The role of herpes simplex virus glycoprotein gE/gI in virus envelopment and egress.

By

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TABLE OF CONTENTS

Tables

Figures

Abbreviations:

α: alpha

a.a.: aminoacid

A: adenine

Ad: adenovirus

AIDS: acquired immunodeficiency syndrome

β: beta

BAC: bacterial artificial chromosome

BHV: bovine herpes virus

bp: base pair

BSA: bovine serum albumin

C: cytosine

C-: carboxyl

CBD: calmodulin-binding domain

DMEM: Dulbecco's modified Eagle's Medium

DOC: deoxycholate

DNA: deoxyribonucleic acid

DS: double stranded

DTT: dithiothreitol

E: Early

EDTA: ethylenediaminetetraacetic acid

EHV: equine herpesvirus

EM: electron microscope

FBS: fetal bovine serum

γ: gamma

g: glycoprotein

G: guanine

GFP: green fluorescent protein

GST: glutathione S-transferase

h: hours

HCMV: human cytomegalovirus

HHV: human herpesvirus

HIV: human immunodeficiency virus

hpi: hours post-infection

HSV: herpes simplex virus

HSV-1: herpes simplex virus type-1

HSV-2: herpes simplex virus type-2

IE: immediate early

INM : inner nuclear membrane

IR: internal repeat

kb: kilobase

kbp: kilobase pairs

L: late

MAb: monoclonal antibody

MOI: multiplicity of infection

N-: Amino

NC: nucleocapsid

NE: nuclear envelope

nm: nanometer

NP-40: nonidet P-40

nt: nucleotide

ONM: outer nuclear membrane

ORF: open reading frame

oriL: origin of replication in the UL segment of HSV

oriS: origin of replication in the US region of HSV

PBS: phosphate buffered saline

PBS-T: phosphate-buffered saline supplemented with .02% tween-20

pi: post-infection

PFU: plaque forming units

PKC: protein kinase-C

PMSF: phenylmethylsulphonyl fluoride

PRV: psuedorabies virus

PSG: penicillin-streptomycin-L-glutamine

PVDF: polyvinylidene fluoride

RBC: red blood cell

RNA: ribonucleic acid

RPMI: Roswell Park Memorial Institute, media used to grow HEC-1A cells

TAP: tandem affinity purification

TEV: tobacco etch virus

TGN: *trans*-Golgi network

UL: unique long

US: unique short

UL#: protein designation in HSV i.e. UL49 refers to the protein product of the 49^{th} ORF contained in the UL segment

US#: protein designation in HSV, i.e. US8 refers to the protein product of the 8^{th} ORF in the US segment

VP: Viral Protein

VP #: coding system describing HSV proteins and relates to their migration on a SDS-page gel i.e. VP22 (UL49) is the 22^{nd} viral protein in an SDS-page gel.

VSV: vesicular stomatitis virus

VZV: varicella zoster virus

x-gal: 5-Bromo-4 Chloro-3-Indolyl-BD-

Galactopyranoside

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Preface

Aaron Farnsworth performed the work outlined in this dissertation under the supervision of Dr. David C. Johnson in the department of Molecular Microbiology and Immunology at Oregon Health & Science University. The results of this work are presented in five chapters three of which have been previously published in scientific journals. Additionally, some unpublished work is presented in an appendix following the body of this text. Chapter 1 is a summary of the life cycle of HSV and a general introduction to this area of study. Chapter 2, titled "Herpes simplex virus glycoproteins gD and gE/gI serve essential but redundant functions during acquisition of the virion envelope in the cytoplasm" was published in the Journal of Virology (77:8481-8494) and describes the effect that the simultaneous deletion of gD and gE has on the envelopment pathway of HSV. Chapter 3, titled "HSVgE/gI Must Accumulate in the *trans*-Golgi Network at Early Times then Redistribute to Cell Junctions to Promote Cell-Cell spread", was published in the Journal of Virology (80:3167-3179). This chapter describes the effect of truncations in the gE CT domain on the trafficking of gE and the subsequent spread and egress of HSV. Chapter 4, titled "HSV glycoprotein gE Cytoplasmic Residues Required for the Secondary Envelopment and Binding of Tegument Proteins VP22 and UL11 to gE and gD", was published in the Journal of Virology (81:319-331). This chapter describes the effects that truncations of the gE CT domain has upon the secondary envelopment of HSV, in the absence of gD. Furthermore this chapter details the interaction of gD and gE with tegument proteins VP22 and UL11 and provides evidence that specific regions of the gE CT domain are required for this interaction.

Chapter 5 is a discussion summarizing these findings and their implications and raises questions for future investigation.

Abstract

Herpes simplex virus (HSV) latently infects a significant proportion of the human population and has been isolated in every human community. The ubiquitous nature and rapid transmission of this pathogen are intimately associated with the envelopment and egress pathways of HSV. Recent work has suggested that alphaherpesviruses acquire their final envelope from a cytoplasmic membrane, such as the *trans*-Golgi network. This envelopment process is mediated by interactions between the cytoplasmic (CT) domain of viral glycoproteins and the tegument-containing nucleocapsid (NC). Although the requirements for secondary envelopment are not identical in all alphaherpesviruses, the glycoprotein gE/gI has a significant role in the envelopment of multiple family members. This raises the possibility that gE/gI may be the primary means by which the envelope is anchored onto the nucleocapsid. In addition to its role in envelopment, gE/gI also participates in the spread and pathogenesis of alphaherpesviruses in both neuronal and epithelial cells.

In this dissertation I have attempted to further characterize the role of gE/gI in the secondary envelopment and cell-to-cell spread of HSV. To this end, I identified two envelope glycoproteins, gD and gE, that are necessary for the envelopment of HSV-1, indicating that these proteins function redundantly in this process. Mutants deleted for both of these proteins accumulated large numbers of nucleocapsids in the cytoplasm but mutants lacking just gE or gD acquired an envelope with only a minor decrease in efficiency. This established three facts regarding the HSV-1 secondary envelopment pathway. First, HSV acquires its final envelope from a host cell membrane in the

cytoplasm mediated by the glycoproteins gD and gE. Second, gD and gE share some functional redundancy in envelope acquisition such that envelopment proceeds if either is present but is abrogated if both are absent. Third, secondary envelopment of HSV-1 follows a similar pathway to other alphaherpesviruses but has distinct molecular requirements.

To further characterize the involvement of gE/gI in secondary envelopment I examined specific contributions of the gE CT domain and identified regions of this domain that specifically contributed to this process. Additionally, I identified two tegument proteins, VP22 and UL11 that bound to both gD and gE suggesting a plausible method by which gD and gE may interact with the nucleocapsid. Finally I characterized the role of specific regions of the gE CT domain in directing both cell-to-cell spread of infectious virions and trafficking of gE/gI. I demonstrated that simply localizing gE/gI to the site of virion envelopment is insufficient to mediate cell-to-cell spread. Furthermore, gE CT domain mutants that do not spread efficiently from cell-to-cell are not targeted to the apical surface. These findings imply that the gE CT domain has an additional role, beyond mediating secondary envelopment, in the trafficking of HSV.

Chapter 1:

Introduction

1.1 Herpesviridae

Herpesviridae classification is based on a combination of virus architecture, biological properties and genetic analysis (Roizman, B. et al. 1992; Davison, A. J., Eberle, R., Haywad, G.S., McGeoch, D.J., Minson, A.C., Pellet, P.E., Roizman, B., Studdert, M.J., Thiry, E. 2005). A typical herpesvirus particle consists of a linear double stranded DNA genome ranging from 125 to 240 kbp inside an icosadeltahedral capsid, which is surrounded by a tegument layer and encased in a host-cell derived lipid envelope (Figure 1.1). During the infectious cycle all herpesviruses: (i) synthesize enzymes involved in nucleotide and DNA metabolism, (ii) synthesize and assemble viral DNA and capsids in the nucleus of the host cell and (iii) have the capacity to establish latency in their natural hosts. Members of the family *Herpesviridae* are divided into one of three subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae* or *Gammaherpesvirinae*. Historically this division was based upon broad biological criteria and further classification into genera was achieved through molecular criteria, primarily analysis of the overall genome structure (Roizman, B. et al. 1992). Herpesviruses included in the subfamily *Alphaherpesvirinae*, such as herpes simplex virus type-1, exhibit a variable host range, rapid reproductive cycle, destructive spread in cultured cells and establish latency in sensory neurons. Members of the *Betaherpesvirinae* subfamily, such as human cytomegalovirus, are characterized by a restricted host range, slow spread in cell culture, enlargement of infected cells and the ability to establish latency in lymphoid and secretory cells. Viruses of the subfamily *Gammaherpesvirinae*, such as the Epstein-Barr virus have an extremely narrow host range and only replicate in lymphoblastoid cells; they establish latency in lymphoid tissues. Currently, classification and phylogenetic

analyses of herpesviruses are based on gene and sequence analysis in addition to morphology and biological characteristics (Davison, A. J., Eberle, R., Haywad, G.S., McGeoch, D.J., Minson, A.C., Pellet, P.E., Roizman, B., Studdert, M.J., Thiry, E. 2005).

1.2 Herpes Simplex Virus, A Brief Overview

The term 'Herpes' is derived from the Greek verb *'Erpein'* (to creep) and was employed in the Hippocratic Corpus to describe a variety of ulcerative lesions ranging from ringworm and eczema to cancer and small pox (Beswick, T. S. 1962). Although shingles was recognized as herpes zoster or zona by Galen and other early physicians the term 'herpes' continued to describe a large number of ulcerative conditions involving the skin or subcutaneous tissues until the early 18th century. In 1714, Daniel Turner provided the first description of herpes simplex using the modern name, and specifically noted the short course and appearance of pustules observed in typical infections (Beswick, T. S. 1962). Histopathological studies in the 19th century led to the grouping of herpetic diseases into three categories: herpes zoster, herpes facialis and herpes progenitalis.

Concurrent with studies into the dermatological nature of herpetic lesions, various medical scientists began to investigate the causative agents behind these outbreaks. Up until the 19th century most herpetic eruptions were believed to occur due to the excretion or attempted excretion of waste through the skin (Beswick, T. S. 1962). Towards the latter half of the 19th century, work by Pasteur and others on 'germ theory' drove investigations into pathogenic causes of human diseases. In 1873, Vidal published the observations that discharge from herpes simplex lesions was infectious and could cause the production of novel lesions (Vidal, J. B. 1873). However, interest in the etiologic agent remained slight until early in the 20th Century when events brought HSV to the

forefront of medical virology. Firstly, reports detailed the isolation of a filterable agent from herpes blisters or keratitis that could be passaged on rabbit corneas, providing researchers with basic tools to study HSV in isolation of its natural host (Lowenstein, A. 1919). Secondly, in 1917 a pandemic of acute encephalitis, known as 'Von Economo's encephalitis', emerged and research focused on identifying the causative agent. HSV was isolated from the brains of several patients who had died following acute encephalitis and the virus was also shown to cause encephalitis in rabbits following corneal scarification (Doerr, R. and Vochting, K. 1920; Levaditi, C. and Harvier, P. 1920). Thus medical interest in HSV and the methodology necessary for its characterization coincided. A large number of laboratories began investigations into the encephalitic nature of HSV and rapid advances were made in understanding the basic virology and HSV biology using rabbits as models of HSV infection. In the 1920s Goodpasture demonstrated that HSV was capable of spreading from the retina to the optic nerve and from the ovary or liver to the spinal cord suggesting a plausible route for herpes induced encephalitis (Goodpasture, E. W. and Teague, O. 1923). Goodpasture concluded that HSV trafficked from the masseter muscle to the trigeminal ganglion by means of the nerve axon and that the virus resided in a latent state within the human body (Goodpasture, E. W. 1925; Goodpasture, E. W. 1929), although formal proof of these concepts would not be published until the 1970s (Bastian, F. O. et al. 1972; Cook, M. L. and Stevens, J. G. 1973). In the 1950s and 60s tissue culture techniques were developed which allowed researchers to examine the HSV particle and electron microscopy which allowed researchers their first glimpses of the particle in the cell body (Enders, E. F. et al. 1949; Morgan, C. et al. 1954a; Morgan, C. et al. 1954b; Hoggan, M. D. and Roizman, B. 1959; Roizman, B. 1961; Roizman, B. et al.

1963; Roizman, B. and Roane, P. R., Jr. 1964) . Molecular biological techniques pioneered in the 1970s and 1980s allowed researchers to ask fundamental questions about the protein and DNA content of HSV resulting in the identification and functional understanding of the major proteins encoded by HSV as well as the molecular sequence of the genes that encode them (Spear, P. G. and Roizman, B. 1970; Gibson, W. and Roizman, B. 1972; Spear, P. G. and Roizman, B. 1972; Gibson, W. and Roizman, B. 1974; McGeoch, D. J. et al. 1985; McGeoch, D. J. et al. 1988). The intertwining of the medical importance of herpes simplex virus with technological advances in molecular biology continues to fuel research of HSV to this day.

1.3 Epidemiology of Herpes Simplex Virus.

HSV-1 is a ubiquitous pathogen that has been isolated from every human community (Nahmias, A. J. et al. 1990). The majority of infections are either asymptomatic or cause recurrent orolabial lesions, however the virus can infect any visceral or mucocutaneous site. HSV-1 is the leading infectious cause of blindness and viral encephalitis and contributes significantly to morbidity and mortality in immunocompromised and immunosuppressed patients (Rantalaiho, T. et al. 2001; Remeijer, L. et al. 2002). Serological studies indicate that 68% of the population over the age of 15 in the USA are infected with HSV-1 (Schillinger, J. A. et al. 2004). Various other countries in the world have HSV-1 infection rates ranging from 50% to 94% of the adult population and in all cases HSV-1 seroprevalence has an inverse correlation to socioeconomic status (Cowan, F. M. et al. 2003; Pebody, R. G. et al. 2004; Schillinger, J. A. et al. 2004). Although HSV-1 has been traditionally associated with oral lesions and HSV-2 with genital lesions this association is changing. In a number of communities

throughout the industrialized world HSV-1 is either an increasing or leading cause of genital herpes (Vyse, A. J. et al. 2000; Roberts, C. M. et al. 2003; Samra, Z. et al. 2003).

The vast majority of HSV infections are asymptomatic in the immunocompetent host. The same is not true in the immunocompromised host where herpes infections and reactivation are much more serious. In HIV-positive or otherwise immunosuppressed patients an increase is observed in the frequency of reactivation as well as in the size of the mucocutaneous area affected (Levin, M. J. et al. 2004; Trope, B. M. and Lenzi, M. E. 2005). The rates of HSV seropositivity in HIV-positive individuals are elevated compared to the surrounding community, with the viruses potentially acting as co-factors for infection (Freeman, E. E. et al. 2006). HIV-positive individuals asymptomatically shed HSV at increased levels and endure an increase in the frequency and duration of herpetic ulcers (Schacker, T. et al. 1998). Conversely, genital ulcers act to increase the risk of HIV acquisition, possibly due to recruitment of CD4+ cells to lesions in the epidermal layer (Schacker, T. et al. 1998). If HSV lesions are left untreated in an immunocompromised host, chronic lesions can result and lead to a variety of complications such as esophagitis tracheobronchitis, pneumonitis or spread to other visceral organs (Perl, T. M. et al. 1992). However, a variety of chemotherapeutic agents exist which effectively control HSV reactivation ensuring that, in general, serious complications are restricted to those individuals suffering from a primary infection (Levin, M. J. et al. 2004).

The neonate has significant morbidity and mortality risks due to HSV infection. Generally, these individuals are exposed to the virus in the birth canal (Yeager, A. S. et al. 1983). Maternal type-specific antibodies appear to offer significant protection such

that the most significant risk is during a primary maternal infection immediately preceding labor or exposure to the virus from another source prior to maternal seroconversion (Yeager, A. S. et al. 1983; Brown, Z. A. et al. 2003). In addition, HSV-1 derived genital ulcers are associated with a high rate of maternal-neonate transmission rate and primary infections of the neonate are associated with a high rate of mortality (Tookey, P. and Peckham, C. S. 1996; Gaytant, M. A. et al. 2000; Brown, Z. A. et al. 2003). Neonatal HSV infection results in one of three conditions: localized mucocutaneous infection, infection of the central nervous system (CNS) or disseminated infection. Of these, the latter two occur in over 70% of neonatal cases in the USA and display a mortality rate of 50%-80% if untreated and 10%-57% with treatment. In spite of intervention 75%-86% of survivors suffer neurological impairment (Toltzis, P. 1991).

HSV-1 is the leading infectious cause of blindness in the industrialized world (Remeijer, L. et al. 2004). The virus can infect a variety of tissues in the eye with most serious being infection of the stroma resulting in herpes stromal keratitis (HSK) (Remeijer, L. et al. 2004). Overall, approximately 50,000 cases of ocular HSV infection or reactivation, requiring medical attention, occur every year in the USA (Remeijer, L. et al. 2004). Initial infection of the cornea usually results from either direct deposit of HSV onto the ocular area or the reactivation of latent virus from the trigeminal ganglia and anterograde transport through the ophthalmic branch (Kaye, S. B. et al. 1992). There is evidence that HSV can reside in the cornea after the acute infection has subsided, possibly establishing latency at this site (Kaye, S. B. et al. 1991). Related to this, the transfer of HSV-1 from infected donor to naive recipient during corneal graft operations has been documented and has led to subsequent loss of the cornea transplant (Remeijer,

L. et al. 2001). In mouse models of HSV-1 eye infection, a single infectious event is sufficient to cause HSK, suggesting that much of the tissue damage may be due to the action of the immune system (Polcicova, K. et al. 2005a).

In addition to causing dermatological lesions and eye infections, HSV is the predominant viral agent associated with encephalitis and herpes simplex encephalitis (HSE) occurs at an annual rate of approximately 2 cases per 100 000 people in the USA (Khetsuriani, N. et al. 2002). Left untreated, HSE has a mortality rate of 70% and even with treatment the mortality rate is 20% with over 50% of recovered individuals showing long term neurological impairment (Tyler, K. L. 2004). HSE may be a result of reactivation of HSV from the trigeminal ganglion or the result of delivery through the olfactory nerve (Whitley, R. J. 2006).

1.4 Herpes Simplex Virus-1 Genome and Structure

1.4.1 Genome

HSV-1, like all herpesviruses, has a large double stranded (DS) deoxyribonucleic acid (DNA) genome contained in an icosadeltahedral capsid. This is surrounded by a dense layer of proteins, termed the tegument, and then by a host cell derived lipid envelope. The HSV-1 genome is in a linear form in the viral capsid and comprises 152 kilobase pairs (kbp) of which 67% are guanine and cytosine (G-C) residues. HSV has a type 4 herpesvirus genome meaning that the DNA molecule is comprised of two unique segments, the long or UL component and the short or US component, each flanked by inverted repeats (Fig. 1.2). The UL segment is 108 kbp in length, encodes at least 56 distinct proteins and contains an origin of replication, referred to as *ori*L (McGeoch, D. J. et al. 1988). 40 of these 56 genes are arranged into discrete gene blocks (Fig. 1.2) and

conserved throughout the *Herpesviridae* family (Davison, A. J., Eberle, R., Haywad, G.S., McGeoch, D.J., Minson, A.C., Pellet, P.E., Roizman, B., Studdert, M.J., Thiry, E. 2005).The US segment is 13 kbp in length and encodes at least 12 proteins of which 6 are embedded in the virion envelope (gG, gJ gD, gE, gI, US9) (McGeoch, D. J. et al. 1985). These unique segments are flanked by the inverted repeat *b* and *c* sequences, respectively with the *c* sequence containing an origin of replication referred to as *ori*S. The *a* sequence is present at the terminal ends of the linear genome as well as in one inverted copy which separates the L and S segments (Fig. 1.2). This sequence contains *cis*-acting packaging elements that result in the cleavage and packaging of the HSV-1 genome. During replication four equimolar isomerization products are made, each capable of acting as a fully functional virus genome (Hayward, G. S. et al. 1975). Homologous recombination events that occur during genome replication appear to be responsible for the generation of these products which, therefore, are a reflection of the repeated elements contained within the HSV-1 genome rather than a specific function required in the virus life cycle. (Szostak, J. W. et al. 1983; Smiley, J. R. et al. 1990; Smiley, J. R. et al. 1992; Sarisky, R. T. and Weber, P. C. 1994; Martin, D. W. and Weber, P. C. 1996).

1.4.2 Capsid

The HSV-1 capsid is comprised of a protein shell that is 15nm thick and 125 nm in diameter (Newcomb, W. W. et al. 1993). The capsid shell has icosadeltahedral symmetry and its chief constituents are 162 capsomeres (150 hexons and 12 pentons) which interact via their proximal ends and protrude outwards forming the capsid floor. Positioned just above the capsid floor are 320 triplexes which lie between adjacent capsomeres and control intracapsomere interactions and assist in capsid morphogenesis

(Saad, A. et al. 1999) (Fig. 1.3). At least nine proteins constitute the virus capsid: VP5 (UL19), VP26 (UL35), VP19C (UL38), VP23 (UL18), VP24 (UL26), VP21 (UL26), VP22a (UL26.5), UL6 and UL25 (see Table 1.1). The first four proteins are the major structural proteins that make up the mature HSV capsid. VP5, the major capsid protein, is the compositional subunit of both hexons and pentons and VP26 constitutes the hexon tip. VP19C and VP23 form the triplex proteins at a ratio of 1:2 (Saad, A. et al. 1999). These four structural proteins and three additional assembly proteins, VP24, VP21 and VP22a, which comprise the protease and scaffolding components, are sufficient for capsid assembly *in vitro* (Section 1.5.5). However, these capsids cannot incorporate genetic material without two minor but necessary constituents, UL6 and UL25. UL6 is the DNA portal, forming a 12 subunit ring through which DNA is inserted into the developing nucleocapsid. The UL25 protein acts to reinforce the capsid upon addition of DNA and is necessary for the full maturation of HSV capsids (Section 1.5.5).

Capsids observed in HSV infected cells are classified into one of three groups. Type A capsids are devoid of both scaffolding proteins and DNA and are the result of a failure to package the HSV genome. Type B capsids contain scaffolding proteins but not DNA and represent the capsid prior to the packaging of genomic DNA and type C capsids are mature nucleocapsids containing HSV DNA and few scaffolding proteins.

1.4.3 Tegument

In HSV, as in all herpesviridae family members, an amorphous layer of proteins is situated between the nucleocapsid and the envelope. Initially this layer was designated the 'tegument layer' and defined as those proteins that were part of the virion but not part of the envelope or capsid compartments (Gibson, W. and Roizman, B. 1972; Gibson, W.

and Roizman, B. 1974; Dolyniuk, M. et al. 1976). Tegument proteins provide a variety of important roles in the virus life cycle and function in events including initiation of infection, capsid transport, DNA transmission, virion envelopment and egress. Currently there are 18 recognized proteins in the tegument layer (Table 1.1). However, the organization of this structure remains poorly understood as structural analysis is difficult due to the complexity and lack of symmetry of this layer. Some structural symmetry has been observed in the tegument layer close to the nucleocapsid possibly due to association of VP1/2 and UL37 with the icosadeltahedral capsid (Zhou, Z. H. et al. 1999). These two tegument proteins remain tightly associated with the nucelocapsid upon virus entry and are termed inner tegument proteins. Outer tegument proteins such as vhs (UL41), VP11/12 (UL46), VP13/14 (UL47), VP16 (UL48) and VP22 (UL49) and are not generally thought to interact directly with the capsid (Wolfstein, A. et al. 2006).

1.4.4 Envelope

The HSV tegument and nucleocapsid are encased by a host cell derived lipid envelope into which a number of virus encoded proteins are embedded. These proteins are the means by which the virus interacts with the extracellular milieu and they function in binding, entry, envelopment and egress. At least 11 glycoproteins and several non-glycosylated proteins encoded by HSV-1 are embedded in the viral membrane (Table 1.1) (Spear, P. G. 1984; Baines, J. D. and Roizman, B. 1991; MacLean, C. A. et al. 1991; Hutchinson, L. et al. 1992b; Baines, J. D. and Roizman, B. 1993). Of these, three (gB (UL27), gD (US6), gH/gL (UL22 and UL1)) are essential for the initial entry and subsequent cell-to-cell spread of HSV (Cai, W. Z. et al. 1987; Ligas, M. W. and Johnson, D. C. 1988; Forrester, A. et al. 1992; Roop, C. et al. 1993). gD is the HSV ligand and is

necessary and sufficient for binding and attachment to cells. gB is capable of binding to heparan sulfate and along with the heterodimer gH/gL is required for fusion between the viral and cellular envelopes. Although these proteins are sufficient to mediate entry other glycoproteins also contribute to entry and intracellular spread. Glycoprotein gM (UL10), which forms a complex with UL49.5, changes the trafficking patterns of various viral and cellular proteins, including gD and gH/gL (Crump, C. M. et al. 2004). HSV gM- mutants have a lower infectious titer compared to wild type viruses suggesting that gM/UL49.5 recruitment of proteins may be important in the virus life cycle (Baines, J. D. and Roizman, B. 1993). Glycoproteins gE (US8) and gI (US7) also function as a heterodimer and have multiple roles in the infectious cycle of the virus, including cell-to-cell spread, protein trafficking as well as virion maturation and egress (Dingwell, K. S. et al. 1994; Wisner, T. et al. 2000; Johnson, D. C. et al. 2001; Collins, W. J. and Johnson, D. C. 2003; Polcicova, K. et al. 2005b).

1.5 Herpes Simplex Virus-1, Infectious Cycle

1.5.1 Attachment and Entry

Herpes simplex virus can utilize two entry pathways. The classical HSV entry pathway, based on tissue culture studies with simian and human kidney cell lines (Vero and Hep-2 cells), describes the entry of HSV via fusion with the plasma membrane in a pH-independent process activated by receptor binding (Wittels, M. and Spear, P. G. 1991). More recently, it has been demonstrated that HSV entry into other epithelial cells (modified CHO or HeLa cells) occurs by a pH-dependent phosphatidyl inositol-3-kinase (PI3-K) endocytic pathway (Nicola, A. V. et al. 2003; Nicola, A. V. et al. 2005). Both pathways appear to be relevant in the life cycle of the virus; different entry routes are

observed in primary human neuronal cells and human epidermal keratinocytes, respectively (Nicola, A. V. et al. 2005). Entry by either process appears to require the identical set of HSV glycoproteins; gD, gB and the gH/gL heterodimer although the mechanistic details of entry have been well studied only for fusion at the plasma membrane (Fig. 1.4). The glycoproteins gB (UL27), gC (UL44), gD (US6) and gH/gL (UL22/UL1) all contribute to some aspect of the entry process. Initial association with the host cell surface is mediated through proteoglycans, specifically their attached glycosaminoglycan (GAG) chains. GAGs are linear, charged polysaccharides that participate in numerous interactions in the host, including cellular adhesion and signaling (Mulloy, B. and Rider, C. C. 2006). The HSV proteins gB and gC bind to the GAG heparan sulfate and deletion of gC reduces the efficiency of entry (Herold, B. C. et al. 1991). A closer association, mediated by gD, is required for HSV entry. gD interacts with at least three cellular receptors including herpesvirus entry mediator (HVEM or HveA), a tumor necrosis family (TNF) receptor, as well as with the cell adhesion molecules nectin-1(HveC) and nectin-2 (HveB), members of the IgG superfamily (Montgomery, R. I. et al. 1996; Whitbeck, J. C. et al. 1997; Geraghty, R. J. et al. 1998; Warner, M. S. et al. 1998) .Nectin-1 is likely the relevant receptor that the virus uses to gain access to the host cell. Unlike the other putative receptors, Nectin-1 is expressed at high levels in neuronal tissues and soluble forms abrogate entry of HSV-1 into primary neurons (Geraghty, R. J. et al. 1998; Richart, S. M. et al. 2003). gD is present in the viral membrane as a homodimer (Krummenacher, C. et al. 2005). Additionally each monomeric portion of the dimer is in a 'closed' confirmation wherein binding between the amino- and carboxyl-terminus of the extracellular domain results in the masking of the gD pro-fusion domain

(PFD) (Fig. 1.4) (Fusco, D. et al. 2005; Krummenacher, C. et al. 2005). Interaction between gD and Nectin-1 leads to the stable attachment of HSV at the cell surface and disrupts the homodimerization of gD. Altogether this binding results in the exposure of the PFD which triggers the fusion cascade by interacting with gB and gH/gL (Cocchi, F. et al. 2004; Fusco, D. et al. 2005). The fusion cascade is thought to proceed via a structural change in gB and gH/gL that results in the insertion of a fusion peptide into the host membrane, however the precise mechanism or sequence of events in this process remains unknown (Fuller, A. O. and Lee, W. C. 1992; Weissenhorn, W. et al. 1999; Krummenacher, C. et al. 2005)(Fig. 1.4). In agreement with this hypothesis a fusion peptide in gH has been isolated (Gianni, T. et al. 2005). Following the fusogenic activities of gB and gH/gL the two bilayers merge and the tegumented nucleocapsid is deposited into the host cytoplasm (Weissenhorn, W. et al. 1999; Okazaki, K. and Kida, H. 2004; Galdiero, S. et al. 2005).

1.5.2 Transport to the Nucleus

Once fusion between the HSV envelope and the host cell membrane occurs the nucleocapsid is trafficked to the nucleus where binding to the nuclear pore complex (NPC) occurs (Batterson, W. et al. 1983; Sodeik, B. et al. 1997). The nucleocapsid is transported in a retrograde direction along microtubules (MT) using the cellular motor protein dynein and its cofactor dynactin (Sodeik, B. et al. 1997). The specific virus encoded mechanisms by which HSV interacts with these proteins are unclear and conflicting reports regarding a role for VP26 (UL35) in this transport have been described (Douglas, M. W. et al. 2004; Dohner, K. et al. 2006). Since capsids devoid of tegument proteins appear to be incapable of retrograde transport a plausible model postulates that

incoming nucleocapsids are stripped of outer tegument proteins and the nucelocapsid and inner tegument proteins interact with the MT network for transport back to the Microtubule Organizing Centre (MTOC) (Wolfstein, A. et al. 2006). Interaction between the nucleocapsids and the NPC results in extrusion of the HSV DNA genome through the pore and into the nucleus (Ojala, P. M. et al. 2000). The tegument protein VP1/2 (UL36) is required for the release of the viral DNA into the NPC and in the absence of cytosolic factors or an energy source the genome does not enter the nucleus as the large HSV genome requires an active transport mechanism (Ojala, P. M. et al. 2000; Shahin, V. et al. 2006). Upon entry into the nucleus the viral DNA circularizes in preparation for DNA replication and amplification (Ojala, P. M. et al. 2000).

1.5.3 DNA Replication

Proteins involved in the replication of herpes simplex virus can be divided into two categories; those that mediate the actual synthesis of viral DNA and a second class that act in nucleic acid metabolism (Chattopadhyay, S. et al. 2006). The latter group of proteins is not necessary for replication in immortalized cell lines and includes the enzymes thymidine kinase (UL23), ribonucleotide reductase (UL39, UL40), uracil glycosylase (UL2) and deoxyuridine triphosphate nucleotidohydrolase (UL50). Seven HSV proteins are essential for viral DNA replication. An origin DNA-binding protein/helicase (UL9); a two component DNA polymerase (UL30 and UL42); a three component helicase/primase protein (UL5, UL8 and UL52) and a single-stranded DNA-binding protein (UL29) (Olivo, P. D. et al. 1988; Crute, J. J. et al. 1991; Falkenberg, M. et al. 2000; Chattopadhyay, S. et al. 2006).

HSV has three different origins of replication, one in the UL segment located between the genes UL29 and UL30, designated *ori*L and two copies of an origin designated *ori*S in the *c* sequences flanking the US segment of the genome (Chattopadhyay, S. et al. 2006). Both *ori*L and *ori*S contain a central adenine and thymine (A-T) rich region that is flanked by large palindromes containing UL9 recognition sequences. In tissue culture two of the three origins can be deleted with little effect on the virus, however it is possible that these sequences are differentially employed during reactivation or in different cell types (Polvino-Bodnar, M. et al. 1987; Igarashi, K. et al. 1993).

Initiation of DNA synthesis is mediated through the binding of UL9 dimers to recognition sites contained within the origins. In conjunction with UL29 this leads to local unwinding of the A-T central region (Olivo, P. D. et al. 1988). Opening of this section of the DNA allows the entry of the DNA helicase/primase heterotrimeric complex composed of UL5, UL8 and UL52 and the creation of a replication fork (Crute, J. J. et al. 1991). RNA primers synthesized by this heterotrimer are extended by the HSV-1 DNA polymerase (UL30 and UL42) (Falkenberg, M. et al. 2000) (Chattopadhyay, S. et al. 2006). Late replication events appear to occur independently of UL9, indicating that *de novo* initiation events are no longer required for continued replication. One plausible explanation for this change is that late replication occurs via a rolling circle mechanism, and therefore does not require further initiation (Skaliter, R. and Lehman, I. R. 1994).

1.5.4 Gene Expression

The genes of HSV-1 are defined by their temporal expression pattern upon host cell infection and are designated immediate early (IE or α), early (E or β) and late (L or γ)

with the latter class divided into two categories γ_1 and γ_2. IE genes are defined as those genes that are expressed at high levels in the absence of HSV protein synthesis although the presence of the viral protein, VP16 (UL48 or αTIF) has a strong stimulatory effect on this expression (Nogueira, M. L. et al. 2004). Five genes expressed by HSV are part of this class, ICP0 (α0), ICP4 (α4), ICP22 (US1), ICP27 (UL54) and ICP47 (US12) all map within or near the inverted repeats with ICP0 and ICP4 present twice in the HSV genome (Fig. 1.2) (McGeoch, D. J. et al. 1985; McGeoch, D. J. et al. 1988). The first four members of this class are involved in the regulation or transactivation of other HSV genes as well as a subset of cellular genes. The fifth member of this class, ICP47, is responsible for inhibiting the presentation of peptides by MHC class I thereby stalling the adaptive immune response of the host organism (York, I. A. et al. 1994; Hill, A. et al. 1995). High level expression of E genes is dependent on the presence of ICP0 and ICP4 but this expression precedes DNA synthesis (Weir, J. P. 2001). In fact E gene expression is enhanced in the absence of DNA synthesis, as might be expected since genes essential for DNA replication and nucleotide metabolism are part of this kinetic class (Weir, J. P. 2001). L gene expression is divided into two classes, the first, leaky-late or γ_1 genes typified by proteins such as gB, are expressed early in infection independently of DNA synthesis while the second class, late or γ_2 genes, typified by proteins such as US11 or gC, are tightly regulated and not expressed in the absence of DNA synthesis (Weir, J. P. 2001). These genes generally encode for structural proteins or proteins involved in virion assembly. Unlike the IE genes the members of the E and L temporal classes are dispersed throughout the genome (Everett, R. 1997).

1.5.5 Capsid Assembly and Packaging

Assembly of the capsid occurs in the nucleus of the infected cell, initially a procapsid structure is formed that contains an internal structural scaffold (Trus, B. L. et al. 1996). Successful packaging of HSV-1 DNA requires viral processing of the scaffold proteins and eventually release of these proteins from the capsid structure. *In vitro*, capsid assembly can be reconstructed through the expression of seven proteins, VP5 (UL19), VP26 (UL35), VP19C (UL38), VP23 (UL18), VP24 (UL26), VP21 (UL26), VP22a (UL26.5) which constitute the four major structural proteins and three proteins which make up the protease and scaffolding elements (Tatman, J. D. et al. 1994). UL26 and UL26.5 encode proteins with identical C-terminal VP5-interaction and oligomerization domains. Condensation interactions between VP5 and full length UL26 and UL26.5 result in the formation of partial procapsid shells, interactions between VP19C and VP23 bring these partial shells together to form a virus procapsid which contains the protease and scaffold components in unprocessed forms (Newcomb, W. W. et al. 1996). UL26 undergoes autocatalytic cleavage, separating the protease domain (VP24) from the oligomerization domain (VP21). The protease then cleaves a 25 a.a. peptide from the C-terminus of both itself and UL26.5 that serves to tether VP22a and VP21 to the capsid shell (Liu, F. and Roizman, B. 1993). These cleavage reactions result in the maturation of the procapsid which becomes an icosadeltahedral capsid containing 150 hexons (VP5), 12 pentons (VP5) and 320 triplexes (VP19C and VP23) (Newcomb, W. W. et al. 1996). Ring proteins composed of six units of VP26 interact with each of the hexons after this maturation step as procapsid structures are devoid of this protein (Zhou, Z. H. et al. 1994). UL6 and UL25 are minor but necessary constituents of the mature HSV-1 capsid.

UL6 comprises the portal protein and it is thought that an interaction between UL6 and VP5 initiates the capsid assembly process (Newcomb, W. W. et al. 2001). UL25 reinforces the nucleocapsid following insertion of the HSV-1 genome. HSV-1 UL25-null mutants assemble capsids that are unable to contain the full genome and the viral DNA is relased from capsids following packaging (Newcomb, W. W. et al. 2006).

Following replication the HSV genomes exist as a mass of concatamers that must be individually inserted into preformed capsids. Seven HSV encoded proteins are essential for the cleavage and packaging step: UL6, UL15, UL17, UL25, UL28, UL32 and UL33. HSV mutants deleted for any of these proteins have large accumulations of DNA and capsids in the nucleus and do not form mature virions (al-Kobaisi, M. F. et al. 1991; Poon, A. P. and Roizman, B. 1993; Tengelsen, L. A. et al. 1993; Chang, Y. E. et al. 1996; Lamberti, C. and Weller, S. K. 1996; McNab, A. R. et al. 1998; Salmon, B. et al. 1998). The functions mediated by UL17 and UL32 remain unclear, however, studies of null mutants demonstrated that viruses lacking either protein were unable to target empty capsids to the site of DNA replication (Lamberti, C. and Weller, S. K. 1998; Taus, N. S. et al. 1998). As previously described, UL6 is the compositional subunit of the portal, a 12 member ring shaped protein through which HSV DNA enters the capsid (Newcomb, W. W. et al. 2001). UL15, UL28 and UL33 are all components of the terminase, a multiunit enzyme that is responsible for the cleavage and translocation of DNA into the capsid as well as the energy generation required for this process (Yang, K. and Baines, J. D. 2006). UL15 acts to cleave the concatameric HSV DNA into genome lengths and UL28 is the DNA-binding portion of the enzyme. As described above, UL25 stabilizes the capsid against the pressure exerted by the packaged DNA. To summarize, capsids containing the

portal protein are recruited to sites of DNA replication via the activity of UL17 and UL32. The terminase protein (UL15, UL28, UL33) then cleaves and binds to both HSV genomes and the portal protein (UL6). Energy dependent translocation of the genome subsequently occurs followed by reinforcement (UL25) of the capsid. The insertion of the HSV-1 genome displaces the scaffolding proteins (VP21 and VP22a) from the internal capsid structure. VP24 levels, however, remain unchanged between the procapsid and the mature DNA filled capsid indicating that there may be a role for the protease following maturation of the nucleocapsid (Tatman, J. D. et al. 1994).

1.5.6 Transport across the Nuclear Envelope

After packaging the herpes genome, mature nucleocapsids must acquire an envelope and exit the cell body to complete the infectious cycle. In 1969 two alternate theories were proposed each attempting to explain how HSV completed this process (Schwartz, J. and Roizman, B. 1969) The first can be designated the nuclear membrane envelopment (NME) model (Schwartz, J. and Roizman, B. 1969). The NME model proposed that HSV acquires a final envelope by budding through the inner nuclear membrane (INM) and subsequent trafficking through the rough endoplasmic reticulum (RER) and the Golgi network results in the processing of viral proteins and the delivery of the virus to the plasma membrane (Schwartz, J. and Roizman, B. 1969). The second was the envelopment->de-envelopment->re-envelopment (EDR) model (Stackpole, C. W. 1969). This stated that nucleocapsids undergo two distinct envelopment events, the primary one at the INM and the secondary one at the Golgi network (Stackpole, C. W. 1969).

A number of biochemical and genetic studies have since been conducted on the maturing HSV and the majority of these results are consistent with the EDR model. The final virion envelope does not resemble either the nuclear or plasma membrane bilayers. The mature virus envelope is devoid of markers for the nuclear membrane or RER but contains markers consistent with an origin at the TGN (Rodriguez, M. and Dubois-Dalcq, M. 1978; Komuro, M. et al. 1989). Viral glycoproteins retained at the ER are excluded from the virion envelope suggesting that viruses do not acquire these proteins, except by budding into the TGN (Browne, H. et al. 1996; Whiteley, A. et al. 1999; Skepper, J. N. et al. 2001). Analysis of viral particles has also demonstrated differences in the protein composition of virions in the perinuclear space compared with mature extracellular particles. Perinuclear HSV-1 contain U_L31 and U_L34 but do not appear to contain VP11/12, VP13/14 or gC. In contrast, extracellular virions are devoid of U_L31 and U_L34 but VP11/12, VP13/14 and gC are all present (Jensen, H. L. and Norrild, B. 1998; Reynolds, A. E. et al. 2002; Naldinho-Souto, R. et al. 2006). Finally, HSV mutants deleted for gD and gE demonstrated a marked inhibition in the final assembly of the virus and resulted in the accumulation of tegumented nucleocapsids in the cytoplasm of infected cells (Chapters 2 and 3). These results are consistent with the EDR model but are not readily explained by the NME model. A third model has recently been proposed which combines elements of both earlier models (Leuzinger, H. et al. 2005). The authors suggest that HSV nucleocapsids either envelop at the INM as proposed by Schwartz *et al.* or leave the nucleus via the nuclear pore and undergo envelopment at the TGN. Although this model explains the presence of enveloped viruses in the perinuclear space and nucleocapsids in the cytoplasm the proposal still fails to account for the differences in

protein and lipid content that exist between extracellular and perinuclear virions. This significant shortcoming leaves the envelopment->de-envelopment->re-envelopment model, which describes the maturation process as consisting of a primary envelopment stage at the INM followed by fusion with the ONM and a subsequent secondary envelopment stage in the cytoplasm, as the only broadly accepted description of HSV envelopment.

Three HSV encoded proteins UL31, UL34 and US3 and several cellular proteins have been demonstrated to have important roles in the process of primary envelopment and de-envelopment. UL34 is a type two integral membrane protein that complexes with UL31, a nuclear matrix-associated phosphoprotein, and this complex is found in both leaflets of the nuclear membrane of infected cells (Reynolds, A. E. et al. 2001). The UL34/UL31 complex binds lamins at the INM resulting in the localized thinning or dissolution of nuclear lamins, a step that involves protein kinase C (PKC) (Reynolds, A. E. et al. 2004; Park, R. and Baines, J. D. 2006). US3 is a virally encoded protein kinase that phosphorylates multiple viral and cellular proteins (Purves, F. C. et al. 1987; Munger, J. and Roizman, B. 2001). US3 interacts with the UL34/UL31 complex and *in vitro* data suggests multiple roles including targeting of the UL34/UL31 complex and regulating lamin disruption (Leopardi, R. et al. 1997; Ogg, P. D. et al. 2004; Ryckman, B. J. and Roller, R. J. 2004; Bjerke, S. L. and Roller, R. J. 2006; Poon, A. P. et al. 2006). In an actin-mediated step, DNA-filled capsids move from sites of herpes genome packaging to the newly thinned sites of the INM where budding into the perinuclear space occurs (Forest, T. et al. 2005). Several studies have found evidence that other HSV proteins, such as UL11, UL20, VP16 and UL25, act in envelopment at the nuclear membrane.

However, as the deletions of each protein have pleiomorphic effects difficulties arises in determining whether the observed defects are due to a direct or indirect consequence of the missing protein (Baines, J. D. et al. 1991; Baines, J. D. and Roizman, B. 1992; Mossman, K. L. et al. 2000; Stow, N. D. 2001).

HSV nucleocapsids bud through the INM resulting in an enveloped nucleocapsid situated between the INM and ONM (primary enveloped virions) (Fig. 1.5). However, in cells infected by wild type virions few particles are observed at this stage implying that the fusion event between the primary enveloped virion and the ONM occurs rapidly. Although it would be teleologically appropriate for HSV to employ similar fusion machinery in this event as is does during virus entry, individual deletions of gB, gD or gH/gL have no apparent effect on egress (Cai, W. Z. et al. 1987; Ligas, M. W. and Johnson, D. C. 1988; Forrester, A. et al. 1992; Roop, C. et al. 1993). Primary enveloped herpes viruses have a different protein composition compared with mature virus particles which may explain some of the differences in fusion requirements between these particle types (Desai, P. J. 2000; Desai, P. et al. 2001; Reynolds, A. E. et al. 2002; Ryckman, B. J. and Roller, R. J. 2004; Naldinho-Souto, R. et al. 2006). Descriptions of primary enveloped virions have indicated the presence of gM, gD, gB, VP16, US3, UL34, UL31, VP1/2, UL37 and UL11 but the absence of gC, VP11/12, VP13/14 and VP22 (Desai, P. J. 2000; Desai, P. et al. 2001; Skepper, J. N. et al. 2001; Ryckman, B. J. and Roller, R. J. 2004; Naldinho-Souto, R. et al. 2006; Wolfstein, A. et al. 2006). Deletion of either VP16 or UL11 from HSV results in some accumulation of enveloped particles between the two leaflets of the nuclear membrane, implying that each of these proteins may function in the de-envelopment process possibly through the recruitment of other factors (Baines, J. D.

and Roizman, B. 1992; Mossman, K. L. et al. 2000). Over-expression in cells of gK, a viral protein that blocks premature activation of the HSV fusogen complex, does inhibit de-envelopment of wild type primary enveloped particles (Hutchinson, L. et al. 1995). Since gK appears to regulate primary de-envelopment one possible explanation is that some common elements do exist between the HSV fusogenic apparatus employed in entry and the means by which primary enveloped particles de-envelop. Recently we began investigating the hypothesis that HSV fusogenic proteins are involved in crossing the nuclear envelope (NE). These unpublished results, presented in Appendix A, demonstrate that in the absence of both gB and gH enveloped virions accumulate in both the perinuclear space and the nucleus, similar to observations of a US3-null HSV. This observation suggests that multiple glycoproteins may be involved in de-envelopment and that gB and gH may operate redundantly in terms of fusion with the ONM.

1.5.7 Assembly in the cytoplasm

Following fusion at the ONM a partially tegumented nucleocapsid is released into the cytoplasmic space. Although the precise composition of this structure is unknown the nucleocapsid is likely associated with VP1/2, UL37 and VP16 (Mettenleiter, T. C. et al. 2006; Naldinho-Souto, R. et al. 2006). Subsequent interactions with viral proteins result in the acquisition of other tegument proteins, (Table 1.1) prior to secondary envelopment at the TGN. The envelopment process is driven by homo- and heterotypic interactions between individual tegument proteins, between tegument proteins and the nucleocapsid as well as between tegument proteins and glycoproteins embedded in the TGN.

Virus-like particles are devoid of capsids and designated L-particles. L-particles are produced on several cell lines and contain many of the tegument and envelope

proteins of a fully formed virus (Szilagyi, J. F. and Cunningham, C. 1991). Therefore, L-particle production occurs independently of capsid formation in these cells (McLauchlan, J. and Rixon, F. J. 1992) and although the functional significance of L-particle production is unclear, their production implies that tegumentaion can occur at both the nucleocapsid and the CT domains of various glycoproteins independently.

The inner tegument protein VP1/2 (UL36) is the largest tegument protein encoded by HSV. VP1/2 interacts with UL37 and binds the capsid directly through an association with VP5 (UL19) (Spear, P. G. and Roizman, B. 1972; McNabb, D. S. and Courtney, R. J. 1992; Vittone, V. et al. 2005). Deletion of either VP1/2 or UL37 abrogates the maturation of HSV and results in the accumulation of capsids in the cytoplasm; it appears likely that these two tegument proteins are the scaffold by which other tegument proteins associate with the nucleocapsid (Desai, P. J. 2000; Desai, P. et al. 2001). Yeast-two hybrid analysis and direct immunoprecipitations have detected a number of heterotypic interactions including UL11-UL16, VP1/2-UL37, VP1/2-VP16 (UL48), vhs (UL41)-VP16, VP11/12 (UL46)-VP16, VP13/14 (UL47)-VP16 and VP16- VP22 (UL49) and homotypic interactions involving US11, UL37 and UL49 (Smibert, C. A. et al. 1994; Elliott, G. et al. 1995; Vittone, V. et al. 2005). Several of these interactions may be important for the acquisition of the second envelope by the nucleocapsid while others mediate the inclusion of various proteins into the developing virion. UL11, VP16 and VP22 are three of the most abundant tegument proteins contained in HSV and have important roles in bringing the nucleocapsid and associated viral proteins together with the envelope.

