Molecular Genetics and Functions of Herpes Simplex Virus Type 1 (HSV-1) Glycoprotein K (gK) in the Morphogenesis of Infectious Virion Particles.

Timothy Paul Foster

Louisiana State University and Agricultural & Mechanical College

Follow this and additional works at: https://digitalcommons.lsu.edu/gradschool_disstheses

Recommended Citation

This Dissertation is brought to you for free and open access by the Graduate School at LSU Digital Commons. It has been accepted for inclusion in LSU Historical Dissertations and Theses by an authorized administrator of LSU Digital Commons. For more information, please contact gradetd@lsu.edu.
INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Bell & Howell Information and Learning
300 North Zeib Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

UMI®
MOLECULAR GENETICS AND FUNCTIONS OF HERPES SIMPLEX VIRUS TYPE 1 (HSV-1) GLYCOPROTEIN K (gK) IN THE MORPHOGENESIS OF INFECTIOUS VIRION PARTICLES

A Dissertation

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

in

The Interdepartmental Program in Veterinary Medical Sciences through the Department of Veterinary Microbiology And Parasitology

By

Timothy P. Foster
B.Sc. Louisiana State University, 1995
B.Sc. Louisiana State University, 1995
December, 1999
DEDICATION

This work is in memory of my grandfather, Charles Gillis Carraway, whom despite quite debilitating circumstances, which took away both his mental and physical capacities, always displayed the qualities of patience and understanding. Although it is the latter years of his life that I most remember, those memories do not do justice to a man who throughout his life struggled to better himself and those around him. His faith in my endeavors and vigilance in asking how my research was progressing will never be forgotten. He is my inspiration and motivation to keep reaching for my dreams in the midst of any and all adversity.
ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my advisor Dr. K. G. “Gus” Kousoulas for his support, mentorship, confidence, and most of all friendship. He has been my father, brother, confidant and friend throughout both the good and the difficult times. He has provided me not only a sound base to build my research efforts upon, but also the knowledge that it takes to acquire funding for those efforts. The state of the art facilities, equipment, reagents and experience that his laboratories provided, gave me the wherewithal to succeed in the face of adversity. His dedication to his students, his laboratory, and his resourcefulness in funding will certainly always be remembered and admired.

All too often Dr. Kousoulas spent long nights away from his family and often took time out of his personal life to help advance my research efforts and graduate studies. For this reason, I must also acknowledge the patience and understanding of his wife, Effie Kousoulas and his daughter Elena Kousoulas.

I am indebted to the efforts of my department head, Dr. Johannes Storz for his guidance throughout my studies in developing important leadership qualities and organizational skills. His attention to detail has helped me develop both as a researcher and as a person. I am also extremely grateful for his efforts in acquiring my graduate fellowship through the Louisiana Board of Regents Fellowship Program.

I send my thanks to my committee members, Dr. Ding Shih, Dr. Kathy O’Reilly, Dr. Elmer Godeny, Dr. Roger Laine, and Dr. Fred Rainey. This is not only for their efforts during my proposal, general exam, and defense, but for their
efforts in the classroom as well. I have not known a more qualified and diligent
group of teachers. Each has contributed their own unique style and interpretations
to the way that I now perceive so many things in life and in science. Their efforts
through courses in the Department of Biological Sciences and the Department of
Veterinary Microbiology and Parasitology has provided me with a diverse and well
rounded background in the biological sciences that will follow me in all my future
endeavors.

I would like to thank Dr. Roger Laine for his insight into the entrepreneurial
workings of both science and academics. I have spent many hours with he and his
family discussing science, business, and life as well as partaking in his incredible
cooking. His efforts at taking basic science into real-world application must be
applauded.

I would like to give special thanks to all those in GeneLab that provided both
personal and professional assistance, Dr. Vladimir Chouljenko, Dr. A. Baghian, Li
Huang, Susan Newman, Rafael Luna, Sukhanaya Jayachandra, Jim Cavacoli, and
Tamara Chouljenko. I have learned so much from each of you and your diverse
cultures and thank you all for your patience and tolerance during some of my worse
moments.

To the most special person in my life during this sometimes trying ordeal, I
would like Galena Rybachuk to know that I have the greatest love and admiration
for her. Despite her seemingly gentle nature, you have been the rock that has
always been there when I needed someone to lean upon. I know I leaned a bit too
hard at times and for this I am sorry. I hope that in our future years together we will
be able to look on this time as not only productive and enjoyable, but as some of the fondest memories that we will ever share.

Last but certainly not least, I would like to especially thank my family. Despite their initial efforts to steer me in some (or any) other direction, my father, Mr. Edwin E. Foster Jr. and mother, Mrs. Cheryl A. Carraway Foster have been extremely supportive throughout these four years. We have certainly grown closer during this time than we ever have been in our lives. Your undying and unfettered support is greatly appreciated and acknowledged. I must especially thank you for all the sacrifices you have made along the way to make my dreams a reality.

My time here would not have been as cherished without the continuous chiming in of my sister, Amy E. Foster. Her unique outlook on life and on me has been a driving force in my life. I have never known someone so opposite and yet so similar as her. I would like to wish her the best in her efforts to follow in my footsteps in attaining an advanced degree, but she is one that must and will blaze her own trails.
# TABLE OF CONTENTS

DEDICATION ........................................................................................................... ii

ACKNOWLEDGEMENTS ..................................................................................... iii

LIST OF TABLES .................................................................................................... xi

LIST OF FIGURES .................................................................................................. xii

ABSTRACT ............................................................................................................. xx

CHAPTER I: INTRODUCTION ............................................................................. 1

STATEMENT OF RESEARCH PROBLEM AND HYPOTHESIS .................. 1

STATEMENT OF RESEARCH OBJECTIVES ................................................... 2

LITERATURE REVIEW ....................................................................................... 4

Historical Perspective of Herpesviruses ......................................................... 4

Taxonomy of *Herpesviridae* ......................................................................... 5

Clinical Significance of Herpes Simplex Viruses ........................................ 8

Epidemiology ...................................................................................................... 8

Pathogenesis ..................................................................................................... 10

Mucocutaneous Infections ............................................................................. 11

Fetal and Neonatal Infections ....................................................................... 12

Keratoconjunctivitis ....................................................................................... 14

Immunocompromised Host ............................................................................ 14

Central Nervous System (CNS) Infections .................................................. 14

Prevention and Treatment ........................................................................... 15

Architecture of the Herpes Virion ................................................................. 16

The Core .......................................................................................................... 16

The Capsid ....................................................................................................... 16

The Tegument ................................................................................................. 19

The Envelope .................................................................................................. 19

Organization of the Viral Genome ................................................................. 20

The Herpesvirus Lifecycle ............................................................................. 23

Attachment ...................................................................................................... 23

Receptor Facilitated Entry .............................................................................. 26

Herpes Virus Entry Mediator A (HveA) ...................................................... 29

Herpes Virus Entry Mediator B (HveB) ...................................................... 33

Herpes Virus Entry Mediator C (HveC) and Herpesvirus Ig-like receptor

(HIgR) .............................................................................................................. 33

Herpes Virus Entry Mediator D (HveD) and Other herpesvirus receptors.... 37

Host Protein Shutoff ..................................................................................... 39

Virion Transport to the Nucleus ................................................................. 40

Transcriptional Regulation ......................................................................... 40

Viral DNA Replication and Metabolism .................................................. 42

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
CHAPTER II: EXPRESSION OF THE ENHANCED GREEN FLUORESCENT PROTEIN BY HERPES SIMPLEX VIRUS TYPE 1 (HSV-1) AS AN IN VITRO OR IN VIVO MARKER FOR VIRUS ENTRY AND REPLICATION

INTRODUCTION ............................................................................................................... 98
MATERIALS AND METHODS .......................................................................................... 100
  Cells and viruses ........................................................................................................ 100
  Reagents ................................................................................................................... 101
  Plasmids .................................................................................................................... 101
  Virus Construction .................................................................................................. 102
  Diagnostic PCR ....................................................................................................... 102
  Western Immunoblots .............................................................................................. 102
  Fluorescent Microscopy ............................................................................................ 103
  FACS Analysis .......................................................................................................... 103

RESULTS ......................................................................................................................... 105
  Construction of the $\Delta$gK-EGFP recombination plasmid ........................................ 105
  Isolation of the recombinant virus $\Delta$gK-EGFP ...................................................... 105
Genetic characterization of recombinant viruses containing EGFP gene cassettes........................................................................................................... 108
Detection of EGFP expression by western immunoblot analysis . 111
Fluorescence and phase contrast microscopy of ΔgK-EGFP infected Vero cells .............................................................................................................. 111
FACS analysis of ΔgK-EGFP-1 infected cells ............................................. 114

DISCUSSION................................................................................................. 114

CHAPTER III: FUNCTIONAL CHARACTERIZATION OF THE HVEA HOMOLOG SPECIFIED BY AFRICAN GREEN MONKEY KIDNEY CELLS THROUGH THE USE OF A HERPES SIMPLEX VIRUS EXPRESSING THE GREEN FLUORESCENCE PROTEIN.............................. 117

INTRODUCTION ............................................................................................... 117
MATERIALS AND METHODS ........................................................................... 119
Cells and viruses .......................................................................................... 119
Reagents ........................................................................................................ 119
Isolation of the HveAs gene and construction of plasmids ..................... 120
Construction of the HSV-1 (KOS)/EGFP recombinant virus ............... 121
Diagnostic PCR .......................................................................................... 121
Construction of Chinese hamster ovary (CHO) cell lines transformed with the HveAs or HveAs:Fc genes ..................................................... 121
Production of anti-HveAs-specific antibodies ......................................... 122
Production of HveA:Fc by CHO-transformed cells ................................. 122
Phase-contrast and fluorescent microscopy ........................................... 123
FACS analysis ........................................................................................... 123
Inhibition of Virus Entry by anti-HveAs antibody and Soluble HveA:Fc protein .............................................................. 123

RESULTS ......................................................................................................... 124
Construction and genetic characterization of the recombinant virus HSV-1 (KOS)/EGFP ...................................................................................... 124
Plaque phenotype, EGFP expression, and growth characteristics of the KOS/EGFP virus ...................................................................................... 126
Isolation of the gene coding for the Vero HveA homolog (HveAs) and comparison of the HveA and HveAs predicted amino acid sequences ........................................................................................................... 129
Functional characterization of the HveAs receptor .................................. 132

DISCUSSION................................................................................................. 137
The KOS/EGFP Virus .................................................................................. 137
HveAs mediates HSV entry ........................................................................ 138
The HveAs Protein ..................................................................................... 139

CHAPTER IV: HERPES SIMPLEX VIRUS TYPE 1 (HSV-1) GLYCO PROTEIN K (gK) IS A STRUCTURAL COMPONENT OF PURIFIED VIRIONS THAT FUNCTIONS TO MODULATE HVEAS RECEPTOR MEDIATED VIRUS ENTRY ......................................................... 141

INTRODUCTION ............................................................................................... 141
MATERIALS AND METHODS ........................................................................... 145
CHAPTER V: GENETIC ANALYSIS OF THE ROLE OF HERPES SIMPLEX VIRUS 1 (HSV-1) GLYCOPROTEIN K (gK) IN INFECTIOUS VIRUS PRODUCTION AND EGRESS

INTRODUCTION

MATERIALS AND METHODS

Cells and viruses

Reagents

Plasmids

Construction and purification of gK mutant viruses

Viral DNA

PCR confirmation and sequencing of gK-mutant viruses

Rescue and complementation of gK-mutant viruses

Electron microscopy of gK-truncated virions in Vero cells

Production of infectious virions

RESULTS

Construction and genetic characterization of HSV-1 (KOS) mutant viruses containing stop codons within the gK gene

Plaque morphologies of virus isolates and viral yields

Electron Microscopy

Alignment of gK specified by alphaherpesviruses

Construction of recombinant viruses specifying amino acid changes within the CXXCC and YXXØ motifs

DISCUSSION

ix

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
LIST OF TABLES

Table 1.1. Classification of Select Members of Herpesviridae............................. 7

Table 1.2. Description of Herpesvirus Entry Mediating Receptors ..................... 38

Table 3.1. Viral yields of KOS and KOS/EGFP viruses. Subconfluent Vero cell monolayers were infected with each virus at an MOI of 5 and at 12, 24, 36, and 48 hours post infection the total number of infectious virions was determined. Viral titers represent one of three experiments in which individual numbers varied by less than 2-fold. ................................................... 130

Table 5.1. Viral yields of KOS and gK-truncated mutant viruses. Subconfluent Vero cell monolayers (approximately 8 x 10^5 cells) were infected with each virus at an MOI of 5, and at 12 and 24 h.p.i. the total number of infectious virions were determined as well as the number of infectious virions within cells and extracellular fluids. The ratio of extracellular (OUT) to intracellular (IN) virions at 24 h.p.i. is also shown. The viral yields represent one of three experiments in which individual numbers varied by less than twofold. .... 184
LIST OF FIGURES

Figure 1.1. **Architecture of Herpes Virus Particles.** Cut away diagram of HSV-1 virion particle showing the viral genomic DNA within the core, the icosahedral capsid, the proteinaceous tegument, the lipid bilayer envelope, and the viral glycoproteins. ................................................................. 17

Figure 1.2. **Herpes Simplex Genome Inversion.** A) Schematic representation of the arrangement of the viral genome depicting the unique long (UL) and unique short (US) regions and their prototypical (P) orientations (designated by directional arrows) and flanked by inverted and terminal repeats (IR and TR, respectively). B) IL: Inversion of the UL segment relative to prototypic arrangement. C) IS: Inversion of the US segment relative to prototypic arrangement. D) ISL: Inversion of both the UL and US segments relative to prototypic arrangement. .................................................................................... 21

Figure 1.3. **Replicative Lifecycle of HSV-1.** The stages of the HSV-1 lifecycle are diagrammed as discussed in the text, including attachment, penetration, coordinated sequential transcription, DNA replication, capsid assembly, packaging, maturation, and egress ................................................................. 24

Figure 1.4. **Model of Herpesvirus Attachment and Entry.** The current model of HSV-1 attachment and penetration is illustrated. A) Infectious virions attach in a “loose” association with heparin sulfate proteoglycans found ubiquitous on cell surfaces. B) A “tight” association between the virion and cell occurs as the virion glycoproteins associate with cellular herpes virus entry mediators. C) Viral glycoproteins mediate penetration through a pH independent fusion event of the herpes virus envelope with the cellular plasma membrane depositing the nucleocapsid into the cytoplasm of the cell. D) The nucleocapsid is actively transported to nuclear pores by means of the cytoskeleton. ................................................................. 27

Figure 1.5. **Mediators of Herpesvirus Entry.** The schematic represents the two broad families of herpes virus entry mediating receptors, TNF-like and Immunoglobulin like. ................................................................. 35

Figure 1.6. **Packaging of Unit Length Herpes Virus Genomic DNA.** The depicted model developed by Frenkel *et al* (1976) and is described in the text. A) Proteins attach to components of the a sequence and the empty capsids scan the genomic concatameric DNA until contact with a Uc sequence. B) The capsid is filled with DNA until (C) filled or contact is made with an a sequence in the same orientation as the first a sequence (one unit length of genomic viral DNA). D) Each strand is nicked at the packaging signal on opposite sides of the DR1 sequence. E) The juxtaposition of the a sequences results in their reiteration. ............................... 48
Figure 1.7. **Diagrammatic Representation of Models of Virion Egress.** Cross-section of a eukaryotic cell depicting the nucleus, endoplasmic reticulum, Golgi, and plasma membranes. The two models of virus maturation and egress as discussed in the text are shown. A) Envelopment/De-envelopment model. B) Vesicular Transport model. ............................................................. 53

Figure 1.8. **Synthesis of lipid linked oligosaccharides in the rough endoplasmic reticulum.** Oligosaccharides are assembled on a dolichol lipid intermediate embedded within the membrane of the ER in a step wise fashion prior to an en bloc transfer to a newly synthesized protein as described in the text. ....... 63

Figure 1.9. **Processing of oligosaccharides within the endoplasmic reticulum and Golgi apparatus.** Processing of oligosaccharides is highly ordered and follows a strict pathway that begins within the ER with the removal of three glucose residues. The remaining steps occur within the stacks of the Golgi apparatus as depicted above. Notably, removal of two mannoses by mannosidase II in the medial cisternae renders the bond between the two N-acetylglucosamines resistant to cleavage by Endoglycosidase H. ............. 67

Figure 1.10. **Relative Positions of HSV Glycoproteins in the Genome.** A) The prototypical arrangement of the HSV-1 genome is depicted with the U_L and U_S regions highlighted. B) The relative positions within the viral genome of each of the eleven HSV-1 glycoproteins are shown. ........................................ 76

Figure 1.11. **Predicted primary sequence for HSV-1 (KOS) gK.** The primary amino acid sequence of gK was deduced from the HSV-1 UL53 gene as published by Debroy et al. (1985). Also shown are the relative positions of the signal sequence, sites for N-linked glycosylation, and four putative hydrophobic domains. ........................................................................................ 82

Figure 1.12. **Predictions of hydrophilicity, surface probability, flexibility, and antigenic index for HSV-1 glycoprotein K.** Analysis was performed using the protein analysis tool box of the MacVector software (IBI-Kodak). A) Representation of the hydrophilic profile of gK. B) Prediction of the surface probability of gK. C) Diagram of the relative flexibility of HSV-1 gK. D) Presentation of the antigenic index of gK. ............................................................. 83

Figure 1.13. **Prediction of helix, sheet, and secondary structure from the primary amino acid sequence of gK.** Analysis was performed using the protein analysis tool box of the MacVector software (IBI-Kodak). CF: Chou-Fasman secondary structure predictions. RG: Robson-Garnier secondary structure prediction. Schematic of these results are presented in Fig. 1.14 .............................................................................................................. 85

Figure 1.14. **Schematic model of the predicted secondary structure of gK.** The gK model of Debroy et al (1985) was modified to have three instead of four
 membrane spanning membrane (lines) are shown as embedded within the membrane. Syncytial mutations that map to gK are indicated by asterisks. 86

Figure 2.1. Construction schematic of the plasmid used to transfer the CMV-EGFP gene cassette into the d27-1 viral genome. (a) PCR amplification of the CMV promoter with primers CMVEco and CMVBam. (b) Construction of the CMV-EGFP gene cassette by cloning the CMV promoter upstream of the EGFP gene to create plasmid pGR8000. (c) Elimination of the BamHI site and amplification of the CMV-EGFP gene cassette with primers CMVEcoRI and EGFPBamHI. (d) Insertion of CMV-EGFP gene cassette within the gK gene to create plasmid pTF9201 ............................................. 104

Figure 2.2. Schematic of ΔgK-EGFP virus construction. (a) The top line represents the prototypic arrangement of the HSV-1 genome with the unique long (UL) and unique short (US) regions flanked by the terminal (TR) and internal (IR) repeat regions and marked in map units. (b) The region of the mutant virus HSV-1 d27-1 genome (between map units 0.7 and 0.8) containing the UL52, UL53, and the partially deleted UL54 open reading frames with relevant restriction endonuclease sites. (c) Plasmid pTF9201 (Fig. 1d) containing the CMV-EGFP gene cassette within the gK gene and bracketed by the UL52 and UL54 genes. This plasmid was used to rescue the ICP27-UL54 deletion of virus d27-1 via homologous recombination as shown. Represented on the HSV-1 (d27-1) genome are the relative positions of PCR primers, UL52KpnI2/gKTr2, used to detect the gene insertion. Represented on the pTF9201 schematic are the relative positions of PCR primers CMVEcoRI and EGFPBamHI used to amplify the CMV-IE-EGFP cassette for insertion into the gK gene. .................................................................................. 106

Figure 2.3. Diagnostic PCR of plaque purified EGFP recombinant viruses. Agarose gel electrophoresis of double stranded DNA PCR products with the UL52KpnI2/gKTr2 primer pair (lanes 2, 3) or the UL52KpnI2/EGFPBamHI primer pair (lanes 4, 5) used to detect the EGFP gene insertion within the UL53 (gK) gene. Lane 1: lambda phage DNA digested with HindIII (marker); Lanes 2 and 4: KOS viral DNA; Lanes 3 and 5: ΔgK-EGFP viral DNA; Lane 6: 1 kbp molecular weight ladder marker. .................................109

Figure 2.4. Western immunoblot analysis of recombinant EGFP viruses. Anti-GFP monoclonal antibody was used to detect EGFP expression in virus-infected cell extracts. Lane 1: KOS, lane2: ΔgK-EGFP1, and lane 3: ΔgK-EGFP2. Molecular mass standards are as indicated. ......................... 110

Figure 2.5. Autofluorescence and phase contrast microscopy of EGFP expressing viruses. Vero cells were infected with DgK-EGFP1 and analyzed by either fluorescence microscopy (panels: a, b) or phase contrast microscopy (panels: a', b') at 24 hours post infections (panels: a, a') or 48 hours post infection (panels: b, b'). The same virus plaque is shown in panels a and a' and a different plaque is shown in both panels b and b'.... 112
Figure 2.6. Fluorescent cytometry profiles of KOS and ΔgK-EGFP infected Vero cells. Vero cells were infected with either KOS (unshaded histogram) or ΔgK-EGFP1 (shaded histogram) at a multiplicity of infection of 3. Cytometric profiles were determined at 48 hours post infection using either unfixed infected Vero cells (panel a) or cells fixed with 3% paraformaldehyde (panel b)..............................113

Figure 3.1. Construction of recombinant KOS and DgK viruses constitutively expressing the EGFP gene. (a) The top line represents the prototypic arrangement of the HSV-1 genome indicating approximate map units. (b) The region of the mutant virus HSV-1 d27-1 genome containing the U152, U153, and partially deleted U154 genes with relevant restriction endonuclease sites. (c) Plasmid pTF9201, which contains the CMV-IE-EGFP gene cassette in place of the gK gene (Foster et al, 1998). (d) Plasmid pTF9202 was produced by inserting the gK gene within the unique KpnI/SpeI sites of plasmid pTF9201. The approximate location of PCR primers UL52KpnI2, gKTr2, and CMVBamHI are shown (b-d)..............125

Figure 3.2. Diagnostic PCR of plaque purified KOS/EGFP recombinant virus. DNA PCR products with the UL52KpnI2/gKTr2 primer pair (lanes 2 and 3) or the UL52KpnI2/CMVBamHI primer pair (lanes 4 and 5) used to detect the EGFP gene. Lanes 6-8: PCR diagnostic with primer pair UL52KpnI2/d27-1α used to determine the purity of viral stocks. Lanes 2, 4, and 6: KOS viral DNA. Lanes 3, 5, and 7: KOS/EGFP viral DNA. Lane 1: lambda phage DNA digested with HindIII (marker). Lane 9: 1 kb molecular weight ladder (marker). ...............................................................127

Figure 3.3. Morphology and fluorescence detection of KOS/EGFP virus infections. Vero cells were infected with either KOS (c) or KOS/EGFP virus (a and b) at a multiplicity of infection of 0.001 in 24 well plastic tissue culture plates. Individual viral plaques were photographed through the use of phase-contrast (a and c) and epi-fluorescence (b) microscopy under live conditions at 48 hours post infection. FACS analysis of KOS- and KOS/EGFP-infected cells (MOI of 5) was performed at 48 h p.i. (d) as described previously (Foster et al, 1998). .........................................................128

Figure 3.4. Comparison of the amino acid sequences of HveAs and HveAh. The HveAs (top lines) and HveAh (bottom lines) amino acid sequences were aligned through the use of computer assisted algorithms and visual inspection. Both proteins are 283 amino acids long. Relevant structural features are marked as indicated.................................................................131

Figure 3.5. Detection of HveAs expression in E. coli., Vero, and HveAs-transformed CHO cells using anti-HveAs serum. (a) Immunoblot of electrophoretically separated HveAs/MBP fusion protein after expression in E. coli using anti-HveAs serum produced by genetic immunization. Pre-immune and immune sera were tested as shown. Lanes labeled 1: MBP
control. Lanes labeled 2: MBP/HveAs fusion protein. (b) Vero cells were analyzed by FACS after reaction with either anti-HveAs antibody followed by reaction with anti-mouse-IgG labeled with fluorescein (filled histogram) or with pre-immune sera as the primary antibody (unfilled histogram). (c) CHO-HveAs- (filled histogram) and CHO/sAevH- (unfilled histograms) transformed cells were analyzed by FACS to detect HveAs expression using anti HveAs antibody.

Figure 3.6. Immunoblot detection of HveAs:Fc expression from CHO/HveAs:Fc transformed cells. Supernatants from either HveAs:Fc transformed CHO cells or naive CHO/sAevH control cells were subjected to protein A sepharose purification and prepared for immunoblot analysis. Immunoblots were reacted with goat-anti-mouse HRP reagent. Lane 1: reaction with supernatant concentrate from control CHO cells. Lane 2: reaction with supernatant concentrate from HveAs:Fc-transformed CHO cells.

Figure 3.7. Blocking of virus entry by HveAs:Fc soluble protein and by anti-HveAs antibodies. (A and C-F) CHO/HveAs cells infected with KOS/EGFP virus at an MOI of 10. (A) Untreated control. (B) CHO/sAevH control cells (transformed with the HveAs gene in the non-coding orientation. (C and D) CHO/HveAs cells were pretreated with pre-immune or anti-HveAs serum, respectively for 30 minutes at room temperature prior to infection. (E and F) KOS/EGFP virus was pre-treated with CHO/sAevH control supernates and soluble HveAs:Fc, respectively for 30 minutes at 4°C prior to infections.

Figure 4.1. Domains of gK expressed as fusion products with GST. Schematic diagram depicting the predicted secondary structure of HSV-1 gK as described previously (Foster and Kousoulas, 1999). Domains II and III of gK that were expressed as a fusion with GST are shaded.

Figure 4.2. SDS-PAGE and western analysis of the expression of GST, GST-gKdomain2 and GST-gKdomain3 proteins. Bacterial lysates were separated on an SDS-PAGE and either A) stained with Gel-Code blue to visualize protein bands or B) electrotransferred to nitrocellulose for immunoblotting with anti-GST antibody. Lane 1: GST expression; Lane 2: GST-gKdomain2; Lane 3: GST-gKdomain3; M: Low molecular weight marker.

Figure 4.3. SDS-PAGE and western analysis of the purification of GST-gKdomain2 and GST-gKdomain3 inclusion bodies. Purified GST-gKdomain2 and GST-gKdomain3 were separated on an SDS-PAGE and proteins were either A) visualized by staining with Gel-CODE or B) electrotransferred to nitrocellulose for western analysis using anti-GST antibody. Lane 1: GST-gKdomain2; Lane 2: GST-gKdomain3.
Figure 4.4. Detection of HSV-1 gK as a structural component of purified virions. Purified HSV-1 KOS or ΔgK virions were normalized to gD protein content and separated on an SDS-PAGE. Proteins were electrophoresed to nitrocellulose and probed with anti-GSTgKdomain2 and anti-GSTgKdomain3 sera pools.

Figure 4.5. Kinetics of HSV-1 ΔgK (Vero), ΔgK (VK302), and KOS virus entry into Vero (VK302) cells. Classical low pH inactivation of extracellular virions was employed to determine entry kinetics at 34°C. All experiments were done in triplicate.

Figure 4.6. Entry of ΔgK/EGFP (Vero), ΔgK/EGFP (VK302), and KOS/EGFP virus into Vero cells. Vero cells were infected with KOS/EGFP (A), ΔgK/EGFP propagated on Vero cells (B) or ΔgK/EGFP propagated on VK302 cells (C) at an MOI of 10 and visualized under fluorescent microscopy at 12 hours post infection.

Figure 4.7. KOS/EGFP and ΔgK/EGFP entry into CHO cells transformed with Hve receptors. CHO-K1 cells transformed with HveAs (panels A, B, C), sAevH (opposite orientation of HveA (panel D), HveB (panel E), HveAh (panel F) or HveC (panel G) were infected with EGFP expressing viruses and analyzed for entry 12 h.p.i. Panel A: KOS/EGFP infected cells. Panel B: ΔgK/EGFP virus propagated on gK null complementing VK302 cells. Panels C-G: ΔgK/EGFP virus propagated on non-complementing Vero cells.

Figure 4.8. Polyethylene glycol mediated penetration of gK null viruses into CHO cell-lines expressing Hve receptors. 5 PFU/cell KOS/EGFP (A) or ΔgK/EGFP (B and C) were absorbed at 4°C for 2 hours on HveAs (A and B) or HveAh (C) transformed CHO cell-lines. Following adsorption, virus was treated with PEG to mediate entry.

Figure 4.9. ΔgK/EGFP entry into HveAs/HveAh and HveAh/HveAs chimeric receptors. HveAh amino terminal/HveAs carboxyl terminal chimera (representative in panel A), HveAs amino terminal/ HveAh carboxyl terminal chimera (representative in panel B), HveAh signal/ HveAs chimeric protein (panel C) or HveAs signal/ HveAh chimeric protein (panel D) expressing CHO cell-lines were infected with ΔgK/EGFP at an MOI of 10. Cells were visualized by fluorescent microscopy 24 hours post infection.

Figure 5.1. Strategy for the isolation and PCR detection of HSV-1 mutants specifying gK truncations. (a) The top line represents the prototypic arrangement of the HSV-1 genome with the unique long (UL) and unique short (US) regions flanked by the terminal (TR) and internal repeat (IR) regions. Shown below is the region of the mutant virus HSV-1 d27-1 genome (between map units of 0.7 and 0.8) containing the UL52, UL53 and the partially deleted UL54 open reading frames with relevant restriction.
endonuclease sites. (b) Plasmid constructs containing each of the truncated gK genes used to generate the ΔAgKhpd-1, -2, -3, and -4 mutant viruses. Represented on the HSV-1 (KOS) genome are the relative positions of PCR primers, UL52KpnI/gKTir, used to detect the truncated gK genes. The hatched segments represent the portions of genes that are expressed after truncation, while segments 3' to the TGA stop codon are portions of the gK genes that are deleted.

Figure 5.2. Diagnostic PCR of recombinant viruses specifying truncations in gK. (a) Agarose gel electrophoresis of ds DNA PCR products with the UL52KpnI/gKTir primer pair used to detect the truncated gK genes. Lane 1: lambda phage DNA digested with HindIII (marker). Lanes 2, 3, 4, 5, and 6: Viral DNA of ΔAgKhpd-1, ΔAgKhpd-2, ΔAgKhpd-3, ΔAgKhpd-4, and KOS viruses, respectively, amplified with PCR primer pair UL52KpnI/gKTir. Lane 7: Molecular mass marker (1-kbp ladder). (b) Agarose gel electrophoresis of double stranded DNA PCR products with the UL52KpnI/d27-1α primer pair used to confirm the purity of the recombinant viruses after extensive plaque purification. Lane 1: lambda phage DNA digested with HindIII (marker). Lanes 2, 3, 4, 5, 6 and 7: Viral DNA of ΔAgKhpd-1, ΔAgKhpd-2, ΔAgKhpd-3, ΔAgKhpd-4, d27-1 and KOS viruses, respectively, amplified with PCR primer pair UL52KpnI and d27-1α. Lane 8: Molecular mass marker (1-kbp ladder).

Figure 5.3. Plaque morphology of KOS, ΔgK, ΔAgKhpd-1, -2, -3, and -4 on Vero cells. Panels A: ΔgK; B: ΔAgKhpd-1; C: ΔAgKhpd-2; D: ΔAgKhpd-3; E: ΔAgKhpd-4; F: KOS. The cells were infected at an MOI of 0.01 PFU/cell and photographed using a phase-contrast microscope at 48 h.p.i.

Figure 5.4. Electron micrographs of Vero cells infected with the ΔAgKhpd-1 mutant virus. Subconfluent Vero cell monolayers were infected at an MOI of 5 PFU/cell, incubated at 37°C for 36 hours and prepared for electron microscopy. The solid arrows in panels C and D mark nucleocapsids. The open arrows in panel C mark the outer and inner nuclear membranes in the cytoplasmic (c) and nuclear (n) compartments. The arrowheads mark membranes surrounding nucleocapsids within the perinuclear space in panels C and D. The scale bar = 0.5 μm for all panels.

Figure 5.5. Electron micrographs of Vero cells infected with different gK mutant viruses. Subconfluent Vero cells were infected with ΔgK virus (panel A1 and A2), ΔAgKhpd-2 (panels B1 and B2), ΔAgKhpd-3 (panels C1 and C2), ΔAgKhpd-4 (panels D1 and D2), and KOS (panels E1 and E2). All cells were infected at an MOI of 5 PFU/cell, incubated at 37°C for 36 hours and prepared for electron microscopy. The arrowheads in panels A2 and B2
mark enveloped virions within cytoplasmic vacuoles. The open arrows mark extracellular virions in panels C1, D1, E1, and E2. The solid arrows mark enveloped virions within electron dense vesicles in panels C2, D2, and E2. The scale bar = 0.5 μm for all panels.

Figure 5.6. Alignment of gK amino acid sequences specified by alphaherpesviruses. Alignment was performed using the MultiAlign Program (Corpet, 1988). Hydrophobic domains (signal peptide, hpd1, hpd2, hpd3, and hpd4) are presented as lightly shadowed. Dark shadowed areas contain conserved amino acid motifs. YXXΦ is a tyrosine-based motif known to function in vesicular transport of membrane embedded glycoproteins. X denotes any amino acid and Φ denotes a bulky hydrophobic amino acid. CXXCC is a cysteine-rich motif. The last line depicts the consensus gK sequence with conserved residues indicated by capital letters. $ depicts either L or M amino acids. % depicts either F or Y residues. # is anyone of D or N. Amino acid residues I or V are depicted with an (!) marking.

Figure 5.7. Plaque morphology of gK-mutant viruses with amino acid changes within conserved gK motifs. Either Vero cells (panels A, B, C, E, and G) or VK302 cells (panels D, F, and H) were infected at an MOI of 0.01 PFU/cell and photographed using a phase contrast microscope at 48 h.p.i. Panels A: KOS; B: gK/C269S; C and D: gK/C304S-C307S; E and F: gK/Y183S; G and H: ΔgK.

Figure 5.8. Schematic model of the predicted secondary structure of gK. The gK model of Debroy et al (1985) was modified to have three instead of four membrane spanning domains as suggested by Mo and Holland (1998), and as predicted by computer-based predictions using the PSORT (Nakai et al, 1992), Tmpred (Hoffman et al, 1993), and SOSUI (Hirokawa et al, 1999) algorithms. The predicted putative hydrophobic domains (hpd) (lightly shaded circles) of gK which transverse the membrane (lines) are shown as embedded within the membrane. The arrows indicate the termination sites for truncated gK specified by the designated viruses. Asterisks mark syncytial mutations. Amino acid motifs that are conserved among alphaherpesviruses are contained within shaded oval-shaped areas. The darkly shaded circles represent the signal peptide.
ABSTRACT

Herpes simplex virus type 1 (HSV-1) specifies at least 11 glycoproteins that are expressed in infected cells and in virions. HSV-1 enters into cells via fusion of the viral envelope with cellular membranes and can spread to adjacent cells via fusion of cellular membranes. Glycoproteins gB, gD, gH, and gL are known to be essential for virus entry, virus spread, and virus induced cell-to-cell fusion. The majority of spontaneous single amino acid changes that cause extensive cell fusion map within the Ul53 gene encoding glycoprotein K (gK); however, the role of gK in virus entry and virus egress had not been defined. Specific antibodies against portions of gK expressed in E. coli were raised in mice and detected gK as a 40-kDa protein in purified virions. To investigate the effect of gK in virus entry via the known HSV-1 HveAh and HveCh receptors, two recombinant viruses were constructed, KOS/EGFP (wild-type) and ΔgK/EGFP (gK null) expressing the enhanced green fluorescent protein (EGFP) constitutively. The simian homolog of HveA (HveAs) was cloned, sequenced, and CHO/HveAs transformed cell-lines were isolated. The ΔgK/EGFP virus failed to enter into CHO/HveAs, while ΔgK/EGFP prepared in complementing Vero (VK302) cells entered efficiently. In contrast, ΔgK/EGFP entered efficiently into CHO/HveAh and CHO/HveCh cells. A series of hybrid receptors were made expressing different portions of HveAh and HveAs and were used to map a short region of HveAh that conferred receptor-mediated entry to an HveAs receptor background. The role of gK in virion morphogenesis and egress was addressed by constructing a panel of recombinant viruses expressing gK carboxyl-terminal truncations and single amino acid
substitutions. Two cellular vesicular transport motifs, a cysteine-rich motif and a tyrosine-based motif were found to be essential for infectious virus production, intracellular virion transport, and virus egress. Therefore, gK may interact with vesicular transport pathways during virion morphogenesis and egress. The overall results from this research suggest that HSV-1 gK is a multifunctional protein involved in receptor utilization during virus entry and transport of virion particles to extracellular spaces through cellular vesicular transport pathways during virus egress.
CHAPTER I
INTRODUCTION
STATEMENT OF RESEARCH PROBLEM AND HYPOTHESIS

Herpes Simplex Virus type I (HSV-1) is a neurotropic and neurovirulent virus that is a causative agent of cold sores, genital lesions, corneal blindness, and viral encephalitis (Whitley et al., 1997). Although herpes viruses have been an area of intense study, some of the basic mechanisms involved in pathogenesis remain unsolved. There is not a clear understanding of virus attachment, entry, intracellular virion transport, envelopment or egress.

HSV-1 specifies at least 11 glycoproteins: gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, and gM, which are expressed during productive virus replication. These glycoproteins function during virus entry, cell-to-cell spread, and egress of infectious virion particles (Roizman and Sears, 1996; Spear, 1993a; Spear, 1993b). Syncytial mutations (syn) that cause extensive virus-induced cell fusion can arise in at least 4 different regions of the viral genome including the Ul20 gene (Baines et al., 1991; MacLean et al., 1991); the Ul24 gene (Jacobson et al., 1989; Sanders et al., 1982); the Ul27 gene encoding glycoprotein B (gB) (Bzik et al., 1984; Pellett et al., 1985) and the Ul53 gene coding for glycoprotein K (gK) (Bond and Person, 1984; Debroy et al., 1985; Pogue-Geile et al., 1984; Ryechan et al., 1979). Syncytial mutations in the Ul53 (gK) gene are more frequently isolated than syncytial mutations in any other genes (Bond and Person, 1984; Bond et al., 1982; Debroy et al., 1985; Dolter et al., 1994; Pogue-Geile et al., 1984; Pogue-Geile and Spear, 1987; Read et al., 1980; Ryechan et al., 1979).
Recently, it was shown that gK is involved in infectious virus production and virion egress (Jayachandra et al., 1997). Furthermore, extrapolation of data from other mutant viruses suggests that gK may also function in virus entry. Therefore, the hypothesis for these investigations is HSV-1 glycoprotein K is a regulator of multiple membrane fusion events during HSV-1 virus entry, intracellular virion transport, and cellular egress.

