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Role of Herpes Simplex Type I Glycoproteins in Entry and Cell-cell Fusion

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ROLE OF HERPES SIMPLEX TYPE I GLYCOPROTEINS IN ENTRY AND CELL-CELL FUSION

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DEDICATION

To my beloved mother, Leila Chowdhury and beloved father, Dipak Kumar Chowdhury; without your love and blessings this goal would not have been accomplished.

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ABSTRACT

Herpes simplex virus type I (HSV-1) is a neurotropic virus that infects primarily mucocutaneous epithelial cells and nervous tissue. Membrane fusion is an important aspect of the HSV-1 lifecycle, that occurs during viral entry (virus-cell fusion), viral spread (cell-to-cell fusion), as well as, during virion morphogenesis (assembly and egress). These membrane fusion steps involve complex interactions between multiple viral glycoproteins and cellular receptors. HSV-1 glycoprotein B (gB) is necessary but not sufficient for membrane fusion events. Despite the fact, that the majority of known hypermorphic mutations which cause extensive virus-induced cell fusion occur within glycoprotein K (gK); yet the role of gK in gB-mediated cell fusion is not well understood. We found that a mutation within the carboxyl terminal of gB that resulted in extensive cell fusion is lost in presence of a mutation in gK amino terminus (deletion of amino acids 31-68). These results suggest that gK may regulate gB mediated virus-induced cell fusion. Co-immunoprecipitation experiments revealed, that a peptide specifying the amino terminus of gK physically interacted with others members of the fusion complex i.e. gB, gH but not gD. Moreover, UL20p, known to interact with gK, interacted with gB as revealed by immunoprecipitation reaction. Virus entry was also modulated by gK, since gK mutants lacking the entire gK gene or a deletion in the amino terminus (amino acids 31-68) failed to enter Chinese hamster ovary cells (CHO) expressing the gB receptor paired immunoglobulin-like type 2 receptor alpha (PILRα). While these gK mutants efficiently entered into CHO cells expressing the gD specific receptors, nectin-1 and HVEM. Co-immunoprecipitation experiments revealed that PILRα formed a multi-protein complex with gB and gK. Thus gK functions in entry in presence of gB specific receptors but not gD specific receptors. Overall, results obtained in this study, show that gK and UL20 are part of the fusion complex (gB, gD, gH/gL) that functions

during virus entry and cell spread, and regulates interactions of gB with gB-specific cellular receptors.

CHAPTER I

INTRODUCTION

STATEMENT OF PROBLEM AND HYPOTHESIS

Herpes Simplex Virus Type-1 (HSV-1) is a neurotropic virus that infects cells of the muco-epethelium and of neuronal origin. A hallmark of herpes infection is that it becomes latent in neurons and persists in the host throughout life. Disease symptoms often interfere with everyday life and occasionally HSV infections are cause of life threatening (e.g. herpes encephalitis) or life impairing diseases (e.g. herpes keratitis). Moreover, recent studies have shown that HSV-1 may be linked to Alzheimer's.

In order, to produce a productive infection the virus has to overcome the physical barrier of the cellular membrane during virus entry, as well as utilize different mechanisms to spread within the host. Glycoproteins expressed on the virion surface of HSV-1 play a major role in virus entry and cell-to-cell spread. Virus entry and cell-to-cell spread involve fusion of the viral envelope with cellular membranes and fusion among cellular membranes, respectively. These membrane fusion steps occur as a result of interaction between viral glycoproteins and cellular receptors and may be important in determining host range of the virus *in vivo*. In this regard, the existence of highly virulent HSV-1 strains may be due to specific amino acids in glycoproteins that promote efficient virus entry and spread *in vivo*. Therefore, to better define the role of glycoproteins in membrane fusion in different HSV-1 strains; comparison of predicted amino acid sequence differences between the membrane proteins is necessary.

Although, specific cellular receptors have been identified that bind to viral glycoproteins gC, gB and gD; the mechanism by which these receptors are involved in virus-associated membrane fusion phenomena is not clearly understood. The X-ray structures of viral glycoproteins gB, gH and gD have been solved for HSV-1 and EBV providing unique insight into how these glycoproteins interact with each other, as well as, with cellular receptors. This knowledge has led to better understanding of the so called "fusion complex" consisting of gD, gB, gH and gL. It is now believed that this core fusion machinery plays an important role during both viral entry as well as cell-to-cell fusion.

Overall, the membrane fusion machinery of HSV-1 is highly complex. Recent studies have designated gB as the sole viral fusogen, while the rest of the glycoproteins involved in fusion are now considered as activators or regulators of gB. HSV-1 induced cell–cell fusion leads to the formation of multinucleated cells that are termed as syncytia. Single amino acid changes within the proteins encoded by the UL53 (gK), UL20 (UL20p), UL24 (UL24p) and UL27 (gB) are known to cause extensive syncytia formation. Specifically, a number of single amino acid changes within the amino termini of gK and UL20p and the carboxyl terminus of gB cause extensive virus-induced cell fusion. The amino terminus of gK is localized extracellulary, while the amino terminus of UL20p is localized intracellularly. Therefore, I hypothesize that gK and UL20p physically interact through cytoplasmic and extracellular domains with glycoproteins gB to modulate gB mediated membrane fusion.

Glycoprotein K and UL20p are expressed on virions and function in virion entry, inasmuch as, HSV-1 strains F and KOS virions devoid of gK enter substantially slower into Vero cells than the parental viruses. Therefore, gK/UL20p function in a similar way in both virus entry and virus-induced cell fusion by modulation of the fusogenic properties of gB. In this

study we have investigated the contribution of individual viral receptors on virion entry regulated by gK/UL20p to gain a better understanding of the mechanism by which gK modulates gB's fusogenicity during virus entry.

STATEMENT OF RESEARCH OBJECTIVES

The main goal of this research was to investigate the role of individual viral receptors in gB-mediated membrane fusion regulated by viral proteins gK and UL20p. The specific aims of this research were:

I To analyze the role of amino terminus of gK in gB mediated virus-induced cell-to-cell fusion:

- i. To evaluate the ability of the amino terminus of gK to complement (in trans) gB-mediated cell-to-cell fusion.
- ii. To characterize the interaction between the amino terminus of gK and amino terminus of gB.
- iii. To characterize the interaction between the amino terminus of gK and amino terminus of gH.
- iv. To determine the interaction between the UL20p and gB.

II To analyze the role of amino terminus of gK in gB mediated virus-cell fusion (entry):

- i. To clone the HSV-1 McKrae strain genome into a bacterial artificial chromosome (BAC) and generate a HSV-1 McKrae BAC virus (McKbac).

ii. To construct gK mutant viruses gKΔ31-68 (encoding gK carrying an in-frame deletion of aa 31 to 68), and gKΔ31-117 (encoding gK carrying an in-frame deletion of aa 31 to 117) by utilizing the McKbac virus.

iii. To characterize recombinant viruses in cell culture.

iv. To characterize the entry phenotype of McKbac and gK mutants into Vero cells and Chinese hamster ovary cells (CHO) expressing single receptors –Nectin-1, HVEM, and PILRα.

v. To characterize the mode of entry (pH dependent or independent fusion) of McKbac and gK mutants into CHO-nectin-1cells and PILRα cells.

vi. To characterize the interaction between PILRα and gK /UL20p complex in infected cells.

III To compare the entry phenotype of HSV-1 strains, McKrae and F:

i. To sequence the viral proteins involved in entry and cell-to-cell fusion of HSV-1 strain Mckrae.

ii. To compare the predicted amino acid sequence of glycoproteins gB, gD, gH, gL. gC, gK and UL20p of HSV-1 strain McKrae with strain F, H129 and KOS.

iii. To compare the entry phenotype of McKrae and F strains into CHO-nectin-1, CHO-HVEM, CHO-PILRα cells and Vero cells.

Overall, the results obtained from this research indicate that:

1. gK and UL20p heterodimer play an important role in virus-induced cell-cell fusion and viral-cell fusion.

2. The amino terminus of gK modulates the conformation of gB by interacting directly with gB and other members of the fusion complex .
3. gK plays an important role during viral entry not only in Vero cells but specifically in cells expressing a gB receptor (PILRα).
4. HSV-1 Mckrae strain enters CHO-nectin -1 cells in a pH independent manner.
5. HSV-1 McKrae strain enters more efficiently than F strain into cells expressing gB specific receptor PILRα.

Thus, it seems that gK is intimately associated with gB and plays a role in gB mediated membrane fusion.

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

CHAPTER II: The amino terminal portions of HSV-1 gK and UL20p interact with gB and gH and regulate gB-mediated membrane fusion.

CHAPTER III: The amino terminus of HSV-1 glycoprotein K (GK) modulates virion entry into cells via the Paired-Immunoglobulin Type-2 Receptor alpha (PILRα).

CHAPTER IV: Amino acid differences in glycoproteins B (gB), C (gC), H (gH) and L (gL) are associated with Enhanced Herpes Simplex Virus type-1 (McKrae) Entry via the Paired Immunoglobulin-like Type-2 Receptor α.

LITERATURE REVIEW

Historical Perspective of Herpesviruses

O'er ladies ' lips, who straight on kisses dream,
Which oft the angry Mab with blisters plagues,
Because their breaths with sweetmeats tainted are.
-- Act 1. Scene IV. William Shakespeare, *"Romeo and Juliet"*.

The blisters that Shakespeare refers to are in fact cold sores produced by the Herpes Simplex Virus (HSV), a common infectious agent. HSV is an ancient virus that can be traced to the Greek era (Kaplan 1973). In fact, herpesviruses derive their name from the Greek word herpes (erpein) meaning "to creep" or "crawl" to describe the lesions found on the skin. Even earlier than the Greeks, descriptions of lesions resembling HSV were found on a Sumerian Tablet, in the 3rd Millenium BC and also in the Ebers Papyrus, circa 1500 BC (Whitley 2001). During the second century A.D. the Greek physician Galen stated that lesions caused by recurrent HSV always erupted at the same anatomical location (Whitley, 2001).

Originally, the term herpes was used in medicine to describe a variety of skin conditions and diseases but its meaning changed overtime. In 1814, Bateman described both herpes liabilis and herpes perputialis disease as a "restricted group of localized vesicles with a short, self-limiting course" (Bateman and Willan 1814). During King Louis XIV reign, his physician John Astruc, recorded in his De Morbis Venereis the link between HSV and the genital organs, after observing the symptoms of the disease in French prostitutes (Astruc 1736). Vidal proved beyond doubt that HSV was infectious and passed from one human to another (Vidal 1873) by noticing transmission of the disease among human volunteers in the early 20th century. Gruter who is widely acknowledged for isolation of HSV, used animal studies proving that HSV infections could be transmitted from rabbit to rabbit (Gruter, 1924). Burnett and Williams provided a

description of HSV latency wherein they stated stated that HSV persists for life and can be reactivated under stressful conditions (Burnet 1939).

Recent advancement in tissue culture technology has helped in isolation of other human herpes virus such as varicella zoster virus (VZV) and cytomegalovirus (CMV) (Weller and Stoddard 1952; Smith 1956). Isolation of the gammaherpesvirus Epstein-Barr virus (EBV) was possible due to in vitro cultivation of lymphoblastoid tumor cells and B lymphocytes (Epstein, Achong et al. 1964). During 1990's, T lymphocytes cultivation led to the isolation of human herpes viruses 6A, 6B and 7 (Salahuddin, Ablashi et al. 1986; Frenkel, Schirmer et al. 1990). In 1994, Chang et al. used Representational Differential Analysis (RDA) to isolate human herpesvirus 8 (Chang, Cesarman et al. 1994).

Thus, HSV can be described as an ancient virus that not only infects man but also other vertebrates and few invertebrates. In the 21st century, the definition and treatment of this disease/virus has evolved rapidly due to better molecular characterization. Genetic engineering, advancements in tissue culture, proteomics as well as microscopy has revealed a better understanding of the herpes virus. These developments have brought about new antiviral drugs, new candidate vaccines and further the use of herpes for genetic therapy.

Taxonomy of Herpesviridae

Herpes virus taxonomy has been addressed since 1971 by the International Committee on Taxonomy of Viruses (ICTV) (Wildy, 1971). An initial step was taken to bestow herpes virus with formal names (Roizman 1973). Subsequently, they were grouped into subfamilies on the basis of their biological criteria (Roizman, Carmichael et al. 1981). Apart from a few erroneous classifications, this was a successful attempt at classifying herpes virus (Roizmann, Desrosiers et

al. 1992). Molecular data was used to delineate genome characteristics such as size and structure, which helped in further classifying subfamilies into genera (Roizmann, Desrosiers et al. 1992). The latest report of the ICTV Herpesvirus Study Group (Davison 2007), subdivides the family Herpesviridae into three subfamilies: *Alphaherpesvirinae* (containing the *Simplexvirus*, *Varicellovirus*, *Mardivirus* and *Iltovirus* genera); *Betaherpesvirinae* (containing the *Cytomegalovirus, Muromegalovirus* and *Roseolovirus* genera) and *Gammaherpesvirinae* (containing the *Lymphocryptovirus* and *Rhadinovirus* genera). Only the genus (*Ictalurivirus*) is unattached to any subfamily and several species have not been assigned any genera. Most of the herpesviruses infect mammals or birds but there are a number of unassigned herpesviruses that infect lower vertebrate (reptilian, amphibian, and fish) or invertebraete (bivalve) host. Table 1.1 shows the classification of the family Herpesviridae as proposed by Davidson et al. 2005. The human Herpesviruses are in bold type (Davison 2007).

Alphaherpesvirinae are classified based on their variable host range, short reproductive cycle, rapid spread in tissue culture, efficient destruction of infected cells, and the ability to establish latent infections primarily in sensory ganglia. *Betaherpesvirinae* were characterized by a limited host range, long reproductive cycle, and slow infection progression in tissue culture. Cells that are infected often become enlarged (cytomegalia), and the viruses can maintain latency in secretory glands, lymphoreticular cells, kidneys, and other tissues. *Gammaherpesvirinae* were characterized by a limited host range and ability to replicate in lymphoblastoid cells, with some viruses also causing lytic infection in few types of epithelial and fibroblastic cells. Viruses are usually specific for either B or T lymphocytes, and latent virus is frequently demonstrated in lymphoid tissue.

Table 1.1: Members of the family herpesviridae

Subfamily	Genus	Formal Name	Common Name	Abbrev.
Alphaherpesvirinae	Simplexvirus			
		Ateline herpesvirus 1	Spider monkey herpesvirus	
		Bovine herpesvirus 2	Bovine mamillitis virus	
		Cercopithecine herpesvirus 1	B-virus	HVB
		Cercopithecine herpesvirus 2	SA8 virus	
		Cercopithecine herpesvirus 16	Herpesvirus papio 2	
		Human herpesvirus 1	**Herpes simplex virus [type] 1**	**HSV-1**
		Human herpesvirus 2	**Herpes simplex virus [type] 2**	**HSV-2**
		Macropodid herpesvirus 1	Parma wallaby herpesvirus	
		Macropodid herpesvirus 2	Dorcopsis wallaby herpesvirus	
		Saimiriine herpesvirus 1	Herpesvirus tamarinus	
	Varicellovirus			
		Bovine herpesvirus 1	Infectious bovine rhinotracheitis virus	BHV-1
		Bovine herpesvirus 5	Bovine encephalitis virus	BHV-5
		Bubaline herpesvirus 1	Water buffalo herpesvirus	
		Canid herpesvirus 1	Canine herpesvirus	
		Caprine herpesvirus 1	Goat herpesvirus	
		Cercopithecine herpesvirus 9	Simian varicella virus	SVV
		Cervid herpesvirus 1	Red deer herpesvirus	
		Cervid herpesvirus 2	Reindeer herpesvirus	
		Equid herpesvirus 1	Equine abortion virus	
		Equid herpesvirus 3	Equine coital exanthema virus	
		Equid herpesvirus 4	Equine rhinopneumonitis virus	
		Equid herpesvirus 8	Asinine herpesvirus 3	
		Equid herpesvirus 9	Gazelle herpesvirus	
		Felid herpesvirus 1	Feline rhinotracheitis virus	
		Human herpesvirus 3	**Varicella-zoster virus**	**VZV**
		Phocid herpesvirus 1	Harbour seal herpesvirus	
		Suid herpesvirus 1	Pseudorabies virus	PRV

Table 1.1 continued

Subfamily	Genus	Formal Name	Common Name	Abbrev.
Alphaherpes-virinae				
	Tentative species in genus Varicellovirus			
		Equid herpesvirus 6	Asinine herpesvirus 1	
	Genus Mardivirus			
		Gallid herpesvirus 2	Marek's disease virus type 1	MDV-1
		Gallid herpesvirus 3	Marek's disease virus type 2	MDV-2
		Meleagrid herpesvirus 1	Turkey herpesvirus	HVT
	Genus Iltovirus			
		Gallid herpesvirus 1	Infectious laryngotracheitis virus	ILTV
	Unassigned subfamily in subfamily Alphavirinae			
		Psittacid herpesvirus 1	Parrot herpesvirus	
Betaherpesvirinae	Cytomegalovirus	Cercopithecine herpesvirus 5	African green monkey cytomegalovirus	SCMV
		Cercopithecine herpesvirus 8	Rhesus monkey cytomegalovirus	RhCMV
		Human herpesvirus 5	**Human cytomegalovirus**	**HCMV**
		Pongine herpesvirus 4	Chimpanzee cytomegalovirus	CCMV
	Tentative species in genus Cytomegalovirus			
		Aotine herpesvirus 1	Herpesvirus aotus 1	
		Aotine herpesvirus 3	Herpesvirus aotus 3	
	Muromegalovirus			
		Murid herpesvirus 1	Mouse cytomegalovirus	MCMV
		Murid herpesvirus 2	Rat cytomegalovirus	RCMV
	Roseolovirus			
		Human herpesvirus 6	Roseolovirus	
		Human herpesvirus 7	Roseolovirus	
	Unassigned species in subfamily Betaherpesvirinae			
		Caviid herpesvirus 2	Guinea pig cytomegalovirus	GPCMV
		Tupaiid herpesvirus 1	Tree shrew herpesvirus	THV

Table 1.1 continued

Subfamily	Genus	Formal Name	Common Name	Abbrev.
Gammaherpes-virinae	Lymphocrypto-virus			
		Callitrichine herpesvirus 3	Marmoset lymphocryptovirus	Marmoset LCV
		Cercopithecine herpesvirus 12	Herpesvirus papio	
		Cercopithecine herpesvirus 14	African green monkey EBV-like virus	
		Cercopithecine herpesvirus 15	Rhesus lymphocryptovirus	Rhesus LCV
		Human herpesvirus 4	**Epstein-Barr virus**	**EBV**
		Pongine herpesvirus 1	Herpesvirus pan	
		Pongine herpesvirus 2	Orangutan herpesvirus	
		Pongine herpesvirus 3	Gorilla herpesvirus	
	Rhadinovirus			
		Alcelaphine herpesvirus 1	Malignant catarrhal fever virus	AHV-1
		Alcelaphine herpesvirus 2	Hartebeest malignant catarrhal fever virus	
		Ateline herpesvirus 2	Herpesvirus ateles	HVA
		Bovine herpesvirus 4	Movar virus	BHV-4
		Cercopithecine herpesvirus 17	Rhesus rhadinovirus	RRV
		Equid herpesvirus 2		
		Equid herpesvirus 5		
		Equid herpesvirus 7	Asinine herpesvirus 2	
		Hippotragine herpesvirus 1	Roan antelope herpesvirus	
		Human herpesvirus 8	**Kaposi's sarcoma-associated herpesvirus**	**KSHV**
		Murid herpesvirus 4	Murine gammaherpesvirus 68	MHV-68
		Mustelid herpesvirus 1	Badger herpesvirus	
		Ovine herpesvirus 2	Sheep-associated malignant catarrhal fever virus	
		Saimiriine herpesvirus 2	Herpesvirus saimiri	HVS
	Tentative species in genus Rhadinovirus			
		Leporid herpesvirus 1	Cottontail rabbit herpesvirus	
		Leporid herpesvirus 2	Herpesvirus cuniculi	

Table 1:1 contimued

Subfamily	Genus	Formal Name	Common Name	Abbrev.
Gammaherpesvirinae				
		Leporid herpesvirus 3	Herpesvirus sylvilagus	
		Marmodid herpesvirus 1	Woodchuck herpesvirus	
	Unassigned species in subfamily Gammaherpesvirinae			
		Callitrichine herpesvirus 1	Herpesvirus saguinus	
	Unassigned genus Ictalurivirus in family Herpesviridae			
		Ictalurid herpesvirus 1	Channel catfish virus	CCV

Clinical Significance of Herpes Simplex Viruses

Epidemiology

There is worldwide prevalence of HSV-1 and HSV-2 (HSV-2) infections in both developed and undeveloped countries (Black 1975). However, poor nutritional status, lack of medical facilities, poor hygiene and abject poverty among the third world nations, increases the risk of herpes infection in developing countries. Pre-existing HSV infection has been known to increase susceptibility of individuals to HIV/AIDS.

In the USA approximately 58% of individuals become infected with herpes simplex virus type 1 (HSV-1) and 17% with herpes simplex virus type 2 (HSV-2) (Anzivino, Fioriti et al. 2009). HSV-1 infection can cause oral lesions, keratitis and encephalitis. HSV-1 keratitis is the

leading infectious cause of blindness in the USA and approximately 500,000 people have a history of recurrent ocular herpes (Dasgupta, Chentoufi et al. 2009).

Neonatal herpes simplex virus (HSV) infection has high mortality and significant morbidity. In case of immunocompromised individuals HSV infection may lead to severe complications. Herpes genitalis which is a sexually transmitted infection is mainly caused by HSV-2. However, in the USA there has been a rise in genital lesions caused by HSV-1 rather than HSV-2. This is attributed to practice of oral sex among partners. Patients suffering from herpes genitalis infection are at a 2-4 fold higher risk of acquiring HIV during sexual intercourse.

Disease symptoms often interfere with everyday life and are a cause of significant social and psychological stigma to the affected individual. The mouth area is the most common location of infection (Whitley and Roizman 2001). Primary infection leads to the shedding of virus from the mouth and stool for an average of 7 to 10 days (Amir, Harel et al. 1997); and neutralizing antibodies appear between 4 and 7 days after the onset of HSV infection; and peak at approximately 3 weeks post infection (Buddingh, Schrum et al. 1953). HSV infection can occur in children less than 5 years old and is most often asymptomatic. The primary factors affecting the rate of HSV infection are location, socioeconomic status, and age. In some studies, by the age of 15, over 95% of children were found to possess antibodies to HSV (Black, Hierholzer et al. 1974; Bader, Crumpacker et al. 1978). HSV-1 seroprevalence is much more common than its counterpart HSV-2 (Fleming, McQuillan et al. 1997) (Nahmias, Lee et al. 1990). Because HSV-2 is usually acquired through sexual contact, antibodies to HSV-2 are rarely found before the onset of sexual activity.

There are no known animal carriers for HSV; therefore, humans remain solely responsible for transmitting virus to other humans. Virus transmission from an infected to a susceptible individual occurs during close personal contact. The frequency of person to person contact appears to be the major mediator of infections (Whitley and Roizman 2001). Since HSV infection rarely cause fatal disease and virus becomes latent, more than half the world's population probably have recurrent HSV infections, enabling the transmission of the virus.

Currently, there is no cure available for HSV-1 and HSV-2. Existing antiviral drugs, such as acyclovir and valacyclovir are effective only in limiting HSV infection and not in preventing recurrence of the disease. Given the worldwide prevalence of this disease it is important to develop effective strategies to combat this virus. The need to develop a vaccine against herpes is of paramount importance especially for developing countries where antiviral drugs, such as acyclovir and/or valacyclovir are not readily available or accessible to the masses.

Pathogenesis

The spectrum of disease caused by Herpes simplex viruses includes primary and recurrent infections of mucous membrane (e.g., gingivostomatis, herpes labialis, and genital herpes infections), keratoconjuctivitis, herpes whitlow and herpetic esophagitis. HSV, also causes systemic disease as is evident in disseminated herpes virus infection of neonates and immunocompromised host, and sporadic encephalitis.

Mucocutaneous Infections

Primary infection occurs when the virus comes in contact with abraded skin (Whitley and Roizman 2001). The virus replicates at the site of entrance (the oropharanyx and the genitals), where it produces infectious virions resulting in the formation of vesicles and pustules. After

primary infection, HSV-1 ascends in a retrograde manner through the perioxonal sheath of sensory nerve to the trigeminal ganglion which harbors the latent HSV-1 virus; whereas in HSV-2 infection the virus resides in the sacral ganglia. A biological feature unique to the herpes virus is latency and reactivation. During the latent infection, the virus does not replicate hence it is able to evade immune recognition. Certain stimuli such as stress, cold, UV exposure can cause reactivation of the virus. On being reactivated the virus travels along the sensory neuron to the mucocutaneous site, and undergoes replication forming clusters of vesciles or ulcers in the vicinity of the initial site of exposure.

Primary, as well as, recurrent HSV infection leads to a number of cellular changes such as enlargement of infected cells and the appearance of condensed chromatin within the nuclei, which ultimately degenerates. Generally, the infected cells lose plasma membrane integrity and form multi nucleated giant cells, with intranuclear inclusion bodies that consist of virus particles. At the dermal infected site, intense inflammatory response takes place initially but decreases with recurrent infections (Whitley, 2001).

After latency, certain stimuli can reactivate the disease causing acute pain, burning, tingling or itching at the mucocutaneous site. These symptoms last about six hours and are followed by vesicles formation and the typical cold sore appears by second day (Spruance, Overall et al. 1977; Spruance and Crumpacker 1982; Spruance, Crumpacker et al. 1984). Initial pain subsides in 4 to 5 days and lesions disappear in about a week (Segal, Katcher et al. 1974; Ship, Miller et al. 1977). In HSV 2, the primary infection occurs in the genital epithileal or mucosal tissues. Systematic signs such as headache, fever and malaise are often present. Within few days of sexual contact characteristic symptoms are revealed as macules and papules, followed by vesicles, pustules and ulcers. Lesions and viral secretions last for three weeks.

Men and women experience similar and dissimilar symptoms (Kaufman, Gardner et al. 1973; Corey 1982). Pre-existing immunity to HSV-1, reduces the severity of HSV-2 primary infections. Recurrent HSV-2 is milder than initial infection and is characterized by the appearance of 3 to 5 vesicles lasting for a week which gradually rupture.

Fetal and Neonatal Infections

HSV infections are highly contagious and could lead to fatal consequences when transmitted to newborns. The highest risk of transmission occurs when the mother has primary HSV infection (rather than recurrence of chronic infection) late in pregnancy. Infection in the skin, eye and mouth of neonates is curable, while infection that is disseminated like in the central nervous system (CNS) is highly fatal. If not treated accurately, it has a mortality rate of 80% and the infants that survive are left with neurological sequelae (Whitley and Roizman 2001).

Acyclovir therapy can be effective in treating neonatal herpes, but is hampered by the early non-specific symptoms of the disease. Even in cases of early therapy, some infants develop disseminated infection or CNS complications and the virus is usually vertically transmitted to the neonate from an infected mother during delivery. Therefore, to combat the fatality associated with neonatal herpes, the neonate has to be prevented from acquiring HSV infection during delivery. The highest risk of neonatal infection occurs when the mother sheds HSV during labour, which is more common with women who acquire genital herpes in the third trimester. Therefore, one approach for reducing maternal-fetal transmissions is to prevent HSV acquisition in late pregnancy (Brown 2004; Jaiyeoba, Amaya et al. 2012).

Keratoconjunctivitis

Herpetic keratoconjunctivitis is associated with unilateral or bilateral conjunctivitis and the healing can take over a month (Whitley and Roizman 2001). Studies show that in developed countries herpes ocular disease ranges from 4.1 to 20.1 cases per 100,000 (Liesegang 2001). The primary infection besides affecting the ocular region may spread and reach the trigeminal ganglion.

Recent, studies have suggested that super infection or infection with second strain, after being exposed to the virus previously, may account for some cases of ocular disease. Several recurrent attacks of herpes keratitis cause the cornea to turn opaque, which eventually leads to blindness. New cornea transplant also fails, since the latency infected trigeminal ganglia attached to the new cornea develops infection (Easty, Shimeld et al 1987; Kaye, Lynas et al, 1991).

Infection in an Immunocompromised Host

Patients with immunodeficiency or treatment-related immunosuppression are at a higher risk of developing severe herpes simplex virus (HSV) infection. These patients can develop progressive disease involving respiratory tract, esophagus, or the gastrointestinal tract (Montgomerie, Becroft et al. 1969; Korsager, Spencer et al. 1975). Repeated treatment with anti-virals to control recurrent infection can lead to viral mutants resistant to antiviral therapy.

Central Nervous System (CNS) Infections

HSV-1 infects limbic system structures in the central nervous system (CNS). It is the most common cause of sporadic, fatal encephalitis in USA (Olson, Buescher et al. 1967). Some studies estimate the rate of incidence as high as 1250 cases per year in the United States

(Whitley, 2001). Encephalitis is caused when the virus spreads past the dorsal root ganglia, in which the virus establishes latency. The mechanisms responsible for this aberrant event in the virus life cycle are unclear. The manifestations of HSV encephalitis include primarily focal encephalitis along with fever, altered behavior, and localized neurological symptoms. There is usually evidence of a localized temporal lobe disease (Whitley, Soong et al. 1977; Whitley, Soong et al. 1981). In untreated patients, mortality exceeds 70% and only 2.5% of the patients return to normal neurological function (Whitley, 2001).

The possibility that HSV-1 reactivation in neurons of the CNS cause chronic progressive damage at cellular level, and alters neuronal function has not been thoroughly investigated. Currently, it is not known whether recurrent reactivation of HSV-1 in asymptomatic patients involves some risk of progressive deterioration of CNS functions. This deterioration could be due to neuroinflammatory response against the virus or by direct toxicity of the virus on the neurons. New reports are indicating a link between HSV-1 and neurological diseases such as schizophrenia and Alzheimers. Recent studies have indicated a link between HSV-1 exposure and some of the cerebral morphological changes often reported in schizophrenia (Prasad, Shirts et al. 2007). Moreover, Herpes simplex virus type 1 has been indicated as a risk factor associated to Alzheimer disease (Mori 2010; Martin, Solis et al. 2011).

Prevention and Treatment of HSV Infection

There are basically two methods for controlling HSV infections i.e. antiviral therapy and prevention. One of the challenges is to develop antiviral drug which will target the viruses during latency. The present drugs act only on replicating viruses. Antivirals such as acyclovir and valaciclovir are effective in limiting the extent of HSV infection and therefore helpful in

limiting spread to uninfected individuals. However, post-exposure antiviral treatment does not prevent lifelong infection in an individual. Acyclovir is available in generic forms as well as sold by GlaxoSmithKline under the brand name Zovirax. Acyclovir (ACV) is an antiviral guanine nucleoside analog. During viral DNA replication acyclovir triphosphate (ACV-TP) is readily incorporated into the viral DNA by HSV-1 DNA polymerase opposite to a cytosine residue. Further, chain elongation of the DNA molecule is prevented since ACV structure lacks a 3'OH group for addition of a new deoxynucleoside triphosphate (Weller 2011). This leads to inhibition of viral DNA replication. Valacyclovir is sold by GlaxoSmithKline under the brand name, Valtrex. It is a prodrug of Acyclovir. It consists of an ACV structure with a valine moiety covalently attached to it. This drug is absorbed three to five times more than ACV. Other anti-viral drugs present in the market are famciclovir-sold by Novartis under the brand name, Famvir and Foscarnet, sold by Clinigen Healthcare under the brand name, Foscavir. Foscavir is administered intravenously and not orally.

There are two new classes of drugs that have been discovered but no clinical trials have been carried out to prove the efficacy of these drugs; they being amino-thiazolyl phenyl - containing drugs and thiazole urea derivatives (TZP/Us). These drugs target the helicase – primase complex which unwinds the double –stranded viral DNA. The mode of action of these drugs is to increase the binding of helicase-primase complex for DNA resulting in inhibition of helicase activity, primase activity and viral DNA synthesis. The two drugs seem promising since there is evidence that resistance to helicase-primase inhibitors occurs less frequently than resistance to acyclovir. A Japanese research group has recently published a report which evaluates and describes the anti- herpes virus effect of ASP2151 (amenamevir), a novel non-

nucleoside helicase–primase complex inhibitor, which possesses anti-viral activity against HSV-1, HSV-2 and VZV (Chono, Katsumata et al. 2010).

Many non-traditional anti-HSV therapeutic or preventive substances have been advocated for use for prevention of herpes, although they have not been clinically tested. Diet that is rich in broccoli, cabbage and Brussel sprouts is said to reduce the severity and occurrence of herpes infection. These vegetables contain the active ingredient DIM (di-indolemethane)/I3C (indole-3-carbinol) which is said to inhibit virus replication in infected cells. Indian Ayurvedic medicines such as Neem and Tulsi have been recommended for control of herpes disease since they possess anti-viral properties.

Structure of the Herpes Virion

The herpes virion size can vary from 120 nm to 300 nm (Roizman and Furlong 1974) and consists of four components; an electron-dense core harboring the double stranded DNA, an icosadeltahedral capsid around the core, an amorphous proteinaceous tegument surrounding the capsid, ultimately enclosed within an outer envelope containing glycoprotein spikes (Roizman and Furlong, 1974). A model of the virion architecture is presented below in Figure 1.1.

The Core

The core of a mature herpes virion contains the viral DNA in the form of a torus that may appear to be suspended by a proteinaceous spindle to the capsid (Falke, Siegert et al. 1959; Roizman, Spring et al. 1967; Furlong, Swift et al. 1972; Nazerian 1974). The toroidal structure is 50 nm high, with an inside diameter of 18 nm and an outside diameter of 70 nm. The arrangement of the viral DNA in the torus is not known.

HSV-1 Virion Structure

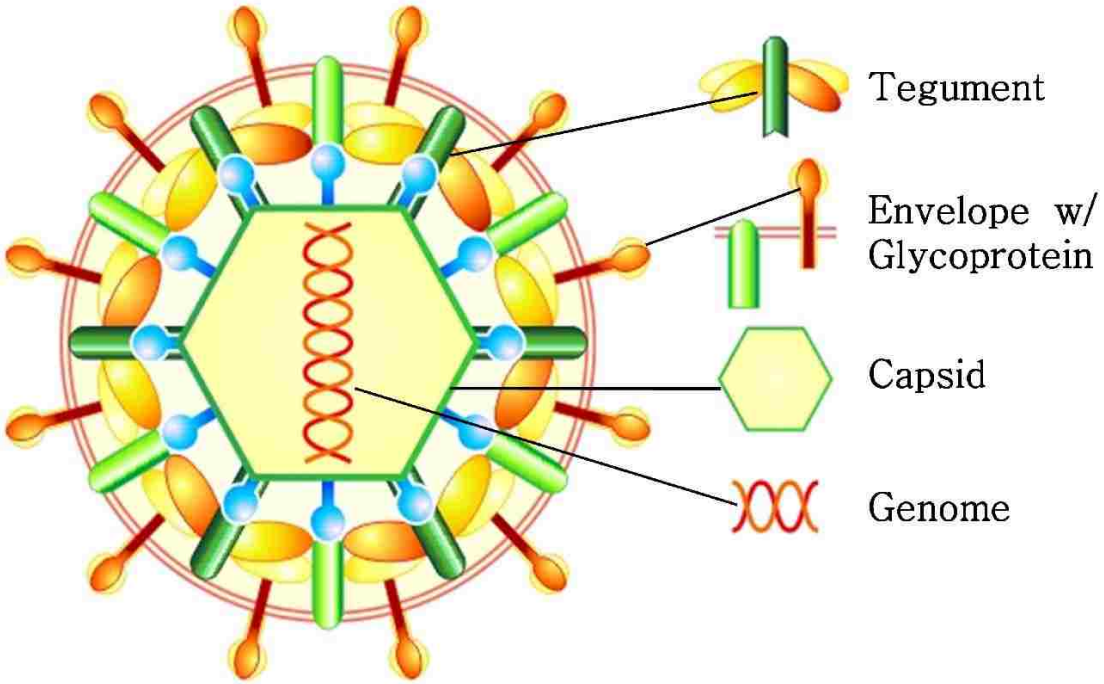

Figure 1.1: Herpesvirus virion structure: Herpes virion consists of four morphologically different components; an electron dense core containing viral genome, an icosahedral capsid shell, a proteinaceous tegument and a lipid envelope decorated with glycoprotein spikes. [Figure from (Lee 2008)].

The Capsid

Most subfamilies of herpes virus possess an icosahedral capsid that contains densely coiled double stranded DNA. The 125 nm diameter capsid consists of 162 capsomeres, 7 different viral proteins (VPs), VP5 (UL19), VP19C (UL38), VP21 (UL26), VP22a (UL26.5), VP23 (UL18), VP24 (UL26), and VP26 (UL35), and the products of the UL6 and UL25 genes (Roizman and Sears, 1996). VP5 is the major protein and, together with VP19C, VP23, and VP26, is present on the surface. In the capsid, the pentons are located on the icosahedral 5-fold

vertices, and the hexons make up the faces and edges. The pentons and hexons contain five and six copies, respectively of protein, VP5 (Newcomb, Trus et al. 1993; Zhou, Prasad et al. 1994). They are surrounded and connected by 320 triplexes, which are heterotrimers formed from two copies of VP23 and a single copy of VP19C (Newcomb, Trus et al. 1993) (Saad, Zhou et al. 1999). Six copies of a 12-kDa protein (VP26) occupy the top of each hexon (Trus, Homa et al. 1995; Chen, Jakana et al. 2001).

The Tegument

The tegument is an amorphous layer present between the envelope and nucleocapsid and appears fibrous on negative staining (Morgan, Rose et al. 1959; Wildy and Watson 1962; Morgan, Rose et al. 1968). The tegument can be distributed asymmetrically and its thickness can vary depending on the location of the virion particle within the infected cell. There are fewer teguments that are more symmetrically arranged in perinuclear virions than in virions in cytoplasmic vesicles that contain more tegument distributed more asymmetrically (Falke, Siegert et al. 1959). There is ordered tegument density around the pentons, suggesting symmetry wherein the capsid and tegument interact (Zhou, Chen et al. 1999). These tegument proteins function during virion assembly and egress of herpes viral articles. Herpes simplex virion tegument contain about 20 proteins, including VP1/2 (UL36), VP11/12 (UL46), VP13/14 (UL47), VP16 (UL48), VP22 (UL49), ICP0, ICP4, and the virion host shutoff protein (UL41) plus the products of genes US2, US3, US10, US11, UL13, UL14, UL16, UL17, UL21, UL37, UL51, and UL56.

The Envelope

The outermost covering of the HSV virion is the lipid envelope which is derived from altered host cellular membrane (Falke, Siegert et al. 1959; Morgan, Rose et al. 1968). The surface of the viral envelope contains 600-750 glycoprotein spikes that vary in length, spacing and the angle of emergence from the envelope (Campadelli-Fiume and Menotti 2007). These glycoproteins are designated as glycoprotein B (gB), glycoprotein C (gC), glycoprotein D (gD), glycoprotein E (gE), glycoprotein H (gH), glycoprotein I (gI), glycoprotein J (gJ), glycoprotein K (gK), glycoprotein L (gL), glycoprotein M (gM) and glycoprotein G (gG). In addition to these 11 glycoproteins there are membrane associated proteins such as UL20, UL34, UL45 and possibly US9. Some of these viral proteins have not been detected on the virion envelope. These membrane proteins play important role at different stages of the HSV-1 life cycle i.e. virus entry, virus spread as well as virus envelopment and egress.

Organization of the Herpes Viral Genome

Herpesviruses possess a linear double stranded viral DNA that circularizes upon entering nucleoplasm of infected cells. The size of the viral genome varies between 120 to 250 kbp, between the various herpesviruses.

There is polymorphism among the genomic length of individual viruses which arises due to presence of terminal and internal repeated sequences which can vary in copy number, leading to variations in genomic length of more than 10 kbp. Herpesviruses have a high GC content and the total G+C content of the herpesviruses varies from 31% to 75%, and this variation is seen across the genome.

The HSV-1 DNA genome is approximately 152,261 base pairs (McGeoch, Dalrymple et al. 1988) and consists of two covalently linked components designated L and S. Each component consists of largely unique sequences (UL and US) flanked by inverted repeats (Figure1.2). The inverted repeat sequences of the L component has been designated as ab and b'a', whereas those of the S component has been designated as c'a' and ca (Hayward, Jacob et al. 1975). A unique property of the HSV-1 genome is the ability of the L and S components to invert relative to each other. As a consequence, viral DNA extracted from wild-type virions or infected cells, consists of four equimolar populations differing in the relative orientation of the two components (Hayward, Jacob et al. 1975).

The majority of herpesvirus genes contain a single open reading frame (ORF). Most viral genes contain a promoter region 50 to 20 bases upstream of the TATA box and a 5'untranslated leader sequence 20 to25 bp downstream of the TATA box. Usually, herpesviruses possess single translational initiation codon and a 10 to 30 bp of 3' untranslated sequence, and a polyadenylation signal with standard flanking sequences (Roizman and Knipe 2001). However, some genes contain a second in-frame initiation codon while there are genes that lack a TATA box (Chou and Roizman 1986; Markovitz, Filatov et al. 1999). Most transcriptional gene products are not spliced, although every herpesvirus expresses a few spliced genes. Interstingly, herpesviruses also produce noncoding RNAs, such as the HSV-1 latency associated transcript (LAT) (Roizman and Pellett 2001). LATs are thought to be introns being derived from the processing of a large 8.3 kb precursor. These are the only viral gene products that accumulate to a high concentration in the trigeminal ganglia (TG) of latently infected animals. The different members of the herpesvirus family encode between 70 and 200 genes, estimated using various

methods (Roizman and Knipe 2001). HSV-1 encodes about 90 gene products, with at least 84 of the transcriptional units encoding proteins (Roizman and Knipe 2001).

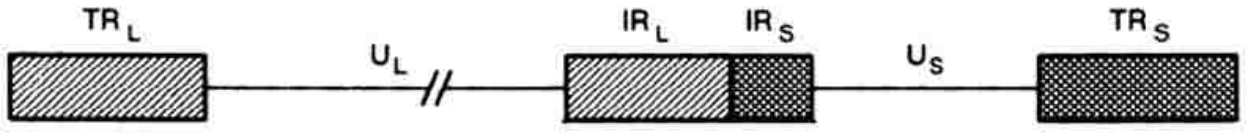

Figure 1.2: A schematic map of the herpes simplex virus type 1 (HSV-1) genome. The HSV DNA consists of two covalently linked components, L (long) and S (short), with unique sequences - unique long ($U_{L)}$ and unique short (Us). The two components are separated by inverted repeats (IR) while the ends are flanked by terminal repeats (TR).

The Herpes Simplex Virus Lifecycle

The herpes virus lifecycle consists of a lytic phase and a latent phase. The virus first infects the epithelial cell and can progress to infect the peripheral nervous system, where it can establish lifelong latency in neurons. Entry as well as establishment of infection requires interaction between viral components and cellular factors.

Once, the virus enters the epithelial cell, it then uses the cellular machinery to create new virion particles. These new virion particles then spread to uninfected neighboring cells either through release of virions into extracellular spaces that infect neighboring cells or through direct cell-cell fusion (infected and uninfected cells) avoiding the immune system. Details of the lytic cycle of replication consisting of entry, viral DNA replication, capsid assembly and virion egress are discussed in the following sections (Figure 1.3). A brief overview of latency is also included.

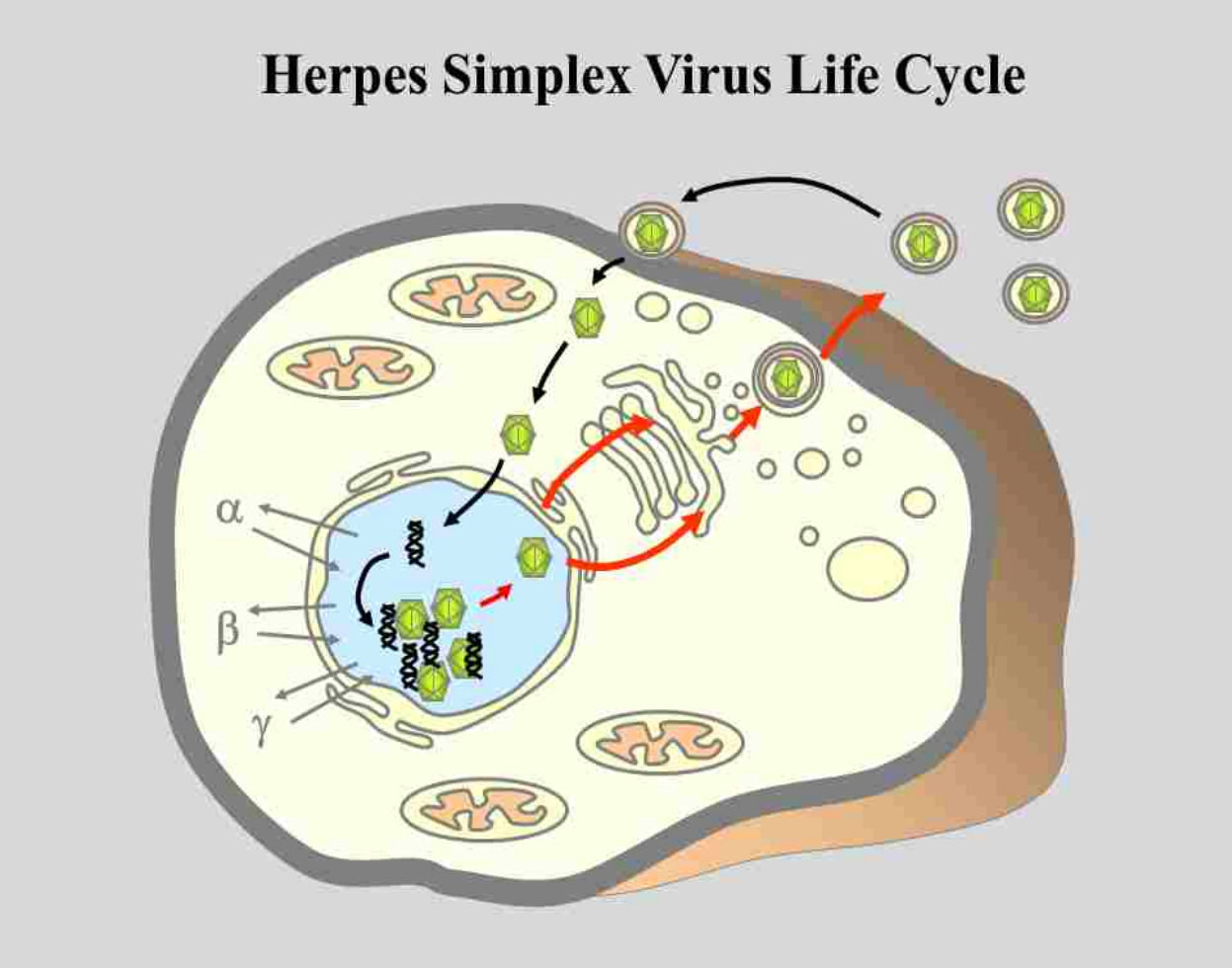

Figure 1.3 Herpes Simplex Virus Life Cycle. Virion particles enter the cell by either endocytosis or fusion. The virion particles lose their envelope at the cell membrane followed by transport of the capsid to the nucleopore. The viral DNA is then released into the nucleoplasm followed by viral DNA replication that involves coordinate expression of alpha, beta and gamma proteins. (black arrows). This is followed by virion morphogenesis that comprises of primary envelopment of the nucleocapsids at the inner nuclear membrane, de-envelopment at the outer nuclear membrane and final envelopment at TGN derived vesicles. The enveloped virions are then released into extracellular spaces by exocytosis. (Melancon, 2003).