UL11 functions in the acquisition of the secondary envelope and is capable of localizing both itself and other proteins to the TGN (Baines, J. D. and Roizman, B. 1992; Kopp, M. et al. 2003; Loomis, J. S. et al. 2006). UL11 is modified with both myristate and palmitate which enable perinuclear accumulation and golgi-membrane association. Furthermore UL11 also contains a dileucine motif and an acidic cluster both of which are required for UL16 association and retrieval from the plasma membrane (Loomis, J. S. et al. 2001; Loomis, J. S. et al. 2003). If UL11 is mutated to prevent fatty acid modification or deleted of either the dileucine or acidic cluster motifs UL11 is not packaged into virions(Loomis, J. S. et al. 2006). These results indicating that both membrane association, and a specific interaction with viral or cellular proteins are required for virion incorporation. Although the myristate and palmitate modification are sufficient to localize UL11 to the site of envelopment and associate with the cellular membrane these modification remain insufficient for incorporation of UL11 into virions. Replacement of the UL11 acidic cluster with a foreign acidic cluster (i.e. cellular furin or HIV Nef) abrogates the binding between UL11 and UL16 but still permits the correct retrieval and packaging of UL11, indicating that the UL11-UL16 interaction is not necessary for UL11 packaging. (Loomis, J. S. et al. 2006).

VP16 is expressed at high levels in the virus, is present in both primary and secondary enveloped virions and is essential for the acquisition of the final envelope. Virions deleted for this protein accumulate large numbers of cytoplasmic nucleocapsids as well as perinuclear virions (Mossman, K. L. et al. 2000). Interaction with VP16 is the means by which several tegument proteins are incorporated into the developing virion (Smibert, C. A. et al. 1994; Hafezi, W. et al. 2005). GST binding assays have been

published which suggest that VP16 can interact with several envelope glycoproteins including gD and gH (Gross, S. T. et al. 2003; Chi, J. H. et al. 2005). However, the lack of negative controls in these experiments makes the interpretations of this data difficult to fairly evaluate. Regardless, the diverse set of interactions with which VP16 is a part, suggest that this protein is involved in the envelopment process at multiple stages and may serve to connect the nucleocapsid and inner tegument proteins with the virion envelope.

While VP22 (UL49) deleted viruses do not display an obvious defect in envelopment the viruses have a number of growth and development defects (Elliott, G. et al. 2005; Duffy, C. et al. 2006). These viruses have a reduced capacity to replicate and spread and difficulty incorporating normal levels of several viral proteins including ICP0, gB, gD and gE (Elliott, G. et al. 2005; Duffy, C. et al. 2006). One plausible hypothesis is that the role of VP22 is to assist in localizing both glycoproteins and tegument proteins to the site of secondary envelopment

While individual tegument proteins necessary for secondary envelopment have been identified including VP1/2, UL11, VP16 and UL37 the same is not true of proteins embedded in the virion envelope (Baines, J. D. and Roizman, B. 1992; Desai, P. J. 2000; Mossman, K. L. et al. 2000; Desai, P. et al. 2001). A mutant HSV-1 deleted for any individual integral envelope protein does not display a marked defect in secondary envelopment (Ligas, M. W. and Johnson, D. C. 1988; Herold, B. C. et al. 1991; Forrester, A. et al. 1992; Hutchinson, L. et al. 1992a; Hutchinson, L. et al. 1995). Recently, studies of the related alphaherpesvirus, pseudorabies virus (PRV), demonstrated that the simultaneous deletion of two glycoproteins, gM and gE, abrogated the envelopment and

maturation process (Brack, A. R. et al. 2000). In cells infected with gM-/gE- PRV large numbers of nucleocapsids immersed in an electron dense array accumulated in the cytoplasm leading the researchers to conclude that gM and gE specifically anchored the envelope onto the nascent virion (Brack, A. R. et al. 1999; Brack, A. R. et al. 2000). The fact that envelopment could occur in the absence of either gM or gE but not in the absence of both glycoproteins implied either (i) the total numbers of glycoproteins are important for envelopment or (ii) that a specific set of interactions were being disrupted through the deletion of gM and gE. Interestingly, similar deletions in other alphaherpesviruses did not result in a similar inhibition of secondary envelopment implying that different viruses in this family have evolved different means to mediate this process (Rudolph, J. and Osterrieder, N. 2002; Browne, H. et al. 2004)(Chapters 2 and 3).

1.5.8 Egress and Cell to Cell Spread.

Once formed, HSV virions must reach the cell surface and extracellular compartments then spread to other cells. Cell-to-cell spread can occur via one of two pathways, either direct or indirect. Direct cell-to-cell spread is defined as the transmission of the virus directly to a neighboring cell without exposure of the particle to the extracellular milieu. In an epithelial monolayer this is observed when the virus infects a cell from the lateral cell junction after egressing into this space from a neighboring cell. Indirect cell-to-cell spread, on the other hand occurs when the virus is exposed to the extracellular milieu prior to initiating entry into a neighboring or distal cell. In an epithelial monolayer this spread is observed when a virus is shed from the apical layer or after cellular lysis.

In a natural HSV infection the virus initially infects the host through epithelial cells and subsequently spreads into the neuron innervating the epithelial layer and enters a latent state in the sensort ganglion. Periodic reactivation in these neurons results in localized replication and the spread of the virus down the axon leading to reinfection of the epithelial tissue. The cycle of spread from the epithelium to the underlying neuron, ganglion and then back suggests that the trafficking and egress of HSV particles occurs in a directed fashion.

A number of HSV proteins are involved in HSV egress and cell-to-cell spread. Proteins such as gD, gB and gH/gL are necessary for entry and are required for any spread of HSV (Cai, W. Z. et al. 1987; Ligas, M. W. and Johnson, D. C. 1988; Forrester, A. et al. 1992; Roop, C. et al. 1993). Unlike these proteins HSV US9 and gE/gI are involved in mediating events in virus egress and cell-to-cell spread but are not required for cell entry.

US9 is a HSV envelope protein that in the related alphaherpesvirus, PRV, is implicated in the trafficking of viral glycoproteins down the axon to the site of secondary envelopment (Tomishima, M. J. and Enquist, L. W. 2001). This mechanism is consistent with observations of US9-null HSV in neurons and epithelial cells (Polcicova, K. et al. 2005a). An HSV mutant deleted for US9 is incapable of spreading in the anterograde direction but spreads between epithelial cells and in the retrograde direction to the neuronal cell body (Polcicova, K. et al. 2005a). This result indicates that US9 is involved in mediating the trafficking of the virus possibly through direct interactions with the host-cell trafficking machinery.

HSV gE/gI mutants are deficient in direct cell-to-cell spread regardless of direction or cell type (Dingwell, K. S. et al. 1994; Dingwell, K. S. et al. 1995; Dingwell, K. S. and Johnson, D. C. 1998). HSV gE/gI participates in cell-to-cell spread by two seemingly distinct mechanisms (Wisner, T. et al. 2000; Johnson, D. C. et al. 2001; Collins, W. J. and Johnson, D. C. 2003; Farnsworth, A. and Johnson, D. C. 2006). The extracellular (ET) domains of gE/gI are necessary for spread, and likely bind receptors in order to promote the movement of HSV across epithelial cell junctions (Johnson, D. C. et al. 2001; Collins, W. J. and Johnson, D. C. 2003; Polcicova, K. et al. 2005b). This hypothesis is based on three key observations: gE/gI accumulates at cell junctions late in infection (Wisner, T. et al. 2000; McMillan, T. N. and Johnson, D. C. 2001); when expressed in *trans*, gE/gI interferes with cell-to-cell spread (Collins, W. J. and Johnson, D. C. 2003); small insertion mutations in the gE ET domain can abolish the capacity of gE/gI to promote cell-to-cell spread (Polcicova, K. et al. 2005b). The fact that gE, expressed independently of HSV at cell junctions, is capable of interfering with HSV cell-to-cell spread suggests that a saturatable gE/gI receptor exists at these junctions. This hypothesis is reinforced by the additional evidence that small insertions in the extracellular domain of the HSV gE result in a reduction of cell-to-cell spread similar to that observed in a gE-null virus. Interestingly these small mutations similarly disrupt the immunoglobulin binding capacity of gE/gI suggesting that the gE/gI receptor and immunoglobulin may share common domains (Polcicova, K. et al. 2005b).

The cytoplasmic (CT) domain of gE also has a significant role in the cell-to-cell spread of HSV. Deletion of the gE CT domain has a similar effect as deleting the entire protein in terms of cell-to-cell spread between cultured epithelial cells and in the corneal

epithelium (Wisner, T. et al. 2000; Polcicova, K. et al. 2005b). The gE CT domain encodes TGN localization signals such that gE/gI accumulates in the *trans*-Golgi network independently of other HSV-1 proteins (McMillan, T. N. and Johnson, D. C. 2001). These gE CT sequences are both necessary and sufficient for the correct localization of gE/gI, and can function independently of the rest of the gE/gI heterodimer indicating that this domain does not require any structural or signaling information from the ET domain to fulfill its trafficking functions (McMillan, T. N. and Johnson, D. C. 2001). A gE CT-null mutation results in a gE/gI protein that fails to localize to the TGN early in infection or specifically to baso-lateral surfaces late in infection and while gE/gI is still incorporated into virions the mutant protein fails to mediate cell-to-cell spread (Wisner, T. et al. 2000; McMillan, T. N. and Johnson, D. C. 2001). While these studies correlate the role of the trafficking of gE/gI and the cell-to-cell spread of the virus with mutations in the CT domain direct evidence of this was provided by electron microscopy analysis of infected epithelial cell monolayers (Johnson, D. C. et al. 2001). In this study the authors grew the epithelial cells on coverslips, subsequently fixing and embedding the cells in resin prior to removal of the coverslip. This technique allowed the preservation of apical and baso-lateral cell morphology including cell junctions which would normally be disrupted using standard fixation and embedding techniques. Electron microscopic analyses were then employed to determine the effects of various gE mutations on the delivery of HSV to either apical, lateral or basal surfaces(Johnson, D. C. et al. 2001) . These results clearly demonstrated that WT HSV was trafficked predominately to lateral cell junctions while in the absence of gE or the gE CT domain the majority of virions were found on apical cell surfaces. Furthermore this study provided direct evidence that

gE and specifically the gE CT domain was directly involved in the egress of HSV (Johnson, D. C. et al. 2001).

A model which sought to explain the involvement of gE/gI in the trafficking, envelopment and cell-to-cell spread of HSV in epithelial cells has been proposed (McMillan, T. N. and Johnson, D. C. 2001). This model describes the role of gE/gI in three stages (See Fig. 1.6). In stage 1, gE/gI accumulates in the TGN based on interactions between the gE CT domain with aspects of the cellular sorting machinery such as clathrin adaptors, PACS-1 or other cellular proteins. Other HSV glycoproteins are also targeted to this compartment and, alongwith gE/gI, drive envelopment of cytosolic nucleocapsids into TGN compartments or vesicles. In stage 2 vesicles containing HSV virions are trafficked to lateral cell surfaces. This trafficking may be dependant on signals in the gE/gI CT domains to sort these vesicles to lateral cell surfaces or may be due to gE/gI directing envelopment a subset of TGN derived vesicles that are destined for lateral cell surfaces. In stage 3, once virions arrive at lateral cell surfaces the ET domain of gE/gI mediates an interaction with a cellular receptor and efficient entry into this neighboring cell (Fig.1.6). In this dissertation I attempted to test various elements of this model and further characterize the involvement of the gE CT domain in gE/gI localization and HSV envelopment and egress.

1.6 Focus of Dissertation

This dissertation is focused on several key questions surrounding envelopment and egress in HSV-1 and the role of gE/gI in these processes. Chapter 2 addresses the role of HSV membrane proteins, specifically gE/gI and gD, in secondary envelopment. In this Chapter I characterized several HSV-1 mutants including gD-/gE-/gI-, gD-/gE- and gB-

/gE- viruses. Electron microscopic analysis demonstrated that gD and gE are required for secondary envelopment in a redundant fashion. Deletion of both glycoproteins dramatically inhibits acquisition of the final envelope. Instead, these mutants accumulate large numbers of nucleocapsids in the cytoplasm of infected epithelial cells.

In Chapter 3 I examined the contribution of the regions of the gE CT domain to the cell-to-cell spread of HSV. I identified the region of the gE CT responsible for the accumulation of gE/gI to the TGN early in HSV infection and delivery to baso-lateral surfaces late in infection. Furthermore I demonstrated that delivery of gE/gI to baso-lateral surfaces was required for the intercellular spread of HSV-1 and that accumulation of gE/gI to the TGN was insufficient to allow for efficient cell-to-cell spread.

The focus of Chapter 4 is on the contribution of the gE CT domain to secondary envelopment. In this Chapter I investigated the contributions this domain by using truncations of the gE CT domain made in Chapter 3 but inserting these into a gD- HSV. Furthermore, I identified two tegument proteins, VP22 and UL11, that bind to gE and gD and defined regions of the gE CT domain contribute to this binding.

Overall, this thesis will describe how: gE/gI together with gD is involved in HSV secondary envelopment (Chapter 2) and that the gE amino acid residues 448-470 are essential for this maturation step (Chapter 4). Additionally these studies will demonstrate that VP22 and UL11 bind to gD and gE in HSV- infected cells. Finally, this thesis will demonstrate that specific regions of the gE CT domain that are necessary for targeting HSV to baso-lateral cell surfaces and direct cell-to-cell spread of the virus (Chapter 3).

Table 1.1 HSV proteins in Virion

Location	Protein	Gene	Function or Characteristics
Capsid		UL6	Portal protein
Capsid		UL17	Capsid localization
Capsid	Vp23	UL18	Triplexes
Capsid	Vp5	UL19	Hexons and Pentons
Capsid		UL25	DNA packaging Capsid stabilization
Capsid	Vp21	UL26	Scaffolding Protein
Capsid	Vp24	UL26	Protease/Scaffolding
Capsid	Vp22A	UL26.5	Scaffolding Protein
Capsid	Vp26	UL35	Capsomere Tips
Capsid	Vp19C	UL38	Triplexes
Tegument		UL11	Envelopment and egress
Tegument		UL13	Protein kinase
Tegument		UL14	Virus transport?
Tegument		UL16	Binds to UL11
Tegument		UL17	Capsid maturation
Tegument		UL21	unknown
Tegument	Vp1/2	UL36	Capsid transport to NPC, envelopment
Tegument		UL37	Phosphoprotein
Tegument	VHS	UL41	Host shut off protein, destabilizes mRNA
Tegument	Vp11/12	UL46	Modulates VP16 activity
Tegument	Vp13/14	UL47	Modulates VP16 activity
Tegument	Vp16	UL48	TranscriptionalActivator
Tegument	Vp22	UL49	Egress and spread
Tegument		UL56	Egress
Tegument		US1	Cell specific replication
Tegument		US3	Protein kinase, capsid egress, anti-apoptosis
Tegument		US10	unknown
Tegument		US11	RNA binding, post-trancriptional regulation
Envelope	gL	UL1	Fusion, assoc. with gH
Envelope	gM	UL10	Spread, Localization, Assoc. with UL49.5
Envelope	gH	UL22	Fusion, binds to gL
Envelope	gB	UL27	Binds to GAGs, Fusion
Envelope	gC	UL44	Binds to GAGs
Envelope		UL49.5	Assoc. with gM
Envelope	gK	UL53	Regulates Fusion
Envelope	gJ	US4	unknown
Envelope	gG	US5	unknown
Envelope	gD	US6	Binds to HVEM, nectin-1,-2
Envelope	gI	US7	Spread, Binds IgG, assoc. with gE
Envelope	gE	US8	Spread, envelopment, binds IgG, assoc with gI.
Envelope		US9	Anterograde Spread in neurons

Figure 1.1 Structure of the HSV-1 virion. (**A**) Surface rendering of a single HSV virion; glycoprotein spikes (yellow) embedded in envelope (blue). (**B**) Cutaway of virion showing interior capsid (light blue), tegument (orange) envelope and glycoproteins (blue and yellow). pp Proximal pole, dp distal pole. (**C**) Segment of virion envelope showing glycoproteins (yellow), embedded in envelope (blue) contacting tegument (orange). (Adapted from (Grunewald, K. et al. 2003)).

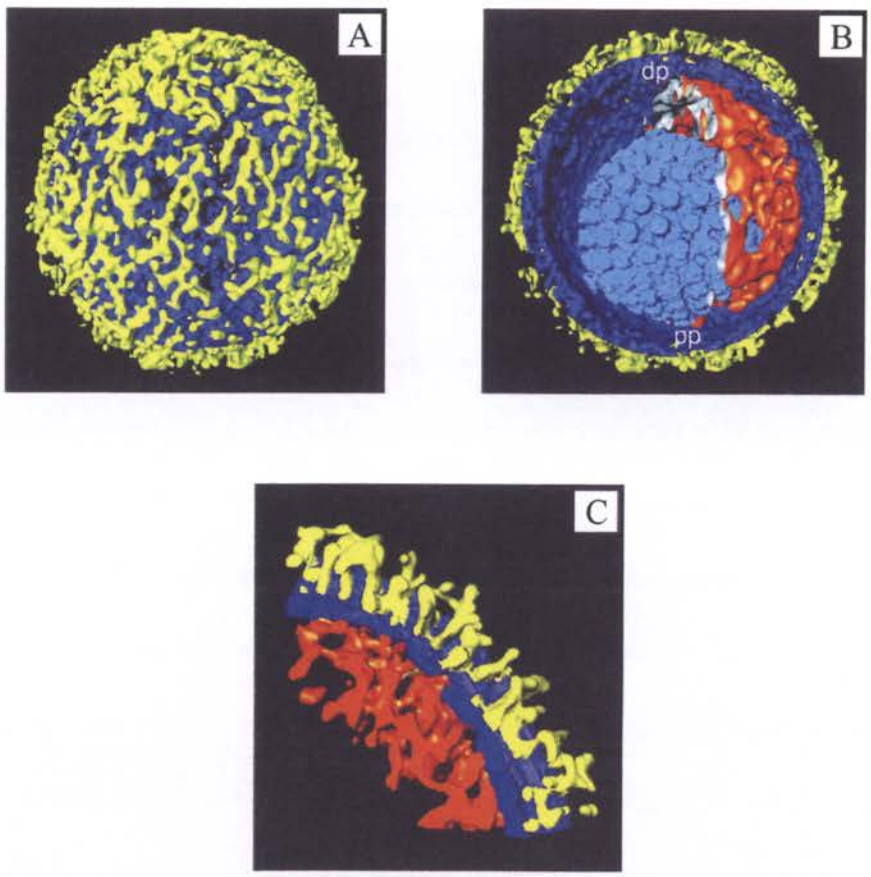
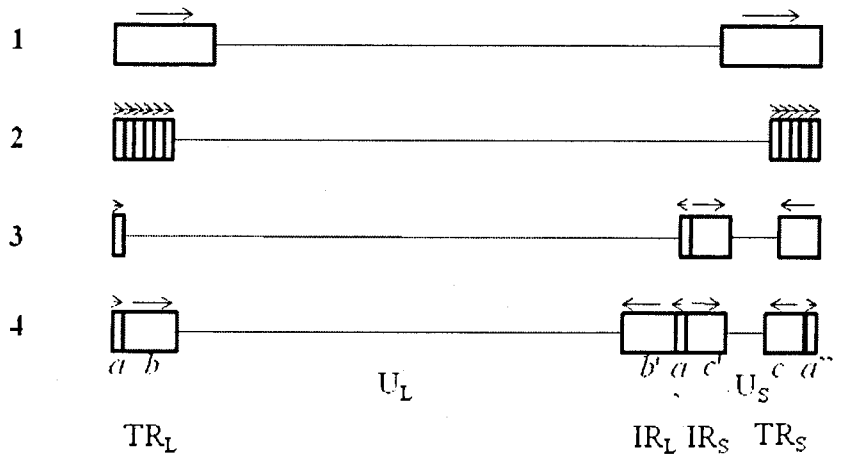

A
B
dp
pp
C

Figure 1.2 Herpesvirus Genome. **(A)** Unique (lines) and repeated (boxes) sequences are shown in schematic form. Type 1-4 genomes are illustrated. Type 1 shows a single unique sequence flanked by a direct repeat this type of genome organization is observed in HHV-6. Type 2 shows a similar organization but flanking sequences are repeated at each terminus. Examples of this structure are associated with some members of *Gammaherpesvirinae* such as HHV-8. The Type 3 structure is composed of two unique sequences flanked by inverted repeated sequences however the repeats flanking the unique long component are very short, leading to only two isomers accumulating during genome replication. This structure is associated with the genus *Varicellovirus* e.g. PRV. Type 4 structure has a unique long and unique short sequence each flanked by inverted repeat sequences. This structure results in the accumulation of 4 isomerization prodicts during genome replication and is observed in HHV-1, HHV-2 and HHV-5. U_L = unique long, U_S = unique short, TR= terminal repeat, IR = internal repeat. **(B)** Organization of conserved gene blocks from representatives of *Alphaherpesvirinae* (HHV-1 or HSV-1), *Betaherpesvirinae* (HHV-5 or HCMV) and *Gammaherpesvirinae* (HHV-5 or EBV). Approximately 40 genes are conserved among mammalian and avian herpesviruses and these are arranged into 7 distinct blocks. Although the gene blocks have different orders and orientations in different herpesvirus subfamilies, gene order within individual blocks are maintained. Gene blocks are identified with roman numerals and with HHV-1 ORFs provided (Adapted from (Davison, A. J., Eberle, R., Haywad, G.S., McGeoch, D.J., Minson, A.C., Pellet, P.E., Roizman, B., Studdert, M.J., Thiry, E. 2005)).

A


1
2
3
4
a b
U_L
b' a c
U_S
c a''
TR_L
IR_L IR_S TR_S

B

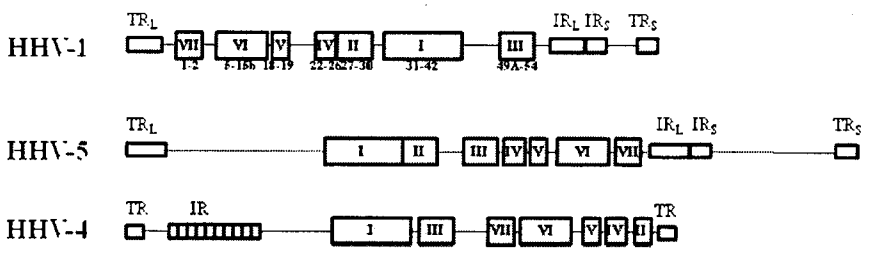

HHV-1
TR_L
IR_L IR_S
TR_S
HHV-5
HHV-4
TR
IR

Figure 1.3 HSV-1 Capsid. Surface rendering of the outer surface of the HSV-1 nucleocapsid. White/orange protrusions are pentons. Adapted from (Grunewald, K. et al. 2003).

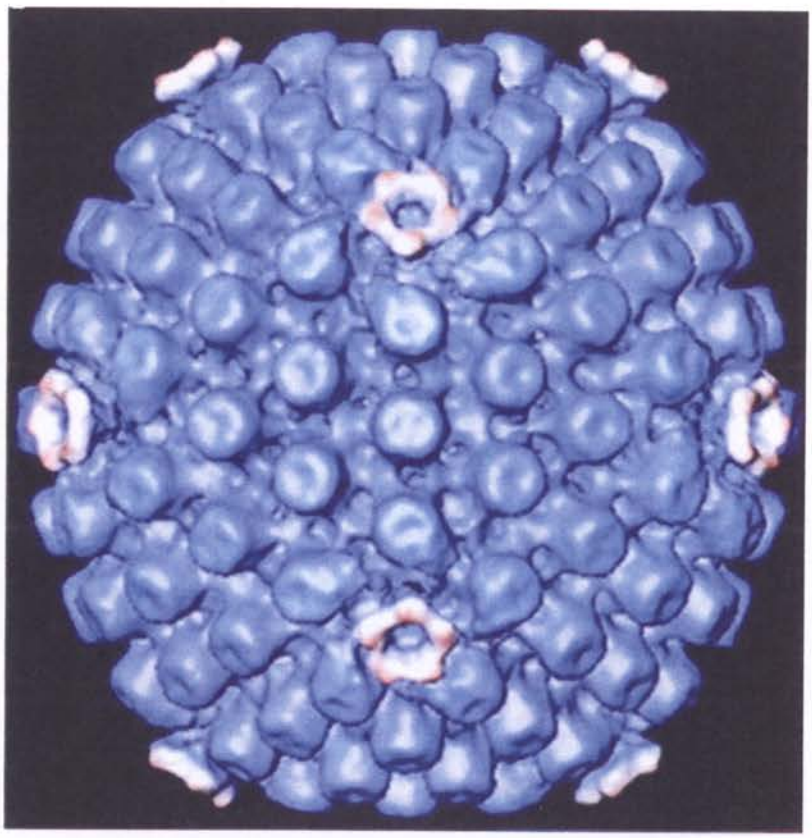


Figure 1.4 Membrane-fusion machinery of HSV (A) gD is shown in its putative dimeric confirmation as would expected in the HSV lipid bilayer prior to interaction with the cellular receptor. **(B)** Interaction with a gD receptor (Nectin-1) disrupts the gD dimer and exposes a pro-fusion domain that activates the fusion activity of gB and gH/gL. The stoichiometry or interactions sites on gD or gB and gH/gL are unclear. N-ter, amino terminus; C-ter, carboxy terminus; PFD, pro-fusion domain. Adapted from (Rey, F. A. 2006).

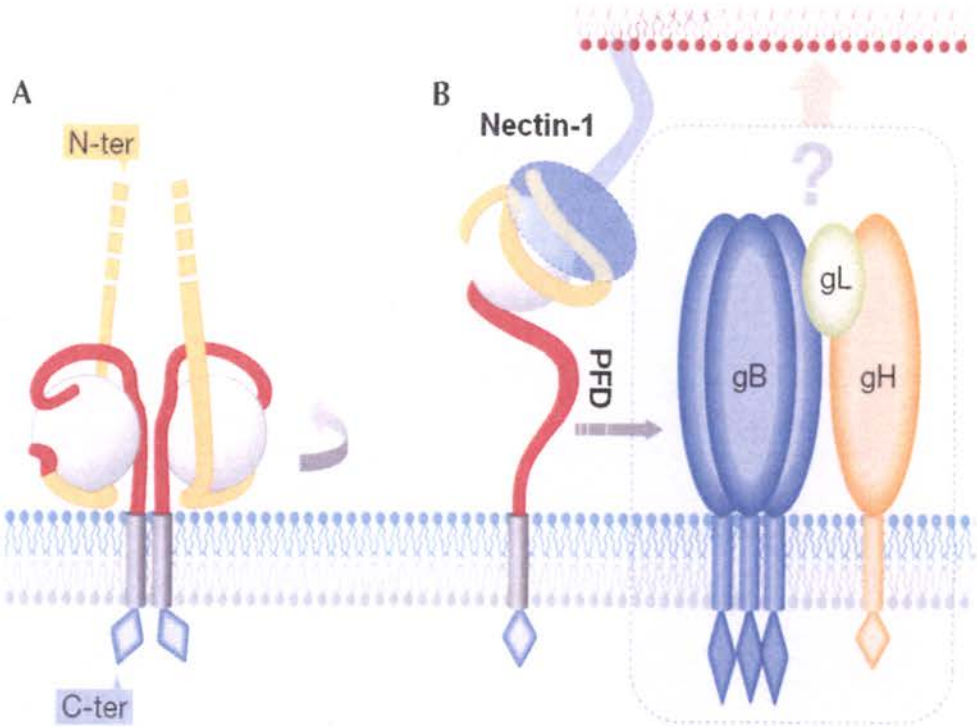

A
N-ter
C-ter
B
Nectin-1
?
gL
gB
gH
PFD

Figure 1.5. Model of HSV maturation pathway. Intranuclear capsids components assemble into preformed capsids (**1-3**). Replicated genomes are packaged into capsids and transported to the inner nuclear membrane (INM) (**4-5**) and bud into the perinuclear space (**6-7**). Fusion with the outer nuclear membrane results in the deposition of a partially tegumented nucleocapsid into the cytosol (**8**) where additional tegumentation occurs (**9-10**) At the TGN or TGN derived vesicle, glycoproteins and outer tegument proteins interact (**11**). Tegumented nucleocapsids (NC) interact with tegument proteins and the CT domains of glycoproteins resulting in the budding or secondary envelopment of the nucleocapsid (**12-13**) which is then transported to the plasma membrane whereupon fusion releases the virus to the cell surface (**14-15**). Adapted from (Mettenleiter, T. C. 2006).

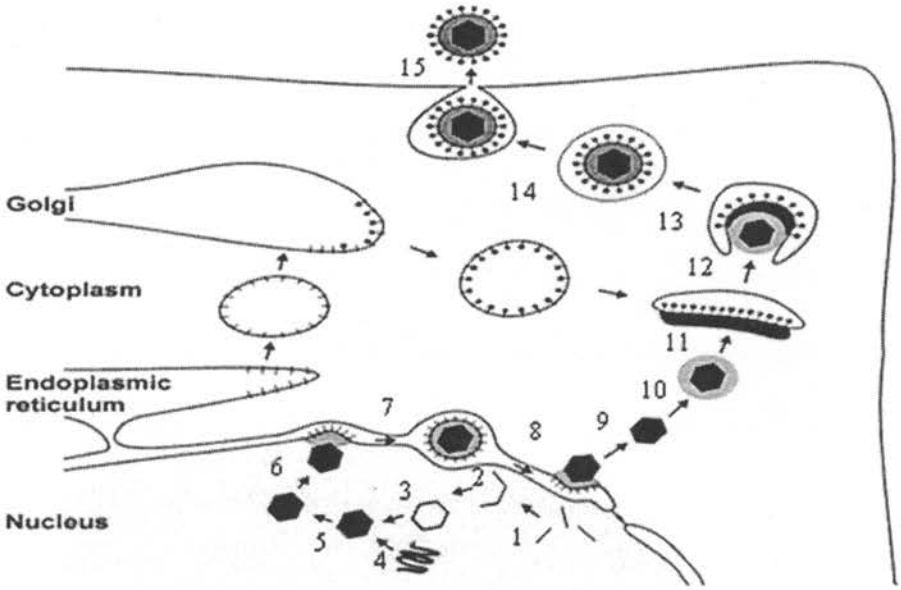
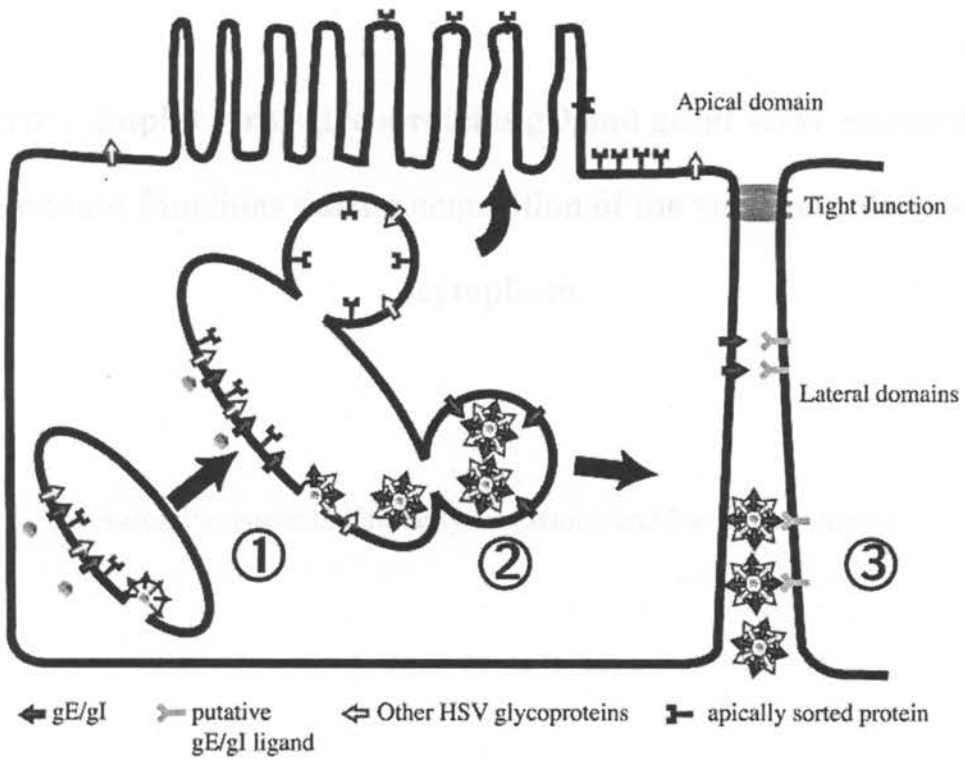

15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
Golgi
Cytoplasm
Endoplasmic reticulum
Nucleus

Figure 1.6 gE/gI in HSV envelopment and cell-to-cell spread This model shows three distint stages of the involvement of gE/gI. (**1**) gE/gI, other HSV glcyoproteins, tegument proteins and the nucleocapsid accumulate in or at the TGN. Cytoplasmic nucleocapsids bind to the CT domains of glycoproteins embedded here leading to secondary envelopment. (**2**) Vesciles-containing virions are sorted to lateral domains and involves the gE CT domain. (**3**) Tranport veicles fuse with the plasma membrane, delivering virions to the extracellular space. (McMillan, T. N. and Johnson, D. C. 2001)


Apical domain
Tight Junction
Lateral domains
①
②
③
gE/gI
putative gE/gI ligand
Other HSV glycoproteins
apically sorted protein

Chapter 2:

Herpes simplex virus glycoproteins gD and gE/gI serve essential but redundant functions during acquisition of the virion envelope in the cytoplasm

Aaron Farnsworth, Kimberly Goldsmith and David C. Johnson

J. Virol. 77:8481-8494

In the following chapter, Aaron Farnsworth carried out all experiments in all figures presented, fixed, stained and processed all samples for EM sectioning and constructed HSV vRR1097/gE-GFP-R and gB-GFP/gEβ viruses. Kimberley Goldsmith constructed the plasmid pgE-GFP as well as HSV F-gE-GFP, F-gDβ/gE-GFP, vRR1097/gEβ, F-US6kan/gE-GFP viruses and made initial observations concerning envelopment defects in the virus F-US6kan/gE-GFP. Mike Webb sectioned all samples for electron microscopy. This chapter was co-authored by David Johnson and Aaron Farnsworth.

2.1 Abstract

The late stages of assembly of herpes simplex virus (HSV) and other herpesviruses are poorly understood. Acquisition of the final virion envelope apparently involves interactions between viral nucleocapsids coated with tegument proteins and the cytoplasmic domains of membrane glycoproteins. This promotes budding of virus particles into cytoplasmic vesicles derived from the *trans*-Golgi network (TGN). The identities of viral membrane glycoproteins and tegument proteins involved in these processes remain unknown. Here, we report that HSV mutants lacking two viral glycoproteins gD and gE accumulated large numbers of unenveloped nucleocapsids in the cytoplasm. These aggregated capsids were immersed in an electron dense layer that appeared to be tegument. More subtle defects were observed with an HSV unable to express gD and gI whereas a triple mutant lacking gD, gE and gI exhibited more severe defects in envelopment. We conclude that HSV gD and the gE/gI heterodimeric complex act in a redundant fashion to anchor the virion envelope onto tegument-coated capsids. In the absence of either one of these HSV glycoproteins envelopment proceeds, however, without both gD and gE, there is profound inhibition of cytoplasmic envelopment.

2.2 Introduction

Herpes simplex virus is the best studied of the herpesviruses, and the paradigm for alphaherpesvirus replication and assembly. As with other herpesviruses, viral DNA is packaged into nucleocapsids in the nucleus (Chang, Y. E. et al. 1996). To escape the nucleus, capsids become enveloped by regions of the inner nuclear membrane (INM) and then move into the cytoplasm by fusion with the outer lamellae of the nuclear envelope (Stackpole, C. W. 1969; Skepper, J. N. et al. 2001). In the cytoplasm, nucleocapsids bind several tegument proteins (reviewed in (Mettenleiter, T. C. 2002)). Tegument-coated capsids bind onto cytoplasmic

membranes, including the TGN and endosomes, enriched in viral glycoproteins (reviewed in (Johnson, D. C. and Huber, M. T. 2002)). Nascent virions bud into the lumen of these cytoplasmic vesicles as the envelope wraps around tegumented capsids. Enveloped virions are transported to the cell surface where fusion of cytosolic vesicles with the plasma membrane delivers virus particles into the extracellular environment or onto the cell surface.

The molecular details of how alphaherpesviruses become enveloped at either nuclear or cytoplasmic membranes are poorly characterized. In the case of cytoplasmic envelopment, it is clear that viral membrane glycoproteins accumulate extensively in the TGN and this likely promotes incorporation into the virion envelope (reviewed in (Johnson, D. C. and Huber, M. T. 2002; Mettenleiter, T. C. 2002)). The cytosolic domains of these membrane glycoproteins probably provide an anchor by which tegumented nucleocapsid interact with the membrane. However, it is not clear if certain viral membrane proteins are more important than others for tethering of tegument-coated capsids onto the envelope. HSV mutants lacking individual glycoproteins known to be essential for virus replication: gD, gB, gH and gL, display no obvious defects in envelopment (Cai, W. H. et al. 1988; Ligas, M. W. and Johnson, D. C. 1988; Forrester, A. et al. 1992; Roop, C. et al. 1993). In each case, normal numbers of enveloped particles are produced, although these particles are not infectious because they lack the machinery to enter cells. When either gD or gH is modified with ER-localization signals so that they do not reach the TGN, the glycoproteins are not incorporated into nascent virions and normal numbers of enveloped particles are produced but these particles lack gD or gH (Browne, H. et al. 1996; Whiteley, A. et al. 1999; Skepper, J. N. et al. 2001). Therefore, any one of these essential glycoproteins is not necessary for cytoplasmic envelopment. Moreover, no major defects in envelopment have been noted with HSV mutants lacking other so-called "nonessential"

glycoproteins: gE, gG, gI, gJ, and gM (Baines, J. D. and Roizman, B. 1991; Balan, P. et al. 1994; Dingwell, K. S. et al. 1994). Recently, clues as to how cytoplasmic envelopment occurs came from studies of the porcine alphaherpesvirus, pseudorabies virus (PRV). Large numbers of nucleocapsids accumulated in the cytoplasm of cells infected with PRV double mutants lacking gE, or just the cytoplasmic (CT) domain of gE, and a second glycoprotein gM (Brack, A. R. et al. 1999; Brack, A. R. et al. 2000). These cytoplasmic capsids were immersed in an electron dense array that appeared to be tegument and which stained with anti-UL49 antibodies (Brack, A. R. et al. 2000). Therefore, it appears that the CT domains of PRV gE and gM act in a redundant fashion to interact with tegument proteins coating the surface of nucleocapsids, when one or other is present there is still significant envelopment.

The tegument proteins that bridge the virion envelope onto nucleocapsids are also ill-defined, although some candidates have recently come to light. Many HSV or PRV tegument proteins can be deleted without affecting virus assembly (Mettenleiter, T. C. 2002). The PRV UL49, a homologue of HSV VP22, interacts with the CT domains of both gE and gM (Fuchs, W. et al. 2002). Nevertheless, PRV UL49 can be deleted with only minor effects on virus envelopment or growth, and without affecting the subcellular localization of gE/gI or gM (Fuchs, W. et al. 2002). An HSV tegument protein VP16, the product of the UL48 gene, interacts with gH in vitro (S. Gross, C. A. Harley, and D. W. Wilson, Proc. 27th Int. Herpesvirus Workshop 2002, abstr. 7.05, 2002). Moreover, an HSV VP16 mutant exhibited significant defects in cytoplasmic envelopment, accumulating unenveloped nucleocapsids (Mossman, K. L. et al. 2000). HSV VP16 was also found to associate with gD in mild detergent extracts of purified preparations of HSV virions (Johnson, D. C. et al. 1984). Therefore, it appears that several

tegument proteins may interact with more than a single alphaherpesvirus membrane protein to promote cytoplasmic envelopment.

HSV glycoprotein gD is essential for virus entry into all cells studied to date and virus mutants lacking gD must be propagated on cells that express gD (Johnson, D. C. and Ligas, M. W. 1988; Ligas, M. W. and Johnson, D. C. 1988). By expression cloning, several gD receptors have been defined and one of these, nectin-1, is expressed extensively on biologically relevant epithelial and neuronal cells (reviewed in (Spear, P. G. et al. 2000)). An HSV-1 mutant that expresses a form of gD lacking the entire CT domain appeared to be able to access virus receptors and enter cells, but produced smaller plaques and reduced yields of infectious virus (Feenstra, V. et al. 1990). This was consistent with a subtle effect of the gD CT domain in virus replication or cell-to-cell spread.

HSV and PRV glycoproteins gE and gI form a heterodimeric complex that promotes cell-to-cell spread viruses in epithelial and neuronal tissues (Johnson, D. C. and Feenstra, V. 1987; Zuckermann, F. A. et al. 1988; Card, J. P. et al. 1992; Whealy, M. E. et al. 1993; Balan, P. et al. 1994; Dingwell, K. S. et al. 1994; Dingwell, K. S. et al. 1995; Babic, N. et al. 1996; Enquist, L. W. et al. 1998; Whiteley, A. et al. 1999). We recently proposed that HSV gE/gI can act in two phases of virus cell-to-cell spread in epithelial cells (Johnson, D. C. et al. 2001; Johnson, D. C. and Huber, M. T. 2002). In one aspect of this, gE/gI interacts with cellular components of epithelial junctions to promote the movement of virions across the cell junctions (McMillan, T. N. and Johnson, D. C. 2001; Collins, W. J. and Johnson, D. C. 2003). By a distinct mechanism, gE/gI promotes virus spread by directing nascent virions to epithelial cell junctions (Johnson, D. C. et al. 2001). Viruses deleted for gE or just the gE CT domain were mislocalized in epithelial cells, moving more substantially to apical surfaces, rather than to lateral surfaces and cell

junctions additionally these mutants revealed subtle defects in envelopment (Johnson, D. C. et al. 2001). We hypothesized that gE/gI promotes assembly of nascent virions into cytoplasmic vesicles that specifically traffic to lateral surfaces of epithelial cells, which would promote HSV cell-to-cell spread (Johnson, D. C. et al. 2001; McMillan, T. N. and Johnson, D. C. 2001).

Recently, we characterized an HSV-1 gD mutant, denoted F-US6kan, in which a kanamycin gene cassette was placed between the gD promoter and coding sequences in an effort to suppress gD expression (Johnson, D. C. and Ligas, M. W. 1988). Although F-US6kan was inhibited from direct cell-to-cell spread on most cells, due to very low levels of gD expression, the virus could spread between keratinocytes (Huber, M. T. et al. 2001). HaCaT keratinocytes express high levels of the gD receptor, nectin-1 which may contribute to the observed spread. However, there still remained the possibility that gE/gI also contributed substantially to the ability of F-US6kan to spread on keratinocytes. In order to test this we replaced the gE gene in F-US6kan with a green fluorescent protein (GFP) gene. F-US6kan/gE-GFP did not spread beyond a single infected cell, an observation that was consistent with the notion that gE/gI promoted cell-to-cell spread in the absence of gD. However, F-US6kan/gE-GFP exhibited major defects in late stages of virus assembly. Large numbers of unenveloped nucleocapsids accumulated in the cytoplasm of cells infected with this mutant. Two other HSV-1 mutants in which gD and gE, or gD, gE and gI were replaced with β-galactosidase and GFP sequences also failed to produce enveloped virus particles. We concluded that gD and gE/gI act in a redundant fashion to promote cytoplasmic envelopment of HSV.

2.3 Results

2.3.1Characterization of a virus derived from F-US6kan and lacking gE.

F-US6kan expresses extremely low levels of gD, yet can spread between human keratinocytes forming small plaques (Huber, M. T. et al. 2001). Anti-gE antibodies reduced the number of plaques formed on these cells, suggesting the possibility that gE/gI might be functioning either in entry or in cell-to-cell spread (Huber, M. T. et al. 2001). To characterize further the effects of gE/gI, we replaced gE coding sequences with GFP sequences in F-US6kan. The virus used to construct this double mutant was derived from a non-syncytial version of F-US6kan (Huber, M. T. et al. 2001). A GFP gene cassette in which GFP coding sequences were coupled to the HCMV IE promoter was inserted in place of most of the gE coding sequences in a plasmid denoted pgE-GFP (Fig. 2.1). Plasmid pgE-GFP was cotransfected along with DNA derived from F-US6kan into VD60 cells which express gD. Viruses derived from this transfection were screened for GFP expression and plaque purified. HSV recombinants were characterized for expression of gB, gD and gE by infecting HaCaT cells and labeling the cells with [^{35}S] methionine-cysteine. HSV glycoproteins were immunoprecipitated with anti-gB, -gD, or -gE monoclonal antibodies (MAb). As expected, a virus derived from this transfection and denoted F-US6kan/gE-GFP expressed gB but not gE, and only a trace of gD that was not readily apparent in these exposures (Fig. 2.2), as was described previously for F-US6kan (Huber, M. T. et al. 2001).

To determine whether F-US6kan/gE-GFP could spread on human keratinocytes, we infected HaCaT cells and determined the sizes of plaques. As previously reported (Huber, M. T. et al. 2001), F-US6kan produced on complementing VD60 cells could replicate in and spread between HaCaT cells forming small plaques (Fig. 2.3). The numbers of plaques were similar in

each case (not shown), suggesting that neither entry nor the production of infectious F-US6kan was compromised. Similarly, F-gE-GFP, which lacks gE, formed small plaques on these cells. By contrast, F-US6kan/gE-GFP was consistently unable to spread beyond a single infected cell. The numbers of these singly infected cells that stained with anti-HSV antibodies was similar to the numbers of plaques formed by F-US6kan. This suggested that F-US6kan/gE-GFP produced relatively normal numbers of infectious viruses and that were able to initiate an infection efficiently in HaCaT cells. However, without gE and with low levels of gD, F-US6kan/gEβ could not replicate or spread beyond a single infected cell.

2.3.2 F-US6kan/gE-GFP is severely restricted in the production of enveloped virus particles.

To determine whether F-US6kan/gE-GFP could replicate normally, cells infected with the mutant virus were examined by electron microscopy. HEC-1A cells infected with wild type HSV-1 displayed relatively large numbers of enveloped virus particles on the cell surfaces, the majority at cell junctions (Fig. 2.4, upper left panel). Relatively few unenveloped or enveloped particles were observed in the cytoplasm of wild type-infected cells suggesting, as in previous studies (Johnson, D. C. and Spear, P. G. 1982; Johnson, D. C. et al. 2001), that late stages of assembly and acquisition of the final envelope are rapid as is movement to the cell surface. Cells infected with F-gE-GFP, a recombinant lacking gE but expressing gD, displayed a similar pattern of enveloped virions at the cell surface, although these were frequently on apical rather than lateral surfaces (Fig. 2.4, upper right panel). In stark contrast, F-US6kan/gE-GFP-infected HEC-1A cells contained large aggregates of virus particles in the cytoplasm and very few enveloped particles (Fig. 2.4, lower panels). These aggregated particles were not enveloped (Fig. 2.4, lower right panel) and the particles were immersed in a electron dense structure which

appeared to be tegument. There were few enveloped particles observed either in the cytoplasm or on the cell surface. In some cases, a few enveloped particles were observed, as in Fig. 2.4, but often no enveloped particles were observed in sections that contained 50 or more unenveloped particles. Therefore, loss of most of gD and all of gE markedly inhibited HSV cytoplasmic envelopment.

