The Pennsylvania State University

The Graduate School

College of Medicine

ACYL DETERMINANTS OF THE HERPES SIMPLEX VIRUS TYPE 1 UL11 TEGUMENT PROTEIN REQUIRED FOR MEMBRANE TRAFFICKING AND VIRION ENVELOPMENT

A Dissertation in

Microbiology and Immunology

by

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Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

May 2009

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Abstract

Herpes simplex virus type 1 (HSV-1) is a complex virus composed of over 50 virally encoded proteins that form 3 distinct physical structures. The innermost structure, the nucleocapsid, contains the viral genome. The outermost structure is a host-derived lipid envelope coated with virally-encoded membrane proteins, many of which are glycosylated. Located between the two regions is the tegument, of which UL11 is a member.

UL11 is a small (96 amino acid) protein that is membrane bound via two covalently attached fatty acids. Myristate is 14-carbons long and is added co-translationally to UL11 via a non-reversible amide bond, whereas palmitate is 16-carbons in length and is added post-translationally via a reversible thioester bond. Myristylation is a prerequisite to palmitylation; however, the former provides only weak membrane binding strength whereas the latter penetrates deeper into the lipid bilayer to form a more stable protein/membrane interaction. The addition of both fatty acids to UL11 is required for membrane trafficking and the subsequent accumulation at the site of virus assembly, where UL11 is predicted to function during the envelopment step, albeit via an unknown mechanism.

The studies in this dissertation were designed to better understand what role UL11 plays during virus envelopment, and specifically to what extent the acylations of UL11 are required for such functions. As a first step, the ability of UL11 to localize to lipid rafts, or detergent resistant membranes (DRMs), was analyzed. Dual acylation of proteins with myristate and palmitate is a classical DRM targeting signal, and UL11 was

found to be no exception. These studies also demonstrated the importance of two additional motifs within UL11 for DRM accumulation. The “di-leucine” (LI, leucine-isoleucine) and acidic cluster (AC) motifs are involved in targeting UL11 from the plasma membrane (PM) to the site of virus assembly, the trans-Golgi network (TGN). Mutants of UL11 that lack the palmitate moiety were reduced for DRM association and mutants that lack both myristate and palmitate were further reduced to background levels. Removal of only a single trafficking motif (LI or AC) did not alter the ability of UL11 to associate with DRMs, but mutants of UL11 that lack both the LI and AC had increased DRM association. Taken together, these data suggest that UL11 has highly dynamic membrane-trafficking properties, and that UL11 may require trafficking through DRMs prior to accumulation at the TGN to ensure proper function during virus envelopment.

To directly examine the hypothesis that UL11 requires both myristate and palmitate for function during virus envelopment, recombinant viruses were created to express variants of UL11 with different acylation patterns. The mutants included a non-acylated UL11 (no myristate or palmitate), a partially-acylated UL11 (myristate only), or one of two UL11 chimeras that contain acylation signals from non-HSV proteins (myristylated only or myristylated and palmitylated). The results showed that only WT UL11 (with both myristate and palmitate moieties) fully rescued the growth properties of a UL11-null virus. However, it was very surprising that the non-acylated UL11 (no myristate or palmitate) rescued some growth defects of the UL11-nulll virus. These experiments demonstrated that although not essential, the acylations are important for the function of UL11 during virus envelopment.

The studies detailed within this dissertation provide new insights into how the

UL11 protein functions during virus assembly and envelopment. With further studies, it should be possible to elucidate the exact mechanism(s) by which UL11 functions during the assembly and envelopment of HSV-1.

Table of Contents

List of Figures ix

List of Tables x

List of Abbreviations xi

Acknowledgements xvi

Chapter I: Overview 1

Chapter II: Literature Review 12

Classification of Herpesviruses 13

Virion Structure 14

Overview 14

The HSV-1 Nucleocapsid 17

The HSV-1 Tegument 20

The HSV-1 Envelope 23

Virion Assembly 24

Genome Remodeling 24

Protein Expression 27

Nuclear Reorganization & DNA Replication 29

Capsid Assembly & DNA Packaging 30

Nuclear Egress & Cytoplasmic Transport 31

Tegumentation 34

Final Envelopment 37

Secretion 39

Entry & Uncoating....40
UL11....42
Myristylation & Palmitylation....42
Di-leucine & Acidic Cluster....52
Detergent Resistant Membranes....53
Discovering and Defining DRMs....53
DRM Size....57
DRMs Vs. Caveolae....58
DRMs & HSV....58
Chapter III: Sequences in the UL11 Tegument Protein of Herpes Simplex Virus that Control Association with Detergent-Resistant Membranes....60
Abstract....61
Introduction....62
Results....65
Flotation Analysis of UL11-GFP....65
Specificity of the UL11 Antibody....69
Role of Fatty Acid-Modification....69
Sequences that Control Levels of DRM Association....75
Chapter IV: Myristylation and Palmitylation of UL11 are Necessary but Not Sufficient to Support Growth of Herpes Simplex Virus....77
Abstract....78
Introduction....79
Results....81

Relocation of the UL11 Coding Sequence....81
UL11 Acylation Mutants....90
Why Did a Non-myristylated UL11 Enhance Growth of Δ30-96?....96
Fusion of Foreign Acylation Signals to UL11....96
Chapter V: Discussion....101
UL11 Acylation & DRM Trafficking....102
UL11 Acylation & Virus Growth....107
Chapter VI: Materials & Methods....110
Cells & Infections....111
Recombinant Viruses....111
Antibodies....113
Membrane Flotation....114
Transferrin Receptor & Methyl-β-Cyclodextrin Treatment....115
Growth Curves....115
Incorporation Assay....116
Myristylation of UL11....117
Quantitative PCR Analysis....117
Appendix A: Permission for use of Figure 2.6....119
Appendix B: Table of All Recombinant BAC Viruses Created....121
References....123

List of Figures

1.1 Structure of an HSV-1 virion 3

1.2 Assembly and egress pathway of HSV-1 5

1.3 UL11 protein from HSV-1 7

2.1 Organization of the HSV-1 genome 18

2.2 HSV-1 gene organization 21

2.3 General replication cycle of HSV-1 25

2.4 Lipid modifications of UL11 44

2.5 Myristyl switches 47

2.6 Two-signal model of membrane binding by myristylated proteins 49

2.7 A schematic representation of the raft flotation assay 55

3.1 Flotation analysis of UL11 66

3.2 DRM association of UL11 mutants 70

3.3 Analyses of N-terminal UL11 chimeras 73

4.1 Relocation of UL11 82

4.2 UL11 expression and virion incorporation 86

4.3 Growth properties of recombinant viruses 88

4.4 UL11 acylation mutants 91

4.5 Membrane binding of UL11 derivatives 94

4.6 Further characterization of M15 97

5.1 Model of proposed UL11 functions 105

List of Tables

2.1 Human Viruses of the Family *Herpesviridae* 15

2.2 Functions of HSV-1 Virion Components 16

4.1 Recombinant HSV Constructs 84

List of Abbreviations

μCi	microcurie
μg	microgram
μl	microliter
μm	micrometer
α-TIF	alpha *trans*-inducing factor
AC	acidic cluster
AP	adaptor protein
BAC	bacterial artificial chromosome
bp	base pair
CCSC	C-capsid-specific component
CD4	cluster of differentiation 4
Ci	curie
CKII	casein kinase II
CPE	cytopathic effect
Cys	Cysteine
d	day
DMEM	Dulbecco's modified eagles medium
DNA	deoxyribonucleic acid
DRM	detergent resistant membrane
dsDNA	double-stranded deoxyribonucleic acid
E	early

EBV	Epstein-Barr virus
ER	endoplasmic reticulum
ESCRT	endosomal sorting complexes required for transport
FBS	fetal bovine serum
gB	glycoprotein B
gC	glycoprotein C
gD	glycoprotein D
gE	glycoprotein E
GFP	green fluorescent protein
GGA	Golgi-localized γ-adaptin ear homology domain ARF-binding protein
gH	glycoprotein H
gI	glycoprotein I
gK	glycoprotein K
gL	glycoprotein L
Gly	Glycine
gM	glycoprotein M
GPI	glycosylphosphatidyl inositol
h	hour
HAT	histone acetyl transferases
HCMV	human cytomegalovirus
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
HIV	human immunodeficiency virus
hpi	hours post-infection

Hsp	heat shock protein
HSV-1	herpes simplex virus type 1
HVEM	herpesvirus entry mediator
ICP	infected cell protein
IE	immediate-early
KSHV	Kaposi’s sarcoma-associated herpesvirus
L domain	late domain
L particle	light particle
L	Late
LAT	latency-associated transcript
LI	leucine-isoleucine, “di-leucine-like”
LL	leucine-leucine, di-leucine
MβCD	methyl-β-cyclodextrin
MAP	methione-aminopeptidase
Met	methionine
MHC-I	major histocompatibility complex 1
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
mmol	millimoles
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid

ND10	nuclear domain 10
nm	nanometer
ORF	open reading frame
oriL	origin of replication within the unique long segment
oriS	origin of replication within the unique short segment
PACS-1	phosphofurin acidic cluster sorting protein-1
PAGE	polyacrylamide gel electrophoresis
PAT	palmityl acyl transferase
PBS	phosphate-buffered saline
PC	phosphatidylcholine
PCR	polymerase chain reaction
PFU	plaque forming units
PKC	protein kinase C
PKD	protein kinase D
PM	plasma membrane
pol	polymerase
PrV	pseudorabies virus
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
TBP	TATA binding protein
TFIIB	transcription factor II B
TfR	transferrin receptor

TGN	trans-Golgi network
TX-100	triton X-100
U_L	unique long
U_S	unique short
UV	ultraviolet
Vhs	virion host shut-off
VP	virion protein
VZV	varicella-zoster virus
w/v	weight/volume
w/w	weight/weight
WT	wildtype

Acknowledgements

The process of graduate school is daunting, to say the least. It is only with the support from others that one can succeed. As such, I owe a great deal of thanks to many people. First, my thanks go to John Wills for providing the environment to grow not only as a scientist, but also as a person. Also, I thank all the members of my committee: Drs. Richard Courtney, Leslie Parent, David Spector, and Ira Ropson. It was the combined effort of all five of these people and their continued support that helped to shape me into the scientist I am today. Second, I thank all the personnel of the Department of Microbiology and Immunology (central lab resources and administrative staff) whose under-recognized efforts make our journey through graduate school incomprehensibly easier. Also, a huge debt of gratitude goes to the members of the Wills Lab that provided an enjoyable work setting and great friendships. While the purpose of graduate school is, well, school, one cannot live entirely within the laboratory. To everyone, especially JWM, whose friendship and support made this time much more enjoyable, I am forever grateful for all of the incredible memories, whether it was an adventure to State College, Assateague, or even Shakey's. As such, I raise my glass to you all and say "THANK YOU!"

I was born and raised in Idaho, where my parents still reside. I then moved to Colorado; in part to attend undergraduate school, but mostly to follow my "big" brother, Shane. Despite wanting to maim each other in our younger years, he has become my best friend and supported me through countless tribulations of life, both scholastically and personally. I often joke that I moved to Pennsylvania because Colorado was not far

enough from my parents; that they could still just drive over and visit. However, nothing is farther from the truth. The only thing more difficult than graduate school for me was not being able to visit my family but a couple of times a year. My parents, Dan and Gail, have provided unconditional love, support, and encouragement, and I am forever thankful to them both. It is with the utmost love and gratitude that I dedicate my dissertation to you guys: Mom, Dad, and Shane.

Chapter I

Overview

Herpes simplex virus type 1 (HSV-1) virions are very complex in their assembly process, coordinating nearly 50 virally encoded proteins and numerous cellular factors into a single particle. Each protein can be grouped into one of three morphologically distinct structures: a nucleocapsid, the tegument, and a glycoprotein-studded lipid envelope (Figure 1.1). Capsid shells are assembled and loaded with the viral genome in the infected cell nucleus. Nucleocapsids are then released into the cytoplasm by means of an envelopment/de-envelopment process at the inner and outer nuclear membranes, respectively. During nuclear egress, the first steps of tegumentation occur and coat the nucleocapsid with viral proteins (49, 153, 271, 309, 314). The process of tegumentation continues as unenveloped capsids are targeted to trans-Golgi network (TGN) derived vesicles via microtubules (215) and results in a complex network of protein-protein interactions. The final tegument proteins and the glycoprotein-containing lipid envelope are acquired during a second envelopment process at the TGN to produce an enveloped virion inside a vesicle. The vesicles traffic to the cell surface via the secretory pathway and fuse with the plasma membrane to release infectious virus into the extracellular milieu. While the major aspects of assembly and egress are known, as outlined above (Figure 1.2), the precise mechanisms are poorly understood. As such, the goal of this dissertation was to study the small, membrane bound tegument protein UL11, which likely functions late during assembly (20), although the exact mechanism is unknown. Below is a brief overview of the knowledge leading up to the experiments, as well as a concise summary of the findings obtained from the work detailed in this dissertation.

To understand UL11, the experiments in this dissertation examined the role of fatty acid modifications of this protein during HSV-1 biogenesis (Figure 1.3). UL11

Figure 1.1

Structure of an HSV-1 virion. The linear double stranded DNA (dsDNA, black lines) is contained within an icosohedral capsid composed of five viral proteins (grey). The nucleocapsid is surrounded by the tegument, an assembly of more than 25 viral proteins, numerous cellular proteins, and molecules of RNA (orange, yellow and green). Encasing the tegument and nucleocapsid is a host-derived lipid envelope (red) that is coated with virus-encoded membrane proteins, 12 of which are glycosylated (blue spikes).

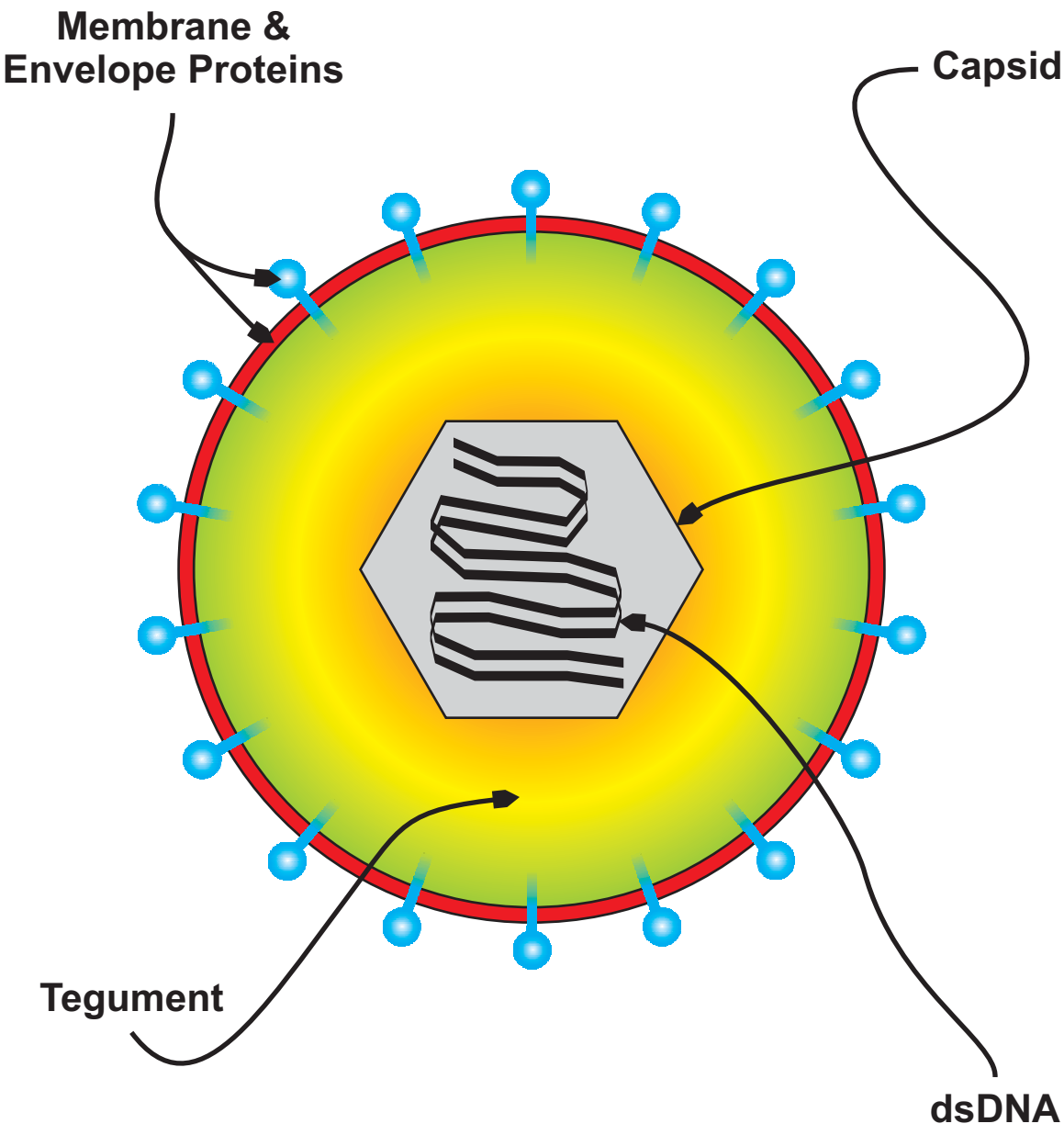

Membrane &
Envelope Proteins
Capsid
Tegument
dsDNA

Figure 1.2

Assembly and egress pathway of HSV-1. Following assembly within the nucleus, the procapsid rearranges to form an icosahedral capsid which is packaged with the double stranded DNA genome (dsDNA). Prior to or during nuclear egress, tegumentation begins by coating the nucleocapsid with inner tegument proteins (orange). Nucleocapsids are released into the cytoplasm following an envelopment/de-envelopment process at the inner and outer nuclear membranes, respectively. After exit from the nucleus, nucleocapsids traverse the cytoplasm to a vesicle of the trans-Golgi network (TGN). Once at the vesicle, the nucleocapsid undergoes a second envelopment step to acquire the final tegument proteins (green) and the host-derived lipid envelope (red) that is studded with viral membrane proteins (blue spikes). Subsequently, the vesicle travels via the host-secretory pathway and fuses with the plasma membrane to release virus into the extracellular milieu.

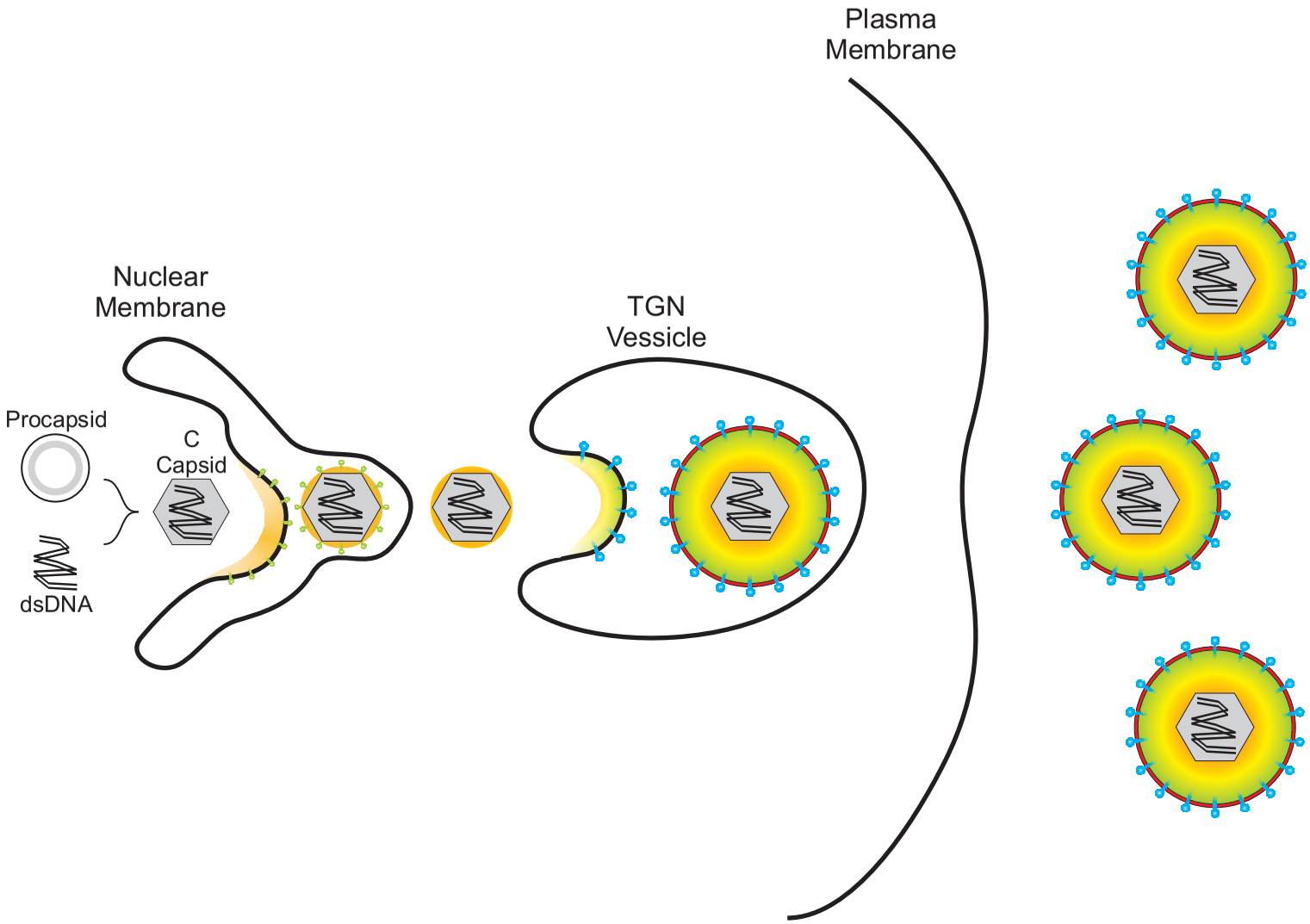
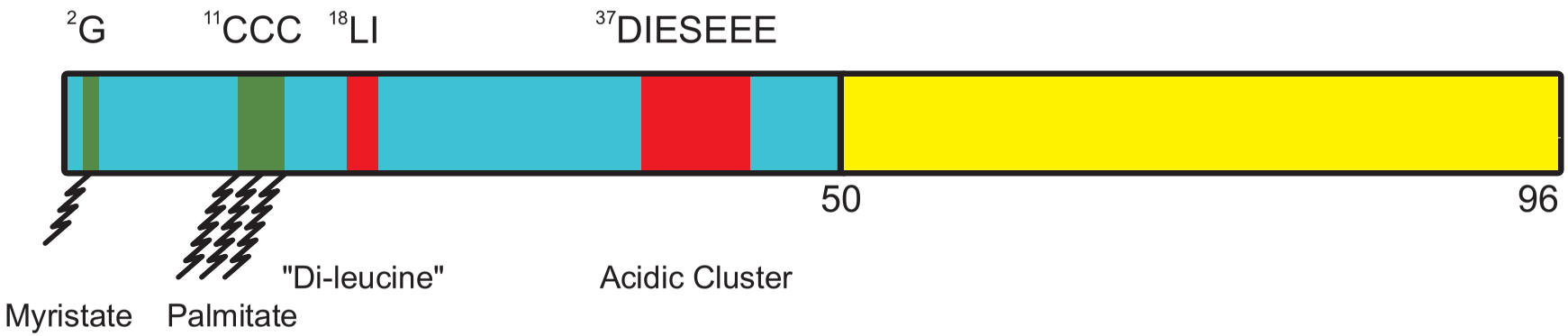

Plasma
Membrane
Nuclear
Membrane
TGN
Vessicle
Procapsid
C
Capsid
dsDNA

Figure 1.3

UL11 protein from HSV-1. (A) A Schematic representation and (B) the amino acid sequence of UL11 from HSV-1. Four motifs have been characterized within the UL11 protein, all residing within the first 50 amino acids (blue). As such, the C-terminal 46 amino acids are dispensable for all *in vitro* studies (yellow). Two acylation signals are present within UL11 (green). Myristylation of the glycine residue at position two precedes, and is required for, the palmitylation of at least one nearby cysteine residue (residues 11-13). "Di-leucine" and "acidic cluster" (red) represent motifs that are involved with protein trafficking, virion incorporation, and interaction with UL16.

