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# Comparative roles of herpes simplex virus type 1 (HSV-1) glycoproteins in cytoplasmic virion egress

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# COMPARATIVE ROLES OF HERPES SIMPLEX VIRUS TYPE 1 (HSV-1) VIRAL GLYCOPROTEINS IN CYTOPLASMIC VIRION EGRESS

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# EPIGRAPH

Research is what I'm doing when I don't know what I'm doing.

-Wernher Von Braun-

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# TABLE OF CONTENTS

EPIGRAPH ……ii

ACKNOWLEDGEMENTS ……iii

LIST OF TABLES ……vii

LIST OF FIGURES ……viii

ABSTRACT ……ix

CHAPTER ONE: INTRODUCTION ……1
STATEMENT OF PROBLEM AND HYPOTHESIS ……2
STATEMENT OF RESEARCH OBJECTIVES ……5
LITERATURE REVIEW ……6
Historical Overview of Herpesviruses ……6
*Hepesviridae* Taxonomy ……7
Epidemiology of Herpes Simplex Virus ……10
Transmission and Pathogenesis ……11
Symptoms of Clinically Significant Herpesviruses ……12
Orofacial Herpes ……12
Genital Herpes ……13
Ocular Herpes ……14
Neonatal Herpes ……14
Central Nervous System (CNS) Herpes ……14
Treatment and Preventative Measures ……15
Herpes Virion Structure and Biological Properties ……16
The Core ……16
The Capsid ……16
The Tegument ……18
The Envelope ……18
Viral Genome ……18
Herpesvirus Lifecycle ……20
Virus Attachment and Fusion ……20
Binding Receptor Heparan Sulfate (Glycosaminoglycans) ……23
Entry Receptors ……23
Tissue Necrosis Factor (TNF) Family ……24
Immunoglobulin (Ig) Superfamily ……24
3-O-sulfated Heparan Sulfate (3-OS HS) ……25
Virus-to-Cell Fusion ……25
Host Protein Shutoff ……26
Virion Transport to the Nucleus ……27
Regulation of Gene Expression ……28
Viral DNA Replication ……31
Capsid Assembly and Packaging ……33

Nuclear Egress: Primary Envelopment ……..……...35
Egress from the Perinuclear Space: De-Envelopment ..……36
Tegumentation in the Cytoplasm ..…….....39
Final Envelopment at the Trans-Golgi
Network/Endosomes and Egress to Extracellular Spaces ……....41
Light (L) Particles ……..……...…..…43
HSV-1 Glycoproteins ……...……..……43
Glycoprotein B (UL27) …..…….....43
Glycoprotein C (UL44) ..…….....44
Glycoprotein D (US6) …..…….....46
Glycoproteins E (US8) and I (US7) …..…….....47
Glycoprotein G (US4) ……....48
Glycoproteins H (UL22) and L (UL1) …..…….....48
Glycoprotein J (US5) …..…….....49
Glycoprotein K (UL53) ……....50
Glycoprotein M (UL10) and Glycoprotein N (UL49.5) ……51
UL20 …..……...……..……53
REFERENCES ……..……...…….....54

CHAPTER TWO: THE HERPES SIMPLEX VIRUS TYPE-1 (HSV-1) GLYCOPROTEINS D (gD) AND E (gE) ARE NOT ESSENTIAL AND DO NOT FUNCTION IN A REDUNDANT MANNER FOR CYTOPLASMIC VIRION EGRESS ……...85
INTRODUCTION ……...……..86
MATERIALS AND METHODS ……....88
Cells ……...……....88
PCR Primer Design ……...……88
Construction of HSV-1 Mutants ΔgD(US6), ΔgE(US8), ΔUL20, ΔgE+gDΔcp, and ΔgE/ΔgM ……..……...……..……...89
Confirmation of the Targeted Mutations……....91
Transfection of HSV-1 bac DNAs ……..…….....93
Plaque Morphology of Mutants ……..…….....93
One-step Growth Kinetics ……....94
Electron Microscopy ……....94
RESULTS ……....95
Construction of Recombinant Viruses Carrying One or More Mutations ……..……95
Molecular Analyses of the Constructed Recombinant Viruses ……..96
Recovery and Characterization of Infectious Virus from HSV-1 bac DNA ……...……98
Plaque Morphology of HSV-1 Mutants ……..……....99
Replication Kinetics of HSV-1 Mutants ……..…….....99
Ultrastructural Characterization of the gDΔcp, ΔgE, ΔgE+gDΔcp, and ΔUL20 Mutant Viruses ……..……..…100
DISCUSSION ……...……....102
REFERENCES ……...……....108

CHAPTER THREE: CONCLUDING REMARKS ……..113
SUMMARY ……..114
CURRENT AND FUTURE PROSPECTS ……..116
FINAL COMMENTS ……..119
REFERENCES ……..121

VITA ……..123

# LIST OF TABLES

Table 1.1 The Herpesviridae Family ……...……..…...9

Table 2.1 Synthetic Oligonucleotide Primers for Red Recombination ……...90

# LIST OF FIGURES


Figure 1.1 HSV-2 Prevalence Among Women ……..12

Figure 1.2 Herpesvirus Induced Lesions ……..15

Figure 1.3 Herpesvirus Virion Structure ……..17

Figure 1.4 Linear Arrangement of HSV-1 Genome ……..19

Figure 1.5 The Replication Cycle of Herpes Simplex Virus ……..21

Figure 1.6 Schematic of Herpesvirus Entry ……..22

Figure 1.7 HSV-1 Cell Surface Entry Receptors ……..23

Figure 1.8 Coordinate Gene Expression in Herpes Simplex Virus ……..30

Figure 1.9 Representation of HSV-1 Virion Morphogenesis and Egress ……39

Figure 2.1 Two-step Red Recombination Mutagenesis System……..92

Figure 2.2 Schematics of the Strategy for the Construction of Mutant bacs ……..96

Figure 2.3 Schematic Diagram of the Carboxyl Terminus of HSV-1 gD……..97

Figure 2.4 Genomic Analysis of bac Mutants ……..98

Figure 2.5 Plaque Phenotypes of Wild-type, ΔgE, gDcp, ΔgE+ΔgM, ΔgE+gDΔcp, ΔUL20……..100

Figure 2.6 Viral Replication Kinetics ……..101

Figure 2.7 Ultrstructural Morphology of Mutant Viruses ……..102

Figure 3.1 Schematics of Cytoplasmic Envelopment ……..117

Figure 3.2 Plaque Morphologies ……..120

# ABSTRACT

HSV-1 acquires its final envelope by budding into cytoplasmic vesicles thought to be derived from Trans-Golgi Network (TGN) membranes. This process is facilitated by interactions among the carboxyl termini of viral glycoproteins and tegument proteins. To investigate the relative importance of different viral glycoproteins in cytoplasmic virion morphogenesis, a set of recombinant viruses were constructed silencing expression of the glycoprotein E (ΔgE), the carboxyl terminus of glycoprotein D (gDΔcp), and the membrane protein UL20 (ΔUL20). In addition, recombinant viruses were constructed having the ΔgE+gDΔcp, and the ΔgE+ΔgM (glycoprotein M) deletions. These recombinant viruses were constructed using the double-red, site-directed mutagenesis system implemented on the HSV-1 genome cloned into a bacterial artificial chromosome (bac). The ΔgE, ΔgE+ΔgM, and gDΔcp viruses produced viral plaques that were approximately 50% smaller to those produced by the wild-type virus HSV-1(F strain). The gDΔcp+ΔgE recombinant virus produced viral plaques that were on the average 50% smaller to those produced by either the ΔgE, ΔgE+ΔgM, or the gDΔcp viruses. However, these viral plaques were substantially larger than those produced by previously constructed UL20-null or gK-null viruses. Kinetics of viral replication revealed that all recombinant viruses appeared to produce similar viral titers at late times post infection. However, both the gDΔcp and the ΔgE+gDΔcp viruses appeared to replicate slower than the wild-type virus or the ΔgE and ΔgE+ΔgM viruses. Electron microscopy revealed that all viruses regardless of their different gene mutations produced enveloped virions that were secreted outside, with no apparent accumulation of unenveloped capsids in the cytoplasm of infected cells. These results suggest that the

gD and gE carboxyl termini, either alone or in a redundant manner, are not essential in cytoplasmic virion envelopment and egress from infected cells. Furthermore, the results show that gK/UL20 complex serves preeminent roles among all viral glycoproteins in cytoplasmic virion morphogenesis and egress.

# CHAPTER ONE

# INTRODUCTION

## STATEMENT OF PROBLEM AND HYPOTHESIS

Herpes Simplex Viruses (HSV) have been a model for the study of pathogenesis, molecular regulation, host-virus interaction and virion morphogenesis for all herpesviruses for many years. Along with the emergence of new techniques and technological advancements, there are several factors of HSV that makes it useful in expanding the understanding of herpesviruses as well as many other viruses. Some of the useful properties include its widespread distribution, ability of viral deoxyribonucleic acid (DNA) to integrate into the host genome, latent tendencies, redundancy of multiple genes, and relative manipulability. The herpesvirus family has a huge socioeconomic impact as they are the etiologic agents for a variety of human diseases ranging from non-lethal mucocutaneous oral and genital lesions to the more severe keratoconjunctivitis and often fatal viral encephalitis. It is estimated that more than 80 percent of the adult population in the United States has HSV-1 (Ohana 2000).

Glycoproteins, which are found in the viral envelope, play a role in both viral entry and egress during the herpesvirus lifecycle. Initial virus to cell fusion is mediated by a multipartite entry-fusion system consisting of glycoproteins (glycoprotein D, B, H, and L) and the receptors; herpesvirus entry mediator (HVEM), nectin 1, and modified heparin-sulfate (Campadelli-Fiume 2006). While much research has been devoted to elucidating the glycoproteins involved in virus attachment and entry, the components involved in the latter stages of virion morphogenesis and infectious virion egress are not well understood. Assembly and the ensuing cleavage of virus capsid, and the encapsidation of newly synthesized viral DNA occur in the nucleus. This is followed by the primary envelopment as the capsid buds through the inner nuclear membrane into the

perinuclear space. Fusion of this primary enveloped virus with the outer nuclear membrane releases naked capsids into the cytoplasm. The nucleocapsids and its associated tegument proteins gain their final envelope as they bud into glycoprotein containing Golgi-derived vesicles (Mettenleiter, Klupp et al. 2006). It remains to be seen whether the glycoproteins involved in virus entry are also involved in the de-envelopment and re-envelopment process during virus egress. Practically all relevant glycoprotein candidates (both essential and non-essential) have been individually deleted, yet none of the deletion viruses were defective in virus transport out of the perinuclear space (Longnecker, Chatterjee et al. 1987; Forrester, Farrell et al. 1992; Dingwell, Brunetti et al. 1994). In the absence of the unique long genome region 31 (UL31) (Chang, Van Sant et al. 1997) or UL34 (Roller, Zhou et al. 2000) tegument proteins, capsids are defective in primary budding, and also are unable to use dilated nuclear pores as alternate pathways to the cytosol. Without the unique short genome region (US3) (Reynolds, Ryckman et al. 2001) or the glycoproteins B and H (Farnsworth 2007), capsids can acquire a primary envelope at the inner nuclear membrane but are stuck in the perinuclear space between inner and outer nuclear membrane, presumably due to an inability to catalyze fusion of the viral envelope with the outer layer of the nuclear membrane.

As for the secondary envelopment process, which occurs in the cytoplasm, gM and gE/gI were shown to be important in pseudorabies virus (PrV) (Brack, Dijkstra et al. 1999). This implies that multiple glycoproteins and tegument proteins may interact with one another to anchor the virion envelope onto tegumented nucleocapsids. In HSV-1, gD and gE/gI triple-null mutants accumulated large numbers of unenveloped

nucleocapsids in the cytoplasm (Farnsworth, Goldsmith et al. 2003). Given that pronounced effects were not observed for the gD and gE single glycoprotein deletions, David C. Johnson's lab (University of Oregon Department of Chemistry) has suggested that gD and gE/gI serve essential but redundant functions in cytoplasmic envelopment (Farnsworth, Goldsmith et al. 2003). Corroborative evidence for this claim is that cytoplasmic residues of HSV-1 gE interact with tegument proteins viral protein 22 (VP22) and UL11 (Farnsworth 2007). GK/UL20 deletion mutants constructed in our laboratory exhibit drastic accumulation of unenveloped virions in the cytoplasm (Foster, Melancon et al. 2004a) strikingly similar to that shown by the gD and gE/gI triple-null mutant. While gD is an essential glycoprotein for virus entry, the regions responsible for this critical function have been attributed exclusively to the first 250~260 amino acids of the glycoprotein (Krummenacher, Nicola et al. 1998). In addition, the cytoplasmic tail of gD is dispensable for growth in cell culture (Feenstra, Hodaie et al. 1990). Therefore if gD and gE serve as essential but redundant components of cytoplasmic envelopment, the deletion of the cytoplasmic tail of gD and the complete deletion of non-essential gE should not prevent successful virus entry or replication events, and only virion egress should be defective. Since, any interaction between capsids and tegument proteins with gD or gE must be mediated by their cytoplasmic termini, the absence of these regions in gD and gE should prevent cytoplasmic virion envelopment, thus, confirming the original observations by the Johnson laboratory. Conversely, if these deletions did not affect cytoplasmic envelopment, then, this could mean that the absence of the entire gD and gE glycoproteins may lead to destabilization of other viral glycoproteins and membrane proteins that are important in cytoplasmic virion envelopment such as gK and UL20.

## STATEMENT OF RESEARCH OBJECTIVES

The primary goal of this research was to assess the roles of Herpes Simplex Virus (HSV-1) Type-1 glycoproteins D (gD), gE, and gM in cytoplasmic virion envelopment, and determine whether they function independently or synergistically. A secondary goal of the work was to compare the relative importance of these glycoproteins to those of gK and UL20, which have been shown to be essential for cytoplasmic virion envelopment.

The specific aims of this research include:

I. To characterize and compare single mutant viruses for UL20, gD, and gE.
   a. Construct nonsense point mutations altering ATG start codon to CTG for UL20 and gE. Since gD is an essential glycoprotein, only the cytoplasmic tail presumed to be important in cytoplasmic envelopment was to be deleted.

II. To characterize and compare effect of double mutant viruses for gE+gM and gE+gD.
   a. Use the single mutant virus constructs mentioned in specific aim I to construct a second null mutation for a different glycoprotein.

III. To determine if the cytoplasmic termini of gE and gD are essential for cytoplasmic virion envelopment.

Overall, the results obtained from this investigation indicate that: gE mutants exhibit smaller plaque phenotype, similar growth kinetics, and slightly impaired secondary envelopment in HSV-1 cytoplasmic morphogenesis. gD mutants exhibit smaller plaque phenotype (comparable to gE mutants) and delayed growth kinetics. The

gE+gM plaque and growth kinetics are similar to the gE null mutant and therefore gM is not essential for cytoplasmic virion envelopment. gE+gD double mutants are able to grow and form plaques on Vero cells, therefore they are not essential for cytoplasmic envelopment in Vero (kidney epithelial cells extracted from African green monkey) cell culture. UL20 deletion mutant viruses accumulate unenveloped capsids in the cytoplasm and do not release infectious virions extracellularly; confirming that UL20 is essential for cytoplasmic virion envelopment.

## LITERATURE REVIEW

### Historical Overview of Herpesviruses

The exact origin of herpes in humans is unknown. However, its presence has been acknowledged as early as ancient Greece when Hippocrates documented sores that seemed to "to creep or crawl" (Miller 2007). Fittingly so, the term "herpes" is derived from the Greek word "to creep". Sumerian tablets dating back to the 3$^{rd}$ millennium B.C. describes abrasions that closely resemble HSV lesions (Whitley 2001). In Roman times, there have been several notable reports of herpes. One well-documented event was Emperor Tiberius' attempt to contain an outbreak by prohibiting kissing at public events and ceremonies. A Roman physician named Celsus suggested that the sores be treated by applying a hot iron (Miller 2007). Another Roman physician by the name of Herodotus was the first to make the association between herpes labialis or "fever blisters" and HSV induced fever (Whitley 2001). Many believe that Shakespeare made a reference to the virus in his play Romeo and Juliet when they talked about "blisters plagues" (Miller 2007). In 1873, Vidal first demonstrated that herpes simplex was infectious and caused by human inoculation (Vidal 1873). Gruter finally isolated HSV in

1924 (Gruter 1924). Between the 1920s and 1930s, HSV infection in the central nervous system was discovered and shortly after, Burnett and Williams described the latent property of HSV as a condition that was lifelong and could cause an outbreak of surface lesions when stimulated (Burnet and Williams 1939). More recent research has focused on antiviral research, differences between the various HSV strains, and using HSV as viral-vectors in vaccine treatments.

The herpesvirus family has grown over the years due to the discovery of several other viruses. Varicella-Zoster Virus (VZV), the causal agent to chickenpox and herpes zoster, was isolated by Weller and Stoddard in 1952 (Whitley 2001). The Epstein-Barr virus was isolated from lymphoblastoid cell lines from established explants of Burkitt's lymphoma (Whitley 2001). Currently, Epstein Barr virus (EBV) is implicated as the etiological agent for many cancers and diseases. Cytomegalovirus (CMV) was also recently discovered as it was first found in patients with congenital cytomegalic inclusion disease (Whitley 2001). All of the diseases associated with CMV are characterized by enlarged cells. In the 1980s and 1990s, T lymphocyte cultivation led to the isolation of human herpesviruses 6A, 6B, and 7 (Salahuddin, Ablashi et al. 1986; Lopez, Pellett et al. 1988; Frenkel, Schirmer et al. 1990). The newest member of the herpesvirus family is the herpesvirus 8, associated with Kaposi's sarcoma (Chang, Cesarman et al. 1994).

### ***Herpesviridae*** **Taxonomy**

Previous methods of delineating families were based on general and relatively arbitrary properties. However, gene sequencing has allowed nucleic acid sequence analysis for Herpesviruses. Furthermore, the organization of herpes viral genome on an evolutionary time scale provides considerable insight to the origin of viruses. Table 1.1

shows the classification of the nine known human Herpesviruses (in bold) along with some of the other known Herpesviruses (Albà 2001).

All members of the Herpesviridae family share some common biological properties:

1) Herpesviruses express a host of enzymes capable of metabolizing nucleic acid (e.g. tymidine kinase), synthesizing DNA (e.g. DNA helicase/primase) and processing proteins (e.g. protein kinase).
2) Viral genome replication and assembly of capsids occurs in the nucleus.
3) Infectious virion leads to inevitable cell death.
4) Virus infections can establish and maintain an indefinite latent state in their host and can become reactivated by cellular duress.

The *Herpesviridae* family was divided into three subfamilies by the herpesvirus study group: *Alphaherpesvirinae, Betaherpesvirinae, and Gammaherpesvirinae* (Roizman, Bartha et al. 1973; Roizman, Desrosiers et al. 1992; Van Regenmortel, Fauquet et al. 2000).

*Herpesviridae* subfamily *Alphaherpesvirinae* were distinguished based on their rapid reproductive cycle and spread in tissue culture on a variety of host cells. They are proficient in promoting cell death upon infection and in establishing latency in sensory ganglia. More notable members of the subfamily includes *Simplexvirus, Varicellovirus, Mardi virus (*e.g. *Marek's disease*)*,* and *Iltovirus* (e.g. avian Infectious *laryngotracheitis)* (Van Regenmortel, Fauquet et al. 2000).

*Betaherpesvirinae* have longer reproductive cycles and are much more restrictive in their permissive host range. Common characteristics of *Betaherpesvirinae* infected cells are that they can expand, and they can remain latent in multiple sites such as

secretory glands, lymphoreticular cells, and kidneys. *Cytomegalovirus, Muromegalovirus, and Roseolovirus* are part of this subfamily (Van Regenmortel, Fauquet et al. 2000).

**Table 1.1 The Herpesviridae Family**

| Subfamily and sublineage | Virus name | Vernacular designation | Pathophysiology |
|---|---|---|---|
| *Alphaherpesviridae* | | | |
| *α1* | ***Human herpesvirus 1 (HHV-1)*** | **Herpes simplex virus type 1 (HSV-1)** | **predominantly orofacial** |
| *α1* | ***Human herpesvirus 2 (HHV-2)*** | **Herpes simplex virus type 2 (HSV-2)** | **predominantly genital** |
| *α2* | ***Human herpesvirus 3 (HHV-3)*** | **Varicella-zoster virus(VZV)** | **chickenpox and shingles** |
| *α2* | *Equine herpesvirus 1 (EHV-1)* | | |
| *α2* | *Equine herpesvirus 4 (EHV-4)* | | |
| *α2* | *Bovine herpesvirus 1 (BHV-1)* | | |
| *α3* | *Gallid herpesvirus 1 (GaHV-1)* | Infectious laryngotracheitis virus | |
| *α3* | *Gallid herpesvirus 2 (GaHV-2)* | Marek's disease herpes virus 2 | |
| | *Cercopithecine herpesvirus* | Herpesvirus B, Simian Herpesvirus | |
| | *Suid herpesvirus 1 (SuHV-1)* | Pseudorabies virus, Aujesky's disease | |
| | *Ictalurid herpesvirus 1 (IcHV-1)* | Channel catfish herpesvirus | |
| *Betaherpesviridae* | | | |
| *β1* | ***Human herpesvirus 5(HHV-5)*** | **Cytomegalovirus(CMV)** | **mononucleosis-like syndrome, retinitis** |
| *β2* | ***Human herpesvirus 6 A*** | | |
| *β2* | ***Human herpesvirus 6 B*** | **Roseolovirus** | **roseola infantum** |
| *β2* | ***Human herpesvirus 7(HHV-7)*** | | |
| | *Cercopithecine herpesvirus 8* | Rhesus monkey cytomegalovirus | |
| | *Murid herpesvirus 1 (MuHV-1)* | Mouse cytomegalovirus | |
| | *Murid herpesvirus 2 (MuHV-2)* | Rat cytomegalovirus | |
| | *Suid herpesvirus 2 (SuHV-1)* | Pig cytomegalovirus | |
| | *Felid herpesvirus 2(FeHV-1)* | Cat cytomegalovirus | |
| *Gammaherpesviridae* | | | |
| *γ1* | ***Human herpesvirus 4(HHV-4)*** | **Epstein-Barr virus (EBV)** | **cofactor in human cancers Burkitt's lymphoma** |
| *γ2* | *Alcelaphine herpesvirus 1* | | |
| *γ2* | *Ateline herpesvirus 3* | | |
| *γ2* | *Macaca mulatta rhadinovirus* | | |
| *γ2* | *Saimiriine herpesvirus 2* | | |
| *γ2* | *Equine herpesvirus 2* | | |
| *γ2* | ***Human herpesvirus 8(HHV-8)*** | **Kaposi's sarcoma-associated herpesvirus (KSHV)** | **Kaposi's sarcoma, effusion lymphoma** |
| *γ2* | *Murine herpesvirus 68* | | |

*Gammaherpesvirinae* are unique in that they establish latency in lymphoblastoid cells and are associated with tumorigenesis (Ackermann 2006). The virus interacts with B or T lymphoctyes abundant in lymphoid tissues. The subfamily is comprised of *Lymphocryptovirus (e.g. Epstein-Barr virus),* and R*hadinovirus (e.g. Equine herpesvirus)* (Van Regenmortel, Fauquet et al. 2000).