2.3.3 Construction of HSV mutants unable to express gD and gE.

F-US6kan expresses low levels of gD which confuses the interpretation of the results. To determine whether similar defects in late virus assembly occurred when both gD and gE were not expressed, we constructed two different gD-/gE- recombinants in which the gD and gE genes were entirely or largely replaced with marker genes as explained in the materials and methods. These recombinant viruses were examined for expression of GFP or β-galactosidase and those that expressed both were characterized further. HEC-1A cells were infected with each virus, the cells radiolabelled with [^{35}S]-methionine/cysteine, and gD, gE or gB immunoprecipitated. As expected, cells infected with these viruses produced proteins of the appropriate size and type (Fig. 2.2) and the relevant genotypes of these viruses are listed in Table 2.1. Previously, Rauch et al. (Rauch, D. A. et al. 2000) constructed a recombinant HSV-1, denoted vRR1097, in which GFP sequences replaced most of the gD gene. VD60 cells were cotransfected with the plasmid pgEβ (Dingwell, K. S. et al. 1994) and vRR1097 DNA. Viruses expressing β-galactosidase and GFP were selected and one isolate denoted vRR1097/gEβ was characterized further. To construct a rescued version of vRR1097/gEβ able to express gD and gE, Vero cells were cotransfected with vRR1097/gEβ DNA and plasmid pSS17 (Ligas, M. W. and Johnson, D. C. 1988), which contains the HSV-1 Bam HI-J fragment including the gD gene and the majority of the gE gene. Recombinants that could express gD were selected by propagation on Vero cells

that do not express this essential glycoprotein. These recombinants were examined for expression of GFP and β-galactosidase and a virus, denoted vRR1097/gEβ-R that expressed neither GFP or β-galactosidase was characterized further.

A second recombinant unable to express gD and gE, and also missing gI coding sequences, was derived from F-gDβ, where all of the gD gene and the 5' end of the gI gene were replaced with β-galactosidase sequences (Ligas, M. W. and Johnson, D. C. 1988). Plasmid pgE-GFP was cotransfected with F-gDβ DNA into VD60 cells and virus isolates which expressed both β-galactosidase and GFP were selected. To characterize all of these recombinant viruses further, HEC-1A cells were infected with each virus, the cells were radiolabeled with [^{35}S]methionine-cysteine, and gD, gE, or gB was immunoprecipitated. As expected, cells infected with vRR1097 did not express gD but expressed gE and gB (Fig. 2.2A-C). vRR1097/gEβ derived from this virus expressed gB but not gD and gE. F-gDβ expressed gB and gE but not gD, and F-gDβ/gE-GFP expressed gB but not gD and gE (Fig. 2.2A-C). The rescued virus, vRR1097/gEβ-R, expressed gD, gE, and gI (Fig. 2.2D). These data are summarized in Table 2.1.

2.3.4 Assembly of HSV gD-/gE- recombinants in HEC-1A endometrial cells.

The double gD-/gE- mutant vRR1097/gEβ was characterized by electron microscopy. As before, HEC-1A cells infected with either wild type HSV-1 displayed numerous enveloped particles largely on the cell surface and at cell junctions (Fig. 2.5, upper left panel). Cells infected with the gD- mutant vRR1097 also exhibited numerous cell surface virions, although in some cells there were slightly more unenveloped particles in the cytoplasm (Fig. 2.5A, upper right panel). By contrast, vRR1097/gEβ-infected cells accumulated large numbers of virus particles in cytoplasmic aggregates (Fig. 2.5A, lower left panel). At higher magnifications, the

aggregated vRR1097/gEβ particles clearly lacked an envelope and were found immersed in an electron dense material, probably composed of tegument proteins (Fig. 2.5B, right panels). There were enveloped virions in vRR1097/gEβ-infeced cells but these were markedly reduced in numbers and some sections showed only unenveloped particles. This was quantified by counting approximately 750-1000 virus particles, unenveloped nucleocapsids and enveloped virions in the cytoplasm and on the cell surface in numerous cell sections (Table 2.2). Cytoplasmic unenveloped nucleocapsids were increased by 23 fold in vRR1097/gEβ-infected cells compared with wild type HSV-1-infected cells and the numbers of cell surface enveloped virions were reduced by 6 fold in vRR1097/gEβ-infected cells. Cells infected with vRR1097/gEβ-R, derived from vRR1097/gEβ by restoring the gD and gE genes, displayed numerous enveloped viruses on the cell surface, similar to the pattern observed with wild type HSV-1 (Fig. 2.5A, lower right panel).

Previously, we reported minor defects in the assembly of F-gEβ, an HSV-1 recombinant in which β-galactosidase sequences replace those of gE, in HEC-1A cells (Johnson, D. C. et al. 2001). In those studies and the present studies, there were more subtle 2-3 fold increases in numbers of unenveloped nucleocapsids observed in the cytoplasm (Table 2.2). Here, we characterized a second gE- mutant, F-gE-GFP, a recombinant in which GFP sequences replace gE sequences, and there were subtle increases in unenveloped particles, although most cells displayed primarily enveloped particles on the cell surface (Fig. 2.4). Somewhat more striking defects in late assembly were observed with the gD-/gI- mutant F-gDβ. HEC-1A cells infected with F-gDβ displayed aggregates of unenveloped particles in many, but not all sections, and enveloped particles were also observed in both the cytoplasm and on the cell surfaces (Fig 2.6A). This was not previously noted in Vero cells (Ligas, M. W. and Johnson, D. C. 1988) and we did

not observe obvious defects in assembly of F-gDβ in HaCaT cells (not shown). Therefore, it appears that deletion of either gE alone or gD and gI causes more subtle but still apparent defects in virus envelopment so that increased numbers of unenveloped particles accumulate but this is dependent on the host cells studied.

HEC-1A cells infected with the triple mutant, F-gDβ/gE-GFP, lacking gD, gE and gI, displayed large aggregates of cytoplasmic nucleocapsids (Fig. 2.6B, lower panels). These aggregates were similar to those observed with vRR1097/gEβ, except in many cases the sizes of these aggregates appeared to be greater with F-gDβ/gE-GFP when compared to vRR1097/gEβ. Moreover, there were few examples of enveloped particles in F-gDβ/gE-GFP-infected cells (Table 2.2). Therefore, it appeared that the additional loss of gI in a virus also missing gD and gE, further decreased the production of enveloped virions and increased the accumulation of unenveloped capsids in the cytoplasm.

2.3.5 Assembly of a gD-/gE- HSV in HaCaT keratinocytes.

To extend these observations to other cells, we infected the human keratinocyte cell line, HaCaT. These cells are derived from primary keratinocytes and are able to form differentiated epidermal tissue when transplanted into nude mice (Boukamp, P. et al. 1988) and we believe that these cells are particularly relevant for studies of HSV replication. HSV gE- mutants produce plaques that are reduced by 5-8 fold in the numbers of infected HaCaT cells compared with wild type HSV (Wisner, T. et al. 2000). HaCaT cells infected with wild type HSV-1 showed extensive numbers of enveloped virions at cell junctions (Fig. 2.7, upper left panel). Similarly, vRR1097 (gD-) also produced relatively normal numbers of virus particles and these were again largely on the cell surface (Fig. 2.7, upper right panel). The double gD-/gE- mutant vRR1097/gEβ produced large numbers of unenveloped virions that accumulated in an electron

dense matrix in the cytoplasm of HaCaT cells (Fig. 2.7, lower panels). Few or no enveloped particles were produced. Therefore, as with HEC-1A cells, a gD-/gE- virus failed to produce unenveloped, cytoplasmic nucleocapsids in HaCaT cells.

2.4 Discussion

Herpes simplex viruses acquire two different envelopes. Nucleocapsids interact with the inner nuclear membrane, budding into the space between the inner and outer nuclear envelopes. Fusion of the virion envelope with the outer nuclear membrane delivers nucleocapsids into the cytoplasm. Tegument-coated proteins interact with the CT domains of HSV membrane glycoproteins that accumulate in the TGN promoting the budding of capsids into cytoplasmic vesicles. This secondary envelopment occurs relatively rapidly and efficiently with HSV-1, as few nucleocapsids are observed in the cytoplasm of most cultured cells (Johnson, D. C. and Spear, P. G. 1982; Campadelli-Fiume, G. et al. 1991; Johnson, D. C. et al. 2001; Skepper, J. N. et al. 2001). Apparently, no single HSV-1 glycoprotein is essential for either nuclear or cytoplasmic envelopment. There are no major defects in assembly of enveloped particles with HSV-1 mutants lacking any membrane glycoproteins (Cai, W. H. et al. 1988; Ligas, M. W. and Johnson, D. C. 1988; Forrester, A. et al. 1992; Roop, C. et al. 1993; Balan, P. et al. 1994; Dingwell, K. S. et al. 1994). The important work of Brack et al. (Brack, A. R. et al. 1999; Brack, A. R. et al. 2000) showing that PRV depends upon two glycoproteins, gM and gE suggested an hypothesis that 2 or more ⟨-herpesvirus membrane proteins interact with more than a single tegument protein to anchor the virion envelope onto nucleocapsids.

Here, we demonstrated that HSV-1 depends upon gD and gE for secondary envelopment. HSV-1 mutants expressing low levels of or no gD and no gE produced very few enveloped virions. Another HSV-1 recombinant lacking gD, gE and gI exhibited a similar, but more

profound, phenotype. In every case, large numbers of unenveloped nucleocapids accumulated in the cytoplasm in an electron dense milieu which appeared to be tegument. There were no obvious effects on nuclear envelopment and no obvious accumulation of capsids in the nucleus or perinuclear space was observed. Therefore, gD and gE serve essential but redundant functions in cytoplasmic envelopment.

Previously, we noted minor defects in envelopment when characterizing HSV-1 mutants lacking gE or just the CT domain of gE (Johnson, D. C. et al. 2001). There were 2-3 fold increased numbers of cytoplasmic nucleocapsids compared with wild type HSV-1 and here we observed similar defects with a second gE- mutant. Moreover, deletion of the CT domain of gD also reduced production of infectious virus and plaque size (Feenstra, V. et al. 1990). However, these effects observed in single null-mutants were relatively mild, compared to the much more severe phenotype of a mutant lacking both gD and gE.

There also appeared to be a contribution of the gI glycoprotein. Careful analysis of a mutant F-gDβ which lacks gD and gI showed that increased numbers of unenveloped nucleocapsids accumulated in HEC-1A cells compared with wild type HSV, although significant numbers of enveloped virions were also observed. Nevertheless, reduced envelopment was not observed in F-gDβ-infected HaCaT (here) or Vero cells (Ligas, M. W. and Johnson, D. C. 1988). Loss of gD and gE produced massive accumulation of unenveloped capsids in virtually every cell, whereas many cells infected with F-gDβ exhibited few unenveloped capsids. However, loss of gD, gE and gI in the triple F-gDβ/gE-GFP lead to a more severe phenotype than was observed with the gD-/gE- mutant, even fewer numbers of enveloped capsids were observed. Therefore, we concluded that gI also contributes, perhaps in a more subtle manner than gE, to cytoplasmic envelopment.

In contrast to these results with HSV-1, a PRV quadruple: gD-, gE-, gI-, gG- mutant did not produce drastically reduced numbers of enveloped virions and they could be purified and characterized by immunoelectron microscopy (Mettenleiter, T. C. et al. 1994). Evidently, HSV and PRV differ in their requirements for envelopment. There have been other differences described for how gD functions for HSV and PRV. PRV gD is not required for spread of viruses between cells, once gD-complemented virus particles are able to enter cells spread occurs both in cultured cells and animal tissues (Rauh, I. and Mettenleiter, T. C. 1991; Peeters, B. et al. 1992; Peeters, B. et al. 1993). In contrast, HSV gD- mutants do not spread beyond a single infected cell either in cultured cells or in animal models (Johnson, D. C. and Ligas, M. W. 1988; Ligas, M. W. and Johnson, D. C. 1988; Peeters, B. et al. 1993; Dingwell, K. S. et al. 1995). Therefore, these differences may be as illuminating as the many similarites between PRV and HSV, but caution against drawing parallels in every case.

To begin to understand how the virion envelope interacts with tegument, Fuchs et al. have characterized interactions between the PRV UL49 gene, a homologue of HSV VP22, and gE and gM (Fuchs, W. et al. 2002). Both gE and gM interacted with UL49 in yeast two hybrid screens and a PRV gE- /gM- deletion virus failed to incorporate UL49 into virions. This might have suggested that UL49 was essential for envelopment, as PRV gE and gM appear to be. However, PRV VP22 can be deleted without affecting envelopment, or even neurovirulence (del Rio, T. et al. 2002; Fuchs, W. et al. 2002). Thus, interactions between gE and gM and other PRV tegument proteins are also apparently important for late assembly. In other ⟨-herpesviruses, bovine herpesvirus type 1 (BHV-1) VP22 is important for virus replication and neurovirulence (Liang, X. et al. 1997). With HSV, the C-terminus of VP22 appears to be critical for incorporation into virions, and for cell-to-cell spread (Pomeranz, L. E. and Blaho, J. A. 2000).

However, there is presently no HSV VP22 null mutant to test the notion that VP22 is essential for envelopment. It appears that HSV gE, like the PRV counterpart, interacts with VP22 as we detected a 37 kDa viral protein, corresponding to the size of HSV-1 VP22, bound onto a glutathione-S-transferase fusion protein containing the gE CT domain (T. Wisner and D.C. Johnson, unpublished results). Recently, the cytoplasmic tail of HSV gD was found to interact with VP22 *in vitro* (Gross et al., Proc. 27th Int. Herpesvirus Workshop 2002) which provides the possibility that HSV gD and gE both interact with VP22. Unfortunately, the contribution of gM in envelopment has not been tested in HSV by the construction of either gE-/gM- or gD-/gM- double mutants.

A second HSV tegument protein VP16 appears to play an important role in cytoplasmic envelopment. An HSV-1 recombinant unable to express VP16 (and the virion host shutoff protein vhs) produced few enveloped virions, and unenveloped particles accumulated in the cytoplasm, suggesting that VP16 is essential or important for cytoplasmic envelopment, but not for nuclear envelopment (Mossman, K. L. et al. 2000). There is evidence that the cytoplasmic domain of HSV gH interacts with VP16 *in vitro* (Gross et al., Proc. 27th Int. Herpesvirus Workshop 2002), supporting the notion that gH may also play a role in envelopment. Moreover, VP16 was found to be physically associated with gD extracted from virions with mild nonionic detergents and purified by antibody affinity chromatography (Johnson, D. C. et al. 1984). Therefore, it is plausible that HSV cytoplasmic envelopment involves interactions between gE and gD with VP22 and between gH and gD with VP16, although loss of any individual interaction may not be entirely fatal. Clearly, loss of both gE and gD dramatically diminishes envelopment. However, it is also reasonable that HSV glycoproteins other than gD and gE/gI are involved in cytoplasmic envelopment e.g. gB and gH and there may be yet other interactions

for nuclear envelopment. It will require other double and triple glycorprotein mutants to define this further.

2.5 Materials and Methods

2.5.1 Cells and viruses.

HEC-1A, human endometrial epithelial cells (Ball, J. M. et al. 1995) were grown in RPMI medium (BioWhittaker Inc., Walkersville, Md.) supplemented with 8% fetal bovine serum (FBS). HaCaT, human keratinocyte cells derived from a culture of primary keratinocyes (Boukamp, P. et al. 1988) and Vero cells were grown in Dulbecco's modified Eagles Medium (DMEM; BioWhittaker Inc., Walkersville, Md.) supplemented with 8% FCS. VD60 cells were maintained in DMEM lacking histidine and supplemented with 0.4-1.0 mM histidinol and 8% FCS (Sigma Chemical Co., St. Louis, Mo.). Wild type HSV-1 strain F (originally from Pat Spear, Northwestern Medical School) and the HSV-1 gE- recombinants derived from F: F-gEβ (Dingwell, K. S. et al. 1995) and F-gE-GFP (produced here) were propagated and titered on Vero cells. gD- recombinants derived from F, F-US6kan (Johnson, D. C. and Ligas, M. W. 1988; Dingwell, K. S. et al. 1995; Huber, M. T. et al. 2001), F-gDβ, which lacks gD and gI sequences (Ligas, M. W. and Johnson, D. C. 1988) and vRR1097 that has much of the gD coding sequences replaced by GFP sequences (Rauch, D. A. et al. 2000), as well as gD-/gE- recombinants described below, were propagated and titered on gD-expressing VD60 cells (Ligas, M. W. and Johnson, D. C. 1988).

2.5.2 Construction of gD-/gE- recombinants.

In order to insert green fluorescent protein (GFP) sequences in place of gE sequences, a plasmid, denoted pgE-GFP was constructed from pUC US7/8, which contains a 3.1 kb fragment of the US region of HSV-1 F DNA encompassing the US7 (gI) and US8 (gE) genes (Wisner, T.

et al. 2000). Plasmid pUC US7/8 was digested to completion with restriction endonuclease *SmaI*, removing the first 700 base pairs of coding sequence of US8, creating the plasmid pUC US7/8Δ*SmaI*. GFP coding sequences were subcloned from the plasmid pEGFP-C1 (Clontech; GenBank Acc. # U55763), in which GFP sequences were coupled to the HCMV immediate early promoter, by using PCR. Two primers were employed:
'ACGCCCCGGGTGCATTAGTTATTAATAGT AATCAAT' and
'TCCCCCCGGGTTACTTGTACAGCTCGTCCATGC' adding Sma I sites at either end of GFP sequences. The PCR product was sequenced, digested with *SmaI* and inserted into the SmaI site of pUC US7/8Δ*SmaI* creating pgE-GFP. Three gE- derivatives were produced using pgE-GFP. VD60 cells were cotransfected with wild type HSV-1 F, F-US6kan or F-gDβ DNA as well as plasmid pgE-GFP and viruses that expressed GFP isolated, producing three recombinants: F-gE-GFP, F-US6kan/gE-GFP and F-gDβ/gE-GFP. vRR1097/gEβ was constructed from vRR1097 (Rauch, D. A. et al. 2000) by cotransfecting VD60 cells with vRR1097 DNA and plasmid pgEβ in which β-galactosidase sequences replace gE coding sequences (Dingwell, K. S. et al. 1994) and viruses that expressed β-galactosidase characterized. A rescued version of this virus was created by cotransfection of vRR1097/gEββ DNA and plasmid pSS17 (Ligas, M. W. and Johnson, D. C. 1988) vero cells.

2.5.3 Staining of HSV-infected cells.

HaCaT cells with HSV plaques were washed, fixed and stained with polyclonal anti-HSV-1 antibodies, peroxidase conjugated secondary antibodies and peroxidase substrate as previously described (Wisner, T. et al. 2000). Recombinant viruses expressing β-glactosidase were identified after overlaying cells with agarose containing X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) as previously described (Ligas, M. W. and Johnson, D. C. 1988).

Recombinant viruses expressing GFP were identified by exposing infected cell monolayers to 521 nm fluorescent light visualized with a Nikon Optiphot fluorescence microscope.

2.5.4 Radiolabelling of infected cells and immunoprecipitation.

HaCaT cells were left uninfected or were infected with wild type or mutant HSV-1 using 5 PFU/cell. After 2 h the cells were labelled with [^{35}S] methionine-cysteine (Amersham) (50μCi/ml) for 5 h, cell extracts were made using NP-40-deoxycholate (DOC) lysis buffer (100mM NaCl, 50mM Tris-HCl [pH7.5], 1.0% NP-40, 0.5% DOC) supplemented with 2 mg of bovine serum albumin/ml and 1 mM phenylmethylsulfonyl fluoride and the extracts frozen at -70°C. HSV-1 gD was immunoprecipitated using monoclonal antibody (MAb) DL6 (Eisenberg, R. J. et al. 1985), gE was precipitated using MAb 3114 (Dingwell, K. S. et al. 1994), and gI was preciptated using MAb 3104 (Johnson, D. C. et al. 1988). The immunoprecipitated cell extracts were eluted and subjected to electrophoresis as described (Tomazin, R. et al. 1996). The dried gels were placed in contact with a phosphoimager and viral proteins were quantified.

2.5.5 Electron Microscopy

HEC-1A or HaCaT cells were infected with wild type or mutant HSV-1 for 16 hr then washed with 0.1M sodium cacodylate buffer (pH 7.2) and fixed in Ito and Karnovskys fixative (1.6% paraformaldehyde, 2.5% glutaraldehyde, 0.5% picric acid all buffered in 0.1M sodium cacodylate). Samples were postfixed in 1.5% osmium tetroxide then rinsed and then postfixed in 4% paraformaldehyde. Samples were dehydrated in a graded acetone series, embedded in epoxy resin and ultrathin sections were double stained in uranyl acetate and lead citrate and viewed with a Philips EM 300 electron microscope.

TABLE 2.1. Virus Expression of relevant proteins

Virus	gD	gE	gI
FUS6kan	Low [a]	WT	WT
FUS6kan/gE-GFP	Low [a]	Null	WT
vRR1097	Null	WT	WT
vRR1097/gEβ	Null	Null	WT
F-gDβ	Null	WT	Null
F-gDβ/gE-GFP	Null	Null	Null
vRR1097/gEβ R	WT	WT	WT
F-1	WT	WT	WT

a As shown in Huber et al gD is expressed at approximately 1/500th the level as observed in wild type HSV (Huber, M. T. et al. 2001)

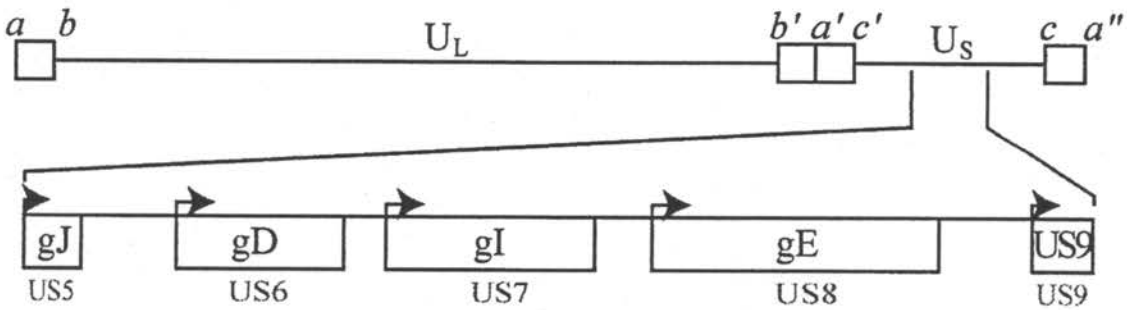
TABLE 2.2. Distribution of virus particles produced by wild-type and mutant HSV-1 in HEC-1A cells[a]

Virus	Nuclear Nucleocapsids[b]	Cytoplasmic Nucleocapsids[b]	Cytoplasmic Enveloped Virions[b]	Cell Surface Enveloped Virions[b]
Wild type	393 (43.9%)	**30 (3.3%)**	36 (4.0%)	**435 (48.6%)**
F-gEβ	335 (44.5%)	75 (9.9%)	97 (12.8%)	246 (32.6%)
vRR1097	414 (52.5%)	36 (4.6%)	45 (5.7%)	293 (37.1%)
vRR1097/gEβ	268 (25.2%)	**695 (65.6%)**	12 (1.2%)	**85 (8%)**
F-gDβ/gE-GFP	374 (22.4%)	**1227 (73.4%)**	32 (1.9%)	**38 (2.3%)**
VRR1097/gEβ-R	389 (56.3%)	44 (6.3%)	38 (5.4%)	220 (31.8%)

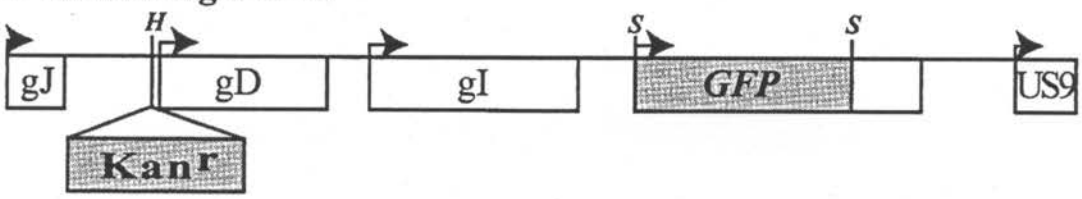
[a] Human HEC-1A cells were infected with wild type or mutant HSV-1 for 16 h. The cells were fixed, sectioned and then examined by electron microscopy.

[b] The numbers of unenveloped nucleocapsids in the nucleus and in the cytoplasm and enveloped virions in the cytoplasm and on the cell surface were counted in approximately 10-15 randomly chosen cells. The numbers in parentheses are the percentages of the total number of particles.

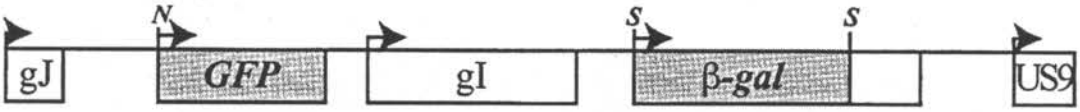
Figure 2.1 Construction of recombinant viruses. The genome of HSV-1 including the US5 (gJ), US6 (gD), US7 (gI), US8 (gE) and US9 genes, is depicted in the upper part of the figure. F-US6kan/gE-GFP was derived from F-US6kan by replacing gE coding sequences with GFP sequences between two SmaI restriction sites. vRR1097/gEβ was derived from vRR1097 (in which GFP sequences replace gD coding sequences) by replacing gE coding sequences with β-galactosidase sequences. vRR1097/gEβ-R was derived from vRR1097/gEβ by restoring both gD and gE sequences. F-gDβ/gE-GFP was derived from F-gDβ (a mutant in which β-galactosidase sequences replace gD and gI coding sequences) by additionally replacing gE coding sequences with GFP sequences. H represents a HindIII restriction site, S represents SmaI sites, B represents BalI and N represents an NcoI restrictions site.



F-US6kan/gE-GFP



vRR1097/gEβ



vRR1097/gEβ-R

F-gDβ/gE-GFP

Figure 2.2 Expression of gD, gE, gB and gI by recombinant HSV-1. Human keratinocyte HaCaT cells were infected with wild-type HSV-1, F-US6kan/gE-GFP, F-US6kan, F-gE-GFP, vRR1097, vRR1097/gEβ, F-gDβ, F-gDβ/gE-GFP, F-gEβ or were left uninfected. The cells were labelled with [35S]-methionine/cysteine and gD, gE, gB or gI immunoprecipitated from cell extracts using monoclonal antibodies: DL6, 3114, 15βB2, or 3104, respectively. Antigen-antibody complexes were collected using protein A-Sepharose and eluted proteins subjected to electrophoresis on 12% polyacrylamide gels. The positions of mature and immature forms of gE, gD, and gB, as well as marker proteins of 200, 97.4, 68, 43, 29 and 18.4 are indicated.

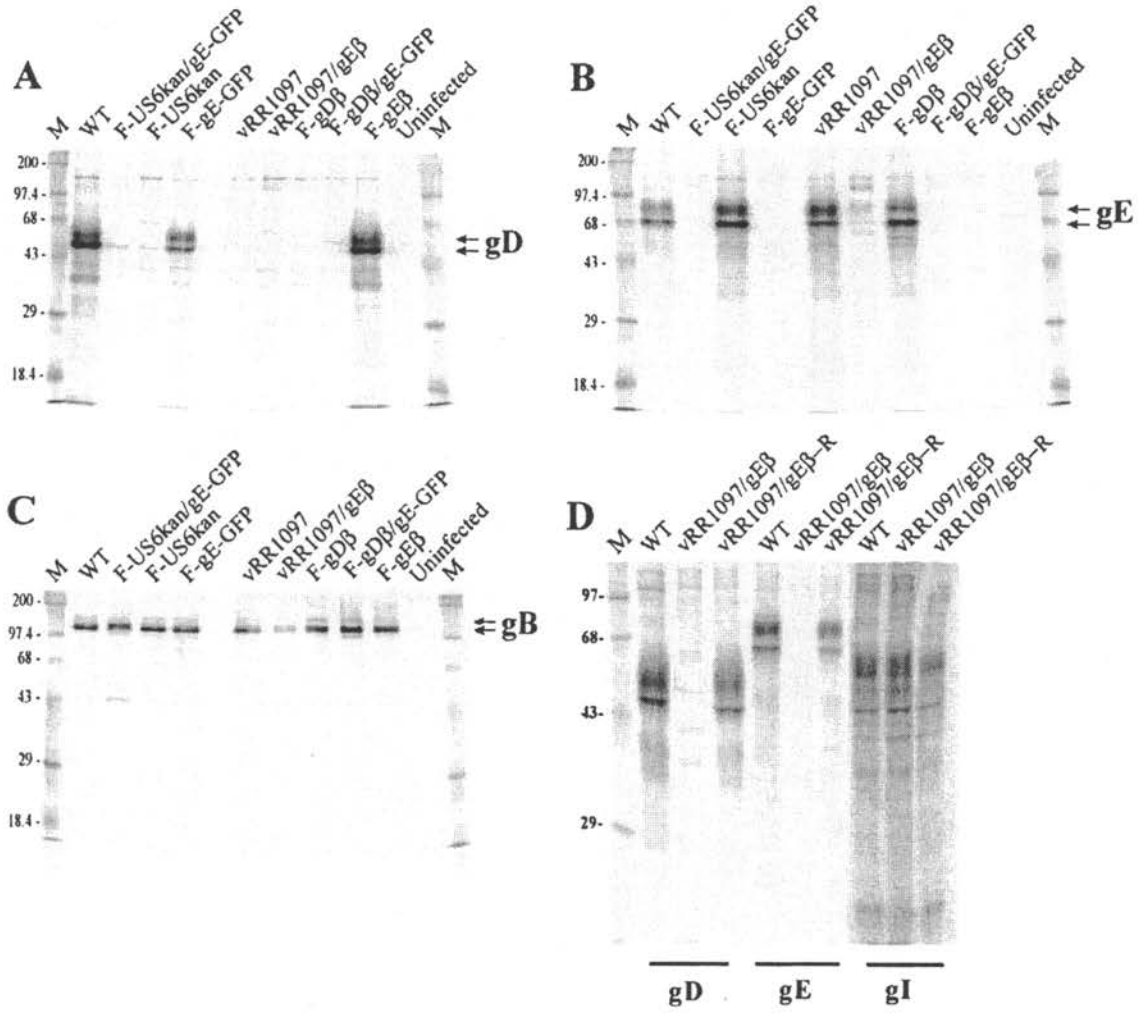

A
M
WT
F-US6kan/gE-GFP
F-US6kan
F-gE-GFP
vRR1097
vRR1097/gEβ
F-gDβ
F-gDβ/gE-GFP
F-gEβ
Uninfected
M
200
97.4
68
43
29
18.4
gD
B
gE
C
gB
D
vRR1097/gEβ
vRR1097/gEβ–R
97
68
43
29
gD
gE
gI

Figure 2.3 Plaques formed by F-US6kan and F-US6kan/gEβ. HaCaT cells were infected with wild-type HSV-1, F-gE-GFP, F-US6kan or F-US6kan/gE-GFP. In the case of F-US6kan and F-US6kan/gE-GFP virus stocks were prepared using gD-complementing VD60 cells. After 2 days, the cells were stained with anti-HSV antibodies and peroxidase-conjugated secondary antibodies.

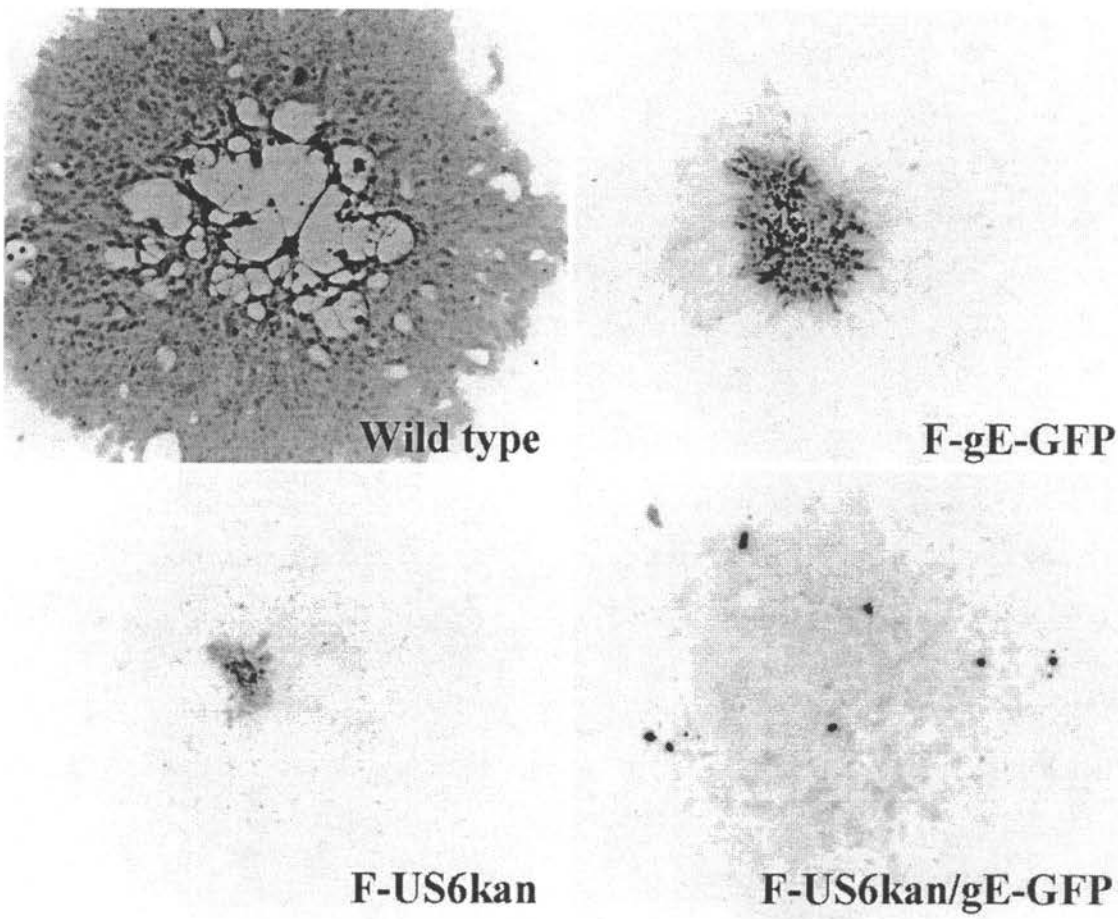
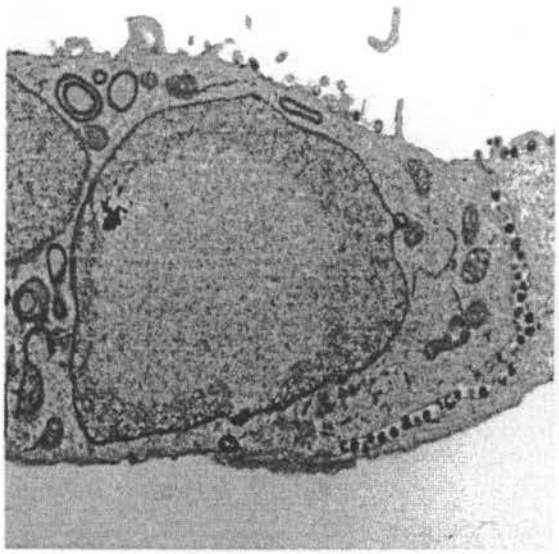
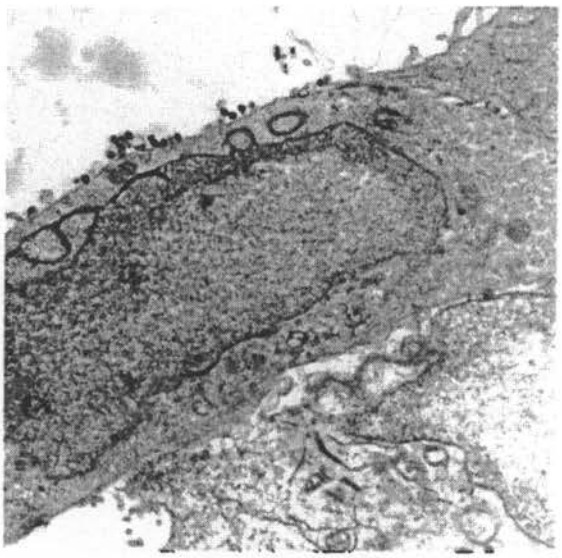

Wild type
F-gE-GFP
F-US6kan
F-US6kan/gE-GFP

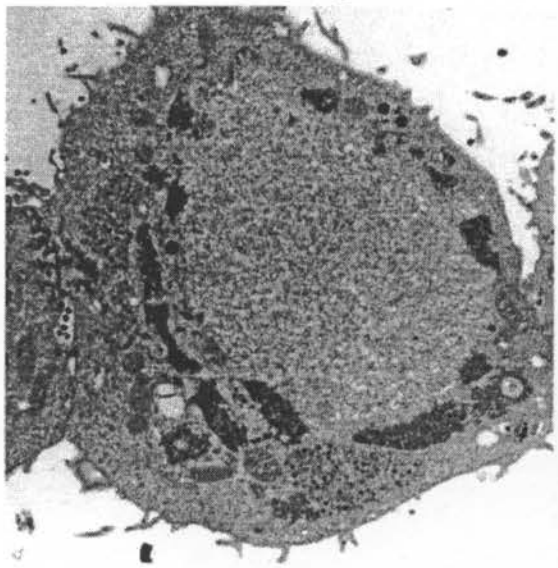
Figure 2.4. Electron micrographs of F-US6kan/gE-GFP-infected HEC-1A cells. HEC-1A cells were infected with wild-type HSV-1 (upper left panel), F-gE-GFP (upper right panel) or F-US6kan/gEGFP (lower panels) for 16 h. The cells were fixed and processed for electron microscopy.

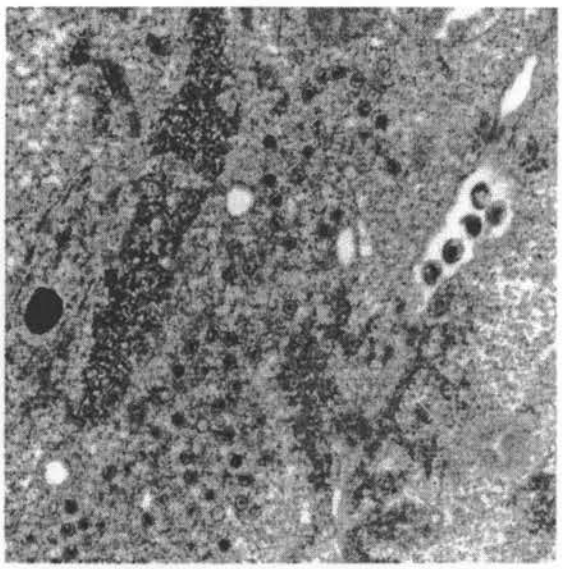


Wild type



F-gE-GFP

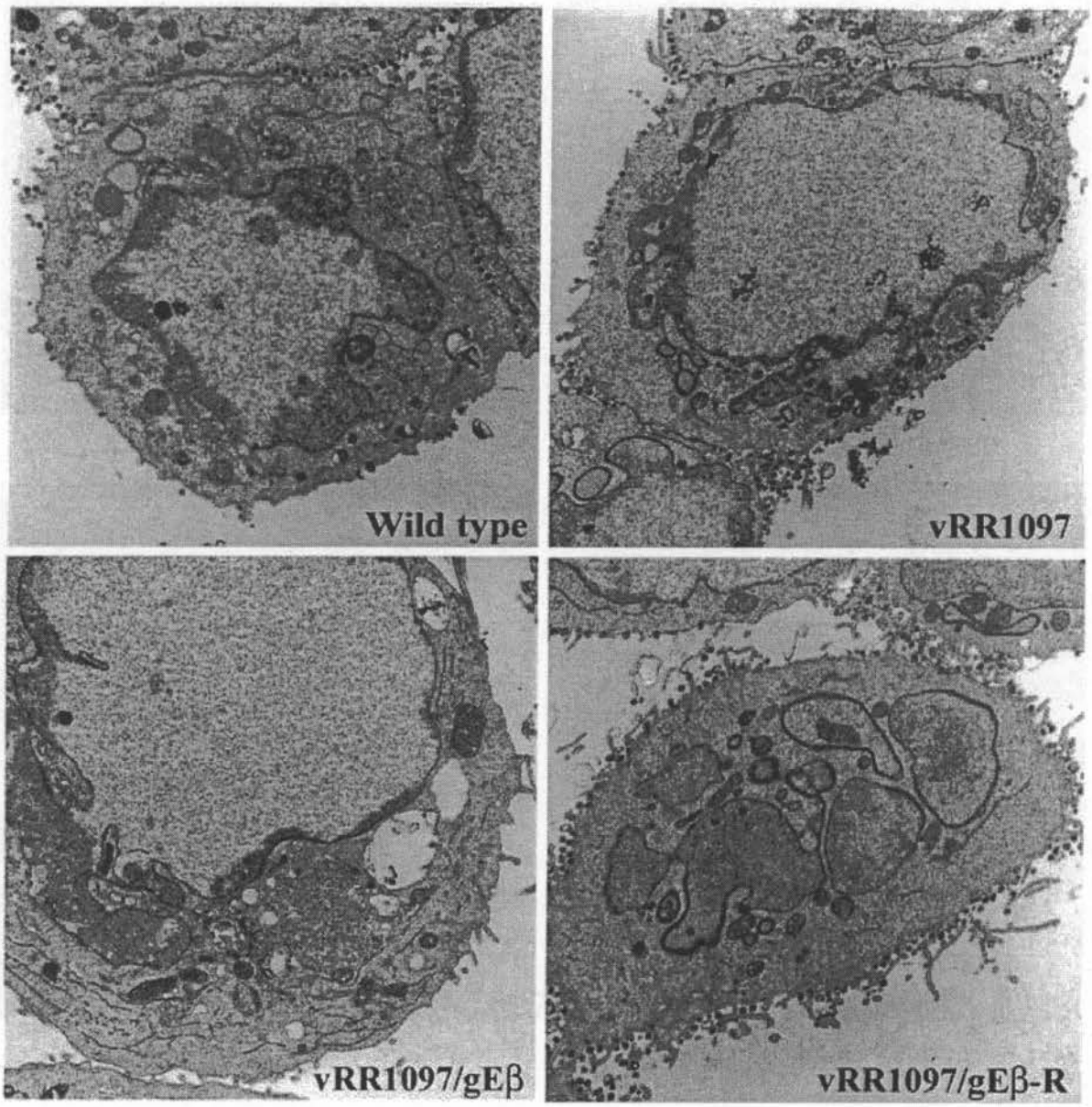




F-US6kan/gE-GFP

Figure 2.5. Electron micrographs of HEC-1A cells infected with F-gDβ/gE-GFP. **(A)** HEC-1A cells were infected with wild type HSV-1, vRR1097 (gD-), vRR1097/gEβ (gD-/gE-) or vRR1097/gEβ-R (a rescued version of vRR1097/gEβ). **(B)** HEC-1A cells were infected with wild type HSV-1 or vRR1097/gEβ. After 16 to 18 h the cells were fixed and processed for electron microscopy.