A



B

M G L S F S G A R P C C C R N N V L I T D D G E V
V S L T A H D F D V V D I E S E E E G N F Y V P P
D M R G V T R A P G R Q R L R S S D P P S R H T H
R R T P G G A C P A T Q F P P P M S D S E

binds cellular membranes via two fatty acids: myristate and palmitate (226, 238). Myristylation of the N-terminal glycine residue is a co-translational addition that follows the removal of the initiator methionine; whereas the palmitylation of at least one nearby cysteine residues is post-translational and occurs on a cytoplasmic membrane. Importantly, myristylation is a prerequisite of palmitylation. As such, a mutant of UL11 that lacks the N-terminal glycine residue is neither myristylated nor palmitylated, even though there are cysteine residues nearby (226). Together, these two adducts strongly localize UL11 to membranes and facilitate the accumulation of UL11 at the TGN, even in the absence of all other viral proteins (226).

Dual acylation of proteins with myristate and palmitate is a classical signal for lipid raft, or detergent resistant membrane (DRM), targeting. Thus, UL11 was predicted to be localized to DRMs, which are membrane-microdomains that are enriched in cholesterol and are resistant to non-ionic detergents, conditions that disrupt non-DRM membranes (46, 47). Because of this property, DRMs were isolated using Triton X-100 (TX-100) and flotation in sucrose gradients, enabling the analysis of resident proteins. As predicted, a population of UL11 does localize to DRMs, a property that is dependent on the presence of both fatty acids.

DRM localization of UL11 was also influenced by two additional motifs, a leucine-isoleucine (LI) and an acidic cluster (AC) (Figure 1.3). These two motifs are responsible for several properties of UL11, including retrieval off of the plasma membrane (PM), accumulation at the TGN, and interaction with the tegument protein UL16 (226, 227). Removal of either the AC or the LI results in an impairment of these functions. However, removal of either motif alone is insufficient to cause a phenotypic

change in regards to the DRM accumulation of UL11. But, when both motifs are removed, there is nearly a 3-fold increase of DRM localized UL11. The function of this membrane-subdomain targeting is still of interest.

UL11 has a strong and regulated membrane binding and targeting mechanism; one that traffics UL11 through DRMs and accumulates the protein at the site of final envelopment even in the absence of other viral proteins (226). Additionally, a UL11-null virus has defects in secondary envelopment, which manifest as an accumulation of unenveloped particles within the cytoplasm of the infected cell and a reduction of virus release (20, 239). This characteristic suggests that UL11 plays a critical role in the assembly process; either by providing a direct function to the envelopment process, or by recruiting another protein to the TGN, possibly obtained as UL11 traffics through DRMs.

Given the requirement of UL11 for efficient virus production and an apparent importance of membrane binding, it was predicted that UL11 would require both acyl modifications to properly function during virus envelopment. However, it is not possible to make mutations of UL11 that result in altered acylation signals due to an overlap with the UL12 coding sequence which is essential for virus production (424). To circumvent this issue, the UL11 coding sequence was deleted and relocated to a different locus within the viral DNA using a bacterial artificial chromosome (BAC) that contains the entire genome of HSV-1 (144). This allowed either wildtype (WT) UL11 or mutants of UL11 that either lack all acylations, are only myristylated, or contain foreign acylation signals to be expressed without disrupting any essential genes. It was predicted that a virus which expresses a mutant of UL11 that is not acylated in any form would parallel a null-virus in growth characteristics. However, this was not the case. The non-acylated

UL11-expressing virus, as well as all other recombinant viruses that expressed a UL11 derivative, grew better than the UL11-null virus. However, none grew as well as the WT virus. Together, these results suggest that a non- or mis-acylated UL11 is better than no UL11, and is capable of partial function during virion assembly.

To better understand the rational for, and significance of, the experiments described in this dissertation, it is necessary to understand the HSV-1 replication cycle as well as protein acylation. A review of the literature pertaining to these subjects is contained in Chapter II.

Chapter II

Literature Review

Classification of Herpesviruses

To date, over 200 herpesviruses have been identified that infect a wide range of life forms, from bivalves to humans. However, only 9 are known to cause disease in humans, including herpes simplex virus type 1, the focus of this dissertation.

As with all virus families, the herpesvirus family has defining characteristics. For example, all herpesviruses encode the necessary machinery for DNA synthesis (e.g., helicase, polymerase) and protein modification (e.g., protein kinase). Additionally, all herpesviruses have an icosohedral capsid that contains the linear double-stranded DNA (dsDNA) genome (Figure 1.1). Immediately adjacent to the capsid is a layer of both viral and cellular proteins termed the tegument. The outermost structure of a herpesvirus is a lipid bilayer that contains the virally-encoded envelope proteins, many of which are glycosylated. The focus of this dissertation is UL11, a tegument protein that is conserved across all herpesviruses.

Aside from the structural properties, possibly the most notable feature of the herpesvirus family is that each member is able to enter a latent state in its natural host and periodically reactivate when triggered by certain stimuli. HSV-1 latency occurs within sensory neurons and is characterized by the lack of viral DNA replication and protein production. Additionally, during HSV-1 latency very limited transcription occurs, producing only the latency associated transcript, or LAT (141). In response to stimuli such as UV radiation, fever, or even mental stress, HSV-1 can reactivate and resume protein expression, DNA replication, and virus production. All herpesviruses cycle between latent and lytic cycles; however, the tissue tropisms vary as well as the rate of virus production during the lytic phase. Based on these biological properties, as well as

the susceptible host range, herpesviruses are categorized into three subfamilies: Alpha-, Beta-, and Gammaherpesvirinae (319) (Table 2.1). At least one virus from each subfamily infects humans.

Alphaherpesviruses are defined as having a wide host range, a relatively short replication cycle, and enter latency within the sensory ganglia. Members of this subfamily that infect humans include the simplexviruses, types 1 and 2 (HSV-1 and -2), and varicella-zoster virus (VZV). Infection with the simplexviruses can result in cold sores, genital herpes, herpetic keratitis, encephalitis, and dermal whitlows; whereas VZV infection results in chickenpox (varicella, primary infection) and shingles (zoster, reactivation). Betaherpesviruses, including human cytomegalovirus (HCMV), have a restricted host range and a long replication cycle as compared to the alphaherpesviruses. Betaherpesviruses are maintained latently in secretory glands, lymphoreticular cells, and the kidneys. The third subfamily of herpesviruses is gammaherpesviruses which includes Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV). These viruses have very restricted host ranges similar to the betaherpesviruses, are usually specific for T- or B-lymphocytes, and result in latent infections within lymphoid tissue.

Virion Structure

Overview

The overall structure of an extracellular herpesvirus virion is conserved across all herpesviruses and is composed of three disparate structures: nucleocapsid, tegument, and envelope (Figure 1.1). In the case of HSV-1, the three structures of the extracellular virion comprise a total of ~50 virally encoded proteins (Table 2.2; each protein is present in multiple copies), numerous cellular proteins, and strands of RNA. Once assembled,

Table 2.1 Human Viruses of the Family *Herpesviridae*

Subfamily	Genus	Designation	Common Name	Site of Latency
α	Simplexvirus	Human herpesvirus 1	Herpes simplex virus 1	Sensory ganglia
		Human herpesvirus 2	Herpes simplex virus 2	
	Varicellovirus	Human herpesvirus 3	Vericella-zoster virus	
β	Cytomegalovirus	Human herpesvirus 5	Cytomegalovirus	Secretory glands, Lymphoreticular cells, Kidneys
	Roseolovirus	Human herpesvirus 6A	HHV-6 variant A	
		Human herpesvirus 6B	HHV-6 variant B	
		Human herpesvirus 7		
γ	Lymphocryptovirus	Human herpesvirus 4	Epstein-Barr virus	Lymphoid tissue
	Rhadinovirus	Human herpesvirus 8	Kapsoi's sarcoma-associated herpesvirus	

Table 2.2 Functions of HSV-1 Virion Components

Virion Localization	Gene Designation (Protein designation in HSV-1)	Function
Capsid	UL6	Portal protein
	UL18 (VP23)	Triplex
	UL19 (VP5)	Major capsid protein
	UL26 (VP24)	Protease
	UL35 (VP26)	Coats hexon tips
	UL38 (VP19c)	Triplex
Tegument	UL4	Unknown
	UL11	Virion egress; secondary envelopment
	UL13 (PK)	Protein kinase
	UL14	Cell to cell spread
	UL15	ATPase subunit of terminase
	UL16	Unknown
	UL17	DNA cleavage; capsid localization within the nucleus
	UL21	Unknown
	UL25	DNA packaging; Caps portal complex
	UL28	Terminase
	UL33	Terminase
	UL36 (VP1/2)	Virion envelopment; egress
	UL37	Virion envelopment; egress
	UL41 (Vhs)	Non-specific mRNA degradation
	UL46 (VP11/12)	Modulates VP16 activity
	UL47 (VP13/14)	Modulates VP16 activity
	UL48 (VP16)	Transactivates α gene expression
	UL49 (VP22)	Unknown
	UL51	Unknown
	UL56	Unknown
	US2	Unknown
	US3	Protein kinase
	US10	Unknown
	US11	Anti-attenuation factor
	ICP0	Transactivator
	ICP4	Required for β and γ gene expression
Envelope	UL1 (gL)	Membrane fusion
	UL10 (gM)	Unknown
	UL20	Virion egress
	UL22 (gH)	Membrane fusion
	UL27 (gB)	Membrane fusion
	UL43	Unknown
	UL44 (gC)	Attachment
	UL45	Unknown
	UL49A (gN)	Unknown
	UL53 (gK)	Virion egress
	US4 (gG)	Virion egress
	US5 (gJ)	Unknown
	US6 (gD)	Post-attachment entry
	US7 (gI)	Virus spread in polarized cells
	US8 (gE)	Virus spread in polarized cells
	US9	Unknown

HSV-1 genes essential for growth in cell culture are underlined.
Vhs, virion host shut-off; PK, protein kinase.

the HSV-1 particle is, on average, 186 nm in diameter (159). The centermost structure of the virion is the nucleocapsid, a shell of virally encoded proteins encasing the dsDNA genome. Surrounding the nucleocapsid is a layer of proteins termed the tegument. Because of the large number of proteins in the tegument, it is arguably the most complex feature of a herpesvirus. In the case of HSV-1, the tegument includes more than 25 individual proteins and each protein is present in multiple copies. For example, the tegument protein UL11 is present in approximately 750 copies per HSV-1 virion (228). Surrounding the tegument is the viral envelope, a host-derived membrane that is coated with viral proteins, most of which are glycosylated.

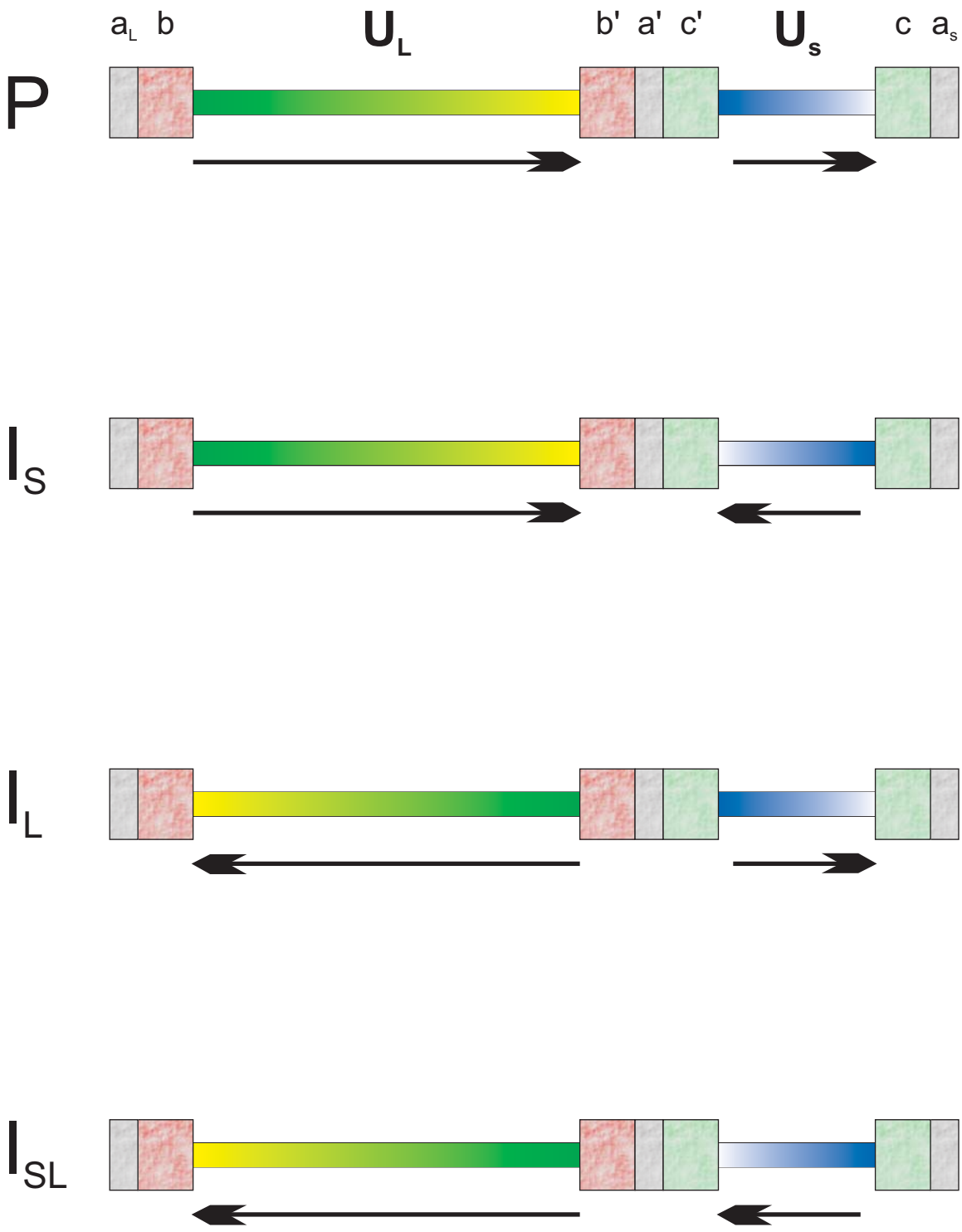
Each of these structures will be examined in more detail in the following sections, with an emphasis on the tegument structure and the UL11 tegument protein.

The HSV-1 Nucleocapsid

The centermost structure of an HSV-1 virion is the nucleocapsid which contains the ~150,000 bp dsDNA, viral genome. The HSV-1 genome can be divided into two regions, the unique long (U_L) and unique short (U_S); each section is flanked by inverted repeats that include a stretch of homologous DNA, designated "a," that allow recombination and subsequent production of up to four isomeric genomes (Figure 2.1). The HSV-1 genome has ~90 predicted open reading frames (ORFs) that are encoded on each strand of DNA and often overlap other ORFs. The gene and corresponding protein product are named based on the unique segment that the gene is located in (gene, U_L or U_S; protein, UL or US), and are increasingly numbered from left to right with respect to the prototypical isomer. For example, U_L11 is the 11^{th} gene in the unique long segment and encodes the UL11 protein. Furthermore, U_L11 is a "reverse" gene that is transcribed

Figure 2.1

Organization of the HSV-1 genome. The double stranded DNA of HSV-1 is linear when contained within a virion. The genome consists of two regions, the unique long (U_L; green/yellow) and unique short (U_S; blue/white). The U_L and U_S regions each contain inverted repeats at their ends, b/b' and c'/c, respectively. Each repeat contains a stretch of DNA that is homologous ("a") to allow recombination of the genome and produce four isomers. P, prototypical isomer; I_S, inverted U_S; I_L, inverted U_L, I_{SL}, inverted U_S and U_L.


a_L b
U_L
b' a' c'
U_s
c a_s
P
I_S
I_L
I_SL

from the "top" strand and overlaps the U_L12, U_L13 and U_L14 genes (Figure 2.2). Using the above nomenclature, it is quite simple to locate a specific gene within the large HSV-1 genome.

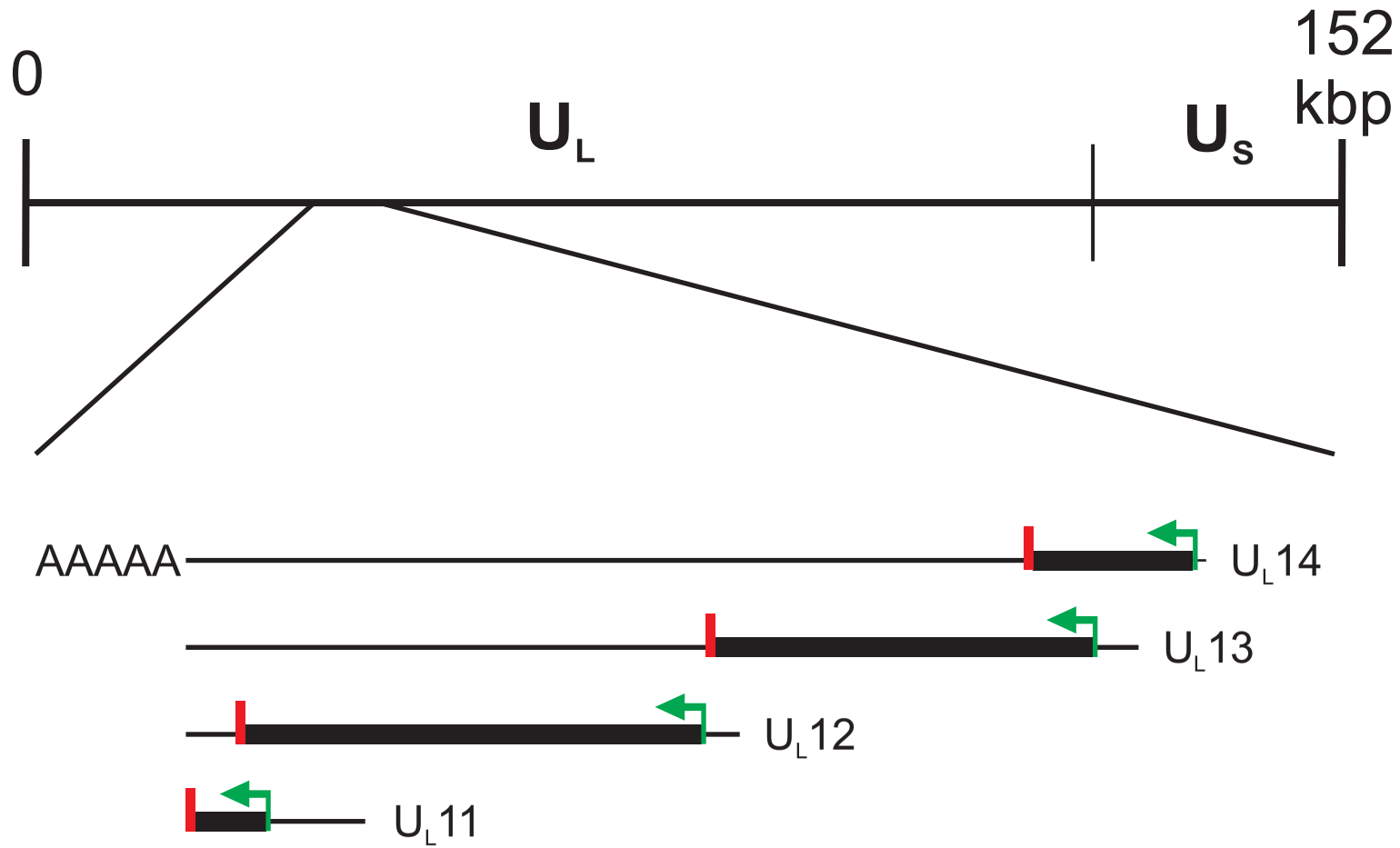
Encasing the viral genome is a capsid shell. For HSV-1, the capsid is composed of 5 proteins (Table 2.2) assembled into 162 capsomers to form an icosohedral structure with T=16 symmetry. The major capsid protein, VP5, forms the basic capsid structure of 150 hexon capsomers (6-VP5 molecules) and 11 pentameric capsomers (5-VP5 molecules). Each penton is located at a vertex of the icosohedron, with the hexons "filling in" the faces. Occupying the twelfth penton is a dodecameric ring of UL6, the portal protein, through which the viral DNA is packaged (403). On top of each VP5 hexon is a hexameric ring of VP26 molecules (446). Connecting the 152 capsomers in a pair-wise fashion is a triplex of VP23 and VP19C proteins in a 2:1 ratio, respectively (445). In addition to the 5 structural proteins listed above, a sixth protein, VP24 (protease), is located within the capsid following a maturation step (discussed later).

The HSV-1 Tegument

Immediately adjacent to the nucleocapsid, but within the lipid envelope, is the tegument, possibly the most complex sub-structure of a herpesvirus virion due to the number of proteins present (Table 2.2). In HSV-1 virions, over 25 individual virally encoded proteins are in the tegument, and each protein is present in multiple copies [e.g., UL11 is present at ~750 copies per virion (228)]. Additionally, the tegument contains numerous cellular proteins that include annexin, heat shock proteins 70 and 90 (Hsp70 and Hsp90, respectively), actin, and tubulin (28, 41, 192, 200, 340, 447). However, given their sheer abundance in an infected cell, it is unknown whether such cellular components

Figure 2.2

HSV-1 gene organization. Open reading frames (ORFs) of HSV-1 are on both strands of DNA. Genes on the same strand often overlap. Shown is the region of HSV-1 that encodes UL11, UL12, UL13, and UL14. All four genes are "reverse" (in the prototypical isomer, Figure 2.1) and overlap, ending co-terminally at the 3' ends. Thin black lines, transcripts; thick black lines, ORFs; AAAAA, poly-A signal.


0
152
kbp
U_L
U_S
AAAAA
U_L14
U_L13
U_L12
U_L11

are specifically packaged or if they represent "filler" material. Finally, RNA molecules are also packaged into the tegument, likely because of the RNA binding-tegument proteins US11, UL47, and UL49 (41, 155, 340, 341).

The protein-protein interactions between tegument proteins are specific and form an amorphous and flexible protein network to connect the nucleocapsid and the envelope, similar to the matrix protein of HIV-1 (164). As a membrane bound protein, UL11 is distal to the capsid, yet may be a critical part of the bridge since it interacts with both envelope proteins and capsid associated proteins (117, 227, 253).

No tegument protein or any other viral protein is necessary for initiating a productive HSV-1 infection. As such, the DNA alone is infectious (151) as exemplified by the latent-to-lytic cycle of HSV-1. However, some tegument proteins are essential for the assembly of virions (discussed below). UL11 is not one of these proteins, and is deemed "non-essential" because UL11-null viruses release infectious particles. However, UL11 must be involved in virion biogenesis at some point since UL11-null viruses have defects during virus assembly, resulting in reduced virus production and accumulation of capsids within the infected cell (20, 136, 239). The role of UL11 during virion assembly is the focus of this dissertation, and the studies of this protein are detailed in the following chapters.

The HSV-1 Envelope

The HSV-1 membrane is a remnant of the host cell and is acquired from a TGN-derived vesicle. As such, the lipid content of HSV-1 virions is similar to that of cytoplasmic membranes (409). On the outer membrane of an HSV-1 virion, over 15 viral proteins are present, at least 12 of which are glycosylated. Like the capsid and tegument

proteins, extracellular virions contain numerous copies of each membrane protein. The glycoproteins function during egress, entry, and immune evasion (56, 370).

With the understanding of the basic HSV-1 virion structure, the next section describes the processes that are coordinated to assemble infectious virus.

Virion Assembly

The life cycle of HSV is circular, therefore a "beginning" point must be decided upon to detail the specifics. For this dissertation, this point will be following the deposition of viral DNA into the host-cell nucleus. The rationale for this choice is one important fact: herpes simplex virus DNA is infectious (151).