Herpes Simplex Virus Entry

HSV uses a myriad of cellular receptors and attachment factors that are ubiquitous, as well as, cell–specific in order to gain entry into cells, to establish a productive infection. Unlike other envelope viruses HSV has distinct glycoproteins that have specific function in each step of viral entry: glycoproteins for attachment, specific glycoprotein for binding to specific cellular receptors, glycoproteins that bring about fusion and regulate fusion. HSV-1 utilizes two mechanisms of entry into cells i.e. direct fusion at the plasma membrane at neutral pH or receptor mediated endocytosis which is either pH dependent or independent.

The virus initially binds to binds to non-specific cellular attachment factors such as carbohydrates, a phenomenon called tethering. This binding is followed by migration of the virus to specific receptors that range from adhesion molecules, immunoglobulin like signaling receptors as well as cytoskeleton receptors to enter host cells (Figure 1.4). Specific cellular receptors exist for each of the glycoproteins involved in entry; gC, gB, gD, gH/gL. The above interactions activate signaling pathways, initiate the movement of the virus to endocytic routes or trigger conformation changes in virus envelope proteins leading to activation of the fusion process. The different cellular receptors involved in binding as well as membrane fusion have been discussed in detail in the following sections. The mechanism of membrane fusion between the viral membrane and host cell membrane involving viral proteins and cellular receptors are further elaborated in the latter part of the chapter.

Cellular Receptors –Attachment Receptors

Heparan sulfate- Heparan sulfate is a cell surface glycosaminoglycan (GAG) that belongs to the proteoglycan family consisting of chondroitin sulfate, and dermatan sulfate.

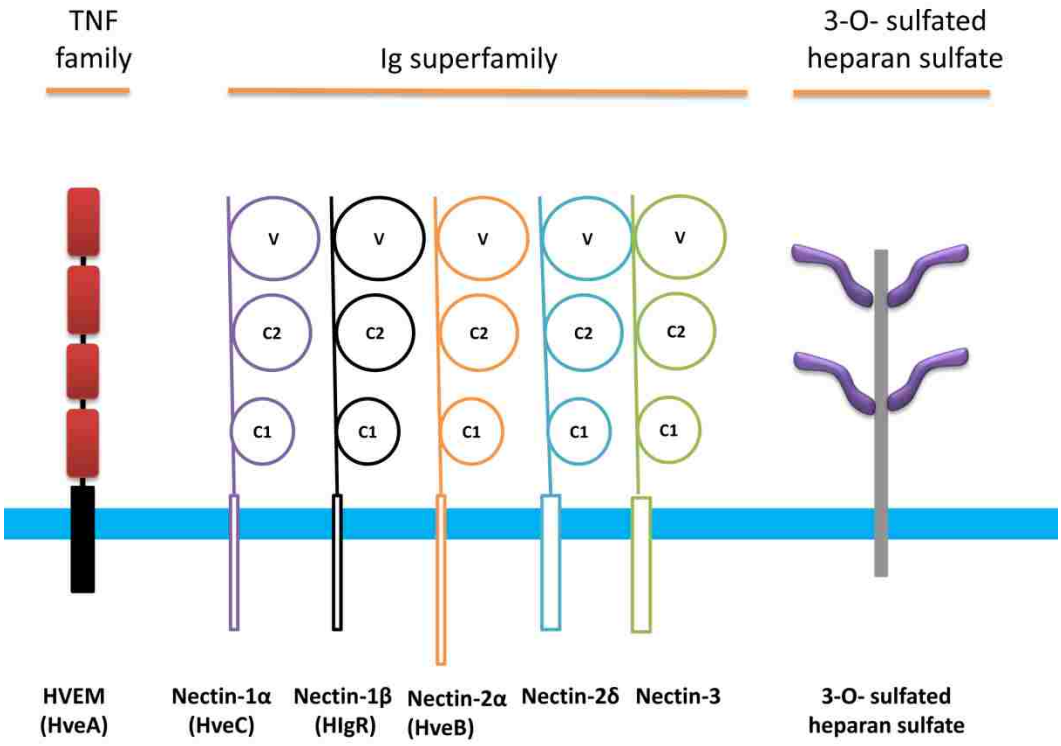

Figure 1.4: Cellular receptors involved in virus entry. There are three classes of cell surface receptors that are involved in HSV entry: the tumor necrosis factor (TNF) receptor family consisting of HVEM, the immunoglobulin superfamily consisting of different types of nectins, and 3-O-sulfated heparan sulfate. [Figure modified (Melancon 2004)]

These arrays of proteoglycans are ubiquitously present on most vertebrate adherent cells. Heparan sulfate is one of the principal binding receptors for HSV. It has been observed that in cell lines defective in HS biosynthesis, but not chondroitin sulfate, there is about 85% reduction in viral attachment (Gruenheid, Gatzke et al. 1993). Further evidence of heparan sulfate as a binding receptor has been demonstrated by inhibition studies in which heparin a closely related molecule to heparan prevents binding of HSV to most cells (Nahmias and Kibrick 1964). It has also been shown that removal of heparan sulfate from human cells , by enzymatic treatment ,

reduces the ability of the cells to bind virus and this renders the cells partially resistant to HSV infection (WuDunn and Spear 1989). Furthermore, the amount of N-sulfation of heparan sulfate determines the amount of virus that binds to the cell (Shieh, WuDunn et al. 1992).

The first step of viral entry is the attachment or the tethering of the virus to heparan sulfate moieties present on cellular membrane. gB and gC are known to interact with heparan sulfate, moieties. This binding step is not an absolute requirement for viral entry, although, in absence of gC or heparan sulfate, the rate of entry is reduced. This tethering helps to concentrate the virus on the cell surface. It has been reported that the highly sulfated negative charge on heparan sulfate particles interact with the positively charged glycoproteins, thereby increasing binding affinity (Cardin and Weintraub 1989). The two different strains of HSV, HSV-1 and HSV-2 differ in their binding affinity to heparan sulfate. HSV-1 gC binds preferentially to heparan sulfate while in case of HSV-2, gB plays an important role (Gerber, Belval et al. 1995; Cheshenko and Herold 2002). The differential binding affinity of the two strains to heparan sulfate influences several biological activities including sensitivity to polyanionic and polycationic substances (Langeland, Holmsen et al. 1987). Moreover, basic amino acid residues on heparan sulfate are known to be critical for HS-binding activity (Trybala, Roth et al. 2002). Previous studies have delineated the HS-binding domain, that interacts with HSV-1 gC amino terminal residues to be located between aa 33 and 123 (Tal-Singer, Peng et al. 1995) and between 129 and 247 (Trybala, Bergstrom et al. 1994). The main function of gC binding to GAGs allows for more stable interaction of gD with an entry receptor.

gD-Binding Entry Receptors

The second step of entry is the penetration of the virus into the cell which requires the interaction of gD with one of the several potential entry receptors. These cellular receptors are designated as binding or attachment receptors or fusion triggers. The cellular entry receptors are of three classes i.e. herpes virus entry mediator (HVEM), which belongs to the tumor necrosis factor receptor family; nectin-1 –nectin-4, members of the immunoglobulin superfamily; and last being 3-*O*-sulfated Heparan Sulfate (3-OS HS).

HVEM-HVEM was the first gD receptor that was identified by Montgomery et al (Montgomery, Warner et al. 1996). They discovered, this receptor while searching for genes in the human cDNA expression library that could mediate entry of HSV into CHO-K1 cells that are non-permissive to HSV entry. This attachment receptor is also referred to as TNFRSF14, Tumor necrosis factor receptor-like 2 (TR2); CD antigen CD270. It is a type I integral membrane protein that regulates immune responses by interacting with signal transduction pathway proteins through its cytoplasmic region. The cytoplasmic domain binds to several members of the TNFR-associated factor family (TRAF1, TRAF2, TRAF3, and TRAF5 but not TRAF6), leading to the activation of targets like NF-κB, Jun N-terminal kinase, and AP-1; and consequently induces T cell activation, proliferation, cytokine release and expression of cell surface activation markers (Marsters, Ayres et al. 1997; Harrop, Reddy et al. 1998). HVEM is expressed on different cell types such as T and B lymphocytes and other leukocytes, epithelial cells, fibroblasts and tissues of the lung, liver, kidney and to a lesser extent the brain tissue (Montgomery, Warner et al. 1996). The cellular natural ligands of HVEM are LIGHT, secreted lymphotoxin-alpha, the immunoglobulindomain-containing cell-surface receptors B and T lymphocyte attenuator (BTLA), CD160 and lymphotroxin - α (LTα) (Mauri, Ebner et al. 1998) (Figure 1.5).

LIGHT-HVEM interactions lead to co-stimulation of several cell types, including T cells and antigen-presenting cells, whereas HVEM ligation to T-cell-expressed BTLA or CD160 results in inhibition of T-cell proliferation and effector function (Sedy, Spear et al. 2008). Although, HSV-1 gD binds to different domain of HVEM in comparison to its natural cellular ligands yet it competes with LIGHT and BTLA for binding to HVEM (Sedy, Spear et al. 2008). Certain studies have shown that HSV-1 entry into activated T cells is principally mediated by HVEM, although in a variety of other cell types HVEM was shown not be the primary mediator of virus entry (Montgomery, Warner et al. 1996).

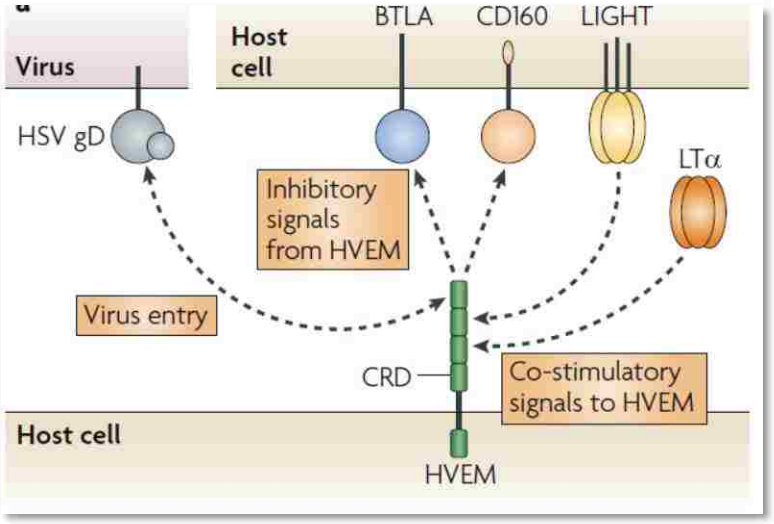

Figure 1.5: HVEM binds to cellular natural ligands i.e. LIGHT, the immunoglobulindomain-containing cell-surface receptors B and T lymphocyte attenuator (BTLA), CD160 and lymphotroxin-α (LTα) (Mauri, Ebner et al. 1998). LIGHT-HVEM interactions leads to co-stimulation of several cell types, including T cells and antigen-presenting cells, whereas ligation of T-cell-expressed BTLA or CD160 by HVEM results in inhibition of T-cell proliferation and effector function. HSV-1 glycoprotein D also binds to HVEM this interaction initiates the process of virus entry (Sedy, Spear et al. 2008).

There are several evidences to demonstrate HVEM as a receptor for glycoprotein D of HSV-1 and HSV-2. It is known that anti-gD Abs completely block the interaction between the virus and HVEM. Moreover, specific antigenic sites on gD (Ib and VII) contain residues important for HVEM binding. Neutralizing MAbs to these specific antigenic sites abolish HSV entry into HVEM-expressing CHO cells since they prevent interaction between gD and HVEM (Nicola, Ponce de Leon et al. 1998). Certain mutations within gD sequence of HSV-1 strains rid1and ANG reduce infectivity on HVEM –expressing cells (Montgomery, Warner et al. 1996) (Dean, Terhune et al. 1994). Mutagenic analysis of HSV-1 gD revealed that the first 32 amino acids of the N-terminal extension have a critical role in functional interactions of gD with all the HSV entry fusion receptors, except for nectin-1 (Yoon, Zago et al. 2003).

The X-ray structure of the soluble portion of gD as well as gD interacting with HVEM has been solved. The structure of gD consists of an ectodomain composed of an N terminus and C terminus region and C terminus extensions that anchors it to the viral envelope. gD assumes two conformations; a closed conformation in absence of the receptor and an open and active conformation upon interacting with receptor (either HVEM or possibly nectin 1) (Campadelli-Fiume, Amasio et al. 2007). The structure of unliganded gD revealed that the N terminus is flexible in the absence of receptor, and the C terminus is anchored near the N terminus, masking the receptor binding site. The interacting region of gD with HVEM have been localized to amino acids 7 to 15 and 24 to 32 within the N-terminal hairpin loop (Spear and Longnecker, 2003). The C-terminal region of gD (encompassing residues 260-310) has been termed the pro-fusion domain as it is required to trigger fusion. gD triggers or transmits a signal through its pro-fusion domain to gB and/or gH/gL thereby activating these glycoproteins (Reske, Pollara et al. 2007).

It has been reported that the soluble portion of gD is both necessary and sufficient to rescue the non-infectious gD null mutant (Cocchi, Fusco et al. 2004).

Nectins-Nectins are Ca^{2+}-independent immunoglobulin (Ig)-like cell-cell adhesion molecules (CAMs). Nectins play an important role in the formation of adheren junctions (AJ) and tight junctions. They comprise of a family of four members, nectin-1, -2, -3 and -4 each of which has multiple splice isoforms. Nectin- 1α, –1β, -1γ, -2α,-2δ, -3α, -3β, and -3γ variants have been identified and nectin-4 too has two splice variants. Nectins interact in *trans* with each other both homophilically and heterophilically to form cell-cell adhesions. In addition, they heterophilically interact in *trans* with other immunoglobulin-like CAMs. The carboxyl-terminal domains of nectins bind to L-afadin, a PDZ binding protein that anchors the receptors to the cytoskeleton and adherens junctions. Nectins and afadin play roles in the formation of a variety of cell-cell junctions such as adheren junction (AJ) as well as tight junctions (TJ), cooperatively with, or independently of, cadherins. In addition, they are involved in the formation of synapses in neurons (Takai, Irie et al. 2003). Many cellular activities such as movement, proliferation, survival, differentiation, polarization, and the entry of viruses are regulated by nectins in conjunction with other CAMs and cell surface membrane receptors (Takai, Ikeda et al. 2008). Nectins are used in cell-to cell spread by viruses in polarized epithelium cells, since progeny virus have been shown to exit the cell in the vicinity of adherens junctions on the lateral surfaces of the cell (Haarr, Shukla et al. 2001).

Nectin-1 is a broad spectrum receptor for both human and animal herpesviruses. This receptor was first designated as Poliovirus receptor-related protein 1 (Prr1) and is also referred to as Herpes Virus Entry mediator C (HveC). Three isoforms of Nectin-1 are produced by mRNA splicing of human Prr1 gene; two of which, α and β are membrane-bound

(Krummenacher, Rux et al. 1999; Campadelli-Fiume, Cocchi et al. 2000) while nectin-1γ is secreted as it lacks a transmembrane domain.

HSV-1 strains, HSV-2, pseudorabies virus (PrV) and bovine herpes virus 1 (BHV-1) can use both nectin-1α and nectin-1β (Geraghty, Krummenacher et al. 1998) to enter cells. In mammals nectins are highly conserved in terms of structure, function and the ability to permit HSV-1 entry. Nectin1 is broadly expressed in human tissues, including tissues and organs targeted by HSV, like CNS, ganglia and muco-epithelia (Cocchi, Menotti et al. 1998) They are expressed in virtually all human cell lines, including epithelial cells, neurons and fibroblasts (Campadelli-Fiume, Cocchi et al. 2000; Spear, Eisenberg et al. 2000).

Nectin1 interacts with gD, this interaction involves the first 250 residues of gD and the V domain of Nectin-1. Specifically the amino acid residues 77 and 85 of Nectin 1are thought to be part of the interface that interacts with gD (Martinez and Spear 2002). Many HSV-1 mutants (ANG, Rid1, Rid2) that have mutations in gD are unable to use HveA as an entry receptor but they can use Nectin-1 for entry (Geraghty, Krummenacher et al. 1998).

The binding affinity between Nectin-1-gD ranges from 10^{-6} to 10^{-8} molar. It has been observed, that in some gD mutants the binding affinity is so low that it may be undetectable. However, these mutants are still able to enter the cells. Therefore, it has been concluded that gD functions in both entry and cell-cell fusion as long as it binds to a functional Nectin-1 receptor, its functions are independent of its receptor binding kinetics with Nectin-1(Milne, Hanna et al. 2003).

Nectin- 2 was first designated as Poliovirus receptor-related protein 2 (Prr2) as is also referred to as Herpes Virus entry mediators B (HveB). Nectin-2α and Nectin-2δ mediate entry of

HSV-2, PrV, and certain viable mutant forms of HSV-1 but not wild type HSV-1(Warner, Geraghty et al. 1998). These viable mutant forms of HSV-1 that can utilize Nectin-2 as an entry receptor, have amino acid substitutions at position 25(L25P) or 27(Q27P) of gD (unrestricted, or rid mutations). The nectin- 2 residues critical for HSV entry are amino acids 75–81 and 89, which lie adjacent to the predicted C′C″ β-strands; the region corresponding to the nectin-1 region involved in interaction with wild-type gD.

Nectin -2 is expressed on a number of human tissues (e.g. placenta, kidney, lung, prostrate, pancreas and thyroid) and various human cell lines.(e.g. epithelial, endothelial, and neuronal).

Nectin-3 is expressed on J cells, which lack HVEM, nectin-1 and nectin-2. Work by Cocchi et al has shown that wild type HSV-1 is unable to infect J cells while a HSV-1 mutant designated as HSV-1 (JMP) is able to infect. The HSV-1 mutant contains mutations in glycoprotein K and glycoprotein D (Cocchi, Fusco et al. 2004).

Nectin-4 or HveD, a fourth receptor is designated as Poliovirus receptor-related protein 4 (Prr4). In contrast to other nectin molecules, detection of nectin4 transcripts is mainly restricted to placenta in human tissues. It serves as receptors for a limited range of alphaherpesviruses.

3-O-sulfated Heparan Sulfate (3-OS HS)- Heparan sulfate is a cell surface glycosaminoglycan (GAG) that contains alternating disaccharide units of uronic acid and N-acetylglucosamine, and serves as an initial attachment site for the viral envelope glycoproteins gB and gC. When heparan sulfate is modified by enzymes such as D-glucosaminyl 3-O-sulfotransferase isoforms-3 and/or -5 during biosynthesis, it results in 3-O-sulfated Heparan Sulfate (3-OS HS). This heparan sulfate (3-OS HS) serves as an entry receptor of HSV-1, and specifically binds to glycoprotein D. This receptor is broadly distributed on human cells

and tissues such as liver, placenta, kidney pancreas and mediates specifically the entry of HSV-1 but not HSV-2. Study by Tiwari et al. has shown that 3-OS HS plays an important role in HSV-1 entry into primary cultures of corneal fibroblasts. This receptor plays an important role in both virus entry and virus induced cell-cell fusion (Tiwari, Clement et al. 2006).

gB-Binding Entry Receptors

Recently a number of co-receptors have been identified in addition to gD cellular receptors that interact with gB i.e. Paired Immunoglobulin-like type 2 receptor alpha (PILR α), Myelin-associated glycoprotein (MAG), Non muscle myosin heavy chain IIA (NMHC-IIA). Details regarding these receptors have been discussed in the following sections.

PILRα –Paired Immunoglobulin-like type 2 receptor **(**PILR**)** is a transmembrane-anchored protein that belongs to a paired immunoglobulin-like type 2 receptor family and is encoded by PILR gene present on chromosome 7. The PILR gene encodes an inhibitory and activating receptor, PILR alpha (PILR α) and PILR beta (PILR β). PILR is part of a cell signaling pathway that regulates both innate and adaptive immune systems by PILR α mediated inhibition and PILR β –mediated activation. PILRα consists of a 303 amino acid protein, which is composed of a immunoglobulin (Ig)-like V (variable)-type domain and a small cytoplasmictail consisting of a immunoreceptor tyrosine-based inhibitory motif (ITIM). Similarly PILRβ also has an Ig-like V-type domain; but the cytoplasmic domain consists of a immunoreceptor tyrosine-based activation motif (ITAM), that associates with DAP12 delivering activating signals (Satoh, Arii et al. 2008). PILRα and PILRβ are expressed on cells of the immune system, such as monocytes, dendritic cells, NK cells, B cells, macrophages, neutrophils, eosinophils, mast cells, and megakaryocyte/platelets, and neurons (Fan and Longnecker 2010).

The natural cellular ligand for PILRα and PILRβ, is the leukocyte-expressed protein CD99, and NKcells that express PILRβ are activated to kill target cells that express CD99. Recognition of CD99 by PILR depends upon the presence of sialyated O linked glycans on CD99. It is postulated that interaction between CD99 and T cells expressing PILRβ and/or PILRα might regulate T-cell activation (Sedy, Spear et al. 2008). Recently, a new ligand for PILRα was discovered called PILR-associating neural protein (PANP); which is well conserved in mammals (mouse, rat, humans), and is specifically found in neural tissues. In humans, it is mostly found in the of cerebellum adult brain. PILRalpha-PANP interaction dependents on O-glycosylation sites present on PANP (Kogure, Shiratori et al. 2011). Two more novel ligands

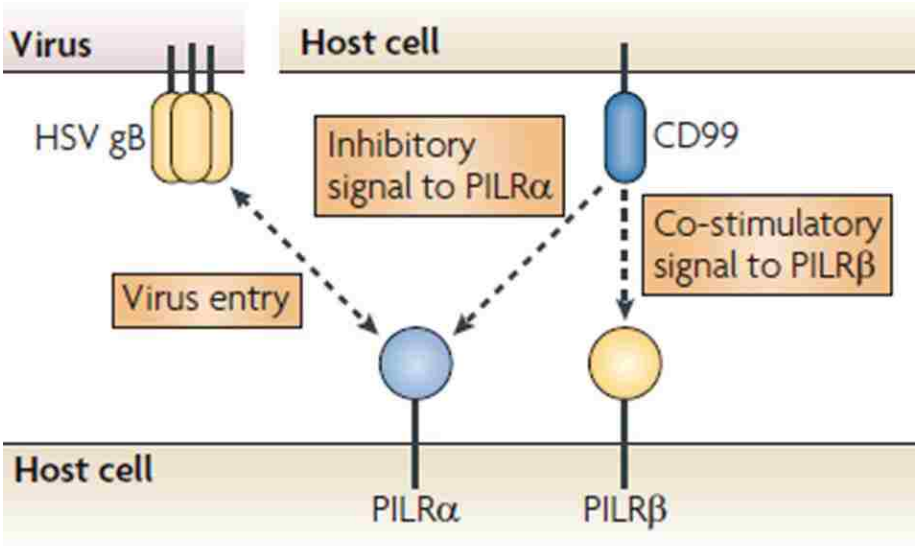

Figure 1.6: Paired Immunoglobulin-like type 2 receptor **(**PILR) are paired immunoglobulin-like receptors consisting of an of an inhibitory (PILR-α) and activating (PILR-β) receptor. The natural ligand for both these cellular receptor are leukocyte-expressed protein CD99. HSV-1 gB (trimer) interacts with PILRα (Sedy, Spear et al. 2008).

have been discovered which interact with PILRα i.e. neuronal differentiation and proliferation factor-1 (NPDC1) and collection -12 (COLEC 12). COLEC12 is a cell surface transmembrane protein that is expressed in vascular endothelial cells and monocytes (Sun, Senger et al. 2012). NPDC-1 is a protein expressed primarily in brain and lung and whose expression can be correlated with the regulation of cellular proliferation and differentiation. The functional role of PILRα interaction with these novel ligands have still to be elucidated.

Recently, it was reported that paired immunoglobulin –like type 2 receptor alpha is an entry co-receptor for HSV that binds to gB. HSV resistant CHO cells become susceptible to HSV entry when they are transduced with PILRα. The virus entry into CHO-PILRα cells is via fusion at the plasma membrane and not in endosomal vesicles. Specifically, PILRα binds to gB at two O glycosylation sites i.e. threonine position T53 and T480 (Wang, Fan et al. 2009); this binding is also dependent upon the conformation of gB. This PILRα mediated entry depends upon interaction between PILRα and gB and also requires gD to interact with one of its cognate receptors. This phenomenon has been experimentally verified wherein; anti-PILRα or anti-HVEM antibodies block HSV-1 entry into susceptible cells expressing both HVEM and PILRα. PILR also plays an important role in cell-cell fusion.

The *in vivo* significance of gB –PILRα interaction is not yet known; but this gB receptor mediates the entry of HSV into different cell types *in vitro*. It also facilitates entry of HSV-2 into human retinal pigment epithelial cells. Since, PILRα is well conserved among mammals, it has been implicated that PILRα mediated entry is quite prevalent mode of entry. However, within alphaherpesvirus subfamilies differences in PILRα mediated entry exist. Interaction between PILRα and gB may have immunomodulatory effect i.e. gB binding to inhibitory

receptor PILRα may potentially deactivate natural killer cells and prevent infected cells from being lysed (Sedy, Spear et al. 2008). PILR α plays a role in entry in HSV-1, PRV but not in HSV-2 and HVEB. Although, the natural ligands of PILRα are divergent the specificity of interaction remains the same i.e. recognition of O-glycan structures as well as protein conformation.

MAG- Myelin-associated glycoprotein (MAG) is a cell surface molecule that is a member of the Sialic-acid-binding Ig-like lectin (SIGLEC) family of proteins. It is preferentially expressed in neural tissues and specifically in glial cells and is a functional ligand of Nogo-66 receptor. MAG plays an important role in regulation of axonal growth, including myelination, initiation, and myelin integrity maintenance. MAG is considered to be a gB receptor that mediates membrane fusion and entry of HSV-1 and VZV into neuronal tissues (Suenaga, Satoh et al. 2010). Epithelial cells and neurons do not express MAG therefore there may be other molecules on these cells that interact with gB. Hence, MAG is not thought to serve as a major receptor for these viruses. It may play a role during acute phase of infection when glial cells are infected with HSV-1 and VZV, suggesting that MAG might be involved in the neurological disorders caused by these neurotropic viruses.

NMHC-IIA–The initial steps of viral infection involve attachment of the virions to the host cell surface, subsequently followed by penetration of the cell membrane to reach the cytoplasm. However, in order to do so, they have to overcome the physical barrier imposed by the cortical actin meshwork, which is ubiquitous in cells. Virion attachment to the cell surface activates signaling events that lead to actin cytoskeleton remodeling. This brings about a change in the plasma membrane, that facilitates or activates virion surfing, virion endocytosis, receptor clustering and fusion of the virion membrane to the plasma membrane

(Connolly, Jackson et al. 2011). Thus, during viral infection the viral proteins interact with the actin cytoskeleton to facilitate reshaping of the cell for virus entry and spread.

Myosin molecules are cytoskeleton motor proteins that propel the sliding or produce tension on actin filaments. Non-muscle myosin II (NM II) is an actin motor protein, which is part of the myosin family of motor proteins. Most myosins belong to class II and, together with actin, they make up the contractile proteins of cardiac, skeletal and smooth muscle. Myosin II molecules present in non-muscle eukaryotic cells are called non-muscle myosin II molecules. NM II plays an important role in cell adhesion, cell migration and tissue architecture. There are three isoforms NM II that share some unique properties. NM II is composed of a globular head domain, two heavy chains and a light chain. There are three different genes which encode for the heavy chain in mammals i.e. myosin heavy chain 9 (MYH9), myosin heavy chain 10 (MYH10) and myosin heavy chain 14 (MYH14). The whole myosin molecule consisting of the heavy and light chain and is referred to as NMII, while the heavy chains alone are referred to as non-muscle myosin heavy chain NMHC. The three different isoforms are referred to as NMHC IIA, NMHC IIB and NMHC IIC (Vicente-Manzanares, Ma et al. 2009). Recently, the NMHCIIA has been identified as a functional entry receptor for HSV-1. It has been shown to physically interact with HSV-1 gB (Arii, Goto et al. 2010), and mediate HSV-1 infectivity both *in vitro* and *in vivo*. HL60 cells, that are relatively resistant to HSV-1 infection, become susceptible when transduced with NMHCIIA. NMHCIIA is ubiquitously expressed in various human tissues and cell types and mostly function in the cytoplasm. It has been reported, that HSV-1 adsorption leads to induction and expression of NMHC-IIA from the cytoplasm to the cell surface.

"Viral surfing" is a strategy HSV-1 uses to enter and spread into cells. It has been observed, that HSV exposure leads to the formation of filopodia-like cell membrane protrusions. The virus uses these protrusions to bind and move towards the cell body, a phenomenon termed as surfing. It is known, that NMHC-IIA binds to actin, and is also involved in many events controlling cell movement and reshaping of the cell. Therefore it is likely that virus surfing on filopodia that requires the involvement of the cytoskeleton is mediated by gB binding to NMHC-IIA. Therefore, Shukla et al. have proposed the involvement of gB in this process of viral surfing (Karasneh and Shukla 2011).

Table 1.2: Function of HSV-1 entry glycoproteins and receptor interactions

Glycoprotein	Function	Cellular receptors
gC	Binds cells Dispensable for fusion	Heparan sulphate
gD	Binds cells Triggers fusion	HVEM Nectin 1 and nectin 2 3-*O*-sulphated heparan-sulphate
gH–gL	Triggers or regulates fusion	Integrins
gB	Catalyses membrane fusion	Heparan sulphate DC-SIGN PILRα MAG NMMHCIIA

gH/gL-Binding Entry Receptors

The role of gH/gL in entry at present is not clearly defined. It was earlier thought that gH/gL are fusion proteins that bring about hemi-fusion. However, gH/gL are now considered as

fusion regulators. There is still debate as to whether gH/gL of HSV-1 interacts with receptors during viral entry. Study by Parry et al. have shown that αVβ3 integrins bind to HSV-1 gH, specifically the RGD motif of gH (Parry, Bell et al. 2005). This interaction facilitates entry but is not absolutely essential. A summary of HSV-1 entry glycoproteins and cellular receptors has been tabulated in table 1.2.

Other Herpesvirus Receptors

In alphaherpesviruses, betaherpesviruses and gammaherpesviruses the core fusion machinery consisting of gB, gH and gL is well conserved. Interestingly, only alphaherpesviruses (except VZV) encode members of the gD family. In case of betaherpesvirus and gammaherpesvirus, other viral proteins serve as ligands for cellular receptors. EBV uses gp42, which forms a trimer with gH-gL-gp42 complex, to serve as a ligand for human leukocyte antigen (HLA) class II molecules on B lymphocytes. Human CMV encodes glycoprotein O (gO), which is unrelated to gp42 but forms a complex with gH/gL (Huber and Compton, 1998). In addition, HHV-6A encodes glycoprotein Q (gQ), which forms a complex with gH-gL (Mori et al., 2003). However, there is still controversy regarding whether gO or gQ are required for entry in CMV or HHV-6A, respectively.

Virus-Cell Fusion

Sequential Events in Entry

HSV-1 viral entry is complex as it involves a number of different glycoproteins and cellular receptors. The mode of entry of the virus varies from cell to cell and depends on various viral and cellular factors (Fig1.7).

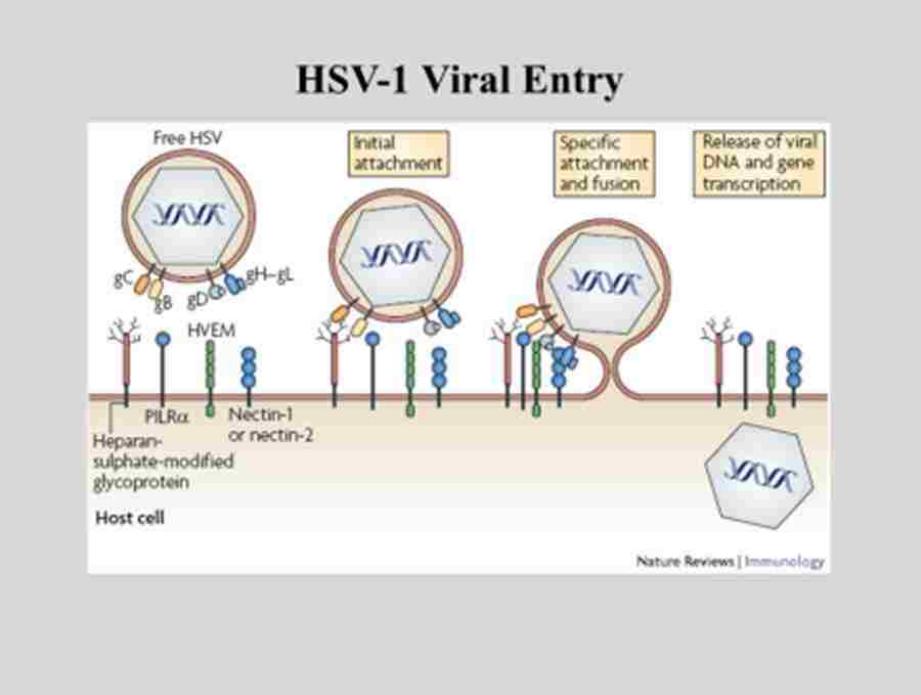

Figure 1.7: Herpes Simplex Virus Entry. The virus enters either by fusion at the plasma membrane or by endocytosis. Interaction of gC with heparan-sulfate moieties on host cell – surface proteoglycans initiates the process of entry. This is followed by interaction between gD with one of its cognate receptors i.e. HVEM, nectin-1, nectin-2 or 3-O-sulfated heparan sulfate. The interaction of gD with its cognate receptors leads to the triggering of the fusion process. gB also interacts with the co-receptor PILRα. Membrane fusion is then brought about by gB with the help of gH/gL heterodimer. Membrane fusion between the viral membrane and cellular membrane ultimately leads to the release of the capsid into the cytoplasm (Connolly, Jackson et al. 2011)

At present, there are two models of HSV-1 fusion that have been proposed. In the first model i.e. the sequential model gD interacts with its cognate receptor and triggers the fusion machinery in which gH/gL function in hemifusion, and ultimately gB brings about fusion between the two membranes (viral and plasmammembrane or viral and endosmal membrane) (Connolly, Jackson et al. 2011). According to the second model of fusion which is referred to as regulatory, structural or complex formation model, gD receptor binding triggers fusion that in turn activates gB which is also activated by gH/gL; ultimately leading to gB mediated fusion

between the membranes. However, neither of these models unable to explain the receptor binding properties of gB and gH/gL. Moreover, it has been suggested that besides the four quartet glycoproteins other viral proteins may be involved in entry. Hence, more extensive study is needed to be carried out in order to propose a model that encompasses all these factors which can adequately explain HSV-1 membrane fusion during entry. A brief summary of the steps that occur during fusion have been described below.

The first step of entry is binding of gC to GAGs on cell surfaces that enables more stable interaction of gD with an entry receptor. This interaction of gC with cell surface GAGs is not essential since entry can occur in absence of gC.

The second step of viral entry is binding of gD to its cognate receptors such as Nectin-1 and HVEM. gD is a dimeric protein, and its domains near the carboxyl termini occlude its receptor-binding site. After binding HVEM or Nectin 1, the amino terminus of gD forms a loop and displaces the gD C terminus exposing the pro-fusion domain. It has been proposed that subsequently, gB trimer also binds with the recently identified co-receptors such as PILR α, MAG and NMHC-IIA (Connolly, Jackson et al. 2011). Further, it has been suggested that gH/gL may also bind to its cognate cellular receptor. The binding of gD to receptor triggers the fusion machinery involving gH/gL and gB. Different sites within the gD pro-fusion domain interact with gH/gL (residues 260 and 310) and gB (resisues 240-260 and resisues 304-305). Moreover, gH/gL and gB may interact with each other in response to the binding of gD to its receptor. These interactions have been revealed by different research groups by bimolecular complementation assays such as split EGFP or split YFP (Atanasiu, Whitbeck et al. 2007; Atanasiu, Saw et al. 2010). Although the correct sequence of events during membrane fusion have not been completely deciphered.

The solved X-ray crystal structure of gH/gL complex reveals that it does not possess characteristics of any known fusion protein (Chowdary, Cairns et al. 2010). It has an unusual 'boot shaped structure' which is composed of three domains; N-terminal domain that binds to gL (domain H1), the central helical domain (domain H2) and the C-terminal β-sandwich domain. The gH/gL complex was earlier thought to act as a fusion protein which brought about hemifusion. However, it has now been proposed that gH/gL complex activates the fusogenic potential of gB by binding to it directly. There are experimental evidences to show that neutralizing antibodies can disrupt the formation of the complex between gH/gL and gB. It has been reported that the antibody LP11 competes with gB to bind to gH/gL. Moreover, it has been proposed that the site on HSV gH/gL complex, that interacts with gB also overlaps with the epitope of the neutralizing antibody (LP11) and thus can inhibit (gH/gL)-gB association (Chowdary, Cairns et al. 2010). Henc, gH/gL can be considered as fusion regulators that are required to change the conformation of gB from a post-fusion to a pre-fusion form. Some resarchers have suggested, that gH/gL –gB interaction occurs before fusion rather than after, since certain antibodies can block fusion without necessarily disrupting the interaction between gH/gL and gB. (Connolly, Jackson et al. 2011). HSV-1 may have evolved such a intricate mechanism in order to control the fusogenic nature of gB, wherein gH/gL act as fusion regulators so as to prevent uncontrolled membrane fusion.

By X-ray crystallography the extracellular structure of gB has been solved facilitating in understanding the role of gB in fusion process. gB crystal structure reveals a trimeric form that consists of three identical protomers designated as A, B and C (Heldwein, Lou et al. 2006). Each protomer further consists of five distinct domains (Domain I to Domain V). Insertion mutations in all five of the gB domains can abrogate fusion, consistent with the concept that gB undergoes

a complex, ordered refolding process to drive fusion. Four functional regions (FR) have also been defined. The Functional Region I (FR1) consists of Domain 1 and V which are predicted to be proximal to the plasma membrane (Atanasiu, Whitbeck et al. 2010). Thus FR1 contains fusion loops that associate with the host cell membrane. Recently, it has been suggested that the binding of gD to its receptor activates gH/gL, which subsequently triggers gB to insert its fusion loops into the target membrane, and ultimately bringing about fusion of the viral envelope and the cellular membrane.

Moreover it has been suggested that a significant amount of energy is released during conversion of gB from its initial pre-fusion form to the very stable, energetically favorable post-fusion form. This energy is required to overcome the high activation energy needed to merge the two lipid bilayers ie. the viral and the cellular membrane (Silverman, Sharma et al. 2010).

Some researchers have suggested otherwise, that gB-gH/gL interaction occurs after activated gB has inserted its fusion loops into the target membrane. This interaction leads to conformation changes in gB that helps to pull the two membranes together.

Once the two lipid bilayers are in close proximity, fusion is thought to proceed through a "hemifusion" intermediate, in which the proximal leaflets of the two bilayers have merged but the viral contents have not yet mixed with the cell cytoplasm. Hemifusion is followed by fusion pore formation, pore enlargement, and complete content mixing. These steps are still being elucidated and it is now believed that all the entry glycoproteins are necessary for both hemifusion and fusion (Jackson and Longnecker 2010).

These complex interactions between the viral glycoproteins as well as cellular receptors ultimately lead to fusion between the viral membrane and host cell membrane and deposition of the naked capsid into the cytosol.

Endocytosis

HSV-1 can enter the host cell by fusion occurring between the viral membrane and the plasma membrane or it can gain access into the cells by utilizing the cellular endocytic machinery. The latter pathway helps the virus to overcome the physical barriers of the plasma membrane and cytoskeleton. This mode of entry is rapid and leads to uptake or internalization of the intact virion particle from the cell surface into intracellular compartments. The virus bound endocytic vescicles are trafficked through the cytoplasm in a highly regulated manner that requires cytoskeletal elements and regulatory molecules, such as kinases and GTPases (Nicola and Straus 2004). Ultimately, fusion occurs between the plasma membrane and endosomal membrane and then the capsid is deposited into the cytoplasm. It has been suggested that fusion occurs in low pH environment in late endosomes. The low pH is believed to trigger conformational changes in fusion peptides of glycoproteins to convert them to fusion-active states. Evidence for this phenomenon has been shown by Dollery et al. wherein low-pH induced conformational changes in HSV-1 glycoprotein B (Dollery, Delboy et al. 2010)

Viruses that enter by endocytosis utilize different endocytic routes such as clathrin-coated pits or caveolae; however it seems that HSV does not enter by either pathway (Gianni, Campadelli-Fiume et al. 2004). Some researchers have suggested that the virus enters by phagocytosis which requires activation of RhoA (Clement, Tiwari et al. 2006). Rahn et al. have observed that HSV-1 enters human keratinocytes via an entry route that requires dynamin

and host cholesterol (Rahn, Petermann et al. 2011). The current theory is that HSV-1 enters the cells through macropinocytosis, although this phenomenon has not been well established (Mercer and Helenius 2009).

Macropinocytosis is a cellular process that leads to formation of macropinosomes that internalize large amounts of fluid and membrane. HSV-1 utilizes these large vesicular structures to enter cells the formation and intracellular trafficking of these macropinosome need phosphatidylinositol 3-kinase (PI3- kinase) activity. Nicola et al. have reported that PI3- kinase inhibitors like wortmannin and LY294002 decrease entry of virus into HeLa ,CHO-nectin-1 and keratinocytes cells that are known to endocytose the virus (Nicola and Straus 2004).

It is well established that Chinese hamster ovary cells (CHO) are not permissive to HSV-1 entry and the endocytosed virus is ultimately degraded in the lysosome. However, when these cells are engineered to express a gD receptor the virus is able to overcome lysosomal degradation and penetrate into the cytoplasm. Thus binding of gD to its cognate receptor has two effects: firstly it directs the internalization of the virus and pushes it towards endosomal pathway, secondly it triggers the fusion machinery leading to conformational changes in gB. These two responses occur simultaneously, however in case of endocytosis the fusion step is delayed.

As discussed earlier, a second trigger such as low pH, may be needed to bring about fusion between viral membrane and endosomal membrane in case of endocytosis. CHO cells have been engineered to constitutively express a single gD receptor such as Nectin-1 or HVEM. Entry into these cells (CHO-HVEM and CHO-nectin1) as well as HeLa and human primary epidermal keratinocytes involves pH-dependent endocytosis. It has been reported that

endocytosis in CHO-nectin-1 cells is rapid and nearly 50% of the virus intake is within the first 10 minutes (Milne, Nicola et al. 2005).

Researchers have utilized various endocytic inhibitors, such as monensin, ammonium chloride and bafilomycin and other endocytic inhibitors to distinguish between pH dependent and independent endocytosis of HSV-1 into different cell types. These inhibitors of endosomal acidification raise the pH of the endosomal vesicle and hence prevent fusion between the two membranes (viral and endosomal) and prevent release of the capsid into the cytosol (Nicola, McEvoy et al. 2003).

A third mode of endocytic entry has also been described i.e. pH-independent endocytosis as seen in mouse melanoma cells (B78H1) expressing nectin1 (C10). This mode of entry has not been well characterized (Milne, Nicola et al. 2005).

Glycoproteins required during endocytosis

Whether it be fusion at the plasma membrane or in endocytic vesicles the same set of glycoproteins (gD, gB, gH and gL) are required. Mutant viruses lacking individual glycoproteins gC, gD, gB and gH/gL when tested on CHO-nectin1 and HeLa cells were shown to be non-infectious. However, mutant viruses lacking gC infected both HeLa and CHO-nectin1 cells. From these observations it was concluded that gC is dispensable for HSV entry via endocytosis while gB, gD, gH and gL are required.

Examples of endocytic mode of entry have been described in other herpes subfamilies. The Epstein-Barr virus (gammaherpesvirus) enters epithelial cells via fusion at the plasma membrane but enters B cells via endocytosis. While human cytomegalovirus, a betaherpesvirus, fuses at the plasma membrane of fibroblasts but enters epithelial cells by endocytosis.

HSV-1 entry route is dependent on both viral factors as well as cellular factors (receptors). Delboy et al. demonstrated that the HSV ANG strain differentially uses Nectin-1 and Nectin -2 to enter CHO cells. It enters CHO-Nectin1 cells by pH-dependent endocytosis while in CHO-Nectin- 2, it enters by fusion (Delboy, Patterson et al. 2006). The determining factor is the presence of glycoprotein D. However, in case of PILR α mediated entry, the cellular receptor determines the entry route of the virus i.e.by fusion at the plasma membrane.

Thus, HSV-1 virus utilizes different strategies and myriad of receptors to enter different types of host cells and hence it has a wide host range and cell tropism.

Host Protein Shutoff

HSV-1 and 2 HSV-2 are capable of suppressing the host cell protein synthesis even without viral gene expression (Matis and Kudelova 2001). This phenomenon is known as the early shutoff or as the virion-associated host shut off (vhs) and is mediated by a structural component of the infecting virion which does not require de novo protein synthesis after infection (Nishioka and Silverstein 1977; Fenwick and Walker 1978; Nishioka and Silverstein 1978; Nishioka and Silverstein 1978). The isolation of *vhs*- mutants which were unable to shutoff host cell protein synthesis in HSV-1 infected cells, led to detailed study of this phenomena (Read and Frenkel 1983). The UL41 ORF was identified as being responsible for the phenotype in the *vhs*- mutants. The major product of this gene is a 58 kDa phosphoprotein which is present in light (L) particles but is not essential for virus replication. There are atleast two forms of UL41 protein differing in the extent of phosphorylation present in HSV-1-infected cells. VHS is an RNase that degrades viral and cellular mRNAs, thereby limiting host and viral antigen production (Walker, Sehgal et al. 2011). *In vitro* studies have shown that VHS functions

at least in part by inducing endoribonucleolytic cleavage of mRNA (Elgadi and Smiley 1999), and the 5' end of mRNA is degraded before the 3' end (Karr and Read 1999). It appears to form a complex with the translation factor eIF-4H, and this interaction is required for RNase activity, possibly through targeting to polyribosomes. The VHS and eIF-4H complex appears to decap cellular mRNA from the 5' end (Roizman and Knipe 2001).

UL41 protein is capable of interacting with a transactivator of an alpha-gene i.e. the alpha-transinducing factor (alpha-TIF) (Schmelter, Knez et al. 1996). This interaction between UL41 protein and alpha-TIF down regulates the UL41 (vhs) gene activity during lytic infection. In the context of a vhs- mutant, host protein synthesis is not shut off early in infection and α and β gene expression is extended in more in comparison to a wild-type virus. These effects are due to the fact that vhs accelerates the degradation of both cellular and viral mRNAs. As a result, during HSV-1 infection, cellular mRNA levels diminish while the turnover of viral mRNAs is accelerated. In this way, vhs may facilitate the transition from α to β to γ gene expression through shortening the life of the viral mRNAs (Kwong and Frenkel 1987). As a γ1 gene product, vhs accumulates late in infection, but does not degrade viral mRNA as would be expected. An interaction between VP16 and vhs at a later stage of infection, blocks the degradation of RNA by vhs (Lam, Smibert et al. 1996). As a result, the accumulation of VHS in tegument complexes accomplishes two crucial functions: it brings vhs into the infected cell to shutoff host protein synthesis, and it regulates the activity of vhs late in infection when mostly viral mRNA is present.