A



B

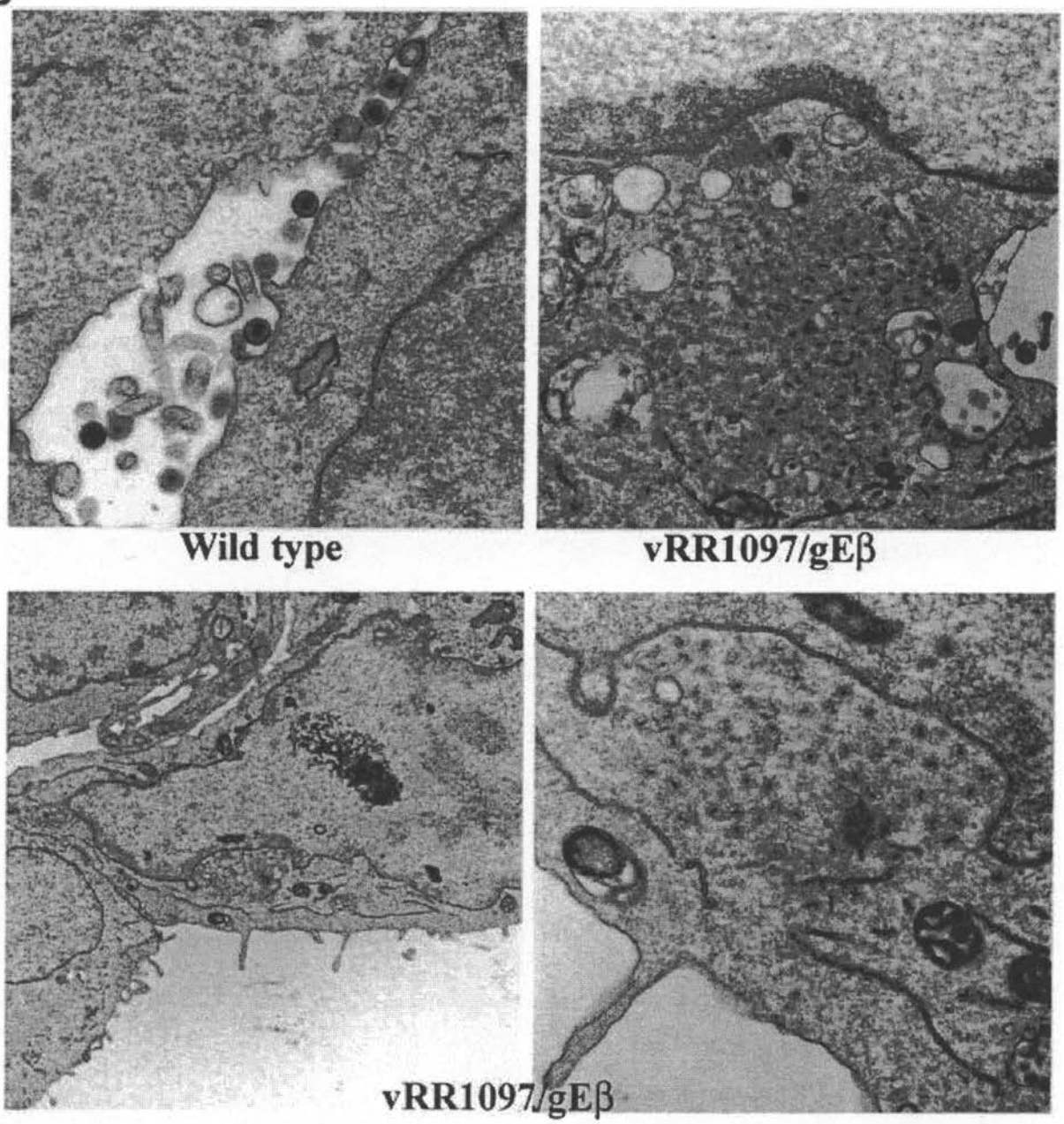
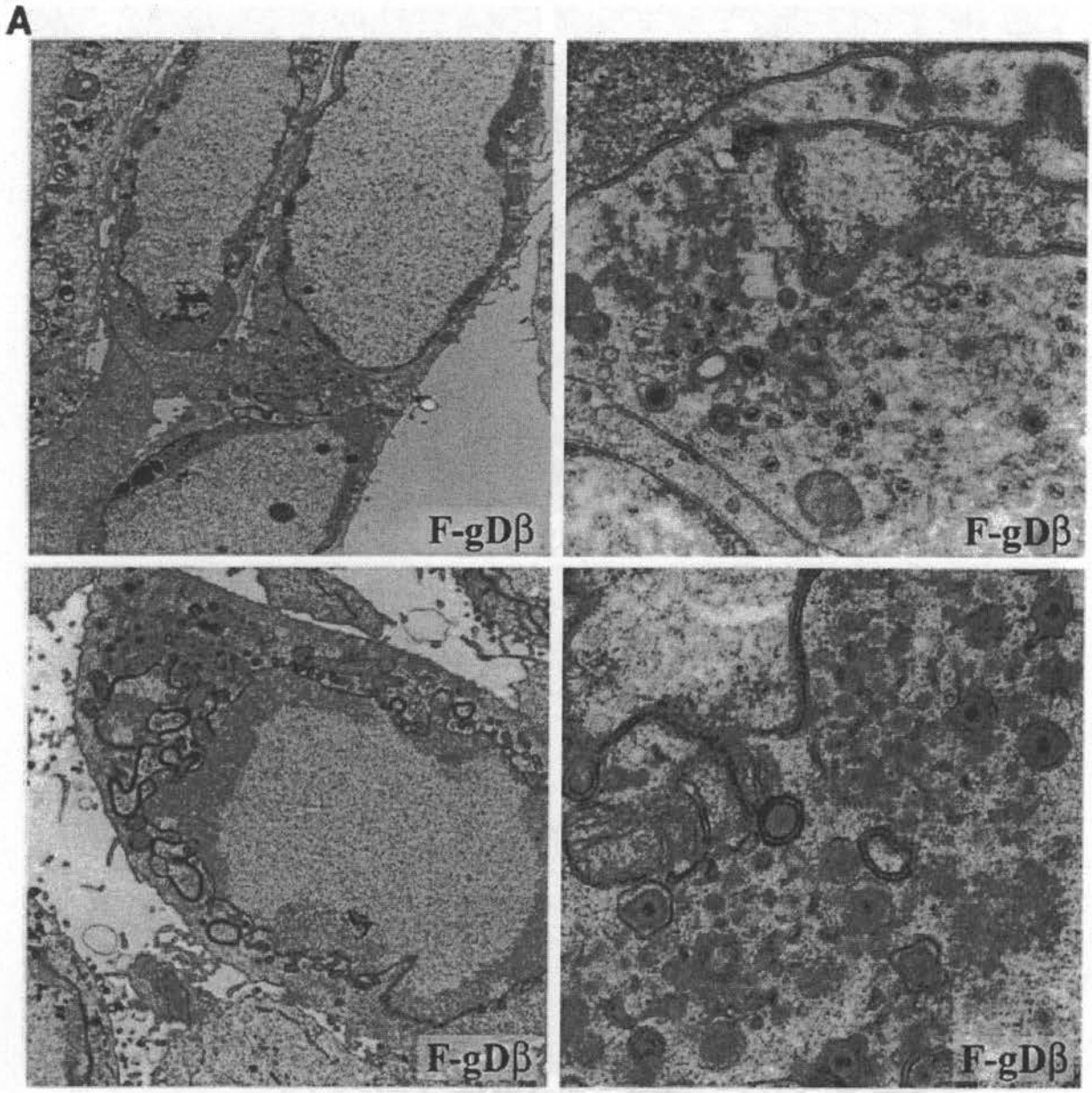
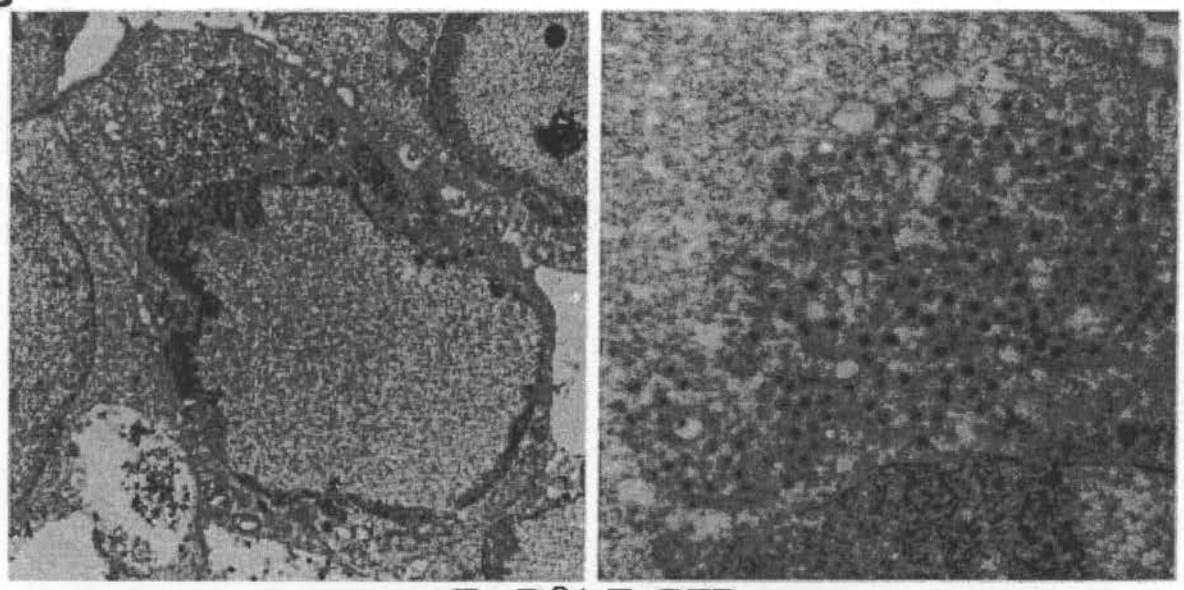


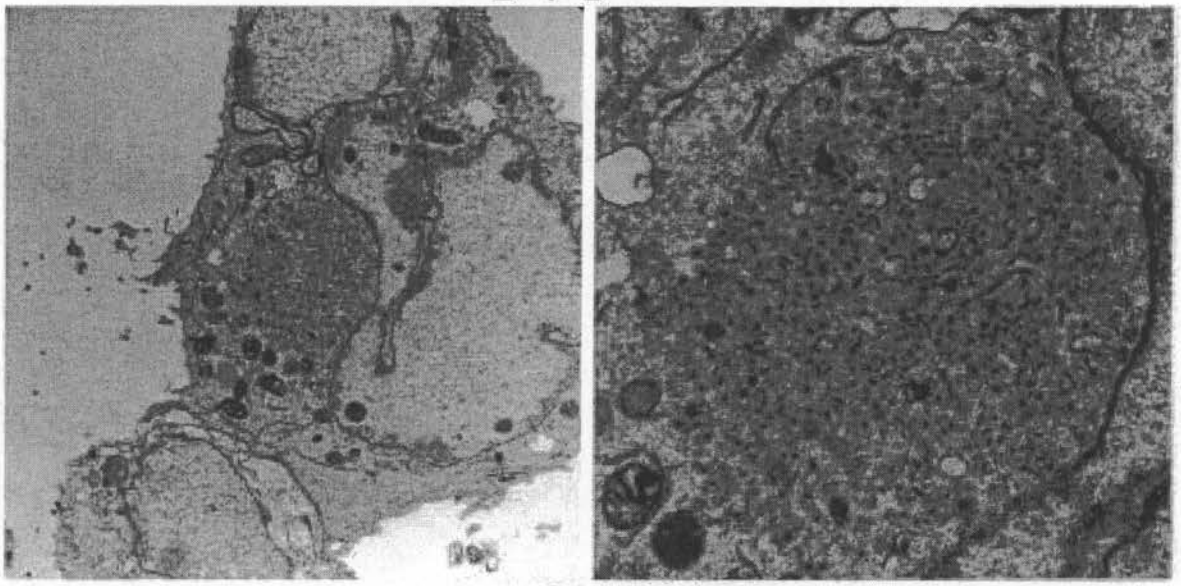
Figure 2.6. Electron micrographs of F-gDβ- and F-gDβ/gE-GFP-infected HEC-1A cells. **(A)** HEC-1A cells infected with the gD-/gI- mutant F-gDβ **(B)** the gD-/gI-/gE- mutant F-gDβ/gE-GFP were fixed and processed for electron microscopy.


A
F-gDβ
F-gDβ
F-gDβ
F-gDβ

B

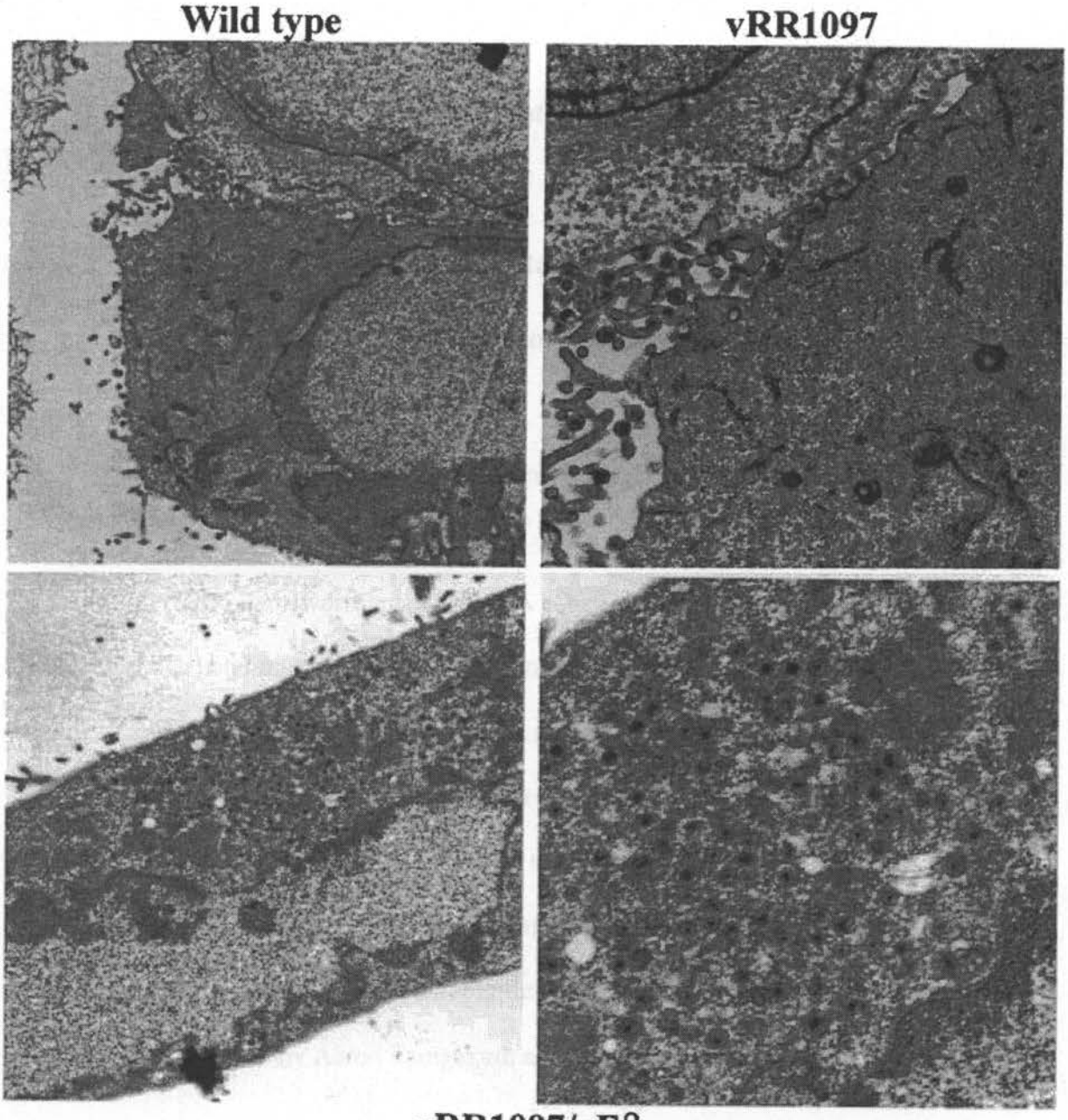


F-gDβ/gE-GFP



F-gDβ/gE-GFP

Figure 2.7. Electron micrographs of vRR1097/gEβ-infected HaCaT cells. HaCaT cells were infected with wild-type HSV-1 (upper left panel), vRR1097 (upper right panel), or vRR1097/gEβ (lower panels) for 16 to 18 h. The cells were fixed and processed for electron microscopy.


Wild type
vRR1097
vRR1097/gEβ

Chapter 3:

HSV gE/gI must accumulate in the TGN at early times then redistribute to cell junctions to promote cell-cell spread

Aaron Farnsworth and David C. Johnson

Aaron Farnsworth made all DNA constructs and viruses, fixed, stained and mounted samples for confocal microscopy and performed all experiments. David C. Johnson radiolabelled Sheep Red Blood cells and designed cartoon in Figure 8. Aurelie Snyder assisted with the capture of images requiring the confocal microscope and performed deconvolution processing on all images. Tiffani Howard drew the cartoon displayed in Figure 8. This chapter was co-authored by Aaron Farnsworth and David Johnson.

3.1 Abstract

Herpes simplex virus (HSV) glycoprotein heterodimer gE/gI is necessary for virus spread in epithelial and neuronal tissues. Deletion of the relatively large gE cytoplasmic (CT) domain abrogates the ability of gE/gI to mediate HSV spread. The gE CT domain is required for sorting gE/gI to the *trans*-Golgi network (TGN) in early stages of virus infection and there are several recognizable TGN sorting motifs near the center of this domain. Late in HSV infection, gE/gI and enveloped virions redistribute from the TGN to epithelial cell junctions, and the gE CT domain is required for this process. Without the gE CT domain, newly enveloped virions are directed to both apical and lateral surfaces. We hypothesized that the gE CT domain promotes virus envelopment into TGN sub-domains from which nascent enveloped virions are sorted to cell junctions, a process that enhances cell-to-cell spread. To characterize elements of the gE CT domain involved in intracellular trafficking and cell-to-cell spread we constructed a panel of truncation mutants. Specifically, these mutants were used to address whether sorting to the TGN and redistribution to cell junctions are necessary, and sufficient, for gE/gI to promote cell-to-cell spread. gE_{519}, lacking 32 C-terminal residues, localized normally to the TGN early in infection then trafficked to cell junctions at late times, and mediated virus spread. By contrast, mutants gE_{495} and gE_{470} accumulated in the TGN but did not traffic to cell junctions and did not mediate cell-to-cell spread. A fourth mutant, gE_{448} (lacking most of the CT domain), did not localize to cell junctions and did not mediate virus spread. The capacity of gE/gI to promote cell-cell spread requires early localization to the TGN, but this is not sufficient for virus spread. Additionally, gE CT sequences between residues 495 and 519, which contain no obvious cell sorting motifs, are required to promote gE/gI traffic to cell junctions and cell-to-cell spread.

3.2 Introduction

Herpes simplex virus commonly infects mucosal and ocular epithelium causing oral and genital lesions. During primary infection in epithelial tissues HSV enters sensory and autonomic neurons where the virus replicates and can establish latency. Periodic reactivation in neurons leads to transient replication and spread along neuronal axons and the reinfection of epithelial tissues. This cycle of HSV spread in epithelial tissues, entry into neurons, spread to sensory ganglia and return to epithelial tissues involves directed intracellular transport to specific cell surfaces and extremely rapid spread between cells (reviewed in (Johnson, D. C. and Huber, M. T. 2002)). As evidence of the speed of this process, HSV can spread from a single infected cell to over 250 cells in the cornea within 48 hours (Polcicova, K. et al. 2005b). HSV is largely cell-associated and spreads across cell junctions and resists the effects of high concentrations of virus-neutralizing antibodies. Titers of antibodies do not predict the severity of disease (Corey, L. and Spear, P. G. 1986). We demonstrated that HSV cell-to-cell spread in cultured epithelial cells involves a process by which progeny virions are targeted specifically to epithelial cell junctions (Johnson, D. C. et al. 2001; Johnson, D. C. and Huber, M. T. 2002). By virtue of being sorted to epithelial cell junctions there is preferential movement of virus between cells, rather than into extracellular fluids. There is also evidence for directed spread of HSV in the nervous system. HSV particles are preferentially sorted into sensory axons and move in the direction of epithelial tissues, rather than into dendrites and toward the central nervous system (Cook, M. L. and Stevens, J. G. 1973). Once HSV reaches epithelial and neuronal cell junctions, virus particles can spread to adjacent cells through interactions with cellular receptors that preferentially accumulate at these junctions (Richart, S. M. et al. 2003).

HSV cell-to-cell spread requires three viral glycoproteins gD, gB and gH/gL that are also required for the related process by which extracellular virions enter cells (Cai, W. Z. et al. 1987; Ligas, M. W. and Johnson, D. C. 1988; Forrester, A. et al. 1992; Roop, C. et al. 1993). However, in contrast to these envelope glycoproteins, HSV glycoprotein gE/gI promotes cell-to-cell spread without any obvious role in entry of extracellular particles (Dingwell, K. S. et al. 1994; Dingwell, K. S. et al. 1995; Johnson, D. C. and Huber, M. T. 2002). HSV gE- or gI-null mutants display markedly reduced spread between cultured epithelial and neuronal cells and in epithelial and neuronal tissues (Dingwell, K. S. et al. 1994; Dingwell, K. S. et al. 1995; Dingwell, K. S. and Johnson, D. C. 1998; Polcicova, K. et al. 2005a). HSV gE-null mutants spread to between 4 and 6% of the epithelial cells compared to wild type HSV infection of the cornea (Polcicova, K. et al. 2005a) and spread of gE-/gI- double mutants is reduced to 2% (D.C. Johnson, unpublished). Additionally, gE- mutants display markedly reduced spread between neurons in the retina, as well as from the retina to retinorecipient regions of the brain (Dingwell, K. S. et al. 1995).

HSV gE/gI appears to participate in at least two processes that promote cell-to-cell spread. The extracellular (ET) domains of gE/gI are necessary for spread, probably to promote virus movement across epithelial cell junctions (Johnson, D. C. et al. 2001; Johnson, D. C. and Huber, M. T. 2002; Collins, W. J. and Johnson, D. C. 2003). This was based on observations that gE/gI accumulates at cell junctions late in infection, apparently tethered there similar to various cell adhesion molecules (Wisner, T. et al. 2000; McMillan, T. N. and Johnson, D. C. 2001; Collins, W. J. and Johnson, D. C. 2003), and when expressed in trans can interfere with cell-to-cell spread (Collins, W. J. and Johnson, D. C. 2003). Moreover, small insertion mutations in the gE ET domain that do not reduce cell surface expression, incorporation into virions or

complex formation with gI, can abolish the capacity of gE/gI to promote cell-to-cell spread (Polcicova, K. et al. 2005b). We hypothesized that gE/gI can promote cell-to-cell spread by binding receptors that are selectively expressed at cell junctions. However, the relatively large cytoplasmic (CT) domain of gE is involved in a second process that promotes cell-to-cell spread. Mutants lacking the gE CT domain behave much like gE-null mutants with dramatically reduced spread between cultured epithelial cells and within the corneal epithelium (Wisner, T. et al. 2000; Polcicova, K. et al. 2005a).

The gE CT domain promotes extensive accumulation of gE/gI in the *trans*-Golgi network (TGN) in HSV-infected cells at early times of infection and when gE/gI is expressed by transfection or with virus vectors (Dingwell, K. S. and Johnson, D. C. 1998; Alconada, A. et al. 1999; Wisner, T. et al. 2000; McMillan, T. N. and Johnson, D. C. 2001; Collins, W. J. and Johnson, D. C. 2003) Swapping the gE CT domain in place of the gD CT domain caused gD to accumulate in the TGN, but this was not the case with the gI CT domain (McMillan, T. N. and Johnson, D. C. 2001). The TGN and endosomes serve as intracellular compartments where secondary envelopment of HSV occurs (van Genderen, I. L. et al. 1994; Whiteley, A. et al. 1999; Dasgupta, A. and Wilson, D. W. 2001; Skepper, J. N. et al. 2001; Johnson, D. C. and Huber, M. T. 2002; Farnsworth, A. et al. 2003; Wisner, T. W. and Johnson, D. C. 2004)(Chapter 2). HSV gE/gI and gD serve essential but redundant functions during acquisition of the virion envelope in the cytoplasm; nucleocapsids accumulate in massive quantities in the cytoplasm when both gE and gD are deleted (Chapter 2). Thus, gE/gI both accumulates extensively in the TGN at times when virus particles are being assembled and, in conjunction with gD, promotes envelopment there. Related to these observations, the gE CT domain is required for the specific sorting of enveloped virions formed in the TGN to epithelial cell junctions (Johnson, D. C. et al. 2001).

When gE, or just the gE CT domain, was deleted virions trafficked to apical surfaces, rather than to lateral cell junctions. These observations supported a working model in which gE/gI affects intracellular sorting decisions promoting HSV envelopment into subdomains of the TGN from which cargo, in this case virus particles, are transported specifically to lateral cell surfaces (Johnson, D. C. et al. 2001; Johnson, D. C. and Huber, M. T. 2002).

Coincident with the relocalization of HSV virions from sites of envelopment in the TGN to lateral cell surfaces late in infection, gE/gI is redistributed from the TGN to epithelial cell junctions (Wisner, T. et al. 2000; McMillan, T. N. and Johnson, D. C. 2001; Wisner, T. W. and Johnson, D. C. 2004). This process appears to involve global rearrangement or redistribution of the TGN because HSV gB and host TGN proteins (TGN46 and carboxypeptidase D) also moved to the plasma membrane (Wisner, T. W. and Johnson, D. C. 2004). This redistribution was specifically to lateral cell surfaces, not to apical surfaces, and did not require the assembly of enveloped virions in the TGN. Apparently, there are viral proteins that function to redistribute enveloped virus particles from the TGN to cell junctions and other TGN components follow.

In polarized epithelial cells, the TGN and endosomes are the major compartments in which membrane and secreted proteins are sorted to either basolateral or apical cell surfaces (reviewed in (Hunziker, W. et al. 1991; Mellman, I. 1996; Campo, C. et al. 2005)). Decisions are made as to whether cellular proteins are sequestered into subdomains of the TGN that then give rise to vesicles, frequently coated with clathrin as well as other proteins, which are directed to basolateral or apical cell surfaces. The cytoplasmic domains of cargo proteins interact with cytosolic adaptor molecules, e.g. the clathrin adaptors AP-1 and AP-3 and PACS-1, that promote coating of vesicles and direct transport (Mellman, I. 1996; Ang, A. L. et al. 2004; Campo, C. et al. 2005). Clathrin adaptors recognize tyrosine (YXXØ) and dileucine motifs in the cytoplasmic

domains of membrane proteins, among other signals. We previously demonstrated that a clathrin adaptor, AP-1B, selectively expressed in polarized epithelial cells, functions to sort alphaherpesvirus to cell junctions (Johnson, D. C. et al. 2001).

The gE CT domain is 106 amino acids in length and contains several obvious TGN sorting motifs and these are also found in PRV and VZV homologues (McGeoch, D. J. et al. 1985; Hunziker, W. et al. 1991; Humphrey, J. S. et al. 1993; Olson, J. K. and Grose, C. 1997; Brack, A. R. et al. 2000; Kurten, R. C. 2003). HSV gE CT contains two tyrosine motifs (YXXØ) as well as an acidic cluster of amino acids, which is phosphorylated (Wisner, T. et al. 2000). Tyrosine motifs bind the μ1 and μ2 components of AP-1 and AP-2 clathrin adaptors to promote incorporation into clathrin-coated transport vesicles (Gu, F. et al. 2001). The HSV gI CT domain also contains a di-leucine motif at the C-terminus that likely binds the β1 component of AP-1 clathrin adaptors. We demonstrated that an epithelial-specific component of the AP-1 complex, μ1B, was important for directing PRV particles to lateral cell surfaces (Johnson, D. C. et al. 2001). Additionally, the gE CT domain interacts with the acidic cluster-binding protein PACS-1 (Colin Crump and Gary Thomas, personal communication) that directs molecules to the TGN (Wan, L. et al. 1998; Gu, F. et al. 2001). TGN sorting sequences, e.g. tyrosine motifs, can also function in endocytosis of HSV gE/gI from the cell surface (Alconada, A. et al. 1999; McMillan, T. N. and Johnson, D. C. 2001). However, the majority of HSV gE/gI accumulates in the TGN through internal recycling loops involving endosomes and the *trans*-Golgi apparatus rather than by endocytosis, as with other TGN proteins (Wan, L. et al. 1998; Gu, F. et al. 2001).

In this study we constructed truncation mutants of the gE CT domain. These mutants allowed us to address whether sorting of gE/gI to the TGN and redistribution to cell junctions was necessary, and sufficient, for gE/gI to promote cell-to-cell spread. A mutant that did not

accumulate in the TGN was unable to mediate cell-to-cell spread. In addition, mutants that localized to the TGN but could not redistribute to cell junctions were also defective for virus spread. Therefore, both processes are necessary for cell-to-cell spread but different sequences in determine TGN localization and redistribution to cell junctions.

3.3 Results

3.3.1 Construction of HSV-1 gE CT domain mutants.

The HSV gE CT domain affects intracellular trafficking of gE/gI, enhances movement of virions to epithelial cell junctions and promotes virus spread (Dingwell, K. S. et al. 1994; Dingwell, K. S. et al. 1995; Dingwell, K. S. and Johnson, D. C. 1998; Wisner, T. et al. 2000). Fig. 3.1 depicts the gE CT domain showing identifiable TGN sorting motifs including two tyrosine motifs at residues 463 and 472, two serine residues at positions 476 and 477 that are phosphorylated (Wisner, T. et al. 2000) and a cluster of acidic residues between residues 478-484 that likely interacts with PACS-1 (Colin Crump and Gary Thomas, personal communication).

To characterize regions of the gE CT domain that are important for intracellular sorting and cell-to-cell spread, we truncated the CT domain by inserting tandem stop codons. Mutations were transferred into the HSV genome using a bacterial artificial chromosome (BAC) copy of HSV-1 strain F (F-BAC) (Horsburgh, B. C. et al. 1999). In mutant gE_{448}, two stop codons were inserted into the gE CT domain C-terminal to three arginine residues that are adjacent to the membrane so that the vast majority of the CT domain was removed (Fig. 3.1). Mutant gE_{470} has two stop codons replacing residue 470 so that only the membrane-proximal YXXØ motif is present and all potential phosphorylation sites are removed. In gE_{495} both tyrosine motifs, the acidic cluster, two of three phosphorylation sites, and an additional 11 amino acids are present.

Mutant gE_{519}, contains all but the final 32 amino acids of the gE CT. F-BAC ΔgE is an additional mutant lacking all gE coding sequences constructed using the BAC system.

3.3.2 Expression of mutant gE proteins and incorporation into virions.

To characterize the expression of gE mutants, Vero cells were infected and then radiolabelled with [^{35}S]-methionine/cysteine. Cell extracts were denatured to disrupt the gE/gI complex and gE was immunoprecipitated using MAb 3114, subjected to electrophoresis and autoradiography. F-BAC expressed immature (66 kDa) and mature (80kDa) forms of gE as observed previously (Polcicova, K. et al. 2005b). Mutants gE_{519}, gE_{495}, gE_{470} and gE_{448} expressed immature and mature gE molecules of the sizes predicted (Fig. 3.2A). As expected, cells infected with F-BAC ΔgE expressed no gE protein. Normal amounts of gB and gD proteins were expressed by all mutants (Fig. 3.2B and C). As with other mutants lacking all gE CT domain sequences (Wisner, T. et al.; Polcicova, K. et al. 2005b), the present gE mutants complexed normally with gI as measured by immunoprecipitation with MAb 3063 that requires an epitope produced only in the gE/gI complex (data not shown).

To ensure that mutant gE proteins were incorporated into the virion envelope, HaCaT cells were infected with each virus, cell culture supernatants harvested and virions partially purified by centrifugation through a sucrose cushion. Pelleted virus particles were examined by Western blotting using anti-gE MAb 3114. In Figure 3.2D, bands corresponding to the size of mature gE, as appropriate for each construct, were observed. In addition to gE there was a cross reacting protein of approximately 72 kDa present in all lanes, including F-BAC ΔgE. These results demonstrated that cells infected by these mutant viruses produced the appropriate gE molecules that were processed to mature forms and incorporated into virion envelopes.

3.3.3 Cell-to-cell spread of HSV-1 gE CT domain mutants.

HSV-1 mutants lacking either gE or just the gE CT domain form plaques that contain 15-20% the number of epithelial cells compared with plaques formed by wild type HSV-1 (Wisner, T. et al. 2000; Polcicova, K. et al. 2005b). This was observed with HaCaT cells, a human keratinocyte cell line that mimics the cells infected in human mucosa, as well as with ARPE-19 cells, a human retinal epithelial cell line that also forms extensive cell junctions. Mutants F-BAC ΔgE, gE_{448}, gE_{470}, and gE_{495} all formed plaques that encompassed 16-18% the number of HaCaT cells compared with plaques formed by F-BAC (Fig. 3.3A). By contrast, F-BAC gE_{519}, formed large plaques that were similar to plaques produced by wild type F-BAC (Fig. 3.3A). Similar results were obtained on ARPE-19 cells (Fig. 3.3B). With both epithelial cell lines, there was a distinct division between large plaques formed by gE_{519} and wild type HSV-1 and small plaques formed by gE_{448}, gE_{470}, and gE_{495} and the gE-null mutant. Therefore, truncation of the C-terminal 56 residues of gE a region that does not contain obvious TGN sorting motifs in mutant gE_{495} abolished the capacity of gE/gI to mediate cell-to-cell spread, yet removal of 32 residues in mutant gE_{519} did not.

3.3.4 Replication of gE CT mutants.

To ensure that the defects observed in plaque formation by gE CT domain mutants were not due to defects in virus replication we characterized production of infectious virus and movement into extracellular compartments. At various times after infection, total virus (cells and media combined) or virus in cell culture supernatants were harvested from HaCaT cells and titered on Vero cells. All five mutants produced similar total amounts of progeny virus as F-BAC (Fig. 3.4A), consistent with the conclusion that replication was not altered. However, F-BAC ΔgE and F-BAC gE_{448} shed significantly more virus into cell culture supernatants at early times

of infection (Fig. 3.4B), as had previously been reported for gE-null and CT domain mutants (Johnson, D. C. et al. 2001). Specifically, there was ≈ 45 fold more F-BAC gE_{448} and F-BAC ΔgE in the media compared with wild type HSV 10 h after infection. Mutants F-BAC gE_{470} and gE_{519} did not display increased numbers of virions in growth media. At late times of infection, e.g. after 14 h, the differences in the quantities of virus in cell culture supernatants disappeared, likely because cell junctions were blown apart and apical and basolateral compartments mixed. Coupled with previous observations (Johnson, D. C. et al. 2001), we concluded that that virions produced by a gE mutant lacking the entire CT domain (gE_{448}) were missorted toward apical cell surfaces, whereas gE_{470}, gE_{495} and gE_{519} were not.

3.3.5 Accumulation of gE/gI in the TGN.

gE/gI accumulates predominately in the TGN at early times after HSV-1 infection (6 h) (Dingwell, K. S. and Johnson, D. C. 1998; Wisner, T. et al. 2000; McMillan, T. N. and Johnson, D. C. 2001; Wisner, T. W. and Johnson, D. C. 2004). gE/gI TGN localization appears to be important for virus assembly and as a first step towards the selective sorting of enveloped particles to cell junctions which promotes cell-to-cell spread. To examine whether gE CT domain mutants accumulated in the TGN at early times, confocal microscopy was used to compare gE/gI localization to TGN46, a cellular component of the TGN (Humphrey, J. S. et al. 1993). In HaCaT cells, wild type gE/gI (F-BAC, green) was found extensively in a perinuclear location co-localizing with TGN46 (red) 6 h after infection (Fig. 3.5A). Similarly, gE/gI was predominately perinuclear and colocalized with TGN46 in cells infected with mutants gE_{519}, gE_{495} and gE_{470} (Fig. 3.5A). With wild type HSV and mutants gE_{519}, gE_{495} and gE_{470} there was also smaller, more punctuate gE/gI vesicles that were nearer the plasma membrane and a fraction of these did not contain TGN46. Still the majority of gE/gI was present in the TGN. By contrast,

gE/gI expressed in gE_{448}-infected cells was significantly different. There was little perinuclear accumulation, most cytoplasmic vesicles containing gE/gI were more peripheral and few contained TGN46 and gE/gI localized to plasma membranes, both lateral and apical (Fig. 3.5A).

These results were extended to include ARPE-19 cells. Again, wild type gE/gI and that of mutants gE_{519}, gE_{495} and gE_{470} primarily accumulated in a perinuclear location colocalizing with TGN46 at 6 h (Fig. 3.5B). There was also a smaller fraction of gE/gI which was found in more peripheral vesicles, some that did not stain with TGN46 antibodies. As in HaCaT cells, gE/gI produced in gE_{448}-infected cells was found in vesicles throughout the cytoplasm, which largely did not contain TGN46, as well as on lateral and apical cell surfaces. We concluded that mutants gE_{519}, gE_{495} and gE_{470} largely retain the ability to localize to the TGN. By contrast, gE_{448}, lacking the majority of the CT domain did not accumulate in the TGN.

3.3.6 Redistribution of gE/gI to lateral cell junctions.

gE/gI accumulates at lateral cell surfaces co-localizing with the cell junction marker, β-catenin late in HSV infections (McMillan, T. N. and Johnson, D. C. 2001). We sought to determine if gE/gI redistributed to lateral cell junctions when the gE CT domain was truncated and whether this correlated with cell-to-cell spread. In HaCaT cells infected with F-BAC and mutant gE_{519}, gE/gI largely accumulated at cell junctions substantially co-localizing with β-catenin (Fig. 3.6A). However in cells infected with gE_{495} and gE_{470}, gE/gI was much less extensively redistributed to cell junctions and, instead, was found largely in cytoplasmic vesicles. A fraction of these cytoplasmic vesicles were just under lateral plasma membranes adjacent to, but not overlapping with, β-catenin (Fig. 3.6A). By contrast, gE/gI accumulated at cell junctions and was also present in vesicles distributed throughout the cytoplasm in cells infected with gE_{448} (Fig. 3.6A). We previously reported that a mutant lacking the gE CT domain was present on

apical surfaces by constructing Z-axis confocal images (Wisner, T. et al. 2000) and a similar phenotype was observed with this mutant (data not shown).

In ARPE-19 cells, wild type gE and gE_{519} were again predominately at cell junctions although there were also cytoplasmic vesicles containing gE/gI (Fig. 3.6B). We have found that gE/gI generally does not traffic as extensively to cell junctions in ARPE-19 cells compared with HaCaT cells. In ARPE-19 cells infected with F-BAC gE_{495} and gE_{470}, gE/gI accumulated in tight perinuclear bundles and was rarely found at cell junctions. Interestingly, there was substantial β-catenin present in the cytoplasmic vesicles that contained gE/gI in these cells (Fig. 3.6B). Again, the gE/gI produced in gE_{448}-infected cells was found on all cell surfaces and in membrane vesicles throughout the cytoplasm while β-catenin remained at cell junctions (Fig. 3.6B). We concluded that gE/gI produced in gE_{519}-infected cells redistributes to cell junctions while gE/gI produced in gE_{495} and gE_{470}-infected cells does not accumulate at junctions and instead remains in cytoplasmic vesicles.

3.3.7 Distribution of gE CT domain mutants onto apical surfaces.

HSV gE/gI binds the Fc domains of IgG on the surfaces of HSV-infected cells (Johnson, D. C. and Feenstra, V. 1987; Bell, S. et al. 1990; Hanke, T. et al. 1991). We recently reported that epithelial cells infected with HSV mutants lacking the CT domain bound approximately 5 fold more IgG-coated sheep red blood cells (RBC) compared with cells infected with wild type HSV-1 (Polcicova, K. et al. 2005b).We concluded that removal of the gE CT domain promotes increased traffic to apical cell surfaces instead of to lateral cell surfaces and produces increased IgG binding at apical surfaces. Thus, this increased Fc receptor activity is a good quantitative measure of mislocalization of gE/gI to apical surfaces. We examined binding of ^{51}Cr labelled IgG-coated RBC to cells infected with gE CT domain mutants. HaCaT cells infected with gE_{448}

exhibited 430% increased binding of IgG-coated RBC compared with cells infected with wild type HSV F-BAC (Fig. 3.7). Cells infected with mutants gE_{470}, gE_{495} and gE_{519} all bound IgG-coated RBC similar to cells infected with F-BAC. Therefore, gE_{519}, gE_{495} and gE_{470} are not extensively present on apical cell surfaces confirming the results of confocal experiments.

3.4 Discussion

Fundamental to the correct subcellular localization of HSV proteins and subsequent assembly, directed egress of virions, and cell-to-cell spread is capacity of the virus to usurp elements of the host cell trafficking systems. HSV proteins are sorted to the TGN for assembly and virions are then directed to cell junctions, processes that increase spread of virus to neighboring cells (Johnson, D. C. et al. 2001; Johnson, D. C. and Huber, M. T. 2002). Most studies of epithelial cell-to-cell spread have involved cultured cells. However, characterization of HSV spread from sensory neurons into the corneal epithelium produced evidence for directed transport of virus. HSV particles moved specifically onto apical surfaces of less differentiated, basal epithelial cells spreading toward the surface of the cornea, then after reaching squamous epithelial cells the virus spreads laterally (Ohara, P. T. et al. 2001). In neurons, similar or related sorting decisions are made, determining whether virions transit down axons, remain in neuron cell bodies, or move into dendrites. HSV and PRV gE/gI mutants spread poorly between cultured epithelial and neuronal cells and within corneal and neuronal tissues *in vivo* and it appears that gE/gI functions to direct HSV particles toward other cells or down neuronal axons (Whealy, M. E. et al. 1993; Dingwell, K. S. et al. 1995; Johnson, D. C. et al. 2001; Tomishima, M. J. and Enquist, L. W. 2001; Ch'ng, T. H. and Enquist, L. W. 2005; Polcicova, K. et al. 2005a).

HSV gE/gI accumulates in the TGN in early phases of infection and is then specifically sorted to cell junctions in late phases of infection and appears to be tethered there (Wisner, T. et

al. 2000; McMillan, T. N. and Johnson, D. C. 2001; Collins, W. J. and Johnson, D. C. 2003; Wisner, T. W. and Johnson, D. C. 2004). This sorting of gE/gI correlates with the directed sorting of HSV virions to cell junctions (Johnson, D. C. et al. 2001). HSV-1 mutants lacking the gE CT domain do not localize gE/gI well to the TGN and virions are subsequently mislocalized to apical cell surfaces and into cell culture supernatants, These mutants spread poorly, exhibiting similar defects in spread as do gE-null mutants (Wisner, T. et al. 2000; Johnson, D. C. et al. 2001). The model that was tested here suggests that the gE CT domain promotes localization of gE/gI to TGN subdomains that sort gE/gI, and by extension virions, to cell junctions (Johnson, D. C. et al. 2001; Johnson, D. C. and Huber, M. T. 2002). Specifically, we tested whether TGN accumulation and sorting to cell junctions required the same gE CT domain sequences and whether one or both of these processes were required for cell-to-cell spread.

Three of the gE CT domain truncation mutants: gE_{470}, gE_{495} and gE_{519} produced gE/gI molecules that largely accumulated in the TGN, colocalizing with TGN46, at early times of infection. By contrast, gE_{448}, that lacks the majority of the CT domain and forms small plaques, produced gE/gI that was found throughout the cytoplasm as well as on both apical and lateral cell surfaces. This supports the conclusion that TGN accumulation is necessary in order for gE/gI to promote cell-to-cell spread.

While wild type HSV and gE_{519} produced gE/gI that redistributed from the TGN to cell junctions at late times, gE/gI produced in cells infected with mutants gE_{495} and gE_{470} remained in cytoplasmic vesicles, frequently underlying the plasma membrane. Since mutants gE_{470} and gE_{495} exhibited a similar deficiency in cell-to-cell spread, we concluded that TGN localization is necessary, but not sufficient, for gE/gI-mediated spread, gE/gI also must redistribute to cell junctions for the virus to efficiently spread. Related to this, we found that virus particles

produced in gE_{448}-infected cells were missorted apically, so that 45 fold more infectious virus was observed in cell culture supernatants at 10 h compared with wild type HSV. By contrast, gE_{470} and gE_{495} produced particles that were not released into cell culture supernatants at these early times, yet these viruses did not spread well. This is consistent with the notion that gE_{470} and $-_{495}$ produce gE/gI dimers that do not traffic to cell junctions and virions produced are not shed into apical compartments but may instead accumulate in cytoplasmic vesicles.

gE TGN sorting motifs, those that can be recognized based on primary sequence, all cluster to a 20 residue region in the N-terminal half of the gE CT domain (Fig. 3.1). Surprisingly, truncation of all of these sequences except the tyrosine motif at residue 463 produced a gE/gI heterodimer (gE_{470}) that retained much of its ability to accumulate in the TGN, yet a mutant lacking the gE CT domain (gE_{448}) did not. Apparently, HSV gE/gI can accumulate in the TGN with just one gE tyrosine motif (gE_{470}) supporting the view that there is ample redundancy built into the gE CT domain in terms of TGN localization motifs. Previous studies of the PRV gE CT domain indicated that replacement of both tyrosine motifs reduced spread in cultured epithelial cells and, to some extent, into the brain (Brack, A. R. et al. 2000). Again, these studies were consistent with the notion that sorting and spread depends on a number of different motifs in the gE CT domain that are redundant.

Redistribution of gE/gI to cell junctions at late times of infection required sequences in the C-terminal half of the gE CT domain between residues 495 and 519 (Fig. 3.1). No recognizable sorting motifs are present in this region, although this region is proline-rich. Eight of the 10 gE CT domain prolines are present between residues 487 and 517. The accumulation of gE/gI just under the plasma membrane in gE_{495} and gE_{470}-infected cells may suggest that movement of gE/gI from the TGN to lateral cell surfaces involves endosomes as intermediates.

There are distinct recycling loops between the TGN and endosomes, and between endosomes and the plasma membrane (Gu, F. et al. 2001). Thus, with these mutants, it is possible that gE/gI becomes trapped in endosomal compartments and is unable to reach the plasma membrane, or is rapidly internalized. However, analysis of this is complicated as HSV disrupts the Golgi apparatus, the TGN, and possibly endosomes dispersing cellular components to other compartments and the plasma membrane (Campadelli, G. et al. 1993; Wisner, T. W. and Johnson, D. C. 2004). As such, the normal architecture of post-Golgi membrane compartments is lost, making determinations of the character of these gE/gI-containing vesicles difficult.

These results add important details to our model for how gE/gI facilitates cell-to-cell spread. In this hypothesis, gE/gI promotes envelopment into specific subdomains of the TGN that then sort nascent virions to cell junctions. Here we showed that TGN accumulation is not sufficient and gE/gI apparently functions at some post-TGN site and must move to cell junctions in order to mediate cell-to-cell spread. These data are consistent with the notion that gE_{495} and gE_{470} have defects in delivery of virions to cell junctions. However, this requires substantiation by electron microscopy, studies that are in progress. It is difficult to understand how the gE CT domain can alter the traffic of viral glycoproteins and particles after gE/gI is incorporated into the virion envelope so that the gE CT domains is buried inside the virus particle. One possibility is that gE/gI, which is not part of the virion envelope but present in membrane vesicles that enclose newly enveloped virions, might couple to cell sorting machinery and thereby move both gE/gI and virions to cell junctions. By this model the gE CT domain affects sorting in both TGN and post-TGN compartments. A related hypothesis suggests that gE/gI accumulates in the TGN, promotes envelopment there, and also interacts with other HSV proteins in a process that unmasks cryptic sorting sequences in the gE CT domain that promote delivery to cell junctions.

This model is attractive because it may explain the requirement for both the gE ET and CT domains in cell-to-cell spread and may explain why gE/gI remains at the TGN compartment when other HSV proteins are not present. However, these models await further validation through identification and characterization of gE/gI-interacting proteins.

3.5 Materials and Methods

3.5.1 Cells and viruses.

HaCaT cells and Vero cells were grown on DMEM media supplemented with 10% fetal bovine serum (FBS). ARPE-19 cells were grown on DMEM/F-12 media containing 10% FBS. F-BAC, an HSV-1 strain F derivative with a BAC inserted into the tk gene (Horsburgh, B. C. et al. 1999) and F-BAC mutants were propagated and titered on Vero cells.

3.5.2 Mutagenesis of the gE gene.

A plasmid, pUC US7/8PA gE_{448}, in which all but three residues the gE CT domain was removed has been described (Polcicova, K. et al. 2005b). Plasmids containing additional gE CT domain truncations were constructed by using PCR using a template involving plasmid pUC-US7/8 (Wisner, T. et al. 2000). Specifically, two stop codons and a *Stu*I restriction site were inserted after residues: 470, 495 or 519. In all cases the sense oligonucleotide, GCAGGCGGCCTCCGTCAATCTG corresponding to codons 340 – 346, was employed. To construct gE_{470} the antisense oligonucleotide was: TTT**AGGCCTCTATTA**GCTGTCGGCCACGCGAATGTA corresponding to codons 463-469 preceeding two stop codons and a *StuI* site. For gE_{495}, the antisense oligonucleotide was CAA**AGGCCTTTATTA**TCTCTCCGGGGGGGCCAG corresponding to codons 489-494 preceeding two stop codons and a *StuI* site. The antisense oligonucleotide used to construct gE_{519} was CAA**AGGCCTTTATTA**ACGGGGGTATACAGACGG, corresponding to codons 513-518

preceeding two stop codons and a *StuI* site. Following PCR, the DNA product was digested with restriction endonucleases *Mlu*I and *Stu*I and bands of the correct size were purified and ligated into pUC-US7/8PA that had been digested with *Mlu*I and *Stu*I. The plasmids produced pUC-US7/8PA gE_{470}, pUC-US7/8PA gE_{495} and pUC-US7/8 gE_{519} were sequenced and then digested with restriction endonucleases *Pacs*I and *Asc*I, the mutated US8 genes were purified and ligated into plasmid pSTPA, a plasmid used to shuttle sequences into the HSV BAC (Polcicova, K. et al. 2005b) creating the plasmids pSTPA gE_{470}, pSTPA gE_{495}, and pSTPA gE_{519}. To construct a gE null mutant the following oligonucleotides were employed: 1) ATGGATCGCGGGGCGGTGGTGGGGTTTCTT**ATT**GTGTAGGCTGGGAGCTGCTTTC corresponding to codons 1-9 of the US8 gene followed by a stop codon and the priming site corresponding to plasmid pKD4 (Datsenko, K. A. and Wanner, B. L. 2000) and 2) TTACCAGAAGACGGACGAATCGGAGGACATATGAATATCCTCCTTAG corresponding to codons 543-551 of the gE gene followed by the reverse priming site for pKD4. PCR using these two oligonucleotides generated a kanamycin gene cassette flanked by 36 b.p. of the gE gene (denoted gE/kanamycin cassette) that was used to recombine the mutation directly into F-BAC.

3.5.3 Construction of F-BAC mutants.

Plasmids pSTPA gE_{470}, pSTPA gE_{495}, and pSTPA gE_{519} contain a temperature sensitive origin of replication, the *SacB* gene encoding sucrose sensitivity, and a kanamycin resistance gene (Polcicova, K. et al. 2005b) and were used to shuttle recombinant forms of gE into the HSV BAC using a modified protocol based on that described by Horsburgh *et al.* (Horsburgh, B. C. et al. 1999). In brief, shuttle plasmids were electroporated into RR1 bacteria containing the HSV-1 BAC (Horsburgh, B. C. et al. 1999) transformants were incubated and restreaked on plates,

containing chloramphenicol (20ug/ml) and kanamycin (30ug/ml), at 43° C. Following this individual colonies were streaked onto chloramphenicol and sucrose (5% w/v) containing plates and incubated at 30°C. Colonies that were replica plated on both kanamycin and chloramphenicol plates to confirm loss of the shuttle plasmid and the presence of mutations were confirmed by PCR amplification of appropriate regions and restriction analyses. Mutant F-BACΔgE, lacking gE coding sequences, was created using a modified protocol of Datsenko *et al.* (Datsenko, K. A. and Wanner, B. L. 2000). Briefly, pKD46 a plasmid encoding ampicillin resistance and the red recombinase, was transferred into RR1 bacteria containing F-BAC. F-BAC and pKD46 containing bacteria were electroporated with the gE/kanamycin gene cassette and 1 ml of SOC media was added for 1 h then chloramphenicol (20 ug/ml) was added and incubated at 25°C overnight. The following day the transformation mixture was plated on chloramphenicol/kanamycin plates and incubated overnight at 37°C. Recombinant colonies were confirmed by screening for kanamycin resistance and sensitivity to ampicillin and by PCR and restriction analysis. Plasmid pCP20 that encodes ampicillin resistance and FLP recombinase were introduced into the bacteria containing the F-BAC. Transformants were selected at 30°C, on chloramphenicol (20ug/ml) and ampicillin (100ug/ml) plates, resistant colonies were streaked on chloramphenicol plates and grown at 43°C to induce FLP recombinase genes. Colonies were replica plated onto chloramphenicol, kanamycin and ampicillin plates and colonies that were $Chl^R/Amp^S/Kan^S$ were analyzed by PCR and restriction analyses to confirm loss of both the US8 gene and the kanamycin cassette. F-BAC that contained mutant gE were sequenced across, upstream and downstream of the mutation site.

3.5.4 Derivation of HSV from BAC DNA.

Bacteria containing F-BAC were grown in LB media containing chloramphenicol and BAC DNA purified using alkaline lysis, isopropanol precipitation and phenol/chloroform extraction. Vero cells in Opti-MEM lacking serum were transfected with HSV-BAC DNA using Lipofectamine (Invitrogen, Carlsbad, CA) and cytopathic effects of viruses observed after 4-5 days.