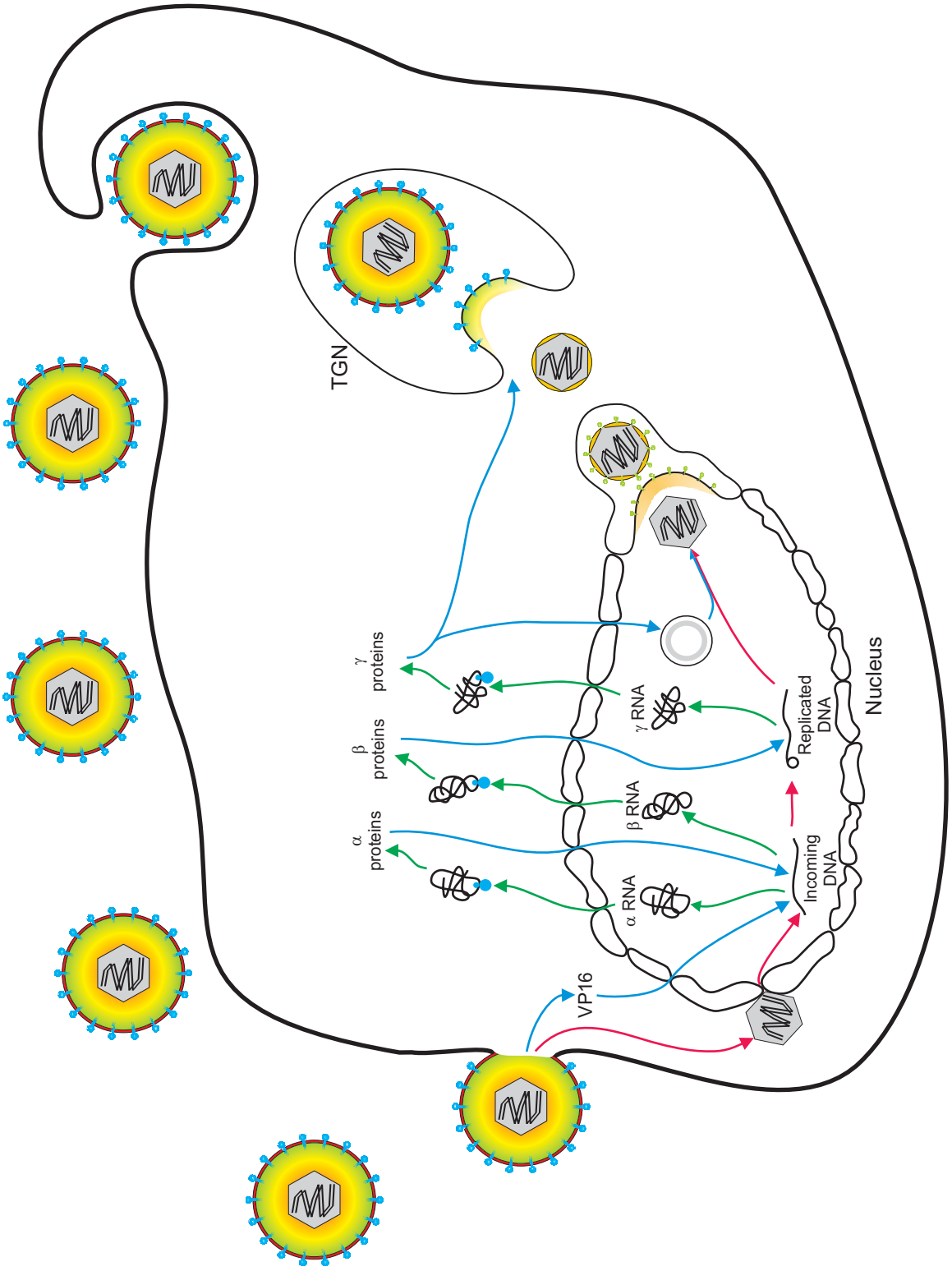
UL11 is a component of the HSV-1 assembly and egress pathway. This section outlines the current knowledge of HSV-1 replication, with an emphasis on the tegumentation and secondary envelopment steps (Figure 2.3).

Genome Remodeling

Rapidly following deposition of the HSV-1 genome into the nucleus, the viral DNA circularizes (140, 304). The host cell adds histone H3 to the incoming viral DNA to form a chromatin-like structure and squelch viral transcription (62, 172, 201). To counteract the host cell, both VP16 and VP22 (provided by the incoming virus) can regulate the formation of nucleosomes (172, 411). VP16 regulates histone modification by two mechanisms. First, VP16 reduces the amount of histone H3 associated with gene promoters that are transcribed immediately after infection. Second, VP16 recruits histone acetyl transferases (HATs) to acetylate the tails of histones that were added to form

Figure 2.3

General replication cycle of HSV-1. Replication of HSV-1 is circular, therefore the "beginning" is arbitrary. For the purposes of this dissertation, replication initiates from a strand of DNA newly deposited into the nucleus. Within the nucleus, VP16 accelerates transcription of the immediate-early (α) genes. Importantly, VP16 is not essential for α gene transcription since the DNA is infectious. The α gene products initiate early gene (β) transcription, which facilitate DNA replication and late gene (γ) expression. The γ genes encode structural proteins to form the new virions. Virus assembly begins in the nucleus as the capsid is assembled and packaged with the linear dsDNA genome. Nucleocapsids leave the nucleus by an envelopment/de-envelopment process at the inner- and outer-nuclear membranes, respectively. After exit from the nucleus, the capsids travel along microtubules to the trans-Golgi network (TGN) where a second envelopment process occurs. This step yields an enveloped virus particle that is studded with viral glycoproteins inside a vesicle. The vesicle is transported to the cell periphery via the secretory pathway where the vesicle fuses with the plasma membrane and releases the virus particle into the extracellular milieu. Newly released virions bind to and fuse with a new cell to release the nucleocapsid and tegument proteins into the cytoplasm. Nucleocapsids are partially uncoated, releasing the transactivator protein VP16, and trafficked to a nuclear pore complex, where the genome is released into the nucleoplasm, beginning a new round of virus replication.


TGN
γ proteins
β proteins
α proteins
γ RNA
β RNA
α RNA
Replicated DNA
Incoming DNA
VP16
Nucleus

euchromatin, or transcriptionally active DNA (172). Gene transcription and protein production initiates following the VP16-induced modifications of the viral genome.

Protein Expression

After viral DNA is deposited and restructured in the host cell nucleus, RNA pol II-mediated transcription begins. HSV genes are divided into three major groups based on temporal expression; each group is transcribed sequentially via a positive feed-back cascade mechanism (77, 180). That is, the initial gene products (also referred to as immediate early, IE, or α) precede the expression of the second set of genes (early, E, or β), which are followed by the final group of gene products (late, L, or γ) (Figure 2.3).

IE proteins are detectable between 2 and 4 hours after deposition of viral DNA into the host cell nucleus. Though no viral proteins are required for *de novo* protein synthesis (HSV-1 DNA is infectious), the transcriptional transactivator VP16 supplied from the tegument of the incoming virus does stimulate the expression of the α genes (1, 384). VP16 binds to specific consensus sequences in the α gene promoters (237) and recruits host transcription factors (for example, TFIIB, TBP, Oct-1, and HCF) that increase transcription (210, 224, 236, 237). Five of the six α gene products [infected cell protein 0 (ICP0), ICP4, ICP22, ICP27, US1.5] trigger the switch to β gene transcription. The remaining α gene product, ICP47, is an immuno-modulating protein that interferes with antigen processing (113, 114).

Transcription of the β genes requires the presence of at least ICP4 and occurs between 5 and 7 hpi. The β proteins make up the DNA replication machinery (described below) and also initiate some γ gene transcription.

Subsequent to the expression of the β genes and concurrent with DNA synthesis, γ gene transcription and translation begins (181) to produce the structural proteins. The γ genes are further divided into two groupings based on their dependence of DNA replication for expression (72, 198). γ1, or leaky-late, gene expression is enhanced by, but does not require, DNA replication whereas γ2, or true late, genes are not expressed in the absence of DNA synthesis. It appears the differential transcription of γ1 and γ2 may be attributable to specific sequences in the promoter regions of the γ genes (76, 178, 179). γ1 gene promoters contain binding sites for several transcriptions factors (YY1, Sp1 and ICP4) (183, 184, 257) in addition to the classical TATA box, whereas the γ2 genes only contain a TATA box (196, 343). However, the exact mechanism of γ gene activation by DNA replication is not fully understood.

In addition to the DNA sequences, both positive and negative transcriptional regulators play a role in γ gene expression, as exemplified by the viral Vhs protein. Vhs is an RNase that suppresses host-gene expression by accelerating the turnover rate of cellular mRNAs (29, 115, 197). Late during an infection, Vhs also increases the turnover of the α and β gene transcripts to facilitate the switch to γ gene expression. Included in the γ genes is the coding sequence for VP16 which complexes with, and dampens, the RNase activity of Vhs (334, 363). Though the viral genes are classified into groupings, it is important to note that a continuum exists, especially for the β and γ genes.

Synchronous with the various stages of gene transcription, HSV-1 reorganizes the nucleus and replicates the viral DNA.

Nuclear Reorganization & DNA Replication

Following the restructuring of viral DNA with histones and transcription of the α genes, HSV remodels the infected cell nucleus to compartmentalize the viral proteins necessary for γ gene transcription and DNA replication. Prereplicative sites are formed near nuclear domain 10 (ND10) structures, but the timing of this compartmentalization is debated, as is the necessity of viral proteins (243, 369, 386). At the prereplicative sites, the replication machinery assembles and the DNA is copied via Θ (theta) replication. Only seven of the 13 β gene products are required for DNA replication and include: UL5, UL8, UL52, ICP8, UL9, UL30, and UL42 (59, 60, 65, 87, 146, 195, 245, 431, 448). The remaining 6 proteins function in nucleotide metabolism in non-dividing cells such as neurons, and repair/modification of synthesized genomes (122, 147, 267, 424).

Briefly, DNA replication occurs by UL9 binding to one of the three origins of replication (2 in the U_S segment, *ori*S; 1 in the U_L segment, *ori*L) to unwind the DNA and recruit ICP8, the single stranded DNA binding protein. The five remaining replication proteins include the helicase/primase complex (UL5, UL8, and UL52) and the polymerase complex [UL30 (polymerase) and UL42 (processivity factor)] which bind to the replication forks and initiate replication. By some unknown mechanism, Θ replication gives way to rolling circle replication to produce concatomeric genomes (344, 441). UL9 is not necessary after the switch to rolling circle replication since this mechanism is independent of origins of replication.

As replication proceeds, the small prereplicative sites enlarge, merge, and fill the nucleus (87, 369, 388, 389). Expansion of the prereplicative sites marginalizes the host

chromatin to the periphery of the nucleus and contributes to the silencing of host transcription.

Following replication of the viral DNA and production of the structural (γ) proteins, the necessary components are available to begin virus assembly.

Capsid Assembly & DNA Packaging

Newly translated capsid proteins must enter the nucleus prior to assembly into a capsid. However, not all capsid proteins have the necessary intrinsic properties to enter the nucleus and require other proteins for such localization (93, 278, 316). After entry into the nucleus, spherical procapsids are formed via interactions between the pre-scaffold protein pre-VP22a and the major capsid protein VP5. UL26 is another scaffold protein present in procapsids. The ORFs of pre-VP22a and UL26 overlap and produce proteins with common C-termini which bind to VP5 (94, 221-223, 394). The triplex proteins VP23 and VP19c then link the pre-VP22a/UL26/VP5 complexes to assemble hexons and pentons and form the spherical procapsid (274). The portal complex of 12-UL6 proteins is assembled into the procapsid through UL6 and pre-VP22a interactions (275).

A series of protein-cleavage events occur shortly after procapsid assembly is completed to cause a structural rearrangement that results in an angular polyhedron (69, 394, 402). First, UL26 is cleaved either autoproteolytically or in *trans* to yield both the VP21 scaffold protein and the VP24 protease (222, 276, 308, 423). Next, VP24 cleaves pre-VP22a to release VP22a from the VP5 capsomers and leave the C-terminal 25 amino acids bound to VP5. Together, these cleavage events result in the energy-independent

transformation to an icosohedral shell (402) with VP22a, VP24, and VP21 "trapped" inside the capsid.

After assembly, the mature capsid is transported to sites of DNA replication by UL17 and UL32 (66, 211, 327, 387) where UL6 recruits UL15, UL28 and UL33. In turn, the UL15/UL28/UL33 protein complex binds to and cleaves the concatomeric viral DNA at "pac" sequences to form unit-lengths of viral DNA that are fed into the capsid through the portal in an ATP-dependent manner (2, 4, 19, 27, 36, 85, 86, 89, 390, 426, 435). Concurrent with DNA packaging, the scaffolding proteins VP21 and VP22a are displaced from the capsid interior, leaving only the VP24 protease in the nucleocapsid. Once encapsidated, the DNA interacts with UL25 to form a "plug" that keeps the genome from leaking back out (251, 284).

Capsid shells that properly undergo DNA packaging are referred to as C-capsids. In the event that DNA packaging was initiated but aborted, an A-capsid is formed that lacks both DNA and scaffold proteins. Like A-capsids, B-capsids lack viral DNA, are non-functional, and are located within infected cell nuclei. However, B-capsids retain the scaffold proteins (143, 273, 300, 349).

Following capsid formation and DNA packaging, the nucleocapsids must traffic to the nuclear membrane and begin capsid egress.

Nuclear Egress & Cytoplasmic Transport

The nucleocapsid must traverse through the nucleus and escape into the cytoplasm to form the complete virion. Nucleocapsids are shuttled to the nuclear membrane via actin filaments in an energy dependent manner (123). However, the 125 nm capsid is too large to be expelled through the nuclear pore and an alternative process

must occur (292, 293). Following many years of debate and several seemingly incorrect models, a model of envelopment/de-envelopment at the nuclear membrane followed by re-envelopment at a site distal to the nucleus has come to acceptance (152, 361, 374) (Figure 1.2). The first step of nuclear envelopment requires the dissolution of the nuclear matrix. The HSV-1 US3 protein kinase recruits UL31 and UL34 to the nuclear rim. The US3/UL31/UL34 complex then recruits the cellular protein kinase C (PKC) to the nuclear rim to phosphorylate lamins A/C and/or B and cause the necessary weakening the matrix (294, 312-314, 360).

Interestingly, despite all forms of capsids (A, B, and C) being equal in morphology and exterior-protein content, C-capsids seem to be preferentially enveloped at the inner nuclear membrane. It has been proposed that the outward pressure of the encapsidated genome causes structural changes on the outer surface of the C-capsids. As a result, UL25 and UL17 (involved in DNA packaging) are believed to form a heterodimeric C-capsid-specific component (CCSC) (404) and specifically target C-capsids towards the nuclear membrane.

Envelopment of HSV-1 capsids at the inner nuclear membrane produces an enveloped particle within the perinuclear space. As outlined by the envelopment/de-envelopment/re-envelopment model, these primary particles must fuse with the outer nuclear membrane to release naked capsids into the cytoplasm. Several lines of evidence support the model of fusion with the outer nuclear membrane. 1), Biochemical studies show the lipid content of the final viral envelope and the nuclear membrane are different (409). 2), Perinuclear particles contain UL31 and UL34, whereas mature virions do not contain these proteins (133, 312). 3), Fusion of the perinuclear particle with the outer

nuclear membrane requires only one of glycoprotein B or H (gB and gH, respectively), whereas membrane fusion during virus entry requires both gB and gH (118). And 4), gC, gD, and gM are incorporated into the primary virion, but cytoplasmic capsids are unenveloped and lack all glycoproteins (21, 191, 295, 397).

De-envelopment at the outer nuclear membranes removes many viral proteins from perinuclear particles. However, the tegument proteins VP16, Vhs, VP1/2, US3, and UL37 are all reported as bound to intranuclear capsids and are also components of mature virions; but only US3 is present in both primary enveloped virions and mature virions in equal molar amounts (49, 203, 271, 309, 314, 338, 404).

The unenveloped, cytoplasmic capsids are transported from the nucleus to the TGN via microtubules (177, 232, 259, 298, 298, 410). Capsid transport requires proteins of the inner tegument. Capsids that have a full compliment of tegument proteins show little motility *in vitro*; but, removal of the outer tegument proteins to expose the inner proteins allows efficient capsid transport (14, 103, 231, 429). In support of the inner proteins playing a role in capsid transport, the large tegument protein VP1/2 of both HSV and the related alphaherpesvirus, pseudorabies virus (PrV) has been shown to be required for the transport of capsids (95, 232, 347). Additionally, the tegument proteins VP16, VP22, and US11 are capable of interacting with the kinesin motor (97, 383, 410). Despite the ability of US11 to interact with the kinesin motor, US11 is not required for microtubule transport *in vitro* (429).

Importantly, one must be careful when considering capsid transport mechanism(s). As mentioned earlier, HSV-1 has the ability to infect neurons, enter latency, and upon stress, reactivate. During reactivation, it is debated whether the capsid,

tegument, and envelope proteins traffic to the axon termini together as a virion or as separate components (15, 57, 63, 64, 90, 177, 298, 325, 367, 368). Despite the different models, axonal transport of nucleocapsids (or virions) appears to be driven on host microtubules as in epithelial cells, albeit using different viral proteins (97, 213, 233-235, 298).

Tegumentation

Tegumentation is not a single event, but rather a continuous event that begins in the nucleus, continues as capsids traffic to the TGN, and culminates with secondary, or final, envelopment at a TGN vesicle.

Immunolabeling experiments show the US3, UL16, UL37, VP1/2, and VP16 tegument proteins are bound to nucleocapsids after exit from the nucleus (131, 253, 260, 271). However, the final virion contains over 25 viral tegument proteins. Nucleocapsids obtain many of these, including UL11, at TGN vesicles which are also coated with the envelope proteins. Incorporation of these constituents requires their ability to target to and remain at the membranes of the TGN. The trans-Golgi network is part of a larger cellular organelle, the Golgi apparatus, which also includes the cis-, medial-, and trans-cisternae (not to be confused with the trans-Golgi network). The apparatus as a whole is the sorting center for membrane bound proteins trafficking to and from the endoplasmic reticulum (ER), the PM, and the endosome/lysosome pathway (119, 145). Given the continuous flow of large numbers of proteins, several distinct mechanisms exist for targeting to and retention at the TGN.

Retention of transmembrane proteins at the TGN is influenced by both the length and composition of the transmembrane domain. However, there is no obvious consensus

for a Golgi retention signal when comparing the amino acid sequence of several cellular glycotransferases and viral glycoproteins (71, 174, 381). Unlike a transmembrane protein, a peripheral membrane protein must utilize alternative mechanisms for TGN binding and targeting. Often, co- and post-translational modifications with fatty acids containing myristyl, farnesyl, palmityl, or geranylgeranyl groups are sufficient (104, 134, 168, 299, 310, 337, 408, 438). It is believed that the hydrophobic nature of the fatty acid adducts results in retention of the protein at the membrane where acylated. Therefore, one could infer that the site of accumulation and the site of acylation are one in the same (430).

Peripheral membrane proteins traffic throughout the cell and require trafficking signals to localize to specific locations. Protein trafficking is achieved by short amino acid sequences that are categorized into three groups: a tyrosine-based (YXXΦ; where Φ is a bulky hydrophobic residue), a di-leucine (LL or LI, leucine-isoleucine), or an acidic cluster (AC) motif. These motifs recruit clathrin adaptor proteins such as adaptor protein-1 (AP-1), AP-2, phosphofurin acidic cluster sorting protein-1 (PACS-1), and Golgi-localized γ-adaptin ear homology domain ARF-binding protein (GGAs) which in turn interact with clathrin and facilitate coat formation and vesicular trafficking (375, 392, 420).

AP-1 and AP-2 are heterotetramers with two large, one medium, and one small subunit (α/β1/μ1/σ1 and γ/β2/μ2/σ2, respectively). Despite the similar structures, AP-1 and AP-2 localize to different membranes and direct vesicles to different locations. For example, AP-1 is found at the TGN and endosomes and traffics cargo between these locations, whereas AP-2 is found at the PM and mainly functions during endocytosis.

The μ subunit of each AP complex recognize the tyrosine-based (290) and di-leucine motifs (188) in cargo proteins, whereas acidic clusters are bound by PACS-1.

An example of this dynamic protein trafficking network is the cellular endoprotease furin, which exits the TGN after AP-1 binds to a tyrosine-based motif (YKGL) (391). Once in a post-TGN endosome, furin is either shuttled back to the TGN or to the cell surface. If furin makes it to the cell periphery, the di-leucine motif is recognized by AP-2 and the protein is internalized into an endosomal compartment (375). Once endocytosed, a serine residue in the furin AC is phosphorylated, recruits PACS-1, and returns furin to the TGN (420).

Utilizing the above mentioned mechanisms, several HSV proteins localize to the TGN and await the arrival of a partially-tegumented nucleocapsid. In HSV-1, gB, gE, and gI are transmembrane but also contain an assortment of YXXΦ, di-leucine, and acidic cluster motifs (5-7, 30, 80, 81, 189, 250, 286, 287, 328). The presence of said motifs, however, is not always sufficient for TGN localization. For example, gI requires an interaction with gE for efficient TGN targeting (5, 7, 193). Similarly, individual expression of gK and UL20 results in ER accumulation, but co-expression shifts their localization to the TGN (16, 17, 125, 126, 128, 421). Also, internalization of gD and the gH/gL complex from the PM to the TGN requires gM, which possesses both a YXXΘ and AC motif (79).

In addition to the envelope proteins, several tegument proteins bind membranes and localize to the TGN. UL11 accomplishes membrane binding via both co- and post-translational modifications with myristate and palmitate, respectively (226). Once on cellular membranes, an acidic cluster (and possibly a di-leucine motif) localize UL11 to

the TGN (226); however, the adaptor proteins involved in this trafficking are unknown. Importantly, UL11 targets to the TGN independently of all other viral proteins (226). The functions of the UL11 AC and LI are discussed in detail later. Not all membrane bound HSV-1 proteins contain transmembrane domains or are modified with fatty acids. For example, the tegument proteins VP22 and Vhs do not bind membranes directly (i.e., no fatty acid modification and no transmembrane domain), yet are associated with membranes, even in the absence of other viral proteins (43, 214, 266).

Given the large number of proteins that are in an HSV-1 virion, the protein-protein interactions and protein trafficking discussed above are critical to ensure proper protein localization and virus production (68, 112, 156, 160, 202, 227, 363, 418, 442). Through this extensive, complicated, and very precise meshwork of proteins, the nucleocapsid and envelope are linked and poised for final envelopment.

Final Envelopment

Upon interaction of the nucleocapsid and the TGN-localized tegument and envelope proteins, a poorly understood envelopment process occurs. Several protein-protein interactions likely contribute to this process to provide a "bridge" between the two complexes. For example, UL11 is a TGN localized, membrane-bound tegument protein that interacts with UL16 (227, 434), a tegument protein which can bind cytoplasmic capsids (253, 289, 418). UL11 also interacts with viral glycoprotein tails (gD and gE) (117). Similarly, the TGN bound VP22 interacts with the capsid-associated VP16 and membrane-bound gD and gE (68, 112, 282, 283). However, none of these interactions are absolutely required in cell culture, since both UL11 and VP22 are deemed "non-essential" for growth *in vitro* (20, 106, 111, 136, 239). The moderate

defects of virus production with the UL11- and VP22-null mutants are likely due to redundancy of function between multiple proteins. An example of protein redundancy is best seen with PrV. A single deletion of either UL11 or gM from PrV has only minor defects, but simultaneous deletion of both severely inhibits virus production (40, 204). Conversely, HSV-1 VP16 does not have a redundant counterpart and deletion of VP16 alone completely blocks secondary envelopment (265, 422). VP16 interacts with both membrane bound proteins (gH, gB, gD and Vhs) and the capsid bound protein VP1/2 (156, 214, 266, 363, 449).

Despite the seemingly critical interactions between the capsid and TGN-bound proteins, the presence of the nucleocapsid is not a prerequisite for secondary envelopment and particle release. Light, or L, particles lack nucleocapsids and are non-infectious, yet are produced by many herpesviruses during the normal replication cycle in cell culture and the natural host (8, 187, 247, 248, 317, 382). L particles contain a large subset of tegument and glycoproteins found within a virus particle as well as phosphoproteins that are absent from infectious virions (382). L particles can bind to and fuse with a new host cell, and *in vivo* may provide additional copies of tegument proteins such as VP16 and Vhs to facilitate the initiation of a new infection (84, 247). L particles have been studied in hopes of determining the minimal proteins required for the process of envelopment; however, such "machinery" has yet to be absolutely defined. Interestingly, PrV L particles are not formed in the absence of UL11 and gM (204), suggesting that UL11 (and gM) is (are) involved with the envelopment process.

Cellular proteins, such as VPS4, are also required for HSV envelopment (54, 82). Vps4 is a component of the cellular ESCRT (endosomal sorting complexes required for

transport) complex that is critical for the assembly and envelopment of many RNA viruses [as reviewed in (33, 91, 263)]. In uninfected cells, the ESCRT complex is involved with vesicular formation and trafficking and sorting of proteins that are destined for degradation in lysosomes (157, 362).

Following secondary envelopment the enveloped virion is contained in a TGN-derived secretory vesicle which is transported to the PM where a fusion event occurs to release the enveloped virion.