STATEMENT OF RESEARCH OBJECTIVES

It was the goal of this research to develop an understanding of the structure and function of HSV-1 gK in the herpesvirus lifecycle by investigating the effects of specific mutations and deletions on both virus entry and egress. The overall research objectives were divided into two main categories. Each of these main objectives was then subdivided into specific research aims, which were designed to address the two main objectives. The specific research aims were:

I. The role of HSV-1 gK in virus entry.

1. To generate a panel of recombinant viruses that express an indicator cassette in order to facilitate the monitoring of successful viral entry into permissive cells.

2. To generate and characterize Chinese hamster ovary (CHO) cell-lines that express the African Green Monkey Kidney (Vero) cells HveA receptor homolog.

3. To determine if HSV-1 glycoprotein K is a structural component of the virion.
4. To determine the role of HSV-1 glycoprotein K in receptor utilization.

II. Characterization of the role of HSV-1 gK in virus egress.

1. To analyze the role of domains of HSV-1 gK in virus egress.

2. To determine if conserved amino acid motifs within gK are essential for HSV-1 gK function.

The results from this research suggest that HSV-1 gK is a multifunctional protein involved in differential receptor utilization and transport of virion particles to extracellular spaces. The results from these examinations are presented as four individual chapters following a review of the current literature in herpes virology. The chapters are presented as follows:

1. Expression of the Enhanced Green Fluorescent Protein by Herpes Simplex Virus Type 1 (HSV-1) as an in Vitro or in Vivo Marker for Virus Entry and Replication.


3. Herpes Simplex Virus Type 1 (HSV-1) Glycoprotein K (gK) is a Structural Component of Purified Virions that Functions to Modulate HveAs Receptor Mediated Virus Entry.

4. Genetic Analysis of the Role of Herpes Simplex Virus 1 (HSV-1) Glycoprotein K (gK) in Infectious Virus Production and Egress.
LITERATURE REVIEW

Historical Perspective of Herpesviruses

The first written documentation of human herpes infections dates back to ancient Greece (Nahmias and Dowdle, 1968). The herpesviruses derive their name from the Greek word, ερπετ, meaning "creep", which Hippocrates used to describe the characteristic spread of the cutaneous lesions (Wildy, 1973). However, throughout history the term herpes was used to describe a number of skin ailments and diseases, not all of which were associated with herpes infections. Astruc, the 18th century physician to the king of France, was first to draw a correlation between herpetic lesions and genital infections (Beswick, 1962; Hutfield, 1966). But it was not until 1893 that Vidal recognized the transmission of herpes infections between individuals.

The beginning of the twentieth century brought several advances in the recognition of the etiology of herpetic lesions. First, in 1896 Unna described giant multinucleated cells associated with all known herpes infections (Unna, 1896). Second, Lownstein (1919) demonstrated the infectious nature of fluid from herpes keratitis and herpes labialis by inoculating the rabbit cornea. Third, the presence of neutralizing antibodies in the serum of infected patients was demonstrated by Andrews and Carmichael (1930). Paradoxically, only patients with neutralizing antibodies developed recurrent lesions, albeit less severe.

Although originally proposed by Lipschitz in 1921, it was not until the 1960's when the existence of two distinct antigenic herpes simplex types, HSV type 1 (HSV-1) and HSV type 2 (HSV-2) was discovered, each being shown to correlate
to particular anatomical sites (Lipschitz, 1921). Nahmias and Dowdle (1968) demonstrated that HSV-1 was more frequently associated with non-genital infections, while HSV-2 was associated most commonly with genital disease. Since these initial characterizations, the biological properties of herpesviruses have been an area of intense study.

**Taxonomy of *Herpesviridae***

The viruses which comprise the family *Herpesviridae* share a number of common structural features which will be described in detail in subsequent sections, but include: 1) a core wound around which is the linear double stranded DNA genome; 2) an icosadeltahedral capsid; 3) a proteinacious tegument; and 4) a cell derived lipid envelope (Wildy *et al.*, 1960; Roizman and Furlong, 1974). Although these structural features aid in the inclusion of virions into the family *Herpesviridae*, the various herpesviruses cannot be differentiated from one another by electron microscopic examination. Instead, viruses are differentiated on the basis of biological properties, number of base pairs in the genome, genome arrangement, immunological specificity, cell tropism and replication characteristics (Roizman, 1982).

The nomenclature, which classifies the herpesviruses, was defined by the Herpesvirus Study Group of the International Committee on the Taxonomy of Viruses (Roizman *et al.*, 1981). Because of the extensive diversity of the various herpesviruses, the viruses have been subdivided into three subfamilies: 1) *Alphaherpesvirinae*-rapidly replicating, highly cytolytic herpesviruses; 2)
Betaherpesvirinae—slowly replicating, cytomegalic herpesviruses; and 3) Gammap herpesvirinae—lymphocyte associated herpesviruses (Roizman et al., 1973; Roizman, 1982). Although these divisions were formulated on the basis of biological properties, such as, cell tropism or replication, they do not represent an accurate grouping of the relatedness of either the characteristics of the viral genome or the structural organization of the viral genome (Bomkamm et al., 1976; Raab-Traub et al., 1980). Thus, each subfamily has been further divided into genera, which reflects the phylogenetic relatedness of each virus. This relatedness is based on several factors including the genomic structure, sequence homology, and serological relationships (Roizman, 1982). A partial listing of selected herpesviruses and their classifications is represented in table 1.1.

Herpesviruses are widely distributed in nature and have been found to infect most animal species. Of the more than eighty distinct herpesviruses described in the past fifty years, eight have been identified as known human pathogens (highlighted in bold in Table 1) and have been implicated in a number of disease states discussed in the subsequent section. Despite the diversity of the family Herpesviridae, the alphaherpesvirus, human herpesvirus 1 (HHV-1) (also known as herpes simplex virus type 1 [HSV-1]) is the prototypical herpesvirus. Its close relatedness to a number of other important human and animal alphaherpesvirus pathogens make it a fairly accurate model for studying alphaherpesvirus replication, and thus it is the focus of this thesis.
<table>
<thead>
<tr>
<th>Subfamily</th>
<th>Provisional Designation</th>
<th>Common Names</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alphaherpesvirinae</strong></td>
<td><strong>Human herpesvirus 1</strong></td>
<td>Herpes simplex virus type 1</td>
</tr>
<tr>
<td></td>
<td><strong>Human herpesvirus 2</strong></td>
<td>Herpes simplex virus type 2</td>
</tr>
<tr>
<td></td>
<td><strong>Human herpesvirus 3</strong></td>
<td>Varicella-zoster virus</td>
</tr>
<tr>
<td></td>
<td><strong>Cercopithecine herpesvirus 1</strong></td>
<td>Herpesvirus B; Simian herpesvirus</td>
</tr>
<tr>
<td></td>
<td><strong>Cercopithecine herpesvirus 2</strong></td>
<td>Infectious bovine rhinotracheitis virus</td>
</tr>
<tr>
<td></td>
<td><strong>Bovine herpesvirus 1</strong></td>
<td>Bovine mamillitis virus</td>
</tr>
<tr>
<td></td>
<td><strong>Bovine herpesvirus 2</strong></td>
<td>Dog herpesvirus</td>
</tr>
<tr>
<td></td>
<td><strong>Canid herpesvirus 1</strong></td>
<td>Equine rhinopneumonitis virus; Equine abortion virus</td>
</tr>
<tr>
<td></td>
<td><strong>Equid herpesvirus 1</strong></td>
<td>Infectious laryngotracheitis virus</td>
</tr>
<tr>
<td></td>
<td><strong>Gallid herpesvirus 1</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Suid herpesvirus 1</strong></td>
<td>Pseudorabies virus; Aujeszky’s disease virus</td>
</tr>
<tr>
<td></td>
<td><strong>Felid herpesvirus 1</strong></td>
<td>Cat herpesvirus; Infectious rhinotracheitis virus</td>
</tr>
<tr>
<td></td>
<td><strong>Ictalurid herpesvirus 1</strong></td>
<td>Channel catfish virus</td>
</tr>
<tr>
<td><strong>Betaherpesvirinae</strong></td>
<td><strong>Human herpesvirus 5</strong></td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td></td>
<td><strong>Cercopithecine herpesvirus 8</strong></td>
<td>Rhesus cytomegalovirus</td>
</tr>
<tr>
<td></td>
<td><strong>Equid herpesvirus 2</strong></td>
<td>Slow growing-cytomegalovirus-like equine virus</td>
</tr>
<tr>
<td></td>
<td><strong>Suid herpesvirus 2</strong></td>
<td>Inclusion body rhinitis virus; Pig cytomegalovirus</td>
</tr>
<tr>
<td></td>
<td><strong>Murid herpesvirus 1</strong></td>
<td>Murine cytomegalovirus</td>
</tr>
<tr>
<td></td>
<td><strong>Murid herpesvirus 2</strong></td>
<td>Rat cytomegalovirus</td>
</tr>
<tr>
<td></td>
<td><strong>Felid herpesvirus 2</strong></td>
<td>Cat cytomegalovirus</td>
</tr>
<tr>
<td></td>
<td><strong>Human herpesvirus 6</strong></td>
<td>Roseolovirus</td>
</tr>
<tr>
<td></td>
<td><strong>Human herpesvirus 7</strong></td>
<td>Unclassified β virus</td>
</tr>
<tr>
<td><strong>Gammaherpesvirinae</strong></td>
<td><strong>Human herpesvirus 4</strong></td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td></td>
<td><strong>Human herpesvirus 8</strong></td>
<td>Kaposi-sarcoma herpesvirus</td>
</tr>
<tr>
<td></td>
<td><strong>Cercopithecine herpesvirus 10</strong></td>
<td>Rhesus leukocyte-associated herpesvirus I</td>
</tr>
<tr>
<td></td>
<td><strong>Gallid herpesvirus 2</strong></td>
<td>Marek's disease herpesvirus</td>
</tr>
</tbody>
</table>
Clinical Significance of Herpes Simplex Viruses

Herpes simplex viruses are distributed worldwide in both developed and undeveloped countries (Black, 1975). Animal vectors for human herpesviruses have not been identified; therefore, man remains the only known reservoir for viral transmission to other humans. Because virus infection is only very rarely fatal and because the virus remains latent with episodes of reactivation, the propensity for those infected to spread the virus is quite high (Whitley et al, 1997). The associated disease of herpes simplex infections ranges from very mild, clinically undetectable vesicular lesions at the site of reactivation or primary infection to a sporadic, severe, and sometimes life-threatening disease. Historically, herpes simplex viruses have garnered much biological, but little clinical interest. However, because of the social significance and the pain that can be associated with the ulcerative lesions, there has recently been a resurgence of investigation into possible treatments (Whitley, 1982). This has been further advanced by a more detailed understanding of the biological and structural properties of the virus, especially with regard to virus-host interactions.

Epidemiology: Herpesviruses are distributed throughout the world with more than one-third of the world's population exhibiting recurrent HSV infections; therefore generating the capacity for transmission to susceptible individuals during episodes of a productive infection. There are many factors that influence the prevalence of infection including geographic location, race, gender, socioeconomic status, and age (Dodd et al, 1938; Rawls and Campione-Piccardo, 198; Scott et al, 1941; Scott, 1957). For those individuals in developing countries or from lower
socioeconomic populations, approximately one-third of the children seroconvert by five years of age and 70-80% of the population converts by adolescence (Rawls and Campione-Piccardo, 1981). Middle class children five years of age and below have a prevalence of seroconversion of 20%, with no significant increase until their twenties or thirties, when the prevalence increases to 40-60%. University students seroconvert at about 5-10% annually (DeGiordano et al., 1970; Dunkle et al., 1979; Whitley et al., 1997).

Caucasians and African-Americans within the United States vary significantly for the prevalence of HSV-1 infections; however, by forty years of age, the differences are negligible (DeGiordano et al., 1970; Juretic et al., 1966). By the age of 5, >35% of African Americans relative to 18% of Caucasians are infected by HSV-1 (Rawls et al., 1969; Nahmias et al., 1970a; Nahmias et al., 1970b). Throughout adolescence, the difference remains approximately two-fold higher for African Americans than for Caucasians (Whitley et al., 1997).

Because HSV-2 is generally acquired through intimate sexual contact, seroconversion is rarely found before the onset of sexual activity. The number of cases of HSV-2 infections is estimated at over 500,000 cases annually, with approximately 40 to 60 million Americans latently infected with HSV-2 (Nahmias et al., 1973; Magder et al., 1989). The seroprevalence of HSV-2 infections is 10% by 29 years of age and 35% by 60 years of age. While race (more African Americans than Caucasians), gender (more females than males), marital status (more divorced than single or married), sexual preference (more homosexuals than heterosexuals), and residence (more city residents than suburbs) all are important factors which
influence acquisition of HSV-2, the best indicator and correlate of prevalence is the
number of sexual partners of an individual (Whitley et al, 1997). Although much
less prevalent, the incidence of genital HSV-1 infections has steadily increased over
recent years (Nahmias, 1990). HSV-1 genital infections, however, are much less
clinically severe and are not prone to reactivation (Whitley et al, 1997).

Pathogenesis: Virus is transmitted from infected to susceptible individuals
(those individuals who are HSV seronegative) during close personal contact
developing a primary infection. A recurrent infection occurs following reactivation
within a latently infected individual and clinically is usually less severe than the
primary infection. An initial infection occurs in individuals with preexisting
antibodies to one type of HSV experiences a first infection with the opposite virus
type (i.e. A latently infected HSV-1 infected individual is exposed to HSV-2 for the
first time). Because of the cross-reactivity of many HSV antibodies, an initial
infection is generally less severe than would be a primary infection of the same type
(Whitley et al, 1997).

Histopathologically, HSV infections reflect the cytopathological changes
initiated by the virus, as well as, the associated inflammatory response to the
infection. Viral infection produces large inflated cells, condensation of the
chromatin, and degeneration of nuclei within the basolateral and intermediate cells
of the epithelium (Roizman and Sears, 1996; Whitley and Gnann, 1993). Upon cell
lysis, a vesicular fluid containing large numbers of infectious virus particles is
generated between the epidermal and dermal layers. The fluid also contains lysed
cellular debris, multinucleated fused cells, and inflammatory cells. Because of the
recruitment of inflammatory cells to the lesion sites, the vesicular fluid becomes pustular and eventually scabs. In mucosal tissue areas, the vesicles are replaced with ulcerative lesions (Whitley et al, 1997).

Following a primary infection, the capsid is transported retrograde by neurons to the dorsal root ganglia where latency is established. Despite replication of virus causing disease and much less frequently encephalitis, the predominant host-virus interaction in herpes infections leads to the establishment of latency. Following the establishment of latency, external stimuli such as stress or UV light, can cause reactivation of the virus from its latent state, during which the virus particles are anterogradely transported along peripheral sensory nerves back to the initial site of infection at epithelial surfaces (Roizman and Sears, 1996; Whitley et al, 1997).

**Mucocutaneous Infections**: Although herpesviruses generate a spectrum of disease states, asymptomatic infections are the rule rather than the exception (Whitley and Gnann, 1993). Incubation periods range from 2 to 12 days, with a mean of 4 days. Primary infections shed virus for up to 23 days, with a mean of 7-10 days and are characterized by fever and intraoral gingival ulceration, but may also present pharyngitis and mononucleosis-like syndromes (Glezen et al, 1975). Lip associated ulcerative lesions are indicative of a recurrent infection.

Recurrent orolabial lesions are characterized by pain, burning, itching, and the appearance of three to five vesicles within the first six hours of onset and persist for only 48 hours. These lesions progress to an ulcerative stage within 72-96 hours, which resolves within 8-10 days. The frequency of recurrence is variable within an
individual and is dependent on several factors (Spruance et al, 1977). Other cutaneous HSV-1 lesions are less common and are generally either manifested as localized eczema herpeticum or as a disseminated Kaposi’s varicella-like eruption. HSV lesions of either type can trigger erythema multiforme (Whitley and Gnann, 1993; Whitley et al, 1997).

Genital herpetic lesions are more severe due to systemic complications. Primary infections are manifested as macules and papules, and are accompanied by fever, dysuria, malaise, and inguinal adenopathy. Systemic complications occur in 70% of patients and are characterized by excruciating pain, aseptic meningitis, neuralgias, and meningoencephalitis. The more severe the primary infection, the more likely the severity and frequency of recurrent infections; however, recurrent infections usually consist of a limited number of vesicular lesions. While the frequency of recurrence varies among individuals, about one-third of the patients have recurrences in excess of eight per year, one-third with 2-3 per year, and the other one-third will have between 4-7 recurrences per year (Corey, 1982; Corey et al, 1983). Although these numbers are for clinical recurrences, virus can be shed while asymptomatic and is readily detectable in genital secretions by PCR at a high frequency between clinical recurrences (Mertz et al, 1992).

**Fetal and Neonatal Infections:** Genital HSV infections can rarely become disseminated during pregnancy and result in necrotizing hepatitis, thrombocytopenia, encephalitis, and in greater than 50% of the cases the fetus does not survive. There is also a greater than 50% mortality for women, which acquire the infection during term. Primary and initial maternal genital infections pose the
greatest risk of dissemination. Therefore, identification of couples for which the mother is seronegative for HSV-2 and her partner is HSV-2 seropositive is essential (Whitley et al., 1997).

Nearly one in every 2,000 to one in every 5,000 deliveries results in neonatal HSV infection each year (Nahmias et al., 1983; Nahmias et al., 1989; Sullivan-Bolyai et al., 1983). Transmission to the neonate from the mother can occur via three routes: 1) in rare cases through in utero infection; 2) in 75 to 80% of the cases through intrapartum contact of the fetus with maternal genital secretions; 3) through postnatal acquisition from relatives, hospital personal, etc. containing orolabial herpetic lesions. Neonatal infections are invariably symptomatic characterized by a triad of skin vesicles, scarring, eye infections, microencephaly, hydraencephaly and frequently death. Herpetic neonatal disease can be divided into the following three categories: 1) Localized disease with lesions in the skin, eye, and mouth occurs in 40% of infected neonates and presents at approximately 10 to 11 days after delivery. Recurrences are frequent and common, regardless of the use of anti-viral therapies; however, if no anti-viral therapies are delivered, nearly 30% will progress to neurological impairment. 2) Encephalitis occurs in 35% of neonates. Virus is readily cultured from cerebral spinal fluid (CSF). Clinical manifestations of encephalitis include tremors, seizures, fever and/or temperature instability, pyramidal tract signs, poor feeding, lethargy, irritability, and bulging fontanelle. 3) In 25% of neonates there is a severe disseminated infection involving multiple organs, the central nervous system (CNS), lungs, skin, and eyes. Encephalitis is a common component of a disseminated infection occurring in 60-75% of patients.
Mortality exceeds 80%; however, survivors are usually both mentally and physically impaired (Whitley et al, 1988; Whitley et al, 1997).

**Keratoconjunctivitis:** Herpes infections of the eye are the second leading infectious cause of corneal blindness in the world. Approximately 300,000 cases of HSV infections in the eye are diagnosed annually with the majority of these occurring in children through neonatal transfer (Binder, 1976; Ostler, 1977; Scott, 1957). Herpetic keratoconjunctivitis may cause tearing, photophobia, eyelid edema, dendritic lesions, and geographic ulceration of the cornea. Recurrent infections are usually unilateral; however, a small percentage is bilateral (Whitley and Gnann, 1993; Whitley et al, 1997).

**Immunocompromised Host:** Immunocompromised or immunosuppressed patients have a greatly increased risk of severe recurrent herpetic infection (Logan et al, 1971; Muller et al, 1972; Pass et al, 1978; Whitley et al, 1984). Organs and tissues normally not involved in recurrent or primary infections may be afflicted including the respiratory tract, esophagus, and gastrointestinal tract (Montgomerie et al, 1969; Korsager et al, 1975). Furthermore, acyclovir or other drug resistant strains of HSV readily evolve within these individuals (Erlich et al, 1989).

**Central Nervous System (CNS) Infections:** CNS infections by HSV which lead to encephalitis is the most devastating of all infections. HSV is the most common cause of sporadic fatal encephalitis, with over 70% of untreated cases being fatal and only 2.5% of survivors regaining normal neurological function (Ward and Roizman, 1994).
Prevention and Treatment: While vaccination against viral infections remains the ideal method for prevention, HSV presents unique problems because of its ability to recur in the presence of both humoral and cellular immunity. Vaccines containing subunit glycoprotein components, which induce strong neutralizing antibody responses, have been extensively studied. In phase two clinical trials, a subunit vaccine to glycoprotein D (gD) demonstrated that the number of culture positive episodes could be reduced by one-third. However, no degree of protection to seronegative sexual partners of persons with known HSV-2 infection was demonstrated during phase three clinical trials. Thus, due to the unsuccessfulness of subunit vaccines, they have been largely abandoned.

Alternative approaches to subunit vaccines that are safe and efficacious are an area of intense study. The popularity of genetic immunization has spurred a new interest in HSV vaccine generation. Recently, genetic immunization against viral glycoproteins has begun phase one clinical trials. While having some advantages over subunit vaccines, naked DNA genetic immunization still has the problem of only representing a limited number of antigens of the virus. Therefore, modified attenuated live vaccines, which express a large repertoire of the viral genome, have also been studied. Until a vaccine has been proven effective, education and prophylaxis are the only prevention. For those that have already acquired herpes infections, there is no known cure for the infection.

Treatment is based on nucleotide analog drugs that become active only in the presence of herpes infections and are based largely on the synthetic acyclic purine nucleoside analog, acyclovir (Whitley and Gnann, 1993). Treatment with acyclovir
or derivatives thereof, including valacyclovir and famciclovir, is associated with very few adverse side effects. Furthermore, no increased risk to mothers or fetuses is associated with these drugs during pregnancy (Andrews et al, 1988). Initial infections can be treated through topical, oral, or intravenous delivery methods. Long-term preventive therapy includes daily oral doses of acyclovir in order to suppress reactivation and severity of the recurrent lesions (Whitley et al, 1997).

Architecture of the Herpes Virion

Inclusion of viruses into the family Herpesviridae is based largely on the architecture of the virion. The herpes virion particle varies in size from 120nm to 300nm in diameter and is comprised of four concentric layers: 1) a cylindrical core around which the linear double stranded viral genome is wound, 2) an icosadeltahedral capsid, 3) an electron dense proteinacious tegument, 4) a lipid bilayer envelope (Figure 1.1) (Roizman and Furlong, 1974; Roizman and Sears, 1996; Wildy et al, 1960).

The core: The innermost core contains the linear double-stranded DNA genome wound in the form of a torus and seemingly suspended by spindles protruding from the underlying walls of the capsid (Figure 1.1) (Roizman and Sears, 1996).

The capsid: The capsid is a protein shell that surrounds and protects the viral genome (Figure 1.1). It consists of 162 capsomeres arranged into an icosadeltahedran approximately 100nm in diameter and 15nm in thickness. HSV-1 infected cells contain three major capsid types, which can be separated by
Figure 1.1. Architecture of Herpes Virus Particles. Cut away diagram of HSV-1 virion particle showing the viral genomic DNA within the core, the icosahedral capsid, the proteinacious tegument, the lipid bilayer envelope, and the viral glycoproteins.
density gradient sedimentation and differentiated by electron microscopy. These major capsid types correspond to the degree to which DNA has been packaged and have been designated “type A” (empty), “type B” (intermediate), and “type C” (full) capsids (Gibson and Roizman, 1972; Gibson and Roizman, 1974). The “A” capsids consists of four viral proteins, viral protein 5 (VP5), VP19c, VP23, and VP26, but are devoid of DNA and lack the internal torroid structure. The major capsid protein VP5 is a component of both the 150 hexameric capsomeres and the 12 pentameric capsomeres (Heine et al, 1974; Schrag et al, 1989). VP19c is disulfide bonded to VP5 in an equimolar ratio and has been shown to bind to viral DNA, suggesting that it may play some role in anchoring the viral genome in the capsid (Braun et al, 1984). Studies have suggested that the type A capsids are not in the pathway of capsid maturation and may actually be a decay product (Sherman and Bachenheimer, 1988). Type B capsids like type A capsids are devoid of DNA, but they differ in that type B capsids contain three additional proteins: VP21, VP22a, and VP24. VP21 and VP22a have been shown to be internal capsid proteins, while VP19 and VP23 are on the surface of capsids and may form intercapsomeric fibers located between each of the capsomeres (Liu and Roizman, 1991a; Liu and Roizman, 1991b). Type B capsids are thought to be intermediates in capsid maturation as pulse chase studies have shown that “B” capsids are capable of packaging viral DNA to form “C” capsids (Braun et al, 1984). Type C capsids are the only capsid type isolated by de-enveloping intact virions and contain the entire linear double stranded DNA genome and an additional protein, VP22 (Gibson and Roizman, 1972; Schrag et al, 1989).
The tegument: The amorphous proteinacious region between the undersurface of the envelope and the outer surface of the capsid has been designated the tegument (Figure 1.1). The least distinctive of the four layers, the tegument is variable in both size and composition. Many regulatory structural proteins, which are necessary in the viral lifecycle, are contained within the tegument (Roizman and Furlong, 1974). Most notably, the α-trans-inducing factor (α-TIF, VP16), which functions in transactivation of alpha protein synthesis, and the virion host shutoff protein (VHS, encoded by UL41) are important constituents within the tegument (Read and Frenkel, 1983). Other components in the tegument include, VP1-2 (UL36), VP11-12 (UL46), VP13-14 (UL47), and the gene product of US11 (Chou and Roizman, 1989; Roizman and Furlong, 1974; Roizman and Sears, 1996).

The envelope: The outermost layer of the herpes virion is comprised of a bilayer lipid envelope and membrane imbedded viral glycoproteins (Figure 1.1). The virion envelope and the imbedded viral glycoproteins are acquired at least initially by budding through the inner nuclear lamellae of infected cells; however, whether or not this initial envelope serves as the mature virus' final envelope is still not known. HSV-1 specifies at least eleven glycoproteins designated as gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, and gM, most of which have been demonstrated to be structural components of the virion (Baines and Roizman, 1993; Hutchinson et al, 1992; Ramaswamy and Holland, 1992; Spear, 1985). These glycoproteins function in a number of important membrane associated events during the virus lifecycle including attachment, penetration, egress, and virus induced cell fusion (Spear, 1985).
Organization of the Viral Genome

All herpesviruses contain a linear double stranded DNA genome that can vary in size depending on the virus from 120kb to 250kb in length. The HSV genome is approximately 150kb and is packaged in the virus in a tightly wound torroid structure (Becker et al, 1968; Kieff et al, 1971; Plummer et al, 1969). The genome consists of two covalently linked segments, designated as the unique long (UL) and unique short (US) sequences. Each segment contains nucleotide sequences distinct from the other and is bracketed on either side by inverted repeats. During viral replication, the UL and US segments can invert relative to one another generating four possible linear isomeric forms of the viral genome (Figure 1.2) (Kieff et al, 1971). These isomers have been designated as P (prototype, Fig 1.2A), IL (Inversion of the UL component, Fig. 1.2B), IS (Inversion of the US component, Fig. 1.2C), and ISL (Inversion of both the UL and US components, Fig. 1.2D). However, all isomers of the HSV viral genome are viable in cell culture (Hayward et al, 1975; Morse et al, 1977).

Initial nucleotide sequencing of the viral genome and prediction of putative open reading frames specified by these unique segments was performed by McGeoch et al (McGeoch et al, 1985; McGeoch et al, 1988a). The HSV-1 genome specifies at least eighty-four open reading frames, of which fourteen are located within the US segment, sixty within the UL segment, four in each of the inverted repeats flanking the UL segment, and one within the inverted repeat flanking the US region (McGeoch et al, 1988b).
Figure 1.2. Herpes Simplex Genome Inversion. A) Schematic representation of the arrangement of the viral genome depicting the unique long (UL) and unique short (US) regions and their prototypical (P) orientations (designated by directional arrows) and flanked by inverted and terminal repeats (IR and TR, respectively). B) IL: Inversion of the UL segment relative to prototypic arrangement. C) IS: Inversion of the US segment relative to prototypic arrangement. D) ISL: Inversion of both the UL and US segments relative to prototypic arrangement.
Herpes Simplex Genome Inversion

A

TR

b' a' c'

IR_L IR_S

U_L

U_S

c a

P

B

TR

b' a' c'

IR_L IR_S

U_L

U_S

c a

I_L

C

TR

b' a' c'

IR_L IR_S

U_L

U_S

c a

I_S

D

TR

b' a' c'

IR_L IR_S

U_L

U_S

c a

I_SL
The Herpesvirus Lifecycle

The principal events in the herpesvirus replicative cycle have been derived from cell culture systems. The herpesvirus lifecycle can be broken down into several stages as diagrammed in figure 1.3. These stages include: attachment of the virion particle to the cell surface, penetration of the nucleocapsid into the cellular cytoplasm, host protein synthesis shut off, transport of the nucleocapsid to the nucleus, regulated cascade of gene transcription, viral DNA replication, packaging of viral genomes into preassembled capsids, maturation of virion particles, and translocation of infectious particles to extracellular spaces (Roizman and Sears, 1996). The replicative cycle within cell culture systems characteristically produces swollen and rounded cells, chromatin condensation and margination, and nuclear inclusions. In some cases limited or extensive cell-to-cell fusion is also observed (Roizman and Sears, 1996).

Attachment: Initial virus attachment events provide loosely associated interactions with cells and are mediated through interactions of virion glycoproteins with heparin-sulfate proteoglycan moieties found ubiquitous on cell surfaces (Fig. 1.4) (Shieh et al, 1992; Wudunn and Spear, 1989). Binding of virion particles to cell surfaces is inhibited by soluble heparin, a molecule similar in structure to heparin sulfate. Similarly, purified virion particles bind heparin-affinity columns under physiological conditions (Herold et al, 1991; Mettenleiter et al, 1990; Wudunn and Spear, 1989). Of the eleven glycoproteins, gC, has shown the highest affinity for heparin binding, indicating that it may play an important role in the initial attachment (Herold et al, 1991; Herold et al, 1994; Spear et al, 1989).
Figure 1.3. Replicative Lifecycle of HSV-1. The stages of the HSV-1 lifecycle are diagrammed as discussed in the text, including attachment, penetration, coordinated sequential transcription, DNA replication, capsid assembly, packaging, maturation, and egress.
HSV-1 Replicative Lifecycle

- HSV-1 Virion
- Penetration
- Attachment
- HSV-1 capsid
- Viral DNA
- Replication
- Packaging
- Assembly
- Nucleus
- ER
- Maturation
- Golgi
- Plasma Membrane
- Extracellular Space
- Infectious Virions
- VHS
- Release of tegument proteins
- Viral Glycoproteins
- Extracellular Space
Furthermore, antibodies to gC, as well as, lack of gC abrogate virus binding to polarized epithelial cells (Fuller and Spear, 1985; Svennerholm et al, 1991). However, virions devoid of gC still attach to non-polarized epithelial cells, suggesting that other virion glycoproteins participate in heparin sulfate attachment. HSV-1 glycoprotein B (gB) has also been shown to have heparin sulfate binding activity and to be responsible for cell attachment in the absence of gC (Spear, 1993).

Receptor Facilitated Entry: Penetration of virion particles is through pH independent fusion of the virion envelope to the cellular plasma membrane or to early endosomes (Wittels and Spear, 1991) (Fig. 1.4). Virion glycoproteins required for this event include gB, gD, gH and gL (Sarmiento et al, 1979; Cai et al, 1988; Ligas and Johnson, 1988; Roop et al, 1993). While the initial observations of HSV attachment to heparin sulfate proteoglycans led many investigators to speculate that these molecules were the receptors for virus entry, evidence began to accumulate that suggested the presence of other specific receptors at cell surfaces that mediated virus entry. This evidence included the observation that certain cell types, such as Chinese hamster ovary (CHO) and swine testis (ST) cells, which exhibited glucosaminoglycan chains on cell surface proteoglycans that allowed virus attachment, but did not allow virus entry (Shieh et al, 1992; Subramanian et al, 1994). Furthermore, normally permissive cell-lines transformed with the gD gene were resistant to viral infection, while gD could not be shown to exhibit any heparin sulfate binding activity (Campadelli-Fiume et al, 1988; Johnson and Spear, 1989). This data suggested that gD may sequester a cellular receptor required for virus entry and that this receptor was not expressed in CHO or ST cells.
Figure 1.4. Model of Herpesvirus Attachment and Entry. The current model of HSV-1 attachment and penetration is illustrated. A) Infectious virions attach in a “loose” association with heparin sulfate proteoglycans found ubiquitous on cell surfaces. B) A “tight” association between the virion and cell occurs as the virion glycoproteins associate with cellular herpes virus entry mediators. C) Viral glycoproteins mediate penetration through a pH independent fusion event of the herpes virus envelope with the cellular plasma membrane depositing the nucleocapsid into the cytoplasm of the cell. D) The nucleocapsid is actively transported to nuclear pores by means of the cytoskeleton.
Herpesvirus Attachment and Entry

A) Loose Association
- Herpesvirus entry mediators
- Viral gC/gB
- Heparin Sulfate Proteoglycans

B) Stable Attachment

C) Penetration
- Viral gD (gK???)
- pH-independent fusion of viral envelope to cellular plasma membrane

D) Transport of Capsid to the nucleus
- Cytoplasma
- Nucleus
Initially, the fibroblast growth factor receptor (FGFr) was implicated in attachment and entry of HSV into the non-permissive CHO cells (Kaner et al., 1990); however, these results could not be confirmed by outside labs (Shieh and Spear, 1991; Mirda et al., 1992). Although other molecules, such as the mannose-6-phosphate/insulin like growth factor II receptor, had been reported to mediate virus entry into non-permissive cells, cell-lines that lacked these receptors remained fully susceptible to viral infection (Brunetti et al., 1995). It was not until Montgomery et al. (1996) screened cDNA expression libraries isolated from HeLa cells that proteins that could definitively transfer susceptibility to the normally resistant CHO and ST cells were identified. A description of each of these receptors follows.

**Herpes Virus Entry Mediator A (HveA):** HveA (initially designated HVEM) was the first in a series of receptors identified that rendered the normally resistant CHO cell-line susceptible to herpes virus infection. HveA, however, differs from the subsequently described receptors in its structure. HveA is a type I integral membrane protein with a cysteine rich extracellular domain that exhibits homology with the tumor necrosis factor receptor (TNFr) family. HveA was found to be distributed in most human tissues, including heart, lung, kidney, placenta, muscle, and pancreas, but not brain or other neuronal tissues. Interestingly, HveA expression was upregulated in activated T lymphocytes, highlighting its possible importance in HSV pathogenesis (Montgomery et al., 1996). It has been well established that HSV replicates in activated T lymphocytes (Pelton et al., 1977; Rinaldo et al., 1978; Teute et al., 1983) and that infected T lymphocytes could be isolated from biopsies of cutaneous lesions (Boddingius et al., 1987). Because
certain isolates of HSV, which specified subtle genetic alterations, were found that were incapable of utilizing the HveA receptor, it may be that in the field, the virulence of the virus may be directly attributable to its ability (or inability) to infect activated T cells.

The ability of HveA to mediate entry of other strains of HSV was determined by infecting HveA transformed CHO cells that also carried a virus inducible β-galactosidase gene. Although, HveA enhanced the ability of most HSV-1 and HSV-2 strains, HveA failed to mediate entry of HSV-1 mutants, HSV-1(ANG), HSV-1(KOS)rid1 or HSV-1(KOS)rid2 (Montgomery et al, 1996). Moreover, soluble HveA was shown to copurify with wild-type HSV-1 (KOS) virions, but not HSV-1(KOS)rid1/2 mutant virions following coincubation and sucrose gradient centrifugation (Nicola et al, 1998). The rid mutants, HSV-1(KOS)rid1 and HSV-1(KOS)rid2, each specify single amino acid substitutions within gD (Q27P or Q27R, respectively) that enable the virus to overcome the interference for entry that is imposed by cell-lines that express gD (Dean et al, 1994). HSV-1(ANG) is also resistant to gD mediated interference through the same amino acid substitution as HSV-1(KOS)rid2, but also has other amino acid substitutions within gD and other proteins (Dean et al, 1994).

In this regard, HSV-1 gD was implicated as the primary virion protein that would physically associate with HveA. This is consistent with previous work that had indicated that gD was the primary virion protein involved in receptor recognition. Expression of gD in normally permissive cell-lines rendered those cell resistant to HSV infection, presumably due to sequestration of a cellular receptor.

30

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
required for entry (Campadelli-Fiume et al, 1988). Incubation of these cells with anti-gD neutralizing antibody prior to infection partially released this inhibition (Brandimarti et al, 1994; Campadelli-Fiume et al, 1990). A further indication of the role of gD in receptor binding was that anti-idiotypic antibodies mimicking gD bound cell surfaces of normally permissive cells and blocked virus infection following binding (Huang et al, 1996). Moreover, soluble gD was capable of binding cells in a saturable manner and could inhibit infection (Johnson et al, 1990; Nicola et al, 1997). Although gB, gH, and gL are essential for virus infectivity, similar lines of evidence for receptor binding do not exist.

Interaction of HSV-1 gD and HveA was shown through direct binding assays. Binding of gD to HveA was conformationally dependent, but was not dependent on the glycosylation state of the protein (Whitbeck et al, 1997). Furthermore, the complex formation appeared to occur in a 1:2 (gD:HveA) stoichiometric ratio as assessed by gel filtration and SDS-PAGE analysis (Whitbeck et al, 1997). This stoichiometry is consistent with receptor oligomerization following ligand interactions for receptors that when bound transmit signals into the cell (Wells, 1994; Wells, 1996). Using optical biosensor technology, the binding affinity of gD with HveA was determined to be in the micromolar range (Willis et al, 1998). However, this interaction data may be somewhat misleading due the use a form of gD that exhibits a higher affinity for HveA than its wild-type counterpart (Willis et al, 1998). Given the rather low affinity for HveA in these experiments, it is plausible that other virion proteins participate in virus binding. On the other hand, HveA is not the sole receptor present on cell surfaces, and it is just as
possible, that contributions from several receptors increase the overall binding affinity given that different receptors appear to bind different regions of gD (Krummenacher et al, 1998; Rux et al, 1998). Another explanation is that it is not advantageous for the virus to exhibit high binding affinities for cell surface receptors because it would prevent the release of virions from cell surfaces upon egress.