3.5.5 Radiolabeling of cells, virus purification and immunoprecipitation of gE.

Vero cells were infected with HSV using 10 PFU/cell and labelled from 6-9 h postinfection with [^{35}S]-methionine/cysteine (75 uCi/ml) in media lacking methionine and cysteine. Radiolabelled cells were lysed in 1% NP40/0.5% deoxycholate (DOC) buffer containing 2mg/ml bovine serum albumin (BSA) and 1.0 mM phenylmethylsulfonyl fluoride (PMSF), and gE/gI, gD or gB were immunoprecipitated with MAb 3114 (anti-gE), DL6 (anti-gD) or 15βB2 (anti-gB) as described (Wisner, T. et al. 2000). Radiolabelled HSV-1 particles were prepared from cell culture supernatants harvested after 18 h by centrifugation through 30% sucrose at 75 000 x *g* for 1 h in a Beckman SW41 rotor. Virus pellets were disrupted in buffer containing 2% SDS, subjected to electrophoresis, and western blotting using anti-gE MAb 3114.

3.5.6 Cell-to-cell spread in cultured epithelial cells.

Spread of F-BAC mutants in cultured HaCaT and ARPE-19 cells was determined as described (Wisner, T. et al. 2000). Briefly, cells were infected with low multiplicities of viruses and after 2 h the virus removed and cells overlaid with media containing 0.4% human gamma globulin for 72 h. The cells were fixed in PBS containing 4% para-formaldehyde for 20 min, washed, permeabilized with 0.2% TX-100 in PBS for 10 min, and then stained with rabbit anti-HSV-1 serum (Dako, Copenhagen, Denmark) and then donkey anti-rabbit IgG antibodies

conjugated with horseradish peroxidase (Amersham, Arlington Ill.). Plaques were visualized after the addition of peroxidase substrate 3,3'-diaminobenzidine hydrochloride (Sigma). The area of 10 plaques was determined by using NIH Image.

3.5.7 Single step HSV replication.

HaCaT cells were infected with HSV using 10 PFU/cell. After 2 h virus was removed and cells washed for 1 min with 0.1M Na citrate, pH 3.0, then the cells washed and overlaid with media containing 1% FBS. After various times, the cells were scraped from dishes, pelleted and either the cells and media or media alone frozen at -70°C, subsequently thawed and sonicated, then virus titered using Vero cells.

3.5.8 Immunofluorescence microscopy.

HaCaT cells or ARPE-19 cells growing on glass coverslips were infected with HSV using 10 PFU/cell and after 6 h or 11.5 h the cells were washed and fixed in PBS containing 4% para-formaldehyde for 20 min. Cells were then washed in PBS, permeabilized with 0.2% TX-100 in PBS for 20 min and washed twice with PBS containing 0.02% Tween 20 (PBS-T) before incubation with PBS-T containing 2% normal goat serum (NGS) for 1 h. Cells were incubated with sheep anti-TGN46 (Serotec) or rabbit anti-β-catenin (Sigma) antibodies and simultaneously with anti-gE MAb 3114 for 2 h, washed three times with PBS-T, qand incubated with Cy5-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) or Alexa-594 donkey anti-sheep IgG and simultaneously with Alexa-488 goat anti-mouse IgG (Molecular Probes, Eugene OR). The cells were washed, mounted using ProLong (Molecular Probes, Eugene OR) and photographed using a Nikon TE200 inverted fluorescent confocal microscope with an Applied Precision Deltavision ^{TM} wide field image restoration system and images

processed by deconvolution software. In the case of Cy-5-conjugated secondary antibodies, the blue signal was converted to a red signal.

3.5.9 Binding of IgG-coated red blood cells (RBC) to HSV-infected cells.

Sheep RBC were labelled with ^{51}Cr (100-150 uCi/ml) then incubated with rabbit anti-sheep RBC IgG, as described (Hanke, T. et al. 1991). HaCaT cells infected with HSV for 14 h were incubated with saturating amounts of IgG-coated RBC for 2 h at 37°C in D-MEM with 1% FBS. The cells were incubated for 10-15 min on ice, washed 3-4 times with PBS containing 1% FBS then lysed in NP40/DOC buffer and radioactivity measured.

Figure 3.1 Map of the gE CT domain and truncation mutants.

HSV-1 gE CT domain is 106 residues and C-terminal to the 25 amino acid transmembrane (TM) domain which ends at amino acid 445. There are three arginine (R) residues next the TM domain, two tryrosine motifs (YXXØ, where Ø is a larger hydrophobic amino acid) at 463-466 and 472-475, three serines that are phosphorylated at residues 476, 477 and 503 and a cluster of acidic residues located between residues 476 to 484. Mutations in the CT domain were constructed by inserting two tandem stop codons in place of residues 519, 495, 470 or 448. The numbering of gE includes the signal sequence and is based on the published sequence of HSV-1 strain 17 gE (McGeoch, D. J. et al. 1985), whereas HSV-1 strain F gE (used here) contains two additional residues in the ET domain (Wisner, T. et al. 2000).

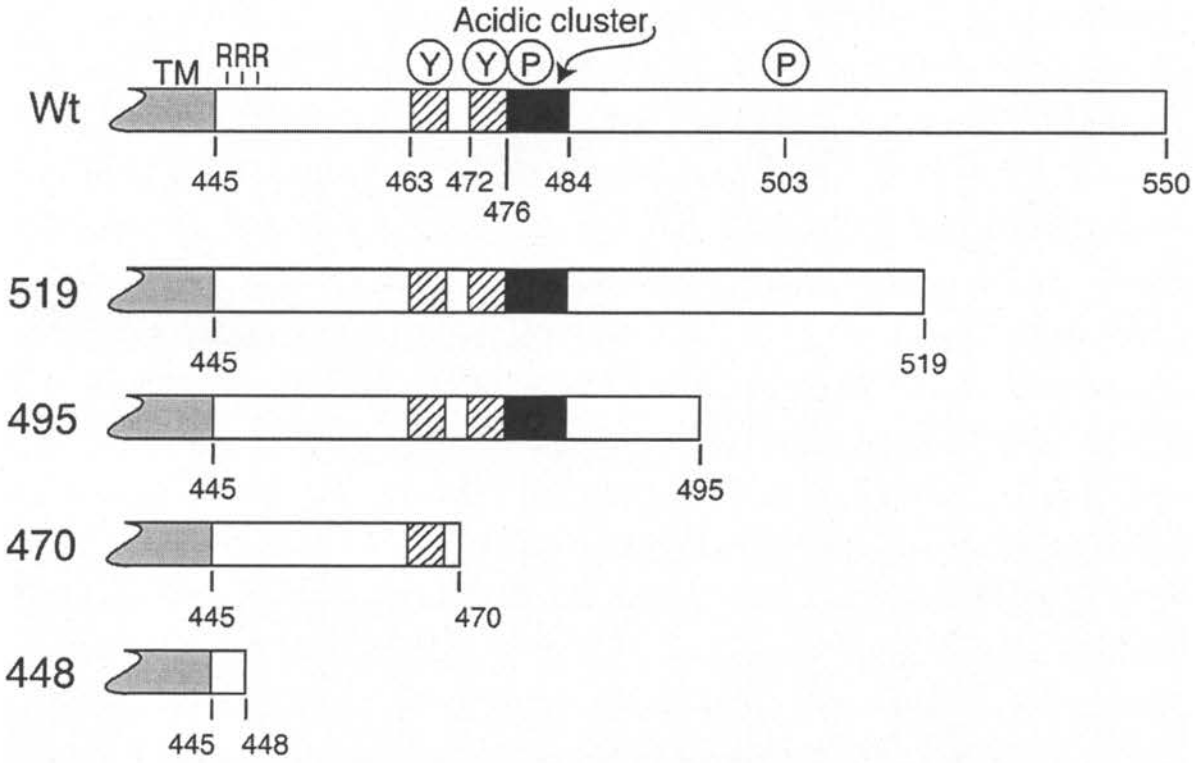

Acidic cluster
TM
RRR
Y
Y
P
P
Wt
445
463
472
476
484
503
550
519
445
519
495
445
495
470
445
470
448
445
448

Figure 3.2 Analysis of mutant gE expression and incorporation into virions.

Vero cells were infected with F-BAC (wild type), F-BACΔgE (gE null), gE_{448}, gE_{470}, gE_{495} or gE_{519} for 9 h then radiolabelled with [35S]-methionine/cysteine for 3h. Glycoproteins gE (**A**), gB (**B**) or gD (**C**) were immunoprecipitated from cell extracts using MAb 3114, MAb 15βB2, or MAb DL6, respectively, and subjected to electrophoresis. (**D**) HaCaT cells were infected with various viruses, cell culture supernatants collected at 16 h and centrifuged through a sucrose cushion for 1 h. Virus pellets were solubilized, subjected to electrophoresis, and gE detected by western blotting with MAb 3114. A non-specific protein was detected even in the absence of gE (F-BAC ΔgE).

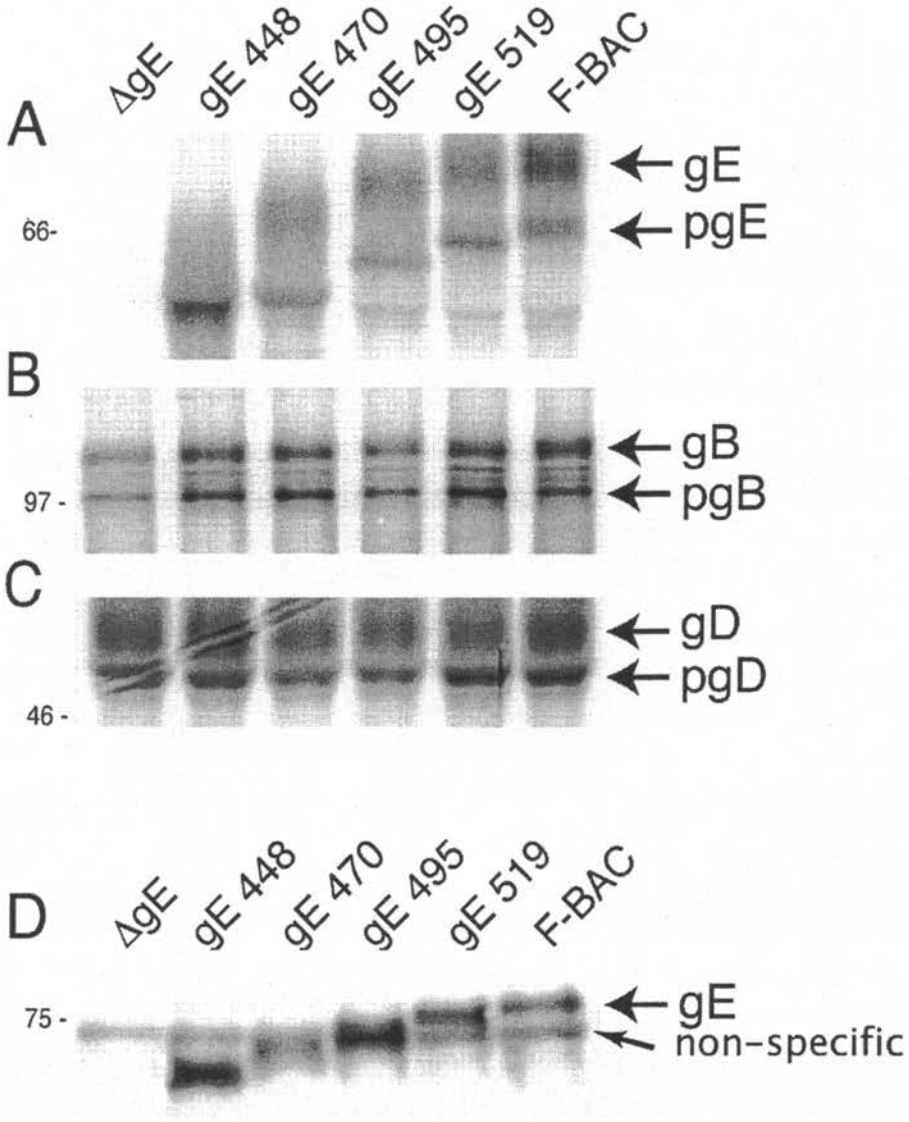

ΔgE
gE 448
gE 470
gE 495
gE 519
F-BAC
A
66-
gE
pgE
B
97 -
gB
pgB
C
46 -
gD
pgD
D
75 -
gE
non-specific

Figure 3.3 Cell-to-cell spread of HSV gE CT domain mutants. Human HaCaT keratinocytes (**A**) or ARPE-19 retinal epithelial cells (**B**) were infected with F-BAC, F-BAC ΔgE, gE_{448}, gE_{470}, gE_{495} or gE_{519} using a low MOI (30-100 PFU/35mm dish). After 72 h cells were stained with anti-HSV polyclonal antibodies and peroxidase-conjugated secondary antibodies. Ten plaques were photographed and NIH Image software used to calculate plaque areas. The numbers shown in each panel are the average areas +/- standard deviation for each set of plaques compared to F-BAC plaques arbitrarily set at 100.

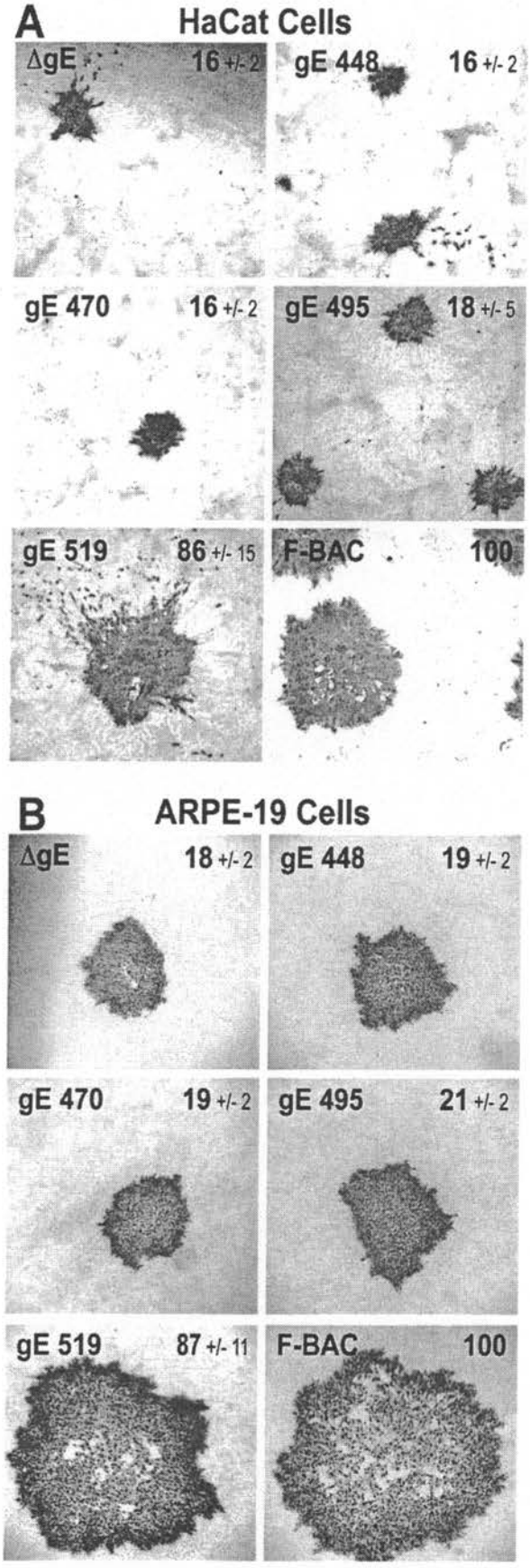

A
HaCat Cells
ΔgE
16 +/- 2
gE 448
16 +/- 2
gE 470
16 +/- 2
gE 495
18 +/- 5
gE 519
86 +/- 15
F-BAC
100
B
ARPE-19 Cells
ΔgE
18 +/- 2
gE 448
19 +/- 2
gE 470
19 +/- 2
gE 495
21 +/- 2
gE 519
87 +/- 11
F-BAC
100

Figure 3.4 Replication of F-BAC mutants. HaCaT cells were infected with F-BAC, F-BAC ΔgE, gE_{448}, gE_{470}, gE_{495} or gE_{519} using 5 PFU/cell for 2h then extracellular virus was inactivated by washing the cells briefly with 0.1M Na citrate, pH 3.0. At various times, the combined cells and media (**A**) or media alone (**B**) were harvested, frozen, sonicated and HSV-1 was titered on Vero cells by plaque titration. The standard deviations were shown as bars and can be seen in a few cases, but for most points, these bars were small and covered by the symbols.

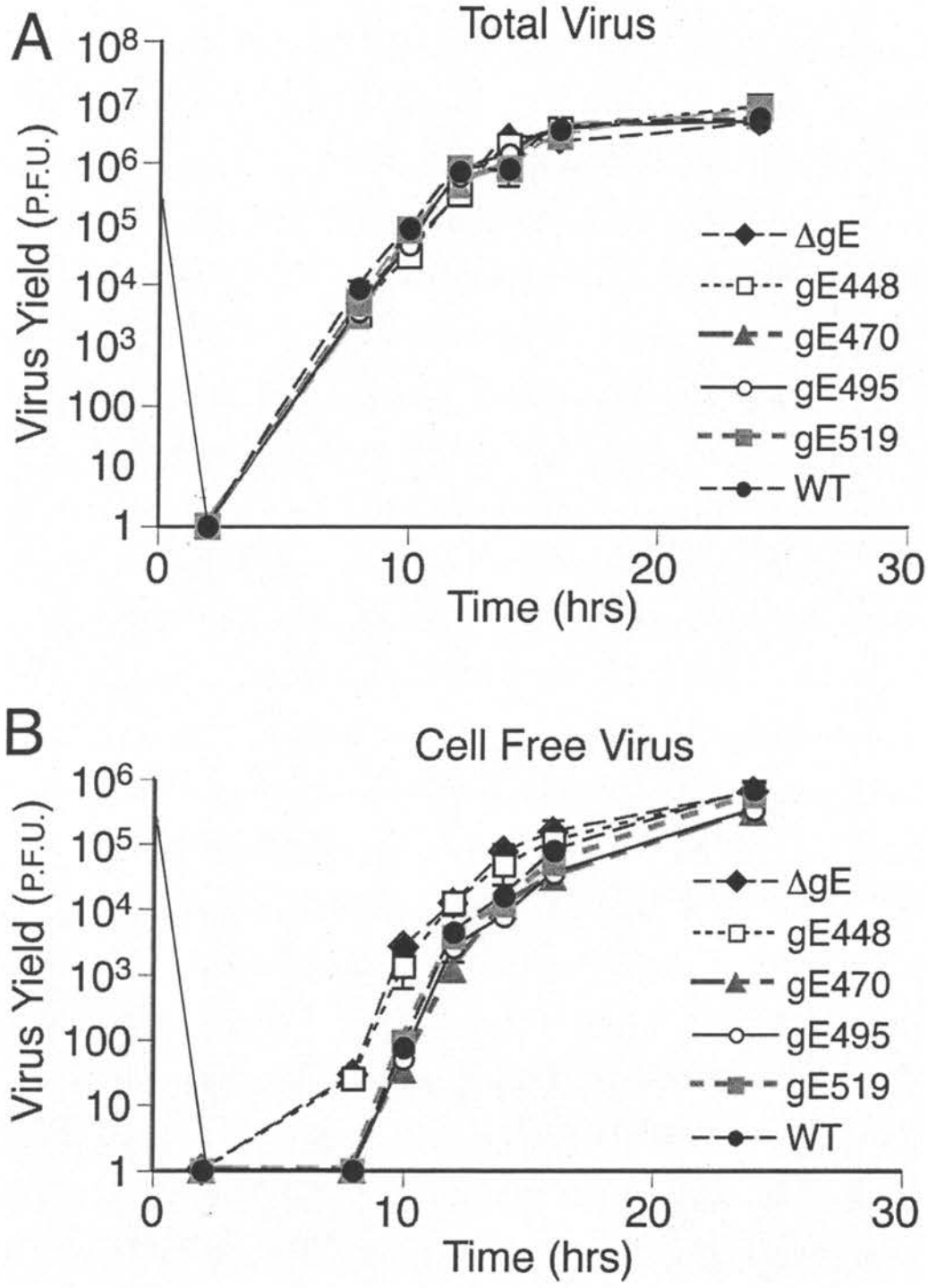
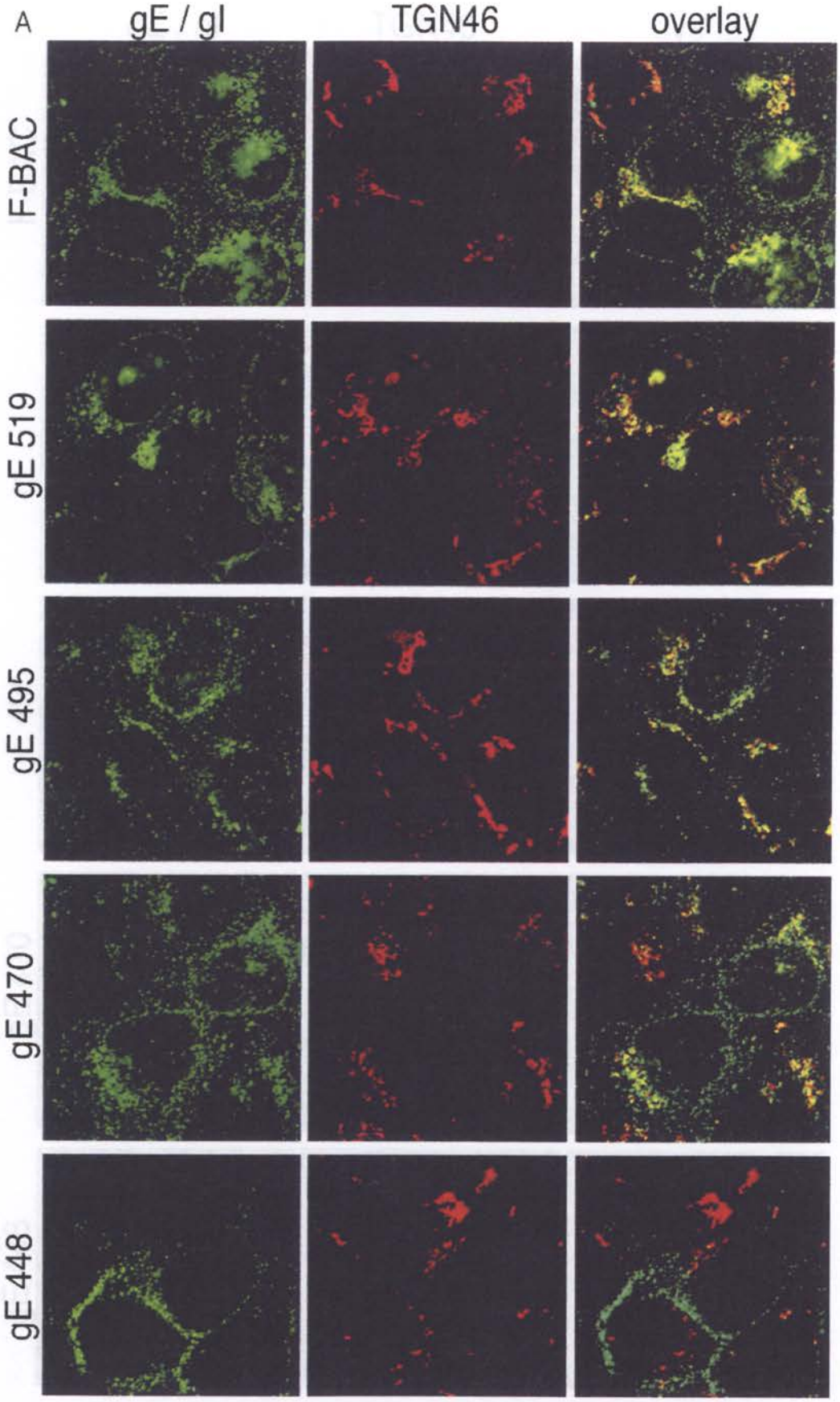

A
Total Virus
Virus Yield (P.F.U.)
10^8
10^7
10^6
10^5
10^4
10^3
100
10
1
0
10
20
30
Time (hrs)
ΔgE
gE448
gE470
gE495
gE519
WT
B
Cell Free Virus
Virus Yield (P.F.U.)
10^6
10^5
10^4
10^3
100
10
1
0
10
20
30
Time (hrs)
ΔgE
gE448
gE470
gE495
gE519
WT

Figure 3.5. Accumulation of gE/gI in the TGN. HaCaT cells (**A**) or ARPE-19 cells (**B**) were infected with F-BAC, F-BAC ΔgE, gE_{448}, gE_{470}, gE_{495} or gE_{519}. After 6 h, the cells were fixed, permeabilized and then stained with MAb 3114 (anti-gE, green) and simultaneously with sheep anti-TGN46 (red). They were then washed and stained with secondary antibodies: Alexa 488-conjugated goat anti-mouse IgG and Alexa 594-conjugated donkey anti-sheep IgG and characterized by confocal microscopy.


A
gE / gI
TGN46
overlay
F-BAC
gE 519
gE 495
gE 470
gE 448

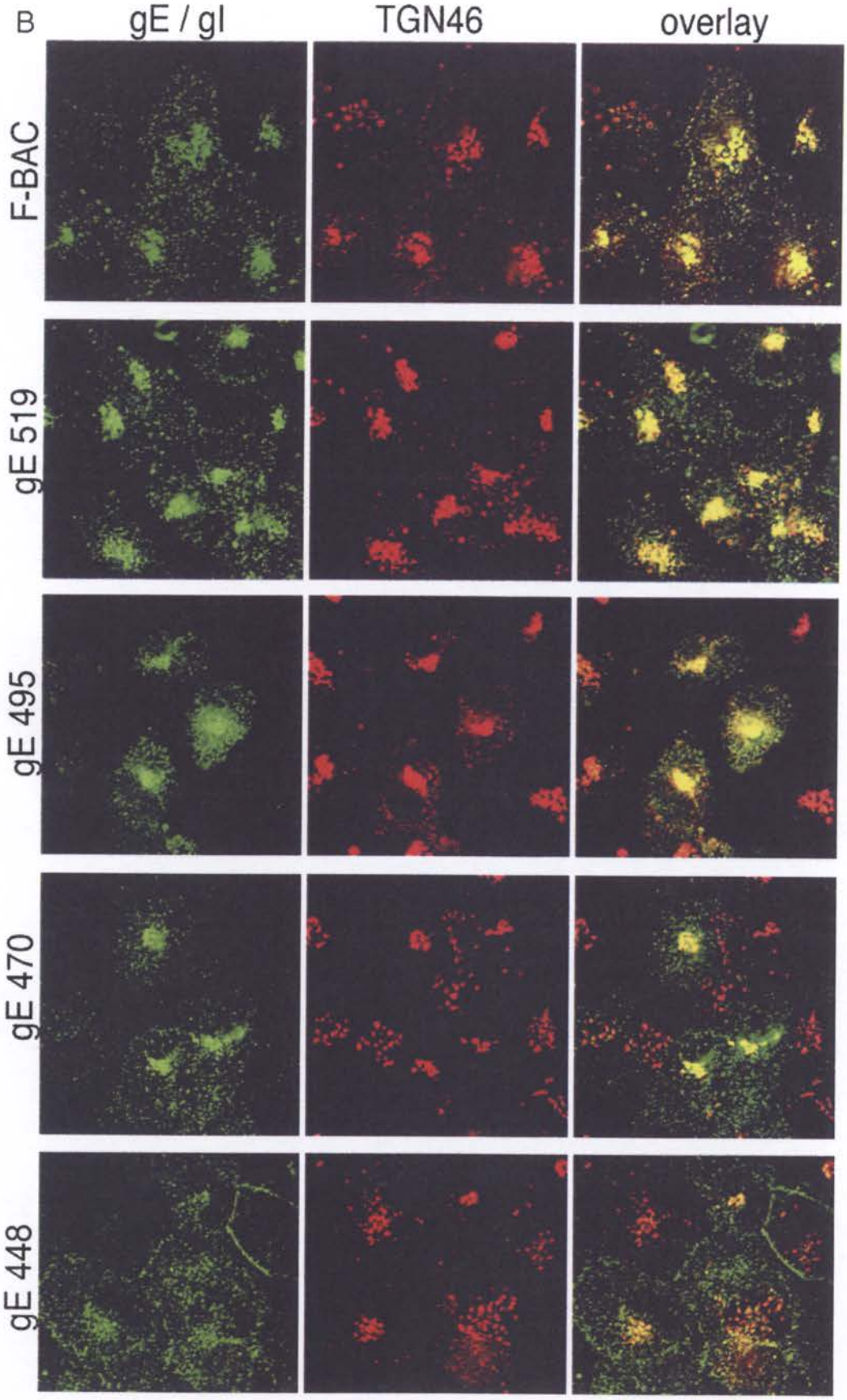
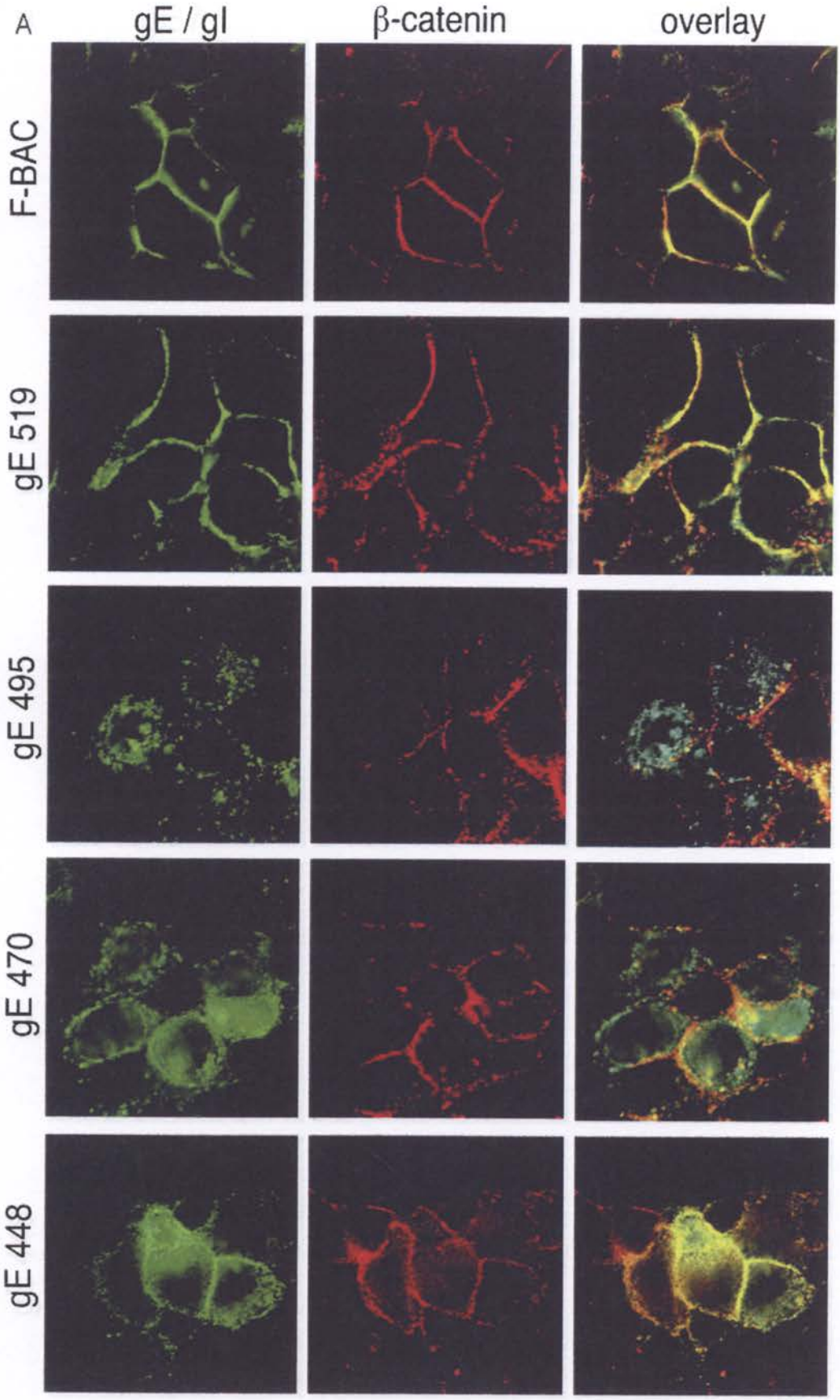

B
gE / gI
TGN46
overlay
F-BAC
gE 519
gE 495
gE 470
gE 448

Figure 3.6 Redistribution of gE/gI to lateral junctions. HaCaT cells (**A**) or ARPE-19 cells (**B**) were infected with F-BAC, F-BAC ΔgE, gE_{448}, gE_{470}, gE_{495} or gE_{519} for 11.5h. The cells were fixed, permeabilized and then stained with MAb 3114 (anti-gE, green) and simultaneously with rabbit anti-β-catenin (red) antibodies. Cells were then washed and stained with secondary antibodies: Alexa 488-conjugated goat anti-mouse IgG and Cy5-conjugated goat anti-rabbit IgG.


A
gE / gI
β-catenin
overlay
F-BAC
gE 519
gE 495
gE 470
gE 448

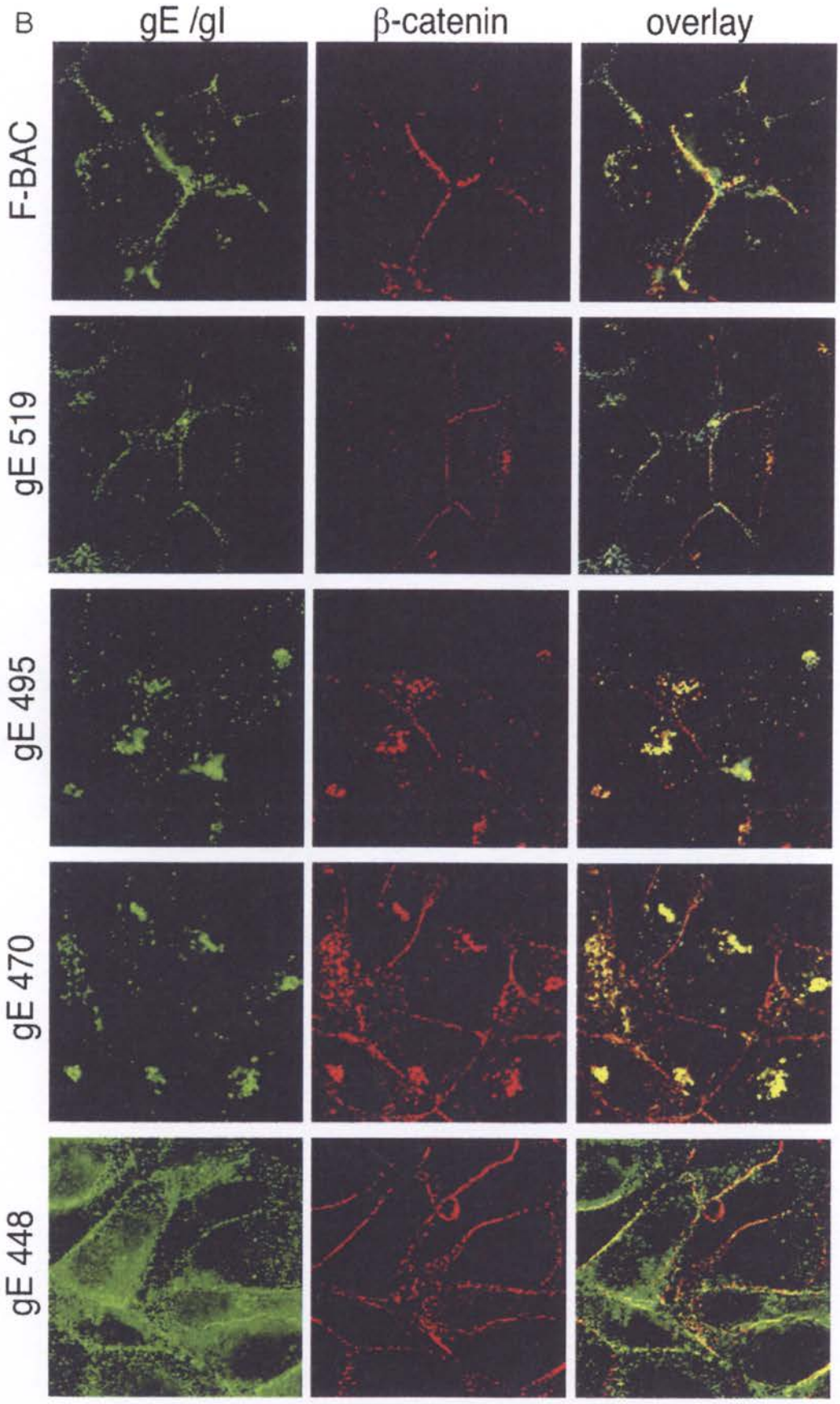

B
gE /gI
β-catenin
overlay
F-BAC
gE 519
gE 495
gE 470
gE 448

Figure 3.7 Binding of IgG-coated RBC to cells infected with gE CT domain mutants.

HaCaT cells were infected with F-BAC, F-BACΔgE, gE_{448}, gE_{470}, gE_{495} and gE_{519} using 5 PFU/cell for 10 h. The cells were incubated with [^{51}Cr] labelled, IgG-coated RBC for 2 h at 37°C, then the cells were washed and total radioactivity bound to cells determined. The amount of radioactivity bound by F-BAC infected cells was arbitrarily set at 100 and triplicate wells infected with F-BAC ΔgE, gE_{448}, gE_{470}, gE_{495} and gE_{519} were compared to this value.

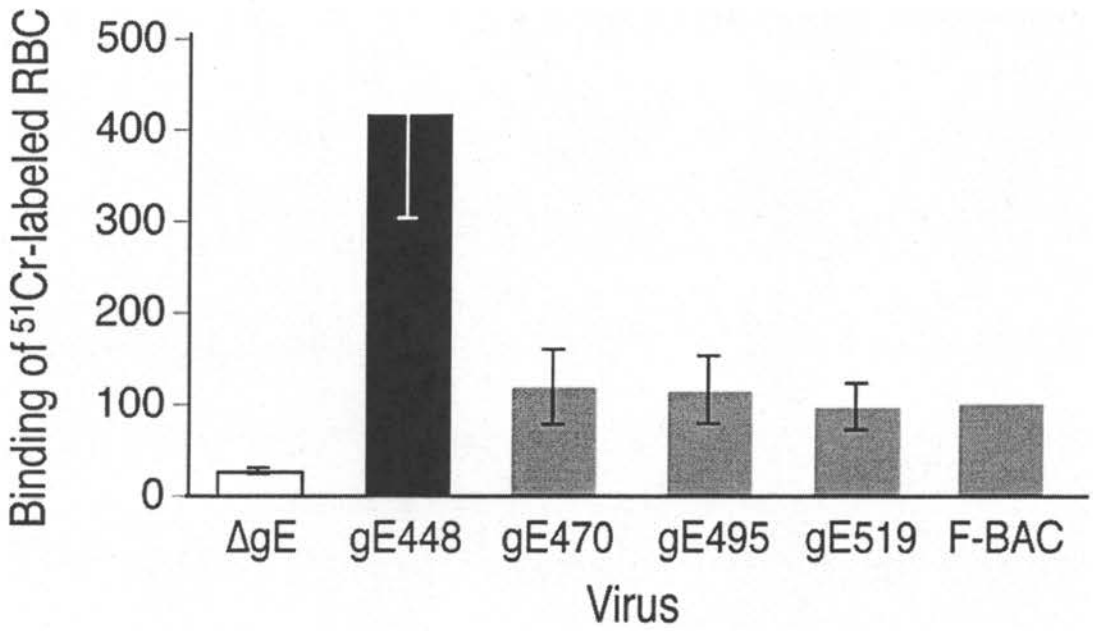
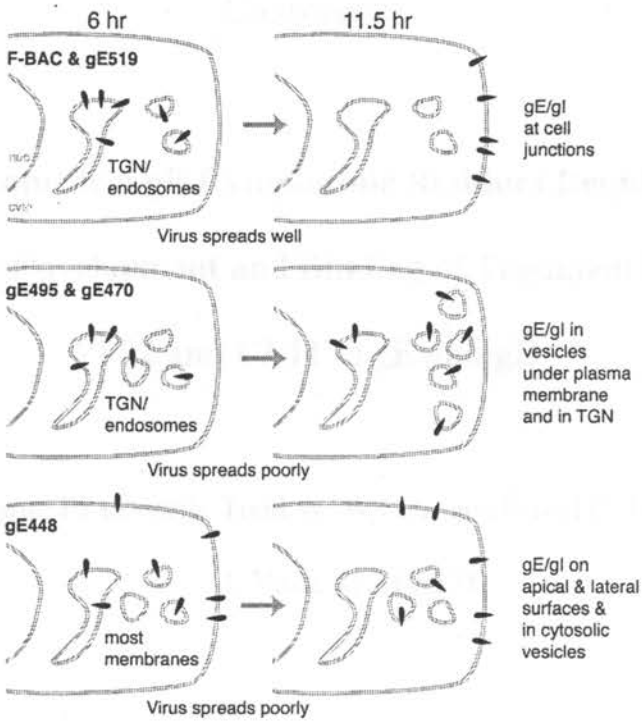

Binding of 51Cr-labeled RBC
500
400
300
200
100
0
ΔgE
gE448
gE470
gE495
gE519
F-BAC
Virus

Figure 3.8 Cartoon summarizing traffic of gE mutants. In cells infected with mutant gE_{519} and wild type HSV-1, gE/gI is initially found in the TGN but the moves to cell junctions, and these viruses spread well. gE/gI expressed in gE_{470} and gE_{495}-infected cells localizes initially to the TGN and then remains primarily in cytosolic vesicles, some underlying the plasma membrane and these viruses spread poorly. In gE_{448}-infected cells, gE/gI does not accumulate in the TGN and remains distributed throughout the cytoplasm and on apical and cell surfaces and this mutant spreads poorly.


6 hr
11.5 hr
F-BAC & gE519
TGN/
endosomes
gE/gI
at cell
junctions
Virus spreads well
gE495 & gE470
TGN/
endosomes
gE/gI in
vesicles
under plasma
membrane
and in TGN
Virus spreads poorly
gE448
most
membranes
gE/gI on
apical & lateral
surfaces &
in cytosolic
vesicles
Virus spreads poorly

Chapter 4:

HSV glycoprotein gE Cytoplasmic Residues Required for Secondary Envelopment and Binding of Tegument Proteins VP22 and UL11 to gE and gD

Aaron Farnsworth, Todd W. Wisner, and David C. Johnson

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In the following chapter Aaron Farnsworth carried out all experiments, prepared and stained samples for electron microscopy sectioning, constructed all BAC-derived HSV viruses, all TAP constructs and Ad-vectors expressing TAP constructs. Todd W. Wisner created the Ad-vector expressing VP22. Mike Web sectioned samples for electron microscopy. This chapter was co-authored by Aaron Farnsworth and David Johnson.

4.1 Abstract

The final assembly of herpes simplex virus (HSV) involves binding of tegument-coated capsids to viral glycoprotein-enriched regions of the *trans*-Golgi network (TGN). We previously demonstrated that HSV gE/gI and gD, acting in a redundant fashion, are essential for this secondary envelopment. In order to define regions of the cytoplasmic (CT) domain of gE required for secondary envelopment, recombinant HSV lacking gD and expressing truncated gE molecules were constructed. A central region of the gE CT domain (a.a. 470 to 495) was necessary for secondary envelopment however, C-terminal residues were also important. Tandem affinity purification proteins including fragments of the gE CT domain were used to identify tegument proteins VP22 and UL11 as binding partners and gE CT residues 470-495 were important in this binding. VP22 and UL11 were also coimmunoprecipitated from HSV-infected cells in conjunction with full-length gE, gE CT domain truncations and gD. However, substantial quantities of VP22 and UL11 bound non-specifically onto gE and gD molecules lacking most of the CT sequences. Expression of VP22 and gD or gE/gI in cells using adenovirus vectors provided evidence that other viral proteins were not necessary for this tegument/glycoprotein interaction. Therefore, there is specific binding of VP22 and UL11 to the CT domains of gE and gD, but VP22, UL11 and VP16 were found to be relatively sticky proteins that also interact non-specifically.

4.2 Introduction

Herpesvirus capsids cross nuclear membranes by being enveloped at the inner nuclear membrane followed by fusion or de-envelopment at the outer nuclear membrane, delivering nucleocapsids into the cytoplasm (reviewed in (Johnson, D. C. and Huber, M. T. 2002; Mettenleiter, T. C. 2002; Mettenleiter, T. C. 2006)). Secondary envelopment occurs as

herpesvirus tegument-coated capsids bind onto viral glycoprotein-enriched regions of the Golgi apparatus, *trans*-Golgi network (TGN) or endosomes. This delivers enveloped virions into cytoplasmic vesicles that are subsequently trafficked to cell surfaces.

How tegument-coated nucleocapsids interact with membranes to promote herpesvirus budding is not well understood. Recent studies have suggested that redundant interactions between tegument proteins and the cytoplasmic domains of specific viral glycoproteins are required. Herpes simplex virus (HSV) produces as many as 12 membrane glycoproteins, as well as other non-glycosylated membrane proteins (Spear, P. G. and Longnecker, R. 2003). Any one of these membrane proteins can be deleted without substantially reducing the numbers of enveloped virions produced. However, HSV mutants lacking both gD and gE did not produce enveloped particles (Johnson, D. C. et al. 2001; Farnsworth, A. et al. 2003)(Chapter 2). Instead, these mutants produced aggregates including thousands of tegument-coated capsids in the cytoplasm. Deletion mutants lacking gD and gI displayed more subtle defects in assembly, while mutants lacking gD, gE and gI displayed more profound defects compared with gD-/gE- mutants (Chapter 2). There were only minor (2-3 fold) defects in secondary envelopment when either gD or gE were individually deleted (Johnson, D. C. et al. 2001; Farnsworth, A. et al. 2003)(Chapter 2). Another alphaherpesvirus, the porcine herpesvirus or pseudorabies virus (PRV) exhibited a different requirement for this process. PRV mutants lacking both gM and gE failed to produce enveloped particles and accumulated capsids in the cytoplasm (Brack, A. R. et al. 1999; Brack, A. R. et al. 2000). By contrast, an HSV mutant lacking gE and gM was not compromised for secondary envelopment (Browne, H. et al. 2004). It has become an important paradigm that herpesviruses utilize viral glycoproteins, and likely tegument proteins, in redundant interactions for assembly.

HSV glycoproteins gE/gI and gD have important functions beyond participating in virus envelopment. gD is essential for entry and spread in all cells tested to date and mutants lacking gD are propagated on complementing cells (Ligas, M. W. and Johnson, D. C. 1988). An HSV mutant lacking the gD CT domain could enter cells normally but produced smaller plaques and reduced yields of infectious virus (Feenstra, V. et al. 1990) possibly representing minor defects in assembly. HSV gE/gI complexes are required for HSV cell-to-cell spread in both neuronal and epithelial cells (Johnson, D. C. and Feenstra, V. 1987; Dingwell, K. S. et al. 1994; Dingwell, K. S. et al. 1995; Dingwell, K. S. and Johnson, D. C. 1998). We proposed that gE/gI functions in HSV cell-to-cell spread by promoting movement of newly assembled virions to epithelial cell junctions (reviewed in (Johnson, D. C. and Huber, M. T. 2002)). The CT domains of gE/gI allow the glycoprotein to interact with TGN sorting machinery and, specifically, the gE CT domain is necessary for TGN accumulation as well as cell-to-cell spread (Wisner, T. et al. 2000; Johnson, D. C. et al. 2001; McMillan, T. N. and Johnson, D. C. 2001; Wisner, T. W. and Johnson, D. C. 2004; Farnsworth, A. and Johnson, D. C. 2006)(Chapter 3). Coupled with observations that the HSV gE CT domain functions in secondary envelopment, this suggested that gE/gI promotes envelopment into specific subdomains of the TGN from which sorting occurs so that nascent virions move specifically to cell junctions (Johnson, D. C. et al. 2001; Johnson, D. C. and Huber, M. T. 2002). In polarized cells the TGN is the major site for plasma membrane sorting (Hunziker, W. et al. 1991; Ang, A. L. et al. 2004). HSV mutants lacking the gE CT domain were mislocalized in epithelial cells and transported apically rather than to basolateral surfaces and cell junctions (Johnson, D. C. et al. 2001). We recently demonstrated that N-terminal, juxtamembrane regions of the gE CT domain promote TGN localization at early times of infection, while sequences nearer the C-terminus promote trafficking to cell junctions

late in infection (Chapter 3). Both segments of the gE CT domain were required for gE/gI-mediated cell-to-cell spread.