Secretion

Secretion of HSV-containing vesicles to the PM via the secretory pathway requires UL20 and gK (127, 132, 186, 190). UL11 has also been implicated in the transportation of vesicles because a UL11-null virus is delayed in the release of infectious virions following final envelopment (20). This defect is in addition to the secondary envelopment defect, suggesting UL11 has multiple roles during virion biogenesis. Additionally, the cellular factor protein kinase D (PKD) regulates vesicle transport and mutations of PKD that abrogate the enzymatic activity result in an accumulation of vesicles at the TGN (218). Concurrent with transport, vesicles are acidified and without the pH drop, infectivity of the virus is abolished (162). A recent study suggests that the acidification process may alter the network of protein-protein interactions within the tegument (253).

In polarized epithelial cells, vesicle fusion at the apical surface releases virus into the extracellular space; but fusion at basolateral membranes releases virions into cell junctions and leads to cell-to-cell spread. The latter method of spread protects the virus from the host's humoral immune response (23, 58, 98, 194).

Cell-to-cell spread is a process that requires gE and gI to target vesicles to epithelial junctions (194), as well as gB, gD, and gH/gL for fusion and entry (53, 124, 217, 320). Mutants of HSV that lack gE and gI fail to spread laterally in both cultured cells and tissues (98-100, 305). Similar to the mechanism of trafficking proteins to the TGN, the cytoplasmic tail of vesicle-bound gE interacts with clathrin adaptor proteins to target the vesicle to a cell junction (116, 194).

Entry & Uncoating

Virions released into either the extracellular space or cell junctions proceed through a two-step process of binding and fusion to introduce the nucleocapsid into the new cell. The initial entry step of HSV is attachment to the host-cell surface by the binding of either gB or gC to a carbohydrate chain such as heparan sulfate (158, 170, 171, 212, 350, 351, 371, 432). Following attachment, the actions of gB, gD, and the heterodimer gH/gL are both necessary and sufficient for fusion and entry. Deletion of any one of these four glycoproteins abrogates entry; however, cell binding still occurs due to the presence of gC (51, 52, 124, 217, 320). The receptors that interact with gD and facilitate entry fall into three categories: HVEM (herpesvirus entry mediator), nectin-1 and -2, and 3-O-sulfotranserases (142, 207, 262, 315, 351, 395, 396, 425). The breadth of HSV entry receptors may account for the ability of HSV to infect a wide range of tissues. Since gD lacks the characteristics of a fusion protein, it is believed the interaction of gD with the cellular receptor signals the gH/gL and gB fusion complex (70, 96, 124, 137, 148, 175, 208); gH/gL initiates the fusion process which is then completed by gB (377). Fusion of HSV and the host cell membrane can occur at three distinct locations: the plasma membrane, an acidified endosome, or a pH-neutral endosome.

Regardless of where membrane fusion occurs, the quartet of glycoproteins B, D, H and L is required (185, 258, 279-281, 352, 428).

Following fusion, three events occur in the cytoplasm. First, kinases such as casein kinase II (CKII, cellular) and UL13 (viral) phosphorylate a subset of tegument proteins to facilitate the disassembly of the tegument from the capsid (264). Uncoating not only releases a population of tegument proteins such as VP16 and VP22 (which help initiate the new round of infection), but also exposes the inner tegument proteins that are required to traffic the nucleocapsid to the nucleus via microtubules (event two) (101, 102, 364). It is believed that a component of the inner tegument is responsible for this transport because capsids with a full complement of tegument have little motility, whereas capsids that are only covered with inner tegument proteins are transported along microtubules quite efficiently (14, 231, 429). After arrival at the nuclear pore, the third and final event occurs to release the DNA through a nuclear-pore in a process that requires Importin-β. Also, cleavage of the portal protein UL6 is concurrent with the ejection of DNA from the viral capsid (272, 285).

Given the numerous steps that must occur during the precise and complex HSV assembly process, let alone the entire replication scheme, is a formidable task. In an attempt to better understand the envelopment mechanism, the small tegument protein UL11 was studied. The following section is a review of the current, albeit limited, knowledge of this protein. Additionally, the acyl modifications that occur to UL11 (myristate and palmitate) are detailed.

UL11

HSV-1 encodes over 80 proteins and at least 26 of these are incorporated into the tegument, including UL11; however, the role of each of these has not been elucidated. Throughout the 1980's, it was discovered that a set of four transcripts aligned co-terminally at the 3' ends and corresponded to the genes U_L11, U_L12, U_L13, and U_L14 (74, 75, 105, 244, 246) (Figure 2.2). The U_L11 transcript is the shortest (0.9kb) of the four transcripts and encodes the myristylated UL11 protein (238, 239, 244, 246) (Figure 1.3). All herpesviruses encode a UL11 homolog, but this protein is deemed "non-essential" for many viruses, including HSV and PrV. Though UL11 is not essential for virus growth, it is clearly involved in the virus life cycle. Removal of UL11 from the HSV proteome causes a 1000-fold decrease of virion production and an accumulation of unenveloped capsids within the cytoplasm of infected cells (20, 136, 239). In contrast, the UL11 homolog (UL99) is essential for growth of the prototypic betaherpesvirus HCMV (355).

HSV-1 UL11 contains four known motifs: one signal each for myristylation and palmitylation, an acidic cluster, and a di-leucine. Each motif is detailed below.

Myristylation & Palmitylation

Possibly the most characteristic features of UL11 are the myristic and palmitic acid adducts. Myristate and palmitate are saturated fatty acids that mediate protein-membrane interactions; and in the case of UL11 are required for membrane binding (226). Furthermore, when UL11 is modified with both fatty acids, the protein localizes to the TGN, even in the absence of all other viral proteins. UL11 mutants that lack both fatty acids or that are only myristylated fail to efficiently target to or bind membranes (226). Furthermore, neither of the above mutants are efficiently packaged when they

have to compete with wild-type UL11 for incorporation into the virus particle (228). The enzymes and reactions involved in myristylation and palmitylation are described below.

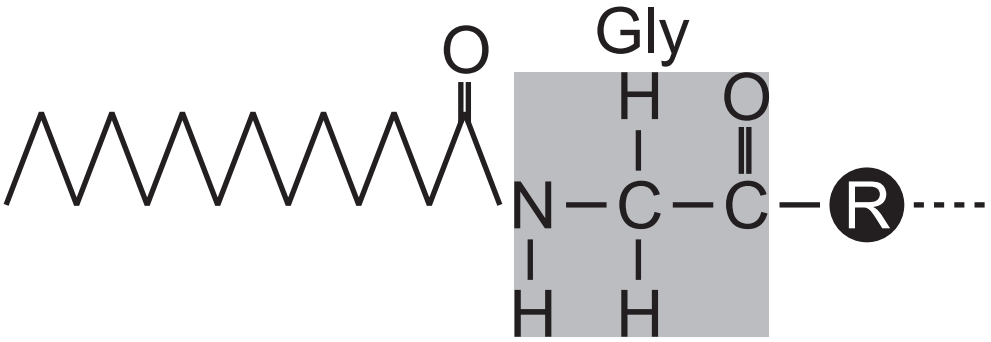
Myristylation is the addition of the 14-carbon myristic acid, or myristate, to an N-terminal glycine residue (61, 291, 337). Generally, this is a non-reversible reaction, though a few cases of de-myristylation have been documented (83, 240). Of all eukaryotic proteins, approximately 0.5% are myristylated (311). Two separate enzymatic processes must occur to add myristate to a protein. First, methione-aminopeptidase (MAP) removes the initiator methionine to expose a glycine residue as the new N-terminus (130, 173, 324, 400, 401). Then, N-myristyltransferase (Nmt) modifies the newly exposed glycine residue with myristic acid via an amide bond (61, 324, 398-401, 405) (Figure 2.4). Nmt is a monomeric enzyme that catalyzes the transfer of myristate from myristyl-CoA (synthesized by acyl-CoA synthetase) to a suitable protein substrate, like UL11 (324, 399). Studies of yeast Nmt show the enzymatic reaction involves five steps: 1) myristyl-CoA binds to Nmt, 2) protein substrate binds to Nmt/Myristyl-CoA, 3) myristate transfers from CoA to the N-terminal glycine of the protein, 4) CoA releases from the enzyme, 5) the myristylated protein releases from the enzyme (324). For the vast majority of proteins, including UL11, these reactions occur co-translationally on free, cytoplasmic ribosomes (88, 291, 337, 427). However, a proteolytic cleavage of the cellular BID protein reveals a "hidden" myristylation motif that leads to a post-translational myristylation (437). More recently, additional proteins that undergo a post-translational myristylation have been identified (326, 407, 416).

Myristylation does not ensure continual membrane binding as a "myristyl switch" occurs for at least one dozen proteins; exposing the myristate group when the protein is in

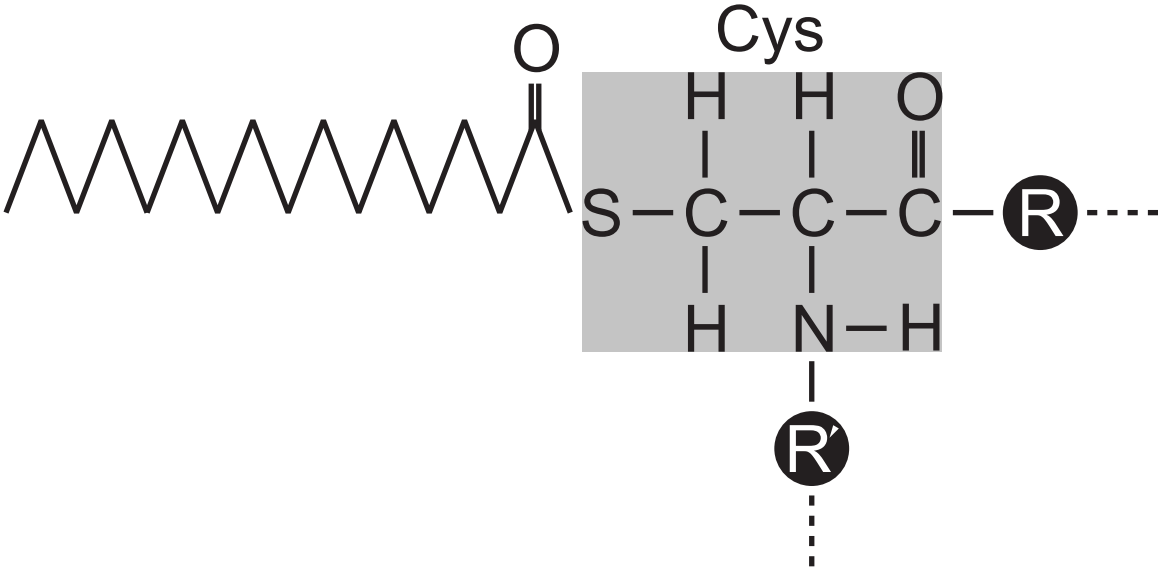
Figure 2.4

Lipid modifications of UL11. Myristylation occurs co-translationally (usually) by the attachment of the 14-carbon, saturated fatty acid myristate to a conserved acceptor glycine residue (grey box) following removal of the initiator methionine via a stable amide bond. Palmitylation occurs post-translationally by the addition of the 16-carbon, saturated fatty acid palmitate to a cysteine residue (grey box) via a labile thioester bond. R', amino terminus; R, carboxy terminus.

MYRISTYLATION



PALMITYLATION



one conformation but a second conformation of the protein sequesters the fatty acid (Figure 2.5). Conformational changes of the myristylated protein may occur under three circumstances: interaction with another protein (10, 11, 37, 163, 385), electrostatic changes such as phosphorylation or pH dependent protonation (161, 249, 270, 345, 393), or protein proteolysis (169, 372, 443, 444). Though it cannot be excluded as a possibility, it seems highly unlikely that UL11 undergoes any of these switches because the protein is further modified with a second fatty acid.

Because myristylation alone only provides weak membrane binding (it is only 14 carbons long, $K_d=10^{-4}M$) (268, 296), many membrane bound proteins increase the membrane binding potential via basic amino acids near the myristate (such as the Src oncoprotein) (50, 269, 353) or like UL11, contain additional fatty acids (9, 34, 138, 139, 226, 346, 412, 430, 450) (Figure 2.6). Following myristylation, UL11 is modified with the 16-carbon fatty acid palmitate via a thioester linkage on at least one of three cysteine residues near the myristyl moiety (Figure 2.4). Importantly, the myristylation of UL11 is a prerequisite to the addition of palmitate; thus a UL11 mutant that cannot be myristylated is not palmitylated either (226).

Unlike myristylation, palmitylation occurs post-translationally on membranes and is readily removed. Palmitylation usually requires the transmembrane protein acyltransferase, or PAT, to catalyze the transfer of palmitate from palmityl-CoA to protein substrates (107, 135, 225, 261, 321, 322, 366, 380). However, in certain cases no PAT is necessary and proteins autopalmitylate *in vitro* (24, 31, 73, 107, 209, 413, 414). Characteristically, PATs contain a DHHC (Asp-His-His-Cys) motif embedded in a cysteine-rich domain (CRD). The cysteine residue in the DHHC is often palmitylated

Figure 2.5

Myristyl switches. Myristyl switches allow myristylated proteins to reversibly bind membranes. A) Myristyl-ligand switch. Binding of a ligand triggers a conformational change that regulates exposure of the myristate moiety. In one conformation, the fatty acid is exposed and promotes membrane binding ("myristate out"). Another conformation sequesters the myristate ("myristate in") (e.g., ADP ribosylation factor). B) Myristyl-electrostatic switch. Phosphorylation of a protein that is membrane bound via myristate and basic residues reduces the electrostatic interactions and releases the protein from the membrane (e.g., MARCKS) C) Myristyl-proteolytic switch. Proteolytic digestion of membrane bound proteins causes a conformational switch of the protein that sequesters the myristate (e.g., Pr55 Gag of HIV-1).

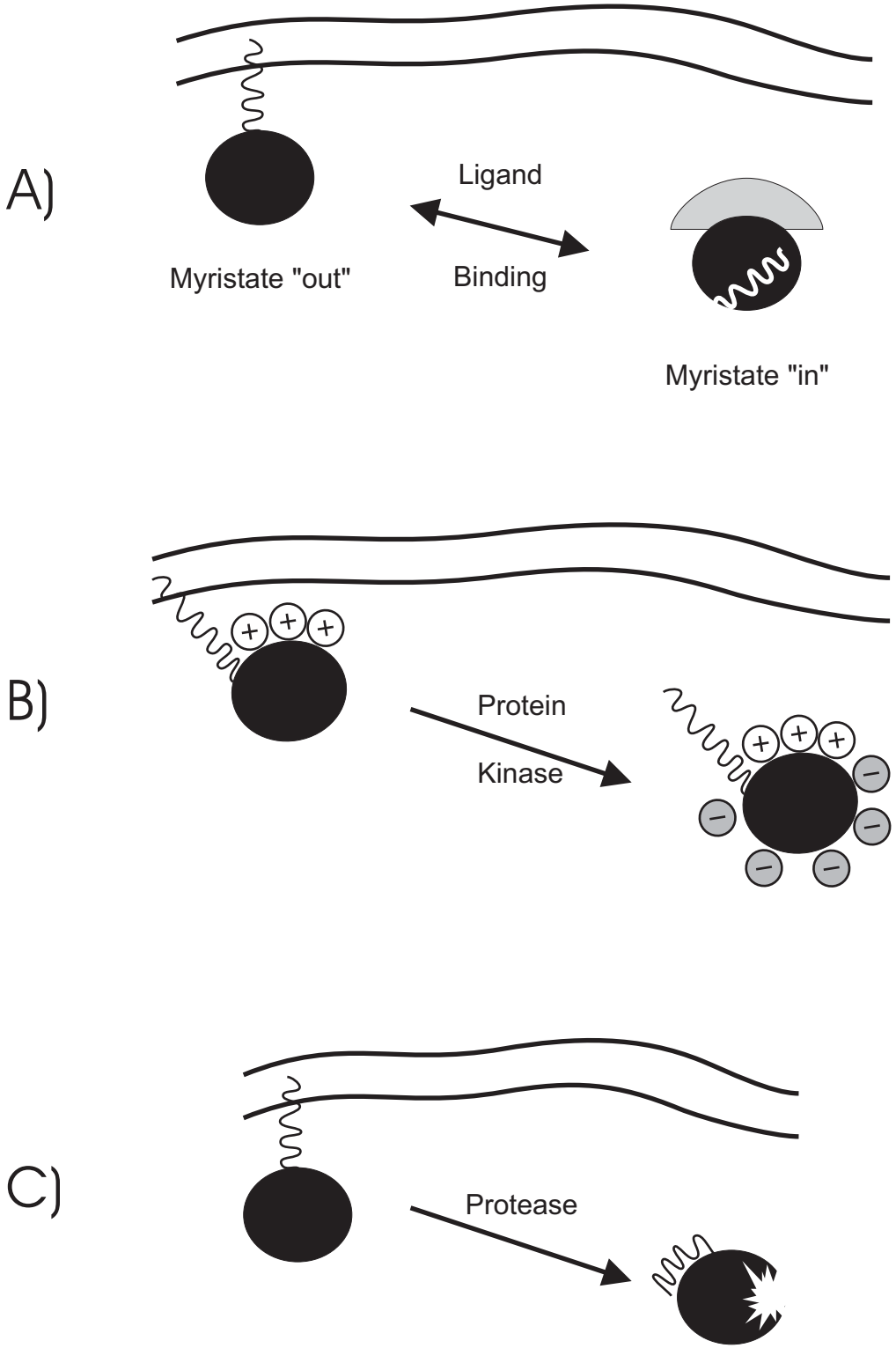
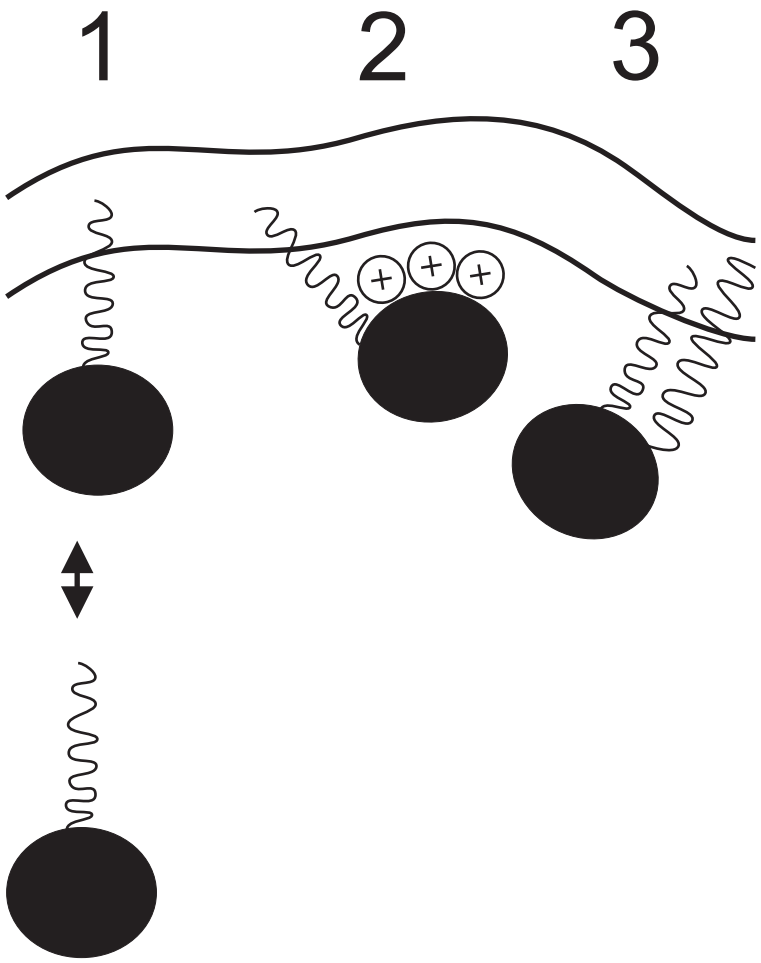

A)
Ligand
Myristate "out"
Binding
Myristate "in"
B)
Protein
Kinase
C)
Protease

Figure 2.6

Two-signal model of membrane binding by myristylated proteins. (1) Myristylated proteins bind membranes weakly and easily dissociate into the cytoplasm. For increased membrane binding strength, myristylated proteins require a second membrane binding signal. (2) A cluster of basic amino acids ("+") interacts with the acidic phospholipid head groups of the membrane. (3) Palmitate moieties increase membrane binding through hydrophobic interactions.


1
2
3

and mutation of this residue inhibits acylation of both the PAT and the substrate protein. This data suggests an acylated-enzyme intermediate is formed during palmitylation of a protein and that the DHHC-motif is directly involved in the palmityl transfer reaction (225, 321). At least 23 proteins in the human proteome are predicted to contain DHHC-CRD motifs (261); however, the number of these that have PAT function is unknown. Additionally, the PAT that is required for UL11 palmitylation is not known.

The reverse reaction, depalmitylation, is catalyzed by a thioesterase (108, 109, 379, 415, 433). Thus far, only two thioesterases have been identified: PPT1 (palmitoylthioesterase 1) and APT1 (acyl protein thioesterase 1). PPT1 is a lysosomal hydrolase that contributes to the degradation of palmitylated proteins by deacylating cysteine residues (55, 415). More pertinent to this dissertation, APT1 is a cytosolic protein that hydrolyses the thioester bond between the palmityl fatty acid and the acylated protein to release palmitic acid (108, 109). The half-life of palmitate on proteins varies. For example, the cellular Fyn protein is palmitylated with a half-life of 1.5-2 h (430) whereas the half-life of palmitate on Lck is 15-30 min (436). However, APT1 does not depalmitylate all proteins, such as caveolin (433), suggesting either additional thioesterases exist or not all proteins cycle between a palmitylated and depalmitylated state.

Because a palmitate moiety can be removed easily, protein palmitylation is often thought of as a lipid counterpart to protein phosphorylation. The cycles of palmitylation and depalmitylation provide a potential regulatory mechanism for shuttling proteins between the cytosol and membranes or between different membranes or membrane domains. For example, the human δ opioid receptor is a transmembrane protein that

requires palmitylation for efficient biosynthetic delivery to the PM (301). Whether UL11 cycles between a palmitylated and de-palmitylated state is unknown.

Di-leucine & Acidic Cluster

Di-leucine (LL) and acidic cluster (AC) motifs are common trafficking signals of cellular and viral proteins (discussed above, Tegumentation section). A cargo protein, such as furin, contains one or more of these motifs to recruit adaptor proteins (e.g., AP-1, AP-2, and PACS-1) that in turn form clathrin-coated vesicles. The vesicles then traffic the cargo protein to a specific destination within the cell depending on the adaptor protein present. In addition to the cellular protein furin, the viral proteins Nef (HIV), US9 (PrV), and gB and UL99 (HCMV) all encode at least one of these motifs, similar to HSV UL11 which contains an AC and possibly an LL-like motif, an LI (leucine-isoleucine) (42, 199, 226-228, 406, 419, 434).

Studies by Loomis et al. (2001) have demonstrated the AC of UL11 functions as an internalization signal. UL11 mutants that lack the AC accumulate at the PM and replacement of the UL11 AC with a known AC from another protein (furin or HIV Nef) restores the PM to TGN targeting. Also, a chimera of the CD4 extracellular- and transmembrane-domains fused to the N-terminus of UL11 is efficiently internalized to the TGN via an AC-dependent mechanism. In contrast to the AC motif, the LI is poorly characterized. Interestingly, the classical signal for a di-leucine is [D/E]XXXL[L/I] and UL11 does not contain the upstream acidic residue. Confocal microscopy of a GFP-tagged UL11 that lacks the LI motif (LI/AA) suggests the protein accumulates at the cell periphery and the LI is a trafficking motif (227). However, a mutant of UL11 that lacks the LI is preferentially incorporated into virions over WT UL11; conversely, removal of

the known trafficking signal (AC) results in an exclusion of the mutant compared to WT UL11 (228). If each motif is indeed a trafficking signal, the packaging data suggest the LI and AC are targeting UL11 differently within the infected cell, similar to furin.

Regardless of their roles in trafficking, both the AC and LI motifs are critical for the interaction of UL11 with UL16 (227, 434). But unlike the PM to TGN targeting of UL11, which is mediated by any AC motif (from UL11, Nef, or furin), only the UL11 AC motif is recognized by UL16.