Two cellular ligands were identified that associated with HveA, the secreted lymphotixin α (LTα) and LIGHT, a new member of the TNF superfamily (Mauri et al, 1998). Each of these proteins was shown to competitively bind HveA and to block the binding of HSV-1 gD (Mauri et al, 1998). Furthermore, soluble HveA and antibodies to HveA inhibited a mixed lymphocyte reaction, suggesting that there may be a possible role of HveA or its ligands in T cell proliferation (Harrop et al, 1998; Kwon et al, 1997; Mauri et al, 1998). In accordance with this, monoclonal antibodies to HveA prevented T cell proliferation, cytokine production, and expression of activation markers following incubation with a soluble recall antigen, tetanus toxoid (Harrop et al, 1998).

Several lines of evidence indicated that HveA was not the only or the primary mediator of HSV entry. Firstly, the fact that HSV is a neurotropic virus and that HveA was not found in neuronal tissue, indicated that other receptors remained to be isolated that could mediate entry into the nervous system. Secondly, although antibodies raised against HveA and soluble HveA blocked virus infection into CHO cells expressing HveA, both failed to block virus entry into normally permissive HeLa cells (Montgomery et al, 1996). Finally, mutant strains of virus, such as
HSV-1(KOS)rid1, as well as other alphaherpesviruses, such as PRV, were unable to enter into HveA expressing cells, but were able to enter into permissive cell-lines (Dean et al., 1994; Montgomery et al., 1996).

**Herpes Virus Entry Mediator B (HveB):** HveB was initially described as poliovirus receptor related protein 2 (Prr2); however, no function or ability to mediate poliovirus entry had been ascribed (Eberle et al., 1995). HveB is a 479 amino acid type I integral membrane protein that is structurally similar to those proteins within the immunoglobulin superfamily. It contains three immunoglobulin-like regions in its extracellular domain and two potential sites for the addition of N-linked sugars (Warner et al., 1998).

HveB was shown to mediate entry of HSV-2 and PRV-1, but not that of wild-type HSV-1 or BHV-1 (Warner et al., 1998). Certain viable mutants of HSV-1, namely HSV-1(KOS)rid1 and HSV-1(KOS)rid2, which were unable to utilize HveA were capable of utilizing HveB for virus entry (Montgomery et al., 1996; Warner et al., 1998). In contrast, the murine homolog of the human HveB receptor was unable to facilitate either HSV-1 or HSV-2 entry, but was able to mediate PRV-1 entry (Shukla et al., 1999). Furthermore, antibodies to this receptor were capable of completely blocking PRV-1 infection into at least some normally permissive murine cell-lines, indicating that the murine HveB is the principal mediator of PRV-1 entry into those cell-lines (Shukla et al., 1999).

**Herpes Virus Entry Mediator C (HveC) and Herpesvirus Ig-like receptor (HlgR):** The HveC receptor, initially described as poliovirus receptor related protein 1 (Prr1), mediates entry of all alphaherpesviruses tested to date.
including HSV-1, HSV-2, PRV-1, and BHV-1 (Geraghty et al, 1998). HveC is a 518 amino acid type I integral membrane protein that exhibits structural properties similar to those proteins in the immunoglobulin (Ig) superfamily. This includes three Ig-like loop domains within the extracellular region (Lopez et al, 1995).

HveC mRNA was found to be distributed across tissue types, including epithelial and neuronal tissues, making it the prime candidate for the primary receptor for virus entry into permissive cells (Geraghty et al, 1998). In accordance with this observation, incubation of virus with soluble HveC blocked infection of many normally permissive cell-lines (Geraghty et al, 1998). Furthermore, incubation of virus with either soluble HveA or HveC abrogated entry into CHO cell-lines expressing either HveA or HveC receptors, indicating that each entry mediator may recognize overlapping but distinct structural domains within gD (Geraghty et al, 1998; Krummenacher et al, 1998).

The HveC receptor was further shown to exist as different splice variants within cells. Cocchi et al (1998a) described a splice variant isoform of HveC, designated HIgR, which functioned to facilitate alphaherpesvirus entry in a manner analogous to HveC. HIgR is a 458 amino acid protein that is structurally similar to HveC and specifies an ectodomain identical to HveC. The homology to HveC does however diverge following a splice donor consensus sequence within the HveC RNA resulting in a different amino acid sequence following amino acid residue 334 (Cocchi et al, 1998a).
Figure 1.5. Mediators of Herpesvirus Entry. The schematic represents the two broad families of herpes virus entry mediating receptors, TNF-like and Immunoglobulin like.
Mediators of Herpesvirus Entry

<table>
<thead>
<tr>
<th>TNF-like</th>
<th>Immunoglobulin-like</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>HveA/TR2</td>
<td>HveB/Prr2</td>
</tr>
<tr>
<td>HveC/Prr1</td>
<td>HlgR</td>
</tr>
<tr>
<td>HveD/PVR</td>
<td></td>
</tr>
</tbody>
</table>
Similar to their HveA counterpart, HveC and H1gR were shown to physically interact with gD (Cocchi et al, 1998b; Krummenacher et al, 1998). Despite the inability of HVS-1 mutant viruses rid1 and ANG mutants to bind HveA (Krummenacher et al, 1998; Whitbeck et al, 1997), these mutants bound HveC with a higher affinity than their wild-type counterpart (Krummenacher et al, 1998). Disruption of the structure of gD abrogated its ability to bind HveC. Furthermore, whereas, mutation or deletion of three of the four functional domains of gD elicited similar effects for both HveA and HveC binding, mutation of the functional domain I had contrasting effects on HveA versus HveC binding. While functional region I is required for interaction of gD with HveA, it is not crucial for HveC binding (Krummenacher et al, 1998). Interaction of gD with the ectodomain of HveC and H1gR was shown to require the V domain (Figure 1.5.) of the receptor. Moreover, the V domain was shown to be necessary and sufficient to mediate HSV entry into non-permissive cells (Cocchi et al, 1998b). Monoclonal antibodies to the V domain of HveC and H1gR competed for HSV-1 gD binding and blocked virus infection. Additionally, chimeric receptors which specified only the V domain fused to a transmembrane region were shown to facilitate virus entry in the absence of other portions of the receptor (Cocchi et al, 1998b). Therefore, the major functional region of both gD binding and receptor facilitated entry lies within the V domain of HveC and H1gR.

**Herpes Virus Entry Mediator D (HveD) and other herpesvirus receptors:** HveD is the poliovirus receptor (Pvr) and has been shown to mediate entry of PRV-1 and BHV-1 but not HSV-1 virus strains (Mendelsohn et al, 1989;
<table>
<thead>
<tr>
<th>Receptor Designation</th>
<th>Other Names</th>
<th>Receptor Class</th>
<th>Cell Distribution</th>
<th>Virus Entry Mediated</th>
</tr>
</thead>
<tbody>
<tr>
<td>HveA</td>
<td>HVEM; TR2; ATAR</td>
<td>TNF-like</td>
<td>Mainly in lymphocytes and keratinocytes; lung; liver; kidney</td>
<td>HSV-1; HSV-2</td>
</tr>
<tr>
<td>HveB</td>
<td>Prr2 (Poliovirus receptor related protein 2)</td>
<td>Ig-like</td>
<td>Most tissues; neurons; keratinocytes</td>
<td>HSV-1 mutants; HSV-2; PRV; MurineHveB only PRV</td>
</tr>
<tr>
<td>HveC</td>
<td>Prr1 (Poliovirus receptor related protein 1)</td>
<td>Ig-like</td>
<td>Most cell types except HEL299; Especially cells of neuronal origin</td>
<td>HSV-1; HSV-2; PRV; BHV-1</td>
</tr>
<tr>
<td>HlgR</td>
<td>Splice variant isoform of HveC</td>
<td>Ig-like</td>
<td>Similar tissue distribution to HveC</td>
<td>HSV-1; HSV-2; BHV-1</td>
</tr>
<tr>
<td>HveD</td>
<td>Pvr (Poliovirus Receptor)</td>
<td>Ig-like</td>
<td>Wide cellular distribution</td>
<td>PRV; BHV-1</td>
</tr>
</tbody>
</table>
Similar to the Pvr related proteins, HveB and HveC, HveD is a member of the immunoglobulin superfamily (Mendelsohn et al, 1989).

A number of other receptors that mediate virus entry have been reported, but are yet to be described. It is however clear that a myriad of different receptors from a number of different structural families exist and that each participate in receptor mediated virus entry. The process by which each receptor functions in this entry event and the role that each may play in a biological model is still to be determined. A diagrammatic summary of the described receptors is presented in Figure 1.5 and Table 1.2.

**Host Protein Shutoff:** A distinguishing characteristic of herpesvirus-infected cells is the rapid shut down of host macromolecular synthesis machinery including DNA synthesis, host protein synthesis, and glycosylation/processing of cellular proteins. The cessation of host macromolecular metabolism occurs in two distinct stages. Initially, structural proteins within the tegument of the virion shut down host protein synthesis in the absence of de novo synthesis. This occurs through the actions of the virion host shutoff (vhs) protein by destabilizing and degrading host mRNA (Kwong and Fenkel, 1987; Kwong et al, 1988; Nishika and Silverstein, 1977). Furthermore, VHS (UL41) functions in downstream lifecycle events in the nondiscriminatory degradation and destabilization of viral mRNA’s (Read and Frenkel, 1983). The second stage of host protein shutoff requires de novo synthesis of virion proteins following infection and coincides with the onset of β protein synthesis.
Virion Transport to the Nucleus: Due to the lack of the necessary machinery for transcription and DNA replication, the nucleocapsid must be transported to the nucleus following penetration. The cytosolic transport of the nucleocapsid specifically to the nucleus has been reported to be mediated by cytoskeletal microtubules (Dales and Chardonnet, 1973; Kristensson et al, 1986). Depolymerization of microtubules prevented transport of nucleocapsids to nuclear pores. Furthermore, Sodeik et al. (1997) showed by immunoelectron microscopy and confocal microscopy that nucleocapsids use dyenin, a minus end directed microtubule dependent molecular motor, to be specifically transported through the cytoplasm towards the centriole. The nucleocapsid is subsequently transported to the nucleopore and the viral DNA unpackaged and inserted into the nucleus in a manner yet to be determined.

Transcriptional Regulation: Cellular RNA polymerase II transcribes viral RNA from the HSV DNA genome (Costanzo et al, 1977). Viral mRNAs are processed similar to cellular RNAs in that they are capped, methylated, and polyadenylated (Bachenheimer and Roizman, 1972; Bartkoski and Roizman, 1976; Silverstein et al, 1973; Silverstein et al, 1976). Splicing of viral RNA occurs in only six of the open reading frames, ICP0, ICP22, ICP47, UL15, UL44 (gC), and UL45. Genes sharing 5' and 3' termini have been described with some genes utilizing several alternate initiation and polyadenylation sites (McLauchlan and Clements, 1982; Wagner, 1985; Wagner and Roizman, 1969). The stability of the transcripts varies; however, α and β gene transcripts appear more stable than γ genes (Wolf and Roizman, 1978).
Viral gene expression is tightly regulated with respect to both abundance of transcripts and timing of expression. Transcription of viral genes is sequentially ordered expressing immediate early or alpha (α), then early or beta (β), and finally late or gamma (γ) genes. The coordinate regulation of each of the virus gene classes occurs through specific viral proteins that turn on the next transcriptional class, while shutting down the previous transcriptional class (Roizman and Sears, 1996). Consequently, viral genes have been shown to have specific trans-activatable regulatory elements that facilitate the direct or indirect binding of viral transcriptional activators (Chou and Roizman, 1986; Chou and Roizman, 1989; Roizman and Sears, 1996).

Classification of each of the kinetic classes of transcription is dependent on whether or not DNA synthesis has occurred (Honess and Roizman, 1974; Honess and Roizman, 1975). The transcription of α genes occurs immediately following infection and does not require prior protein synthesis. Five α genes have been identified, ICP0, ICP4, ICP22, ICP27, and ICP47 and have been shown to function in regulation of subsequent transcriptional events. Both ICP4 and ICP27 have been shown to be essential for viral replication in tissue culture (Dixon et al, 1983; Sacks et al, 1985). Initiation of α gene transcription is stimulated by the virion structural protein α-TIF (alpha-transinducing factor) through the coordination of cellular transcriptional factors Oct-1, TFIID, and SP1 binding to cis-acting sequences upstream of viral genes (Gerster and Roeder, 1988; Spector et al, 1990).

Accumulation of α gene products activates β gene transcription, many of which are enzymes involved in nucleotide metabolism and DNA synthesis/
replication (Roizman and Sears, 1996). β gene transcription has the capacity to be active in the context of the cellular genome in the absence of viral gene products (McKnight and Kingsbury, 1982; McKnight et al, 1984). However, in the context of the viral genome, β gene transcription is dependent on and greatly increased by α gene expression, especially ICP4 (Krisitie and Roizman, 1984). Trans-activation of β genes involves both release from the repressive state and transcriptional transactivation. Production of β gene products initiates viral DNA synthesis and replication (Roizman and Sears, 1996).

β gene transcription in turn leads to the final kinetic class of gene transcription-the γ kinetic class. The γ genes can be subdivided into γ1 and γ2 gene types. Expression of the γ1 gene products is enhanced, but not dependent, on the completion of viral DNA replication; whereas, the γ2 kinetic class is stringently dependent on viral DNA replication. Regulation of γ genes is quite complex and has been shown to involve: a) α proteins; b) repression during early stages of infection; c) 5’ response elements in transcribed coding and non-coding domains; d) activation of gene expression following the onset of viral DNA synthesis. Many of the HSV γ gene products comprise the structural components of virion particles. Therefore, γ gene product accumulation leads directly to virion assembly and eventually to virion maturation (Roizman and Sears, 1996).

Viral DNA Replication and Metabolism: Following entry into the nucleus, the linear double stranded DNA viral genome circularizes into a head to tail concatamer (Jacob et al, 1979; Jacob and Roizman, 1977) and begins transcription of α genes; however, only a small portion of the total input viral DNA undergoes
replication. Viral DNA synthesis is detected as early as 3 hours post infection and roughly corresponds with the appearance of β gene products. Although DNA synthesis occurs from 3 to 15 hours post infection, the bulk of the viral DNA is made relatively late in infection.

Seven virus-specified proteins (UL5, UL8, UL29, UL30, UL42, and UL52) are essential and sufficient for HSV origin dependent replication in transfection assays (Wu et al, 1988). The UL30 and UL42 gene products form a heterodimeric DNA dependent DNA polymerase of which the UL30 protein forms the catalytic subunit, while the double stranded DNA binding UL42 protein confers processivity to the complex. DNA synthesis is further facilitated by a single stranded DNA binding protein (UL29), an origin of replication binding protein (UL9) that possesses helicase activity, as well as a heterotrimeric complex (UL5, UL8, UL52) that possesses both 5' and 3' helicase and primase activities (Crute and Lehman, 1991; Crute et al., 1989).

The HSV genome specifies three origins (ori) of replication. These origins have been deduced from the structures of defective genomes and defined by the necessity of their presence in a HSV DNA fragment to allow for DNA amplification in permissive cells. Two of the three origins (oriS1 and oriS2) map within the c reiterated sequences (see figure 1.2 for localization) of the Us component, while the third origin (oriL) maps in the middle of the UL component. All three origins are situated between transcriptional initiation sites, suggesting that initiation of DNA synthesis may be enhanced by trans-activation due to the local environment of the genome during transcriptional initiation events. Replication of the viral genome
may originate in either direction from any of the three origins of replication (Roizman and Sears, 1996).

Early in DNA synthesis, only parental circular DNA and linear branched forms can be detected. These are replaced at later times in DNA synthesis by rapidly sedimenting tangled DNA consisting of large tandemly repeated viral genomes arranged in head to tail concatamers. Circular duplex DNAs generally replicate in a theta (Θ) structure (Elson et al, 1995) or through a rolling circle mechanism (Becker et al, 1978; Jacob et al, 1979). Although rolling circle DNA replication may be involved in the generation of the concatameric HSV genomic complex, it is also possible that HSV DNA replication occurs by an inherently recombinogenic mechanism. This may account for the presence of intermediate structures found at early hours after infection. T4 bacteriophage DNA replication similarly results in intermediate branched forms of viral DNA that are resolved prior to packaging by the T4 endonuclease VII (Kemper et al, 1984; Mizuuchi et al, 1988). HSV mutants defective in the alkaline nuclease (U₁₂) gene, believed to function in a manner analogous to the T4 endonuclease VII, were defective in the production of mature DNA containing capsids. Furthermore, the complex branched structures within the viral genomes are not resolved in U₁₂ defective viruses, suggesting that the HSV alkaline nuclease does indeed function in resolving the branched DNA structures (Martinez et al, 1996).

In addition to alkaline phosphatase, there are several other virally encoded proteins that while not essential for DNA synthesis, undoubtedly play a role in the processing, cleavage, and packaging of genomic viral DNA. These proteins include
thymidine kinase, ribonucleotide reductase, uracil DNA glycosylase, and dUTPase reductase (Roizman and Sears, 1996).

Although the U\textsubscript{L}23 thymidine kinase (TK) gene is dispensable in cell culture, it is vital for virus replication in natural in vivo infections (Field and Wildy, 1978). TK functions in phosphorylation of purine pentosides and a wide diversity of other nucleotide analogs that are not phosphorylated by cellular kinases (Jamieson and Subak-Sharpe, 1974; Klemperer, et al, 1967). This unique property of TK is the basis of herpesvirus antiviral treatments. Antivirals such as the guanosine acyclic analog, acyclovir, is mono-phosphorylated by the viral TK, making it susceptible to further phosphorylation by cellular kinases. Acyclovir triphosphate binds viral DNA polymerase and acts as a DNA chain terminator (Elion et al, 1977; Schaeffer et al, 1978).

The ribonucleotide reductase is a heterodimeric protein consisting of both a large (U\textsubscript{L}39) and small (U\textsubscript{L}40) subunit that together function to reduce ribonucleotides to deoxyribonucleotides. This process effectively creates a large pool of substrates for DNA synthesis (Hones and Roizman, 1974; Hones and Roizman, 1975; Huszar and Bachetti, 1981; Preston et al, 1984). Deletion of the HSV ribonucleotide reductase is deleterious only in non-dividing cells or in cells maintained above 39.5°C, indicating that actively dividing cells are capable of complementing this viral function (Daikoku et al, 1991; Goldstein and Weller, 1988).

HSV also encodes for a proofreading and DNA repair enzyme, uracil DNA glycosylase (U\textsubscript{L}2). Uracil DNA glycosylase functions to correct insertion of dUTP
and deamination of cytosine residues in DNA, two of the most common mutational events that occur within DNA. This proofreading activity is an important element in the context of the herpes virus genome, which has an extremely high G+C content (Caradonna and Cheng, 1981; Caradonna et al, 1987; Mullaney et al, 1989).

The UL50 viral dUTPase (deoxyuridine triphosphate nucleotidohydrolase) functions to hydrolyze dUTP to dUMP providing an additional method to prevent the incorporation of dUTP into viral DNA. Furthermore, hydrolysis of dUTP to dUMP generates a pool of dUMP for conversion to dTMP by thymidylate synthetase (Wohlrab and Francke, 1980).

Capsid Assembly and Packaging: Herpes capsids are assembled and packaged exclusively within the host cell nucleus (Darlington and Moss, 1968; Schwartz and Roizman, 1969) at distinct regions of dense nuclear structures designated as “assemblons.” These regions are localized to the periphery of the nucleus and are separate from the viral DNA replication centers.

Capsid assembly begins upon the transport of capsid proteins to the nucleus. Genetic and biochemical analysis suggests that seven proteins (VP5, VP19c, VP22a, VP23, VP24, and VP26) encoded by six viral genes (UL19, UL38, UL26.5, UL18, UL26, and UL35, respectively) are necessary and sufficient for HSV capsid assembly. Three basic capsid forms are evident within thin sections of infected cell nuclei: a) empty capsids with no internal structure (A capsids); b) intermediate capsids that lack DNA, but contain a proteinaceous core within the outer capsid shell (B capsids); c) capsids which contain the viral genome (C capsids) (Roizman and Furlong, 1974; Roizman and Sears, 1996).
The empty A capsids consist of four proteins, VP5, VP19c, CP23, and VP26. It has been suggested by Sherman and Bachenheimer (1988) that A type capsids are not in the virion morphogenesis pathway, but rather that A type capsids represent a terminal decay product of B capsids (Sherman and Bachenheimer, 1988).

B type capsids, however, are the progenitors of both A and C type capsids, the latter of which is awaiting DNA packaging. B capsids contain three additional proteins, VP21, VP22a, and VP24. Both VP21 and VP22a are absent from mature C type capsids and may account for the appearance of the proteinaceous core within B type capsids that form the scaffolding for proper capsid assembly.

Localization of the major capsid protein VP5 to the nucleus is dependent on its interaction with the scaffolding protein VP22a (Matusick-Kumar et al, 1994; Nicholson et al, 1994). Further association of VP22a with itself and proteolytic processing form a structure to which other capsid proteins can attach and assemble (Thomsen et al, 1995) into large-cored B capsids. The outer icosadeltahedral shell is formed by VP5, VP19c, VP23, and VP26, while VP22a forms the internal scaffold structure. Proteolytic cleavage of VP22a yields two proteins, VP21 and VP24, and alters the morphology of the capsid structure to that of a small-core B capsid. C capsids are formed following the insertion of the viral DNA genome and removal of the scaffolding proteins VP22a and VP21 (Roizman and Sears, 1996).

Packaging of the viral genome into preformed B capsids occurs immediately following or concurrent with DNA replication. Cleavage of concatameric DNA into unit length viral genomes occurs at specific recognition sequences and is intimately
Figure 1.6. Packaging of Unit Length Herpes Virus Genomic DNA. The depicted model developed by Frenkel et al (1976) and is described in the text. A) Proteins attach to components of the a sequence and the empty capsids scan the genomic concatameric DNA until contact with a U_c sequence. B) The capsid is filled with DNA until (C) filled or contact is made with an a sequence in the same orientation as the first a sequence (one unit length of genomic viral DNA). D) Each strand is nicked at the packaging signal on opposite sides of the DR1 sequence. E) The juxtaposition of the a sequences results in their reiteration.
linked with the packaging process. The inverted repeats, present at the termini of the UL and US segments of the viral genome, contain two highly conserved site specific cleavage sequence elements designated pac1 and pac2 within the “a” sequences (Figure 1.2). The cleavage-packaging reaction involves two site-specific breaks on either side of the “a” sequences producing a net result of a free S component terminus containing one “a” sequence and a free L component terminus consisting of a variable number of repeated “a” sequences. The length of the packaged DNA is therefore determined by the distance between two directly repeated “a” sequences (Deiss et al, 1986a; Deiss et al, 1986b).

Detection and monitoring of the “a” sequences within the concatameric DNA in order to generate unit length genomes consists of several steps (Figure 1.6). a) The cleavage-packaging protein attaches to the Uc sequence within the DNA. b) A protein or protein sequence on the surface of the capsid complexes with the cleavage-packaging protein bound to the Uc sequence. c) The viral DNA is looped into the capsid structure by the complex and scanned from the original “a” sequence (a1) across the L and S components. d) A Uc domain within an “a” sequence in the identical orientation to a1 is detected. e) Cleavage occurs within the shared juxtaposed direct repeat (DR1) regions of the two “a” sequences and generates a headfull capsid containing one unit length viral genome (Roizman and Sears, 1996).

Envelopment, Maturation, and Translocation of Infectious Virions to Extracellular Spaces: The final stage in the replicative cycle of herpesviruses is the process of envelopment of nucleocapsids, maturation of glycoproteins on the virion envelope, and transport of virions to extracellular spaces. Several herpesvirus
genes code for proteins that are known to be involved in HSV-1 virion envelopment and egress. Defects in certain tegument proteins such as UL11, UL48 (α-TIF), and ICP34.5 inhibit virus egress (Ace et al., 1988; Baines et al., 1992; Weinheimer et al., 1992; Browne et al., 1994); whereas, deletion of other tegument proteins do not affect virus maturation (Fenwick et al., 1990; Post et al., 1981; Zhang et al., 1991). In addition, membrane associated proteins such as UL20 and UL53 (gK) have been implicated in virion egress. Deletion of the UL20 gene caused accumulation of virions within perinuclear spaces, while deletion of gK caused an accumulation of virions within the cytoplasm (Baines et al., 1992; Hutchinson et al., 1995; Jayachandra et al., 1997). Interestingly, mutant viruses exhibiting egress defects could be partially complemented by certain cellular functions, because the UL20 null virus replicated well on 143TK− cells and the replication of the gK null virus was enhanced in actively replicating cells (Baines et al., 1992; Jayachandra et al., 1997).

Primary envelopment occurs as nucleocapsids bud through the inner nuclear lamella at areas of high electron density, which is believed to represent viral tegument proteins (Nii et al., 1968; Roizman and Furlong, 1974). Immature viral glycoproteins imbedded within nuclear membranes are initially acquired during this budding process. Capsids that do not contain DNA or contain only fragments of less than full length genomic viral DNA, rarely become enveloped (Vlazny et al., 1982). A possible explanation for the inability of these capsids to become enveloped is that packaging of full length genomic viral DNA alters the capsid structure or composition, which in turn alters the nucleocapsids affinity for viral tegument proteins. Therefore, capsids lacking this putative modification would not

51

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
interact with the tegument proteins juxtaposed with viral membrane proteins within nuclear membranes.

Although there is general agreement that initial virion envelopment occurs in this manner, there is considerable debate as to the processes of final virion envelopment, maturation and transport to extracellular spaces. While envelopment of virus at the inner nuclear lamella does provide the virion with its tegument, envelope, and viral glycoproteins, the glycoproteins within the envelope are immature, as they have not yet been transported to or processed by the Golgi apparatus. Ultrastructural examination of Vero cells infected with a U120 null virus indicated that enveloped capsids accumulated within the perinuclear spaces (Baines et al, 1991). U120 null virions isolated from these cells contained glycoproteins of the immature type, while fully mature glycoproteins were present within Golgi or cell surface membranes (Avitabile et al, 1994). Mature virions present in extracellular fluids contain fully processed glycoproteins that have been significantly modified by Golgi enzymes. Therefore, virions that initially acquire their envelopes at the inner nuclear lamella must either acquire new envelopes at Golgi membranes that contain fully processed glycoproteins, or must be transported through the Golgi pathway for subsequent in situ processing of viral glycoproteins. These hypotheses have led to two contrasting models of herpesvirus maturation and egress, known as the envelopment/de-envelopment model and the vesicular transport model (Fig. 1.7).

Stackpole et al (1990) originated the envelopment/de-envelopment model of herpesvirus egress wherein capsids are initially enveloped at the inner nuclear
Figure 1.7. **Diagrammatic Representation of Models of Virion Egress.** Cross-section of a eukaryotic cell depicting the nucleus, endoplasmic reticulum, Golgi, and plasma membranes. The two models of virus maturation and egress as discussed in the text are shown. A) Envelopment/De-envelopment model. B) Vesicular Transport model.
Models of Virion Egress

A

Golgi

Endoplasmic Reticulum

Nucleus

B

Plasma Membrane

Cytoplasm

Nuclear Membrane

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
lamella, de-enveloped at the outer nuclear lamella, and finally re-enveloped at Golgi membranes for transport to extracellular spaces. Ultrastructural examinations of infected cells have revealed the presence of un-enveloped capsids within the cytoplasm juxtaposed to electron dense cytoplasmic membranes in the process of envelopment. Enveloped virions within cytoplasmic vesicles were also observed fusing to these vesicles depositing naked capsids into the cytosol (Stackpole et al, 1990).

In this model, naked nucleocapsids within the cytoplasm represent a prerequisite step for budding into Golgi-derived vacuoles, generating enveloped virions containing fully processed glycoproteins. Enveloped virions are then transported to extracellular spaces within Golgi derived vesicles that fuse to the cellular plasma membrane depositing infectious virions into the extracellular spaces. In support of this model, recombinant viruses have been constructed that specify virion glycoproteins modified to be retained within the endoplasmic reticulum by means of the ER retention motif, KKXX. This targeting signal appended to a glycoprotein H (gH), conferred the predicted localization properties on gH in recombinant virus infected cells. Furthermore, the gH polypeptide failed to be processed to a mature form by Golgi modification enzymes, indicating that it was indeed retained within the ER (Browne et al, 1996). Glycoprotein H is an essential virion glycoprotein. Virus isolated from these cells was non-infectious, indicating that gH was not incorporated on the virion. By the envelopment/de-envelopment model, because gH is not transported to the Golgi apparatus, recombinant virions purified from extracellular spaces should not contain gH. Consistent with this
hypothesis, purified recombinant virus particles contained normal amounts of other virion proteins; however, they did not contain any detectable amounts of gH (Browne et al, 1996). Targeting wild-type gH to the Golgi in *trans* restored virus infectivity, suggesting that gH was incorporated into the excreted virions (Browne et al, 1996). One critique of this model is based on how cells differentiate between cytoplasmic capsids following an initial infection that are selectively transported to nuclear pores, versus de-enveloped capsids that must be transported to Golgi membranes in order to acquire their envelopes.

A second model of virus egress, designated here as the vesicular transport model, suggests that virions follow the cellular secretory pathway during translocation to extracellular spaces (Figure 1.7). The process of transport would therefore be similar to that described in subsequent sections of this dissertation for protein transport. The vesicular transport model diverges from the envelopment/de-envelopment model following envelopment at the inner nuclear membrane. Whereas enveloped virions are predicted to fuse to the outer nuclear lamella in the envelopment/de-envelopment model, virions within the perinuclear space are thought to bud from the outer nuclear lamella into transport vesicles in the vesicular transport model. In a manner analogous to vesicular transport of proteins in cells from the ER to the Golgi, virions would then be trafficked to the Golgi for processing and modification of immature glycoproteins *in situ* during translocation to extracellular spaces (Di Lazarro et al, 1995; Johnson and Spear, 1982; Torrisi et al, 1992). Fusion of the transport vesicle to Golgi membranes deposits enveloped virions into the lumen of the Golgi for modification. The two models do however
re-converge at this point, as cellular vesicular transport mechanisms traffic the
enveloped virions containing fully mature glycoproteins to cell surfaces.
Nucleocapsids devoid of viral envelopes, which are frequently found in the
cytoplasm of infected cells, are thought to represent a nonproductive population of
viruses (Campadelli-Fiume et al, 1991). In support of this model, specific inhibitors
of glycoprotein processing and transport, such as monensin, similarly inhibit
infectious virus production and transport (Spear and Johnson, 1982). Furthermore,
cells defective in the processing of high-mannose to mature oligosaccharides
accumulate infectious virus, but do not allow its transport to extracellular spaces
(Banfield and Tufaro, 1990; Campadelli-Fiume et al, 1982). Opponents to this
model suggest that it is unlikely that virions could be efficiently transported through
a sequential Golgi system that is fragmented following viral infection. It is however
interesting to note that in either model, cellular trafficking pathways are involved
following processing of virion glycoproteins.

**Herpes Simplex Latency:** The most unique feature of herpes viruses are
their ability to remain latent through the majority of an individual’s lifetime, while
exhibiting limited episodes of recurrent lesions. Following a primary infection of
cells at the mucosal membranes, sensory nerves innervating mucosal tissue
become infected and transport the virus back to the trigeminal ganglia where latency
is established (Stevens and Cook, 1971). In latently infected neurons, the viral
genome is maintained in a circular episomal state (Mellerick and Fraser, 1987; Rock
and Fraser, 1983) with very little to no viral protein expression. Reactivation of
HSV is precipitated by several factors including, stress, fever, overexposure to
sunlight, and the host’s immune competency (Roizman and Sears, 1996). Periodic reactivation of HSV occurs in a small fraction of neurons harboring latent virus and is manifested as lesions often at the same peripheral site as the primary infection (Goodposture, 1929; Carton and Kilbourne, 1952; Roizman, 1966). The severity of recurrent lesions is largely dependent on the host’s immune system, but is usually less severe than the primary infection (McKendall, 1979; Whitley et al, 1997).

Herpes viruses have evolved through an intimate host-virus relationship. The major selective pressure guiding HSV evolution towards neuronal latency was most likely the host’s immune response. Neuronal latency provides several major advantages in evasion of the host’s immune system. First, during establishment of latency, virus is transported intraaxonally within cytoplasmic processes of the neurons to the neural bodies (Lycke et al, 1984). Therefore, the virus is not exposed to the immune system during transport to the sites of latency establishment. Second, HSV does not require replication in order for latency to be established. This limits the availability of antigenic markers that can be displayed by the cell to the immune system. Third, by definition, during latency little or no protein is produced and replication is silenced (Doerig et al, 1991; Rodahl and Stevens, 1992). Neuronal latency provides an immunologically privileged site for maintenance of the viral genome. Furthermore, the lack of protein expression enhances the ability of the virus to evade the host’s immune response by preventing or limiting the display of viral antigens. Finally, even upon reactivation in the ganglion when infectious virus is produced, the virus remains within the cytoplasmic processes of
neurons and is delivered by axonal flow to target cells of the epithelium (Cook and Stevens, 1973).

There are three primary animal models of HSV latency—the mouse, the rabbit, and the guinea pig. In the mouse, latency is established following inoculation of the eye, footpad, or ear; however, the rate of reactivation is extremely low (Hill et al, 1982). In contrast, the rabbit eye model exhibits spontaneous reactivation and pathogenesis similar to that of a natural infection (Nesburn, 1967). The guinea pig is used primarily for HSV-2 vaginal studies. Recurrent lesions are observed only with a high dose infection; however, there has been some debate of whether this is a truly latent state or a festering chronic infection (Stanbury, 1982).

Following infection of HSV in animal models, the virus replicates at or near the site of inoculation ensuring entry into the innervating sensory nerve endings. The capsid containing the viral genome is then transported by rapid retrograde axonal transport to the neuronal nucleus along microtubules (Kristensson et al, 1986). Drugs that either disrupt microtubule structure or inhibit retrograde transport similarly block the ability of capsids to be transported to the nuclei. Replication often occurs within the ganglia for a short period of time; however, this may be an artifact of the large amount of virus used in infectious inoculums and most probably does not occur in humans (Klein et al, 1978).

Approximately 0.1 to 1 latent viral genomes exist per 1 ganglionic cellular genome equivalent with only 0.1% to 3% of neurons within ganglia harboring virus (Rock and Fraser, 1985; Cabrera et al, 1980). Intriguingly, neurons only account for about 10% of the total cell mass of ganglia (Rodahl and Stevens, 1992).
Therefore, unless every cell within the ganglion, including glial cells, contains a viral genome, it is obvious that each latently infected neuron must contain more than one copy of the genome. This copy number must however be reached without the necessity for viral genome replication by viral enzymes. Indeed, it has been shown that within neurons that exhibited no viral antigen expression, the copy number of viral genomes was greater than twenty genomes (Roizman and Sears, 1996). Thus, either more than one viral genome can enter a single neuron during the establishment of latency or viral genomes can be amplified by cellular machinery during latency.

Two to four weeks post infection, no replicating virus can be detected within sensory ganglia that innervate the site of inoculation. Co-culture of extracted latently infected ganglia with permissive epithelial cells produces infectious virus only if the ganglia are fully intact. Production of infectious virus following maceration indicates that infectious virus is present and the virus never truly established latency.

To date, no gene sequence or viral protein has been implicated in the maintenance of the latent state. In fact, only one family of transcripts, designated LAT (Latency Associated Transcripts), is detected during latency (Stevens et al, 1987). LAT is relatively abundant during latency and accumulates within the cell’s nucleus; however, the transcript does not specify a protein. LAT is a nuclear RNA of approximately 2 Kb in length derived from a primary transcript of 8.3 Kb (Mitchell et al, 1990). It was originally postulated that the 3’ terminus of the LAT transcript, which is antisense to the major regulatory ICP0 gene, functioned to
preclude the expression of ICP0 and therefore preclude the initiation of gene expression. Although this theory is quite logical, it did not prove to be the case. In the absence of LAT viral gene expression is still silenced and the latent state is similarly maintained. It is not conceivable that a transcript with all its regulatory elements has been conserved throughout evolution in both HSV-1 and HSV-2 if it does not perform some function. This function however, remains an enigma (Roizman and Sears, 1996).

External stressors, including stimuli such as physical and emotional stress, peripheral tissue damage, or immune suppression results in the reactivation of virus and transport to a site near the portal of entry. Most commonly, reactivation is induced experimentally by physical trauma, iontophoresis of epinephrine or other drugs, transient hyperthermia, corneal scarification, or injection of immuno-suppressants. Reactivation by these means allows for the detection of infectious virus near the sites of the portal of entry. Similar to the in vivo situation, exhibited lesions are observed that are generally less severe than that seen upon initial inoculation (Roizman and Sears, 1996; Whitley et al, 1997).

Glycoprotein Processing and Transport

N-linked glycoprotein synthesis and processing: The majority of proteins directed into the rough endoplasmic reticulum are destined for the covalent addition of oligosaccharides and transport to the Golgi apparatus, lysosomes, the plasma membrane, or the extracellular spaces. Cytosolic proteins seldom are glycosylated; however, those that contain only simple oligosaccharide modifications.
Although some O-linked glycosylation occurs at the hydroxyl group of serine or threonine residues within the Golgi apparatus, the most common oligosaccharide modification of proteins is N-linked or Asparagine-linked (Asn-linked) glycosylation (Hubbard and Ivatt, 1981; Kornfeld and Kornfeld, 1985).

Asn-linked glycosylation consists of the en bloc transfer of a preformed fourteen sugar residue complex to the side chain NH₂ group of an Asn residue contained within the amino acid motif Asn-X-Ser/Thr (where X is any amino acid except proline). The oligosaccharide complex is assembled by sequential addition of sugars onto a dolichol lipid embedded within the ER membrane prior to its transfer to the newly synthesized protein. Attachment of the sugar residues to the dolichol lipid is mediated through a high-energy pyrophosphate bond. Nucleotide-sugar intermediates (UDP-GlcNAc or UDP-Man) then assemble within the cytosol the core sugar, (GlcNAc)₂(Man)₅, onto the dolichol lipid in a strictly ordered fashion (Bergnan and Kuehl, 1978; Roberts et al., 1978; Ronnet and Lane, 1981). Following assembly of the core sugars within the cytoplasm, the dolichol-sugar complex is flipped into the lumen of the ER where the sequential incorporation of four additional mannose residues and three glucose residues occurs (Fig. 1.8). These seven sugar residues are transferred to the complex through a dolichol phosphate intermediate (Dol-P-Man or Dol-P-Glc). Transfer of the complex to the newly synthesized protein occurs en bloc and is facilitated by the presence of the terminal three glucose residues (Snider and Rogers, 1984; Snider and Robbins, 1982; Turko et al., 1977; Spiro et al., 1979).
Figure 1.8. Synthesis of lipid linked oligosaccharides in the rough endoplasmic reticulum. Oligosaccharides are assembled on a dolichol lipid intermediate embedded within the membrane of the ER in a step wise fashion prior to an en bloc transfer to a newly synthesized protein as described in the text.
Processing and trimming of the primary oligosaccharide structure accounts for the vast diversity of carbohydrates exhibited by glycoproteins. Initial trimming occurs rapidly within the ER with the removal of three glucose residues and one mannose residue (Atkinson and Lee, 1984). This process serves as a checkpoint for signaling that the glycoprotein is correctly folded. Removal of these residues is essential for the glycoprotein to be transported from the ER to the Golgi apparatus where oligosaccharides are further trimmed and processed. If however the glycoprotein becomes unfolded or misfolded following trimming, glucose is added back to the protein preventing transport from the ER. Generally, there are several rounds of removal and addition of glucose residues prior to the protein being properly folded and prepared for transport (Parodi et al, 1984). The ER resident membrane anchored chaperone protein calnexin is pivotal in assuring that the protein is correctly folded. Any glycoprotein within the ER that contains one or more glucose residues remains bound to calnexin until it is properly folded and the glucosylation ceases. Therefore, the final quality of the glycoproteins being transported from the ER is assured (Lodish and Kong, 1984; Schlesinger et al, 1984).

