

2014

Genetics and Functions of Herpes Simplex Virus Type-1 Membrane Proteins UL20/gK in Virion Envelopment and Entry

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GENETICS AND FUNCTIONS OF HERPES SIMPLEX VIRUS TYPE-1
MEMBRANE PROTEINS UL20/gK IN VIRION ENVELOPMENT
AND ENTRY

A Dissertation

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

in

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

by
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December 2014

Dedicated to my Parents,
Mrs. Susamma Charles and Dr. Charles Philip

ACKNOWLEDGEMENTS

First and foremost I would like to thank my Parents, Susamma Charles and Charles Philip and my sister Linu for their love, support and encouragements during my education. I am also grateful to my husband Pratheesh, sons Joel and Noah, and parents-in-law Jemini George and Thomas George for being understanding and supportive during my pursuits.

I would sincerely extent my gratitude to Dr. Gus Kousoulas for giving me an opportunity to pursue Ph. D under his guidance and without his constant support and advice I would not be able to succeed in graduate studies along with family commitments. I specially thank Dr. Vladimir Chouljenko for all his help and technical assistance. I am grateful to all post docs Drs Nithya Jambunathan, Jason walker, Arun Iyer, Ramesh Subramanian and Paul Rider and to fellow graduate students Sona, Dimitry, Andrew, Beth, Ahmed, Misagh and Brent for their help and also making our laboratory a happy work place. I am also thankful to administrative staffs Ms. Pam Stratton and Mr. Anand Ramachandran and members of the GeneLab, Ms. Thaya Guedry and Tori for their sequencing and lab supplies ordering help.

I would like to thank the faculty members that served on my committee, Drs Nobuko Wakamatsu, Maria Antonieta Guerrero Plata, Christopher N. Mores and Rendy Kartika for their advice and recommendations.

I am also indebted to all teachers in Mount Carmel School, Bishop Choolaparambil Memorial College and College of Veterinary and Animal Sciences, Kerala who gave the best education out of the available resources.

Last but not least, I thank God almighty for all his blessing and grace throughout my life.

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ABSTRACT

The envelopment and egress of the Herpes Simplex Virus is an important event in the life cycle of the virus. The important membrane proteins required for the envelopment and egress of the virus are glycoproteins gM, gK, gE and non-glycosylated membrane proteins UL20p and UL11. Among them one of the most critical protein is the UL20 protein which has four transmembrane domains with amino and carboxyl termini are predicted to lie within the cytoplasmic side of cellular membranes. Studies done in our laboratory have shown that deletion of UL20 or other lethal mutations have an adverse effect on envelopment and results in accumulation of unenveloped capsids in the cytoplasm of infected cells. The carboxyl termini of UL20 protein was shown to be extremely important for viral egress. Mutant viruses were made with mutations in the carboxyl termini of UL20p especially targeting phenylalanine residues as they are known to be an important target for protein-protein interactions through stacking of the aromatic structure. The two membrane proximal phenylalanines were very critical for efficient replication as well as envelopment whereas, the middle phenylalanine at the carboxyl terminus may have a negative effect with regard to the virus since the mutation helped the virus to replicate, envelop and egress better.

Another glycoprotein shown to be playing a role in viral entry to susceptible cells or neurons is Glycoprotein K (gK) especially the amino terminus of the protein. If the virus has entered the cell but failed to enter nucleus and start protein synthesis, it is very difficult to assess the entry of virus. In this scenario, the virus may be either stuck at the cell membrane or the transport to the nucleus is adversely affected. In order to demonstrate the entry of virus at a very early time point is quite challenging, but through

Proximity Ligation assay (PLA) we have showed the importance of gK, the defect or less efficiency of gK mutant virus in entry to epithelial cultured cells as well as in neurons at a very early time point post infection.

CHAPTER I INTRODUCTION

Statement of Problem and Hypothesis

The most common manifestation of HSV-1 infection is primary herpetic gingivostomatitis (PHGS) characterized by transient painful vesicles in and around mouth which often affects the quality of life rather than a serious infection. Ocular herpes viral infection is considered as the leading cause of infectious blindness in developed countries. The herpes virus is capable of establishing latency in the trigeminal ganglia. So once infection occurs, then they are incurable and persist throughout the lifetime of the host. Apart from recurrence that occur spontaneously, there are a many extrinsic and intrinsic factors that can cause viral reactivation like stress, exposure to sunlight, menstrual cycle, immunosuppression, treatment with corticosteroids, and trauma. The double-stranded viral DNA genome is enclosed within an icosahedral nucleocapsids, surrounded by proteinaceous tegument layer and lipid envelop embedded with glycoproteins.

Envelop proteins play an important role in the life-cycle events of HSV-1 like envelopment/egress and entry. It is believed that the virus acquire a primary envelope when mature capsid bud from nucleus to perinuclear space. De-envelopment results from the fusion between outer nuclear membrane and viral envelopes and the capsids released to the cytoplasm of infected cells undergo secondary envelopment in cytoplasmic vesicles derived from trans-golgi network (TGN) or endosomes and final egress happen to extracellular space [1]. Till date nobody has proved the exact mechanism and viral proteins involved in envelopment and egress.

Studies conducted in our laboratory have shown that UL20 protein serves crucial roles in cytoplasmic virion envelopment in comparison to other viral glycoprotein [2]. Deletion of just either 6 (UL20 Δ 216t) or 11 amino acids (UL20 Δ 211t) allowed efficient UL20p intracellular transport and cell surface expression, but failed to complement for infectious virus production affecting the envelopment and egress. Phenylalanine residues are aromatic and hydrophobic amino acids that have been shown to play an important role in respiratory syncytial virus assembly [3]. We hypothesize that phenylalanine at the carboxyl terminus of UL20p are important for virion envelopment and egress.

Another important Glycoprotein in the virion envelop is gK which plays multiple roles in viral entry, virion fusion, egress and envelopment [2, 4-6]. It has been shown that gK is important for viral corneal spread and neuroinvasiveness [7] as well as for both retrograde and anterograde spread along axons of neurons [8]. Deletion of amino terminus of gK (gK31-68) slowed the entry of virus into epithelial cells [6]. PLA has been a novel technique to explore protein interactions using DNA along with processes like ligation, cleavage, and polymerization [9]. We also hypothesize that through PLA experiments showing association between inner tegument protein UL37 and motor protein dynein can be utilized to show the delay in the entry of gK amino terminus mutant to epithelial and neuronal cells.

Statement of Research Objectives

The main aims of this research was to investigate the importance of carboxyl terminal of UL20p in HSV-1 with regard to virion envelopment and egress with special emphasis on aromatic amino acid phenylalanine and also to investigate the role of gK with regard to entry in both epithelial and neuronal cell culture.

The research objectives were:

- I. To investigate the role of carboxyl terminus amino acids of UL20p especially phenylalanine residues in virion replication and spread:
 1. To generate and validate a set of mutant viruses having deletion of phenylalanine residues at the carboxyl terminus or replacement of them with alanine residues in HSV-1 (F) VC-1 genome cloned on bacterial artificial chromosome (BAC) using two-step red double recombination method.
 2. To characterize the phenotypes of mutant viruses using plaque morphology, fusogenic capacity using luciferase assay, expression profiling using immunoprecipitation/western blotting and virus replication curves.
- II. To investigate the role of phenylalanine residues at the carboxyl terminus of UL20p of HSV-1 with regard to viral envelopment and egress:
 1. To analyze the ability of mutants to envelop and egress using q-PCR, and to study the transport of UL20p to TGN, an important step during envelopment, using confocal microscopy through colocalization.

2. To compare the interaction of UL20p with an important tegument protein UL37 during envelopment of mutant viruses with wild-type virus using proximity ligation assay (PLA).
 3. To investigate the ultra structural phenotype of viruses with phenylalanine mutations on carboxyl-terminus of UL20p exhibiting defects in virion egress and spread.
- III. To investigate the role of amino terminus HSV-1 gK with regard to entry in both epithelial and neuronal cell culture.
1. To standardize and utilize the PLA technique in studying the entry of HSV-1 virus to cells.
 2. To compare the defects of gk mutant viruses for entry in both epithelial cells and neuronal cells by acquiring signals of PLA in confocal microscope.

CHAPTER II REVIEW OF LITERATURE

History of Herpesviruses

The occurrence of human herpes simplex virus infections was documented in the ancient Greek times. The Greek scholar Hippocrates described the lesions using the word “herpes” which means to creep or crawl along the skin [10]. The link between cutaneous lesions caused by HSV and the related fever was pointed out by Herodotus and recurrence of the lesion at same anatomic site was recognized by Galen [11]. In 1736 John Astruc, a physician for King Louis XIV of France published *De Morbis Veneris* after he found a connection between HSV and the genital organs after studying the disease in French prostitutes [12]. Later in the early 19th century Thomas Bateman described herpes as “a restricted group of localized vesicles with a short, self-limiting course” (Bateman, 1814). In 1873, Vidal demonstrated that the disease was infectious and can cause reinfection in a new anatomical site by re-inoculating the herpetic lesion materials.

During early 20th century, Cushing from his observations came up with the concept of latency by suggesting that posterior root ganglia were responsible for the occurrence of herpes [13]. Gruter is credited for using an animal model for showing the infectious nature of virus by studying the spread of HSV among rabbits and he is acknowledged for the isolation of the virus [14]. As a therapeutic measure herpetic vesicular fluid was inoculated into forearm skin in both animals well as in humans [15][16].

Later new technologies like tissue culture accelerated the isolation and identification of other viruses in the herpes family. In 1950’s, Varicella Zoster Virus (VZV), causative

agent of chicken pox, and cytomegalovirus (CMV) [17], immune system associated herpes viruses like human herpesvirus 4 (HHV-4; Epstein-Barr virus) [18] and human herpesviruses 6A, 6B, and 7 [19, 20] were isolated as a result of cultivation of lymphoblastoid tumor cells, B cells and T cells respectively. Newly, human herpesvirus 8 was isolated through Representational Differential Analysis (RDA) [21]

Taxonomy of Herpesviruses

Herpesviruses are complex viruses due to their large genomic size and a complicated lifecycle. The human Herpesviridae family consists of eight members: HSV-1 and 2 as well as varicella-zoster virus (VZV) under subfamily Alphaherpesviruses, human cytomegalovirus (HCMV) and roseoloviruses (HHV-6 and -7) under Betaherpesviruses and Epstein-Barr virus (EBV), Kaposi-sarcoma herpesvirus (KSHV) under Gammaherpesviruses. Table 1.1 summarizes all the nine known human herpesviruses along with commonly studied herpesviruses [22, 23].

Alphaherpesvirinae subfamily was classified on factors like diverse host range, short reproductive cycle, fast spread in tissue culture, efficient lysis of susceptible cells and establishment of latency mainly in sensory ganglia. Genera's included under the subfamily include *Simplexvirus*, *Varicellovirus*, *Marek's disease-like virus*, and *Infectious laryngotracheitis-like virus* [23].

Betaherpesvirinae subfamily was classified on factors like small host range, long reproductive cycle, and slow progression of infection on tissue culture. Infected cells are characterized by enlargement called cytomegaly, and viruses are capable of going latent

Table 1.1: Members of the family Herpesviridae

Subfamily	Designation	Vernacular Name
Alphaherpesvirinae	Human herpesvirus 1 (HHV-1) Human herpesvirus 2 (HHV-2) Human herpesvirus 3 (HHV-3) Gallid herpesvirus 1 (GaHV-1) Gallid herpesvirus 2 (GaHV-2) Suid herpesvirus 1 (SuHV-1) Felid herpesvirus 1 (FeHV-1) Ictalurid herpesvirus 1 (IcHV-1) Bovine herpesvirus 1 (BoHV-1) Equid herpesvirus 1 (EHV-1)	Herpes simplex virus type 1 (HSV-1) Herpes simplex virus type 2 (HSV-2) Varicella-zoster virus (VZV), shingles, chicken pox. Infectious laryngotracheitis virus Marek's disease virus Pseudorabies virus, Aujeszky's disease virus Feline herpesvirus 1, Feline rhinotracheitis virus Channel catfish herpesvirus Infectious bovine rhinotracheitis virus Equine HV-1, Equine abortion virus
Betaherpesvirinae	Human herpesvirus 5 (HHV-5) Human herpesvirus 6A (HHV-6A) Human herpesvirus 6B (HHV-6B) Human herpesvirus 7 (HHV-7)	Cytomegalovirus (CMV) Roseolovirus
Gammaherpesvirinae	Human herpesvirus 4 (HHV-4) Human herpesvirus 8 (HHV-8) Canid herpesvirus 1 (CaHV-1) Ovine herpesvirus 2	Epstein-Barr virus (EBV) Kaposi's sarcoma-associated herpesvirus (KSHV) Canine herpesvirus Sheep-associated malignant catarrhal fever

in secretory glands, kidneys and other tissues. Genera's included in the subfamily are *Cytomegalovirus*, *Muromegalovirus*, and *Roseolovirus* [23].

Gammaherpesvirinae subfamily was classified on factors like limited host range and ability to replicate in lymphoblastoid cells. Latency has been frequently shown in lymphoid tissues and viruses are specifically for B cells or T cells. The genera's included are *Lymphocryptovirus* (EBV), and *Rhadinovirus* [23].

Structure of Virus

HSV-1 genome consists of double-stranded DNA enclosed within an icosahedral nucleocapsid, which is surrounded by a lipid envelop embedded with glycoproteins.

Between nucleocapsids and envelope lies a proteinaceous layer called tegument (Fig 1.1). The nucleocapsid houses DNA, in a densely coiled liquid crystalline arrangement [24] and it is one of the similarities HSV-1 shares with bacteriophages [25]. There are three types of capsids isolated from the viral infection, A, B, C capsids. They are icosahedral in shape and were made of 162 capsomers, but the difference was in their core content. Only capsid C contains viral DNA and are able to mature to infectious virus [26, 27]. An additional structure found in the C capsids or the capsids in the complete virion is the C capsid specific component (CCSC) and is absent in the A or B capsids of virus.[28]. Works done both *in vivo* and *in vitro* has shed light on the pathway for HSV-1 capsid assembly [26, 29, 30]. Capsid shell is made of proteins U_L19, U_L35, U_L18, U_L38 and the scaffolding protein U_L26.5 which is lost during maturation of procapsid to mature capsid. Portal system is a ring shaped structure made of which viral DNA enters and exit from the capsid [31, 32]. The viral envelop is made up of 11 glycoproteins and the tegument is made of 20 virally encoded proteins.

Mostly viruses are observed as round shape although they could be in different shapes. The lipid bilayer is around ~5 nm thick and the diameter ranged between 170 to 200 nm. The diameter taking into account membrane proteins decorating the envelope averages ~225 nm. Nucleocapsid occupied an eccentric position inside the envelope and linkers or ~4 nm connected tegument cap with the envelope. There number of protein spikes on the envelope varies from 600 to 750. The spikes are 10 to 25 nm long, ~4 nm wide and ended in a globular structure ~6 nm across. There are variation in the angles of protrusion and straightness of spikes [25]

HSV-1 Virion Structure

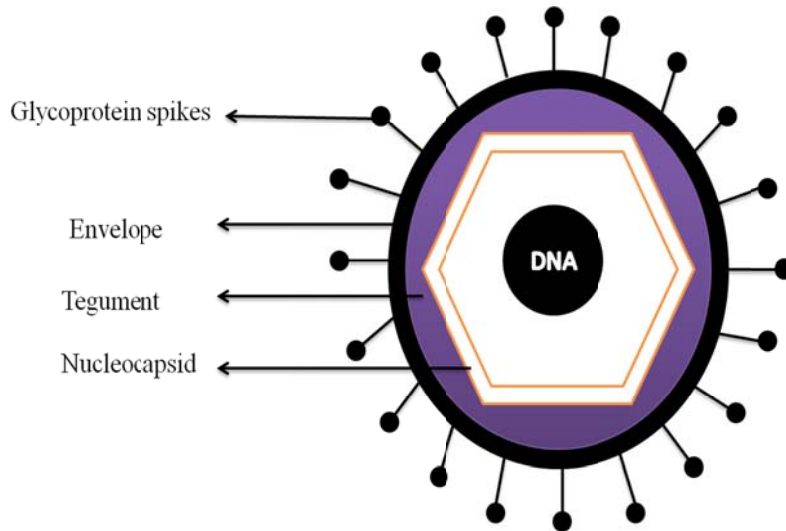


Figure 1.1: Virion Structure of HSV-1. The genome of virus is made of DNA which is encapsulated within a nucleocapsids surrounded by proteinaceous tegument and outermost lipid envelop with glycoprotein spikes. The total diameter of the virion taking into account of membrane proteins decorating the envelope averages ~ 225 nm.

HSV-1 genome and origins of DNA replication

The structurally complex HSV-1 genome consists of two unique regions, unique long and unique short (UL and US) flanked by inverted repeat sequences (Fig 1.2) [33].



Figure 1.2. Arrangement of the HSV-1 genome. Graphic representation of HSV-1 genome with the unique long (UL) and unique short (US) regions flanked by the terminal repeat (TR) and internal repeat (IR) regions with long (IR_L) and short (IR_S).

The HSV-1 genome exist in four equimolar isomeric forms and that are P (prototype), I_{LS} (Both L and S are inverted), I_L (L is inverted) and I_S (S is inverted)[34]. There are three origins of replication in the genome: There are two OriS in the viral genome in the repeated c region and one OriL is present in UL. They have recognition sites for origin binding protein, the product of the UL9 gene. UL9 protein is a homodimer that binds the

two inverted pentanucleotide repeats, boxes I and II of the HSV-1 origin of replication, OriS. UL9 protein also possesses DNA-dependent ATPase and 3'-5' helicase activities [35]. The six important proteins for replication are: a single-strand DNA-binding protein (known as ICP8 or UL29), a two-subunit DNA polymerase (catalytic subunit Pol and processivity subunit UL42), and a three-subunit helicase/primase complex (H/P:UL5, UL8, and UL52)[36]. H/P complex is recruited once ICP8 and UL9 have unwound the DNA at the site of replication. Subsequently polymerase complex is recruited for DNA synthesis leading to the formation of tandem head to tail concatemeric DNA which accumulate in the nuclei which are later cleaved at regular intervals to monomeric units and finally encapsidation to generate progeny virus. There is a higher rate for recombination among herpes viruses [37].