There are also numerous (15 or more) HSV tegument proteins that form a complex lattice divided into at least two coats: i) an inner layer including VP1/2 and UL37 that associate directly with capsids and ii) a more peripheral layer composed of proteins that interact with the envelope (reviewed in (Mettenleiter, T. C. 2002; Mettenleiter, T. C. 2006). Among the peripheral tegument proteins are three polypeptides: VP22 (UL49), VP16 (UL48), and UL11 that have been implicated in secondary envelopment. VP22 is one of the most abundant HSV tegument proteins (Heine, J. W. et al. 1974) that associates with cellular membranes and localizes to the TGN and endosomes when expressed without other HSV proteins (Brignati, M. J. et al. 2003). A mutant HSV lacking VP22 was able to assemble enveloped virions that reached extracellular compartments and produced virions with reduced immediate early proteins: ICP0 and ICP4 and displayed delayed viral protein synthesis (Elliott, G. et al. 2005). Another VP22- mutant described recently displayed defects in reaching extracellular compartments and in cell-to-cell spread (Duffy, C. et al. 2006). Thus, VP22 plays some role in the final assembly of HSV, although likely in a redundant fashion with other tegument proteins.

VP16 is also an abundant component of the tegument (Heine, J. W. et al. 1974) and interacts with VP22 (Elliott, G. et al. 1995). HSV and PRV VP16 mutants display increased numbers of cytoplasmic unenveloped capsids and few or no enveloped capsids (Lam, Q. et al. 1996; Mossman, K. L. et al. 2000; Fuchs, W. et al. 2002). VP16- mutants accumulate capsids in the cytoplasm but these are not highly aggregated, as in the case of gD-/gE- mutants. This implies that, without VP16, tegument proteins responsible for aggregation do not assemble around these capsids.

The HSV UL11 protein is modified with myristate and palmitate and contains an acidic cluster that is necessary for association with cellular membranes (MacLean, C. A. et al. 1991; Baines, J. D. et al. 1995; Loomis, J. S. et al. 2001). An HSV UL11- mutant exhibited fewer enveloped virions and a 2-3 fold increase in unenveloped cytoplasmic capsids (Baines, J. D. and Roizman, B. 1992). Similarly, a PRV UL11- mutant displayed defects in secondary envelopment (Kopp, M. et al. 2003) and a UL11-/gM- double mutant displayed large cytoplasmic capsid aggregates (Kopp, M. et al. 2003). Consistent with the notion that UL11 has a conserved role in multiple herpesvirus families, a mutant human cytomegalovirus (HCMV) unable to express the UL11 homologue, UL99, accumulated large numbers of tegument-coated, cytoplasmic capsids (Silva, M. C. et al. 2003).

Interactions between tegument proteins and glycoproteins have been described by biochemical analyses. Early studies involving affinity purification and crosslinking indicated that VP16 interacts with glycoproteins gB, gD and gH (Johnson, D. C. et al. 1984; Wang, Z. H. et al. 2001). Two hybrid analyses have indicated that PRV VP22 interacts with the CT domains of gE and gM (Fuchs, W. et al. 2002). In vitro experiments indicated that the HSV gD CT domain fused to glutathioine-S-transferase (GST) interacts with VP22 as well as with tegument-coated nucleocapsids and VP22 was immunoprecipitated with gD from HSV-infected cells (Chi, J. H. et al. 2005). Moreover, VP16 can interact with glycoprotein H (gH) *in vivo* and by coimmunoprecipitation (Gross, S. T. et al. 2003; Kamen, D. E. et al. 2005). Genetic confirmation of certain of these interactions has been reported. HSV VP22-null mutants incorporate reduced amounts of gB, gD and gE into the virion envelope (Elliott, G. et al. 2005; Duffy, C. et al. 2006). Moreover, a mutant PRV lacking both gE/gI and gM incorporated less VP22 into virions (Fuchs, W. et al. 2002).

In order to better understand the final assembly of HSV, we attempted to define regions within the relatively large gE CT domain that are essential for secondary envelopment. A region in the middle of gE CT domain, including residues 470-495, played a key role in envelopment, although other residues nearer the C-terminus also contributed. Using TAP fusion proteins containing the full-length gE CT domain, we identified HSV tegument proteins UL11 and VP22 as binding partners of the CT domain and less UL11 and VP22 bound to truncated gE CT molecules. VP22 and UL11 were precipitated in conjunction with gE/gI and also with gD from HSV-infected cells.

4.3 Results

4.3.1 Construction of gD and gE mutant viruses.

We previously demonstrated that HSV-1 gD and gE/gI act in a redundant fashion to promote secondary envelopment (Chapter 2). Tegument proteins likely interact with the CT domains of these glycoproteins in order to assemble an envelope around capsids as virions bud into the TGN. The gE CT domain is large, extending from three juxtamembrane arginine residues beginning at residue 445 to residue 550, whereas the gD CT domain is 30 amino acids in length. The N-terminal half of the gE CT domain (residues 448-495) contains numerous recognizable TGN sorting motifs and we demonstrated that these sequences were necessary for gE/gI complexes to localize to the TGN in early stages of virus replication (Chapter 3). Residues nearer the C-terminus of the CT domain (residues 495-519) were required for redistribution of gE/gI complexes to cell junctions a process that occurs at late times coincident with movement of virions to cell junctions.

Here, we sought to determine which gE CT domain sequences were important for secondary envelopment. For these studies, it was necessary to construct HSV expressing gE

truncation mutations but unable to express gD. This was initiated with previously described bacterial artificial chromosomes (BACs) expressing HSV-1 (strain F) mutant genomes containing various gE truncations (Chapter 3). For each of these mutant HSV/BAC genomes gD (US6) sequences were replaced with a kanamycin gene cassette (Fig. 4.1A). Additionally, two other BAC were produced as controls: i) BAC gD-, a BAC in which the kanamycin gene cassette replaced gD sequences and ii) BAC gD-/gE-, derived from BAC gE- (Chapter 3) lacking both gE coding sequences and gD sequences. These BACs were transfected into cells to produce viruses: F-BAC gD-/gE_{519} expressing gE truncated at residue 519, F-BAC gD-/gE_{495} truncated at gE residue 495, F-BAC gD-/gE_{470} truncated at gE residue 470, and F-BAC gD-/gE_{448} truncated at gE residue 448 (with only 3 residues of the CT domain), F-BAC gD- expressing wild type gE and lacking gD, and F-BAC gD-/gE- lacking both gD and gE. Because gD is essential for entry, these BAC DNAs were transfected into VD60 cells which express gD (Ligas, M. W. and Johnson, D. C. 1988).

Mutant viruses were characterized for expression of gD, gB and gE by infecting Vero cells and subsequently radiolabeling them with [^{35}S]-methionine/cysteine and immunoprecipitating gD with MAb DL6, gE with MAb 3114 and gB with MAb 15βB2. F-BAC (w.t. in Fig. 4.1B) expressed immature (51 kDa) and mature (56 kDa) forms of the protein gD (Fig. 4.1B). F-BAC gD-, F-BAC gD-/gE519, F-BAC gD-/gE_{495}, F-BAC gD-/gE_{470}, F-BAC gD-/gE_{448} and F-BAC gD-/gE- did not express gD and expressed gE molecules of the sizes predicted from previous work (Chapter 3). All viruses produced similar quantities of gB.

4.3.2 Secondary envelopment of F-BAC gD-/gE CT mutants.

Viruses deleted for both gD and gE undergo final envelopment inefficiently, instead, large numbers of nucleocapsids aggregates in the cytoplasm of infected cells (Chapter 2). As

expected, HEC-1A cells infected with F-BAC gD-/gE- accumulated large aggregates of non-enveloped capsids in the cytoplasm (Fig. 4.2, filled arrow) as previously described with other gD-/gE- and gD-/gE-/gI- mutants (Chapter 2). As before, these aggregates appeared to be immersed in an electron-dense material, presumably containing tegument proteins. These observations were quantified by counting approximately 1000 virus particles in multiple cell sections for each mutant. Particles were categorized as: unenveloped capsids in the nucleus or cytoplasm or enveloped particles in the perinuclear space, cytoplasm or on the cell surface. There were 12-14 fold increases in the numbers of unenveloped nucleocapsids comparing F-BAC gD-/gE- with wild type F-BAC (Fig. 4.3). F-BAC gD-/gE_{448}, missing the entire gE CT domain (gE_{448}), also exhibited aggregates of unenveloped capsids (Fig. 4.2, filled arrow), confirming that the gE CT domain was responsible for this defect. F-BAC gD-/gE_{470}, that expresses a gE with only 25 residues of the CT domain, also displayed a similar defect in maturation and few enveloped virions were observed (Fig 4.2, 4.3). F-BAC gD-/gE_{495} and F-BAC gD-/gE519 primarily produced enveloped virions in the cytoplasm and on cell surfaces (Fig. 4.2, empty arrows) and unenveloped capsids in the cytoplasm were less common, as was the case with wild type (w.t.) F-BAC. However, there were obvious differences between F-BAC gD-/gE_{495} and F-BAC gD- which expresses wild type gE; more unenveloped particles were observed with F-BAC gD-/gE_{495} (see the filled arrow in the lower part of the panel, Fig. 4.2 and Fig. 4.3) as well as enveloped particles in the cytoplasm. F-BACgD-/gE519 displayed an intermediate phenotype between that of F-BAC gD-/gE_{495} and F-BAC gD- (Fig. 4.2 and 4.3). These observations supported a role for the C-terminal sequences (495-550) as a contributing factor in secondary envelopment. We also observed relatively minor defects in assembly with F-BAC gD- compared with F-BAC, as had been described before (Feenstra, V. et al. 1990; Farnsworth, A. et al.

2003)(Chapter 2). We concluded that a region of the gE CT domain between amino acids 470 and 495 is important for secondary envelopment, although sequences between 495 and 550 significantly influence this process.

4.3.3 Construction of gE CT domain tandem affinity purification (TAP) fusions.

The CT domain of gE functions in three related process: TGN accumulation, in secondary envelopment and to promote HSV cell-to-cell spread (reviewed in (Johnson, D. C. and Huber, M. T. 2002). As a method to identify both cellular and viral proteins involved in these processes, we fused the gE CT domain onto a TAP (tandem affinity purification) domain. This TAP domain included two protein A (IgG-binding) sequences separated by a TEV protease site from a calmodulin-binding domain (Fig. 4.4A) allowing sequential purification by using IgG and calmodulin affixed to Sepharose (Rigaut, G. et al. 1999). Truncated versions of the gE CT domain: gE_{519}, gE_{495}, gE_{470} were fused onto the C-terminus of the TAP domain, as well as a control, TAP scrambled that contains no gE sequences but instead has a random sequence of amino acids (Fig. 4.4A). To achieve various levels of expression in different cells, the TAP-tagged proteins were expressed using replication-defective adenovirus (Ad) vectors under the control of a tetracycline -inducible promoter (Tomazin, R. et al. 1996; Hardy, B. et al. 1997; Collins, W. J. and Johnson, D. C. 2003). While, it might have been reasonable to express the TAP fusion proteins in the context of the HSV genome, Ad vectors had the benefit of allowing identification of interacting cellular proteins in future studies.

To assess expression of the TAP::gE fusions, Vero cells were coinfected with Ad vectors expressing TAP::gE proteins and Adtet-trans and western blots performed on cell extracts using an anti-calmodulin binding domain (CBD) antibody. Proteins of the expected size were expressed (Fig. 4.4B). Adtet TAP::gE_{550}, Adtet TAP::gE_{519} and Adtet TAP::gE_{495} all produced a

protein doublet, likely related to phosphorylation events that occur on the gE CT domain (Wisner, T. et al. 2000). There were also faster-migrating species of approximately 20-25 kDa observed with all the TAP fusion proteins including TAP scrambled that may be degradation products lacking the protein A domains, as these bands were not observed with IgG-Sepharose (not shown).

4.3.4 Binding of tegument proteins to TAP gE CT domain.

To characterize the binding of viral proteins to gE CT, the human keratinocyte cell line, HaCaT, was chosen because these cells display the most profound phenotype with gE-null mutants (Wisner, T. et al. 2000). HaCaT cells were infected with Ad vectors expressing TAP::gE_{550} (full-length gE CT) or TAP::gE_{470} that included only 25 gE CT residues (a domain insufficient for secondary envelopment). After 18 h, these cells were infected with an HSV gE-null mutant, F-gEΔCT (Wisner, T. et al. 2000), for an additional 7-8 h, or not infected with HSV, and then radiolabelled with [^{35}S]-methionine/cysteine for 3 h. Under these conditions, there was HSV-induced host protein shutoff and radiolabelled proteins were predominately of HSV origin, although the TAP::gE proteins expressed by the Ad vectors continued to be expressed at high levels (not shown). Cell extracts were made using mild nonionic detergents: 0.5% NP40 or 1% digitonin and two concentrations of NaCl: 100 mM or 500 mM NaCl were used. Extracts were centrifuged at 60,000 X g to remove insoluble or aggregated material and then cell extracts mixed with IgG-Sepharose that binds the protein-A domains of TAP proteins. The extracts were centrifuged at low speeds (200-500 X g) for ≈30 sec to pellet IgG-Sepharose, conditions that did not pellet any significant quantities of viral proteins (not shown). TAP::gE_{550} pulled down two sets of protein bands that were not observed, or observed in lower quantities, with TAP::gE_{470} (Fig. 4.5). These proteins were observed with 0.5% NP40 and 1% digitonin lysis buffers and

were similar to the sizes of tegument proteins VP22 and UL11. Thus, these proteins were denoted VP22* and UL11* at this point. VP22* and UL11* were not observed with high salt concentrations or when the cells were not infected with F-gEΔCT (Fig. 4.5).

To identify the viral proteins observed in Fig. 4.5, HaCaT cells were infected with Ad vectors expressing TAP::gE fusions or TAP scrambled then superinfected with HSV F-gEΔCT then TAP proteins purified from 0.5% NP40 cell extracts using IgG-Sepharose before blotting with anti-tegument proteins. VP16 was observed in similar quantities whether a TAP::gE fusion protein, TAP scrambled or no TAP protein was expressed in cells (Fig. 4.6A). Thus, it was impossible to observe any specific binding of VP16 to these TAP proteins, although this non-specific interaction provided a positive control for HSV infection in subsequent experiments. VP22 binding to TAP::gE_{550} was observed and there was approximately a third as much binding to TAP::gE_{470} and TAP scrambled (Fig. 4.6B). TAP::gE_{519} and TAP/gE_{495} were intermediate. The non-specific component of VP22 binding was illustrated by the levels of VP22 observed with TAP scrambled and where no TAP protein was expressed and likely reflect non-specific binding to IgG-Sepharose. UL11 also bound to TAP::gE_{550} and approximately as well to TAP::gE_{519} and TAP::gE_{495} and there was less binding to TAP::gE_{470}, TAP scrambled and in the absence of TAP proteins. Expression of the TAP proteins was measured by blotting with anti-protein A antibodies (Fig. 4.6D).

In other experiments, we assessed how well VP22, VP16 and UL11 were solubilized under these extraction conditions by using western blots to probe pelleted material versus protein in supernatants after centrifugation at 60,000 X g. Approximately 35% of the VP22 in cells was soluble, i.e. present in the supernatant fraction, with both 1% digitonin and 0.5% NP40 extraction buffers containing 100 mM NaCl. Similarly, ≈25% of UL11 and VP16 were

solubilized with both these buffers. In all these assays, and in subsequent experiments below, cell extracts were not frozen and experiments carried out quickly under conditions in which tegument proteins did not aggregate or pellet under the low centrifugal forces (200-500 X g) used to harvest Sepharose beads (not shown). Therefore, insolubility during our assays cannot account for the tegument proteins observed. Instead, the non-specific component of binding with these proteins must be explained by binding to these TAP domain or IgG-Sepharose. Other experiments in which proteins associated with these TAP fusion proteins were immunoblotted with antibodies specific for VP1/2, vhs (UL41) and UL24 were consistent with very low or no binding of these tegument proteins (data not shown). VP1/2, vhs and UL24 were partially solubilized in these cell extracts (not shown). Together, these results indicated that the gE CT domain can interact with VP22 and UL11 in the context of HSV-infected cells. Furthermore, the region of the gE CT domain most important for envelopment (470-495), as well as more C-terminal domains, contributed to VP22 and UL11 binding.

4.3.5 Binding of VP22 and UL11 to gE expressed by HSV.

We next attempted to determine whether VP22 and UL11 could also interact with wild type gE and truncated gE expressed by HSV. HaCaT cells were infected with F-BAC or HSV expressing truncated gE molecules, the cells extracted with 0.5% NP40 and extracts centrifuged at 25,000 X g then preincubated with protein A Sepharose beads which were removed by low speed centrifugation. Cell extracts were incubated with anti-gE MAb 3114 and protein A Sepharose for 1 hr then immunoprecipitated proteins subjected to electrophoresis, transferred to PVDF membranes, and blots probed with antibodies specific to VP16, VP22 or UL11. Again, VP16 was non-specifically precipitated with antibody and protein A-Sepharose, i.e. in samples from cells that did not express gE (Fig. 4.7A, top panel). VP22 was observed with wild type gE

and there was less VP22 detected with gE_{519}, gE_{495}, gE_{448} and a gE-null mutant (Fig 4.7A, middle panel). Expression of the different gE molecules was similar in each case (Fig. 4.7B). Therefore, a substantial fraction of the specific binding of VP22 to gE was to a region including residues 519-550 at the extreme C-terminus of gE. UL11 also bound specifically to the full-length gE, there was approximately 5 fold more UL11 compared with a gE-null mutant and 2 fold more UL11 comparing full length gE CT domain with gE_{448} (Fig. 4.7A, lower panel). In this case, the specific binding component of UL11 primarily involved residues near the C-terminus of the gE CT domain between residues 495-550. There was also substantial non-specific binding, with gE_{448} that lacks most of the CT domain. This non-specific binding was not due to insolubility during the assay because these extracts had been centrifuged at high speed for longer periods then preincubated with protein A-Sepharose and centrifuged at low speed (200-500 X g) before the pull down assays that involved low speed centrifugation for ≈30 sec. Instead, these non-specific interactions involved tegument protein binding to regions of gE other than the CT domain, with IgG, or with protein A Sepharose. Binding to gE extracellular domain is illustrated by the levels of UL11 observed with gE_{448} compared with a gE-null mutant (Fig. 4.7A). Although non-specific binding was observed, there was also clearly specific binding to VP22 and UL11 to gE that required the gE CT domain and specifically sequences in the C-terminal half of the gE CT (residues 495 to 550) that contributes to secondary envelopment.

4.3.6 Binding of VP22 and UL11 to the CT domain of HSV gD.

HSV gD and gE apparently interact with multiple tegument proteins where the glycoproteins and the tegument proteins serve redundant functions in secondary envelopment. We extended our analyses to gD, by immunoprecipitating gD and blotting with anti-tegument antibodies. VP22 was precipitated extensively with gD, and much less (≈ 6%) VP22 was

observed when gD was absent (Fig. 4.8A). Additionally, UL11 was precipitated from extracts of HSV-infected cells with gD antibodies and less (≈ 4%) was detected when gD was absent (Fig. 4.8A). Again, VP16 was precipitated by anti-gD MAb whether cell extracts contained or did not contain gD.

Given our observations that VP22 and UL11 precipitated in substantial quantities with gE lacking the CT domain (Fig. 4.7), we also compared binding of these tegument proteins to gD molecules with and without the CT domain. F-dl2 expresses a gD molecule lacking the CT domain and produces slightly smaller plaques compared with wild type HSV-1 (Feenstra, V. et al. 1990). HaCaT cells were infected with F-dl2, HSV-1 strain F (the parental virus), or vRR1097, a gD-null virus derived from F (Rauch, D. A. et al. 2000). gD was immunoprecipitated and precipitated proteins blotted with VP22-specific or UL11-specific antibodies. There were significantly less (11-16%) VP22 and UL11 precipitated from extracts of the gD-null mutant vRR1097 compared with wild type HSV expressing gD (Fig 4.8B). However, substantially more VP22 and UL11 were observed when gD was immmunoprecipitated from F-dl2-infected cells, compared with the gD-null mutant. VP22 binding to gD lacking a CT domain (F-dl2) was reduced to 26% that observed with wild type gD and UL11 was reduced to only 76% that observed with wild type gD. Expression of wild type and F-dl2 gD was characterized by western blotting using a rabbit anti-gD serum. Unfortunately, anti-rabbit secondary antibodies cross-reacted with the mouse MAb DL6 (anti- gD) and IgG heavy and light chains were observed (Fig. 4.8B). Nevertheless, it was clear that F-dl2 gD, a smaller molecule, was expressed at similar levels as wild type gD.

To confirm interactions between VP22 and UL11 and gE and gD, reciprocal pull down experiments were performed. It was impossible to perform these experiments with gE/gI

because gE/gI is an IgG Fc receptor and binds rabbit IgG. The only available anti-tegument antibodies are rabbit antibodies which precipitate gE/gI. However, precipitation of VP22 from extracts of wild type HSV infected cells followed by blotting with anti-gD MAb DL6 produced a strong band corresponding to gD (Fig. 4.8D). There was a fainter, faster migrating band with extracts from a gD-null mutant, which resulted from low level cross reactivity of the anti-mouse IgG secondary antibody. Attesting to this, the IgG light chain was also detected in this blot (not shown). Moreover, gD was detected in precipitates using anti-UL11 antibodies against an extract of wild type HSV-infected cells but not a gD- null virus (Fig. 4.8E). We concluded that VP22 interacts substantially and in a specific fashion with gD, interactions that require the CT domain of gD. There was more limited specific binding of UL11 to gD. In our assays, as much as 50-75% of the binding of UL11 to gD occurred with a mutant lacking the CT domain.

4.3.7 Binding of VP22 to gD and gE in the absence of other HSV proteins.

Given that tegument proteins exist in a matrix with other viral proteins in infected cells, it was of interest to determine whether VP22 could bind to gD and gE/gI in the absence of other HSV proteins. A non-replicating adenovirus (Ad) vector expressing VP22 was constructed as described (Tomazin, R. et al. 1996) and Ad vectors expressng gE/gI and gD have been described (Brunetti, C. R. et al. 1998) . VP22, gE/gI and gD were expressed in HaCaT cells using the Ad vectors then gE/gI or gD were immunoprecipitated from cell extracts. Precipitated proteins were subjected to electrophoresis and probed with VP22-specific antibodies. Approximately 3 fold more VP22 was precipitated from extracts of cells expressing gE/gI compared with cells not expressing gE/gI (Fig. 4.9, upper panel). This was observed despite lower levels of expression of VP22 in cells that were also expressing gE/gI (Fig. 4.9 lower panel). Similarly, there was ≈5 fold more VP22 precipitated from cells expressing gD compared with those not expressing gD

when extracts were immunoprecipitated with an anti-gD antibody. The lower levels of VP22 expressed in cells coinfected with Ad vectors expressing both gE and gI, and to a lesser extent with gD, relates to competition for transactivator proteins. (Huber, M. T. et al. 2001). We concluded that VP22 can interact with gE/gI and gD without the requirement of other viral proteins.

4.4 Discussion

The final stages of HSV assembly begin with the targeting of certain membrane glycoproteins and tegument proteins to the TGN. Prominent among the viral membrane proteins that accumulate in the TGN when expressed without other viral proteins are gE/gI, gB and gM. gM/UL49A causes gD and gH/gL to relocalize from the plasma membrane to the TGN and can relocalize several cellular proteins to the TGN (Crump, C. M. et al. 2004). HSV gE/gI and gB localize to the TGN in early phases of virus replication but then redistribute to epithelial cell surfaces and cell junctions late in infection (McMillan, T. N. and Johnson, D. C. 2001; Wisner, T. W. and Johnson, D. C. 2004). The CT domains of these glycoproteins contain TGN sorting motifs that cause them to accumulate at sites of virus assembly (Wisner, T. et al. 2000; McMillan, T. N. and Johnson, D. C. 2001; Beitia Ortiz de Zarate, I. et al. 2004; Crump, C. M. et al. 2004). Apparently there are other HSV proteins that then redistribute TGN membranes to cell surfaces at late times of infection in order to promote egress of assembled virions (Wisner, T. W. and Johnson, D. C. 2004). The CT domains of gE/gI and gD, acting in a redundant fashion, are necessary to tether tegument-coated capsids onto TGN membranes (Chapter 2). Tegument proteins such as UL11, VP16 and VP22 also accumulate at TGN assembly sites either by directly interacting with TGN sorting machinery through acidic domains or other motifs, by interacting with other tegument proteins, or by interacting with membrane proteins e.g. gD, gE/gI or gM/gN.

Presumably, interactions between tegument proteins and the CT domains of gE and gD drive the wrapping of a virion envelope around capsids as HSV particles bud into TGN-derived vesicles. Given the requirement for gE/gI or gD in this process and the phenotypes of HSV tegument mutants, it is very likely that tegument proteins interacting with gE/gI and gD also act in a redundant fashion in secondary envelopment.

We dissected the gE CT domain required for secondary envelopment in an attempt to better understand HSV envelopment at the TGN. Of interest was whether gE CT sequences closer to the C-terminus and not required for TGN sorting but required for HSV spread (Chapter 3) were necessary for envelopment. HSV recombinants lacking gD and the entire gE CT domain produced large cytoplasmic accumulations of nucleocapsids and few enveloped virions, as had been previously shown with double gD-/gE- null mutants (Chapter 2). This established what we presumed, that the gE CT domain was necessary for secondary envelopment. F-BAC gD-/gE_{470} exhibited similar defects but gE_{470} localizes to the TGN at early times of infection (Chapter 3). Therefore, accumulation of gE/gI at assembly sites is not sufficient in order to promote envelopment. F-BAC gD-/gE_{495} exhibited a substantial improvement in secondary envelopment compared with F-BAC gD-/gE_{470}, there were largely enveloped instead of unenveloped capsids. We concluded that a region of the gE CT domain between residue 470 and 495 is important for secondary envelopment. However, sequences nearer the C-terminus of the CT domain also contributed significantly, more enveloped virons and fewer unenveloped capsids were observed with gE_{519} and with gE_{550} (full length gE) compared to gE_{495}.

To identify tegument proteins that bound to the gE CT domain, full-length and truncated CT domains were fused onto a TAP domain and expressed using Ad vectors in HSV-infected cells. This protocol had some advantages over some previous efforts to characterize

glycoprotein/tegument interactions involving GST proteins that were purified and subsequently incubated with cell extracts (Gross, S. T. et al. 2003; Chi, J. H. et al. 2005; Kamen, D. E. et al. 2005) and yeast two hybrid analyses (Fuchs, W. et al. 2002; Vittone, V. et al. 2005). In our experiments, TAP proteins were able to interact with viral proteins within HSV-infected cells. Radiolabelled proteins with sizes similar to UL11 and VP22 were observed with TAP::gE550, and less so with so with TAP::gE_{470} and the identity of these proteins was confirmed by western blotting. Other tegument proteins could well have been missed for a number of reasons: i) certain tegument proteins might interact with the first 25 residues of the gE CT domain, ii) mild nonionic detergents might potentially disrupt interactions dependent on membrane structure or requiring hydrophobic protein surfaces, iii) these tegument proteins may not be solubilized under the conditions used. VP22 and UL11 bound best to the entire gE CT domain fused to TAP (TAP::gE_{550}) and less well to TAP::gE_{470} (Fig 4.5). Coinciding with our observations that a region between 470 and 495 was important in secondary envelopment, TAP::gE_{495} bound more UL11 and VP22 compared with TAP::gE_{470} (Fig. 4.6). However, larger differences are observed when TAP::gE_{550} is compared to TAP::gE_{470}, suggesting that C-terminal residues 495-550 also contribute to binding. Background binding to TAP scrambled or where no TAP protein was expressed was substantial.

We extended the analysis of VP22 and UL11 binding to the gE CT domain in pull down experiments involving HSV-infected cells expressing gE truncations immunoprecipitated with anti-gE MAb and blotted with anti-VP22 or -UL11 antibodies. gE_{470} and gE_{495} bound approximately as much VP22 and UL11 as with gE_{448} which lacks all but three residues of the gE CT domain. Substantially more UL11 was observed with gE_{519} and with full-length gE_{550}. VP22 binding was highest with gE_{550} and low with all the other truncations. Thus, in these

experiments, the binding of VP22 and UL11 to gE was specific but depended primarily on more C-terminal residues (a.a. 495 to 550) than the TAP assays suggested. It is important to note that these residues contribute significantly to secondary envelopment. Thus, the two assays of tegument protein binding to gE indicated the importance of different gE residues in binding tegument proteins. TAP::gE fusion proteins primarily required the central 470-495 residues to bind VP22 and UL11, although the addition of C-terminal residues (495-550) increased VP22 binding. However, gE precipitated from HSV-infected cells primarily depended on the presence of C-terminal residues for both VP22 and UL11 binding. These disparities may be related to differences in subcellular localization, gE proteins associate with membranes and are incorporated into the nascent virion and this is unlikely to be the case with TAP fusion proteins. Furthermore, gE interactions with gI contribute to secondary envelopment (Chapter 2) and may influence binding of tegument proteins. It is unlikely that TAP::gE proteins form complexes with gI. Whatever the origin of these differences in the two assays, it is clear that C-terminal gE residues (a.a. 470-550) contribute to both secondary envelopment and binding of VP22 and UL11.

Binding of VP22 and UL11 to gD was also investigated. Previous experiments involving GST::gD CT domain fusion proteins and immunoprecipitation from HSV-infected cells were consistent with the conclusion that gD binds VP22 (Chi, J. H. et al. 2005). We confirmed and extended these results, by characterizing gD null mutants, as well as F-dl2, a mutant lacking the gD CT domain. VP22 bound well to gD. In side-by-side experiments approximately 2-3 fold more VP22 was observed with gD compared with gE/gI (not shown). However, given that more gD may be expressed and the affinities of the anti-gD and anti-gE MAb may be different, this comparison may accurately reflect the situation in cells. Compared with wild type gD, less

(≈25%) VP22 was precipitated with gD lacking the CT domain and ≈11% with a gD null mutant. Thus, there was clearly specific binding of VP22 that required the CT domain of gD. UL11 also bound to gD, although in this case as much as 75% of the binding observed with wild type gD was observed with the gD mutant lacking the CT domain. We concluded that both VP22 and UL11 can bind specifically to gD, but again there was a substantial non-specific component in these assays. Consistent with the notion that VP22 and UL11 bound specifically to gE and gD, other viral proteins: VP1/2, UL24 and vhs (UL41) were partially solubilized, but did not substantially bind to either gE/gI or gD in parallel pull down experiments.

Many of our experiments investigating glycoprotein/tegument interactions were carried out using HSV-infected cells. These assays had major advantages compared with in vivo experiments in which extracts are mixed with GST proteins or experiments done by transfection or using virus vectors because the tegument proteins were in their native form expressed along with other HSV proteins and properly assembled into larger structures. The majority of these tegument proteins (65-75%) remained associated with the insoluble fraction following cell lysis in either 0.5% NP40 or 1% digitonin and centrifugation at 60,000 x g. Stronger detergents or salts would be expected to perturb protein-protein interactions. It is important to note that the soluble fraction of VP22, UL11 and VP16 did not aggregate and pellet during the course of our pull-down assays. Extracts were centrifuged at high speed, not frozen and immediately precleared with Sepharose beads involving low speed centrifugation before incubation with antibody and protein A-Sepharose and centrifugation at low speed (200-500 X g) for ≈ 30 sec. We concluded that the non-specific binding of UL11, VP22 and VP16 involved stickiness or non-specific binding of proteins to other proteins, not by insolubility that developed during our assays.

The relative amounts of non-specific binding of VP22 and UL11 to gE and gD were often substantial, especially with extracts of HSV-infected cells. U L11 and VP22 bound to tailless gD and gE molecules at quantities amounting to 20-75% of that observed with full-length gD and gE. By comparison, amounts of UL11 and VP22 observed with gE -or gD- null mutants were often substantially lower. Thus, it appeared that a significant fraction of UL11 and VP22 may have interacted with the extracellular domain of the glycoprotein following extraction, a process unlikely to occur *in vivo*. Coupled with observations involving VP16, these results provide an important note of caution when interpreting studies involving tegument proteins. Our observations also suggest that these tegument proteins form higher order structures, perhaps involving other tegument proteins in HSV-infected cells, and it is these higher order structures (still soluble at 60,000 X g) that interact with glycoproteins. By contrast, in cells expressing the proteins after transfection or use of virus vectors such structures lack other viral proteins. It was important to document this non-specific binding to tailless glycoproteins, as these results underscore the notion that these tegument proteins readily adhere onto other viral or cellular proteins. This fits with known properties and functions of tegument proteins that are involved in extensive protein/protein interactions during assembly.. Consequently, efforts to characterize interactions between tegument proteins and other viral proteins require extreme care and numerous controls.

Nevertheless, our studies also strongly support the conclusion that VP22 and UL11 bind specifically onto the CT domains of gE and gD. These interactions were observed with TAP-tagged constructs, in HSV-infected cells, and when glycoproteins and tegument proteins were expressed by using Ad vectors. VP22 binding to gD was the better example of these tegument/glycoprotein interactions. VP22 binding to both gD and gE/gI was also observed when

other viral proteins were absent. However, binding of VP22 and UL11 to gE/gI, although weaker, should not be discounted. Both gE/gI and gD contribute to secondary envelopment and our *in vitro* assays may not reflect the affinity *in vivo*.

gD and gE are apparently the major membrane proteins that link the envelope onto tegument-coated capsids and, thus, our results support the notion that VP22 and UL11 may function in secondary envelopment. VP22 is highly conserved among alphaherpesviruses, although not among other herpesvirus families. Although VP22-null mutants produce relatively normal amounts of enveloped virions, there are defects in the incorporation of gB, gD and gE into virions, movement of virus particles into extracellular compartments, and cell-to-cell spread (Elliott, G. et al. 2005; Duffy, C. et al. 2006). Moreover, a PRV gE-/gI-/gM-null mutant failed to incorporate VP22 into virions (Fuchs, W. et al. 2002). Together, these observations with mutant viruses coupled with our results provide strong support for the hypothesis that VP22 interacts with gD and gE/gI to help promote secondary envelopment. However, UL11 and other tegument proteins may also be involved in this process. HSV and PRV UL11 mutants display defects in secondary envelopment (Baines, J. D. and Roizman, B. 1992; Kopp, M. et al. 2003), although these defects are not as profound as with gD-/gE- HSV. An HCMV mutant lacking the UL11 homologue, UL99 protein, produces few or no enveloped particles (Silva, M. C. et al. 2003) suggesting that UL11 may be more important in other herpesvirus families. To date, there are no reports of HSV VP22-/UL11- double mutants to test the hypothesis that these two proteins are the primary tegument proteins that bridge the tegumented nucleocapsid and the envelope. Certainly, other tegument proteins may also bind to gE and gD and there is the potential that other HSV membrane proteins may be involved in secondary envelopment.

4.5 Materials and Methods

4.5.1 Cells and viruses.

HEC-1A cells (Ball, J. M. et al. 1995) were grown in RPMI medium (BioWhittaker, Inc., Walkersville Md.) supplemented with 10% fetal bovine serum (FBS). HaCaT cells (Boukamp, P. et al. 1988) and Vero cells were grown in Dulbecco's modified Eagle's medium (DMEM) (BioWhittaker) supplemented with 10% FBS. VD60 cells, derived from Vero cells and capable of expressing gD (Ligas, M. W. and Johnson, D. C. 1988), were maintained in DMEM lacking histidine and supplemented with 10% FBS and 0.5 mM histidinol (Sigma, St. Louis, Mo.) 293M and 293-Cre4 cells (Ng, P. et al. 1999) were obtained from Microbix (Toronto, Canada) and maintained in Eagle minimal essential medium (EMEM) supplemented with 10% FBS. Wild type HSV-1 strain F (originally from Pat Spear, Northwestern Medical School), F-dl2 (Feenstra, V. et al. 1990), F-gEΔCT (Wisner, T. et al. 2000), F-BAC HSV-1 (Horsburgh, B. C. et al. 1999) and F-BAC gE CT domain mutants (Chapter 3) were propagated and titered on Vero cells. vRR1097, mutant lacking gD coding sequences (Rauch, D. A. et al. 2000), F-BAC gD-, an HSV-1 derived from a BAC lacking gD sequences, and F-BAC gD-/gE-, lacking gD and gE sequences were propagated and tittered on VD60 cells. Adenoviruses expressing gD (AdgD1(E1-) (Brunetti, C. R. et al. 1998), gE (Ad(E1-) gE), gI (Ad(E1-)gI) (Dingwell, K. S. and Johnson, D. C. 1998), VP22 or TAP fusion proteins were propagated and tittered on 293 cells.

4.5.2 Construction of F-BAC mutants.

HSV-1 mutants containing single and double mutations in gE and gD were constructed as previously described (Chapter 3), using a modification of a protocol developed by Datsenko *et al.* (Datsenko, K. A. and Wanner, B. L. 2000). Briefly, bacteria containing BAC with wild type HSV-1 strain F, gE- or gE truncations were further mutagenized to remove gD coding sequences.

PCR primers which included the first or last 18 nucleotides of the gD coding sequences as well as sequences homologous to the kanamycin gene flanked by FRT (FLP recognition target) sites in the plasmid pKD4 (Datsenko, K. A. and Wanner, B. L. 2000) were used: ATGGGGGGGGCTGCCGCCGTGTAGGTCGGAGCTGCTTC and CTAGTAAAACAAGGGCTGCATATGAATATCCTCCTTAG to produce a DNA fragment in which the majority of the gD coding sequences being replaced by a kanamycin cassette. This DNA was transformed into RR1 bacteria containing HSV BAC (containing wild type HSV sequences, gE- or gE truncations) and pKD46 a plasmid encoding ampicillin resistance and the red recombinase,. Selection for recombinant BAC was performed as described (Farnsworth, A. and Johnson, D. C. 2006). BAC containing mutant HSV-1 genomes were sequenced across, upstream and downstream of the mutation site. BAC DNA was produced and transfected into Vero or VD60 cells as described (Chapter 3).

4.5.3 Construction of recombinant adenovirus (Ad) expression vectors.

The plasmid pAdTet7 contains Tet-responsive enhancer sequences, the simian virus 40 late poly(A) cassette, adenovirus E1A, and a single loxP site to increase recombination frequency (Tomazin, R. et al. 1996). The sense oligonucleotide UL49F GAATTCATGTGATTCCGTGTTCGTGGAACCATGA and the antisense oligonucleotide UL49R GGATCCTTAGTGGATCCGTTGGTGCTTTATTGTCT were used to amplify VP22 sequence from HSV-1 strain F using *Pfu* DNA polymerase (Stratagene) and nucleotides from Promega. The product of this amplification reaction was cloned into pAdTet7 and the resulting construct pAdTet7-VP22 was sequenced to verify VP22 coding sequences. Plasmid pAdTet7-NTAP was identical to pAdTet7 except that there is a tandem affinity purification (TAP) domain cloned upstream of the multiple cloning site of pAdTet7 and was a gift from Dan Streblow

(Vaccine and Gene Therapy Institute, Portland OR). The CT domains of various gE truncation mutants were amplified as described previously (Chapter 3) and subcloned in frame at the C-terminus of the TAP domain in pAdTet7-NTAP. Recombinant adenoviruses were produced by cotransfection of pAdTet7-VP22 or pAdTet7-NTAP/gE plasmids with adenovirus DNA (Ad5-Ψ5) into 294-Cre4 cells that express Cre recombinase (Ng, P. et al. 1999). Recombinant adenoviruses were expanded for 4 passages on Cre-4 cells to remove Ad5-Ψ5, and the titers determined using 293M cells.

4.5.4 Antibodies

3104 a gI-specific HSV MAb and 3114, a gE-specific MAb were gifts from Anne Cross and Nigel Stow (Institute of Virology, Glasgow, United Kingdom). DL6, a MAb specific for HSV-1 gD was a gift from Gary Cohen and Roselyn Eisenberg (University of Pennsylvania, Philadelphia). The rabbit polyclonal anti-VP22 antibody AGV 030 was a gift from Gillian Elliott (Marie Curie Research Institute, Surrey, United Kingdom) (Elliott, G. and O'Hare, P. 1997), The rabbit polyclonal anti-UL11 antibody, (Rbt #73) was a gift from John W. Wills (College of Medicine, Pennsylvania State University, Hershey) and the rabbit polyclonal anti-VP16 antibody (Cat #3844-1) was purchased from BD biosciences (San Jose, California). An anti-calmodulin binding domain antibody was obtained from Upstate Cell Signalling (Lake Placid NY)

4.5.5 Electron Microscopy

HEC-1A cells were infected with wild type F-BAC HSV-1 or F-BAC mutants for 16 h, washed with 0.1M sodium cacodylate buffer (pH 7.2) and fixed in Ito and Karnovsky's fixative for 30 min at room temperature (1.6% paraformaldehyde, 2.5% glutaraldehyde and 0.5% picric acid in 0.1 M sodium cacodylate). Samples were postfixed in 1.5% osmium tetroxide, rinsed and then postfixed in 4% paraformaldehyde. Samples were dehydrated in a graded acetone series,

embedded in epoxy resin and ultrathin sections were double stained in uranyl acetate and lead citrate and viewed with a Philips EM 300 Electron Microscope.

4.5.6 Immunoprecipitation of radiolabelled HSV proteins

Vero cells were infected with HSV-1 (using 10 PFU/cell) in DMEM containing 1% FBS for 2 h then fresh media was added for an additional 6-7 h. Cells were washed twice with DMEM lacking methionine and cysteine and containing 1% dialyzed FBS and then labelled in this medium containing [^{35}S]-methionine-cysteine (150 μCi/ml; NEN) for a further 3 h. Cells were lysed in NP-40-deoxycholate (DOC) extraction buffer (1% NP-40, 0.5% DOC, 50 mM Tris-HCl [pH 7.5], 150 mM NaCl) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and stored at -70°C. Cell extracts were thawed, centrifuged at 50,000 x g for 45 min, and anti-gE antibody (MAb 3114), anti-gD antibody (MAb DL6), or anti-gB antibody (MAb 15βB2) added for 1-2 h at 4°C followed by incubation with protein A-Sepharose. Immunoprecipitated proteins were subjected to electrophoresis on polyacrylamide gels followed by analysis by autoradiography (Wisner, T. et al. 2000).

4.5.7 Immunoprecipitation of radiolabelled or unlabelled TAP constructs

HaCaT cells were initially coinfected with various Ad vectors expressing TAP/gE fusion proteins using 50 PFU (defined using 293 cells)/ cell and simultaneously with AdTet-Trans using 10 PFU/cell in DMEM supplemented with 1% FBS for 18 h. The cells were subsequently infected with F-gEΔCT or left uninfected for a further 7.5 h. For producing radiolabelled viral proteins, the cells were washed twice with media lacking methionine and cysteine, containing 1% dialyzed FBS and then labelled for 3 h with [^{35}S]-methionine/cysteine as described in the previous section. Cells were harvested, briefly washed and incubated in either 0.5% NP40 lysis buffer (0.5% NP40, 50mM Tris/HCl pH 7.5, 0.5mM EDTA, 2mM DTT, protease inhibitor

tablets (Roche diagnostics) containing either 100 mM or 500 mM NaCl or 1% digitonin lysis buffer (1% digitonin, 50mM Tris/HCl pH7.5, 0.5mM EDTA, 2mM DTT complete protease inhibitor tablets) containing either 100 or 500 mM NaCl for 20 min. Cell extracts were centrifuged at 60,000 x g for 30 min and incubated with IgG-Sepharose beads (Amersham-BioSciences, Piscataway, NJ) for 1 h at 4°C. Sepharose beads were pelleted at low speed (200-500 X g) for ≈30 sec, washed 4 times in dilute wash buffers (0.1% NP40 or 0.1% digitonin, 50mM Tris/HCl) containing 150mM or 500mM NaCl and bound proteins were released by boiling in buffer containing 2% SDS and 2% β-mercaptoethanol before electrophoresis on SDS-polyacrylamide gels (Wisner, T. et al. 2000) and detection of proteins by autoradiography. In other cases unlabelled cells infected as above were extracted using the same buffers, centrifuged at 25,000 x g for 20 min and subsequently incubated with IgG-Sepharose for 1 hr at 4 °C, the beads washed 4 times in dilute NP40 or digitonin wash buffers and pelleted at 200-500 X g for ≈30 sec, and subjected to electrophoresis in polyacrylamide gels. Proteins were transferred to PVDF membranes then the membranes immunoblotted with anti-tegument antibodies as described (Wisner, T. et al. 2000).

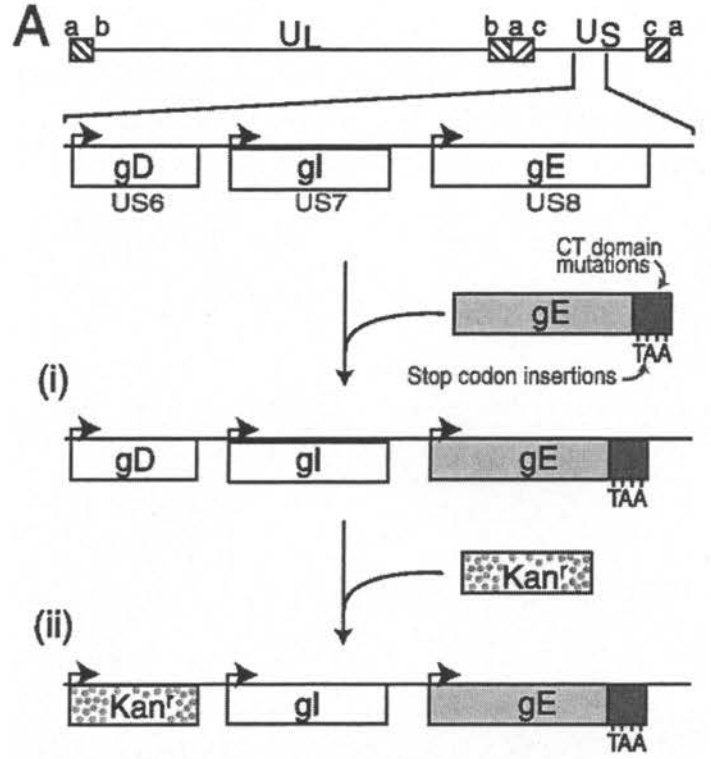
4.5.8 Immunoprecipitation of gD and gE from HSV-1 or Ad-infected cells

Confluent HaCaT cells were infected with HSV-1 for 12 h or with Ad vectors expressing gE/gI or gD and VP22 for 24 h. Cells were harvested, briefly washed and incubated in 0.5% NP-40 lysis buffer containing 150 mM NaCl on ice for 20 min. Cell extracts were centrifuged at 25,000 x g for 20 min then supernatants precleared by incubation with protein A-Sepharose for 15 min. These beads were removed by centrifugation and anti-gE (3114) or anti-gD (DL6) MAb and protein A-Sepharose added to extracts for an additional 20 min at 37°C. The beads were

washed in a dilute NP40 buffer, subjected to electrophoresis in polyacrylamide gels then proteins were transferred to PVDF membranes and immunoblotted as described (Wisner, T. et al. 2000)

Figure 4.1 Construction of recombinant viruses.

(**A**) The genome of HSV-1, including the US6 (gD), US7 (gI) and US8 (gE) genes is depicted (McGeoch, D. J. et al. 1985). (i) The insertion of stop codons in the gE CT domain at residues 448, 470, 495 and 519 were described (Farnsworth, A. and Johnson, D. C. 2006). A kanamycin resistance gene cassette was inserted into the coding sequences of gD and this was transferred into BAC containing truncated versions of gE. (**B**) Vero cells were infected with F-BAC gD-, F-BAC gD-/gE_{519}, F-BAC gD-/gE_{495}, F-BAC gD-/gE_{470}, F-BAC gD-/gE_{448}, F-BAC gD-/gE- or wild type F-BAC HSV-1 (w.t.). The cells were labelled with [^{35}S]-methionine-cysteine and gB, gD or gE was immunoprecipitated from cell extracts using MAb15βB2, DL6 or 3114 respectively. The positions of gB, gD and gE are indicated on the right side of the panel.