**Epidemiology of Herpes Simplex Virus**

Herpes infection is widespread throughout the world (Halioua 1999). Eight of the more than 80 Herpesviruses that have been identified are known human pathogens (Fatahzadeh 2007). HSV-associated diseases are the most prevalent, estimated to have affected 60% to 95% of human adults (Ohana 2000). Infections are incurable and are persistent throughout a host's lifetime, alternating between latent and active states. The mode of viral entry and the level of host immune competence are determinants of the severity of clinical symptoms for each individual (Brady 2004). HSV-1 prevalence has shown correlation with several factors such age, race, geographic location, and socioeconomic status (with a higher rate of seropositivity in less industrialized nations) (Whitley 2001). HSV-2 is the most numerous cause of genital ulcerations worldwide (Steben 2005). Approximately 500,000 new symptomatic cases of genital HSV infections are yearly accounted for in the US and studies have shown that older age, female gender (Figure 1.1), black race, poor socioeconomic status, low level of education, prior sexually transmitted disease, early age at first intercourse, and a higher number of lifetime sexual partners are some of the factors that increase the risk of acquiring HSV-2 (Corey 2000).

**Transmission and Pathogenesis**

HSV spread is dependent on direct exposure of mucous membranes or abraded skin to the virus. Infected individuals shed the virus through saliva, tears, genital, and other secretions (Beauman 2005). Since humans are the only known hosts for HSV, the most common method of transmission is through a kiss from a person who is shedding the virus. There are three types of herpetic episodes: 1) Primary episode refers to the first occurrence after a 5-15 day incubation period from time of infection. 2) Reactivation indicates the latent virus becoming active. 3) Initial or first episode describes infection on an anatomical site naïve to prior viral exposure. Primary infection initiates viral replication and subsequently infects sensory nerve endings (Chilukuri 2003). The virus ends up migrating to the dorsal root ganglia. In HSV-1, infection colonizes the trigeminal ganglia (which can then harbor latent virus) and in HSV-2, the latent virus can be found in the sacral ganglia (Whitley 2001). Shedding of infectious virion particles can begin 7-10 days post infection (Amir, Harel et al. 1997). Neutralizing antibodies can be seen a week after initial HSV infection, and peak three weeks post infection (Buddingh, Schrum et al. 1953). Symptoms are subclinical in most individuals and are rare after the age of 30. After the establishment of latency, certain factors can stimulate reactivation, and virus can resurface in vesicles. Of those infected, approximately 45% of orally infected individuals and 60% of patients with genital herpes will experience recurrences.

On a cellular level, viral infection destabilizes plasma membranes resulting in the enlargement of infected cells that often forms giant multinucleated cells. Within the cell, the chromatin is condensed, and the nucleus undergoes complete degradation. In

infected dermal regions, the immune response is an intense inflammatory reaction that decreases with each subsequent occurrence (Whitley 2001).

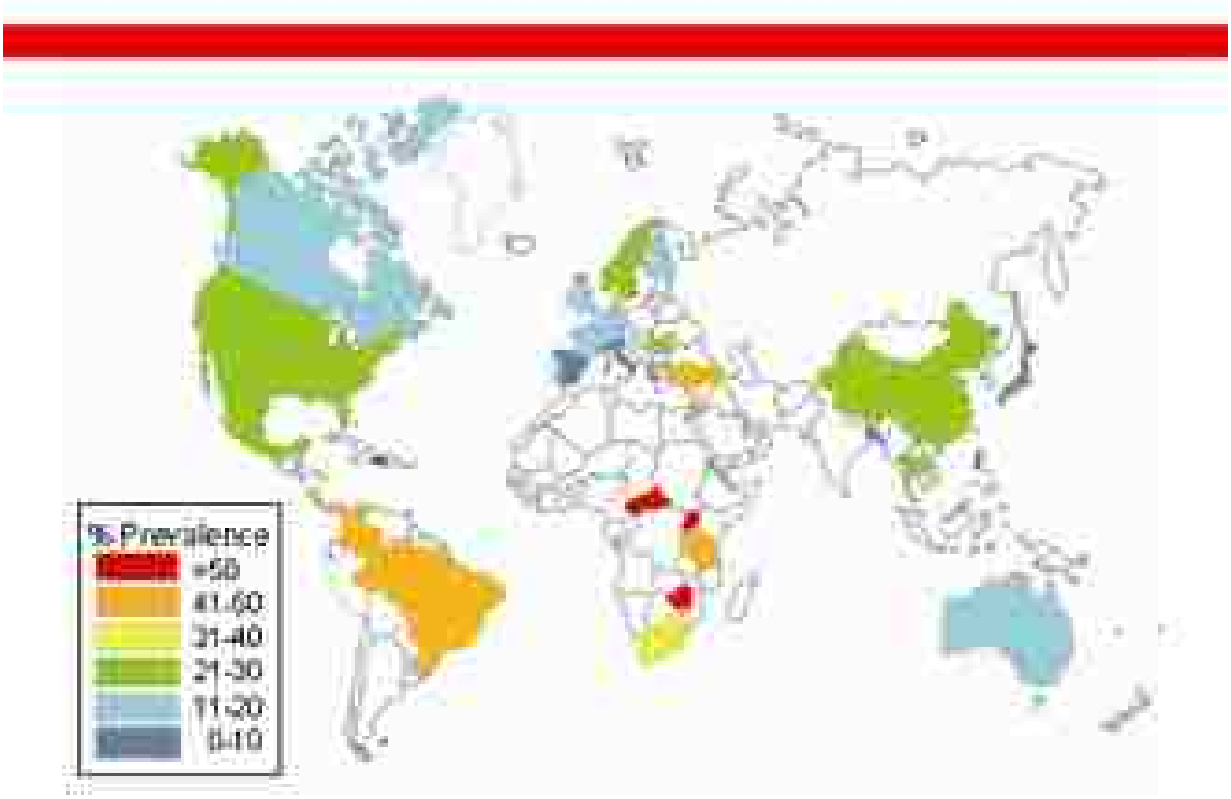


**Figure 1.1 HSV-2 Prevalence Among Women** (adapted from the global epidemiology of genital herpes and the interaction of herpes simplex virus with HIV)

**Symptoms of Clinically Significant Herpesviruses**

Orofacial Herpes

HSV-1 is the primary cause of orofacial infections (Fatahzadeh 2007). Herpes simplex labialis (HSL), typically referred to as fever blisters or cold sores, are usually found on the outer vermilion border and adjacent cutaneous regions (Sciubba 2003). The lesions are often preceded by a burning or tingling sensation prior to development

(Nadelman and Newcomer 2000). These symptoms can last around 6 hours and are an indication that viral replication products are being localized to sensory nerve endings, innervating the mucocutaneous dermatomes (Whitley, Kimberlin et al. 1998). Within 24 hours, multiple vesicles appear then rupture to form tender abrasions which crust over quickly (Fig 1.2: A). Pain and discomfort are greatest during the first few days but within two weeks, lesions heal without scarring (Young, Rowe et al. 1976). Viral shedding continues for 3 to 5 days after the lesions have healed.

Genital Herpes

HSV-2 is the most common cause of genital herpes. Contrary to popular belief, the majority of HSV-2 infections are subclinical and unrecognized by those infected (Wald, Zeh et al. 2000). For those that exhibit symptoms, however, macules and papules emerge within a few days of sexual contact. These vesicles of varying sizes can be located on the labia minora, introitus, and urethra meatus in women, and on the shaft and glans of the penis in men (Fig 1.2: B). The duration of pain, tingling, or burning sensation associated with primary infection lasts up to a day (Beauman 2005). Headache, fever, depression, and inguinal lymphadenopathy are often systemic signs and symptoms of genital herpes infection. Recurrent episodes are often less severe than the initial reaction (Corey 1990). Although there is a public misconception that HSV-1 is the “good” herpes and HSV-2 is “bad”, studies show that certain HSV-1 infections are a major cause of sporadic lethal encephalitis and blindness in humans (Mansur, Kroon et al. 2005). Furthermore, the clinical appearance, severity, and duration of HSV-1 and HSV-2 genital diseases are practically indistinguishable (Brugha, Keersmaekers et al. 1997).

Ocular Herpes

With over 300,000 diagnosed cases of HSV eye infections yearly, ocular HSV infection is one of the leading causes of corneal blindness (Binder 1977). Ocular HSV may lead to keratoconjunctivitis in one or both eyes and may require surgical cleansing of the cornea along with local antiviral therapy (Sudesh 1999). When recurrent ocular ulcerations fail to heal, they can spread and perforate leading to severe complications (Figure 1.2: C).

Neonatal Herpes

Transmission of HSV to neonates takes place in utero, intrapartum, or postnatally (Rudnick and Hoekzema 2002). In vertical transmission, HSV-infected infants are often born prematurely. The initial signs and symptoms of neonatal herpes are often nonspecific but are localized to the skin (Figure 1.2: D), eye, mouth, or in the case of disseminated infection it may involve multiple organs (Nahmias, Josey et al. 1970). Neonatal herpes is of devastating consequence, and even with therapy, mortality rates for neonatal HSV encephalitis or disseminated disease are high (Rudnick and Hoekzema 2002).

Central Nervous System (CNS) Herpes

Herpes simplex encephalitis (HSE) is the most common form of sporadic, fatal encephalitis in the United States (Whitley 2005). Patients may experience fever, malaise, headaches, and personality fluctuations. Their condition may progress into seizures, visual defects, paresis, speech defects, behavioral changes, stupor, and coma. When the virus migrates to the CNS, it results in chronic cerebral edema and damages critical neurological systems (Whitley 2001).

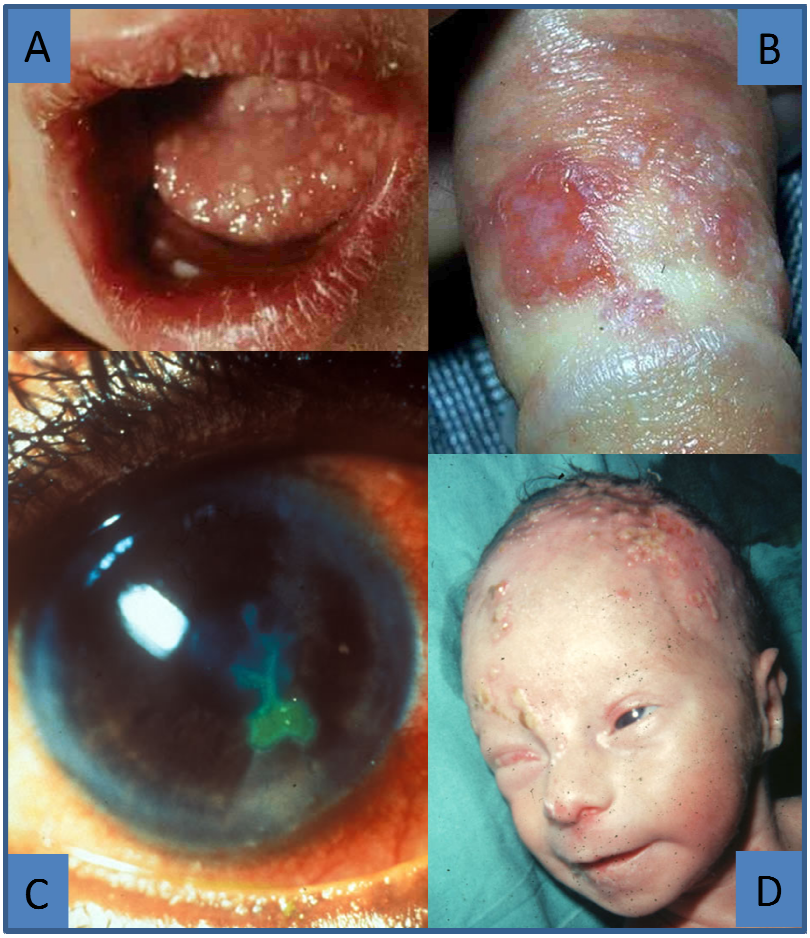


**Figure 1.2: Herpesvirus Induced Lesions.** [A] oral herpes (HSV-1) (adapted from University of California San Francisco Department of Dermatology); [B] genital herpes (HSV-2) (adapted from University of Erlangen Department of Dermatology); [C] ocular herpes (stained with fluorescence) (adapted from The Eye in Primary Health Care Teaching Set); [D] neonatal herpes (adapted from Hospital Materno Infantil La Paz).

### Treatment and Preventative Measures

A cure has yet to be discovered for herpes. The virus is ubiquitous, and all attempts to prevent the transmission of infection have proven ineffective. The most frequent method of treatment is through antiviral drugs such as acyclovir, valaciclovir, and famciclovir (Martinez, Caumes et al. 2008). As for preventive measures, prophylactic chemotherapy is a costly alternative that offers limited success. Several

recombinant subunit vaccines are being evaluated for methods to effectively reduce the frequency and severity of recurrence (Rajcáni and Durmanová 2006). Control of herpes recurrence is important not only because of the potential to improve the quality of life for millions of people, but also due to its association with other sexually transmitted diseases; especially those that compromise the immune system of the host like human immunodeficiency virus (HIV).

**Herpes Virion Structure and Biological Properties**

Herpes viruses are spherical shaped with a diameter ranging from 120 to 300 nanometers (Roizman and Furlong 1974). The virion is composed of an icosadeltahedral capsid that stores an electron-dense core. The capsid is surrounded by amorphous tegument proteins and an outer membrane envelope where glycoproteins can be found. Members within the herpesvirus family have unique combinations of tegument and envelope proteins. A model of virion structure is represented in Figure 1.3.

The Core

The core of a mature herpes virion retains toroidal form and appears to be suspended by a proteinaceous spindle (Nazerian 1974). The structure is 50 nm high with an inside diameter of 18nm and an outside diameter of 70nm (Furlong, Swift et al. 1972). Double-stranded viral DNA in the torus is wound around the proteinaceous core.

The Capsid

The icosadeltahedral capsid stores DNA in a densely coiled, liquid crystalline configuration (Booy, Newcomb et al. 1991). The shell is 15 nm thick and 125 nm in diameter (Plomp, Rice et al. 2002). Four proteins form the capsid shell: the major capsid

protein (VP5), the scaffolding protein (pre-VP22a), VP19C, and VP23 (Zhou, Dougherty et al. 2000). The capsid features 162 capsomeres (12 pentons and 150 hexons) conserved for all Herpesviruses. The pentons are located on the vertices of the capsid and the hexons are found on the faces and edges. A heterotrimeric complex consisting of two VP23 and one VP19C connects the capsomeres and act as a scaffold for the capsid (Spencer, Newcomb et al. 1998). Hexons also have 6 copies of VP26, forming a ring around each capsomere (Zhou, He et al. 1995).

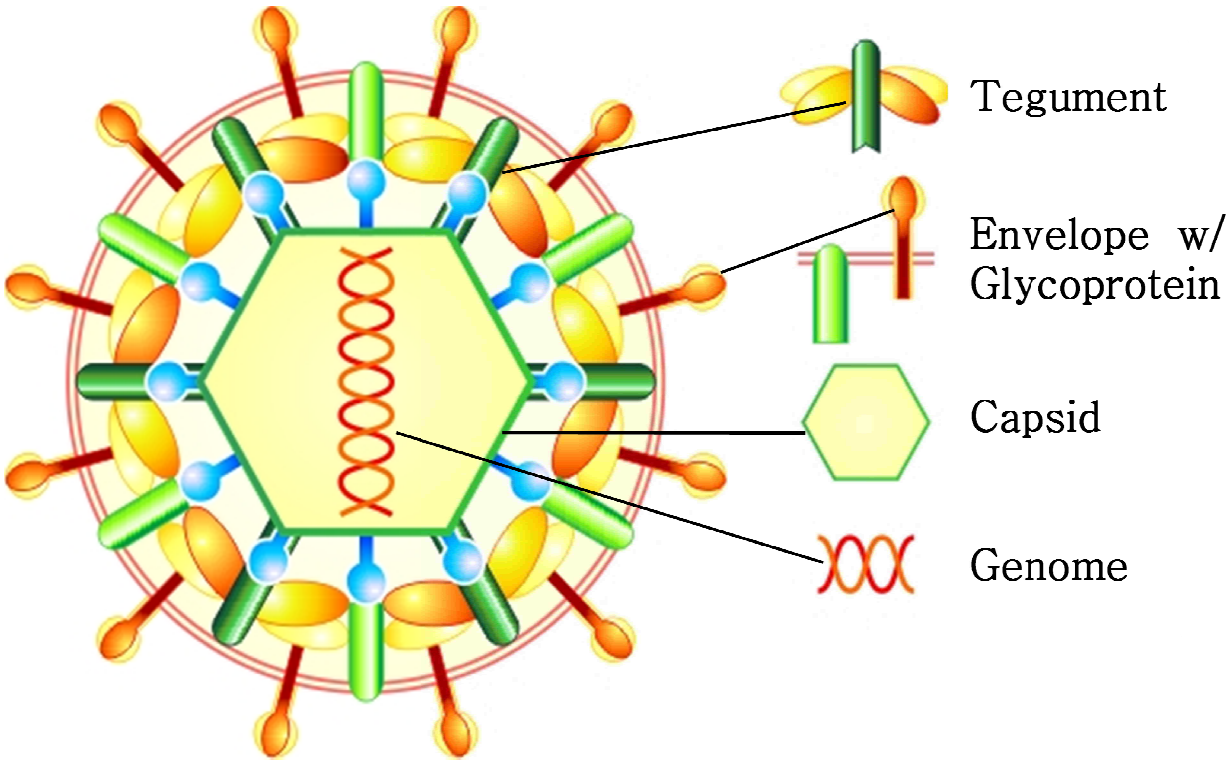


**Figure 1.3: Herpesvirus Virion Structure.** The virion is composed of an icosadeltahedral capsid that stores an electron-dense core. The capsid is surrounded by amorphous tegument proteins and an outer membrane envelope where glycoproteins can be found.

The Tegument

The tegument, which is made up of approximately 20 proteins, is the most diverse structural element of the virus in terms of polypeptide composition and functions (Desai 2000). It has a particulate substructure with several short actin-like filaments (Arduino and Porter 2008). It functions as a delivery compartment for proteins during the early course of infection and also plays a role in virion assembly. Three major proteins of the tegument (VP22, VP16, and VP13/14) have exhibited a range of distribution in infected cells (Hutchinson, Whiteley et al. 2002). Fluorescent tagging shows VP13/14 targeting the nucleus (Donnelly and Elliott 2001). The localization of VP16 and VP22 to nuclear compartments and the cytoplasm suggest that they have multiple roles in replication and egress (Elliott, Mouzakitis et al. 1995; Pomeranz and Blaho 1999; LaBoissiere and O'Hare 2000).

The Envelope

The outermost section of herpesvirus is an envelope (Epstein 1962) of modified cellular membranes (Ramos-Kuri 1992). At least 11 viral glycoproteins have been found in the envelope, with gD, gH, gL, and gB being accepted as essential for HSV entry into cells (Reske, Krummenacher et al. 2007). The envelope accommodates 600–750 glycoprotein spikes that vary in length, spacing, and in the angles at which they extrude from the lipid membrane (Grunewald, Desai et al. 2003). The glycoproteins involved in virion morphogenesis are currently not well defined.

**Viral Genome**

Herpesviruses range from 120 – 250 kbp in length and 31% - 75% in their total G+C content (Roizman and Pellett 2001). The HSV-1 genome is a linear double-

stranded DNA molecule of about 152, 261bp (McGeoch, Dalrymple et al. 1988) that becomes circularized immediately after being released from capsid in to the nucleus of the cell. The herpesvirus genome has two covalently joined segments L (long) and S (short), flanked by inverted repetitions of terminal repeat (TR) and internal repeat (IR) regions for both segments (Figure 1.4). At the end of each terminus and at the L／S joint, an a-sequence is present as a direct repeat (DR) in an inverted form (Arduino and Porter 2008). This a-sequence allows for internal recombination between the L and S regions and can present four isomeric forms (Quinn, Dalziel et al. 2000). A typical herpesvirus genome has a promoter region 50 to 200bp upstream and a transcription initiation site 20 to 25bp downstream of the TATA box. A 5' untranslated leader sequence of 30 to 300bp is followed by a single major open reading frame (ORF) and 10 to 30bp of 3' untranslated sequence is prototypical of the genome. A polyadenylation signal can be found in the end regions (Roizman and Pellett 2001). Of the 90 genes present in the genome, HSV-1 encodes at least 84 proteins (Whitley 2001) that have various functions for virus growth in culture, entry of virus into hosts, regulation of gene transcription and translation, and final assembly of virions (McGeoch 1987). The establishment of viral latent state does not require any specific virally encoded functions, however, the complete package of viral gene products are obligatory for efficient reactivation (Ward and Roizman 1994).


**Figure 1.4: Linear Arrangement of HSV-1 Genome.** HSV-1 genome has unique long (UL) and unique short (US) regions that are flanked by the terminal repeat (TR) and internal repeat (IR) sequences.

## Herpesvirus Lifecycle

The herpesvirus lifecycle (Figure 1.5) begins as the virus particle recognizes and binds to receptor proteins on the surface of cell membrane. The viral envelope then fuses with the plasma membrane of the cell and the viral capsid containing the viral genome enters the cytoplasm. The capsid then traverses along microtubules to the nuclear pores where it docks. The viral DNA enters the nucleus through the pore and viral DNA is released, circularizing prior to replication. Virion morphogenesis is a multistep process that involves new daughter genomes being assembled into capsids in the nucleus. Mature capsids bud through the inner nuclear envelope and gain a temporary primary envelope as they move in to the perinuclear space. They become de-enveloped as they fuse with the outer nuclear envelope and release naked capsids into the cytoplasm. Cytoplasmic envelopment takes place as these capsids and associated tegument proteins bud into exocytotic Golgi vesicles, embedded with all the glycoproteins associated with the adult virion. The vesicle delivers infectious virion to the cell surface where it fuses with the plasma membrane and the new virus particle can spread to uninfected cells.

### Virus Attachment and Fusion

Entry is essential to the successful pathogenesis of HSV. First, the virus attaches to cell membranes by interaction of gC (Herold, WuDunn et al. 1991) and possibly gB (Laquerre, Person et al. 1996) with glycosaminoglycans (GAGs) moieties of heparan sulfate. This attachment process is fully reversible, and although gC-initiated attachment greatly improves the virus' ability for infection, it is nonessential for virus entry (Gruenheid, Meadows et al. 1993). Thereafter, the receptor-binding domain of gD

recognizes one of the alternative receptors: nectin-1, nectin-2, and HVEM (herpesvirus entry mediator) (Montgomery, Warner et al. 1996; Cocchi, Menotti et al. 1998; Lopez, Cocchi et al. 2000). This step is absolutely required for virus infection.