Clinical Manifestation

HSV-1 is more important causative agent than HSV-2 in causing ocular disease, characterized by conjunctivitis, iridocyclitis, acute retinal necrosis and keratitis. HSV-2 is the main culprit behind various genital infections and is a sexually transmitted disease [38, 39]. We could notice an endemic pattern of distribution for herpes virus infection in the natural human host of the virus throughout the world irrespective of level of civilization that is from highly developed urban population to native tribal population [40]. Predilection sites for latency occurs in trigeminal ganglia for HSV-1 and sacral ganglia for HSV-2 as they cause infections in the face and genital areas respectively. Recurrent infection of herpes virus is not considered as reinfection by virus, but it is due to viral reactivation from latency [41]. Apart from recurrence that occur spontaneously, there are many extrinsic and intrinsic factors that can cause viral reactivation like stress,

exposure to sunlight, menstrual cycle, immunosuppression, treatment with corticosteroids, tissue damage and trauma [42]. Viral reactivation not always leads to clinical manifestation of disease, but can cause asymptomatic shedding of virus known as recurrence [39, 43] and viral reactivation with clinical signs of disease are known as recrudescence. Transmission of HSV-1 occurs mainly through oral secretions and lesions are mainly associated with eyes, face, pharynx, and central nervous system infections. The ability of virus to remain viable on fomites and skin helps in horizontal transmission through social contacts like kissing and shaking hands [44, 45]. There are two kinds of initial herpes virus infections; a) True primary infection and b) nonprimary infection. True primary infection occurs when a HSV-1 seronegative individual gets infected with the virus and the infection usually more severe and having a prolonged duration of viral shedding [38, 46, 47]. Nonprimary infection occurs when a new strain of HSV-1 infects an already infected person with another strain of HSV-1 [38, 48]. Primary infection is characterized by elevated levels of IgM, while in the case of nonprimary infection there is an elevated level of IgG. The most common manifestation of HSV-1 infection is primary herpetic gingivostomatitis (PHGS) characterized by transient vesicles in and around mouth [49]. Spread of virus to new hosts or different regions of the body is facilitated by rupture of the vesicles. The most common form of recurrent herpes infection is Herpes simplex labialis characterized by the presence of painful vesicles on the outer vermilion border and adjacent cutaneous region [50] which heal in a span of 2 weeks, but dissemination of virus continues for 3 to 5 days after healing [51].

Herpes infection is considered as the leading cause of infectious blindness in the developed countries [52]. Ocular manifestation can be the result of primary infections or

recurrent infections. HSV epithelial keratitis initiates as a superficial punctate lesion, developing to a stellate erosion and ultimately leading to dendritic ulcer [53, 54]. Although immune system is capable of clearing the infection, virus can travel retrograde axonal transport through sensory neurons before establishing latency in the trigeminal ganglia. So once infection occurs, then they are incurable and persist in latent infection throughout the lifetime of the host. Primary evidence for the transport of virus to central nervous system came to notice as the virus causes herpes encephalitis [55]. Areas of brain damaged by the virus are the anterior perforated substance, amygdala, insula, hippocampus, mammillary bodies and cingulate gyrus [56-58]. There are two schools of thoughts regarding entry of the virus to brain. A) Entry may be through trigeminal nerve and reach meninges covering the temporal lobe [59], from there infecting limbic system structures. B) Entry to brain through the olfactory system [60]. HSV-1 and -2 and VZV can cause facial, genital ulcers and in rare cases encephalitis. CMV infection is of concern in immunocompromised people and HHV-6 as well as HHV-7 cause exanthum subitum or roseola infantum in infants. Whereas, EBV causes infectious mononucleosis and can exist in latency in individual's B cells and KSHV causes Kaposi's sarcoma. HSV-1 and HSV-2 share similarities in their genomic sequence, but since their envelope proteins differ they are considered antigenically distinct [38, 44].

Epidemiology

Studies have shown that seroprevalence of HSV-1 is greater than 50%, 75% and 90% in the general adult populations of the countries the United States, Germany, and Tanzania respectively [61, 62]. Thus most of the people are infected with HSV-1 and carry a latent

infection in them. In the United States, around 500,000 people have ocular HSV and the country has to spend 17.7 million US\$ annually for the treatment of the disease [63, 64]. Additionally, incidence of HSV-1 infection is greatly influenced by race as well as socio-economic condition. By the age of 5 years, 35% of black kids are seropositive for HSV-1 antibodies in comparison to 18% of white kids [65]. Whereas, 33% of kids in lower socioeconomic background are seropositive for the infection by the age of 5 years and percentages reach up to 70 -80 % by late puberty [65, 66]. In contrast, only 20% of kids in higher socioeconomic background are seropositive for HSV-1 by age 5 and 40% to 60% by late adolescence [42, 65].

The world wide prevalence of HSV-1 comes to be around 90%, whereas prevalence in United State of America is 65% [67] and in northern Europe 52-67% [68]. The generally accepted facts are 1) Latency is due to failure to start the expression of immediate early genes (IE); 2) Latent DNA is maintained in a non-linear configuration as a circular episome; 3) The latency-associated transcripts (LATs) are the only viral gene products produced in latently infected cells; 4) Finally reactivation causes activation of virus gene expression and entry into lytic phase [69].

The molecular basis of Herpes Simplex Virus latency

All herpesviruses have the unique characteristic ability to establish life-long latency in the infected host. The latency can be periodically interrupted leading to reactivation and dissemination of infection to newer host upon instances like immunosuppression. This is a highly efficient survival system they have evolved to maintain as well as to perpetuate infection. After primary replication in the oral mucosa, HSV-1 virus enters the sensory

neuronal termini innervating the site of infection and is carried in a retrograde direction to establish latency in the trigeminal ganglia.

An important event for lytic cascade is the association of VP16 viral protein with two cellular factors, Octamer binding protein-1 (Oct-1) and host cell factor (HCF) and binding to TAATGARAT region in the promoter sequence for the five IE genes ICP4, ICP0, ICP22, ICP27, and ICP47 [70]. The basic requisite for latency is failure of IE gene activation. This could be due to inadequate quantity of VP16 reaching the neuronal nucleus after the virus traverses long distance through the axon [71]. HSV latency associated promoter has been associated with acetylated histone H3, whereas decreased association have been seen for IE as well as early gene promoters through chromatin immunoprecipitation studies [72, 73]. Only expressed viral gene product during latency are LATs, which are polyadenylated non-coding RNA. They are transcribed from repeat regions flanking the unique long regions of herpes viral genome[74]. The size of primary transcript is 8.3kb and which then subsequently spliced to 2.0 kb intron and finally into 1.5 kb intron [75]. Studies have shown that the of 3' end of the first exon and 5' end of the stable 2.0 kb LAT intron protects the cells from going into apoptosis [76].

Herpes virus encoded miRNAs play important roles in latent/lytic cycle control, evasion of immune system, and cellular survivability [77]. HSV-1 encodes 16 siRNAs and of which six miRNAs (miR-H2, H3, H4, H5, H7 and H8) are encoded within the 8.3 kb primary LAT [78]. Co-transfection studies have shown that miR-H2 and miR-H6 decreased the amount of proteins ICP0 and ICP4, showing the part played by miRNA in suppressing the lytic phase and supporting the latent state of infection. The virus has

shown to encode several proteins gD and gJ, as well as protein kinase U_s3, ICP27 and ICP10 to prevent apoptosis of cells [33, 79-81].

As mentioned before, the key factor for a cell to enter lytic cycle is counting on the transactivating function of the viral protein VP16 and formation of a complex with HCF-1 and Oct-1 [70]. There has been a school of thought that in case of neurons VP16 may be inefficiently transported through the axons leading to an insufficient accumulation in the neuronal cell body [71]. The unique cytoplasmic localization of HCF-1 [82] and down regulation of Oct-1 [83] in neuronal cells were roadblocks in the formation of VP16-induced complex leading to suboptimal activation of IE promoters and subsequent latency stage. However, till now no study is able to find the reason behind why the virus cannot go latent *in vitro*. Most of the latency studies have been done in mice, but reactivation does not cause reinfection at the site of infection like in humans. Most common stimuli used for *in vivo* reactivation are the use of adrenergic agents like epinephrine.

Immune Response against HSV-1

Viral replication in the peripheral epithelial cells activates the innate immune response through type 1 interferons (IFNs) released by macrophages and the interferons help to protect neighboring susceptible cells from infection. Neutrophils invading the site of infection cause apoptosis of infected cells and also phagocytose them. Macrophages in the primary site of infection and in the trigeminal ganglia play a vital role in controlling viral replication [84, 85]. Table 1.2 has summarized the HSV-1 pathogen recognition receptors (PRRs) and the corresponding pathogen associated molecular patterns (PAMPs).

Dendritic cells (DCs) take up the viral proteins and process them to present to lymphocytes to activate adaptive immune system [86]. Activated T lymphocytes, T-helper ($CD4^+$) and cytotoxic T cells ($CD8^+$) take part in clearing infection. Whereas, B-cells play the role of presenting antigens and secreting cytokines rather than producing neutralizing antibodies [87]. The unmethylated CpG motifs on HSV-1 genome is detected by Toll-like receptor 9 (TLR-9) in the membrane of endosomal compartments. Whereas, PRR TLR-9 on plasmacytoid DCs and B cells can detect endocytosed DNA of HSV-1 [88, 89]. Recognizing herpes virus by TLR2 causes an influx of inflammatory cytokines in the brain leading to encephalitis [90]. Pathogen recognition receptor (PRR) TLR-3 can recognize dsRNA in the endosomes which are intermediate in herpes viral replication [91]. Studies have shown that susceptibility for herpes encephalitis is more in children who are genetically deficient in TLR-3 receptor [92-94]. Most of the TLRs signal through adaptor myeloid differentiation primary response gene 88 (MyD88) for producing downstream type I IFNs [95, 96]. Innate immune response is critical for controlling viral replication at the site of infection. Studies have shown that in IFN A1 receptor knockout mice where type I IFN signaling is absent results in higher host morbidity and mortality [97, 98]. Cytosolic short dsRNA and longer dsRNA of herpes virus are detected by retinoic acid inducible gene (RIG)-like receptors (RLRs) and melanoma differentiation-associated gene 5 (MDA-5) [99, 100]. All Toll-like receptors and nucleic acid sensors activate intracellular signaling cascade that leads to the induction of pro-inflammatory cytokines such as IL-8, RANTES and IL- 1β [101] and type I IFNs (IFN α and IFN β) promoting antimicrobial functions and inflammations. [102]. Table showing PRRs and HSV-1 are shown in table 1.2.[103]. IFN α and IFN β are responsible for the activation of

the Jak/STAT pathway which leads to the expression of ISGs (Interferon Stimulated Genes) that encode antiviral factors. Inflammasomes are activated by herpes viruses leading to the activity of caspase-1. AIM₂ (An interferon-inducible protein, absent in melanoma 2) act as sensor of viral genomes in the cytoplasm leading to caspase-1 activation [104] and interacts with apoptosis-associated speck-like protein containing a CARD (Caspase Recruitment Domain) recruiting pro-caspase-1, followed by production of active caspase-1 and mature IL-1 β and IL-18.

Life cycle of HSV-1

HSV-1 enter cells by membrane fusion between viral and cell membrane. Viral proteins gB or gC interact with heparan sulfate proteoglycans (HSPGs) present on the filopodia of cells [105]. It has been shown that cells lacking HSPGs, can get infected through another receptor called paired immunoglobulin like type 2 receptor alpha (PILR α) through gB envelop protein [106]. When PILR α is expressed on Chinese hamster ovary (CHO) cells, viral entry happens through fusion at the plasma membrane. On the other hand, entry happened through endocytosis when CHO cells are expressing nectin1 or herpesvirus entry mediator (HVEM) [107, 108]. In human cells, changes happen in the cytoskeleton for facilitating viral entry through cdc42 (Cell Division Control Protein 42)[109], FAK-1 (Focal Adhesion Kinase) [110] and phosphoinositide 3 kinase [111].

Glycoprotein D of herpes virus interacts with three different families of cellular receptors and this interaction determines the tropism of the virus as well as the fusion mechanism. HVEM helps in entry apart from its role in apoptosis through the tumor necrosis factor (TNFR) [112]. Other receptors include nectin-1 and nectin-2, which are the members of

Table 1.2: Herpes Simplex Type-1 associated pathogen associated molecular patterns (PAMPs) and the corresponding pathogen recognizing receptors (PRRs)

Pathogen Recognizing Receptors (PRRs)	Pathogen (HSV-1) associated molecular patterns (PAMPs)
TLR2	Viral component and dsRNA
TLR7	Genomic DNA
TLR9	Endocytosed DNA
MDA5	Replication intermediate and Genomic DNA
Pol III / RIG-I	Genomic DNA
IFI16	Genomic DNA

immunoglobulin (Ig) family, and 3-O sulfated heparan sulfate (3-OS-HS), a polysaccharide belonging to the Heparan Sulphate family [113]. Nectins are transmembrane proteins abundantly found on nervous system [114] and interacts through afadin with actin, thus functions in intercellular adhesion system found in synaptic as well as adherens junctions [115]. Receptors used by virus depend on the type of the uninfected susceptible cells. For example, entering epithelial cells nectin-1 is preferred by the virus [116], while for T lymphocytes HVEM is the major receptor. Interaction with receptors acts like a catalyst [117] triggering conformational changes and ultimately causing fusion with the help of envelop proteins gB and gH/gL. During fusion “hemifusion intermediate” is formed by the mixing of lipids as well as the membranes of the cell and virus, ultimately forming a fusion pore allowing mixing of cytoplasmic

content of cell with viral contents leading to the entry of nucleocapsids with tegument proteins into the cytoplasm of the cell [118]. Of all proteins involved, gB is the membrane fusogen and most conserved among viral membrane proteins involved in entry [119]. Interactions have been demonstrated between gD:gH/gL, gD:gB, gH/gL:gB [120-122]. Multinucleated cells or syncytia are produced when all the four glycoproteins were expressed together in COS cells and syncytial formation failed when only one or two proteins were expressed [123, 124]. Another major pathway for HSV entry into HeLa and CHO-K1 cell lines is by endocytosis and this can be prevented by reagents that increase endosomal pH, indicating the importance of passage through acidic compartments [125]. After endocytosis of enveloped viruses, fusion between viral and cellular membranes leads to release of the nucleocapsid into the cytoplasm. Whatever be the route of entry, tegument protein VP16 promotes transcription of immediate early or α viral genes leading to a cascade of activation leading to transcription of early or β genes and finally those β genes products helps in viral DNA replication as well as activate the late or γ genes which encode viral structural proteins [126]. ICP0, 4, 22 and 27 are the α viral proteins responsible for the transcription herpes genome. In some cases ICP4 has been shown to have a negative effect on transcription of genes [127]. ICP22 functions along with virus associated protein kinase UL13 by influencing phosphorylation and affecting RNA polymerase II functions [128]. ICP27 may also associate with RNA polymerase II and influence its functions [129]. Virion host shut-off (Vhs) is a tegument protein, which has RNase or RNAase component in association with the translation initiation factor eIF4H, which chew off the cellular mRNAs and thus favor expression of viral transcripts [130]. DNA viruses including herpes viruses replicates in the subnuclear structures called

ND10 [131] and cause disruption of ND10, which may enhance viral transcription/replication by making use of its components or to prevent its negative effect on viral lifecycle. ICP0 acts as a promiscuous transactivator and it separates HDACs 1 and 2 from the CoREST/REST repressor complex, thus prevents viral DNA silencing [132]. There is circularization of linear genome of herpes virus within 30 minutes of entry to cells [133]. Spread of infection to susceptible neighboring cells happens through virus released to infected cells or through direct cell-to-cell spread [134]. During egress the virus nucleocapsid first acquires tegument proteins and then envelope. The most supported school of thought is the envelopment-de-envelopment theory put forward by Stackpole [135]. The herpes virion morphogenesis and egress occurs through a complex mechanism characterized by: a) primary envelopment of capsids at the inner nuclear membrane; b) de-envelopment of them at the outer nuclear membrane; c) cytoplasmic capsids acquire sets of tegument proteins; d) re-envelopment of tegumented capsids into TGN-derived membranes [1].

Transport and Egress of HSV-1

If the transport is only through diffusion, HSV-1 capsid could take around 230 years to move just 1 cm through cytoplasm [136]. However in the case of *in vitro* infection of cultured cells, capsids may move by diffusion to reach nucleus since at the apical surface it lies very close to cytoplasm [137]. On the other hand virus will be adopting more sophisticated transport mechanism for traveling through long sensory neurons during its life cycle. For the transport of cargos like organelles or vesicles, cells make use of microtubules and motor proteins like kinesin or dynein.

Microtubules are made of alphabeta-tubulin heterodimers. Dynein motors move cargos from minus end of microtubule towards the nucleus to microtubule organizing center (MTOC), whereas kinesin motors transport cargos towards periphery that is to the plus-ends of the microtubules [138]. Viruses encode only limited number of proteins required to complete its life cycle since their genomes are small. So they make use of cellular mechanisms for their own benefit. The size of dynein is Mr 1.2×10^6 and is made of heavy chains, intermediate chains, light intermediate chains and light chains. The different combinations of the light and intermediate chains decide the specificity of cargo binding [139]. Dynactin is a co-factor of dynein which regulates its functions [140]. The retrograde transport of capsids requires microtubular network and the transport is disrupted by microtubular inhibitors like colchicine, vinblastine, or nocodazole [141]. Speed for retrograde transport is roughly 200 ± 300 mm/day in animals and 3 ± 5 mm/h in culture [142, 143].

