized after immunohistochemical staining (see Materials and Methods). Representative viral plaques of HSV-1(McKrae) and the derived mutant viruses are shown (A-D & I-L). Representative fluorescent plaques were visualized using fluorescence microscopy (E-H & M-P).

Mouse eye infections

Mice corneas were scarified and infected with 10^5 pfu for each eye. The ensued development of clinical ocular changes and disease progression were monitored and scored. The first signs of ocular changes started to appear at 4 dpi characterized by blepharospasms and small amounts of ocular discharges. These symptoms progressed and peaked at 7-8 days post infection. The clinical symptoms regressed and started to wane until relative resolution at 30 dpi. Specifically, the ocular clinical symptoms of blepharoconjunctivitis, hyperemia, keratitis and ocular discharge were the criteria used for scoring (see Materials and Methods section).

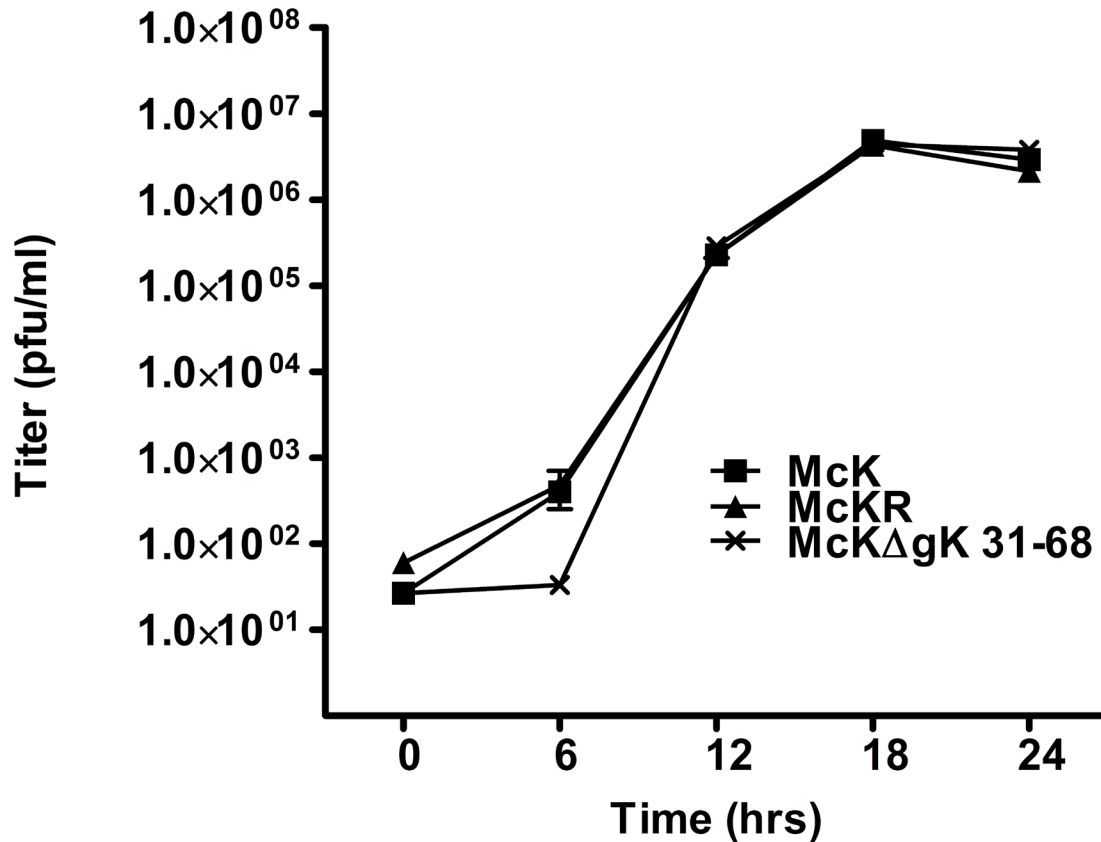


Figure 4.3: Replication kinetics of parental and mutant viruses. Confluent monolayers of Vero cells were infected at an approximate MOI of 1 with McK, McKR, or McKΔgK31-68 viruses. Infection was halted at the indicated time points and viral titers were obtained.

Infection of mouse eyes with the McK virus produced progressive ocular changes characterized as mild clinical ocular symptoms (score 1) at 3 days post infection (dpi), moderate blepharitis, hyperemia and ocular discharge (score 2) at 6 dpi, and acute ocular disease (score 3) at 9 dpi. In contrast mock-infected eyes, or eyes infected and observed immediately after infection, had no apparent ocular changes and were scored as 0 (Figure 4.4 A, B, C, D). Mouse eyes infected with the McKΔgK31-68 virus did not show any significant ocular disease symptoms throughout the thirty-day-long observation post infection (not shown).