Despite the small size of UL11 (96 amino acids) all of the above motifs are contained within the first 50 amino acids (Figure 1.3). As such, *in vitro* expression of only these 50 residues suffices for all the functions discussed above. As a final note, a recent study demonstrated that a homolog of UL11 from HCMV, UL99, multimerizes (342). It remains to be determined whether other homologs of UL11 also self-interact.

Detergent Resistant Membranes

Discovering & Defining DRMs

Many proteins are inserted into membranes via a membrane spanning peptide. However, not all membrane associated proteins possess this property and require post-translational modifications for membrane binding. Glycosylphosphatidyl inositol (GPI) serves as such a membrane anchor (78, 229) and is covalently linked to proteins as they traffic through the ER. The modified protein is then exposed on the exoplasmic face of cell membranes. A hallmark study by Brown and Rose (47) shows GPI anchored proteins are selectively localized in "glycolipid-enriched membrane subdomains." These

subdomains are resistant to solubilization in cold non-ionic detergents such as Triton X-100, have a low density profile, and can be isolated on sucrose gradients (Figure 2.7). Several other groups quickly published data confirming this observation (318, 335, 336). Examination of the lipids in the cellular subdomains revealed an increased sphingolipid and cholesterol concentration and an exclusion of glycerophospholipids (47). A half decade later, the term "raft" was used to describe the lipid domains (356), and today the term detergent resistant membrane (DRM) is used interchangeably with raft.

Further studies revealed tandem acylation with myristate and palmitate is also capable of targeting proteins to DRMs (256, 348). These additions occur when a Met-Gly-Cys motif is present on the N-terminus of a protein and the initiator methionine is removed, the glycine is myristylated, and the cysteine is palmitylated (discussed above). Also, as seen in the T cell adaptor protein LAT, dual palmitylation of nearby Cys residues is sufficient and necessary for DRM targeting (440).

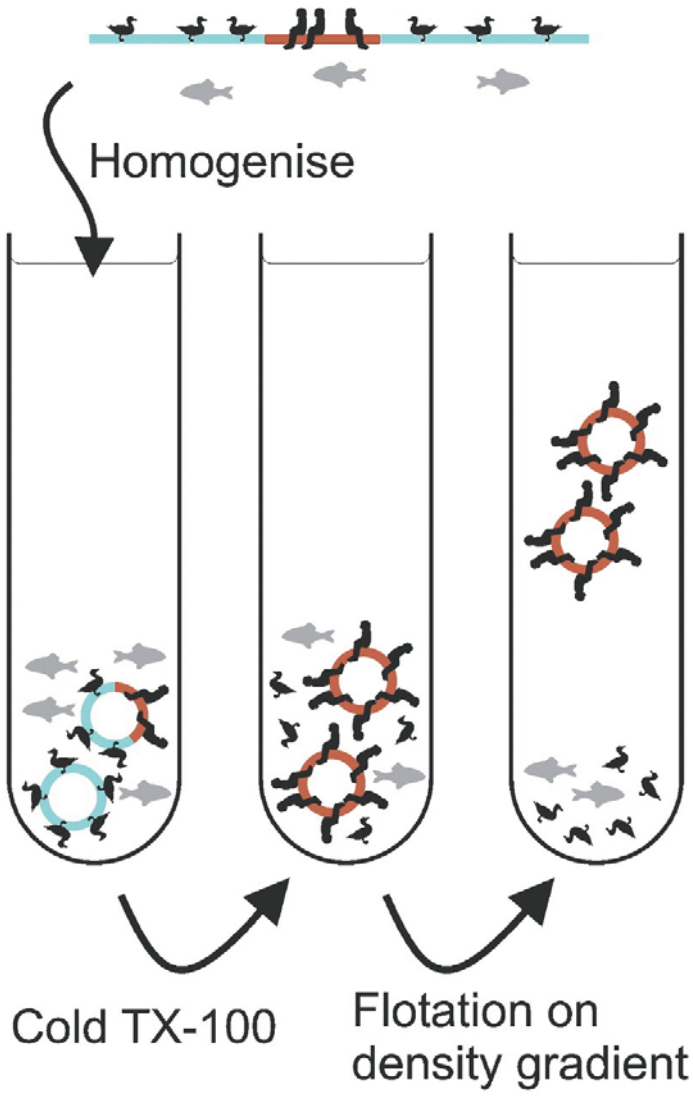
The common theme among all three lipid modifications (GPI-modification, myristylation and palmitylation, or dual palmitylation) is the addition of saturated, fatty acyl chains. The long tails of the lipid anchors interdigitate and interact with the sphingolipids in DRMs through van der Waals interactions and extensive hydrogen bonding (35, 356). However, the presence of a lipid modification does not guarantee DRM association, as many palmitylated proteins do not reside in DRMs (254).

DRMs have been suggested to function in several cellular processes. Possibly the most notable function for DRMs is as "signaling platforms" that couple events on the outside of the cell with signaling pathways inside the cell (45, 46, 356, 357). Numerous cellular factors utilize DRMs for signaling including T cell receptors, B cell receptors,

Figure 2.7

A schematic representation of the raft flotation assay. The plasma membrane (blue) contains lipid rafts (brown). Rafts contain proteins (men) with affinity for their particular lipid composition. The bulk phase of the plasma membrane also contains membrane proteins (ducks). Fish indicate cytoplasmic proteins. Homogenization leads to the formation of membrane vesicles. Cold Triton X-100 treatment solubilizes the bulk phase of the plasma membrane, but the raft domains remain insoluble. The raft fraction floats upon centrifugation from the bottom of a sucrose gradient, whereas cytoplasmic and dissolved plasma membrane proteins remain at the bottom of the tube.

Image and legend taken from Briggs et al., 2003. J. Gen. Virol. 84: 757-768. See appendix A for copyright approval.


Homogenise
Cold TX-100
Flotation on
density gradient

IgE receptors, growth factors, chemokines, and interleukins (22, 48, 121, 176, 182, 302, 373). DRMs have also been implicated in the sorting of lipids and proteins in both the endocytic and secretory pathways. After synthesis in the ER, GPI-linked proteins are transported to the Golgi complex, where they are incorporated into DRMs (47, 451). Lipid sorting via DRMs is supported by the observation that apical membranes are enriched in sphingolipids (a major component of DRMs) whereas basolateral membranes have reduced sphingolipids (358).

DRM Size

Most groups agree that DRMs exist *in vivo*, but now the size of DRMs is debated. Using single molecule microscopy, domain size is estimated to be 0.7μm (0.2 – 2μm) which corresponds to approximately 13% of the cell surface (47, 339). One caveat to this experiment is that the analysis was done at ambient temperatures; and studies preformed at physiological temperatures show a reduced size of DRMs compared to the room temperature studies (339). Another method of studying DRM size is to use a laser trap to monitor local diffusion of a DRM targeted protein. In doing so, it was estimated DRMs are approximately 50 nm in diameter (307). The issue of DRM size is still unresolved, and measurements vary depending on the method of analysis. However, it is likely that DRMs are heterogeneous in structure and function and that their sizes and protein contents are transient.

DRMs Vs. Caveolae

Caveolae are small, flasked shape invaginations on the cell surface. A classical marker of caveolae is caveolin (VIP21), a ~22 kDa type II transmembrane protein. Initial isolation of caveolin with detergent resistant membrane fractions led to the belief that DRMs and caveolae were synonymous (323, 330). However, more recent studies demonstrate this is not the case and that DRMs can be isolated in cells lacking caveolae (129, 149). The current consensus is that DRMs exist both inside and outside of caveolae.

DRMs & HSV

Many pathogens utilize DRMs for entry, exit, or assembly. Some examples are Semliki Forest virus, influenza, respiratory syncytial virus, HIV, measles, simian virus 40, Ebola, Marburg, murine leukemia virus, human enterovirus, the non-enveloped rotavirus, and herpesviruses (3, 12, 13, 25, 26, 67, 166, 167, 216, 220, 230, 241, 242, 277, 288, 297, 303, 329, 331, 332, 359, 376, 439).

HSV entry is inhibited in a dose dependent manner when DRMs are disrupted by cholesterol depletion (32); however, this inhibition is not due to a mislocalization of the receptors HVEM and necti-1 since neither receptor is constitutively DRM associated. Immediately after the addition of soluble glycoproteins or infectious virus to uninfected cells, a fraction of HSV-1 gB but not gC, gD, or gH associates with DRMs; a similar result was obtained for gB from PrV (120). Together, these data suggest gB interacts with a cellular receptor, rapidly mobilizes to DRMs, and serves as a platform for virus entry (32).

HSV also uses DRMs during virion assembly. As discussed earlier, the Vhs protein of HSV-1 regulates translation during the early stages of an infection as well as degrades host mRNAs (29, 110, 115, 197). Interestingly, a population of Vhs localizes to DRMs and this population is believed to be assembled into the tegument of new virions (214, 266).

Chapter III

Sequences in the UL11 Tegument Protein of Herpes Simplex Virus that Control Association with Detergent-Resistant Membranes

Adapted from:

Baird, N.L., P.C. Yeh, R.J. Courtney, and J.W. Wills. 2008

Virology 374: 315-321

Abstract

The product of the UL11 gene of HSV-1 is a small, membrane-bound tegument protein with features that are conserved among all herpesviruses. For all viruses examined, mutants lacking this protein (or its homolog) have secondary-envelopment defects and accumulate capsids in the cytoplasm of the infected cell. UL11 binds to the cytoplasmic faces of host membranes via N-terminal myristate and nearby palmitate moieties. These fatty-acid modifications are typical of proteins that localize to detergent-resistant membranes (DRMs), and the experiments described here revealed that a small amount (~10%) of UL11 retains the ability to float in sucrose gradients following treatment of cells with Triton X-100. However, mutants lacking sequences previously shown to be involved in the trafficking of UL11 from the plasma membrane (LI and acidic cluster motifs) were found to have a dramatically increased association with DRMs. These findings emphasize the dynamic properties of this poorly-understood but conserved tegument protein.

Introduction

The UL11 protein of herpes simplex virus type 1 (HSV-1) is necessary for the efficient production of virions in cell cultures (20, 136, 239). This small, 96-amino-acid molecule is thought to be made on cytoplasmic ribosomes, where it is co-translationally modified with myristate on its N-terminal glycine following removal of the initiator methionine (238, 310). UL11 subsequently binds to the cytoplasmic faces of cellular membranes and becomes palmitylated on at least one of the three cysteines located near the amino terminus (18, 226). Modifications with myristate and palmitate are needed for membrane binding of UL11 and subsequent accumulation at the trans-Golgi network (39, 226), the site of secondary envelopment (255). Approximately 700 molecules of UL11 are packaged into the virion (228), and these are thought to extend from the membrane into the tegument, the region located between the envelope and the capsid (255). Mutants having large deletions in the UL11-coding sequence exhibit an accumulation of unenveloped capsids in the cytoplasm (20, 136, 239). Moreover, all herpesviruses encode a homolog of UL11, and in those cases where this gene has been disrupted, replication defects and cytoplasmic capsid accumulations also result (44, 204, 205, 333, 354, 355). However, in contrast to human cytomegalovirus, where null mutants are completely defective for the release of extracellular virions, HSV mutants have been reported to be reduced at most ~1000 fold, perhaps due to redundancy. Alternatively, this could be due to incomplete removal of the UL11-coding sequence to avoid the overlap with the U_L12 gene, thereby leaving large portions of the N-terminus of UL11 intact (20, 136, 239).

Although UL11 mutants have defects in secondary envelopment, the actual function of this protein is unknown. Insight was provided by the discovery of an interaction between UL11 and UL16 (227, 418), a tegument protein that has been reported to be associated with capsids (253, 289). This led to a model that is reminiscent of the function of viral matrix proteins in which UL11 links capsids (via UL16) to host membranes to promote the envelopment process (227). However, it is clear that this is not the only mechanism of UL11 incorporation because UL11 mutants have been found that are incorporated into virions even though they lack the LI (leucine-isoleucine) and acidic cluster (AC) motifs needed for the interaction with UL16 (226, 228, 434). These motifs are particularly interesting because they are important for the recovery of UL11 from the plasma membrane back to internal membranes. That is, when either motif is absent, a portion of UL11 accumulates at the cell periphery (226). Moreover, chimeras that have foreign acidic clusters (from Nef or furin) do not accumulate on the plasma membrane and are packaged, even though they do not interact with UL16 (226-228). Based on these observations, another model for the function of UL11 in virus assembly can be imagined in which passage through a particular trafficking pathway is needed (228). This might enable the recruitment of other proteins (virus or host encoded) from the plasma membrane, or enable post-translational modifications of UL11 that are essential for envelopment.

In light of the apparent importance of membrane trafficking for the function of UL11, the ability of this tegument protein to associate with detergent-resistant membranes (DRMs) was examined. DRMs are microdomains within cellular membranes that are enriched in cholesterol and sphingolipids, yielding “platforms” that are thought to

be important for several functions including signal transduction, cytoskeletal organization, and pathogen entry and exit (13, 67, 166, 216, 288, 303, 359). DRMs are insoluble in non-ionic detergents such as Triton X-100 (TX-100), conditions that disrupt non-DRM membranes (216, 356, 417). Because of this property, DRMs can be released by adding TX-100 and purified by flotation in sucrose gradients, enabling the resident proteins to be analyzed (Figure 2.5). Proteins that are dually modified with myristate and palmitate are typically associated with DRMs (303, 310, 359), and therefore, it was predicted that UL11 would be, too. The results described below suggest that a population of UL11 molecules traffic through DRMs under the control of the LI and acidic cluster motifs.

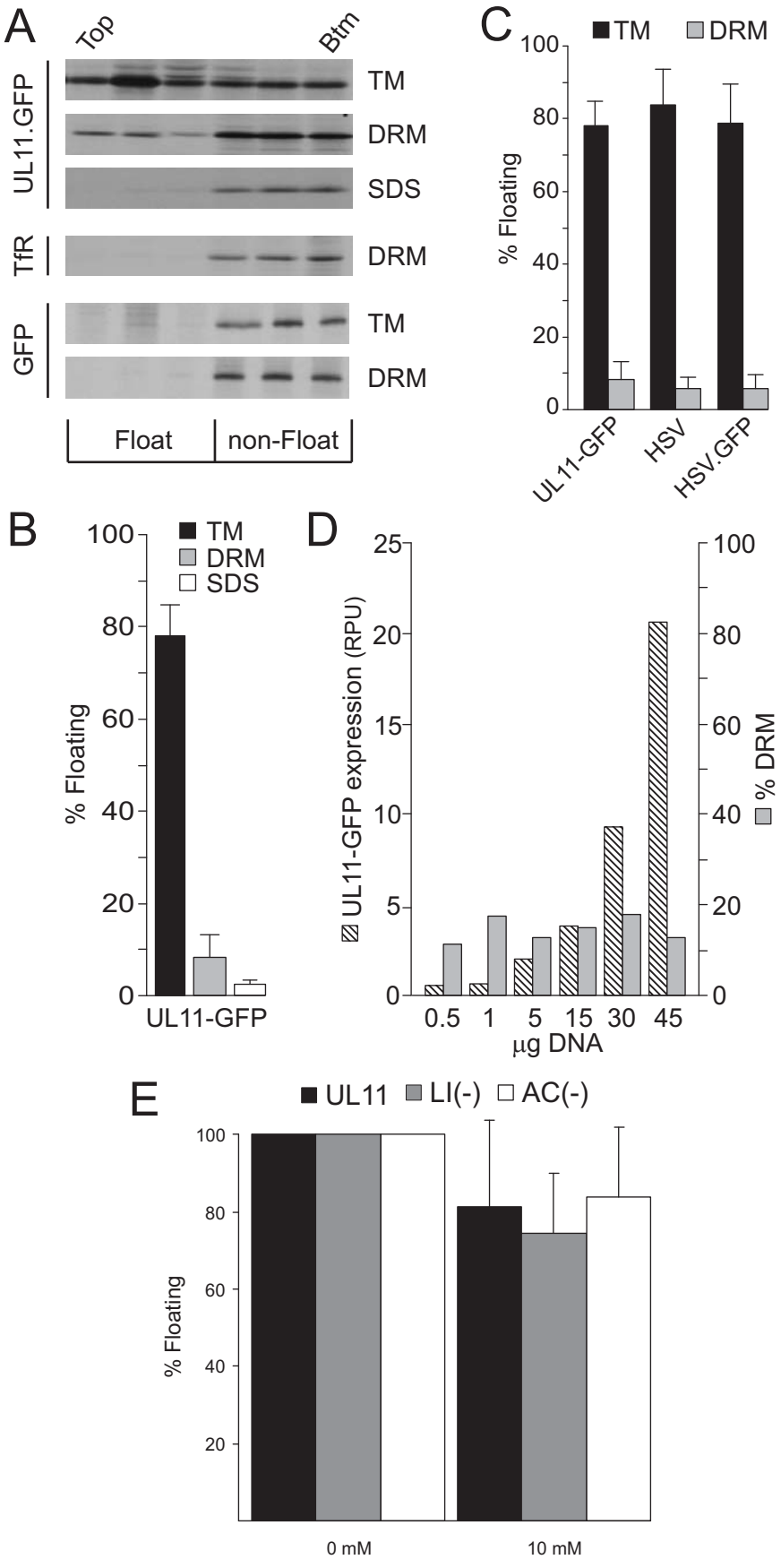
Results

Flotation Analysis of UL11-GFP

Initially, the ability of UL11 to associate with DRMs in the absence of other viral proteins was examined using a UL11-GFP fusion protein (UL11 tagged at the C-terminus with the green fluorescent protein, GFP). This construct has been studied extensively and appears to behave identically to the untagged protein in all assays used (226-228). Using metabolic labeling, membrane binding was determined in the absence of detergent. Labeling and immunoprecipitations allow an increased sensitivity, reproducibility, and ability to be quantitative over a wider range of expression levels compared to Western blots. As expected from previous studies of UL11-GFP (226), about 80% of the protein was found to float in the absence of detergent, indicating that it was stably membrane bound (Figure 3.1A and B). After treatment with TX-100, only about 10% of the protein could float into the top three fractions, and this represents the DRM-associated molecules present during 2.5 h of continuous radiolabeling. Although low, this amount was above background levels, as determined by flotation in the presence of 0.5% SDS to solubilize all membranes (Figure 3.1A and B). While this is not a statistically significant difference from TX-100 (Student T-test, $P = 0.10$), if the definition of floating protein is restricted to the top two fractions (thereby eliminating the possibility of contamination from the large amount of underlying material that does not float), then a dramatic increase in statistical significance is seen ($P = 0.003$). As will be seen below, mutants that disrupt the trafficking of UL11 result in dramatic differences in DRM localization, even with the less restrictive definition, and hence, it was used for the remainder of the experiments.

Figure 3.1

Flotation analysis of UL11. (A) A7 cells transfected with a UL11-GFP expression vector were metabolically labeled for 2.5 h and osmotically disrupted, as described in the text. Cytoplasmic membranes were treated with nothing (TM, total membranes), 0.5% TX-100 (DRM, detergent-resistant membranes), or 0.5% SDS (negative control). The ability of UL11-GFP to float to the upper fractions of sucrose step gradients during centrifugation was examined, and representative autoradiograms, obtained following immunoprecipitation and SDS-PAGE analysis, are shown. As a control for DRM disruption, endogenous transferrin receptor (TfR) was monitored in one experiment following radiolabeling, TX-100 treatment, and flotation. The tops and bottoms of the gradients are indicated. (B) Phosphorimager analysis was used to quantitate the flotation results, which are shown as the percentage of floating protein (top three fractions) relative to the total protein (all fractions). The averages of four experiments are shown, along with the standard deviations. (C) Flotation assays were used to compare the membrane-binding properties of radiolabeled UL11-GFP produced in transfected cells with untagged UL11 and UL11-GFP produced by wild-type and recombinant viruses (HSV and HSV.GFP, respectively). Cells were labeled for the final 2.5 h of infection, harvested, and floated as described in the text. The averages from at least four experiments are shown, along with the standard deviations. (D) To examine the saturability of DRMs, cells were transfected with increasing amounts of plasmid DNA, metabolically labeled, and subsequently analyzed for UL11-GFP expression levels (hatched bars; RPU = relative phosphorimager units) and DRM localization (grey bars). A repeat of this experiment gave comparable results. (E) DRMs were disrupted by cholesterol depletion using the drug methyl-β-cyclodextrin as indicated in the text. Following treatment, cells were harvested and floated as before.


A
Top
Btm
UL11.GFP
TfR
GFP
TM
DRM
SDS
DRM
TM
DRM
Float
non-Float
B
TM
DRM
SDS
% Floating
UL11-GFP
C
TM
DRM
% Floating
UL11-GFP
HSV
HSV.GFP
D
UL11-GFP expression (RPU)
% DRM
0.5
1
5
15
30
45
µg DNA
E
UL11
LI(-)
AC(-)
% Floating
0 mM
10 mM

Two additional experiments provided evidence for the association of wild-type UL11 with DRMs. In the first, the flotation property of UL11-GFP was compared with endogenous transferrin receptor (TfR) present in the transfected cells. TfR is a membrane-bound but DRM-excluded protein, and when TX-100 was present, the amount of floating material was found to be only 25% that of UL11-GFP, a difference that was similar to that seen for UL11-GFP when comparing SDS and TX-100 treated samples (Figure 3.1A and B). Second, when DRMs were disrupted with the cholesterol chelating drug methyl-β-cyclodextrin (MβCD; 10mM), the amount of WT UL11-GFP localized to DRMs decreased ~20% (Figure 3.1E). While this is not a complete abolishment of DRM-bound UL11, not all DRM resident proteins are completely sensitive to MβCD (378).

To ascertain whether the association of UL11-GFP with DRMs depends on expression levels, increasing amounts of the expression vector were transfected into A7 cells. If the capacity of the cell to create DRMs was saturable, then the amount of UL11-GFP floating would plateau as expression continued to increase, and consequently, the percentage of DRM-associated protein would decrease. This was not found. Instead, the percentage of DRM-associated protein remained constant over a 20-fold range of expression (Figure 3.1D). Thus, any variation in UL11-GFP expression between experiments appears to be unimportant for the studies below. Nevertheless, attempts were made to keep expression-levels equal by transfecting consistent amounts of DNA (15μg) for each construct in each experiment.

Because UL11 interacts with other HSV tegument proteins (118, 227, 418) and such interactions may alter the localization of UL11, the membrane association of UL11-

GFP during an HSV infection was examined. To this extent, a recombinant virus was created to express the fusion protein. For the flotation analyses, A7 cells were infected with the wild-type or recombinant virus at a multiplicity of infection of 10, radiolabeled for the final 2.5 h of infection, and osmotically disrupted at early (10-12 h) or late (18-20 h) times post-infection. In all cases, the flotation properties for virus-encoded UL11 and UL11-GFP were similar to that of transfected-only UL11-GFP (Figure 3.1C). These results demonstrate that other viral proteins do not influence the DRM distribution of UL11; nor does the attachment of GFP (which by itself did not float; (Figure 3.1A). Given this, all subsequent experiments were performed in the absence of all other viral proteins and in the context of a GFP fusion protein.