Virion Transport to the Nucleus

After the virion attaches to the cell plasma membrane the virion envelope fuses with the cell membrane releasing the capsid and the associated glycoproteins into the cytoplasm. This is followed by the release of most of the outer tegument proteins while others, such as VP16 (αTIF) are transported to the nucleus to affect their functions. The nucleocapsid coated with the inner tegument proteins (pUL36, pUL37 and pUS3) is then transported along the cell's microtubule network to a nuclear pore. Studies have shown that dynein, the microtubule dependent motor, is bound to capsids after entry (Sodeik, Ebersold et al. 1997). These results have initiated the theory that incoming capsids bind to microtubules and utilize the cell's dynein motor to transport them to nuclear pores. Since most of the tegument proteins are lost during entry, the candidate viral protein(s) that directly interact with dynein/dynactin complex are thought to be inner tegument proteins and capsid proteins. Although, no direct interaction between inner tegument protein and dynein has been identified, the inner tegument proteins pUL36 and pUL37 are the likely dynein –binding viral proteins (Antinone, Shubeita et al. 2006) .

After intracytoplasmic transport to the nucleus, capsids accumulate at the nuclear envelope and become associated with nuclear pore complexes. Experiments have shown that UL36 is required for DNA release at the nuclear pore, specifically; N-terminus of pUL36 binds to the NPC followed by proteolytic cleavage leading to release of viral genomic DNA into the nucleus (Jovasevic, Liang et al. 2008), (Copeland, Newcomb et al. 2009). The N-terminus of HSV-1 pUL36 has also been shown to contain a nuclear localisation signal which may play a role in both these early and late events of viral replication (Abaitua and O'Hare 2008)

Coordinate Gene Expression

HSV-1 produces 80 gene products; the transcription of these genes occurs in a highly coordinated manner and is tightly regulated. This process of transcriptional involves expression of specific proteins at different times post infection and can be divided into three phases of gene expression, "immediate-early" or α phase, "early" or (β) phase and "late" or (γ) phase. The host RNA polymerase II is responsible for mRNA synthesis of the viral DNA. Transcription of the α genes occurs in the absence of de novo viral protein synthesis and is initiated by binding of, α gene transactivating factor (α-TIF) which is part of the virion (Batterson and Roizman 1983). α-TIF encodes for the protein VP16 which binds to a motif TAATGArATT in the promoter region of alpha genes. In absence of VP16 basal transcription continues, however it plays an important role in viral transcription in resting cells such as neuronal cells where the availability of transcription factors is low. After release from the tegument complex, VP16 binds to a cellular protein called the host cell factor (HCF) or C1 (Katan, Haigh et al. 1990; Kristie and Sharp 1990), and HCF carries VP16 into the nucleus, during which the VP16-HCF complex binds to Oct-1 that is bound to viral DNA. This event forms the activator complex which is responsible for transactivation of α genes (La Boissiere, Hughes et al. 1999). In addition to the Oct-1 binding sites, the promoters for α genes also contain binding sites for other cellular transcriptional activators upstream of a TATA box that may contribute to a basal level of α gene expression in rapidly dividing cells in the absence of VP16 (Roizman and Knipe 2001).

The viral DNA after entry localizes to PML bodies or ND10 structures where the transcription of α genes is initiated (Maul, Ishov et al. 1996). Two to four hours post infection the six alpha genes are expressed at high levels. These genes are designated as ICP0, ICP4, ICP22, ICP27, ICP47 and US1.5. Five of the six α genes stimulate viral β gene expression in

at least some cell types. Recent findings have suggested that both ICP0 and ICP4 are present on intranuclear capsids, suggesting that they may at least partially be recruited on the viral particles during early stages of virion morphogenesis (Loret and Lippe 2012).

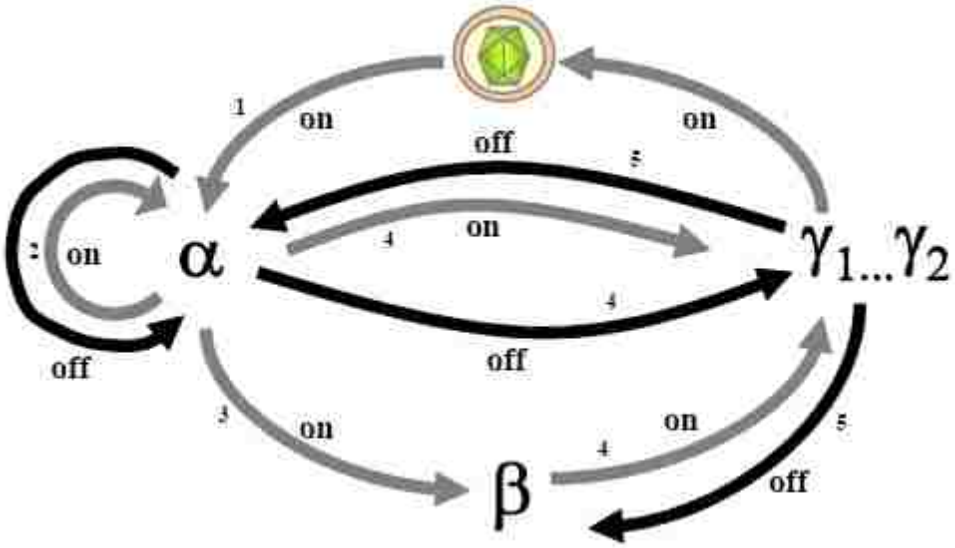

Figure 1.8: Coordinate gene expression in Herpes Simplex Virus. 1) Transcription of the genes occurs in the absence of de novo viral protein synthesis and is highly stimulated by α *trans*-inducing activity of virion of tegument protein α-TIF. 2) Autoregulation of gene expression. 3) The early (β) gene products are synthesized next and are principally involved in viral DNA synthesis. 4) The last set of genes expressed is the late (γ) genes, which encode proteins involved in virion structure and assembly. These genes are activated by α and β genes. 5) Repression of α and β gene expression by γ genes late in infection. (Roizman and Knipe 2001)

One of the most important alpha gene is ICP4 which is required for all post-α gene expression (Clements, Watson et al. 1977; Dixon and Schaffer 1980) and is a major transcriptional regulator. It has been suggested that ICP4 must interact with components of the

pol II machinery (TATA box-binding protein (TBP), TFIIB, TFIID and Mediator complex) to regulate viral gene expression (Lester and DeLuca 2011).

The mechanism by which ICP4 exerts its transcriptional control over β gene expression is unclear. ICP4 is also responsible for down regulation of α gene products including itself and ICP0, and the "pre α" gene products ORF P and ORF O. In this case, specific consensus binding sites appear to be responsible for ICP4 mediated transcriptional regulation (Faber and Wilcox 1986; Kristie and Roizman 1986; Muller 1987). In addition, ICP4 has different isoforms depending on different post translational modifications, and possibly the different functions may be somewhat dependent on the specific isoform present at different times during the course of the infection. ICP0 promotes viral infection and gene expression, especially at a low multiplicity of infection (MOI) where its absence leads to a virus yield that is 100 fold less than a wild type virus (Sinclair, McLauchlan et al. 1994).

ICP0 is a nonspecific transactivator that induces the expression of the HSV α, β, and γ genes. Because ICP0 does not bind DNA directly, it appears to act indirectly in modulation of transcription (Everett, Orr et al. 1991).

In the second phase or the early phase, expression of viral β genes is highest between 4 to 8 hours post infection. The β gene expression is dependent on the presence of ICP4 but not on viral DNA synthesis. The β genes code for proteins that are involved in viral DNA replication and nucleotide metabolism. As a result of viral DNA replication, a third class of proteins called γ genes is produced. The β class of genes can be divided into two groups: β1 and β2 that are expressed at different time points. β1 class of genes are expressed either immediately or simultaneously after synthesis of α proteins and are characterized by the single-stranded DNA

binding protein, ICP8 and the large subunit of ribonucleotide reductase. The β2 gene product such as thymidine kinase is produced after gamma gene expression (Roizman and Knipe 2001).

The third set of genes the γ (late) genes are expressed at high levels after initiation of viral DNA synthesis. Transcription of these gamma genes is dependent on expression of ICP4, ICP27 and ICP8. These set of genes are subdivided into two groups γ1 and γ2, which encode for structural proteins, glycoproteins and tegument proteins (Weir 2001). Viral DNA synthesis controls/ determines the relative rate of expression of γ1 and γ2 genes. The γ1 (leaky –late) genes are expressed relatively early in infection and include the major capsid protein ICP5, gB, gD,and ICP34.5, while the γ2 genes are expressed only after DNA replication has occurred and accumulate to appreciable amounts whereas inhibitors of viral DNA synthesis prevent their expression The γ2 genes include gC, UL41 (VHS), UL36, UL38, UL20, and gK.

Viral DNA Replication

Infection by HSV leads to remodeling of the host cell nucleus. Viral DNA are localized to sites near ND-10 structures which are disrupted eventually by ICPO (Infected cell protein O). Viral DNA replication proteins and viral DNA assemble near ND10 structures to form prereplicative sites. The order of these steps mentioned above is still not well defined. Viral DNA replication leads to the conversion of prereplicative sites to small replicationn compartments. Initially, viral DNA replication initiates on the circular viral DNA, creating a "theta" structure, which as replication progresses transits to a rolling circle mechanism of replication producing head-to-tail concatemers of viral DNA (Jacob, Morse et al. 1979).

HSV DNA replication requires seven essential viral proteins, as well as a variety of enzymes involved in DNA precursor metabolism. These proteins comprise components of a

helicase-primase complex (composed of three proteins: UL5, UL8, and UL52) (Challberg 1986; Wu, Nelson et al. 1988), the major single stranded DNA-binding protein (ICP8), DNA polymerase (UL30) (Purifoy, Lewis et al. 1977), and its accessory protein (UL42) (Conley, Knipe et al. 1981). Viral DNA synthesis also requires host cell factors and host enzymes that include the DNA polymerase α-primase, DNA ligase, and topoisomerase II. The HSV-1 genome also contains three origins of replication, one within the unique long region (oriL) and two within the c repeats bounding the unique short region (oriS). oriS, a palindromic sequence of 45 bp that is located in c sequence bounding the S component and present in two copies; and oriL, a palindromic sequence of 144bp that is located between the transcriptional units of ICP8 and DNA polymerase (UL29 and UL30, respectively) (Mocarski and Roizman 1982; Weller, Spadaro et al. 1985; Deb and Doelberg 1988; Lockshon and Galloway 1988). OriS and oriL share extensive nucleotide sequence homology within the 45-bp oriS palindrome and within sequences extending to the left of the palindrome, whereas sequences extending to the right of the palindrome show essentially no homology (Lockshon and Galloway 1986).The reason for the presence of three origins of replication is not clear, although it may reflect the evolutionary history of the virus. However, only one origin of replication is needed for replication to occur (Igarashi, Fawl et al. 1993).

The basic steps of HSV viral DNA replication is as follows. The parental viral DNA circularizes on entering the nucleus of the infected cell. The alpha and the β genes are transcribed and slowly the viral DNA is unwound. This unwinding is brought about by UL9 that binds to specific motifs on either *ori*L or *ori*S. ICP8 (ss DNA binding protein) is recruited by UL9 to unwound portion of the DNA. These two proteins then recruit the remaining five proteins involved in replication to replication forks. The helicase–primase and viral DNA

polymerase complexes assemble at each replication fork and initiates theta form replication. Replication switches between theta form and rolling circular replication. As a result of rolling circular replication long head to tail concatamers of viral DNA are formed which are then cleaved into individual units during packaging of viral DNA into capsids (Roizman and Knipe 2001).

Capsid Assembly

HSV-1 capsid is an icosahedral shell that consists of 162 capsomers. VP5 is the structural subunit of all 162 capsomers while VP19C and VP23 (triplex protein) are located in the space between the capsomers (Homa and Brown 1997). Capsid proteins are transcribed by γ genes just after DNA replication has started. Initial stages of capsid assembly occur in the cytoplasm of infected cells (Nicholson et al 1994 Rixon et al 1996) followed by final maturation of the capsid occurring in the nucleus of infected cells. The major capsid protein (VP5), the outer tip of hexons (VP26), and a triplex protein (VP23) are not capable of nuclear localization on their own; however, VP5 can be carried into the nucleus by VP19C, another capsid triplex protein, or by pre-VP22a, a scaffolding protein. VP23 localizes to the nucleus only in the presence of VP19C, while VP26 localizes to the nucleus only when it is expressed with both VP5 and VP19C or pre-VP22a (Nicholson et al., 1994; Rixon et al., 1996). Considerable advances have been made in understanding the structure an assembly of the capsid shell, largely as the result of applying cryoelectron microscopy techniques as well as recombinant baculoviruses. The *in vitro* system has identified a fragile roundish procapsid which matures into the polyhedral capsid in a transition similar to that undergone by bacteriophage proheads (Homa and Brown 1997).

Three types of capsids, called *A*, *B*, and *C* capsids have been identified by sucrose density gradient ultracentrifugation (Gibson and Roizman, 1972) in the nucleus of infected cells. These procapsids, and anglularized types A, B and C capsids accumulate to form structures called assemblons at the periphery of the replication compartment. All three types of capsids are about 120 nm in diameter with an outer shell composed of hexons and pentons made up of VP5. The individual capsomeres are linked by triplex structures consisting of VP19C and VP23, the two minor capsid proteins. Each triplex consists of one molecule of VP19C and two molecules of VP23 (Newcomb et al., 1993). The *C*-capsids are the mature capsids that contain packaged viral DNA and can proceed to become infectious virions by budding through the nuclear membrane into the perinuclear space (Perdue et al., 1976). In contrast, *A* and *B* capsids lack viral DNA, but *B* capsids are filled with VP22a and VP21, the cleaved scaffolding proteins, and a viral protease VP24 (Gibson and Roizman, 1972; Newcomb et al., 1993). The internal proteins VP22a, VP21, and VP24 are removed upon packaging of viral DNA to form *C* capsids (Davison, Rixon, and Davison, 1992; Gibson and Roizman, 1972). The A capsids do not contain viral DNA or scaffolding proteins and are believed to be an abortive result of failed attempts at DNA packaging.

In terms of the order of assembly, once in the nucleus, VP5-pre-VP22a complexes come together as a result of self-assembly of pre-VP22a. The triplex proteins VP19C and VP23 are then added to form a partial capsid. As hexons and pentons are added, the structure assembles into a round procapsid (Newcomb et al., 1996), ultimately transforming into polyhedral structures (Thomsen, Newcomb et al. 1995; Trus, Booy et al. 1996; Church and Wilson 1997).

Encapsidation of Viral DNA

DNA packaging is initiated with a procapsid that contains outer capsid shell and inner scaffold and requires participation of viral DNA and atleast seven additional HSV-1 encoded proteins. The transformation of empty procapsids into DNA-filled C capsids is the result of an elegant molecular machine that cleaves individual genomes from concatemeric substrate and translocates them into the capsid precursor. Proper functioning of this machine requires specific cleavage sequences on the DNA substrate, a DNA packaging enzyme called terminase, and capsid structural elements such as the portal protein. To initiate cleavage and packaging, the terminase complex composed of pUL28, pUL15 and pUL38 cleave the concatemeric DNA substrate in the DR1 sequence that joins the sequence between the US and UL segments. The mechanism of viral DNA packaging is complex and is not yet well defined.

Virion Egress

Virion morphogenesis begins with the packaging of the replicated DNA into pre-formed capsids which then bud out through the inner nuclear membrane into the perinuclear space. These capsids then exit from the perinuclear space into the cytoplasm. There are different theories regarding egress of the virus from the perinuclear space into the cytoplasm and the site of final envelopment.

Primary Envelopment

Studies by various reseach groups have shown that multiple deletions of glycoprotein genes may it be essential or non-essential do not affect virus transport to the perinuclear space (Sandri-Goldin 2006). It has been documented that tegument proteins UL31 and UL34 are involved in primary envelopment. UL34 protein is likely a type II membrane protein that

localizes within the inner and outer nuclear membrane (Klupp, Granzow et al. 2000), while UL31 is a nuclear matrix-associated phosphoprotein also present in the nuclear membrane of infected cells (Reynolds, Ryckman et al. 2001; Fuchs, Klupp et al. 2002). Localization of UL31 protein at the nuclear rim requires the presence of UL34 protein, whereas the UL34 protein appears to possess an intrinsic nuclear targeting signal (Klupp, Granzow, and Mettenleiter, 2000). However, the nuclear targeting of UL34 is increased by the presence of UL31. Several groups have shown that deletion of either UL31 or UL34 results in drastic impairment in primary envelopment with capsids trapped within the nucleus(Chang, Van Sant et al. 1997; Klupp, Granzow et al. 2000; Roller, Zhou et al. 2000). Therefore, UL31 and UL34 proteins form a complex that accumulates at the nuclear membrane and play an important role in nucleocapsid envelopment at the inner nuclear membrane. Additionally, it has been reported that HSV infection disrupts the nuclear lamina which may facilitate capsids to reach the inner nuclear membrane (Scott and O'Hare 2001). UL31 and UL34 complex may play a role in disruption of nuclear lamina since overexpression of the UL31 protein in uninfected cells was sufficient to relocalize lamin A/C from the nuclear rim into nucleoplasmic aggregates, while overexpression of UL34 was sufficient to relocalize some lamin A/C into the cytoplasm (Reynolds, Liang et al. 2004). It has been reported that both UL31 and UL34 physically interact with lamin A/C and this complex is responsible for altering the conformation of the nuclear lamin to promote nucleocapsid egress from the nucleus. The UL11 protein has also been proposed to function in primary envelopment and increases the efficiency of envelopment at this step (Baines and Roizman 1992).

De-envelopment

At the outer nuclear membrane de-envelopment occurs by fusion of the viral envelope with the outer nuclear membrane resulting in capsid release into the cytoplasm. There is controversy regarding the mechanism of egress of the virus from the perinuclear space into the cytoplasm. Two models have been suggested –a) luminal" model or single envelopment model and b) the de-envelopment re-envelopment" model of egress. According the first model the perinuclear virions retain their primary envelope and integrity as they leave the cell through the secretory pathway (Johnson and Spear 1982; Campadelli-Fiume, Farabegoli et al. 1991). According to this model, virion glycoproteins are modified in transit to the plasma membrane and perinuclear virions contain the entire complement of tegument and envelope proteins present on mature extracellular virions. The second model envisions that the primary envelope of perinuclear virions fuses with the outer nuclear lamellae, resulting in loss of the primary envelope and some tegument components and translocation of the naked capsid into the cytoplasm of the infected cells (Mettenleiter 2002; Mettenleiter, Klupp et al. 2006b). Therefore according to the de-envelopmet and re-envelopment model, final tegumentation and re-envelopement occurs in cytoplasmic vesicles resulting in intracytoplasmic/extracellular virions that differ in composition from perinuclear virions.

Genetic and biochemical studies support de-envelopmet and re-envelopment model of virion morphogenesis however, other models are a possibility (Johnson and Baines 2011). Few of the evidences have been cited. pUL31 and pUL34 are present in perinuclear particles , but pUL34 is not detected in cytoplasmic particles by immunoelectron microscopy, suggesting that the protein remains in the ONM after de-envelopment (Reynolds, Wills et al. 2002).

UL46 and UL49 are present on intracytoplasmic/extracellular virions but they are absent from perinuclear virions (Mettenleiter 2002). Moreover it has been experimentally shown that the phospholipid composition of the final virion envelope differs substantially from the composition of the nuclear membrane. This difference in composition can only be explained by the two-step envelopment model of HSV virion egress (van Genderen, Brandimarti et al. 1994). Thus the above observations from various experiments favor the de-envelopment and subsequent re-envelopment model of herpesvirus virion morphogenesis (Mettenleiter 2002).

It has been reported that the glycoproteins involved in membrane fusion do not play a significant role in the de-envelopmemt process as deletion of these glycoproteins do not affect the process. Works by Melancon et.al have shown that membrane proteins gK and UL20p are also not involved in this process (Melancon, Luna et al. 2005). Thus it has been postulated that de-envelopment involves interaction between tegument proteins and other membrane proteins. However, recent studies reveal the involvement of gB and gH during de-envelopment at the outer nuclear membrane (Farnsworth, Wisner et al. 2007). However gB deleted alone resulted in minor defects in nuclear egress. In other herpes viruses such as EBV and KSHV, gB plays a major role in virion egress as mutants lacking gB alone exhibit severe defects in virion egress (Krishnan, Sharma-Walia et al. 2005) .

Tegumentation

On reaching the cytoplasm the naked capsids undergo a two-step tegumentation process that involves interaction between tegument and capsid proteins. The tegument layer of herpesvirus particles is complex and is made up of at least 23 different proteins that are present in abundance (200 copies - 2,000 copies). The tegument of other herpesviruses such as CMV and VZV are even more

complex as they contain even greater number of tegument proteins (Gibson 1996). The tegument proteins interact with the capsid on one side and the viral envelope proteins on the other side in order to link the structural components to the final envelope of the HSV-1 virion and also to secure the integrity of the virus particle. Recent data indicate that tegumentation follows an intrincate pattern of protein-protein interactions that contains significant levels of redundancy built in, at least as observed in cell culture (Mettenleiter 2002). The innermost portion of the tegument located adjacent to the capsid exhibits icosahedral symmetry, resulting from the interaction of a large tegument protein, presumably UL36, with the pentons of the capsid (Zhou et al., 1999). The UL36 gene product has been shown to interact with the major capsid protein VP5, which forms both the pentons and hexons (Newcomb, Trus et al. 1993). Hence, it appears that the first layer of tegument around the capsid is structured and composed of UL36. UL37 has been shown to interact with UL36 in co-immunoprecipitation and yeast two-hybrid experiments, and capsids that accumulate in the absence of UL37 contain UL36 (Klupp et al., 2002). UL36 and UL37 appear to be the only tegument proteins conserved in all herpesvirus subfamilies, and the absence of the HSV-1 UL36 and UL37 proteins abolishes virus maturation (Desai, Sexton et al. 2001). Unfortunately, the subsequent steps in capsid tegumentation are still largely undefined. Virion morphogenesis can still proceeds in the absence of several other tegument proteins, including UL13, US3 (Purves et al., 1987), UL41, UL46, UL47 (Rafield and Knipe, 1984; Roizman and Knipe, 2001), and UL49. So far the most dramatic effect has been seen upon deletion of UL48 (α-TIF), which is responsible for transducing α gene promoters and makes up a major part of the virus tegument (Batterson and Roizman, 1983; Heine et al., 1974). The absence of UL48 may interfere with a later step in virion assembly, presumably affecting tegumentation and reenvelopment in the cytoplasm (Mossman, Sherburne et al. 2000). Interestingly, UL48 has

been shown to interact with other tegument components, UL49 and UL41 (VHS) (Smibert et al., 1994). Mutant forms of UL41 unable to bind to UL48 are not part of the mature virion (Read, Karr, and Knight, 1993). Cross-linking studies have indicated that UL48 may interact with gB, gD, and gH (Zhu and Courtney 1994), although these interactions have not been confirmed through more stringent methods.

Tegumentation of the nucleocapsid begins in the nucleus but most of the tegument proteins are incorporated onto the nucleocapsid in the cytoplasm. Tegument proteins VP1-2, pUL37, vhs, VP22 and VP16 are added in the nucleus as well as additional quantities of these tegument proteins are also added in the cytoplasm. Other tegument protein that are found exclusively in the cytoplasm are incorporated either during the transport of the nucleocapsid from the nuclear membrane to the TGN or during secondary envelopment at the TGN membranes.

Secondary Envelopment

These tegumented capsids then bud into trans-Golgi network (TGN) derived vesicles that are embedded with viral glycoproteins on their luminal side. The TGN is considered the site for secondary envelopment, a process by which the virus acquires the full complement of envelope glycoproteins. Cytoplasmic envelopment or secondary envelopment is a process which is not yet well understood. In literature, there are different opinions regarding the function of membrane proteins and glycoproteins involved in this process.

Viral proteins involved in secondary virion envelopment-Viral glycoproteins play a role during cytoplasmic virion envelopment. gB does not play a role in virion envelopment and egress since gB-null viruses undergo cytoplasmic virion envelopment and can infect cells if

treated with polyethylene glycol. However work by Calistri et al, have revealed deletions in the carboxyl terminus of gB results in substantial reduction in cytoplasmic envelopment and egress (Calistri, Sette et al. 2007). Membrane proteins gD, gM, gE/gI, and gK/UL20p and membrane associated protein UL11 are thought to play a role in the final envelopment stage. The process of cytoplasmic envelopment involves various interactions between the cytoplasmic domains of the viral glycoproteins with tegument and membrane proteins. The most profound effect on cytoplasmic envelopment has been documented when either gK or UL20 have been deleted. The cytoplasm of cells infected with these mutant viruses (gK null or UL20 null) show accumulation of aberrant enveloped capsids and unenveloped capsids. UL20p carboxyl terminus is involved in cytoplasmic virion envelopment as it may function to prevent de-envelopment of virions contained within cytoplasmic vesicles (Melancon, Fulmer et al. 2007). However, work by Johnson et. al has shown that glycoproteins gD and gE/gI are important for cytoplasmic virion envelopment although they function in a redundant manner since deletion of single glycoproteins alone did not cause any deleterious effect (Farnsworth, Goldsmith et al. 2003). According to their findings, deletion of gD and gE, or gD, gE and gI lead to accumulation of unenveloped capsids. These finding are contrary to data published by our laboratory (Lee, Chouljenko et al. 2009). Work by our group reveals that mutant viruses lacking carboxyl terminal of gD (gDΔct) or entire gE (ΔgE), or double mutant gDΔct+ΔgE are able to grow on Vero cells and have no dramatic defect in virion envelopment. Works by other groups have indicated that gM/gN may work in a redundant manner in cytoplasmic virion envelopment (Crump, Bruun et al. 2004). Deletion of UL11 gene produces mild defects in cytoplasmic virion egress while simultaneous deletion of UL 11and gM caused drastic inhibition of cytoplasmic virion envelopment. Recent data from our laboratory has further revealed that a triple null mutant (gDΔct, ΔgE and ΔgM),

replicated 1 log unit less efficiently than HSV-1 wild type virus and produced viral plaques one-third the size of HSV-1 (F). The recombinant virus ΔUL11-ΔgM, replicated more than 1 log unit less efficiently and produced significantly smaller plaques than UL11-null or gM-null viruses alone; this is in agreement with the results of Leege et. al. Comparison of the triple null mutant (gDctg, gM and gE) with UL20 virus reveals that deletion of the UL20 gene is substantially more deleterious to infectious virus production and causes most severe inhibition of cytoplasmic virion envelopment. Work from our laboratory has shown that gK and UL20 interact with both gB and gH. Recently, it was reported that gB may function in a redundant manner with gD to facilitate cytoplasmic virion envelopment. Therefore, lack of gK or UL20 may affect the binding ability of gB, gD and gH to tegument proteins. Moreover, gK and UL20 may interact directly with gM, UL11 and tegument proteins to facilitate cytoplasmic virion envelopment. These observations unequivocally show that UL20p (or gK) are the main players in cytoplasmic virion envelopment because deletion of these two membrane proteins practically abolishes virion envelopment at cytoplasmic sites.

Virion Egress to Extracellular Spaces

The last step of virion morphogenesis is the egress of the enveloped capsids into extracellular spaces. Cytoplasmic secondary envelopment leads to enveloped virions within secretory vesicles. These secretory vesicles or transporter vesicles travel to the plasma membrane where they fuse with the plasma membrane and release the mature virions into extracellular spaces. Not much is known regarding the role of viral proteins involved in transport or egress. It is thought, that this process of exocytosis of virions is brought about by the cellular fusion machinery rather than the viral glycoproteins. However, it has been shown that mutant viruses. However, it has been shown that mutant viruses that lack gK and UL20p

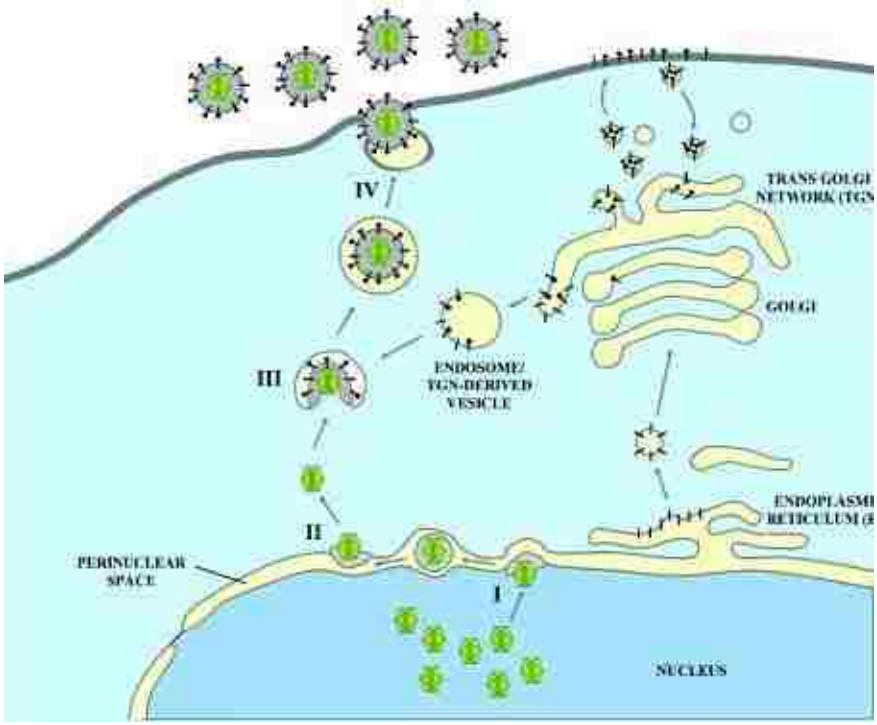

Figure 1.9: Depiction of different steps in HSV-1 virion morphogenesis (assembly and egress). After viral DNA replication the genome is packaged into newly formed capsids in the nucleus. (I) The DNA filled capsids bud into the perinuclear space and attain a primary envelope from the inner nuclear membrane. (II) De-envelopments of the virions occur at the outer nuclear membrane and virions are release into the cytosol. Naked capsids are tegumented in the cytosol. (not shown). (III) Tegumented capsids bud into TGN derived vesicles that are decorated with glycoproteins. The mature cytoplasmic capsids finally attain an envelope (re-envelopment). (IV) Egress of the mature virions into extracellular spaces by process of reverse exocytosis.

have a defect in virion egress with most of the virion particles stuck in the cytoplasm (Jayachandra, Baghian et al. 1997; Foster and Kousoulas 1999).

Latent Phase

The most outstanding feature of herpes virus is its ability to become latent in the host and later to reactivate from latency and start producing infectious virions at the site of initial infection. The site of latency depends upon the type of virus, HSV1 remains latent in the

trigeminal ganglion while HSV-2 in the sacral ganglion. When the virus enters the latent state no infectious virions are produced but the viral genome is retained intact within specific cells. A recurrent infection occurs when the virus reactivates from latent state because of an external stimulus such as heat, stress and sunlight (UV rays).

HSV-1 Glycoproteins and Their Putative Functions

The surface of the HSV viral envelope contains 600-750 glycoprotein spikes that vary in length, spacing and the angle of emergence from the envelope. These glycoproteins are designated as glycoprotein B (gB), glycoprotein C (gC), glycoprotein D (gD), glycoprotein E (gE), glycoprotein I (gI), glycoprotein J (gJ), glycoprotein H (gH), glycoprotein K (gK), glycoprotein L (gL), glycoprotein M (gM) and glycoprotein G (gG). In addition to these 11 glycoproteins, there are membrane associated proteins such as UL20, UL34, UL45 and possibly US9. Some of these viral proteins have not been detected on the virion envelope. These membrane proteins play important role at different stages of the HSV-1 life cycle i.e. virus entry, virus spread, virus envelopment and egress.

Common structural features of HSV-1 glycoproteins:

a) These integral membrane proteins (glycoproteins) are mostly structural proteins that are encoded by gamma late genes. Mostly, these glycoproteins are type I membrane proteins that have certain common features such as a signal sequence, hydrophobic membrane spanning domain and potential sites for N-glycosylation.

b) Another unique feature of HSV-1 membrane protein is that they form multimers which are either homomeric or heteromeric in nature. For example, gC has been found to

form multimers, while gB forms homotrimers and gH/gL, gK/Ul20p, gE/gI, gM/gN form heterodimers.

c) HSV-1 infected cells (*in vitro*) normally do not fuse with uninfected neighbouring cells. Although certain spontaneous mutations in the viral genome cause the formation of large multinucleated cells called syncytia. These HSV-1 mutants (*syn* mutants) have either point mutations or deletions that map to atleast four genes of the viral genome; UL27 (gB), UL53 (gK), UL20 (UL20p) and UL24. Glycoproteins with syncytial mutations play an important role in virus induced cell-cell fusion.

Glycoprotein B

gB is one of the most conserved glycoproteins found in all herpes virus subfamilies. It is a type I integral membrane protein encoded by UL27 gene, consists of 904 amino acids. The ectodomain of gB (696 amino acids) consists of a secretory signal and a number of N-glycosylation sites, whereas the intracellular carboxyl terminus consists of 109 amino acids. Surprisingly, most of the syncytial mutations are located exclusively in this intracellular region. gB is a class III viral fusion protein. The X-ray crystal structure of ectodomain of gB reveals striking similarity to glycoprotein G of vesicular stomatitis virus and baculovirus gp64. However, unlike other members of its class, it cannot function alone and needs gH/gL to bring about fusion . From the X-ray structure it has been determined that gB assumes two conformations, a pre-fusion and post-fusion form, one of which is essential for membrane fusion. It appears that gD, gH/gL may be responsible for bringing about this conformational change in gB, facilitating its fusogenic ability. The extracellular portion of gB as determined by X-ray crystallography, reveals a trimeric form that consists of three identical protomers designated as

A, B and C. Each protomer further consists of five distinct domains (Domain I to Domain V). Four functional regions (FR) have also been defined, based on mapping of anti-gB neutralizing mAbs to the crystal structure. The Functional Region I (FR1) consists of Domain 1 and V which are predicted to be proximal to the plasma membrane. Neutralizing mAbs to FR1, blocks the association of gB with lipid membranes. Thus FR1 contains fusion loops that associate with the cell membrane.

The carboxyl terminal of gB is located in the cytosol and most of the syncytial mutations (point mutations, truncations as well as deletions) have been mapped to this region. Deletion of 28 aa from the carboxy terminal of gB leads to hyperfusion while ceratain muations withinin this region lead to a fusion null phenotype. Thus the carboxy terminal of gB seems to act as a fusion regulator either to reduce or to enhacing fusion (Chowdary and Heldwein 2010).

Glycoprotein C

gC is encoded by UL44 gene and is a mucin-type glycoprotein because of its high content in N-linked and O-linked oligosaccharides. This membrane protein consist of 511 amino acids, a 25 aa signal sequence at the N terminus, a long 453 aa extracellular domain, a 23 aa transmembrane anchoring domain, and a short 10 aa C-terminal cytoplasmic tail (Homa et al., 1986). The initial contact between the virus and the cell is through interactions between heparan sulfate moieties on GAGS and gC.

Previous studies have delineated the HS-binding domain that interacts with gC amino terminal residues to be located between aa 33 and 123, as well, as between 129 and 247 (Trybala, Bergstrom et al. 1994). The basic aa residues present in gC amino acid sequence are known to

be critical for HS-binding activity. Although, gC is dispensable for the infection of cultured cells but its presence can increase the efficiency of virus binding almost 10-fold.

The role of glycoprotein C in virus –induced cell fusion is debatable. It was found that HSV strain MP lacking gC (gC-) produces large syncytial (Syn) plaques on many cell types , whereas a sibling strain mP with a full copy of gC (gC+) produces wild-type, small, nonsyncytial (Syn+) plaques (Pogue-Geile, Lee et al. 1984). Several other spontaneously arising syncytial virus strains have been found to be gC-deficient. Sequencing of the MP strain variant MP10311 revealed that it lacks gC but contained a syncytial mutation in gK (Bartoletti, Tognon et al. 1985). Hence it may be in the absence of gC the syncytial phenotype is enhanced, although this phenotype is not well defined.

gC is also involved in modulating complement activation by blocking C5 and C3. i (Eisenberg, Ponce de Leon et al. 1987). The presence of gC protects the virus and infected cell from antibody (Ab)-independent complement neutralization and cell lysis (Friedman, Wang et al. 2000). gC is also considered an important virulence factor *in vivo* (Sahu et al., 1998). Thus, presence of gC provides the virus with various mechanisms to escape the host immune system and establish a productive infection.

Glycoprotein D

US6 gene is located in the unique short region of the herpes simplex genome and consisting of 1182 bases. It encodes gD, a membrane protein that consists of a 25 aa signal peptide, 15 aa ectodomain containing three glycosylation sites (McGeoch, Dolan et al. 1985) , a 22 aa transmembrane domain, and a 32 aa C-terminal cytoplasmic domain (Minson, Hodgman et al. 1986). The ectodomain of gD has 6 cysteine residues at positions 66, 106, 118, 127, 189

and 202 forming disulfide bonds (Long, Wilcox et al. 1992). gD is considered as the principal determinant of cell recognition during viral entry and is known to interact with all known HSV-1 entry receptors (Spear, Eisenberg et al. 2000). gD is absolutely necessary for virus entry and virus-induced cell-to-cell fusion. HSV-1 gD has the ability to bind HVEM, nectin-1, nectin-2, and other cell surface receptors to mediate virus entry. In addition, cells that express gD are resistant to HSV infection in a dose-dependent manner due to a saturation of the corresponding entry receptors (Campadelli-Fiume et al. 1988; Johnson, Burke, and Gregory, 1990). The X-ray structure of the soluble portion of gD as well as gD interacting with HVEM has been solved. The crystal structure of HSV-1 gD reveals an Ig-like fold with unconventional disulfide bonding patterns (Carfi, Willis et al. 2001). There is an N-terminal extension from the Ig-like fold that forms a hairpin loop in the complex with HVEM, but is disordered in the crystals of gD alone. It has been reported that the soluble ectodomain of gD is both necessary and sufficient to rescue the non-infectious gD null mutant . The contacts in gD for HVEM have been localized to amino acids 7 to 15 and 24 to 32 within the N-terminal hairpin (Spear and Longnecker, 2003). Mutagenic analysis of HSV-1 gD revealed that the first 32 amino acids of gD in the N-terminal extension plays a critical role in functional interactions with all the HSV entry fusion receptors, except for nectin-1 (Yoon, Zago et al. 2003).

gD assumes two conformations; a closed conformation in absence of the receptor and an open and active conformation upon interacting with receptor (either HVEM – and possibly nectin 1). In absence of the receptor, the N terminus is flexible and regions near the C terminal region occlude the receptor binding site. On binding to a receptor the N terminus forms a loop and displaces the C terminus that contains the pro-fusion domain. The pro-fusion domain

consists of amino acids 260-310 that transmits a signal through this domain to gB and/or gH/gL thereby activating these glycoproteins.

Glycoprotein E and I

The gI protein is a type I membrane protein 390 aa in length with a single transmembrane domain and a cytosolic tail at the C terminus (Norberg, Olofsson et al. 2007). It is encoded by US7 gene which is 117 bp long. The function of gI is not yet known, but it forms a non-convalently linked heterodimeric complex with glycoprotein E (gE). 550 aa glycoprotein E is encoded by US8 gene which is 1652 bp long. This heterodimeric complex (gE/gI) is found in HSV, PrV and VZV and plays a role in cell-to-cell spread of virus in epithelial and neuronal tissues. However, in transformed cell lines as well as in unpolarized cells the gE/gI complex do not function in cell-cell spread as these cells lack cellular junctions. Experiments done by Wisner et. al. have shown that there is a distinctive difference in spread of the virus in keratinocytes and HeLa cells infected with gE negative virus. In absence of gE there is eight fold decrease in plaque formation in keratinocytes in comparison to HeLa cells (transformed cell line) (Wisner, Brunetti et al. 2000). The importance of the gE-gI heterooligomer in neuron-to-neuron transmission of HSV-1 was shown by Dingwell et.al (Dingwell, Doering et al. 1995). In PRV too it has been reported that gE/gI complex is important for spread at synaptically linked regions in the peripheral and central nervous system.

It has also been reported that gE/gI binds to Fc portion of IgG and thus reduces antibody-dependent cellular cytotoxicity (ADCC) in infected host.

Glycoprotein H and L

The heterodimeric complex of gH/gL is well conserved within the herpes virus subfamilies and is a major target for neutralizing antibodies. gH, a type I membrane glycoprotein encoded by UL22 gene is essential for viral infectivity as deletion of gH results in non-infectious progeny and abolishment of cell-cell fusion (Westra, Verjans et al. 2000). gL encoded by UL1 gene is not an integral membrane protein as it lacks a transmembrane domain. gH/gL are always found together and forms a stable (1:1) complex that is present on virus particles as well as surface of infected cells. gH consists of 18 amino acid signal peptide and a 785 amino acid long ectodomain while gL consists of a 25 amino acid signal peptide and 224 amino acid residue. The H1 domain of gH interacts extensively with the N-terminal region of gL. This interaction is important for their proper folding since absence of either partner leads to misfolding of proteins and lack of surface expression. A major role of gH, a glycoprotein which was earlier thought to have inherent fusogenic properties and also functioned in bringing about fusion between membranes during both viral and cell-cell fusion. However, recent X-ray structure of gH ectodomain revealed a crystal structure that did not resemble any of the known viral fusogen but rather had an unusual boot shape structure (Chowdary, Cairns et al. 2010). Therefore, it has been suggested that instead of acting as a fusogen, gH/gL activates the fusogenic potential of gB by binding it directly. The carboxyl terminus of gH has been shown to be important for virus-induced cell fusion (Jackson, Lin et al. 2010).

Glycoprotein J

The 279 bp US5 gene encodes the 92 aa glycoprotein J (gJ) (Ghiasi, Nesburn et al. 1998). gJ contains a predicted signal peptide and a hydrophobic transmembrane domain. Deletion

viruses that lack the US5 ORF show no phenotypic deficiencies either in vitro or in vivo (Balan et al., 1994). It has been reported by Jerome et al. that HSV-1 inhibits apoptosis of infected cells, presumably to ensure that the infected cell survives long enough to allow completion of viral replication. Therefore, it has been suggested that presence of gJ helps the virus to evade the host immune system by preventing apoptosis induced by cytotoxic lymphocytes (Jerome, Chen et al. 2001).

Glycoprotein M and UL49.5 (gN)

Glycoprotein M, encoded by UL10 is considered by certain research groups as a non-essential glycoprotein. It is an extremely hydrophobic glycoprotein with eight transmembrane segments and forms a complex with UL49A (gN). gM is a component of the virion envelope and is present in the cytoplasmic membranes of infected cells (MacLean, Robertson et al. 1993). Viruses deficient in the expression of gM exhibit a 10 to 20 fold reduction in virus titers in Vero or BHK cells and form slightly smaller plaques (MacLean, Efstathiou et al. 1991). The 276 bp long UL49.5 (UL49A) gene, encodes a 91 aa integral membrane protein, gN (Baines and Roizman 1993). The UL49.5 polypeptide contains a 23 aa cleaved signal peptide and a C-terminal hydrophobic transmembrane domain (Barnett et al., 1992). There are also potential sites for O-glycosylation, leading to the naming of UL49.5 as glycoprotein N (gN) in some cases. However studies have revealed that the UL49.5 gene product shows no evidence of glycosylation in HSV-1 infected cells, and appears to be abundantly present in virions, linked to the tegument by disulfide bonds (Adams et al., 1998). While initial attempts at deletion of UL49.5 have been unsuccessful (Barker and Roizman, 1992); a more recent attempt utilizing a more precise deletion resulted in a UL49.5-deficient virus that showed no phenotypic differences in plaque yield or morphology but only a marginal two-fold difference in virus yield (Adams et al., 1998).

Interestingly, in all herpesvirus subfamilies gM/gN heterodimer is well conserved. The PrV gM has been shown to act as an inhibitor of cell fusion as reported by Klupp et al. (Klupp, Nixdorf, and Mettenleiter, 2000). Although, no definite interaction is seen between HSV-1 gM and UL49.5, an enhancement of function has been demonstrated, when both are present; inasmuch as cotransfection of the HSV-1 gM and UL49.5 significantly inhibited cell fusion caused by the HSV-1 gB, gD and gH/gL or the Molony murine leukemia virus envelope protein; while transfection of the HSV-1 gM alone did not inhibit membrane fusion (Koyano et al., 2003). At present, the mechanism by which gM and UL49.5 mediate inhibition of cell-to cell fusion is unknown.

Glycoprotein K

HSV-1 gK is one of the 11 glycoproteins encoded by the virus. However, the role of HSV-1 gK has been debated extensively in the last two decades. Over the past ten years, considerable advancements have been made regarding the structure and function of this glycoprotein.

Glycoprotein K is encoded by UL53 gene and is a viral structural protein expressed during the "late phase" of transcription. gK consists of 338 amino acids and consist of several hydrophobic domains (Fig. 1.10). It consists of a signal sequence of 30 aa as well as two asparagine residues at positions 48 and 58 that are glycosylated by N-linked mannose (Hutchinson, Goldsmith et al. 1992). The hydrophobicity of gK has hampered the detection of this glycoprotein since it is difficult to generate anti-sera against this protein in animals.