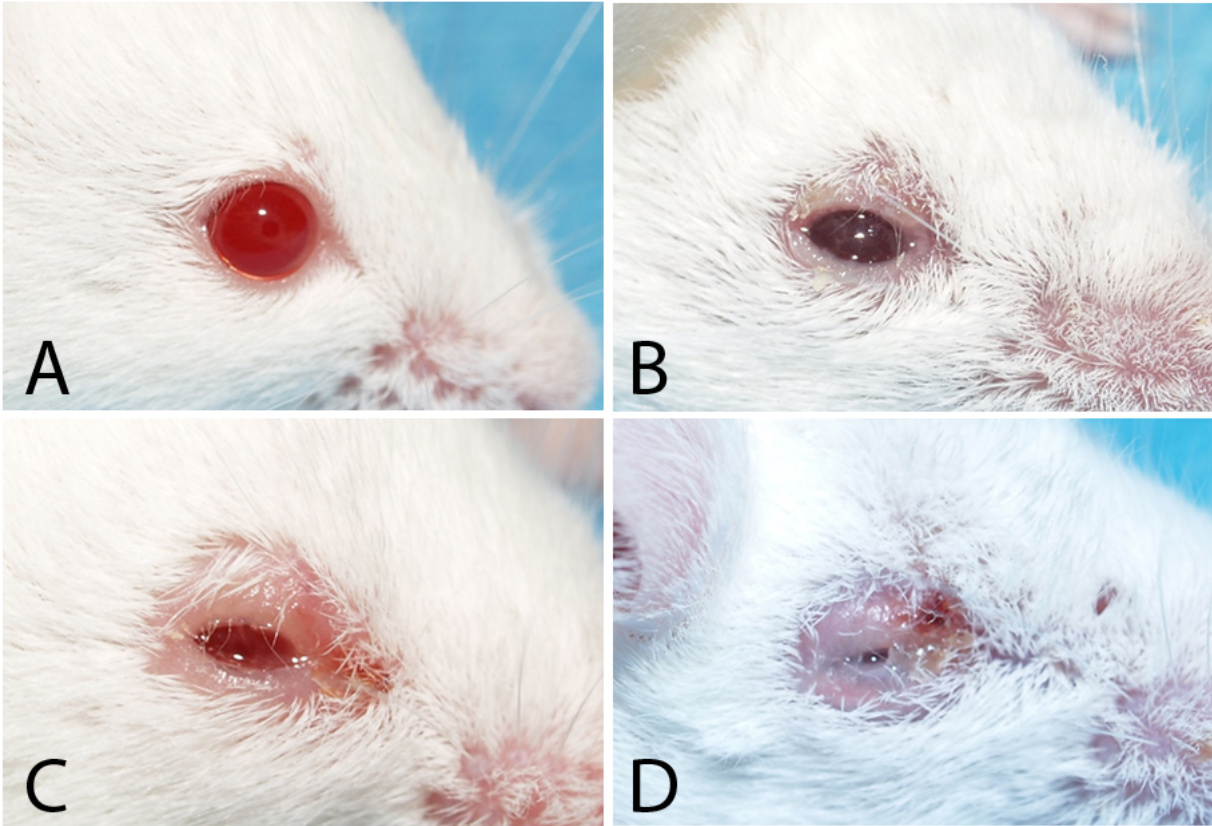


Figure 4.4: Clinical ocular disease development and scoring: Representative photographs of eyes exhibiting different lesion scores are shown. Scoring criteria included the degree of blepharoconjunctivitis, hyperemia, keratitis and ocular discharge (A) Score 0: Normal. (B) Score 1; Mild. (C) Score 2; Moderate. (D) Score 3; Severe.

TG-associated viral DNA and infectious virions

The TG were collected from mice at 30 dpi and tested for the presence of viral DNA genomes by qPCR, as we have described previously (David et al., 2012). Over 600 viral DNA genomes were detected in the TGs of mice infected with McK, or the McKR viruses; however, no viral DNA was detected in the TGs of mice infected with McK Δ gK31-68, or mice in the mock-infected groups (Figure 4.5 A).

TG explants were co-cultured with permissive cells (Vero cells) for 48 hrs to allow for viral reactivation. Infectious viral particle recovery was assessed by the standard plaque assay. All TGs from mice infected with McK and 70% of TGs from mice infected with McKR produced high numbers of infectious viral particles. However, substantially lower numbers of infectious virions were recovered only from two-of-ten of the mice infected with the McKΔgK31-68 virus (Figure 4.5 B).

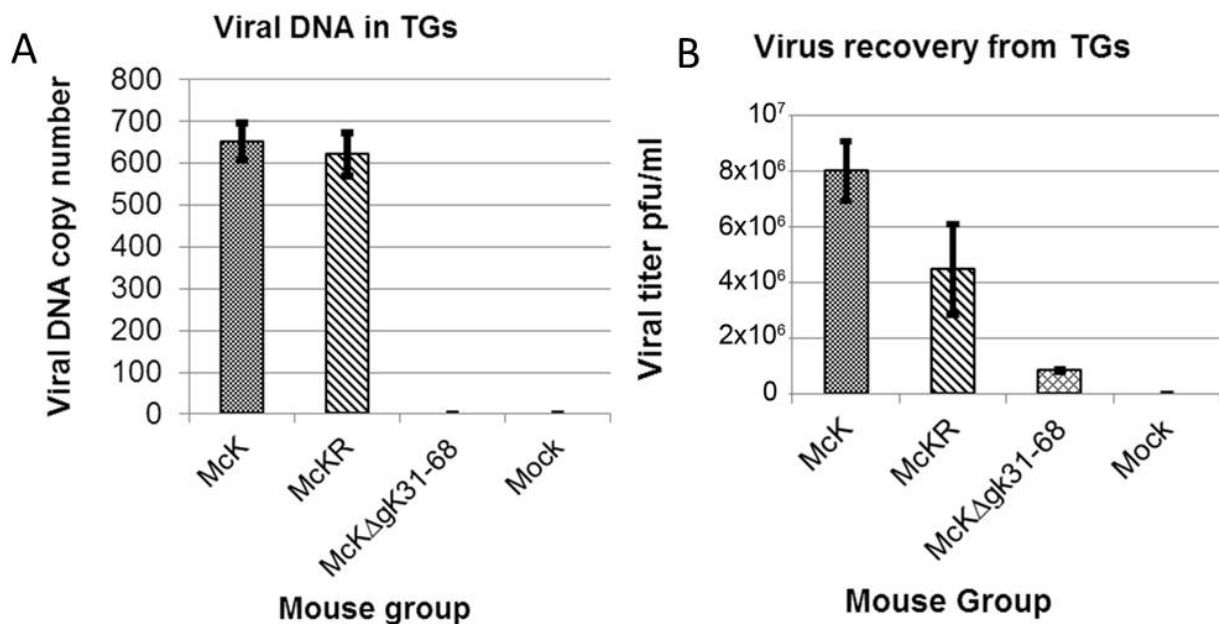


Figure 4.5: Viral DNA genome detection by qPCR and infectious viral particle recovery from TGs. A) TG samples were taken 30 dpi. The Viral DNA copy numbers were determined by qPCR. Purified plasmid containing the gD gene was used to generate the standard curve. B) Trigeminal ganglia explants were trituated and co-cultured with permissive cells (Vero cells) and viral titers were obtained by standard titration plaque assay on Vero cells. TG samples were collected 30 dpi.