Vesicular transport, as discussed in a subsequent section of this dissertation, mediates the transport of glycoproteins from the ER to the cis phase of the Golgi apparatus, as well as from the Golgi apparatus to the cellular surfaces and other cellular organelles (Jamieson and Palade, 1968). The Golgi apparatus consists of several flattened membranous cisternae that each specify within their lumen an ordered and specialized oligosaccharide processing function. These cisternae can be
differentiated into three distinct processing compartments: the cis compartment or face of entry; the medial compartments or central compartments; and the trans compartment where glycosylation processes are completed. Furthermore, the trans compartment is thought to be continuous with the lumen of the trans Golgi network, where proteins are segregated and sorted into vesicles for transport to their final destinations (Kornfeld and Kornfeld, 1985).

Following processing by Golgi enzymes, there are two broad classes of N-linked oligosaccharide-containing proteins, the complex oligosaccharide and the high mannose oligosaccharides containing glycoproteins. High mannose oligosaccharides contain two N-acetylglucosamine residues and a number of mannose residues. In contrast, complex oligosaccharides contain several N-acetylglucosamine residues and may further contain a variable number of galactose, sialic acid, and fucose residues. The structures of the complex oligosaccharides are generated through a combination of both trimming and addition of sugars. However, despite the extensive trimming and processing that occurs within the Golgi, all glycoproteins maintain a common pentasaccharide core consisting of three mannose residues anchored to the protein backbone by two N-acetylglucosamine residues (Kornfeld and Kornfeld, 1985).

The determination of whether or not oligosaccharides remain high mannose or are subsequently processed to complex oligosaccharides is due to the conformation of the protein. Inaccessibility of oligosaccharides by Golgi processing enzymes prevents further trimming and additions. Therefore, it is less likely to be converted to a complex form. This process accounts for the presence of both high

65

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
mannose and complex oligosaccharides at different sites on the same glycoprotein (Kornfeld and Kornfeld, 1985).

The glycoprotein enters the cis face of the Golgi containing two N-acetylglucosamine residues and eight mannose residues. If the conformational environment of the protein permits, Golgi mannosidase I removes three mannose residues. Immediately following this removal and subsequent transport to the medial cisternae of the Golgi, N-acetylglucosamine transferase I adds N-acetyl glucosamine allowing Golgi mannosidase II to remove an additional two mannoses. This generates the pentameric core sugar with an additional N-acetylglucosamine that is ubiquitous for all complex oligosaccharides. Moreover, the oligosaccharide structure is now rendered resistant to cleavage by Endoglycosidase H or F treatment (Kornfeld and Kornfeld, 1985). The oligosaccharide complex is further processed through the incorporation of two additional N-acetylglucosamines, three galactose residues, and three N-acetylneuraminic acid residues within the trans cisternae of the Golgi (Fig. 1.9). Within the trans Golgi network, glycoprotein cargo is sorted and segregated for transport to the extracellular spaces or to other cellular organelles through an extensive and complex vesicular trafficking system that will be discussed in a subsequent section of this dissertation.

Cargo Loading and Vesicle-Mediated Transport: Transport between organelles and a cellular membrane is a highly ordered and directed process that is dependent on an array of proteins, lipids, and enzyme complexes. Each stage in the series of transport steps requires the assembly and disassembly of complex structures, as well as the regulation of their transport. This ordered process assures
Figure 1.9. Processing of oligosaccharides within the endoplasmic reticulum and Golgi apparatus. Processing of oligosaccharides is highly ordered and follows a strict pathway that begins within the ER with the removal of three glucose residues. The remaining steps occur within the stacks of the Golgi apparatus as depicted above. Notably, removal of two mannoses by mannosidase II in the medial cisternae renders the bond between the two N-acetylglucosamines resistant to cleavage by Endoglycosidase H.
that cargo within vesicles reaches targeted organelles, cell surfaces, or extracellular spaces. During transport through the secretory pathway, each molecule passes through multiple cellular compartments, where the molecule can be modified in a series of controlled steps, stored until needed, or packaged for delivery to a specific site or region of the cell surface.

Although similar transport events also occur during endocytosis, the vast majority of this section will focus on the process of transport to and between organelles that eventually leads to exocytosis of vesicular cargo. This focus is because during a herpes infection, the majority of the processes that are occurring are exocytotic in nature. This includes the transport of virion glycoproteins to cell surfaces, as well as the transport of other macromolecules, including virion particles to extracellular spaces. In this regard, a portion of this section may explain the possible mechanisms of transport of infectious virions to extracellular spaces, as discussed in an earlier section of this dissertation. This section will however briefly review the mechanisms for recycling of proteins from the cell surface. Protein recycling is of recent interest as many herpesvirus proteins have been shown to be cycled between cell surfaces and cellular organelles through processes analogous to many cellular proteins. The biological relevance for this recycling during infection is in its infancy, but it is becoming increasingly important in understanding herpes viral pathogenesis.

**Protein Transport between the Endoplasmic Reticulum (ER) and Golgi:**
As discussed in the previous section of this dissertation, newly synthesized proteins enter the biosynthetic-secretory pathway by crossing the ER membrane from the
cytosol (Hubbard and Ivatt, 1981; Kornfeld and Kornfeld, 1985). All subsequent transport steps between organelles are facilitated through transport vesicles in a series of vesicle budding and fusion events. Once a protein has entered the ER, transport from the ER to the Golgi and finally to the cell surface occurs without the requirement for specific transport signals. In contrast, proteins that are destined to remain within these organelles or are destined to be transported to other organelles specify retention or transport motifs within their amino acid sequence. Retention motifs therefore selectively cause the retention of ER resident proteins from vesicles when cargo is transported to the next compartment (Hardwick and Pelham, 1992). However, proteins that direct the vesicular transport between organelles and regulate the fusion process of vesicles with the next compartment must be recycled in retrograde vesicles back to the previous compartment.

Two types of protein coated vesicles shuttle cargo between the ER and Golgi and are defined by the type of cytosolic proteins that are coalesced with the vesicles surface (Bednarek et al, 1995; Scheckman and Orci, 1995). These two distinct protein complexes are termed coatamer (or Coat Protein I [COPI]) and COPII. Both COPI and COPII coated vesicles include fusogenic proteins designated as v-SNAREs (soluble NSF attachment protein receptor) that act in coordination with the Golgi t-SNARE or the ER t-SNARE to facilitate fusion of the vesicle to the targeted organelle membrane (Lewis and Pelham, 1996; Lupashin et al, 1996). Although both COPI and COPII coated vesicles bud from the ER, only COPII has been shown to be involved in anterograde transport to the Golgi. COPI coated vesicles are associated with cargo that contains ER retention/retrieval motifs (KKXX, KKKKKK,
K/HDEL, where X is any amino acid). In this manner, COPI coated vesicles may shuttle cargo back to the ER (retrograde transport) in order to replenish proteins necessary for budding and fusion of vesicles (Kuehn and Schekman, 1997).

The high concentration of a given cargo in COPII coated vesicles favors the active selection of such molecules, which would envisage specific cargo receptors (Balch et al, 1994; Bednarek et al, 1995; Mizuno et al, 1993;). This would entail cargo receptors or coat adapters interacting with one or more transport signals on cargo molecules; however, signals for ER to Golgi transport of cargo have yet to be identified. Interestingly, cyclohexamide treatment of cells, which inhibits protein synthesis and therefore depletes the available cargo, does not inhibit vesicle budding, suggesting that COPII vesicle formation is independent of the presence of cargo (Yueng et al, 1995).

Assembly of COPII components occurs sequentially on a specific cytosolic portion of the ER, beginning with the recruitment of a small GTP binding protein. Following sequential assembly of the COPII proteins, the cargo destined for transportation is selected and ER resident proteins are excluded through a yet to be identified mechanism. Proteins necessary for recognition of Golgi membranes as well as the fusion event with those membranes, such as the v-SNARE protein, are also included during vesicle morphogenesis (Shekman and Orci, 1996). Following vesicle morphogenesis, the COPII coated vesicle is directed to the cis phase of the Golgi where the v-SNARE protein within the vesicle recognizes the t-SNARE protein of the Golgi. Docking of the two SNARE proteins is necessary, but not sufficient for vesicle fusion. A GTPase component of the COPII protein complex,
Rab proteins, recognize the binding of complimentary SNARE proteins and lock the
docked vesicle onto the target membrane by hydrolyzing the bound GTP. Fusion of
the vesicle to target Golgi membranes deposits the cargo into the Golgi for
additional processing, sorting, or transport (Shekman et al., 1995; Kuehn and
Shekman, 1997).

**Protein Trafficking between the Trans Golgi and Cell Surfaces:**
Transport vesicles containing membrane proteins and lipids traffic from the Trans
Golgi network (TGN) to cellular surfaces providing new components for the cell’s
plasma membrane, while soluble proteins present within these vesicles are secreted
to the extracellular spaces. This process, known as the constitutive secretory
pathway provides for the majority of the cell’s proteoglycans and glycoproteins of
the extracellular matrix. In polarized cells, a variation of the constitutive cellular
surface transport route occurs, such that proteins destined for basolateral delivery
are differentially sorted from that cargo which is destined for apical surfaces
(Cosson and Letourneur, 1997).

Separation, sorting, and packaging of proteins for transport to their final
destination occurs within the Trans Golgi network. Despite extensive efforts to
characterize the pathways of transport from the TGN to cell surfaces, sorting and
trafficking mechanisms from the TGN have proven to be more complex and more
numerous than initially anticipated. The process of transport from the TGN to the
cell surface is similar to transport from the ER to the Golgi in that coated vesicles
mediate the transport of the cargo. However, the composition of the coat proteins
remains to be established. Several coatlike proteins, including p62 (Jones et al,
1993), p200 (Narula et al., 1992; Narula and Stow, 1995); and p230 (Kooy et al., 1992), have been identified. Furthermore, these proteins have been shown to cycle on and off TGN membranes and are localized to coated buds and vesicles in the TGN region (Kooy et al., 1992; Simon et al., 1996). Unfortunately, the function of these proteins in vesicle transport is yet to be identified.

Although little is known about the transport through the constitutive secretory pathway, there are well-identified cytosol-oriented signals that mediate the transport of cargo in the selective transport pathway through the intracellular endosomal membrane system. Curiously, the basolateral and lysosomal targeting signals both contain similar tyrosine- and dileucine-based signals; however, these signal motifs interact with the coat proteins with different affinities. Therefore, minor differences within these signals separate proteins selectively either into basolateral-destined vesicles or lysosomal destined vesicles. Moreover, these same motifs function in the recycling of cell surface proteins back to the TGN and prevent the delivery of these proteins to clathrin coated lysosome-destined vesicles.

In fact, the \( \mu \) subunits of AP-1, AP-2, and AP-3 (adapter proteins 1, 2, and 3) do exhibit different binding affinities for certain sorting signals (Ohno, et al., 1995; Ohno et al., 1996). In this manner, adapter proteins (AP) interact with cargo directly and facilitate the coalescence of specific coat proteins on the emerging vesicle. Therefore, it is the cargo that selects for the type of coated vesicle that will be formed and by this selection the destination of the vesicle (Cosson and Letourneur, 1997). It is however not yet possible to assign specific coats to a specific sorting route from the TGN. Moreover, it is apparent that vesicular trafficking is subject to
additional levels of regulation, including protein phosphorylation (Simon et al., 1996a; Simon et al., 1996b).

**Proteins Recycle from the Cell Surface via Endocytosis: Internalization**

of proteins from the cellular surface occurs in a manner analogous to endocytosis of extracellular materials, except that the signals required for internalization are different. Cytosolic signals direct the rapid internalization, as well as the intracellular targeting to specific membranes. Two common signal motifs contain a critical tyrosine residue (YXXØ, where Ø is any bulky hydrophobic amino acid) or a dileucine motif (Trowbridge et al., 1993; Mellman 1996; Marks et al., 1997). Although these signals function in internalization from the plasma membrane, subsets of these signals are involved in the targeting of proteins to lysosomes, the TGN, basolateral surfaces of the plasma membrane, and endosomal/lysosomal compartments (Mellman, 1996; Marks et al., 1997).

The tyrosine-based signal motif, YXXØ, has shown a high affinity for the adapter protein (AP) family of proteins. As would be expected for interactions reliant on a limited number of recognition molecules, internalization through these signals is a saturable process (Marks et al., 1996). Phosphorylation abrogates the ability of the AP2/µ2 subunit to bind to the tyrosine-based motif. Thus, phosphorylation provides a means to regulate the interactions and subsequent vesiculation (Shiratori et al., 1997). Regulation of µ2 interactions is regulated by other methods as well. Phosphorylation of residues adjacent to the signal modifies the local environment and the conformational context of the signal, making the signals more or less accessible for interaction with the adapter proteins. Another
important determinant in these interactions is the oligomeric state of the membrane proteins. Oligomeric complexes of proteins, which may occur at a greater frequency when being localized into coated pits prior to internalization, would be expected to dramatically increase the avidity for the adapter proteins.

Clathrin-based vesiculation from the plasma membrane is the best described system for internalization of plasma membrane receptors. Binding of adapter proteins to the signal motifs within membrane proteins facilitates clathrin coat assembly and regulates the recruitment of other coat components. Membrane proteins specifying internalization motifs are concentrated within coated pits at the surface of the cell. The main structural component of these pits is clathrin, a trimeric scaffold protein organized into cagelike lattices on cytosolic surfaces of the plasma membrane (Kirchhausen, 1993). Clathrin functions as the organizing framework for proteins that facilitate the sorting, membrane budding, and other steps in vesicle assembly, uncoating and fusion.

Although most adapter proteins recruit clathrin as the framework for vesiculation, other adapter proteins, such as AP3, are non-clathrin associated (Dell'Angelica et al, 1997; Simpson et al, 1997). The process of transport is further complicated by similar adapter protein complexes mediating transport from the TGN to cellular surfaces, as well as from cellular surfaces back to the TGN. In either process, during or after signal-adapter interaction, the coat is assembled in a coordinated manner; however, the precise sequence of events in vesicle formation is still largely unknown.
Herpes Simplex Glycoproteins and their Putative Functions

The herpes simplex virus genome specifies at least eleven glycoproteins, designated gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, and gM (Figure 1.10). These glycoproteins function in a number of important events during the virus replicative cycle including virus attachment, penetration, cell-to-cell spread, egress, and virus induced cell-to-cell fusion. This section will summarize the important features of these glycoproteins and briefly describe the putative functions associated with each. Conspicuously absent from this discussion is glycoprotein K (gK), which will be discussed extensively in the subsequent section.

**Glycoprotein B (gB):** Glycoprotein B, encoded by the Ul27 ORF, is the largest glycoprotein specified by HSV-1. The precursor 904 amino acid polypeptide contains a cleavable 30 amino acid signal peptide (Claesson-Welsh and Spear, 1987) and three putative transmembrane regions (Gilbert et al., 1994). The transmembrane regions anchor and orient gB such that 696 amino acids are located extracellularly and encode five potential sites for N-linked glycosylation (Cai et al., 1988), while a relatively large 109 amino acid carboxyl terminus faces the lumen or intracellular regions of the cell. (Pellet et al., 1985). Following synthesis, gB forms homodimers within the endoplasmic reticulum (ER) through interaction of two regions of the polypeptide at amino acid residues 92-282 and 596-711 (Highlander et al., 1991; Quadri et al., 1991).

Glycoprotein B initially functions with gC in virus attachment to heparin sulfate containing proteoglycans on the surfaces of permissive cells. Although virions deficient for gB are capable of attaching to cell surfaces, these virions can

75
Figure 1.10. Relative Positions of HSV Glycoproteins in the Genome. A) The prototypical arrangement of the HSV-1 genome is depicted with the $U_L$ and $U_S$ regions highlighted. B) The relative positions within the viral genome of each of the eleven HSV-1 glycoproteins are shown.
not penetrate cells. However, addition of fusogenic reagents, such as polyethylene glycol (PEG), following attachment allows the virus to enter cells and initiate a productive infection. Collectively, these experiments indicate that gB is required for the virion to cell fusion event in virus penetration.

**Glycoprotein C (gC):** The U_{L44} gene specifies the 511 amino acid precursor of gC, that contains a 25 amino acid signal peptide, a 435 amino acid extracellular domain, a 23 amino acid transmembrane region, and a 28 amino acid cytoplasmic domain (Fink et al., 1983; Homa et al., 1986; Holland et al., 1984). Although gC has a predicted molecular mass of only 55 kDa, gC migrates at 120-130 kDa on SDS-PAGE due to extensive N-linked and O-linked glycosylation (Kikuchi et al., 1987).

While not essential for a productive infection in tissue culture cells, gC is believed to function in the initial attachment of virion particles to cells through interactions with heparin sulfate moieties ubiquitous on cell surfaces (Wudunn and Spear, 1989). This attachment event can however be mediated by gB in the absence of gC.

**Glycoprotein D (gD):** The 394 amino acid precursor of gD is specified by the US6 ORF and contains three sites of N-linked glycosylation to generate a 52kD precursor gD molecule (Watson et al., 1982). Cleavage of a 25 amino acid signal peptide, as well as conversion of the carbohydrate additions to complex forms through further glycosylation and sialylation, yields a protein with an apparent molecular weight of 59kD. The cytoplasmic carboxyl terminus is 32 amino acids in length specified by amino acids 338-369 (Minson et al., 1986). The majority of
neutralizing antibodies to HSV-1 and HSV-2 react strongly with several discontinuous epitopes of gD. In accordance with this, gD has been shown to be essential for virus entry into permissive cells. Furthermore, gD mediated entry is through receptor specific interactions, as gD has been shown to interact both with the HveA and HveC receptors (Krummenacher, 1998). Although no syncytial mutations have been mapped to gD, it may also function in membrane fusion events. In the absence of gD, virus mediated or in vitro initiated syncytia formation does not occur.

Glycoproteins E and I (gE and gI): Herpes simplex virus gE and gI are encoded by the neighboring US8 and US7 genes, respectively. In infected cells, they form a single membrane anchored heterodimeric complex. Virions deficient in the gE/gI complex formed small plaques on epithelial cells, but showed no defect in virus entry or infectious virus production indicating a possible role of these proteins in cell to cell spread (Dingwell et al, 1994). Accordingly, infections of in vitro cultured trigeminal ganglionic neurons with gE/gI mutants indicated that there was a defect in virion crossing of cytoplasmic membranes (Dingwell et al, 1995). Corresponding results were observed with in vivo neural pathway models (Racjciani et al, 1990; Kudelova et al, 1991). The gE/gI complex is also capable of binding the Fc portion of IgG antibodies, providing the virus an in vivo evasion mechanism from specific antibody action. More recently, gE and gI have been shown to localize to tight junctions of cellular membranes, presumably to facilitate virus spread to adjacent cells (Dingwell and Johnson, 1998).
Glycoprotein G (gG): The product of the US4 gene, gG is localized to both nuclear and cytoplasmic membranes (Ackeman et al., 1986; Frame et al., 1986; Sullivan and Smith, 1987). In tissue culture, gG is not essential for virus entry or translocation of virus to extracellular spaces; however, virions devoid of gG are arrested through an unknown mechanism post-adsorption (Roizman and Sears, 1996).

Glycoproteins H and L (gH and gL): The products of the UL22 and UL1 genes, gH and gL respectively, form a heterodimeric complex that is essential for virus cell-to-cell spread, egress, and entry (Hutchinson et al, 1992a). Expression of gH in the absence of gL results in a polypeptide that is both incorrectly folded and processed due to its inability to be transported from the endoplasmic reticulum. Interestingly, expression of the gL monomer results in its secretion from cells to extracellular spaces (Hutchinson et al, 1992a; Roop et al, 1993). Therefore, gL appears not to be an integral membrane protein, but one that is dependent on interactions with gH for its anchoring to membranes (Dubin et al, 1995). Virions devoid of gH and gL can attach but not enter into permissive cells. Attachment of these virions blocked superinfection by wild type virus, indicating that gH was involved in membrane fusion, but not receptor binding (Forrester et al, 1992).

Glycoprotein J (gJ): Glycoprotein J is encoded by the US5 gene and is the most recently described and characterized of the HSV glycoproteins. Baculovirus expressed gJ exhibited an apparent molecular weight of 10 kD and 16-17kD and was shown to be glycosylated. Moreover, gJ was expressed at cellular surfaces and
was incorporated into mature virion particles (Ghiasi et al, 1998). There has been no functional data described for this protein to date.

**Glycoprotein M (gM):** Glycoprotein M is a highly hydrophobic glycoprotein specified by the UL10 gene. Once synthesized, the protein is tightly associated with membranes transversing the membrane eight times (Baines and Roizman, 1993). Glycoprotein M has been shown to be a component of the virion particle and has been shown to be expressed at cell surfaces. Deletion mutant analysis has suggested that gM functions in cell-to-cell spread as deletion of gM produced a virus that replicated to lower titers in certain cell lines and exhibited a small plaque phenotype (MacLean et al, 1993; Davis-Poyter et al, 1994).

**Characterization of Glycoprotein K (gK)**

The vast majority of this dissertation will focus on understanding the structure and function of a single glycoprotein encoded by HSV. Therefore, it is only fitting that this section provides a detailed overview of the experimental work to date on glycoprotein K (gK). The UL53 open reading frame (ORF) was initially described nearly fifteen years ago and was predicted to encode a putative glycoprotein with multiple hydrophobic regions. It garnered much research interest because of the propensity for mutations that caused extensive cell-to-cell fusion (syncytia) to map to the gK gene. However, despite extensive efforts to characterize the UL53 gene product and its functions, until recently gK has remained quite elusive.
The Ul53 ORF: The Ul53 ORF coding sequences have a GC content of 61%, which is relatively similar to the 67% GC content of the viral genome (Kieff et al., 1971). The coding sequences of the Ul52 and Ul53 ORFs overlap within the viral genome, in that the Ul52 ORF runs through the first 43 nucleotides of Ul53. Contained within the Ul52 ORF are several putative transcriptional signals including a TATA box, a CAAT signal, and a GGGCGG signal (McKnight et al., 1984; Gidoni et al. 1984), as well as two copies of its complement (CCCGCC). Approximately 10 nucleotides upstream of the predicted start codon, there is a sequence that is similar to the ribosomal binding sequences (TCCTCCTACA). Ninety-one nucleotides downstream of the putative termination sequence there is a consensus polyadenylation addition signal sequence that may function to signal polyadenylation for both the Ul52 and Ul53 transcripts (Debroy et al., 1985).

There are conflicting reports as to which kinetic class (β or γ) the transcript of the Ul53 gene belongs. Initial reports by Debroy et al. (1985) showed that Ul53 mRNA could not be detected in infected cells in the presence of phosphonoacetic acid (PAA), a potent viral DNA synthesis inhibitor. This would tend to indicate that the kinetics of transcription for the Ul53 gene would be within the γ class due to its dependence on viral DNA replication. However, Hutchinson et al. (1992b) reported that transcription of the Ul53 gene was not sensitive to PAA treatment, and thus, it was most likely regulated as a β class protein.

Characteristics of the Ul53-gK protein: The Ul53 ORF is predicted to encode a 338 amino acid protein (Figure 1.11/primary sequence) with very hydrophobic characteristics (Figure 1.12). In vitro transcription/translation analysis
Figure 1.11. Predicted primary sequence for HSV-1 (KOS) gK. The primary amino acid sequence of gK was deduced from the HSV-1 UL53 gene as published by Debroy et al (1985). Also shown are the relative positions of the signal sequence, sites for N-linked glycosylation, and four putative hydrophobic domains.
Figure 1.12. Predictions of hydrophilicity, surface probability, flexibility, and antigenic index for HSV-1 glycoprotein K. Analysis was performed using the protein analysis tool box of the MacVector software (IBI-Kodak). A) Representation of the hydrophilic profile of gK. B) Prediction of the surface probability of gK. C) Diagram of the relative flexibility of HSV-1 gK. D) Presentation of the antigenic index of gK.
revealed that the UL53 gene product had characteristics of an integral membrane glycoprotein including: 1) a 30 amino acid signal sequence on the amino terminus that was shown to be cleaved between the Gly and Ala residues following translation; 2) two potential sites for N-linked glycosylation within the amino terminal one-third of the protein at amino acids 48 and 58; 3) four hydrophobic domains that have potential for transversing membranes and thus, anchoring the glycoprotein to the cellular membranes. However, it was recently determined by membrane protection assays that for in vitro transcribed/translated gK only three of the four hydrophobic domains transversed membranes (Mo et al., 1997). This was consistent with computer predictions and experimental data from our laboratory generated during the course of these investigations. Analysis of the predicted secondary structure of gK reveals much information as to its topology and possible mechanisms of function in infected cells (Figure 1.13). A modification of the original Chou-Fasman prediction by Debroy et al. is schematically presented in Figure 1.14 and discussed later in this dissertation. We have operationally divided gK into four specific domains, each separated physically by transmembrane regions of the protein. In this modified representation, the amino and carboxyl terminuses of gK are placed on opposite sides of the cellular membranes (Figure 1.14). An interesting consequence of this model is that syncytial mutations that map within gK fall within two specific domains (I and II) and are localized to the same side of the membrane (lumen/extracellular). Previously, mutations mapped to opposite sides of the membrane. Therefore, for alteration of amino acids within intracellular/cytoplasmic domains to induce syncytia formation, the mutation would have to
Figure 1.13. Prediction of helix, sheet, and secondary structure from the primary amino acid sequence of gK. Analysis was performed using the protein analysis tool box of the MacVector software (IBI-Kodak). CF: Chou Fasman secondary structure predictions. RG: Robson-Garnier secondary structure prediction. Schematic of these results are presented in Fig. 1.14.
Figure 1.14. Schematic model of the predicted secondary structure of gK. The gK model of Debroy et al (1985) was modified to have three instead of four membrane spanning domains are shown as embedded within the membrane (lines). Syncytial mutations that map to gK are indicated by asterisks.
somehow transduce a signal to extracellular domains. In our revised model of gK, all known syncytial mutations now map to the two extracellular domains of gK. Therefore, it can more easily be envisioned that either mutations directly participate in causing syncytia formation or alternatively, that the two domains (domain I and domain III) cooperate in the regulation of syncytia formation. An extensive review of HSV induced cell fusion will be presented within the next section to further define the current knowledge of the role of gK in cell fusion.

The gK polypeptide has been difficult to characterize because of the lack of immunological reagents (see antigenic index, Figure 1.12) and the high hydrophobicity of the protein (Figure 1.12). Therefore, characterization of gK has been for the most part done by assessing the properties of *in vitro* transcribed/translated protein (Hutchinson *et al.*, 1992b; Mo and Holland, 1997; Ramaswamy and Holland, 1992). *In vitro* translation of the U53 ORF indicated that the gene product was unable to enter the separating gel of an SDS-PAGE following heat treatment at 100°C; however, the protein samples could be successfully separated in the absence of boiling. Aggregation of the protein products was abrogated by truncation of the carboxyl terminal one-third (truncation at amino acid 239) of the polypeptide indicating that the domain that affected aggregation following heating resided between amino acids 240-298 (Ramaswamy and Holland, 1992).

Glycoprotein K contains two possible sites (amino acid residues 48 and 58) for the addition of N-linked carbohydrates (Figure 1.14). Addition of canine pancreatic microsomal membranes to *in vitro* translated protein reactions increased
the molecular weight of the polypeptide by approximately 9000 Daltons relative to the unprocessed forms. Endoglycosidase treatment of microsomally treated peptides produced similar albeit not identical banding patterns to that of the unprocessed forms. Furthermore, truncation of these peptides to as little as 170 amino acids did not affect the relative shift due to glycosylation, demonstrating that glycosylation only occurred within the amino terminal one-third of the protein (Ramaswamy and Holland, 1992).

Treatment of microsomally processed in vitro translated protein with sodium carbonate results in conversion of the closed vesicles to open sheets of membranes (Fujiki et al., 1982). As a consequence, membrane associated proteins remain membrane bound, while soluble proteins enclosed within the microsomes are released. Membranes and associated proteins can be isolated by high-speed centrifugation, while soluble proteins can be precipitated with trichloroacetic acid. Therefore, by this method it can be readily assessed whether or not a protein is membrane associated. In vitro translated U153 protein and all truncated forms greater than 170 amino acids were shown to associate with membranes. However, truncation of the peptide to 112 amino acids resulted in a soluble form of the protein indicating that at least one transmembrane region resided between amino acid residues 112 and 170 (Ramaswamy and Holland, 1992; Mo and Holland, 1997). Furthermore, because the 112-residue protein was soluble in these fractions, an amino terminal signal cleavage must have occurred to release the protein, as the signal sequence is a transmembrane domain prior to cleavage (Shaw et al., 1988).
N-terminal sequencing demonstrated that this cleavage had occurred between the Gly and Ala residues of the polypeptide (Ramaswamy and Holland, 1992).

Hutchinson et al. (1992b) were successful in generating antipeptide antisera in rabbits to three stretches of linear amino acids within gK. To date, these sera have been the sole method for characterizing gK in infected cells (Hutchinson et al., 1992b; Hutchinson et al., 1995). However, this methodology and the results derived from them have several disadvantages. Firstly, all antipeptide sera were generated within rabbits. Herpes virus glycoproteins gE and gI are notorious for binding the Fc portion of rabbit antibodies. Thus, sera generated within rabbits are cross-reactive and non-specific in herpes infected cells—a problem easily visualized in all radioimmunoprecipitations (RIPS) published to date. Secondly, reaction of these antibodies with protein is poor at best. This may be due to several factors including the relatively low levels of expression for gK, the inability of the protein to enter into SDS-PAGE, inaccessibility of the protein due to the hydrophobic nature of the protein, and/or a low affinity of these antibodies to their target epitopes.

In any case, the use of antipeptide sera was essential for the initial characterization of infected cell protein (Hutchinson et al., 1992b; Hutchinson et al., 1995). In contrast with the in vitro translated gK protein, infected cell gK detected with antipeptide sera was not sensitive to heating. Furthermore, infected cell gK had an apparent molecular weight of approximately 40 kDa compared to 36 kDa for in vitro translated and microsomal processed protein. This difference could not however be attributed to Golgi modification within infected cells as the peptide was endoglycosidase H/F sensitive, indicating that contrary to all other HSV
glycoproteins, gK had not undergone extensive golgi processing. Moreover, using antipeptide sera, gK could not be detected as a structural component from purified virion extracts (Hutchinson et al., 1992b; Hutchinson et al., 1995).

**Localization of HSV-1 gK:** All HSV glycoproteins except gK appear as two protein species by SDS-PAGE analysis, differing in the composition of N-linked and O-linked oligosaccharides. The fact that gK was shown to exist as a single 40 kDa species and to be endoglycosidase H/F sensitive indicated that gK was neither processed to a mature form during transit through the Golgi apparatus nor transported to the surface of cells (Hutchinson et al., 1992). This assessment was, however, somewhat paradoxical to the role of gK in the regulation of syncytia formation. Hutchinson et al. (1995) demonstrated by immunofluorescence assays (IFA) using anti-peptide sera that gK did not localize to cell surfaces. Instead, gK was only detected in perinuclear and nuclear membranes. In contrast, all other HSV glycoproteins have been shown to be distributed throughout cytoplasmic and surface membranes. Interestingly, localization of gK did not change if syncytial mutations were introduced into gK. Thus, the effect exerted by gK in the formation of cell to cell fusion would most likely be a regulatory one.

Ghiasi et al. (1994) generated antisera to gK expressed in baculovirus infected cells. Although not discussed by the authors, IFA’s of either gK expressing baculovirus infected cells or HSV-1 infected cells appear to depict gK distributed throughout the cytoplasm and at cell surfaces. This localization pattern is consistent with the architecture and the cell fusion characteristics of the protein, and the lack of endoplasmic reticulum (ER) retention signals within the gK polypeptide. Although
this scenario seems most plausible, proper controls were not provided for the needed assessment and this antisera has not been made available for testing.

Despite initial indications to the contrary, HSV gK has been reported not to be a structural component of the virion (Hutchinson et al., 1994; Hutchinson et al., 1995). Therefore, gK cannot function in membrane fusion events other than syncytia formation, during entry of the virion into cells. However, gK has been detected as a structural component of several other closely related herpes viruses, including pseudorabies virus (PRV) (Klupp et al., 1998) and varicella-zoster virus (VZV) (Mo et al., 1999). It is possible that the observed differences in detection of gK in PRV and VZV but not HSV, are solely dependent on the availability of quality immunological reagents. Detection of gK as a structural component of both PRV and VZV was achieved using sera generated in mice against bacterially expressed gK protein (Klupp et al., 1998; Mo et al., 1999). However, expression of HSV-1 gK in bacterial systems prior to this dissertation has not been possible.

Function of gK in the HSV-1 Lifecycle: Although the lack of immunological reagents has placed limitations on the characterization of gK, recent genetic studies have begun to unravel the mystery of the function of gK. Initial attempts to isolate U₅3 null virions were unsuccessful leading many to theorize that the U₅3 gene was essential for virus replication in tissue culture (MacLean et al., 1991). Similarly, Hutchinson and Johnson (1995) isolated the mutant virus, designated FgKβ that contained an insertionally inactivated U₅3-gK gene which was unable to efficiently replicate in cell culture. The FgKβ virus was constructed through the insertion of a β-galactosidase gene cassette within the coding region of
the Ul53-gK gene; however, the design of this insertion allowed the amino terminal one-third of the gK protein to be expressed. Electron microscopic examination of infected Vero cells revealed unenveloped capsids dispersed throughout the cytoplasm indicating that gK most likely functions during egress of virion particles (Hutchinson and Johnson, 1995).

Concurrent with these investigations, Jayachandra et al (1997) constructed the gK-null virus ΔgK, in which the entire coding region of Ul53 was deleted. Although the ΔgK virus was significantly inhibited in both infectious virus production and virion egress, the virus was still capable of being propagated on the non-complementing Vero cell line, indicating that gK was not essential for infectious virus production. The difference in results between these two viruses was attributed to the expression of the amino terminal one-third of gK in the FgKβ virus. In support of this hypothesis FgKβ produced large syncytial plaques on 143TK- cells, while the ΔgK virus did not. Furthermore, melitin a specific inhibitor of gK mediated syncytia formation, inhibited FgKβ syncytia formation (Jayachandra et al, 1997).

**Glycoprotein K and Viral Pathogenesis:** There has been very little work done to characterize the function of gK in HSV pathogenesis. Furthermore, although some of the work arrives at very interesting conclusions, the premise of many of those conclusions is questionable. Moyal et al. (1992) reported that mutations that mapped within the Ul53-gK gene abolished neurovirulence in mice that were infected by an intracerebral route. The authors’ conclusions were based on sequence comparisons of two strains of HSV, R15 (apathogenic) and R19
Four mutations within the U53 gene were found that were thought to be responsible for the apathogenic phenotype of HSV strain R15. Moyal et al. failed to sequence the pathogenic controls, but instead compared the apathogenic sequences to those of published wild-type (wt) pathogenic virus strain sequences. Of those four mutations, three were described within the Debroy et al. (1985) reference as “either/or” due to compressions on the sequencing gel that made determination of the exact sequence difficult. This sequence was subsequently corrected to the exact sequence, which Moyal et al. mistakenly described to be the changes that abolish neurovirulence. The fourth and final mutation was not a determinate of neurovirulence, as other apathogenic strains also specify this mutation.

Recently, Ghiasi et al. (1994; 1996; 1997) have used baculovirus expressed HSV-1 gK to investigate the role gK plays in HSV pathogenesis. Although immunization of mice with baculovirus expressed gK did not elicit neutralizing antibodies to gK, mice were protected against lethal intraperitoneal (i.p.) challenge. Immunization did not however protect against establishment of latency within the trigeminal ganglia (TG) (Ghiasi et al., 1994). Following intraocular infection with HSV, latency is established within the TG resulting in the absence of detectable infectious virus (need reference here). Because the virus within the TG is normally latent (existing in an episomal DNA state), reactivation following co-cultivation of the TG with Vero cells usually takes a week or more to detect. Mice immunized with gK were shown to constantly shed infectious virus; therefore, co-cultivation with Vero cells resulted in cytopathic changes within the first three days. Moreover,
incubation of TG cellular extracts from gK-immunized mice resulted in similar cytopathic effects (CPE); whereas, cellular extracts from the TGs of mock vaccinated mice produced no cytopathic changes (Ghiasi et al., 1996). These results indicate that following immunization with HSV-1 gK, clearance of virus from the TG is impaired resulting in a chronic productive infection.

It could only follow that if there is constant shedding of virus from gK-vaccinated animals, that there would be a significant exacerbation of the disease state. In accordance with this, Ghiasi et al. (1994) demonstrated that the severity of HSV-1 induced eye disease in vaccinated mice was exacerbated relative to that of control vaccinated mice. Challenge of gK vaccinated BALB/c mice with KOS resulted in corneal scarring, despite the fact that KOS does not normally produce any corneal scarring. Furthermore, challenge of gK vaccinated C57BL/6 mice, which are normally refractory to HSV induced corneal scarring, with HSV-1 McKrae strain produced significant corneal scarring. The increased severity of HSV-1 induced corneal scarring was attributed to non-neutralizing antibodies against gK; therefore, total anti-gK mouse sera or purified anti-gK mouse IgG was passively transferred to BALB/c mice four hours prior to ocular challenge.