A
a b
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c a
gD
US6
gI
US7
gE
US8
CT domain mutations
gE
TAA
Stop codon insertions
(i)
gD
gI
gE
TAA
Kanr
(ii)
Kanr
gI
gE
TAA

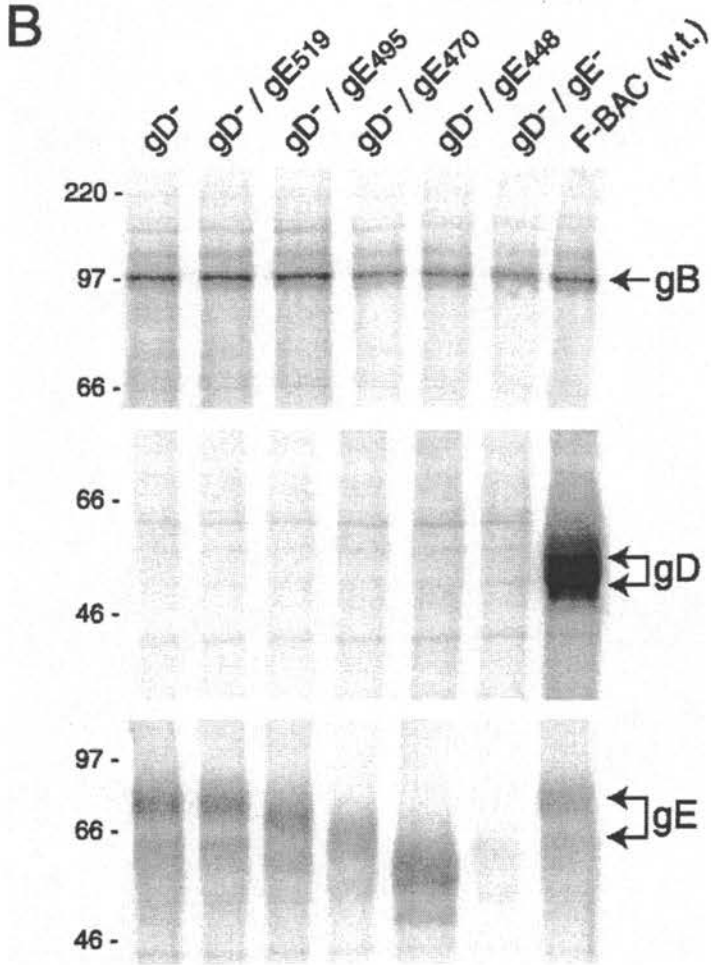

B
gD-
gD- / gE519
gD- / gE495
gD- / gE470
gD- / gE448
gD- / gE-
F-BAC (w.t.)
220 -
97 -
66 -
gB
66 -
46 -
gD
97 -
66 -
46 -
gE

Figure 4.2 Electron micrographs of cells infected with F-BAC gD-/gE CT mutants.

Human HEC-1A epithelial cells were infected with F-BAC gD-/gE-, F-BAC gD-/gE_{448}, F-BAC gD-/gE_{470}, F-BAC gD-/gE_{495}, F-BAC gD-/gE_{519} or F-BAC (w.t.) for 16 h. The cells were fixed and processed for electron microscopy. Filled arrows point to aggregates of unenveloped capsids while empty arrows point to enveloped virions on the surfaces of cells.

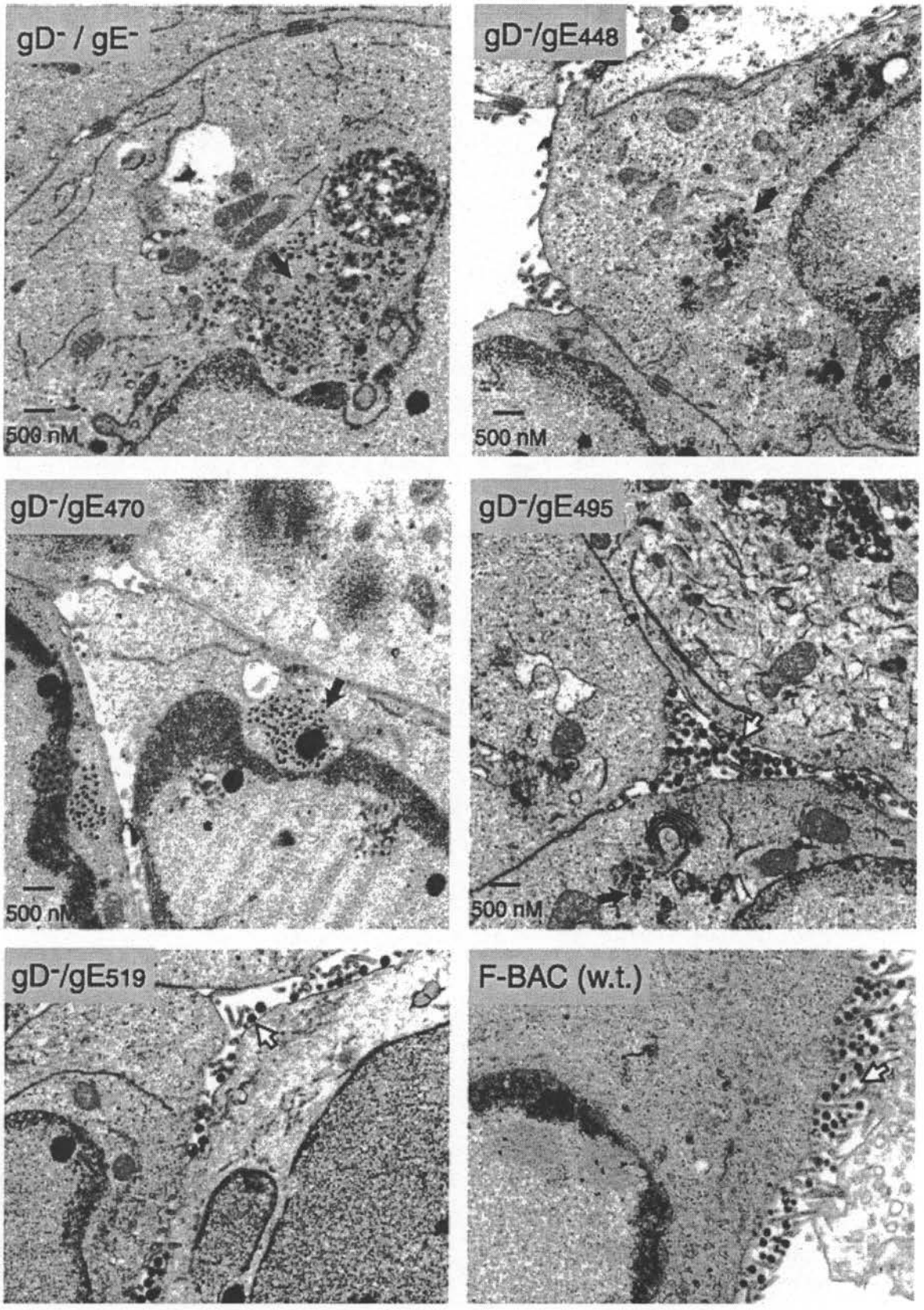

gD⁻ / gE⁻
500 nM
gD⁻/gE448
500 nM
gD⁻/gE470
500 nM
gD⁻/gE495
500 nM
gD⁻/gE519
F-BAC (w.t.)

Figure 4.3 Distributions of virus particles in cells infected by gD-/gE CT mutants. Randomly selected sections of HSV-infected HEC-1A cells were characterized by electron microscopy, counting unenveloped nucleocapsids in the nucleus and cytoplasm and enveloped virions in the perinuclear space, cytoplasm and on cell surfaces. In the figure, unenveloped capsids in the cytoplasm (open bars) were compared to enveloped virions in the cytoplasm combined with virions on cell surfaces (filled bars).

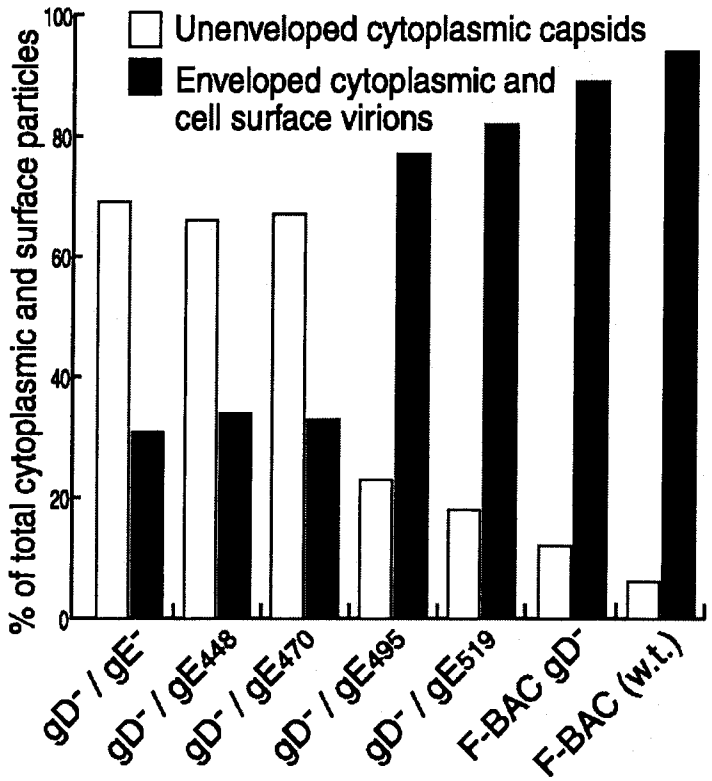

Unenveloped cytoplasmic capsids
Enveloped cytoplasmic and cell surface virions
% of total cytoplasmic and surface particles
100
80
60
40
20
0
gD- / gE-
gD- / gE448
gD- / gE470
gD- / gE495
gD- / gE519
F-BAC gD-
F-BAC (w.t.)

Figure 4.4 Construction of TAP::gE fusion proteins.

(**A**) The TAP domain was composed of two tandem protein A (IgG-binding) domains separated from a calmodulin binding domain (CBD) by a Tobacco Etch Virus protease (TEV) cleavage site. The TAP domain was fused N-terminal of the entire gE CT domain beginning with three arginine residues that are adjacent to the gE transmembrane domain. Other TAP fusion proteins included truncated versions of the gE CT domain: gE_{519}, gE_{495}, and gE_{470}. Also shown is a construct denoted TAP scrambled that contains 25 random amino acids unrelated to the gE CT domain. (**B**) Vero cells were coinfected with Ad vectors expressing TAP::gE_{550}, TAP::gE_{519}, TAP::gE_{495}, TAP::gE_{470}, or TAP scrambled and Adtet-trans, or with Adtet-trans alone. Cell extracts were subjected to electrophoresis on polyacrylamide gels then proteins transferred to PVDF membrane and probed with an anti-CBD rabbit antibody. Molecular mass markers of 50, 37, 25 and 20 kDa are indicated.

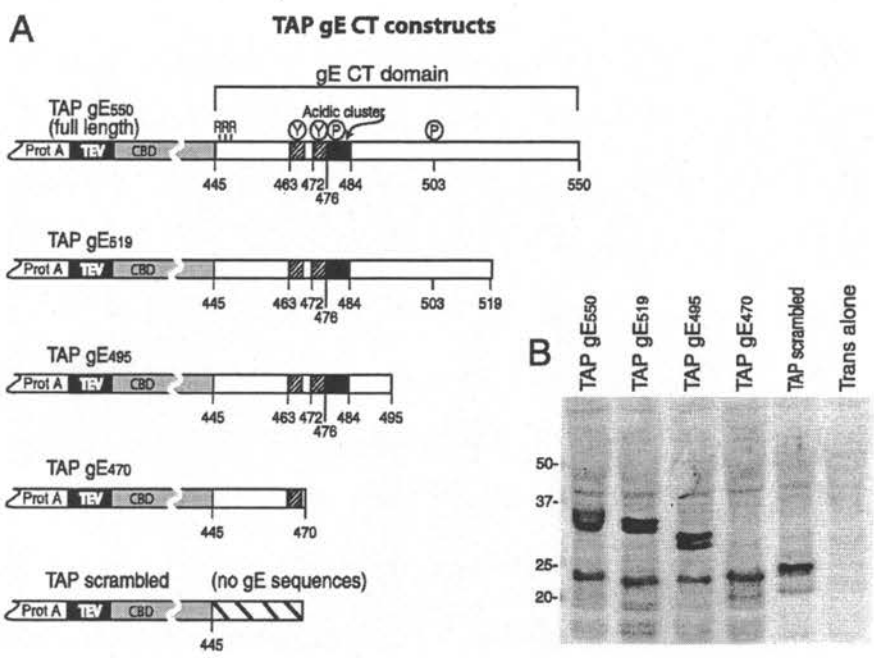

A
TAP gE CT constructs
gE CT domain
TAP gE550
(full length)
RRR
Acidic cluster
Prot A
TEV
CBD
445
463 472
476
484
503
550
TAP gE519
519
TAP gE495
495
TAP gE470
470
TAP scrambled
(no gE sequences)
B
TAP gE550
TAP gE519
TAP gE495
TAP gE470
TAP scrambled
Trans alone
50-
37-
25-
20-

Figure 4.5 Interactions between TAP::gE fusion proteins and HSV proteins.

Human HaCaT keratinocytes were infected with Ad expressing either TAP::gE_{550} or TAP::gE_{470} for 18 h then infected with F-gEΔCT (a gE-null mutant) for an additional 7-8 h. The cells were radiolabelled with [^{35}S]-methinonine-cysteine from 3 h then cell extracts made using 0.5% NP40 lysis buffer or 1% digitonin lysis buffer and low salt (L, 100 mM NaCl) or high salt (H, 500 mM NaCl). Extracts were centrifuged at 60,000 X g and supernatants incubated with IgG-Sepharose at 4°C. Proteins were eluted and subjected to electrophoresis. Two sets of bands, denoted VP22* and UL11* were observed when cells were infected with TAP::gE_{550} and F-gEΔCT but not with TAP/gE_{470} and F-gEΔCT and not without F-gEΔCT. Molecular mass markers of 97, 66, 46 and 30 kDa are indicated.

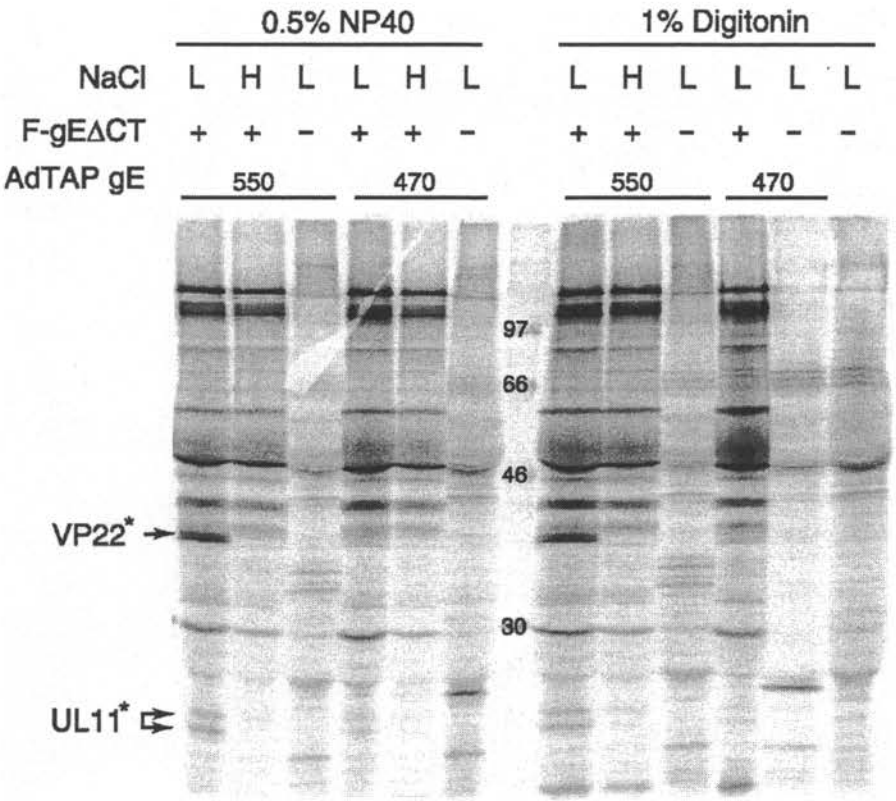

0.5% NP40
1% Digitonin
NaCl L H L L H L L H L L L L
F-gEΔCT + + − + + − + + − + − −
AdTAP gE 550 470 550 470
97
66
46
30
VP22*
UL11*

Figure 4.6 Coimmunoprecipitation of VP16, VP22 or UL11 with TAP/gE fusion proteins. HaCaT cells were coinfected with Ad vectors expressing TAP::gE_{550}, TAP::gE_{519}, TAP::gE_{495}, TAP::gE_{470}, TAP scrambled and AdTet trans or were not infected with an Ad vector (no Adeno) for 18 h. The cells were subsequently infected with HSV F-gEΔCT and harvested 12 h later in 0.5% NP40 lysis buffer. Cell extracts were incubated with IgG-Sepharose, washed and proteins eluted and subjected to electrophoresis before transfer to PVDF membranes. Membranes were probed with rabbit polyclonal antibodies specific for VP16 **(A)**, VP22 **(B)**, UL11 **(C)** or anti-protein A antibodies to detect TAP proteins. Note that IgG eluted from IgG-Sepharose and the heavy chain comigrated with TAP proteins as indicated in panel D. VP22 and UL11 bands were quantified using the IP Lab Gel software.

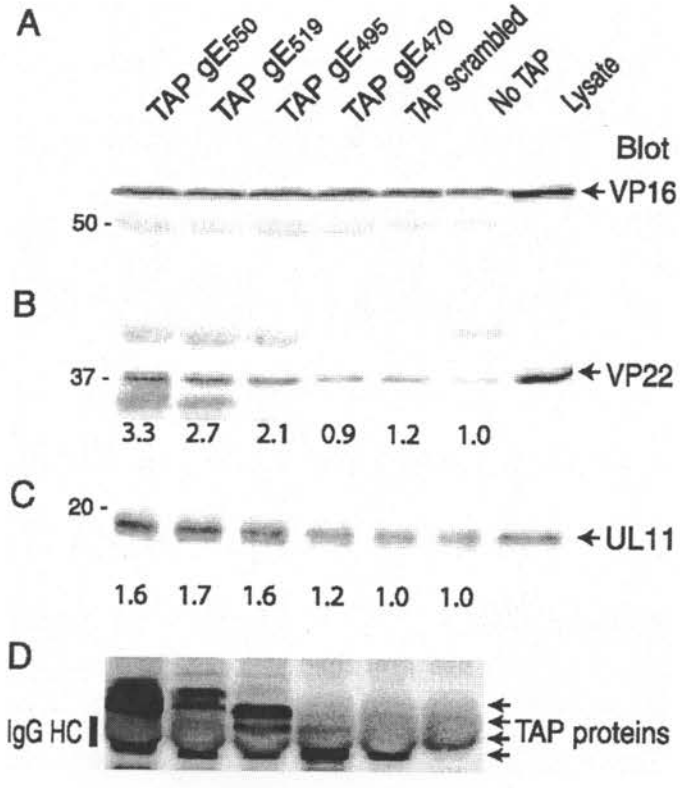

A
TAP gE550
TAP gE519
TAP gE495
TAP gE470
TAP scrambled
No TAP
Lysate
Blot
VP16
50 -
B
37 -
VP22
3.3 2.7 2.1 0.9 1.2 1.0
C
20 -
UL11
1.6 1.7 1.6 1.2 1.0 1.0
D
IgG HC
TAP proteins

Figure 4.7. Coimmunoprecipitation of VP16, VP22 or UL11 with gE from HSV-infected cells. HaCaT cells were infected with HSV: F-BAC (w.t.) expressing wild type gE, F-BAC gE_{519}, F-BAC gE_{495}, F-BAC gE_{470}, F-BAC gE_{448}, or F-BAC gE- for 12 h. Cell extracts were made using 0.5% NP40 lysis buffer and gE immunoprecipitated with anti-gE MAb 3114. Precipitated proteins and a sample representing 20% of the cell lysate were subjected to electrophoresis, proteins transferred to membranes and then western blotted with rabbit polyclonal antibodies specific for: anti-VP16 (**A,** upper panel), anti-VP22 (**A,** middle panel), anti-UL11 (**A,** lower panel) or gE (**B**). VP22 and UL11 were quantified using the IP Lab Gel software.

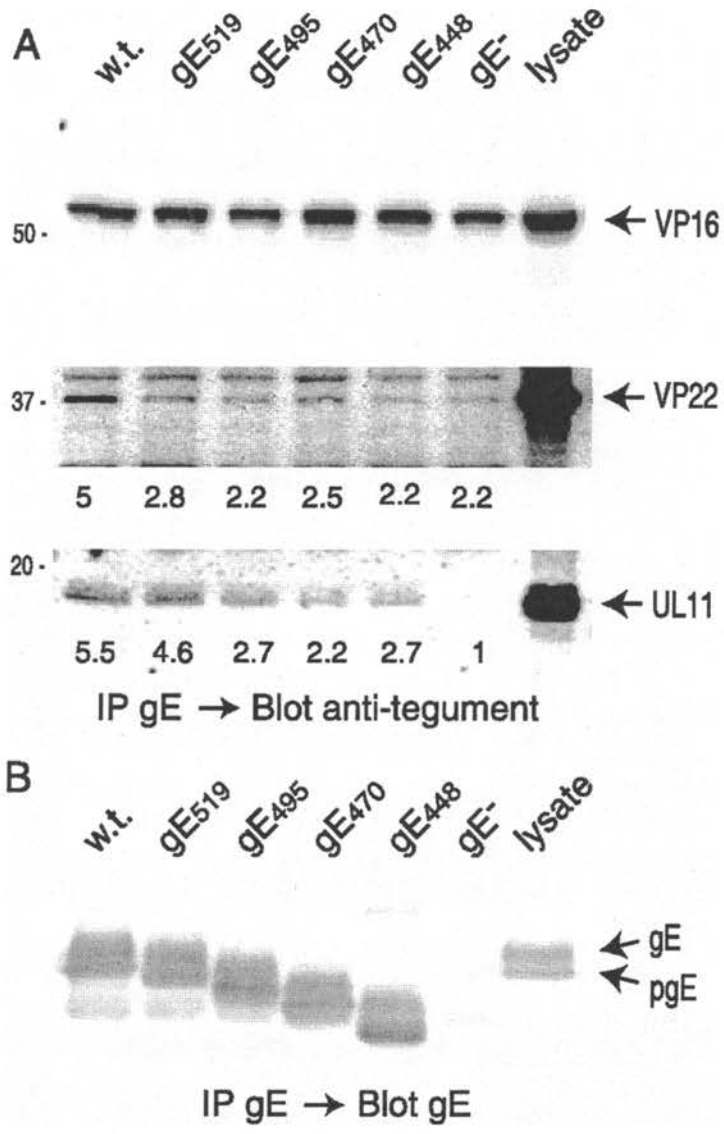

A
w.t.
gE519
gE495
gE470
gE448
gE-
lysate
50 -
VP16
37 -
VP22
5 2.8 2.2 2.5 2.2 2.2
20 -
UL11
5.5 4.6 2.7 2.2 2.7 1
IP gE → Blot anti-tegument
B
w.t.
gE519
gE495
gE470
gE448
gE-
lysate
gE
pgE
IP gE → Blot gE

Figure 4.8. Coimmunoprecipitation of VP22 and UL11 with gD from HSV-infected cells. (**A**) HaCaT cells were infected with F-BAC (w.t.) or F-BAC gD- for 12 h then cell extracts made using 0.5% NP40 lysis buffer. gD was immunoprecipitated using MAb DL6, proteins subjected to electrophoresis, then transferred to membranes and the membranes probed with rabbit polyclonal anti-VP16 (upper panel), anti-VP22 (middle panel) or anti-UL11 antibodies (lower panel) (**B**) HaCaT cells were infected with wild type HSV-1 strain F, vRR1097 (a gD-null mutant), F-dl2 (expressing a gD lacking the CT domain), F-BAC, or F-BAC gD- for 12 h or were left uninfected. Cells extracts were made using 0.5% NP-40 lysis buffer and gD was immunoprecipitated using anti-gD MAb DL6. Precipitated proteins and a sample representing 20% of the cell extract (Lysate) were subjected to electrophoresis, transferred to membranes and probed with anti-VP22 or anti-UL11 rabbit antibodies. (**C**) Samples immunoprecipitated in A and B were also probed with rabbit anti-gD antibodies. The IgG heavy and light chains (indicated by HC and LC) derived from mouse MAb DL6 used to precipitate gD were detected through crossreaction with secondaryantibodies and were most obvious in samples from cells lacking gD. (**D and E**) VP22 or UL11 were precipitated using rabbit polyclonal antibodies and samples blotted with mouse anti-gD MAb DL6. In D some IgG heavy chain was detected by the secondary antibodies and most obviously in samples from the gD- mutant.

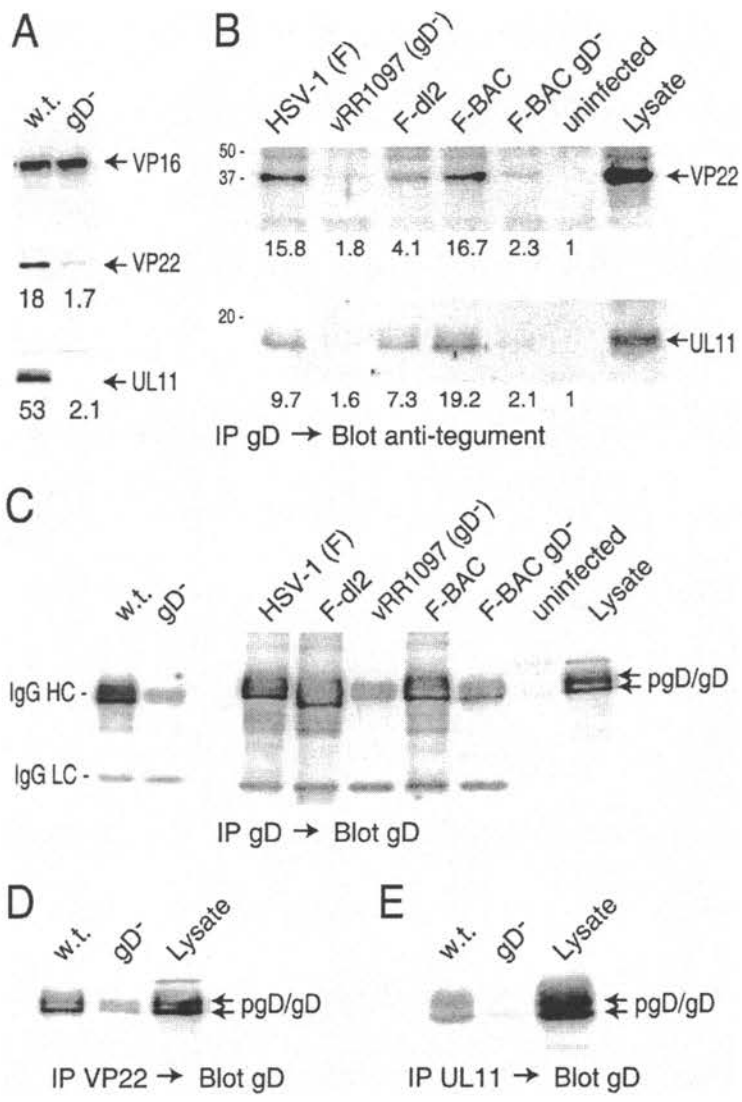
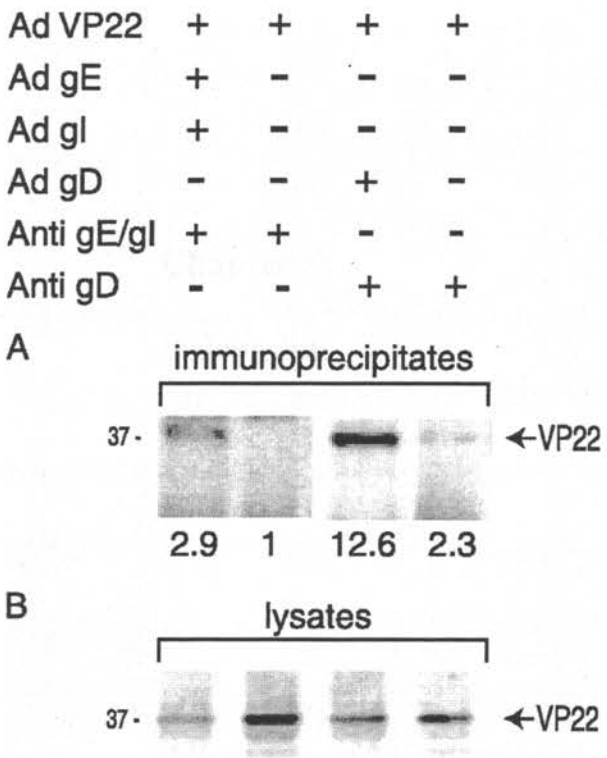

A
w.t.
gD-
VP16
VP22
18 1.7
UL11
53 2.1
B
HSV-1 (F)
vRR1097 (gD-)
F-dl2
F-BAC
F-BAC gD-
uninfected
Lysate
50 -
37 -
VP22
15.8 1.8 4.1 16.7 2.3 1
20 -
UL11
9.7 1.6 7.3 19.2 2.1 1
IP gD → Blot anti-tegument
C
w.t.
gD-
HSV-1 (F)
F-dl2
vRR1097 (gD-)
F-BAC
F-BAC gD-
uninfected
Lysate
IgG HC -
IgG LC -
pgD/gD
IP gD → Blot gD
D
w.t.
gD-
Lysate
pgD/gD
IP VP22 → Blot gD
E
w.t.
gD-
Lysate
pgD/gD
IP UL11 → Blot gD

Figure 4.9 VP22 binds to gE/gI and gD in the absence of other HSV proteins.

HaCaT cells were infected for 24 h with non-replicating Ad vectors expressing VP22, gE/gI and gD in conjunction with AdTet-trans as indicated in the upper panel. Cell extracts were made using 0.5% NP40 lysis buffer. (**A**) gE/gI immunoprecipitated with pooled gE-specific MAb 3114 and gI-specific MAb 3104 (Anti-gE/gI) or gD precipitated with anti-gD MAb DL6 as indicated. Immunoprecipitated proteins were probed using antibodies specific to VP22. (**B**) Approximately 5% of the cell extract was subjected to electrophoresis, transferred to membranes then blotted with anti-VP22 antibodies.


Ad VP22 + + + +
Ad gE + - - -
Ad gI + - - -
Ad gD - - + -
Anti gE/gI + + - -
Anti gD - - + +
A
immunoprecipitates
37 -
←VP22
2.9 1 12.6 2.3
B
lysates
37 -
←VP22

Chapter 5:

Discussion

5.1 General principles of Virus Envelopment and Spread

This dissertation has focused on the contribution of gE/gI to three processes: envelopment, egress and cell-to-cell spread of HSV. These processes are intimately linked in all enveloped viruses. Viral proteins necessary for envelopment accumulate and direct envelopment into a specific cellular membrane. This site of envelopment largely or wholly determines the mechanism of viral egress, which in turn dictates the way in which viral particles spread to other cells, tissues and hosts. Therefore, the localization of viral proteins is a major determinant of viral pathogenicity and disease. In the following chapter I will discuss some general principles of virus envelopment and cell-to-cell spread. I will then address the current understanding of the mechanisms by which HSV targets viral proteins to specific membranes, undergoes envelopment and spreads cell-to-cell, with emphasis on my thesis work. Finally I will describe future directions for these studies.

Many eukaryotic viruses are surrounded by a lipid bilayer. For virions to be infectious, it is essential that the final envelopment or 'budding' mechanism results in the incorporation of sufficient numbers of viral proteins into the mature virus. In order to be incorporated the viral proteins must accumulate at the site of envelopment. Viral proteins are targeted to the correct cellular membrane in one of three ways. First, signaling motifs that interact with the host cell trafficking machinery can directly target the viral protein to the correct subcellular location. Second, viral proteins may be recruited to these membranes through interactions with other viral proteins. Third, there may be combination of both specific sequences that interact with cellular trafficking machinery and interactions with other viral proteins. All three methods may be employed by any particular virus.

Regardless of the means by which viral proteins reach the appropriate membrane these proteins are embedded in the membrane alongside cellular proteins. The fluid mosaic model predicts that both viral and cellular proteins will be incorporated into viral envelopes in amounts proportional to their overall quantities in the membrane (Singer, S. J. and Nicolson, G. L. 1972). By concentrating viral proteins to specific patches within the membrane and budding at these patches the mature virus expresses lower concentrations of cellular proteins than would otherwise be predicted . A model, based on the envelopment of Semliki Forest Virus, has been proposed to explain this observation (Garoff, H. and Simons, K. 1974). Garoff *et al.* suggested that interactions between viral glycoproteins embedded in the lipid bilayer and the viral nucleocapsid (NC) lead to the lateral recruitment of additional viral glycoproteins into a patch of the membrane (Garoff, H. and Simons, K. 1974). This lateral recruitment results in the incorporation of viral proteins into the virion envelope at higher concentrations than observed in the cellular membrane. The progressive binding of glycoproteins to the NC results in the bending of the lipid bilayer around the NC and eventually in the budding of an enveloped virion from this membrane. This simple model is attractive as it explains a number of events in virus budding. First, the model describes how a viral NC might be targeted to the correct patch of the host cell membrane. Second, how a virion envelope contains high concentrations of viral glycoproteins but lower levels of host cell proteins. Although this model is too simplistic to apply to every enveloped virus the notion that interactions between viral proteins drive the envelopment process is always relevant, as the following thermodynamic argument demonstrates.

The envelopment process can generally be defined in terms of the forces that favor or oppose virus budding. Defining the membrane as existing in only two states, either planar (normal cellular membrane) or curved (portion of the membrane bending around the NC) and

using the Garoff and Simons model we can define three forces that contribute significantly to this process. The first is the increase in potential energy associated with protruding a portion of the planar membrane. The second is the decrease in entropy associated with the assymetrical concentration of proteins in a lipid envelope. These two forces must be overcome by the free energy released by the interaction between viral glycoproteins and the nucleocapsid. As envelopment proceeds, a point is reached when the opposing forces are nearly equal in magnitude and a thermodynamically unstable situation arises where the membrane is protruded and viral proteins are concentrated in a small surface area. The membrane can return to a lower energy state when either the interactions between the viral proteins driving envelopment are disrupted or when the enveloped virus is pinched off from the membrane. Cellular proteins, such as the scission protein TSG101, may have a necessary role in promoting virus budding rather than readsorption during this final stage.

Enveloped virions have evolved more than one method for envelope acquisition. The above thermodynamic model applies to most virus envelopment events, with the free energy provided by interactions between different viral proteins in different systems. Thus far, viral envelopment has been classified into four distinct types based upon the viral proteins necessary *in vitro* to produce virus-like particles (VLP). Type I budding is the classical model proposed by Garoff and Simon and is mediated by interactions between envelope and nucelocapsid proteins. Type II viral budding is observed in cells infected by retroviruses and is mediated by interactions between capsid proteins and the viral core (as reviewed in (Garoff, H. et al. 1998)). Type III budding is typified by coronaviruses, and occurs based on interactions between specific viral envelope proteins (as reviewed in (Garoff, H. et al. 1998)). Finally type IV budding, as observed in rhabdoviruses, depends on only the matrix and core viral proteins although the envelope

proteins contribute significantly to virion morphology and budding efficiency (as reviewed in (Garoff, H. et al. 1998)).

5.2 HSV trafficking and envelopment

HSV replicates rapidly and specific, immediate cell-to-cell spread is a key component of the virus life cycle. Primary infection of HSV usually occurs at mucosal epithelial cells and the virus spreads from these cells to the underlying neuron which innervates the infected tissue. In these epithelial cells, newly synthesized genomes are packaged into preformed capsids which egress from the cell via an envelopment->de-envelopment->re-envelopment pathway (Stackpole, C. W. 1969; Skepper, J. N. et al. 2001)(Section 1.5.5-1.5.8). According to this model, HSV particles acquire a final envelope at an internal membrane, generally agreed to be the TGN or an early endosomal compartment. Since the virus spreads directly from cell-to-cell, HSV is dependant on the host cell trafficking machinery to move virions from this internal site to baso-lateral cell surfaces. HSV nucleocapsids must bud therefore into specific subcompartments of the TGN that are either destined for baso-lateral surfaces or HSV must engage the host cell trafficking to direct these vesicles to baso-lateral surfaces. This dissertation examines the involvement of gE/gI in secondary envelopment, viral egress and cell-to-cell spread, however, fundamental to all of these processes is the targeting and accumulation of gE/gI and other viral proteins.

5.2.1 Accumulation of HSV glycoproteins at the TGN

In order for a virus to acquire an envelope from a cellular membrane, viral membrane proteins must first accumulate in the cellular membrane in question. Thus, both secondary envelopment and subsequent transport and spread are dependent on viral protein accumulation at the correct site of viral envelopment and a model detailing the involvement of gE/gI and

sequences specifying its localization has been proposed (McMillan, T. N. and Johnson, D. C. 2001)(Fig. 1.6). Accordingly, gE/gI accumulates in the TGN based on interactions between the gE CT domain and the cellular sorting machinery such as clathrin adaptors, PACS-1 or other cellular proteins. Other HSV glycoproteins are also targeted to this compartment and, along with gE/gI, drive envelopment of cytosolic nucleocapsids into TGN compartments or vesicles. Next, vesicles containing HSV virions are trafficked to lateral cell surfaces in a step that is either directly mediated by the gE CT domain or is a result of gE/gI driving envelopment into vesicles that are destined for lateral cell surfaces. Following fusion between the virus containing vesicle and the plasma membrane the ET domain of gE/gI mediates an interaction with a cellular receptor and efficient entry into the neighboring cell (McMillan, T. N. and Johnson, D. C. 2001).

HSV employs three general methods to accumulate viral proteins at the TGN (Section 5.1). The HSV glycoproteins gB, gC and gE/gI all accumulate in the TGN in HSV infections and when expressed exogenously (Koga, J. et al. 1986; Wisner, T. et al. 2000; McMillan, T. N. and Johnson, D. C. 2001; Beitia Ortiz de Zarate, I. et al. 2004). Furthermore, deletion of specific sequences within these proteins results in their mislocalization meaning that each contains sufficient and necessary signals for their accumulation to the TGN. gD, on the other hand, accumulates in the TGN in infected cells but, when expressed without other viral proteins, gD accumulates on cell surfaces (Koga, J. et al. 1986). Co-expression of HSV gM/UL49.5 is sufficient for the retrieval of gD to the TGN indicating that recruitment of gD to the correct subcellular compartment can be mediated by other viral glycoproteins (Crump, C. M. et al. 2004). Trafficking of gK represents the third type of targeting discussed in Section 5.1. In cells in which gK is expressed independently of other HSV proteins, gK is retained in the ER (Foster, T. P. et al. 2004b). Co-expression of UL20 results in the release of gK from this compartment

and the co-trafficking of gK and UL20 to the TGN. However, deletion of specific sequences in the gK CT domain results in the accumulation of both of these proteins at the plasma membrane (Foster, T. P. et al. 2004b).

gE/gI localizes to the TGN in HSV infected cells and when expressed in the absence of other viral proteins. The gE CT domain is both necessary and sufficient for this localization (Wisner, T. et al. 2000; McMillan, T. N. and Johnson, D. C. 2001). The gE CT domain is 106 amino acids in length and contains several cell sorting motifs which are found in PRV and VZV homologues, as well as in cellular proteins such as furin and mannose-6-phosphate receptors (McGeoch, D. J. et al. 1985; Hunziker, W. et al. 1991; Humphrey, J. S. et al. 1993; Molloy, S. S. et al. 1994; Olson, J. K. and Grose, C. 1997; Brack, A. R. et al. 2000; Kurten, R. C. 2003). Both furin and gE/gI exhibit similar trafficking patterns dependant on respective CT domains and appear to contain similar trafficking motifs, suggesting that these two proteins interact with the host cell trafficking machinery by a similar mechanism. Both the HSV gE CT and furin contain canonical tyrosine motifs (YXXØ) as well as an acidic cluster of amino acids, phosphorylated by casein kinase II (Molloy, S. S. et al. 1994; Wisner, T. et al. 2000). The μ1 and μ2 components of AP-1 and AP-2 clathrin adaptors bind to tyrosine motifs resulting in the incorporation of furin into clathrin-coated transport vesicles (Schafer, W. et al. 1995; Gu, F. et al. 2001) and μ1B directs PRV particles to lateral cell surfaces (Johnson, D. C. et al. 2001). The acidic cluster of both the gE and furin CT domain interacts strongly with PACS-1 (Wan, L. et al. 1998)(Colin Crump and Gary Thomas, personal communication) which is important in recycling molecules from early endosomes to the TGN (Wan, L. et al. 1998; Gu, F. et al. 2001).

In Chapter 3 I examined regions of the gE CT domain necessary for the correct localization of gE/gI. gE proteins missing all or part of the cytoplasmic tail (CT), gE_{448} (gE CT-

null), gE_{470}, gE_{495} and gE_{519} and were inserted into a WT HSV background (Chapter 3). While the gE CT domain was necessary for TGN accumulation as reflected by the trafficking of gE/gI in HSV gE_{448} (gE ΔCT), we found that gE_{470} was sufficient for accumulation of gE/gI to the TGN. Furthermore we did not find a significant difference between gE_{470}, gE_{495} gE_{519} or WT gE in terms of gE/gI accumulation at the TGN (Chapter 3 and Table 5.1). These results indicate that one tyrosine motif, located at a.a. 463, was sufficient for the TGN localization gE/gI (Chapter 3 and Table 5.1). Interestingly, the acidic cluster and phosphorylation domains were unimportant for the accumulation of gE/gI at the TGN. gE mutants lacking these domains did not cause a substantial mislocalization of gE/gI (Chapter 3). This is distinct from the trafficking of furin, which requires the acidic cluster and PACS-1 for TGN accumulation while the tyrosine motif acts in a secondary fashion to recycle furin from the cell surface to endosomes (Schafer, W. et al. 1995; Wan, L. et al. 1998). Since the gE CT domain acidic cluster was not necessary for TGN localization of gE/gI (Chapter 3), PACS-1 is unlikely to contribute to gE/gI trafficking in polarized epithelial cells. Comparing these results to the model presented in Figure 1.6, we observe that that only the first 25 a.a. residues of the CT domain are necessary for accumulation at the TGN. These results also indicate that HSV, and potentially other alphaherpesviruses, only require a minimal amount of sequence information to correctly localize and accumulate viral proteins. This may reflect a certain amount of pliability or redundancy in the trafficking requirements of HSV proteins.

5.2.2 HSV Glycoporteins involved in Budding at the TGN

Once viral membrane proteins have accumulated in the TGN or endosomes, the cytoplasmic tails of these glycoproteins can interact with tegument-coated capsids to promote budding into TGN-derived vesicles. Deletion of any single envelope glycoprotein does not

significantly inhibit this secondary envelopment (Cai, W. Z. et al. 1987; Ligas, M. W. and Johnson, D. C. 1988; Forrester, A. et al. 1992; Roop, C. et al. 1993; Dingwell, K. S. et al. 1994; Schafer, W. et al. 1995; Wan, L. et al. 1998). Studies on the related alphaherpesvirus, PRV, have suggested that membrane proteins may have redundant roles in the envelopment of viruses in this subfamily, masking the involvement of any one glycoprotein (Brack, A. R. et al. 1999).

As detailed in Chapter 2, I demonstrated that an HSV mutant lacking gD and gE failed to produce enveloped particles. Instead, gD-/gE- HSV mutants accumulated large numbers of nucleocapsids in the cytoplasm (Chapter 2). This phenotype was observed on multiple cell lines, indicating that the defect was reflective of a general involvement of these proteins and not due to specific requirements in one cell line. Other HSV mutants lacking pairs of glycoproteins were also constructed and examined for defects in assembly or egress. A gB-/gE- virus had no defect in secondary envelopment whereas a gD-/gI- HSV mutant had comparatively mild defects (Chapter 2 and Chapter 4). However, infections with a gD-/gE-/gI- HSV resulted in the accumulation of very large aggregates in the cytoplasm of infected cells, even compared with gD-/gE- mutants. These findings led to several important conclusions about the mechanism of HSV envelopment. First, unenveloped capsids accumulated in the cytoplasm in the absence of gD and gE indicating that the virus was trapped at an intermediate stage in the envelopment pathway and this supports the envelopment->de-envelopment->re-envelopment model of HSV envelopment (Stackpole, C. W. 1969; Skepper, J. N. et al. 2001). Second, gD and gE/gI appear to function in a redundant fashion in envelopment. Finally, although the gI glycoprotein contributes to the envelopment of HSV it appears to have a lesser role compared with gE.

Deletion of both gE and gD abrogated secondary envelopment and firmly established that HSV envelope glycoproteins are integrally involved in viral envelopment (Chapter 2 and

Chapter 4). In the nuclear membrane envelopment (NME) model proposed by Schwartz et al., the HSV envelope is acquired from the inner nuclear membrane and viruses then traffick through the rough endoplasmic reticulum, transported to the TGN and to the site of viral egress. However, the results in Chapter 2 can not be explained by this model as virions prevented from acquiring a final envelope would remain in the nucleus and not enter the cytoplasm. The observed results strongly support re-envelopment at the TGN.

The deletion of gD and gE specifically inhibited secondary envelopment while the simultaneous deletion of gB and gE/gI or gM and gE had no significant effect on HSV assembly or maturation (Chapters 2 and 4) (Browne, H. et al. 2004). Since deleting other pairs of glycoproteins does not appear to compromise secondary envelopment this result suggests that only gD and gE specifically anchor the envelope onto the tegumented nucleocapsid. However, it remains possible that other combinations of glycoprotein deletions may reveal other effects on virus maturation or envelopment.

The glycoproteins gE and gI exist functionally as a single glycoprotein dimer, gE/gI, in HSV infected cells. The participation of gI in secondary envelopment was demonstrated by comparing cells infected with a gD-/gE-/gI- triple mutant versus a gD-/gE- double mutant. Cells infected with the gD-/gE-/gI- triple mutant displayed greater accumulation of cytoplasmic nucleocapsids and very few enveloped particles when compared to those infected by gD-/gE- virions (Chapter 2). gD-/gI- deleted virions were not as defective in maturation as were gD-/gE- viruses. Thus, gI appears to play a role in the envelopment process although contributes less than gE. As previously demonstrated, the gE CT contains the information necessary for the accumulation of gE/gI at the TGN. In a gD-/gI- virus, gE accumulates at the site of virion envelopment while in a gD-/gE- virus, gI accumulates on the cell surface and is largely

unavailable for interaction with the developing virus. This difference in protein localization makes the interpretation of the relative activities of gE and gI difficult and may partially explain the more substantial contribution of gE.

The research in Chapter 2 was the first demonstration that multiple HSV envelope proteins participate in a redundant fashion in envelopment and provided further support to the model of cytoplasmic envelopment. This paper, therefore, was an important contribution to our understanding of how HSV assembles. However, this work left several unanswered questions regarding the means by which gD and gE/gI interact with tegument-coated capsids and which tegument proteins mediate this event.

5.2.3 Role of gE CT domain in HSV Envelopment

To further define the role of gE in the secondary envelopment of HSV I began to examine the contributions of the gE CT domain to HSV envelopment (Chapter 4). A panel of truncated gE proteins missing all or part of the cytoplasmic tail (CT), gE_{448} (gE CT-null), gE_{470}, gE_{495} and gE_{519} were inserted into either WT or gD-null HSV-1 backgrounds (Chapter 3 and 4). These investigations resulted in several important findings. First, secondary envelopment of the gD-/gE_{448} mutant did not produce large numbers of enveloped virions and was similar to the gD-/gE- virus, demonstrating that the gE CT domain is critical for secondary envelopment (Chapter 4). Second, gD-/gE_{470} HSV was defective in secondary envelopment but the gD-/gE_{495} mutant produced primarily enveloped virions, indicating that a region between amino acids 470 and 495 is necessary for secondary envelopment. Furthermore as gE_{470} is expected to localize with gI to the TGN, the absence of envelopment in this mutant suggests that the accumulation of gE/gI at the TGN is insufficient to mediate virion maturation. Finally, although the truncations at gE_{495} and gE_{519} did not abrogate secondary envelopment these mutations did impact viral maturation.