Discussion

We have demonstrated previously that HSV-1 gK plays a pivotal role in neuronal infection and the ability of the virus to translocate in a retrograde manner from infected mouse corneas to the TG, while lack of gK also prevents virus to be transported in an anterograde manner (David et al., 2008; David et al., 2012). The purpose of the present study was to investigate whether a relatively small deletion in the amino terminus of gK, which is known to bind to the amino terminus of gB and modulate gB's fusogenic properties, could confer similar defects in neuronal infection and spread observed by viruses lacking gK gene expression. The results presented herein show that the amino terminal segment of gK spanning amino acids 31-68 that includes two N-glycosylation sites is required for neuronal infection and acute inflammatory responses responsible for herpes keratitis.

Previous studies using the HSV-1(F) genome cloned as a bac have identified the amino terminus of gK as an important determinant for virus-induced cell fusion, virus entry and virus spread (Chouljenko et al., 2009; Chowdhury et al., 2013; Foster et al., 2008; Foster et al., 2001b; Jambunathan et al., 2011; Jayachandra et al., 1997). However, HSV-1(F) does not cause significant ocular disease in the mouse eye model (not shown). Therefore, we have chosen to work with the human HSV-1(McKrae) viral strain, because it causes acute disease and death in experimental infections of mice via the eye route with or without cornea scarification (David et al., 2008). The HSV-1(McKrae) was cloned as a bac (McKbac) to facilitate the isolation of recombinant viruses using the double-red mutagenesis procedure (Tischer et al., 2006). Insertion of the bac between the UL3 and UL4 genes appeared to reduce virus-induced

mortality in an infectious dose dependent manner (not shown); however the McK remained highly pathogenic inducing acute herpes keratitis in infected mouse eyes. In agreement with previous findings using the HSV-1(F) virus, deletion of aa 31-68 from the amino terminus of gK did not drastically impair the virus ability to produce infectious virions (Chouljenko et al., 2009).

We chose to scarify the mouse eyes prior to infection in order to expose a significant number of neuronal axonal termini embedded within mouse corneas to direct infection by the virus. The cornea is one of the most densely innervated tissues in the mouse and human body. This highly innervated tissue protects the cornea and the eye from the external environment (Belmonte and Gallar, 1996; Wang et al., 2012). Cross-hatching scarification exposes a significant number of axons to direct viral infection. Surprisingly, infection of scarified mouse eyes with the McKΔgK31-68 virus failed to efficiently infect mouse TG, in comparison to the parental virus. Specifically, qPCR failed to detect viral DNA in mouse TGs; however, very low numbers of virions were recovered in 2-of-10 mice infected with the McKΔgK31-68 virus. This discrepancy is attributed to the inability of qPCR to reproducibly and reliably detect less than 50 copies of viral genomic DNA in ganglionic extracts (not shown), while explant-reactivation co-culture experiments can amplify a low number of virions during the ganglia incubation at 37° C (48 hours) and during the subsequent co-culture on Vero cells (48 hours). Thus, few reactivating genomes can result in the observed titers produced by explant culture of the two McKΔgK31-68 virus-positive mouse ganglia.

Importantly, mice infected with McK developed severe blepharoconjunctivitis, hyperemia, keratitis and marked ocular discharge 7-8 days post infection. Whereas, mice infected with McKΔgK31-68 virus did not show significant clinical ocular changes. These results suggest that efficient translocation of the virus to TG may be prerequisite to the causation of acute inflammatory responses and herpes keratitis. It has been reported that immunization of mice with gK significantly exacerbated inflammation, corneal scarring and facial dermatitis following ocular HSV-1 infection (Ghiasi et al., 1995; Ghiasi et al., 1997; Ghiasi et al., 1994a; Ghiasi et al., 1994b). Therefore, it is also possible that the amino terminus of gK is involved in the induction of innate immune responses that result in acute inflammation and herpes keratitis. This hypothesis is also supported by the observations that a recombinant virus expressing extra copies of *gK* caused elevated levels of inflammation and corneal scarring in both mice and rabbits (Mott et al., 2007b). Moreover, it was reported that elevation of anti-gK antibody in individuals with a history of HSV-1 recurrence was correlated with increased severity of eye disease (Mott et al., 2007a). Additional experiments are required to elucidate the role of gK in neuronal infection and herpes keratitis.

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CHAPTER V: CONCLUDING REMARKS

Summary

Herpes simplex virus type 1 (HSV-1) is a neurotropic pathogen of humans responsible for a wide spectrum of clinical disease, ranging from vesicular lesions on the lips to sight threatening keratitis, and fatal encephalitis and disseminated disease. The hallmark of the HSV-1 life cycle is infection of sensory neurons, where the virus establishes a latent infection for the life of the host (Roizman et al., 2007).

HSV-1 viral particle consists of a double stranded DNA genome enclosed in an icosahedral capsid. The capsid is surrounded by a proteinaceous tegument and an envelope derived from cell membranes. The viral envelope is studded with 12 glycoproteins that function in multiple aspects of viral life cycle and host interaction (Roizman et al., 2007).

Glycoprotein K (gK) is an envelope glycoprotein that has been the main focus of my PhD research work. Specifically, I studied the role of gK and its functional domains in neuroinvasion and immunopathogenesis of keratitis. Glycoprotein K has been shown to be involved in viral envelopment, fusion, egress and spread (Hutchinson et al., 1992; Hutchinson and Johnson, 1995; Jambunathan et al., 2011; Jayachandra et al., 1997; Melancon et al., 2005). Other glycoproteins such as gE, gM and the membrane protein UL11 have also been shown to play roles in viral envelopment, fusion, egress and spread (Chouljenko et al., 2012; Han et al., 2012; Kim et al., 2013; Legee et al., 2009). To explore the relative importance of these membrane proteins (gK, gE, gM, and UL11) in infection of mouse corneas and establishment of latency in ganglionic neurons, we constructed recombinant mutant viruses that lack the expression of