Following challenge, anti-gK sera recipient mice exhibited increased corneal scarring relative to either those mice that received transfer from naïve animals or those that received transfer from KOS vaccinated animals. As had been demonstrated previously, mice that had received passive transfer from HSV KOS strain (live avirulent virus) vaccinated animals exhibited no corneal scarring indicating that the passive transfer was effective. While adoptive transfer of T cells

94
from mice vaccinated with KOS showed complete protection from corneal scarring, adoptive transfer of T cells from gK vaccinated mice neither protected nor exacerbated the disease state (Ghiasi et al., 1997).

**Virus Induced Syncytia Formation**

Both HSV-1 and HSV-2 cause infected cells to round up and adhere to one another in tissue culture; however, some mutant viruses induce extensive cell-to-cell fusion forming large polykaryocytes. Although syncytia formation by wild-type virus strains does not usually occur in cell culture, syncytia formation within *in vivo* herpetic lesions is common. The specific mechanism by which herpes viruses elicit polykaryocytosis is largely unknown. Some have suggested that the observed cell fusion may be a biologically irrelevant event, elicited by herpes expressed membrane proteins with altered structure or conformation causing an aberrant manifestation of the interactions of juxtaposed membranes. Others have argued that the syncytia formation is biologically relevant, providing a manner in which the virus can avoid the host’s immune response and insure virion spread to adjacent cells. In either case, virus induced cell fusion is studied for a number of reasons: as a probe into the structure and function of cellular membranes, as a tool to analyze the structure and function of cellular and viral membrane proteins, and as a model of virus to cell fusion events during virus entry and egress.

In contrast to most other viruses that induce cell fusion, herpes viruses have a number of proteins that the syncytial phenotype is associated. Whereas expression of the fusogenic protein from other viruses generally has the propensity to cause cell
fusion to occur, expression of a single or several herpes virus syncytial proteins does not elicit syncytia formation. Therefore, it has been postulated that a complex of proteins function collectively as fusion machinery, opening a fusion pore between adjacent cells.

Mutations, which cause extensive virus induced cell-to-cell fusion can arise in at least four genes of the HSV genome: the UL53-gK gene (Bond and Person, 1984; Debroy et al, 1985; Pogue-Geile et al, 1984), the UL27-gB gene (Bzik et al, 1984; Pellet et al, 1982), the UL20 gene (Baines et al, 1991; MacLean et al, 1991), and the UL24 gene (Jacobson et al, 1989; Sanders et al, 1982). However, syncytial mutations within the UL53-gK gene are more frequently isolated than any other gene (Bond and Person, 1982; Bond and Person, 1984; Dolter et al, 1994). HSV-1 gB, gD, gH, gL, and UL45 have all been shown to be required for syncytia formation to occur, while other viral glycoproteins including gI, gE, and gM may enhance syncytia formation. In contrast, gC may inhibit syncytia formation of some cultured cells and this may account for the absence or altered expression of gC by many syncytial mutants.

Glycoprotein K and HSV-1 Induced Cell Fusion: Although mutations that cause extensive virus induced syncytia formation map to the UL53-gK gene more than any other gene, little is understood of how gK participates in the fusion event. The story has become even more complicated by studies indicating that gK is absent from the surface of infected cells (Hutchinson et al, 1995). It is difficult to contemplate how a membrane-associated protein, such as gK, can induce cell-to-cell fusion if it does not localize to juxtaposed regions of cell surfaces. Due to the
hydrophobic nature and intimate membrane association of gK (Fig. 1.12), it is a prime candidate for the fusogenic protein in a multiple protein complex. Therefore, it is more likely that the lack of adequate gK-specific reagents has limited characterization of the role of gK plays in this process.

Amino acid changes within the gK polypeptide that result in virus induced syncytia formation are concentrated to two domains of gK, domain I and domain III (Fig. 1.14). Initial topology predictions localized each to opposite sides of cellular membranes with domain I localized to the luminal/extracellular sides of membranes, while domain III localized to cytoplasmic/intracellular sides of membranes (Debroy et al, 1985). Consequently, mutations within domain III could not directly participate in the fusion event, but rather, those mutations would have to initiate a conformational change within domain I that then could induce cell fusion. Thus, domain III would only play a regulatory role in gK mediated cell fusion.

More recent topological studies have repredicted the structure of gK to that shown in Figure 1.14. Whereas, domain I and III were first proposed to lie on opposite sides of cellular membranes, the new model depicts these domains on the same side of membranes (Mo and Holland, 1997). This orientation allows the ability for direct interaction between these two domains either through disulfide linkages or protein-protein interactions. Direct interaction between domains I and III may be involved in the regulation of fusion events. Indeed, mutations within a cysteine residue of domain III has been shown to elicit gK mediated cell fusion.
CHAPTER II

EXPRESSION OF THE ENHANCED GREEN FLUORESCENT PROTEIN BY HERPES SIMPLEX VIRUS TYPE 1 (HSV-1) AS AN IN VITRO OR IN VIVO MARKER FOR VIRUS ENTRY AND REPLICATION

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) specifies at least 11 glycoproteins: gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, and gM, which are expressed during productive virus replication. These glycoproteins function during virus entry, cell-to-cell spread, and egress of infectious virion particles (Spear, 1993a; Spear, 1993b; Roizman and Sears, 1996). Syncytial mutations (syn) that cause extensive virus-induced cell fusion can arise in at least four different regions of the viral genome including the UL20 gene (Baines et al, 1991; MacLean et al, 1991); the UL24 gene (Jacobson et al, 1989; Sanders et al, 1982); the UL27 gene encoding glycoprotein B (gB) (Bzik et al, 1984; Pellett et al, 1985) and the UL53 gene coding for glycoprotein K (gK) (Bond and Person, 1984; Pogue-Geile et al, 1984; Ryechan et al, 1979; Debroy et al, 1985). Syncytial mutations in the UL53 (gK) gene are more frequently isolated than syncytial mutations in any other genes (Ryechan et al, 1979; Read et al, 1980; Bond et al, 1982; Bond and Person, 1984; Pogue-Geile et al, 1984; Debroy et al, 1985; Dolter et al, 1994). Recently, it was shown that gK is involved in infectious virus production and virion egress (Jayachandra et al, 1997). Furthermore, domains of gK, which are involved in infectious virus production and egress, were delineated through the construction and characterization of mutant viruses expressing sequential gK truncations (Foster and Kousoulas, 1999).

The β-galactosidase (β-gal) gene has been used for insertional inactivation of HSV-1 genes (Goldstein and Weller, 1988), for monitoring the kinetics of HSV virus infection and spread in cell culture (Montgomery et al, 1996), as well as for tracing HSV-1 infections in vivo (Ho and Mocarski, 1988). However, β-gal detection requires the addition of exogenous chromophoric reagents, and quantitative spectrophotometric measurements of β-gal expression requires lysis of cells. In general, detection of fluorescence is more sensitive than spectrophotometric methods and enables convenient quantitation, analysis and sorting of labeled cells using fluorescent activated cell sorting (FACS) instruments. Recently, a PRV recombinant virus was constructed which carried the green fluorescence protein (GFP) gene under the promoter control of glycoprotein gG. PRV infected cells emitted green fluorescence, which enabled the detection of virus plaque formation, but the fluorescence did not appear to be stable over time (Jons and Mettenlieter, 1997).

The green fluorescent protein was first isolated from the jellyfish Aequorea victoria and has since been modified through codon optimization for use in eukaryotic mammalian systems (Zolotukhin et al, 1996). The enhanced green fluorescent protein (EGFP) chromophore can form a either a monomer, dimer or trimer and fluorescence emission is dependent upon proper folding and oxidation. The critical amino acid sequence for fluorescence emission is a Ser\textsuperscript{65}-Tyr\textsuperscript{66}-Gly\textsuperscript{67} peptide (Cody et al, 1993). The fluorescence of GFP is independent of co-factors, substrates, or other cellular gene products (Chalfie et al, 1994; Chalfie, 1995; Cubitt et al, 1995). The EGFP is a modified (Ser\textsuperscript{65} replaced with Thr) 238 amino acid peptide, which exhibits excitation maxima at 488 nm and emission maxima at 511 nm and fluoresces 35 times more intensely than that of its GFP counterpart (Cubitt et al, 1995; Zolotukhin et al, 1996). These modifications facilitate EGFP detection.
by fluorescent microscopy, plate fluorometry, and FACS, using standardized FITC filter sets (Cormack et al, 1996).

To assess the role of gK in virus entry, virus spread, and in the in vivo pathogenicity of HSV-1, we constructed a recombinant virus expressing the EGFP gene under the control of the cytomegalovirus (CMV) immediate early promoter. This virus expressed relatively large amounts of EGFP causing infected cells to emit bright green fluorescence.

MATERIALS AND METHODS

Cells and viruses. African green monkey kidney (Vero) cells were obtained from ATCC (Rockville, MD). Cells were propagated and maintained in Dulbecco’s Modified Eagles Medium (DMEM; Sigma Chemical Company, St. Louis, MO) containing sodium bicarbonate, 15mM Hepes and supplemented with 7% heat inactivated fetal bovine serum (FBS). The VK302 cell-line (Hutchinson and Johnson, 1995) is a gK transformed cell line obtained from Dr. D.C. Johnson, Oregon Health Sciences University. These cells were propagated in DMEM lacking histidine (GIBCO-BRL Laboratories, Grand Island, NY) supplemented with 7% FBS and 0.3mM histidinol (Sigma Chemical Co., St. Louis, MO). The ICP27 transformed cell-line, V27 (Rice and Knipe, 1990) was provided by Dr. D.M. Knipe and was passaged in DMEM with 7% FBS. All cells were passaged in DMEM with 7% FBS without selection prior to virus infections. The parental wild-type strain, HSV-1 (KOS), was originally obtained from Dr. P.A. Schaffer (Dana-Faber Cancer Institute, Boston, MA). Virus d27-1 (Rice and Knipe, 1990), which contains a 1.6kb deletion of the ICP27 gene, was provided by Dr. D.M. Knipe, Harvard Medical School, and was propagated in V27 cells. Recombinant viruses were propagated in VK302 cells.
Reagents. Restriction enzymes and DNA modification enzymes were obtained from New England Biolabs (Beverly, MA). Prep-a-gene gel fragment purification matrix and buffers were purchased from BioRad (Hercules, Calif.). Sequenase and dideoxynucleotides for dideoxy chain termination sequencing reactions were obtained from United States Biochemical (Cleveland, Ohio). $^{35}$S-dATP was obtained from Dupont/NEN (Wilmington, Dela.). AmpliTaq, XL Polymerase, and deoxynucleotide triphosphates were purchased from Perkin Elmer (Foster City, Calif.). All synthetic oligonucleotide primers were synthesized in our laboratory using phosphoramidite chemistry on an Applied Biosystems ABI 394 DNA/RNA synthesizer utilizing Perkin Elmer reagents.

Plasmids. The cytomegalovirus (CMV) immediate early promoter was polymerase chain reaction (PCR) amplified from the plasmid vector pcDNA3.1 (Invitrogen, Carlsbad, Calif.) using primer CMVEcoRI (5'CTCTCCGAATTCTCGTTTTCGCTGCTTC3') as the sense primer and primer CMVBamHI (5'CTCTCCGGATCCCTATAGTGGAGTCG3') as the antisense primer. The PCR product was digested with EcoRI and BamHI and cloned into the similarly restricted promoterless plasmid pEGFP-1 (Clontech, Pala Alto, Calif.) generating plasmid pGR8000. PGR8000 was digested with BamHI and T4 polymerase was used to fill in overhangs and inactivate the BamHI restriction site. This plasmid was designated pTF9200. Primers CMVEcoRI and EGFPBamHI (5'CGCGCGATCCCTAAGCAGCTACGCTC') which flank the upstream and downstream regions of the CMV-EGFP gene cassette respectively, were used in PCR. The final product was restricted with SpeI and BamHI and cloned into the previously described plasmid pSJ1723 (Jayachandra et al., 1997), which was also digested SpeI and BamHI, generating plasmid pTF9201.
Virus Construction. Plasmid pTF9201 specifying the CMV-EGFP gene cassette flanked by homologous HSV-1 sequences was transfected into 50% confluent VK302 cells using Lipofectamine (GIBCO-BRL, Gaithersburg, Md.) as per the manufacturer's instructions. Twenty-four hours post transfection (h.p.t.), the cells were infected at an MOI of 10 with d27-1 virus (ICP27 null) as described previously for the generation of the gK null virus, ΔgK (Jayachandra et al, 1997). At forty-eight hours post infection (h.p.i.), the cells were lysed by three freeze-thaw cycles and the resultant virus stocks were plated onto VK302 cells and overlaid with agarose. Virus plaques were visualized by fluorescent microscopy, picked and plaque purified five times on VK302 cells.

Diagnostic PCR. Primers UL52KpnI2 (5’TAGTCGCGTGCATCGAAACCCG 3’) and gKTr2 (5’ATCACATACCCCGTTCCGCTTCCG 3’) were used to amplify the region of the UL53 gK gene locus containing the CMV-EGFP gene cassette. To confirm the EGFP gene cassette insertion into the gK gene, the UL52KpnI2 primer was used in conjunction with the EGFPBamHI primer to confirm the isolated virus construction. Reaction conditions for both primer sets were 98°C for 3 sec., 72°C for 2 min, for 30 cycles.

Western immunoblots. Vero cells were infected at an MOI of 1. At 24 h.p.i., infected cells were collected by centrifugation and cell pellets were resuspended in SDS-PAGE sample buffer (0.1M Tris, 5% SDS, 20% glycerol, 0.2% bromophenol blue, 10% β-mercaptoethanol). Samples were heated for 10 minutes at 100°C and electrophoretically separated in a 10% SDS-polyacrylamide gel (Laemmli, 1970). Following separation, the proteins were electrotransferred to nitrocellulose membranes, visualized with Ponceau S (0.1% Ponceau S in 3% trichloroacetic acid), and destained. Blots were blocked against non-specific
binding for 2 hours using 10% skim milk in TBS. Blots were then incubated with anti-GFP monoclonal (Clontech, Pala Alto, Calif.) overnight at a 1:5,000 dilution in TBS with 0.1% Tween 20 (TBS-T), washed five times for ten minutes each with TBS-T, incubated for 1 hour with horse radish peroxidase conjugated goat antimouse secondary antibody (Pierce, Rockford, IL) at a 1:50,000 dilution in TBS-T, and washed five times in TBS-T for 15 minutes each. Blots were visualized by autoradiography using the Pierce SuperSignal chemiluminescent detection kit (Pierce, Rockford, IL) as per the manufacturer’s instructions.

**Fluorescent Microscopy.** Subconfluent Vero cell monolayers in 24-well plates were infected with ΔgK-EGFP1 at an MOI of 0.01. After one hour room temperature adsorption, cells were overlaid with DMEM containing 2% FCS and 0.5% methylcellulose and incubated at 37°C for the times indicated. Infected cells were visualized directly by epi-fluorescence using a standardized FITC filter set on a Nikon Optiphot-2 fluorescence microscope. Phase-contrast microscopy was also used to visualize plaque morphology in the absence of fluorescence.

**FACS Analysis.** Vero cells were infected at an MOI of 3 with either KOS or ΔgK-EGFP. Forty-eight h.p.i. cells were pelleted, washed in PBS, and either fixed in 3% paraformaldehyde for five minutes or resuspended in PBS without fixation. All samples were analyzed using a Becton Dickinson FACScan flow cytometer equipped with a 15-mW air-cooled 488-nm argon ion laser. The green fluorescence from the GFP was detected with a 530/30-nm band pass filter. FACS data was acquired and analyzed on a Hewlett-Packard (San Diego, Calif.) 340 computer. Log green fluorescence histograms were illustrated using the Lysys II software package (Becton Dickinson, San Jose, Calif.).
Construction of a CMV-EGFP gene cassette

Elimination of BamHI restriction site

Insertion of CMV-EGFP gene cassette into transfer plasmid pSJ1723

Figure 2.1. Construction schematic of the plasmid used to transfer the CMV-EGFP gene cassette into the d27-1 viral genome. (a) PCR amplification of the CMV promoter with primers CMVEcoRI and CMVBamHI. (b) Construction of the CMV-EGFP gene cassette by cloning the CMV promoter upstream of the EGFP gene to create plasmid pGR8000. (c) Elimination of the BamHI site and amplification of the CMV-EGFP gene cassette with primers CMVEcoRI and EGFPBamHI. (d) Insertion of CMV-EGFP gene cassette within the gK gene to create plasmid pTF9201.
RESULTS

Construction of the ΔgK-EGFP recombination plasmid. To facilitate the insertion of the EGFP gene cassette within the ΔgK viral genome by homologous recombination, a recombinant plasmid was engineered in which the EGFP gene cassette was cloned between UL52 and UL54 DNA sequences. The HCMV immediate early promoter sequences from plasmid pcDNA3.1 were PCR amplified using primers CMVEcoRI and CMVBamHI (Fig. 2.1a). The PCR product was restricted with EcoRI and BamHI, cloned into plasmid pEGFP-1 generating plasmid pGR8000 (Fig. 2.1b). Subsequently, the BamHI site was deleted using T4 polymerase after BamHI restriction creating plasmid pTF9200, and the CMV-EGFP gene cassette was PCR amplified using primers EGFPBamHI and CMVEcoRI and restricted withSpeI and BamHI (Fig. 2.1c). Plasmid pSJ1723 containing the UL52, UL53, and UL54 open reading frames (ORF) was as described previously (Jayachandra et al., 1997). The CMV-EGFP cassette was cloned into the plasmid pSJ1723 to create plasmid pTF9201 (Fig. 2.1d). Plasmid pTF9201 was confirmed to contain the EGFP gene cassette inserted within the UL53 gene with multiple restriction endonucleases and PCR. Transfection of plasmid pTF9201 into Vero cells resulted in bright fluorescence emission (data not shown).

Isolation of the recombinant virus ΔgK-EGFP. HSV-1 (KOS) d27-l carries a deletion in the essential ICP27-UL54 gene rendering the virus unable to replicate in Vero cells; however, it can replicate in the complementing cell line V27, which is transformed with the ICP27 gene (Rice and Knipe, 1990). A recombination system was designed to transfer the CMV-EGFP gene cassette into the gK locus of the d27-l viral genome by rescuing a deletion within the ICP27-UL54 gene. Successful rescue of the deleted ICP27-UL54 region of the d27-l viral...
Figure 2.2. Schematic of ΔgK-EGFP virus construction. (a) The top line represents the prototypic arrangement of the HSV-1 genome with the unique long (UL) and unique short (US) regions flanked by the terminal (TR) and internal (IR) repeat regions and marked in map units. (b) The region of the mutant virus HSV-1 d27-1 genome (between map units 0.7 and 0.8) containing the UL52, UL53, and the partially deleted UL54 open reading frames with relevant restriction endonuclease sites. (c) Plasmid pTF9201 (Fig. 1d) containing the CMV-EGFP gene cassette within the gK gene and bracketed by the UL52 and UL54 genes. This plasmid was used to rescue the ICP27-UL54 deletion of virus d27-1 via homologous recombination as shown. Represented on the HSV-1 (d27-1) genome are the relative positions of PCR primers, UL52KpnI2/gKTr2, used to detect the gene insertion. Represented on the pTF9201 schematic are the relative positions of PCR primers UL52KpnI2 and EGFPBamHI used to detect the CMV-IE-EGFP cassette within the gK gene.
genome by plasmid pTF9201 ensures that recombinant viruses that form plaques on Vero or VK302 cells contain the CMV-EGFP gene cassette (Fig. 2.2b, c). A similar method was used to construct the gK-null virus ΔgK (Jayachandra et al., 1997).

Purified pTF9201 plasmid was transfected into VK302 cells, and the cells were subsequently infected with d27-1 virus. Virus stocks were collected and individual viral plaques formed on Vero cells were plaque purified and tested by PCR for the presence of the EGFP gene cassette. Several viral isolates expressing EGFP were picked, plaque purified five times, and final virus stocks were made for further analysis. Overall, this recombinational scheme transferred the EGFP gene cassette into the d27-1 viral genome with 100% frequency as evidenced by the fact that all virus plaques formed on Vero cells expressed bright fluorescence.

**Genetic characterization of recombinant viruses containing EGFP gene cassettes.** Viral DNA from the HSV-1 wild-type strain KOS or the plaque purified recombinant virus ΔgK-EGFP1 was isolated and used in a diagnostic PCR to determine the presence or absence of the EGFP gene cassettes within the UL53-gK gene. Primer pair UL52KpnI2/ gKTr2 was designed to amplify the UL53 gene region (Figure 2.2b). The insertion of the EGFP gene cassette was predicted to produce a PCR product of 1865 base pairs (bp) (Fig. 2.3, lane 3) instead of the 1569 bp predicted to be produced by PCR amplification of the wild-type genome (Fig. 2.3, lane 2). Furthermore, the EGFP specific primer, EGFPBamHI (Fig. 1, panel e), and the HSV-1 specific primer, UL52KpnI2, (Fig. 2.2d) yielded an expected PCR product of 1759 bp for the ΔgK-EGFP1 viral genome (Fig. 2.3, lane 5), whereas, no PCR product was visualized with HSV-1 KOS viral DNA as the PCR template (Fig. 2.3, lane 4). The precise insertion of the EGFP gene cassette was confirmed by dideoxy-nucleotide DNA sequencing of DNA sequences bracketing the EGFP gene (data not shown).
Figure 2.3. Diagnostic PCR of plaque purified EGFP recombinant viruses. Agarose gel electrophoresis of double-stranded DNA PCR products with the UL52KpnI2/gKTr2 primer pair (lanes 2, 3) or the UL52KpnI2/EGFPBamHI primer pair (lanes 4, 5) used to detect the EGFP gene insertion within the UL53 (gK) gene. Lane 1: lambda phage DNA digested with HindIII (marker); Lanes 2 and 4: KOS viral DNA; Lanes 3 and 5: ΔgK-EGFP viral DNA; Lane 6: 1 kbp molecular weight ladder marker.
Figure 2.4. Western immunoblot analysis of recombinant EGFP viruses. Anti-GFP monoclonal antibody was used to detect EGFP expression in virus-infected cell extracts. Lane 1: KOS, lane 2: ΔgK-EGFP1, and lane 3: ΔgK-EGFP2. Molecular mass standards are as indicated.
Detection of EGFP expression by western immunoblot analysis. Anti-GFP monoclonal antibody (Clontech, Pala Alto, Calif.) was used in western blots of infected cell lysates to detect EGFP expression by recombinant viruses. Vero cells were infected with KOS (Fig. 2.4, lane 1), ΔgK-EGFP1 (Fig. 2.4, lane 2), and ΔgK-EGFP2 (Fig. 2.4, lane 3) at an MOI of 1. Twenty-four hours post infection, cellular extracts were prepared and EGFP expression was detected by western immunoblot analysis using anti-EGFP monoclonal antibody. The enhanced green fluorescent protein is expressed in cells as a 27 kDa monomeric protein which forms post-translationally an extremely stable homotrimERIC complex (Cody et al., 1993). EGFP was detected as a 27-kDa protein (Fig. 2.4, lanes 2 and 3), as well as a protein species with an apparent molecular weight corresponding to the predicted trimeric form (Fig. 2.4, lanes 2 and 3) in ΔgK-EGFP virus infected cell lysates. In contrast, the anti-GFP antibody did not detect any proteins in KOS infected cellular extracts (Fig. 2.4, lane 1).

Fluorescence and phase contrast microscopy of ΔgK-EGFP infected Vero cells. Vero cells were seeded in 15.5 mm culture dishes and infected at an MOI of 0.01. Cells were monitored by either fluorescence or phase contrast microscopy at different times post infection. Four to five h.p.i., individual cells emitted fluorescence, while there were no apparent cytopathic effects (CPE) including non-adherent cells or cell rounding. Fluorescence intensity increased drastically at later times after infection achieving a maximum intensity at 12-18 h.p.i. (not shown). All cells recruited into viral plaques emitted bright green fluorescence at 24 or 48 h.p.i. (Fig. 2.5: a, b). At 24 h.p.i., fluorescence microscopy detected viral plaque formation readily, while plaques were not discernible by phase contrast microscopy (Fig. 2.5: a, a'). At 48 h.p.i., cells within the center of viral
Figure 2.5. Autofluorescence and phase contrast microscopy of EGFP expressing viruses. Vero cells were infected with ΔgK-EGFP1 and analyzed by either fluorescence microscopy (panels: a, b) or phase contrast microscopy (panels: a’, b’) at 24 hours post infections (panels: a, a’) or 48 hours post infection (panels: b, b’). The same virus plaque is shown in panels a and a’ and a different plaque is shown in both panels b and b’.
Figure 2.6. Fluorescent cytomtery profiles of KOS and ΔgK-EGFP infected Vero cells. Vero cells were infected with either KOS (unshaded histogram) or ΔgK-EGFP1 (shaded histogram) at a multiplicity of infection of 3. Cytometric profiles were determined at 48 hours post infection using either unfixed infected Vero cells (panel a) or cells fixed with 3% paraformaldehyde (panel b).
plaques emitted bright green fluorescence, while newly infected cells in the periphery of viral plaques appeared to fluoresce less intensely (Fig. 2.5: b, b').

**FACS analysis of ΔgK-EGFP-1 infected cells.** EGFP has a single red-shifted excitation peak at 488 nm, which is ideal for automated analysis by fluorescence-activated cell sorting (FACS) instruments utilizing argon ion lasers and standard FITC filter sets (Cormack et al., 1996). To assess the ability of recombinant virus infected cells to be detected by FACS analysis, Vero cells were infected at an MOI of 3 with either KOS or ΔgK-EGFP1 viruses. Forty-eight h.p.i., the cells were pelleted by centrifugation, washed with PBS, and analyzed either directly (Fig. 2.6, panel a), or after fixation with 3% paraformaldehyde (Fig. 2.6, panel b). Overlay of histograms produced by KOS (unshaded histograms) and of ΔgK-EGFP1 infected cells (shaded histograms) (Fig. 2.6, panels a and b) revealed that greater than 97% of the EGFP-1 cells were intensely stained with EGFP, while KOS-infected cells appeared unstained. Paraformaldehyde fixation (panel b) did not significantly diminish fluorescence intensity relative to the unfixed cells (panel a).

**DISCUSSION**

Recombinant viruses were constructed expressing the EGFP gene cassette under the promoter control of the HCMV immediate early promoter. High levels of EGFP expression were achieved in virus-infected cells, which was easily detected by fluorescence microscopy and quantified by FACS analysis.
EGFP is capable of emitting bright green fluorescence within cells in cell culture or within animals under physiological conditions without the requirement of exogenous substances. In addition, EGFP is highly stable under varying pH conditions, detergents and reducing agents enabling the direct measurement of EGFP expression (Chalfie et al., 1994; Chalfie, 1995). Most importantly, EGFP labeling of infected cells did not affect the production of infectious HSV-1. These EGFP characteristics provide significant advantages over the detection of conventional enzymatic reactions such as that produced by the widely used β-gal protein, which requires the addition of toxic chromophoric substrates and other reagents that inactivate cells and viruses.

Recently, a PRV recombinant virus was constructed expressing the EGFP gene under the promoter control of the PRV G glycoprotein. It is important to note that there are significant differences in the amount and intensity of EGFP fluorescence reported for the PRV virus in comparison to the ΔgK-EGFP virus. The PRV EGFP cassette enabled the detection of newly infected cells during plaque formation, while initially infected cells ceased to fluoresce at later times after infection. This loss of fluorescence was probably due to down regulation of the PRV gG promoter at later times post infection. In contrast, EGFP expressed by the ΔgK-EGFP virus recombinant was detected early and increased at later times after infection without any loss of fluorescence intensity. This increased fluorescence emission must be due to high levels of EGFP expression under the HCMV-IE promoter control. The HCMV-IE promoter functions constitutively expressing EGFP immediately after the delivery of viral DNA into the nuclei of infected cells. EGFP expression by surrounding cells indicates viral replication into newly infected cells, and provides a sensitive monitoring method for virus spread. Alternatively, productive infection within individual cells could be achieved by the use of promoter sequences controlling the expression of structural proteins. It is worth
noting that EGFP gene cassettes under different HSV-1 promoter controls would be a valuable tool for the assessment of differential promoter utilization during viral infection \textit{in vitro} and \textit{in vivo}.

The \(\Delta gK\)-EGFP virus as well as other viruses constructed in this laboratory expressing EGFP genes will be instrumental in determining the role of \(gK\) and other glycoproteins in virus attachment, entry, virion maturation and egress. Furthermore, virions capable of expressing high levels of EGFP should be valuable tools in the tracing of viral infections in animals. In this regard, it is important to note that \(gK\) has been implicated in neurovirulence and increased herpetic keratitis disease (Moyal \textit{et al}, 1992; Ghiasi \textit{et al}, 1996). EGFP expression should enable the monitoring of viruses with or without \(gK\) in animal infections and the determination of the role of \(gK\) in neuroinvasiveness and herpetic eye disease.
CHAPTER III

FUNCTIONAL CHARACTERIZATION OF THE HVEA HOMOLOG SPECIFIED BY AFRICAN GREEN MONKEY KIDNEY CELLS THROUGH THE USE OF A HERPES SIMPLEX VIRUS EXPRESSING THE GREEN FLUORESCENCE PROTEIN

INTRODUCTION

Early steps during productive infection of eukaryotic cells by herpes simplex virus include initial binding of virions to cell surfaces mediated by binding of viral glycoproteins gB and gC onto glycosaminoglycan chains that are ubiquitous on most cell surfaces (Spear, 1993). Virion penetration into cells occurs via the pH-independent fusion of the viral envelope with the cell membrane or an early endosome (Wittels and Spear, 1991) and is mediated by at least viral glycoproteins gB, gD and gH-gL hetero-oligomers (Cai et al., 1988; Forrester et al., 1992; Ligas and Johnson, 1988; Roop et al., 1993; Sarmiento et al., 1979).

A gD receptor for virus entry, HveA, is an additional member of the tumor necrosis factor receptor family (Geraghty et al., 1998; Montgomery et al., 1996; Whitbeck et al., 1997). HveA is expressed in various tissues, including liver, lung, kidney, spleen, and peripheral leucocytes, and it is the principal receptor for entry into human lymphoid cells but not into other cell types (Montgomery et al., 1996). The cytoplasmic region of HveA binds to several members of the tumor necrosis factor-associated factor (TRAF) family, such as TRAF1, TRAF2, TRAF3, and TRAF5, but not to TRAF6. Transient transfection of HveA into human 293 cells causes activation of nuclear factor-kB (NF-kB), a transcriptional regulator of multiple immunomodulatory and inflammatory genes as well as activation of the

2 Reprinted by permission of Virology.
Jun-containing transcription factor AP-1, a regulator of cellular stress-response genes. These results suggest that HveA is associated with signal transduction pathways that activate the immune response (Marsters et al., 1997).

A second receptor for virus entry, HveB, is the poliovirus receptor-related protein 2 (Prr2). HveB mediates HSV-2, but not wild-type HSV-1 viral entry (Eberle et al., 1995). Apparently, certain mutants of HSV-1 utilize HveB for viral entry, and HveB but not HveA mediates pseudorabies virus entry. A third receptor, HveC, is the poliovirus receptor-related protein (Prr1), which is a human member of the immunoglobulin superfamily (Lopez et al., 1995). HveC mediates entry of several alphaherpesviruses, including HSV-1 and -2, pseudorabies virus (PRV) and bovine herpesvirus-1 (BHV-1). HveC is expressed in human cells of epithelial and neuronal origin, and is the primary candidate for the coreceptor that mediates HSV-1 and -2 viral entry into epithelial cells on mucosa surfaces and virus spread to cells of the nervous system (Geraghty et al., 1998). A fourth receptor, HveD, is the poliovirus receptor Pvr (Mendelsohn et al., 1989). HveD mediates the entry of PRV and BHV-1 but not of HSV-1 (Geraghty et al., 1998).

African green monkey kidney (Vero) cells are commonly used for the propagation of HSV-1 in many herpesvirus laboratories as well as for the determination of HSV-1 virus entry and egress. Therefore, we cloned, sequenced, and characterized the Vero HveA homolog, designated here as simian HveA (HveAs). Functional characterization of HveAs for HSV-1 virus entry was accomplished through the construction and use of a recombinant virus constitutively expressing the enhanced green fluorescence protein (EGFP), while retaining wild-type KOS-like characteristics. Our results indicate that HveAs is the functional homolog of HveA.
MATERIALS AND METHODS

Cells and viruses. African green monkey kidney (Vero) cells were obtained from ATCC (Rockville, MD). Cells were propagated and maintained in Dulbecco's Modified Eagles Medium (DMEM; Sigma Chemical Co., St. Louis, MO) containing sodium bicarbonate, 15 mM Hepes, and supplemented with 7% heat inactivated fetal bovine serum (FBS). V27 cells carry a stably integrated copy of the HSV-1 (KOS) ICP27 gene and were kindly provided by Dr. D. M. Knipe, Harvard Medical School. These cells were cultured in DMEM supplemented with 7% FBS (Rice and Knipe, 1990). HSV-1 (KOS), the parental wild-type strain used in this study, was originally obtained from Dr. P. A. Schaffer (Dana-Faber Cancer Institute, Boston, MA). HSV-1 (KOS) d27-1 which has a 1.6 kb BamHI-Stul deletion of the ICP27 gene was kindly provided by Dr. D. M. Knipe, Harvard Medical School and was propagated in V27 cells (Rice and Knipe, 1990).

Reagents. Restriction and DNA modification enzymes were obtained from New England BioLabs (Beverly, MA). RNase and Proteinase K were purchased from Boehringer Mannheim (Indianapolis, IN). Gel fragment purification matrix and buffers (Prep-a-gene) were obtained from BioRad (Hercules, CA). Sequencing grade [\(^{35}\text{S}\)] dATP was obtained from DuPont/NEN (Wilmington, DE). Amplitaq, rTth XL Polymerase, and deoxynucleotide triphosphates were purchased from Perkin Elmer (Foster City, CA.). All synthetic oligonucleotide primers were synthesized by the LSU Gene Probes and Expression Systems Laboratory “GeneLab” using phosphoamidite chemistry on an Applied Biosystems ABI394 DNA/RNA synthesizer with Perkin Elmer (Foster City, CA) reagents.
Isolation of the HveAs gene and construction of plasmids. Total RNA was extracted from Vero cells using TRIReagent (Molecular Research Center, Inc., Cincinnati, OH). A cDNA library was constructed using Ready-To-Go You-Prime First-Strand Beads (Pharmacia Biotech Inc., Uppsala, Sweden). All PCR amplifications were performed using the Gene-Amp PCR system 9600, AmpliTaq, and other Perkin Elmer reagents (Perkin-Elmer, Norwalk, CT). PCR products were derived using HveA-specific primers (Montgomery et al., 1996), and were cloned into plasmid pCR2.1 using the TA-cloning kit (Invitrogen Inc., Carlsbad, CA) producing plasmid pCR/HveAs. The cloned HveAs gene was sequenced using dideoxy-chain termination methodology, and the predicted primary structure of the protein was obtained (GeneBank Accession number AF147720). The HveAs gene was isolated from plasmid pCR/HveAs after restriction with EcoRI and cloned into plasmid pcDNA3.1Zeo+ in the coding and non-coding orientations producing plasmids pCMV/HveAs and pCMV/sAevH, respectively. The extracellular domain of HveAs (first 202 amino acids) was fused to a DNA fragment specifying the Fc portion of the mouse IgG heavy chain using PCR-assisted splice-overlap-extension (Choulenko et al., 1996), and cloned into plasmid pCR3.1 using the eukaryotic TA-cloning kit (Invitrogen, Inc.) producing plasmid pCR/HveAs:Fc. This portion of the HveAs gene was also cloned into plasmid pMALc2 (NEB, Beverly, MA) producing plasmid pMAL/HveAs. Plasmid pTF9202 was constructed by inserting the gK gene, after amplification with primer pair UL52KpnI2/gKSpeI (5'-AACACCAACACT AGTGGTGGATGTCCCTTATACC-3'), into the unique KpnI and SpeI sites of
pTF9201 (Foster et al., 1998). This plasmid was used to transfer the EGFP gene into the d27-1 viral genome.

Construction of the HSV-1 (KOS)/EGFP recombinant virus. Plasmid pTF9202 specifying the CMV-EGFP gene cassette flanked by homologous HSV-1 sequences was transfected into 50% confluent Vero cells using Lipofectamine (GIBCO-BRL, Gaithersburg, MD) according to the manufacturer's instructions. Twenty-four hours post transfection (h.p.t.), the cells were infected at a multiplicity of infection (MOI) of 10 with d27-1 virus (ICP27- null) as described previously for the generation of the gK null virus, ∆gK (Jayachandra et al., 1997). At forty-eight hours post infection (h.p.i.), the cells were freeze-thawed three times, and the resultant virus stocks were plated onto Vero cells and overlaid with agarose. Virus plaques were visualized by fluorescent microscopy, individual virus plaques were isolated, and virus was plaque purified five times on Vero cells.

Diagnostic PCR. Primer pairs UL52KpnI2/gKTr2, and UL52KpnI2/CMVBamHI were used to confirm the EGFP gene insertion as described previously (Foster et al., 1998). Primer pair UL52KpnI2/d27-1α was used to detect the presence of contaminating d27-1 viral DNA. Reaction conditions for UL52KpnI2/gKTr2 and UL52KpnI2/d27-1α pairs were 98°C for 3 sec., 72°C for 7 min, repeated for 30 cycles using rTth XL-Polymerase (Perkin-Elmer, Inc.). Reaction conditions for the UL52KpnI2/CMVBamHI primer pair were 98°C for 3 sec. and 72°C for 3 min, repeated for 30 cycles using rTth XL-Polymerase.

Construction of Chinese hamster ovary (CHO) cell lines transformed with the HveAs or HveAs:Fc genes. Plasmid pCMV/HveAs, pCMV/sAevH, and pCR/HveAs:Fc were transfected into CHO cells using LipofectAmine™ and
transformed cells were selected using Zeocin for the first two plasmids (Invitrogen, Carlsbad, CA) and G418 (Sigma, Inc.) for the pCR/HveAs:Fc plasmid. Individual CHO colonies were isolated, expanded, and tested for the presence of the HveAs gene using PCR and Southern blotting.

**Production of anti-HveAs-specific antibodies.** Plasmid pCMV/HveAs was used to generate mouse anti-HveAs antibodies by direct injection into mice (genetic immunization). Purified plasmid DNA was inoculated intramuscularly into mice five times approximately every four weeks. HveAs was expressed in *E. coli* as a fusion protein with the maltose binding protein (MBP) by plasmid pMAL/HveAs and purified according to the manufacturer's instructions (New England Biolabs, Beverly, MA). Anti-HveAs antibodies were tested in immunoblots against the MBP/HveAs fusion protein essentially as described previously (Foster *et al.*, 1998).