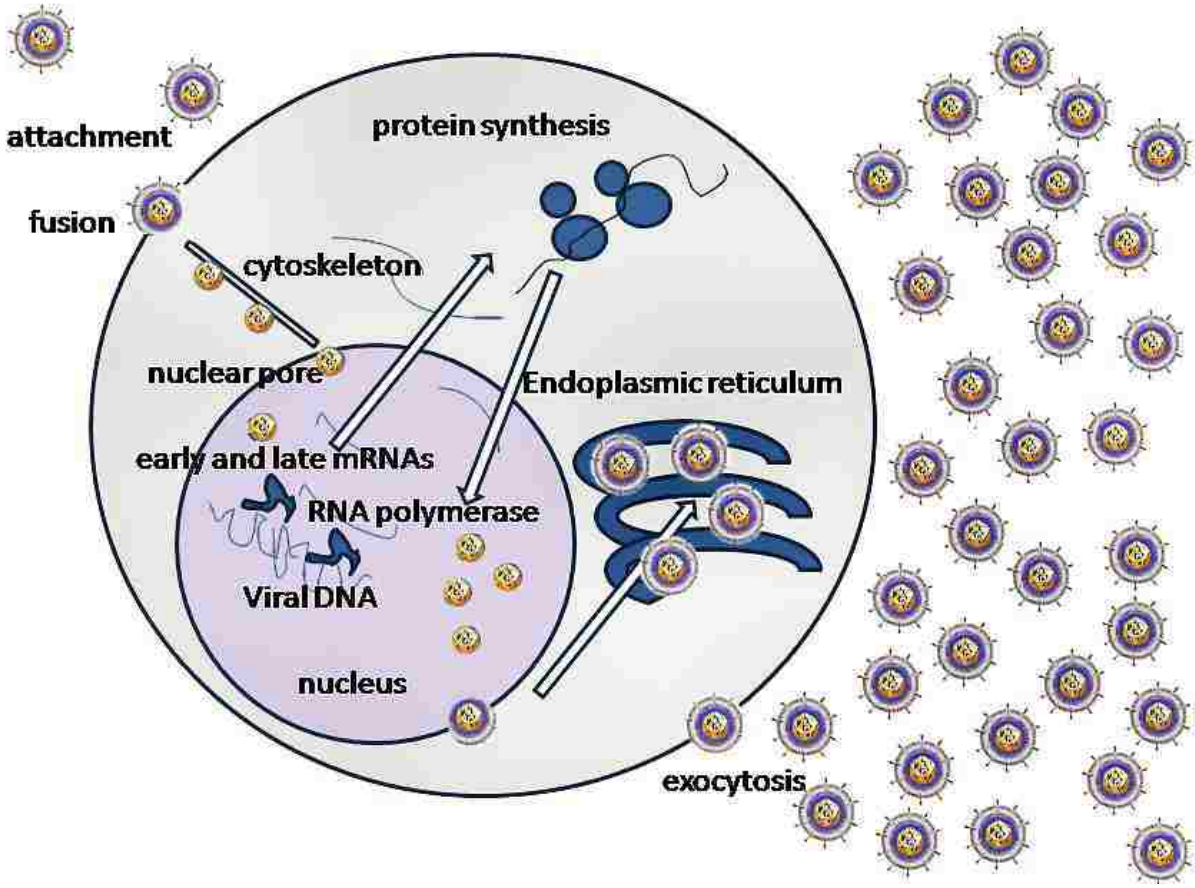


**Figure 1.5: The Replication Cycle of Herpes Simplex Virus.** The herpesvirus life cycle consists of virus entry, capsid transport to the cell nucleus, deposition of viral DNA into the nucleoplasm, coordinate gene expression and viral DNA replication, primary envelopment at the inner nuclear membrane, de-envelopment at the outer nuclear membrane, final envelopment into cytoplasmic vesicles, and transport to extracellular spaces

Successful binding triggers a separate pro-fusion domain in gD (Cocchi, Menotti et al. 2000; Cocchi, Menotti et al. 2004b) to recruit other glycoproteins: possibly gB, gH, gL, to

complete this membrane fusion step (Ligas and Johnson 1988; Cai, Gu et al. 1988a; Forrester, Farrell et al. 1992). Viral envelope to cellular membrane fusion remains the least understood step of virus entry. The ectodomain of gH contains hydrophobic alpha-helix acting as an interfusion peptide, and two heptad repeats that are important to fusion (Gianni, Martelli et al. 2005a; Gianni, Menotti et al. 2005b). Fusion may take place either at the plasma membrane or in endocytic vesicles (Nicola, McEvoy et al. 2003; Gianni, Campadelli-Fiume et al. 2004; Nicola and Straus 2004). A schematic representation of entry is shown in Figure 1.6.

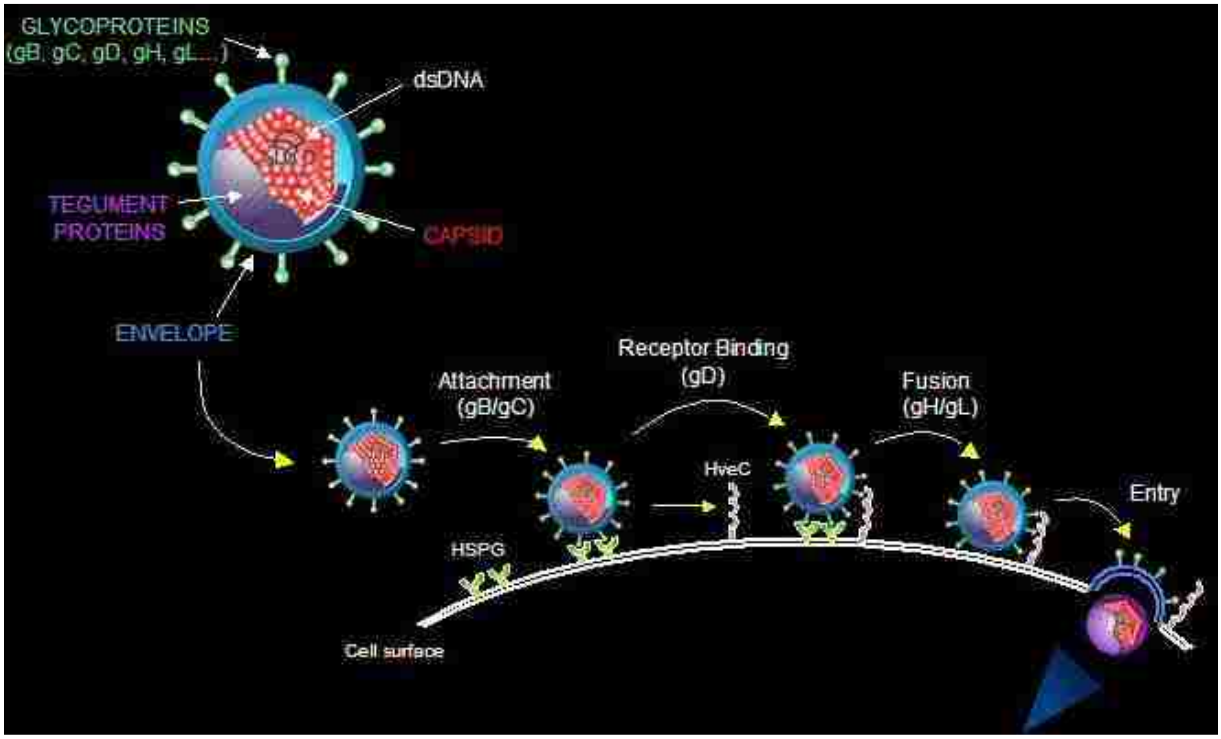


**Figure 1.6: Schematic of Herpesvirus Entry.** HSV entry starts with the recognition of virus to the plasma membrane through interaction of gC (ligand for binding) with cell surface receptor heparan sulfate (HSPG). Fusion of the viral envelope with the cell plasma membrane occurs after gD (ligand for fusion) attaches to HveC or a nectin on cell surfaces. Fusion requires the presence of gD and an entry receptor, as well as gB and the gH/gL heterodimer.

Binding Receptor Heparan Sulfate (Glycosaminoglycans)

Heparan sulfate (HS), a member of the glycosaminoglycan family of carbohydrates, is a linear proteoglycan widely distributed on cell surfaces of all mammals.

**Entry Receptors**

Viral entry involves multiple alternative receptors for different cell types and tissues, making the role of each receptor difficult to clearly assess (Taylor, Lin et al. 2007). Studies indicate that there is no specific minimum affinity for gD and receptor, just that the interaction takes place (Milne, Hanna et al. 2003; Zhou, Avitabile et al. 2003). HSV entry receptors are classified into three groups (Figure 1.7).

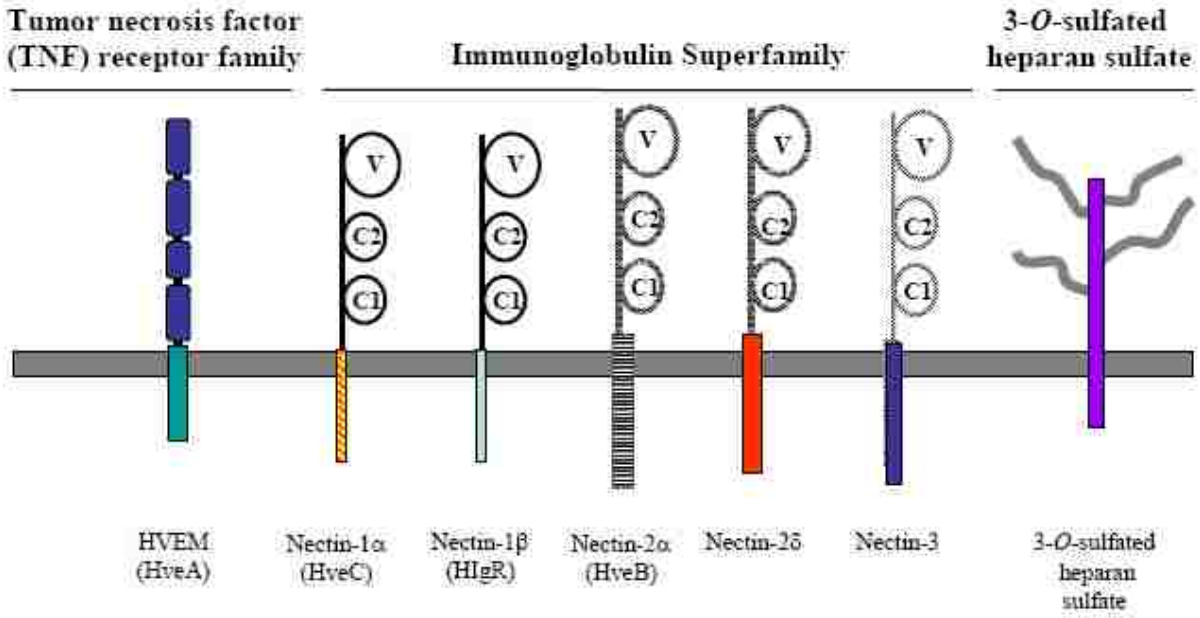


**Figure 1.7: HSV-1 Cell Surface Entry Receptors.** The three classes of cell surface receptors for HSV entry are: the tumor necrosis factor (TNF) receptor family consisting of HVEM, the immunoglobulin superfamily consisting of the nectins, and 3-O-sulfated heparin sulfate.

Tissue Necrosis Factor (TNF) Family

Herpes Virus Entry Mediator A (HVEM/HveA) belongs to the TNF receptor family and is a component of lymphocytes and a variety of other cell types such as epithelial cells, and fibroblasts. Its ectodomain consists of 5 cysteine-rich domains (CRD), and within CRD1, 2, and 4, are 17 residues directly involved in the physical interaction with gD (Montgomery, Warner et al. 1996). Light (L) particles and lymphotoxin-alpha are the ligands for HVEM (Mauri, Ebner et al. 1998), and their involvement in the regulation of immune response are being investigated (Kwon, Kim et al. 2003). HVEM serves as a high affinity entry mediator for both HSV-1 and HSV-2 (Willis, Rux et al. 1998; Whitbeck, Muggeridge et al. 1999), but studies have shown that in some cell types, HVEM was not the primary mediator of virus entry (Montgomery, Warner et al. 1996; Krummenacher, Baribaud et al. 2004).

Immunoglobulin (Ig) Superfamily

Nectins are intercellular adhesion molecules that act as key components of adhesion junctions (Reymond, Borg et al. 2000; Takai, Irie et al. 2003).Their Ig-like structure and role in mediating HSV entry are highly conserved throughout (Shukla, Dal Canto et al. 2000; Milne, Connolly et al. 2001). The distribution of nectin receptors in epithelial, endothelial, fibroblastic, lymphocytic, and neuronal cells is indicative of virus infection susceptibility (Roizman and Knipe, 2001). Isoforms derived from alternative mRNA splicing have been found (Campadelli-Fiume 2006).

Nectin-1 alpha (alternatively designated Herpes Virus Entry Mediator C, HveC) and nectin-1 beta (Herpesvirus Immunoglobulin-like receptor, HIgR) serve as entry receptors for HSV-1, HSV-2, Pseudorabies virus (PrV), and Bovine herpesvirus 1

(BHV-1) (Geraghty, Krummenacher et al. 1998). The CC'C'' ridge of the nectin-1 receptor V domain interact with gD in virus entry (Krummenacher, Baribaud et al. 2000; Menotti, Cocchi et al. 2002). They are localized on the brain, spinal cord, trachea, prostate, placenta, skin, kidney, lung, pancreas, thyroid, liver, myelomonocytic tissues, and their associated cell lines (Cocchi, Menotti et al. 1998; Campadelli-Fiume 2006).

Nectin-2 alpha (Herpes virus Entry Mediator B, HveB) and nectin-2 beta are similar in their target and distribution to nectin 1, but differ in that they do not mediate virus entry for HSV-1 (Warner, Geraghty et al. 1998; Lopez, Cocchi et al. 2000). They are expressed on the placenta, prostate, kidney, lung, pancreas, thyroid, liver, myelomonocytic, and endothelial cells.

3-O-sulfated Heparan Sulfate (3-OS HS)

The third class of receptors for HSV gD is the 3-O-sulfated heparan sulfate. Heparan sulfate is catalyzed by the enzyme D-glucosamyl 3-O-sulfotransferase to generate gD-binding sites on HS (Shukla, Liu et al. 1999). 3-OS HS are distributed on human, rat, and mouse endothelial cells. They have only been found to be involved in HSV-1 entry (Campadelli-Fiume 2006).

Virus-to-Cell Fusion

Fusion of virion envelope with the host cell membrane is the final step in entry. Prior to receptor binding, gD adopts a closed conformation as the C-terminus of the ectodomain fold back to wrap its own N-terminus. At receptor binding, the C terminus is displaced from the binding site and interacts with the receptor, and the opened gD is thought to create a structure for the recruitment of downstream glycoproteins (Fusco, Forghieri et al. 2005; Krummenacher, Supekar et al. 2005). Cell-cell fusions assays

(Turner, Bruun et al. 1998) indicate gD, gB, and gH/gL as the critical components of virus-to-cell fusion. HSV entry has two distinct pathways of fusion that may take place either at the plasma membrane or in acidic endosomes (Nicola, McEvoy et al. 2003; Nicola and Straus 2004). Nevertheless, the same sets of viral glycoproteins are required in both modes of entry.

Host Protein Shutoff

During lytic HSV infections, the virion host shutoff (VHS) protein shuts off the synthesis of cellular protein products by destabilizing host mRNAs in the cytoplasm (Read 1997). VHS (UL41) polypeptides enter the cell upon virus-to-cell fusion and thus do not require de novo protein synthesis (Nishioka and Silverstein 1977; Fenwick and Walker 1978; Nishioka and Silverstein 1978a; Nishioka and Silverstein 1978b). The inhibition of pre-mRNA splicing by the immediate-early polypeptide infected cell protein (ICP) 27 (Hardwicke and Sandri-Goldin 1994; Hardy and Sandri-Goldin 1994) also contributes to redirecting the host cell protein synthesis machinery to expressing viral proteins. After the initiation of viral transcription, the VHS protein helps determine newly synthesized viral mRNA levels and facilitates the sequential viral gene class expressed (Read 1997). The VHS phosphoprotein is approximately 58 kDa and expressed as a gamma-1 gene (Frink, Anderson et al. 1981; Read, Karr et al. 1993). VHS accelerates endoribonucleolytic cleavage of cellular and viral mRNA *in vitro* (Elgadi and Smiley 1999), and with the translation factor eIF-4H, forms a complex targeting polyribosome RNAase (Doepker, Hsu et al. 2004).

VHS accelerates the degradation of both cellular and viral mRNAs while sparing other cytoplasmic RNA species (Everly and Read 1999). This preferential translation

process may stimulate the sequential transition of viral proteins (Kwong and Frenkel 1987; Oroskar and Read 1987). The interaction between VP16, a tegument protein, and VHS after the late expression of the gamma-1 gene blocks the degradation of RNA by VHS (Lam, Smibert et al. 1996). Overall, VHS facilitates two critical functions during a herpesvirus lifecycle: it is capable of suppressing the host cell protein synthesis even without viral gene expression, and when new UL41 proteins are synthesized late in infection, it regulates virus-host interactions at a stage when viral mRNA is predominant within the host (Matis and Kúdelová 2001).

Virion Transport to the Nucleus

Following fusion, at which point the tegumented nucleocapsid is released in to the cytoplasm of the cell, the capsid interacts with dynein and the minus ends of microtubules to rapidly travel along the cell's microtubule network to the nuclear pores where the viral DNA can be deposited into the nucleus (Sodeik, Ebersold et al. 1997). The viral proteins that mediate this centripetal transport are largely unknown, but studies have shown that some tegument proteins such as VP16 (α-TIF), VHS, and UL48 are released at this time, while VP1/2 remains associated with the capsid and is localized at the nucleus (Antinone, Shubeita et al. 2006 ; Jovasevic, Liang et al. 2008). VP1/2 is the largest tegument protein (270-kDa) in HSV-1 and is encoded by UL36 (Sodeik, Ebersold et al. 1997). The 55 kDa N-terminal proteolytic cleavage fragment of VP1/2 creates conformational changes in the capsid that allows the release of the HSV-1 DNA into the nucleus (Jovasevic, Liang et al. 2008). Importin-α and Ran GTPase are also essential in the attachment of the capsid to the nuclear pores and the subsequent release of viral DNA into the nucleus (Ojala, Sodeik et al. 2000).

Regulation of Gene Expression

Like other DNA viruses, HSV-1 transcription of viral DNA takes place in the nucleus, and the host RNA polymerase II is utilized for transcription of all viral genes during productive infection (Alwine, Steinhart et al. 1974; Costanzo, Campadelli-Fiume et al. 1977). During its productive infection, more than 80 viral genes are tightly regulated (Roizman and Knipe 2001). These viral genes have been categorized into three groups based on when they are coordinately expressed post infection (Figure 1.8): α/immediate-early [IE; approximately 2-5 hours post infection(hpi)], β/delayed-early (DE; approximately 4-8 hpi), and γ/ late (L; 6hpi and onwards).

Upon infection, 500–1000 VP16 (Campbell, Palfreyman et al. 1984) molecules are delivered from virus to the host cell and freed from tegument to bind to cellular protein host cell factor (HCF-1), and are carried into the nucleus (Katan, Haigh et al. 1990; Kristie and Sharp 1990). There, a VP16-induced complex is formed (Roizman and Knipe 2001). The HSV virion supplies its own transcription initiation factor (α-TIF), which co-operates with VP16-induced complex to regulate virus-specific immediate early (IE) transcription (Rajcáni and Durmanová 2006). α-TIF contains the 'TAATGARAT' regulatory element which binds octamer 1 (Oct-1), a broadly expressed and versatile transcription factor (Wysocka and Herr 2003). This event completes the activator complex, hat I, which transactivates IE genes (La Boissiere, Hughes et al. 1999). In addition, the promoters for IE genes also contain binding sites upstream of a TATA box that may bind to other transcriptional activators to regulate IE gene expression in the absence of VP16 (Roizman and Knipe 2001). Therefore,

transcriptional requirements for VP16 may be different for various cell types especially those that do not have cellular transcription factors available such as neuronal cells.

IE proteins play an active role in viral gene transcription regulation and the expression of delayed early (DE) and late (L) genes are largely dependent on the five immediate early (IE) proteins (Honess and Roizman 1974). Four of the five IE proteins, infected-cell protein No. 0 (ICP0), ICP4, ICP22, and ICP2, have shown localization in the nucleus and are responsible for regulation of viral gene activity (Roizman and Knipe 2001). The last member of IE proteins, ICP47, remains in the cytoplasm and acts to inhibit major histocompatibility complex (MHC) class I antigen presentation (Hill, Jugovic et al. 1995). After being deposited into the nucleus of the infected cell, the HSV-1 viral genome localizes to nuclear structures known as nuclear domain (ND) 10 where IE gene transcription occurs (Maul, Ishov et al. 1996). Of the IE proteins, ICP4 is essential for stimulation of DE and L mRNA pre-initiation complexes (Clements, Watson et al. 1977; Dixon and Schaffer 1980). ICP4 physically interacts with Pol II general transcription factors (GTFs) in a sequence-specific manner (Zabierowski and De Luca 2004). The major regulatory protein ICP4 also down-regulates at least two HSV-1-encoded proteins, ORF O and ORF P, during productive infection (Roizman 1999). In this case, specific consensus binding sites appear to be responsible for ICP4-mediated transcriptional regulation (Kristie and Roizman 1984; Faber and Wilcox 1986; Kristie and Roizman 1986; Gelman and Silverstein 1987; Muller 1987). However, little of the mechanism by which ICP4 exerts its transcriptional control has been defined.

ICP0 is a promiscuous transactivator that induces the expression of the HSV genes by indirectly modulating their transcription (Everett, Orr et al. 1991). As a member

of the family of E3 ubiquitin ligand enzymes carrying a zinc-binding domain, it is able to induce proteasome-dependent degradation of cellular proteins. In this manner, it disrupts ND10. Because of its role in determining the lytic or latent progression of infection (Everett 2000; Hagglund and Roizman 2004), and its pronounced effects on the host cell (Stow and Stow 1986), ICP0 is the subject of a wide range of ongoing research.

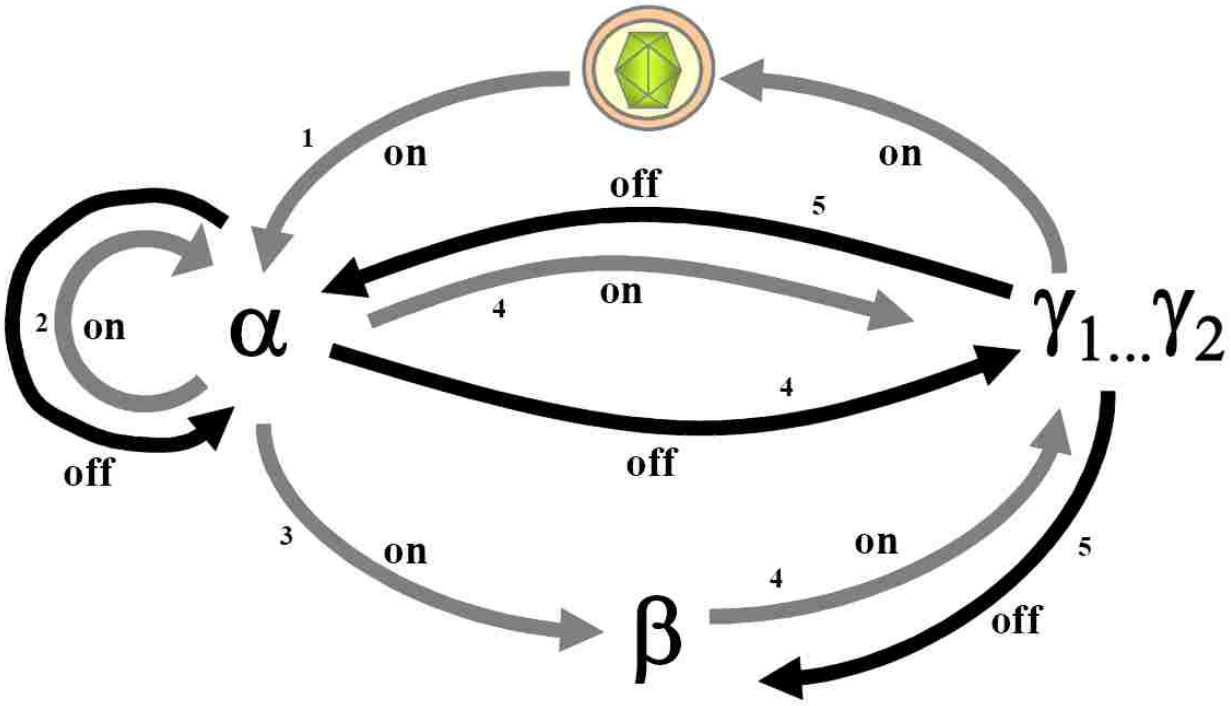


**Figure 1.8: Coordinate Gene Expression in Herpes Simplex Virus.** 1) α-TIF, a γ gene tegument protein, activates initial transcription of the α genes. 2) Autoregulation of gene expression. 3) Activation of β gene expression. 4) Activation of γ gene expression by α and β genes, release of repression of γ genes, and replication of the viral genome. 5) γ genes turn off α and β genes late in infection (Roizman and Knipe 2001).