either gK, gE, gM, and UL11 and compared them to the wild type parental virus. The recombinant viruses were constructed using a markerless two-step Red-mediated recombination system implemented on a bacterial artificial chromosome (BAC). The mutant viruses were sequenced to assure that no collateral extraneous mutations were introduced. The wild type and the recombinant viruses were used to infect mice via the ocular route. The mutant viruses were also characterized by using plaque morphology to assess virus spread, viral growth kinetics to measure infectious virus production, viral shedding in tear film after ocular inoculation, quantitative PCR analysis for the quantification of viral genome copy numbers in the trigeminal ganglia. The wild type virus and all the recombinant viruses except gK-null mutant were able to infect axonal termini embedded in the cornea and establish latency in ganglionic neurons albeit with varied efficiency. This result suggests that gK plays the most important role in neuroinvasiveness. UL11-null virus replicated in the eyes less efficiently than gM-null and gE null mutants, however, it infected ganglionic neurons and established latency more efficiently than either the gM or gE-null viruses (Kim et al., 2014).

Recent work in this research laboratory has shown that gK is essential for viral corneal spread and neuroinvasiveness (David et al., 2008). Previously, I participated in the development of a primary neuronal culture system to study the role of gK in axonal infection and viral transport in retrograde and anterograde manner in neurons. Primary neuronal cultures were prepared from the dorsal root ganglia of embryonic rats. A single cell suspension of neurons was plated in microfluidic devices that separate neuronal bodies from pure axonal fraction. We utilized this microfluidic system to show that gK is required for infection of axonal termini and egress from neuronal cell bodies in primary neuronal cultures (David et al., 2012).

The above mentioned investigations revealed that deletion of the entire gK gene resulted in drastic decrease in viral replication and spread efficiencies. A viral backbone that lacks gK constitutes a safe viral vector; however, low replication and spread efficiencies would be considered major disadvantages. Therefore, I sought to delineate gK domains responsible for neuroinvasion and replication as well as immunopathogenesis of keratitis. The highly pathogenic and neuroinvasive clinical ocular isolate of HSV-1 McKrae strain, was used to construct a recombinant mutant virus lacking 37 amino acids from the amino terminus of gK, amino acids (31-68). This recombinant virus and its parent wild type were characterized by using plaque morphology to assess virus spread, viral growth kinetics to measure infectious virus production, and by quantitative PCR analysis for the quantification of viral genome copy numbers in the trigeminal ganglia. Experiments in this investigation (outlined in chapter IV of this dissertation) showed that the amino terminus of gK is dispensable for replication in tissue culture, but important for neuroinvasion and establishment of latency after ocular inoculation of mice (Saied et al., 2014).

One important observation from the above investigation is that mice infected with the wild type virus developed severe keratitis, conjunctivitis, blepharitis and ocular discharge. On the other hand, mice infected with the recombinant virus lacking the amino terminus of gK did not exhibit significant ocular changes (Saied et al., 2014). This observation suggests a potential role for the amino terminus of gK in immunopathogenesis of keratitis. The investigation of the role of the amino terminus of gK in immunomodulation of keratitis is currently in progress. The preliminary results obtained so far are included in Appendix I.

In conclusion, the research work in this dissertation is considered a significant contribution to our understanding of alphaherpesvirus virulence factors and neurotropic determinants. It may lead to the development of a safe, replication competent viral vector backbone for oncolytic viral therapy and vaccine. The research work presented in this dissertation may also lead the discovery of new therapeutics to treat herpes keratitis, and to find new ways to combat neuronal infections.

Current and Future Research

Current work is focused on answering another critical question; is gK that lacks the 37 amino acids from the amino terminus expressed on the virion particle? To answer this question, I have constructed a recombinant virus that has a deletion in the amino terminus of gK and also carries a V5 tag on its carboxyl terminus. gK is extremely hydrophobic, and notoriously difficult to detect. The purpose of tag insertion is to facilitate detection in various types of experiments. Sequencing of this virus showed that the V5 tag is inserted in the intended location and that no amino acid changing mutations were introduced. We are currently working on showing the expression of tagged gK (31-68) in the virion particle by western blot analysis on purified virions. Concurrently, I am studying the mechanism by which gK is involved in immunomodulation of keratitis (please see Appendix I).

The work in this dissertation raised important research questions about the critical amino acid requirements in the amino terminus of gK that are responsible for neuroinvasion and immunopathogenesis. Glycoprotein K is post-translationally modified by N-linked carbohydrate addition at the amino-terminus. The segment spanning amino acid (aa) 31 to aa

68 in the amino terminus of gK contains two N-linked glycosylation sites at aa 48 and aa 58. Future research could delineate the gK critical amino acids and the role of N-glycosylation in gK function.

Another interesting and very important question to address is the mechanism by which gK functions in virion entry at the axonal termini. This research question can be investigated by studying the effects of gK mutations on gB conformation, and by assessing the role of cellular receptors and signaling pathways in gK-mediated virus entry into axonal termini.

The secondary structure of gK presents Domain II as a loose cytoplasmic domain with a couple of membrane anchored amino acids. However, the modeled tertiary structure of gK predicts that Domain II is composed of two connected alpha helices. This is particularly interesting because it reveals that this portion may not be a mere structural domain. It may also have an important function which merits further investigation.

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APPENDIX I: ADDITIONAL WORK

Introduction

HSV-1 causes multiple medical disorders such as cold sore, keratitis, encephalitis and systemic disease (Roizman et al., 2007; Whitley et al., 1998). It is the leading infectious cause of corneal blindness in the US. An estimated 400,000 people in the US suffer from herpes simplex ocular disease, with about 50,000 new or recurrent cases occurring annually. HSV-1 infects sensory neurons and establishes a latent infection in the trigeminal ganglia (Roizman et al., 2007; Whitley et al., 1998). Reactivation of latent virus from the trigeminal ganglia can cause spread to corneal transplants causing corneal graft failure (Cockerham, 2001).

