HOST AND VIRAL FACTORS INVOLVED IN REGULATION OF NUCLEAR EGRESS OF HERPES SIMPLEX VIRUS

A Dissertation
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by
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The nascent nucleocapsids of Herpes Simplex Virus (HSV) egress from the infected cell nuclei by budding at the inner nuclear membrane (INM), and become enveloped primary virions in the perinuclear space. The envelope then fuses with the outer nuclear membrane (ONM) and the capsids are released into the cytosol, where they acquire tegument proteins and the final envelope at the trans-Golgi network and are delivered to the extracellular space via secretory vesicles. This complicated process involves interactions between both viral and host factors.

The products of viral UL31 and UL34 genes form a complex on the INM that is essential to initiate the primary envelopment reaction. The proper positioning of this complex requires a kinase encoded by the viral US3 gene. In the absence of pUS3 kinase activity, pUL31/pUL34 complexes aggregate on the nuclear rim and perinuclear virions accumulate within invaginations of the INM. I discovered that the nuclear lamina was dramatically perforated near those pUL31/pUL34 aggregations, and identified lamin A/C as a putative substrate of US3 kinase \textit{in vitro}. The kinase activity was found to be sufficient to induce partial dissolution of lamin A/C from permeabilized cell nuclei. Two-dimensional electrophoretic analyses confirmed that lamin A/C was phosphorylated in HSV-infected cells, and the optimal phosphorylation required US3 kinase activity. These data suggest that US3 kinase activity regulates HSV-1 capsid nuclear egress at least in part by phosphorylation of lamin A/C.
Lamins A/C and B1 were shown to interact with pUL34. To determine the roles of these interactions on viral infectivity and pUL34 targeting, the localization of pUL34 was examined in lamin knockout mouse embryonic fibroblasts (MEFs) in the presence or absence of pUS3 kinase activity. It was determined that both lamin proteins directly or indirectly modified pUL34 distribution but neither was required for its INM targeting during viral infection. The elimination of lamin B1 made cells less permissive for viral replication, whereas lamin A/C was dispensable for viral infection. Furthermore, in cells infected by the US3 defective virus, the lack of lamin A/C precluded accumulation of perinuclear virions and partially restored replication of this virus. These observations reveal different roles of specific lamins in HSV infection, suggesting that lamin A/C normally impedes viral nuclear egress and that US3 kinase helps alleviate this impediment, whereas lamin B1 is necessary for efficient viral replication, probably through its effects on many cellular signaling pathways.

pUL31, the binding partner of pUL34, is also a substrate of pUS3. The N-terminus of pUL31 was found to be critical for the protein’s normal function and contains multiple phosphorylation sites for US3 kinase. The phosphorylation was not essential for productive infection, but was necessary for optimal viral growth kinetics. Phosphorylation-deficient pUL31 caused pUL31/pUL34 complex aggregation as well as perinuclear virion accumulation, similar to the phenotype caused by abolishing US3 kinase activity. Mimicking phosphorylation of pUL31 by replacement of phosphorylated residues with glutamic acid largely restored the smooth distribution of pUL34/pUL31, and precluded the perinuclear virion herniations, regardless of US3 kinase activity. However, the pseudo-phosphorylated protein hindered the primary envelopment of capsids. These results indicate that pUL31 phosphorylation is a dynamic event, which mediates pUS3 regulatory functions in both primary envelopment of nucleocapsids and subsequent de-envelopment of perinuclear virions.
BIOGRAPHICAL SKETCH

Fan Mou was born and raised in Enshi, a beautiful town surrounded by mountains in central China. At age of 16, she left home and went to Wuhan, where she spent four years of undergraduate life at Huazhong Agricultural University. In May 2003, she graduated with a Bachelor of Science degree in Animal Science. Later this year, she was awarded a fellowship to attend Cornell University to pursue a doctorate degree in the field of Comparative Biomedical Sciences. After one year rotation, she joined the lab of Dr. Joel Baines in 2004 and began the thesis research on Herpes Simplex Virus. The degree work was completed by January of 2009.
This work is dedicated to my dear mom and dad
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CHAPTER I

INTRODUCTION

Herpes simplex viruses are members of the Herpesviridae family. Members of this family are enveloped DNA viruses that produce life-long infections (168). Based upon details of tissue tropism, pathogenicity, and behavior under conditions of culture in the laboratory, the herpesviruses have been classified into three groups (171, 172): the alpha-herpesviruses which are neurotropic, have a rapid replication cycle and usually a broad host and cell range, and the beta- and gamma-herpesviruses which differ in genome size and structure. Both beta- and gamma-herpesviruses replicate more slowly and in a much more restricted range of cells of glandular and/or lymphatic origin. The human herpesviruses include herpes simplex virus type 1 and type 2 (HSV-1 and HSV-2, respectively), varicella-zoster virus (VZV), human cytomegalovirus (HCMV) and Epstein-Barr virus (EBV) (168).