Delayed Early (DE) gene products also promote viral DNA replication, which results in expression of the late class of genes. The single-stranded DNA-binding

protein, ICP8 (UL29 product), is required for viral DNA synthesis. It co-localizes with other replication proteins at pre-replicative sites, but becomes redistributed into large, globular replication compartments once viral DNA synthesis is initiated (Uprichard and Knipe 2003). ICP8 and ICP6 (the large subunit of ribonucleotide reductase UL39 product) are expressed shortly after IE production, and thymidine kinase encoded by UL23 is expressed with more of a delay (Roizman and Knipe 2001).

Late gene products include the major viral structural proteins, tegument components, and glycoproteins required for further processing of cells for productive infection. They require ICP4, ICP27, and ICP8 for efficient levels of transcription, and thus peak after viral DNA replication has started. The change in the nuclear localization of transcription from sites near ND10 domains to replication compartments marks the transition from DE to L gene expression (Knipe, Senechek et al. 1987; Rice, Long et al. 1994; Leopardi, Ward et al. 1997). The late genes have been divided into groups based on when they are expressed. Leaky-late genes (ICP5, gB, gD, and ICP34.5) are expressed relatively early when transcription is increased only a few fold after DNA replication has occurred. The other Late gene group members (gC, UL41, UL36, UL38, UL20, and gK) do not accumulate in appreciable amounts until well into DNA replication and are absent while viral DNA synthesis is ongoing (Wagner 1985).

Viral DNA Replication

Herpesviruses employ a unique strategy of DNA replication which takes advantage of both viral and host recombination and repair machinery. There are three origins of replication (Ori) located on the HSV genome: Two copies of OriS, a 45bp-palindromic sequence in which an A/T rich region is flanked by two recognition sites, is

present in the c repeat region of the viral genome. OriL, a 144bp near perfect palindrome sequence located between the transcriptional units of ICP8 and the DNA polymerase (UL29 and UL30), is present in the unique long region. Both contain high affinity binding sites for UL9 (Frenkel, Locker et al. 1976; Locker, Frenkel et al. 1982; Mocarski and Roizman 1982; Stow 1982; Vlazny, Kwong et al. 1982; Weller, Spadaro et al. 1985; Knopf, Spies et al. 1986; Lockshon and Galloway 1986; Deb and Doelberg 1988). HSV DNA synthesis is believed to initiate at one of the three viral origins of replication (Polvino-Bodnar, Orberg et al. 1987; Igarashi, Fawl et al. 1993).

The HSV-1 genome encodes seven viral proteins absolutely required for viral DNA replication (Knipe 1989; Weller 1995; Lehman and Boehmer 1999; Marintcheva and Weller 2001). Six have core functions: the single stranded DNA binding protein ICP8 (UL29), the two subunit viral DNA polymerase UL30 (Purifoy, Lewis et al. 1977), and its accessory protein UL42 (Conley, Knipe et al. 1981), the helicase-primase complex consisting of UL5, UL8, and UL52 (Challberg 1986; Wu, Nelson et al. 1988a), and origin-binding protein UL9. Host cell factors and enzymes that are believed to be involved in DNA synthesis include the DNA polymerase alpha-primase, DNA ligase, and topoisomerase II. Replication units localize in pre-replicative sites proximal to nuclear ND10 structures (Ishov and Maul 1996; Uprichard and Knipe 1996). Upon initiation, newly synthesized DNA and replication complexes are found in replication compartments (Quinlan, Chen et al. 1984).

The basic model for the replication of HSV viral DNA occurs in two stages. The first stage involves an origin-UL9-dependent step. Upon release into the host nucleus, parental viral DNA is circularized. UL9 binds to specific recognition sites in either oriL or

oriS and begins to unwind the viral DNA. UL9 then recruits the single-stranded (ss) DNA binding protein ICP8 to that site. Together, they recruit the five other essential replication proteins to the replication site. The three subunit helicase-primase complex (UL5, UL8, and UL52) and viral DNA polymerase complexes assemble at the replication fork to initiate theta form replication (Jacob, Morse et al. 1979). The second stage of DNA replication is driven in an origin-independent manner via a recombination and/or rolling circle mechanism. In this stage, UL9 is no longer required, and replication switches from theta form to the rolling circle form via an unknown mechanism. The rolling circle replication forms longer-than-unit length concatemers, which become packaged into monomeric units by cleavage. In conjuction, viral genomes are packaged into the preassembled capsids (Roizman and Knipe 2001).

Capsid Assembly and Packaging

Herpes simplex virus capsid assembly can be ordered into several steps. The empty spherical procapsid is the first capsid formed and consists of an inner and outer shell. The internal scaffold is cleaved then expulsed or degraded and viral DNA is inserted (Desai, Homa et al. 1994; Tatman, Preston et al. 1994; Thomsen, Newcomb et al. 1995; Homa and Brown 1997). Once in the nucleus the HSV-1 scaffold protein, preVP22a, associates with the portal protein (pUL6), the major capsid protein (VP5), the viral protease (UL26), and itself to form the basic capsid (Thomsen, Newcomb et al. 1995; Desai and Person 1996; Hong, Beaudet-Miller et al. 1996; Pelletier, Do et al. 1997; Newcomb, Homa et al. 1999; Preston and McDougall 2002; Newcomb, Thomsen et al. 2003). The triplex proteins VP19C and VP23 are also added to the partial capsid. As hexons and pentons are added, the structure assembles into a round procapsid

(Newcomb, Homa et al. 1996). Proteolytic cleavage of the scaffold proteins causes a conformational alteration on the outer shell of the procapsid as it angularizes and matures into stable polyhedral icosahedrons (Thomsen, Newcomb et al. 1995; Trus, Booy et al. 1996; Church and Wilson 1997). The mature, DNA-filled nucleocapsid is then actively transported to the nuclear membrane where it attaches and buds into the perinuclear space to become a virion.

Electron microscopic studies have shown that capsid assembly occurs within the voided center of the nucleus in a virus-specific compartment called the DNA replication compartment (Quinlan, Chen et al. 1984). Three types of capsids, called procapsid A, B, and C have been identified (Gibson and Roizman 1972). C-capsids contain viral DNA and eventually form mature enveloped capsids as they bud though the nuclear membrane in to the perinuclear space (Perdue, Cohen et al. 1976). A and B capsids do not contain viral genome, but B capsids are filled with cleaved scaffolding proteins, VP22a and VP21 (Rixon, Cross et al. 1988; Newcomb and Brown 1991), and a viral protease VP24 (Gibson and Roizman 1972; Newcomb, Trus et al. 1993). VP22a, VP21, and VP24 are all absent in C-capsids (Gibson and Roizman 1972; Davison, Rixon et al. 1992). A-type capsids have lost scaffolding proteins and do not contain viral DNA. They form as a result of failed attempts at DNA packaging and their capsid assembly is aborted (Sherman and Bachenheimer 1988).

Encapsidation of viral DNA involves sequence specific cleavage of concatamers into unit-length-monomers as they are packaged into preformed B-capsids (Ladin, Blankenship et al. 1980; Ladin, Ihara et al. 1982). Additionally, the DNA packaging enzyme terminase and capsid structural elements such as portal protein, and pac1 and

pac2 packaging signals are required (Varmuza and Smiley 1985; Deiss, Chou et al. 1986; Smiley, Duncan et al. 1990). Also, UL15, UL25, UL28, UL32, UL33, UL36, and UL378 gene products (Mocarski and Roizman 1982; Spaete and Frenkel 1982; Vlazny, Kwong et al. 1982; Stow and McMonagle 1983a; Stow, McMonagle et al. 1983b) are thought to be involved, but the precise mechanism has not been well defined (Roizman and Knipe 2001).

Nuclear Egress: Primary Envelopment

Successful encapsidation of viral genome in mature nucleocapsids is followed by budding through the inner nuclear membrane into the perinuclear space (the space between the inner and outer nuclear membrane) to acquire the primary envelopment (Vlazny, Kwong et al. 1982). Two conserved herpesvirus proteins, UL31 and UL34 are involved in the initiation of budding (Chang, Van Sant et al. 1997; Klupp, Granzow et al. 2000; Roller, Zhou et al. 2000; Reynolds, Ryckman et al. 2001; Fuchs, Granzow et al. 2002). The UL31 protein contains a nuclear localization signal that allows it to enter the nucleus via nuclear pores, then it is C-terminally anchored in the lamina through its interaction with lamin A/C (Klupp, Granzow et al. 2000). The type II nuclear phosphoprotein UL34 is originally incorporated into the endoplasmic reticulum (ER), diffuses into the outer nuclear membrane (ONM), bypasses the nuclear pore, and finally localizes to the inner nuclear membrane (INM) where its nucleocapsid domain (amino acids 137-181) engages UL31 protein in the lamina (Ye, Vaughan et al. 2000; Reynolds, Ryckman et al. 2001; Liang, Tanaka et al. 2004) to promote envelopment (Reynolds, Ryckman et al. 2001; Fuchs, Granzow et al. 2002). The nuclear lamina is a fibrous meshwork of filaments on the inner surface of the nuclear membrane and denies

nucleocapsids direct access to the INM (Gruenbaum, Wilson et al. 2000). Differentiated cells contain three types of lamins: A, B, and C. Types A and C is derived from alternative splicing of the lamin transcript (Fisher, Chaudhary et al. 1986; Hogger, Krohne et al. 1991; Lin and Worman 1993). The UL31 protein partially dismantles the nuclear lamina by competing with lamin/lamin and lamin/chromatin interactions to allow intranuclear capsids direct access to the INM (Scott and O'Hare 2001). Other viral membrane proteins are thought to play lesser roles in primary envelopment. The UL11 protein associates with the UL31/UL34 complex through N-terminal myristylation. Protein kinase C is recruited to phosphorylate lamins and mediates their localized disassembly. Nucleocapsids engage the UL34 protein at the INM and this initiates the budding reaction. Completion of the budding reaction occurs through a function of the UL11 protein (Baines and Roizman 1992; MacLean, Dolan et al. 1992). The UL31 and UL34 proteins remain on the virion membrane as a part of primary envelope after budding completes.

Egress from the Perinuclear Space: De-Envelopment

After assembly of capsid in the nucleus, the ensuing primary envelopment and budding in to the perinuclear space, the virion is trapped and requires a mode of exit from the closed compartment to continue egress and ultimately be released into the extracellular space as infectious virions. Initially, two models of egress have been proposed (Enquist, Husak et al. 1998; Skepper, Whiteley et al. 2001; Johnson and Huber 2002; Mettenleiter 2002) with a possible third model shown for bovine herpesvirus 1 (BHV-1) and HSV-1 (Wild, Senn et al. 2004). In the first model, termed “luminal” pathway or the single envelopment model (Johnson and Spear 1982),

virions retain their primary envelope and leave the perinuclear space via a vesicle/vacuole derived from the outer nuclear membrane (Darlington and Moss 1968; Johnson and Spear 1982; Campadelli-Fiume, Farabegoli et al. 1991; Johnson, Webb et al. 2001). Once in the cytoplasm, the vesicle-vacuole interacts with membranes of the exocytic pathway of the Golgi apparatus and gain the glycoproteins present in mature virions. As a final step, the glycoprotein enriched vesicle-vacuole fuses with the cytoplasmic region of the host cell plasma membrane to release adult virions. The second and more widely accepted model of egress is the envelopment de-envelopment paradigm (Stackpole 1969). Here the mode of exit involves the primary envelope of perinuclear virions fusing with the outer nuclear lamellae, resulting in de-envelopment and release of nucleocapsids in to the cytoplasm (Enquist, Husak et al. 1998; Mettenleiter 2000) (Figure 1.9: II). Maturing capsids undergo re-envelopment at the Trans Golgi Network and acquire the full complement of glycoproteins (Figure 1.9:III). In the recently proposed third model, nucleocapsids exit though the modified nuclear pores and thus pass directly from the nucleoplasm to the cytoplasm. Here they undergo their first envelopment at late Golgi or TGN (Wild, Senn et al. 2004).

There is data to support each of the three proposed models, but the majority of evidence favors the de-envelopment re-envelopment pathway of egress. Electron microscopic studies of assembly show that the primary envelope and primary tegument of perinuclear virions clearly differ from the final envelope and final tegument of extracellular virions (Gershon, Sherman et al. 1994; Granzow, Klupp et al. 2001). For example, the UL31/ UL34 complex found on nuclear membranes and perinuclear virions are not detected in extracellular virions (Reynolds, Wills et al.

2002). Furthermore, the major tegument proteins UL46 and UL49 present in intracytoplasmic/extracellular virions are absent from perinuclear virions (Klupp, Granzow et al. 2000; Mettenleiter 2002). In addition, the phospholipids of the final virion envelope carry pronounced compositional differences from the nuclear membrane (van Genderen, Brandimarti et al. 1994). The fusion of primary envelopes with the outer nuclear membrane with many herpesviruses shown via EM analysis cannot be accounted for by the luminal model of virion egress (Harms, Ren et al. 2000; Granzow, Klupp et al. 2001). Therefore, based on the biochemical and morphological data presented by various experiments, the two-step de-envelopment re-envelopment model is currently the preferred mode of virion egress (Mettenleiter 2002).

As for the mechanism of virion morphogenesis, much remains unclear. Fusion of the perinuclear virion envelope with the outer nuclear membrane occurs in a process that is facilitated by the US3 and UL13 encoded kinases. The virion loses UL31 and UL34 as it becomes de-enveloped, and it may be possible that the UL31/UL34 complex is then recycled to the inner nuclear membrane. The single deletion of the major glycoproteins thought to be involved in membrane fusion events do not substantially affect the de-envelopment process (Cai, Person et al. 1987; Jayachandra, Baghian et al. 1997; Steven and Spear 1997; Granzow, Klupp et al. 2001). However, studies have shown that certain glycoproteins function in a redundant manner, and only multiple glycoprotein deletions have a pronounced effect on virion de-envelopment. This possibility is a major component of this thesis and will be addressed in Chapter II.

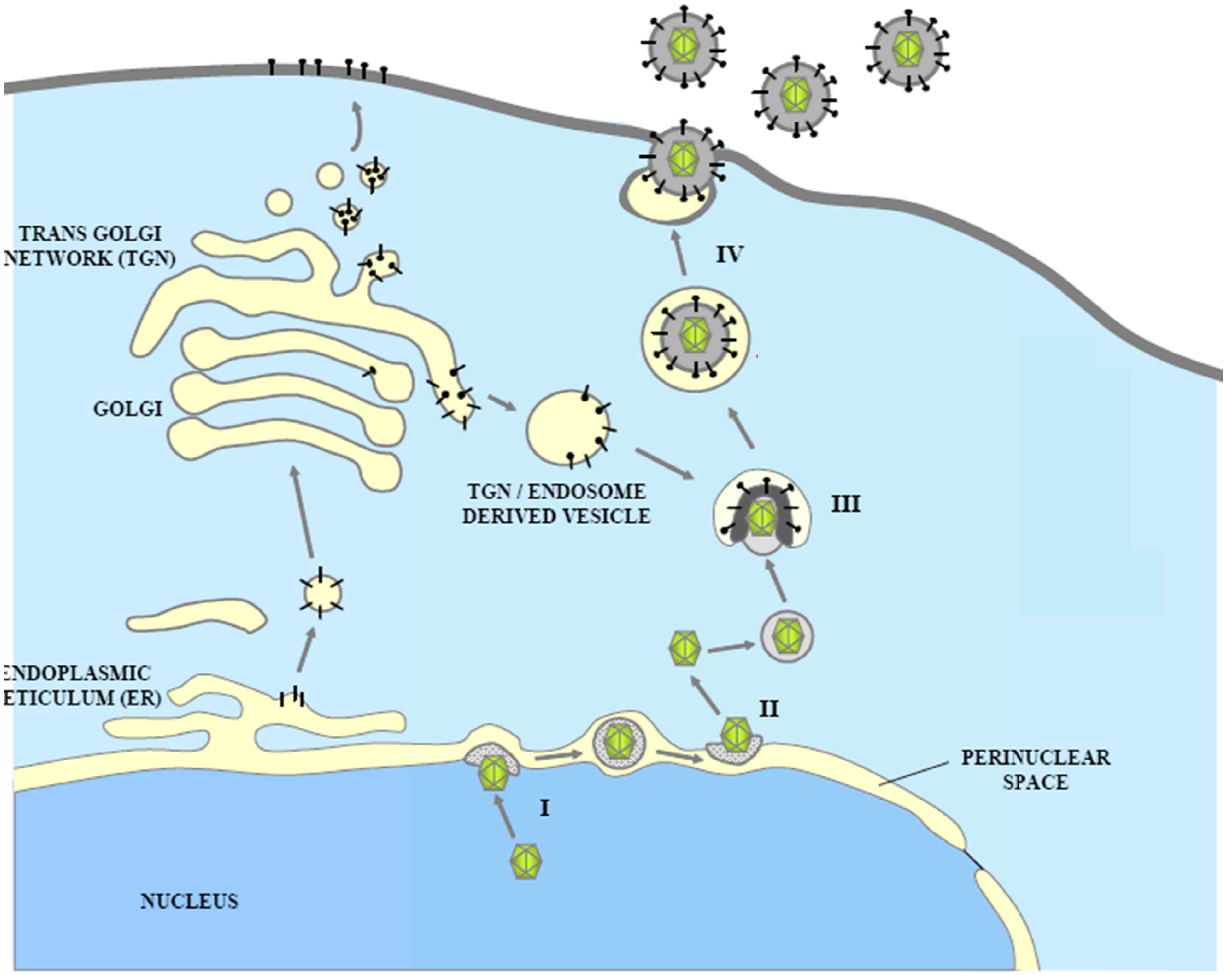


**Figure 1.9: Representation of HSV-1 Virion Morphogenesis and Egress.** (I) Mature capsids bud through the inner nuclear membrane and into the perinuclear space; (II) De-envelopment of perinuclear virions at the outer nuclear membrane; (III) Re-envelopment of cytoplasmic capsids by budding into cytoplasmic vesicles; (IV) Final egress to the extracellular space.

Tegumentation in the Cytoplasm

The tegument is an amorphous protein layer that contains approximately 20 proteins (Yamauchi, 2008), including VP1/2 (UL36), VP11/12 (UL46), VP13/14 (UL47), VP16 (UL48), VP22 (UL49), ICP0, ICP4, the virion host shutoff protein (UL41), and the

products of genes US2, US3, US10, US11, UL11, UL13, UL14, UL16, UL17, UL21, UL37, UL51, and UL56 (Yao and Schaffer 1994; Homa and Brown 1997; Mettenleiter 2002). Tegumentation is believed to be a highly ordered pattern of adding “layers” of protein (Mettenleiter 2002) to the capsid during assembly, with different portions of the tegument being added in the nucleus, in the cytoplasm, and at the trans-Golgi network (TGN) (Mettenleiter 2004). Recent work done with UL16 (Meckes and Wills 2007) has challenged this widely accepted model by claiming that the tegumentation process is much more dynamic than previously realized. The tegument proteins act as a link between capsid and viral envelope proteins by interacting with each on opposite sides (Figure 1.9: IV) (Mettenleiter 2002). UL36 (VP1/2) is found attached to the penton vertices of the capsid in the inner most layer of the tegument (Zhou, Chen et al. 1999) and interacts with the major capsid protein VP5 (Machtiger, Pancake et al. 1980; McNabb and Courtney 1992; Newcomb, Trus et al. 1993). The UL37 interacts with residues F593 and E596 of UL36 (Klupp, Fuchs et al. 2002; Mijatov, Cunningham et al. 2007), and are essential to virus maturation (Desai 2000; Desai, Sexton et al. 2001; Klupp, Granzow et al. 2001a). UL36 and UL37 are the only tegument proteins conserved for all members of the herpesvirus family known to date. The ensuing steps in tegument assembly is still being defined, however, tegument proteins that are present in high amounts (1,000 to 2,000 copies per virion), such as UL46, UL47, UL48, and UL49, are structural candidates (Heine, Honess et al. 1974; Zhang and McKnight 1993), and the lower-copy-number ICP0 and ICP4 (100 to 150 copies per virion) probably constitute regulatory roles (Yao and Schaffer 1994). UL48 (α-TIF), also responsible for mediating alpha gene promoters (Heine, Honess et al. 1974; Batterson and Roizman

1983), may interfere with late stages of virion assembly by affecting tegumentation and the subsequent cytoplasmic re-envelopment process (Mossman, Sherburne et al. 2000). UL48 interacts with other tegument components, namely UL41 (VHS) and UL49 (Smibert, Popova et al. 1994). In PrV, the carboxyl terminus of tegument protein VP1/2 anchors to capsids, and the multifunctional capsid protein UL25 is also involved in tegumentation as evidenced by their interaction with VP1/2 (Coller, Lee et al. 2007).