The envelope of the HSV-1 virion particle contains 12 glycoproteins as well as several membrane-associated proteins, which play important roles in viral entry and virus-induced cell fusion (Roizman et al., 2007). HSV-1 glycoprotein K (gK) is a structural component of the virion particle and functions in virus entry into epithelial cells (Foster et al., 2001; Jambunathan et al., 2011) cytoplasmic virion envelopment, virion egress, and virus-induced cell fusion (Chouljenko et al., 2009). Glycoprotein K exerts its function in virus entry by modulation of gB, the bona fide fusogen of HSV-1 (Chouljenko et al., 2009). HSV-1 gB has been shown to interact with TLR-2 which leads to pro-inflammatory cytokine production via MyD88 dependent signaling (Cai et al., 2013).

Corneal epithelial cells are one of the most important participants in herpes simplex keratitis (HSK). They not only take part in pathogen recognition via the expression of a wide range of pattern recognition receptors (PRR) including Toll-like receptors (TLR) TLR-2, and TLR-

7, but also secrete β -defensins, cytokines and chemotactic factors upon microbial invasion (Johnson et al., 2005; Kumar et al., 2006).

We have reported that mice infected with wild type virus (wt) exhibited significant clinical ocular changes and keratitis while mice infected with a recombinant virus lacking the amino terminus of gK (McK Δ gK 31-68) did not show significant ocular disease (Saied et al., 2014) (Figure AI.1). In this work, we describe a novel immuno-modulatory role for gK in the immunopathogenesis of HSK.

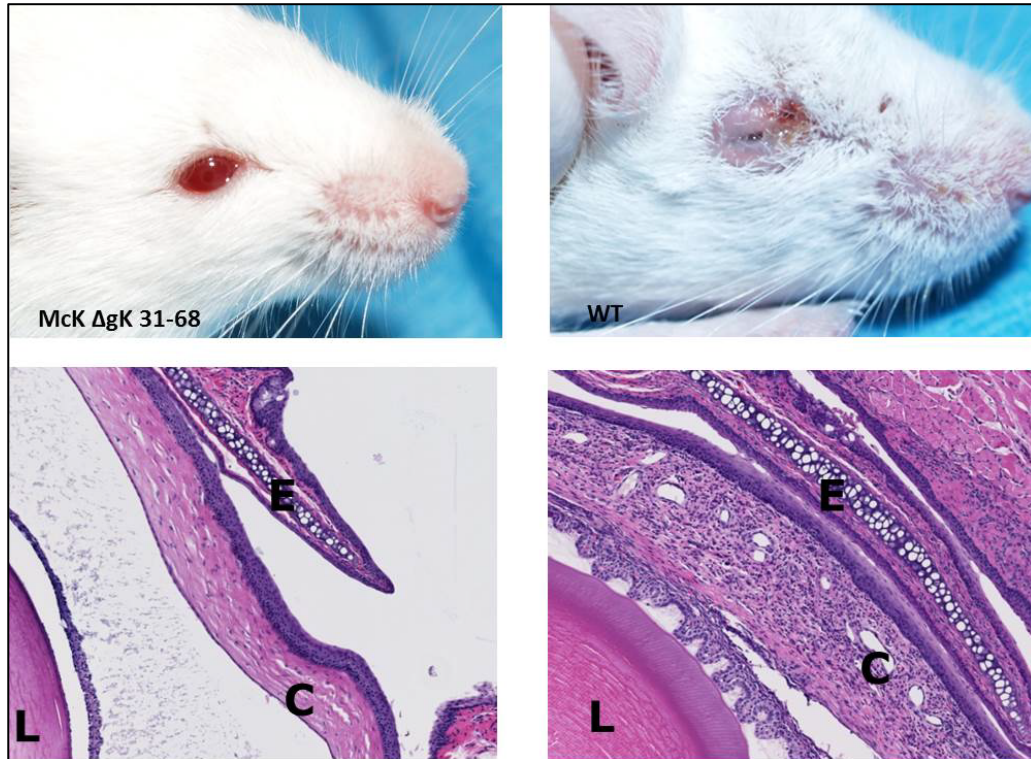


Figure AI.1: Ocular infection of mice. Infection of mice eyes with the wt virus produced progressive ocular changes characterized by severe blepharitis, keratitis, hyperemia and ocular discharge seen in top right panel. Histopathological examination of the eyes revealed that the cornea is infiltrated by a large number of neutrophils, lymphocytes, plasma cells and macrophages and neovascularization is noted, bottom right panel. Infection of mice eyes with McK Δ gK 31-68 virus produced no significant ocular disease shown in top left panel. The cornea shows no significant histopathological changes in eyes infected with McK Δ gK 31-68 virus, bottom left panel. L: lens, C: cornea, E: 3rd eyelid.

Objectives and Hypothesis

The objective of this work is to investigate the role of the amino terminus of gK in the induction of the innate signaling pathways that are involved in herpes immunopathogenesis of keratitis. We hypothesize that the lack of the amino terminus of gK alters gB binding to TLR-2 which leads to TLR-2 mediated immunomodulation.

Materials and Methods

Viruses

The highly pathogenic and neuroinvasive wild type HSV-1 virus McKrae strain cloned onto a bacterial artificial chromosome plasmid vector (designated as wt) was used as a backbone to construct a recombinant virus that lacks the amino terminus of glycoprotein K (designated as McK Δ gK 31-68) virus. Two-step red mutagenesis implemented on the wt virus was performed to construct McK Δ gK 31-68 virus.

Toll-like receptor-2 (TLR2) stimulation

HEK-Blue™-hTLR2 cells (bought from InvivoGen) were used to study the stimulation of TLR2 by monitoring the activation of NF- κ B. HEK-Blue™-hTLR2 cells are HEK293 cells co-transfected with human TLR2 (hTLR-2) and SEAP (secreted embryonic alkaline phosphatase) reporter genes. Levels of SEAP were determined using a detection medium with SEAP substrate QUANTI-Blue™. The hydrolysis of the substrate by SEAP produces a purple/blue color that can

be measured with a spectrophotometer. HEK-Blue™ Null1 cell line (bought from InvivoGen) is the parental cell line of HEK-Blue™ hTLR2. HEK-Blue™ Null1 cells lack the expression of TLR-2 but express the SEAP reporter gene.