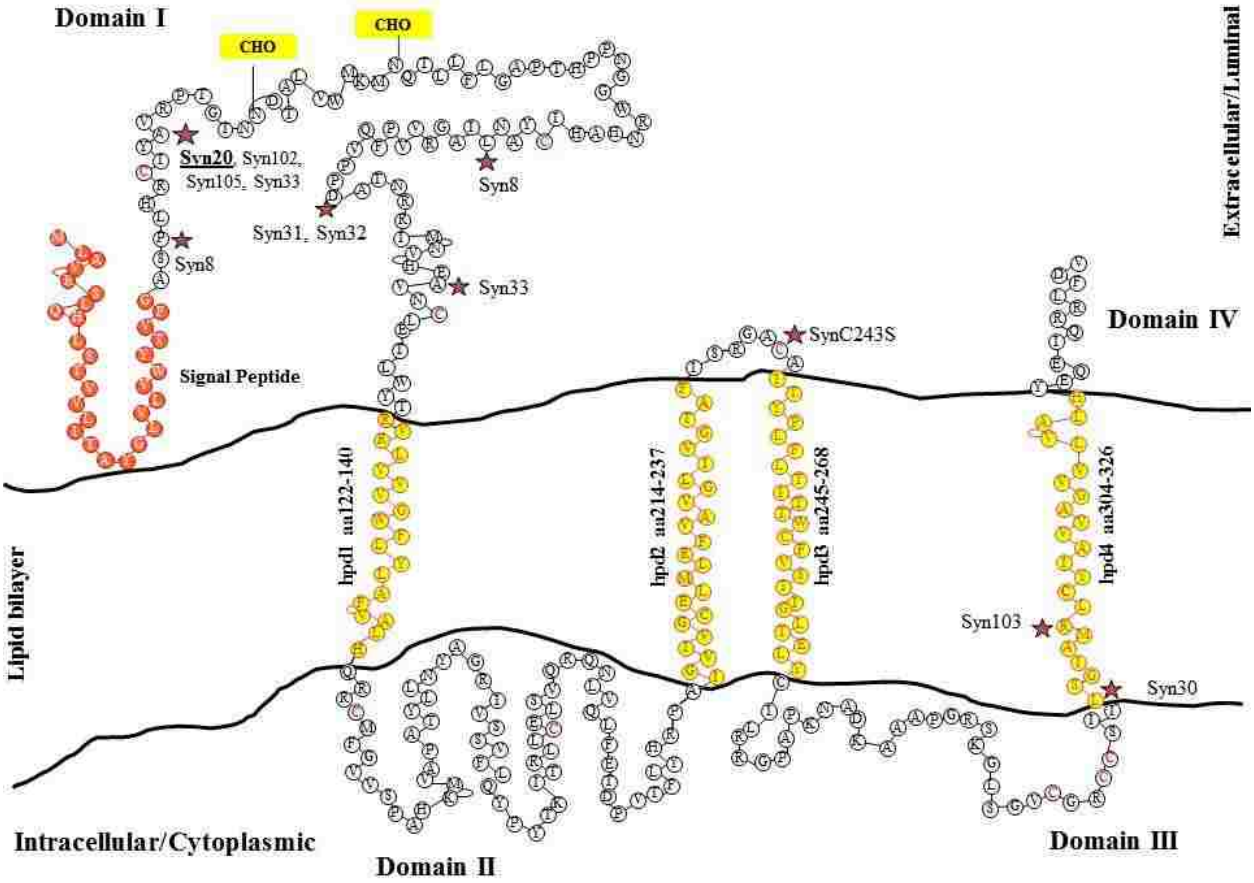

Figure 1.10: HSV-1 gK membrane topology. (A) The experimentally validated topology of gK is shown in conjunction with the secondary predicted structure of gK. Features of the gK topology include four membrane-spanning hydrophobic domains (hpd1 to -4), a signal sequence of 30 aa, and two N-linked glycosylation sites (CHO). The locations of different syncytial mutations published previously are indicated by stars. Chouljenko, V. N., A. V. Iyer, et al. (2009).

Predicted secondary structure of glycoprotein K

A number of studies have been done to predict the secondary structure of gK. Work by Debroy et. al predicted gK topology consisting of four transmembrane domains, with both the N-terminus and C-terminus placed in the extracellular side of cellular membranes (Debroy, Pederson et al. 1985). To further understand the transmembrane topology of gK, experiments

with *in vitro* translated gK were done in the presence of microsomal membranes. Data generated from these studies demonstrate that gK contains three transmembrane domains (amino acids 125-139, 226-239, and 311-325) (Debroy, Pederson et al. 1985; Mo and Holland 1997). However, work in our laboratory confirmed the topology of gK by utilizing epitope tags inserted into specific regions of gK that revealed, gK domain I and domain IV were located extracellulartly; whereas domain II and III were located intracellularly and moreover, it consisted of four transmembrane domains (Foster, Alvarez et al. 2003).

gK part of the virion

There is controversy as to whether HSV-1 gK is a structural component of the virion. Previous studies have indicated that gK is not specified on the virion surface. Lorret et al. were unable to detect gK on the virion surface by mass spectrometry. However, the authors concluded that since gk is extremely hydrophobic it could be difficult to detect this glycoprotein since it may aggregate upon boiling and not reach the mass spectrometer (Loret, Guay et al. 2008). Our laboratory has successfully reported that gK is a structural component of virions as a Golgi complex-dependent glycosylated species present on HSV-1 (KOS) virion. In 2010 we further reported that gk and UL20p can be readily detected on double-gradient purified HSV-1 (F) virion preparations. For these studies a recombinant virus with in-frame epitope tags fused to gK and UL20p were used. Moreover, immunoelectron microscopy of this virus showed the presence of gK and UL20p on the surface of virions (Jambunathan, Chowdhury et al. 2011).

gK is expressed on surface of infected cells

It was earlier thought that gK was mainly present in perinuclear membranes as well as endoplasmic reticulum of infected cells and not expressed on the surface of infected cells.

These findings were inconsistent with the role of gK in syncytia formation that requires the surface expression of gK. Experiments done by Foster et al. were able to solve this paradigm by showing that gK is expressed on infected cell surfaces. Moreover, in absence of UL20p, gK is unable to reach infected cell surfaces. They observed that when Vero cells were infected with either gK null or UL20 –null viruses, UL20 or gK respectively were entrapped in endoplasmic reticulum (ER). By confocal co-localization studies it has been shown that gK and UL20p are interdependent for post-ER-to –TGN intracellular transport and localization (Foster, Melancon et al. 2004).

Role of gK in virus-induced cell-cell fusion

It has been well established that gK plays a role in virus –induced cell-to-cell fusion. Several syn mutants have been isolated in which point mutations within the extracellular domain of gK (Domain I) leads to extensive cell-cell fusion (Foster, Alvarez et al. 2003). Several of these syn mutants have mutations in codon 40 and this loci is designated as gKsyn 1. gKsyn1 is known to cause extensive virus induced cell fusion in all cell types; while gB syncytial mutations have a restricted cell type range. Work carrid out in our laboratory showed that in absence of gB a recombinant virus carrying a gKsyn1 mutation cannot cause fusion. Moreocer, gB syncytial mutation gBsyn3 or gB amb1511; lacking gK and UL20 gene, fail to cause virus induced cell fusion. These observations further confirm the role of gK and UL20 in gB mediated virus-induced cell fusion (Foster, Melancon et al. 2004).

gK is important for virion envelopment and egress

Glycoproteins gD,gE, gK, gM, the membrane protein UL20 and membrane associated protein UL11 are important players in the process of cytoplasmic virion envelopment and egress from

infected cells. Recombinant viruses possessing single, double, triple deletion of these above mentioned viral proteins in various combinations show differential defect in virion envelopment. However, the most profound effect on cytoplasmic virion envelopment has been documented when either gK or UL20 have been deleted. The cytoplasm of cells infected with these mutant viruses (gK null or UL20 null) show accumulation of aberrant enveloped capsids and unenveloped capsids. Therefore gk and UL20p play a critical role during cytoplasmic virion envelopment and egress (Jayachandra, Baghian et al. 1997; Foster and Kousoulas 1999; Lee, Chouljenko et al. 2009).

gK interacts with UL20p

Series of recent studies have shown that HSV-1 gK and UL20 functionally and physically interact and that these interactions are necessary for their coordinate intracellular transport and cell surface expression. Specifically, direct protein-protein interactions between the amino terminus of HSV-1 UL20 and gK domain III, both of which are localized intracellularly, were recently demonstrated by two-way coimmunoprecipitation experiments (Foster, Chouljenko et al. 2008).

Role of gK in entry

In 2001 experiments done by Foster et al. showed, gK null (HSV-1 (KOS)) virus entry was slower in Vero cells in comparison to the wild type virus. Recently our laboratory reported that in absence of the amino terminus (31-68 aa) of gK HSV-1(F) there is a 30% reduction in viral entry in Vero cells. However, it should be noted that virus particles lacking gK also show drastic defects in cytoplasmic virion envelopment. In order to overcome this issue we utilized a strategy wherein the amino terminus of gK was cleaved off after formation of fully enveloped

mature virion particle. This was achieved by generating a recombinant virus that encoded a TEV protease site immediately after gK aa 68. Treatment of this recombinant virus with enterokinase resulted in cleavage of amino terminus of gK that was subsequently used for entry assay. Vero cells infected with gK cleaved virion particles showed 30-34% decrease in virus entry. gK is involved in viral virulence (Jambunathan, Chowdhury et al. 2011).

Recently, our laboratory has reported that gK plays an important role in virus spread in the cornea of mice, neuroinvasiveness and establishment of latency in ganglionic neurons (David, Baghian et al. 2008). HSV-1 glycoprotein K may be particularly important for the causation of eye disease in humans because it has been reported to exacerbate eye disease mediated by anti-glycoprotein K humoral and cellular immune response. Vaccination with gK also appears to block viral clearance from trigeminal ganglia, which results in a chronic, productive infection.

Homologs of gK

Glycoprotein K is conserved among the alphaherpesviruses and homologous proteins have been reported for HSV-2 , bovine herpesvirus 1, equine herpesvirus 1, varicella-zoster virus (VZV), infectious laryngotracheitis virus (ILTV), and Marek's disease virus. Comparison of HSV-1 gK sequence with that of gK from other alphaherpesviruses have shown that there is 46% sequence homology with HSV-2, 44% with EHV-1, 53% with Marek disease virus and 43% with VZV. Further it has been reported that gK is a structural virion component of PRV,VZV and EHV-1 (Klupp, Baumeister et al. 1998) . It has been reported that Equine herpes virus type I (EHV-1) glycoprotein K is important for virus growth in vitro and it is required for efficient cell-to-cell spread as well as envelopment and virus egress. Similar, to its HSV-1 counterpart VZV

gK plays an important role in membrane fusion and cytoplasmic virion morphogenesis (Mo, Suen et al. 1999).

Characterization of the Membrane Protein UL20 (UL20p)

UL20p is encoded by the UL20 gene which lies in the unique long region of the HSV-1 genome. It is 222 aa in length and is a gamma late protein conserved in alphaherpes viruses. It is an integral unglycosylated membrane protein that consists of multi-membrane spanning domains. The topological orientation of UL20p has been worked upon by several groups. It was first thought that UL20p consists of two-three hydrophobic transmembrane domains but later it was shown by computer algorithm to contain four transmembrane domains lacking a signal sequence . The predicted secondary structure of UL20p was later confirmed in our laboratory by epitope tagging different predicted domains of UL20p (Foster, Melancon et al. 2004). According to the present model of UL20p it consists of four transmembrane domains with the amino terminus and carboxyl terminus situated within the cytoplasm.

UL20p plays a crucial role in cytoplasmic virion morphogenesis. Vero cells infected with UL20-null virus exhibit defect in virion egress; and there is accumulation of un-enveloped capsids in the cytoplasm (Foster et al. 2004). It has been suggested that UL20p may function in the reenvelopment step or to prevent de-envelopment of virions contained within cytoplasmic vesicles.

As discussed earlier UL20p is needed for proper trafficking of gK beyond the endoplasmic reticulum (Foster, Melancon et al. 2004). Recent studies have shown, that these two proteins physically interact and this interaction is necessary for intracellular protein trafficking,virus egress and virus-mediated cell-to-cell fusion (Foster, Chouljenko et al. 2008).

Recently our laboratory has confirmed the presence of UL20p on virions that interact with both gK and gB (Jambunathan, Chowdhury et al. 2011).

UL20p plays an important role in virus-induced cell fusion. Most of the syncytial mutations in UL20 lie in the amino terminus that lies intracellularly. It has been revealed that in absence of the UL20p syncytial mutations in gK and gB are unable to cause virus-induced cell fusion. Experiments done by Melancon et al. have shown that UL20p domains involved in cytoplasmic virion envelopment are distinct from domains involved in virus-induced cell fusion. Specifically, UL20 mutations within the amino and carboxyl termini of UL20p allowed cotransport of gK and UL20p to cell surfaces, virus-induced cell fusion, and TGN localization, while effectively inhibiting cytoplasmic virion envelopment (Melancon, Foster et al. 2004).

VIRUS-INDUCED CELL-CELL FUSION

Wild type HSV-1 causes limited fusion in culture, mostly infection leads to rounding of cells. The virus can spread from one cell to another either by release of infectious virion particles or by virus-induces cell-cell fusion. Cell–cell fusion is a mechanism which facilitates spread of the virus without being recognized by the immune system. There are a number of HSV-1 mutants that cause virus induced cell-to-cell fusion also referred to as syncytia formation (i.e. fusion between neighboring uninfected and infected cells). Genetic studies have revealed that these mutations are present in at least four loci of the viral genome: the UL20 gene (encoding UL20p), the UL24 gene, the UL27 gene (encoding gB), and the UL53 gene (coding for gK)(Bzik, Fox et al. 1984; Debroy, Pederson et al. 1985; Baines, Ward et al. 1991; Jacobson, Chen et al. 1998). Viruses possessing these mutations are called syncytial mutants or syn mutants. Syncytia formation occurs by the interaction between surface expressed viral proteins

on infected cells with cellular receptors or other factors on uninfected neighboring cells. Thus fusion ensues between altered membranes of infected cells with that of unaltered membranes of uninfected cells, ultimately resulting in multinucleated cells.

The concept that gB is responsible or is part of the fusion process was derived from the observation of syncytial phenotype of virus strains with point mutation in the cytoplasmic tail of gB. An interesting observation is that syncytial mutations (point mutations, truncations as well as deletions) that enhance virus-induced cell fusion are located primarily in the carboxy terminus of gB, which lies in the cytoplasm (Chowdary and Heldwein 2010). A single point mutation from Arg-His at aa position 857 in HSV-1 (KOS) leads to extensive syncytia formation and is designated as gBsyn3 (Baghian, Huang et al. 1993).

One of the most striking phenotypes is observed when 28 aa from the carboxy terminal of gB is deleted. It results in extensive virus-induced cell fusion and also extensive fusion in a virus-free transient cell –cell fusion system consisting of gD, gH/gL and gB plasmids.

Cell-cell fusion has been well studied in a virus free transient in vitro system. In this system four glycoproteins gB, gD, gH and gL when expressed transiently are sufficient to bring about fusion (Turner, Bruun et al. 1998). However, in the context of virus induced cell-cell fusion surface expression of gK and UL20p are essential. It has been demonstrated that recombinant viruses containing gB syncytial mutation gBsyn3 or gB amb1511 but lacking gK and UL20 gene fail to cause virus induced cell fusion (Foster, Melancon et al. 2004).

Furthermore, a recombinant virus carrying a mutation gKsyn1, cannot bring about fusion in absence of UL20. These observations further suggest the role of gK and UL20 in gB mediated virus-induced cell fusion.

REFERENCES

Abaitua, F. and P. O'Hare (2008). "Identification of a highly conserved, functional nuclear localization signal within the N-terminal region of herpes simplex virus type 1 VP1-2 tegument protein." J Virol **82**(11): 5234-5244.

Amir, J., L. Harel, et al. (1997). "Treatment of herpes simplex gingivostomatitis with aciclovir in children: a randomised double blind placebo controlled study." BMJ **314**(7097): 1800-1803.

Antinone, S. E., G. T. Shubeita, et al. (2006). "The Herpesvirus capsid surface protein, VP26, and the majority of the tegument proteins are dispensable for capsid transport toward the nucleus." J Virol **80**(11): 5494-5498.

Anzivino, E., D. Fioriti, et al. (2009). "Herpes simplex virus infection in pregnancy and in neonate: status of art of epidemiology, diagnosis, therapy and prevention." Virol J **6**: 40.

Arii, J., H. Goto, et al. (2010). "Non-muscle myosin IIA is a functional entry receptor for herpes simplex virus-1." Nature **467**(7317): 859-862.

Astruc, J. (1736). De Morbis Venereis Libri Sex. Paris, G.Cavelier.

Atanasiu, D., W. T. Saw, et al. (2010). "Cascade of events governing cell-cell fusion induced by herpes simplex virus glycoproteins gD, gH/gL, and gB." J Virol **84**(23): 12292-12299.

Atanasiu, D., J. C. Whitbeck, et al. (2007). "Bimolecular complementation reveals that glycoproteins gB and gH/gL of herpes simplex virus interact with each other during cell fusion." Proc Natl Acad Sci U S A **104**(47): 18718-18723.

Atanasiu, D., J. C. Whitbeck, et al. (2010). "Bimolecular complementation defines functional regions of Herpes simplex virus gB that are involved with gH/gL as a necessary step leading to cell fusion." J Virol **84**(8): 3825-3834.

Bader, C., C. S. Crumpacker, et al. (1978). "The natural history of recurrent facial-oral infection with herpes simplex virus." J Infect Dis **138**(6): 897-905.

Baghian, A., L. Huang, et al. (1993). "Truncation of the carboxy-terminal 28 amino acids of glycoprotein B specified by herpes simplex virus type 1 mutant amb1511-7 causes extensive cell fusion." J Virol **67**(4): 2396-2401.

Baines, J. D. and B. Roizman (1992). "The UL11 gene of herpes simplex virus 1 encodes a function that facilitates nucleocapsid envelopment and egress from cells." J Virol **66**(8): 5168-5174.

Baines, J. D. and B. Roizman (1993). "The UL10 gene of herpes simplex virus 1 encodes a novel viral glycoprotein, gM, which is present in the virion and in the plasma membrane of infected cells." J Virol **67**(3): 1441-1452.

Baines, J. D., P. L. Ward, et al. (1991). "The UL20 gene of herpes simplex virus 1 encodes a function necessary for viral egress." J Virol **65**(12): 6414-6424.

Bartoletti, A. M., M. Tognon, et al. (1985). "Characterization of virus obtained from MDBK cells persistently infected with a variant of herpes simplex virus type 1 strain MP [HSV-1(MP)]." Virology **141**(2): 306-310.

Bateman, T. and R. Willan (1814). A practical synopsis of cutaneous diseases according to the arrangement of Dr. Willan, exhibiting a concise view of the diagnostic symptoms and the method of treatment. London,, Longman, Hurst, Rees, Orme, and Brown.

Batterson, W. and B. Roizman (1983). "Characterization of the herpes simplex virion-associated factor responsible for the induction of alpha genes." J Virol **46**(2): 371-377.

Black, F. L. (1975). "Infectious diseases in primitive societies." Science **187**(4176): 515-518.

Black, F. L., W. J. Hierholzer, et al. (1974). "Evidence for persistence of infectious agents in isolated human populations." Am J Epidemiol **100**(3): 230-250.

Brown, Z. (2004). "Preventing herpes simplex virus transmission to the neonate." Herpes **11 Suppl 3**: 175A-186A.

Buddingh, G. J., D. I. Schrum, et al. (1953). "Studies of the natural history of herpes simplex infections." Pediatrics **11**(6): 595-610.

Burnet, F. M., and Williams, S. W. (1939). "Herpes simplex: new point of view." Med J Aust **1**: 637-640.

Bzik, D. J., B. A. Fox, et al. (1984). "Nucleotide sequence of a region of the herpes simplex virus type 1 gB glycoprotein gene: mutations affecting rate of virus entry and cell fusion." Virology **137**(1): 185-190.

Calistri, A., P. Sette, et al. (2007). "Intracellular trafficking and maturation of herpes simplex virus type 1 gB and virus egress require functional biogenesis of multivesicular bodies." J Virol **81**(20): 11468-11478.

Campadelli-Fiume, G., M. Amasio, et al. (2007). "The multipartite system that mediates entry of herpes simplex virus into the cell." Reviews in Medical Virology **17**(5): 313-326.

Campadelli-Fiume, G., F. Cocchi, et al. (2000). "The novel receptors that mediate the entry of herpes simplex viruses and animal alphaherpesviruses into cells." Rev Med Virol **10**(5): 305-319.

Campadelli-Fiume, G., F. Farabegoli, et al. (1991). "Origin of unenveloped capsids in the cytoplasm of cells infected with herpes simplex virus 1." J Virol **65**(3): 1589-1595.

Campadelli-Fiume, G. and L. Menotti (2007). Entry of alphaherpesviruses into the cell. Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis. A. Arvin, G. Campadelli-Fiume, E. Mocarskiet al. Cambridge.

Cardin, A. D. and H. J. Weintraub (1989). "Molecular modeling of protein-glycosaminoglycan interactions." Arteriosclerosis **9**(1): 21-32.

Carfi, A., S. H. Willis, et al. (2001). "Herpes simplex virus glycoprotein D bound to the human receptor HveA." Mol Cell **8**(1): 169-179.

Challberg, M. D. (1986). "A method for identifying the viral genes required for herpesvirus DNA replication." Proc Natl Acad Sci U S A **83**(23): 9094-9098.

Chang, Y., E. Cesarman, et al. (1994). "Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma." Science **266**(5192): 1865-1869.

Chang, Y. E., C. Van Sant, et al. (1997). "The null mutant of the U(L)31 gene of herpes simplex virus 1: construction and phenotype in infected cells." J Virol **71**(11): 8307-8315.

Chen, D. H., J. Jakana, et al. (2001). "The pattern of tegument-capsid interaction in the herpes simplex virus type 1 virion is not influenced by the small hexon-associated protein VP26." J Virol **75**(23): 11863-11867.

Cheshenko, N. and B. C. Herold (2002). "Glycoprotein B plays a predominant role in mediating herpes simplex virus type 2 attachment and is required for entry and cell-to-cell spread." J Gen Virol **83**(Pt 9): 2247-2255.

Chono, K., K. Katsumata, et al. (2010). "ASP2151, a novel helicase-primase inhibitor, possesses antiviral activity against varicella-zoster virus and herpes simplex virus types 1 and 2." J Antimicrob Chemother **65**(8): 1733-1741.

Chou, J. and B. Roizman (1986). "The terminal a sequence of the herpes simplex virus genome contains the promoter of a gene located in the repeat sequences of the L component." J Virol **57**(2): 629-637.

Chowdary, T. K., T. M. Cairns, et al. (2010). "Crystal structure of the conserved herpesvirus fusion regulator complex gH-gL." Nat Struct Mol Biol **17**(7): 882-888.

Chowdary, T. K. and E. E. Heldwein (2010). "Syncytial phenotype of C-terminally truncated herpes simplex virus type 1 gB is associated with diminished membrane interactions." J Virol **84**(10): 4923-4935.

Church, G. A. and D. W. Wilson (1997). "Study of herpes simplex virus maturation during a synchronous wave of assembly." J Virol **71**(5): 3603-3612.

Clement, C., V. Tiwari, et al. (2006). "A novel role for phagocytosis-like uptake in herpes simplex virus entry." J Cell Biol **174**(7): 1009-1021.

Clements, J. B., R. J. Watson, et al. (1977). "Temporal regulation of herpes simplex virus type 1 transcription: location of transcripts on the viral genome." Cell **12**(1): 275-285.

Cocchi, F., D. Fusco, et al. (2004). "The soluble ectodomain of herpes simplex virus gD contains a membrane-proximal pro-fusion domain and suffices to mediate virus entry." Proc Natl Acad Sci U S A **101**(19): 7445-7450.

Cocchi, F., L. Menotti, et al. (1998). "The ectodomain of a novel member of the immunoglobulin subfamily related to the poliovirus receptor has the attributes of a bona fide receptor for herpes simplex virus types 1 and 2 in human cells." J Virol **72**(12): 9992-10002.

Conley, A. J., D. M. Knipe, et al. (1981). "Molecular genetics of herpes simplex virus. VII. Characterization of a temperature-sensitive mutant produced by in vitro mutagenesis and defective in DNA synthesis and accumulation of gamma polypeptides." J Virol **37**(1): 191-206.

Connolly, S. A., J. O. Jackson, et al. (2011). "Fusing structure and function: a structural view of the herpesvirus entry machinery." Nat Rev Microbiol **9**(5): 369-381.

Copeland, A. M., W. W. Newcomb, et al. (2009). "Herpes simplex virus replication: roles of viral proteins and nucleoporins in capsid-nucleus attachment." J Virol **83**(4): 1660-1668.

Corey, L. (1982). "The diagnosis and treatment of genital herpes." JAMA **248**(9): 1041-1049.

Crump, C. M., B. Bruun, et al. (2004). "Alphaherpesvirus glycoprotein M causes the relocalization of plasma membrane proteins." J Gen Virol **85**(Pt 12): 3517-3527.

Dasgupta, G., A. A. Chentoufi, et al. (2009). "New concepts in herpes simplex virus vaccine development: notes from the battlefield." Expert Rev Vaccines **8**(8): 1023-1035.

David, A. T., A. Baghian, et al. (2008). "The herpes simplex virus type 1 (HSV-1) glycoprotein K(gK) is essential for viral corneal spread and neuroinvasiveness." Curr Eye Res **33**(5): 455-467.

Davison, A. J. (2007). Overview of classification. Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis. C.-F. G. Arvin A, Mocarski E. Cambridge, Cambridge University Press; 2007.

Dean, H. J., S. S. Terhune, et al. (1994). "Single amino acid substitutions in gD of herpes simplex virus 1 confer resistance to gD-mediated interference and cause cell-type-dependent alterations in infectivity." Virology **199**(1): 67-80.

Deb, S. and M. Doelberg (1988). "A 67-base-pair segment from the Ori-S region of herpes simplex virus type 1 encodes origin function." J Virol **62**(7): 2516-2519.

Debroy, C., N. Pederson, et al. (1985). "Nucleotide sequence of a herpes simplex virus type 1 gene that causes cell fusion." Virology **145**(1): 36-48.

Delboy, M. G., J. L. Patterson, et al. (2006). "Nectin-2-mediated entry of a syncytial strain of herpes simplex virus via pH-independent fusion with the plasma membrane of Chinese hamster ovary cells." Virol J **3**: 105.

Desai, P., G. L. Sexton, et al. (2001). "A null mutation in the gene encoding the herpes simplex virus type 1 UL37 polypeptide abrogates virus maturation." J Virol **75**(21): 10259-10271.

Dingwell, K. S., L. C. Doering, et al. (1995). "Glycoproteins E and I facilitate neuron-to-neuron spread of herpes simplex virus." J Virol **69**(11): 7087-7098.

Dixon, R. A. and P. A. Schaffer (1980). "Fine-structure mapping and functional analysis of temperature-sensitive mutants in the gene encoding the herpes simplex virus type 1 immediate early protein VP175." J Virol **36**(1): 189-203.

Dollery, S. J., M. G. Delboy, et al. (2010). "Low pH-induced conformational change in herpes simplex virus glycoprotein B." J Virol **84**(8): 3759-3766.

Eisenberg, R. J., M. Ponce de Leon, et al. (1987). "Complement component C3b binds directly to purified glycoprotein C of herpes simplex virus types 1 and 2." Microb Pathog **3**(6): 423-435.

Elgadi, M. M. and J. R. Smiley (1999). "Picornavirus internal ribosome entry site elements target RNA cleavage events induced by the herpes simplex virus virion host shutoff protein." J Virol **73**(11): 9222-9231.

Epstein, M. A., B. G. Achong, et al. (1964). "Virus Particles in Cultured Lymphoblasts from Burkitt's Lymphoma." Lancet **1**(7335): 702-703.

Everett, R. D., A. Orr, et al. (1991). "High level expression and purification of herpes simplex virus type 1 immediate early polypeptide Vmw110." Nucleic Acids Res **19**(22): 6155-6161.

Faber, S. W. and K. W. Wilcox (1986). "Association of the herpes simplex virus regulatory protein ICP4 with specific nucleotide sequences in DNA." Nucleic Acids Res **14**(15): 6067-6083.

Falke, D., R. Siegert, et al. (1959). "[Electron microscopic findings on the problem of double membrane formation in herpes simplex virus]." Arch Gesamte Virusforsch **9**: 484-496.

Fan, Q. and R. Longnecker (2010). "The Ig-like v-type domain of paired Ig-like type 2 receptor alpha is critical for herpes simplex virus type 1-mediated membrane fusion." J Virol **84**(17): 8664-8672.

Farnsworth, A., K. Goldsmith, et al. (2003). "Herpes simplex virus glycoproteins gD and gE/gI serve essential but redundant functions during acquisition of the virion envelope in the cytoplasm." J Virol **77**(15): 8481-8494.

Farnsworth, A., T. W. Wisner, et al. (2007). "Herpes simplex virus glycoproteins gB and gH function in fusion between the virion envelope and the outer nuclear membrane." Proc Natl Acad Sci U S A **104**(24): 10187-10192.

Fenwick, M. L. and M. J. Walker (1978). "Suppression of the synthesis of cellular macromolecules by herpes simplex virus." J Gen Virol **41**(1): 37-51.

Fleming, D. T., G. M. McQuillan, et al. (1997). "Herpes simplex virus type 2 in the United States, 1976 to 1994." N Engl J Med **337**(16): 1105-1111.

Foster, T. P., X. Alvarez, et al. (2003). "Plasma membrane topology of syncytial domains of herpes simplex virus type 1 glycoprotein K (gK): the UL20 protein enables cell surface localization of gK but not gK-mediated cell-to-cell fusion." J Virol **77**(1): 499-510.

Foster, T. P., V. N. Chouljenko, et al. (2008). "Functional and physical interactions of the herpes simplex virus type 1 UL20 membrane protein with glycoprotein K." J Virol **82**(13): 6310-6323.

Foster, T. P. and K. G. Kousoulas (1999). "Genetic analysis of the role of herpes simplex virus type 1 glycoprotein K in infectious virus production and egress." J Virol **73**(10): 8457-8468.

Foster, T. P., J. M. Melancon, et al. (2004). "The herpes simplex virus type 1 UL20 protein modulates membrane fusion events during cytoplasmic virion morphogenesis and virus-induced cell fusion." J Virol **78**(10): 5347-5357.

Foster, T. P., J. M. Melancon, et al. (2004). "Herpes simplex virus type 1 glycoprotein K and the UL20 protein are interdependent for intracellular trafficking and trans-Golgi network localization." J Virol **78**(23): 13262-13277.

Frenkel, N., E. C. Schirmer, et al. (1990). "Isolation of a new herpesvirus from human CD4+ T cells." Proc Natl Acad Sci U S A **87**(2): 748-752.

Friedman, H. M., L. Wang, et al. (2000). "Novel mechanism of antibody-independent complement neutralization of herpes simplex virus type 1." J Immunol **165**(8): 4528-4536.

Fuchs, W., B. G. Klupp, et al. (2002). "Physical interaction between envelope glycoproteins E and M of pseudorabies virus and the major tegument protein UL49." J Virol **76**(16): 8208-8217.

Furlong, D., H. Swift, et al. (1972). "Arrangement of herpesvirus deoxyribonucleic acid in the core." J Virol **10**(5): 1071-1074.

Geraghty, R. J., C. Krummenacher, et al. (1998). "Entry of alphaherpesviruses mediated by poliovirus receptor-related protein 1 and poliovirus receptor." Science **280**(5369): 1618-1620.

Gerber, S. I., B. J. Belval, et al. (1995). "Differences in the role of glycoprotein C of HSV-1 and HSV-2 in viral binding may contribute to serotype differences in cell tropism." Virology **214**(1): 29-39.

Ghiasi, H., A. B. Nesburn, et al. (1998). "The US5 open reading frame of herpes simplex virus type 1 does encode a glycoprotein (gJ)." Intervirology **41**(2-3): 91-97.

Gianni, T., G. Campadelli-Fiume, et al. (2004). "Entry of herpes simplex virus mediated by chimeric forms of nectin1 retargeted to endosomes or to lipid rafts occurs through acidic endosomes." J Virol **78**(22): 12268-12276.

Gibson, W. (1996). "Structure and assembly of the virion." Intervirology **39**(5-6): 389-400.

Gruenheid, S., L. Gatzke, et al. (1993). "Herpes simplex virus infection and propagation in a mouse L cell mutant lacking heparan sulfate proteoglycans." J Virol **67**(1): 93-100.

Haarr, L., D. Shukla, et al. (2001). "Transcription from the gene encoding the herpesvirus entry receptor nectin-1 (HveC) in nervous tissue of adult mouse." Virology **287**(2): 301-309.

Harrop, J. A., M. Reddy, et al. (1998). "Antibodies to TR2 (herpesvirus entry mediator), a new member of the TNF receptor superfamily, block T cell proliferation, expression of activation markers, and production of cytokines." J Immunol **161**(4): 1786-1794.

Hayward, G. S., R. J. Jacob, et al. (1975). "Anatomy of herpes simplex virus DNA: evidence for four populations of molecules that differ in the relative orientations of their long and short components." Proc Natl Acad Sci U S A **72**(11): 4243-4247.

Heldwein, E. E., H. Lou, et al. (2006). "Crystal structure of glycoprotein B from herpes simplex virus 1." Science **313**(5784): 217-220.

Homa, F. L. and J. C. Brown (1997). "Capsid assembly and DNA packaging in herpes simplex virus." Reviews in Medical Virology **7**(2): 107-122.

Homa, F. L. and J. C. Brown (1997). "Capsid assembly and DNA packaging in herpes simplex virus." Rev Med Virol **7**(2): 107-122.

Hutchinson, L., K. Goldsmith, et al. (1992). "Identification and characterization of a novel herpes simplex virus glycoprotein, gK, involved in cell fusion." J Virol **66**(9): 5603-5609.

Igarashi, K., R. Fawl, et al. (1993). "Construction and properties of a recombinant herpes simplex virus 1 lacking both S-component origins of DNA synthesis." J Virol **67**(4): 2123-2132.

Jackson, J. O., E. Lin, et al. (2010). "Insertion mutations in herpes simplex virus 1 glycoprotein H reduce cell surface expression, slow the rate of cell fusion, or abrogate functions in cell fusion and viral entry." J Virol **84**(4): 2038-2046.

Jackson, J. O. and R. Longnecker (2010). "Reevaluating herpes simplex virus hemifusion." J Virol **84**(22): 11814-11821.

Jacob, R. J., L. S. Morse, et al. (1979). "Anatomy of herpes simplex virus DNA. XII. Accumulation of head-to-tail concatemers in nuclei of infected cells and their role in the generation of the four isomeric arrangements of viral DNA." J Virol **29**(2): 448-457.

Jacobson, J. G., S. H. Chen, et al. (1998). "Importance of the herpes simplex virus UL24 gene for productive ganglionic infection in mice." Virology **242**(1): 161-169.

Jaiyeoba, O., M. I. Amaya, et al. (2012). "Preventing neonatal transmission of herpes simplex virus." Clin Obstet Gynecol **55**(2): 510-520.

Jambunathan, N., S. Chowdhury, et al. (2011). "Site-specific proteolytic cleavage of the amino terminus of herpes simplex virus glycoprotein K on virion particles inhibits virus entry." J Virol **85**(24): 12910-12918.

Jayachandra, S., A. Baghian, et al. (1997). "Herpes simplex virus type 1 glycoprotein K is not essential for infectious virus production in actively replicating cells but is required for efficient envelopment and translocation of infectious virions from the cytoplasm to the extracellular space." J Virol **71**(7): 5012-5024.

Jerome, K. R., Z. Chen, et al. (2001). "HSV and glycoprotein J inhibit caspase activation and apoptosis induced by granzyme B or Fas." J Immunol **167**(7): 3928-3935.

Johnson, D. C. and J. D. Baines (2011). "Herpesviruses remodel host membranes for virus egress." Nat Rev Microbiol **9**(5): 382-394.

Johnson, D. C. and P. G. Spear (1982). "Monensin inhibits the processing of herpes simplex virus glycoproteins, their transport to the cell surface, and the egress of virions from infected cells." J Virol **43**(3): 1102-1112.

Jovasevic, V., L. Liang, et al. (2008). "Proteolytic cleavage of VP1-2 is required for release of herpes simplex virus 1 DNA into the nucleus." J Virol **82**(7): 3311-3319.

Kaplan, A. S. (1973). The herpesviruses. New York,, Academic Press.

Karasneh, G. A. and D. Shukla (2011). "Herpes simplex virus infects most cell types in vitro: clues to its success." Virol J **8**: 481.

Karr, B. M. and G. S. Read (1999). "The virion host shutoff function of herpes simplex virus degrades the 5' end of a target mRNA before the 3' end." Virology **264**(1): 195-204.

Katan, M., A. Haigh, et al. (1990). "Characterization of a cellular factor which interacts functionally with Oct-1 in the assembly of a multicomponent transcription complex." Nucleic Acids Res **18**(23): 6871-6880.

Kaufman, R. H., H. L. Gardner, et al. (1973). "Clinical features of herpes genitalis." Cancer Res **33**(6): 1446-1451.

Klupp, B. G., J. Baumeister, et al. (1998). "Pseudorabies virus glycoprotein gK is a virion structural component involved in virus release but is not required for entry." J Virol **72**(3): 1949-1958.

Klupp, B. G., H. Granzow, et al. (2000). "Primary envelopment of pseudorabies virus at the nuclear membrane requires the UL34 gene product." J Virol **74**(21): 10063-10073.

Kogure, A., I. Shiratori, et al. (2011). "PANP is a novel O-glycosylated PILRalpha ligand expressed in neural tissues." Biochem Biophys Res Commun **405**(3): 428-433.

Korsager, B., E. S. Spencer, et al. (1975). "Herpesvirus hominis infections in renal transplant recipients." Scand J Infect Dis **7**(1): 11-19.

Krishnan, H. H., N. Sharma-Walia, et al. (2005). "Envelope glycoprotein gB of Kaposi's sarcoma-associated herpesvirus is essential for egress from infected cells." J Virol **79**(17): 10952-10967.

Kristie, T. M. and B. Roizman (1986). "DNA-binding site of major regulatory protein alpha 4 specifically associated with promoter-regulatory domains of alpha genes of herpes simplex virus type 1." Proc Natl Acad Sci U S A **83**(13): 4700-4704.

Kristie, T. M. and P. A. Sharp (1990). "Interactions of the Oct-1 POU subdomains with specific DNA sequences and with the HSV alpha-trans-activator protein." Genes Dev **4**(12B): 2383-2396.

Krummenacher, C., A. H. Rux, et al. (1999). "The first immunoglobulin-like domain of HveC is sufficient to bind herpes simplex virus gD with full affinity, while the third domain is involved in oligomerization of HveC." J Virol **73**(10): 8127-8137.

Kwong, A. D. and N. Frenkel (1987). "Herpes simplex virus-infected cells contain a function(s) that destabilizes both host and viral mRNAs." Proc Natl Acad Sci U S A **84**(7): 1926-1930.

La Boissiere, S., T. Hughes, et al. (1999). "HCF-dependent nuclear import of VP16." EMBO J **18**(2): 480-489.

Lam, Q., C. A. Smibert, et al. (1996). "Herpes simplex virus VP16 rescues viral mRNA from destruction by the virion host shutoff function." EMBO J **15**(10): 2575-2581.

Langeland, N., H. Holmsen, et al. (1987). "Evidence that neomycin inhibits binding of herpes simplex virus type 1 to the cellular receptor." J Virol **61**(11): 3388-3393.

Lee, H. C. (2008). COMPARATIVE ROLES OF HERPES SIMPLEX VIRUS TYPE 1 (HSV-1) VIRAL GLYCOPROTEINS IN CYTOPLASMIC VIRION EGRESS. M.S., Louisiana State University and Agricultural and Mechanical College.

Lee, H. C., V. N. Chouljenko, et al. (2009). "The herpes simplex virus type 1 glycoprotein D (gD) cytoplasmic terminus and full-length gE are not essential and do not function in a redundant manner for cytoplasmic virion envelopment and egress." J Virol **83**(12): 6115-6124.

Lee, H. C., V. N. Chouljenko, et al. (2009). "The herpes simplex virus type 1 glycoprotein D (gD) cytoplasmic terminus and full-length gE are not essential and do not function in a redundant manner for cytoplasmic virion envelopment and egress." J Virol **83**(12): 6115-6124.

Lester, J. T. and N. A. DeLuca (2011). "Herpes simplex virus 1 ICP4 forms complexes with TFIID and mediator in virus-infected cells." J Virol **85**(12): 5733-5744.

Liesegang, T. J. (2001). "Herpes simplex virus epidemiology and ocular importance." Cornea **20**(1): 1-13.

Lockshon, D. and D. A. Galloway (1986). "Cloning and characterization of oriL2, a large palindromic DNA replication origin of herpes simplex virus type 2." J Virol **58**(2): 513-521.

Lockshon, D. and D. A. Galloway (1988). "Sequence and structural requirements of a herpes simplex viral DNA replication origin." Mol Cell Biol **8**(10): 4018-4027.

Long, D., W. C. Wilcox, et al. (1992). "Disulfide bond structure of glycoprotein D of herpes simplex virus types 1 and 2." J Virol **66**(11): 6668-6685.

Loret, S., G. Guay, et al. (2008). "Comprehensive characterization of extracellular herpes simplex virus type 1 virions." J Virol **82**(17): 8605-8618.

Loret, S. and R. Lippe (2012). "Biochemical analysis of infected cell polypeptide (ICP)0, ICP4, UL7 and UL23 incorporated into extracellular herpes simplex virus type 1 virions." J Gen Virol **93**(Pt 3): 624-634.

MacLean, C. A., S. Efstathiou, et al. (1991). "Investigation of herpes simplex virus type 1 genes encoding multiply inserted membrane proteins." J Gen Virol **72 (Pt 4)**: 897-906.

MacLean, C. A., L. M. Robertson, et al. (1993). "Characterization of the UL10 gene product of herpes simplex virus type 1 and investigation of its role in vivo." J Gen Virol **74 (Pt 6)**: 975-983.

Markovitz, N. S., F. Filatov, et al. (1999). "The U(L)3 protein of herpes simplex virus 1 is translated predominantly from the second in-frame methionine codon and is subject to at least two posttranslational modifications." J Virol **73**(10): 8010-8018.

Marsters, S. A., T. M. Ayres, et al. (1997). "Herpesvirus entry mediator, a member of the tumor necrosis factor receptor (TNFR) family, interacts with members of the TNFR-associated factor family and activates the transcription factors NF-kappaB and AP-1." J Biol Chem **272**(22): 14029-14032.

Martin, C., L. Solis, et al. (2011). "[Herpes simplex virus type 1 as risk factor associated to Alzheimer disease]." Rev Med Chil **139**(6): 779-786.

Martinez, W. M. and P. G. Spear (2002). "Amino acid substitutions in the V domain of nectin-1 (HveC) that impair entry activity for herpes simplex virus types 1 and 2 but not for Pseudorabies virus or bovine herpesvirus 1." J Virol **76**(14): 7255-7262.

Matis, J. and M. Kudelova (2001). "Early shutoff of host protein synthesis in cells infected with herpes simplex viruses." Acta Virol **45**(5-6): 269-277.

Maul, G. G., A. M. Ishov, et al. (1996). "Nuclear domain 10 as preexisting potential replication start sites of herpes simplex virus type-1." Virology **217**(1): 67-75.

Mauri, D. N., R. Ebner, et al. (1998). "LIGHT, a new member of the TNF superfamily, and lymphotoxin alpha are ligands for herpesvirus entry mediator." Immunity **8**(1): 21-30.

McGeoch, D. J., M. A. Dalrymple, et al. (1988). "The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1." J Gen Virol **69 (Pt 7)**: 1531-1574.

McGeoch, D. J., A. Dolan, et al. (1985). "Sequence determination and genetic content of the short unique region in the genome of herpes simplex virus type 1." J Mol Biol **181**(1): 1-13.

Melancon, J. M. (2004). GENETICS AND FUNCTIONS OF HERPES SIMPLEX VIRUS TYPE 1 MEMBRANE PROTEINS IN VIRUS-INDUCED CELL FUSION, VIRION MORPHOGENESIS AND EGRESS. Ph.D., Louisiana State University and Agricultural and Mechanical College.

Melancon, J. M., T. P. Foster, et al. (2004). "Genetic analysis of the herpes simplex virus type 1 UL20 protein domains involved in cytoplasmic virion envelopment and virus-induced cell fusion." J Virol **78**(14): 7329-7343.

Melancon, J. M., P. A. Fulmer, et al. (2007). "The herpes simplex virus UL20 protein functions in glycoprotein K (gK) intracellular transport and virus-induced cell fusion are independent of UL20 functions in cytoplasmic virion envelopment." Virology Journal **4**(1): 120.

Melancon, J. M., R. E. Luna, et al. (2005). "Herpes simplex virus type 1 gK is required for gB-mediated virus-induced cell fusion, while neither gB and gK nor gB and UL20p function redundantly in virion de-envelopment." J Virol **79**(1): 299-313.

Mercer, J. and A. Helenius (2009). "Virus entry by macropinocytosis." Nat Cell Biol **11**(5): 510-520.

Mettenleiter, T. C. (2002). "Herpesvirus assembly and egress." J Virol **76**(4): 1537-1547.

Mettenleiter, T. C., B. G. Klupp, et al. (2006b). "Herpesvirus assembly: a tale of two membranes." Curr Opin Microbiol **9**(4): 423-429.

Milne, R. S., S. L. Hanna, et al. (2003). "Function of herpes simplex virus type 1 gD mutants with different receptor-binding affinities in virus entry and fusion." J Virol **77**(16): 8962-8972.

Milne, R. S., A. V. Nicola, et al. (2005). "Glycoprotein D receptor-dependent, low-pH-independent endocytic entry of herpes simplex virus type 1." J Virol **79**(11): 6655-6663.

Minson, A. C., T. C. Hodgman, et al. (1986). "An analysis of the biological properties of monoclonal antibodies against glycoprotein D of herpes simplex virus and identification of amino acid substitutions that confer resistance to neutralization." J Gen Virol **67 (Pt 6)**: 1001-1013.

Mo, C. and T. C. Holland (1997). "Determination of the transmembrane topology of herpes simplex virus type 1 glycoprotein K." J Biol Chem **272**(52): 33305-33311.

Mo, C., J. Suen, et al. (1999). "Characterization of Varicella-Zoster virus glycoprotein K (open reading frame 5) and its role in virus growth." J Virol **73**(5): 4197-4207.

Mocarski, E. S. and B. Roizman (1982). "Structure and role of the herpes simplex virus DNA termini in inversion, circularization and generation of virion DNA." Cell **31**(1): 89-97.

Montgomerie, J. Z., D. M. Becroft, et al. (1969). "Herpes-simplex-virus infection after renal transplantation." Lancet **2**(7626): 867-871.

Montgomery, R. I., M. S. Warner, et al. (1996). "Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family." Cell **87**(3): 427-436.

Morgan, C., H. M. Rose, et al. (1959). "Electron microscopic observations on the development of herpes simplex virus." J Exp Med **110**: 643-656.

Morgan, C., H. M. Rose, et al. (1968). "Electron microscopy of herpes simplex virus. I. Entry." J Virol **2**(5): 507-516.

Mori, I. (2010). "Herpes simplex virus type 1 persists in the aged brain through hypothetical expression of accessory genes." J Neurovirol **16**(3): 203-207.

Mossman, K. L., R. Sherburne, et al. (2000). "Evidence that herpes simplex virus VP16 is required for viral egress downstream of the initial envelopment event." J Virol **74**(14): 6287-6299.

Muller, M. T. (1987). "Binding of the herpes simplex virus immediate-early gene product ICP4 to its own transcription start site." J Virol **61**(3): 858-865.

Nahmias, A. J. and S. Kibrick (1964). "Inhibitory effect of heparin on herpes simplex virus." J Bacteriol **87**(5): 1060-1066.

Nahmias, A. J., F. K. Lee, et al. (1990). "Sero-epidemiological and -sociological patterns of herpes simplex virus infection in the world." Scand J Infect Dis Suppl **69**: 19-36.

Nazerian, K. (1974). "DNA configuration in the core of Marek's disease virus." J Virol **13**(5): 1148-1150.

Newcomb, W. W., B. L. Trus, et al. (1993). "Structure of the herpes simplex virus capsid. Molecular composition of the pentons and the triplexes." J Mol Biol **232**(2): 499-511.