### Final Envelopment at the Trans-Golgi Network/Endosomes and Egress to Extracellular Spaces

After virion de-envelopment and release into the cytoplasm, HSV-1 nucleocapsids make their way toward cytoplasmic vesicles derived from the trans-Golgi Network (TGN) or endosomes, and gain their secondary envelopment (Figure 1.9: III). Inner tegument proteins UL36 and UL37 mediate transport of nucleocapsids to the envelopment site where envelope glycoproteins and the remaining tegument proteins await them (Mettenleiter, Klupp et al. 2006). At the site of envelopment in the trans-Golgi network, glycoprotein M and pUL11 play critical roles in the assembly of other glycoproteins and outer tegument proteins. gM may function in either retaining envelope glycoproteins at the envelopment site or retrieve them from the cell surface deposits (Crump, Bruun et al. 2004). UL11 localizes with the Golgi apparatus via intrinsic targeting properties and is involved with directing tegument proteins (Bowzard, Visalli et al. 2000; Loomis, Courtney et al. 2003). In some alphaheresviruses, pUL48 possibly mediates the interaction between nucleocapsid and envelopment site (Vittone, Diefenbach et al. 2005). The physical process of cytoplasmic envelopment is not well understood, but believed to result from interactions between tegument proteins of the immature virion with the cytoplasmic tails of several glycoproteins. With the exception of UL20/gK complex, the absence of

single glycoproteins exhibit minimal effects in cytoplasmic virion envelopment. Cells infected with either UL20 or gK deletions lack extracellular virus and accumulates unenveloped and sporadically enveloped capsids in the cytoplasm (Jayachandra, Baghian et al. 1997; Foster, Melancon et al. 2004a). Meanwhile, studies have shown that the deletion of multiple glycoproteins may affect cytoplasmic envelopment, with the specific combination dependent on different members of the herpesvirus family. For example, simultaneous deletion of gE/gI and gM in PrV results cytoplasmic envelopment defects (Brack, Dijkstra et al. 1999; Kopp, Granzow et al. 2004). However, the deletion of the same set of glycoproteins in HSV-1 does not exhibit the same drastic inhibition of plaque formation and replication (Browne, Bell et al. 2004). On the other hand, gD + gE/gI triple mutants had a severe defect in the final envelopment step in HSV-1 (Farnsworth, Goldsmith et al. 2003). The authors proposed that in HSV-1, gD and the gE/gI heterodimeric complex may act in a redundant fashion to anchor the cytoplasmic envelope on to tegument-coated capsids. However, it is also possible that the deletion of multiple glycoproteins may have widespread effects and indirectly disrupt the integrity of many other protein-protein interactions required for cytoplasmic envelopment. Therefore, the resulting phenotype is actually reflective of compounded effects of an abnormal glycoprotein profile. This controversy will be discussed in further detail in Chapter II. The acquisition of the second membrane results in a mature herpesvirus particle, which is then transported to the plasma membrane. There, fusion of the exocytotic vesicle with the host plasma membrane released the fully assemble herpes virion. As alluded to earlier, mechanisms for cytoplasmic envelopment and fusion events leading to the release of virus remain an enigma.

### Light (L) Particles

Capsids are not a necessary component in cytoplasmic envelopment. The extracellular herpesvirus light (L) particles embody the full array of tegument and envelope proteins, but completely lack the capsid segment (McLauchlan, Addison et al. 1992; Rixon, Addison et al. 1992). Studies in PrV have shown that L particle formationis independent of UL36 and UL37, which are tegument components critical for capsid tegumentation. When the appropriate capsids are not available, tegument assembly could proceed abnormally by UL49 interactions with glycoproteins, resulting in the formation of L particles (Mettenleiter 2002).

## HSV-1 Glycoproteins

HSV glycoproteins were discovered in the early 1970s (Spear and Roizman 1970), and soon after were identified as targets of neutralizing antibodies, suggesting a role in virus infection (Spear 1976; Spear, Sarmiento et al. 1978). As improvements in technology led to the complete sequencing of the HSV genome, at least 12 glycoproteins (gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, gM, and gN) have been identified. Since then, much effort has been devoted to delineating specific function of each glycoprotein in entry and egress. The multigene system for entry and egress is a unique characteristic of HSV and understanding this complex system remains a challenging feat.

### Glycoprotein B (UL27)

The HSV-1 glycoprotein B is encoded by UL27 gene and is primarily involved in attachment through a lysine-rich region (Laquerre, Argnani et al. 1998). gB is a 904-amino acid (aa) glycoprotein that consists of a 696-aa ectodomain N-glycosylated at multiple

sites, a 69-aa transmembrane domain, and a 109-aa carboxyl-terminal domain (Claesson-Welsh and Spear 1986; Claesson-Welsh and Spear 1987; Highlander, Goins et al. 1991; Spear 1993; Laquerre, Person et al. 1996; Whitley 2001). Of all the HSV-1 glycoproteins, gB has the largest cytoplasmic tail, which suggests that this domain is important in mediating gB related activities. Throughout the years, it has been determined that gB plays an important role in membrane fusion for both virus entry and virus-induced cell fusion (Cai, Person et al. 1987). gB deletion mutants fail to enter the host due to a post-attachment defect (Cai, Gu et al. 1988a). Various single amino acid substitutions and truncations of the carboxyl terminus of gB induced extensive virus-induced cell fusion (Bzik, Fox et al. 1984; Cai, Person et al. 1988b; Baghian, Huang et al. 1993; Gage, Levine et al. 1993). Cell-to-cell fusion is an ordered event involving gB, gD, gH, and gL (Foster, Melancon, and Kousoulas, 2001; Haan, Lee, and Longnecker, 2001; (Foster, Rybachuk et al. 2001; Haan, Lee et al. 2001; Klupp, Granzow et al. 2001b; Pertel 2002). This sequential phenomenon can be divided into three phases based on glycoprotein involvement: Phase I - gD, Phase II – gH/gL, and Phase III - gB (Gianni, Piccoli et al. 2006; Subramanian and Geraghty 2007). The crystal structure of HSV-1 gB has been resolved and shows a multidomain trimer homologous to glycoprotein G from vesicular stomatitis virus (VSV gG). Class I fusion protein-like alpha-helical coiled-coil core, and two extended beta hairpins with hydrophobic tips indicative of class II fusion proteins, imply that gB has intricate relevance in virus cell fusion events (Heldwein, Lou et al. 2006).

Glycoprotein C (UL44)

The highly N- and O- glyosylated protein gC is a product of the UL44 gene and is a major determinant of HSV attachment to cells (Herold, WuDunn et al. 1991).

The UL44 gene (1,536 bp) (Frink, Eisenberg et al. 1983) contains a 25-aa signal sequence at the N-terminus, a 453-aa extra-cellular domain, a 23-aa transmembrane anchoring domain, and a 10-aa C-terminal cytoplasmic tail (Homa, Purifoy et al. 1986). Initial contact between virion and target cell occurs as gC binds cell surface glycosaminoglycan (GAGs) heparan sulfate (HS) at its 120-aa N-terminal (Tal-Singer, Peng et al. 1995). Although HSV-1 gC is dispensable for successful infection of cultured cells, its presence promotes a 10 fold increase in virus binding efficiency. Target recognition by gC mediated events may stabilize the essential interaction between gD with host receptor in viral entry. Another function of gC is to facilitate immune evasion by binding to C3b, inactivating the complement system (Friedman, Cohen et al. 1984; Eisenberg, Ponce de Leon et al. 1987; Huemer, Larcher et al. 1993; Huemer, Nowotny et al. 1995). gC protects both virus and infected cell from antibody (Ab)-independent complement neutralization and cell lysis (Lubinski, Wang et al. 1998; Lubinski, Wang et al. 1999), antibody dependant complement neutralization (Hook *et al*., 2006), and is an important virulence factor *in vivo* (Kotwal, Isaacs et al. 1990; Isaacs, Kotwal et al. 1992; Sahu, Isaacs et al. 1998). Meanwhile, the extent of gC function in relation to virus-induced syncytia formation is debatable (Bartoletti, Tognon et al. 1985). In general, the syncytial phenotype was more abundantly expressed in the absence of gC (Manservigi, Spear et al. 1977; Bond, Person et al. 1982; Tognon, Bartoletti et al. 1984; Goodman and Engel 1991; Pertel and Spear 1996). It is possible that when gC is absent, the virus does not bind to cell surfaces as rapidly and in turn, cell-to-cell spread of HSV-1 primarily results from syncytial formation.

Glycoprotein D (US6)

Glycoprotein D is the receptor-binding glycoprotein of HSV. It is the universal viral ligand for HVEM, nectin-1, nectin-2, and all other HSV-1 entry receptors (Spear, Eisenberg et al. 2000). The 394-aa (1182bp) product of US6 consists of a 25-aa signal peptide, a 315-aa ectodomain with three glycosylation sites (Watson, Weis et al. 1982; McGeoch, Dona et al. 1985), a 22-aa transmembrane domain, and a 32-aa C-terminal cytoplasmic domain (Minson, Hodgman et al. 1986). The first 250-260 residues of the ectodomain correspond to the receptor binding domain (Krummenacher, Nicola et al. 1998). Located just downstream within the ectodomain, encompassing residues 260-315, is the pro-fusion domain believed to be involved with virus-induced cell-to-cell fusion (Cocchi, Fusco et al. 2004a). X-ray imaging of HSV-1 gD by itself and in a complex with HVEM depict gD forming unconventional disulfide-bonding patterns reminiscent of Ig (Carfi, Willis et al. 2001). For HVEM, the specific residues of contact with gD are amino acids 7 to 15 and 24 to 32 within the N-terminal hairpin (Spear and Longnecker 2003). The first 32 amino acids of the N-terminal extension of gD function in all HSV entry-fusion receptors, except to nectin-1 (Yoon, Zago et al. 2003; Zago and Spear 2003). Mutational analysis of gD has shown that gD null without the TM and C-tail regions can be successfully rescued in cell culture (Cocchi, Fusco et al. 2004a). This is supported by the observation that substitution of the TM and C-tail regions with heterologous sequences leave gD entry function unaltered (Browne, Bruun et al. 2003; Cairns, Milne et al. 2003). Therefore, whilst gD is essential in both viral entry and pro-fusion, the absence of its TM and C-tail regions exhibit no defects in those essential steps. This observation is relevant in the design of experiments found in Chapter II.

Glycoproteins E (US8) and I (US7)

gE and gI form a heterodimer that mediates cell-to-cell spread in certain tissues of HSV, varicella zoster virus (VZV), and PrV (Johnson and Feenstra 1987; Neidhardt, Schroder et al. 1987; Mettenleiter, Zsak et al. 1987a; Johnson and Ligas 1988; Kudelova, Kostal et al. 1991; Card, Whealy et al. 1992; Jacobs, Mulder et al. 1993; Whealy, Card et al. 1993; Balan, Davis-Poynter et al. 1994; Dingwell, Brunetti et al. 1994; Kritas, Pensaert et al. 1994; Mulder, Jacobs et al. 1994; Cohen and Nguyen 1997; Tirabassi, Townley et al. 1997; Dingwell and Johnson 1998; Kimura, Wang et al. 1998; Tirabassi and Enquist 1998; Polcicova, Goldsmith et al. 2005). The US8 gene encodes a 550-aa (1652bp) glycoprotein E (Lee, Para et al. 1982), and the US7 gene encodes a 390-aa (1172bp) glycoprotein I (Longnecker, Chatterjee et al. 1987). The gE/gI heterodimer is required for efficient spread of viruses between polarized cells with extensive cell junctions, such as epithelial cells and neurons, but are not needed for spread between highly transformed, nonpolarized cells that do not form cell junctions, such as Vero or HeLa (immortal cell line derived from cervical cancer cells taken from Henrietta Lacks) cells (Darlington and Moss 1968; Mettenleiter, Zsak et al. 1987a; Mettenleiter, Schreurs et al. 1987b; Zsak, Sugg et al. 1992; Dingwell, Brunetti et al. 1994; Dingwell, Doering et al. 1995; Tirabassi, Townley et al. 1997; Wisner, Brunetti et al. 2000). The gE/gI complex localizes to the TGN during early stages of infection but is translocated to cell junctions during intermediate to late times. gE/gI is known to sort newly assembled virions to lateral cell surfaces and cell junctions. In contrast to their effects on cell-to-cell spread, gE/gI complexes do not appear to be required for virus entry in to cells (Mettenleiter, Zsak et al. 1987a; Dingwell, Brunetti et al. 1994). Through

high affinity binding of gE/gI with the Fc portion of IgG, the virus reduces the effectiveness of antibody dependent cellular cytotoxicity (ADCC) targeting (Eberle, Dubreuil et al. 1995), and thus contributes to the immune evasion mechanism of virus or virus infected cells.

Glycoprotein G (US4)

The 238-aa (716bp) glycoprotein G is encoded by US4 and can be found in nuclear and cytoplasmic membranes of the virus infected cell (Frame, Marsden et al. 1986; Sullivan and Smith 1987). The precise role of gG during infection is not clear, but recombinant viruses devoid of gG showed no major differences from the wild-type virus (Atkinson, Barr et al. 1978). However, in the gC-dependent infection of the apical surfaces of polarized epithelial cells and corneal epithelial cells, mutants lacking gG showed significant phenotypic defects (Tran, Kissner et al. 2000). If gG were to be involved in gC functions, it could be during the gC initial binding stage.

Glycoproteins H (UL22) and L (UL1)

The 838-aa (2517bp) membrane glycoprotein H is encoded by the UL22 gene. gH is a consists of a 18-aa signal peptide, a 785-aa ectodomain, a 21-aa hydrophobic transmembrane domain, and a 14-aa C-terminal cytoplasmic tail. Glycoprotein L is a 224-aa (675bp) product of the UL1 gene. gL carries a 25-aa signal peptide but is unique from other glycoproteins in that it does not have a transmembrane domain needed for it to be an integral membrane protein. Infected cell membranes contain the heterodimer of gH/gL and it is only through this complex that gL is membrane associated (Dubin and Jiang 1995). In the absence of gL, gH fails to form or process in the typical manner. The resulting malformed gH remains in the endoplasmic reticulum and undergoes self-

aggregation (Foa-Tomasi, Avitabile et al. 1991; Forrester, Sullivan et al. 1991; Roberts, Ponce de Leon et al. 1991). In a similar fashion, cells infected with only gL and no gH results in unprocessed gL that cannot embed into the plasma membrane nor the viral envelope (Hutchinson, Browne et al. 1992a; Roop, Hutchinson et al. 1993). Due to the lack of a transmembrane region, the gL polypeptide is secreted into the medium (Dubin and Jiang 1995). The cleavage of the 25-aa signal peptide region in gL permits the first 69-aa N-terminal residues (Roop, Hutchinson et al. 1993) to interact with the central region of gH extra-cellular domain. In addition, the C-terminal cysteine residues are necessary components of gH/gL interactions (Cairns, Landsburg et al. 2005). Viruses lacking in both gH and gL are unable to enter cells but are able to attach to the cell surface, confirming the notion that properly formed gH/gL is required for both virus entry and virus-induced cell-to-cell fusion.

Glycoprotein J (US5)

The 92-aa (279bp) glycoprotein J is encoded by the US5 gene (Ghiasi, Nesburn et al. 1998). gJ is predicted to contain a signal peptide as well as a hydrophobic transmembrane domain. The only known function of gJ is the inhibition of cytotoxic lymphocyte induced apoptosis (Jerome, Chen et al. 2001). Otherwise, the deletion mutants for gJ show no phenotypic deficiencies (Balan, Davis-Poynter et al. 1994). One possible explanation is that the region of sequence homology shared by gJ and the protein encoded by UL27.5 (Chang, Menotti et al. 1998) acts in redundant fashion and thus phenotypic changes can only be seen if both are deleted. The implication that gJ is a multifunctional protein that modulates other cellular process has gained support from

a recent study showing that gJ localizes to multiple cellular organelles and induces generation of reactive oxygen species (Aubert, Chen et al. 2008 ).

Glycoprotein K (UL53)

The 339- aa (1017bp) glycoprotein K is encoded by the UL53 gene (Debroy, Pederson et al. 1985; Pertel and Spear 1996). The hydrophobic gK consists of a 30-aa signal sequence and two asparagine residues that are glycosylated by N-linked mannose at positions 48 and 58 (Ramaswamy and Holland 1992; Hutchinson, Goldsmith et al. 1992b). In the past, gK was thought to traverse the membrane three or four times, with mixed results showing both the N-terminus and C-terminus on the luminal/extra-cellular side of cellular membranes (Debroy, Pederson et al. 1985) or the C-terminal tail located intracellularly (Debroy, Pederson et al. 1985; Ramaswamy and Holland 1992; Mo and Holland 1997). Data from our laboratory using epitope tags inserted into specific regions of gK confirmed both ends of the glycoprotein on the luminal/extra-cellular portion of cell membranes. (Foster, Alvarez et al. 2003). gK was determined to be essential for virus growth (MacLean, Efstathiou et al. 1991). An F-strain gK-null mutant resulted in aberrant virion accumulation of naked capsids in the cytoplasm, and in conjunction with virion failure to reach the extra-cellular space, produced a greatly attenuated virus (Hutchinson, Roop-Beauchamp et al. 1995). Additionally, cells infected with the F-strain gK-null virus caused cell fusion in 143TK- cells. Later, a different aa deletion (N-terminal 112-aa) of the KOS strain gK corresponding UL53 gene produced a slightly different outcome (Jayachandra, Baghian et al. 1997). While the egress of virions in the KOS gK-null virus remained defective, the recombinant virus did not cause cell fusion in 143TK- cells as was shown previously.

Further investigation of the role of gK in cell-fusion was done by constructing a KOS strain virus to mirror the F strain mutant. This resulted in syncytia formation, therefore the syncytial properties of the gK viruses was accredited to the regions beyond the N-terminal 112-aa region (Jayachandra, Baghian et al. 1997; Melancon, Luna et al. 2005). Previously, gK was thought to localize exclusively in the perinuclear spaces and the rough endoplasmic reticulum, never reaching the trans-Golgi network or the cell surface (Hutchinson, Roop-Beauchamp et al. 1995). But the large number of syncytial mutations associated with gK seemed paradoxical to gK being able to induce cell-to-cell fusion. This contradiction was resolved when observations from our laboratory utilizing viruses with epitope tags in the putative extra-cellular domains of gK indicated that gK does indeed reach the surface of infected cells (Foster, Alvarez et al. 2003). As with previously discovered glycoprotein interactions, gK does not function in a solitary fashion. In a UL20 deletion mutant, gK was not correctly processed (Dietz, Klupp et al. 2000). Furthermore, co-expression of gK and UL20 in transfected cells restored the transport of gK to cell surfaces (Foster, Alvarez et al. 2003). However, syncytia formation did not result in these experiments, implying that activation of gK's ability to facilitate membrane fusion may require additional viral proteins (Foster, Alvarez et al. 2003).

Glycoprotein M (UL10) and Glycoprotein N (UL49.5)

The 473-aa (1422bp) glycoprotein M is encoded by the UL10 gene (Baines and Roizman 1993; Babic, Klupp et al. 1996). As a component of the virion envelope, gM contains eight predicted hydrophobic transmembrane regions and can be found in the cytoplasmic membranes of infected cells (MacLean, Robertson et al. 1993). The

role of gM is unclear, but evidence suggests that it may function in nuclear egress (Baines, Wills et al. 2007). gM deficient mutants result in a one-log reduction of virus titers in Vero cells and form slightly smaller plaque phenotype (MacLean, Efstathiou et al. 1991; MacLean, Robertson et al. 1993). The 91-aa (276bp) integral membrane protein gN (UL49.5) is potentially O-glycosylated in PrV (Jons, Granzow et al. 1996), and thus described as glycoprotein N (Baines and Roizman 1993). The gene contains a 23-aa cleaved signal peptide and a C-terminal hydrophobic transmembrane domain (Barnett, Dolan et al. 1992). In HSV-1 infected cells however, the UL49.5 protein was not glycosylated, and appeared to be linked to the tegument via disulfide bonds (Adams, Cunningham et al. 1998). Originally, unsuccessful attempts at recovering virus from UL49.5 deletion mutants generated the belief that the gene was necessary for virus replication (Barker and Roizman 1992), but this notion has been challenged by a site-specific deletion of UL49.5 that showed no significant phenotypic differences in plaque morphology and only a marginal difference in virus yield (Adams, Cunningham et al. 1998). gM and UL49.5 (gN) are conserved throughout the herpesvirus family and form a complex with each other (Jons, Dijkstra et al. 1998; Lake, Molesworth et al. 1998; Wu, Zhu et al. 1998b; Mach, Kropff et al. 2000; Koyano, Mar et al. 2003) to possibly function in fusion inhibition (Klupp, Granzow et al. 2000). While the precise region for interaction has not been defined for this complex, their functions were enhanced when both were present, as opposed to the when either gM or UL49.5 were absent (Koyano, Mar et al. 2003). The mechanism by which gM and UL49.5 mediate inhibition of cell-to-cell fusion remains undefined.

**UL20**

The alphaherpesvirus conserved 669bp membrane protein UL20 is expressed as a gamma 1 (late) gene and is expressed independently of viral DNA replication (Ward and Roizman 1994). The membrane topology of UL20 calculated by using the TMPred and SOSUI computer algorithms (Hofmann and Stoffel 1993; Hirokawa, Boon-Chieng et al. 1998) predicts four hydrophobic transmembrane regions and no signal peptide, and observations from our lab confirms this model of pUL20. The two cytoplasmic regions (domains I and V) include the 66-aa N-terminus and the 14-aa C-terminus. A 10-aa domain is located intracellularly (domain III), while domain II containing 7-aa and domain IV containing 32-aa are located extracellularly. Interactions between pUL20 and other viral proteins are currently being defined. Our laboratory has shown that N-terminus of pUL20, most likely interact with intracellular domain III of gK (Foster, Chouljenko et al. 2008) UL20-null effects, originally shown for the recombinant virus R7225, include viral egress defects via a perinuclear block to accumulate virions between the inner and outer nuclear lamellae and an abnormal level of un-enveloped capsids in the cytoplasm. In addition, the R7225 virus formed small syncytia on 143TK- cells (Baines and Roizman 1991). However, theR7225 virus did not have a specific deletion of the UL20 gene, but also partially deleted the UL20.5 ORF, which is adjacent to the 3' half of the UL20 gene. In an effort to resolve the true phenotype of a HSV-1 UL20 null mutants, our laboratory constructed a more precise UL20 gene insertion-deletion mutant in HSV-1 KOS-strain to remove the 5' region of the UL20 ORF yet maintain the integrity of both UL20.5 gene and the promoter for the major capsid protein, UL19. This time, the recombinant virus showed defects in virion egress but

seemed to be blocked at a different stage of morphogenesis, as un-enveloped capsids accumulated in the cytoplasm and not in the perinuclear region (Foster, Melancon et al. 2004b). Furthermore, the HSV-1 KOS UL20-null virus did not form syncytial plaques on 143TKcells; much like the PrV virus lacking UL20 (Fuchs, Klupp et al. 1997). In all likelihood, the phenotype for the R7225 virus does not accurately represent UL20 functions. Localization of the UL20 protein occurs in nuclear membranes, Golgi apparatus, cytoplasm (Ward and Roizman 1994), and plasma membranes of infected cells (Foster, Melancon et al. 2004a). However, our laboratory has shown that the transport of pUL20 to TGN and cell surface is interdependent on gK. Therefore, gK and pUL20 interaction is important in retaining their functions during the virus life cycle (Foster, Melancon et al. 2004b). In addition, the UL20 protein was required for virus induced cell-to-cell fusion resulting from syncytial mutations in either gB or gK, implying that UL20 may serve to regulate membrane fusion events in HSV-1 (Foster, Melancon et al. 2004a).