QPCR analysis

qPCR analysis to determine the levels of mRNA transcription of pro-inflammatory cytokines and interferon genes was performed on; HEK-Blue™-hTLR2 cells, HEK-Blue™ Null1 cells and Human corneal epithelial (HCE) cells lysates. The pro-inflammatory cytokines and interferon genes; TNF- α , IL-6, IL-10, and IFN- β were selected for the qPCR procedure.

Detection of NF- κ B activation by western blot

Western blot analysis was performed on human corneal epithelial cells to determine the activation of the transcription factor NF- κ B using I κ B- α as a proxy for NF- κ B activation. Antibodies for I κ B- α detection were purchased from abcam, USA.

Results

Toll-like receptor-2 (TLR2) stimulation

McK Δ gK 31-68 virus induces marked NF- κ B activation in HEK-Blue-TLR2 cells compared to wt virus. There is no marked difference in NF- κ B activation between wt virus and McK Δ gK 31-68 virus in infected HEK-Blue Null1 cells (Figure AI.2).

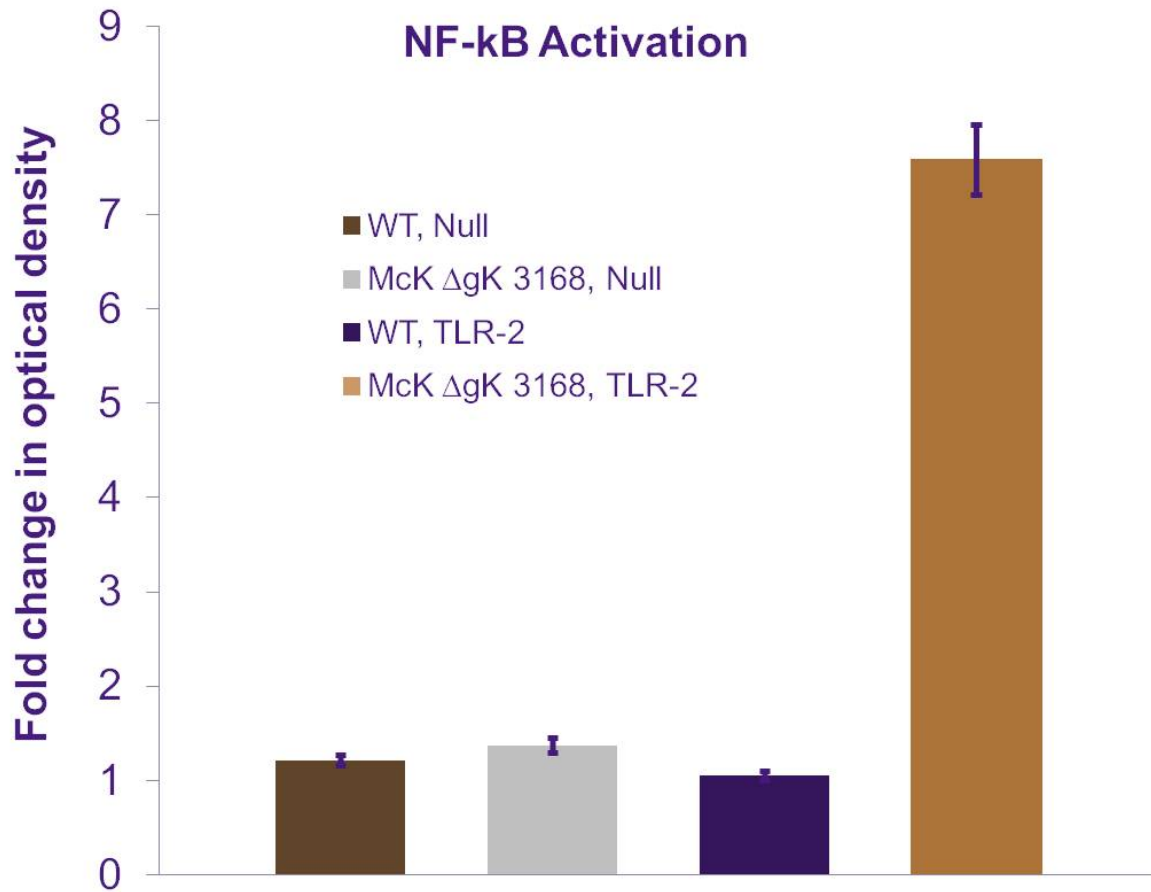


Figure AI.2: NF-kB activation. HEK-Blue-TLR2 and HEK-Blue Null1 cells were infected with either McK ΔgK 31-68 virus or wt virus, a detection medium with SEAP substrate was added to the Supernatants from infected cells were added 18 hrs post infection. Changes in optical density were measured by a spectrophotometer. McK ΔgK 31-68 virus causes 8-8.5 fold increase in optical density in HEK-Blue-TLR2 cells compared to wt virus. Activation levels of NF-kB in HEK-Blue Null1 cells are similar for both viruses. TLR-2: HEK-Blue-TLR2 cells. Null: in HEK-Blue Null1 cells.

QPCR analysis for pro-inflammatory cytokine mRNA transcripts in HEK blue cells

Lack of the amino-terminus of gK is associated with increased pro-inflammatory cytokine mRNA levels in HEK-Blue-TLR2 cells (Figure AI.3). Greater mRNA levels of TNF- α , IL-6 and IFN- β were detected in HEK-Blue-TLR2 cells infected with McK ΔgK 31-68 compared to wt virus. Generally, mRNA levels of TNF- α and IFN- β were similar in HEK-Blue™ Null1 cells

infected with McK ΔgK 31-68 compared to wt virus. The production of large numbers of pro-inflammatory cytokine and IFN-β mRNA transcripts after NF-kB activation is overwhelmingly due to TLR-2 pathway engagement in HEK Blue cells (Figure AI.4).