The viral protein first shown to bind with dynein was pUL34 [144] and the capsid protein VP26 (pUL35) is important for retrograde transport of virus [145]. Proteins predicted to interact with dynein are in the inner tegument or outer capsid. For example capsid protein pUL35 (VP26) has been shown to interact with dynein through colocalization by microinjecting recombinant capsids into live cells and transport of capsids towards nucleus [145]. Dystonin is a cytoskeletal linker protein present at the plus end and function in both retrograde as well as anterograde transport in neurons. Travel to the centrosome by capsid is through minus-ended transport while further towards nucleus is by plus-ended transport. Depletion of dystonin did not affect the transport of capsid to the

centrosome, but had a deleterious effect on further transport towards nucleus [146]. Same group have also shown that dystonin interacts with herpes tegument protein pUL37 and is indispensable for efficient egress of capsids [147]. Membrane glycoprotein gK is required throughout the life cycle of the virus from entry to egress [148]. An *in vitro* transport study in neurons using microfluidic device showed that gK is essential for infection of neuronal axons, may be due to lack of regulatory role of gK on gB during entry process caused by the fusion between viral membrane with axonal synaptic membrane [8]. Anterograde transport refers to transport from the neuronal cell body to axons and there are models proposed for herpes virus anterograde transport through neurons. In cultured sensory neurons newly formed capsids are moving in both directions with a net movement towards the plus-end direction [149]. In separate model, naked nucleocapsids devoid of any membrane proteins are transported down the axons, whereas in the married model complete virions together with the membrane proteins are transported [150]. Brefeldin-A (BFA) is a chemical which can cause disruption of Golgi apparatus and thus affect egress by interfering assembly of virions [151]. A study has shown that BFA caused inhibition in the anterograde transport of glycoproteins and had no effect in the transport of nucleocapsids supporting the separate model [152]. Whereas, transmission electron microscopic images of the proximal and mid-axons of primary superior cervical ganglion (SCG) neurons showed that complete virions were present in case of HSV-1 NS and 17, HSV-2 2.12 and PRV Becker infections supporting the married model for transport [153]. On the contrary, another group has supported the separate model of anterograde transport by showing in HSV infected murine retinal ganglion cell model that US9 is necessary for the efficient anterograde transport

specifically of viral capsid and DNA, but not required for the viral envelope glycoproteins anterograde transport [154]. UL36 (VP1/2) encodes the largest HSV1 tegument protein having a molecular weight of 273 kDa and has been shown to be important for binding to microtubule and also transport down the axon [155]. Motors that function in transporting cargoes from inside to plus end of microtubules located at the periphery of cells are kinesins [156] and make use of energy from ATP for conformational changes to generate motile force [157]. Kinesin protein superfamily consist of 15 members named kinesin 1 to kinesin 14 B. First kinesin discovered is the tetrameric Kinesin 1 [158], made of two identical light chains of Mr 64000 and two identical heavy chains of Mr 120000 [159]. Based on the location of the motor domain they are classified into three types; N-kinesins, M-kinesins and C-kinesins if motor domain is in the amino-terminal, middle or carboxyl-terminal region of the protein respectively. N-kinesins and C-kinesins are involved in the microtubule plus and minus end transport, whereas M-kinesins depolymerize microtubules. For anterograde transport cell make use of N-kinesins and for retrograde transport other than dynein, C-kinesins can also help [156]. In polarized non-neuronal cells, enveloped complete viruses are transported from the Golgi network to cell surface [160, 161]. Interaction has been demonstrated between viral proteins and proteins of cell important for secretory and exocytosis pathways in neurons like TGN-46 (marker protein of trans-Golgi network), Kinesin-1, GAP-43 (neuron-specific protein), Rab3A (regulatory protein in intracellular trafficking), and SNAP-25 (Q-SNARE protein) [162].

gK/UL20 Role in Envelopment and Egress

HSV-1 nucleocapsid in the cytoplasm acquires envelop by budding into *trans*-Golgi network (TGN)-derived membranes. The two important membrane associated proteins at the TGN are picked up by the virus at the TGN. Mature viruses depend on cellular secretory pathway to be transported to cell surface to egress [1]. Genes shown to be important for secondary envelopment are PrV gB [163], HSV-1 gK, UL20p, gE, gM, gD, UL11, UL48 [164-169], and PRV US3 [170]. gK/UL20 proteins were found to be the most vital components for cytoplasmic virion envelopment, egress and infectious virus production when compared to gM, gD, UL11, and gE [2]. gK and UL20p have multiple membrane spanning domains and are highly conserved among the members of alphaherpesviruses [171]. gK is made of 338 amino acids and UL20p is made of 222 amino acids. Interactions between membrane and tegument proteins are required for proper envelopment and egress and this may be affected in gK/UL20 mutant virus causing problems in secondary envelopment. Studies have also shown that UL20p in PRV functions in cell-cell spread and egress from infected cells. Electron micrographs showed that in UL20p mutant PrV virus, there was accumulation of enveloped viral particles in the cytoplasmic vesicles of Vero cells. This observation is different in HSV-1 since deletion of UL20p caused accumulation in the perinuclear space of infected Vero cells [172]. These observations point out the fact the UL20p function definitely in egress but may be at different step during the process. Similarly HSV-1 gK was also shown to be important for regulation of egress of new virion particle from cells since the HSV-1 mutant F-gK β lacking gK protein causes accumulation of enveloped and unenveloped virus particles in the cytoplasm of cells [173]. Apart from cellular viral envelopment and

egress, the two proteins of HSV-1 gK and UL20p are important for virus-induced cell fusion and mutations in either genes can cause extensive fusion between cells forming syncytia [174-176]. There were different experimental results suggesting the interdependence of gK and UL20p to function together indicating a physical and functional interaction between them. Confocal colocalization experiments showed that both proteins are essential for transport within cells, cell surface expression, cell induced fusion and cytoplasmic envelopment and egress [177-179]. The predicted structures of the proteins are mirror images of each other with amino and carboxyl termini of gK facing extracellular region while that of UL20p facing intracellular. Immunoprecipitation and western blot have demonstrated the physical interaction between gK and UL20p and most specifically between cytoplasmic domain III of gK and amino-terminal of UL20p [180]. The role of gK /UL20p in nuclear egress is not suspected as retention of UL20p resulted in accumulation of nucleocapsids in the cytoplasm of cells [174].

Membrane Proteins of HSV-1

UL20 Protein/ Glycoprotein K (UL20/gk)

UL20p is a structural protein made of 222 amino-acids which is non-glycosylated and has transmembrane domains. In infected cells distribution of the protein is mainly perinuclear and cytoplasmic [181]. Glycoprotein K (gK) like UL20p has multiple transmembrane regions and is made of 338 amino acids encoded by the UL53 open reading frame. The protein has two potential sites for N glycosylation and has a 30-amino acid long cleavable signal sequence at the amino terminal [182]. Among alpha herpesviruses like HSV-1, PrV, and varicella-zoster virus, gK mutant virus showed a defect in virion envelopment and egress.

Both gK as well as UL20p is highly conserved among all alphaherpesviruses [183, 184]. The structures of gK/UL20p were predicted based on the results of experiments which tagged the suspected intracellular and extracellular domains with epitopes [5, 176]. gk plays a role in viral fusion since gB Δ syn28 mutation, which is known to cause extensive virus-induced cell fusion, was inhibited in absence of amino terminus of gK (gK Δ 31-68 mutation) pointing out the interdependence and interaction between gK and gB [5]. Mutation of UL20 gene by deletion has resulted in weakly syncytial plaques in some cell lines and also affected proper envelopment showing the importance of gene with regard to cell fusion and virion morphogenesis [176]. So the role of both genes in virus-induced cell fusion was shown by proving the protein-protein interaction between UL20p and amino terminus of gK with gB in infected cells [185].

Studies have found that UL20 null virus accumulate in the space between nuclear membranes in certain types of cells like Vero and HEp-2. However, in the case of 143TK cells virions reach the cytoplasm. This phenomenon depended upon the presence or absence of intact Golgi networks. Cells complemented UL20-null virus was able to maintain intact Golgi network, while the network in those that failed in complementation were fragmented and dispersed [186]. The role of UL20p in fusion is reiterated by the absence of fusion event in syncytial mutants of gB or gK [179] and also UL20p is required for cell surface expression of gK [177-179]. Deletion of UL20 or lethal UL20 mutations affects envelopment and leads to accumulation of a few completely enveloped capsids in the cytoplasmic vesicles and also unenveloped capsids in the cytoplasm of infected cells [172, 179].

According to computer-assisted prediction of UL20p showed that there are four membrane spanning domains and amino (domain I) and carboxyl (domain V) terminals were facing towards cytoplasm of cellular membrane and inner to the envelop of the virus. There domains II made of 5 amino acids and domain IV made of 25 amino acids are facing extracellular of cellular membranes and external to the envelop of the virus. As

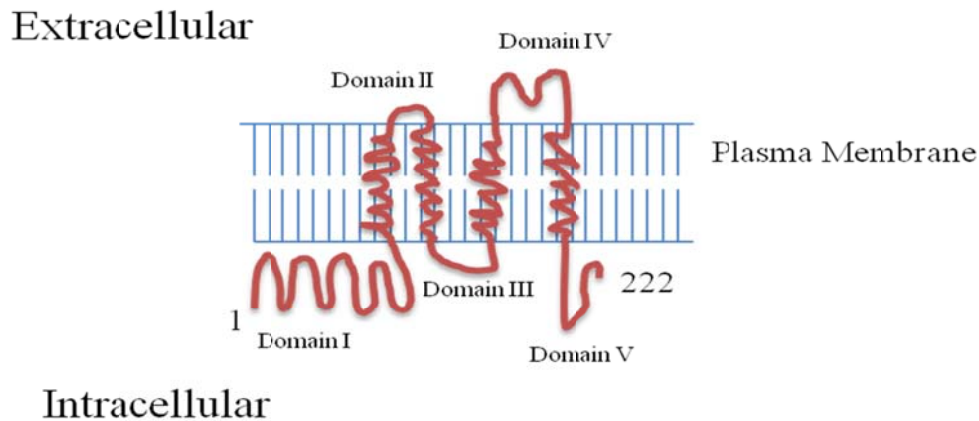


Figure 1.2 Predicted membrane topography of UL20p. UL20p is a structural protein made of 222 amino-acids which is non-glycosylated and has transmembrane domains indicated by arrow. There are four domains, I, III, V facing the intracellular region and II, IV facing extracellular region.

in HSV-1 UL20 null virus, deletion of UL20p in pseudorabies virus (PRV) caused accumulation of enveloped viruses in the cytoplasmic vesicles and nonenveloped capsids in the cytoplasm preventing the escape of viruses to extracellular spaces [172]. In both HSV-1(KOS) and PRV deletion of UL20 only affected efficient transportation of gK but not other viral glycoproteins like gB, gD, and gH [172, 177]. Glycoproteins gM, gK, gE and non-glycosylated membrane proteins UL20p and UL11 play critical roles in

cytoplasmic virion envelopment and egress. When single, double or triple mutant viruses were generated deleting these membrane proteins, the most defective ones in terms of cytoplasmic envelopment, egress, and production of infective viruses were the mutants with deletion of gK or UL20p [2]. There are other herpes proteins with which UL20p interact and functions in various steps in the life-cycle of the virus. Apart from its role in egress and envelopment, UL20p can also modulate virus-induced fusion since the protein can physically interact with major fusogenic protein gB as proved by two-way immunoprecipitation studies [185]. UL20p pairs with another important membrane glycoprotein gK and this is supported by the lack of cell surface expression of gK as well as cellular fusion mediated by gK in UL20-null mutant virus infected cells [179]. Colocalization of the proteins in confocal microscopy showed the proteins were interdependent for intracellular transport, cell surface expression and localization at the TGN, virus-induced cell fusion, and cytoplasmic virion envelopment [176-179]. The physical interaction between them most likely is between-terminal portion of UL20p with cytoplasmic gK domain III and has been shown through glutathione S-transferase (GST) pull down experiments and reciprocal coimmunoprecipitation method. It was also shown that retention of UL20p in the ER leads to retention of gK pointing out that may be their transport is interdependent on each other and also UL20p retention prevents cytoplasmic virion envelopment and virus-induced cell fusion [180]. Studies showed that proteins UL20p and UL11 are transported independently to TGN, but UL20p functions precedes and are required for UL11 function in the cytoplasmic virion envelopment [187].

Glycoprotein B (gB)

The most highly conserved protein in the core fusion machinery among all herpes viruses is glycoprotein B (gB) and the crystal structure reiterates the fusogenic character of the protein [119]. The protein is made of 904 amino acids [188]. In addition to binding with heparin and heparan sulfate, paired immunoglobulin-like type 2 receptor (PILR α) can function as a receptor for gB for viral entry as well as for fusion, but the glycoprotein D should also bind to its respective receptor [106, 189]. There is a 30-amino acid residue N-terminal signal sequence which is cleaved during processing, external domain made of a 743-residue, transmembrane domain of 22 amino acids and a 109-residue cytoplasmic domain [188, 190]. There is structural similarity with fusion proteins of vesicular stomatitis virus (VSV) G and baculovirus gp64 [191, 192]. In case of Epstein-Barr virus (EBV) protease cleavage is required to activate the protein, but in case of HSV this has not yet been proved [193]. It is believed that low pH in the endosomes may be helping gB in reaching the fusogenic form in HSV [194].

Glycoprotein C (gC)

In HSV-1 gC play an important role in attaching to its receptor heparan sulfate (HS) and chondroitin sulfate (CS) [195, 196]. Open reading frame of gC is made of 511 amino acids with a hydrophobic signal sequence, a hydrophobic membrane anchoring sequence and has 9 sites for N-linked glycosylation [197]. Molecular weight of gC calculated by SDS-PAGE is around 130kD, but from amino acid sequence is only 55kD and this difference is due to N-linked oligosaccharides [198], and O-linked oligosaccharides [199]. This heavy glycosylation is important for the protein to act as the receptor for the

complement factor c3b [200]. It was shown that the protein requirement in the life-cycle of HSV-1 is dependent on cell types, as gC is not essential for binding of virus to Vero cells and its replication was similar to wild-type in Vero, BHK and epithelial cells [201], whereas in case of mammalian neurons lack of gC reduced efficiency in replication to 5-10 fold when compared to wild-type [202]. gC can act as an immune modulator by inhibiting complement cascade by binding to C3b, a pivotal component of classical and the alternative complement pathways [203] and preventing binding of C5 and properdin to C3b [204].

Glycoprotein D (gD)

gD is a type I integral membrane glycoprotein made of 394 amino acids and cleaving of a signal peptide of 25 amino acids during processing yield a mature protein of 369 amino acids [205]. There are 3 sites for N-linked glycosylation [205, 206], o-linked oligosaccharides [207] and a hydrophobic transmembrane anchor sequence near the carboxy terminus [208, 209]. Several labs were able to show that gD can act as a ligand for HSV receptor. For instance, inactivated wild-type virions blocked infection with HSV, but UV-inactivated virions lacking gD failed in inhibiting infection [210]. Then scientists have shown that wild-type HSV failed to infect gD expressing cells [211]. This inhibition is based on the structure of the protein in the infecting virus and occurs during the step of penetration [212-214]. Finally, infection is prevented when cells are incubated in the presence of soluble truncated gD [215-217]. Native conformation is critical for gD function and is not dependent on the three N-glycosylation [218-220]. Cell surface has many receptors for gD for allowing entry of HSV [221].

The herpesvirus entry mediator (HVEM), the first herpes receptor found is a member of Tumor Necrosis Factor Receptor (TNFR) family and is used by both type 1 and type 2 of HSV [222]. HVEM has low levels of expression on fibroblasts and on ocular tissue [223, 224]. Whereas, Nectin-1 receptor is for both type 1 and 2 on neurons are present on keratinocytes, and epithelial cells [224]. Heparan sulfate (HS) modified by 3-O-sulfotransferases (3-OS-HS) can also act as a receptor for gD [225].

Glycoprotein G (gG)

US4 gene made of 716 bp encodes 238 aa glycoprotein G (gG). The function of glycoprotein G has been a difficult task to explore. Studies have shown that mutations in the gene whether deletion or insertions did not affect the replication of virus in nonpolarized cells [226, 227]. Whereas, in case of polarized cells viral gG is required for efficient infection of apical membranes of the cells and gG deleted mutant infected basal membranes similar to wild-type virus. The first surface virus getting exposed is the apical membranes of epithelial cells on the mucosal surface of the susceptible host. On the other hand, scarification of corneas allowed viral exposure to other cell surface and gG deleted virus could infect as similar to wild-type virus [228].

Glycoprotein E and I (gE/gI)

gE/gI is a heterodimer and have extracellular portions and cytoplasmic regions made of 100 amino acids have tyrosine motifs, dileucine, acidic clusters which may tend to localize the protein in the trans-Golgi network [229, 230]. gE and gI are found bound to each other and formation of a heterodimer happen quickly and they function as a complex

[231, 232]. The protein complex has a very important role to play with regard to spread of virus between tight junctions of neighboring cells especially in case of epithelial cells, fibroblasts, and neurons. The heterodimer localized on the lateral surfaces of epithelial cells, but was absent on both apical and basal surfaces. In case of less confluent cell culture monolayers, gE/gI was found at cell junctions but lacked completely on lateral cell surfaces which were not in contact with adjacent cells [233]. There was spread defects in mutant HSV-1 viruses lacking gE or gI in epithelial and neuronal cell cultures [233, 234]. A study have shown that in corneal cells there was a spread defect of 4 to 6 % on epithelial cells when compared to the wild-type HSV virus [235]. Spread among susceptible cells is through sorting of new progeny virions to lateral cell surface and cell junctions [236]. In an experiment when the entire gE protein or the cytoplasmic (CT) domain of the protein was deleted caused accumulation of virions in the cytoplasm, at the apical surfaces, and also in cell cultural supernatants whereas in case of wild-type virus accumulation was concentrating at cell junction. This showed the importance of cytoplasmic domain of gE protein in cell-cell viral spread [237]. Apart from spread the heterodimer functions include immune evasion and virulence [238-240].

Glycoproteins H and L (gH/gL)

The most important glycoproteins for viral entry and fusion among herpesviruses other than gB and gD are glycoprotein H (gH) and L (gL). Both gH and gL are required for posttranslational modifications and transport. Studies have shown that if only gH is expressed, then the protein processing is affected and the misfolded protein is trapped intracellularly [241]. Similarly, for proper expressions and posttranslational modifications

of gL is dependent on gH. Since gL lacks any hydrophobic domain to act as a membrane anchor, the association with gH may be helping the protein to remain membrane associated [242]. Another school of thought is that the hydrophobic amino terminus act as a membrane anchor and also as a signal peptide. gH is a type 1 membrane protein with the signal sequence directing it to the biosynthetic pathway and the transmembrane domain of the protein helps in attaching gH/gL protein complex to the membrane [243]. There have been emphasis on specific amino acids on the cytoplasmic domain of gH required for membrane fusion [244]. Antibodies to gH inhibited both cellular spread and virus infectivity [245], while antibodies to gL only affected cellular fusion and not virion infectivity [246]. Neutralizing antibodies were made when mice were vaccinated with gH/gL complex and the vaccinated group showed less primary infections and upon challenge there were no secondary lesions [247].