While both gE_{470} and gE_{495} accumulate in the TGN (Chapter 3) there is a distinct difference in the maturation phenotype of the respective gD- viruses (Chapter 4). This suggests that sequences between amino acids 470 and 495 in the CT domain of gE mediate an interaction that is essential for the acquisition of a final virion envelope. The lesser, but significant, importance of gE amino acid residues C-terminal to 495 may be explained in one of two ways. The interaction between the CT domain of gE and the tegumented nucleocapsid may occur via multiple tegument proteins. Therefore, if one interaction is abrogated because of a deletion of the gE C-terminal 31 residues, other interactions might still occur between the remaining gE CT domain and different tegument proteins. Alternatively, only the short stretch of amino acid residues between gE_{470} and gE_{495} may be necessary for the interaction between the tegumented nucleocapsid and the CT domain of gE. In this case, truncation of C-terminal residues may disrupt the efficiency with which the region between gE_{470} and gE_{495} interacts with the tegumented nucleocapsid. In either case the efficiency of secondary envelopment would decrease as the C-terminus of the gE CT domain is truncated, as observed.

Overall, these results demonstrate the involvement of specific regions of the gE CT domain in secondary envelopment (Table 5.1). Specifically, a region between a.a. 448 and a.a. 470 is essential for accumulation at the TGN as well as HSV envelopment. However, it is unclear if the defects observed in secondary envelopment are due to a failure to interact with specific tegument proteins. To further understand how the gE CT and gD mediate secondary envelopment it is necessary to understand what teguments proteins are involved in HSV envelopment and with which of these gD and gE interact.

5.2.4 Tegument Proteins in Secondary Envelopment

The tegument layer of HSV is comprised of many viral proteins that serve roles throughout the infectious cycle (Table 1.1). Six tegument proteins play a significant effect on the envelopment and maturation of HSV particles: VP1/2, UL37, VP16, UL11, UL20 and VP22 (Baines, J. D. and Roizman, B. 1992; Desai, P. J. 2000; Mossman, K. L. et al. 2000; Desai, P. et al. 2001; Foster, T. P. et al. 2004a; Elliott, G. et al. 2005; Duffy, C. et al. 2006). We hypothesized that gE and gD function in secondary envelopment by interacting with one or more tegument proteins, juxtaposing membrane-bound glycoproteins with the tegument-coated nucleocapsids and driving forward secondary envelopment.

Chapter 4 described the interactions of gD and gE with two tegument proteins, UL11 and VP22, previously shown to be involved in the maturation of HSV-1. The interactions between gE and tegument proteins were characterized using a construct where the gE CT domain was fused to a heterologous tag. The tag was affinity purified and the associated tegument proteins were identified as VP22 and UL11. These results indicated that a region of the gE CT domain between amino acids 470 and 495 interacted with UL11 and VP22, a region that was also necessary for envelopment (Chapter 4). The gE CT fails to interact with the tegument proteins UL24, VP1/2 or vhs. Attempts at immunopreciptating VP16 were uninterpretable as this tegument proteins interacted non-specifically with the IgG or beads used for immunoprecipitation. To confirm these interactions we immunoprecipitated endogenously expressed wild type and mutant forms of gE/gI and characterized these for tegument protein interactions (Chapter 4). Specific interactions between the gE CT domain and VP22 and UL11 were observed, although the interaction was attributed to a different segment of the CT. We also confirmed the specific

interaction between gE and VP22 through the expression of gE/gI and VP22 in the absence of other viral proteins (Chapter 4).

The discrepancy between the results of immunoprecipitations of TAP::gE CT fusion and gE CT domain mutants suggests that one of the two assays failed to accurately describe the interactions. As the TAP::gE CT fusion proteins contain only a small fragment of the native gE protein this assay might be considered more susceptible to artefacts. HSV gE is a transmembrane protein that functions as part of a heterodimer with its partner, gI (Johnson, D. C. et al. 1988). Removal of the ET and TM sections of gE and fusion of the CT domain onto a large soluble protein fundamentally changed both of these characteristics. Furthermore, deletion of the ET and TM domains of gE may affect the conformation and therefore the binding characteristics of the CT domain. However, the results with the TAP::gE CT fusion protein may accurately describe the binding characteristics of the gE CT domain. If these results are accurate then truncation of the gE CT domain while the protein is anchored in the membrane may prevent the formation of the binding site located between residues 470 and 495.

In both Chapters 2 and 4, gD and gE were shown to play redundant roles in secondary envelopment of HSV. The observed redundancy could arise if gD and gE had the same tegument binding partners or if each of their respective binding partners were each sufficient to mediate envelopment (Section 5.3). To differentiate between these two hypotheses, we examined the binding of the gD CT domain to tegument proteins by immunoprecipitation of gD from HSV-infected cells and from cells that co-expressed gD and VP22 in the absence of other HSV proteins. As seen for gE, gD was found to specifically interact with VP22 and UL11. Furthermore, gD did not interact with UL24, VP1/2 or vhs. While the interactions between UL11 and gD as well as VP22 and gD were specific, our results suggested that only the interaction

between VP22 and gD was dependant on the CT domain. The interaction between gD and UL11 was only decreased by 25% in the absence of the CT domain (Chapter 4). UL11 is myristoylated and interacts with membranes dependant on an acidic cluster, gD on the other hand is an integral membrane protein that exists as a homodimer (Fig 1.4), furthermore the gD CT mutant employed in Chapter 4 still expresses three cytoplasmic arginine residues (Feenstra, V. et al. 1990). While the ET domain of gD is completely inaccessible to the cytoplasm, the molecular make-up of these two proteins may mean that portions of the TM domain are able to interact with UL11. For example, dimerization of the gD CT mutant may allow the TM domain to interact with the acyl chains of the fatty acid on UL11. Alternatively, the gD CT mutant, when dimerized, would provide six basic residues which may interact with the acidic cluster of UL11. Conceivably, an interaction mediated by these residues could be an artifact of removing the more C-terminal residues of the gD CT domain. Regardless of the precise mechanisms by which this portion of the UL11-gD interaction occurs some specificity of binding between UL11 and gD was observed. Therefore we conclude from these studies that the gE and gD CT domains may be redundant in mediating secondary envelopment as they appear to interact with an identical or similar subset of tegument proteins (VP22 and UL11).

VP16 may be involved in both primary and secondary envelopment (Mossman, K. L. et al. 2000). Unfortunately, the results contained herein were not capable of determining whether any interactions occur between VP16 and gD or gE. Under the conditions we employed VP16 was precipitated through non-specific interactions with either the antibody or beads. This high background binding may be due to the nature of VP16 or the large amounts of this protein produced in infected cells or both. While a previous publication used similar conditions to generate VP16 that interacted with a GST::gH CT fusion protein, insufficient negative controls

were included such that the specificity of these interactions is unclear (Gross, S. T. et al. 2003). Subsequent analysis from the same lab demonstrated that under identical conditions a similar interaction could be observed with a GST::gD CT construct (Chi, J. H. et al. 2005). However, since none of the fusion proteins tested failed to interact with VP16, the specificity of the interaction is impossible to ascertain.

The work presented in this dissertation is the first to demonstrate interactions between gE and gD, and specific tegument proteins observed in extracts of HSV-infected cells (Chapter 4). Despite the involvement of VP22 and UL11 in HSV envelopment and egress there may be other tegument proteins which gD and gE interact with to mediate secondary envelopment (Baines, J. D. and Roizman, B. 1992; Elliott, G. et al. 2005; Duffy, C. et al. 2006). These other tegument proteins may not have been detected in the experiments described herein due to a lower affinity of interaction, lower protein expression levels or another component of the experimental design. For example, an unidentified interacting protein may remain insoluble under the conditions used in our assays. Alternatively, an unidentified interacting protein may require certain conditions for binding to gD and/or gE that were not provided in this experiment. Changing the assay conditions, however, would be difficult for immunoprecipitation of gE and gD from HSV-infected cells. The reaction conditions are optimized to allow the interaction between the removal of the viral glycoproteins from the membrane, the interations between these glycoproteins and their respective antibodies and the preservation of other protein-protein interactions. Changing the reaction conditions drastically may disrupt one or more of these parameters. Alternatively, additional tegument proteins may not bind gE and gD directly, but instead may be recruited through UL11 or VP22.

5.3 Cell-to-cell Spread of Enveloped Viruses

Following the acquisition of a membrane envelope the necessary next step for all viruses is egress from the original cell and spread to other cells, tissues or hosts. In cases where envelopment occurs at the plasma membrane such as in virus families orthomyxoviridae or paramyxoviridae, the nature of this spread is predetermined by the site of envelopment. In virus families which undergo envelopment at an internal membrane such as coronaviridae or herpesviridae, the progeny virus must be transported to the site of viral egress prior to spreading to distal cells. In all cases, the accumulation of necessary viral proteins at a specific membrane mediates envelopment and largely determines cell-to-cell spread (Section 1.5.8 and Fig.1.6). The type of spread that a virus undergoes determines the nature of the infection and the course of the disease. For example, Sendai virus (SV), a member of the paramyxoviridae family, normally buds from the apical surface of infected epithelial cells and spreads indirectly. The resulting infection is restricted to these cells and the disease is limited to a pneumontropic infection in rats that is largely non-lethal (Mochizuki, Y. et al. 1988). An SV isolate (F1-R) has been identified that sheds virions from both apical and baso-lateral domains of the plasma membrane (Tashiro, M. et al. 1990). This mutant strain infects subepithelial tissues and causes systemic infections leading to a significant increase in the mortality of infected rodents (Tashiro, M. et al. 1992).

Cell-to-cell spread can occur directly or indirectly. Direct cell-to-cell spread is defined by a virion directly infecting a neighboring cell without release into the extracellular fluid. Indirect cell-to-cell spread occurs when the virus gains access to the extracellular milieu either by lysis of the infected cell or egress from the apical membrane. Indirect spread can result in the infection of both adjacent and distal cells, but is blocked by neutralizing antibodies in the extracellular space.

5.4 Cell-to-cell Spread of HSV-1

Following envelopment HSV-1 spreads rapidly to the site of viral egress. In epithelial tissues HSV spreads both laterally to other epithelial cells and basally to both epithelial cells and the underlying neuron. The virus subsequently travels in a retrograde direction through the neuronal axon to the ganglion where it establishes latency. Reactivation triggers new virus replication and progeny virions are trafficked in the anterograde direction resulting in the introduction of infectious HSV-1 into the epithelial tissues in which the infection was originally established. In these studies we measure the direct cell-to-cell spread of respective HSV viruses by infecting polarized epithelial monolayers and, prior to the production of infectious progeny, the addition of antibody to the media. This antibody prevents indirect cell-to-cell spread, therefore the resulting 'viral plaques' are a measurement of the capability of the respective virus to engage in direct cell-to-cell spread.

5.4.1 Role of the glycoprotein gE in HSV cell-to-cell spread

The HSV glycoprotein gE/gI is a heterodimeric envelope glycoprotein that functions in both envelopment and cell-to-cell spread. gE/gI mutants are deficient in direct cell-to-cell spread in neuronal and epithelial cells as well as spread into and from the ganglion (Dingwell, K. S. et al. 1994; Dingwell, K. S. et al. 1995; Dingwell, K. S. and Johnson, D. C. 1998). HSV gE/gI participates in cell-to-cell spread by two distinct mechanisms (Wisner, T. et al. 2000; Johnson, D. C. et al. 2001; Collins, W. J. and Johnson, D. C. 2003; Farnsworth, A. and Johnson, D. C. 2006). The extracellular (ET) domains of gE/gI are necessary for spread, and likely bind receptors in order to promote the movement of HSV across epithelial cell junctions (Johnson, D. C. et al. 2001; Collins, W. J. and Johnson, D. C. 2003; Polcicova, K. et al. 2005b). This hypothesis is based on three key observations: gE/gI accumulates at cell junctions late in

infection (Wisner, T. et al. 2000; McMillan, T. N. and Johnson, D. C. 2001); when expressed in *trans*, gE/gI interferes with cell-to-cell spread (Collins, W. J. and Johnson, D. C. 2003); small insertion mutations in the gE ET domain can abolish the capacity of gE/gI to promote cell-to-cell spread (Polcicova, K. et al. 2005b). The cytoplasmic (CT) domain of gE also has a significant role in the cell-to-cell spread of HSV. Deletion of the gE CT domain has the same effect as deleting the entire protein in cell-to-cell spread between cultured epithelial cells and in the corneal epithelium (Wisner, T. et al. 2000; Polcicova, K. et al. 2005b). The gE CT domain encodes TGN localization signals such that gE/gI accumulates in the *trans*-Golgi network independently of other HSV-1 proteins (McMillan, T. N. and Johnson, D. C. 2001). Since gE, in conjunction with gD, promotes envelopment at this site it two possible means by which gE CT domain may contribute to cell-to-cell spread have been proposed (Section 1.5.8 and Fig. 1.6)(McMillan, T. N. and Johnson, D. C. 2001). First, gE may be inserted in vesicles containing HSV and thereby the gE CT domain may interact with host cell trafficking machinery. Alternatively, through directing envelopment into vesicles destined for lateral cell membranes the gE CT domain may indirectly control the site of viral egress (McMillan, T. N. and Johnson, D. C. 2001).

5.4.2 Role of gE CT sequences in cell-to-cell spread

In Chapter 3 we attempted to test this part of the model of HSV-1 egress by examining the role of the gE CT domain in mediating the spread of HSV-1 virions in epithelial cell layers and the trafficking of gE/gI. Initially, we demonstrated that the HSV mutants F-BAC ΔgE, gE_{448} (gEΔCT), gE_{470} and gE_{495} made equally small plaques on cultured epithelial cells (Chapter 3). If the only role of the gE CT domain in cell-to-cell spread is to direct envelopment of HSV into the correct TGN subcompartment then one would expect these mutants to behave similarly in terms

of protein trafficking and virus egress. However, HSV F-BAC ΔgE and gE_{448} shed elevated numbers of virions into the supernatant consistent with a shift in the direction of viral egress, while F-BAC gE_{470} and gE_{495} shed relatively low number of virions into the supernatant, similar to wild-type HSV. These results indicated that HSV expressing these various mutant gE proteins behaved differently in terms of viral egress consistent with the gE CT domain having multiple effects on HSV spread and maturation. Furthermore, F-BAC gE_{448} did not localize gE/gI to the TGN, and this mutant trafficked significantly more gE/gI to the apical surface whereas F-BAC gE_{470} and gE_{495} were similar to wild-type gE in gE/gI trafficking (Chapter 3). Indeed, the only difference observed between gE_{470} and gE_{495} mutants and wild type HSV was in their capacity to traffic gE/gI to lateral junctions late in infection and in mediating the cell-to-cell spread of the virus (Chapter 3 and Table 5.1)

These results appear to separate early accumulation of gE/gI at the TGN from the efficient cell-to-cell spread of HSV, suggesting that gE/gI is directly involved in the trafficking of vesicles to lateral cell surfaces, however other possibilities exist. Firstly, the gE CT domain might affect the host cell trafficking network, such that truncations of gE interfere with normal cellular trafficking events. Alternatively, while WT gE might not influence trafficking decisions *in vivo* substantial truncations such as contained in gE_{470} or gE_{495} could hypothetically reveal cryptic sites through which the mutant proteins interfere with the host cell trafficking machinery. Secondly, truncations introduced into the gE CT domain may actually interfere in the activity of the gE ET domain. Communication between the CT and ET domains of HSV proteins has previously been noted (Wanas, E. et al. 1999; Jones, N. A. and Geraghty, R. J. 2004). Additionally, these possibilities are not exclusive, such that the gE/gI CT domain may be directing virion-containing vesicles and truncation of this domain may impact the activity of the

gE ET domain. Unfortunately, the results presented in Chapter 3 do not distinguish between these scenarios and future investigations are warranted.

5.5 Future Directions

Simultaneous deletion of the envelope glycoproteins gD and gE from HSV-1 disrupts secondary envelopment of HSV. Unenveloped nucleocapsids accumulate in the cytoplasm immersed in an electron dense matrix. We have also found that specific regions of the gE CT domain are necessary for gE/gI localization and interaction with tegument proteins as well as HSV envelopment and cell-to-cell spread. Yet a number of questions remain unanswered. (1) What are the molecular sequences by which gD contributes to secondary envelopment and interacts with the tegument proteins VP22 and UL11 (2) Which specific residues of the gE CT domain are important for secondary envelopment, for interactions with VP22 and U_L11? (3) How are other glycoproteins (e.g. gC, gB etc.) incorporated into the virion envelope and do gE and gD contribute to this recruitment? (4) Does the gE CT domain contribute directly to post-envelopment trafficking of HSV-1 containing vesicles through interactions with the host cell trafficking machinery? (5) Is communication between the gE ET and CT domains required for appropriate targeting of virions to the lateral surface?

(1) What are the molecular sequences by which gD contributes to secondary envelopment and interacts with the tegument proteins VP22 and UL11?

The characterization of sequences in the gD CT domain important for secondary envelopment of HSV will require the construction of several gD mutants. HSV gD has a CT domain of 30 amino acid residues (McGeoch, D. J. et al. 1985). Mutant gD proteins with truncations in the gD CT domain at the membrane (a.a. 340) and at the midway point (a.a. 355) could be created and inserted into HSV gE- viruses to measure the overall contribution of the gD

CT domain to secondary envelopment. In an effort to more precisely identify important sequences in this domain alanine substitutions in 5 amino acid stretches over the length of the gD CT domain could also be inserted into a gE- HSV virus. Both gD truncation mutations and alanine substitutions could also be expressed by gD- HSV as well as Ad-vectors in order to identify sequences involved in VP22 interaction.

While these same mutants could be employed to identify regions of the gD CT important for the interaction of the gD CT domain with UL11, our work has suggested that much of this interaction occurs via the TM or CT domains (Chapter 4 and Section 5.3.2). This result could indicate that much of the interaction we detect in these assays is biologically irrelevant. Alternatively this signal may be being produced through an experimental artifact as discussed in section 5.3.2. To test how this interaction is mediated at least four constructs would be required. The first construct would be a soluble gD protein; the second one in which gD is attached to the membrane via a gp I anchor; the third would express the gD ET domain fused to the CD4 TM domain with no CT domain and the fourth construct would be a fusion of the gD ET and TM domains to the CD4 CT domain domain. These constructs will be expressed both in gD- HSV and in Ad-vectors and will be used to probe any UL11 interaction through immunoprecipitation from HSV infected cells as well as direct interactions with interactions Ad expressed UL11.

(2) Which specific residues of the gE CT domain are important for secondary envelopment, for interactions with VP22 and UL11?

A more precise determination of the CT residues important in secondary envelopment would require the construction of two more mutants which express large deletions in the gE CT domain; one would be deleted of only those sequences between gE_{470} and gE_{495} and the other only those sequences between gE_{495} and gE_{519}. These gE mutants would be expressed in a gD-

background to determine the defects in envelopment associated with missing those specific domains. If defects in envelopment are observed in either of these mutants alanine amino acid substitutions in 5 amino acid sections could be used to measure the involvement of specific sequences along these stretches. These same DNA constructs would be expressed in a gE- HSV background and used to re-examine the interactions with VP22 and UL11. Finally Ad vectors expressing gE mutations as described above would be used to examine the specific interaction between VP22, UL11 and gE.

(3) How are other glycoproteins (e.g. gC, gB etc.) incorporated into the virion envelope and do gE and gD contribute to this recruitment?

Glycoproteins other than gD or gE could be incorporated into the virion envelope in one of two ways. Incorporation of other membrane proteins may be based on interactions between their respective CT domains and the tegumented NC, as seen for gD and gE, or these other viral proteins could be recruited to the envelopment patch through interactions with other glycoproteins, via their ET, TM or CT domains. Research has demonstrated that gB, gC and gH/gL can all be chemically crosslinked to gD and have been postulated to exist as higher order structures in the virion envelope (Handler, C. G. et al. 1996). These results raise the possibility that gD or gE is involved in the recruitment of additional glycoproteins during secondary envelopment. Due to the redundancy in gD and gE function, chimeric CD4::gD CT and CD4::gE CT proteins would be expressed in the absence of the other glycoprotein (i.e. CD4::gD CT/gE- or gD-/CD4::gE_{495} CT HSV-1) in order to test the role of the gD and gE ET and TM domains in secondary envelopment. If the acquisition of a virus envelope is only dependant on the presence of the CT domains of gD or gE, as we have hypothesized, then these mutants should still produce enveloped particles. If HSV undergoes efficient secondary envelopment then these particles will

be compared with wild-type HSV for the relative expression levels of other glycoproteins. Measuring the relative expression levels of glycoproteins in these mutant virus envelopes will provide a clear indication of whether any recruitment is mediated through the activities of the ET and TM domains of gD or gE.

(4) Does the gE CT domain contribute directly to post-envelopment trafficking of HSV-1 containing vesicles through interactions with the host cell trafficking machinery?

Chapter 4 demonstrates that certain truncations of the gE CT domain abrogate HSV spread in conjunction with the mistrafficking of gE/gI. The fundamental question remains, however, are the viruses getting to the lateral junctions? By inserting these gE truncations into an HSV mutant containing a VP26::GFP fusion we can track the transport of the virus independently of various glycoproteins. Using these constructs we can then ascertain where the virus is in cells infected by HSV gE_{470}/VP26::GFP and HSV gE_{495}/VP26::GFP. If the localization is different than for WT HSV then mutants with the gE truncations must be interfering with the transport of the virus. If trafficking of the virus particle is changed then the question of whether this is a phenomenon associated only with HSV transport or are the trafficking of other proteins are also inhibited. To determine the relative efficacy of the trafficking of non-HSV proteins we could measure the transportation of Ad-VSV G protein in the context of HSV WT and gE truncation infections. Additionally, the effects of various gE constructs in isolation of HSV infection would be examined to ascertain whether expression of these constructs alone interferes with the trafficking other proteins as measured by the transport of VSV G protein.

Instead if the HSV gE_{470}/VP26::GFP viruses are localized to lateral junctions similarly to HSV VP26::GFP viruses then some aspect of the truncation of the gE CT domain may be interfering with the functioning of the gE ET domain.

(5) Is communication between the gE ET and CT domains required for appropriate targeting of virions to the lateral surface?

To test the hypothesis that communication between the ET and CT domains of gE is necessary for WT protein function we need to separate the domains from each other but still preserve their expression in the context of the virus particle. This is difficult as gE functions as a heterodimer with its partner, gI. First, we could swap the CT domains of gE and gI. This has the advantage of preserving all of the molecular information that gE/gI encodes. If this mutant, HSV gE::gICT/gI::gECT, remains capable of WT cell-to-cell spread then the hypothesis that the CT and ET domains communicate appears unlikely.

5.6 Summary

All enveloped viruses acquire their membrane from the host cell through interactions between viral proteins. Defining the proteins involved in this process has been difficult, especially in complex viruses such as those of the alphaherpesvirus subfamily. The finding that simultaneous deletion of gM and gE in PRV led to the accumulation of large numbers of cytoplasmic nucleocapsids led to significant insight into the means by which herpesviruses acquire an envelope (Brack, A. R. et al. 1999; Brack, A. R. et al. 2000). However, subsequent studies demonstrated that deletion of gM and gE did not abrogate secondary envelopment in other alphaherpesviruses, whereas deletions of other pairs of glycoproteins did, indicating that the molecular requirements were not constant throughout this sub-family (Rudolph, J. and Osterrieder, N. 2002; Browne, H. et al. 2004). Investigating the role of glycoproteins in

mediating HSV-1 envelopment I described the involvement of gD and gE in the assembly of HSV-1 and suggested that these two glycoproteins have a redundant role in envelope acquisition (Chapter 2). Furthermore, I identified specific regions of the gE CT domain involved in virus assembly and suggested that these regions critical for the envelopment of HSV-1 interact with proteins in the tegument layer (Chapter 4). Finally, I defined regions of the gE CT domain involved in the efficient cell-to-cell spread of HSV-1 (Chapter 3). As a whole, the work presented in this dissertation has significantly expanded our understanding of the maturation and egress of HSV-1. From this work it can be concluded that the development of HSV-1 is clearly consistent with the envelopment->de-envelopment->re-envelopment model (Stackpole, C. W. 1969; Skepper, J. N. et al. 2001). In addition, the gE CT domain, in conjunction with gD, is necessary for the efficient envelopment of HSV-1 (Chapter 4). Thirdly, both gD and gE interact via their CT domains with tegument proteins involved in the maturation of HSV-1 (Chapter 4). Finally, the gE CT domain is necessary for the efficient spread of HSV-1 which suggests an additional role for this domain at a post-assembly stage of the virus life-cycle (Chapter 3).

Table 5.1 Properties of gE CT domain mutants

gE mutation	TGN accumulation of gE/gI	Redistribution of gE/gI to lateral junctions	Cell-to-cell spread of HSV	Secondary Envelopment of HSV[a]	Interaction with UL11[b]	Interaction with VP22[b]
WT	+	+	+	+	+/+	+/+
gE-null	-	-	-	-	-/-	-/-
gE_{448}	-	-	-	-	-/-	-/-
gE_{470}	+	-	-	-	-/-	-/-
gE_{495}	+	-	-	+	-/+	-/+
gE_{519}	+	+	+	+	+/+	-/+

a gE CT domain contribution as measured in a gD- background.

b The first value is interaction measured by immunoprecipitation of endogenous gE, the second is immunoprecipitation of TAP::gE CT fusion constructs.

Appendix A:

Herpes simplex virus glycoproteins gB and gH/gL mediate a necessary step in primary de-envelopment.

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Unpublished

Aaron Farnsworth made the gB/gH complementing cell line and all viruses. Michael Webb processed, stained, and sectioned all samples for EM. David C. Johnson performed all experiments.

A.1 Abstract

Herpes simplex virus replicates and packages its genome in the nucleus of infected cells. The virus envelope, however, is acquired from a Golgi-derived membrane making it necessary for these filled capsids to enter the cytoplasm. This process is accomplished by the capsid interacting with the inner nuclear membrane (INM) and budding into the perinuclear space. These particles, designated primary envelopedvirions, rapidly fuse with the outer nuclear membrane (ONM) and deposit the DNA-filled virus capsid into the cytoplasm (Skepper, J. N. et al. 2001). Although a variety of deletion mutations in HSV result in the accumulation of primary enveloped virions at this stage, little evidence exists as to the precise molecular mechanisms involved. Expression in *trans* of gK, a glycoprotein known to regulate virion fusion, results in the accumulation of wild-type HSV particles in the perinucelar space implying a role for HSV fusogenic proteins in this process. Since neither gB-, gH- nor gL-null HSVs accumulate significant numbers of virions in the perinuclear space of infected cells it has generally been assumed that envelope glycoproteins do not have a substantial role in primary de-envelopment. This assumption ignores the fact that multiple alphaherpesviruses demonstrate a substantial defect in maturation processes only upon the deletion of multiple envelope proteins involved in the pathway (Brack et al. 1999, Corse and Machamer 2003)(Chapters 2 and 4). To test whether similar redundancy occurred in the process of egress from the nuclear compartment we created a gB-/gH- virus and examined cells infected by these mutants HSVs through electron microscopy. We find that the gB-/gH- HSV virus accumulates significant numbers of particles in the perinuclear space in addition to enveloped virions in intranuclear vessicles in the nucleoplasm. These results indicate that either gH or gB

are able to mediate some aspect of fusion with the ONM and in the absence of both proteins the resultant virus is inhibited in efficiently completing this step.

A.2 Results

A.2.1 Construction of a cell line that complements both gB and gH.

To directly test the hypothesis that gB and gH are each capable of mediating a necessary aspect of nuclear de-envelopment we sought to create a HSV mutant deleted for both proteins. The creation of such a virus required a cell line which could complement both genes in *trans*. F6 cells, (Forrester, A. et al. 1992) which express gH, were transfected with the plasmid pSV2HISgB, which contains the gB gene and flanking sequences from the plasmid pKBXX (Cai, W. Z. et al. 1987). Cell transformants capable of growth in histidinol concentrations toxic for untransformed cells were grown up and screened for expression of both gB and gH. Screening was performed by growing and titering small stocks of gH- and gB- viruses on these transformants, and comparing them to the titers of small stocks of identical viruses grown on F6 cells and VB38 cells, a cell line which expresses gB in *trans*. Two cell transformants, F6/gB1 and F6/gB12 were capable of complementing gH and gB stocks to a similar level to F6 and VB38 cell lines.

A.2.2 Construction of mutant HSV

To examine the role of gB and gH in primary envelopment and de-envelopment we constructed a panel of bacterial artificial chromosome (BAC) based HSV-1 viruses. F-BAC gB-, F-BAC gH- and F-BAC gB-/gH- were constructed as described in the materials and methods and the mutant F-BAC gB-/gE-/gI- was constructed previously (Chapter 2).

A.2.3 gB-/gH- virus accumulates in the herniations in HaCaT cells

A human keratinocyte cell line, HaCaT, was employed to make the initial observations of HSV-1 at various stages of maturation (Boukamp, P. et al. 1988). F-BAC gB-/gH- HSV-1 displayed a serious defect in primary de-envelopment as reflected by the accumulation of enveloped particles in the perinuclear space in addition to the accumulation of enveloped particles in intranuclear vesicles adjacent to the INM (Table A.1 and Fig. A.1). In these experiments there was a 50 fold increase in the numbers of enveloped virions in the nuclear compartment (perinuclear and intranuclear enveloped capsids) and a 5 fold decrease in the number of cell-surface enveloped virions, when compared to w.t. HSV. F-BAC gB- and F-BAC gB-/gE-/gI- each displayed a 6 fold increase in enveloped particles in the nucleus and a small decrease in cell-surface associated particles compared to w.t. HSV. Since F-BAC gH- viruses evidenced little accumulation of virions in the nuclear compartment (Table A.1 and Fig. A.1) gB may have a more significant role in nuclear egress. A similar phenotype, to that observed on cells infected by F-BAC gB-/gH- has been previously noted in studies of US3-deleted HSV. These studies suggested that in the absence of US3-mediated phosphorlyation, fusion events were inhibited or misdirected (Reynolds, A. E. et al. 2002; Ryckman, B. J. and Roller, R. J. 2004). However, no evidence has currently been published to suggest any direct interaction between gB or gH and US3.

A.2.4 gB-/gH- virus accumulates in the perinuclear space of Vero cells.

Fusion between gB-/gH- viruses and the outer nuclear membrane was also examined in Vero cells, a monkey kidney cell line. Although intranuclear vesicles containing enveloped virions were observed in cells infected with gB-/gH- viruses (17-fold increase

over w.t. HSV) the majority of enveloped particles in the nuclear compartment remained in the perinuclear space (58 fold increase over w.t. HSV, Table A.1 and Fig. A.2). The F-BAC gB-/gH-virus was further characterized on F6/gB12 cells to ensure that these results were not the result of secondary site mutations introduced during virion constructions (Table A.1).

A.2.5 Enveloped intranuclear gB-/gH- HSV express UL34

The intranuclear vesicles containing gB-/gH- viruses were sometimes contiguous with the INM and othertimes adjacent to it (Fig A.1, B and C). To characterize these vesicles immunoelectron analyses were performed on these infected cells. As demonstrated in Fig. A.3, both viruses and the lipid envelope surrounding them stained heavily with antibodies to UL34. This result is indicative that all of these membranes are similarly derived from the inner nuclear membrane.

A.3 Materials and Methods

A.3.1 Cells and viruses.

HaCaT cells (Boukamp, P. et al. 1988) and Vero cells were grown in Dulbecco's modified Eagle's medium (DMEM) (BioWhittaker) supplemented with 10% FBS. F6 cells, derived from Vero cells and capable of expressing gH (Forrester, A. et al. 1992), were maintained in DMEM containing G418 (400 μg/ml) (Invitrogen, Carlsbad, Ca.) and supplemented with 10% FBS. VB38 cells, derived from Vero cells and capable of expressing gB were maintained in DMEM containing 10% FBS and 0.5 mM histidinol (Sigma, St. Louis, Mo.) F6/gB12 cells, derived from F6 cells and capable of expressing both gB and gH were maintained in DMEM containing G418 (400 μg/ml), 0.5 mM histidinol and 10% FBS. The construction of HSV-1 mutants F-BAC gB-, F-BAC gH-

kan and F-BAC gB-/gH-kan are described here and were each propagated and titered on VB38, F6 and F6/gB12 cell lines respectively.

A.3.2 Plasmids.

A 4-kilobase (kb) insert (*Bam*HI-*Xho*I fragment of HSV-1 KOS (0.345-0.372 map units)), containing the 2.7 kb gB-coding sequence, from pKBXX (Cai, W. Z. et al. 1987)was ligated into pSV2HIS (Hartman, S. C. and Mulligan, R. C. 1988) to create the plasmid pSV2HISgB. The plasmid pKD46 (Datsenko, K. A. and Wanner, B. L. 2000) contains the red recombinase genes γ, β and *exo* of phage λ under control of the arabinose-inducible *ParaB* promoter. The template plasmid, pKD4 (Datsenko, K. A. and Wanner, B. L. 2000) contains a FLP recognition target- (FRT) flanked kanamycin (*kan*) resistance gene. The plasmid pCP20 (Cherepanov, P. P. and Wackernagel, W. 1995)contains the FLP gene from *Saccharomyces cerevisiae* as well as ampillicin (bla) and chloramphenicol resistance genes and has a temperature sensitive origin of replication.

A.3.3 Construction of F6/gB12 cell line.

Subconfluent monolayers of F6 cells contained in 6-well dishes were transfected with 15 μg of pSV2HISgB using the $CaPO_4$ technique (Graham, F. L. and van der Eb, A. J. 1973). Transfected cells were treated, after 5 h, for 90s with DMEM containing 15% glycerol and 10% FBS, and then washed three times in DMEM containing 10% FBS. After 24 hours medium was replaced with DMEM containing G418 (400 μg/ml) and 10% FBS. Two days later cell monolayers were trypsinized, resuspended in DMEM containing G418 (400 μg/ml), 0.4 mM histidinol and 10% FBS and divided into 20 100-mm dishes and, 21 days later, 76 individual colonies of cells were trypsinized and reseeded into 24-

well dishes. These transformants were were screened for the capacity to complement both gB- and gH-null HSV mutants, of these the clonal transformant F6/gB12, could grow high titre stocks of both gB- and gH-null viruses.

A.3.4 Construction of F-BAC mutants.

HSV-1 mutants containing single gene deletions in gB or gH were constructed as previously described (Farnsworth, A. and Johnson, D. C. 2006)using a modification of a protocol developed by Datsenko *et al.* (Datsenko, K. A. and Wanner, B. L. 2000). Briefly, bacteria containing BAC with wild type HSV-1 strain F, were mutagenized to replace gB or gH coding sequences with a kananmycin cassette. The PCR primers gBF-FRT ATGCGCCAGGGCGCCCCCAC

GCGGGGGTGCCGGTGGTTCGGTGTAGGTCGGAGCTGCTTC and gBR-FRT

TCACAGGTCGTCCTCGTCGGCGTCACCGTCTTTGTTGGGA

CATATGAATATCCTCCTTAG

were used to make a FRT-*kan*-FRT cassette flanked by 40 nt of gB coding sequences. This DNA was transformed into RR1 bacteria containing both HSV BAC DNA and pKD46 and recombinant BACs in which the gB coding sequences were replaced by this FRT-*kan*-FRT cassette were selected as previously described (Farnsworth, A. and Johnson, D. C. 2006). Additional PCR primers gHF-FRT

ATGGGGAATGGTTTATGGTTCGTGGGGGTTATTATTTTGG

GTGTAGGTCGGAGCTGCTTC and gHR-FRT

TTATTCGCGTCTCCAAAAAAACGGGACACTTGTCCGGAGACATATGAATATC

CTCCTTAG

were used to create a FRT-*kan*-FRT cassette flanked by 40nt of gH coding sequences and treated as above. BACs containing mutant HSV-1 genomes were sequenced across, upstream and downstream of the insertion site and were analyzed by agarose gel electrophoresis. To construct the mutant F-BAC gB-/gH-kan mutant, RR1 bacteria containing the BAC gB-kan DNA (Ap^R, Kn^R, Cm^S) were transformed with plasmid pCP20 and FLP-mediated removal of the kan-cassette was performed as previously described (Datsenko, K. A. and Wanner, B. L. 2000), creating the BAC gB-. BAC gB- DNA was subsequently transformed with the plasmid pKD46 and deleted for gH using the gH-FRT cassette described above. BACs containing gB-/gH-kan DNA were analyzed as previously and through analysis of several additional individual genes (UL11, UL24, UL49 and US6) using PCR, agarose gel electrophoresis and partial sequence analysis. Infectious HSV-1 mutants were produced by transfecting appropriate BAC DNA into either F6, VB38 or F6/gB12 cells as described (Farnsworth, A. and Johnson, D. C. 2006).

A.3.5 Antibodies.

DL6, a MAb specific for HSV-1 gD was a gift from Gary Cohen and Roselyn Eisenberg (University of Pennsylvania, Philadelphia). LP1, a MAb specific for HSV-1 gH was a gift from Tony Minson (Cambridge University, UK) 15βB2 a MAb specific for HSV-1 gB has been previously described (Hanke, T. et al. 1991). Chicken anti-UL34 antibodies (Reynolds, A. E. et al. 2002)were used in immunoelectron analyses.

A.3.6 Electron Microscopy.

HEC-1A cells were infected with wild type F-BAC HSV-1 or F-BAC mutants for 16 h, washed with 0.1M sodium cacodylate buffer (pH 7.2) and fixed in Ito and

Karnovsky's fixative for 30 min at room temperature (1.6% paraformaldehyde, 2.5% glutaraldehyde and 0.5% picric acid in 0.1 M sodium cacodylate). Samples werepostfixed in 1.5% osmium tetroxide, rinsed and then postfixed in 4% paraformaldehyde. Samples were dehydrated in a graded acetone series, embedded in epoxy resin and ultrathin sections were double stained in uranyl acetate and lead citrate (Farnsworth, A. et al. 2003)and viewed with a Philips EM 300 Electron Microscope. For immunoelectron microscopy infected cells were fixed Ito and Karnovsky's fixative, dehydrated in acetone before being embedded in LR White resin. Sections were cut, stained with primary antibodies followed by secondary antibodies conjugated to 6 or 15 nm gold beads then poststained as described above.

Table A.1 Distribution of virus particle produced by wild type and mutant HSV-1 on HEC-1A and Vero cells

Virus	Cells	Enveloped nuclear capsids	Enveloped perinuclear capsids	Cytoplasmic enveloped virions	Cell surface virions	Total
F-BAC (wild type)	HaCaT	10 (0.8)	9 (0.7)	303 (24)	923 (74)	1245
F-BAC gB-/gH-	HaCaT	***631 (49)***	***315 (24)***	157 (12)	194 (15)	1297
F-BAC gB-	HaCaT	*45 (6.5)*	*31 (4.5)*	152 (22)	461 (67)	689
F-BAC gB-/gE-/gI-	HaCaT	32 (6)	16 (3)	196 (36)	298 (55)	542
F-BAC gH-	HaCaT	36 (2.4)	14 (1.0)	378 (25)	1038 (71)	1462
F-BAC (wild type)	Vero	3 (0.5)	6 (1.0)	171 (29)	401 (69)	581
F-BAC gB-/gH-	Vero	57 (8.5)	390 (58)	122 (18)	101 (15)	670
F-BAC (wild type)	F6/gB+12	2 (0.3)	7 (1.1)	181 (29)	445 (70)	635
F-BAC gB-/gH-	F6/gB+12	6 (1.0)	14 (2.2)	143 (23)	457 (74)	620

Figure A.1 Electron Micrographs of F-BAC gB-/gH- infected HaCaT cells.

HaCaT cells were infected with F-BAC gB-/gH- virus for 16 h and then fixed and processed for electron microscopy. **(A)** Displays numerous intranuclear vesicles containing enveloped capsids. **(B and C)** Show a close up these vesicles at the INM. Solid arrow show a particle in the perinuclear space attempting to fuse with the ONM forming stalk like projection.

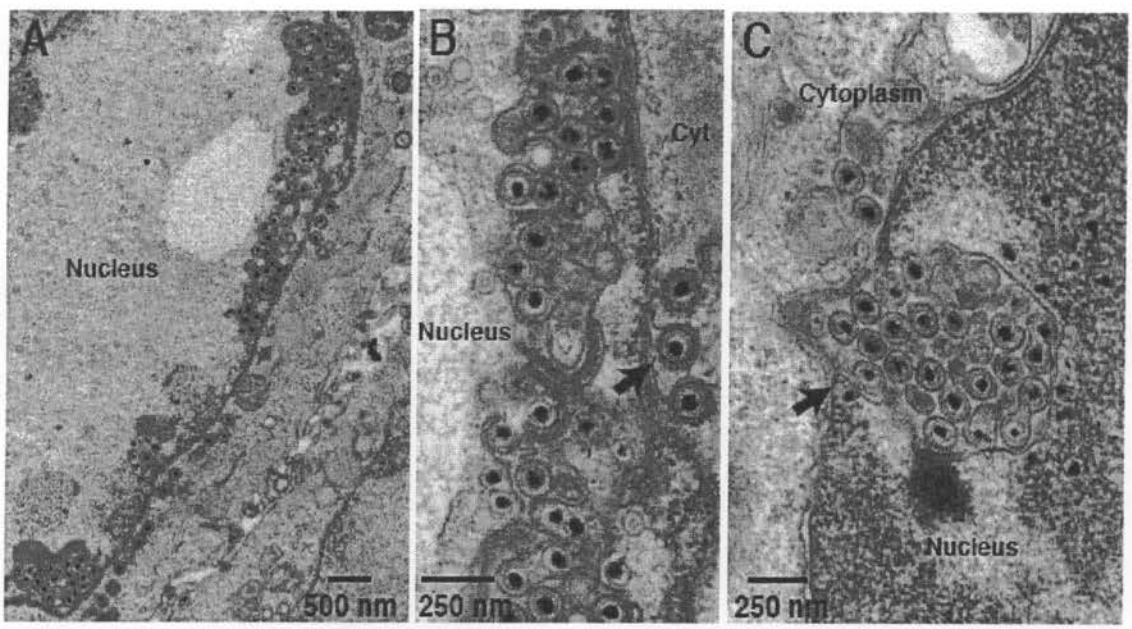

A
Nucleus
500 nm
B
Cyt
Nucleus
250 nm
C
Cytoplasm
Nucleus
250 nm

Figure A.2 Electron Micrographs of F-BAC gB-/gH- infected Vero cells.

Vero cells were infected with F-BAC gB-/gH- virus for 16 h and then fixed and processed for electron microscopy. **(A)** As described in text and Table A.1 enveloped particles accumulated in the perinuclear space more than observed in Figure A.1. **(B)** Close up of primary enveloped virion attempting to fuse with the ONM.

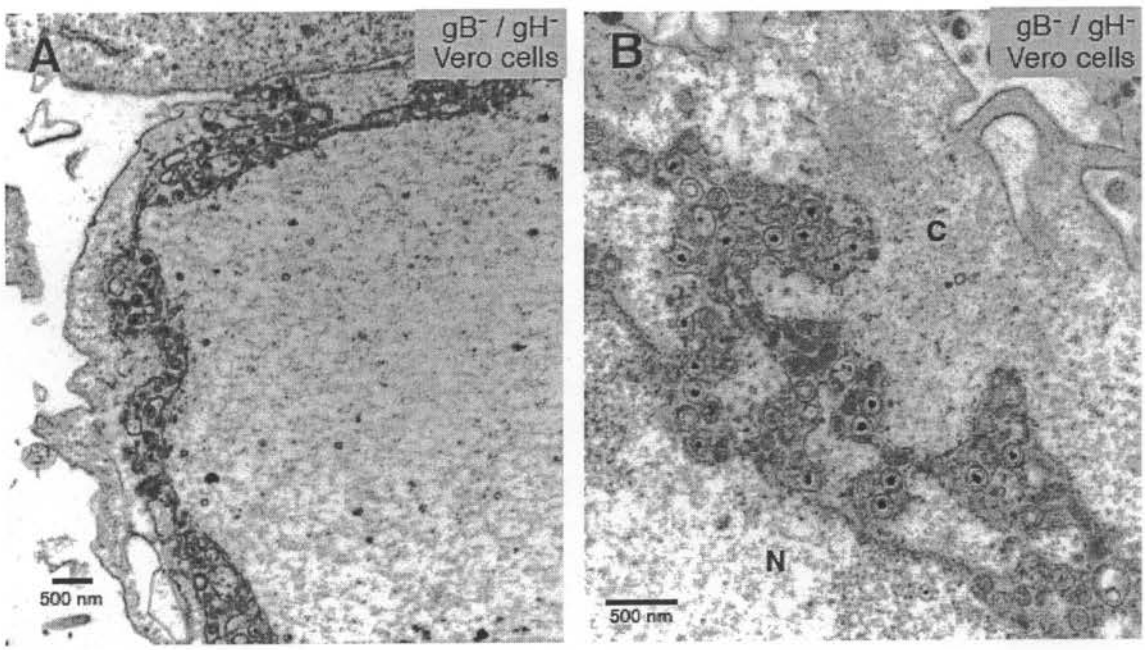
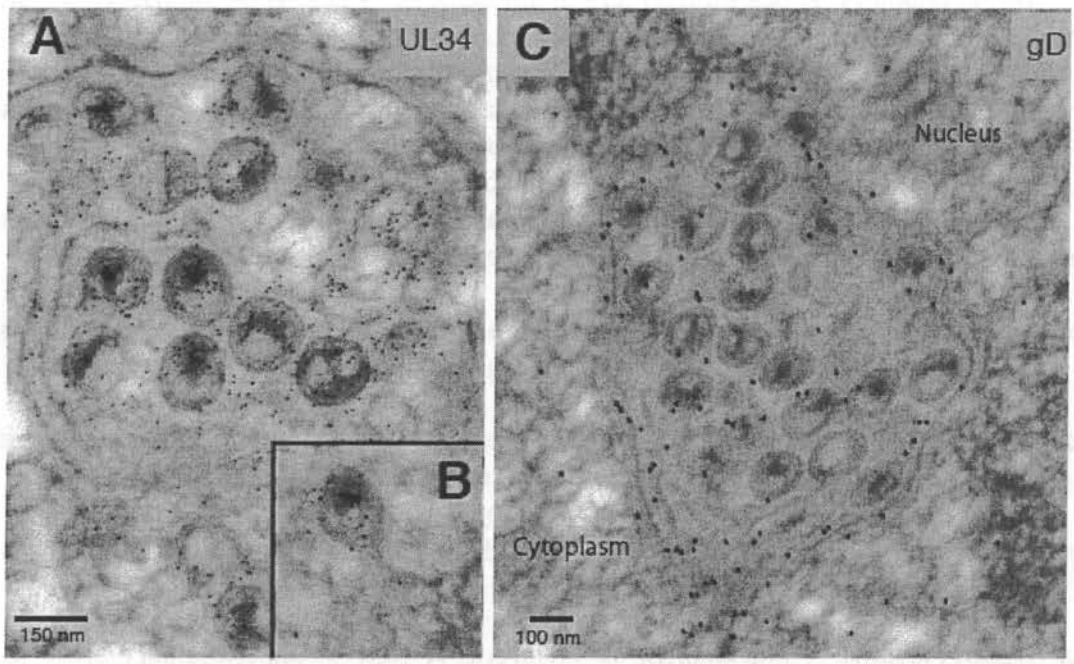

A
gB⁻ / gH⁻
Vero cells
500 nm
B
gB⁻ / gH⁻
Vero cells
C
N
500 nm

Figure A.3 Immunoelectron Micrographs of F-BAC gB-/gH- infected HaCaT cells. HaCaT cells were infected with F-BAC gB-/gH- for 16 h cells were fixed, incubated with primary and secondary antibodies and processed for electron microscopy. **(A)** virions contained within vesicle are extensively labelled with 6nm gold particles displaying sharp association with UL34 compared with area surrounding vesicle. **(B)** Close up of gB-/gH- virion marked with anti-UL34 antibodies and 6nm gold particles. **(C)** 15 nm gold particles label ONM and INM and gB-/gH- virions indicating presence of UL34.


A
UL34
B
150 nm
C
gD
Nucleus
Cytoplasm
100 nm

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