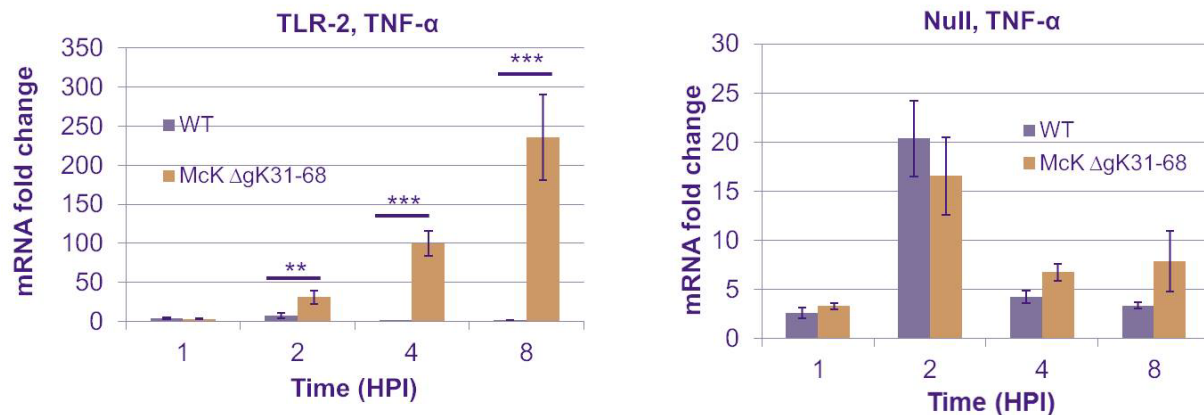


Figure AI.3: QPCR analysis-HEK blue cells. HEK Blue TLR-2 and HEK Blue Null1 cells were infected at a multiplicity of infection (MOI) of 5 with either wt or McK ΔgK 31-68 virus. RNA was extracted at multiple different points post infection and mRNA transcripts copy numbers of pro-inflammatory cytokines were determined by QPCR. TLR-2: HEK-Blue-TLR2 cells. Null: HEK-Blue Null1 cells. Asterisks denote statistical significance. Threshold significance level is 0.05. HPI: hours post infection.

Determination of NF-kB activation in HCE by western blot

McK ΔgK 31-68 virus prompts more NF-kB activation than the WT virus at early time points in HCE cells (Figure AI.5).

QPCR analysis for pro-inflammatory cytokine mRNA transcripts in HCE cells

Lack of the amino-terminus of gK is associated with increased pro-inflammatory cytokine mRNA levels in HCE cells (Figure AI.6). TNF- α and IL-6 mRNA transcripts were significantly higher in HCE cells infected with the McK ΔgK 31-68 than cells infected with the wt virus.

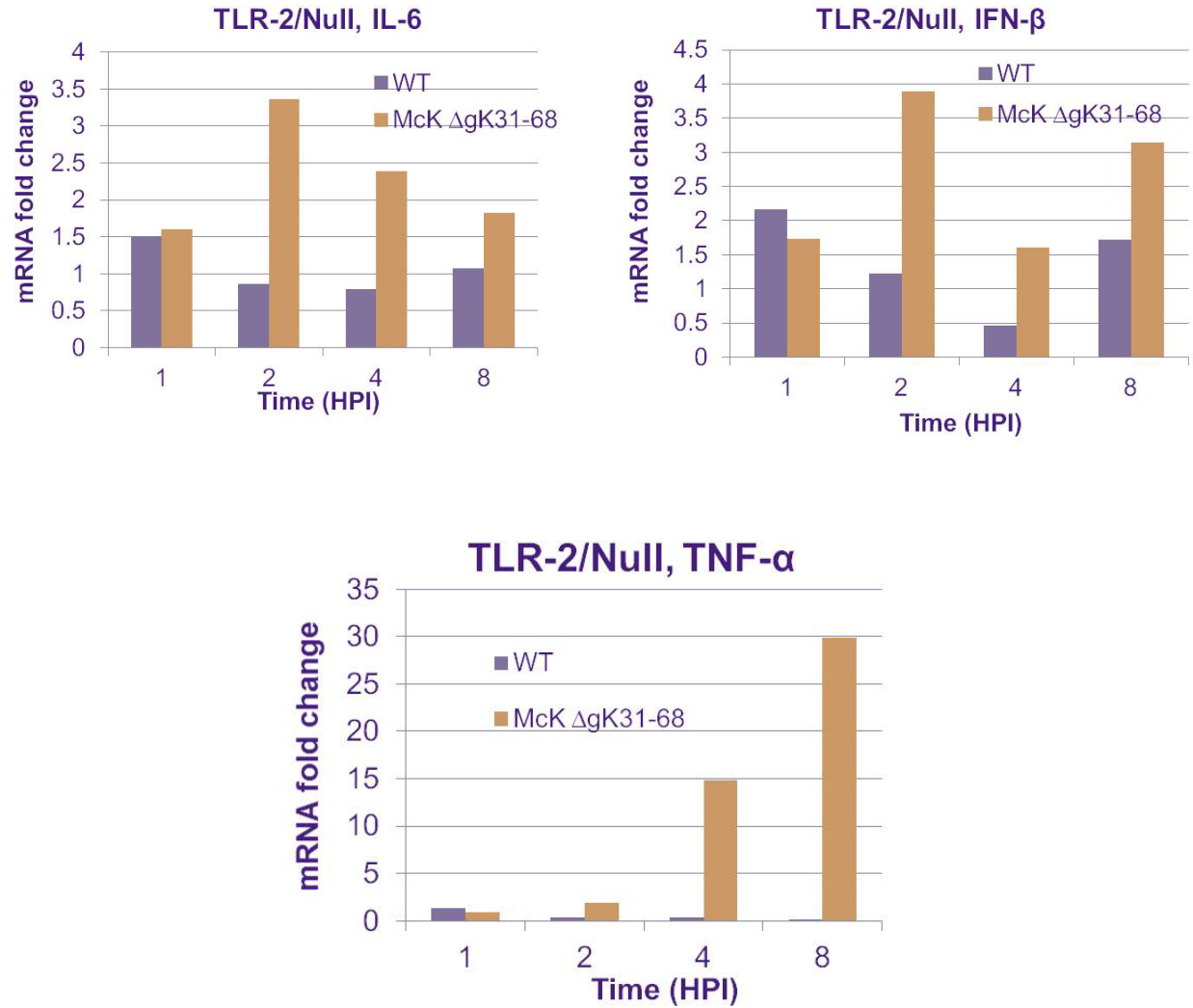


Figure AI.4: QPCR analysis, TLR-2 engagement. Since NF- κ B can be activated by TLR-2 engagement as well as other pathways, we divided mRNA transcription levels obtained from infection of HEK Blue-TLR-2 cells by the mRNA transcription levels obtained from infection of HEK Blue Null1 cells. This will help distinguish the impact of TLR-2 engagement in the activation of NF- κ B and the production of inflammatory cytokine and IFN- β . TLR-2: HEK-Blue-TLR2 cells. Null: HEK-Blue Null1 cells. Asterisks denote statistical significance. HPI: hours post infection.