Glycoprotein J (gJ)

Glycoprotein J is encoded by US5 gene and their major function is in the ability to inhibit apoptosis [248]. It was in 1998 the ability of US9 genes sequence to encode gJ was demonstrated [249]. Soon it was shown that the us9 deletion abolished UV- or anti-fas-induced apoptosis [79]. The gene is initially expressed 4h after infection and can be detected at 6 h after infection. There are two forms, glycosylated and unglycosylated. The distribution was bound to endoplasmic reticulum, *trans*-Golgi network and early endosomes. Further works were able to show that just the expression of gJ can protect infected cells from apoptosis induced by cytotoxic T lymphocyte killing mechanism [248]. The protein is produced in limited quantities and localizes to membranes in the

cells. gJ can produce reactive oxygen species (ROS) in transfected and infected cells and may be influencing HSV lifecycle in different ways [250].

Glycoprotein M (gM)

The glycoprotein M (gM) is a hydrophobic type III membrane protein of 475 amino acids encoded by the UL10 gene. It has eight transmembrane domains with amino and carboxy termini directed towards cytosol [251, 252]. The open reading frame of the gene has two sites for N-linked glycosylation, one at the amino acid position 71 and the other at amino acid position 247. The first site is conserved among homologs of the gene in human cytomegalo virus (CMV) and varicella-zoster virus [253]. gM localizes to ER in infected cells [254]. The protein is highly hydrophobic and aggregates when boiled with buffers having SDS similar to UL20 protein [165, 251]. gM has also shown moderate antagonistic activity against tetherin, an antiviral effector that tethers newly enveloped virions to cell membrane preventing the egress [255]. Even though the gene is highly conserved among the members of family *herpesviridae*, it is a non-essential gene for HSV-1 [256], pseudorabies virus (PRV) [257], bovine herpes virus type 1 [258], Varicella-zoster virus [259], infectious laryngotracheitis virus [260], equine herpes virus type-1 (EHV-1) [261], and EHV type 4 [262]. In HSV-1, deletion of gM leads to small reduction in viral replication titers and smaller plaque sizes [169, 256]. gM can redirect other glycoproteins like gD and gH/gL to TGN following transfection due to the presence of classical endocytic signals at the carboxyl terminus [263]. Interaction between gM and VP22 encoded by UL49 has also been demonstrated [264]. gM can also associate with another membrane glycoprotein gN in various herpes viruses [265-268].

Glycoprotein N (UL49.5)

The open reading frames of HSV-1 and HSV-2 UL 49.5 is comprised of 91 and 87 codons respectively and protein has a transmembrane domains and expressed from a Y2 transcript [269, 270]. In case of PrV UL49.5 protein is made of 98 amino acids with the molecular weight around 10 kDa and have a signal peptide. The molecular mass will be reduced by 2 kDa if the signal sequence is cleaved. In HSV-1 the UL49.5 gene is separated from UL50 by 23 bp. This gene is conserved in Varicella zoster virus (VZV) [271].

Tegument Proteins

Initially HSV-1 virion proteins including tegument proteins were named viral protein 1 (VP1) to viral protein 24 (VP24) on the criteria of migration on a denaturing protein gel [272, 273]. Later tegument proteins were named based on the open reading frames and are called unique short (US) or unique long (UL) with prefix p in order to distinguish between a protein and a gene. The total number of tegument proteins determined by proteomic analysis in HSV-1 is 23 [274]. Some of the tegument proteins like pUL46 (VP11/12), pUL47 (VP13/14), pUL48 (VP16), and pUL49 (VP22) are abundantly present with copy numbers ranging from 1000-2000 copies. The four tegument proteins critical for viral growth in cell lines are pUL36 (VP1/2), pUL37, pUL48 and ICP4 [275]. During entry of virus to nonneuronal cells most of the envelop as well as tegument proteins are lost and nucleocapsids with a few inner tegument proteins reach host cell nucleus [276, 277]. It has been confirmed that the inner tegument proteins pUL36 and pUL37 are conserved among the *herpesviridae* family and associated with capsids after entry to Vero cells [278]. The incoming HSV-1 nucleocapsids depend on microtubular

transport based on dynein/dynactin complex to reach nucleus. The most suspected candidates for interacting with dynein motor proteins are pUL36 and pUL37, though nobody has yet proved through experiments [279]. Multiple studies have shown the importance of pUL36 in directing nucleocapsids to the nuclear pore complex (NPC) for the release of viral DNA [280-282]. Another tegument protein pUL14 has also been shown in targeting capsids to the nucleus [283]. pUL48 plays role in the formation of transcriptional complex and for formation of new virions [284]. Virion host shut-off (vhs) proteins (pUL41) inhibit both host and viral protein synthesis by degrading mRNA [285]. pUS11 binds to dsRNA through RXP repeats and interacts with nucleoli and ribosomes [286] and inhibit cellular protein synthesis through regulation of protein kinase R-activated host protein shutoff and decay of mRNA [287]. For envelopment and egress, virions require both pUL36 and pUL37 which are conserved among members of *Herpesviridae* family [288]. Both these proteins along with pUS3 forms the inner tegument layer of the newly formed nucleocapsids in the cytoplasm [289]. It has been shown that transport of pUL37 to golgi is affected in the absence of pUL36 in a capsid-independent manner [290]. It has been shown that pUL37 is only essential for replication of HSV-1, whereas in PrV they require both proteins UL36 and UL37.

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CHAPTER III
PHENYLALANINE RESIDUES AT THE CARBOXYL-TERMINUS OF THE
HERPES SIMPLEX VIRUS TYPE-1 UL20 MEMBRANE PROTEIN
REGULATE CYTOPLASMIC VIRION ENVELOPMENT AND
INFECTIOUS VIRUS¹ PRODUCTION

Importance

The herpes simplex virus type-1 (HSV-1) UL20 gene encodes a 222 amino acid long non-glycosylated envelope protein which forms a complex with viral glycoprotein K (gK) that functions in virion envelopment, egress, and virus-induced cell fusion. To investigate the role of the carboxyl-terminus of the UL20 protein (UL20p) in cytoplasmic virion envelopment, a cadre of mutant viruses was constructed and characterized. Deletion of six amino acids from the carboxyl terminus of UL20p caused approximately one-log reduction in infectious virus production compared to the wild-type virus. Surprisingly, a phenylalanine-to-alanine replacement at amino acid position 210 caused a gain-of-function phenotype increasing infectious virus production up to one log more than the wild-type virus. In contrast, replacement of two membrane proximal phenylalanines with alanines caused drastic inhibition of infectious virion production and cytoplasmic virion envelopment. Prediction of the membrane topology of UL20p revealed that these two amino acid changes cause retraction of the carboxyl-terminus of UL20p from the intracellular space. Confocal microscopy revealed that all the engineered UL20 mutations did not affect intracellular transport of UL20p to trans-Golgi Network membranes.

¹ This chapter previously appeared as Charles et al., 2014. Phenylalanine residues at the carboxyl-terminus of the Herpes Simplex Virus type-1 UL20 membrane regulate cytoplasmic virion envelopment and infectious virus production. *J Virol.* 2014 Jul 1;88(13):7618-27. It is reprinted with the permission of American Society of Microbiology.

In addition, proximity ligation assay showed that none of the UL20 mutations affected UL20p colocalization and potential interactions with the UL37 protein recently found to interact with the gK/UL20 protein complex. Collectively, these studies show that phenylalanine residues within the carboxyl terminus of UL20p are involved in the regulation of cytoplasmic virion envelopment and infectious virus production.

We have shown that the UL20/gK protein complex serves crucial roles in cytoplasmic virion envelopment and that it interacts with the UL37 tegument protein to facilitate cytoplasmic virion envelopment. In this manuscript, we investigated the role of phenylalanine residues within the carboxyl terminus of UL20p, since aromatic and hydrophobic amino acids are known to be involved in protein-protein interactions through stacking of their aromatic structures. Characterization of mutant viruses carrying phenylalanine (phe) to alanine (ala) mutations revealed that the two membrane proximal phe residues were critical for the proper UL20p membrane topology, and efficient virion envelopment and infectious virus production. Surprisingly, a phe-to-ala change located approximately in the middle of the UL20p carboxyl terminus substantially enhanced cytoplasmic envelopment and overall production of infectious virions. This work revealed that phe residues within the UL20p carboxyl terminus are involved in the regulation of cytoplasmic virion envelopment and infectious virus production.

Introduction

Herpes Simplex Virus type 1 (HSV-1) is a neurotropic herpes virus that after replication in epithelial cells enters into ganglionic neurons via distal axons and establishes latent infection [1]. HSV-1 is the primary infectious cause of blindness in the western hemisphere primarily because periodic reactivation causes recurrent keratitis and scarring

of cornea [2, 3]. Herpes virus particles consist of a linear double-stranded DNA genome containing an icosahedral capsid, surrounded by a proteinaceous tegument, and an outermost cell-derived lipid envelope. The virus encodes at least eleven glycosylated and several non-glycosylated proteins expressed on the viral envelope and in infected cell membranes, which are important for the life cycle of the virus especially during virus entry, intracellular virion morphogenesis and cell-to-cell spread. HSV-1 virion morphogenesis and egress involves complex mechanisms, which are characterized by sequential morphogenetic steps including: a) primary envelopment of capsids at the inner nuclear membrane; b) de-envelopment of enveloped capsids at the outer nuclear membrane; c) acquisition of tegument proteins in the cytoplasm of infected cells; d) re-envelopment of tegumented capsids at the TGN-derived membranes [4-6]. Cytoplasmic virion envelopment is facilitated by multiple interactions among viral glycoproteins embedded in TGN membranes and tegument proteins [6-10].

The UL20 gene encodes a 222 amino acid long non-glycosylated envelope protein, which plays a crucial role in cytoplasmic virion envelopment and egress of HSV-1. The UL20 gene is highly conserved among alphaherpesviruses including varicella-zoster [11], pseudorabies virus (PRV) [12] and the gammaherpesvirus Marek's disease virus type 2 [13]. The UL20 protein has been shown to be important for infectious virion production for both PRV and HSV-1 [14, 15]. The UL20 protein forms a functional complex with glycoprotein K (gK), which physically binds with gB and gH to modulate virus-induced cell fusion [16].

Deletion of 18 amino acids from the carboxyl terminus of UL20p (UL20 mutant virus 204t) inhibited intracellular transport and cell surface expression of both gK and UL20p,

and failed to complement infectious virus production. On the other hand, deletion of either 6 (UL20 216t) or 11 amino acids (UL20 211t) allowed efficient UL20p and gK intracellular transport and cell surface expression, but failed to complement for infectious virus production [17]. Phenylalanine residues are aromatic and hydrophobic amino acids that are known to be involved in protein-protein interactions and have been shown to play an important role in respiratory syncytial virus assembly [18]. Therefore, we sought to determine the potential role of phenylalanine residues in the carboxyl terminus of UL20p in cytoplasmic virion envelopment and egress under the hypothesis that cytoplasmic domains of UL20p may interact with tegument proteins to facilitate cytoplasmic virion envelopment. The UL37 protein is a 120 kDa phosphorylated protein [19] that assumes a predominantly diffused cytoplasmic distribution in infected cells [20-22] and can be transported to TGN membranes as a complex with the UL36 protein in the absence of capsid formation [23]. The UL37 tegument protein specified by HSV-1 and PRV has been shown to play an important role in cytoplasmic virion envelopment, since deletion of the UL37 gene resulted in accumulation of capsids in the cytoplasm of infected cells [24-26]. Recently, we showed that the HSV-1 UL37 protein interacts with gK and UL20p to facilitate cytoplasmic virion envelopment [27]. Herein, we show that a UL20 phenylalanine residue at position 210 plays a critical regulatory function in cytoplasmic virion envelopment and infectious virus production, as evidenced by the surprising one log higher infectious virus production of the mutant virus containing a phe to ala substitution at aa residue 210, when compared to wild-type virus. Moreover, two other phenylalanine residues proximal to the membrane of the carboxyl-terminus of UL20p are required for proper UL20p membrane topography and function. These phenylalanine to

alanine mutations did not appreciably alter the co-localization and potential interactions with the UL37 protein suggesting that they do not play major roles in the overall interactions between the UL37 protein and the gK/UL20 protein complex.

Materials and Methods

Cells.

African green monkey kidney (Vero) cells were obtained from the American Type Culture Collection (Rockville, MD) and Vero based UL20 Protein complementing FRT Cells were maintained in Dulbecco's modified Eagle's medium (Gibco-BRL; Grand Island, N.Y.) supplemented with 10% fetal calf serum and antibiotics.

Construction of HSV-1 mutant viruses

Mutagenesis was accomplished in *Escherichia coli* using the markerless two-step Red recombination mutagenesis system and synthetic oligonucleotides [28, 29] implemented on the bacterial artificial chromosome (BAC) plasmid pYEbac102-VC1 carrying the HSV-1(F) genome with proteins gK and UL20 tagged with V5 and 3xFLAG antigenic epitopes, respectively. Unless otherwise specified, the wild-type virus used in all experiments is the VC-1 strain. HSV-1 mutants UL20 Δ 6 and UL20 Δ Phe were constructed by adding a stop codon TAA in front of last amino acid or last 6 amino acids from the carboxyl end of UL20p respectively. Whereas, mutants UL20F222A, UL20F210A, and UL20F205-206A were created by changing codons for Phenylalanine at respective positions to Alanine codon GCA (Table 1). HSV-1 BAC DNAs were purified from 50 ml of overnight bacterial cultures with a Qiagen large-construct kit (Qiagen; Valencia, CA). Using PCR test primers designed to lie outside the target mutation site (s), all mutated DNA regions were sequenced to verify the presence of the

desired mutations in BACs. Similarly, viruses recovered from cells transfected with BACs [28] were sequenced to confirm the presence of the desired mutations. The entire genomes of the wild-type and the UL20F210A mutant viruses were sequenced using the Ion Torrent Next Generation Sequencing equipment (Life Technologies-Invitrogen; Carlsbad, CA), as we have described previously [30]. Briefly, total genomic DNA (gDNA) was extracted from the virus-infected Vero cells using the PureLink Genomic DNA Mini Kit (Life Technologies-Invitrogen; Carlsbad, CA). High-quality fragment libraries of each virus were prepared from the extracted total gDNAs using the Ion Xpress Plus Fragment Library Kit (Life Technologies-Invitrogen; Carlsbad, CA). The fragment libraries were subsequently applied to Ion 316 chips and were sequenced on the Ion Personal Genome Machine System (Life Technologies-Invitrogen; Carlsbad, CA).

SDS-PAGE and Western Immunoblot Assay

Western immunoblot analysis was carried out essentially as described earlier [31]. Confluent monolayers of Vero cells were infected with the indicated viruses at an MOI of 2. At 24 hpi, cells were washed with PBS and lysed on ice for 30 min in NP-40 lysis buffer (Life Technologies) supplemented with a cocktail of protease inhibitors (Roche, LLC, Basel, Switzerland). The collected samples were mixed with SDS-PAGE sample buffer (NuPAGE) at 3:1 ratio and were electrophoretically separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tris-HEPES-SDS gradient 4-20% gels, Thermo scientific). Following electrophoresis, the proteins were transferred onto a nitrocellulose membrane under a constant current. Membrane was blocked in Phosphate-buffered saline containing 0.1% Tween 20 (PBST) plus 5% nonfat milk for 1 hour at room temperature (RT) and was probed with primary mouse anti-Flag mAbs (Abcam;

Cambridge, England) for over-night at 4°C. Goat anti-mouse secondary antibody conjugated with HRP and Immobilon chemiluminescent HRP substrate (Millipore) were used for detection purposes.

Plaque Morphologies, Replication Kinetics and Electron Microscopy.

For plaque morphology, confluent monolayers of Vero or FRT cells were infected at a MOI of 0.001 with the viruses and fixed with methanol 48 h post infection. Immunohistochemistry was performed with primary rabbit anti-HSV antibodies (1:1,000) (Dako) and goat anti-rabbit horseradish peroxidase-labeled antibody (1:1,000) (Dako), and the reactions were developed using the NovaRed substrate (VectorLabs). Images were taken with an inverted light microscope (Olympus) using relief contrast. Growth kinetics studies of viruses were performed as we have described earlier [32, 33]. Approximately 90% confluent Vero cells were infected with each virus at 4°C for 1 hour at a low or high multiplicity of infection (MOI) of 0.2 or 2 respectively. Afterwards, plates were incubated at 37°C for 1 hour to allow virus penetration. Any remaining extracellular viruses were inactivated by low pH treatment using PBS at pH 3 and plates were incubated till its time point and subsequently stored at -80°C till the virus titers were obtained by endpoint titration on FRT cells. The ultrastructural morphology of virions within infected cells was examined by transmission electron microscopy as described previously [16, 28, 32, 34]. All flasks with infected cells were incubated at 37°C and 5% CO₂ until processed at 18 hours post infection (hpi) and infected cell samples were visualized by transmission electron microscopy.

Quantification of cell-to-cell fusion

Chemiluminescence-based assay system was used to quantify cellular fusion during viral infection [35-37]. Around 80-90 % confluent Vero cells in 12-well plates were transfected separately with plasmids expressing T7 polymerase gene or luciferase gene under the T7 promoter at amounts 1µg/well each. Lipofectamine 2000 (Invitrogen) was used for all transfections. Media was changed six hours post-transfection and after 12 hours of incubation at 37°C and 5% CO₂, cells were detached using trypsin-EDTA. Both populations of cells were washed, further resuspended in complete medium and mixed in 1:1 ratio prior to seeding in 24-well plate. Fifteen hours post seeding cells were infected at a MOI of 0.5. At 24 hpi cells were washed with PBS, lysed with lysis buffer and supernatants were collected. Supernatant was mixed with luciferase assay substrate and the amount of relative fusion was obtained by measuring the relative level of luminescence (RLU) emitted measured using TD-20/20 luminometer (Turner Designs).

Quantitative PCR

Quantitative PCR (qPCR) was utilized to derive the total number of viral genomes within the lysate. Specifically, the primers and probe (6-carboxytetramethylrhodamine [TAMRA]) for the real-time PCR were designed to detect HSV-1 US6 (gD). Lysates were collected at 18 hpi, and 200 µl of each suspension was used for the extraction of viral DNA. Viral DNA was extracted using a DNeasy blood and tissue kit (Qiagen, Inc.) as per the manufacturer's instructions. Equal volumes of viral DNA were used for TaqMan PCR analysis. Purified HSV-1 bacterial artificial chromosome (YE102) DNA was used to generate the standard curve.