Nicola, A. V., A. M. McEvoy, et al. (2003). "Roles for endocytosis and low pH in herpes simplex virus entry into HeLa and Chinese hamster ovary cells." J Virol **77**(9): 5324-5332.

Nicola, A. V., M. Ponce de Leon, et al. (1998). "Monoclonal antibodies to distinct sites on herpes simplex virus (HSV) glycoprotein D block HSV binding to HVEM." J Virol **72**(5): 3595-3601.

Nicola, A. V. and S. E. Straus (2004). "Cellular and viral requirements for rapid endocytic entry of herpes simplex virus." J Virol **78**(14): 7508-7517.

Nishioka, Y. and S. Silverstein (1977). "Degradation of cellular mRNA during infection by herpes simplex virus." Proc Natl Acad Sci U S A **74**(6): 2370-2374.

Nishioka, Y. and S. Silverstein (1978). "Alterations in the protein synthetic apparatus of Friend erythroleukemia cells infected with vesicular stomatitis virus or herpes simplex virus." J Virol **25**(1): 422-426.

Nishioka, Y. and S. Silverstein (1978). "Requirement of protein synthesis for the degradation of host mRNA in Friend erythroleukemia cells infected wtih herpes simplex virus type 1." J Virol **27**(3): 619-627.

Norberg, P., S. Olofsson, et al. (2007). "Glycoprotein I of herpes simplex virus type 1 contains a unique polymorphic tandem-repeated mucin region." J Gen Virol **88**(Pt 6): 1683-1688.

Olson, L. C., E. L. Buescher, et al. (1967). "Herpesvirus infections of the human central nervous system." N Engl J Med **277**(24): 1271-1277.

Parry, C., S. Bell, et al. (2005). "Herpes simplex virus type 1 glycoprotein H binds to alphavbeta3 integrins." J Gen Virol **86**(Pt 1): 7-10.

Pogue-Geile, K. L., G. T. Lee, et al. (1984). "Fine mapping of mutations in the fusion-inducing MP strain of herpes simplex virus type 1." Virology **136**(1): 100-109.

Prasad, K. M., B. H. Shirts, et al. (2007). "Brain morphological changes associated with exposure to HSV1 in first-episode schizophrenia." Mol Psychiatry **12**(1): 105-113, 101.

Purifoy, D. J., R. B. Lewis, et al. (1977). "Identification of the herpes simplex virus DNA polymerase gene." Nature **269**(5629): 621-623.

Rahn, E., P. Petermann, et al. (2011). "Entry Pathways of Herpes Simplex Virus Type 1 into Human Keratinocytes Are Dynamin- and Cholesterol-Dependent." PLoS One **6**(10): e25464.

Read, G. S. and N. Frenkel (1983). "Herpes simplex virus mutants defective in the virion-associated shutoff of host polypeptide synthesis and exhibiting abnormal synthesis of alpha (immediate early) viral polypeptides." J Virol **46**(2): 498-512.

Reske, A., G. Pollara, et al. (2007). "Understanding HSV-1 entry glycoproteins." Reviews in Medical Virology **17**(3): 205-215.

Reynolds, A. E., L. Liang, et al. (2004). "Conformational changes in the nuclear lamina induced by herpes simplex virus type 1 require genes U(L)31 and U(L)34." J Virol **78**(11): 5564-5575.

Reynolds, A. E., B. J. Ryckman, et al. (2001). "U(L)31 and U(L)34 proteins of herpes simplex virus type 1 form a complex that accumulates at the nuclear rim and is required for envelopment of nucleocapsids." J Virol **75**(18): 8803-8817.

Reynolds, A. E., E. G. Wills, et al. (2002). "Ultrastructural localization of the herpes simplex virus type 1 UL31, UL34, and US3 proteins suggests specific roles in primary envelopment and egress of nucleocapsids." J Virol **76**(17): 8939-8952.

Roizman, B., L. E. Carmichael, et al. (1981). "Herpesviridae. Definition, provisional nomenclature, and taxonomy. The Herpesvirus Study Group, the International Committee on Taxonomy of Viruses." Intervirology **16**(4): 201-217.

Roizman, B. and D. Furlong (1974). The replication of herpesviruses. Comprehensive Virology. H. F.-C. R. R. Wagner. New York, Plenum Press.

Roizman, B. and D. M. Knipe (2001). Herpes Simplex Viruses and Their Replication. Fields Virology. D. M. Knipe and P. M. Howley. Philadelphia, PA, Lippincott-Williams & Wilkins. **2:** 2399-2459.

Roizman, B. and P. E. Pellett (2001). The Family Herpesviridae. Fields Virology. a. P. M. H. D. M. Knipe. Philadelphia, PA., Lippincott-Williams & Wilkins. **2:** 2381-2397.

Roizman, B., S. B. Spring, et al. (1967). "Cellular compartmentalization of herpesvirus antigens during viral replication." J Virol **1**(1): 181-192.

Roizman, e. a. (1973). "Editorial: Provisional labels for herpesviruses." J Gen Virol **20**(3): 417-419.

Roizmann, B., R. C. Desrosiers, et al. (1992). "The family Herpesviridae: an update. The Herpesvirus Study Group of the International Committee on Taxonomy of Viruses." Arch Virol **123**(3-4): 425-449.

Roller, R. J., Y. Zhou, et al. (2000). "Herpes simplex virus type 1 U(L)34 gene product is required for viral envelopment." J Virol **74**(1): 117-129.

Saad, A., Z. H. Zhou, et al. (1999). "Roles of triplex and scaffolding proteins in herpes simplex virus type 1 capsid formation suggested by structures of recombinant particles." J Virol **73**(8): 6821-6830.

Salahuddin, S. Z., D. V. Ablashi, et al. (1986). "Isolation of a new virus, HBLV, in patients with lymphoproliferative disorders." Science **234**(4776): 596-601.

Sandri-Goldin, R. M. (2006). Alpha herpesviruses : molecular and cellular biology. Wymondham, Caister Academic.

Satoh, T., J. Arii, et al. (2008). "PILRalpha is a herpes simplex virus-1 entry coreceptor that associates with glycoprotein B." Cell **132**(6): 935-944.

Schmelter, J., J. Knez, et al. (1996). "Identification and characterization of a small modular domain in the herpes simplex virus host shutoff protein sufficient for interaction with VP16." J Virol **70**(4): 2124-2131.

Scott, E. S. and P. O'Hare (2001). "Fate of the inner nuclear membrane protein lamin B receptor and nuclear lamins in herpes simplex virus type 1 infection." J Virol **75**(18): 8818-8830.

Sedy, J. R., P. G. Spear, et al. (2008). "Cross-regulation between herpesviruses and the TNF superfamily members." Nat Rev Immunol **8**(11): 861-873.

Segal, A. L., A. H. Katcher, et al. (1974). "Recurrent herpes labialis, recurrent aphthous ulcers, and the menstrual cycle." J Dent Res **53**(4): 797-803.

Shieh, M. T., D. WuDunn, et al. (1992). "Cell surface receptors for herpes simplex virus are heparan sulfate proteoglycans." J Cell Biol **116**(5): 1273-1281.

Ship, II, M. F. Miller, et al. (1977). "A retrospective study of recurrent herpes labialis (RHL) in a professional population, 1958-1971." Oral Surg Oral Med Oral Pathol **44**(5): 723-730.

Silverman, J. L., S. Sharma, et al. (2010). "Fusion-deficient insertion mutants of herpes simplex virus type 1 glycoprotein B adopt the trimeric postfusion conformation." J Virol **84**(4): 2001-2012.

Sinclair, M. C., J. McLauchlan, et al. (1994). "Characterization of a herpes simplex virus type 1 deletion variant (1703) which under-produces Vmw63 during immediate early conditions of infection." J Gen Virol **75 (Pt 5)**: 1083-1089.

Smith, M. G. (1956). "Propagation in tissue cultures of a cytopathogenic virus from human salivary gland virus (SGV) disease." Proc Soc Exp Biol Med **92**(2): 424-430.

Sodeik, B., M. W. Ebersold, et al. (1997). "Microtubule-mediated transport of incoming herpes simplex virus 1 capsids to the nucleus." J Cell Biol **136**(5): 1007-1021.

Spear, P. G., R. J. Eisenberg, et al. (2000). "Three classes of cell surface receptors for alphaherpesvirus entry." Virology **275**(1): 1-8.

Spruance, S. L. and C. S. Crumpacker (1982). "Topical 5 percent acyclovir in polyethylene glycol for herpes simplex labialis. Antiviral effect without clinical benefit." Am J Med **73**(1A): 315-319.

Spruance, S. L., C. S. Crumpacker, et al. (1984). "Early, patient-initiated treatment of herpes labialis with topical 10% acyclovir." Antimicrob Agents Chemother **25**(5): 553-555.

Spruance, S. L., J. C. Overall, Jr., et al. (1977). "The natural history of recurrent herpes simplex labialis: implications for antiviral therapy." N Engl J Med **297**(2): 69-75.

Suenaga, T., T. Satoh, et al. (2010). "Myelin-associated glycoprotein mediates membrane fusion and entry of neurotropic herpesviruses." Proc Natl Acad Sc USA **107**(2): 866-871.

Sun, Y., K. Senger, et al. (2012). "Evolutionarily Conserved Paired Immunoglobulin-like Receptor alpha (PILRalpha) Domain Mediates Its Interaction with Diverse Sialylated Ligands." J Biol Chem **287**(19): 15837-15850.

Takai, Y., W. Ikeda, et al. (2008). "The immunoglobulin-like cell adhesion molecule nectin and its associated protein afadin." Annu Rev Cell Dev Biol **24**: 309-342.

Takai, Y., K. Irie, et al. (2003). "Nectins and nectin-like molecules: roles in cell adhesion, migration, and polarization." Cancer Sci **94**(8): 655-667.

Tal-Singer, R., C. Peng, et al. (1995). "Interaction of herpes simplex virus glycoprotein gC with mammalian cell surface molecules." J Virol **69**(7): 4471-4483.

Thomsen, D. R., W. W. Newcomb, et al. (1995). "Assembly of the herpes simplex virus capsid: requirement for the carboxyl-terminal twenty-five amino acids of the proteins encoded by the UL26 and UL26.5 genes." J Virol **69**(6): 3690-3703.

Tiwari, V., C. Clement, et al. (2006). "Role for 3-O-sulfated heparan sulfate as the receptor for herpes simplex virus type 1 entry into primary human corneal fibroblasts." J Virol **80**(18): 8970-8980.

Trus, B. L., F. P. Booy, et al. (1996). "The herpes simplex virus procapsid: structure, conformational changes upon maturation, and roles of the triplex proteins VP19c and VP23 in assembly." J Mol Biol **263**(3): 447-462.

Trus, B. L., F. L. Homa, et al. (1995). "Herpes simplex virus capsids assembled in insect cells infected with recombinant baculoviruses: structural authenticity and localization of VP26." J Virol **69**(11): 7362-7366.

Trybala, E., T. Bergstrom, et al. (1994). "Localization of a functional site on herpes simplex virus type 1 glycoprotein C involved in binding to cell surface heparan sulphate." J Gen Virol **75 (Pt 4)**: 743-752.

Trybala, E., A. Roth, et al. (2002). "Glycosaminoglycan-binding ability is a feature of wild-type strains of herpes simplex virus type 1." Virology **302**(2): 413-419.

Turner, A., B. Bruun, et al. (1998). "Glycoproteins gB, gD, and gHgL of herpes simplex virus type 1 are necessary and sufficient to mediate membrane fusion in a Cos cell transfection system." J Virol **72**(1): 873-875.

van Genderen, I. L., R. Brandimarti, et al. (1994). "The phospholipid composition of extracellular herpes simplex virions differs from that of host cell nuclei." Virology **200**(2): 831-836.

Vicente-Manzanares, M., X. Ma, et al. (2009). "Non-muscle myosin II takes centre stage in cell adhesion and migration." Nat Rev Mol Cell Biol **10**(11): 778-790.

Vidal, J. B. (1873). *Ann Dermatol Syphiligr*.

Walker, J. D., I. Sehgal, et al. (2011). "Oncolytic herpes simplex virus 1 encoding 15-prostaglandin dehydrogenase mitigates immune suppression and reduces ectopic primary and metastatic breast cancer in mice." J Virol **85**(14): 7363-7371.

Wang, J., Q. Fan, et al. (2009). "Binding of herpes simplex virus glycoprotein B (gB) to paired immunoglobulin-like type 2 receptor alpha depends on specific sialylated O-linked glycans on gB." J Virol **83**(24): 13042-13045.

Warner, M. S., R. J. Geraghty, et al. (1998). "A cell surface protein with herpesvirus entry activity (HveB) confers susceptibility to infection by mutants of herpes simplex virus type 1, herpes simplex virus type 2, and pseudorabies virus." Virology **246**(1): 179-189.

Weir, J. P. (2001). "Regulation of herpes simplex virus gene expression." Gene **271**(2): 117-130.

Weller, S. K. (2011). Alphaherpesviruses : molecular virology. Norfolk, UK, Caister Academic Press.

Weller, S. K., A. Spadaro, et al. (1985). "Cloning, sequencing, and functional analysis of oriL, a herpes simplex virus type 1 origin of DNA synthesis." Mol Cell Biol **5**(5): 930-942.

Weller, T. H. and M. B. Stoddard (1952). "Intranuclear inclusion bodies in cultures of human tissue inoculated with varicella vesicle fluid." J Immunol **68**(3): 311-319.

Westra, D. F., G. M. Verjans, et al. (2000). "Natural infection with herpes simplex virus type 1 (HSV-1) induces humoral and T cell responses to the HSV-1 glycoprotein H:L complex." J Gen Virol **81**(Pt 8): 2011-2015.

Whitley, R. J. (2001). Herpes simplex Virus. Fields Virology. a. P. M. H. D.M. Knipe. Philadelphia, PA, Lippincott-Williams & Wilkins. **2:** 2461-2509.

Whitley, R. J. and B. Roizman (2001). "Herpes simplex virus infections." Lancet **357**(9267): 1513-1518.

Whitley, R. J., S. J. Soong, et al. (1977). "Adenine arabinoside therapy of biopsy-proved herpes simplex encephalitis. National Institute of Allergy and Infectious Diseases collaborative antiviral study." N Engl J Med **297**(6): 289-294.

Whitley, R. J., S. J. Soong, et al. (1981). "Herpes simplex encephalitis: vidarabine therapy and diagnostic problems." N Engl J Med **304**(6): 313-318.

Wildy, P. and D. H. Watson (1962). "Electron microscopic studies on the architecture of animal viruses." Cold Spring Harb Symp Quant Biol **27**: 25-47.

Wisner, T., C. Brunetti, et al. (2000). "The extracellular domain of herpes simplex virus gE is sufficient for accumulation at cell junctions but not for cell-to-cell spread." J Virol **74**(5): 2278-2287.

Wu, C. A., N. J. Nelson, et al. (1988). "Identification of herpes simplex virus type 1 genes required for origin-dependent DNA synthesis." J Virol **62**(2): 435-443.

WuDunn, D. and P. G. Spear (1989). "Initial interaction of herpes simplex virus with cells is binding to heparan sulfate." J Virol **63**(1): 52-58.

Yoon, M., A. Zago, et al. (2003). "Mutations in the N termini of herpes simplex virus type 1 and 2 gDs alter functional interactions with the entry/fusion receptors HVEM, nectin-2, and 3-O-sulfated heparan sulfate but not with nectin-1." J Virol **77**(17): 9221-9231.

Zhou, Z. H., D. H. Chen, et al. (1999). "Visualization of tegument-capsid interactions and DNA in intact herpes simplex virus type 1 virions." J Virol **73**(4): 3210-3218.

Zhou, Z. H., B. V. Prasad, et al. (1994). "Protein subunit structures in the herpes simplex virus A-capsid determined from 400 kV spot-scan electron cryomicroscopy." J Mol Biol **242**(4): 456-469.

Zhu, Q. and R. J. Courtney (1994). "Chemical cross-linking of virion envelope and tegument proteins of herpes simplex virus type 1." Virology **204**(2): 590-599.

CHAPTER II

THE AMINO TERMINAL PORTIONS OF HSV-1 gK AND UL20p INTERACT WITH gB AND gH AND REGULATE gB-MEDIATED MEMBRANE FUSION

INTRODUCTION

HSV-1 induced membrane fusion is a complex phenomenon which is regulated by multiple control mechanisms involving a number of viral proteins. Wild type virus infection leads to rounding and aggregation of cells but limited amount of virus induced cell-to-cell fusion. However, certain point mutations in at least four viral genes lead to extensive virus-induced cell–to-cell fusion (*in vitro*); resulting in the formation of large multinucleated cells or syncytia. These syncytial mutations have been mapped to UL20 gene (Baines, Ward et al. 1991; Foster, Melancon et al. 2004), UL24 gene (Jacobson, Chen et al. 1998), UL27 gene encoding for glycoprotein B (gB) (Bzik, Fox et al. 1984) and UL53 gene encoding for glycoprotein K (gK) (Bond and Person 1984; Pogue-Geile, Lee et al. 1984; Debroy, Pederson et al. 1985; Pogue-Geile and Spear 1987). Syncytial mutations in gK have been shown to cause fusion of a variety of cell types, unlike mutations in gB and UL24 that cause extensive fusion only a limited number of cell types in cell culture. There is accumulating evidence that membrane fusion is mediated by the concerted actions and interactions among glycoproteins gD, gB and the gH/gL heterodimer (Atanasiu, Whitbeck et al. 2007). According to the most recent fusion model for virus-cell and cell-to-cell fusion mechanism; gD, gH/gL and gB act in an orchestrated fashion to bring about fusion. gD is first activated by binding to its cellular receptor and then undergoes conformational changes that in turn activates gH/gL. Activated gH/gL then interacts with gB; up-regulating its

fusogenic capability (Atanasiu, Whitbeck et al. 2010). Most likely, the gH/gL heterodimer triggers conversion of the pre-fusion form of gB into a post–fusion conformation which then leads to fusion of the viral envelope with cellular membranes during virus entry or fusion of cellular membranes during cell-to-cell fusion (Chowdary, Cairns et al. 2010).

It is known that surface expression of two additional viral proteins, glycoprotein K (gK) and the membrane protein UL20 (UL20p) are needed to bring about virus–induced cell fusion (Foster, Melancon et al. 2004; Melancon, Luna et al. 2005). UL20p and gK are multipass transmembrane proteins that form a heterodimeric complex and are coordinately transported to the trans-Golgi Network (TGN) and cell surfaces. These two viral proteins physically interact and play important roles in cell-to-cell fusion, cytoplasmic virion envelopment and egress.

The role of UL20 and gK in virus-induced cell fusion is not well documented. Although gB is known to be a fusogenic glycoprotein, the mechanism by which gK is involved in virus-induced cell fusion remains elusive. We have recently published that the amino terminus of gK is involved in gB mediated cell fusion (Chouljenko, Iyer et al. 2009). A mutant virus gBΔ28, lacking the carboxyl-terminal 28 amino acids of gB loses it extensive syncytial phenotype in presence of a truncation in the amino terminus of gK. My contribution to this paper was to understand the functional role of the amino terminus of gK in cell–to-cell fusion and to further decipher interactions between the amino terminus of gK and other viral proteins that are part of the fusion complex i.e. gB, gH/gL, gD and UL20p. Thus, the main hypothesis of this work is that gK and UL20p physically interact through cytoplasmic and extracellular domains with glycoproteins gB, gH or gL to modulate gB mediated membrane fusion.

MATERIAL AND METHODS

Cells

African green monkey kidney (Vero) cells and human embryonic kidney (HEK293T) cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal calf serum and antibiotics. *Spodoptera frugiperda* (Sf9; Invitrogen, Carlsbad, CA) insect cells were maintained in monolayer and/or suspension cultures at 27°C using serum-free Sf-900 II culture medium (SFM) with antibiotics (Pen Strep at a 1:200 dilution; Gibco).

Plasmids

A gene cassette encoding the gKa peptide was constructed to include a 3× FLAG epitope inserted in frame after gK aa 82 and cloned into transient expression vector p3XFLAG-CMV14 (Invitrogen, Inc.). DNA sequences encoding the ORF of gB and gD were cloned into pcDNA 3.1/V5/His (Invitrogen) expression vector in frame with theV5 epitope at the carboxyl end.

Recombinant Virus Construction

The mutant viruses gKΔ31-68, gBΔ28syn (deletion of 28 aa from the carboxyl terminus of gB), and gKΔ31-68/gBΔ28syn were created as described earlier (Chouljenko, Iyer et al. 2009), using a markerless two-step red recombination mutagenesis system implemented on the bacterial artificial chromosome plasmid pYEbac102 carrying the HSV-1(F) genome (a kind gift from Y. Kawaguchi, Japan) (Tischer, von Einem et al. 2006). All ΔgK recombinant viruses specified gK carrying in-frame deletions spanning the amino terminus of gK immediately after the gK signal sequence (aa 1 to 30). The recombinant virus gBΔ28syn was constructed by deleting the carboxyl-terminal 28 aa of gB and inserting one stop codon at the site of the

deletion. This viral genome was utilized for construction of the double mutant virus gKΔ31-68/gBΔ28syn.

Plaque Morphology

Confluent monolayers of Vero were infected with an MOI of 0.01 with the indicated viruses. 48 hpi the cells were fixed and immunohistochemistry was performed with primary rabbit anti-HSV antibodies (1:1000) (Dako, Carpinteria, CA) and the reactions were developed with NovaRed substrate (VectorLabs; Burlingame, CA). Images were taken with an inverted light microscope (Olympus) using relief contrast.

Complementation Assay and Immunohistochemistry

Nearly confluent Vero cell monolayers were transfected with plasmids expressing gKa. Twenty-four hours posttransfection, cells were infected at an MOI of 0.2 with gKΔ31-68/gBΔ28syn. Thirty hpi, monolayers were washed with Tris-buffered saline (TBS)-Ca-Mg and were fixed with 100% methanol for 10 min or left unfixed (live). Immunohistochemistry was performed by utilizing Vector Laboratories Vectastain Elite ABC kit as described in the manufacturer's manual. Cell fusion was visualized by immunostaining using either anti-FLAG or anti-HSV-1 antibody.

Transfection, Western Immunoblot and Immunoprecipitation

Subconfluent Vero cells were transfected with gKa plasmid using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's directions. Thirty hours posttransfection, cells were processed for Western blot analysis. Samples were further processed for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were subsequently probed with anti-FLAG antibody.

Immunopreipitation: 293 cell monolayers were co-transfected with plasmid expressing the extracellular portion of either gB or gD, along with gKa or transfected with gKa alone. Cellular extracts were prepared at 36 hours posttransfection for immunoprecipitation. In these set of reactions Anti-FLAG M2 Affinity Gel beads were used for immunoprecipitation, as per manufacturer's directions. The anti-FLAG immunoprecipitates were eluted and loaded on sodium dodecyl sulfate-polyacrylamide gel followed by immunoblot analysis. The membranes were probed with. either primary monoclonal anti-V5 antibody (1:2000, Invitrogen) or monoclonal anti-FLAG antibody (1:4000, Sigma). Goat anti-mouse secondary antibody conjugated with HRP and ECL (GE) substrate were used for detection purposes.

Construction of Recombinant Baculoviruses and Immunoprecipitation

Recombinant baculoviruses were generated using the Bac-to-Bac baculovirus expression system (Invitrogen, Carlsbad, CA). To create all the viral constructs pFastbac Dual vector was used that contains two multiple cloning sites to allow expression of two heterologous genes; one controlled by the polyhedrin (P_H) promoter and the other by the p10 promoter. Fail Safe DNA polymerase (Epicentre) was used for all DNA amplifications. The first 82 amino acids of gK was cloned in frame with the 3×FLAG epitope using forward primer gK-Flag-EcoRI-F and reverse primer gK-Flag-9His-HindIII-R. This PCR product was then cloned into the pFast Dual vector (Invitrogen, Inc.) at restriction sites, EcoRI and HindIII under the control of the P_{PH} promoter. This gKa gene cassette resulted in the transfer vector pBD-gKa. DNA sequences encoding truncated ectodomains of gH (amino acids 1-792), gH (amino acids 1-603), and gH (amino acids 1-343) were first cloned into pcDNA 3.1/V5/His (Invitrogen) expression vector in frame with theV5 epitope. Expression of each protein was confirmed by Western immunoblotting of transiently transfected 293 cells. Plasmid pBD-gKa was used as a backbone

for constructing these additional transfer vectors pgKa/gH792, pgKa/gH603, pgKa/gH343 under the baculovirus P_{p10} promoter. Nsi I and Sph I restriction sites were used to clone the PCR amplified gH gene cassette into pBD-gKa. The recombinant pFastBac constructs were individually transformed into *Escherichia coli* DH10Bac cells (Invitrogen) to generate the corresponding recombinant bacmids. Sf9 insect cells were transfected with the recombinant bacmid DNA with Cellfectin II (Invitrogen), and the recombinant baculovirus vectors were amplified by repeated passages. The recombinant viruses produced are as follows pgKa, gH792(pgKa/gH792), gH603(pgKa/gH603), gH363pgKa/gH363.

Immunoprecipitation:Sf9 cell lysates were prepared by harvesting the cells 72 hrs post infection. The cells collected from one T75 flask were washed with PBS and lysed for 30 min on ice with NP-40 lysis buffer (Invitrogen) supplemented with PMSF (Sigma) and cocktail of protease inhibitors (Roche). Immunoprecipitation reactions were performed using Dynabeads Protein G (Invitrogen) according to the manufacturer's instructions. Briefly, 50 µl of Dynabeads was incubated 10 min at room temperature with 2ul of anti- V5 (Invitrogen) antibody for each reaction. The clarified supernatant of mock or infected cell lysates were incubated for 15 min with the protein G antibody complex. After immunoprecipitation and several washes, the eluted samples were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis. Membranes were probed with either primary monoclonal anti -V5 antibody (1:2000, Invitrogen) or monoclonal anti-FLAG antibody (1:4000, Sigma). Goat anti-mouse secondary antibody conjugated with HRP and ECL (GE) substrate were used for detection purposes.

Live Cell Microscopy and FRET Imaging

To study the interaction between UL20 and gB, Fluorescence resonance energy transfer (FRET) technique was employed. CFP and YFP flourophore pairs were used in this study. To generate YFP/CFP fusion proteins, gB and UL20 DNA sequence were amplified by PCR and ligated to CFP and YFP epitope tags. The fusion proteins generated were as follows gB-CFP, gB-YFP, UL20-CFP and UL20-YFP. 293 cells grown on chamber slides were transfected with plasmids expressing various YFP/CFP-tagged proteins. To visualize CFP and YFP protein expression, consecutive images were acquired through corresponding filter channels. The method of sensitized FRET measurement was followed as described previously (Galperin and Sorkin 2003). Briefly, images were acquired sequentially through YFP, CFP, and FRET filter channels. The background images were subtracted from the raw images prior to carrying out FRET calculations. Corrected FRET (FRETC) was calculated on a pixel-by-pixel basis for the entire image using Equation 1: $FRET_c = FRET_{raw} – D_f/D_d[CFP] – D_f/D_a[YFP]$ where $FRET_{raw}$, [CFP], and [YFP] are the signals visualized through the FRET, CFP, and YFP filter sets, respectively. The constants D_f/D_d and D_f/D_a are the transmissivity, or bleed-through, constants describing donor emission visible in FRET channel and direct excitation of acceptor. Normalized sensitized FRET (FRETN) values were obtained according to the following equation Equation 2: FRETN=FRETc/ √(YFP XCFP), where $FRET_C$, CFP, and YFP are the mean intensities of $FRET_C$, CFP, and YFP fluorescence in the selected sub-region. $FRET_C$ images are presented in pseudocolor mode. NFRET values were compared by One-way analysis of Variance (ANOVA) followed by Tukey's post-hoc test.

Expression and Purification of Glutathione S-transferase (GST) Fusion Protein

The cytoplasmic domain of UL20p consisting of the first 66 amino acids was cloned into

pGEX-4T-3 vector (Amersham). *Escherichia coli* cells, BL21 DE3 star (Invitrogen, CA) were transformed with either the GST vector alone or the GST-UL20p amino fusion (GST-UL20am) vector. A single colony of *E. coli* was grown in 2 ml of LB (Luria-Bertani) medium with ampicillin (50 μg/ml). Next day, 2 ml of the overnight culture was added to 98 ml of fresh LB medium and grown at 30°C until the optical density (OD_{600}) reached 0.5-0.6. When the optical density (OD_{600}) approached 0.5, IPTG (isopropyl-β-d-thiogalactopyranoside) was added (final concentration of 1 mM) to induce expression; the culture was incubated for an additional 3 hours at 30°C. The harvested cells were lysed by sonication in B-PER (Pierce Chemical) with 20 mg/liter lysozyme and protease inhibitor cocktail. The induced protein was then passed over glutathione-Sepharose 4B columns for purification purposes as described by the manufacturer (Pierce Chemical). The protein concentrations was determined by the Bradford method relative to bovine serum albumin (BSA) protein standards.

Expression and Purification of Histidine (His) Tagged Fusion Protein

To express the carboxyl terminal of gB, C-terminal 109 aa of gB was amplified by PCR using following primers gB-C F (5-'GAC GAA TTC CAC CAT CAC CAT CAC CAT CAC CAT CGC TAC GTC ATG CGG CTG CAG-3') and gB-C R (5'-AGA CTC GAG AAA CAG GTC CTC GTC GGC GTC ACC GTC TTT-3') containing EcoRI and XhoI sites respectively (restriction sites are indicated by underline). The PCR products was digested with the above enzymes and cloned into the same restriction sites of pET-21a expression vector (EMD Biosciences). In this construct, the C-terminal had a 6X His sequence in frame with the gene to facilitate the purification. *Escherichia coli* BL21 DE3 star (Invitrogen, CA) cells were transformed with the above gene construct. A single colony of *E. coli* was grown in 2 ml of LB medium overnight at 37°C. Next day, 2 ml of overnight culture were added to 98 ml of fresh LB

medium and grown at 37°C until the optical density (OD_{600}) reached 0.5-0.6. Expression of the fusion proteins was induced with 0.5 mM IPTG (isopropyl-β-d-thiogalactopyranoside) and the culture was further incubated at 37°C for 3 hours. The induced bacterial culture was harvested by centrifugation and the pellet was frozen at -20°C. Pellets were lysed in lysis buffer (10 mM Tris-HCL, PH 8.0, 300 mM NaCl and 10 mM imidazole) containing 0.5 % N- Lauroylsarcosine (sarkosyl). Triton-X 100 was added to the clarified lysate at a final concentration of 1-2%. The target protein was purified by affinity purification by the Profinia system (Bio-Rad) loaded with Immobilized metal affinity chromatography (IMAC) column, as recommended by the supplier.

GST Pull Down Assay

To test whether the GST-UL20am protein physically interacted with the carboxyl terminal of gB equimolar amounts of both protein were mixed and allowed to interact for 2 hours in binding buffer (100mM KCl+10mM Tris-HCL+0.2% Triton X-100 +1mM DDT) at 4°C. The protein mixture was then mixed with glutathione-linked agarose beads and rocked for another 8 hours at 4°C. The GST-beads were then recovered by centrifugation, washed thrice, and suspended in sample buffer for SDS-PAGE analysis. Proteins were transferred to nitrocellulose membranes,and detected by immunoblotting with the anti-His and anti-GST antibody.

RESULTS

Plaque Phenotype of the different Mutant Viruses on Vero Cells

Vero cells were infected with wild type and mutant viruses. The wild type virus produced the characteristic plaque with limited fusion (2.1 A). Deletion of the carboxyl-terminal 28 aa of gB caused extensive virus-induced cell-to –cell fusion, producing a distinctive syncytial

virus plaque (Fig.2.1B). In contrast, the gKΔ31-68/gBΔ28syn mutant virus produced non-syncytial, substantially smaller plaque (Fig.2.1D), while mutant virus gKΔ31-68 produced a plaque that was smaller than the wild type but larger than mutant virus gKΔ31-68/gBΔ28syn (Fig.2.1C).

Expression of the gKa Peptide Consisting of Amino Acid (aa) 1-82 of gK

A gene cassette encoding the gKa peptide was constructed to include a 3x FLAG epitope inserted in frame after gK aa 82 and cloned into transient expression vector p3xFLAGCMV14 (Invitrogen, Inc.). Transient expression of gKa in Vero cells produced protein species migrating with an apparent molecular mass of approximately 12 to 22 kDa, readily detected by anti-FLAG antibody (Fig.2.2).

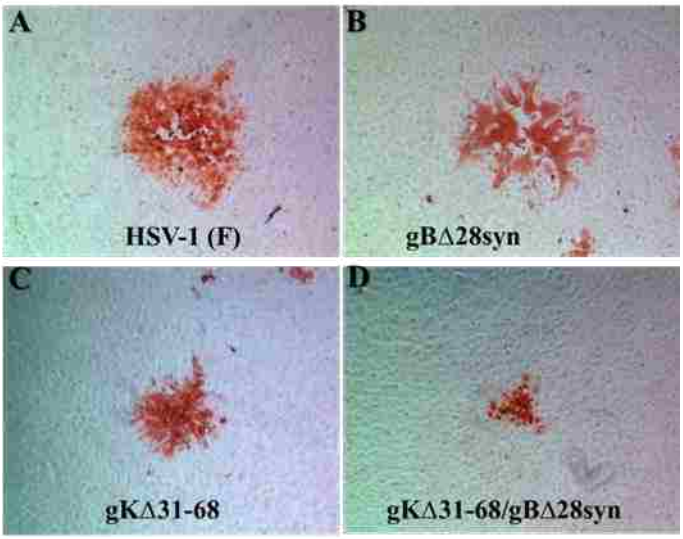

Figure 2.1: Plaque phenotype of the different mutant viruses on Vero cells. The plaque morphology of recombinant viruses; HSV-1 (F), gBΔ28syn, gKΔ31-68, gKΔ31-68/gBΔ28syn are produced on Vero cells. HSV-1gKΔ31-68/gBΔ28syn is a double mutant virus which has a mutation in amino terminus of gK (deletion of 31-68 aa) and carboxyl terminus of gB (28 aa truncated) (Chouljenko, Iyer et al. 2009).

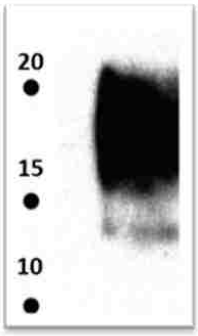

Figure 2.2: Expression of the gKa peptide consisting of aa 1-82 of gK. Vero cells were transfected with the gKa peptide that encodes the first 82 amino acids of gKa and a 3xFLAG epitope. Cell lysate were collected and processed for SDS page and immunoblot assay. gKa species were detected by anti-FLAG antibody.

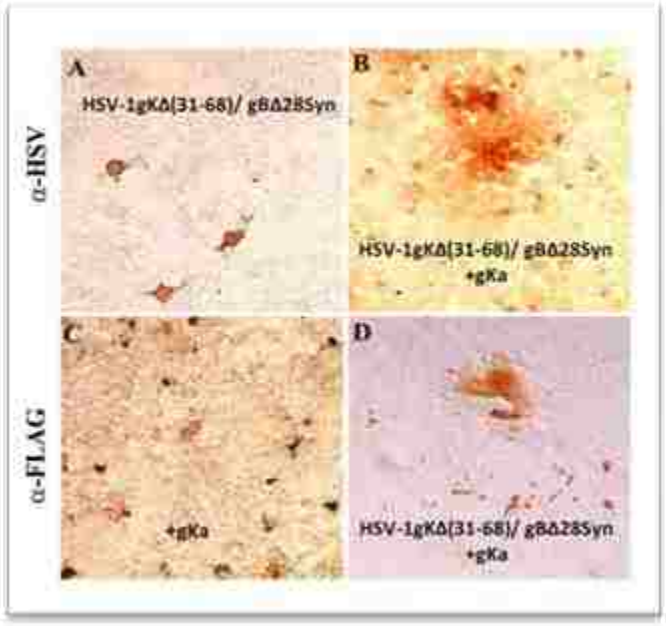

Figure 2.3: Ability of gKa peptide to complement HSV-1gKΔ (31-68)/ gBΔ28Syn induced cell fusion. Vero cells were transfected with gKa, 24 hrs after transfection cells were superinfected with gKΔ31-68/gBΔ28syn virus. 24 hpi the cells were fixed and stained with anti-FLAG (C, D) or anti-HSV antibody (A, B). Cells transfected with gKa peptide restored syncytia formation that was positive for both anti-HSV and anti-FLAG antibody (B,D).

The gKa Peptide Complements in *trans* gKΔ31-68/gBΔ28syn-Induced Cell Fusion

Vero cells were transfected with gKa peptide (Fig. 2.3: B, D, and C). Twenty-four hours posttransfection cells were infected with mutant virus gKΔ31-68/gBΔ28syn (Fig. 2.3: A, B, D) under live conditions cells were probed with anti-HSV (Fig. 2.3: A, B) and anti-FLAG antibody (Fig. 2.3: C, D). Only in presence of the gKa plasmid syncytia formation was observed in Vero cells infected with gKΔ31-68/gBΔ28syn virus (Fig. 2.3: B, D). The plaques were positive for both anti-HSV and anti-FLAG antibody.

The gKa Peptide Interacts with Extracellular Portion of gB

293 cells were co-transfected with gKa plasmid and plasmids expressing full length gB and gD glycoproteins according to the following combinations gKa + gB and gKa + gD. Viral glycoproteins gB and gD were clearly detected by anti-V5 antibody in transfected 293 cellular extracts (Fig.2.4: B, lane 1 and 2), while gKa protein species were detected by anti-FLAG antibody (Fig.2.4: B, lane 1 and 2). Cellular extracts were immunoprecipitated with anti-FLAG beads. Anti-FLAG immunoprecipitates probed with anti-V5 antibody detected gB but not gD (Fig. 2.4: C lane 2). These results indicate that the amino terminus of gKa may interact with the amino terminus of gB.

The gKa Peptide Interacts with Extracellular Portion of gH.

Recombinant baculoviruses gH792, gH603, gH343 were constructed to simultaneously express the gKa peptide in combination with truncated portion of gH (Fig.2.5). Western immunoblots of infected Sf9 cellular extracts probed with anti-V5 monoclonal antibody detected gH in all three baculovirus constructs (Fig. 2.4.: A). The truncated viral glycoproteins gH;

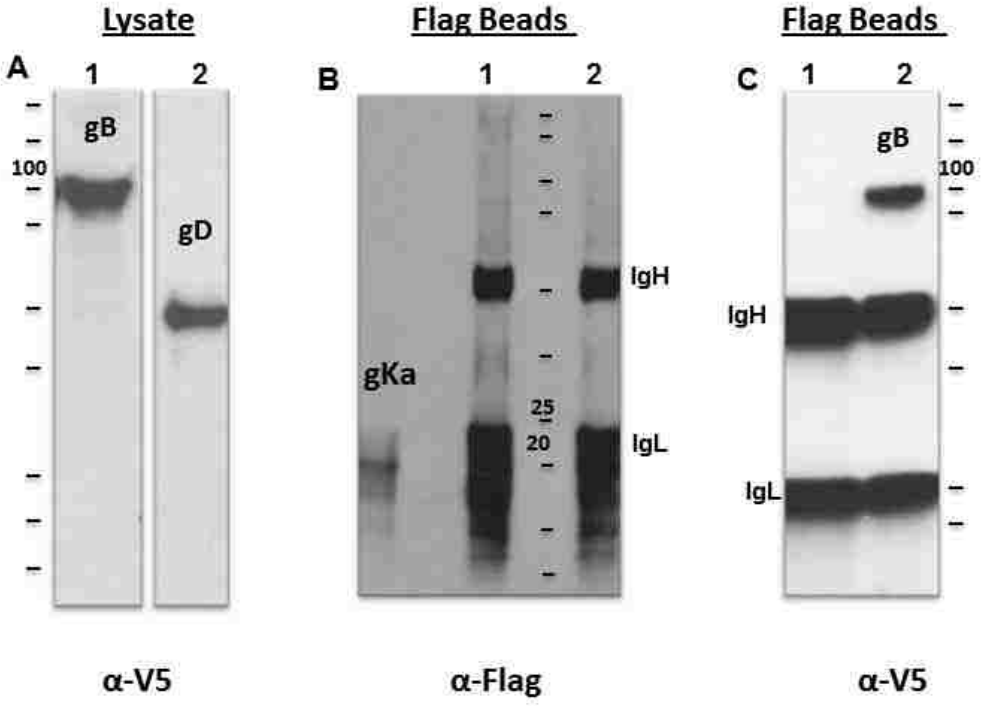

Figure 2.4: gKa interacts with extracellular portion of gB. 293 cellular extracts obtained after transfection with following plasmid combinations (gB+gKa) and (gD +gKa) were immunoprecipitated with anti-V5 antibody. Immunoprecipitates were separated by SDS page gel electrophoresis followed by immunoblotting with anti-V5 (B) or anti-FLAG antibody (A). Molecular mass standards are shown with dashes on each panel (250, 150, 100, 75, 50, 37,25, 20, 15, and 10 kDa).

gH792, gH603, gH343 exhibited apparent molecular masses of approximately 95 to 110 kDa, 75kDa and 45 to 55 kDa, respectively. The anti-FLAG (anti-gKa) antibody was used to detect the expression of the gKa peptide in all four recombinant constructs (Fig. 2.4.: B). The gKa peptide was efficiently expressed, in all recombinant baculoviruses migrating in SDS gels with an apparent molecular mass of approximately 15 to 22 kDa. Cellular extracts were immunoprecipitated with anti-V5 antibody. These immunoprecpitates, when probed with anti-V5 antibody revealed the presence of truncated gH proteins with apparent molecular masses of

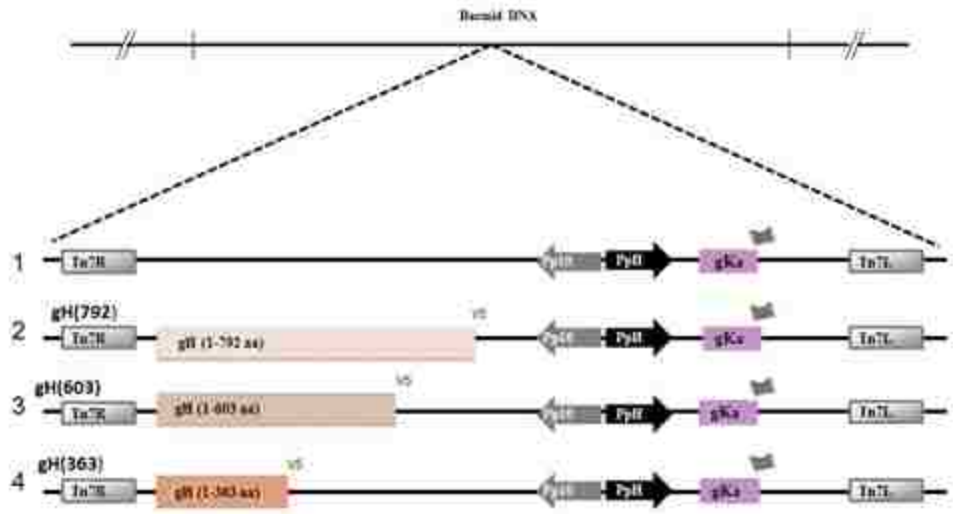

Figure 2.5: Construction of recombinant gH baculoviruses. The portion of gH, expressed by the baculoviruses. Schematic of the gene cassettes expressing the truncated gH792, gH603, gH363, and gKa inserted in recombinant baculovirus genomes. Pp10 and PpH are baculovirus promoters for the p10 and polyhedrin genes, respectively. V5 epitope was inserted at the 3' end of truncated gH gene cassettes and 3xFLAG epitope at 3' of gKa.

approximately 95 to 100 kDa, 60 to 65 kDa, 45 to 50 kDa, and 18 to 20 kDa, respectively (Fig. 2.6:C). The gH (363) protein species co-migrated with the IgG heavy chain, but it was readily detected due to the substantial increase in band intensity over the IgG heavy-chain background. The gKa protein species (detected by anti-FLAG) was present only in anti-V5 immunoprecipitates containing truncated versions of gH but not in the sample that expressed gKa alone (Fig. 2.6:D). This suggests that the amino terminus of gK interacts with the truncated versions of the amino terminus of gH.

Interactions between UL20p and gB.

293 cells were transfected with gB and UL20 fusion proteins according to the the following combination, (gB-YFP / gB-CFP), (gB-YFP / UL20-CFP),(gB-CFP /UL20-YF) and (YFP /CFP). 36hrs post-transfection FRET microscopic analysis was performed on all

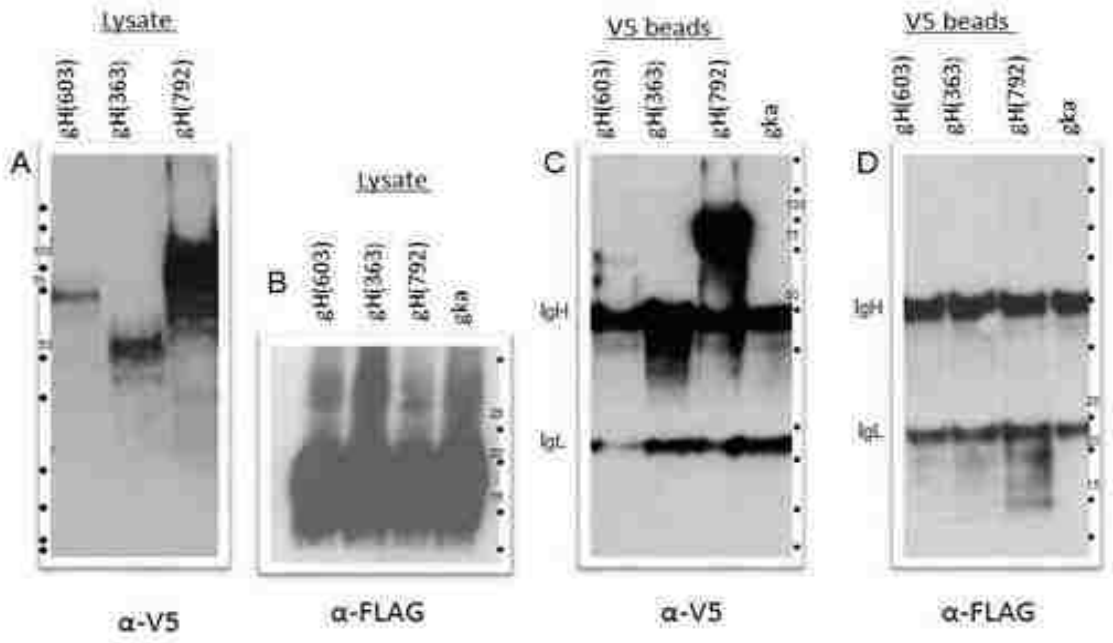

Figure 2.6: gKa interacts w/h extracellular portion of gH. SF9 cellular extracts obtained after infection with recombinant baculoviruses expressing gKa alone or in combination with truncated versions of gH (gH 792,gH 603 and gH363) were immunoprecipitated with anti-V5 antibody. Immunoprecipitates were separated by SDS page gel electrophoresis followed by immunoblotting with anti-V5 (C) or anti-FLAG antibody (D.) Molecular mass standards are shown with dots on each panel (250, 150, 100, 75, 50, 37,25, 20, 15, and 10 kDa

sample combinations under live conditions. Briefly, images were acquired sequentially through YFP, CFP, and FRET filter channels. Sensitized FRET calculations were performed as described under material and method section. As a control for FRET analysis, FRET signals were measured for cells co-expressing CFP-YFP protein combination. The normalized FRET (NFRET) values were compared between the sample groups (Fig. 2.7). High FRET signals were obtained in cells that co-expressed gB-YFP and gB-CFP fusion proteins suggesting that gB forms a protein complex. FRET signal obtained from CFP-YFP were minimal and were

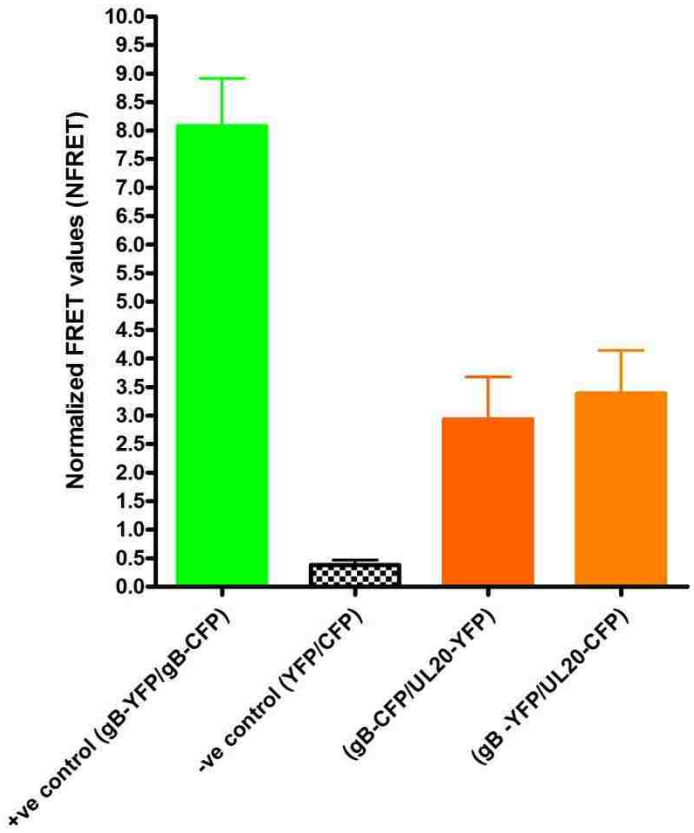

Figure 2.7: Plausible interaction between gB and UL20p. Normalized FRET (NFRET) values were compared between sample groups and control groups. gB-YFP/gB-CFP produced high FRET signal. gB-YFP/UL20-CFP protein combination produced FRET signal more than the negative control group (YFP/CFP).

considered insignificant. FRET signals were detected for cells co-expressing (gB-YFP / UL20-CFP) or (gB-CFP / UL20-YF) fusion proteins. The measured FRET signal for this protein combination (gB/UL20p) was higher than (CFP-YFP). These results suggest that UL20p and gB are in close proximity and interact with each other and to form a protein complex (Fig.2.7).