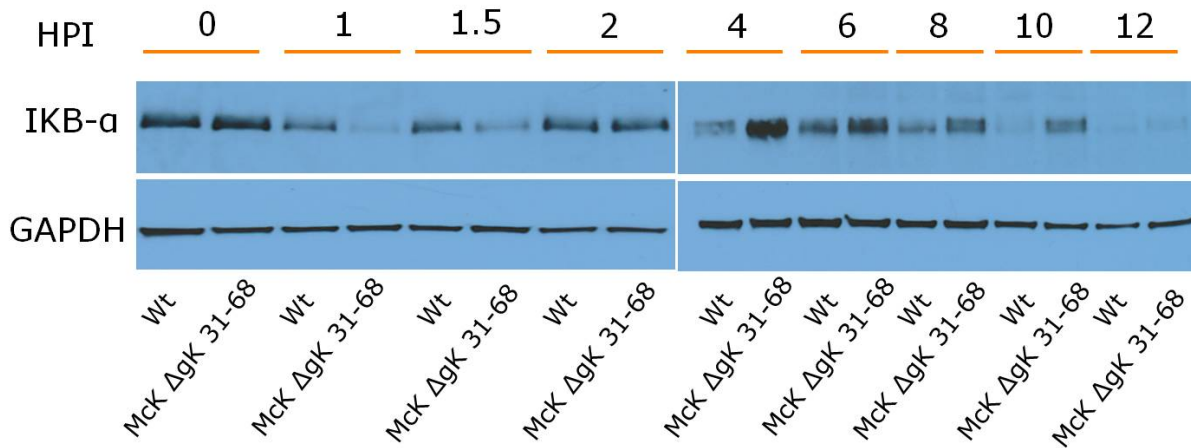


Figure AI.5: Western blot for NF- κ B activation in HCE cells. IKB- α was used as a proxy for NF- κ B activation. McK Δ gK 31-68 virus induces more NF- κ B activation than wt virus at early time points. HCE cells: Human corneal epithelial cells.

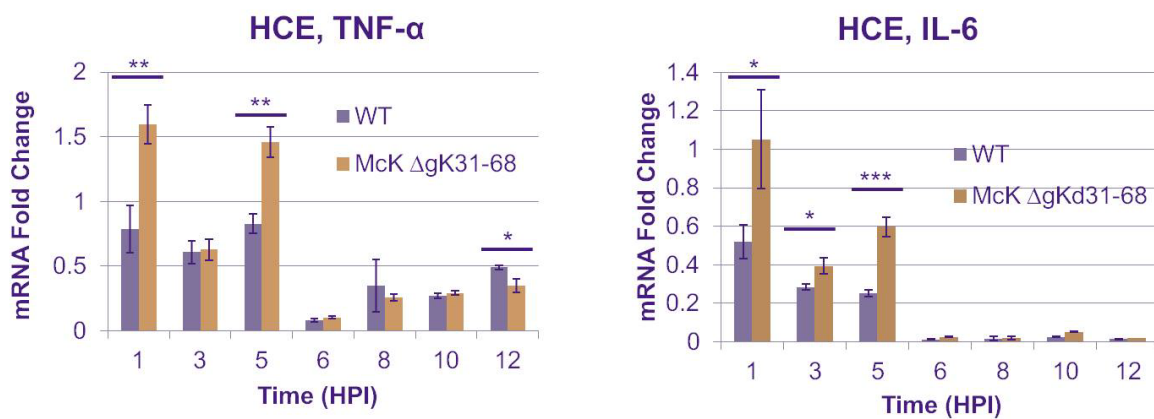


Figure AI.6: QPCR analysis-HCE cells. HCE cells were infected at a multiplicity of infection (MOI) of 5 with either wt or McK Δ gK 31-68 virus. RNA was extracted at multiple different time points post infection and mRNA transcripts copy numbers of pro-inflammatory cytokines were determined by QPCR. Asterisks denote statistical significance. Threshold significance level is 0.05. HPI: hours post infection. HCE: human corneal epithelial cells.

Summary

Lack of the amino-terminus of gK alters NF- κ B activation which indicates that gK is potentially involved in the immunomodulation of herpes keratitis. Mck Δ gK 31-68 induces marked NF- κ B activation than the wt virus. Subsequently, greater pro-inflammatory cytokine mRNA transcripts were detected in cells infected with Mck Δ gK 31-68 than the wt virus at early time points. The global gene expression dynamics of viral as well as host mRNA transcripts are being studied using digital PCR and RNA seq. Lack of the amino terminus of gK may alter gB binding to TLR-2 which leads to TLR-2 mediated immunomodulation. Future studies may involve the detection of conformational changes in gB using a cadre of monoclonal antibodies and confirm of the engagement of TLR-2 pathway.

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Journal: Current Eye Research 2014 Jun;39(6):596-603. doi: 10.3109/02713683.2013.855238.
Epub 2014 Jan 8

Article II

Authors: In-Joong Kim*, Ahmad A. Saied*, Vladimir N. Chouljenko, Ramesh Subramanian and Konstantin G. Kousoulas
*These authors contributed equally to this manuscript.

Title: Functional Hierarchy of Herpes Simplex Virus Type-1 Membrane Proteins in Corneal Infection and Virus Transmission to Ganglionic Neurons

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