Confocal Microscopy

Vero cell monolayers grown on eight-well glass *Lab-Tek* II chamber slide were infected with the indicated virus at an MOI of 10. Uninfected cells were used as negative controls. Cells were washed once with PBS and fixed with ice-cold 100% methanol for 10 minutes at 18 hpi. Monolayers were subsequently blocked for 1 h with 2% fetal calf serum in PBS. Further staining was done using mouse monoclonal anti-FLAG IgG1 (Sigma-Aldrich, St. Louis, MO) for recognition of UL20p and TGOLN2 IgG2a (Abnova, Taiwan) for recognition of trans-Golgi network diluted 1:200 in PBS blocking buffer followed by Alexa Fluor 555-conjugated goat anti-mouse IgG1 and Alexa Fluor 488-conjugated goat anti-mouse IgG2a secondary antibodies. Nucleus was stained using 4', 6-diamidino-2-phenylindole (DAPI). Specific immunofluorescence was examined using an Olympus FluoView confocal microscope. Image analysis and subcellular scatter plot graphs were generated and analyzed using the Olympus FluoView FV1000 confocal microscope software interface. Scatterplot graphs with colocalization of two fluorophores within the TGN were generated by specifically defining the regions of interest with a bounding box of equal area based on pixel enumeration for each captured image. To determine an approximate percentage of subcellular colocalization, pixel enumeration and intensity statistics within the Olympus software package were accumulated for each sample image. The percentage of upper right quadrant pixels from the total sample pixels was displayed. Finally images were compiled and rendered with Adobe Photoshop.

Proximity Ligation Reaction (PLA)

In situ PLA detection was carried out using the appropriate DUOLINK II In Situ kit components obtained from Sigma-Aldrich, St. Louis, MO. Vero cell monolayers grown

on eight-well glass *Lab-Tek* II chamber slide were infected with the indicated viruses at a MOI of 10. At 18 hpi cells were fixed using methanol for 10 minutes and blocked using DUOLINK blocking solution at 37°C in a wet chamber for 2 h. Primary antibodies used are anti-FLAG mouse (Sigma-Aldrich, St. Louis, MO) and anti-UL37 rabbit for the negative controls (mock infection and untagged wild type F strain) and for the wild type as well as the mutants. Whereas, primary antibodies used for positive control (gK-UL20p interaction) are anti-V5 mouse (Invitrogen Life Technologies) and anti-FLAG rabbit (Sigma-Aldrich, St. Louis, MO). Antibodies were diluted in blocking buffer and incubated in a wet chamber at 4°C overnight. The slides were washed two times with 1X TBS + 0.05% Tween 20 for 5 min each, then secondary antibodies (DUOLINK anti-rabbit PLA-plus probe, DUOLINK anti-mouse PLA-minus probe) were added and incubated at 37°C for 1 h. Two washes with Duolink In situ wash buffer A for 5 min each were then followed by addition of the ligation mix and incubation in wet chamber at 37°C for 30 min. After ligation slides were washed twice for 2 min using Duolink In situ wash buffer A before adding amplification reaction mix for 90 min at 37°C. Texas red-labeled oligonucleotide detection probes (Olink Bioscience) were used. Subsequently, the slides were washed twice with Duolink In situ wash buffer B, and once with 0.1× Duolink In situ wash buffer B. Slides were mounted with Duolink *In Situ* Mounting Medium with DAPI and analyzed in a Olympus FluoView confocal microscope.

Results

Construction and Molecular Analysis of recombinant viruses

We have previously shown that deletion of the UL20p carboxyl terminal six-amino acids resulted in drastic inhibition of cytoplasmic virion envelopment and egress [17]. To

further investigate the potential role of phenylalanine residues within the carboxyl terminus of the UL20 protein, we constructed a cadre of mutant viruses for which one or more phenylalanine residues were replaced with alanine residues. All mutant viruses were constructed using the markerless two-step Red recombination mutagenesis system implemented on the bacterial artificial chromosome (BAC) plasmid pYBac102-VC1 carrying the HSV-1(F) genome (see Materials and Methods). This virus expresses gK and UL20p tagged with V5 and 3xFLAG antigenic epitopes, respectively [30, 38]. The recombinant mutants included viruses having a deletion of the terminal phenylalanine residue (UL20 Δ Phe), deletion of six amino acids from the carboxyl terminus (UL20 Δ 6), and replacement of one or two internal phenylalanine residues with alanines (UL20F222A, UL20F210A, and UL20F205-206A) (Fig. 3.1 and Table 3.1). The deletion mutants were made by the insertion of stop codon TAA in front of the last amino acid for UL20 Δ Phe and before the last 6 amino acids for UL20 Δ 6. Similarly, for the amino acid replacement mutants, codons for phenylalanine (TTT or TTC) were replaced with the codon for alanine (GCA). Virus stocks were prepared after BAC transfection on FRT cells. Mutant viral genomes were characterized using PCR-assisted DNA sequencing, as well as whole genome sequencing approaches (see Materials and Methods section).

Replication and Phenotypic characteristics of the mutant viruses

The plaque morphologies of all mutant and wild-type viruses were examined on Vero cells (Fig. 3.2a) and FRT cells (Fig. 3.2b). As expected, the HSV-1(VC1) wild-type virus produced the largest virus plaques, while the UL20F205-206A, UL20 Δ 6 produced smaller viral plaques when compared to the parental wild-type virus on Vero cells, while the recombinant viruses UL20 Δ Phe, UL20F222A produced syncytial plaques on Vero

cells (Fig. 2a). All mutants produced wild-type-like plaques on FRT (Vero cells transformed to express the UL20 gene) cells (Fig. 2b). Western immunoblot analysis revealed that all viruses expressed similar levels of the UL20 protein (Fig. 3.3). To investigate the role of these mutations on the kinetics of viral replication, replication curves were produced for infections at both low (0.2) and high (2) MOI (Fig.3.4a, b). At both high and low MOI, the UL20F205-206A mutant yielded the lowest end titer and UL20F210A mutant yielded the highest end titer when compared to wild-type virus. Importantly, the UL20F210A virus reproducibly produced approximately one log higher viral titers, when compared to the wild type virus at high MOI. At low MOI of 0.2, terminal viral titers of UL20 Δ 6 and UL20F205-206A were 1 and 2 logs less, respectively in comparison to wild-type, while the UL20F210A mutant virus produced significantly higher viral titers than the wild-type virus. At MOI 2, the UL20F205-206A mutant virus titer was more than a log less than the wild-type virus.

Quantification of cell-to-cell fusion.

Mutant viruses UL20 Δ Phe and UL20F222A produced syncytial plaques on Vero cells. The extent of virus-induced cell fusion was evaluated using a previously described luciferase assay [30] (see Materials and Methods). The gB Δ 28syn mutant HSV-1 virus carrying a 28 amino acid deletion of the carboxyl terminus of gB causes extensive virus-induced cell fusion [31] and was used as positive control. The relative levels of luminescence were measured for all samples at 24 hours hpi. Among mutant viruses, the UL20F222A produced the highest extent of fusion exhibiting RFU levels that were similar to those of the positive control gB Δ 28syn virus (Fig. 3.5).

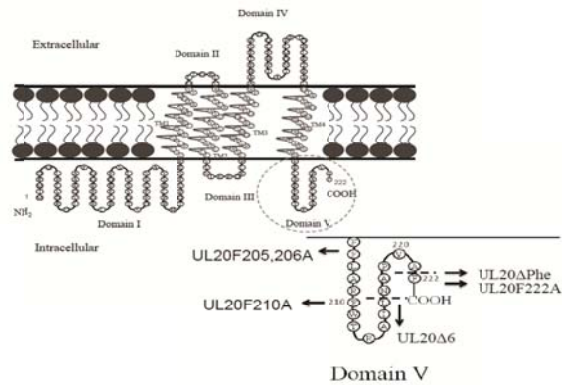


Figure 3.1 Predicted membrane topology of UL20p and location of mutations. Amino acid(s) replacements are indicated with the amino acid position(s) bracketed on the left by the targeted phenylalanine(s) and on the right by the changed alanines (s) i.e. UL20F222A. (TM-transmembrane region).

Table 3.1: Names of mutant viruses and amino acids in the wild type and mutants shown.
* Indicates stop codons

Mutation	Amino acid sequence	
	Wild type	Mutant
UL20ΔPhe	NAPVAF	NAPVA*
UL20Δ6	RAILNA	RAIL*
UL20F222A	NAPVAF	NAPVAA
UL20F210A	FFLARF	FFLARA
UL20F205-206A	FFLARF	AALARF

carrying a 28 amino acid deletion of the carboxyl terminus of gB causes extensive virus-induced cell fusion [31] and was used as positive control. The relative levels of luminescence were measured for all samples at 24 hours hpi. Among mutant viruses, the UL20F222A produced the highest extent of fusion exhibiting RFU levels that were similar to those of the positive control gB Δ 28syn virus (Fig. 3.5).

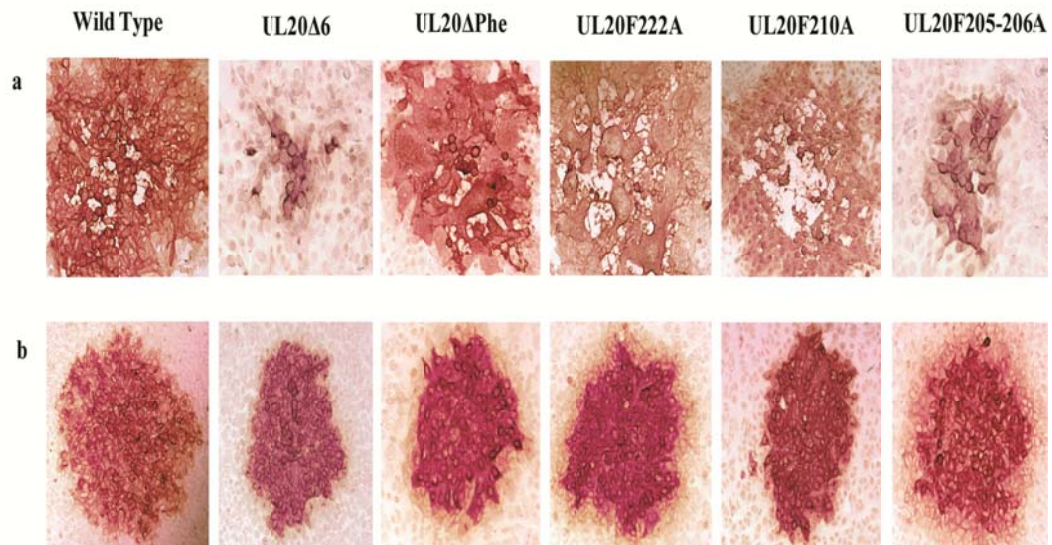


Figure 3.2: Plaque morphology of mutant viruses in comparison to HSV-1(F)-YE102-VC1. Confluent monolayers of Vero cells were infected with 0.0001 MOI of wild-type and mutant viruses and immunohistochemically was performed using polyclonal anti-HSV rabbit sera at 48 hpi, as described in Materials and Methods. Representative viral plaques of HSV-1(F)-YE102-VC1 wild-type virus and mutant viruses on Vero cells (a) and on FRT cells (b).

degrees of cytoplasmic defects in virion envelopment, as evidenced by the presence of numerous unenveloped or partially enveloped capsids in the cytoplasm of infected cells (Fig. 3.6).

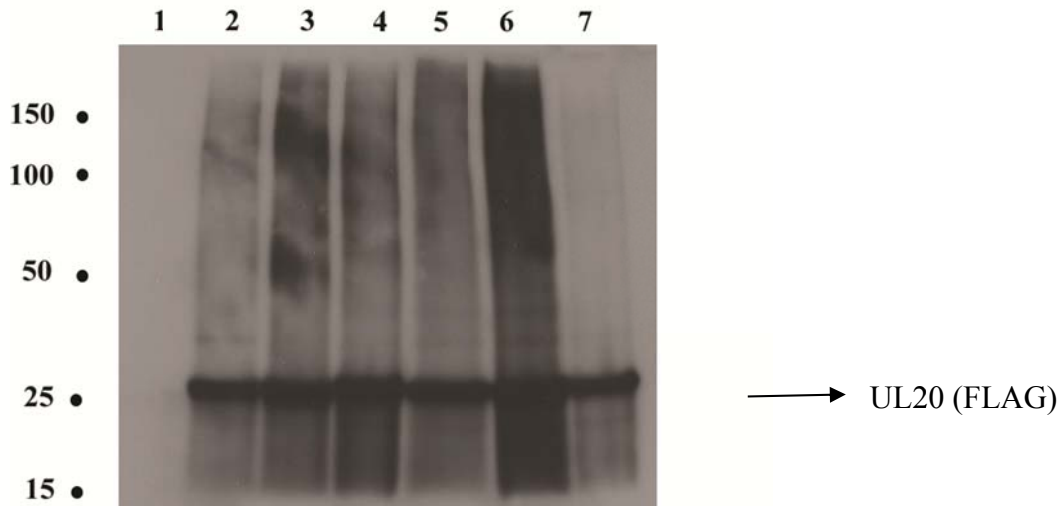


Figure 3.3. Western immunoblots of UL20 protein (FLAG epitope) in lysates from cells infected with the indicated viruses. Confluent monolayers of Vero cells were infected with the viruses at an MOI of 2. At 24 hpi cell lysates were collected and ran on SDS PAGE and probed with anti-FLAG antibody. Lanes 1) Vero cell Lysate, 2) Wild-type lysate, 3) UL20 Δ 6 lysate, 4) UL20 Δ PHE lysate, 5) UL20F222A lysate, 6) UL20F210A lysate, 7) UL20F205-206A lysate.

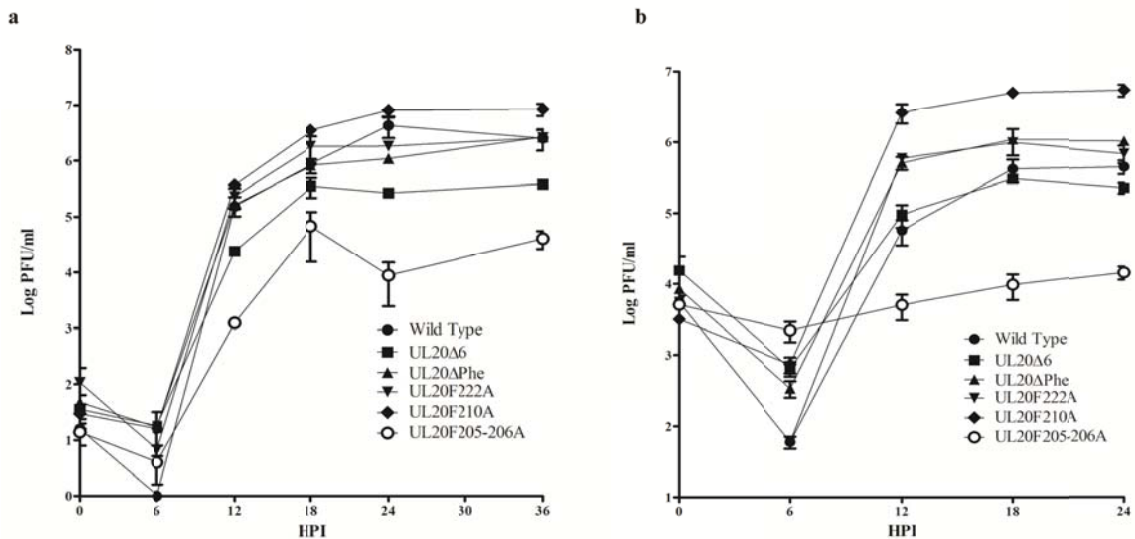


Figure 3.4. Replication kinetics of the wild-type and mutant viruses. Confluent Vero cell monolayers were infected with each virus either at low MOI (0.2) (a) or high MOI (2.0) (b) and viral titers were obtained by plaque assay on FRT cells at different time points post infection. Error bars represent standard error.

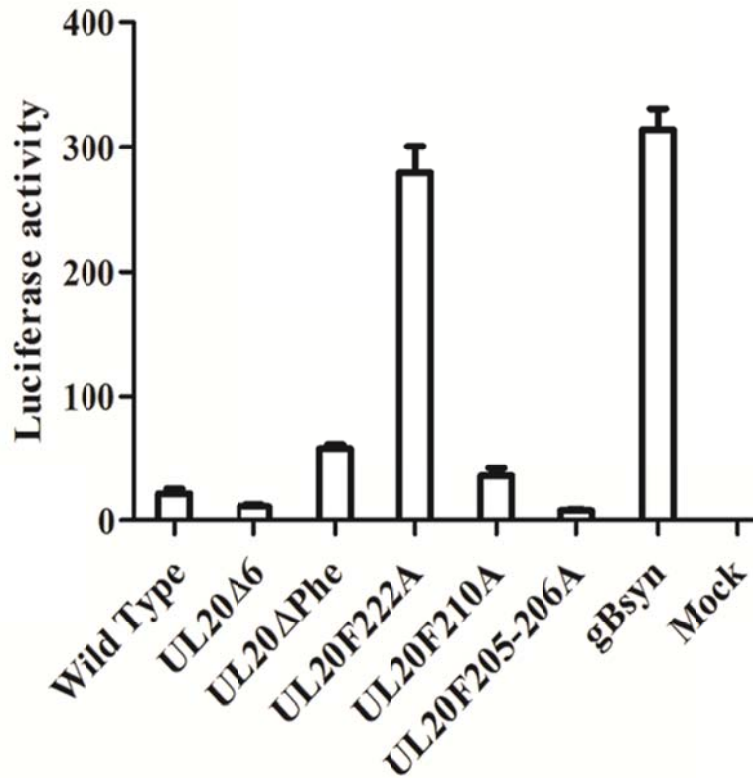


Figure 3.5. Fusion activity of each virus was quantified by luciferase-based assay (see Materials and Methods). The extent of fusion was assessed at 24 hpi for the wild-type virus and all mutant viruses. Error bars indicate the standard error.