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# CHAPTER TWO

# THE HERPES SIMPLEX VIRUS TYPE-1 (HSV-1) GLYCOPROTEINS D (gD) AND E (gE) ARE NOT ESSENTIAL AND DO NOT FUNCTION IN A REDUNDANT MANNER FOR CYTOPLASMIC VIRION EGRESS

## INTRODUCTION

Herpes simplex virus type 1 (HSV-1) virion assembly is a multi-step process (Mettenleiter, Klupp et al. 2006b). This process initiates in the nucleus where procapsid proteins assemble around scaffolding proteins (Walters, Sexton et al. 2003; Singer, Newcomb et al. 2005), followed by the digestion of these scaffolding proteins creating empty capsids to be filled with DNA (Tatman, Preston et al. 1994; Thomsen, Newcomb et al. 1995; Homa and Brown 1997). HSV capsids then undergo primary envelopment as they cross the inner nuclear membrane, into the perinuclear space (Vlazny, Kwong et al. 1982 1982; Mettenleiter 2000), and then fuse with the outer membrane (de-envelopment) delivering nucleocapsids into the cytoplasm (Stackpole 1969; Vittone, Diefenbach et al. 2005). In the cytoplasm, secondary virion envelopment occurs after HSV capsids are coated with tegument proteins and subsequently bind to viral glycoprotein-enriched regions of the Trans Golgi network (TGN) (Fuchs, Klupp et al. 2002; Chi, Harley et al. 2005; Kamen, Gross et al. 2005). This final virion morphogenesis step delivers fully enveloped virions into cytoplasmic vesicles, which are ultimately transported out of the cell within vesicles using exocytosis.

Multiple glycoproteins have been associated with cytoplasmic virion envelopment, with different herpesviruses utilizing a different set of glycoproteins. For example, in pseudorabies virus (PrV) the absence of the heterodimer glycoprotein E (gE)/gI and gM (Minson, Hodgman et al.) inhibits cytoplasmic virion envelopment (Brack, Dijkstra et al. 1999; Brack, Klupp et al. 2000), whereas HSV-1, gM (UL10) and gE (US8)/gI (US7) ( triple knockout mutant viruses do not show significant defects in virion envelopment (Browne, Bell et al. 2004). Instead, a concurrent deletion of both HSV-1 gD (US6) and

gE/gI causes accumulation of unenveloped capsids in the cytoplasm (Farnsworth, Goldsmith et al. 2003), presumably due to the lack of appropriate interactions with viral tegument proteins. There are several other membrane proteins including UL11, UL20, and UL53 (gK), that have been shown to be involved in cytoplasmic virion envelopment for both PrV and HSV-1, suggesting that they may also play important roles in cytoplasmic virion envelopment (Jayachandra, Baghian et al. 1997 1997; Bowzard, Visalli et al. 2000; Loomis, Courtney et al. 2003; Foster, Melancon et al. 2004).

The working model for cytoplasmic virion envelopment calls for multiple interactions between tegumented capsids in the cytoplasm and viral glycoproteins and membrane proteins embedded in TGN-derived membranes. Such interactions have been described between various glycoproteins and tegument proteins UL16, UL48, and UL49 (VP22). In addition, the membrane protein UL11, which intrinsically targets to the Golgi apparatus, is believed to direct tegument proteins to the glycoprotein embedded envelopement site (Bowzard, Visalli et al. 2000; Loomis, Courtney et al. 2003). Furthermore, UL11 has been shown to interact with the UL16 tegument protein (Meckes and Wills 2007). Interestingly, deletion mutants in some of these tegument proteins do not appreciably affect cytoplasmic virion envelopment (Fuchs, Klupp et al. 2002; Mettenleiter 2002) arguing for a potential redundant role for different tegument proteins in cytoplasmic virion envelopment.

It is unclear at this point, which viral glycoproteins and/or membrane proteins are the most important determinants of cytoplasmic virion envelopment, as well as how they interact with tegument proteins to facilitate cytoplasmic virion envelopment. To address the relative roles of UL20, gD, gE, and gM in cytoplasmic re-envelopment, we sought to

construct single and double-null mutants in these genes in the same viral genetic background of the HSV-1(F) strain to assess their phenotypic and replication properties. Mutant viruses for UL20, gD, gE and gM were constructed using a markerless two-step Red-mediated recombination mutagenesis protocol implemented using the HSV-1 (F) genome cloned into a bacterial artificial chromosome (bac). The results clearly demonstrated that gD and gE do not appreciably function either alone or in a redundant manner in cytoplasmic envelopment and virion egress. Direct comparison with the UL20 or gK-null viruses showed that the UL20/gK protein complex is the preeminent determinant for cytoplasmic virion egress.

## MATERIALS AND METHODS

### Cells

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

### PCR Primer Design

Mutations are accomplished by marker-less Red recombination in *E. coli* using specific novel PCR primers (Tischer, von Einem et al. 2006) (Table 1). The 5' end of the forward primer contains ~40bp homologous sequence upstream of the site of mutation, followed by the mutant DNA sequence(s). An extra 20bp downstream of the target site is added. The 3' end of the forward primer anneals to pEPkans-S so that it overlaps an *I-SceI* self-homing endonuclease site (Figure 2.1: A). The 5' end of the reverse primer was designed so that it contains the reverse complement 40bp downstream of the

target site which is also homologous to the 5' end of the forward primer. This was followed by the reverse complement of the target mutation site, and then an additional 20bp upstream of the target site. The 3' end of each primer is designed to anneal to the reverse complement sequences downstream of the *AphA1* conferring positive selection marker Kanamycin (Kan) encoded by pEPkans-S plasmid. Using these primers to amplify the *AphA1* gene located on pEPkan-S produces a PCR product that contains the desired mutation and a Kanamycin resistant cassette (Figure 2.1: B). Primer sequences used in this study are given in Table 2.1.

**Construction of HSV-1 Mutants ΔgD(US6), ΔgE(US8), ΔUL20, ΔgE+gDΔcp, and ΔgE/ΔgM**

Mutagenesis was accomplished in *Escherichia coli* using the markerless two-step RED recombination mutagenesis system (Tischer, von Einem et al. 2006) (Figure 2.1). ΔgE recombinant virus was constructed by changing the initiation codon from ATG to CTG (Table 1). gDΔcp was a truncation mutant that deleted the last 87bp (29 amino acids) of the cytoplasmic tail region (based on SOSUI and DAS secondary transmembrane predictions), but left the natural stop codon and immediate downstream sequences still intact. ΔUL20 altered two possible initiation codon sites (from ATG to CTG) located 6bp apart in open reading frame (ORF) UL20. ΔgE recombinant virus was used as backbone for construction of the double mutant ΔgE+ΔgM by silencing the initiation codon for glycoprotein M (via ATG to CTG) as well as to create ΔgE+gDΔcp by introducing gD-specific deletions. Maintenance and mutagenesis of the constructs were performed in E. coli strain EL250 which contains a λ prophage encoding recombination enzymes Exo, Beta, and Gam under a heat inducible promoter (Lee, Yu et al. 2001). EL250 cells were made electrocompetent

**Table 2.1 Synthetic Oligonucleotide Primers for Red Recombination**

| Primer | Name | Primer Sequence | Purpose |
|---|---|---|---|
| *I* | 5'gE-EnPas | GTTGGGCTCCCATTTTACCCGAAGATCGGCTGCTATCCCCGGGACCTGGATCGC<br>GGGGCGGTGGTGGGGTTTCTT***AGGATGACGACGATAAGTAGGG*** | **gE null**<br>(start codon point mutation) |
| *II* | 3'gE-EnPas | AACACAAACACCGAGAAGAAACCCCACCACCGCCCCGCGATCCAGGTCCCGG<br>GGATAGCAGCCGATCTTCGGGTA***CAACCAATTAACCAATTCTGATTAG*** | |
| *III* | 5'gD-366Del | GGCAGCCCTGGTCATTTGCGGAATTGTGTACTGGATGCGC_TAGATACCCCCCCT<br>TAATGG***AGGATGACGACGATAAGTAGGG*** | **gD truncation**<br>(Deletion from amino acid 366, stop codon intact) |
| *IV* | 3'gD-366Del | CAGACCTGACCCCCCCCGCACCCATTAAGGGGGGGTATCTA_GCGCATCCAGTAC<br>ACAATTC ***CAACCAATTAACCAATTCTGATTAG*** | |
| *V* | 5'gM-EnPas | GATACGCTCGACGTGTACTGTTCGCACTCGTCGTCCCCACTGGGACGCCCGGCC<br>CCCAG***AGGATGACGACGATAAGTAGGG*** | **gM null**<br>(start codon point mutation) |
| *VI* | 3'gM-EnPas | GCGCGGAGTCGGGAGATCCTCTGGGGGCCGGGCGTCCCAGTGGGGACGACGA<br>GTGCGAAC***CAACCAATTAACCAATTCTGATTAG*** | |
| VII | 5'UL20-EnPas | GCGACCCTTTGCGGTTTCGGTCTCCCCACCTCCACCGCACACCCCCTGACCCT<br>GCGGGATGACCTTCCTCTGGTGGATCGA***AGGATGACGACGATAAGTAGGG*** | **UL20 null**<br>(start codon & downstream site point mutation) |
| VIII | 3'UL20- EnPas | CTCGTCGACCAGATCTCGATCCACCAGAGGAAGGTCATCCCGCAGGGTCAGGG<br>GGTGTGCGGTGGAGGTGGGGAGACCGAA***CAACCAATTAACCAATTCTGATTAG*** | |

***** For Red recombination primers the region homologous to HSV-1 DNA is in upper case, the underlined sequence denotes site of mutation, the underscore represents deletion, and the region which binds to the marker gene is in bold italics.

and were transformed with HSV-bac containing the entire HSV genome (Fulmer, Melancon et al. 2007) to produce EL250-HSV-bac. Electrocompetent EL250-HSV-bac was then transformed with the mutant-Kan PCR product (Figure 2.1: B) and pBAD-*I-SceI* plasmid, containing an arabinose inducible *I-SceI* self-homing endonuclease. The transformation was spread on plates containing chloramphenicol (12.5 µg/ml), ampicillin (100 µg/ml), and kanamycin (50 µg/ml), derived from bac, pBAD, and pEP-KanS respectively. Colonies were screened for positive integration of the correct mutant+Kan PCR product (Figure 2.1: C) using PCR test primers designed to lie outside of the target mutation sites (not shown). Clones that were confirmed to have the correct integration were used for resolution step by growing (from a 1:1000 dilution) for 2 hours in LB media without kanamycin (chloramphenicol$^{+}$ and ampicillin$^{+}$) at 32°C/225rpm, followed by the addition of arabinose (1% final concentration), and an additional hour of incubation. Cultures were then placed in a 42°C water bath for 30 minutes followed by a final 1 hour of growth at 32°C. Aliquots of resolved culture were plated on chloramphenicol and ampicillin plates containing 1% arabinose. After 24 hours, colonies were screened by replica plating for the loss of kanamycin resistance (Figure 2.1: E). DNA from kanamycin sensitive colonies were prepared and sent for sequencing to confirm the presence of the desired mutation.

**Confirmation of the Targeted Mutations**

HSV-1 BAC DNAs (plasmid gDΔcp, pΔgE, pΔUL20, pΔgE/gDΔcp, pΔgE/ΔgM) were purified from 50 ml of BAC cultures with the Qiagen large-construct kit (Qiagen; Valencia, CA). Using PCR test primers designed to lie outside of the target mutation site(s), all mutation regions were sequenced to verify the presence of the desired

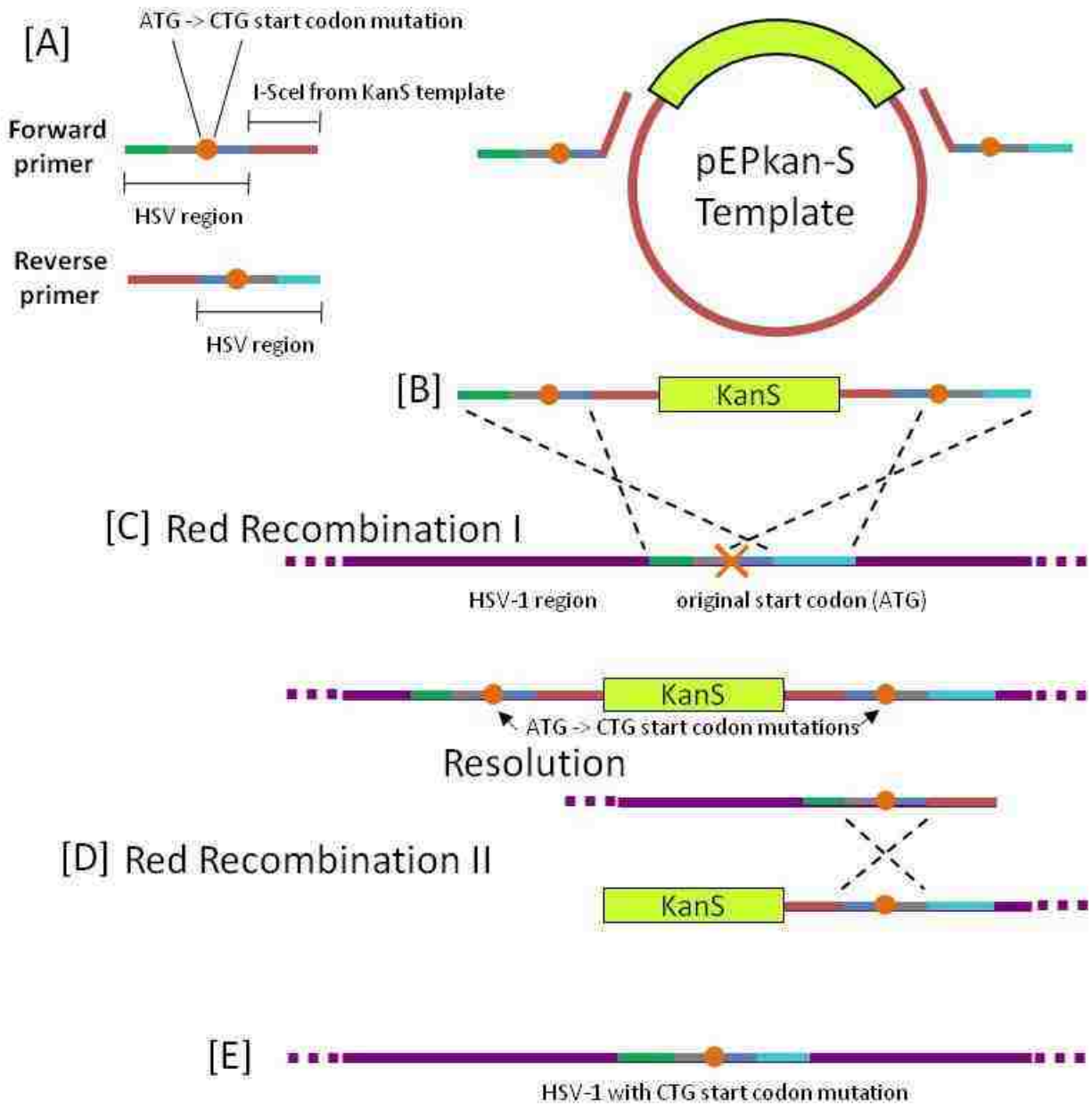


**Figure 2.1: Two-step Red Recombination Mutagenesis System.** [A] are the forward and reverse primers designed to carry the desired mutation (CTG) and homologous sequences upstream and downstream of the gene of interest. Sequences that are homologous to the pEPkan-S template at the 3' end of each primer allows them to anneal. [B] PCR fragment that carries the KanS resistant cassette flanked by two identical mutations. [C] First red recombination integrates the PCR product into targeted HSV-1 region. [D] The second homologous recombination event is a resolution step preceded by the self cleavage induced by the *I-SceI* endonuclease. [E] HSV-1 with CTG start codon mutation.

mutations in bacs. To confirm that no spurious mutations were introduced into the target genes of interest during mutagenesis or during the transfection procedure, cells from virus infected culture for each mutation were collected and DNA was isolated to be checked by sequencing. Each mutant confirmed the correct alterations.

**Transfection of HSV-1 bac DNAs**

Vero cells were grown to 95% confluency in twelve-well plates. Cells were transfected with BAC DNA mixed with Lipofectamine 2000 in Opti-MEM medium as recommended by the manufacturer (Invitrogen). After 6 h of incubation at 37°C, the medium was removed from the transfected cells, and the transfected cells were washed with phosphate buffered saline (PBS) before fresh Dulbecco's modified Eagle's medium with 10% fetal calf serum was added. At 42-72 h post transfection, virus stocks were collected and designated as passage 0 ($P_0$). Passage $P_1$ viruses were used for all experiment described in this manuscript.

**Plaque Morphology of Mutants**

Analysis of plaque morphology of mutant viruses was performed as previously described by our lab (Melancon, Foster et al. 2004; Melancon, Luna et al. 2005; Fulmer, Melancon et al. 2007). Briefly, near confluent Vero cell monolayers in 6 well plates were infected with the indicated virus at an MOI of 0.5. After 1hr at room temperature, media was discarded and cells were washed with PBS before adding methylcellulose. 48 hours post infection (hpi) at 37°C /5% $CO_2$, cells were washed several times with PBS to remove methylcellulose media, and fixed with ice cold methanol for 15 minutes. PBS containing a 1:500 dilution of the polyclonal HRP conjugated anti-HSV-1 antibody (Dako

Cytomation) was added to the cells and placed on a rocker at room temperature for 1 h. Virus plaques were visualized using the VECTOR NovaRED peroxidase substrate kit as directed by the manufacturer (VECTOR, Inc). Plaques were imaged using the Leica DMIRB inverted wide-angle microscope.

**One-step Growth Kinetics**

Analysis of one-step growth kinetics was as described previously by our lab (Foster, Rybachuk et al. 2001 2001; Foster, Alvarez et al. 2003 2003). Briefly, each virus at an MOI of 0.5 was adsorbed in one well of near confluent six-well plate of Vero cells at 4°C for 1 h. Thereafter, plates were incubated at 37°C/5% $CO_2$ and virus was allowed to penetrate for 1 h at 37°C. Any remaining extra-cellular virus was inactivated by low-pH treatment (pH 3.0), and cells were incubated at 37°C/5% $CO_2$. The virus infected cells were taken out of incubation and put into -80°C immediately (0 h) and 6,12,18,24 hours thereafter. Virus stocks were made by two freeze-thaw cycles of the samples followed by collection of supernatants from centrifugation. Virus titers were determined by endpoint titration of virus stocks on Vero cells.

**Electron Microscopy**

Cell monolayers were infected with the indicated virus at an MOI of 5. All cells were prepared for transmission electron microscopy (TEM) examination at 18 h post infection. Infected cells were fixed in a mixture of 2% paraformaldehyde and 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3. Following treatment with 1% OsO4 and dehydration in an ethanol series, the samples were embedded in Epon-Araldyte resin and polymerized at 70°C. Thin sections were made on an MTXL ultratome (RMC Products), stained with 5% uranyl acetate and citrate/nitrate/acetate

lead, and observed with a Zeiss 10 transmission electron microscope as described previously by our lab (Foster, Melancon et al. 2004; Foster, Melancon et al. 2004a 2004b).

## RESULTS

### Construction of Recombinant Viruses Carrying One or More Mutations

The complete HSV-1(F) genome has been cloned into a bacterial artificial chromosome (bac) enabling the rapid and efficient genetic manipulation of the HSV-1 genome in *E. coli*. Site-directed mutagenesis of gene targets was accomplished using the double-red site-directed mutagenesis methodology implemented on to the HSV-1(F) genome cloned into a bacterial artificial chromosome (bac). Specifically, initiation codons for gE and gM were altered using specific oligonucleotide primers (Table 2.1) to silence their synthesis (see Materials and Methods). In addition, a recombinant virus was made to silence UL20 gene expression. This UL20-null virus was constructed by converting two initiation codons (from ATG to CTG) six base pairs apart that may be used to synthesize UL20. None of these mutations affected any of the adjacent or overlapping genes (Fig. 2.2).

Glycoprotein D (gD) is essential for HSV-1 virus entry and spread (Feenstra, Hodaie et al. 1990). However, extracellular domains of gD are necessary and sufficient for gD-mediated virus entry. Therefore, a recombinant virus was constructed to code for gD lacking its predicted carboxyl cytoplasmic terminus (Figure 2.2). Different prediction algorithms show the cytoplasmic terminus to be approximately 30 amino acids in length (see Materials and Methods). To delete the gD cytoplasmic terminus, while ensuring that

the intramembrane sequence remains intact, we chose to delete a 87 bp DNA fragment coding for 29 amino acids of the carboxyl terminus of gD (Fig. 2.3).

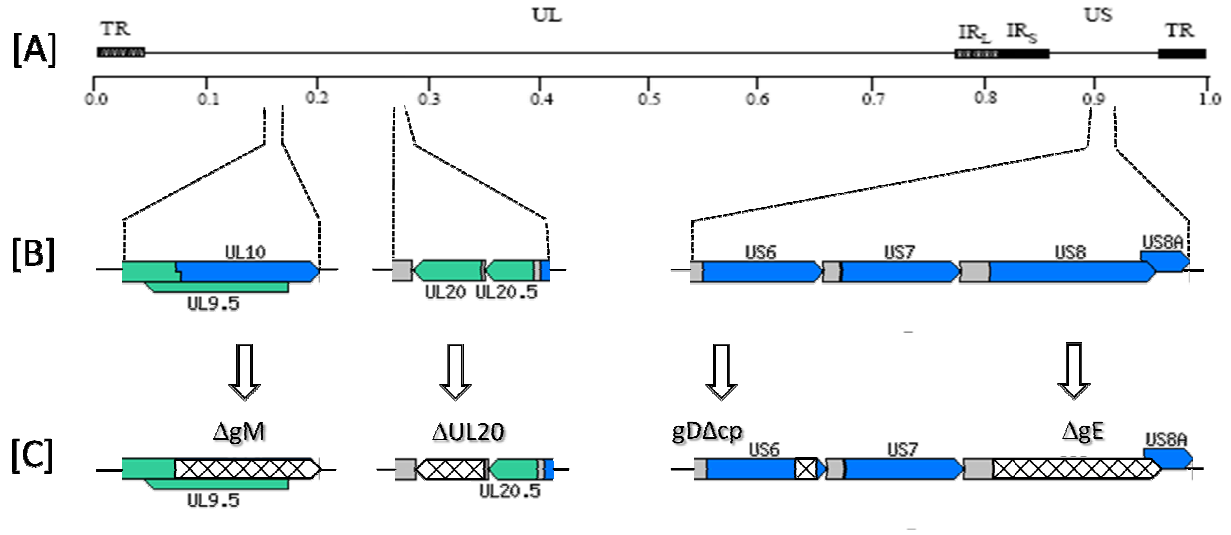


**Figure 2.2: Schematic of the Strategy for the Construction of Mutant bacs.** [A] The top line represents the prototypic arrangement of the HSV-1 genome with the unique long (UL) and unique short (US) regions flanked by the terminal repeat (TR) and internal repeat (IR) regions. [B] Shown below are the expanded genomic regions of the UL10, UL20, US6, and US8 ORFs. [C] The effect of mutations (ΔgM, ΔUL20, gDΔcp, ΔgE) to the same regions shown above. The white regions represent silenced regions.

**Molecular Analyses of the Constructed Recombinant Viruses**

The bac-based genomic constructs were tested for the presence of the intended mutations via diagnostic PCR. Test primers located outside and bracketing the appropriate genes were used to amplify the targeted DNA fragments. Diagnostic PCR against the integrates of ΔgE, gDΔcp, ΔgE+ΔgM, ΔUL20, ΔgE+gDΔcp bac DNAs using appropriate test primers produced PCR-amplified DNA fragments greater than 1kb, as predicted due to the insertion of the kanamycin gene cassette (Figure 2.4: A). Once

confirmed for the positive integration, the constructs were resolved and amplified with the same appropriate test primers via PCR. This generated DNA fragments less than 1kb, as the constructs had lost their kanamycin resistance in the second RED recombination event (Figure 2.4: B).