**Production of HveA:Fc by CHO-transformed cells.** HveAs:Fc protein secreted in supernatant fluids of approximately $10^7$ CHO/HveAs:Fc-transformed cells was purified using protein A sepharose beads. Samples were resuspended in SDS-PAGE sample buffer and electrophoretically separated in a 10% SDS-polyacrylamide gels. Following electrophoretic separation, proteins were electrotransferred to nitrocellulose membranes. Blots were blocked against non-specific binding and incubated with horseradish peroxidase conjugated goat anti-mouse secondary antibody (Pierce, Rockford, IL) at a 1:50,000 dilution in TBS-T. Blots were visualized by autoradiography using the Pierce SuperSignal.
chemiluminescent detection kit (Pierce, Rockford, IL) as per the manufacturer’s instructions.

**Phase-contrast and fluorescent microscopy.** Subconfluent cell monolayers in 24-well plates were infected with the KOS/EGFP virus at an MOI of 0.02 for visualization of individual viral plaques by either phase contrast or fluorescence microscopy essentially as described previously for the ΔgK/EGFP virus (Foster *et al.*, 1998). Viral entry experiments were performed at an MOI of 10 and visualized by fluorescence microscopy at 24 h.p.i.

**FACS analysis.** CHO-transformed cells expressing HveAs or Vero cells were fixed with paraformaldehyde and incubated with anti-HveAs antibody for 1 hour at 4°C. Next, cells were washed three times with PBS buffer, incubated with goat-anti-mouse FITC-conjugated secondary antibody (Sigma, Inc.), and analyzed by FACS. KOS and KOS/EGFP-infected Vero cells were analyzed by FACS at 24 h.p.i. as described previously (Foster *et al.*, 1998).

**Inhibition of virus entry by anti-HveAs antibody and soluble HveA-Fc protein.** CHO/HveAs cells were incubated with either pre-immune or anti-HveAs serum for 1 hour at room temperature immediately prior to infection with KOS/EGFP at an MOI of 10. One ml of supernatant fluids from either CHO/HveAs:Fc or CHO cells transformed with pCR3.1 (control) was mixed with one ml of KOS/EGFP virus stock (1 x 10^8 PFU/ml) and incubated at 4°C for 1 hour

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
immediately prior to infection. The amount of virus used in these experiments was approximately $10^6$ PFU per $10^5$ CHO/HveAs cells.

RESULTS

Construction and genetic characterization of the recombinant virus HSV-1 (KOS)/EGFP. Recently, we constructed the recombinant virus ΔgK/EGFP expressing an engineered enhanced green fluorescent protein (EGFP) from a gene cassette inserted in place of the glycoprotein K (gK) gene (Foster et al., 1998). To facilitate our studies on HSV-1 entry, we engineered the virus KOS/EGFP constitutively expressing EGFP, by inserting the HCMV-EGFP gene cassette into the intergenic region between the UL53 (gK) and the UL54 (ICP27) genes. First, plasmid pTF9202 was constructed to contain the HCMV-EGFP cassette in the intergenic region between the UL53 and UL54 genes. Next, the deleted ICP27-UL54 region of the d27-1 viral genome was rescued by plasmid pTF9202, while simultaneously transferring the CMV-EGFP gene cassette into the viral genome (Fig. 3.1). Similar methodologies were used to construct the ΔgK virus (Jayachandra et al., 1997) and the ΔgK/EGFP virus (Foster et al., 1998). Virus stocks from marker-rescue experiments were collected, individual virus plaques were plaque purified five times, and virus isolates were tested by diagnostic PCR. Primer gKTr2 is located within the deleted portion of the UL54 gene (Fig. 3.1c). Amplification with this primer is possible only if the UL54 gene has been rescued by the pTF9202 plasmid. Complementary DNA sequences to primer gKTr2 are present in plasmid
Figure 3.1. Construction of recombinant KOS and ΔgK viruses constitutively expressing the EGFP gene. (a) The top line represents the prototypic arrangement of the HSV-1 genome indicating approximate map units. (b) The region of the mutant virus HSV-1 d27-1 genome containing the UL52, UL53, and partially deleted UL54 genes with relevant restriction endonuclease sites. (c) Plasmid pTF9201, which contains the CMV-IE-EGFP gene cassette in place of the gK gene (Foster et al, 1998). (d) Plasmid pTF9202 was produced by inserting the gK gene within the unique KpnI/SpeI sites of plasmid pTF9201. The approximate location of PCR primers UL52KpnI2, gKTr2, and CMVBamHI are shown (b-d).
pTF9202 immediately upstream and downstream of the CMV/EGFP gene cassette. (Fig. 3.1: c, d). Therefore, amplification with primer pair UL52KpnI2/gKTr2 generated two DNA fragments of 1,569 bp and 3,261 bp against KOS/EGFP viral DNA (Fig. 3.2, lane 3), while only the 1,569 bp DNA fragment was generated against KOS viral DNA (Fig. 3.2, lane 2). Primer pair UL52KpnI2/CMVBamHI detected the presence of the EGFP gene cassette within the KOS/EGFP viral genome as evidenced by the PCR-amplified DNA fragment of 2,401 bp (Fig. 3.2, lane 5), whereas it did not produce a PCR product against the KOS viral genome (Fig. 3.2, lane 4). Additional PCR testing was performed to ensure that the KOS/EGFP virus was not contaminated with the d27-1 virus using primer pair UL52KpnI2/d27-1α (Fig. 1b). This primer pair produced the predicted size DNA fragments of 3,241 bp and 1,613 bp against KOS and d27-1 viruses (Fig. 3.2, lanes 6, and 8, respectively), and the predicted DNA fragment of 4,889 bp against the KOS/EGFP virus (Fig. 3.2, lane 7). A second DNA fragment of 650 bp was of non-specific origin.

**Plaque phenotype, EGFP expression, and growth characteristics of the KOS/EGFP virus.** The KOS/EGFP virus produced viral plaques similar in appearance to those produced by the wild-type virus KOS (Fig 3.3: a, c). Bright green fluorescence was detected in all infected cells, when viral plaques were examined under the fluorescent microscope (Fig. 3.3b). Fluorescence from Vero cells infected with either KOS or KOS/EGFP viruses at an MOI of 5 was detected using FACS at 24 h.p.i. Nearly, all KOS/EGFP-infected cells emitted bright
Figure 3.2. Diagnostic PCR of plaque purified KOS/EGFP recombinant virus. DNA PCR products with the UL52KpnI2/gKTr2 primer pair (lanes 2 and 3) or the UL52KpnI2/CMVBamHI primer pair (lanes 4 and 5) used to detect the EGFP gene. Lanes 6-8: PCR diagnostic with primer pair UL52KpnI2/d27-1α used to determine the purity of viral stocks. Lanes 2, 4, and 6: KOS viral DNA. Lanes 3, 5, and 7: KOS/EGFP viral DNA. Lane 8: d27-1 viral DNA. Lane 1: lambda phage DNA digested with HindIII (marker). Lane 9: 1 kb molecular weight ladder (marker).
Figure 3.3. Morphology and fluorescence detection of KOS/EGFP virus infections. Vero cells were infected with either KOS (c) or KOS/EGFP virus (a and b) at a multiplicity of infection of 0.001 in 24 well plastic tissue culture plates. Individual viral plaques were photographed through the use of phase-contrast (a and c) and epi-fluorescence (b) microscopy under live conditions at 48 hours post infection. FACS analysis of KOS- and KOS/EGFP-infected cells (MOI of 5) was performed at 48 h p.i. (d) as described previously (Foster et al, 1998).
fluorescence, whereas KOS-infected cells did not emit any fluorescence (Fig. 3.3d).

To determine the replication characteristics of the KOS/EGFP virus, Vero cell monolayers were infected in triplicates with either KOS or KOS/EGFP virus at an MOI of 10, and virus stocks were prepared at 12, 24, 36, and 48 h.p.i. The KOS/EGFP virus replicated to high titers approaching those of the KOS parental virus (Table 1).

Isolation of the gene coding for the Vero HveA homolog (HveAs) and comparison of the HveA and HveAs predicted amino acid sequences. Primers specific for the HveA gene were used to isolate and clone the HveAs cDNA specified by Vero cells. Translation of the largest open reading frame of the HveAs cDNA sequence produced a putative protein of 283 amino acids sharing a high degree of homology with the previously described HveA receptor gene (Montgomery et al., 1996). On the basis of the homology to HveA, this protein was designated the HveA simian homolog (HveAs). A comparison of the predicted amino acid sequences of HveA and HveAs is shown in Figure 3.4. The two proteins were of equal length (283 amino acids), but contained a number of amino acid differences including: i) Forty-one amino acid substitutions of which twenty-four were to similar amino acids and seventeen were to dissimilar amino acids; ii) Amino acid deletions and insertions principally located within the intramembrane sequences. Specifically, HveAs contained a single amino acid insertion (aa 187 of HveAs) and a single amino acid deletion (aa 212 of HveA), and a segment of deleted amino acids (aa 216-218 of HveA) and inserted amino acids (aa 216 and aa
Table 3.1. Viral yields of KOS and KOS/EGFP viruses.

<table>
<thead>
<tr>
<th>Virus</th>
<th>12 hpi</th>
<th>24 hpi</th>
<th>36 hpi</th>
<th>48 hpi</th>
</tr>
</thead>
<tbody>
<tr>
<td>KOS</td>
<td>$5.0 \times 10^7$</td>
<td>$1.5 \times 10^8$</td>
<td>$2.0 \times 10^8$</td>
<td>$2.0 \times 10^8$</td>
</tr>
<tr>
<td>KOS-EGFP</td>
<td>$3.0 \times 10^7$</td>
<td>$1.0 \times 10^8$</td>
<td>$1.4 \times 10^8$</td>
<td>$1.5 \times 10^8$</td>
</tr>
</tbody>
</table>

Note: Subconfluent Vero cell monolayers (approximately $8 \times 10^5$ cells) were infected with each virus at an MOI of 5 and at 12, 24, 36, and 48 hours post infection the total number of infectious virions was determined. Viral titers represent one of three experiments in which individual numbers varied by less than 2-fold.
Figure 3.4. Comparison of the amino acid sequences of HveAs and HveAh. The HveAs (top lines) and HveAh (bottom lines) amino acid sequences were aligned through the use of computer assisted algorithms and visual inspection. Both proteins are 283 amino acids long. Relevant structural features are marked as indicated.
Characteristically, the carboxyl terminus of HveA contained three extra amino acids (P-N-H) in comparison with HveAs.

**Functional characterization of the HveAs receptor.** The ability of HveAs to mediate HSV-1 virus entry was assessed through the use of HveAs-transformed CHO cells, anti-HveAs antibody, and soluble HveA protein. Anti-HveAs serum was produced by genetic immunization using plasmid pCMV/HveAs, and it was tested for its ability to react with HveAs expressed in *E. coli* as a fusion protein with the maltose-binding protein (MBP). Anti-HveAs serum reacted with a protein species migrating with an apparent molecular mass of 70 kDa in agreement with the expected apparent molecular mass of electrophoretically separated MBP/HveAs fusion protein (Fig. 3.5a, lane 2 of immune samples). Anti-HveAs serum did not react against MBP expressed in *E. coli* (Fig. 3.5a, lane 1 of immune samples). Similarly, pre-immune sera did not react with either MBP or MBP/HveAs proteins (Fig. 3.5a, lanes 1 and 2, respectively, of pre-immune samples).

Next, CHO cells were transformed with either pCMV/HveAs or pCMV/sAevH. Plasmid pCMV/sAevH contains the HveAs gene in the opposite (noncoding) orientation. HveAs expression by CHO/HveAs and Vero cells was assessed using anti-HveAs serum and FACS analysis. Anti-HveAs serum reacted strongly with paraformaldehyde-fixed Vero cells and CHO/HveAs cells (Fig. 3.5, solid histograms in panels b and c, respectively). No background fluorescence was observed in control experiments, in which Vero cells were incubated with pre-immune sera prior to incubation with goat-anti-mouse FITC-conjugate (Fig. 3.5,
Figure 3.5. Detection of HveAs expression in *E. coli*, Vero, and HveAs-transformed CHO cells using anti-HveAs serum. (a) Immunoblot of electrophoretically separated HveAs/MBP fusion protein after expression in *E. coli* using anti-HveAs serum produced by genetic immunization. Pre-immune and immune sera were tested as shown. Lanes labeled 1: MBP control. Lanes labeled 2: MBP/HveAs fusion protein. (b) Vero cells were analyzed by FACS after reaction with either anti-HveAs antibody followed by reaction with anti-mouse-IgG labeled with fluorescein (filled histogram) or with pre-immune sera as the primary antibody (unfilled histogram). (c) CHO-HveAs- (filled histogram) and CHO/sAevH- (unfilled histograms) transformed cells were analyzed by FACS to detect HveAs expression using anti-HveAs antibody.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Figure 3.6. Immunoblot detection of HveAs:Fc expression from CHO/HveAs:Fc transformed cells. Supernatants from either HveAs:Fc transformed CHO cells or naïve CHO/sAevH control cells were subjected to protein A sepharose purification and prepared for immunoblot analysis. Immunoblots were reacted with goat-anti-mouse HRP reagent. Lane 1: reaction with supernatant concentrate from control CHO cells. Lane 2: reaction with supernatant concentrate from HveAs:Fc-transformed CHO cells.
unfilled histogram of panel b). Anti-HveAs antibody did not react with CHO/sAevH cells (Fig. 3.5, unfilled histogram of panel c).

To facilitate virus entry blocking experiments, soluble HveAs protein was produced by first, constructing a gene cassette coding for the extracellular portion of HveAs fused to the Fc portion of mouse IgG. Next, CHO cells were transformed with plasmid pCR/HveAs:Fc capable of constitutively expressing HveAs:Fc protein, and HveAs:Fc protein was purified from supernatant fluids of these cells using protein A sepharose beads (see Materials and Methods). The authenticity of HveAs:Fc protein was tested in immunoblots using anti-mouse-IgG antibody. A protein species with an apparent molecular mass of approximately 60 kDa was detected in immunoblots of electrophoretically separated HveAs:Fc protein (Fig. 3.6, lane 2), while anti-mouse IgG antibody did not react with supernatants from CHO/sAevH control cells (Fig. 3.6, lane 1). The HveAs:Fc purified protein was sensitive to digestion by PNGase F, indicating that it contained N-glycosylated carbohydrates (not shown). A glycosylated protein with similar apparent mass was produced by a HveA:Fc fusion protein (Montgomery et al., 1996).

Virus entry experiments were performed by monitoring EGFP expression specified by KOS/EGFP virus. KOS/EGFP infection (MOI 5) of CHO/HveAs cells resulted in most cells emitting green fluorescence at 24 h.p.i. indicating successful viral entry (Fig. 3.7A). In contrast, practically none of the KOS/EGFP-infected CHO/sAevH control cells emitted fluorescence (Fig. 3.7B). Next, experiments were performed using the soluble HveAs:Fc protein. Pre-incubation of KOS/EGFP virions with supernatant fluids from CHO/sAevH control cells did not reduce the number of fluorescent cells, while pre-incubation with purified HveAs:Fc protein
Figure 3.7. Blocking of virus entry by HveAs:Fc soluble protein and by anti-HveAs antibodies. (A and C-F) CHO/HveAs cells infected with KOS/EGFP virus at an MOI of 10. (A) Untreated control. (B) CHO/sAevH control cells (transformed with the HveAs gene in the noncoding orientation). (C and D) CHO/HveAs cells were pretreated with pre-immune or anti-HveAs serum, respectively, for 30 minutes at room temperature prior to infection. (E and F) KOS/EGFP virus was pre-treated with CHO/sAevH control supernates and soluble HveAs:Fc, respectively, for 30 minutes at 4° C prior to infections.
reduced the number of fluorescent cells substantially (Fig. 3.7: C, D, respectively).
Similarly, treatment of the CHO/HveAs cells with pre-immune control serum prior to infection with KOS/EGFP virus did not reduce the number of fluorescent cells, while treatment with anti-HveAs antibody serum caused a substantial decrease in the number of fluorescent cells (Fig. 3.7: E, F, respectively).

DISCUSSION

We report here the functional characterization of the herpes simplex HveA receptor homolog specified by African green monkey kidney cells, designated here as HveAs. These studies were undertaken because Vero cells constitute our primary cells in which virus entry and egress are studied. Investigation of the ability of HveAs to mediate HSV-1 virus entry was achieved through the use of a novel wild-type KOS-like herpesvirus constitutively expressing the EGFP protein immediately after viral entry into cells.

The KOS/EGFP virus. We inserted the EGFP gene cassette within the intergenic region between the UL53 and UL54 genes, immediately after the poly-A sequence of the UL53 gene. The purpose of this construction was to enable the monitoring of viral entry through the detection of EGFP fluorescence. In addition, EGFP could serve as a fluorescent marker for the isolation of recombinant viruses containing gK mutations by simultaneously transferring the mutated gK genes and the EGFP gene cassette to viral genomes. EGFP was selected because this protein is capable of emitting bright green fluorescence within cells in cell culture under physiological conditions without the requirement of exogenous substances. In
addition, EGFP is highly stable under varying pH conditions, detergents and reducing agents enabling the direct measurement of EGFP accumulated expression (Chalfie, 1995; Chalfie et al., 1994). In this regard, EGFP detection provides a useful alternative to β-galactosidase and other enzyme detection method because it does not require addition of toxic reagents that inactivate cells and viruses.

The growth characteristics and plaque morphology of the resultant KOS/EGFP virus were similar to wild-type KOS virus, indicating that insertion of the EGFP gene cassette between the two essential genes, UL52 and UL54 as well as expression of the EGFP proteins did not adversely affect virus replication. High levels of EGFP expression were achieved in KOS/EGFP virus-infected cells that was easily detected by fluorescence microscopy and quantified by FACS analysis. KOS/EGFP fluorescence was detected through the use of a fluorescent microscope as early as 5-6 hours post infection and fluorescent plaques were readily visualized at 12 h.p.i. Fluorescence emitted from cells located within the center of each plaque increased with time, whereas newly recruited cells appeared weakly fluorescent until EGFP accumulated at later times. This radial feature of fluorescence emission enables an improved, time-dependent visualization of KOS/EGFP plaque formation in comparison to phase-contrast microscopy.

**HveAs mediates HSV entry.** The functionality of the HveA receptor was demonstrated principally by showing that CHO/HveA-transformed cells facilitated HSV-1 entry, and that HSV-1 entry into these cells was blocked by either soluble HveA:Fc protein or anti-HveA antibodies (Montgomery et al., 1996). We performed similar experiments with the exception that viral entry into cells was monitored through fluorescence detection of the EGFP protein specified by the KOS/EGFP virus. Collectively, these experiments showed that HveAs mediates HSV virus entry in a specific manner. Undiluted amounts of anti-HveAs antibody and CHO cell
supernatants containing soluble HveAs:Fc protein reduced viral entry by approximately 40%, and 20%, respectively, as measured by FACS (not shown). The inability of anti-HveAs antibody and soluble HveAs:Fc to fully inhibit virus entry into Vero cells is either due to the use of the blocking reagents at non-saturating conditions or the presence of the HveC and other receptors on Vero cell surfaces (Geraghty et al., 1998).

The HveAs protein. Comparison of the cDNA-predicted amino acid sequences of HveA and HveAs proteins revealed conservation of all cysteine residues with the exception of a single cysteine missing in the membrane-spanning portion of HveAs with respect to HveA. Furthermore, overall structural features, which identified the HveA protein as a homolog to the TNF family of proteins, were conserved (Montgomery et al., 1996). However, there was a significant divergence at the amino acid level between the HveA and HveAs proteins. Most amino acid substitutions were concentrated within specific regions of the amino terminus of the molecule and were to similar amino acids that are not expected to drastically alter the secondary structure of the molecule. However, certain amino acid substitutions were to dissimilar amino acids, which may alter structural properties of the protein. These were: S9 to P9, R12 to W12, P14 to S14, T22 to R22, S33 to P33, Q70 to L70, S79 to P79, T113 to R113, A115 to E115, A140 to R140, S172 to P172, G182 to Q182 and N183 to T183 in the extracellular portions of the proteins, and S233 to P230, I256 to T253, T258 to I254, and E276 to I273 in the cytoplasmic portions of the proteins. Significant insertions and deletions were noted in the predicted intramembranous sequences of the HveAs and HveA proteins including a deletion, which eliminated one cysteine residue in the HveAs sequence in comparison to the HveA sequence. However, both intramembranous sequences exhibited similar hydrophobic potential indicating that both proteins should be effectively anchored.
into membranes. The carboxyl terminus of HveA ended in amino acids P-N-H, which were not present in the carboxyl terminus of the HveAs protein. Deletion of the terminal 26 amino acids of the HveA protein did not adversely affect the ability of this protein to mediate herpes simplex virus entry (Montgomery et al., 1996); therefore, the carboxyl terminal differences between HveAs and HveA are not expected to significantly alter the ability of HveAs to mediate HSV virus entry in comparison to HveA.

The primary sequences of gD specified by HSV and simian herpes B virus (SHBV) are 58% identical. However, regions of gD that are important for HSV-1 virus entry into Vero cells are highly conserved in HSV-1, HSV-2, and SHBV (Bennett et al., 1992; Chiang et al., 1994). Furthermore, anti HSV-1 gD monoclonal antibodies that block HSV-1 binding to soluble HveA recognize epitopes that overlap or are adjacent to these conserved regions (Nicola et al., 1998). HSV enters into both HveA and HveAs-transformed cells efficiently, suggesting that conserved domains in the extracellular portions of HveA and HveAs proteins may serve as common functional sites for binding gD during viral entry.
CHAPTER IV

HERPES SIMPLEX VIRUS TYPE 1 (HSV-1)
GLYCOPROTEIN K (gK) IS A STRUCTURAL COMPONENT
OF PURIFIED VIRIONS THAT FUNCTIONS TO MODULATE
HVEAS RECEPTOR MEDIATED VIRUS ENTRY

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) encodes eleven glycoproteins, designated gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, and gM, that function at various stages during a productive infection of eukaryotic cells (Roizman and Sears, 1996). At least four of these glycoproteins, gB, gD, and the gH/gL hetero-oligomer, participate in HSV-1 entry into cells (Cai et al., 1988; Forrester et al., 1992; Roop et al., 1993). Initial binding of virions to cell surfaces is mediated through the interactions of viral gC and/or gB with heparin sulfate proteoglycans found ubiquitous on most cell surfaces; however, binding of virus to cells is not sufficient to facilitate penetration (Herold et al., 1991; Herold et al., 1994). Binding events that lead to virus penetration and uncoating can be distinguished experimentally as heparin sulfate sensitive (initial binding) and heparin sulfate resistant (stable attachment) (WuDunn and Spear, 1989; Lycke et al., 1991; Shieh et al., 1992). Stable attachment therefore requires additional non-heparin sulfate receptors found at cell surfaces.

Several human cellular receptors have been identified as herpesvirus receptors by their ability to mediate virus entry into normally HSV resistant Chinese hamster ovary (CHO) cells. These receptors include HveA, a member of the tumor
necrosis factor receptor family (Montgomery et al, 1996), as well as several members of the immunoglobulin superfamily, HveB, HveC, HveD, and H\(\text{IgR}\) (Cocchi et al, 1998a; Geraghty et al, 1998; Warner et al, 1998). Various homologs of some of these receptors have also been isolated from other cell-types (Foster et al, 1999; Shukla et al, 1999). HveA is expressed in various tissues including liver, lung, kidney, spleen, and peripheral leukocytes, and it is the principal mediator for HSV-1 entry into human lymphoid cells, but not other cell types. However, HveA failed to mediate the entry of certain viable mutants of HSV-1 (those carrying mutations in gD), as well as other alphaherpesviruses (Montgomery et al, 1996). HveC and the splice variant isoform of HveC, H\(\text{IgR}\), are capable of mediating entry of human and animal alphaherpesviruses, including HSV-1 and -2, pseudorabiesvirus (PRV-1), and bovine herpesvirus (BHV-1). HveC and H\(\text{IgR}\) are expressed in human cells of epithelial and neuronal origin and are therefore the primary candidates for co-receptors that mediate entry into epithelial cells at the initial site of infection and into neuronal cells for the establishment of latency (Cocchi et al, 1998a; Geraghty et al, 1998).

Soluble forms of HveA, HveC, and H\(\text{IgR}\) have all been shown to bind to HSV-1 gD (Cocchi et al, 1998b; Krummenacher et al, 1998; Nicola et al, 1998; Rux et al, 1998; Willis et al, 1998). However, whereas the amino terminal of gD was important for interaction with HveA, it was not directly involved in HveC binding (Krummenacher et al, 1998), indicating that different regions of gD are capable of interacting with different receptors. Although interaction of gD with these receptors does facilitate stable attachment of virions, the molecular mechanisms that dictate
virus penetration following receptor interaction remain obscure. Penetration of virion particles into cells occurs through a pH-independent direct fusion of the virus envelope with cellular membranes and is mediated by viral glycoproteins gB, gD, and gH/gL, although other viral glycoproteins may also be involved.

Despite being two distinct processes, fusion of the virion particle to cell surfaces is often paralleled with virus induced cell-to-cell fusion. This correlation is drawn due to the involvement of many of the same viral and cellular components in each of these processes and because both involve membrane fusion. Mutations, which cause extensive virus induced cell-to-cell fusion can arise in at least four genes of the HSV genome: the UL53 (gK) gene (Bond and Person, 1984; Debroy et al, 1985; Pogue-Geile et al, 1984), the UL27 (gB) gene (Bzik et al, 1984; Pellet et al, 1982), the UL20 gene (Baines et al, 1991; MacLean et al, 1991), and the UL24 gene (Jacobson et al, 1989; Sanders et al, 1982). HSV-1 gB, gD, gH, gL, and UL45 have all been shown to be required for syncytia formation to occur, while other viral glycoproteins including gI, gE, and gM may enhance syncytia formation. In contrast, gC may inhibit syncytia formation of some cultured cells and this may account for the absence or altered expression of gC by many syncytial mutants. Although little is known about its function in cell-to-cell fusion, syncytial mutations within the UL53 (gK) gene are more frequently isolated than any other gene (Bond and Person, 1982; Bond and Person, 1984; Dolter et al, 1994).

HSV-1 gK has characteristics of a membrane-spanning protein including an N-terminal signal sequence, two potential sites for N-linked glycosylation, and three putative membrane spanning domains (Debroy et al, 1985; Mo and Holland, 1997;
Glycoprotein K exists as a single 40 kDa species in infected cells (Hutchinson et al., 1992), while gK translated in vitro had an apparent molecular mass of 36 kDa and N-linked glycosylation occurred within the first 112 amino acids, consistent with glycosylation at residues 48 and 58 (Ramaswamy and Holland, 1992). Although gK has been described as a structural component of other alphaherpesviruses including PRV-1 (Klupp et al., 1998) and varicella-zoster virus (VZV) (Mo et al., 1999), detection of HSV-1 gK in purified virions has been elusive. Recombinant viruses that lack gK or that contain mutations within critical amino acid motifs replicated inefficiently and were unable to egress from infected cells, indicating that gK plays a crucial role infectious virus production and translocation of infectious particles to extracellular spaces (Foster and Kousoulas, 1999; Hutchinson and Johnson, 1995; Jayachandra et al., 1997).

African Green Monkey Kidney (Vero) cells constitute the primary cell-line for propagation of HSV-1, as well as the determination of virus entry and egress. Recently, we described the isolation from Vero cells of a simian homolog (HveAs) to the human HveA (HveAh) receptor (Montgomery et al., 1996) that was capable of mediating entry of wild-type HSV-1 that expressed the enhanced green fluorescent protein (Foster et al., 1999). Although there was significant amino acid homology, the HveAs specified several amino acid substitutions, deletions, and insertions relative to HveAh (Foster et al., 1999; Montgomery et al., 1996). In this study, we generated antibodies against domains of gK and identified HSV-1 gK as a structural component of purified virions. To characterize the role of gK in virus entry, we tested the ability of gK null viruses to enter into Vero cells, as well as to utilize
various cellular receptors. Our results indicate that in the absence of gK, the kinetics of HSV-1 entry into Vero cells are delayed. Furthermore, gK null virions cannot efficiently utilize currently characterized individual HSV-1 receptors for virus entry.

MATERIALS AND METHODS

Cells and Viruses. African green monkey kidney (Vero) cells were obtained from ATCC (Rockville, MD). Cells were propagated and maintained in Dulbecco's Modified Eagles Medium (DMEM; Sigma Chemical Co., St. Louis, MO) containing sodium bicarbonate, 15 mM Hepes, and supplemented with 7% heat inactivated fetal bovine serum (FBS). The gK-transformed cell line VK302 was obtained from D. C. Johnson, Oregon Health Sciences University, and was maintained in DMEM lacking histidine (GIBCO Laboratories, Grand Island, NY) supplemented with 7% FBS and 0.3 mM histidinol (Sigma Chemical Co. St. Louis, MO) (Hutchinson and Johnson, 1995). CHO cells that were transformed with HveAh, HveB, and HveC were a gift of P.G. Spear, University of Northwestern, Chicago, IL and were maintained in Ham's F12 supplemented with 10% FCS, 500μg/ml G418, and 75μg/ml purimycin. CHO cells transformed with the HveAs gene were described previously (Foster et al, 1999). All cells were passaged once in the absence of selection prior to infection. The parental wild-type strain used in this study HSV-1 (KOS) was originally obtained from P. A. Schaffer (University of Pennsylvania, Philadelphia, PA). The gK-null virus ΔgK was propagated either on Vero cells and
was as described previously (Jayachandra et al, 1997). KOS-EGFP and ΔgK-EGFP are viruses that express the enhanced green fluorescent protein upon entry and were described previously (Foster et al, 1998; Foster et al, 1999).

**Construction of expression plasmids.** The sequence coding for domain II of gK (amino acids 140 to 225) or domain III of gK (amino acids 240 to 310) (Figure 4.1) were amplified by PCR using the primer pairs gKdom25’ (5’TTCTTCGGA TCCCAACACGCGATGTAT3’)/gKdom23’ (5’TTCTTCGAAATTGC TTAGCATCAACTCGCA3’) and gKdom35’ (5’TTCTTCGGAATCCGCGGGGGGCG ATGTGCG3’)/ gKdom33’ (5’TTCTTCGGAATTGCGGATGCGGATGCC3’) respectively. The 5’ primers, gKdom25’ and gKdom35’, included BamHI restriction sites; whereas, the 3’ primers, gKdom23’ and gKdom33’, included EcoRI restriction sites. The amplified PCR fragments were restriction digested with BamHI and EcoRI (New England Biolabs) and cloned into the BamHI and EcoRI sites of pGEX2T (Pharmacia) generating pGEX-dom2 and pGEX-dom3. Plasmids pGEX-dom2 and pGEX-dom3 specified GST fusions with domain II and domain III of gK, respectively. Each DNA construct was verified by dideoxy chain termination sequencing to ensure that the gK gene fragments were in frame with the GST peptide coding sequence.

**Expression and purification of fusion proteins.** *Escherechia coli* (*E. coli*) strain DH5α (GIBCO-BRL) was transformed with either pGEX2T or the pGEX-gK derivatives pGEX-dom2 or pGEX-dom3. A single colony of each was inoculated into 5ml LB broth (ampicillin 100μg/ml) and grown overnight at 37°C. The
Figure 4.1. Domains of gK expressed as fusion products with GST.
Schematic diagram depicting the predicted secondary structure of HSV-1 gK as described previously (Foster and Kousoulas, 1999). Domains II and III of gK that were expressed as a fusion with GST are shaded.
overnight culture was diluted 1:100 into a total of 50 ml fresh culture media and incubated at 37°C until A600=0.500. Protein expression was induced with 0.5mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 3h at 37°C. Cells were harvested by centrifugation, and the pellets were resuspended in Tris buffered saline (TBS). Cell lysates were aliquoted for SDS-PAGE analysis.

**Inclusion body purification and production of antisera.** GST-gKdomain2 and GST-gKdomain3 were expressed as insoluble inclusion bodies in *E. coli.* Pelleted bacteria was centrifuged and incubated in a 10% lysis buffer (100mM NaCl, 1mM EDTA, 50 mM Tris, and 1 mg/ml lysozyme) at room temperature for 20 minutes. Pelleted spheroplasts were resuspended in ice cold solution (100mM NaCl, 1mM EDTA, 0.1% deoxycholate, 50 mM Tris) and incubated on ice for 10 min. MgCl2 (final concentration of 8mM) and DNase I (final concentration 10μg/ml) were added and allowed to incubate until viscosity disappeared. Inclusion bodies were removed by centrifugation at 8,000 g for 10 min and washed five times in 2M urea, 2% SDS, 50mM Tris followed by a final wash in 100mM NaCl, 1mM EDTA, 50mM Tris. Hyperimmune sera were generated against GST-gKdomain2 and GST-gKdomain3 by immunizing mice with four doses of purified inclusion body protein. Freund’s complete adjuvant supplemented the first injection, while Freund’s incomplete adjuvant supplemented each subsequent injection. Injections were administered subcutaneously and intramuscularly at approximately two and one half week intervals.
Gradient Purification of HSV-1 virions. Vero cells were infected with KOS and AgK viruses at an MOI of 10 and prepared for density gradient centrifugation. Cultures were scraped into medium and both extracellular and intracellular virions were harvested from infected cells by three freeze-thaw cycles and sonication. Cell debris was pelleted at 2,000 g for 30 min and discarded. The virus within supernatants was pelleted at 25,000 g for 2 hours through a 10% sucrose cushion. Virus was resuspended in TBS and double gradient purified on 15-60% continuous sucrose gradients. Final purified virus stocks were concentrated by centrifugation through a 10% sucrose cushion.

SDS-PAGE and immunoblotting. Cell lysates were placed in sample buffer and heated for 5 minutes at 100°C prior to electrophoretic separation in a 10% SDS-polyacrylamide gel (Laemmli, 1970). Following separation, the proteins were stained with Gel Code-Blue (Pierce, Rockford, IL) or electrotransferred to nitrocellulose membranes, visualized with Ponceau S (0.1% Ponceau S in 3% trichloroacetic acid), and destained. Blots were blocked against non-specific binding for 2 hours using 10% skim milk in TBS and incubated with the primary antibody indicated for 1 hour. The blots were washed five times for ten minutes each with TBS-Tween (TBS-T), incubated for 1 hour with horse radish peroxidase (HRP) conjugated secondary antibody at a 1:50,000 dilution in TBS-T and washed five times in TBS-T for 15 minutes each. Blots were visualized by autoradiography using the Pierce SuperSignal chemiluminescent detection kit (Pierce, Rockford, IL) as per the manufacturer’s instructions.
Kinetics of Virus Entry into Vero (VK302) cells. VK302 cells were seeded at 3.5 x 10^5 cells/cm² in 6 well plates. Subconfluent monolayers were infected in multiples of six with 200-300 plaque forming units per well of AgK propagated on Vero, AgK propagated on VK302, or KOS propagated on Vero and incubated at 4°C for 2 hours. Following adsorption, cells were washed with pre-warmed media, overlaid with DMEM with 2% FCS, and shifted to 34°C for the times designated. At each time point extracellular virions were inactivated in triplicate using a low pH buffer (0.1M glycine in TBS, pH3.0) wash, as described previously (Huang and Warner, 1964; Highlander et al, 1987). The other three wells were mock treated with media and served as a control for the determination of percent survival. Subsequently, plates were washed with media and monolayers were overlaid with DMEM, 2% FCS, 1% methylcellulose. Plates were incubated at 37°C for forty-eight hours, at which time the number of plaques in each well was determined and the % survival at each time point was calculated as the average of the number of plaques formed in inactivated wells divided by the number of plaques formed in mock treated wells.

Fluorescent Microscopy of Infected Cells. Subconfluent cell monolayers in 24-well plates were infected with AgK/EGFP or KOS/EGFP at an MOI of 5. After a two-hour adsorption at 4°C, cells were washed, overlaid with DMEM containing 2% FCS and 0.5% methylcellulose, and incubated at 37°C. Infected cells were visualized directly by epi-fluorescence using a standardized FITC filter set on a Nikon Alphaphot-2 fluorescence microscope.
Polyethylene Glycol (PEG) Induced Virus Entry. Subconfluent cell monolayers were infected with ΔgK/EGFP or KOS/EGFP at an MOI of 5 and allowed to adsorb at 4°C for two hours. Following attachment, cells were washed with medium, and treated with polyethylene glycol solutions to facilitate entry essentially as described previously (Cai et al., 1988). Cells were exposed briefly to PEG 8000 (50% in TBS or 1g/2 ml), then washed twice in TBS and treated again with PEG (33% in TBS or 1g/3ml). Control cells were treated identically except for exposure to PEG. Finally, cells were washed with media, and overlaid with DMEM supplemented with 2% FBS and 1% methylcellulose. At the indicated time points, cells were observed by fluorescent microscopy for virus penetration.

Construction of Chimeric HveAh/HveAs expressing CHO cell-lines.
HveaH/HveAs chimeric genes were generated by splice overlap extension PCR essentially as described previously (Choljenko et al., 1996; Foster et al., 1999; Foster and Kousoulas, 1999). Briefly, primers HveA5’ and HveA3’ that recognize specific sequences within the amino and carboxyl termini, respectively of both HveAs and HveAh were used in PCR reactions against HveAs or HveAh template DNA with 3’ and 5’ primers that represented the sites at which chimeras were to be generated. 7 µl of PCR product from the coding regions of HveAs or HveAh were mixed with 7 µl of PCR product from the carboxyl coding regions of HveAh or HveAs, respectively and extended with 10 cycles: 96°C 30 sec; 72°C 10 min. The chimeric genes from this extension were amplified by PCR utilizing primers HveA5’ and HveA3’. Final chimeric PCR products were cloned into pcr3.1TA (Invitrogen) and
checked for orientation relative to the CMV promoter. Chimeric expression plasmids were introduced into CHO-IE8 cells (Montgomery et al., 1996) by Lipofectamine (GIBCO-BRL) transfection. Forty-eight hours post transfection, cells were selected in Ham's-F12, 75mg/ml puromycin, 500mg/ml G418, and 10% FCS as described previously (Foster et al., 1999).