Ultrastructural Characterization of wild-type and mutant viruses

The ultrastructural phenotypes of all recombinant viruses were compared to the wild-type parental virus after infection of Vero cells at an MOI of 3 at 18 hpi post infection (Fig. 6). As expected, the wild-type virus did not exhibit any defects in cytoplasmic virion envelopment and egress, as fully enveloped viruses were readily seen via electron microscopy outside the infected cells. In contrast, mutant viruses exhibited varying

degrees of cytoplasmic defects in virion envelopment, as evidenced by the presence of numerous unenveloped or partially enveloped capsids in the cytoplasm of infected cells (Fig. 3.6).

Characterization of the relative efficiency of cytoplasmic virion envelopment

A measure of the relative efficiency of infectious virion production is indicated in the ratio of total viral genomes produced in infected cells to the number of infectious virions determined by the standard plaque assay. In this regard, The UL20F205-206A mutation caused the most severe defect in the relative efficiency of infectious virion production followed by UL20 Δ 6 mutant virus (Table3. 2).

Intracellular localization of the UL20 mutant proteins and UL20 interactions with the UL37 protein

The intracellular location of wild-type and mutant UL20 proteins was investigated using confocal microscopy. All mutant viruses expressed UL20p that colocalized with the TGN marker TGOLN2. These visualization results were further supported by determination of the approximate percentage of subcellular co-localization using pixel enumeration and intensity statistics as detailed in Materials and Methods (Fig. 3.7). Recently, we showed that the UL37 protein physically interacted with the gK/UL20 protein complex to facilitate cytoplasmic virion envelopment. To investigate whether the engineered UL20mutations affected interactions between UL20p and UL37 proteins, we utilized the proximity ligation assay [39, 40] in conjunction with the anti-FLAG monoclonal antibody detecting the UL20-tagged protein and the anti-UL37 polyclonal antibody. The known interactions between the UL20 protein and gK was also tested using the anti-FLAG (UL20) and anti-V5 (gK) antibodies, as a positive PLA control. PLA produced vivid mutations affected interactions between UL20p and UL37 proteins, we utilized the

proximity ligation assay [39, 40] in conjunction with the anti-FLAG monoclonal antibody detecting the UL20-tagged protein and the anti-UL37 polyclonal antibody. The known

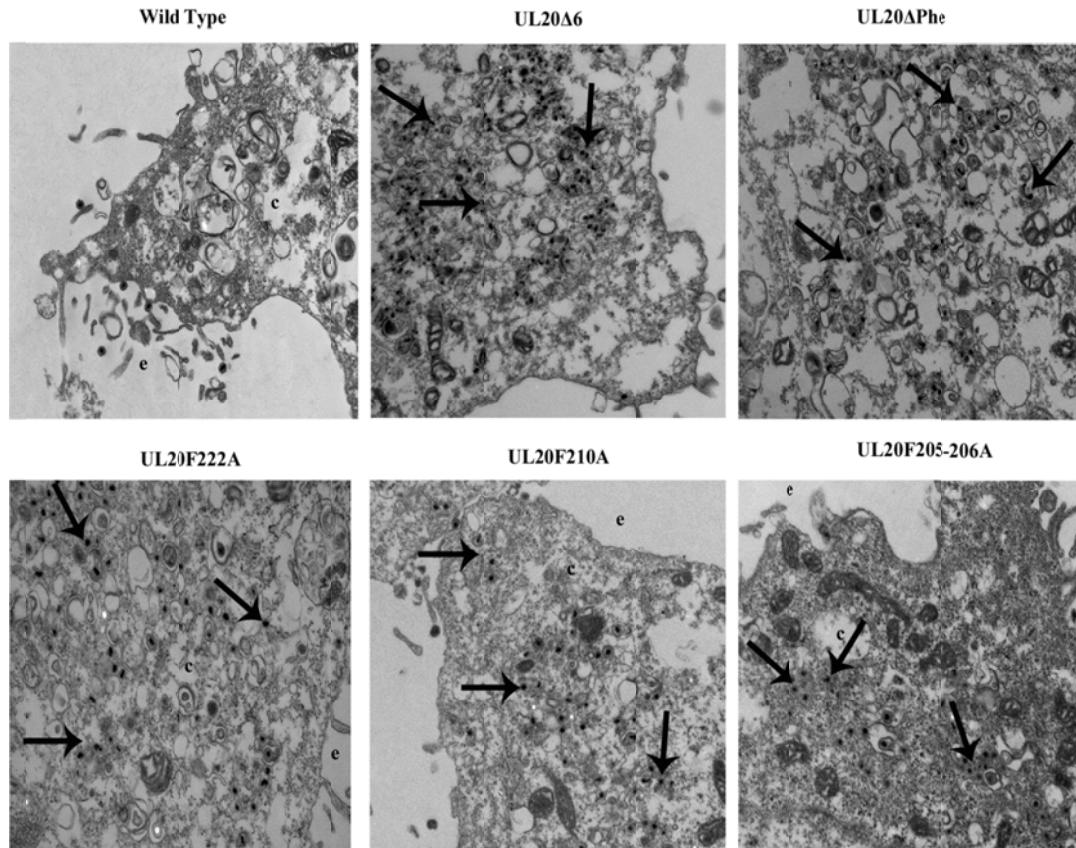


Figure 3.6. Ultrastructural morphology of wild-type and mutant viruses. Electron micrographs of Vero cells infected with different viruses at an MOI of 2 and processed for electron microscopy at 18 hpi are shown. The extracellular space (e) and cytoplasm (c) are marked. Arrows show viral particles with envelopment defect.

interactions between the UL20 protein and gK was also tested using the anti-FLAG (UL20) and anti-V5 (gK) antibodies, as a positive PLA control. PLA produced vivid fluorescent signals showing efficient colocalization between the interacting gK and UL20p, as well as between UL20p and UL37 in wild-type as well as in all mutant virus

infected Vero cells. In contrast, mock-infected cells or cells infected with the HSV-1(F) strain (expresses untagged gK and UL20 proteins) did not exhibit any appreciable fluorescent signals (Fig. 3.8).

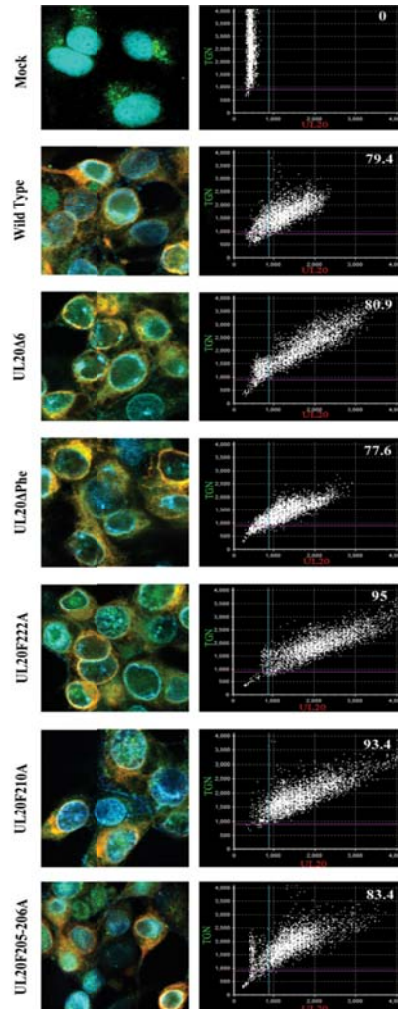


Figure 3.7. Colocalization of UL20p and TGN in the wild type virus and mutant viruses. Vero cells fixed at 18 hpi after infecting with each virus at an MOI of 2 were stained with anti-FLAG antibody for UL20p (red), TGOLN2 antibody for TGN (green) and DAPI for nucleus (blue). Scatterplot graphs, which depict pixel intensity and distribution, and an approximate percentage of subcellular colocalization, are shown for each image.

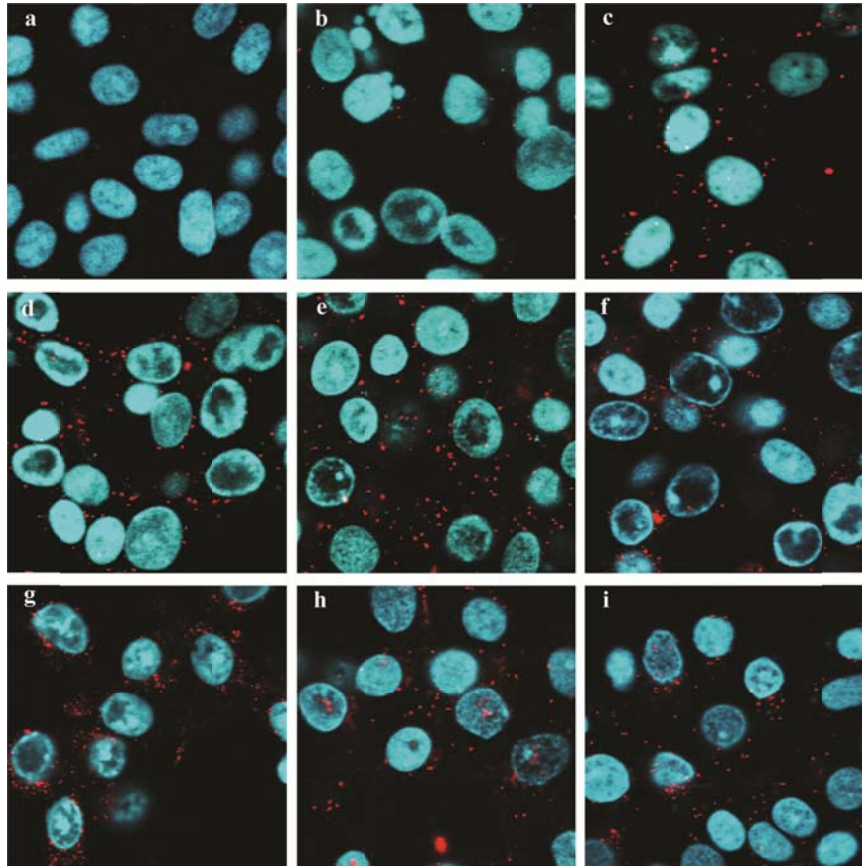


Figure 3.8. Proximity ligation assay to determine UL20p and UL37 interactions. Vero cells were infected with viruses at a high MOI of 10 and PLA was performed at 18 hpi. **a)** Negative control mock infection, **b)** Negative control with HSV-1 F strain infection, **c)** Positive control showing gK-UL20p interaction, **d)** Wild Type, **e)** UL20 Δ 6, **f)** UL20 Δ Phe, **g)** UL20F222A, **h)** UL20F210A, **i)** UL20F205-206A.

fluorescent signals showing efficient colocalization between the interacting gK and UL20p, as well as between UL20p and UL37 in wild-type as well as in all mutant virus infected Vero cells. In contrast, mock-infected cells or cells infected with the HSV-1(F) strain (expresses untagged gK and UL20 proteins) did not exhibit any appreciable fluorescent signals (Fig. 3.8).

Table 3.2. Determination of viral genomes-to-PFU ratios. The total number of viral genomes infected Vero cells at 24 hpi were obtained by qPCR. The total number of intracellular infectious virions was obtained by plaque assay on FRT cells. Ratios reflecting relative efficiency of envelopment and infectious virion production were obtained by dividing the average of the number of viral genomes within infected cells by number of PFU.

Viruses	Total HSV-1 genomes/PFU (in thousands)
Wild- Type	48
UL20 Δ 6	538
UL20 Δ Phe	170
UL20F222A	166
UL20F210A	38
UL20F205-206A	5648

Discussion

We have previously shown that the gK/UL20 protein complex serves crucial roles in cytoplasmic virion envelopment [9]. Our laboratory and others have shown that the UL37 protein localizes to the TGN membranes and functions in cytoplasmic virion envelopment [41]. Recently, we showed that the UL37 protein interacts with the gK/UL20 protein complex and facilitates cytoplasmic virion envelopment and infectious virus production [27]. Herein, we show that phenylalanine residues located at the

carboxyl terminus of the UL20 protein are involved in the regulation of cytoplasmic virion envelopment and infectious virus production.

We have previously reported the use of extensive alanine-scanning mutagenesis of the UL20 protein to delineate domains of the UL20 protein that function in cytoplasmic virion envelopment and virus-induced cell fusion [42]. Specifically, we showed that the amino terminus of the UL20 protein functioned in virus-induced cell fusion, while the carboxyl terminus was important for cytoplasmic virion envelopment [42]. Of specific interest to the current study was that deletion of the terminal six amino acids of the UL20 protein drastically inhibited cytoplasmic virion envelopment, while it did not affect UL20 protein interactions with gK and virus-induced cell fusion [42]. Based on our recent findings that the gK/UL20 protein complex interacts with the UL37 protein, we hypothesized that this interaction may occur between the carboxyl terminus of the UL20 protein and the UL37 protein. These interactions may involve phenylalanine residues, which are known to facilitate protein-protein interactions through residue stacking and hydrophobic interactions. Significantly, the UL20F210A mutation caused more than one log higher replication kinetics and final infectious virus production than wild-type virus on Vero cells at high MOI. Moreover, the ratio of total viral genomes to PFU was lower than that of the wild-type virus indicating that this mutation increases the efficiency of infectious virus production. The observed increase in the production of infectious virions reflects enhanced cytoplasmic virion envelopment, since it is unlikely that UL20 mutations would affect other aspects of virion assembly such as capsid assembly in the nucleus. Moreover, the UL20F210A mutant virus produced higher infectious virus titers at high versus low MOI in comparison to the parental wild-type virus suggesting that

enhancement in infectious virus production is dose-dependent on the expression of the UL20F210A protein.

The UL20F210A “gain-of-function” mutation reveals that the phenylalanine (210) residue is involved in regulation of cytoplasmic virion envelopment and infectious virus production. The purpose of this inhibitory mechanism is not immediately apparent. Infectious virus titers and particle to PFU ratios are affected by temperature with optimum virus titers and particle-to-PFU ratios produced at 34°C presumably because the entire process of virion assembly is more efficient at reduced assembly speed (Kousoulas, unpublished observations). Thus, it is possible that the process of cytoplasmic virion envelopment is down-regulated by UL20/gK to ensure efficient virion envelopment at 37°C or higher. Alternatively, the replacement of the phenylalanine (210) may cause more efficient interaction of UL20p with other viral proteins or cellular proteins involved in cytoplasmic virion envelopment.

The UL20F222A mutant virus caused extensive virus-induced cell fusion on Vero cells, which was completely inhibited on the UL20 complementing FRT cells. This result suggests that the phenylalanine residue at amino acid position 222 is involved in the regulation of membrane-fusion presumably because it alters the interactions of the gK/UL20 protein complex with the fusogenic glycoprotein gB. All mutant viruses produced similar plaques with respect to size and fusogenic characteristics on FRT cells indicating that wild-type UL20p expression by FRT cells efficiently reverted all mutant phenotypes to that of the wild-type. Overall, these results suggest that the wild-type UL20p expressed by the FRT cells acts in a dominant manner to reverse the effect of these UL20 mutations presumably because it interacts more efficiently with gK, gB and

UL37. Reversion of the phenotypic and growth properties of the UL20 mutant viruses indicates the absence of any other spurious mutations within the mutant viral genomes. This conclusion is further supported by PCR-assisted sequencing of targeted genomic areas and genomic sequencing of the wild-type and the UL20F201A genomes by next generation sequencing methodologies, which did not detect any spurious mutations.

Phenylalanine replacement or deletion of carboxyl terminal regions of the UL20 protein that contained phenylalanine residues caused drastic defects in virion envelopment without affecting colocalization of the UL20 mutant proteins with the UL37 protein, as evidenced by similar PLA-produced fluorescent signals. Of particular interest is the mutagenesis of two membrane proximal phenylalanine residues to alanines. This double amino acid replacement is predicted to cause retraction of the carboxyl terminus of the UL20 protein, so that it is no longer exposed to intracellular spaces (Fig. 3.9). These results suggest that the carboxyl terminus of the UL20 protein is not a major contributor to overall interactions with the UL37 protein. Alternatively, mutagenesis of the carboxyl terminus of the UL20 protein may negatively affect other functional aspects of the overall interactions of the gK/UL20 complex, as well as its potential domain-specific affinity to UL37 or other tegument proteins causing inhibition or enhancement of cytoplasmic virion envelopment and infectious virus production.

Cytoplasmic virion envelopment has been thought to involve a series of complex interactions between viral glycoproteins embedded in the TGN membranes and tegument proteins found on cytoplasmic capsids, or bound to TGN membranes. Results, herein, show that cytoplasmic virion envelopment is a dynamic process where

interactions can affect cytoplasmic virion envelopment in either a positive or negative manner. These dynamic interactions may provide an advantage for virus replication in

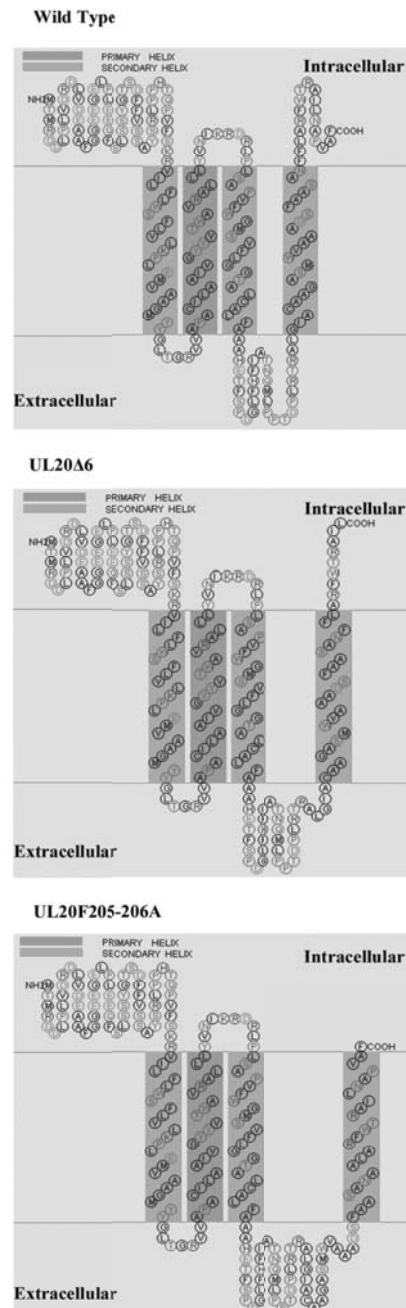


Figure 3.9. SOSUI programme predicted structure of UL20p in wild type and mutant viruses UL20 Δ 6 and UL20F205-206A.

certain cells, where the virus may need to control the rate of infectious virus production. The UL20F210A gain of function mutation can be incorporated into other mutant herpes viruses engineered for anticancer or gene therapy purpose to enhance their beneficial functions or to alleviate their defects in replication efficiencies and produce higher titers of virus. Further studies are required to explore this gain of function behavior of the mutant virus and to investigate whether the mutation has any effect on the pathogenesis of the virus in experimental animal infections.