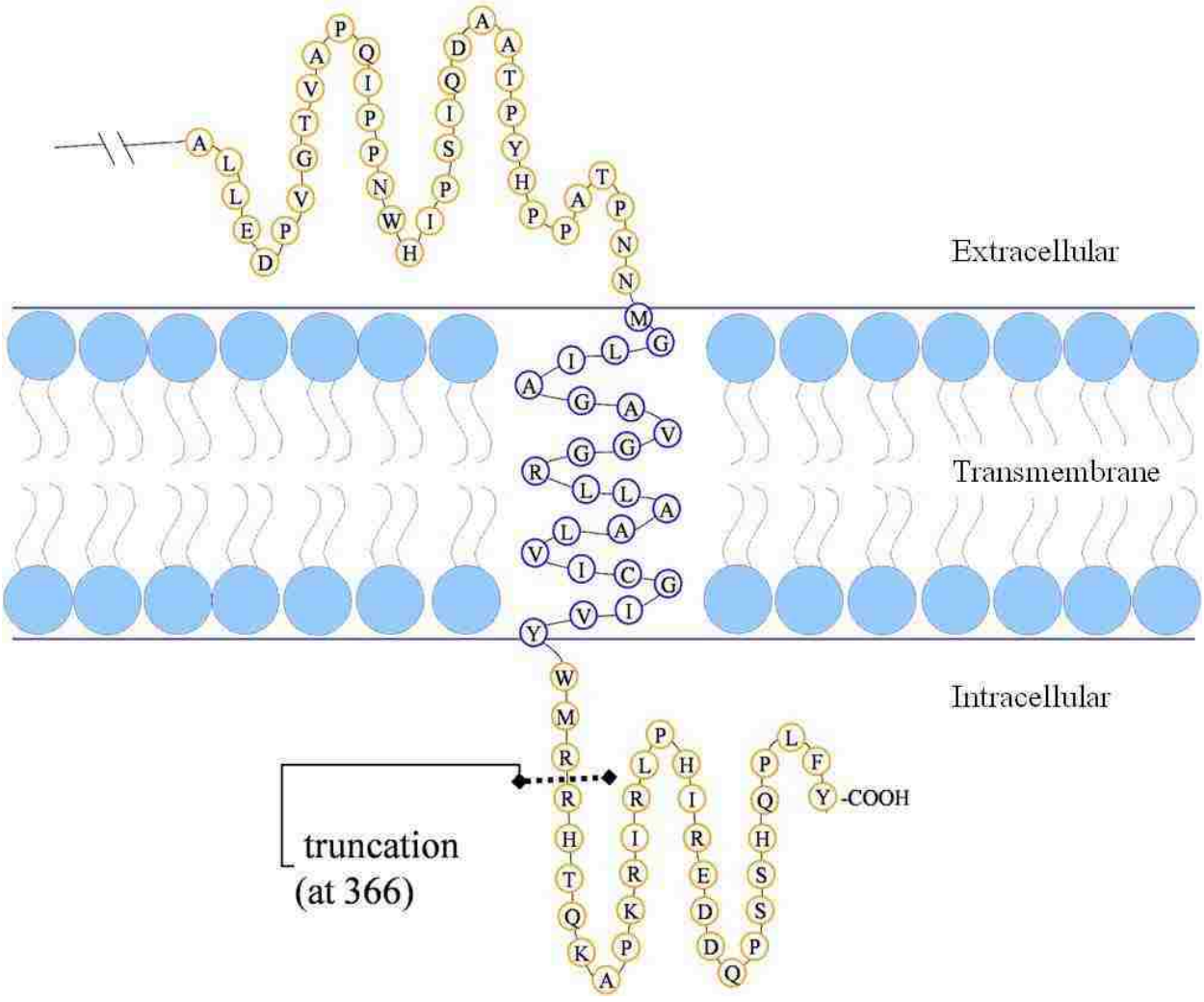


**Figure 2.3: Schematic Diagram of the Carboxyl Terminus of HSV-1 gD.** The predicted secondary structure of the gD carboxyl terminal is shown. The blue circles represent the amino acids(aa) in the transmembrane region. In ΔgD mutants, the cytoplasmic tail was truncated at 366-aa, effectively deleting 29-aa (87bp) of ORF US6.

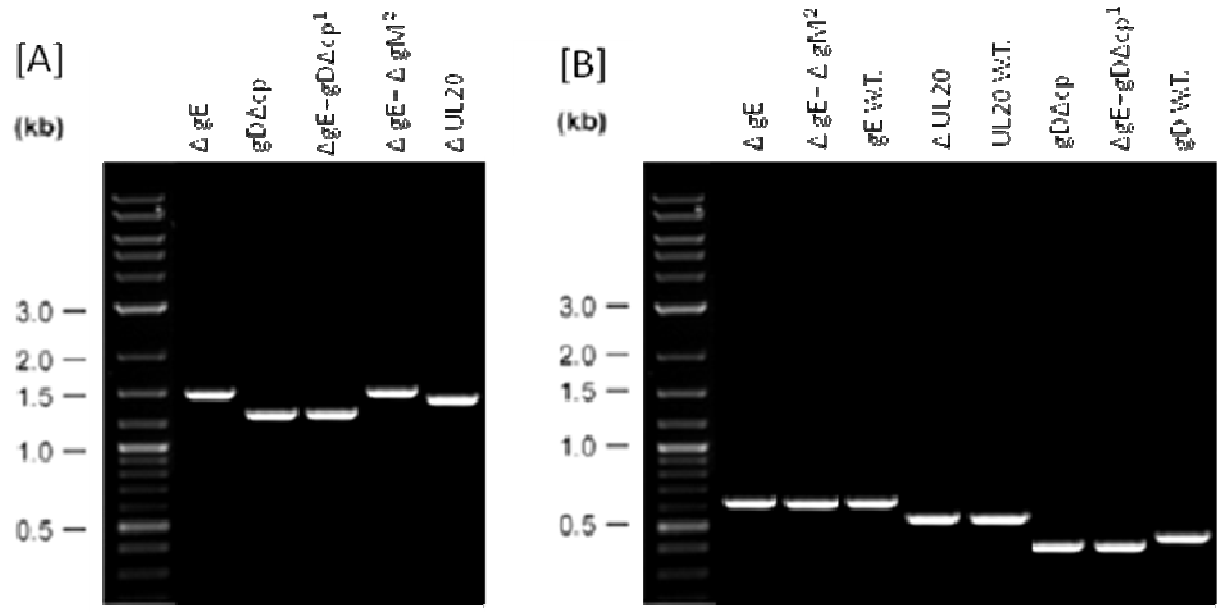


**Figure 2.4: Genomic Analysis of bac Mutants. [**A] Diagnostic PCR to confirm the insertion of the relevant positive selection marker Kanamycin gene cassette. Greater than 1kb band size indicates successful integration of fragment carrying mutant sequences. [B] Diagnostic PCR to confirm the resolution of the Kanamycin gene cassette. Bands less than 1kb indicate the loss of the integrate in mutant bac; mutagenesis system leaves no genetic footprints altering sequences effectively. [1] denotes that gD test primers were used and [2] denotes that gM test primers were used.

**Recovery and Characterization of Infectious Virus from HSV-1 bac DNA**

To generate virus stocks from the mutant bac genomic constructs, transient transfections of individual bac DNAs were performed into Vero cells producing virus stocks designated as passage 0 ($P_0$). Working virus stocks were generated by infecting fresh Vero cells ($P_1$), and the presence of individual nucleotide substitutions or deletions were confirmed by PCR-assisted DNA sequencing of the mutated genomic regions as well as for possible spurious mutations within UL20, gK, gD, gE or gM (not shown).

**Plaque Morphology of HSV-1 Mutants**

The plaque morphologies of the gDΔcp, ΔgE, ΔUL20, ΔgE+ΔgM, ΔgE+gDΔcp mutant viruses were examined in Vero cells (Figure 2.5). As expected, the HSV-1(F) wild-type virus produced large plaques (Fig 2.5). The ΔgE and gDΔcp mutant viruses formed plaques that were similar in size and on average approximately 50% smaller than the wild-type virus plaques (Figure 2.5). The double-mutant virus ΔgE+ΔgM formed plaques that were similar to those produced by the ΔgE virus (Figure 2.5). The ΔgE+gDΔcp double-mutant virus formed plaques, which were on average 50% smaller than either single mutants, ΔgE or gDΔcp (Figure 2.5). The ΔUL20 mutant virus produced the smallest plaques of all mutants composed of 3-5 cells per plaque as it has been described previously for a UL20-null mutant virus in which most of the UL20 gene was deleted (Foster, Melancon et al. 2004) (Figure 2.5).

**Replication Kinetics of HSV-1 Mutants**

To examine the effect of the various mutations on virus replication, Vero cells were infected at an MOI of 0.5 with either the wild-type or each mutant virus and one-step growth kinetics were determined as described in the Materials and Methods. All mutant viruses appeared to efficiently replicate approaching similar titers at 24 hpi to those produced by the gE+ (rescued) virus (Fig. 2.6) and the wild-type virus (not shown), with the exception of the ΔgE+ΔgM virus that appeared to replicate up to five-fold more efficiently than all other viruses. Both ΔgD and ΔgE+gDΔcp double-mutant viruses appeared to initially replicate substantially slower than the other mutant viruses and the wild-type virus (Fig. 2.6)

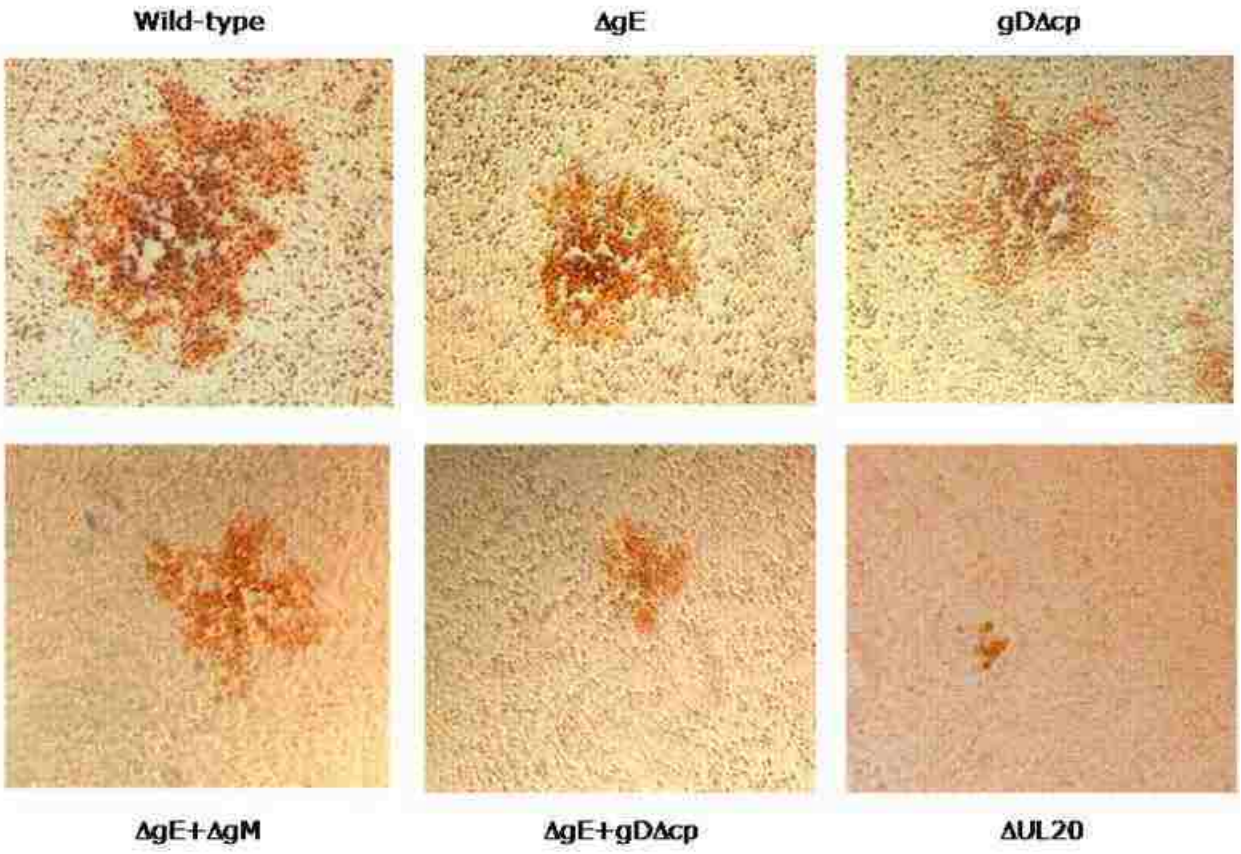


**Figure 2.5: Plaque Phenotypes of Wild-type, ΔgE, gDΔcp, ΔgE+ΔgM, ΔgE+gDΔcp, ΔUL20.** Plaque phenotypes of wild-type and mutant viruses were observed on Vero. Confluent cell monolayers were infected with the viruses at an MOI of 0.5, and viral plaques were visualized at 48 hpi by immunohistochemistry.

### Ultrastructural Characterization of the gDΔcp, ΔgE, ΔgE+gDΔcp, and ΔUL20 mutant viruses

The ultrastructural phenotypes of the gDΔcp, ΔgE, ΔgE+gDΔcp, and ΔUL20 viruses relative to the wild-type parental virus were investigated utilizing transmission electron microscopy at 18 hpi. As expected, the wild-type virus exhibited no apparent defects in virion egress as exemplified by the presence of fully enveloped virions extracellularly (Figure 2.7), as well as the presence of fully enveloped virions intracellularly as reported previously (Melancon, Foster et al. 2004).

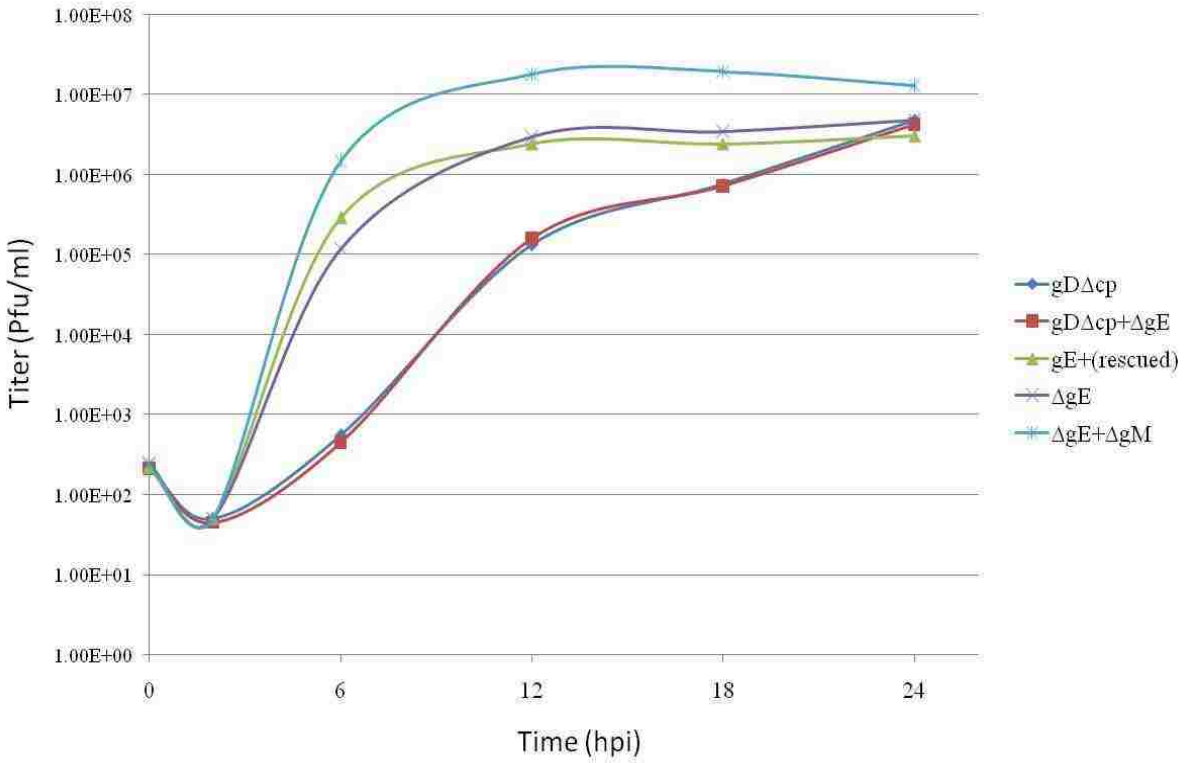


**Figure 2.6: Viral Replication Kinetics.** Comparison of virus replication characteristics of bac mutants. One step kinetics of infectious virus production were calculated after infection at an MOI of 5 followed by incubation at 37°C.

The ultrastructural visualization of Vero cells infected with the different mutant viruses revealed a diverse range of cytoplasmic defects in virion envelopment. The most pronounced effects were produced by the ΔUL20 mutant, which produced unenveloped capsids in the cytoplasm embedded within morphologically darker stained areas that may be caused by the accumulation of tegument proteins (Figure 2.7). In contrast, the ΔgE, gDΔcp, and ΔgE+gDΔcp ultrastructural morphologies were similar to each other in that most of the virions appeared to produce fully

enveloped virions in extracellular spaces, while very few viral capsids or enveloped virions were found in the cytoplasm of infected cells. All virus-infected cells appeared to contain arrays of capsids within the nuclei of infected cells (Figure 2.7).

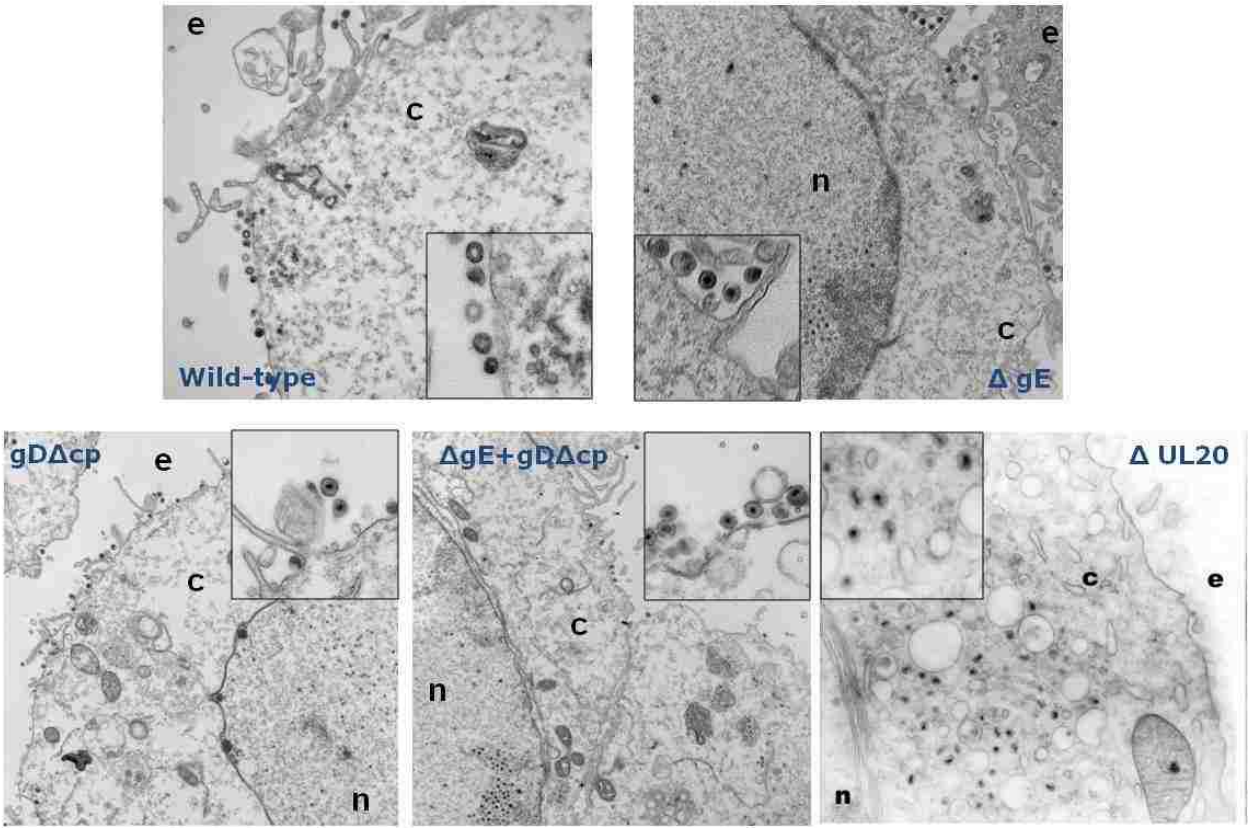


**Figure 2.7: Ultrastructural Morphology of Mutant Viruses.** Electron micrographs of Vero cells infected with bac mutant viruses ΔgE, gDΔcp, ΔgE+gDΔcp, ΔUL20, and wild-type. Confluent cell monolayers were infected at an MOI of 5, incubated at 37°C for 18 hours, and prepared for transmission electron microscopy. Nuclear (n), cytoplasmic (c) and extracellular (e) spaces are marked.

## DISCUSSION

HSV-1 cytoplasmic virion envelopment is thought to be mediated by complex interactions among tegument proteins and the cytoplasmic portions of viral

glycoproteins and membrane proteins embedded in TGN membranes (Miranda-Saksena *et al.,* 2002; Skepper *et al.,* 2001; Whiteley *et al.,* 1999). Consequently, a number of viral glycoproteins have been shown to either interact with tegument proteins or otherwise affect cytoplasmic envelopment (Fuchs *et al.,* 2002; Loomis, 2003; O'Regan *et al.,* 2006; Vitonne *et al.,* 2005). Similarly, mutations within certain tegument proteins are known to inhibit cytoplasmic virion envelopment. Glycoproteins gD and gE (Farnsworth, Goldsmith et al. 2003) and the gK/UL20p heterodimer (Jayachandra, Baghian et al. 1997; Melancon, Foster et al. 2004) have been reported to play essential roles in cytoplasmic virion envelopment. The main purpose of the work presented herein was to compare the relative importance of these glycoproteins in cytoplasmic virion envelopment. It was found that a recombinant virus that carried both the deletion of the carboxyl terminus of gD (gDΔcp) and a deletion of the entire gE gene had no appreciable defect in cytoplasmic virion envelopment, and the double gene mutant accumulated enveloped virions in extracellular spaces with similar efficiencies to either ΔgE or gDΔcp viruses. In contrast, ΔUL20 viruses constructed using similar methods (see Materials and Methods) appeared to have drastic defects in cytoplasmic envelopment as reported previously (Foster, Melancon et al. 2004; Melancon, Foster et al. 2004). It was concluded that the gK/UL20 protein complex serves unique and preeminent functions in cytoplasmic virion envelopment, while other glycoproteins such as gD and gE are of secondary importance in cytoplasmic virion envelopment.

Over the years, a number of mutant viruses carrying deletions or other mutations within viral genes have been constructed and characterized. These mutant viruses have been constructed utilizing different methodologies including insertional inactivation with

dominant selectable markers, partial gene deletions, etc. Furthermore, viruses have been constructed utilizing different strains of viruses, complicating the interpretation of relative importance of affected viral proteins in the virus lifecycle. The availability of the HSV-1(F) genome cloned into a bac vector enables the rapid construction of recombinant viruses in the same genetic background, thus allowing direct comparison of the different mutational effects during specific stages of the virus lifecycle.