RESULTS

Expression and purification of fusion proteins. Fusion proteins (GST-gKdomainII and GST-gKdomainIII) and GST were expressed in bacterial cells transformed with the recombinant plasmids (GSTdom2 and GSTdom3) or with parental plasmid vector pGEX2T. Transformed cells were induced with 0.5mM IPTG at 37°C for 3 hours. Protein bands corresponding to the respective expressed protein products were observed by Gel-Code Blue stained SDS-PAGE (Fig. 4.2A). Expression of GST (≈26kDa), GSTgKdomain2 (≈35.5kDa), and GSTgKdomain3 (≈33.8kDa) were readily detected by SDS-PAGE and were further confirmed by western immunoblot analysis using anti-GST antibodies (Fig. 4.2B). Analysis of soluble and insoluble proteins following sonication indicated that under these conditions the expressed protein products were insoluble (data not shown).

Attempts to solubilize expressed protein products proved unsuccessful; however, inclusion bodies are easily purified and particulate antigens make
Figure 4.2. SDS-PAGE and western analysis of the expression of GST, GST-gKdomain2 and GST-gKdomain3 proteins. Bacterial lysates were separated on an SDS-PAGE and either A) stained with Gel-Code blue to visualize protein bands or B) electrotransferred to nitrocellulose for immunoblotting with anti-GST antibody. Lane 1: GST expression; Lane 2: GST-gKdomain2; Lane 3: GST-gKdomain3; M: Low molecular weight marker.
**Figure 4.3. SDS-PAGE and western analysis of the purification of GST-gKdomain2 and GST-gKdomain3 inclusion bodies.** Purified GST-gKdomain2 and GST-gKdomain3 were separated on an SDS-PAGE and proteins were either A) visualized by staining with Gel-CODE or B) electrotransferred to nitrocellulose for western analysis using anti-GST antibody. Lane 1: GST-gKdomain2; Lane 2: GST-gKdomain3.
excellent immunogens. Therefore, proteins within inclusions were purified by solubilizing the bacterial proteins, while the expressed fusion products within the inclusions remained in an insoluble pellet. Analysis by SDS-PAGE indicated that these protein products were purified to near homogeneity (Fig. 4.3A). The purified proteins were again confirmed by western immunoblots using anti-GST antibodies to be GST fusion proteins (Fig. 4.3B).

Detection of gK as a structural component of purified virions.

Glycoprotein K has been characterized as a structural component of other alphaherpesviruses, including PRV-1 and VZV (Klupp et al, 1998; Mo et al, 1999). To determine if gK was a structural component of HSV-1 virions, ΔgK and KOS viruses were double purified on continuous sucrose gradients. The virion protein content was normalized by the amount of gD detected through immunoblot analysis with an anti-gD monoclonal antibody (data not shown). Utilizing a pool of the anti-GST-gKdomain2 and anti-GST-gKdomain3 antibodies, we detected a 40-kDa protein species for KOS but not ΔgK electrophoretically separated normalized virions (Fig. 4.4). This 40 kDa species appeared to correspond to the predicted and previously described glycosylated gK (Ramaswamy and Holland, 1992; Hutchinson et al, 1995) and was not detected with pre-immune or anti-GST sera. A second protein species of approximately 80 kDa could also be faintly visualized in these immunoblots. This protein species may represent gK homodimers as it was not detected in gK-null virion preparations and the protein products could not
Figure 4.4. Detection of HSV-1 gK as a structural component of purified virions. Purified HSV-1 KOS or ΔgK virions were normalized to gD protein content and separated on an SDS-PAGE. Proteins were electrotransferred to nitrocellulose and probed with anti-GSTgKdomain2 and anti-GSTgKdomain3 sera pools.
be fully dissociated due to problems with protein aggregation upon heating (Ramaswamy and Holland, 1992).

**Entry of ΔgK and KOS viruses into Vero (VK302) cells.** Glycoprotein K null virions were tested for their ability to enter into normally permissive Vero cells. Classical pH inactivation entry kinetics were performed to determine the kinetics of virus entry into Vero (VK302) cell-lines. Although HSV-1 virions are capable of attachment to cell surfaces at 4°C, virus cannot enter into these cells (Huang and Warner, 1964). Therefore, shifting of cultures to an entry permissive temperature (i.e. 34-37°C) following attachment synchronizes virus penetration and thus allows for the quantitative measurement of virus entry. Because extracellular virus are inactivated by low pH buffers, low pH treatment of cells at various time points post adsorption shift generates the kinetics of virus entry by the number of plaques formed (i.e. those virus particles that were capable of entry at the given time point) (Highlander et al, 1987). ΔgK virions entered into VK302 cells in a delayed manner relative to that of wild-type KOS virus (Fig. 4.5). Furthermore, at the farthest time point (180 min), ΔgK has not yet achieved an equivalent entry efficiency compared to KOS. It does however appear that entry is still occurring, since the slope of the line has not reached a plateau (Fig. 4.5). ΔgK virions propagated on the gK-null complimenting VK302 cells had entry kinetics that were similar to those of wild-type KOS (Fig. 4.5), indicating that *trans* complementation of gK restored the defect in entry caused by deletion of gK.
Figure 4.5. Kinetics of HSV-1 ΔgK (Vero), ΔgK (VK302), and KOS virus entry into Vero (VK302) cells. Classical low pH inactivation of extracellular virions was employed to determine entry kinetics at 34°C. All experiments were done in triplicate.
Despite the delay in entry kinetics, we had previously observed that ΔgK virions are capable of entering into Vero cells. Therefore, to assess the ability of these viruses to penetrate Vero cells rather than establish a productive infection, Vero cells were infected at an MOI of 5 by viruses that constitutively express the enhanced green fluorescent protein (EGFP) (Foster et al., 1998; Foster et al., 1999). Consistent with previous observations, ΔgK/EGFP virus propagated on Vero cells (Fig. 4.6B) penetrated cell surfaces as efficiently as ΔgK propagated on VK302 (Fig. 4.6C) and wild-type KOS/EGFP virus (Fig. 4.6A).

Receptor mediated entry of gK null virions. To investigate if the delayed entry kinetics exhibited by ΔgK were due to failure to utilize different Hve receptors, ΔgK/EGFP virus stocks were prepared in both Vero and VK302 cells. An equal number of infectious virions were used to infect CHO-K1 cells transformed with HveAs (Fig. 4.7A, B, C), sAevH (Fig. 4.7D), HveAh (Fig. 4.7F), HveB (Fig. 4.7E), or HveC (Fig. 4.7G). ΔgK/EGFP virus prepared in Vero cells did not enter into CHO/HveAs cells (Fig. 4.7C); whereas, ΔgK/EGFP virus propagated on VK302 cells (Fig. 4.7B) entered at a similar efficiency to that of KOS/EGFP (Fig. 4.7A). Interestingly, the human homolog of the HveAs receptor, HveAh, did facilitate ΔgK/EGFP virus entry, albeit inefficiently (Fig. 4.7F). The ΔgK/EGFP virus propagated on Vero cells was then tested for its ability to utilize other Hve receptor expressing cell-lines. As expected, ΔgK/EGFP did not enter into either the control CHO/sAevH cells or the CHO/HveB cells (Fig. 4.7. D and E, respectively).
Figure 4.6. Entry of ΔgK/EGFP (Vero), ΔgK/EGFP (VK302), and KOS/EGFP virus into Vero cells. Vero cells were infected with KOS/EGFP (A), ΔgK/EGFP propagated on Vero cells (B) or ΔgK/EGFP propagated on VK302 cells (C) at an MOI of 10 and visualized under fluorescent microscopy at 12 hours post infection.
However, AgK/EGFP did enter into CHO/HveC cells with some efficiency (Fig. 4.7G).

Mutant viruses that are capable of attachment, but defective in penetration of cell surfaces are enhanced by PEG, an agent that promotes fusion between membranes. KOS/EGFP enters into CHO cells expressing various Hve receptors with 100% efficiency and as expected KOS/EGFP entry was neither enhanced nor inhibited by PEG treatment (Fig. 4.8A). Contrastingly, the gK null virus AgK/EGFP, which was unable to utilize the HveAs receptor for virus entry, was enhanced several fold by PEG treatment (Fig. 4.8B). Similarly, inefficient entry into CHO cell-lines expressing other Hve receptors could be enhanced by PEG treatment (Fig. 4.8C). Hence, it gK-null viruses are able to attach to CHO cells expressing receptors at their surfaces, but are defective in virus penetration and entry.

Domains of HveAh/s that facilitate AgK entry. Recently, our characterization of the HveAs receptor revealed that although there was significant homology to HveAh, there were several amino acid substitutions, deletions, and insertions (Foster et al., 1999). Thus, we investigated whether these differences accounted for the inability of HveAs to facilitate AgK virus entry. Genes that specified chimeric proteins were generated that when expressed in CHO cells could be used to rapidly identify regions of the HveAh receptor that mediated AgK/EGFP virus entry. Chimeric proteins were designed to address specific regions of HveA where the greatest differences in amino acid composition resided. Furthermore, for
Figure 4.7. KOS/EGFP and ΔgK/EGFP entry into CHO cells transformed with Hve receptors. CHO-K1 cells transformed with HveA (panels A, B, C), sAevH (opposite orientation of HveA (panel D), HveB (panel E), HveAh (panel F) or HveC (panel G) were infected with EGFP expressing viruses and analyzed for entry 12 h.p.i. Panel A: KOS/EGFP infected cells. Panel B: ΔgK/EGFP virus propagated on gK null complementing VK302 cells. Panels C-G: ΔgK/EGFP virus propagated on non-complementing Vero cells.
Figure 4.8. Polyethylene glycol mediated penetration of gK null viruses into CHO cell-lines expressing Hve receptors. 5 PFU/cell KOS/EGFP (A) or ΔgK/EGFP (B and C) were absorbed at 4°C for 2 hours on HveAs (A and B) or HveAh (C) transformed CHO cell-lines. Following adsorption, virus was treated with PEG to mediate entry.
each chimeric receptor generated, a reciprocal was similarly generated, which in
theory should always function in contrast to its sister receptor. Chimeric receptors
were generated to isolate: 1) the signal peptide motif (11 amino acid substitutions);
2) the transmembrane and carboxyl terminus (9 amino acid substitutions, 4 amino
acid deletions, 6 amino acid insertions, and a 3 amino acid shorter carboxyl
terminus); 3) the amino terminus prior to amino acid 100 (8 amino acid
substitutions); 4) the amino terminus between amino acids 100 and 203 (13 amino
acid substitutions and 1 insertion).

All HveAh amino terminal chimeric receptors containing HveAs after amino
acid 100 functioned to facilitate ΔgK/EGFP virus entry in a similar manner to
HveAh (Fig. 4.9A); however, the HveAh signal peptide attached to HveAs did not
mediate ΔgK/EGFP virus entry (Fig. 4.9B). Conversely, all HveAs amino terminal
chimeric receptors containing HveAh after amino acid 100 did not facilitate
ΔgK/EGFP virus entry (Fig. 4.9C); whereas, addendum of the HveAs signal peptide
to HveAh receptor did mediate ΔgK/EGFP virus entry (Fig. 4.9D). All chimeric
receptors facilitated KOS/EGFP virus entry, indicating that chimeric receptors did
not adversely affect their ability to mediate HSV-1 entry.

DISCUSSION

Although many of the alphaherpesvirus glycoproteins are well characterized,
information on gK is limited. Due to the highly hydrophobic nature of gK and the
lack of immunological reagents, structural studies of gK have been mostly limited
Figure 4.9. ΔgK/EGFP entry into HveAs/HveAh and HveAh/HveAs chimeric receptors. HveAh amino terminal/HveAs carboxyl terminal chimera (representative in panel A), HveAs amino terminal/HveAh carboxyl terminal chimera (representative in panel B), HveAh signal/ HveAs chimeric protein (panel C) or HveAs signal/ HveAh chimeric protein (panel D) expressing CHO cell-lines were infected with ΔgK/EGFP at an MOI of 10. Cells were visualized by fluorescent microscopy 24 hours post infection.
to characterization of in vitro translated and processed protein products. However, recent genetic studies have begun to unravel the mysteries of HSV-1 gK function. Previously, we showed that the gK-null virus ΔgK replicated inefficiently and failed to be translocated through the cytoplasm of infected cells to extracellular spaces (Jayachandra et al., 1997). We recently extended these observations by generating a series of truncations and mutations within the UL53 gK gene. Many of these mutations indicated that gK may function in vesicular transport events from the Golgi apparatus to the cellular plasma membrane (Foster and Kousoulas, 1999). Consistent with the original theories of HSV-1 gK functioning in multiple membrane fusion events within the herpesvirus lifecycle, in this study we report that gK is a structural component of purified virions that appears to function in entry by modulating receptor mediated penetration events.

Structural characterization of HSV-1 gK. Glycoprotein K has been characterized as a structural component of other alphaherpesviruses, including PRV-1 and VZV (Klupp et al., 1998; Mo et al., 1999); however, according to Hutchinson et al. (1995) it did not appear to be present in purified virion preparations. This was in contradiction to the popular belief that HSV-1 gK functioned in multiple membrane fusion events during HSV-1 infection, based in large part on earlier studies describing several mutations in the UL53 (gK) gene that resulted in syncytial phenotypes (Dolter et al., 1994). Moreover, viruses that specified mutations in gK exhibited delayed entry into non-complementing cells (Pertel and Spear, 1996).

Prokaryotically expressed proteins of GST fused with domains of HSV-1 gK were used to generate monospecific anti-gK sera. Pools of these sera detected a 40
kDa protein species in gradient purified KOS virions, but not purified ΔgK virions. Furthermore, a second protein species was detected at approximately 80 kDa, the expected molecular mass of a gK homodimer. Thus, HSV-1 gK clearly represents a virion structural component that may function in virus entry events. Considering our observations, it is possible that the lack of reactivity of previously generated anti-gK peptide sera or that low levels of gK present in purified virions prevented detection. Alternatively, the inability of peptide antisera to recognize homodimeric protein complexes, as gK appears to exist as in purified virions, may have accounted for this oversight. In either case, the presence of gK as a structural component of virions can now account for the phenotypic alterations in penetration exhibited by viruses that contained mutations in the U₅₃ (gK) gene (Dolter et al, 1994; Hutchinson et al, 1993; Pertel and Spear, 1996).

**Entry profiles and receptor utilization of the ΔgK virion.** Although KOS/EGFP virions were capable of utilizing all herpes simplex virus entry mediating receptors at 100% efficiency, ΔgK/EGFP was capable of only using a limited subset of these receptors. Specifically, while HveAh and HveCh were capable of mediating ΔgK/EGFP virus entry, HveAs was not. Despite the ability of these two receptors to mediate ΔgK/EGFP virus entry, they did so at a lower efficiency relative to KOS/EGFP virus entry. Complementation of ΔgK/EGFP virus through propagation on a cell-line that expressed gK in *trans*, restored the ability of the gK-null virion to efficiently utilize these receptors, indicating that gK and not another virion component was most likely responsible for this phenotype. Moreover, the inability of the gK null virus to utilize the HveAs receptor was shown
not to be attributable to an inability of the virus to attach to cell surfaces as PEG mediated virus entry. Therefore, gK may function in coordination with HveAs in either receptor-mediated attachment or more likely in gK/HveAs receptor mediated virus to cell fusion during penetration. There is some evidence for the latter scenario. The HveA receptor has been shown to be essential for virus mediated cell-to-cell fusion for viruses that specified a syncytial mutation in glycoprotein K.

Domains of HveA involved in ΔgK/EGFP entry. Although our recent characterization of the simian homolog of the HveAh receptor revealed a high degree of homology between the two proteins, there were several amino acid substitutions, deletions, and insertions. Specifically, HveAs contained: 1) 41 amino acid substitutions of which 24 were to similar amino acids and 17 were to dissimilar amino acids; 2) amino acid deletions within the transmembrane sequence; 3) amino acid insertions within the transmembrane sequence and the amino terminal (Foster et al, 1999). The inability of the HveAs receptor to mediate ΔgK/EGFP virus entry, while the HveAh receptor was able to facilitate virus ΔgK/EGFP penetration, indicated that the amino acid differences between these two receptors were critical to its function. A panel of chimeric HveAh/HveAs and HveAs/HveAh protein expressing CHO cell-lines were generated in order to map the functional region of HveA that facilitated ΔgK/EGFP virus entry. From these results, it can be inferred that the domain of HveAh, which mediates entry of gK null viruses, lies between amino acids 38 of the signal peptide and 100 of the amino terminus. Moreover, due to the significant homology of these proteins, this region can be further narrowed to
8 amino acid substitutions between amino acids 59 and 87. These amino acid substitutions are: G58 to S58, F61 to Y61, H62 to R62, R64 to K64, Q65 to E65, Q70 to L70, S80 to P80, and F87 to L87. Of these 8 amino acid substitutions, only Q70 to L70 and S80 to P80 represent changes to dissimilar amino acids and therefore, are the most likely candidates to function in ΔgK/EGFP virus receptor mediated entry.
CHAPTER V

GENETIC ANALYSIS OF THE ROLE OF HERPES SIMPLEX VIRUS 1 (HSV-1) GLYCOPROTEIN K (gK) IN INFECTIOUS VIRUS PRODUCTION AND EGRESS

INTRODUCTION

Glycoproteins specified by herpes simplex virus (HSV) are synthesized in the endoplasmic reticulum (ER) and are thought to be transported to the plasma membrane via the Golgi apparatus (Roizman and Sears, 1996; Spear, P.G., 1993a; Spear, P.G., 1993b), presumably following cellular vesicular transport pathways (Griffiths et al, 1986; Palade, G, 1975; Pfeffer et al, 1987; Rothman, J.E., 1996; Rothman et al, 1996). Herpes virions assemble their nucleocapsids within the nucleus and acquire an envelope containing viral glycoproteins by budding through the inner nuclear lamellae (Morgan et al, 1959; Roizman and Sears, 1996; Schwartz et al, 1969; Torrisi et al, 1992). The process by which enveloped virions are transported from the perinuclear spaces through the cytoplasm to extracellular spaces and adjacent cells is not completely understood. According to one hypothesis, virions are transported as enveloped particles from the ER to the Golgi within vesicles derived from the outer nuclear lamellae and from the Golgi to extracellular spaces via Golgi-derived vesicles in a manner analogous to vesicular transport of viral and cellular glycoproteins. In this model, viral glycoproteins are modified in situ during transport before they are released to extracellular spaces.

---

3 Reprinted by permission of the Journal of Virology.
Nucleocapsids devoid of viral envelopes, which are frequently found in the cytoplasm of infected cells, are thought to represent a non-productive population of viruses (Campadelli-Fiume et al., 1991). An alternative model suggests that after virions acquire their envelopes from the inner nuclear membrane, they fuse with the outer nuclear lamellae releasing unenveloped nucleocapsids into the cytoplasm of infected cells (Browne et al., 1996; Gershon et al., 1994; Granzow et al., 1997; Klupp et al., 1998; Whealy et al., 1992). In contrast to the previous model, free nucleocapsids in the cytoplasm represent a prerequisite step for subsequent budding into Golgi-derived vacuoles, generating enveloped virions containing fully processed glycoproteins. In both egress models, virions are released from the infected cells via fusion of vacuoles containing enveloped virions with the plasma membranes (Komuro et al., 1989; Roizman et al., 1996; Stackpole, C.W., 1969).

At least three genes, UL11, UL20 and UL53 (glycoprotein K [gK]), code for proteins that are known to be involved in HSV type 1 (HSV-1) virion egress (Baines et al., 1992; Baines et al., 1991; Hutchinson et al., 1995; Jayachandra et al., 1997). Deletion of the UL20 gene caused accumulation of enveloped virions within the perinuclear spaces (Baines et al., 1992), while deletion of the gK gene led to accumulation of enveloped virions within the cytoplasm (Jayachandra et al., 1997). Both UL20- and gK-null mutant viruses were partially complemented by cellular factors, because the UL20-null virus replicated in 143 TK⁻ cells, and the replication
of the gK-null virus was enhanced in actively replicating cells (Baines et al.,
Jayachandra et al., 1997).

HSV-1 gK is encoded by the UL53 open reading frame (Debroy et al., 1985;
McGeoch et al., 1988), and it has characteristics of a membrane protein, including a
N-terminal signal sequence, and two potential sites for N-glycosylation 10 amino
acids apart (Debroy et al., 1985; Pogue-Geile et al., 1987). It exists as a single 40
kDa protein species in infected cells (Hutchinson et al., 1992), while gK translated in
vitro had an apparent molecular mass of 36 kDa, and N-linked glycosylation
occurred in the first 112 residues of the protein consistent with glycosylation at
residues 48 and 58 (Ramaswamy et al., 1992). Initially, gK was predicted to have
four membrane spanning regions (Debroy et al., 1985); however, recent,
experiments with in vitro-translated gK in the presence of microsomal membranes
suggested that gK contained three instead of four membrane-spanning regions (Mo
and Holland, 1997).

To investigate the role of gK in infectious-virus production and virion
egress, we constructed mutant viruses containing stop codons within the gK gene
immediately after gene segments coding for each of the four putative hydrophobic
domains (hpd) predicted by Debroy et al. (1985) as well as mutant viruses
containing mutated codons causing single amino acid changes within gK amino acid
motifs conserved among all alphaherpesviruses. Characterization of these viruses in
cell culture revealed structural features of gK that are important for infectious virus
production and egress.
MATERIALS AND METHODS

Cells and viruses. African green monkey kidney (Vero) cells were obtained from American Type Culture Collection (Manassas, Va.). The cells were propagated and maintained in Dulbecco's Modified Eagle's Medium (DMEM; Sigma Chemical Co., St. Louis, Mo.) containing sodium bicarbonate and 15 mM HEPES and supplemented with 7% heat-inactivated fetal bovine serum (FBS). V27 cells carry a stably integrated copy of the HSV-1 (KOS) ICP27 gene and were kindly provided by D. M. Knipe, Harvard Medical School. These cells were propagated in DMEM supplemented with 7% FBS and 500μg/ml of G418 (Rice and Knipe, 1990). The gK-transformed cell line VK302 was obtained from D. C. Johnson, Oregon Health Sciences University, and was maintained in DMEM lacking histidine (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 7% FBS and 0.3 mM histidinol (Sigma Chemical Co.). All cells were passed once in DMEM plus 7% FBS without selection prior to infection with virus (Hutchinson and Johnson, 1995). HSV-1(KOS), the parental wild-type strain used in this study, was originally obtained from P. A. Schaffer (University of Pennsylvania, Philadelphia). HSV-1(KOS) d27-1, which has a 1.6 kb BamHI-StuI deletion of the ICP27 gene, was kindly provided by D. M. Knipe and was propagated in V27 cells (Rice and Knipe, 1990). The AgK virus was propagated on VK302 cells and was as described previously (Jayachandra et al, 1997).
Reagents. Restriction enzymes and DNA modification enzymes were obtained from New England BioLabs (Beverly, Mass.). RNase and proteinase K were purchased from Boehringer Mannheim (Indianapolis, Ind.). Gel fragment purification matrix and buffers (Prep-a-gene) were obtained from BioRad (Hercules, Calif.). Sequencing grade $[^{35}S]$ dATP was obtained from DuPont/NEN (Wilmington, Del). AmpliTaq, XL Polymerase, and deoxynucleotide triphosphates were purchased from PE Biosystems (Foster City, Calif.). All synthetic oligonucleotide primers were synthesized by the Louisiana State University Gene Probes and Expression Systems Laboratory “GeneLab” using phosphoamidite chemistry on an Applied Biosystems ABI394 DNA/RNA synthesizer with PE Biosystems Inc. reagents.

Plasmids. Truncations in gK were generated by the insertion of stop codons in the gK gene by PCR. Antisense oligonucleotides, which contained a stop codon and a BamHI restriction site at their 5' termini were synthesized. Antisense primers were: $\Delta$gKhp-1 (5'-TCGGGATCCTCAGAGGGCGACGAACG-3'); $\Delta$gKhp-2 (5'-CAGGATCCTCAGGAATATGAAAGCGG-3'); $\Delta$gKhp-3 (5'-GCCGGGATCCTCAATACAGCTCTGTCAGGC-3'); and $\Delta$gKhp-4 (5'-GCCGGGATCCTCAGCGCCAGCAGG-3'). The sense primer for all PCR reactions was UL52Kpn I (5'-TAGTCGCGTGCATCGAAACCC-3'). PCR was performed as described previously with HSV-1 (KOS) viral DNA as a template (Chouljenko et al, 1996; Jayachandra et al, 1997). Reaction conditions for the XL PCR were as follows: 98°C for 3 seconds, and 72°C for 2 minutes for 30 cycles.
The PCR products were precipitated, restricted with KpnI and BamHI, and gel purified. Plasmid pSJ1723, containing the UL52, UL53, and UL54 genes was described previously (Jayachandra et al., 1997). PCR-derived DNA fragments containing stop codons within the gK gene were cloned into the unique KpnI and BamHI sites of plasmid pSJ1723 to produce plasmids pTF9101, pTF9102, pTF9103, and pTF9104 coding for gK truncations after the first, second, third, and fourth putative hydrophobic domains, respectively (Fig. 5.1). Single-codon changes within the gK gene were produced by splice-overlap extension with synthetic oligonucleotides, and the PCR-derived gK gene containing single-codon changes was cloned into the acceptor plasmid pSJ1723. Plasmid pTF9120 specified gK with two C-to-S mutations within the CXXCC gK motif. Plasmid pTF9121 specified gK with a C-to-S change at amino acid position 269. Plasmid pTF9122 specified gK with a Y-to-S change within the YTKØ motif. Plasmid pTF9105 was derived by insertion of a PCR-amplified wild-type KOS gK DNA fragment into pSJ1723.

**Construction and purification of gK mutant viruses.** Plasmids specifying truncations in the UL53 gK gene were transfected into 50% confluent VK302 cells with Lipofectamine (GIBCO-BRL, Gaithersburg, Md.) according to the manufacturer’s instructions. Twenty-four hours posttransfection, the cells were infected at a multiplicity of infection (MOI) of 10 with the d27-1 virus (ICP27-null) as described previously for the generation of the ΔgK virus (Jayachandra et al., 1997). At 48 hours post infection (h. p. i.), the cells were lysed by three freeze-thaw cycles, and the resultant virus stocks were plated onto VK302 cells and overlaid.
with agarose. Virus plaques were picked, and plaque-purified five times on VK302 cells. Viruses specifying amino acid changes in gK were produced following a similar protocol with the exception that Vero cells were used instead of VK302 cells to eliminate the possibility of revertants arising from rescue of mutants by the resident wild-type gK gene.

**Viral DNA.** Viral DNA was prepared from infected Vero cells as described previously (Kousoulas et al., 1984). Briefly, Vero cells were infected with plaque purified virus at a MOI of 5. At 2 days post infection cells were lysed with 1% NP40, 0.5% deoxycholate in 10 mM Tris, and 1 mM EDTA (TE). The lysates were treated with RNase (10 μg/mL) for 10 min. at 37°C, followed by addition of sodium dodecyl sulfate (1% final concentration) and proteinase K (10 μg/mL) at 55°C overnight. DNA was purified with two phenol-ether extractions and precipitated with 3M sodium acetate and ice-cold ethanol.

**PCR confirmation and sequencing of gK-mutant viruses.** The UL53 region encoding the truncated gK was PCR amplified from plaque-purified recombinant viral DNA with the UL52KpnI oligonucleotide as the sense primer and the gKTr oligonucleotide (5’-CATACCCCGTTCGGCTTC- 3’), which binds downstream of the UL53 gene, as the antisense primer. The size differences of each gK gene truncation were determined by agarose gel electrophoresis followed by ethidium bromide staining. Truncated gK genes as well as specific codon changes were confirmed by direct sequencing using the fmol® DNA Cycle Sequencing...
System (Promega, Madison, Wis.) according to the manufacturers' directions. Sequencing reactions were resolved in a Gel-Mix 6 polyacrylamide gel (GIBCO-BRL). Mutant viruses were tested for the absence of any contaminating d27-1 virus by diagnostic PCR using primers UL52KpnI and d27-1α. These PCR reactions were performed under long-PCR conditions using XL-Polymerase (Perkin Elmer, Inc.) essentially as described previously (Chouljenko et al., 1996; Foster et al., 1999; Foster et al., 1998).

Rescue and complementation of gK-mutant viruses. Isolated viruses specifying truncations or mutations in the UL53 gene were rescued by transfecting plasmid pTF9105 into Vero cells and superinfecting with each of the truncated virus isolates at 24 hours post transfection. At 48 h. p. i., the cells were freeze-thawed three times, plated to confluent Vero cells, and the number of wild-type plaques in relation to ΔgK-like plaques was determined. The wild-type plaques were picked, plaque purified, and tested by PCR and sequencing to determine whether the wild-type UL53 gene was present. All gK mutant viruses were tested for their ability to form KOS-like plaques on the complementing cell line VK302, which complements the ΔgK virus (Jayachandra et al., 1997).

Electron microscopy of gK-truncated virions in Vero cells. Vero cells were infected with truncated viruses prepared from VK302 cells or KOS at an MOI of 5 and incubated at 37°C for the time designated. The infected cells were prepared for negative staining electron microscopy as described previously.
(Jayachandra et al, 1997). All sections were examined with a Phillips 410
transmission electron microscope.

Production of infectious virions. Different subconfluent Vero monolayers
containing approximately 8 x 10⁵ cells per 9.62 cm² well at the time of infection
were infected with each mutant virus at an MOI of 5. After adsorption for 2 hours
at 4°C, viruses were removed and cultures were washed with medium. Fresh pre-
warmed medium was added to the cells, and cultures were incubated at 37°C for the
duration of the study. At 12 and 24 h.p.i., the combined cells and supernatant fluid
samples were frozen and thawed three times and sonicated, and the number of
infectious virions was determined by standard end-point plaque assays on VK302
cells.

RESULTS

Construction and genetic characterization of HSV-1 (KOS) mutant
viruses containing stop codons within the gK gene. Specific portions of the gK
gene were generated by PCR with a 3'-oligonucleotide primer containing an in-
frame stop codon. Stop codons were inserted immediately after the gK gene
sequences coding for each of the four putative hydrophobic domains of gK,
resulting in predicted truncated gKs of 139, 239, 268 and 326 amino acids.
Plasmids pTF9101, pTF9102, pTF9103, and pTF9104 contained the truncated gK
genes between the UL52 and UL54 genes, reconstructing the prototypic sequence of
Figure 5.1. Strategy for the isolation and PCR detection of HSV-1 mutants specifying gK truncations. (a) The top line represents the prototypic arrangement of the HSV-1 genome with the unique long (UL) and unique short (US) regions flanked by the terminal (TR) and internal repeat (IR) regions. Shown below is the region of the mutant virus HSV-1 d27-1 genome (between map units of 0.7 and 0.8) containing the UL52, UL53 and the partially deleted UL54 open reading frames with relevant restriction endonuclease sites. (b) Plasmid constructs containing each of the truncated gK genes used to generate the ΔgKpd-1, -2, -3, and-4 mutant viruses. Represented on the HSV-1 (KOS) genome are the relative positions of PCR primers, UL52KpnI/gKTr, used to detect the truncated gK genes. The hatched segments represent the portions of genes that are expressed after truncation, while segments 3' to the TGA stop codon are portions of the gK genes that are deleted.
the genes (UL52-UL53-UL54). Each plasmid was used to rescue the mutant virus d27-1 (KOS), which has a lethal deletion within the UL54 gene specifying the immediate early-protein ICP27 as shown in figure 5.1 and described previously for the construction of the ΔgK virus (Jayachandra et al, 1997). Putative recombinant virus isolates were plaque-purified and tested by PCR for both the presence of contaminating d27-1 virus and the engineered gK truncations. Primers UL52KpnI/gKTr, which flank the gK-coding region, were used to amplify the gK genes. This set of primers will not produce a PCR product against the d27-1 virus, because primer gKTr is located within the deleted ICP27 gene portion of the d27-1 viral genome (Fig. 1). Amplification of the gK gene specified by each of the four different mutant viruses with the UL52KpnI/gKTr primer set generated the predicted DNA fragments of 730 bp, 1,030 bp, 1,117 bp, and 1,291 bp confirming the presence of the predicted truncated gK genes (Fig. 5.2a). The presence of the engineered stop codons was confirmed by DNA sequencing (not shown). To confirm that there was no contaminating d27-1 virus present, additional diagnostic PCR was performed using primer pair UL52KpnI/d27-1α. The parental d27-1 virus generated a single PCR product of 1,613 bp (Fig. 5.2b, lane 6), while the wild-type strain KOS generated the predicted 3,241 bp DNA fragment (Fig. 5.2b, lane 7). The mutant viruses ΔgKhpd-1, -2, -3, and -4, produced PCR-amplified DNA fragments of 2,358 bp, 2,658 bp, 2,745 bp, and 2,919 bp, respectively (Fig. 5.2b, lanes 2-5). None of the mutant viruses generated the d27-1 specific DNA fragment of 1,613 bp, indicating that there was no contaminating viral DNA in the mutant virus stocks (Fig. 5.2b, lanes 2-5).
Figure 5.2. Diagnostic PCR of recombinant viruses specifying truncations in gK.
(a) Agarose gel electrophoresis of ds DNA PCR products with the UL52KpnI/gKTr primer pair used to detect the truncated gK genes. Lane 1: lambda phage DNA digested with HindIII (marker). Lanes 2, 3, 4, 5, and 6: Viral DNA of ΔgKhpΔ-1, ΔgKhpΔ-2, ΔgKhpΔ-3, ΔgKhpΔ-4, and KOS viruses, respectively, amplified with PCR primer pair UL52KpnI/gKTr. Lane 7: Molecular mass marker (1-kbp ladder).
(b) Agarose gel electrophoresis of double stranded DNA PCR products with the UL52 KpnI/d27-1α primer pair used to confirm the purity of the recombinant viruses after extensive plaque purification. Lane 1: lambda phage DNA digested with HindIII (marker). Lanes 2, 3, 4, 5, 6 and 7: Viral DNA of ΔgKhpΔ-1, ΔgKhpΔ-2, ΔgKhpΔ-3, ΔgKhpΔ-4, d27-1 and KOS viruses, respectively, amplified with PCR primer pair UL52KpnI and d27-1α. Lane 8: Molecular mass marker (1-kbp ladder).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Plaque morphologies of virus isolates and viral yields. The plaque morphologies of mutant viruses were compared to that of the ΔgK and KOS viruses on Vero cell monolayers. The ΔgKhpdl and ΔgKhpds viruses produced viral plaques that were reproducibly smaller (on average, approximately 20 to 30% fewer cells per plaque) than those of the ΔgK virus, while the ΔgKhpds virus produced plaques, which were slightly larger (on average, approximately 40 to 50% more cells per plaque) than those of the ΔgK virus. ΔgKhpds viral plaques were similar in size to those of the wild-type KOS virus (Fig. 5.3). The ΔgK virus produces wild-type KOS-like viral plaques on the complementing cell line VK302 (Jayachandra et al., 1997). Similarly, all four gK-mutant viruses produced KOS-like plaques on the complementing cell line VK302. Rescue of the hpdl, -2, and -3 mutant viruses by plasmid pTF9105 containing the wild-type KOS gK gene produced viral yields similar to those of the KOS virus (not shown).

Subconfluent Vero cell monolayers were infected in parallel with viruses KOS, ΔgK, ΔgKhpdl, ΔgKhpds, ΔgKhpds, and ΔgKhpds and the total number of infectious virions (intracellular and extracellular) was determined at 12 and 24 h.p.i. The viral yields of ΔgKhpdl and ΔgKhpds viruses were similar to that of ΔgK virus at 24 h.p.i. The viral yield of the ΔgKhpds virus was approximately five-fold higher than that of ΔgK, ΔgKhpdl, and ΔgKhpds viruses, while the viral yield of the ΔgKhpds virus was approximately ten-fold higher than that of ΔgKhpds and identical to that of KOS virus. The viral yields of all four gK-mutant viruses were substantially higher in VK302 cells, approaching those of KOS virus. The ratios of extracellular to intracellular virus at 24 h.p.i. were similar for ΔgK,
Figure 5.3. Plaque morphology of KOS, ΔgK, ΔgKhpΔ-1, -2, -3, and -4 on Vero cells. Panels A: ΔgK; B: ΔgKhpΔ-1; C: ΔgKhpΔ-2; D: ΔgKhpΔ-3; E: ΔgKhpΔ-4; F: KOS. The cells were infected at an MOI of 0.01 PFU/cell and photographed using a phase-contrast microscope at 48 h.p.i.
Table 5.1. Viral yields of KOS and gK-truncated mutant viruses.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Total Yield</th>
<th>Out/in (24 h.p.i.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 h.p.i.</td>
<td>24 h.p.i.</td>
</tr>
<tr>
<td>ΔgK</td>
<td>3.2 x 10^5</td>
<td>3.7 x 10^6</td>
</tr>
<tr>
<td>ΔgKhp-1</td>
<td>8.6 x 10^5</td>
<td>2.1 x 10^6</td>
</tr>
<tr>
<td>ΔgKhp-2</td>
<td>1.6 x 10^6</td>
<td>2.6 x 10^6</td>
</tr>
<tr>
<td>ΔgKhp-3</td>
<td>2.1 x 10^6</td>
<td>1.6 x 10^7</td>
</tr>
<tr>
<td>ΔgKhp-4</td>
<td>3.0 x 10^6</td>
<td>3.0 x 10^8</td>
</tr>
<tr>
<td>KOS</td>
<td>3.5 x 10^6</td>
<td>3.0 x 10^8</td>
</tr>
</tbody>
</table>

Note: Subconfluent Vero cell monolayers (approximately 8 x 10^5 cells) were infected with each virus at an MOI of 5, and at 12 and 24 h.p.i. the total number of infectious virions were determined as well as the number of infectious virions within cells and extracellular fluids. The ratio of extracellular (OUT) to intracellular (IN) virions at 24 h.p.i. is also shown. The viral yields represent one of three experiments in which individual numbers varied by less than twofold.
AgKhpdl, AgKhpdl-2, and AgKhpdl-3, while the AgKhpdl-4 ratio was identical to that of KOS virus (Table 5.1).