Interaction between the Amino Terminus of UL20p and Carboxyl Terminal of gB

Bacterial extracts revealed the presence of UL20 protein species as overexpressed protein species in induced bacterial samples after Coomassie blue staining of

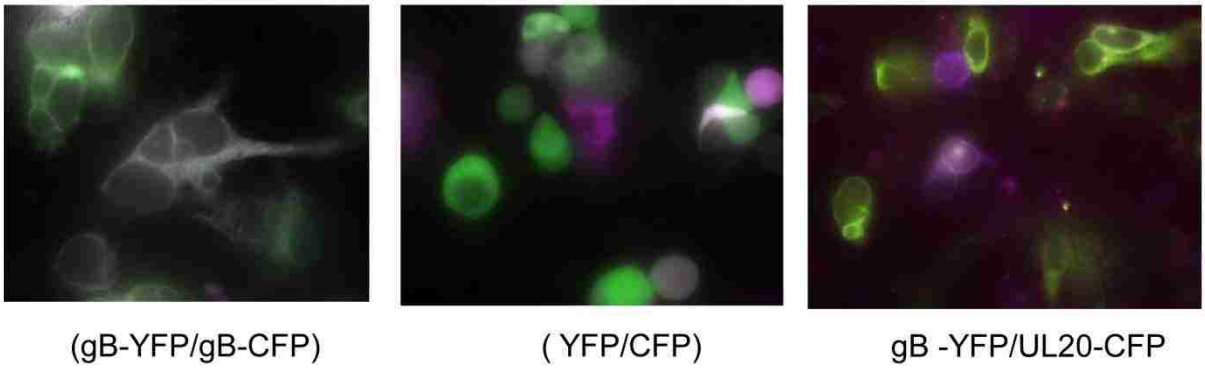

Figure 2.8: Live cell microscopy and FRET imaging. 293 cells were co-transfected with following combination of plasmid gB-YFP/gB-CFP, gB-YFP / UL20-CFP and YFP/CFP. Representative images of samples showing FRET signal.

SDS-polyacrylamide gels (Fig.2.9:A). The GST protein alone migrated with a molecular weight of 25kDa while the GST-UL20am fusion proteins migrated with a molecular mass of 34kDa. Both proteins were purified on glutathione columns, and the purified proteins were detected by Western immunoblot assay probed with anti-GST antibody (Fig. 2.9: B). Bacterial extracts revealed the presence of the carboxyl terminal of gB (cloned in frame with a Histidine-tag epitope at the C terminal) as a overexpressed protein species in induced bacterial samples, after staining of SDS-polyacrylamide gel. Purified gBc protein species migrated with a molecular weight of 20kDa (Fig. 2.10). To test, whether the GST-UL20am protein physically interacted with the carboxyl terminal of gB, equimolar amounts of both protein were mixed and subsequently precipitated with glutathione-linked agarose beads. These immunoprecipitates when probed with anti-GST anti-His antibodies revealed the presence of GST and GST-UL20am with apparent molecular masses of 25KDa and 34KDa; and His-gBc fusion protein with apparent molecular mass of 20KDa. The GST-UL20am precipitation specifically co-precipitated the carboxyl terminal of gB while GST precipitation failed to precipitate gB,

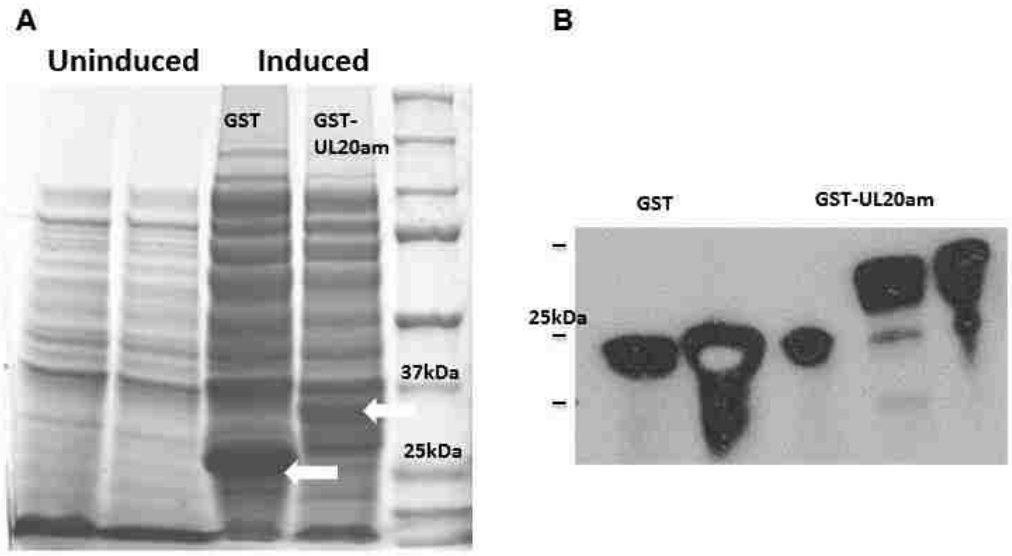

Figure 2.9: SDS-PAGE and Coomassie blue staining of crude cell lysates from bacterially expressed GST and GST fused with the amino terminus of UL20p (A). Immunoblot specific detection of purified GST or GST fused with the amino terminus of UL20p using anti-GST antibody (B).

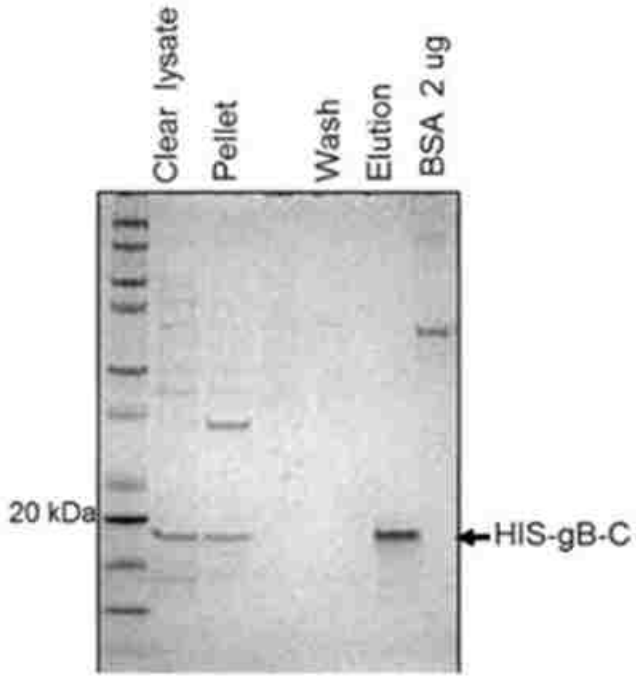

Figure 2.10: SDS-PAGE and Coomassie blue staining of bacterial cell lysates from bacterially expressed Histidine fused with the carboxy terminal of gB (His-gBc). Purified gB-His fusion protein migrated with a molecular mass of 20KDa .

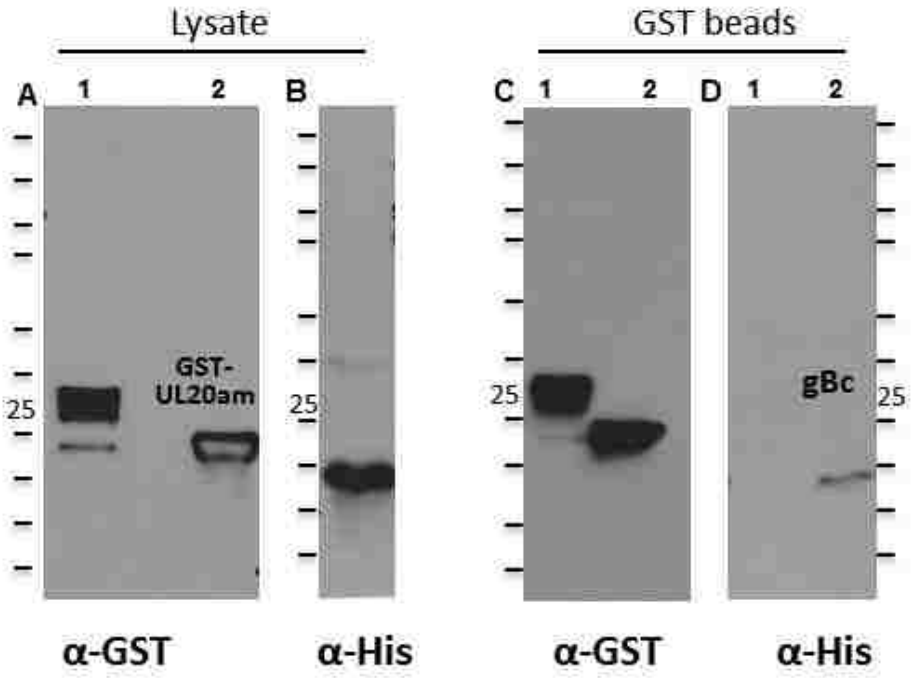

Figure 2.11: Amino terminus of UL20p interacts with the carboxyl terminus of gB. Purified GST-UL20am (amino terminus of UL20p) and HisgBc (carboxyl terminal of gB) proteins were mixed and subsequently precipitated with glutathione-linked agarose beads. Immunoprecipitates were separated by SDS page gel electrophoresis followed by immunoblotting with anti-GST or anti-His antibody. Molecular mass standards are shown with dashes on each panel (250, 150, 100, 75, 50, 37,25, 20, 15, and 10 kDa).

indicating that the amino terminus of UL20p specifically interacts with the carboxyl terminal of gB (Fig 2.11:D, lane 2).

DISCUSSION

HSV-1 enters cells by catalyzing a membrane fusion reaction between the viral membrane and cellular membrane; a mechanism which needs a concerted action of viral glycoproteins and cellular receptors. This fusion process occurs during both viral entry, as well as, cell-to-cell fusion and needs the quartet of glycoproteins gD, gB and gH/gL.

These glycoproteins are sufficient to bring about cell-cell fusion when expressed from plasmid vectors in absence of any other viral glycoprotein. However extensive cell-cell fusion (multinucleated cells) has also been observed in the context of viral infection in an in vitro system. These multinucleated cells have been designated as syncytia and are a result of point mutations within four viral genes; UL20 gene, UL24 gene, UL27 gene encoding for glycoprotein B (gB) and UL53 gene encoding for glycoprotein K (gK). It is also known that certain mutations in the carboxyl terminus of gB and the amino terminus of gK cause extensive virus-induced cell fusion (Chowdary, Cairns et al. 2010) (Foster, Alvarez et al. 2003). Previous work from our laboratory has shown that gK *syn1* mutations do not cause virus-induced cell-to-cell fusion in the absence of gB, and that gB *syn3* mutations do not cause fusion in the absence of UL20p (Foster, Melancon et al. 2004). Moreover, certain mutations within the amino terminus of the UL20p protein (located intracellularly) cause extensive virus-induced cell fusion, while others inhibit gB-mediated cell fusion (Foster, Melancon et al. 2004; Melancon, Fulmer et al. 2007) . Therefore, it is obvious that surface expression of gK and UL20p are needed for virus-induced cell-to-cell fusion. The recently solved X-ray crystal structure of gB has implicated it, as the sole viral fusogen in HSV-1 that orchestrates the final step of fusion in which the viral membrane and cell membrane are pulled close together and ultimately fuse. The role of gK and UL20p in this process of virus-induced cell-to-cell fusion is not well documented. We have shown that the amino terminus of gK is involved in virus-induced cell fusion, since its absence drastically inhibits the syncytial phenotype of mutant virus gBΔsyn28. When this amino terminus was provided *in trans*, the syncytial phenotype of mutant virus gBΔsyn28 was restored. This observation suggested that the amino terminus of gK may directly or indirectly interact with gB and /or other viral proteins. Immunoprecipitation results revealed that the gKa peptide physically interacted with gB and gH but not gD. Recombinant baculoviruses were created to

specifically delineate the extracellular portion of gH that interacts with gKa. Co-immunoprecipitation results revealed that the gKa peptide directly interacted with all three truncated versions of the amino terminus of gH [(1-792aa), (1-603aa), and (1-363aa)].

Moreover, FRET analysis revealed that UL20p, the binding partner of gK interacts with gB. Similar results showing the interaction between gB and UL20p was obtained in infected cells.Specifically the amino terminus of UL20 (GST-tag) interacted with the carboxyl terminal of gB (His-tag).

These results indicate that the amino terminus of gK plays a role in gB mediated cell fusion and is absolutely essential for virus-induced cell fusion. The role of gK can be envisioned as a fusion regulator that controls virus-induced cell fusion as seen in cells infected in the wild type virus. This regulation of fusion may be important for the virus in order to prevent uncontrolled cell fusion by gB. In fact, the exaggerated syncytial phenotype observed when Vero cells were infected with mutant virus gBdeltasyn28 is lost in presence of a deletion in the amino terminus of gK. Furthermore, the amino terminus of gK interacts directly with the extracellular portion of gB and gH that are needed for gB mediated cell fusion. It was also revealed that the amino terminus of gB interacts with the carboxyl terminal of UL20 situated in the cytosolic phase of the membrane. Based on these observations a new fusion complex can be proposed. This complex consists of glycoproteins gB.gD, gH,gK and gL as well as the membrane protein UL20p. gD the receptor binding glycoprotein interacts with its cognate receptors and triggers the fusion machinery consisting of gH/gL and gB. The gH/gL complex then interacts with gB resulting in conformational changes in gB. In this model, the gB conformation is also controlled or regulated by the heterodimer gK/UL20p, ie. gK and UL20p are like “tongs” that control the conformation of gB by interactions occurring at both

cytoplasmic and extracellular portions of gB. In this regard, syncytial mutations in either gK or UL20 enhance virus-induced cell fusion by physically altering conformational restrictions on gB imposed by gK and UL20.

REFERENCES

Atanasiu, D., J. C. Whitbeck, et al. (2007). "Bimolecular complementation reveals that glycoproteins gB and gH/gL of herpes simplex virus interact with each other during cell fusion." Proc Natl Acad Sci U S A **104**(47): 18718-18723.

Atanasiu, D., J. C. Whitbeck, et al. (2010). "Bimolecular complementation defines functional regions of Herpes simplex virus gB that are involved with gH/gL as a necessary step leading to cell fusion." J Virol **84**(8): 3825-3834.

Baines, J. D., P. L. Ward, et al. (1991). "The UL20 gene of herpes simplex virus 1 encodes a function necessary for viral egress." J Virol **65**(12): 6414-6424.

Bond, V. C. and S. Person (1984). "Fine structure physical map locations of alterations that affect cell fusion in herpes simplex virus type 1." Virology **132**(2): 368-376.

Bzik, D. J., B. A. Fox, et al. (1984). "Nucleotide sequence of a region of the herpes simplex virus type 1 gB glycoprotein gene: mutations affecting rate of virus entry and cell fusion." Virology **137**(1): 185-190.

Chouljenko, V. N., A. V. Iyer, et al. (2009). "The amino terminus of herpes simplex virus type 1 glycoprotein K (gK) modulates gB-mediated virus-induced cell fusion and virion egress." J Virol **83**(23): 12301-12313.

Chowdary, T. K., T. M. Cairns, et al. (2010). "Crystal structure of the conserved herpesvirus fusion regulator complex gH-gL." Nat Struct Mol Biol **17**(7): 882-888.

Debroy, C., N. Pederson, et al. (1985). "Nucleotide sequence of a herpes simplex virus type 1 gene that causes cell fusion." Virology **145**(1): 36-48.

Foster, T. P., X. Alvarez, et al. (2003). "Plasma membrane topology of syncytial domains of herpes simplex virus type 1 glycoprotein K (gK): the UL20 protein enables cell surface localization of gK but not gK-mediated cell-to-cell fusion." J Virol **77**(1): 499-510.

Foster, T. P., J. M. Melancon, et al. (2004). "The Herpes Simplex Virus Type 1 UL20 Protein Modulates Membrane Fusion Events during Cytoplasmic Virion Morphogenesis and Virus-Induced Cell Fusion." J Virol **78**(10): 5347-5357.

Galperin, E. and A. Sorkin (2003). "Visualization of Rab5 activity in living cells by FRET microscopy and influence of plasma-membrane-targeted Rab5 on clathrin-dependent endocytosis." J Cell Sci **116**(Pt 23): 4799-4810.

Jacobson, J. G., S. H. Chen, et al. (1998). "Importance of the herpes simplex virus UL24 gene for productive ganglionic infection in mice." Virology **242**(1): 161-169.

Melancon, J. M., P. A. Fulmer, et al. (2007). "The herpes simplex virus UL20 protein functions in glycoprotein K (gK) intracellular transport and virus-induced cell fusion are independent of UL20 functions in cytoplasmic virion envelopment." Virol J **4**: 120.

Melancon, J. M., R. E. Luna, et al. (2005). "Herpes simplex virus type 1 gK is required for gB-mediated virus-induced cell fusion, while neither gB and gK nor gB and UL20p function redundantly in virion de-envelopment." J Virol **79**(1): 299-313.

Pogue-Geile, K. L., G. T. Lee, et al. (1984). "Fine mapping of mutations in the fusion-inducing MP strain of herpes simplex virus type 1." Virology **136**(1): 100-109.

Pogue-Geile, K. L. and P. G. Spear (1987). "The single base pair substitution responsible for the Syn phenotype of herpes simplex virus type 1, strain MP." Virology **157**(1): 67-74.

Tischer, B. K., J. von Einem, et al. (2006). "Two-step red-mediated recombination for versatile high-efficiency markerless DNA manipulation in Escherichia coli." Biotechniques **40**(2): 191-197.

CHAPTER III

THE AMINO TERMINUS OF HSV-1 GLYCOPROTEIN K (gK) MODULATES VIRION ENTRY INTO CELLS VIA THE PAIRED IMMUNOGLOBULIN-LIKE TYPE-2 RECEPTOR ALPHA (PILRα)

INTRODUCTION

The HSV-1 entry mechanism is unique among enveloped viruses, as well as complex, since it involves specific glycoproteins for each step of virus entry i.e. attachment, binding and fusion. Specific glycoproteins embedded in the viral envelope interact with various cellular receptors to facilitate virus entry into different types of cells. Initial attachment of the virus to cellular membranes is mediated by interaction of glycoproteins gB and gC with glycosaminoglycan (GAG) moieties of cell surface proteoglycans (Herold, WuDunn et al. 1991; Shukla and Spear 2001). This initial attachment of virions to cellular membranes is thought to facilitate subsequent binding of gD with one or more of its specific receptors, including the herpesvirus entry mediator (HVEM or HveA), nectin-1 (HveC), or 3-O-sulfated heparan sulfate (Montgomery, Warner et al. 1996; Geraghty, Krummenacher et al. 1998; Shukla, Liu et al. 1999). Recent reports have shown that gB can also bind to additional receptors (co-receptors), including paired immunoglobulin-like type 2 receptor alpha (PILR α), nonmuscle myosin heavy chain IIA (NMHC-IIA), and myelin-associated glycoprotein (MAG), that play a pivotal role in virion attachment and virus entry (Satoh, Arii et al. 2008; Arii, Goto et al. 2010; Suenaga, Satoh et al. 2010). The sequence of events that ultimately lead to fusion of the viral envelope with cellular membranes is triggered by the interaction of gD to its cognate receptors

(nectin-1, HVEM, and other receptors) and also, gB binding to its cognate receptors. These events are thought to cause conformational changes, first in gD ,then in gH/gL, and eventually in gB leading to gB mediated membrane fusion (Heldwein, Lou et al. 2006; Connolly, Jackson et al. 2011).

Depending on the cell type, the virus can enter by three possible entry pathways: (I) entry mediated by fusion of the viral envelope with the host cell membrane in a pH-independent manner as seen in Vero and HEp-2 cells (Nicola, McEvoy et al. 2003); (II) receptor-mediated endocytosis followed by pH dependent fusion between the viral envelope with endocytic membranes, as evident in HeLa and Chinese hamster ovary (CHO) cells expressing the nectin-1 gD receptor (Nicola, McEvoy et al. 2003); (III) entry mediated via low-pH-independent endocytosis as shown for C10 murine melanoma cells (Milne, Nicola et al. 2005). Recently, it has become apparent that gB-specific receptors play important roles in viral entry (Arii, Uema et al. 2009). Moreover, gB-specific receptors such as PILRα or other cellular factors may determine whether the virion enters predominantly via fusion at the plasma membrane, or via receptor-mediated endocytosis, (pH-dependent or pH-independent) followed by fusion of the viral envelope with endosomal membranes (Arii, Uema et al. 2009).

We have shown, gK is a structural component of virions and it interacts with UL20 protein on virion particles. Furthermore gK plays a role in entry inasmuch as virions lacking entire gK or the amino terminal of gK (aa 31-68) enter susceptible cells substantially slower than the wild-type virus (Foster, Rybachuk et al. 2001; Jambunathan, Chowdhury et al. 2011). Furthermore, HSV-1 gK and UL20 functionally and physically interact, which is most essential for their coordinate intracellular transport, cell surface expression, and also other related functions in the HSV-1 life cycle (Foster, Melancon et al. 2004; Foster, Chouljenko et al. 2008).

Recently, we showed that the amino terminus of gK interacts with gB and can complement gB-mediated cell fusion (Chouljenko, Iyer et al. 2009; Chouljenko, Iyer et al. 2010). These results have strongly suggested that the gK/UL20 protein complex can bind gB and gH and modulate membrane fusion phenomena occurring during both virus entry and virus-induced cell fusion.

In this chapter, we show that the gK amino terminus is necessary for efficient virus entry via the PILRα gB-specific receptor, but not for entry via the gD-specific receptors, nectin-1 and HVEM. Moreover, PILRα is shown to form a multi-protein complex with gB and gK, but not gD. HSV-1 (McKrae) receptor-specific entry into CHO-nectin-1 occurs via pH-independent mechanisms in contrast to previous reports (Nicola, McEvoy et al. 2003). These results strongly implicate gK/UL20 as contributing factors in the utilization of the PILRα receptor for entry.

MATERIALS AND METHODS

Cells and Plasmids

African green monkey kidney (Vero) cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and grown and propagated in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. VK302 cells permanently expressing gK were originally obtained from David Johnson (Oregon Health Sciences University, Portland, OR). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal calf serum and antibiotics. CHO-nectin 1 cells was a gift from Dr. Richard Longnecker, (Northwestern, University) and were propagated in Ham's F12 medium supplemented with 10% fetal calf serum (FCS) and 200 µg/ml G418. The CHO-hPLIRα cells were also obtained from Dr. Longnecker and were grown in Ham's F12 medium supplemented with 10% fetal calf serum

(FCS) and 700 µg/ml G418. The human CHO-HVEM was created by using PiggyBac Transposon system (System Biosciences) as described earlier (Chowdhury, Naderi et al. 2012). All cells were cultured in non-selective medium prior to use in infectivity assay. 293 PEAK TM Rapid (gift from Dr. Longnecker) was grown in DMEM supplemented with 10%FBS. Plasmid expressing the soluble human PILRα-IgG Fc hybrid protein (pME 18S PILRα-Ig) and CD200-Ig Fc fusion protein were kind gifts from Dr. Hishashi Arase, (Osaka University, Osaka Japan) and Dr. Kawaguchi respectively. Plasmids expressing the Ig fusions proteins were constructed as described in by Satoh et.al (Satoh, Arii et al. 2008).

Viruses

The clinical ocular isolate and neuroinvasive strain of HSV-1 (the parental wild-type), McKrae strain, was obtained from Dr. J. M. Hill (Louisiana State University Health Sciences Center, New Orleans, LA, USA). This McKrae genome has been cloned into the bacterial artificial chromosome vector that also contains an excisable EGFP expression cassette creating McKrae-EGFP-BAC virus. The recombinant mutant YE102-VC1 (HSV-1, F strain) has double tags, gK and UL20 containing the V5 and 3xFLAG antigenic epitopes, respectively. GFP-tagged Vesicular stomatitis virus (VSV-GFP) was a kind gift from Dr. John Rose (Yale University, New Haven, Conn.). The mutant viruses gKΔ31-68, gBΔ28syn (deletion of 28 aa from the carboxyl terminus of gB), and gKΔ31-68/gBΔ28syn have been described earlier (Chouljenko, Iyer et al. 2009).

Construction of Recombinant HSV-1 Mutant Viruses

The HSV-1 (the parental wild-type), McKrae strain was cloned into the bacterial artificial chromosome (BAC) plasmid pBeloBAC11(NEB) as described : A gene construct was created wherein the LoxP recombination site was fused together with DNA fragments of approximately

1kb of McKrae DNA sequence, encompassing portions of the UL3-UL4-targeted genomic region by PCR-based overlap-extension. This gene construct was subsequently cloned into the pCR 2.1-TOPO plasmid vector (Invitrogen). Similarly, another gene expression cassette containing the EGFP gene flanked by FRT recombination sites (for future potential removal of the EGFP gene cassette), was constructed and cloned into pEF6/V5-His TOPO plasmid vector (Invitrogen) under control of the EF1α promoter. This gene cassette was excised from the vector by using EcoRI endonuclease and blunt-ended by T4 polymerase. It was cloned into the blunt-ended BamHI site of pBeloBAC11. Cre recombinase enzyme (NEB) was used for *in vitro* reaction to fuse together the L-LoxP-R Amp resistant pCR 2.1- based plasmid and LoxP-EGFP Cm resistant pBeloBAC-based plasmid. The plasmid DNA created after fusion with Cre recombinase was used to transfect Vero cells using Lipofectamine 2000 (Invitrogen). The transfected cells were infected with HSV-1(McKrae) at six hours post transfection. Fluorescent microscopy was used to select fluorescent viral plaques that contained the inserted McKrae genome in the bac plasmid (McKbac). The presence of the bac plasmid was verified by PCR-assisted DNA sequencing. The recovered McKbac virus was utilized for the construction of gK mutant viruses. The mutant viruses gKΔ31-68 (encoding gK carrying an in-frame deletion of aa 31 to 68), and gKΔ31-117 (encoding gK carrying an in-frame deletion of aa 31 to 117), were created as described earlier for the HSV-1(F) genome cloned into a bacterial artificial chromosome using a markerless two-step red recombination mutagenesis system (Tischer, von Einem et al. 2006).

Replication Kinetics, Plaque Morphologies of McKbac and gK Mutant Viruses and Electron Microscopy

Viral plaques were visualized by immunohistochemistry, as we have previously described (Melancon, Foster et al. 2004; Melancon, Luna et al. 2005; Fulmer, Melancon et al.

2007; Lee, Chouljenko et al. 2009). Analysis of one-step growth kinetics was performed as described earlier (Foster, Rybachuk et al. 2001; Foster, Alvarez et al. 2003). Confluent Vero cell monolayers were infected with each virus at 4°C for 1 hour at an MOI of 0.2 and 2. Thereafter, plates were incubated at 37°C and 5% CO_2 and virus was allowed to penetrate for 1 h at 37°C. Any remaining extracellular virus was inactivated by low-pH treatment (pH 3.0), and cells were subsequently incubated at 37°C and 5% CO_2. Supernatants and cell pellets were separated at different times post infection and stored at -80°C. Viral titers were obtained on the VK302 cell line that expresses the HSV-1(KOS) gK gene. The ultrastructural morphology of virions within infected cells was examined by transmission electron microscopy as described previously (Jayachandra, Baghian et al. 1997; Foster and Kousoulas 1999; Foster, Melancon et al. 2004; Melancon, Luna et al. 2005; Lee, Chouljenko et al. 2009). All infected cells processed for electron microscopy were prepared at 18 hpi and visualized by transmission electron microscopy.

Virus Entry Assay

Confluent monolayers of CHO-neo, CHO-HVEM, CHO-PILRα, CHO-human nectin-1 and Vero cells were infected with McK(bac), McKrae (gKΔ31-68), McKrae (gKΔ31-117) at a multiplicity of infection (MOI) of 1 for 1 hr, at 34°C. The virus inoculum was subsequently removed, and the cultures were shifted to 37°C. Eight to twelve hpi, the cells were fixed and stained with anti-ICP4 antibody (Virusys, Inc., Taneytown, MD), and Alexa Fluor 647 goat anti-mouse IgG1(Life Technologies, Grand Island, NY), for flow cytometry analysis (FACS). The relative efficiency of virus entry was calculated as the percentage of cells expressing ICP4. Mean values and standard deviations of three independent experiments were calculated and normalized to CHO-neo entry values.

Treatments with Lysosomotropic Agents

Stock solutions of ammonium chloride (1.5 M) was prepared in distill water immediately prior to use. Monensin (75 mM; Sigma) and bafilomycin A1 (100 mM; Sigma) were dissolved in ethanol and dimethyl sulfoxide, respectively, and stored at −20°C. The pH of all solutions was adjusted to 7.4, prior to use. Growth medium was removed from cells and replaced by medium containing inhibitors and the plate was incubated for 1 hour at 37°C. After incubation, virus was added in presence of the inhibitor for 1 hour at 34°C and subsequently removed; cells were incubated again in presence of inhibitor until they were collected for FACS analysis.

Immunohistochemistry

Nearly confluent CHO-neo, CHO-nectin-1, CHO-hPILRα cell monolayers were infected with an MOI of 1 with mutant viruses gKΔ31-68, gBΔ28syn, and gKΔ31-68/gBΔ28syn and HSV-1 (F). 14 hpi, monolayers were washed with Tris-buffered saline (TBS)-Ca-Mg and fixed with 100% methanol for 10 min. Cell fusion was visualized by immunostaining using anti-HSV-1 antibody**.**

Preparation of Purified Ig Fusion Proteins and Immunoprecipitation Assay

293 PEAK Rapid cells were transfected with the plasmid pME18S-PILRα-Ig and a control plasmid expressing human CD200-Ig Fc fusion protein with 293 Fectin (Invitrogen). Supernatants from transfected cells were collected at 48 and 72 h after transfection and pooled. Ig fusion proteins were purified by protein A affinity chromatography and were subsequently used for immunoprecipitation. Vero infected cells were ruptured with NP40 lysis buffer (Invitrogen) and subsequently used for immunoprecipitation. Lysates were immunoprecipitated with PILRα-Ig or CD200-Ig (control) and the immunoprecipitates were eluted with elution buffer. Immunoprecipitates were separated on 5–20% polyacrylamide gels and were transferred

onto PVDF membranes (Millipore) which were blotted with anti-gB or anti-gD or anti-FLAG or anti-V5 antibody (Ab). Ig fusion proteins used for immunoprecipitation were detected by anti-human IgG Ab.

RESULTS

Construction and Characterization of Recombinant HSV-1 (McKrae) Viruses

To facilitate the construction of recombinant viruses carrying specific mutations in gK and other genes, the HSV-1(McKrae) genome was cloned as an artificial bacterial chromosome essentially as described previously. Specifically, the bac plasmid was inserted within the intergenic region between the UL3 and UL4 genes. The bac plasmid contained a gene cassette encoding the green fluorescence protein (GFP) under the human cytomegalovirus immediate early promoter (HCMV-IE) (Fig.3.1: I). The cloned McKrae genome (McKbac) was subsequently utilized for the construction of recombinant viruses carrying deletions of 37 (ΔgK31-68) and 86 (ΔgK31-117) using the two-step markerless red recombination mutagenesis system (Tischer, von Einem et al. 2006), as previously described for the construction of similar mutant viruses in the HSV-1(F) genomic background (Chouljenko, Iyer et al. 2009)

The McKbac virus produced viral plaques on Vero cells that were similar to the HSV-1(McKrae) wild-type virus (not shown). The McK(gKΔ31-117) recombinant virus produced very small viral plaques containing on an average 2-7 cells per plaque. In contrast, the McK(gKΔ31-68) virus produced viral plaques that were approximately half-the-size of the McKbac plaques. Infection of VK302 cells, that express the HSV-1(KOS) gene under the gD promoter control resulted in efficient complementation for plaque morphology and virus spread for the McK (gKΔ31-68) virus, but not for the McK(gKΔ31-117) virus (Fig. 3.2:I).

These plaque phenotypes were consistent with similar plaque morphologies and cellular spread defects, exhibited by the gKΔ31-117 and gKΔ31-68 mutations, constructed on the HSV-1(F) genetic background with the following exceptions: 1) the McK(gKΔ31-68) virus produced partially syncytial plaques in VK302 cells, unlike the HSV-1(F) (gK31-68) virus characterized

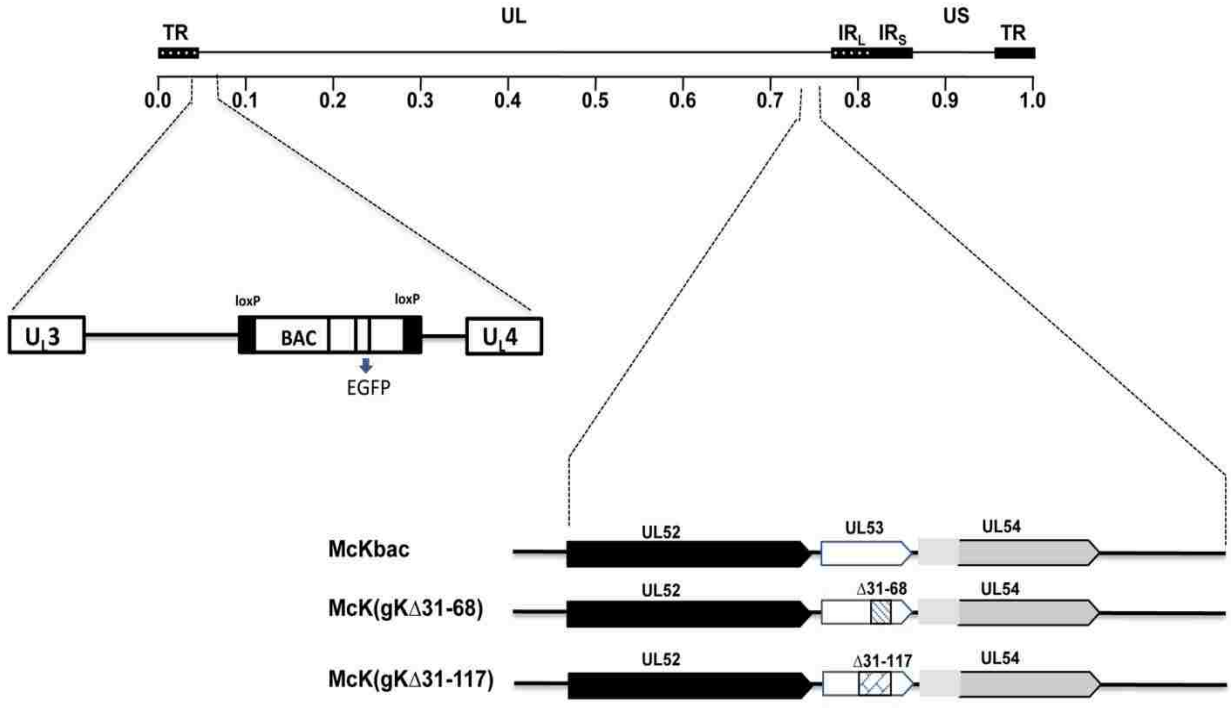

Figure 3.1: Cloning of the HSV-1 (McKrae) genome into a BAC and schematic representation of mutant recombinant viruses. Linear representation of the HSV-1 genome structure, with two unique regions (long and short), each flanked by a pair of terminal repeats. An expanded region of the HSV-1 genome between intergenic region U_L3 and U_L4 is shown. The BAC containing an EGFP sequence was inserted between the intergenic region U_L3 and U_L4 to create a recombinant virus McKrae BAC (McKbac). The McKbac virus was utilized for the construction of gK mutant viruses. The mutant viruses gKΔ31-68 (encoding gK carrying an in-frame deletion of aa 31 to 68), and gKΔ31-117 (encoding gK carrying an in-frame deletion of aa 31 to 117).

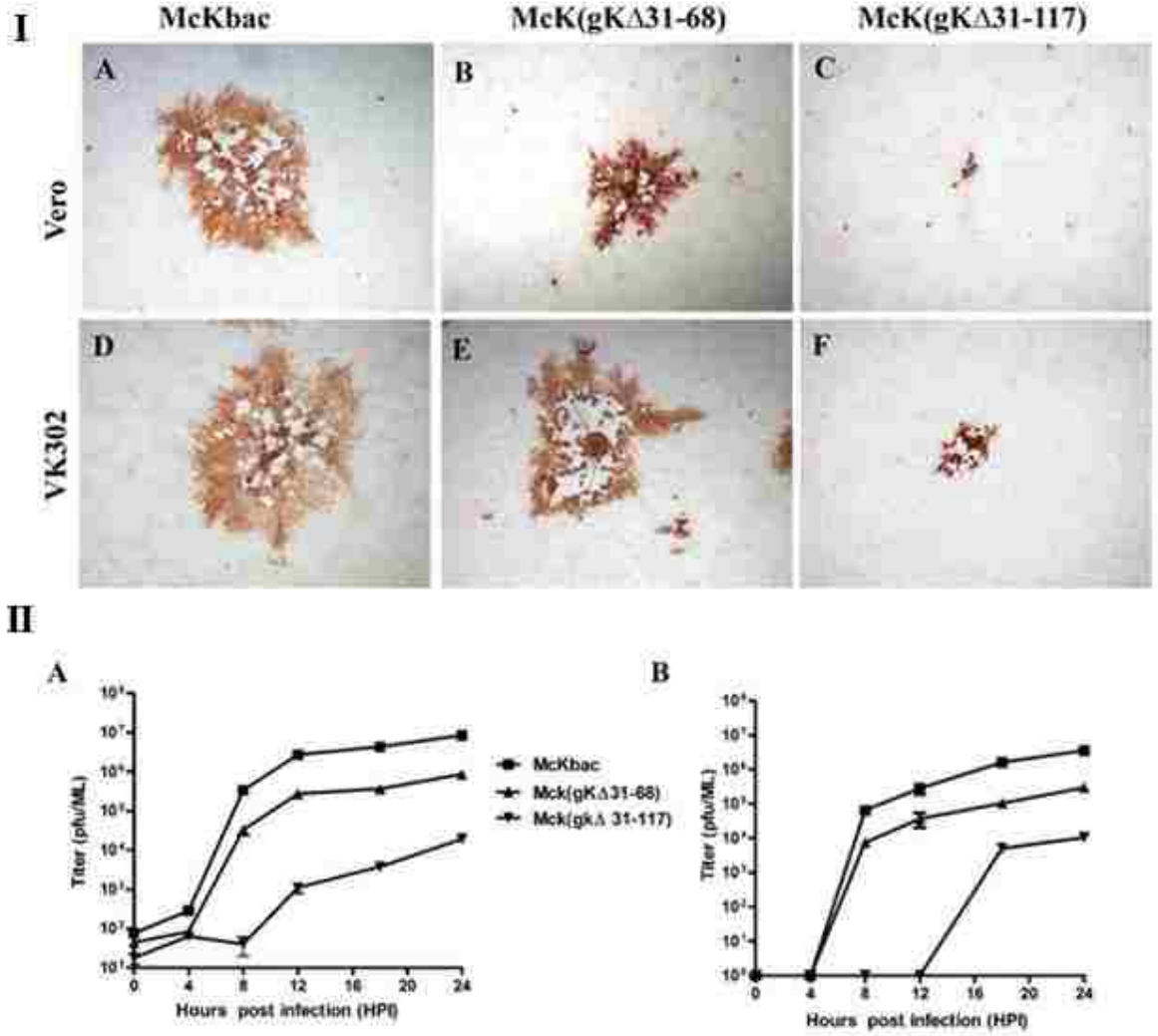

Figure 3.2: (I) Plaque morphologies of gK mutant and wild-type HSV-1(McKrae) viruses. Vero cells were infected with an MOI of 0.001, and viral plaques were fixed with methanol and stained with anti-HSV antibodies as described in Materials and Methods. Representative viral plaques of all gK mutant viruses and the HSV-1(McKrae) wild-type virus are shown on both Vero (A to C) and VK302 (D to F) cells. **(II) One-step replication kinetics of HSV-1(Mckrae) wild-type (wt) and gK mutant viruses.** Vero cells were infected at either a low MOI (MOI of 0.1) or a high MOI (MOI of 2), and the numbers of infectious viruses produced were determined on VK302 cells at different times post infection. Viral titers after high -MOI infection are shown in panel A. Low-MOI titers are shown in panel B.

previously (Chouljenko, Iyer et al. 2009), 2) the McK(gKΔ31-117) virus was not efficiently complemented in VK302 cells, unlike the HSV-1(F)(gKΔ31-117) virus (Chouljenko, Iyer et al. 2009).

The replication kinetics of both McKbac and McKrae gK mutant viruses revealed that the gKΔ31-68 mutant virus replicated quite efficiently in Vero cells achieving viral titers of approximately one log less than the parental McKbac virus. While the gKΔ31-117 virus replicated inefficiently producing viral titers that were more than 3 logs less than the McKbac virus at both low MOI (0.2) and relatively high MOI (2) (Fig.3.2:II).

Ultrastructural Characterization of Wild-type and Mutant Viruses

The ultrastructural phenotypes of all mutant viruses relative to the wild-type parental virus were investigated, utilizing transmission electron microscopy at 18 hpi by visually examining fifty individual virus-infected Vero cells. The wild-type virus did not exhibit any apparent defects in cytoplasmic virion envelopment and egress. Fully enveloped virion particles were observed both in intracellular compartments as well as in extracellular spaces. (Fig.3.3). Similarly, mutant virus Mck(gKΔ31-68) did not show any apparent defect in envelopment and fully enveloped virion particles were excreted out of infected cells. Ultrastructural visualization of Vero cells infected with mutant virus Mck(gKΔ31-117) showed severe defects in virion envelopment and egress, characterized by the presence of numerous unenveloped capsids in the cytoplasm. These results are in agreement with the ultrastructural morphologies of mutant viruses carrying the same gK mutations in the HSV-1(F) genetic background (Chouljenko, Iyer et al. 2009) .

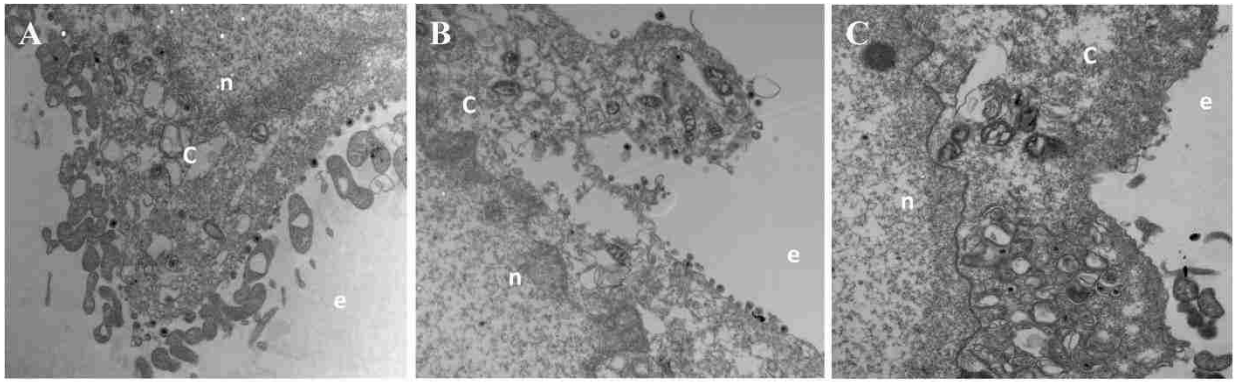

Figure 3.3: Ultrastructural morphology of wild-type and mutant viruses. Electron micrographs of Vero cells infected at an MOI of 2 with A) McKbac B) McK(gKD31-68) C) McK(gKD31-68) and processed for electron microscopy at 18 hpi are shown. Nucleus (n), cytoplasm (c), and extracellular space (e) are marked**.**

Characterization of McKbac and gK Mutant Viruses Entry into Vero and Chinese Hamster Ovary (CHO) Cells Expressing Different HSV-1 Cellular Receptors

We have shown previously that recombinant viruses gKΔ31-68 and gKΔ31-117 show substantial defect in virus entry into Vero cells (Chouljenko, Iyer et al. 2009). To ascertain the relative efficiencies of virus entry into cells, viruses were allowed to enter into their target cells for an hour at 34°C and virus entry was detected by monitoring the level of ICP4 protein expression at 8-12 hpi, as described previously (Jambunathan, Chowdhury et al. 2011) (see also Materials and Methods). Both gK mutant viruses entered into Vero cells, albeit with decreased efficiencies as compared to the McKbac virus (Fig.3.4: A). The entry defect associated with these gK mutations was particularly pronounced in PILRα expressing CHO cells in which both gK mutant viruses entered up to 9-fold less efficiently than the McKbac virus (Fig.3.4: B). In contrast, all viruses entered efficiently into both CHO-nectin-1 and CHO-HVEM cells, while K-mutant viruses appeared to enter slightly more efficiently than the McKbac virus (Fig.3.4: C, D).