We have shown previously that the UL20 protein (UL20p) physically interacts with gK and that this interaction is absolutely essential for UL20p/gK functions in virus-induced cell fusion and cytoplasmic virion envelopment (Foster, Melancon et al. 2004; Melancon, Foster et al. 2004). Specifically to cytoplasmic virion morphogenesis, deletion of gK or UL20, as well as specific mutations within either gene, practically abrogates all cytoplasmic virion envelopment (Melancon, Foster et al. 2004). A separate study reporting that gD+gE/gI deletion mutants accumulating unenveloped capsids in the nucleus (Farnsworth, Goldsmith et al. 2003) identical to that of the cytoplasmic defects shown by our lab, it was thought that gK/UL20p and gD+gE/gI were equally important components for cytoplasmic virion envelopment. Since no substantial defects were seen for the single deletions of gD and gE, it was implied that gD and gE/gI act in essential but redundant fashion for cytoplasmic virion envelopment (Farnsworth, Goldsmith et al. 2003; Farnsworth 2007). Much of the mechanism of cytoplasmic virion envelopment is unclear. However, there has been no previous record or any indication to date suggesting the aforementioned relationship between gK/UL20 and gD+gE/gI. Therefore, it was to our belief that an alternate explanation was more plausible. To resolve this issue, we constructed appropriate sets of mutant viruses in the HSV-1(F)

genetic background to compare the relative importance of gD, gE, and gK/UL20 in cytoplasmic virion egress.

It has been shown that only the gD extracellular domain, but neither its intramembrane nor its intracellular domains, is essential in virus infectivity and spread (Feenstra, Hodaie et al. 1990). In addition, it is the carboxyl terminus of gD that function in cytoplasmic virion envelopment to interact with capsid associated tegument proteins (Johnson, Wittels et al. 1984; Farnsworth 2007) Therefore, the gD recombinant virus gDΔcp was constructed having the cytoplasmic terminus of gD deleted. The rationale behind this mutant virus construction was to preserve the gD receptor domains pertaining to virus-to-cell binding functions, while severing any chance that the carboxyl terminus of gD could bind tegument proteins during cytoplasmic virion envelopment. This gD gene cytoplasmic tail deletion was accomplished on the HSV-1(F) bac genome without the insertion of any potentially interfering foreign DNA sequences. As expected, the virus was able to replicate with relative efficiency (Feenstra, Hodaie et al. 1990). In addition, the gDΔcp infection produced smaller viral plaques in Vero cells, most likely because the deletion mildly affected viral infectivity, as well as virus spread by fusion among adjacent cells. Similar results were obtained with the ΔgE virus lacking the entire gE gene, explained by the understanding that gE is known to affect virus-cell-to-cell spread (Johnson and Feenstra 1987; Dingwell, Brunetti et al. 1994; Dingwell, Doering et al. 1995; Dingwell and Johnson 1998).

Ultrastructurally, both gDΔcp and ΔgE viruses exhibited no appreciable defects in cytoplasmic virion envelopment as evidenced by the presence of fully enveloped virions in extracellular spaces and the lack of non-enveloped capsids in cytoplasms of infected

cells. Surprisingly, the double-mutant virus ΔgE+gDΔcp exhibited no appreciable defect in cytoplasmic virion envelopment, while it produced viral plaques that were on average 50% smaller than either the ΔgE or gDΔCp viruses. In accordance with the known effects of gD in viral infectivity and spread, as well as the known role of gE in virus cell-to-cell spread (Campadelli-Fiume 2006), it is probable that the smaller plaques produced by ΔgE+gDΔcp virus infection was actually an additive effect on virus spread due to the individual gDΔcp and ΔgE mutations.

Previous work has demonstrated that gD and gE function in a redundant manner in cytoplasmic virion egress (Farnsworth, Goldsmith et al. 2003; Farnsworth 2007). Specifically, an earlier report showed that simultaneous deletion of both gD and gE/gI genes caused drastic accumulation of unenveloped capsids into the cytoplasm of infected cells (Farnsworth, Goldsmith et al. 2003). The recombinant virus that carried the gD and gE/gI gene deletions used in these studies was produced by insertional inactivation of gD using a kanamycin gene cassette inserted downstream of the gD promoter, while the gE gene was inactivated by insertion of a GFP gene cassette replacing 705bp (of the total 1653bp) of the amino terminal coding sequence of gE (Farnsworth, Goldsmith et al. 2003). To complicate matters, this virus expressed low levels of gD. And along with the expression of the inserted kanamycin and the EGFP proteins, it is possible that the observed virion morphogenesis defects may have been due to the ancillary toxic effects of the kanamycin and EGFP proteins that amplified the potential effect of gD and gE deletions in cytoplasmic virion envelopment. In a subsequent report, a similar deletion of the gD gene by insertion of the kanamycin gene cassette within the HSV-1(F) bac genome appeared to produce drastic reduction in

cytoplasmic virion envelopment in the absence of gE. Furthermore, studies using gE mutants with progressive deletions of its carboxyl terminus showed that the terminal 50 amino acids of gE were dispensable for cytoplasmic morphogenesis in the absence of gD, while conversely, gE amino acids beyond the carboxyl terminal 50 amino acids appeared to be essential for cytoplasmic virion envelopment in the absence of gD (Farnsworth 2007).

One possible explanation for these disparate findings is that that the extracellular domain of gD is indirectly involved in cytoplasmic virion envelopment. Recently, it was found that both gH and gB physically interact with gD (Avitabile, Forghieri et al. 2007 ), paving the way for defining a well-coordinated "fusion machine" that involves complex interactions among gD, gB and gH glycoproteins, and possibly other viral proteins and glycoproteins for virus entry and virus-induced cell fusion. Therefore, the absence of gD may cause aberrant gB and gH conformations that negatively impact cytoplasmic virion envelopment. This scenario seems more likely, especially since UL20 physically interacts with gB and gH (unpublished data from our lab) However, neither gB or gH are known as important determinants in cytoplasmic virion egress, casting some doubt whether these changes would have any relevant effects. Given that the gK/UL20p protein complex is unequivocally important in cytoplasmic virion envelopment, it is also possible that the absence of the extracellular portion of gD may affect gK/UL20p functions involved in cytoplasmic virion envelopment by either affecting their transport to TGN membranes or altering their conformations.

In summary, it is clear that the carboxyl terminus of gD does not function in a redundant manner with gE in cytoplasmic virion egress, although both gD and gE

appear to function in virus spread. Direct comparison of gDΔcp+ΔgE with ΔUL20-null mutant viruses reveal the UL20p/gK protein complex as the most important determinant of cytoplasmic virion egress. Despite shedding some light to the complex mechanism cytoplasmic virion egress, additional experiments are required to elucidate the interactions between viral membrane proteins and glycoproteins as well as their interaction with viral tegument proteins.

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# CHAPTER THREE

# CONCLUDING REMARKS

## SUMMARY

Herpes simplex virus type-1 (HSV-1) morphogenesis is a multi-step process that promotes virus assembly in a sequential and ordered manner. The virion capsid assembles within the nucleus and newly synthesized viral DNA is encased within. The virion acquires a primary envelope as it buds through the inner nuclear membrane and into the perinuclear space (Roizman and Sears 2001). The stay in the perinuclear space is only temporary, and the virion becomes de-enveloped as it buds into the outer nuclear membrane and into the cytoplasm. The unenveloped capsids are joined by additional associated tegument proteins, and together they bud into cytoplasmic vesicles derived from the trans-Golgi network (TGN). This step provides the virion with a secondary envelope that contains the complete repertoire of glycoproteins found in the mature virus. Enveloped virions are ultimately secreted to extracellular spaces through the utilization of cellular vesicular trafficking systems (Zhu, Gershon et al. 1995; Browne, Bell et al. 1996; Granzow, Klupp et al. 2001; Harley, Dasgupta et al. 2001 2001; Skepper, Whiteley et al. 2001; Mettenleiter 2002).

The envelopment, de-envelopment model of virion egress requires specific interaction between tegumented cytoplasmic capsids and the glycoproteins embedded within the TGN membranes. This model is supported by the inhibitory functions that specific mutations within tegument proteins, multiple membrane proteins, and glycoproteins have in cytoplasmic envelopment (Mettenleiter 2004; Mettenleiter, Klupp et al. 2006). To further investigate the roles of glycoproteins in cytoplasmic virion envelopment, we constructed mutants of gD (US6), gE (US8),

gE+ gM(UL10), gE+gD, and UL20. These proteins were selected specifically because they were the ones known to be associated with cytoplasmic virion envelopment.

In HSV-1, the gK/UL20 deletion mutant constructed from our laboratory accumulated large numbers of unenveloped nucleocapsids in the cytoplasm (Melancon, Foster et al. 2004). gD and gE/gI triple-null mutants also accumulated unenveloped virions in the cytoplasm (Farnsworth, Goldsmith et al. 2003) strikingly similar to the ultrastructural phenotype shown by the gK/UL20 deletion mutant. Given that similar defects were not observed for the gD and gE single glycoprotein deletions, Farnsworth et al. suggested that gD and gE/gI serve essential but redundant functions in cytoplasmic envelopment (Farnsworth, Goldsmith et al. 2003). In a similar manner, gM and gE/gI were shown to be important for PRV cytoplasmic virion envelopment (Brack, Dijkstra et al. 1999), but not for HSV-1 (Browne, Bell et al. 2004). Because the HSV-1 mutant viruses were constructed by different methodologies and on different genetic backgrounds, we considered important to generate a new set of mutant viruses with glycoprotein deletions in the same genetic background and with newer mutagenesis protocols to validate previous results obtained by others.

To assess the roles of the aforementioned membrane protein and glycoproteins in the context of cytoplasmic virion morphogenesis and egress, the gDΔcp, ΔgE, ΔgE+ ΔgM, ΔgE+gDΔcp, and ΔUL20 viruses were constructed using a recently described RED markerless recombination system (Tischer, von Einem et al. 2006). This modified method of RED recombination allowed us to silence targeted gene

expression without inserting foreign DNA sequences, leaving manipulation footprints, or disrupting neighboring/overlapping genes.

Our results confirm prior findings that the UL20 membrane protein is essential for cytoplasmic virion morphogenesis, since mutant viruses lacking UL20p accumulate capsids within the cytoplasm that are unable to acquire envelopes by budding into TGN-associated membranes (Fuchs, Klupp et al. 1997; Dietz, Klupp et al. 2000; Foster, Alvarez et al. 2003 2003; Foster, Melancon et al. 2004; Melancon, Foster et al. 2004 2004). In contrast, the deletion of both the cytoplasmic tail of gD and the complete deletion of non-essential gE did not prevent virus egress. Therefore, the carboxyl terminus of gD does not function in a redundant manner with gE in cytoplasmic virion egress in contrast to previous suggestions (Farnsworth, Goldsmith et al. 2003; Farnsworth 2007). Direct comparison of ΔgE+gDΔcp with ΔUL20-null mutant viruses has made clear that the UL20p/gK protein complex is the most important determinant of cytoplasmic virion egress (Figure 3.1).

In conclusion, this thesis has made contributions to understanding the complex mechanism of cytoplasmic virion egress. Furthermore, it has shown that current bac mutagenesis protocols can be used to efficiently and effectively manipulate any gene of interest in the HSV-1 genome. The later will be sure to facilitate future research concerning cytoplasmic envelopment and egress, and also be instrumental in demystifying other unclear mechanisms of the virus lifecycle.

## CURRENT AND FUTURE PROSPECTS

Additional experiments can be done to support the conclusions derived from Chapter II. It is impossible to completely rule out the chance of spurious mutations being

introduced into the viral genome of the individual bac mutants during mutagenesis or during the transfection procedure unless the entire genome of each HSV-1 mutant is sequenced and compared to the parental genome. However, the same conclusion can be deduced via an aggregate of less laborious methods. On the DNA level, PCR analysis testing for relevant glycoproteins and membrane protein (gK, gM, gE, gD,and UL20) have been done and the DNA fragments have been sequenced. Protein work

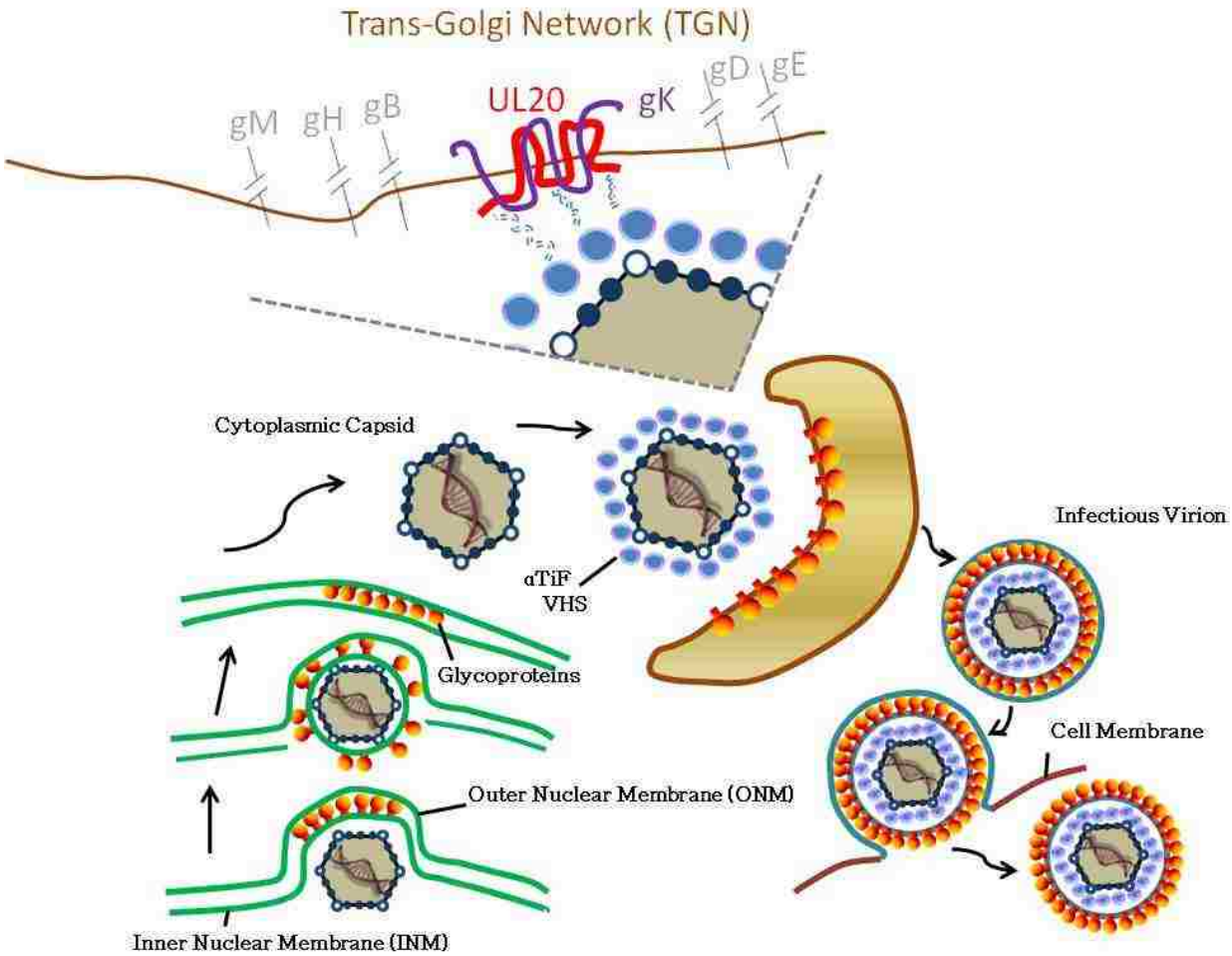


**Figure 3.1: Schematic of Cytoplasmic Envelopment.** A representation of interaction between HSV-1 gK/UL20 with tegumented virus capsids in the cytoplasm at the TGN junction.

using antibodies specific to gD, gE, gM, and gK/UL20 in HSV-1 can also be used for Western blot analysis to verify the proper expression/lack of expression of mutant genes. Additionally, the mutant viruses can be grown in complementing cells that constitutively express the appropriate glycoprotein or membrane protein. Successful complementation on cells lines will provide strong evidence that the observed defects exclusively resided within the mutated gene. Even in the presence of spurious mutations, these experiments can show that they are of negligible importance in virion egress.

Based on previous experiments, our original hypothesis was that only UL20 was essential in cytoplasmic events of the HSV-1 lifecycle, and that the phenotype shown by the gE+gD double-null mutant was actually an indirect effect of multiple glycoprotein deletions disrupting the integrity of UL20. It will be interesting to investigate the viral phenotypes and replication characteristics of the gE+gD double-null mutant grown on UL20 complementing cells (FRT), and vice versa; UL20 null mutants grown on gD and gE complementing cells. This work is currently in progress, and preliminary plaque morphology data shows that gDΔcp mutant viruses grown on the FRT cell line are larger than plaques on non gD complementing cells (Figure 3.1). On average, these plaques were about 3-4 greater in size when grown on FRT vs. when grown on Vero cells. The same did not apply to ΔgE+gDΔcp grown on FRT, as it exhibited similar plaque size to Vero cells. One explanation for this phenomenon can be attributed to the over-expression of UL20 proteins being able to make up for the loss of one glycoprotein but not for multiple glycoproteins in plaque formation, suggesting the potential for direct or indirect interactions between UL20 and gD or gE. These potential interactions need

to be addressed in the future, in addition to conducting similar experiments using ΔgE on UL20 and gE complementing cell lines.

The progressive step to understanding cytoplasmic virion egress is to assess whether the UL20 protein, shown to be essential for virion egress, works individually or synergistically with other proteins in secondary re-envelopment. Recently, our lab has been working with FRET (Förster resonance energy transfer) analysis to check for protein-protein interactions in live cells. UL20 and capsid associated tegument proteins could be tagged with variants of green fluorescent protein (GFP) such as cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) to determine the molecular dynamics of these proteins. Once interaction has been confirmed, different organelles of the infected cells can also be tagged, and infected cells can be fixed at various points of the lifecycle to determine the specific locations of these interactions.

Overwhelming evidence suggests that a large complex of protein-protein interactions work ensemble or in a sequential manner to facilitate cytoplasmic virion envelopment. It is foreseen that when such interactions are completely defined, it may be possible to assemble enveloped virions in vitro synthetic systems in which individual components are added without the need for an intact cell.

## FINAL COMMENTS

Cytoplasmic virion envelopment is but one-step in virion morphogenesis, and it can be easy to temporarily lose sight of the bigger picture when focused on a very specific problem. However, unearthing the nitty-gritty details is prerequisite to the comprehensive understanding of all things if the knowledge is to be applied. In the near future, cellular extracts may be utilized to reconstitute intracellular cytoplasmic

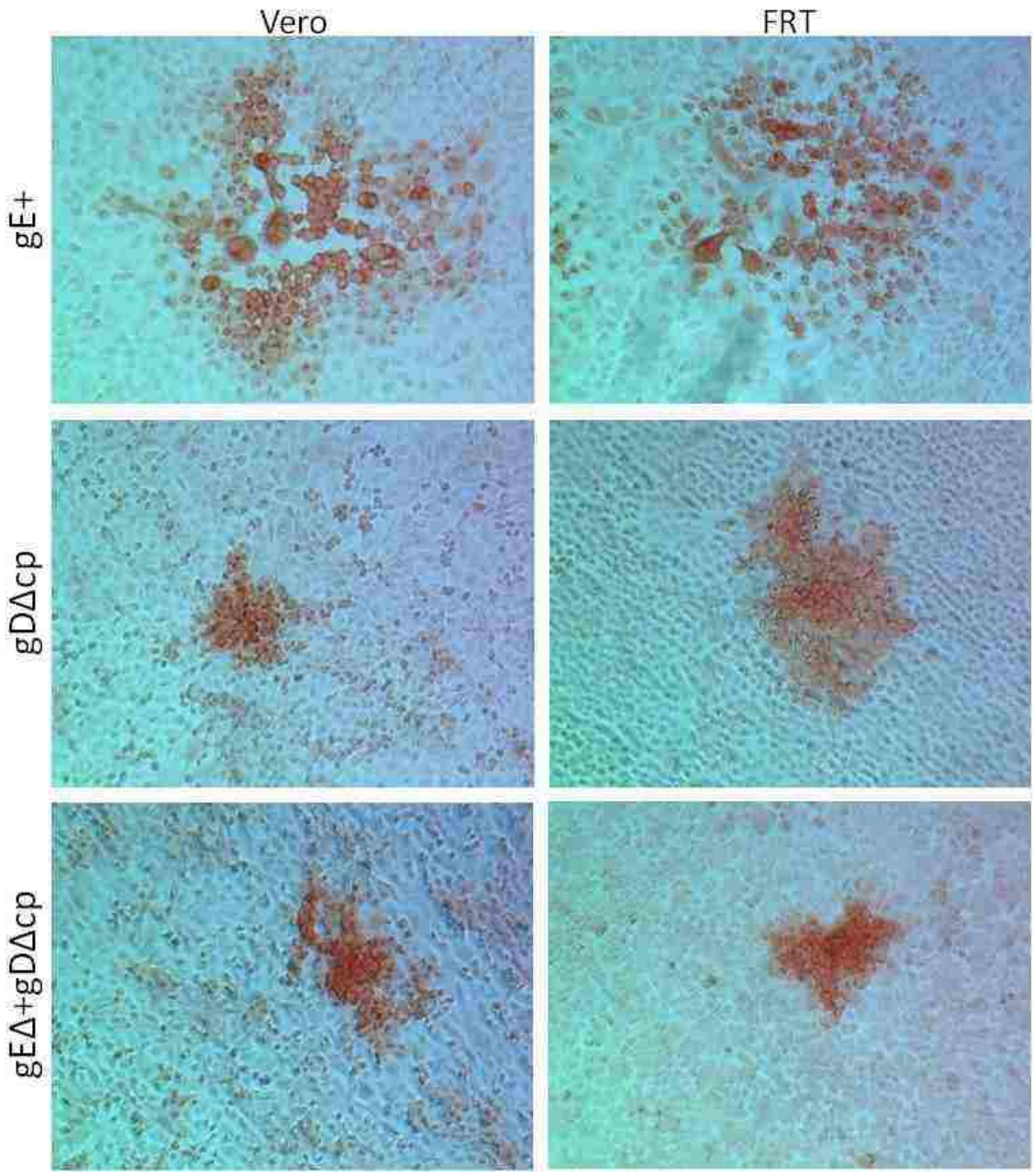


**Figure 3.2: Plaque Morphologies.** Comparison of plaque morphologies of Vero and FRT cells.

envelopment. Specifically, this may be accomplished through the testing of individual mutant virion particles for their ability to acquire envelopes by budding into TGN-derived

vesicles. Ultimately, complete knowledge of virion assembly may enable researchers and bio-engineers to build custom “nanomachines” that have the ability to deliver specific payloads and/or perform specific tasks. With this in mind, it remains a challenge that so many questions still remain unsolved. It is a characteristic of science that every discovery that sheds light on a particular phenomenon raises more questions than it answers.

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# VITA

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