**Electron Microscopy.** Conventional fixation-embedding electron microscopic analysis was undertaken to examine the intracellular localization of AgK mutant viruses in Vero cells as described previously (Jayachandra et al., 1997). Examination of Vero cells infected with the AgKhpdl-1 mutant virus revealed the presence of large double-membrane vesicles containing tens to hundreds of nucleocapsids in the cytoplasm located proximal to the nuclear membrane (Fig. 5.4: A, B, D). Single-membrane vesicles containing numerous nucleocapsids were also visualized within the perinuclear space in the process of budding through the outer nuclear lamella at regions of high electron density (Fig. 5.4: C). In these micrographs it appeared that the outer membrane of the cytoplasmic vesicles was derived from the outer lamellae of the nuclear membrane. Herpes virions are thought to acquire their envelopes by budding through the inner nuclear lamella; therefore, it is hypothesized that the internal membrane of the double-membrane cytoplasmic vesicles must be derived from the inner nuclear lamella (Fig. 5.4: C, D). In contrast to AgKhpdl-1, only a few membrane vesicles were observed with AgKhpdl-2, while no such vesicles were found in AgKhpdl-3-, AgKhpdl-4-, AgK-, or KOS- infected Vero cells. The AgKhpdl-2 and AgKhpdl-3 viruses accumulated virion particles in the cytoplasm of infected Vero cells (Fig. 5.5: B2 and C2). AgKhpdl-1 (Fig. 5.4: A and B), AgK (Fig. 5.5: A2), and AgKhpdl-2 (Fig. 5.5: B2)
Figure 5.4. Electron micrographs of Vero cells infected with the ΔgKhpd-1 mutant virus. Subconfluent Vero cell monolayers were infected at an MOI of 5 PFU/cell, incubated at 37°C for 36 hours and prepared for electron microscopy. The solid arrows in panels C and D mark nucleocapsids. The open arrows in panel C mark the outer and inner nuclear membranes in the cytoplasmic (c) and nuclear (n) compartments. The arrowheads mark membranes surrounding nucleocapsids within the perinuclear space in panels C and D. The scale bar = 0.5 μm for all panels.
Figure 5.5. Electron micrographs of Vero cells infected with different gK mutant viruses. Subconfluent Vero cells were infected with ΔgK virus (panel A1 and A2), ΔgKhpd-2 (panels B1 and B2), ΔgKhpd-3 (panels C1 and C2), ΔgKhpd-4 (panels D1 and D2), and KOS (panels E1 and E2). All cells were infected at an MOI of 5 PFU/cell, incubated at 37°C for 36 hours and prepared for electron microscopy. The arrowheads in panels A2 and B2 mark enveloped virions within cytoplasmic vacuoles. The open arrows mark extracellular virions in panels C1, D1, E1, and E2. The solid arrows mark enveloped virions within electron dense vesicles in panels C2, D2, and E2. The scale bar = 0.5 μm for all panels.
infected cells contained enveloped virion particles within cytoplasmic vacuoles, while ΔgKhpd-3, -4 and KOS contained single enveloped virions within well-defined, electron dense vesicles (Fig. 5.5: C2, D2, and E2, respectively). The outside surfaces of ΔgKhpd-1 (Fig. 5.4A) and ΔgKhpd-2 (Fig. 5.5B1) infected cells were devoid of virion particles, while only a few viruses per cell were detected in ΔgKhpd-3 infected cell surfaces (Fig. 5.5: C1). In contrast, a high number of virions were detected on the outside surfaces of ΔgKhpd-4 and wild-type KOS-infected cells (Fig. 5.5: D1 and E1, respectively).

Alignment of gK specified by alphaherpesviruses. The gK gene is highly conserved among different herpesviruses. Motivated by the hypothesis that domains important in the structure and function of gK should be conserved among different herpesviruses, we investigated whether there are conserved amino acid motifs within gK domains II and III. The gK primary structures of six alphaherpesviruses were aligned using the MultAlign Program (Corpet, 1988) (Fig. 5.6). The HSV-1 gK amino acid sequence contains 13 cysteine residues at positions 37, 82, 114, 144, 187, 220, 243, 257, 269, 296, 299, 300, 312. Cysteines 114, 243, 296, 299, and 300 were conserved among all gK sequences. Cysteine 114 is located within domain I, while cysteines, 243, 296, 299, and 300 are located within domain III, and both domains face the lumen or extracellular side (see Fig. 5.8). The alignment revealed conservation of two short amino acid sequences. In the predicted luminal side of gK, domain III contained the cysteine-rich motif (CXXCC). Domain II, predicted to be oriented toward the cytoplasm, was the most
Figure 5.6. Alignment of gK amino acid sequences specified by alphaherpesviruses. Alignment was performed using the MultiAlign Program (Corpet, 1988). Hydrophobic domains (signal peptide, hpd1, hpd2, hpd3, and hpd4) are presented as lightly shadowed. Dark shadowed areas contain conserved amino acid motifs. $X\#X\#X\#X\#$ is a tyrosine-based motif known to function in vesicular transport of membrane embedded glycoproteins. X denotes any amino acid and $\#$ denotes a bulky hydrophobic amino acid. CXXCC is a cysteine-rich motif. The last line depicts the consensus gK sequence with conserved residues indicated by capital letters. $\%$ depicts either L or M amino acids. $\%$ depicts either F or Y residues. $\#$ is anyone of D or N. Amino acid residues I or V are depicted with an (!) marking.
conserved among different herpesviruses and contained a conserved tyrosine-based amino acid motif (YXX\(\varnothing\)), where \(X\) denotes any amino acid and \(\varnothing\) denotes a bulky hydrophobic amino acid. The tyrosine-based and cysteine-rich motifs were also conserved in the alphaherpesviruses Marek’s disease virus and gallid herpesvirus 1 (not shown).

Construction of recombinant viruses specifying amino acid changes within the CXXCC and YXX\(\varnothing\) motifs. To investigate the role of the conserved amino acid motifs in the structure and function of gK, mutant viruses were constructed specifying single and double amino acid changes within these two motifs, respectively. Mutant virus gK/Y183S (gK/YS) specified gK with a single amino acid change (Y to S) within the YT\(\varnothing\) motif, and mutant virus gK/C304S-C307S (gK/CSCS) specified gK having two cysteines changed to serine residues within the cysteine-rich motif (CXXCC changed to SXXSC) of domain III. The mutant virus gK/CSCS produced small plaques (Fig. 5.7C) similar to those of the \(\Delta\)gK virus (Fig. 5.7G); however, gK/CSCS viral yields were similar to \(\Delta\)gKhp\(d\)3 viral yields. Similarly, the mutant virus gK/YS (Fig. 5.7E) formed plaques that appeared to be similar in size to \(\Delta\)gK viral plaques (Fig. 5.7G). In contrast to all other gK mutant viruses, viral yields of the gK/YS mutant were consistently lower by ten- to one hundred-fold than those of the \(\Delta\)gK virus (Table 5.1). The mutant virus, gK/C269S (Fig. 5.7B) specifying a C-to-S amino acid change exhibited plaque morphology and viral egress characteristics similar to those of the wild-type KOS strain (Fig. 5.7A). All of the gK-mutant viruses described above produced
Figure 5.7. Plaque morphology of gK-mutant viruses with amino acid changes within conserved gK motifs. Either Vero cells (panels A, B, C, E, and G) or VK302 cells (panels D, F, and H) were infected at an MOI of 0.01 PFU/cell and photographed using a phase contrast microscope at 48 h.p.i. Panels A: KOS; B: gK/C269S; C and D: gK/C304S-C307S; E and F: gK/Y183S; G and H: ΔgK.
substantially larger plaques in VK302 cells (Fig. 5.7 D, F, and H), approaching the KOS viral plaque in size (Fig. 5.7A), and their viral yields in VK302 approached those of the ΔgK virus on VK302 cells.

**DISCUSSION**

Previously, we showed that the mutant virus ΔgK, which lacked the entire gK gene, replicated inefficiently and was unable to egress from infected cells (Jayachandra *et al.*, 1997). To improve our understanding of the role of gK in virus replication and egress, we engineered either stop codons at different sites of the gK gene or mutations specifying single-amino-acid changes altering amino acid motifs that are conserved among all alphaherpesviruses. Our results support and extend previous observations that gK plays an important role in virus replication and egress, and furthermore, they suggest that gK is a multifunctional protein involved in virion envelopment, intracellular virion transport and egress.

The initial prediction of gK secondary structure prediction indicated that gK may possess four hydrophobic domains that transverse cellular membranes (Debroy *et al.*, 1985). Recently, differential protection experiments with gK expressed *in vitro* in the presence of microsomal membranes suggested that gK might have three membrane spanning regions (Mo *et al.*, 1997). In support of this model, computer-assisted predictions using different computer algorithms available through the Internet including: PSORT (Nakai *et al.*, 1992), Tmpred (Hoffman *et al.*, 1993),

196
Figure 5.8. Schematic model of the predicted secondary structure of gK. The gK model of Debroy et al (1985) was modified to have three instead of four membrane spanning domains as suggested by Mo and Holland (1998), and as predicted by computer-based predictions using the PSORT (Nakai et al, 1992), Tmpred (Hoffman et al, 1993), and SOSUI (Hirokawa et al, 1999) algorithms. The predicted putative hydrophobic domains (hpd) (lightly shaded circles) of gK which transverse the membrane (lines) are shown as embedded within the membrane. The arrows indicate the termination sites for truncated gK specified by the designated viruses. Asterisks mark syncytial mutations. Amino acid motifs that are conserved among alphaherpesviruses are contained within shaded oval-shaped areas. The darkly shaded circles represent the signal peptide.
and SOSUI (Hirokawa et al, 1999), revealed that the third hydrophobic domain predicted by Debroy et al (1985) has a low probability for transversing cellular membranes (not shown). Based on these considerations, and to facilitate the discussion of results, we present a modified version of the secondary structure model initially proposed by Debroy et al (1985), having the third hydrophobic domain of gK located extracellularly (Fig. 5.8). A striking consequence of having three instead of four membrane spanning domains is that a substantial portion of gK is predicted to lie within the lumen of cellular organelles that is functionally equivalent to the outside of the cell. Based on this gK model, we have subdivided the gK primary structure into four domains: domain I is the amino-terminal portion of gK terminating with the last amino acid of the putative hydrophobic domain hpd1; domain II includes the entire intracellular portion of gK and terminates with the last amino acid of the putative hydrophobic domain hpd2; domain III starts with the first hydrophilic amino acid immediately after hpd2 and terminates with the last hydrophobic amino acid of the putative hydrophobic domain hpd4; domain IV includes the carboxyl-terminal 13 amino acids of gK (Fig. 5.8). Characteristically, this model predicts that all syncytial mutations are on the external portion of gK located within either domain I or domain III suggesting that these domains may cooperate in virus-induced cell fusion (Dolter et al, 1994; Mo et al, 1997). The mutant viruses ΔgKhpdt-1 or ΔgKhpdt-2 produced substantially lower viral yields than the ΔgK virus, indicating that gK-truncations specified by these viruses interfered with infectious virus production. In contrast, the viral yield of the gK-mutant virus ΔgKhpdt-3 was approximately ten-fold higher than that of ΔgK.
virus, indicating that this truncated gK retained partial function in the production of infectious virus. Expression of the entire domain III in gK specified by mutant virus ΔgKhpd-4 restored entirely wild-type viral replication and plaque morphology. Collectively, these results suggest that domain III is important for the structure and function of gK. Furthermore, the first 28 amino acids of domain III must contain gK elements that contribute to the structure and function of gK, since viral yields of ΔgKhpd-3 were higher than those of ΔgK. Deletion of the terminal 12 amino acids in gK specified by the hpd4 mutant virus indicated that the carboxyl terminus of gK (domain IV) is not required for virus replication and virus spread.

Electron microscopic examination of over one hundred Vero cells infected with ΔgKhpd-1 revealed the presence of many large, double-membrane vesicles containing numerous capsids. Considering that each electron micrograph represented a cross-section of an infected cell, it was estimated that each double-membrane vesicle contained hundreds of capsids. These vesicles were found adjacent to nuclear membranes as well as throughout the cytoplasm. The outer membrane of each vesicle appeared electron dense and morphologically similar to that of the outer nuclear lamellae, while an inner membrane of each vesicle appeared less electron dense and otherwise morphologically similar to the inner nuclear membrane. Additional vesicles containing numerous capsids were found within the perinuclear spaces of infected cells. The overall appearance of these vesicles suggested that they constituted precursor forms of the double-membrane vesicles found in the cytoplasm of infected cells. It is not clear from the electron microscopic data how these vesicles were formed. One possibility is that
simultaneous budding of multiple nucleocapsids may be responsible for the production of the large vesicles, indicating that the expression of truncated gKs (ΔgKhpd-1 and ΔgKhpd-2) interferes with viral envelopment mechanisms.

Alternatively, the inner membrane of the double-membrane vesicles may be derived from the fusion of virion envelopes after budding into the perinuclear space. In this scenario, fusion of enveloped virions within the perinuclear space must occur rapidly, because we could not find single enveloped virions within perinuclear spaces.

Based on the assumption that conserved amino acid sequences among all herpesviruses may represent functional domains of gK, we aligned gK sequences specified by alphaherpesviruses. This analysis revealed that the five cysteine residues which are conserved in all alphaherpesviruses are located either in domain I or III, suggesting that these cysteine residues may be involved in cooperative interactions between domain I and III. Domain III contained a CXXCC motif that was conserved among all alphaherpesviruses. Mutating the CXXCC motif to SXXSC caused the appearance of small plaques, while viral yields were similar to the ΔgKhpd-3 virus. In contrast, a single C-to-S change at position 269 did not adversely affect plaque formation nor viral yield. These results indicate that both the CXXCC motif and the first 28 amino acids of domain III contribute to the structure and function of gK, while the Cys269 does not affect gK functions. Domain II, predicted to be oriented toward the cytoplasm, was the most conserved among different herpesviruses and contained the tyrosine-based amino acid motif (YXXØ). Similar motifs are known to serve as putative signals for post-Golgi

201

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
glycoprotein transport, and receptor-specific endocytosis (Canfield et al., 1991; Kirchhausen et al., 1997; Marks et al., 1996; Mellman, 1996; Trowbridge, et al., 1993). Proper subcellular localization of the varicella-zoster virus glycoprotein I (gI) was shown to depend on two different determinants, one of which is a tyrosine containing tetrapeptide related to endocytosis sorting signals (Alconada et al., 1996). Mutation of the YXXL endocytosis motif in the cytoplasmic tail of pseudorabies virus gE, inhibited endocytosis of gE and caused a small-plaque phenotype, while it did not alter in vivo virulence (Tirabassi et al., 1999). A single amino acid change of Y to S within this motif caused the formation of small viral plaques and reduced viral yields drastically in comparison to ΔgK virus, indicating that the mutated gK interfered with virus replication.

It is conceivable that gK truncations and the other mutations described here may destabilize gK, causing its rapid degradation. Attempts to detect truncated gK in infected cellular extracts by radioimmunoprecipitation with rabbit antibodies raised against gK peptide antigens were inconclusive due to the high background reactivity of these sera. However, the different phenotypic properties and viral yields of gK-mutant viruses strongly suggest that mutated gK proteins are expressed in biologically active forms. Specifically, the severe truncations of ΔgKhpdl and ΔgKhpdl2 reduced viral titers substantially in comparison to those of the ΔgK virus, and produced large vesicles containing virions that were readily detected by electron microscopy. Similarly, we noted previously that expression of 112 amino acids by FgKβ resulted in gK-specific virus-induced cell fusion supporting our hypothesis that this amino terminal truncation of gK is expressed (Jayachandra et al., 1997).
The tyrosine-to-serine change within gK domain II produced small plaques and reduced viral yields drastically in comparison to those of ΔgK, indicating that this truncated gK exerts a negative effect on infectious virus production. Mutations within the CXXCC motif of gK domain III produced viruses, which replicated more efficiently than the ΔgK virus, indicating that the mutant gK protein is expressed in a partially functional form. It is important to note, that all gK-mutant viruses produced viral yields and plaques which were similar to those of the wild-type KOS virus when propagated in VK302 cells, indicating that VK302 cells complemented gK-mutant viruses in a trans-dominant manner overcoming the negative effect of the hpd1, hpd2, and gK/Y183S mutations. This cellular complementation indicates that gK-mutant viruses do not contain any secondary mutations that may affect their phenotypic and replication properties. It is unclear at this point why VK302 cells complement all gK-mutant viruses. One of the possible explanations for the trans-dominant complementation of gK mutations by VK302 cells is that gK expressed by the cellular gene complexes with cellular proteins found in limiting amounts within infected cells. In this scenario, mutated gK expressed by the virus cannot displace preformed gK-cellular protein complexes.

Our electron microscopic data is consistent with the hypothesis that enveloped virions are transported to the Golgi via vesicles, which originate from the outer nuclear lamellae. Such vesicles were readily observed in wild-type KOS-infected cells containing single-enveloped virions. Furthermore, expression of truncated gKs (hpd1 and hpd2) resulted in the accumulation of hundreds of virion particles within vesicles, which, except for their large size, otherwise appeared to be
morphologically similar to those containing single KOS virions. The intracellular transport of various intracellular cargo, including soluble and membrane-bound proteins, is achieved in cells through the use of intricate systems of targeted vesicular transport. These systems dictate a well-orchestrated cascade of molecular events, which control the formation of vesicles from the endoplasmic reticulum and their bi-directional transport to the Golgi, intracellular organelles, and extracellular spaces. The hallmark of this cellular transport system is that specific targeting of vesicles is achieved through the use of pilot and sorter proteins embedded within the transport vesicle membrane and the receiving membrane, respectively (Rothman, 1996; Rothman and Wieland, 1996; Teasdale and Jackson, 1996). It is tempting to consider that herpes simplex virions, which have evolved to use cellular systems masterfully to their advantage, may utilize specific elements of the vesicular transport pathways for intracellular virion transport and virion egress. In this regard, differences in the virion egress pathways of HSV in comparison to those of pseudorabies virus and varicella-zoster virus may be due to the differential localization and functions of gK and other viral proteins involved in virion egress.
Chapter VI

CONCLUDING REMARKS AND SUMMARY

The evolution of HSV with its host has produced a remarkably efficient human pathogen that expresses a limited set of 70-100 proteins upon infection. The ability of the virus to accomplish so much with so little is greatly due to the multifunctionality of each of its proteins. HSV-1 gK was initially hypothesized to function during membrane fusion events because of the propensity for single point mutations that caused extensive cell-to-cell fusion to map to its loci. Although initial characterizations of HSV-1 gK by many laboratories has led to some confusion as to its role in the HSV-1 lifecycle, the genetic characterization of U₅₃ (gK) deletions and mutations has begun to clear up some of these misunderstandings. Furthermore, the development of immunological reagents reported in this dissertation provides the unique ability to begin characterizing the gK protein in the context of the cell, whereas previously, this characterization was limited to in vitro translated protein product.

The role of HSV-1 gK in virus egress was initially reported by Hutchinson et al (1995) and Jayachandra et al (1997). Hutchison et al (1995) generated a U₅₃ (gK) null virus by insertionally inactivating the U₅₃ gene with a β-galactosidase expression cassette; however, the construction of this virus allowed for the amino terminal 1/3 of the protein to be expressed. This virus, designated FgKβ, was shown to be essential for virus replication and egress. Infection of African green monkey kidney (Vero) cells with this virus propagated on a cell-line (Vero [VK302]) that expressed gK in trans, led to a non-productive infection and failure to
isolate infectious virus. Ultrastructural examination revealed that virion particles were absent from extracellular spaces and large numbers of unenveloped capsids were distributed throughout the cytoplasm (Hutchinson et al, 1995). Jayachandra et al (1997) significantly extended and revised these results and laid the groundwork for the contents of this dissertation. Utilizing a novel strategy for the generation of deletions and truncations in HSV-1 gK, Jayachandra et al generated a gK null virus, designated ΔgK that specified an entire deletion of the UL53 coding region. Comparison of the ΔgK virus to the FgKβ virus revealed many striking differences that were attributed to the ability of the FgKβ virus to express the amino terminal one-third of gK. The first distinguishing characteristic of ΔgK was its ability to form plaques, albeit to a much lesser extent than wild-type HSV, on Vero cells, indicating that gK was not essential for infectious virus production. Similar to FgKβ, ΔgK failed to egress efficiently from Vero cells; however the ability to form infectious virus was greatly increased relative to FgKβ (Jayachandra et al, 1997). These results would suggest that expression of the amino terminal one-third of gK had a deleterious effect on infectious virus production. It was also shown that the deletion of gK could be partially complemented by infecting cells that were in log phase growth (Jayachandra et al, 1997). Therefore, the function of gK could be at least partially complemented by cellular factors.

In continuation of this work, chapter V of this dissertation examined the effects of truncations and mutations on infectious virus production and virus egress in a syngeneic system. Truncation of gK after the first and second hydrophobic domain produced deleterious effects that were similar to that produced by FgKβ.
This included a reduction in infectious virus production relative to ΔgK.

Interestingly, ultrastructural analysis of ΔgKhpd1, which specified a truncation after the first hydrophobic domain, produced large multi-laminar vesicles that contained numerous unenveloped capsids. Furthermore, while enveloped virions were found within fragmented Golgi vacuoles for both ΔgKhpd1 and ΔgKhpd2 viruses, enveloped virions were not observed within electron dense vesicles. In contrast, these structures were observed in ΔgKhpd4, wild-type KOS, and to a lesser extent in ΔgKhpd3 infected cells. The appearance of extracellular enveloped virions coincided with these observed structures, which may represent vesicles that traffic enveloped virus to extracellular spaces. Therefore, it is interesting to hypothesize that it is gK that functions in this trafficking process. By either egress model (see Fig. 1.7 and corresponding discussion in introduction) trafficking of infectious virus from the Golgi to extracellular spaces would occur within these vesicles. It is therefore intriguing that mutation of a putative transport motif (YXXΦ to SXXΦ) abolished the ability of the virus to egress from infected cells. The YXXΦ motif is predicted to lie within a cytoplasmic domain of gK, thereby allowing for the recruitment and interaction of cytoplasmic cellular vesicular transport proteins. However, it still remains to be resolved whether or not it is the inability of HSV-1 gK to be trafficked to cell surfaces that accounts for this phenotype or if gK participates directly in the trafficking of viruses to cell surfaces.

The observations from the truncations and mutations in gK support both the envelopment/de-envelopment and vesicular transport models of virus egress.

However, in either regard, the phenotypes exhibited suggest that lack of gK prevents
post-Golgi trafficking of infectious virus to extracellular spaces. In support of the
envelopment/de-envelopment model, the numerous un-enveloped capsids found
within the cytoplasm would represent a backlog of virions awaiting final
envelopment at Golgi membranes. Because Golgi vacuoles are being occupied by
enveloped virions that fail to be transported, capsids would accumulate within the
cytoplasm. Furthermore, the large multi-laminar vesicles observed for ΔgKhpdl
virus infections would represent a failed virus to vesicle fusion event and thus
preventing the release of unenveloped nucleocapsids to the cytoplasm.

Contrastingly, in the vesicular transport model, the appearance of numerous
unenveloped capsids within the cytoplasm would most likely be accredited to the
unregulated fusion of enveloped virus to transport vesicles due to the aberrant nature
of truncated gK. The appearance of large multi-laminar vesicles observed in
ΔgKhpdl infections could be attributed to an inability of these vesicles to be
transported due to their immense size. This assumption is supported by the apparent
capture by electron microscopy of the initial formation of these vesicles. The
directional budding of enveloped virions at areas of high electron density is very
reminiscent of endoplasmic reticulum to Golgi vesicular transport. Moreover, the
presence of the large multi-laminar vesicles appeared to be morphologically similar,
except for their large size, to those containing single KOS virions. The intracellular
transport of various intracellular cargo, including soluble and membrane-bound
proteins, is achieved in cells through the use of intricate systems of targeted
vesicular transport. These systems dictate a well-orchestrated cascade of molecular
events, which control the formation of vesicles at the ER and their bi-directional
transport to the Golgi, intracellular organelles, and extracellular spaces. The hallmark of this transport system is that specific targeting of vesicles is achieved through the use of pilot and sorter proteins embedded within the transport vesicle membrane and the receiving membrane, respectfully. It is tempting to consider that the phenotype of the transport motif (YXXØ) mutant is directly attributable to a defect in this transport and therefore, a direct association between the vesicular transport model of egress and gK could be derived.

The determination that HSV-1 gK was a structural component of virion particles as described in Chapter IV was possible due to the generation of anti-gK sera that was specific for domains II and III. Unfortunately, this sera reacted well with purified virions, but not cellular extracts, limiting its usefulness in the structural characterization of gK. It did however confirm that like its alphaherpesvirus counterparts, PRV-1 and VZV, glycoprotein K was indeed a structural component of purified virions. It will be interesting to determine if the future production of sera using this expressed protein is able to generate antibodies that react well with cellular extracts of HSV-1 gK.

The characterization of gK as a structural component of purified virions was an important first step in determining further the functions of gK, for without its detection on purified virions, its role in virus entry could only be presumptive. We had known from our studies that the ΔgK virus could in fact infect Vero cells and produce infectious virus; however, the ability of this virus to infect cells always seemed somewhat restricted. Following the examination of virus entry kinetics into Vero cells, there was little doubt that gK null viruses had at least a delay in their
ability to enter into this cell type; however the reason for this delay could not be explained.

In 1996, Montgomery et al, described the expression cloning isolation of a human co-receptor for virus entry, HveA. Because Vero cells constituted the primary cell-line for our studies, we isolated and cloned the homolog to this receptor, HveAs, as described in Chapter III of this dissertation. Characterization of HveAs revealed significant homology to its human counterpart, HveA; however, there were several deletions, insertions, and substitutions within the predicted amino acid sequence. HveAs facilitated wild-type virus entry into the normally resistant Chinese hamster ovary (CHO) cells as assayed by a virus that was generated to express the enhanced green fluorescent protein (EGFP). Moreover, expression of soluble HveAs as well as antibodies to HveAs blocked entry into HveAs transformed CHO cells, but not Vero cells. The inability of these reagents to block virus infection into Vero cells can be attributed to the presence of other mediators of virus entry, namely HveC and HlgR.

In order to rapidly characterize the role of HSV-1 gK in virus entry, the gK null virus, ΔgK-EGFP, was constructed to constitutively express the green fluorescent protein as described in Chapter II. A wild-type KOS-EGFP counterpart was also constructed such that the EGFP expression cassette was inserted within the intergenic region between the Ul53 (gK) and Ul54 (ICP27) genes. Therefore, the wild-type KOS-EGFP and gK null, ΔgK-EGFP viruses differed only in the presence or absence of the Ul53 (gK) open reading frame, respectively. Each of these viruses
had plaque and replication phenotypes that resembled that of their non-fluorescent counterparts.

The role of HSV-1 gK in virus entry and receptor utilization was established in Chapter IV. It was hypothesized that the delay in the kinetics of ΔgK virus entry into Vero cells was due to an altered recognition of herpes virus entry mediating receptors. Therefore, we tested the ability of the ΔgK-EGFP and KOS-EGFP to enter into CHO cells transformed with different herpesvirus receptors. While the wild-type KOS-EGFP virus entered into all the receptor types, except HveB, ΔgK-EGFP utilized all the receptors less efficiently. *Trans*-complementation of ΔgK-EGFP on a cell-line that expressed gK complimented virus entry into these cells indicating that the presence of gK during infectious virus production was necessary for assembly of infectious virions that were capable of utilizing these receptors. Interestingly, although ΔgK-EGFP entered into CHO-HveAh cells at approximately 50% efficiency, HveAs was unable to facilitate ΔgK virus entry into CHO transformed cells. Complementation of the gK null effect or use of a gK-rescued virus reversed this phenotype. This phenotype was exhibited despite quite significant homology between the human HveA and the simian HveA (Fig. 3.4, in Chapter III).

In order to determine the region of HveAh that facilitates entry of gK null viruses, chimeric receptors were constructed using splice overlap extension PCR. Although the final amino acids that functioned in gK-modulated virus entry are still yet to be determined, the ability to facilitate entry was mapped to the amino terminal one hundred amino acids that contain only twelve amino acid replacements. It will
be of great interest to eventually determine if it is a single amino acid change within this region that alters the ability of gK null viruses to utilize this receptor.

The original hypothesis posed by researchers that HSV-1 gK functions during membrane fusion events of the virus lifecycle is at first glance a broad theory of where its effects are actually exerted. However, the broadness of this theory is now justified by the identification of the multifunctionality of gK. This dissertation emphasizes that even a small 338 amino acid protein such as gK can exert its effects at several stages in the HSV lifecycle including: receptor recognition and utilization, virus entry, infectious virus assembly and production, translocation of virion particles through the cellular pathways and virus egress to extracellular spaces. Although it is tempting to assign a direct correlation of the phenotypes we have observed, the possible connections between these processes cannot be ignored or completely dissociated. Therefore, in order to further our understanding of the role gK plays in these processes, it will be essential that these stages eventually be dissected from one another. As technology and reagents advance, the roles of HSV-1 gK in the virus lifecycle will be definitively determined.

FUTURE CONSIDERATIONS AND CHALLENGES

Characterization of HSV-1 gK remains elusive in many respects. It has been fifteen years since Debroy et al (1985) initially published the sequence of a gene with a high propensity for syncytial mutations. In these fifteen years, information has not been forthcoming, despite extensive efforts from many laboratories. Only in recent years has there been descriptions of the function of gK in the virus lifecycle.
and yet there is still little information as to its structure. Characterization of HSV-1 gK will remain a challenge for some time to come. Those challenges aside, it has becoming increasingly apparent that gK provides a unique probe into both virus and cellular functions.

Herpes simplex viruses have evolved to masterfully utilize cellular systems to their advantage. This dissertation has only begun to probe into these virus-cell interactions with respect to gK. The finding that gK may associate both with cellular receptors upon entry and cellular transport proteins upon egress, opens a wide field of potential research. Furthermore, this dissertation has failed to address the rudimentary understanding of the structure of gK. However, the development of immunological and viral reagents does facilitate the rapid generation of information in this arena. With this new understanding of gK, there are three areas that sorely need addressing: 1) the structure and localization of gK in the context of the cell; 2) the understanding of the role gK plays in modulating virus entry and receptor utilization; 3) the interactions of gK and the cell in the vesicular trafficking of virus particles to extracellular spaces.

**HSV-1 gK and virus entry:** This may be one of the most controversial areas of research with regards to HSV-1 gK. Through our research, it has become quite apparent that gK null viruses fail to utilize the HveAs receptor for virus entry and that this failure may account for the delays in the entry kinetics associated with ΔgK infection of Vero cells. However, in contrast to other entry glycoproteins, it is also quite apparent that gK null viruses are capable of entering into Vero cells with a similar efficiency given time to wild-type KOS virus. It could therefore be
hypothesized that other viral receptors on Vero cells facilitate entry into these cells. In this regard, the receptors would have to function to a greater degree than those of the identified human receptors, because these receptors could only partially facilitate ΔgK entry. Therefore, the identification of other simian receptors for virus entry, and in particular for ΔgK virus entry is warranted.

Utilizing reagents generated to investigate domains of gK involved in egress, it would also be beneficial if the domains of gK were resolved for virus entry. This may aid in the understanding of how gK functions in receptor utilization by identifying domains of gK that participate in this process. This is not however a guarantee that gK is directly involved, just as the determination that gK is a structural component of infectious virions does not directly associate its function with the observed differences in virus entry. Therefore, the structural composition of these viruses must be identified further.

It is readily apparent that these virions are quite different from their wild-type counter-parts simply by looking at their sedimentation in sucrose gradients. In these assays it will be important to determine: 1) the degree of glycosylation of these virions; 2) the relative amounts of glycoproteins between these virions; 3) the particle to plaque forming unit ratio.

**HSV-1 gK and virus egress:** The determination that cellular transport motifs are contained within gK and that modification of these motifs altered infectious virus production and virion egress in a manner as deleterious as deleting the protein in its entirety, may be the single most important finding of this research. Cellular proteins that are known to interact with these motifs and facilitate transport
have been identified and are well characterized. It only remains to be resolved whether or not these proteins in fact do interact with this transport motif.

If indeed there is interactions between the transport motif and cellular adapter proteins, it then remains to be resolved are the proteins only effecting the transport of the protein itself to cell surfaces or are they also functioning to traffic virus within vesicles to cell surfaces. These two processes are not easily dissociated; however, with the emergence of cell-lines defective in particular transport pathways and with better imaging techniques and immunological reagents, these pathways may soon be resolved.

The determination of the trafficking and recycling of gK from cell surfaces is critical in these studies. Immunological and viral reagents developed during this study can determine the effect on transport of mutation of the transport motif. Increasing this understanding may also aid in our understanding of the role of gK in virus induced cell-to-cell fusion.

**Structural characterization of HSV-1 gK:** It is not often that a protein's function is characterized prior to any significant information being available as to its structure. The phenotypes discussed within this dissertation have all been based on alteration of the Ul53 gene and observation of the effect in infected cells. While this approach was necessary due to the limitations of immunological reagents, it is by far not an ideal situation without similarly characterizing the protein product. Questions about the structure of gK remain to be answered including: 1) To what extent is gK processed through glycosylation; 2) Is gK phosphorylated at sites adjacent to transport domains; 3) Are their other carbohydrate modifications that
occur besides N-linked glycosylation; 4) Is the topology predictions that are presented accurate in the context of the cell.
REFERENCES


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


APPENDIX:
LETTERS OF PERMISSION

October 19, 1999

To: Journal's Permission Department
   Elsevier Science, Subsidiary Rights Department

From: Timothy P. Foster
       Candidate for Doctorate of Philosophy Degree
       Department of Veterinary Microbiology and Parasitology
       School of Veterinary Medicine
       Louisiana State University
       Baton Rouge, LA 70803
       (225) 346-3345 fax: (225) 346-5715

Re: Request for permission to include published work in dissertation

I have recently published in one of your journals and need to include the
information contained within this manuscript as a portion of my dissertation. In
accordance with the guidelines of the Louisiana State University Graduate School,
as well as your journal I need to get a letter of permission in order to include this
information. The copyright transfer agreement that we signed excluded its inclusion
in dissertations within the terms of its agreement.

The relevant bibliographical information for this manuscript is:

enhanced green fluorescent protein by herpes simplex virus type 1 (HSV-1) as an in
vitro or in vivo marker for virus entry and replication. *Journal of Virological
Methods.* Nov;75(2):151-60.

I would greatly appreciate if you could please fax the letter of permission to
the above fax number and/or mail it to the above address. Thank you in advance for
your time and assistance.
FRC/ik/oct99.122
29 October 1999

Timothy Foster
Dept of Veterinary Microbiology and Parasitology
School of Veterinary Medicine, Louisiana State University
South Stadium Rd
Baton Rouge, LA 70803
USA

Dear Dr Foster

JOURNAL OF VIROLOGICAL METHODS, Vol 75, No 2, 1998, pp 151-160,
“Expression of the enhanced green fluorescent protein...”

As per your email dated 19 October 1999, we hereby grant you permission to reprint the
aforementioned material in your thesis at no charge subject to the following conditions:

1. If any part of the material to be used (for example, figures) has appeared in our
   publication with credit or acknowledgement to another source, permission must
   also be sought from that source. If such permission is not obtained then that
   material may not be included in your publication/copies.

2. Suitable acknowledgment to the source must be made as follows:
   "Reprinted from Journal title. Volume number. Author(s), Title of article, Pages
   No., Copyright (Year). with permission from Elsevier Science".

3. Reproduction of this material is confined to the purpose for which permission is
   hereby given.

4. This permission is granted for non-exclusive world English rights only. For other
   languages please reapply separately for each one required. Permission excludes use
   in an electronic form. Should you have a specific electronic project in mind please
   reapply for permission.

5. This includes permission for UMI to supply single copies, on demand, of the
   complete thesis. Should your thesis be published commercially please reapply for
   permission.

Yours sincerely

Mary White

P P Frances Rothwell (Mrs)
Subsidiary Rights Manager

250

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
To: Journal’s Permission Department  
Academic Press

From: Timothy P. Foster  
Candidate for Doctorate of Philosophy Degree  
Department of Veterinary Microbiology and Parasitology  
School of Veterinary Medicine  
Louisiana State University  
Baton Rouge, LA 70803  
(225) 346-3345 fax: (225) 346-5715

Re: Request for permission to include published work in dissertation

I have recently published in one of your journals and need to include the information contained within this manuscript as a portion of my dissertation. In accordance with the guidelines of the Louisiana State University Graduate School, as well as your journal I need to get a letter of permission in order to include this information. The copyright transfer agreement that we signed excluded its inclusion in dissertations within the terms of its agreement.

The relevant bibliographical information for this manuscript is:


I would greatly appreciate if you could please fax the letter of permission to the above fax number and/or mail it to the above address. Thank you in advance for your time and assistance.
Dear Requestor:

Thank you for your request to use material from your work published in an Academic Press publication.

It is now the policy of Academic Press that authors need not obtain permission in the following cases: (1) to use their original figures or tables in their future works; (2) to make copies of their papers for their classroom teaching; and (3) to include their papers as part of their dissertations/theses.

Sincerely,

Ana Merced
Paralegal Department
Academic Press - Permissions
407 345 3994
407 345 4058
amerced@harcourthrace.com
To: Linda Illig  
Director of Journals, ASM  
(202) 942-9355

From: Timothy P. Foster  
Candidate for Doctorate of Philosophy Degree  
Department of Veterinary Microbiology and Parasitology  
School of Veterinary Medicine  
Louisiana State University  
Baton Rouge, LA 70803  
(225) 346-3345 fax: (225) 346-5715

Re: Request for permission to include published work in dissertation

I have recently published in the Journal of Virology and need to include the information contained within this manuscript as a portion of my dissertation. In accordance with the guidelines of the Louisiana State University Graduate School, as well as your journal I need to get a letter of permission in order to include this information.

The relevant bibliographical information for this manuscript is:


I would greatly appreciate if you could please fax the letter of permission to the above fax number and/or mail it to the above address. Thank you in advance for your time and assistance.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
VITA

Timothy P. Foster was born November 9, 1972, in Fairfield, California, on Travis Airforce Base. He is the son of Edwin Earl Foster Jr. and Cheryl Ann Carraway Foster and has one younger sister, Amy Elizabeth Foster. Soon after his birth, his family moved to Baton Rouge, Louisiana. The author grew up in Baton Rouge and Denham Springs, Louisiana, and attended and graduated with honors from Denham Springs High School. He obtained two undergraduate Bachelor of Science degrees from Louisiana State University (LSU) in May of 1995: one in biochemistry with a minor in psychology and one in microbiology with a minor in zoology. Following his graduation, Timothy initially enrolled in the Master of Science degree program through the Department of Veterinary Microbiology and Parasitology in the School of Veterinary Medicine, Louisiana State University. However, in January of 1997 Timothy was awarded a three year Board of Regents Fellowship and decided to forgo his master’s and pursue a doctorate under the guidance of Dr. Konstantin Kousoulas studying the molecular biology of herpes simplex viruses. He earned his degree of Doctor of Philosophy in December of 1999. The author enjoys scuba diving, fishing, camping, marine aquaria, cooking, and most of all science.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Timothy P. Foster

Major Field: Veterinary Medical Sciences

Title of Dissertation: Molecular Genetics and Functions of Herpes Simplex Virus Type 1 (HSV-1) Glycoprotein k (gK) in the Morphogenesis of Infectious Virion Particles

Approved:

[Signatures]

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

Date of Examination:

20 October 1999

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.