Specificity of the UL11 Antibody

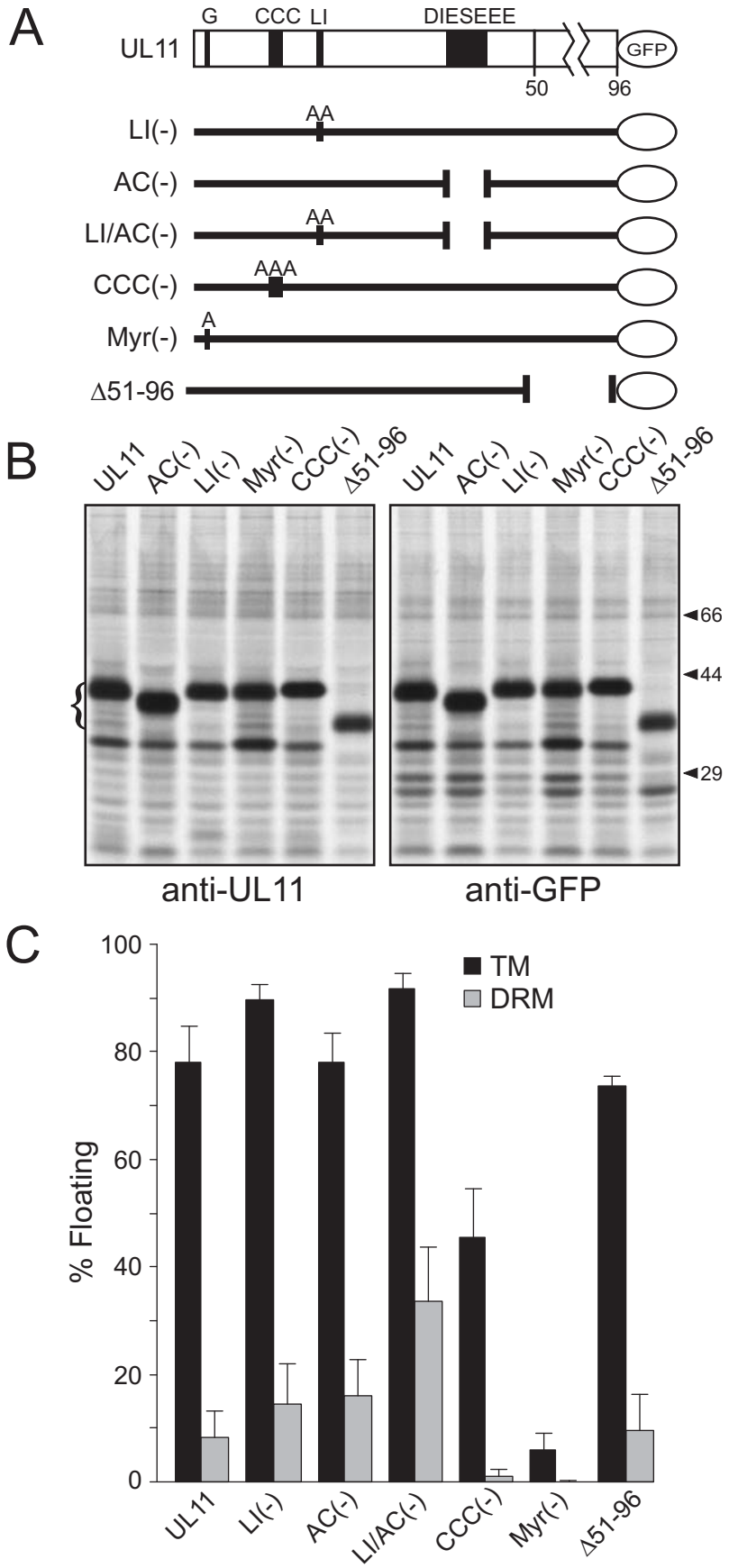
It was of concern that one or more of the mutants used in these studies (Figure 3.2A) would lack epitopes needed for efficient recognition by the previously-described, UL11-specific antibody employed here (227). Therefore, the ability of this antibody to immunoprecipitate the various UL11-GFP derivatives was compared to that of an anti-GFP antibody. All constructs were immunoprecipitated with equal efficiency using either antibody (Figure 3.2B).

Role of Fatty Acid-modification

Based on studies of other proteins, dual modification with myristate and palmitate is predicted to be essential for DRM targeting of UL11 (303, 310, 359). To test this, mutants that lack these modifications were analyzed. Mutant Myr(–), which lacks the site for myristylation and therefore fails to reach membranes where palmitylation occurs (154, 219, 310), behaved as expected and was not associated with any membranes,

Figure 3.2

DRM association of UL11 mutants. (A) Diagram of UL11-GFP and the mutants that were analyzed. The motifs of interest are shown: G, myristylation site; CCC, palmitylation site; LI, di-leucine-like; DIESEEE, acidic cluster (AC). Sites of alanine substitutions are indicated. (B) To examine the reactivity of the mutants to anti-UL11 and anti-GFP sera, transfected cells were metabolically labeled and immunoprecipitated proteins were analyzed by SDS-PAGE. The positions of the UL11-GFP species are indicated with a bracket to the left. Positions of markers (in kDa) are indicated to the right. (C) Constructs depicted in panel A were analyzed for their ability to float in the absence (TM) and presence (DRM) of 0.5% TX-100. Each construct was analyzed a minimum of three times.

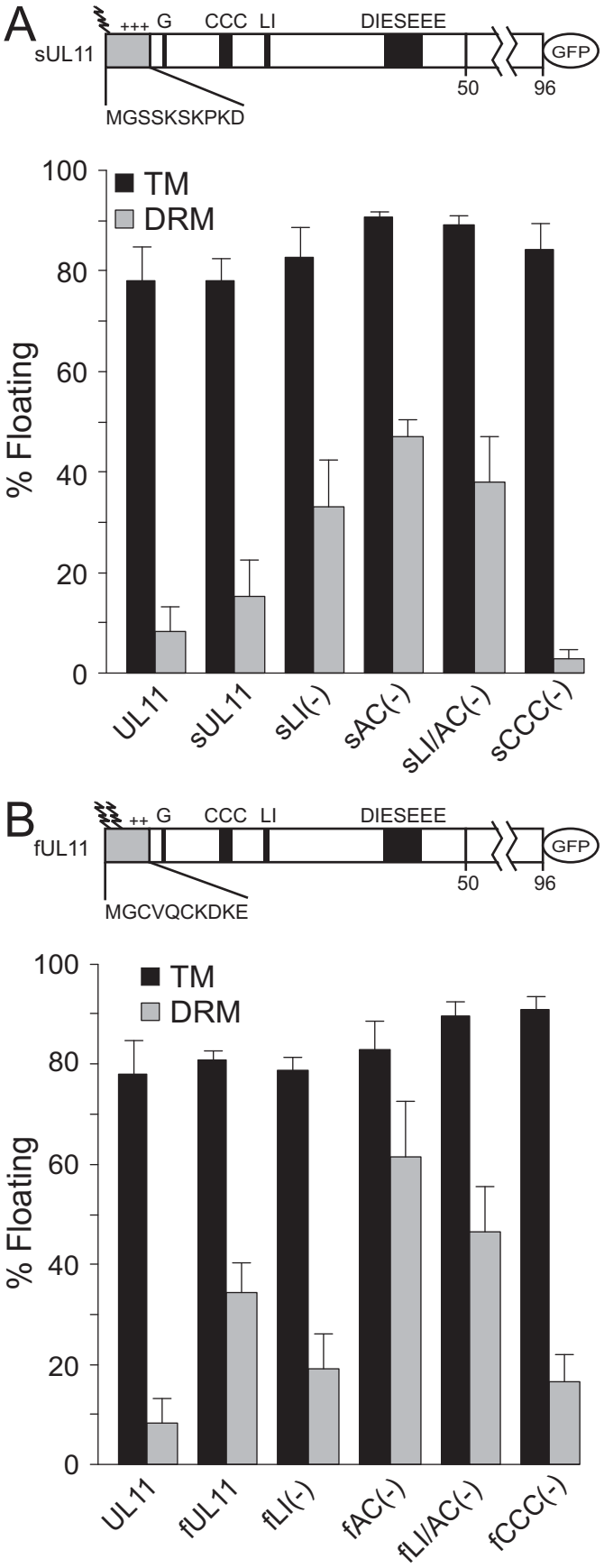

A
G
CCC
LI
DIESEEE
UL11
GFP
50
96
LI(-)
AA
AC(-)
LI/AC(-)
AA
CCC(-)
AAA
Myr(-)
A
Δ51-96
B
UL11
AC(-)
LI(-)
Myr(-)
CCC(-)
Δ51-96
UL11
AC(-)
LI(-)
Myr(-)
CCC(-)
Δ51-96
66
44
29
anti-UL11
anti-GFP
C
100
80
60
40
20
0
% Floating
TM
DRM
UL11
LI(-)
AC(-)
LI/AC(-)
CCC(-)
Myr(-)
Δ51-96

including DRMs (Figure 3.2C). Likewise, mutant CCC(–), which is myristylated but lacks sites for palmitylation (226), would be expected to retain some capacity to associate with membranes but not to be associated with DRMs, and that is what was found. Even though 45% of the CCC(–) molecules were able to float in the absence of TX-100, only 1% was DRM-associated (Figure 3.2C), which is equivalent to the background levels seen for wild type when SDS was used (Figure 3.1B). These data support the hypothesis that myristate alone is capable of directing UL11 to membranes, whereas both palmitate and myristate are required for the targeting of UL11 to DRMs.

To attempt rescue of the Myr(–) and CCC(–) mutants into DRMs, previously-constructed chimeras (226) were examined that have the first 10 amino acids of the v-Src or Fyn proteins added to the N-terminus of UL11-GFP (mutants sUL11 and fUL11, respectively; Figure 3.3). Attachment of these peptides precludes myristylation of the N-terminal glycine of UL11, however, both foreign sequences have their own site for this modification (310). In addition, each has characteristics that increase membrane-binding. The v-Src peptide has three basic residues that interact with acidic phospholipids on the cytoplasmic faces of membranes (310). In addition to basic residues, the Fyn peptide has two cysteine residues that can be palmitylated (9, 310, 348). The membrane-binding properties of these two chimeras (i.e., without other alterations to UL11) were examined first. As expected, sUL11 and fUL11 were about 80% membrane associated in the absence of detergent (Figure 3.3A and B, respectively). When the membranes were treated with TX-100, sUL11 was found to be associated with DRMs to an extent similar to wild-type UL11-GFP. The ability of this chimera to target DRMs is apparently due to palmitylation of one or more of the UL11 cysteines because when these were eliminated

Figure 3.3

Analyses of N-terminal UL11 chimeras. N-terminal extensions corresponding to the first 10 amino acids of (A) v-Src or (B) Fyn were attached to UL11-GFP, as represented by the shaded boxes in the diagrams. Wavey lines denote fatty acid modifications and "+" indicates basic residues. These N-terminal chimeras and the indicated mutants (defined in Fig 2A) were analyzed for their ability to float in the absence (TM) and presence (DRM) of 0.5% TX-100. Each construct was analyzed a minimum of three times


A
sUL11
+++ G CCC LI DIESEEE
GFP
50
96
MGSSKSKPKD
100
80
60
40
20
0
% Floating
TM
DRM
UL11
sUL11
sLI(-)
sAC(-)
sLI/AC(-)
sCCC(-)
B
fUL11
++ G CCC LI DIESEEE
GFP
50
96
MGCVQCKDKE
100
80
60
40
20
0
% Floating
TM
DRM
UL11
fUL11
fLI(-)
fAC(-)
fLI/AC(-)
fCCC(-)

in mutant sCCC(–), DRM association dropped to background levels. In contrast, the association of fUL11 with DRMs was increased ~3-fold compared to UL11-GFP, perhaps due to palmitylation of the extra cysteine residues present in the Fyn peptide. However, even in this case, the cysteines in UL11 appear to contribute to DRM association because when these were eliminated in mutant fCCC(-), levels dropped to that of wild-type UL11-GFP.

Sequences that Control Levels of DRM Association

Although myristate and palmitate are essential for the accumulation of UL11-GFP within DRMs, it is possible that other parts of the protein actually control its trafficking through these membrane locations (e.g., by enabling interactions with cellular factors to enable endocytosis). If so, these functions must reside in the first half of UL11 because mutant Δ51-96, which lacks the second half, was indistinguishable from wild type in the flotation assays (Figure 3.2C). The obvious elements to examine were the LI and the acidic cluster motifs. To analyze these motifs, previously characterized mutants lacking either the leucine-isoleucine motif [mutant LI(-), changed to alanines] or the acidic cluster motif [mutant AC(-), deletion of the seven-residue cluster] were used (226, 227). Additionally, a third variant of UL11, which combines both mutations, was used (mutant LI/AC(-), Figure 3.2A). Elimination of either of these motifs alone from UL11-GFP had small enhancing effects on DRM association, but when both motifs were missing, a striking 3-fold increase occurred (Figure 3.2C). These results are consistent with the hypothesis that either motif is sufficient to enable UL11 to exit DRMs, perhaps through either of two different pathways.

The LI and acidic cluster mutants of UL11-GFP were also examined in the context of the chimeras. Addition of the v-Src peptide appears to make UL11 more sensitive to removal of the trafficking motifs in that enhanced DRM association occurred even with the single mutations (Figure 3.3A). In contrast, DRM association of the Fyn chimera appears to be controlled primarily by the acidic cluster. That is, removal of the acidic cluster (alone or in combination with the LI substitution) resulted in an increase, whereas removal of the LI resulted in at most a small reduction in DRM accumulation. Collectively, these complex results suggest that the foreign sequences may interfere with the normal trafficking properties of UL11, possibly by altering the conformation and hence the ability of the LI and acidic cluster motifs to be properly recognized by the sorting machinery.

Chapter IV

Myristylation and Palmitylation of UL11 are Necessary but Not Sufficient to Support Growth of Herpes Simplex Virus

Abstract

All herpesviruses encode a UL11 tegument protein, and UL11-null viruses have defective envelopment processes which cause capsid accumulation within the cytoplasm and reduced virus release. HSV UL11 requires covalent modification with myristate and palmitate for membrane binding, lipid raft trafficking, and accumulation at the site of virus envelopment. Like HIV-1 Gag, which requires myristylation to support virus production, it was predicted that UL11 acylation would be necessary for efficient virion envelopment. To test this, recombinant viruses were created to express UL11 derivatives that are not acylated, partially acylated, or UL11-chimeras that contain foreign acylation signals. Unexpectedly, a non-acylated UL11 rescued some growth defects of a UL11-null, despite the protein being highly unstable. Furthermore, a myristylated and palmitylated chimera did not fully rescue the null-virus. These results suggest that 1) UL11 maintains function(s) even when not membrane-bound, and 2) the context of the UL11 acylations is important for function.

Introduction

UL11 is a conserved tegument protein among all herpesviruses, and each homolog contains amino acid motifs that allow covalent modifications with two fatty acids, myristate and palmitate. Without both modifications, UL11 lacks all membrane binding potential and is free in the cytoplasm [Chapter III and (226)]. UL11 is co-translationally modified with myristate on the N-terminal glycine following removal of the initiator methionine (238, 310). After release from ribosomes, the myristylated UL11 binds to the cytoplasmic face of cellular membranes and is subsequently palmitylated on at least one of three cysteines near the amino terminus (226). Initial co-translational modification of UL11 with myristate is a prerequisite to the post-translational palmitylation; hence, a mutant of UL11 that lacks the N-terminal glycine residue is also defective for palmitylation even though the cysteine residues are present (226). Following covalent attachment of the fatty acids, UL11 accumulates on membranes of the trans-Golgi network (TGN), the site where DNA-filled capsids are enveloped. Additionally, the dual acylation of UL11 is both sufficient and necessary for UL11 to traffic to lipid rafts, or detergent resistant membranes (DRMs) (Chapter III).

Once UL11 is localized to the TGN, it has been suggested the envelopment process is promoted by interactions between the membrane-bound UL11 and the capsid-bound tegument protein UL16 to form a "bridge" (227). This hypothesis is supported by two lines of evidence. First, UL16 can directly interact with UL11 *in vitro* (434). Second, all UL11-null herpesviruses have defects during virion envelopment (20, 44, 136, 204, 205, 239, 333, 354, 355). As a result of the envelopment defects, naked capsids accumulate within the cytoplasm of the infected cells and fewer virions are released into

the extracellular space.

In retrovirology, it is well known that fatty acids are required to anchor viral proteins to membranes during virion formation. In the case of HIV-1, myristate-minus Gag polyproteins fail to interact with membranes and consequently are severely defective for virus production (150). Similarly, when HSV UL11 acylation mutants [tagged with GFP (green fluorescent protein)] have to compete with the WT UL11 for incorporation into virus particles, they are not efficiently packaged (228). Given the HIV data and the above properties of UL11 that require acylation with both myristate and palmitate, it was predicted that the ability of UL11 to function properly and promote virion envelopment would be completely abolished in the absence of both acyl modifications. Unexpectedly, this was not the case and expression of a non-acylated UL11 derivative rescued some growth defects of a UL11-null virus. Furthermore, a UL11-chimera that contains foreign myristylation and palmitylation signals at the N-terminus failed to fully rescue a UL11-null virus, suggesting acylations alone are not sufficient for protein function and that the context of the modifications is also critical.

Results

Relocation of the UL11 Coding Sequence

The acylated residues of UL11 reside in the first few amino acids, but the coding sequence for these residues overlaps the coding region of the essential U_L12 gene (Figures 2.2 and 4.1A). Consequently, it is not possible to make substitutions of the N-terminal acylation signals in UL11 without disrupting UL12. To circumvent this problem, a recombinant virus (BAC) that contains the KOS-strain HSV-1 genome within a bacterial artificial chromosome (bac) (144) was used to relocate the UL11 ORF (open reading frame) (Figure 4.1A). The first step of the relocation deleted the UL11 sequence that does not overlap the U_L12 ORF to create the UL11-null virus, Δ30-96. Second, the entire UL35-coding sequence was replaced with the UL11 ORF to make the recombinant virus U1. The U_L35 gene, which encodes the minor capsid protein VP26, was chosen because it: 1) is not essential *in vitro* (92), 2) does not overlap any other reading frames which allowed N-terminal mutations of UL11, 3) is approximately the same size as UL11 (~100 amino acids), and 4) is expressed late during an infection similar to UL11 (252). Both Δ30-96 and U1 retained the N-terminal 29 codons of the original UL11 ORF (Figure 4.1A and Table 4.1); however, no peptide was detectable from either the cell lysates or purified virions (data not shown), consistent with previous UL11-null viruses which also left a short piece of the reading frame intact (20, 136, 239). As expected, Δ30-96 lacked the UL11 protein but did express VP26, whereas U1 did not express VP26 but did produce UL11 (Figure 4.1B). Also, the UL11 produced from virus U1 was incorporated into virus particles at levels comparable to BAC, even though it was

Figure 4.1

Relocation of UL11. (A) Schematic of the recombineering strategy used to create UL11 recombinant viruses. "KOS-37" is the KOS strain genome of HSV-1 contained in a bacterial artificial chromosome ("bac," 11.5 kbp) inserted between U_L37 and U_L38. Δ30-96 was created using homologous recombination in bacteria and removed all nucleotides of the UL11 ORF that do not overlap with the essential UL12 ORF to leave the coding sequence for the N-terminal 29 amino acids of UL11. Using Δ30-96 as the parent, the UL35 ORF was completely replaced with the entire UL11 coding sequence by recombination to create U1. (B) Vero cells were infected with indicated virus or mock infected and harvested 24 hpi. Equal numbers of infected cells were lysed in sample buffer, separated by SDS-PAGE, and proteins were transferred to nitrocellulose. Indicated proteins were detected by Western blot analysis using corresponding antibodies.

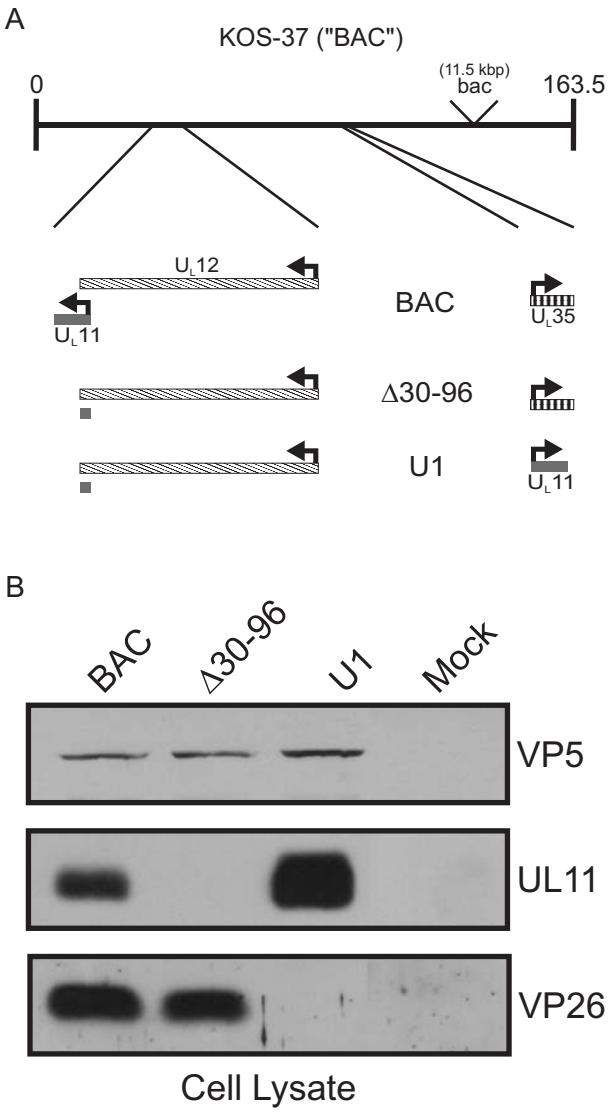

A
KOS-37 ("BAC")
0
(11.5 kbp)
bac
163.5
UL12
UL11
BAC
UL35
Δ30-96
U1
UL11
B
BAC
Δ30-96
U1
Mock
VP5
UL11
VP26
Cell Lysate

Table 4.1 Recombinant HSV Constructs

VIRUS	U_L11 LOCUS	U_L35 LOCUS
KOS	UL11	UL35
BAC	UL11	UL35
Δ30-96	Δ30-96	UL35
U1	Δ30-96	UL11
M15	Δ30-96	Myr(-)
C8	Δ30-96	CCC(-)
sC6	Δ30-96	sCCC(-)
f9	Δ30-96	fUL11
stopM15	stopΔ30-96	Myr(-)

expressed at much higher levels in the cell (Figure 4.2). Similar to KOS, the wild-type virus that lacks any "bac" sequence, extracellular virions from BAC contained UL16 (Figure 4.2 and data not shown), a tegument protein which is detectable as both a soluble and a capsid-bound protein within the cytoplasm of infected cells (253). Interestingly, UL16 was not packaged into Δ30-96 virions but was incorporated into U1 particles, suggesting that UL11, but not VP26, is necessary for UL16 incorporation (Figure 4.2).

Prior to inserting various UL11 acylation mutants into the U_L35 gene, it was necessary to ensure the "bac" sequence was not deleterious to virus growth and also that expression of UL11 from the U_L35 locus would rescue the UL11-null. To do so, single-step growth curve analyses were performed and the size of plaques produced by each virus was studied. The two wild-type viruses, KOS and BAC, were compared to determine whether the presence of the "bac" sequence would alter the growth characteristics of HSV-1 in tissue culture. There were no differences between the two viruses when comparing either the growth curve data (Figure 4.3B) or plaque sizes (data not shown).

Removal of UL11 resulted in drastic growth defects compared to BAC. The plaques produced by Δ30-96 were much smaller than those of BAC (Figure 4.3A). Δ30-96 was also much slower than BAC for production of cell-associated virus and consequently had a delayed release of virus into the medium (Figure 4.3C). Release of Δ30-96 was not only delayed, but was much slower than BAC (Figure 4.3C, slope of "Medium" line), a result similar to what has been reported for other UL11-null viruses (20). As a combined result of these growth defects, the concentration of Δ30-96 released into the medium at 24 hpi (hours post-infection) was reduced from BAC by nearly 4-logs

Figure 4.2

UL11 expression and virion incorporation. Vero cells were infected with the indicated virus and at 24 hpi extracellular virions were collected from the medium by centrifugation through a 30% (w/v) sucrose cushion. Collected virions and infected-cell lysates were separated by SDS-PAGE and transferred to nitrocellulose. Indicated proteins were detected using the corresponding anti-serum. VP5, the major capsid protein, was used as a loading control. To achieve equal VP5 levels in the "Medium" samples, additional cells (indicated as "2X" or "4X") were infected and the media collected as compared to BAC. Equal numbers of infected cells were loaded for the "Cell" sample of all viruses.