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CHAPTER IV
ENTRY CHARACTERISTICS OF HSV-1 gK 31-68 MUTANT IN VERO CELLS
AND NEURONS STUDIED USING UL37 PROTEIN/DYNEIN PLA ASSAY

Significance

Cytoplasmic dyneins are known to transport cargoes in a retrograde manner, which is towards the center of the cell. We show here for the first time utilizing PLA, that in both epithelial cells and neuronal ganglia, the motor protein dynein potentially interacts with the HSV-1 inner tegument protein UL37 upon release of the cytosolic capsids in to the cytosol of the host cell. PLA, is a valuable tool to detect protein-protein interaction that overcomes the non-specificity of antibody approach or the difficulties associated with co-immunoprecipitation assay when relatively large hydrophobic membrane proteins are involved. The interaction between UL37 and dynein was seen as early as 30 minutes after entry and continued till 14 hrs. However, it was considerably reduced and almost completely absent at 16 hours post entry. This interaction was much pronounced in the wild type virus after one hour post infection, when compared to the gK 31-68 mutant virus which has been shown to enter relatively slowly in Vero cells. This clearly implies that upon losing the envelope on the cell surface, the viral capsids with the tegument proteins interact with dynein and utilize the microtubular network to be transported to the nucleus.

Introduction

Herpes Simplex Virus-1 (HSV-1) predominantly enters Vero cells by the fusion mechanism, where the viral envelope fuses with the cellular plasma membranes in a pH-independent manner. While fusion remains the only mode of entry of HSV-1 in neuronal

cells[1], entry of the virus in non-neuronal cells can also occur by receptor-mediated endocytosis where the low pH milieu of the endosomes causes the fusion to take place between the viral envelope and the endocytic membranes [2]. Virus-induced cell fusion mediated by viral glycoproteins helps the virus to spread from infected cells to uninfected cells without being exposed to extracellular spaces [3].

HSV-1 encodes at least 26 tegument proteins and 11 virally encoded glycoproteins, as well as several non-glycosylated membrane associated proteins. The viral glycoproteins gD, gB, gH, and gL have been known to be involved in virion entry, while gC has been known to enhance initial binding of the virus to host cell membranes [4-8]. The virion entry is thought to be initiated by the binding of glycoproteins gB and gC to glycosaminoglycan (GAG) moieties of cell surface proteoglycans [9]. This initial attachment causes the interaction of gD with one or more of its specific receptors, including the herpesvirus entry mediator (HVEM, or HveA), nectin-1 (HVeC), 3-O-heparan sulfate and additionally PILR- α , NMHC-IIA, and myelin associated glycoprotein (MAG), that facilitates virion attachment and virus entry[10].

After fusion of the viral glycoproteins with the host plasma membrane, the tegument proteins and the capsid containing the DNA are released into the cytosol. For efficient replication of the viral DNA in the nucleus of the infected cell, the capsid has to be transported from the plasma membrane across the cytosol to the nuclear membrane. Diffusion cannot be utilized by the HSV-1 capsid across the cytoplasm as it has been calculated that a HSV capsid would take over 200 years to travel just 1cm if diffusion was the only mode of transport[11]. In case of neurons that have the cell body, dendrites and an axon, where the dendrites are highly branched and elongated and an axon which

can be as long as one meter, diffusion cannot be utilized as the optimal means of transport. These cytosolic capsids are ably transported to the nucleus along the microtubules [12]. HSV-1 an alphaherpesvirus undergoes replication in the epithelial cells of the host at the site of infection before entering the nervous system. The viral particles enter the peripheral nervous system where they are transported towards the cell body in a retrograde manner to initiate latent infection in the sensory neurons of the dorsal root and trigeminal ganglia. However, reactivation of the virus from latency causes the viral particles to be transported to the periphery in an anterograde manner. In case of neuronal cells as well, the capsids are transported along the axons to the neuronal cell body, which utilizes the microtubule network. The capsids have been shown to move in minus end direction towards the microtubular organizing centers (MTOC)[13]. Cytoplasmic dyneins are known to transport payloads towards the minus end of microtubules (retrograde transport), which is usually the center of the cell, while kinesin motors usually transport payloads towards the plus end of microtubules (anterograde transport) which is the cell periphery [14-16]. The retrograde transport of the capsids has been shown to be disrupted when the cells are pretreated with microtubule depolymerizing agents such as nocodazole or colchicine suggesting that the microtubule network is required for entry into the nucleus[13, 17].

These cargo transporters, are usually made up of multiple subunits with their molecular weights ranging from 1.5 MD for dynein, 1.2 MD for dynactin, 340-380 kDa for kinesin-1 and 260-280 kDa for kinesin-2 (Plus and minus end paper). Dynein belongs to the AAA+ superfamily (ATPase associated with various cellular activities), and cytoplasmic dynein is a large complex made of two heavy chains (DHCs), two intermediate chains

(ICs), two light intermediate chains (LICs) and several light chains (LCs) [14, 18]. The heavy chain consists of the cargo binding domain (N-terminus) and the motor domain (C-terminus) made up of six AAA ATPase domains in the form of a hexameric ring that causes the movement by utilizing the energy released during ATP hydrolysis [19] and has a stalk that binds to microtubule [20]. Alpha herpes viruses such as HSV-1, pseudorabies virus (PrV) and the retrovirus human immunodeficiency virus type 1 (HIV-1) and adenovirus, exploit cytoplasmic dynein of the host cell which along with its co-factor dynactin, causes their directional movement towards the MTOC during their initial stages of infection [21, 22]. Majority of the outer tegument proteins remain at the plasma membrane along with the glycoproteins upon fusion of the virus at the cell membrane causing the inner tegument proteins attached to the viral capsids to be exposed in the cytoplasm [23, 24]. Experiments have shown that purified dynein motor can bind to tegumented capsids and do not depend on the presence of any host proteins for this binding. Lack of capsid proteins such as VP26, pUS11 or VP11/12 did not seem to affect the transport of these capsids to the nucleus and thus may less likely have the binding sites for the dynein motors. These interactions are further supported by in vitro experiments in which capsids complexed with inner tegument proteins were able to associate with the microtubule motors, while the capsids lacking the tegument proteins did not [25]. Also, the failure of nuclear capsids to bind to the MAPs (microtubule associated proteins) would suggest that the tegument proteins are more likely the candidates for binding the motor proteins. Studies using recombinant viruses having different tagged viral proteins for tracking showed that the major tegument proteins UL36 and UL37 can be detected in capsids undergoing retrograde transport in neuronal cells

and this protein complex along with the capsid persisted together even after reaching the nuclear membrane, while majority of the other tegument proteins are lost from the capsids during this transport, similar to the findings in epithelial cells [21, 26, 27]. Thus these inner tegument proteins UL36 and UL37 may be a few of other proteins that serve as strong candidates that potentially recruit dynein motor [28, 29]. The HSV-1 UL37 is a 120kDa phosphorylated tegument protein expressed in both mature virions and light particles [30]. UL37 protein is known to interact with proteins pUL46, pUL35 [31, 32] and pUL36 and this interaction of pUL37 with pUL36 is known to be critical for transport to the TGN [33, 34]. The pUL36 interacts with the outer tegument protein pUL48 [32] and the minor capsid protein pUL25 [35, 36]. In addition to envelopment, which is associated with egress, pUL36 and pUL37 have also been implicated in intracellular transport, as it has been shown in both HSV1 and PrV that lack of either one of these proteins impairs the intracellular trafficking of viral capsids to the nucleus [37].

Here we show by proximity ligation assay (PLA), a sensitive method to detect protein-protein interaction, that pUL37 potentially interacts with dynein motor protein during entry. This interaction is visualized as bright red spots of fluorescence and the interaction seems to occur starting as early as 30 minutes post infection and seem to persist 2 hrs post entry, while very minimal to no signal is detected after 16 hrs, indicating that may be at 16 hpi the dynein motors may not be engaged with the viral capsids and the nuclear capsids might be associated with kinesin motors which aid in the transport of the capsids towards the plasma membrane of the cell.

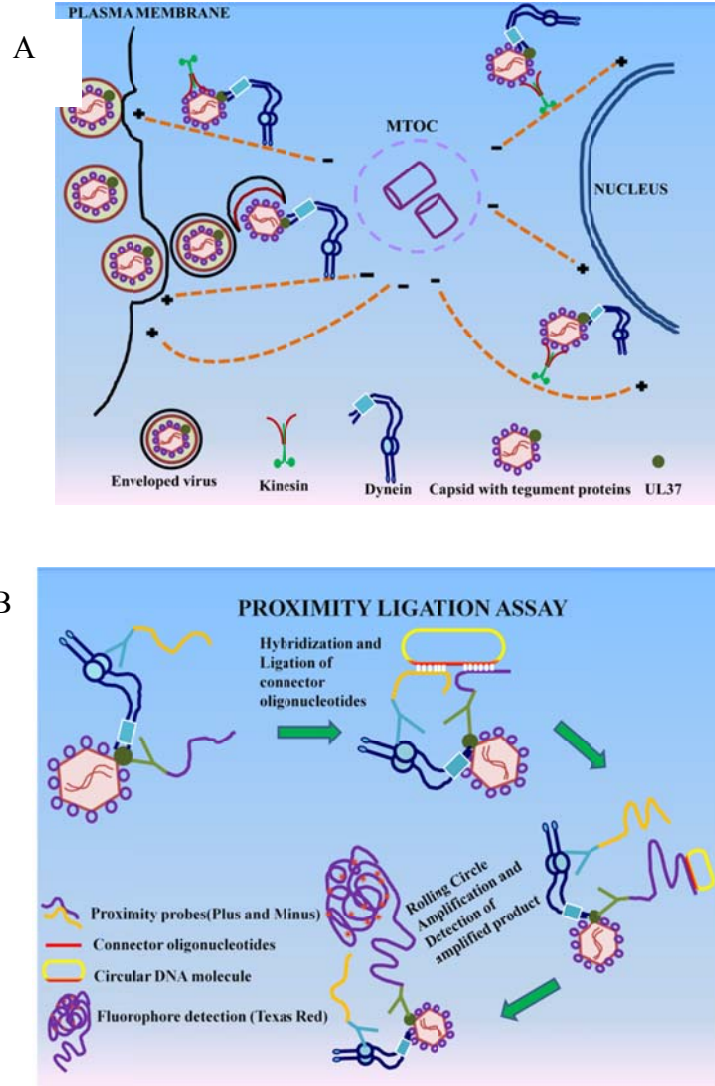


Figure 4.1. Schematic representation of transport of cargoes across the cell. The virus enters the cell via fusion or endocytosis and is transported by dynein motors towards the nucleus (retrograde transport) presumably by the interaction of dynein with one or more inner tegument proteins, in our case pUL37. Kinesin transports the cargoes towards the cell membrane (anterograde transport) (A), Schematic representation of the proximity ligation assay between UL37/Dynein. The interaction of the tegument protein UL37 with the motor protein Dynein after the virus enters the cell is detected by proximity ligation assay. After the binding of the primary bodies to their respective proteins (UL37 and dynein), two antibodies which have oligonucleotides attached to them, referred to as proximity probes are added. The interaction of dynein and UL37 causes the two probes to come closer to each other causing the connector oligonucleotides to circularize leading to the rolling circle amplification by the polymerase. This amplification is detected as fluorescent signals (red dots) (B).

Materials and Methods

Cell Lines

African green monkey kidney (Vero) cells were obtained from the American Type Culture Collection (Rockville, MD) and was maintained in Dulbecco's modified Eagle's medium (Gibco-BRL; Grand Island, N.Y.), supplemented with 10% fetal calf serum and antibiotics. CHO-nectin-1(human) cells were a gift from Richard Longnecker (Northwestern University, Chicago, IL) and CHO-neo cells were a gift from Yasushi Kawaguchi (The University of Tokyo, Japan). Both these cell lines were propagated in Ham's F12 growth medium supplemented with 10% FBS and 200 µg/ml G418 and 250 µg/ml G418 respectively.

In situ proximity ligation assay analysis of protein interactions in virus infected cells.

Vero cells were grown on 8 well chamber slides (Nunc Lab-Tek II chamber slide system) and infected with F strain virus at an MOI of 10. At 18 hours post infection, the cells were fixed with ice cold methanol for 10 minutes at -20°C. After three washes with PBS, the samples were blocked for 2 hrs at 37°C with the Duolink blocking buffer in a humidity chamber. Primary antibodies that were raised in two different species were diluted in antibody diluting buffer and added to the samples and were incubated overnight at 4°C. Mouse anti-dynein antibody against intermediate chain I (Abcam) and rabbit anti-UL37 antibody (a gift from Dr. Frank J. Jenkins, University of Pittsburgh Cancer Institute) were used for dynein/UL37 detection, respectively in Vero, CHO-neo and CHO-nectin cells. Mouse anti-gD antibody and rabbit anti-nectin-1 antibody (Santa Cruz) was used for positive control for gD/nectin-1 interaction in Vero cells. Mouse anti-dynein antibody and rabbit anti-gM antibody was used as negative control for dynein/gM

interaction on Vero, CHO-neo and CHO-nectin-1 cells. Anti-microtubule antibody (Abcam) was used for microtubule detection. Unbound primary antibodies were removed by washing with 1X TBS-Tween20 (0.05%) three times, five minutes each. The Duolink insitu PLA probes, Anti-Rabbit Plus and Anti-Mouse Minus, were added to the samples (1:5 dilution) and incubated at 37°C for 1hr. After the incubation step, washes were done with Duolink wash buffer A, twice for five minutes each. The ligation stock was diluted 1:5 in high purity water and added to the wells (40 to 80ul) and the slides were incubated at 37°C for 30 minutes. The slides were washed with buffer A three times, five minutes each and amplification solution (40 to 50ul) was added and were incubated for 1.5 hrs at 37°C. The slides were then washed with wash buffer B twice for 10 minutes each and washed once with 0.01% of buffer B. The slides were then mounted with mounting media (Duolink II) and the slides were stored at -20°C and protected from light until confocal images were taken. The confocal images were taken using a 60X objective on an Olympus FluoView FV10i confocal laser scanning microscope.

The virus was allowed to attach to confluent monolayer of vero cells at 4°C for 1hour and shifted to 37°C to allow virus entry and the chamber slides were removed at various time points 0, 30, 2 hrs, 3 hrs, 6 hrs, 9 hrs, and 12 hrs and fixed with ice-cold methanol and proceeded with proximity ligation assay.

In situ proximity ligation assay analysis of protein interactions in virus infected dorsal root ganglia

Dorsal Root Ganglia were dissected from fetal Sprague-Dawley rats at embryonic days 8 to 10 and seeded on Poly-D-Lysine 8-well Culture Slides (Catalog number: 354632 BD). Similarly aged Rat DRG was also obtained commercially (Catalog number: DRG –

BrainBits). Maintenance tissue culture medium consisted of neural basal medium with B-27 supplement, at the manufacturer's recommended concentration (Invitrogen, Grand Island, NY). Medium was supplemented with 50 ng/ml neural growth factor 2.5s (Invitrogen), 2% normal rat serum (Invitrogen), 1_ GlutaMAX (Invitrogen), and 0.2% Primocin (InvivoGen, San Diego, CA). The ganglia were monitored regularly for axonal growth and supplemented with neuro-basal media supplemented with neuronal growth factors. A healthy extension of axons was observed 7 days post seeding and the ganglia were ready to be infected. The media was then removed and 200,000 PFU of either wild-type McKrae or mutant gK Δ 31-68 virus was added to the ganglia. The virus was removed after 1 hour incubation time at 37C and the slides were fixed with ice cold methanol for 10 minutes at -20⁰C. PLA assay was performed on these slides as described earlier.

Results

The inner tegument protein UL37 interacts with the motor protein dynein during transport of the viral capsids to the nucleus

Proximity ligation assay shows the interaction of the tegument protein UL37 with the motor protein dynein. This interaction is visualized as bright red spots of fluorescence which can be seen starting from 30 minutes post entry. After the initial attachment of the virus to the cell, glycoprotein D is known to interact with one or more of its specific receptors causing virion attachment and entry. We utilized nectin-1 which is one of its specific receptors for our positive control. Bright red fluorescent signals were visualized which shows the interaction of gD with nectin-1 at zero time point. The gM /dynein combination was used as negative control for both zero and 16 hrs time points, because

after the fusion of viral glycoproteins with the host cell membrane only the tegument proteins and the capsid containing DNA are released in to the cytosol of the host cell. Consequently, gM dynein interaction is physically improbable. As expected, no red fluorescence signals were seen indicating the absence of gM-dynein interaction.

For the pUL37-dynein interaction, the virus was allowed to attach to the cells for 1 hr at 4°C followed by incubation times 0 min, 30 min, 2 hrs, 6 hrs, 9 hrs, 12 hrs, 14 hrs and 16 hrs at 37°C to allow virus entry. The signals were seen at all-time points except for 0 minutes. Visually, the signals seemed to be a maximum at 30 minutes with some seen at 2 and 6 hrs. At 9 hrs post entry, the signals seemed to have a pattern where they accumulated around the nucleus. Although, at 12, 14 and 16 hrs post entry the signals seemed to dissipate and were minimal (Fig 2 A & B).

We have shown previously that the gK 31-68 virus (the first 68 aa deleted which includes the first 30aa signal sequence) enters very slowly in the Vero cells when compared to the wild type virus. Here we saw only a few signals for the pUL37-Dynein interaction for the gK 31-68 virus showing the slow entry of the virus into the cells compared to the wild type, however the signals for gD-nectin-1 interaction in both the viruses appeared to be similar.