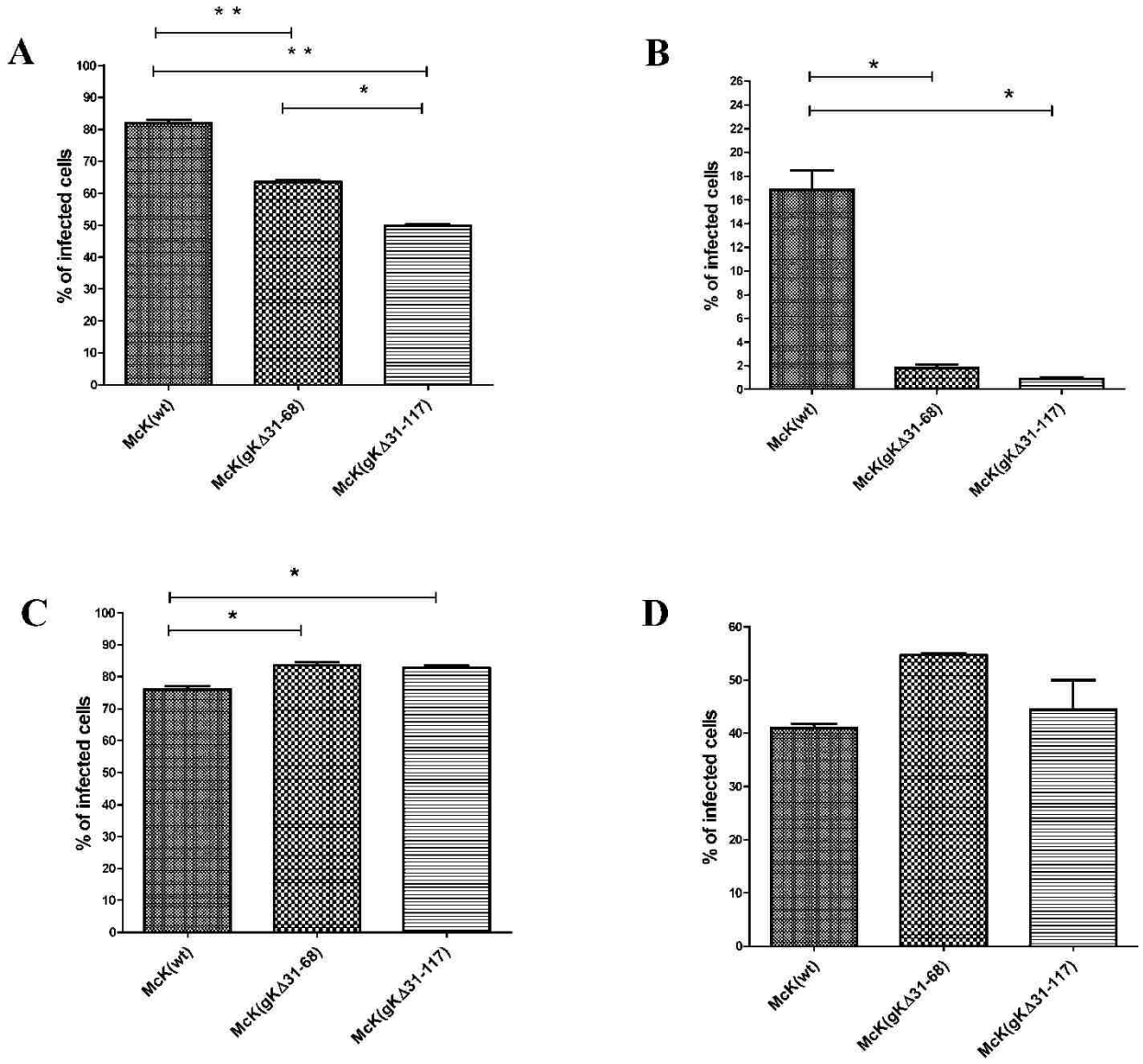

Figure 3.4: Comparison of entry efficiencies of McKbac wild type and gK-mutant viruses. (A) Entry into Vero cells. (B) Entry into CHO-PILRα. (C) Entry CHO-nectin-1. (D) Entry into CHO-HVEM (D). All cells were infected with Mckbac and gK mutant viruses gKΔ31-68 and gK Δ31-117 with an MOI of 1. At 8 h post-infection, the cells were stained with anti-ICP4 antibody and analyzed by flow cytometry to determine the percentage of infected cells. (Statistical difference, $p \leq 0.05$; * represents statistically different, ** represents significant statistical differences)

The Effect of Lysosomotropic Agents on Entry of McKbac and gK Mutant Viruses, into Dfferent Cell Types

It has been reported that HSV-1 enters into HeLa and CHO-nectin-1 cells via pH-dependent endocytosis (Nicola, McEvoy et al. 2003). To investigate the pH dependence of McKbac and gK mutant viruses into different cells, lysosomotropic inhibitors, NH4Cl, monensin and bafilomycin (inhibit the acidification of endosomes) were utilized and compared to virus entry of vesicular stomatitis virus (VSV), which is known to enter via pH-sensitive endocytosis (Le Blanc, Luyet et al. 2005). Monensin and NH_4Cl inhibited VSV entry into CHO-nectin-1 cells in a dose-dependent manner. However, both inhibitors did not inhibit McKbac nor gK mutant virus entry (Fig.3.5: A, B, C, D). Similarly, monensin, NH4Cl, as well as bafilomycin did not inhibit McKbac or gK mutant virus entry into CHO-PILRα cells (Fig.3.5: E, F, G).

The Effect of gK Mutations on the Ability of gB to Cause Virus-Induced Cell Fusion in CHO Cells Expressing gB or gD-Specific Receptors.

To investigate the relationship between gB-induced cell fusion and specific viral receptors, a previously characterized set of viruses that contain deletions in the carboxyl terminus of gB and amino terminus of gK was utilized (gKΔ31-68, gBΔ28syn, gKΔ31-68/gBΔ28syn). CHO nectin-1, CHO-HVEM and CHO-PILRα cells were infected with these mutant viruses and the wild type virus (HSV-1 F). The gBΔ28syn virus caused extensive fusion in all cells, as it has been previously shown for Vero cells (Chouljenko, Iyer et al. 2009). In contrast, the gKΔ31-68/gBΔ28syn that does not cause fusion in Vero cells caused extensive cell fusion only in CHO-nectin-1 cells, but limited fusion in CHO-PILRα cells (Fig. 3.6).

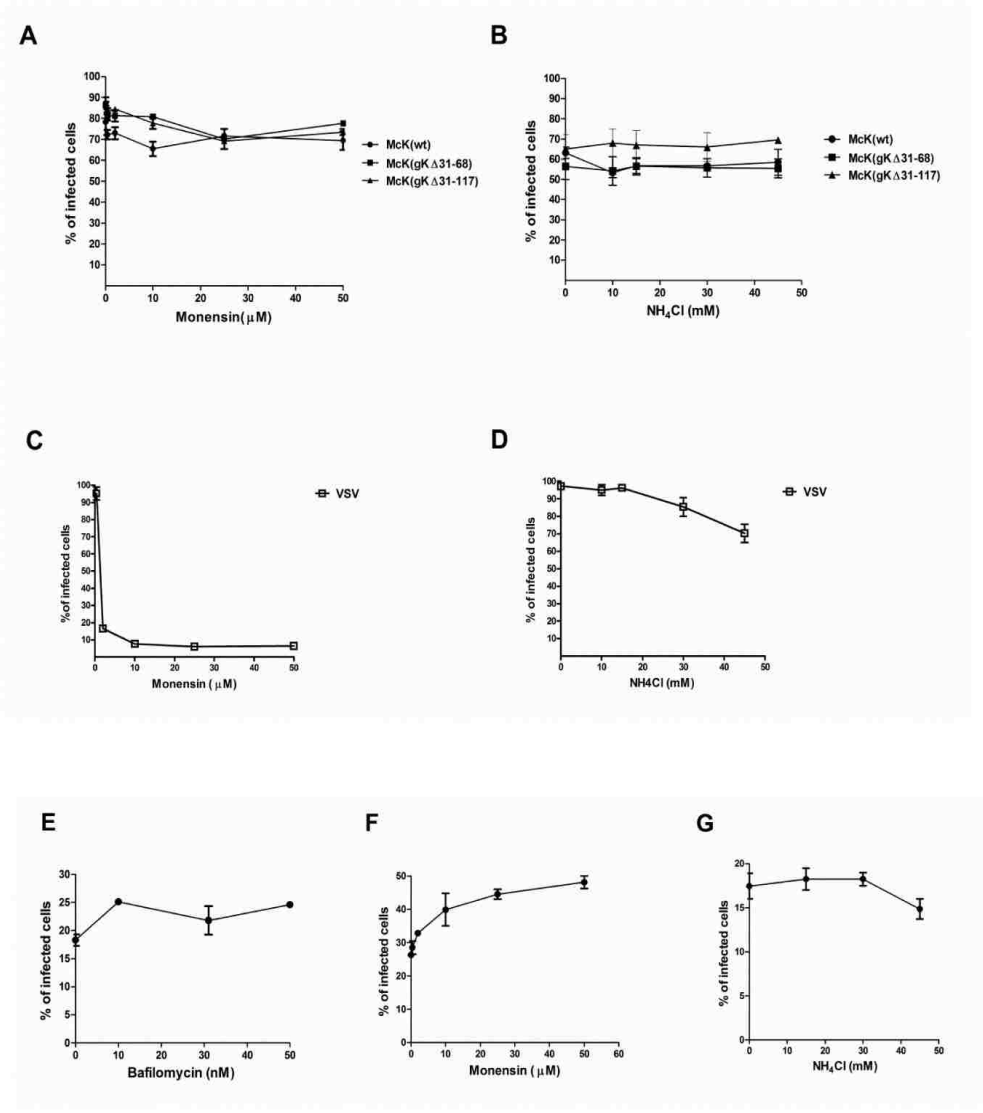

Figure 3.5: The effect of lysosomotropic agents on McKbac and gK mutant virus entry. Cells (CHO-nectin-1) were pretreated with different concentrations of ammonium chloride (Panel B,D) and monensin (Panel A,C) and infected with Mckbac and Mckbac gK mutants (Panel A and B) or VSV (Panel C and D) for 1 h at 34°C in the presence of the inhibitor. One hour after infection the inoculum was removed and the cells were incubated in continued presence of the inhibitor at 37°C until the cells were processed for flow cytometry. The cells were stained with anti-ICP4 antibody and analyzed by flow cytometry to determine the percentage of infected cells. Cells (CHO-PILRα were pretreated with different concentrations of ammonium chloride (G), monensin(E) and bafilomycin(F) and infect with HSV-1(Mckrae) as described above. 6 hours post infection the percentage of GFP positive cells were calculated by FACS analysis.

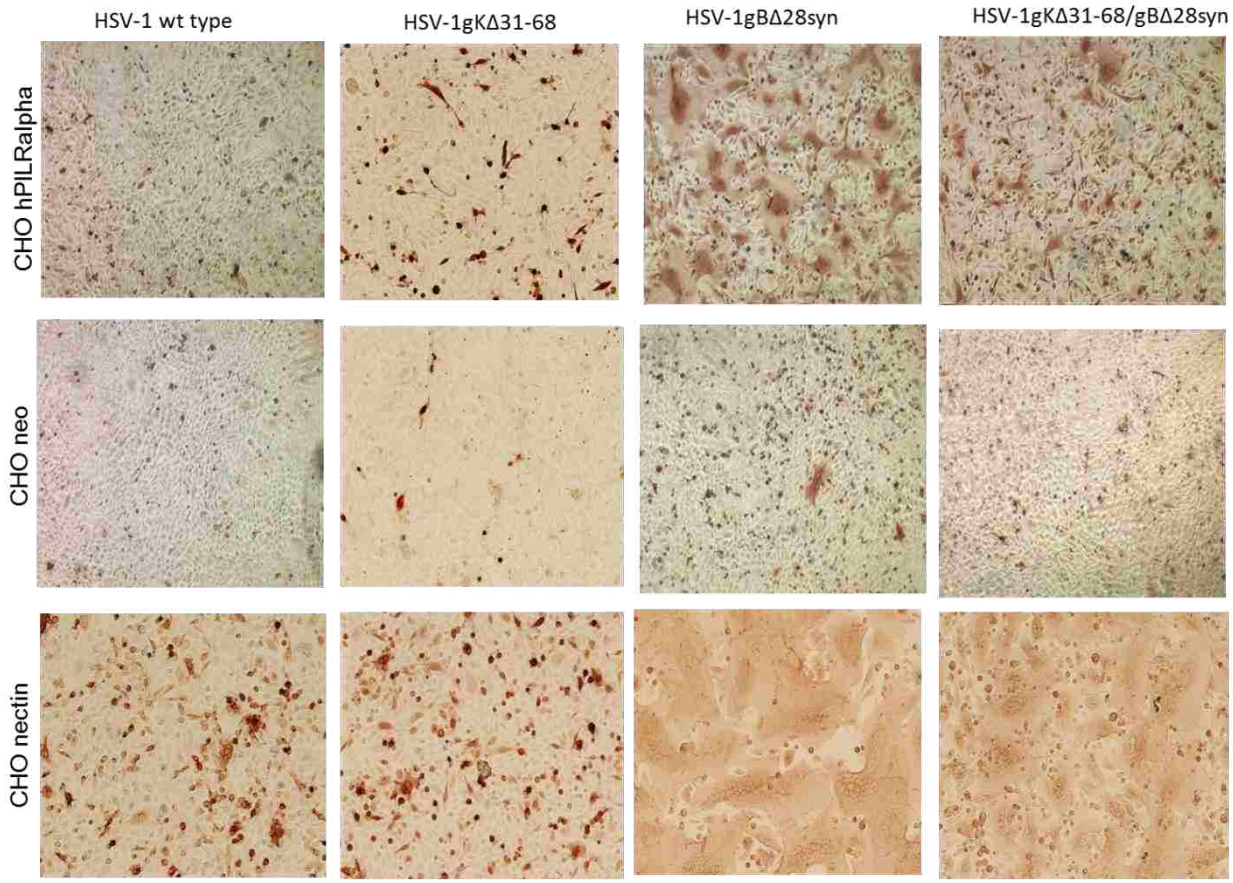

Figure 3.6: Visualization of relative extent of virus-induced cell fusion in CHO cells expressing different cellular receptors. CHO- nectin1, CHO-hPILRα and CHO neo were infected with HSV-1 (F), HSV-1 gKΔ31-68, HSV-1Δ28 gBsyn and HSV-1gKΔ31-68/gBΔ28syn. 14 hpi the cells were fixed and stained with anti-HSV antibody.

PILRα Interacts with gB, gK and UL20p in Virus-Infected Cells.

Glycoprotein B (gB) is known to interact with the amino terminus of PILR (Satoh, Arii et al. 2008). Moreover, we have shown that the amino terminus of gK, as a free peptide, interacted with the amino terminus of gB and could complement gB-mediated cell fusion (Chouljenko, Iyer et al. 2009). To investigate whether PILRα interacted simultaneously with both gB and gK, the purified PILRα-Ig protein was mixed with cellular extracts obtained from infected cells and

immunoprecipitated using magnetic beads coated with protein A (see Materials and Methods). Immunoprecipitates were subsequently tested for the presence of gB, gD, gK and UL20 using specific antibodies for each protein in western immunoblots. Detection of gK and UL20 was accomplished through the use of the YE102-VC1 virus, which expresses gK tagged with the V5 epitope and UL20 tagged with the 3xFLAG epitope (Jambunathan, Chowdhury et al. 2011). Viral infected lysates were immunoprecipitated with soluble PILRα (Fig. 3.7: A, C, E, G, I) and control IgG fusion protein CD200 IgG (Fig.3.7: B, D, F, H J). PILRα-Ig fusion protein immunoprecipitated gB (C, lane 2), UL20p (E, lane 2) and glycoprotein K (G, lane 2) but not gD (A, lane 2). The control CD200 IgG fusion protein did not react with any of the viral proteins (Fig.3.7).

DISCUSSION

Glycoprotein K and UL20p are highly conserved among alpha herpesviruses, suggesting that they serve similar functions in the lifecycle of these viruses. Both these proteins of HSV-1 have been intimately associated with membrane fusion phenomena since mutations in either gK or UL20 cause extensive virus-induced cell fusion. Moreover, both gK and UL20 are structural components of the virion particle and function during virus entry in which the viral membrane fuses with either the plasma or endosomal membranes. Recent evidence suggests that gK and UL20 modulate these membrane fusion phenomena by direct interactions with viral glycoprotein B (gB), which is considered the main fusogenic viral glycoprotein (Heldwein, Lou et al. 2006). In this researchwork, we have utilized CHO cells expressing individual receptors to show that gK is functionally associated with gB-specific receptors for both virus entry and virus-induced cell fusion.

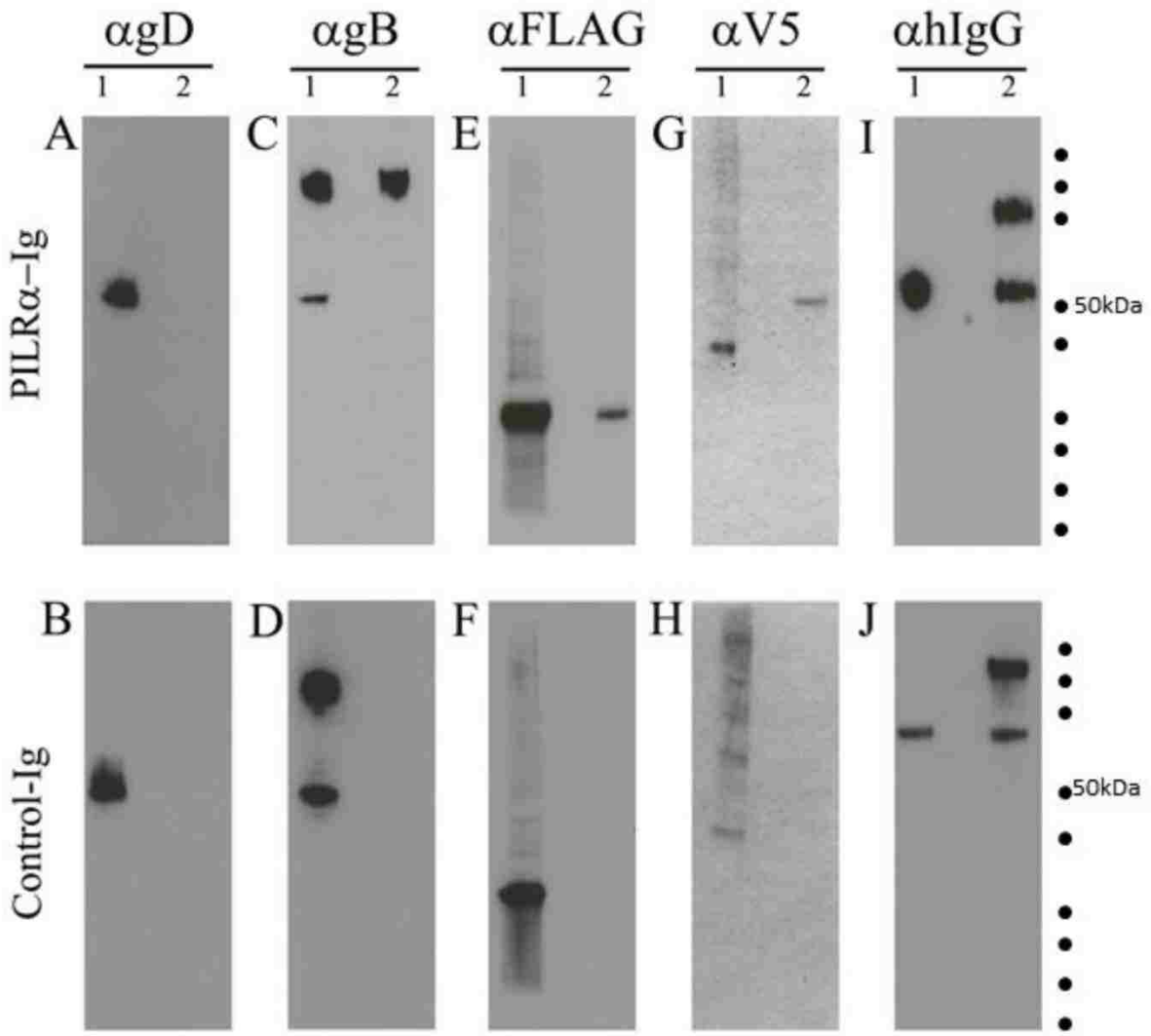

Figure 3.7: Soluble PILRα interacts with gB, gK and UL20p in virus infected cells. Western blot analysis of immunoprecipitates of PILRα ligand from HSV-1-infected cells. Vero cells were infected with a double tagged HSV-1 virus (YE102-VC1). Infected lysate were immunoprecipitated with PILRα-Ig or CD200-Ig (control) and the immunoprecipitates were separated by SDS-PAGE, followed by blotting with anti-gB ,anti-gD,anti-V5 and anti-FLAG Ab. The Ig fusion proteins used for immunoprecipitation were detected by anti-human IgG Ab (Pane I and J). Molecular mass standards are shown with dots on each panel (250, 150, 100, 75, 50, 37,25, 20, 15, and 10 kDa; Precision Plus protein standards; Bio-Rad). Lane1 –lysate from infected cells and Lane 2-lysates immunoprecipitated with PILRα-Ig or CD200-Ig.

The McKrae viral strain was isolated from an ocular clinical sample of a patient suffering from acute infection (Kaufman, Ellison et al. 1969). This viral strain is particularly virulent in mice and rabbits in comparison to other laboratory and clinical strains (Halford, Balliet et al. 2004). Initial investigations suggested that the McKrae viral strain is able to utilize the PILRα receptor more efficiently than other viral strains. Therefore, the McKrae genome was cloned into a bacterial artificial chromosome to facilitate the construction of additional recombinant viruses with defined mutations in gK and UL20. The McKbac appeared to have similar characteristics with regard to viral growth and plaque formation. Moreover, initial results suggest that insertion of the bac plasmid did not substantially affect the neurovirulence characteristics of the parental McKrae virus in mice (not shown).

Recombinant McKbac viruses containing the gKΔ31-68 and gKΔ31-117 mutations appeared to replicate in a similar manner to the previously reported identical mutations in the HSV-1(F) pYE102 bac genetic background. Plaque phenotypes, replication kinetics and ultrastructure morphologies were similar irrespective of the viral genetic background (Chouljenko, Iyer et al. 2009). However, the gKΔ31-68 mutation appeared to cause cell fusion in the gK complementing cell line VK302, while the HSV-1(F) gKΔ31-68 virus did not (Chouljenko, Iyer et al. 2009). Moreover, growth in VK302 cells did not efficiently complement the McK gKΔ31-117 defect although the HSV-1(F) gKΔ31-68 was efficiently complemented (Chouljenko, Iyer et al. 2009). Recently, we sequenced all major McKrae viral glycoprotein (gB, gC, gD, gH, gL, gK, UL20p) genes involved in membrane fusion and compared their primary structures to other viral strains. These results showed that the McKrae and F viral strains have identical gK and UL20 amino acid sequences; however there are specific amino acid differences between their respective gB glycoproteins including specific amino acid changes located at the amino terminus of gB.

Moreover, there are substantial amino acid differences between the KOS gK expressed in VK302 cells and the McKrae and F strain gK sequences (Chowdhury, Naderi et al. 2012). There are number of amino acid changes in the McKrae gB amino acid sequence when compared to that of the F gB sequence. Therefore, the KOS gK specified by the VK302 complementing cell line may be able to complement the gK mutants in the F genomic background more effectively than the same mutations in the McKrae genetic background.

Virus entry and virus-induced cell fusion are considered to be mediated by similar membrane fusion mechanisms involving viral glycoproteins gB, gD, gH and gL (Atanasiu, Saw et al. 2010). gK and UL20 are intimately associated with virus-induced cell fusion, since mutations in either gK or UL20 cause extensive cell fusion of a variety of cell types, unlike mutations in gB and UL24 that cause extensive fusion only a limited number of cell types in cell culture. A largely unexplained phenomenon is that either gK and UL20 are absolutely required for virus-induced cell fusion even in the presence of highly fusogenic mutations in gB (Chouljenko, Iyer et al. 2009). However, deletions of the amino terminus of gK or the entire gK gene caused delayed entry into Vero cells suggesting that gK and UL20 are required for entry into cells in cell culture. Here we show for the first time, that gK functions in both virus entry and virus-induced cell fusion which are intimately associated with the gB-specific receptor PILRα; since gK mutant viruses entered efficiently into CHO-nectin-1 and CHO-HVEM cells, but not CHO-PILRα cells. Furthermore, this functional association between gK and gB-specific receptor PILRα was also demonstrated by the gBΔ28syn mutant virus that caused extensive cell fusion of CHO-nectin-1 cells, but not PILRα cells in the presence of the gK mutation Δ31-68. Therefore, a potential explanation of the previously reported inability of the gKΔ31-68/gBΔ28Syn virus to cause fusion in Vero cells (Chouljenko, Iyer et al. 2009), suggests that

Vero cells express one or more gB-specific receptors that function in conjunction with gK in modulation of the fusogenic properties of gB. Vero cells do not appear to express PILRα (Chowdhury and Kousoulas, unpublished), however, they are known to express a recently discovered gB-specific receptor, the non-muscle myosin heavy chain IIA (NMHC-IIA) receptor that may function in a similar manner as PILRα.

HSV-1 virions can enter by fusion at the plasma membrane or by fusion with endosomal membranes in acidic or neutral endosomes after endocytosis (Nicola, McEvoy et al. 2003). Previous work by others researchers have indicated that HSV-1 enters via pH-dependent endocytic pathway into CHO-nectin-1 cells (Nicola, McEvoy et al. 2003). However, testing a variety of lysosomotropic inhibitors to prevent acidification of endosomes revealed that the McKbac entered into all cells in presence of increasing concentration of inhibitors i.e. through a pH-independent entry mechanism. It is possible that pH-dependence is dependent on the viral strain and specific cell line utilized in different studies. Alternatively, death of inhibitor-treated cells at higher inhibitor concentrations may appear to cause decrease in virus entry. In this regard, the relative viability of cells treated with the various inhibitors should be carefully monitored to ensure the validity of the relevant results. Clearly, HSV-1 McKbac and gK mutant viruses enter into all cells via a pH-independent mechanism which is significantly different from the entry of VSV. Additional studies are required to discern whether the different receptors influence whether the virus enter via fusion at the plasma membrane versus fusion with endosomal membranes intracellularly.

The amino terminus of gK was shown to physically and functionally interact with the amino terminus of gB (Chouljenko, Iyer et al. 2010). Here we show that soluble PILRα was able to precipitate gB, gK and UL20 suggesting that PILRα, gB, gK and UL20 form a functional protein

complex in infected cells. Based on the fact that PILRα binds to the amino terminus of gB, it is possible that gB binds both PILRα and the gK/UL20 protein complex independently of each other utilizing distinct gB domains. Alternatively, PILRα may bind gK/UL20 in the presence or absence of gB. These possibilities will be discerned in future experiments. The observed functional association between gK/UL20 and the gB-specific receptor PILRα and potentially other gB-specific receptors suggest that gK/UL20 may to some extent determine host range in various cell types that express variable amounts of gD and gB-specific receptors (Arii, Goto et al. 2010).

REFERENCES

Arii, J., H. Goto, et al. (2010). "Non-muscle myosin IIA is a functional entry receptor for herpes simplex virus-1." Nature **467**(7317): 859-862.

Arii, J., M. Uema, et al. (2009). "Entry of herpes simplex virus 1 and other alphaherpesviruses via the paired immunoglobulin-like type 2 receptor alpha." J Virol **83**(9): 4520-4527.

Atanasiu, D., W. T. Saw, et al. (2010). "Cascade of events governing cell-cell fusion induced by herpes simplex virus glycoproteins gD, gH/gL, and gB." J Virol **84**(23): 12292-12299.

Chouljenko, V. N., A. V. Iyer, et al. (2009). "The amino terminus of herpes simplex virus type 1 glycoprotein K (gK) modulates gB-mediated virus-induced cell fusion and virion egress." J Virol **83**(23): 12301-12313.

Chouljenko, V. N., A. V. Iyer, et al. (2010). "The herpes simplex virus type 1 UL20 protein and the amino terminus of glycoprotein K (gK) physically interact with gB." J Virol **84**(17): 8596-8606.

Chowdhury, S., M. Naderi, et al. (2012). "Amino Acid Differences in Glycoproteins B (gB), C (gC), H (gH) and L(gL) are Associated with Enhanced Herpes Simplex Virus type-1 (McKrae) Entry via the Paired Immunoglobulin-like Type-2 Receptor." Virol J **9**(1): 112.

Connolly, S. A., J. O. Jackson, et al. (2011). "Fusing structure and function: a structural view of the herpesvirus entry machinery." Nat Rev Microbiol **9**(5): 369-381.

Foster, T. P., X. Alvarez, et al. (2003). "Plasma membrane topology of syncytial domains of herpes simplex virus type 1 glycoprotein K (gK): the UL20 protein enables cell surface localization of gK but not gK-mediated cell-to-cell fusion." J Virol **77**(1): 499-510.

Foster, T. P., V. N. Chouljenko, et al. (2008). "Functional and physical interactions of the herpes simplex virus type 1 UL20 membrane protein with glycoprotein K." J Virol **82**(13): 6310-6323.

Foster, T. P. and K. G. Kousoulas (1999). "Genetic analysis of the role of herpes simplex virus type 1 glycoprotein K in infectious virus production and egress." J Virol **73**(10): 8457-8468.

Foster, T. P., J. M. Melancon, et al. (2004). "The Herpes Simplex Virus Type 1 UL20 Protein Modulates Membrane Fusion Events during Cytoplasmic Virion Morphogenesis and Virus-Induced Cell Fusion." J Virol **78**(10): 5347-5357.

Foster, T. P., J. M. Melancon, et al. (2004). "Herpes simplex virus type 1 glycoprotein K and the UL20 protein are interdependent for intracellular trafficking and trans-Golgi network localization." J Virol **78**(23): 13262-13277.

Foster, T. P., G. V. Rybachuk, et al. (2001). "Glycoprotein K specified by herpes simplex virus type 1 is expressed on virions as a Golgi complex-dependent glycosylated species and functions in virion entry." J Virol **75**(24): 12431-12438.

Fulmer, P. A., J. M. Melancon, et al. (2007). "UL20 Protein Functions Precede and Are Required for the UL11 Functions of Herpes Simplex Virus Type 1 Cytoplasmic Virion Envelopment." J. Virol. **81**(7): 3097-3108.

Geraghty, R. J., C. Krummenacher, et al. (1998). "Entry of alphaherpesviruses mediated by poliovirus receptor-related protein 1 and poliovirus receptor." Science **280**(5369): 1618-1620.

Halford, W. P., J. W. Balliet, et al. (2004). "Re-evaluating natural resistance to herpes simplex virus type 1." J Virol **78**(18): 10086-10095.

Heldwein, E. E., H. Lou, et al. (2006). "Crystal structure of glycoprotein B from herpes simplex virus 1." Science **313**(5784): 217-220.

Herold, B. C., D. WuDunn, et al. (1991). "Glycoprotein C of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells and in infectivity." J Virol **65**(3): 1090-1098.

Jambunathan, N., S. Chowdhury, et al. (2011). "Site-specific proteolytic cleavage of the amino terminus of herpes simplex virus glycoprotein K on virion particles inhibits virus entry." J Virol **85**(24): 12910-12918.

Jayachandra, S., A. Baghian, et al. (1997). "Herpes simplex virus type 1 glycoprotein K is not essential for infectious virus production in actively replicating cells but is required for efficient envelopment and translocation of infectious virions from the cytoplasm to the extracellular space." J Virol **71**(7): 5012-5024.

Kaufman, H. E., E. D. Ellison, et al. (1969). "Double-stranded RNA, an interferon inducer, in herpes simplex keratitis." Am J Ophthalmol **68**(3): 486-491.

Le Blanc, I., P. P. Luyet, et al. (2005). "Endosome-to-cytosol transport of viral nucleocapsids." Nat Cell Biol **7**(7): 653-664.

Lee, H. C., V. N. Chouljenko, et al. (2009). "The herpes simplex virus type 1 glycoprotein D (gD) cytoplasmic terminus and full-length gE are not essential and do not function in a redundant manner for cytoplasmic virion envelopment and egress." J Virol **83**(12): 6115-6124.

Melancon, J. M., T. P. Foster, et al. (2004). "Genetic analysis of the herpes simplex virus type 1 UL20 protein domains involved in cytoplasmic virion envelopment and virus-induced cell fusion." J Virol **78**(14): 7329-7343.

Melancon, J. M., R. E. Luna, et al. (2005). "Herpes simplex virus type 1 gK is required for gB-mediated virus-induced cell fusion, while neither gB and gK nor gB and UL20p function redundantly in virion de-envelopment." J Virol **79**(1): 299-313.

Milne, R. S., A. V. Nicola, et al. (2005). "Glycoprotein D receptor-dependent, low-pH-independent endocytic entry of herpes simplex virus type 1." J Virol **79**(11): 6655-6663.

Montgomery, R. I., M. S. Warner, et al. (1996). "Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family." Cell **87**: 427-436.

Nicola, A. V., A. M. McEvoy, et al. (2003). "Roles for endocytosis and low pH in herpes simplex virus entry into HeLa and Chinese hamster ovary cells." J Virol **77**(9): 5324-5332.

Satoh, T., J. Arii, et al. (2008). "PILRalpha is a herpes simplex virus-1 entry coreceptor that associates with glycoprotein B." Cell **132**(6): 935-944.

Shukla, D., J. Liu, et al. (1999). "A novel role for 3-O-sulfated heparan sulfate in herpes simplex virus 1 entry." Cell **99**(1): 13-22.

Shukla, D. and P. G. Spear (2001). "Herpesviruses and heparan sulfate: an intimate relationship in aid of viral entry." J Clin Invest **108**(4): 503-510.

Suenaga, T., T. Satoh, et al. (2010). "Myelin-associated glycoprotein mediates membrane fusion and entry of neurotropic herpesviruses." Proc Natl Acad Sc USA **107**(2): 866-871.

Tischer, B. K., J. von Einem, et al. (2006). "Two-step red-mediated recombination for versatile high-efficiency markerless DNA manipulation in Escherichia coli." Biotechniques **40**(2): 191-197.

Tischer, B. K., J. von Einem, et al. (2006). "Two-step red-mediated recombination for versatile high-efficiency markerless DNA manipulation in Escherichia coli." Biotechniques **40**(2): 191-197.

CHAPTER IV

AMINO ACID DIFFERENCES IN GLYCOPROTEINS B (gB), C (gC), H (gH) AND L(gL) ARE ASSOCIATED WITH ENHANCED HERPES SIMPLEX VIRUS TYPE-1 (MCKRAE) ENTRY VIA THE PAIRED IMMUNOGLOBULIN-LIKE TYPE 2 RECEPTOR α*

INTRODUCTION

Herpes simplex type 1 (HSV-1), Herpes simplex type 2 (HSV-2) and Varicella-zoster virus (VZV) are human neurotropic viruses that belong to the *Alphaherpesvirinae* subfamily and are a major cause of worldwide morbidity (Fisman, Lipsitch et al. 2002; Stahl, Mailles et al. 2011; Steiner 2011; Zamora 2011). Neurovirulence, establishment of latency in sensory neurons and intermittent reactivation are some of the unique properties of these viruses (Whitley, Kimberlin et al. 1998). Reactivation of latent virus from trigeminal ganglia can lead to recurrent ocular infections and is a leading cause of blindness in developed countries (Liesegang, Melton et al. 1989; Liesegang 2001). In very rare cases HSV-1 can spread spontaneously to the brain, causing life threatening herpes encephalitis (Steiner 2011).

Herpes virus initiates infection by binding to heparan sulfate (HS) moieties on cell surfaces using viral glycoproteins gC and gB (Herold, WuDunn et al. 1991). Moreover, viral

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glycoprotein D (gD) binds to different cellular receptors including the herpesvirus entry mediator (HVEM, or HveA), nectin-1 (HveC), or 3-O-sulfated HS (Geraghty, Krummenacher et al. 1998; Shukla, Liu et al. 1999). Apparently, gB can also bind to additional receptors including the paired immunoglobulin-like type 2 receptor alpha (PILRα), non-muscle myosin heavy chain IIA (NMHC-IIA), and myelin-associated glycoprotein (MAG) that function in virion attachment and virus entry (Satoh, Arii et al. 2008; Suenaga, Satoh et al. 2010). HSV-1 enters into epithelial and neuronal cells via a pH-independent fusion of the viral envelope with plasma membranes, while it can enter into a wide range of non-neuronal cells via either pH-independent or pH-dependent endocytosis. Binding of gD and gB to their cognate receptors is thought to trigger sequential conformational changes in gH/gL and gB causing gB-mediated fusion of the viral envelope with cellular membranes during virus entry, as well as fusion among cellular membranes (Heldwein, Lou et al. 2006; Hannah, Heldwein et al. 2007; Connolly, Jackson et al. 2011).

HSV-1 clinical isolates, such as the McKrae and H129 strains, are known to be highly virulent in rodents and rabbits in comparison to other laboratory strains such as KOS (Hill, Rayfield et al. 1987; Hill 1987; Perng, Mott et al. 2002). Several viral proteins and glycoproteins contribute to neurovirulence and latency *in vivo*, however their mode of action is not well elucidated (Cameron, McDougall et al. 1988; Kosovsky, Vojvodova et al. 2000) (Gordon, Simon et al. 1984; Kurachi, Daikoku et al. 1993; Nishiyama, Kurachi et al. 1993; Yuhasz and Stevens 1993; Perng, Thompson et al. 1995; Rajcani and Kudelova 1999; Imai, Arii et al. 2011). HSV-1 gK is known to be involved in neurovirulence (Rajcani and Kudelova 1999; Mott, Perng et al. 2007; Osorio, Mott et al. 2007; David, Baghian et al. 2008; Mott, Chentoufi et al. 2009), and is a structural component of the virion particle functioning in virus entry into epithelial cells (Foster,

Rybachuk et al. 2001; Jambunathan, Chowdhury et al. 2011), cytoplasmic virion envelopment, virion egress and virus-induced cell fusion (Hutchinson, Roop-Beauchamp et al. 1995; Jayachandra, Baghian et al. 1997; Foster and Kousoulas 1999). Recently, we showed that HSV-1 gK and UL20 physically bind to gB and gH and modulate gB-mediated membrane fusion (Chouljenko, Iyer et al. 2009; Chouljenko, Iyer et al. 2010). Also, we reported that gK is essential for virus spread in the cornea of mice, neuroinvasiveness and establishment of latency in ganglionic neurons (David, Baghian et al. 2008).

In this manuscript, we compared the relative efficiency of virus entry between HSV-1 McKrae and F strains and found that McKrae entered substantially more efficiently into Chinese hamster ovary (CHO) cells expressing the hPILRα receptor. We sequenced all viral genes encoding viral glycoproteins involved in entry and cell-to-cell fusion and identified aa differences between McKrae and F strains that may cause the observed enhanced entry of McKrae over other viral strains.

MATERIAL AND METHODS

Cells and Viruses

The clinical ocular isolate and neuroinvasive strain of HSV-1 (the parental wild-type), McKrae strain, was obtained from Dr. J. M. Hill (Louisiana State University Health Sciences Center, New Orleans, LA, USA). African green monkey kidney (Vero) cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and grown and propagated in Dulbecco's modified Eagle Medium (DMEM) supplemented with 7% fetal bovine serum (FBS) and antibiotics. The HSV-1 McKrae strain was maintained as a low passage stock on Vero cells. CHO-neo cells and CHO hnectin-1 were a kind gift from Dr. Yasushi Kawaguchi,

(The University of Tokyo, Tokyo, Japan) and were propagated in Ham's F12 medium supplemented with 10% fetal calf serum (FCS) and 250 μg/ml G418. The CHO-hPLIRα cells were obtained from Dr. Hishashi Arase, (Osaka University, Osaka Japan), and were grown in Ham's F12 medium supplemented with 10% fetal calf serum (FCS) and puromycin, 1ug/mL. The human CHO-HVEM was created in our laboratory using PiggyBac Transposon system (System Biosciences). HVEM gene was cloned into PB514BL-1 PiggyBac Dual Promoter Vector and CHO cells expressing HVEM were selected using puromycin, 12 ug/mL. All cells were cultured in non-selective medium prior to use in infectivity assays.

Viral DNA Extraction and DNA Sequencing

Viral DNA was isolated from infected Vero cells as described previously for tissue samples (David, Baghian et al. 2008). Briefly, confluent monolayers of Vero cells were infected at a multiplicity of infection of 2 and harvested by scraping at 24 hour post-infection. Cell pellets were rinsed with PBS and viral DNA was extracted using the DNAeasy Blood and Tissue Kit according to manufacturer's instruction (Qiagen, Valencia, CA, USA).

Purified McKrae DNA and Fail Safe DNA polymerase (Epicentre Biotechnologies, Madison, WI) were used for PCR. Due to the high GC content of the genomic DNA, PCR was performed using a series of primers which generated overlapping products encompassing the entire gene to be sequenced. PCR products were column-purified (Zymo Research Corp., Orange, CA) and sequencing reactions were prepared using the Big Dye Terminator v3.1 Cycle sequencing Kit (Life Technologies). DNA sequencing was performed in both directions by a primer walking strategy for each gene using an automated DNA sequencer (3130 Genetic

Analyzer, Applied Biosystems). The list of all synthetic oligo-nucleotides used for amplification of the McKrae genes and sequencing is shown in the supplementary Table 1.

Sequence Assembly and Nucleotide and Amino Acid Alignments

The Sequencher (4.10.1) software package was used to assemble the overlapping fragments using default parameters. Sequence assembly was performed using the HSV-1 (F) strain as the reference sequence (Gene Bank: GU734771.1). Nucleotide sequences of the following McKrae genes: gB, gC, gD, gH, gL, gK and UL20 were submitted to GenBank (accession numbers: JQ320080.1, JQ359758.1, JQ320083.1, JQ320081.1, JQ359759.1, JQ320082.1, JQ320079.1).

The CLC sequence Viewer version 6 software was used to align the aa sequences of H129 (GenBank: GU734772.1), F (GenBank: GU734771.1) and KOS strain (gB: AAG34116.1; gC: AAA45779.1; gL: AAA99790.1) against the derived McKrae sequences. Alignment was performed using the default parameters of the Clustal W alignment program (CLC sequence viewer software). To determine nucleotide and aa sequence variation between the McKrae and F strains, the sequences were aligned by the MegAlign program in the DNASTAR software package (Lasergene). The differences in nucleotides and aa in the coding region of each sequenced gene were tabulated in Tables 1, Table 2, Table 3 and Table 4.

Virus Entry Assays

Confluent monolayers of CHO-neo, CHO-hHVEM, CHO-hPILRα, CHO-human nectin-1 and Vero cells were infected with HSV-1 strains (McKrae and F) at a multiplicity of infection of 1 for 1 hr at 34 °C. The virus inoculum was subsequently removed, and the cultures were shifted

to 37 °C. Twelve hours post-infection (hpi), the cells were fixed and stained with anti-ICP4 antibody (Virusys, Inc., Taneytown, MD) and Alexa Fluor 647 goat anti-mouse IgG1 (Life Technologies, Grand Island, NY). The relative efficiency of virus entry was calculated by flow cytometry as the percentage of cells expressing ICP4 normalized to the CHO-neo entry values. Mean values and standard deviations of three independent experiments were calculated.

RESULTS

Both McKrae and F viruses appeared to enter with similar efficiency into Vero and CHO cells expressing either nectin-1 or HVEM (Fig. 4.1: A, C, D, respectively). In contrast, F entered into CHO cells expressing hPILRα substantially less efficiently than McKrae (Fig. 4.1: B). Comparison of McKrae, F, KOS and H129 gB aa sequences (Fig. 4.2) revealed that most of the aa differences between the four strains were located within the N-terminal 80 aa of gB. Amino acid comparison of four strains revealed that KOS had a number of aa that differed from the other HSV-1 strains. McKrae gB had a unique aa (A28V) within its predicted signal sequence (Figure 4.2). Comparison of F and McKrae gB sequences showed the following aa changes: C10R, A28V, P61A, A62P, T67P, N77P, and P79K (Table 1).

Comparison of the aa sequence of gC of HSV-1 McKrae, F, KOS and H129 revealed that aa variations between the four strains were distributed throughout the protein (Fig. 4.3). Although McKrae and KOS were more similar to each other, McKrae differed from all three strains at aa positions 289 (A289D) and 299 (F299L). Specifically, there were 20 nucleotide differences between gC of F and McKrae strains.

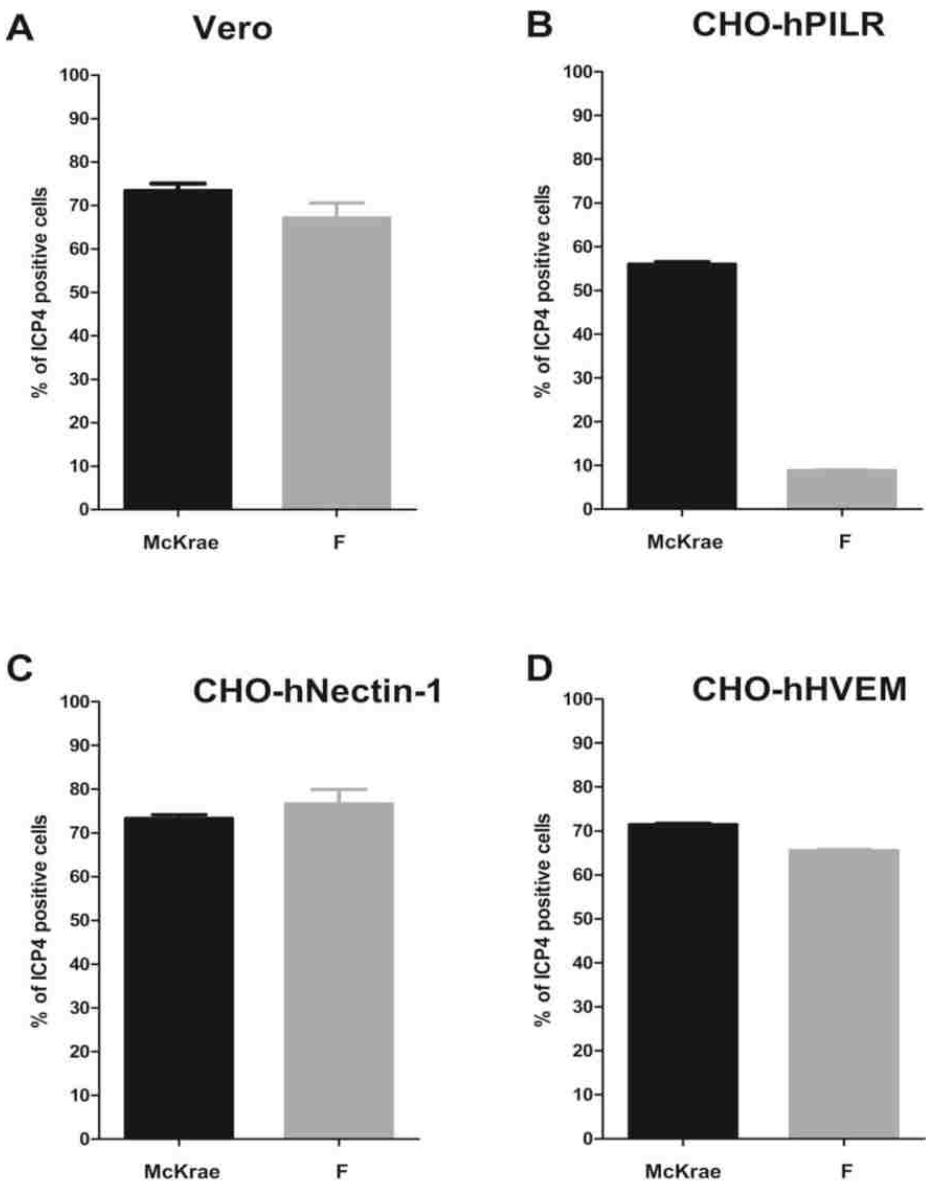

Figure 4.1: Comparison of HSV-1(F) and McKrae entry efficiencies. (A) Entry into Vero cells. (B) Entry into CHO-hPILRα. (C) Entry into human CHO-hNectin-1. (D) Entry into CHO-hHVEM (D). All cells were infected with HSV-1(F) or McKrae at an MOI of 1. At 12 h post-infection, the cells were stained with anti-ICP4 antibody and analyzed by flow cytometry to determine the percentage of infected cells.