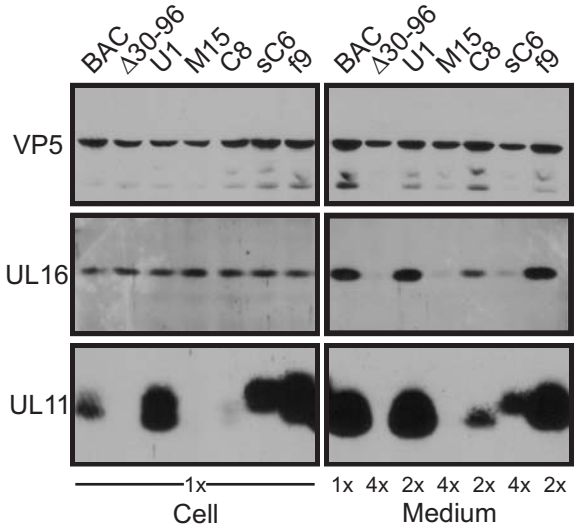
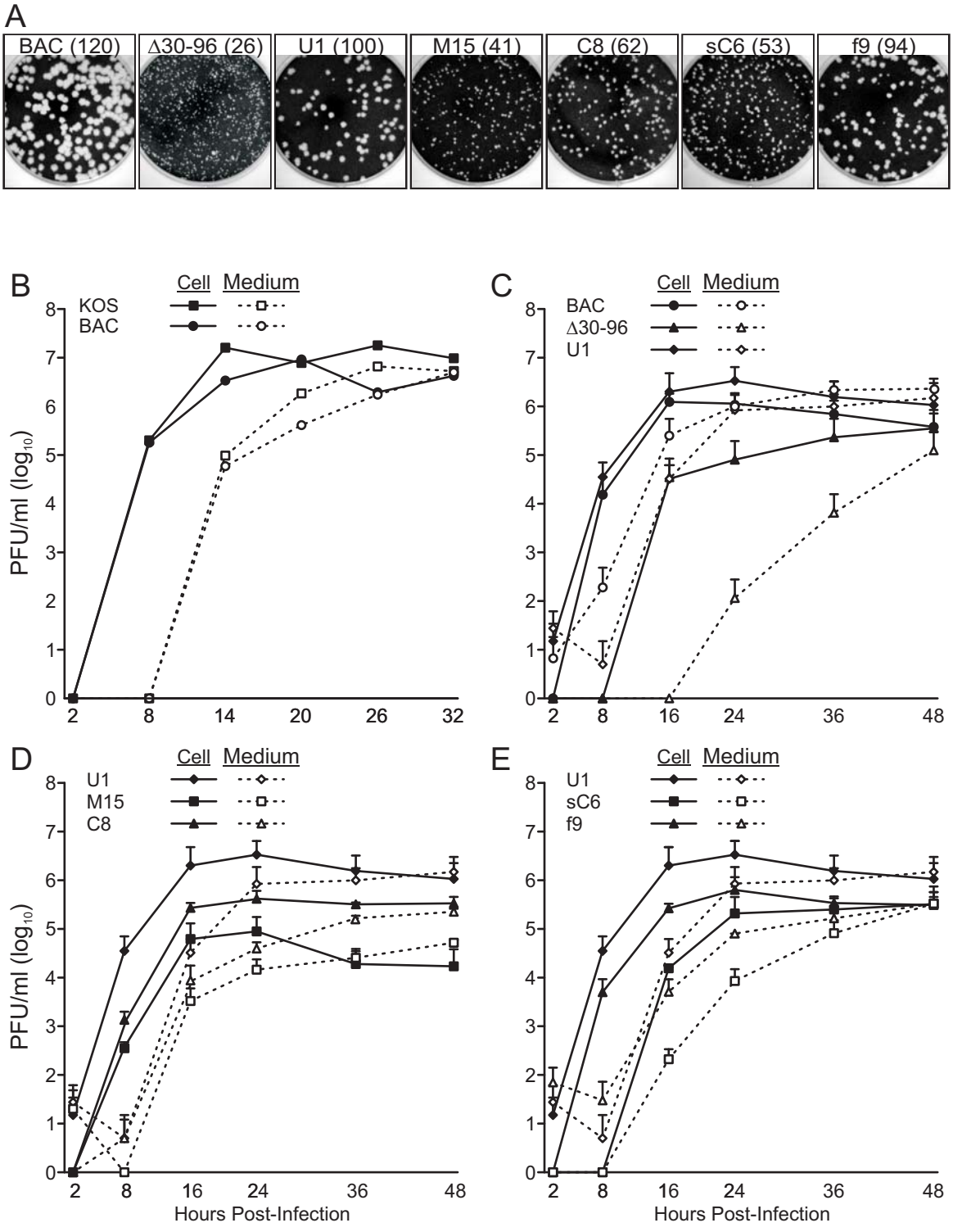

BAC
Δ30-96
U1
M15
C8
sC6
f9
BAC
Δ30-96
U1
M15
C8
sC6
f9
VP5
UL16
UL11
1x
1x 4x 2x 4x 2x 4x 2x
Cell
Medium

Figure 4.3

Growth properties of recombinant viruses. (A) Plaque sizes of UL11 recombinant viruses. Confluent monolayers of Vero cells were infected with indicated virus for 1 h at 37°C, washed with 1% FBS in PBS, then overlayed with 0.5% methylcellulose. 4 days post-infection, cells were stained with crystal violet and imaged. Plaque size was determined by measuring 10 randomly selected plaques and represented as a percent of U1. (B-E) Single-step growth curve analyses of recombinants. Vero cells were infected at an MOI of 1 with indicated virus and after 1 h of adsorption, cells were acid-washed to inactivate any input virus that had not fused with the cellular membrane. At indicated times post-infection, media was collected, cleared, and frozen at -80°C whereas the cells were collected and freeze/thawed three times. During the final thaw cycle of the cells, the clarified media was thawed, and virus titers were determined on Vero cells by plaque assay as detailed in the text. (B) Comparison of WT viruses. Each virus contains the same KOS strain of the HSV genome; the only difference is the absence ("KOS") or presence ("BAC") of the bacterial artificial chromosome. (C) Examination of recombinants that lack UL11 expression ("Δ30-96") or express UL11 from the UL35 locus ("U1"). (D and E) Analysis of recombinant viruses that encode UL11 derivatives with altered acylation signals. These include UL11 alleles that: (D) limit the endogenous acylation of UL11 or (E) contain foreign acylation signals as a consequence of fusing the membrane binding domains of v-Src or Fyn to the N-terminus of UL11. Solid lines denote PFU associated with the cell, whereas dashed lines represent PFU released into the medium. Panels C-E are representative of at least two independent experiments. The limit of detection by plaque assay is 10 PFU/ml.


A
BAC (120)
Δ30-96 (26)
U1 (100)
M15 (41)
C8 (62)
sC6 (53)
f9 (94)
B
Cell
Medium
KOS
BAC
PFU/ml (log10)
C
BAC
Δ30-96
U1
D
U1
M15
C8
Hours Post-Infection
E
U1
sC6
f9
Hours Post-Infection

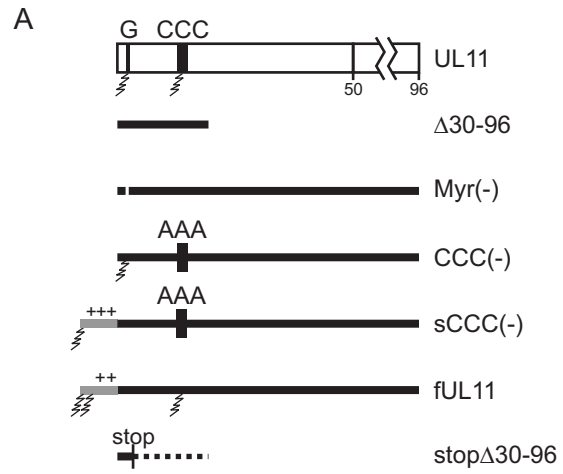
(Figure 4.3C). Interestingly, by 48 hpi, Δ30-96 was only reduced from BAC by about 1-log. Re-introduction of the UL11 ORF in place of the UL35 coding sequence rescued Δ30-96 to near WT levels of growth. The minor growth differences between BAC and U1 in both the growth curve analyses (Figure 4.3C) and plaque sizes (Figure 4.3A) were within the expected range (<2-fold) based on previous studies of a VP26-null virus (92).

UL11 Acylation Mutants

Studies of HIV-1 show that myristylation of the membrane binding domain of Gag is required for virion formation (150). Furthermore, UL11 requires both myristate and palmitate for complete membrane binding, TGN targeting, and DRM localization [Chapter III and (39, 226)]. Given these data, it was predicted that the ability of UL11 to function during virion envelopment would also require acylation for virus growth. As such, only a fully-acylated UL11 was expected to rescue Δ30-96, whereas partially- and non-acylated UL11 derivatives were not expected to rescue Δ30-96. To test this hypothesis, recombinant viruses were constructed to express UL11 acylation mutants form the U_L35 locus; the same methodology used to make recombinant U1. Using Δ30-96 as the parent virus, the UL35 coding sequence was replaced by one of two ORFs that encode UL11 variants with mutated acylation signals. The previously described U_L11 alleles lack either the signal for myristylation [Myr(-)] or palmitylation [CCC(-)] (Figure 4.4A) [Chapter III and (226)], and their insertion into the viral genome created the recombinant viruses M15 and C8, respectively (Table 4.1). Recall that myristylation is a prerequisite for palmitylation. Therefore, even though Myr(-) only removes the myristylated-glycine residue, the protein lacks both acyl modifications; whereas the CCC(-) allele encodes a protein that is myristylated but not palmitylated (Figure 4.4A)

Figure 4.4

UL11 acylation mutants. (A) Schematic of WT UL11 and mutant UL11 alleles that were inserted into the UL35 ORF. Acylation motifs are indicated: G, myristylation; CCC, palmitylation. Sites of alanine substitution are indicated. N-terminal extensions corresponding to the first 10 amino acids of v-Src [sCCC(-)] or Fyn (fUL11) attached to UL11 are indicated by shaded lines. Wavy lines denote fatty-acid modifications and "+" indicates basic residues. "stop" indicates location of 2-nucleotide change that created a stop codon in the remaining UL11 ORF without altering UL12. Dotted line represents the remaining UL11 coding sequence downstream from the introduced stop codon. (B) Alignment of the UL11 and essential UL12 ORFs. The two nucleotide substitution that introduces a stop codon in the UL11 reading frame, but does not interrupt UL12, is indicated.


A
G CCC
UL11
50
96
Δ30-96
Myr(-)
AAA
CCC(-)
AAA
+++
sCCC(-)
++
fUL11
stop
stopΔ30-96
B
UL11
M G L S F S
a t g g g c c t c t c g t T C t c c g
UL12
W A S R S F
stop
Δ30-96
M G L S stop
a t g g g c c t c t c g t A G t c c g
UL12
W A S R S F

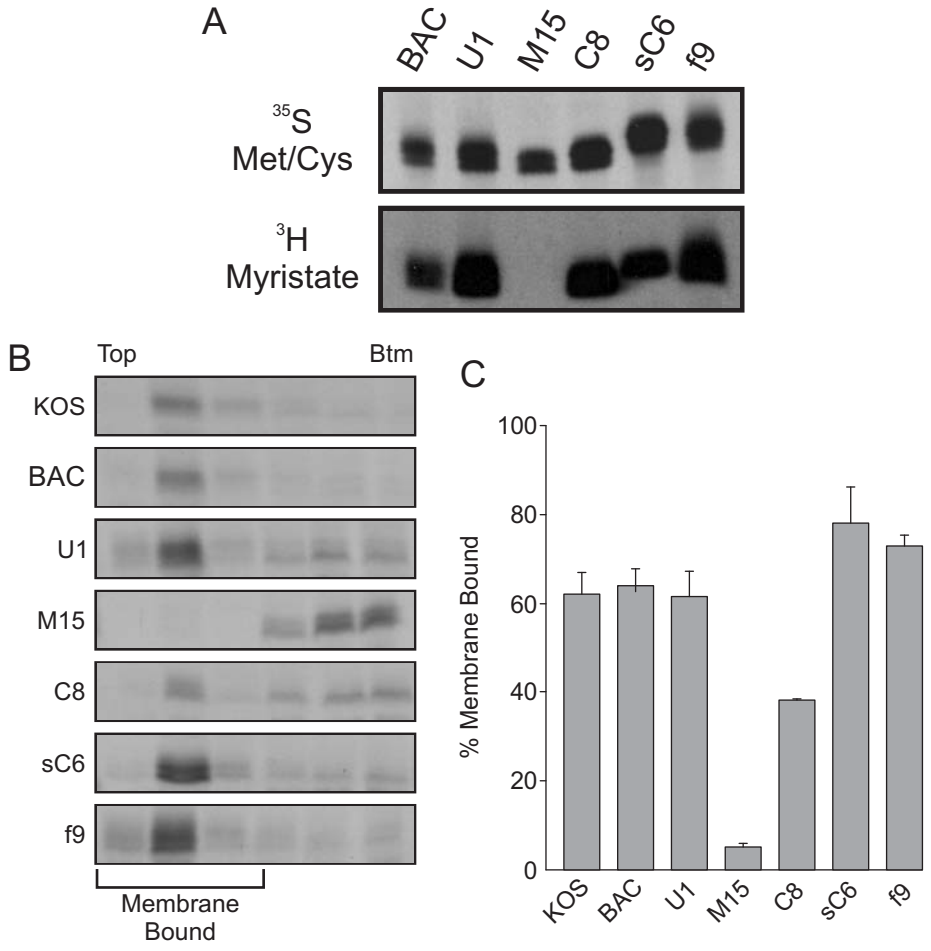
(226).

As expected from previous reports that transfected expression vectors containing the above mutant alleles [Chapter III and (226, 228)], CCC(-) expressed from C8 was myristylated and did have reduced membrane binding compared to WT UL11, whereas the Myr(-) expressed from M15 was not myristylated and did not bind membranes (Figure 4.5). The stability of UL11 in the infected cell appeared to be proportional to its acylation. Despite efficient radiolabeling and immunoprecipitation of both CCC(-) and Myr(-) (Figure 4.5A), the non-acylated Myr(-) was not detectable and the partially-acylated CCC(-) was barely detectable at steady-state levels as measured by immunoblotting (Figure 4.2, Cell). Accordingly, extracellular virus particles had decreased CCC(-) and no Myr(-) proteins when examined by immunoblot. Also, UL16 incorporation was reduced and eliminated with C8 and M15, respectively (Figure 4.2, Medium).

The growth curves and plaque sizes of M15 and C8 show the UL11 derivatives support virus growth proportionally to the amount of UL11 acylation. As such, a growth hierarchy was evident for the recombinants as follows: U1 > C8 > M15 > Δ30-96 (Figures 4.3A, C and D). The moderately enhanced growth of M15 over Δ30-96 was very surprising because Myr(-) is not acylated and had severely reduced protein stability, virion incorporation, and membrane binding. Though the titers of M15 and Δ30-96 in the medium at 48 hpi were nearly equal, M15 did not have the large lag-period of virus production that Δ30-96 did and the plaques produced by M15 were ~50% larger than those of Δ30-96.

Figure 4.5

Membrane binding of UL11 derivatives. (A) Myristylation of UL11 derivatives. Vero cells were infected with indicated virus and radiolabeled with either [^{35}S]methionine/cysteine for 2.5 h or [^{3}H]myristic acid for 30 min as detailed in the text. All labeling concluded at 9 hpi, at which time cell lysates were prepared and UL11 was immunoprecipitated, resolved by SDS-PAGE, and visualized by autoradiography. (B and C) Membrane flotation of UL11 proteins. Vero cells were infected with indicated virus and metabolically labeled for 2.5 h (6.5-9 hpi) with [^{35}S]methionine/cysteine. Immediately after labeling, cells were scraped from the plate and osmotically disrupted as described in the text. The ability of UL11 to float to the upper fractions of sucrose step-gradients during centrifugation was monitored. (B) Representative autoradiographs of proteins separated by SDS-PAGE following immunoprecipitation with UL11-specific antiserum. Tops and bottoms of the gradients are indicated. (C) Phosphorimager analysis was used to quantitate the amount of membrane bound UL11, represented as the percent membrane bound (top three fractions) relative to the total (all fractions). All data is representative of at least two independent experiments.


A
BAC
U1
M15
C8
sC6
f9
35S
Met/Cys
3H
Myristate
B
Top
Btm
KOS
BAC
U1
M15
C8
sC6
f9
Membrane
Bound
C
% Membrane Bound
100
80
60
40
20
0
KOS
BAC
U1
M15
C8
sC6
f9

Why Did a Non-myristylated UL11 Enhance Growth of Δ30-96?

It was very surprising that Myr(-) retained any function and at least two possibilities existed for the UL11-dependent growth properties of M15: 1) the 29-amino acid N-terminal peptide was produced from the U_L11 locus and interacted with the full length Myr(-) expressed from the U_L35 locus to enable the non-myristylated protein to bind membranes, and 2) M15 had greater specific infectivity than Δ30-96.

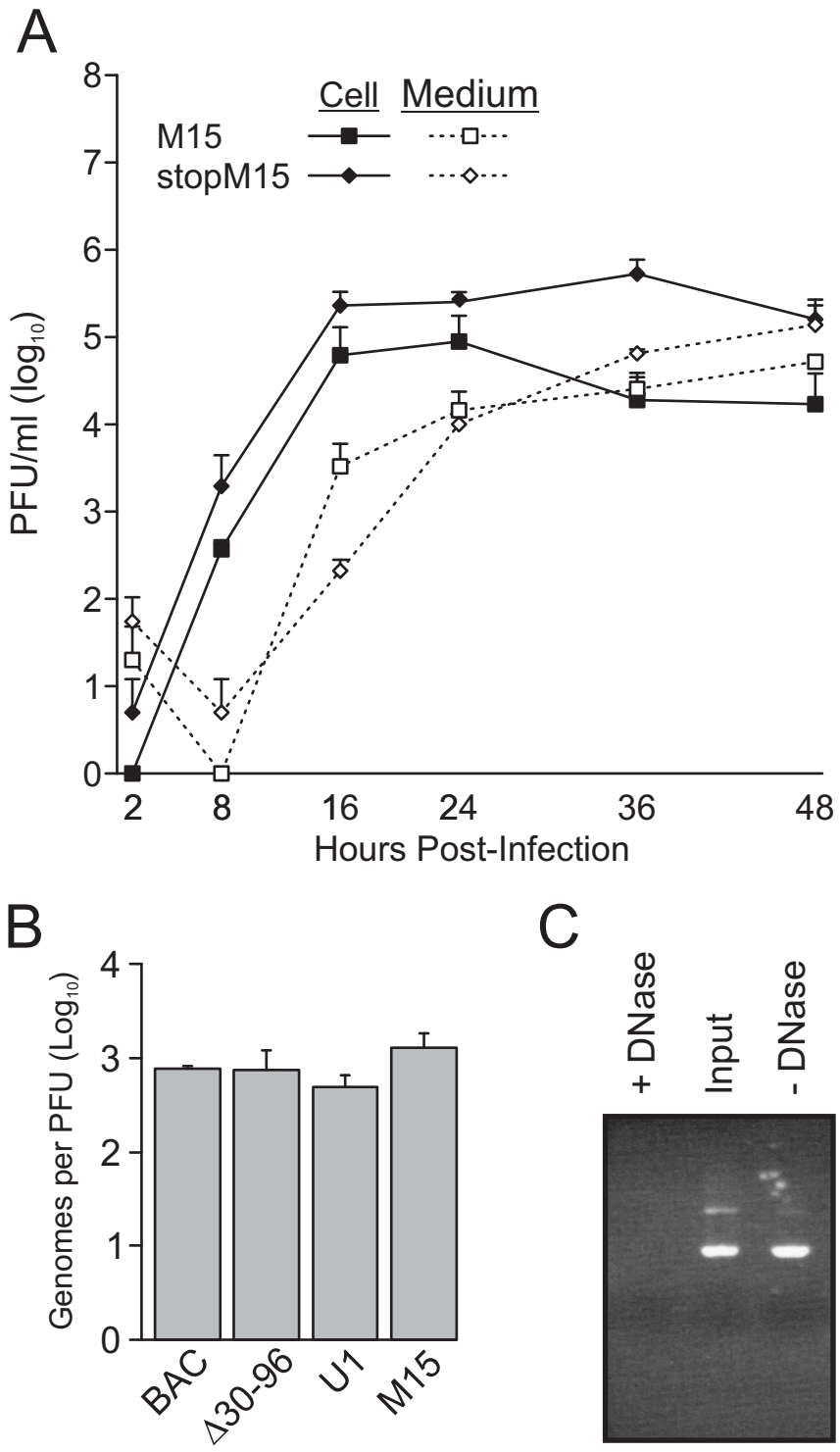
To test the trans-complementation hypothesis, a stop codon was introduced into the original UL11 reading frame of M15, creating the virus stopM15 (Figure 4.4A and Table 3.1). Importantly, this change was silent in the essential UL12 ORF (Figure 4.4B). M15 and stopM15 grew with similar properties which implied an acylated-UL11 peptide was not acting *in trans* to complement Myr(-) expressed from the UL35 locus (Figure 4.6A). To test the specific infectivity hypothesis, quantitative PCR (qPCR) was used and it was determined that the ratio of genome-containing particles to PFU for M15 was not significantly different than Δ30-96 (Figure 4.6B).

Fusion of Foreign Acylation Signals to UL11

Unexpectedly, the ability of UL11 to rescue growth of Δ30-96 correlated with the extent of UL11 acylation as described above. However, the number of acylations on UL11 also correlates with membrane binding (Figure 4.5)(Chapter III). Therefore, it is possible that UL11 simply requires membrane binding and not necessarily myristylation and palmitylation to function. To test this hypothesis, a previously described chimera of UL11 that efficiently binds membranes independently of palmitylation, sCCC(-) (Chapter III), was inserted into the U_L35 locus to create virus sC6 (Table 4.1). sCCC(-) has the 10 amino acid membrane binding domain of the Rous sarcoma virus oncoprotein v-Src fused

Figure 4.6

Further characterization of M15. (A) An acylated UL11-peptide does not trans-compliment Myr(-). The recombinant virus "stopM15" has a 2 nucleotide substitution in the original UL11 ORF that creates a stop codon at position 5 of UL11 but does not alter the essential UL12 ORF (see Figure 4.4 B). Growth properties were then examined as detailed in the text and Figures 4.4 B-E. (B and C) Specific infectivity of virus stocks. (B) 1 X 10^7 PFU were DNase treated and purified from virus stock preparations as detailed in the text. DNA was purified and HSV-1 genomes were detected by quantitative PCR (qPCR). The limit of detection for HSV genomes was 100 copies per PCR reaction. (C) Control of DNase enzymatic activity. In reactions parallel to the virions of (B), a random PCR product was incubated with ("+") or without ("-") DNase enzyme. "Input" is the initial amount of PCR product in each reaction. Data in panels (A) and (B) is representative of at least two independent experiments. Panel (C) shows a representative agarose gel


A
Cell
Medium
M15
stopM15
PFU/ml (log10)
Hours Post-Infection
B
Genomes per PFU (Log10)
BAC
Δ30-96
U1
M15
C
+ DNase
Input
- DNase

to the N-terminus of CCC(-) (Figure 4.4). Use of CCC(-) limits the membrane binding potential to just the myristate and basic residues of the v-Src peptide. To restore the membrane binding mechanism to myristate and palmitate but retain a chimeric protein, the previously described fUL11 was used [Chapter III and (226)]. fUL11 fuses the 10 amino acid membrane binding domain of the cellular protein Fyn to the N-terminus of WT UL11 and replacement of the UL35 ORF with fUL11 created virus f9 (Table 4.1). fUL11 binds to membranes via both myristate and basic residues but is also palmitylated on cysteines in both the Fyn and UL11 sequences (Figure 4.4).

Following infection of cells with either sC6 or f9, cell lysates contained chimeric-UL11 proteins that were detectable at steady state levels (Figure 4.2) and myristylated (Figure 4.5A). Both sCCC(-) and fUL11 were ~20% more membrane bound than WT UL11 (Figures 4.5B and C), but only the myristylated and palmitylated fUL11 was incorporated into virions as well as U1 (Figure 4.2), suggesting the dual acylation of UL11 is a "signal" (possibly for DRM trafficking, Chapter III) and is not just a means membrane binding. Like C8 and M15, the amount of UL16 incorporated into sC6 and f9 virions was proportional to UL11 (Figure 4.3).

To determine the ability of the chimeric proteins to rescue Δ30-96, the plaque sizes and growth curves of sC6 and f9 were examined. As seen in Figure 4.4A, expression of either sCCC(-) or fUL11 increased the plaque size of Δ30-96, but only fUL11 restored the plaques to the size of U1, again implying dual acylation of UL11 is necessary for function of the protein. However, examination of the sC6 and f9 growth curve data revealed that the dually-acylated fUL11 was not functionally equivalent to WT UL11. f9 did not produce cell-associated virus nor release virus into the medium as

efficiently as U1 (Figure 4.3E); but, fUL11 did rescue growth of Δ30-96 better than (16-24 hpi) or equal to (48 hpi) sCCC(-), implying the dual acylation is better than mono-acylation.

The most distinct and unexpected result obtained from the chimeras was the accelerated release of sC6 into the medium. The lag-time between virus production in the cell and release of virus into the medium of sC6 was reduced to less than 8 h, whereas the lag-times of all other recombinants were greater than 8 h. That is, at 8 hpi no sC6 virus was detected from either the cell or medium sample, but at 16 hpi both samples had infectious virus (limit of detection was 10 PFU/ml).