Dorsal root ganglia resected from 8-10 day old Sprague-Dawley rat embryos were seeded on PLL coated 8 well chambered slides and cultured in neuro basal medium supplemented with neuronal growth factors. The Ganglia exhibited robust growth of axons 7 days post seeding and were used for infection studies. As with Vero cells gM-

Dynein interaction was used negative control. Red fluorescent signals representing gD-Nectin interaction were observed on the axonal surface in the case of both wild type

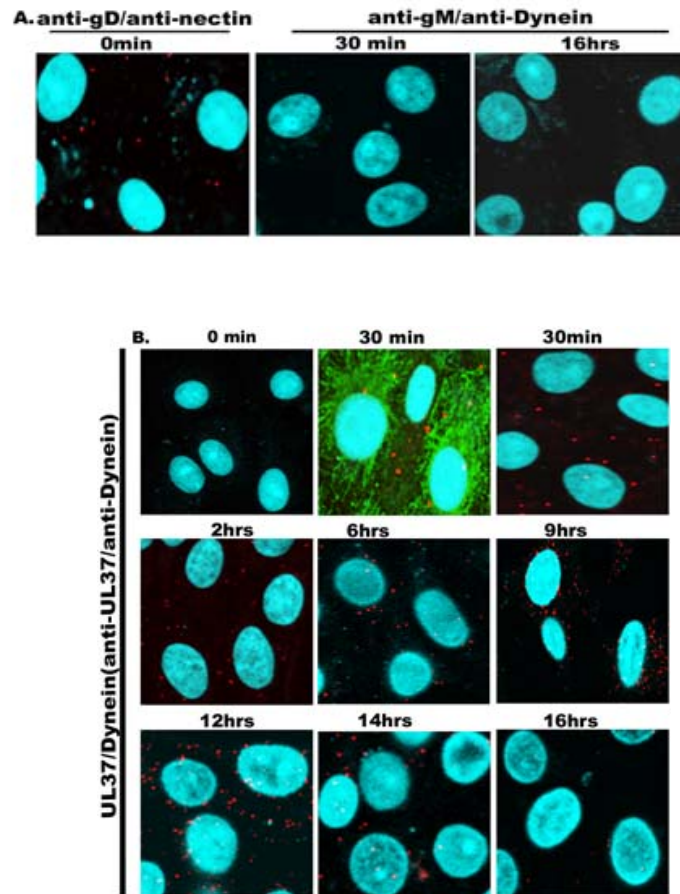


Figure 4.2. Proximity ligation assay of UL37- dynein interaction on Vero cells. Confluent Vero cells were grown on 8 well chamber slides. HSV-1 F strain virus at an MOI of 10 was used to infect the Vero cells and the slides were rocked at 4°C for one hour for the binding of virus to the cells and shifted to 37°C to allow entry. The cells were fixed with ice-cold methanol for 10 minutes at -20°C followed by proximity ligation reaction as described in the materials and methods. gM/dynein antibody pair was used as a negative control and it was used for 30 min and 16 hrs time point while gD/nectin-1 antibody pair was used as positive control at zero time point (A), The remaining time points utilized pUL37/dynein antibody pair to determine the interaction of these two proteins at different time points post entry. The virus was allowed to enter at varying time points, 0, 30 min, 2 hrs, 6 hrs, 9 hrs, 12 hrs, 14 hrs and 16 hrs (B)

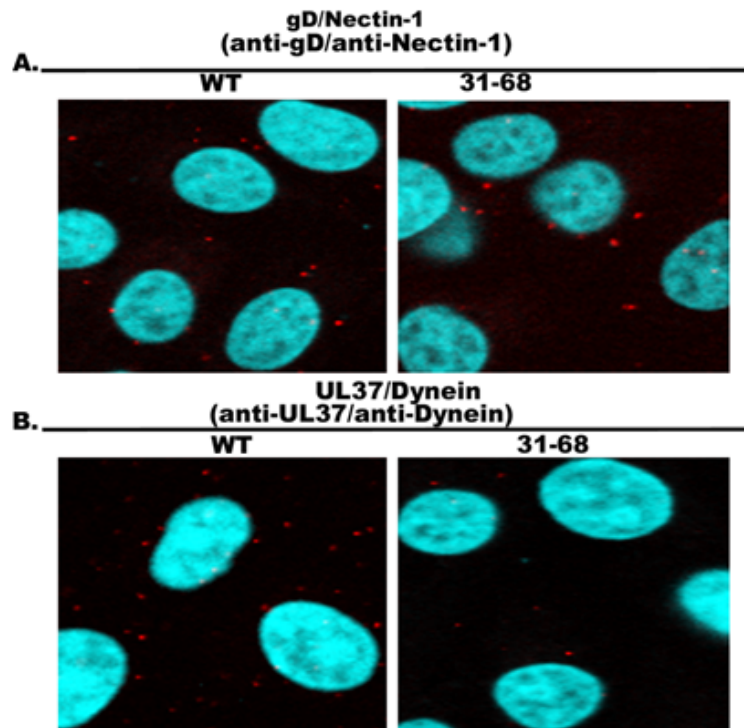


Figure 4.3. Comparison of UL37/dynein interaction in WT and gK 31-68 viruses. Confluent Vero cells grown on 8 well chamber slides were infected with HSV-1 F strain virus at an MOI of 10. The slides were rocked at 4C for binding of virus to the cells for one hour and shifted to 34C to allow entry. At one hour post infection, the virus was removed and cells were fixed and PLA assay was performed as described in materials and methods. Interaction between gD and nectin-1 was studied in both these viruses as control (A). UL37/dynein antibody pair was used to study the interaction between these proteins in the cells (B).

Dorsal root ganglia resected from 8-10 day old Sprague-Dawley rat embryos were seeded on PLL coated 8 well chambered slides and cultured in neuro basal medium supplemented with neuronal growth factors. The Ganglia exhibited robust growth of axons 7 days post seeding and were used for infection studies. As with Vero cells gM-Dynein interaction was used negative control. Red fluorescent signals representing gD-Nectin interaction were observed on the axonal surface in the case of both wild type

McKrae as well as gK31-68 infections. In DRG axons infected with wild type virus the pUL37-Dynein probe combination yielded Red signals indicating the entry of the virus and interaction of UL37 with dynein. However no such signal was observed in DRG axons infected with gK Δ 31-68 which suggest that the virus was stuck on the surface. As with Veros the gM-Dynein combination did not yield any signals (Fig 4 A, B, C, & D).

Discussion

Many new techniques and methods come in to existence in the field of proteomics that help us to understand and acquire insights in protein structure, functions, modifications and interactions and so on.

PLA is a relatively simple tool to detect protein-protein interactions. This technique has also been utilized for detecting infectious agents and proteins with a sensitivity rated higher than much established technique like PCR [38]. Here we have used the technique to understand the entry and transport of HSV-1 towards nucleus. This technique can be utilized to detect the entry of virus to the cytosol of infected cells even if the virus fails to enter nucleus and start protein synthesis Thus is helpful in differentiating between virus stuck at the cell plasma membrane or inside the cell giving more insight into which steps of viral life-cycle are affected. The HSV-1 proteins shown to be interacting with dynein are nuclear protein UL34 through GST pulldown experiment and capsid protein VP5 and helicase UL9 using Pepscan [39]. Through biochemical analysis and live imaging suggest VP1/2 (UL36) and UL37 are the two most suspected candidates for interacting with dynein [37]. We are able to report the proximity between cellular motor protein dynein and the herpes viral tegument protein UL37. Our data suggested close physical interaction between dynein and UL37 throughout replication cycle. Studies showing

interactions between viral proteins and cellular motors are very limited and part of the reason may be because dyneins are tremendously large multimeric proteins with molecular mass ranging from 1,000K-2,000K [40]. In this scenario, PLA can be used to unfold the mysteries of protein-protein interactions rather than opting for the difficult techniques of immunoprecipitation and western blots. It is shown that at zero min there is no interaction between the two proteins as the virus has not even entered the cells and the interaction shows up at later time points. Negative control experiments using membrane protein gM and dynein did not show any interaction at both early and late time points. This clearly supports the belief that it is not the membrane proteins, but the outer capsid or inner tegument proteins are involved in cellular transport of virus. The interaction between pUL37 and dynein starts from the time of viral entry to cells, then intensifies and cluster around nine hours and finally disseminate and vanishes towards later time point. Clustering of interaction during egress suggest that dynein may have a passive role during exit by being part of the motor system and also more pUL37 is present as new viruses are being made Further works need to be done to dissect where the interactions between dynein and UL37 is happening.

Our lab has shown that the gK mutant virus, gK Δ 31-68 lacking 68 aa from its amino terminus (including the 30-aa signal sequence) entered vero cells less efficiently compared to wild-type virus [3]. Through PLA we have supported the observation by showing fewer signals for pUL37-Dynein interaction for gK Δ 31-68 when compared to wild-type virus though the interaction between gD-nectin remained similar for both the viruses showing that the attachment between virus and cell was not affected by the mutation on gK . A critical aspect of the life cycle of HSV-1 is the entry into axons and

establishment of latency in the ganglia. With the development of HSV-1 as a highly potent oncolytic vector/vaccine, it is of critical importance to develop recombinant HSV-1 strains capable of delivering oncolytic payloads, without infecting axons and establishing latency in the ganglia. We believe that 31-68 aa region of the amino-terminal region of HSV-1 glycoprotein K plays a critical role in modulating gB mediated entry into axons. In order to prove this hypothesis we have seeded dorsal root ganglia resected from 8-10 Sprague-Dawley rat embryos to seed them on poly-L Lysine coated * well chambered slides. In presence of Neuronal growth factors, a robust growth of axonal fibers extending away from the neuronal bodies within the ganglionic cluster was observed. Infection of these axonal regions with both wild type McKrae and gK Δ 31-68 virus followed by the proximity ligation assay similar to the vero studies described earlier reinforced our hypothesis. In the case of the wild type infections, red fluorescent signals were observed for both gD/Nectin as well pUL37-Dynein combination. However in the case of gK Δ 31-68 red fluorescent signals were observed only for the gD/Nectin interaction. The absence of pUL37-Dynein interaction in gK Δ 31-68 infections suggest that i) either the virus is unable to enter the axons and is stuck on the surface or ii) gK Δ 31-68 is altering the configuration of UL37 rendering it incapable of interacting with Dynein axons. The latter is a highly unlikely scenario with no empirical evidence to support it and also as virus loses its membrane proteins and many tegument proteins subsequent to the entry into cell after fusion This interaction study demonstrates one of the practical applications of PLA. Thus PLA can be used as a very convenient and useful tool in exploring the life-cycle events of the virus.

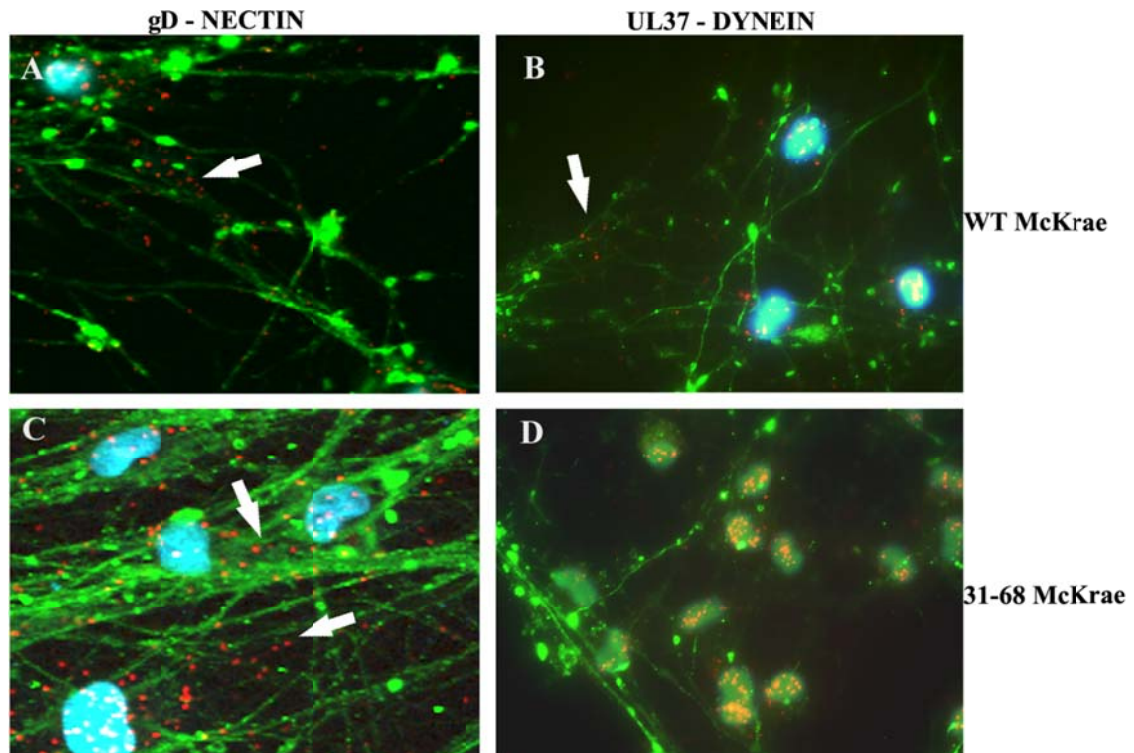


Figure 4.4. The McKrae 31-68 mutant is unable to enter DRG axons. DRG from 9-10 day old rat embryos were collected and seeded on Polylysine coated 8 well chambered slides. Six- days post seeding they were infected with HSV-1 strain McKrae WT or 31-68. The wells were probed for interaction of UL37/Dynein and gD/Nectin, using Proximity Ligation Assay. Panels A and C show interaction between gD and Nectin on DRG axons infected with WT and 31-68 mutant respectively, as seen by the presence of red spots along the axons (arrows). Panel B shows the interaction between UL37/Dynein in DRG axons infected with WT virus. However no interaction was observed between UL37/Dynein in DRG axons infected with 31-68 mutant indicating lack of viral entry (panel D). Neurofilament marker (green) and DAPI (blue) were used to identify axons and the nuclei of glial cells respectively. Both WT and 31-68 mutants were able to enter glial cells, as seen by red spots around DAPI stained nuclei.

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CHAPTER V CONCLUDING REMARKS

Summary

The Herpes Simplex virus has a very complex life cycle and in which envelopment and egress are important events. Viral membrane proteins and tegument proteins functions together in order to get the virus successfully envelop and egress out of the cell. The important membrane proteins required for the step are glycoproteins gM, gK, gE and non-glycosylated membrane proteins UL20 and UL11. Among all the most important is the UL20/gK complex [1]. UL20p is a structural protein made of 222 amino-acids which is non-glycosylated and has transmembrane domains. It is highly conserved among alphaherpesviruses like varicella-zoster [2], pseudorabies virus (PRV) [3] and the gammaherpesvirus Marek's disease virus type 2 [4]. Works done in my laboratories have show that UL20p deletion resulted in accumulation of unenveloped capsids in the cytoplasm of infected cells [5]. Mutant viruses were made with mutations in the carboxyl termini of UL20p especially targeting Phenylalanine residues which may be an important target for protein-protein interactions through stacking of the aromatic structure. The mutation of approximately middle phenylalanine to alanines has an advantageous effect on the virus as evidence by an increased titer at end the replication study and also a lower particle to PFU ratio reflecting the higher efficiency of viral envelopment. Another important observation of the study was the importance of the double phenylalanine residue proximal to the membrane. Replacement of those phenylalanines to alanines affected the replication capabilities of the virus and also the envelopment efficiencies. The protein structure predicted by SOSUI programme showed that the carboxyl terminal

is retracted from the intracellular space and this might have affected the ability of the protein to interact with tegument proteins during envelopment.

In the second study, we have showed that the deletion of amino terminus of viral envelop protein gK has affected the entry of HSV-1 into Vero cells and neurons through PLA technique. gK like UL20p has multiple transmembrane regions and is made of 338 amino acids encoded by the UL53 open reading frame . PLA is a sensitive method to detect protein-protein interaction [6]. After entry to cell, virus recruits cellular motor proteins for their transport to the nucleus and for the retrograde transport dynein is the motor protein of interest. The most suspected tegument protein interacting with dynein is the inner tegument protein of virus UL37. PLA technique has been standardized to show the interaction between UL37 protein and dynein. Here the technique has been successfully shown the entry and transport of HSV-1 towards the nucleus and can be used as a method to evaluate viral entry to the cytoplasm of infected cell. Using the technique it has been shown that the gK Δ 31-68 lacking 68 aa from its amino terminus entered slowly into vero cells and in case of neurons there is complete absence of signals indicating the inability of the virus to enter neurons. It is interesting to note that the viral attachment to cell surface is not affected in either case suggesting the later step after entry is affected and deletion of amino terminus of gK is not hindering the attachment step.

Future Studies

In chapter II, phenylalanine residues at the carboxyl terminus of UL20p has been shown have regulator effect on infectious virus production and envelopment. Additional work need to be done to further understand the how the deletion or replacement is affecting

envelopment and egress of the mutant viruses. Peptides of UL20 carboxyl terminus can be used to study whether they are able to complement the behavior of mutant viruses. Plans are there to use the viruses in an invitro neuronal system to study the anterograde and retrograde transport and to have a better understanding about the behavior of virus in neurons. Furthermore, mice can be infected with the viruses to study pathogenesis of infection.

In chapter III, PLA was used to support and explore the role of amino terminus of gK in entry to Vero cells and neurons respectively. This technique is a valuable tool to detect the entry of virus to cells. It can be used to study similar entry defects in different membrane proteins mutant viruses and used to explore which all domains are critical for entry. Apart from entry, egress can also be studied by using the different motor protein kinesins, which are important for anterograde transport. Thus PLA can be used as a very convenient and useful tool in exploring the life-cycle events of the virus.

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APPENDIX
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VITA

Anu Susan Charles was born to Mrs. Susamma Charles and Dr. Charles Philip in Kerala, India. Anu has a younger sister Linu Susan Charles. From Kindergarten to grade 10, she attended Mount Carmel School, Kottayam, and Kerala. After graduating from school, she joined Bishop Choolaparambil Memorial College (BCM), Kottayam, Kerala for pre-degree studies (grade 11 and 12). Then she joined College of Veterinary and Animal Sciences, Mannuthy, Kerala for her DVM degree and Anu was a recipient of Kerala Agricultural University Scholarship for 5 years for academic excellence during her undergraduate studies. After receiving the DVM degree, she joined University of Connecticut (UCONN) for her masters in Animal Science. Upon graduating from UCONN, she joined Louisiana State University (LSU) for her doctoral degree in the research of Herpes Simplex virus under the guidance of Dr. K. G Kousoulas.