Table 4.1 HSV-1 (F) and McKrae gB Nucleotide and Amino acid Differences

Base#	F	McKrae	Amino acid #	Amino acid change
28	T	C	10	*Cys → Arg*
83	C	T	28	*Ala → Val*
150	A	C		
181	C	G	61	*Pro → Ala*
184	G	C	62	*Ala → Pro*
199	A	C	67	*Thr → pro*
229	A	C	77	*Asn → Pro*
230	A	C	77	*Asn → Pro*
235	C	A	79	*Pro → Lys*
236	C	A	79	*Pro → Lys*
966	T	C		
1,513	C	A		
1,899	C	T		
1,932	C	T		

Out of 20 nucleotide differences only 8 resulted in the aa changes V16L, Q75K, D116G, L132P, A289D, F299L, H306R, R421H (Table 2). Nucleotide comparison between gD of HSV-1 F and McKrae strain revealed no aa differences between the McKrae and F strains (data not shown). Comparison of the McKrae, F and H129 gH aa sequences revealed that these proteins were highly conserved (Fig.4.4).

There were only three aa differences in the H1 domain of gH and two aa differences within the C terminal H3 domain of gH. There were two unique aa substitutions (S670N and C720R) in McKrae gH that were not present in either strain F or H129. Moreover, HSV-1(F) and McKrae strains differed by four aa in gH (Y147H, V150A, N670S, and R720C) (Table 3).

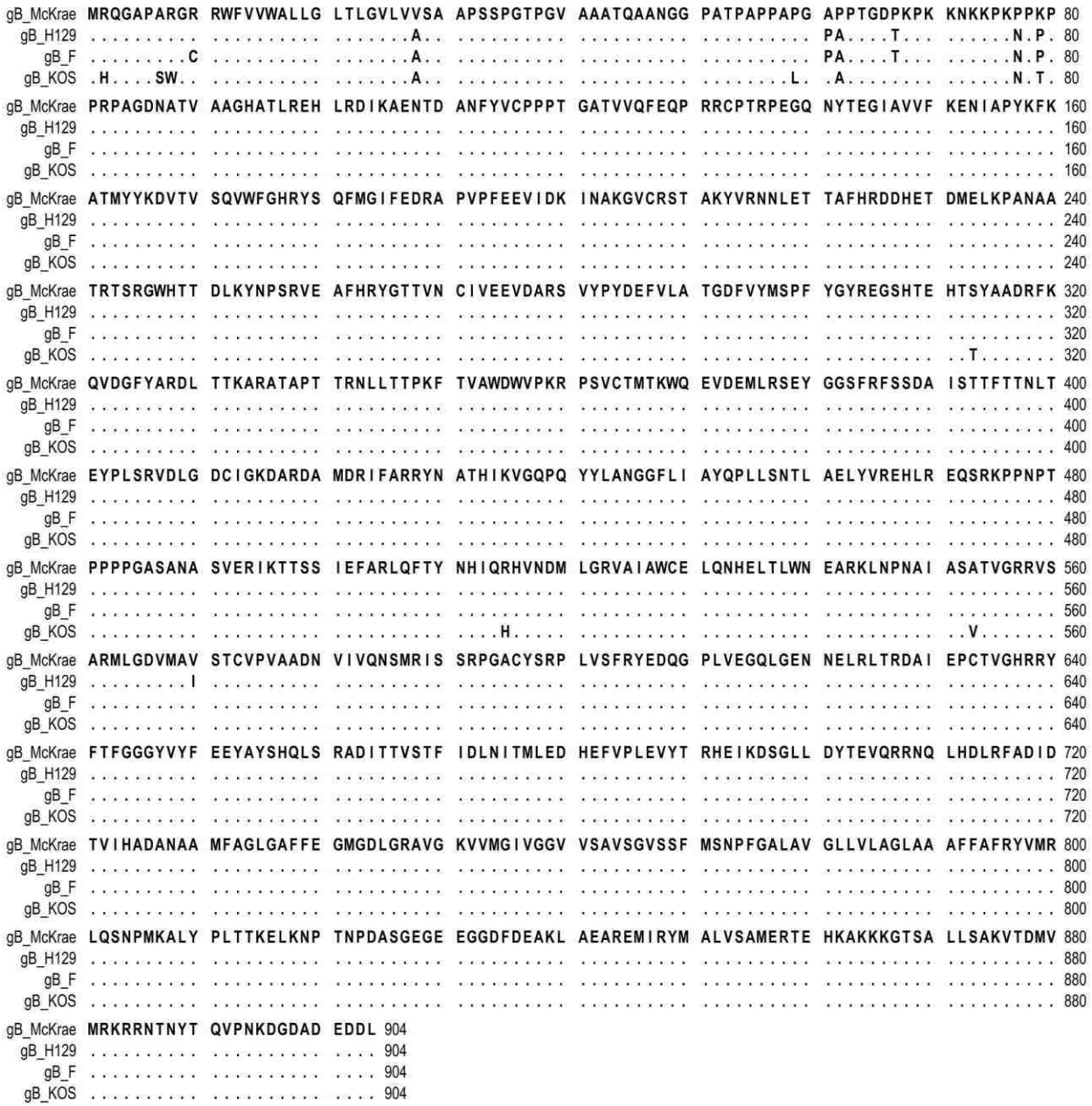

Figure 4.2: Alignment of the predicted glycoprotein B (gB) amino acid sequences specified by HSV-1 strains McKrae, H129, F and KOS. Amino acids that match the McKrae sequence are represented by dots. Amino acid changes that are different with respect to the HSV-1 McKrae strain are shown. The region of gB (aa 1–640), which contains aa substitutions is shown

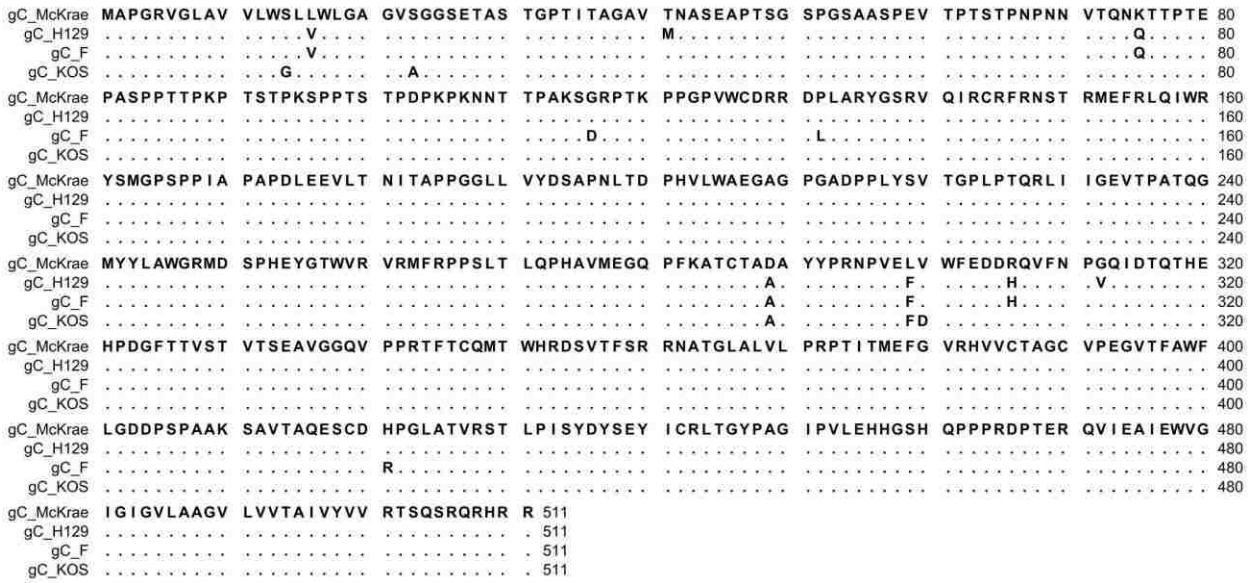

Figure 4.3: Alignment of the predicted glycoprotein C (gC) amino acid sequences specified by HSV-1 strains McKrae, H129, F and KOS. Amino acids that match the McKrae sequence are represented by dots. Amino acid changes that are different with respect to the HSV-1 McKrae strain are shown

Amino acid alignment of gL encoded by McKrae, F, H129 and KOS revealed a number of aa differences that were spread across the entire protein (Fig. 4.5). The aa sequence of strain McKrae was similar to strain H129 except at aa residues 171,181 and 212. Comparison between nucleotide sequence of gL of HSV-1 F and McKrae strain revealed 19 nucleotide differences, ten of which coded for different aa (S22P, K90R, V100G, N115D, P168L, G171R, P181S, P196S, L202S, A212T) (Table 4).

Seven of these aa changes were conserved in the H129 strain (P22, R90, G100, D115, L168, S196, S202) (Table 4). F and McKrae gK sequences were absolutely conserved despite four nucleotide changes within their genes. Similarly, there were four nucleotide changes between the UL20 gene of HSV-1 F and McKrae strains without causing any aa changes

Table 4.2 HSV-1 (F) and McKrae gC Nucleotide and Amino acid Differences

Base#	F	McKrae	Amino acid #	Amino acid change
46	G	T	16	*Val → Leu*
177	G	A		
223	C	A	75	*Gln → Lys*
279	A	G		
347	A	G	116	*Asp → Gly*
393	T	C		
394	T	C	132	*Leu → Pro*
395	T	C		
594	A	G		
619	G	A		
636	C	T		
866	C	A	289	*Ala → Asp*
897	T	G	299	*Phe → Leu*
917	A	G	306	*His → Arg*
981	A	C		
1,071	G	A		
1,254	G	A		
1,262	G	A	421	*Arg → His*
1,365	G	A		
1,485	A	G		

DISCUSSION

HSV-1 utilizes multiple receptors to attach and enter into a variety of cells including neurons. Recently, it was shown that gB binds to cellular receptors that are required for gB-mediated membrane fusion during virus entry and virus-induced cell fusion. We show here that the HSV-1 McKrae strain utilizes the gB receptor hPILRα more efficiently than HSV-1(F). DNA sequencing of all viral glycoprotein genes involved in membrane fusion indicates that there are a number of aa differences between HSV-1(F) and McKrae in gB, gC, gH and gL that may affect PILR α mediated virus entry.

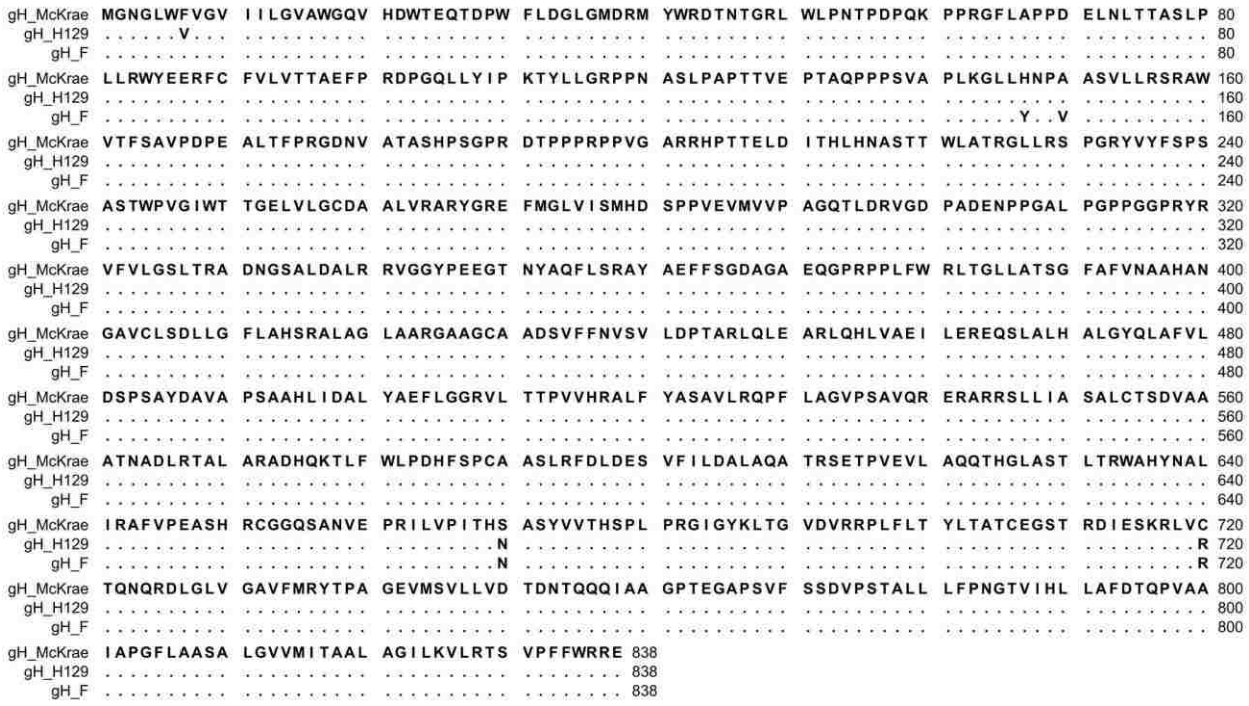

Figure 4.4: Alignment of the predicted glycoprotein H (gH) amino acid sequences specified by HSV-1 strains McKrae, H129 and F. Amino acids that match the McKrae sequence are represented by dots. Amino acid changes that are different with respect to the HSV-1 McKrae strain are shown

Table 4.3 HSV-1 (F) and McKrae gH Nucleotide and Amino acid Differences

Base#	F	McKrae	Amino acid #	Amino acid change
439	T	C	147	*Tyr → His*
449	T	C	150	*Val → Ala*
558	A	G		
1,596	G	T		
1,650	A	C		
1,899	C	T		
2,009	A	G	670	*Asn → Ser*
2,067	C	G		
2,158	C	T	720	*Arg → Cys*
2,217	A	G		
2,301	G	A		

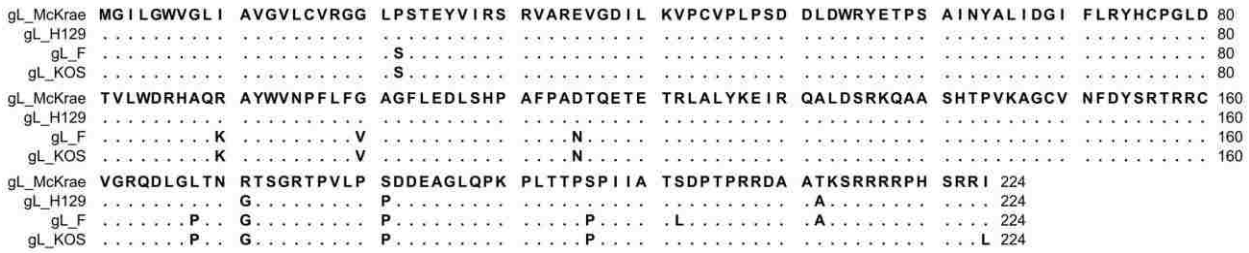

Figure 4.5: Alignment of the predicted glycoprotein L (gL) amino acid sequences specified by HSV-1 strains McKrae, H129, F and KOS. Amino acids that match the McKrae sequence are represented by dots. Amino acid changes that are different with respect to the HSV-1 McKrae strain are shown

Table 4.4 HSV**-1 (F) and McKrae gL Nucleotide and Amino acid Differences**

Base#	**F**	**McKrae**	**Amino acid #**	**Amino acid change**
21	C	T		
64	T	C	22	*Ser → Pro*
165	T	C		
261	T	C		
269	A	G	90	*Lys → Arg*
273	A	G		
294	A	G		
299	T	G	100	*Val → Gly*
327	C	T		
333	A	G		
343	A	G	115	*Asn → Asp*
489	A	C		
503	C	T	168	*Pro → Leu*
511	G	A	171	*Gly → Arg*
541	C	T	181	*Pro → Ser*
586	C	T	196	*Pro → Ser*
605	T	C	202	*Leu → Ser*
634	G	A	212	*Ala → Thr*
660	C	T		

Recently, we cloned the HSV-1(McKrae) strain as a bacterial artificial chromosome (bac) that has enabled us to rapidly produce mutant viruses, as we have previously done with HSV-1(F) bac (Melancon, Luna et al. 2005; Melancon, Fulmer et al. 2007). We have found that this viral strain efficiently enters into a variety of cells including CHO cells constitutively expressing hPILRα. hPILR α binds gB and membrane fusion can be affected by interactions of gB with viral glycoproteins gD, gH/gL and gK/UL20 (Chouljenko, Iyer et al. 2009; Chouljenko, Iyer et al. 2010; Connolly, Jackson et al. 2011). Therefore, we compared the predicted aa sequences between F and McKrae strains and found a number of aa that could contribute to the observed increased efficiency of entry of McKrae over F and other strains. Of particular importance were aa changes between F and McKrae located within the amino terminus of gB, known to bind to PILRα and gK (Wang, Fan et al. 2009; Chouljenko, Iyer et al. 2010). Specifically, gB binds to PILR α via O-linked glycans located at aa positions 53 and 480 (Wang, Fan et al. 2009). However, gB binding to PILRα is also dependent on the conformation of the amino terminus of gB, since aa insertions in gB could reduce this binding (Fan, Lin et al. 2009). Insertional mutagenesis has revealed that the structure and function of gB is not particularly flexible in tolerating aa insertions (Lin and Spear 2007). The McKrae gB contains additional proline residues at aa positions 67 and 77, while other proline residues have been re-arranged. Specifically, the F gB has a proline at aa 61, which is an alanine for the McKrae strain, but the McKrae gB contains a proline at aa 62 instead of alanine. The structure of the amino terminus of gB is not known, although the x-ray structure of gB (aa 111–726) was obtained (Heldwein, Lou et al. 2006). The additional proline residues suggest that the amino terminus of gB assumes a conformation that may affect binding to hPILR α (Williamson 1994; Tabata, Kuroki et al. 2008; Wang, Shiratori et al. 2008; Wang, Fan et al. 2009). In addition, this altered conformation of gB

may affect interactions with gK, which binds to the amino terminus of gB and regulates gB-mediated membrane fusion (Chouljenko, Iyer et al. 2010). Interestingly, six aa changes seen in McKrae versus F gB were conserved in the gB specified by the neurovirulent strain ANG (Kosovsky, Vojvodova et al. 2000) suggesting that these aa may contribute to neurovirulence.

Viral glycoproteins gD and gH have been shown to bind gB and modulate its ability to cause membrane fusion (Connolly, Jackson et al. 2011). Therefore, mutations within the extracellular portions of gD and gH, as well as gL may affect the ability of gB to utilize the hPILRα receptor. In addition, since gH forms a functional heterodimer with gL (Fan, Lin et al. 2009), it is possible that the observed aa differences between McKrae and F within gH domain H1 may affect interaction with gL, known to bind exclusively to this domain (Chowdary, Cairns et al. 2010). The carboxyl terminus of gH has been shown to be important for virus-induced cell fusion (Jackson, Lin et al. 2010). Therefore, the observed aa changes N670S and R720C may alter virus entry kinetics. Multiple aa changes in gL between McKrae and F (S22P, K90R, V100G, N115D) are within the gL domain known to interact with gH and may affect gH/gL cell-surface expression, cell fusion and virus entry.

Positively charged aa residues are known to be critical for interactions with negatively charged sulfate/carboxylate groups of the HS chain (Cardin and Weintraub 1989). Moreover, basic aa residues are known to be critical for HS-binding activity (Trybala, Roth et al. 2002). Therefore, the gC Q75K and H306R aa changes in McKrae compared to F may cause increased HS binding. Previous studies have delineated the HS-binding domain that interacts with gC amino terminal residues to be located between aa 33 and 123 (Tal-Singer, Peng et al. 1995), and between 129 and 247 (Trybala, Bergstrom et al. 1994). Therefore amino acid changes in

McKrae gC (aa 75, 116 and 132) may result in increased initial binding of McKrae gC to HS moieties on cell surfaces.

The hPILR α gene is expressed on cells of the immune system (monocytes, dendritic cells, NK cells, B cells, macrophages, neutrophils, eosinophils, mast cells), as well as neurons (Satoh, Arii et al. 2008; Fan and Longnecker 2010). Moreover, HSV-1 enters into human corneal epithelial cells (HCE) via the nectin-1, HVEM and PILRα receptors (Shah, Farooq et al. 2010). Additional experiments are needed to determine the biological and pathogenic implications of increased utilization of the PILRα receptor by the HSV-1(McKrae). The availability of the McKrae strain as a bacterial artificial chromosome will enable the rapid construction of mutant viruses that could be used to elucidate the role of each viral glycoprotein in PILR α mediated virion entry.

REFERENCES

Cameron, J. M., I. McDougall, et al. (1988). "Ribonucleotide reductase encoded by herpes simplex virus is a determinant of the pathogenicity of the virus in mice and a valid antiviral target." J Gen Virol **69 (Pt 10)**: 2607-2612.

Cardin, A. D. and H. J. Weintraub (1989). "Molecular modeling of protein-glycosaminoglycan interactions." Arteriosclerosis **9**(1): 21-32.

Chouljenko, V. N., A. V. Iyer, et al. (2009). "The amino terminus of herpes simplex virus type 1 glycoprotein K (gK) modulates gB-mediated virus-induced cell fusion and virion egress." J Virol **83**(23): 12301-12313.

Chouljenko, V. N., A. V. Iyer, et al. (2010). "The herpes simplex virus type 1 UL20 protein and the amino terminus of glycoprotein K (gK) physically interact with gB." J Virol **84**(17): 8596-8606.

Chowdary, T. K., T. M. Cairns, et al. (2010). "Crystal structure of the conserved herpesvirus fusion regulator complex gH-gL." Nat Struct Mol Biol **17**(7): 882-888.

Connolly, S. A., J. O. Jackson, et al. (2011). "Fusing structure and function: a structural view of the herpesvirus entry machinery." Nat Rev Microbiol **9**(5): 369-381.

David, A. T., A. Baghian, et al. (2008). "The herpes simplex virus type 1 (HSV-1) glycoprotein K(gK) is essential for viral corneal spread and neuroinvasiveness." Curr Eye Res **33**(5): 455-467.

Fan, Q., E. Lin, et al. (2009). "Differential effects on cell fusion activity of mutations in herpes simplex virus 1 glycoprotein B (gB) dependent on whether a gD receptor or a gB receptor is overexpressed." J Virol **83**(15): 7384-7390.

Fan, Q. and R. Longnecker (2010). "The Ig-like v-type domain of paired Ig-like type 2 receptor alpha is critical for herpes simplex virus type 1-mediated membrane fusion." J Virol **84**(17): 8664-8672.

Fisman, D. N., M. Lipsitch, et al. (2002). "Projection of the future dimensions and costs of the genital herpes simplex type 2 epidemic in the United States." Sex Transm Dis **29**(10): 608-622.

Foster, T. P. and K. G. Kousoulas (1999). "Genetic analysis of the role of herpes simplex virus type 1 glycoprotein K in infectious virus production and egress." J Virol **73**(10): 8457-8468.

Foster, T. P., G. V. Rybachuk, et al. (2001). "Glycoprotein K specified by herpes simplex virus type 1 is expressed on virions as a Golgi complex-dependent glycosylated species and functions in virion entry." J Virol **75**(24): 12431-12438.

Geraghty, R. J., C. Krummenacher, et al. (1998). "Entry of alphaherpesviruses mediated by poliovirus receptor-related protein 1 and poliovirus receptor." Science **280**(5369): 1618-1620.

Gordon, Y. J., P. L. Simon, et al. (1984). "Neurovirulence of an herpes simplex type 1 thymidine kinase negative mutant determined by virus biochemical defect and host immune system in mice. Brief report." Arch Virol **80**(2-3): 225-229.

Hannah, B. P., E. E. Heldwein, et al. (2007). "Mutational evidence of internal fusion loops in herpes simplex virus glycoprotein B." J Virol **81**(9): 4858-4865.

Heldwein, E. E., H. Lou, et al. (2006). "Crystal structure of glycoprotein B from herpes simplex virus 1." Science **313**(5784): 217-220.

Herold, B. C., D. WuDunn, et al. (1991). "Glycoprotein C of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells and in infectivity." J Virol **65**(3): 1090-1098.

Hill, J. M., M. A. Rayfield, et al. (1987). "Strain specificity of spontaneous and adrenergically induced HSV-1 ocular reactivation in latently infected rabbits." Curr Eye Res **6**(1): 91-97.

Hill, T. J. (1987). "Ocular pathogenicity of herpes simplex virus." Curr Eye Res **6**(1): 1-7.

Hutchinson, L., C. Roop-Beauchamp, et al. (1995). "Herpes simplex virus glycoprotein K is known to influence fusion of infected cells, yet is not on the cell surface." J Virol **69**(7): 4556-4563.

Imai, T., J. Arii, et al. (2011). "Role of the herpes simplex virus 1 Us3 kinase phosphorylation site and endocytosis motifs in the intracellular transport and neurovirulence of envelope glycoprotein B." J Virol **85**(10): 5003-5015.

Jackson, J. O., E. Lin, et al. (2010). "Insertion mutations in herpes simplex virus 1 glycoprotein H reduce cell surface expression, slow the rate of cell fusion, or abrogate functions in cell fusion and viral entry." J Virol **84**(4): 2038-2046.

Jambunathan, N., S. Chowdhury, et al. (2011). "Site-specific proteolytic cleavage of the amino terminus of herpes simplex virus glycoprotein K on virion particles inhibits virus entry." J Virol **85**(24): 12910-12918.

Jayachandra, S., A. Baghian, et al. (1997). "Herpes simplex virus type 1 glycoprotein K is not essential for infectious virus production in actively replicating cells but is required for efficient envelopment and translocation of infectious virions from the cytoplasm to the extracellular space." J Virol **71**(7): 5012-5024.

Kosovsky, J., A. Vojvodova, et al. (2000). "Herpes simplex virus 1 (HSV-1) strain HSZP glycoprotein B gene: comparison of mutations among strains differing in virulence." Virus Genes **20**(1): 27-33.

Kurachi, R., T. Daikoku, et al. (1993). "The pathogenicity of a US3 protein kinase-deficient mutant of herpes simplex virus type 2 in mice." Arch Virol **133**(3-4): 259-273.

Liesegang, T. J. (2001). "Herpes simplex virus epidemiology and ocular importance." Cornea **20**(1): 1-13.

Liesegang, T. J., L. J. Melton, 3rd, et al. (1989). "Epidemiology of ocular herpes simplex. Incidence in Rochester, Minn, 1950 through 1982." Arch Ophthalmol **107**(8): 1155-1159.

Lin, E. and P. G. Spear (2007). "Random linker-insertion mutagenesis to identify functional domains of herpes simplex virus type 1 glycoprotein B." Proc Natl Acad Sci U S A **104**(32): 13140-13145.

Melancon, J. M., P. A. Fulmer, et al. (2007). "The herpes simplex virus UL20 protein functions in glycoprotein K (gK) intracellular transport and virus-induced cell fusion are independent of UL20 functions in cytoplasmic virion envelopment." Virol J **4**: 120.

Melancon, J. M., R. E. Luna, et al. (2005). "Herpes simplex virus type 1 gK is required for gB-mediated virus-induced cell fusion, while neither gB and gK nor gB and UL20p function redundantly in virion de-envelopment." J Virol **79**(1): 299-313.

Mott, K. R., A. A. Chentoufi, et al. (2009). "The role of a glycoprotein K (gK) CD8+ T-cell epitope of herpes simplex virus on virus replication and pathogenicity." Invest Ophthalmol Vis Sci **50**(6): 2903-2912.

Mott, K. R., G. C. Perng, et al. (2007). "A recombinant herpes simplex virus type 1 expressing two additional copies of gK is more pathogenic than wild-type virus in two different strains of mice." J Virol **81**(23): 12962-12972.

Nishiyama, Y., R. Kurachi, et al. (1993). "The US 9, 10, 11, and 12 genes of herpes simplex virus type 1 are of no importance for its neurovirulence and latency in mice." Virology **194**(1): 419-423.

Osorio, Y., K. R. Mott, et al. (2007). "Epitope mapping of HSV-1 glycoprotein K (gK) reveals a T cell epitope located within the signal domain of gK." Virus Res **128**(1-2): 71-80.

Perng, G. C., K. R. Mott, et al. (2002). "Herpes simplex virus type 1 mutants containing the KOS strain ICP34.5 gene in place of the McKrae ICP34.5 gene have McKrae-like spontaneous reactivation but non-McKrae-like virulence." J Gen Virol **83**(Pt 12): 2933-2942.

Perng, G. C., R. L. Thompson, et al. (1995). "An avirulent ICP34.5 deletion mutant of herpes simplex virus type 1 is capable of in vivo spontaneous reactivation." J Virol **69**(5): 3033-3041.

Rajcani, J. and M. Kudelova (1999). "Glycoprotein K of herpes simplex virus: a transmembrane protein encoded by the UL53 gene which regulates membrane fusion." Virus Genes **18**(1): 81-90.

Satoh, T., J. Arii, et al. (2008). "PILRalpha is a herpes simplex virus-1 entry coreceptor that associates with glycoprotein B." Cell **132**(6): 935-944.

Shah, A., A. V. Farooq, et al. (2010). "HSV-1 infection of human corneal epithelial cells: receptor-mediated entry and trends of re-infection." Mol Vis **16**: 2476-2486.

Shukla, D., J. Liu, et al. (1999). "A novel role for 3-O-sulfated heparan sulfate in herpes simplex virus 1 entry." Cell **99**(1): 13-22.

Stahl, J. P., A. Mailles, et al. (2011). "Epidemiology of viral encephalitis in 2011." Med Mal Infect **41**(9): 453-464.

Steiner, I. (2011). "Herpes simplex virus encephalitis: new infection or reactivation?" Curr Opin Neurol **24**(3): 268-274.

Suenaga, T., T. Satoh, et al. (2010). "Myelin-associated glycoprotein mediates membrane fusion and entry of neurotropic herpesviruses." Proc Natl Acad Sc USA **107**(2): 866-871.

Tabata, S., K. Kuroki, et al. (2008). "Biophysical characterization of O-glycosylated CD99 recognition by paired Ig-like type 2 receptors." J Biol Chem **283**(14): 8893-8901.

Tal-Singer, R., C. Peng, et al. (1995). "Interaction of herpes simplex virus glycoprotein gC with mammalian cell surface molecules." J Virol **69**(7): 4471-4483.

Trybala, E., T. Bergstrom, et al. (1994). "Localization of a functional site on herpes simplex virus type 1 glycoprotein C involved in binding to cell surface heparan sulphate." J Gen Virol **75 (Pt 4)**: 743-752.

Trybala, E., A. Roth, et al. (2002). "Glycosaminoglycan-binding ability is a feature of wild-type strains of herpes simplex virus type 1." Virology **302**(2): 413-419.

Wang, J., Q. Fan, et al. (2009). "Binding of herpes simplex virus glycoprotein B (gB) to paired immunoglobulin-like type 2 receptor alpha depends on specific sialylated O-linked glycans on gB." J Virol **83**(24): 13042-13045.

Wang, J., I. Shiratori, et al. (2008). "An essential role of sialylated O-linked sugar chains in the recognition of mouse CD99 by paired Ig-like type 2 receptor (PILR)." J Immunol **180**(3): 1686-1693.

Whitley, R. J., D. W. Kimberlin, et al. (1998). "Herpes simplex viruses." Clin Infect Dis **26**(3): 541-553; quiz 554-545.

Williamson, M. P. (1994). "The structure and function of proline-rich regions in proteins." Biochem J **297 (Pt 2)**: 249-260.

Yuhasz, S. A. and J. G. Stevens (1993). "Glycoprotein B is a specific determinant of herpes simplex virus type 1 neuroinvasiveness." J Virol **67**(10): 5948-5954.

Zamora, M. R. (2011). "DNA viruses (CMV, EBV, and the herpesviruses)." Semin Respir Crit Care Med **32**(4): 454-470.

CHAPTER V

CONCLUDING REMARKS

SUMMARY

Membrane fusion plays an important role in several aspects of the lifecycle of HSV-1; viral entry, cell-to-cell fusion, viron morphogenesis. The crystal structure of glycoprotein B that was recently solved reveals that it is the sole viral fusogen of HSV-1. The function of gB is to bring about fusion between the viral membrane and cell membrane (virus entry) or between cell membranes (cell-cell fusion) by inserting its fusion loops into the target membrane and "pulling" the two membranes together. Apposition of these two membranes is followed by the formation of hemifusion intermediates, and ultimately formation of a fusion pore. This fusion pore leads to mixing of viral and cellular contents. However, unlike other members of its class, gB is unable to bring about fusion alone and is regulated by coordinated action of other viral proteins such as gD, heterodimer gH/gL and other membrane proteins. gD binding to its cognate receptor leads to conformational changes in gD that are thought to trigger the fusion machinery. According to the sequential model of fusion, gD in turn activates gH/gL and ultimately leads to conformational changes in gB from an initial pre-fusion form to its corresponding post fusion form. It is now believed that the gH/gL complex, rather than functioning as a fusogenic glycoprotein itself, interacts with gB and enables it to attain its proper conformation to mediate fusion.

Previous record from our laboratory has shown that glycoprotein K and its partner UL20p form a complex which is expressed on the surface of infected cells and plays an important role in

virus-induced cell-to-cell fusion. Recently, we reported that mutant virus gBΔ28syn known to cause extensive cell fusion, is unable to cause fusion in Vero cells in presence of a mutation in the amino terminus of gK (gKΔ31-68/gBΔ28syn). This finding supported for the role of glycoprotein K in virus induced cell fusion (Chouljenko, Iyer et al. 2009). When the missing amino terminus of gK (amino termini of gK, gKa) was provided as a peptide, the syncytial phenotype of mutant virus gKΔ31-68/gBΔ28syn is restored (Chapter II). Restoration of the syncytial phenotype of mutant virus gKΔ31-68/gBΔ28syn by the gKa peptide provided strong evidence for a functional linkage between the two glycoproteins gK and gB. This data suggested that the amino terminus of gK interacts with gB and/or other viral proteins. In this thesis, I have provided evidence, using immunoprecipitation, for direct interaction between the amino terminus of gK (gKa) and the amino terminus of gB, for the first time in a transient system (Chapter II). Subsequent experiments have revealed similar results wherein the gKa peptide interacts with gB in HSV-1 infected cells (Chouljenko, Iyer et al. 2010). Furthermore, the amino terminus of gK (gKa) can interact with other members of the fusion complex that bring about virus induced cell fusion. Three additional recombinant baculoviruses were constructed expressing gKa and truncated gHs encompassing aa; 1 to 792, 1 to 603 and 1 to 363. Co-immunoprecipitation experiments showed that gKa physically interacted with all the three truncated versions of gH (Chapter II). It is well established that UL20 and gK functionally and physically interact to form a heterodimeric complex. Therefore, to determine whether direct interactions exist between gB and UL20p, the functional partner of gK, Förster (Fluorescence) resonance energy transfer (FRET) was utilized to study plausible interaction between gB and UL20p. FRET analysis revealed that gB interacted directly with UL20p. To further map the domains of UL20p and gB that interact I focused on the amino terminus of UL20 and the carboxyl terminus of gB both of

which are predicted to be situated on the cytosolic side of the plasma membrane. By GST pull down assay I demonstrated that the aminoterminus of UL20p interacted with the carboxyl terminus of gB(Chapter II). These results show that multiple interactions exist between the viral surface proteins forming a protein complex which we have designated as the fusion complex. The function of gK/UL20p in this fusion complex is likely to be regulatory, i.e. gK/UL20p modulate, the conformation of gB such that gB assumes fusion conformation at the proper time and place. Therefore, HSV-1 has evolved this highly regulated membrane fusion mechanism in order to prevent aberrant fusion between membranes expressing fusogenic glycoproteins with adjacent cellular membranes during membrane fusion events. This control is exemplified during virus egress wherein, the virion encasing vesicles are prevented from fusing with the virion envelope. Such a regulatory mechanism may be advantageous to the virus during entry and spread and may dictate HSV-1 host range; ie, the virus fuses with only certain kind of cells that specify particular cellular receptors.

Fusion that occurs between cells can be considered analogous to fusion that occurs during viral entry whether it is at the site of the plasma membrane or in endosomal membranes. While the same sets of glycoproteins are involved in both, however, subtle differences exist between the two membrane fusion events.

We have shown that glycoprotein K plays an important role in gB mediated cell fusion by genetically deleting the amino terminus of gK and observing its effect on gB mediated cell fusion. The next logical step was to decipher whether gK plays a role in viral entry which is another membrane fusion event. Previous work by Foster et. al shows, that gK is expressed on virion surfaces (Foster, Rybachuk et al. 2001). Similairly, in recent studies have shown that, gK and UL20 are structural components of the mature virion and play a role in entry (Jambunathan,

Chowdhury et al. 2011), further supporting earlier findings. To better understand the role of gK in gB mediated entry, various cellular receptors were used to examine receptor dependent entry, Experiments performed as detailed in Chapter III of this dissertation reveal that in the presence of gB cellular receptors such as PILRα, the absence of gK's amino terminal leads to a substantial decrease in virus entry. However, in presence of gD receptors such as Nectin-1 such a differential entry is not noticed. Moreover, co-immunoprecipitation experiments revealed that PILRalpha formed a multi-protein complex with gB and gK, but not gD. Thus sit eems, that in presence of the amino termini of gK, efficiency of gB binding to PILRα increases and this leads to increased entry of the virus. My next goal was to decipher the mode of entry of gK mutant viruses into different receptor bearing cells. Contrary to published data the finding of this dissertation establishes, that HSV-1 enters CHO-nectin 1 cells in a pH independent manner. The results provide strong evidence for McKbac virus and gK mutant virus entry into CHO-nectin-1 cells through a route that does not require low pH to trigger fusion as it is not affected by endocytic inhibitors.

For viral entry studies into different receptor bearing cells, the HSV-1 McKrae strain was used, which was earlier isolated from a human ocular herpes infection, and represents a highly neurovirulent strain. Using a clinical isolate rather than a laboratory strain for entry studies is advantageous as it is more relevant to natural HSV infection. All major McKrae viral proteins that are part of the fusion complex (gB,gC,gD, gH,gL, gK and UL20p) were sequenced and their predicted secondary aa structure was compared with other clinical and laboratory strains. Comparisons revealed the McKrae and F viral strains have identical gK and UL20 amino acid sequences; however, there are specific amino acid differences between their respective gB, including specific amino acid changes located at the amino terminus of gB (Chapter IV).

Comparison of gC, gH and gL aa sequences between McKrae and F strain revealed multiple aa differences. Additionally, comparison of the entry phenotype of HSV-1 strains F and McKrae, showed that the McKrae strain entered substantially more efficiently than viral strain F in Chinese hamster ovary (CHO) cells expressing hPIRLα but not CHO-human nectin-1, CHO-human HVEM or Vero cells. This differential entry phenotype between McKrae and F on CHO-PILR α cells can be attributed to predicted amino acid sequences differences between HSV-1, F and McKrae membrane proteins (Chapter IV). These amino acid differences between McKrae and F strain may contribute to the increased virulence of McKrae versus F strain as observed *in vivo* (Unpublished data).

The results presented in this thesis further extend our knowledge and understanding of the role of gK and UL20p in virus-induced cell-cell fusion and viral-cell fusion. This research also reveals the important role of the gk amino terminus in modulating the conformation of gB by directly interacting with gB as well as other members of the fusion complex. We have found that gK plays an important role during viral entry not only in Vero cells but specifically in cells expressing a gB receptor. These results taken together indicate that gK is intimately associated with gB and plays an important role in regulating gB mediated membrane fusion.

CURRENT AND FUTURE RESEARCH CHALLENGES

The findings of this research have raised several new queries which would need to be addressed in future experiments. Specifically, viral entry and cell fusion experiments strongly implied that specific amino acid changes within the amino terminus of gB may influence binding to and utilization of the PIRLα receptor. This possibility can be directly examined by engineering McKrae gB specific mutations within other viral strains that do not efficiently

utilize this receptor. Alternatively, gB genes encoded by these viruses could be replaced by the McKrae gB gene to ascertain whether this phenomenon is gB-specific. Similar gene "swapping" experiments can be performed to ascertain whether gK and UL20p encoded by different virus strains show differential utilization of gB-specific receptors and are responsible for these differences.

The emerging interpretation of the role of both gD and gB-specific receptors in virus entry and virus-induced cell fusion is that both types of receptors are required. The theoretical explanation for entry into CHO cells engineered to express only one type of receptor is that other unknown receptors fulfill this requirement for the presence of both gD and gB viral receptors. Thus, CHO-nectin-1 cells are considered to have low levels of unknown gB-specific receptors, while CHO-PILRα cells are considered to have low levels of unknown gD-specific receptors. Presumably, the inefficient entry of viruses into CHO-PILRα cells is largely due to the critical function of gD-specific receptors, which are found in low-abundance in these cells. These receptors may function upstream of gB receptors and may be required for efficient utilization of gB-specific receptors. This hypothesis can be directly tested by engineering CHO cells that express different amounts of gD and gB specific receptors. Alternatively, transient expression of different combinations of receptors may shed additional light in the requirements for both receptors during virus entry.

The ultimate goal of understanding the functions of gK and UL20 in virus entry and spread must be addressed in the context of neuronal infections. These two proteins are uniquely expressed in all alpha herpesviruses suggesting that they serve important roles in neuronal entry and spread. In this regard, recent results from our laboratory indicate that gK-null virions are unable to enter into axonal termini of neurons in cell culture despite the apparent presence of

high levels of nectin-1; suggests the existence of gB-specific receptors on axonal membranes that are unable to function in virion entry in the absence of gK. Future experiments are needed to investigate whether PILRα or other gB-specific receptors are expressed in axonal membranes and function during virus entry in conjunction with gK and UL20p.

FINAL COMMENTS

A historic review of publications regarding gK and UL20 reveal that despite their discovery and association with extensive virus-induced cell fusion more than 30 years ago, we are still investigating the precise role of these membrane proteins in HSV-1 associated membrane fusion phenomena. It is understandable that seemingly disparate results have been obtained over the years by different laboratories largely due to the high hydrophobic character of both proteins that renders them very difficult to detect and to analyze their structure and function.

The present, research work has further defined the role of glycoprotein K and membrane protein UL20p in virus induced cell-cell fusion and entry. One of the many possibilities is that the virus may have evolved this intricate and complicated mechanism of fusion, in order to infect different cell types. gK's role in entry may be more emphatic at the neuronal ending where the virus is hypothesized to enter strictly by fusion at axonal synaptic membranes.

The ultimate aim of these entry and cell–cell fusion studies is to create fusion inhibitors that will prevent or block interaction between cellular receptors and glycoproteins as well as prevent conformational changes in gB. Prevention of fusion between the viral membrane and cell membrane will prevent viral entry and spread and thus control HSV infection. Such fusion inhibitors have been successful in controlling HIV. Enfuvirtide and T-1249 are two HIV fusion inhibitor peptides that bind to gp41 and prevent its fusogenic conformation, inhibiting viral entry

into host cells. Moreover, it may help in the discovery of new antiviral approaches and improved viral vaccines.

In conclusion, this research work clearly shows that gK and UL20 function by physically interacting with gB and gH and it has specifically for the first time provided a mechanism to show how these proteins function in modulating gB-mediated membrane fusion. The requirement for multiple viral receptors during membrane fusion has added an additional complexity in the overall membrane fusion phenomenon. New technologies that are currently undergoing development for the visualization of protein complexes will help in determining interactions between viral proteins and receptors that facilitate membrane fusion in the HSV-1 life cycle.

REFERENCES

Chouljenko, V. N., A. V. Iyer, et al. (2009). "The amino terminus of herpes simplex virus type 1 glycoprotein K (gK) modulates gB-mediated virus-induced cell fusion and virion egress." J Virol **83**(23): 12301-12313.

Chouljenko, V. N., A. V. Iyer, et al. (2010). "The herpes simplex virus type 1 UL20 protein and the amino terminus of glycoprotein K (gK) physically interact with gB." J Virol **84**(17): 8596-8606.

Foster, T. P., G. V. Rybachuk, et al. (2001). "Glycoprotein K specified by herpes simplex virus type 1 is expressed on virions as a Golgi complex-dependent glycosylated species and functions in virion entry." J Virol **75**(24): 12431-12438.

Jambunathan, N., S. Chowdhury, et al. (2011). "Site-specific proteolytic cleavage of the amino terminus of herpes simplex virus glycoprotein K on virion particles inhibits virus entry." J Virol **85**(24): 12910-12918.

VITA

Sona Chowdhury was born in Kolkata, India and is the youngest daughter of Dipak and Leila Chowdhury. Sona imbibed her love for science from her mother, a retired science teacher and father a retired Mechanical engineer. She has two elder sisters Reena Chowdhury, an IT professional and Kavita Chowdhury, a journalist. She grew up in Kolkata, and her schooling was in Loreto House a convent school. She earned her bachelor's degree (B.Sc) from the University of Calcutta, India, and majored in Botany. She pursued her master's degree in Botany with specialization in Microbiology from the University of Calcutta, India. She then worked as research fellow in Indian Institute of Indian Institute of Horticultural Research (IIHR), Bangalore, India, under Indian Council of Agricultural Research (ICAR).

Sona then went onto pursue her doctoral degree in USA. She joined the Biological Science Department of Louisiana State University, Baton Rouge and she subsequently transferred to the Department of Pathobiological Sciences, School of Veterinary Medicine and joined the laboratory of Dr. K. G. Kousoulas. Sona plans to work as a post-doctoral fellow following graduation. Alongside her abiding interest in science, Sona is passionate about Indian classical dance and she is an accomplished dancer.