Chapter V

Discussion

UL11 has been implicated in several functions during the HSV-1 life cycle. These include secondary envelopment, virus-containing vesicle transport, and cell-to-cell spread (20, 136). However, no mechanism has ever been defined for any of these functions. The studies within this dissertation examined the relationship between UL11 acylation and function during virus envelopment. Furthermore, this dissertation describes a newly discovered aspect of UL11 - the ability to traffic through DRMs. While the reason for this latter property is unknown, it may be required for any of the potential UL11 functions listed above.

UL11 Acylation & DRM Trafficking

The experiments described in Chapter III demonstrate the association of UL11 with DRMs requires the addition of both myristate and palmitate. This is consistent with HSV-2 UL11 (206); however, these studies also show that the LI and AC motifs further mediate the DRM association of HSV-1 UL11. The increased DRM accumulation of UL11 when the LI and AC motifs were deleted could be due to either enhanced entry or inhibited exit from DRMs; however, the latter seems more reasonable for two reasons. First, the combination of myristate and palmitate often function as "entry" signals into DRMs (303, 310, 359). Second, acidic clusters and di-leucine motifs are well established signals involved in exit and recovery of various proteins off the plasma membrane (38, 165). In the case of UL11, removal of the AC in the context of a CD4-UL11 chimera results in accumulation of the mutant on the cell surface (226). And, though the LI in UL11 does not follow the consensus [D/E]XXXL[L/I], the data of Chapter III suggest this motif is also a trafficking signal that mediates DRM localization of UL11.

Interestingly, the LI and AC appear to have redundant functions since removal of either motif alone did not change the DRM localization, but removal of both sequences increased the DRM localization over WT UL11.

Loomis et al. (2006) suggest the LI and AC are not redundant and that each motif internalizes UL11 through different pathways. Their data show GFP-tagged variants of UL11 that lack either the LI or the AC compete differently with WT UL11 for incorporation into virions: the AC(-) variant is packaged at reduced levels whereas the LI(-) mutant is incorporated at levels greater than WT UL11. Given the two sets of data, a model can be drawn that both motifs are capable of trafficking UL11 out of DRMs, but through different routes (similar to furin) and results in the differential packaging of the mutants.

To accurately determine whether the LI and AC are both trafficking signals and to understand their role in UL11 packaging, the BAC recombineering methodology described in Chapter IV should be used to express the mutants from the virus and in the absence of WT UL11. It is possible that similar trends of UL11 incorporation would occur [less AC(-) and more LI(-) packaged] even when the mutants are not transiently expressed from plasmids and do not have to compete with WT UL11. If the mutants are packaged as predicted by the experiments of Loomis et al. (2006), then no growth defects are expected by removing the LI motif. Conversely, the AC(-) expressing virus should be decreased for virus production since UL11 is required for efficient envelopment as shown in Chapter IV. Conversely, if the recombinant viruses do not recapitulate the packaging data and the AC(-) is packaged to WT levels, it is expected that UL16 would *not* be incorporated. This model is based on two observations. One, the AC motif of UL11 is

required for the interaction with UL16 (227, 434) and two, UL11 is required for UL16 packaging (Chapter IV).

Regardless of whether the LI and the AC are redundant trafficking motifs, it is clear that some population of UL11 traffics to DRMs and the PM. At least three reasons can be imagined that involve PM and/or DRM localization and trafficking of UL11. The first is that UL11 has no function at the PM and is only there as a result of some protein "escaping" the TGN. DRM localization may therefore only be a means to concentrate UL11 prior to its targeting back to the TGN for virus envelopment. The second is that UL11 travels to the PM and DRMs to recruit another protein back to the site of envelopment required for the envelopment process. The recruited protein could be either another tegument protein that is required to bridge capsids and TGN-membranes or could be a cellular factor that HSV "hi-jacks" for use as envelopment machinery, similar to the way HIV-1 recruits the ESCRT-1 complex (306). Such binding partners could be isolated using a step-wise procedure of DRM flotation to purify UL11, then co-immunoprecipitations or other pull-down methods to isolate proteins bound to UL11. A third possibility is that UL11 travels to the plasma membrane to promote cell-to-cell spread of infection; either by enabling the egress of virion-containing vesicles to the cell surface or by promoting a direct interaction of a capsid with the plasma membrane (possibly via UL16, gD, and/or gE).

Three separate models have been presented, but each is not mutually exclusive. Separate populations of UL11 may function during various steps of virion biogenesis at different locations throughout the infected cell, as shown in Figure 5.1.

Figure 5.1

Model of proposed UL11 functions. UL11 has been suggested to function during (A) virion envelopment, (B) transport of virus-containing vesicles, and (C) cell-to-cell spread. UL11 may also function to (D) aid vesicle fusion at the plasma membrane through protein/protein interactions and/or (E) retrieve a protein from the plasma membrane to the TGN that is necessary for the envelopment process. DRMs (orange) are depicted.

UL11
Glycoprotein
UL16
HSV receptor
Uninfected Cell
DRM
A
B
C
D
D
E
TGN
Golgi
ER

UL11 Acylation & Virus Growth

The experiments of Chapter IV were designed to test the hypothesis that dual acylation of UL11 is required for function of the protein during virus envelopment and release. The data suggest this hypothesis is both correct and incorrect because expression of the dually acylated fUL11 did not fully rescue the growth of Δ30-96, but the non-acylated Myr(-) did rescue some growth properties of Δ30-96.

Most interesting is why did the non-myristylated UL11 enhance growth of Δ30-96 even though the protein did not bind membranes, did not accumulate at the TGN or in DRMs, and was highly unstable? One possible explanation is that UL11 interacts with another protein, either cellular or viral, to promote virus production and Myr(-) retains some of this interaction potential even though it is not membrane bound. Support for this model comes from studies that show bacterially-produced UL11, which is not acylated, can interact with UL16 (227, 434), a tegument protein that binds to cytoplasmic capsids (253). UL11 has also been reported to interact with the tails of gD and gE (117), which localize to the TGN during an infection. Therefore, Myr(-) may interact with UL16 (which then interacts with capsids) and/or the glycoprotein tails at the TGN to act as a "bridge" during final envelopment. Two obvious arguments against this model are that no UL11 is packaged into M15 virions (nor is UL16) and that the rescue of Δ30-96 is minimal. But, both of these situations can be attributed to the instability of Myr(-). To test this model, stabilization of Myr(-) [possibly with a GFP-tag] is expected to enhance the rescue of Δ30-96 compared to untagged Myr(-). If the "stability model" is correct and a stable Myr(-) rescues Δ30-96, one could argue that membrane binding of UL11 is a means of protecting the protein from rapid degradation. Additionally, the growth

properties of sC6 and f9 suggest the mechanism of membrane binding is also important for UL11 function.

Both sCCC(-) and fUL11 are membrane bound, DRM associated, and in virions, but neither protein fully rescued Δ30-96. The most obvious explanation of why sC6 and f9 did not grow like U1 is that addition of the peptides altered the structure of UL11 and inhibited necessary protein-protein interactions. Another possibility is that the basic residues in the membrane binding domains of each foreign peptide altered the ability of the protein to traffic properly. WT UL11 relies on myristate and palmitate for membrane binding and the latter modification is reversible and may be transient. Depalmitylation of WT UL11 would leave only a myristyl group, which has low membrane binding affinity, and would allow the non-palmitylated protein to "slip" on and off membranes easily. As such, a function of UL11 may actually require release of the protein from membranes, but fusion of the peptides bound the chimeras to membranes irreversibly. The ability of UL11 to slip off membranes may be required during virus entry to release the nucleocapsid into the cytoplasm of the new cell.

More interesting than the inability of the chimeras to rescue Δ30-96 is that sCCC(-) reduced the lag-time of virus release into the medium to less than eight hours, and possibly eliminated the lag. Since UL11 is suggested to be involved with transport of TGN vesicles (20), it is possible that sCCC(-) accelerated the rate of secretion of virus-containing vesicles towards the PM. Alternatively, it is known that fusion of the v-Src to WT UL11 causes a population of the protein to strongly localize to the PM (226). Therefore, it is possible that expression of sCCC(-) caused a small amount of virus envelopment to occur at the PM, thus eliminating any lag-time between envelopment and

release into the medium. Electron microscopy studies should be used to examine sC6 infected cells and look for virion formation at the PM.

What the function(s) of UL11 are during virus assembly, and where such function(s) occur, remain elusive. Regardless, Chapter IV demonstrates that 1) deletion of UL11 causes defects in virus production, 2) a non-acylated UL11 retains some function(s), and 3) the context of the UL11-acylations is important for protein function.

Chapter VI

Materials & Methods

Cells & Infections

Vero and A7 melanoma cells were grown in Dulbecco's Modified Eagles Medium (DMEM, Invitrogen) supplemented with 5% fetal bovine serum (FBS), penicillin, and streptomycin (Gibco, 15140-148).

All infections were preformed in DMEM supplemented with 2% FBS, 25mM HEPES buffer, glutamine (0.3μg/ml), penicillin, and streptomycin. All viruses contained the KOS genome of HSV-1 (144, 365).

Virus stocks were titered on Vero cells in 24-well plates (1.5 x 10^5/well) and infected in duplicate wells with 10-fold serial dilutions for 1 hour at 37°C. After incubation, cells were washed with either 1% FBS in PBS (all titers in Chapter III; "PBS titer" in Chapter IV) or an acid wash (135 mM NaCl, 10 mM KCl, 40 mM citric acid, pH 3.0) followed by 1%FBS in PBS (not used in Chapter III; "acid titer" in Chapter IV). Cells were overlayed with 0.5% (w/v) methylcellulose for 4 days, stained with crystal violet, and plaques counted.

Recombinant Viruses

A recombinant virus expressing UL11-GFP was created by homologous recombination. Sequences from upstream and downstream of U_L11 (350 bp each) were PCR amplified from the viral genome and ligated to the 5' and 3' termini of U_L11-*gfp*, respectively. The composite DNA fragment was inserted into the pSP72 vector (Promega) and then transfected into A7 cells, which were infected 16 h later with the KOS strain of HSV-1. Recombinant virus was selected by five rounds of plaque purification. The resulting virus was confirmed by a combination of PCR analyses using primers that flank the U_L11-*gfp* coding sequence (yielding a larger product than untagged

U_L11) and the failure to express the wild-type, untagged UL11, as determined by Western blotting and radiolabeling/immunoprecipitating for UL11. The recombinant was analyzed for specific infectivity and plaque size, as well as localization and kinetics of UL11-GFP expression. All characteristics examined were undiminished compared the parental virus (data not shown; work performed by P.C. Yeh).

To construct recombinant viruses that express acylation-defective forms of UL11, a bacterial artificial chromosome (bac) containing the HSV-1 KOS strain genome ("BAC") was used [KOS-37, kindly provided by David Leib (144)]. Recombinant HSV-1 clones were made using "BAC recombineering," a *galK*-based positive/negative selection system, as specifically outlined in Warming et al., 2005. In creating the UL11 deletion virus ("Δ30-96"), the first 29 amino acids of UL11 were left intact due to the overlap with the essential UL12 gene. Δ30-96 did not express any detectible peptide as determined by Western blot or radiolabeling and immunoprecipitation assays (data not shown). Using Δ30-96 as the parental strain, a second recombinant virus was created by inserting the full-length UL11 ORF in place of the UL35 ORF ("U1"), which is dispensable for cell culture systems (92). Similarly, a collection of UL11 mutants that differ in their N-terminal acylation signals were inserted into the U_L35 gene. All mutant UL11 coding sequences were PCR amplified from plasmids that have been described previously (Chapter III) (226, 226, 227, 227, 228). The recombinant virus "stopM15" was derived from the virus "M15" as follows. A two nucleotide change was made at codon 5 of the "UL11Δ30-96" gene, introducing a stop codon into the remaining UL11 coding sequence at the native UL11 locus. This created a stop codon within the UL11 reading frame but was a silent change within the essential UL12 reading frame.

All viral clones were analyzed for DNA integrity. First, PCR was used to amplify the locus of interest, as well as flanking sequences from both upstream and downstream. The resulting product was purified and sequenced. Second, to monitor that gross deletions of the genome had not occurred, total DNA was isolated for each virus, digested with *Hind*III, and separated on a 0.8% SeaKem agarose gel.

High quality BAC DNA was obtained using the Qiagen Large Construct kit (cat. no. 12462). Purified DNA was resuspended in 100 µl TE overnight at room temperature. The next day, Vero cells (35-mm dish, ~50% confluent) were transfected with 90 µl of the prepared DNA using Lipofectamine 2000 (Invitrogen, cat. no. 11668-019). 5 days post-transfection, cells were scraped, freeze/thawed 3 times, and cell debris cleared by centrifugation. New Vero cells were infected with the cleared lysate to produce a viral stock. Virus stock preparations were titered on Vero cells as described above.

In all cases, the BAC sequence was left intact within the genome of the recombinant viruses, as no defects were seen in any assay when comparing the WT viruses, KOS and BAC.

Antibodies

UL11-specific antibodies were developed in rabbits and have been described previously (227). GFP-specific antibodies, produced by Cocalico Biologicals, Inc., were obtained from rabbits injected with purified His_6-GFP. The specificity of this antibody was tested in both immunoblot and immunoprecipitation assays. Transferrin receptor antibodies were purchased from BD Pharmingen (555534). Antibodies against VP5 and VP26 were kindly provided by R.J. Courtney.

Membrane Flotation

As performed in Chapter III: DRMs were isolated by using a slight modification of an established protocol (372). Briefly, the calcium phosphate method was used to transfect human melanoma (A7) cells with a plasmid containing U_L11-*gfp*, derived using the KOS strain of HSV-1 (226). After 16-18 h, the cells were radiolabeled with an L-[^{35}S]methionine-cysteine mix (75 μCi/ml, >1,000 Ci/mmol) for 2.5 h, scraped off the plates, and washed in cold PBS. After pelleting, the cells were resuspended and swollen in hypotonic lysis buffer (10 mM Tris-HCl, pH 7.4, 0.2 mM $MgCl_2$) on ice for 30 min. They were lysed at 4°C by 35 strokes with a dounce homogenizer and then centrifuged at low speed to remove unbroken cells and nuclei. Post-nuclear supernatants were split into two equal aliquots, one untreated (for total membranes) and the other adjusted to a concentration of 0.5% TX-100 (for DRMs). In some experiments, samples were adjusted to 0.5% SDS to disrupt all membranes (negative control), and incubated on ice for 30 min. Samples were mixed with 65% (w/w) sucrose (58% final, 2.0 ml total), placed in the bottom of a Beckman SW55 Ti tube, and sequentially overlaid with 2.0 ml of 45% and 1.0 ml of 2.5% sucrose. Sucrose solutions were made in NTE buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA). The samples were centrifuged for 18 h at 200,000 x g and 4°C in a Beckman ultracentrifuge, and six equal-volume fractions were collected from the top. UL11-GFP was immunoprecipitated using rabbit anti-UL11 antibodies (227), separated by SDS-PAGE, and quantitated by phosphorimager analysis.

As performed in Chapter IV: Membranes were isolated using a flotation protocol described previously (372). Briefly, Vero cells (1.2 X 10^7) were infected at an MOI of 10 ("PBS" titer). At 5.5 hpi, cells were starved in DMEM lacking methionine and cysteine for 30 min. Post-starvation, cells were radiolabeled with an L-[^{35}S] methionine-cysteine

mix (300uCi/plate, >1000 Ci/mmol) for 2.5 h, scraped off the plates, and washed in cold NTE. After pelleting, cells were resuspended and swollen in hypotonic lysis buffer (10 mM Tris-HCl, pH 7.4, 0.2 mM $MgCl_2$) on ice for 30 min, then lysed at 4°C by 30 strokes with a dounce homogenizer and then centrifuged at low speed to remove unbroken cells and nuclei. Post-nuclear supernatants were mixed with sucrose, layered in gradients, centrifuged, and analyzed as above.

Transferrin Receptor & Methyl-β-Cyclodextrin Treatment

To examine membrane localization of the endogenous transferrin receptor (TfR), confluent monolayers of A7 cells were radiolabeled with an L-[^{35}S]methionine-cysteine mix (300 μCi/ml, >1,000 Ci/mmol) for 3 h. Following labeling, cells were lysed, TX-100 treated, and floated as before. TfR was immunoprecipitated from fractions using anti-TfR antibodies, separated by SDS-PAGE, and quantitated by phosphorimager analysis. Methyl-β-cyclodextrin (10 mM; Sigma, C4555) was added to A7 cells expressing either wild-type or mutant UL11-GFP during the final hour of a 2.5 h label (75 μCi/ml, >1,000 Ci/mmol). DRM localized UL11 was then immunoprecipitated and examined as before.

Growth Curves

A 6-well tissue culture plate of Vero cells (5 X 10^5/well) was infected with specified virus at a multiplicity of infection (MOI) of 1 (based on "acid titer") at 37°C. After 1h of adsorption, free virus was aspirated and cells were washed sequentially with an acid wash and 1%FBS in PBS, then overlayed with 1ml DMEM containing 2% FBS. At indicated times post infection, medium and cells were harvested from a single well as follows. Medium was cleared of cells, and frozen. Cells were scraped into PBS, washed

1X with PBS, and freeze/thawed 3 times to release virus. Each sample ("Medium" and "Cell") was titered on Vero cells.

Incorporation Assay

To compare the ability of WT and recombinant viruses to incorporate tegument proteins into virus particles, a published protocol was used, slightly modified as described below (228). Confluent monolayers of Vero cells were infected at an MOI of 1 ("PBS" titer) and 24 hpi, media was collected and cleared of cellular debris. Virions were pelleted from the supernatant by centrifugation at through a 30% (w/v) sucrose cushion (1 ml) at 4°C using a Beckman SW41 Ti at 83,500 xg for 1 h. To monitor expression of viral proteins, infected-cell lysates were scraped into PBS, pelleted, and resuspended in sample buffer containing 2-mercaptoethanol. To shear the genomic DNA, samples were sonicated using a cup attachment, at maximum power, for 3 min. Cell lysates and collected virions were separated by SDS-PAGE and analyzed by Western blot using specified antibodies. The major capsid protein, VP5, was monitored to control for equal sample loading. To achieve equal VP5 levels in the "Medium" samples, additional Vero cells were infected, and the medium collected, as compared to WT BAC (6×10^6 cells). For mutants Δ30-96, M15, and sC6, a 4-fold increase of cells were infected (2.4×10^7), whereas all other recombinants only required a two-fold increase (1.2×10^7). It is of importance to note, however, that equal numbers of infected cells were loaded for the "Cell" sample of all viruses.

Myristylation of UL11

To monitor the state of myristylated UL11 and mutants, infected cells were labeled with [^{3}H]-myristic acid. Vero cells were seeded into parallel plates: a 100-mm (3 x 10^{6}) and a 35-mm (6 X 10^{5}). The next day, the cells were infected with designated virus at an MOI of 10 ("PBS" titer). At 6 hpi, the 35-mm dishes were starved for 30 min, then radiolabeled with an L-[^{35}S] methionine-cysteine mix (300 uCi/ml, >1000 Ci/mmol) for 2.5 h (3 h total, 6 – 9 hpi). Concurrent with the final 0.5 h of the [^{35}S] label (8.5 – 9 hpi), the 100-mm dishes were radiolabeled with [^{3}H] myristic acid (300 uCi/ml, 30 Ci/mmol) for 30 min in serum-free DMEM. After labeling, cells were mixed with lysis buffer and the UL11 proteins were immunoprecipitated with polyclonal anti-UL11 antiserum. Immunoprecipitated proteins were separated by 15% SDS-PAGE. Gels were treated with EN^{3}HANCE (PerkinElmer) as per manufacturer's instructions prior to drying and exposure to autoradiography film.

Quantitative PCR Analysis

Using prepared virus stocks, DNA was extracted from 1 X 10^{7} PFU ("PBS" titer) as follows. Virus was mixed with PBS to a final volume of 500µl, layered onto a 100µl sucrose cushion (30%, W/V), and centrifuged at 4°C in a Beckman TLA100.3 at 83,500 xg for 1 h. Viral pellets were resuspended in water and treated with DNase I (0.1U/µl; New England Biolabs, M0303) for 30 min at 37°C. The enzyme was inactivated by incubation at 75°C for 10 min. Treated samples were pelleted as above but without a sucrose cushion. Next, virions were resuspended in PBS and DNA was isolated using the Qiagen DNeasy Blood and Tissue Kit (Cat no. 69504).

All DNA concentrations were determined by UV spectrophotometry. Quantification of viral DNA copy number was made using an Opticon 2 real-time PCR machine (BioRad). Amplification was performed in 20 μl reaction volumes with iQ SYBR Green Supermix (BioRad, 170-8880). HSV-1 specific primers (forward, 5'-CACAGGCGGGACACCAGC; reverse, 5'-CCTCCGCAATCCCAAGATTC) were used to amplify a 97 bp fragment from the U_L13 gene. The HSV-1 copy number was determined against a standard curve constructed by serial dilution of DNA isolated from the WT virus, BAC. Limit of detection for viral DNA was 100 copies per PCR reaction.

For qPCR studies, experimental design, sample preparation, and data analysis were performed by N.L. Baird. qPCR reactions were setup and run by D.J. Hughes.

Appendix A

Permission for use of Figure 2.6

E-mail correspondence dated December 12, 2008:

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Appendix B

Table of All Recombinant BAC Viruses Created

Common Name of Virus Used	Parent Virus	Open Reading Frames (UL11/UL35 loci)	Bacterial Stock (Clone Number)	Virus Stock (Clone Number)
BAC	N/A	UL11/UL35	N/A	N/A
Δ30-96 + GalK	BAC	Δ30-96+GalK/UL35	1	none
Δ30-96	Δ30-96+GalK, Clone 1	Δ30-96/UL35	2,3,5,8	2*,5
Δ30-96/ΔUL35 + GalK	Δ30-96, Clone 2	Δ30-69/GalK	B,C,D	none
U1	Δ30-69/ΔUL35+GalK, Clone B	Δ30-69/UL11	1	1
UG4	Δ30-69/ΔUL35+GalK, Clone B	Δ30-69/UL11GFP	4	4
C8	Δ30-69/ΔUL35+GalK, Clone B	Δ30-69/CCC(-)	8,11	8
CG6	Δ30-69/ΔUL35+GalK, Clone B	Δ30-69/CCC(-)GFP	6	6
M15	Δ30-69/ΔUL35+GalK, Clone B	Δ30-69/Myr(-)	15,21	15
stopM15	M15	stopΔ30-69/Myr(-)	2	2
sC6	Δ30-69/ΔUL35+GalK, Clone B	Δ30-69/SrcCCC(-)	6	6
sCG15	Δ30-69/ΔUL35+GalK, Clone B	Δ30-69/SrcCCC(-)GFP	15	none
f9	Δ30-69/ΔUL35+GalK, Clone B	Δ30-69/FynUL11	9**,10	9
fG13	Δ30-69/ΔUL35+GalK, Clone B	Δ30-69/FynUL11GFP	13	none

* Clone 2 of "Δ30–96" was used for experiments in Chapter IV.
** "f9" has a point change 38nt before the ATG of FynUL11 within the UL35 locus; mutation is not in any reading frame.

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Education

The Pennsylvania State University College of Medicine 2002-2009
Hershey, Pennsylvania
Ph.D., Microbiology and Immunology

Colorado State University 1999-2002
Ft. Collins, Colorado
B.S., Microbiology

Selected Honors and Awards

Academic Achievement Award	Colorado State University, 2002
Golden Key National Honor Society	Colorado State University, 2002
Phi Beta Kappa Honor Society	Colorado State University, 2002
University Honors, Participant and Scholar	Colorado State University, 1999-2002
Beckman Scholars Award	Colorado State University, 2001-2002
Eagle Scout	Boy Scouts of America, 1998

Research Experience

The Pennsylvania State University 2002-2009
Doctoral Dissertation: *Acyl Determinants of the Herpes Simplex Virus Type 1 UL11 Tegument Protein Required for Membrane Trafficking and Virion Envelopment*

Colorado State University 1999-2002
Undergraduate Thesis: *Investigation of Signal Transduction Pathways that Regulate Reactivation of Herpes Simplex Virus Type 1*

Publications

1. **Baird, N. L.,** D. J. Hughes, and J. W. Wills. Unexpected Growth Properties of a Recombinant Herpes Simplex Virus that Expresses a Non-myristylated UL11 Tegument Protein. In Preparation.
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