Herpes simplex viruses belong to alpha-herpesviruses (168). The infection by HSV-1 and -2 initiates at oral and genital mucosa respectively (226, 227). Viruses enter sensory nerve termini and travel through neuronal axons in a retrograde manner to the dorsal root and trigeminal ganglia, where latent infection is established (8, 9, 42, 65). During latency, viruses maintain their quiescent genomes in the neuronal nucleus (41, 122, 167). However, lytic infection can be reactivated by a variety of stimuli including mental stress, menstruation, UV light etc (170, 227). Once activated, viruses resume replication and can disseminate progeny into nearby cells, peripheral nerve endings in skin, and/or the central nervous system (65, 212, 226). The release of viruses from the nerve terminals permits reinfection of the original epithelial tissues (e.g., creation of “cold sore” for HSV-1 or “genital herpes” for HSV-2). These lesions can be painful and recur for the host’s entire life. If the infection spreads into the cen-
tral nervous system, it may result in life threatening, or fatal viral encephalitis. There is no cure for herpes infection currently, as neither vaccines nor antiviral drugs can affect the latent virus.

**HSV-1 virion structure**

The herpesvirus virion is composed of four elements (Fig. 1.1A): a core containing a linear double-stranded DNA genome, an icosahedral capsid protecting genetic material, a tegument protein matrix surrounding the capsid, and an outer lipid bilayer envelope. The HSV-1 particle is approximately 186nm in diameter (73, 169). Twelve different viral glycoproteins and at least 2 nonglycosylated proteins are embedded in the envelope (170). The tegument contains around 20 different proteins and is largely unstructured. Abundance of individual components varies from 100 to 2000 copies per virion (74, 112). The 125nm diameter capsid is not centrally positioned (73). It is composed of 150 hexons and 12 penton capsomeres, which are composed of four proteins. VP5, the major capsid protein, is present in five copies in each penton and six copies in hexon (179). Six copies of VP26 form a ring on the top of VP5 in each hexon (240). The adjacent capsomers are linked together by triplexes formed by one copy of VP19C and two copies of VP23 (239). There are also a number of minor capsid-associated proteins such as U_{L6} protein and VP24; they are not capsid building blocks, but are involved in DNA encapsidation (209, 222).

**Genome organization**

The 152 Kbp genome of HSV is packed in a liquid crystalline array in the capsid (16). As represented in Fig 1.1B, it contains two covalently linked segments of nonrepeated DNA designated unique long (U_L) (128 Kbp) and unique short (U_S) (25 Kbp) (169). These unique components are flanked by inverted repeats of DNA designated R_{L} and R_{S} respectively. A common nomenclature for HSV genes refers to their
Figure 1.1 Structure of herpes virion and viral genome. (A) Electron micrograph (left) of a herpesvirus particle and schematic representation (right) of virion structural components [reprinted from reference (124)]. (B) Schematic illustration of HSV genome. The genome is divided into a $U_L$ and $U_S$ region. The unique sequences are separated by inverted repeats (shown as boxes and designated as $R_L$ and $R_S$).
location in the genome, e.g. UL1-56, US1-11. Genes mapped within the inverted repeats are present in two copies e.g. ICP4 (RS1).

The RL sequences can be more specifically indicated as ab at the genome terminus and b’a’ in the opposite orientation at the L/S junction, whereas RS is represented as a’c’ and ca. More than one “a” sequence can be present in the terminal RL, while the terminal RS invariably contains a single “a” sequence (220). The “a” sequence structure is highly conserved but its sequence composition and size vary from strain to strain. The “c” sequences harbor two copies of a viral DNA replication origin (oriS). A third origin, oriL, was identified in the middle of the UL component. The three origins function redundantly such that deletion of oriL or both oriS have only slight impacts on viral replication (5, 169).

Until now, more than 90 unique transcriptional units have been identified on both strands of the genome; 84 of them encode proteins (170). Of the proteins potentially encoded, it is estimated that more than 40 accompany the viral particle itself, while the remainder are thought to modulate infectivity. With few exceptions, most viral genes do not contain introns and each produces a single protein (169). So far, more than two gene clusters have been noted. For example, a set of beta genes involved in viral DNA synthesis is located near the oriL, and a set of gamma genes encoding glycoproteins is positioned within the US component (170).

**HSV-1 replication**

HSV infection begins when virus penetrates into a host cell by fusion of its envelope with the cell membrane. The de-enveloped particle is then transported to the nuclear pore, through which genomic DNA is released into the nucleus for transcription and genome replication. Assembly of new capsids and genome packaging take place in the nucleus. Following that, nucleocapsids bud across the nuclear membrane into
the cytoplasm, where they acquire tegument and viral envelopes at the Golgi network to become a mature virion. Afterwards, the progeny virus can be released into the extracellular space by exocytic vesicle transport, or can spread to neighboring cells through virus-induced cell fusion or direct cell-cell spread.

**Viral entry**

Two pathways coexist for HSV to enter cells, but which serves as a primary route is cell-type dependent (65). In cells like Hep2 cells, Vero cells and neurons, viral entry is mainly mediated by direct fusion at the plasma membrane and subsequent release of the capsid into cytosol. For keratinocytes and HeLa cells, the virus is first endocytosed and later fuses with the membrane of endosomal vesicles (141). Whatever pathway is used, recognition of and binding to target cells and subsequent fusion are key events, which depend upon interactions between cell surface receptors and viral glycoproteins on the viral envelope. By current count, there are at least 12 glycoproteins on the viral envelope, and four of these glycoproteins: gB (UL27), gD (US6), and the complex of gH (UL22) and gL (UL1) are known to be essential for entry in both pathways (161).

Attachment to the cell surface involves at least three glycoproteins—gB, gC (UL44), and gD. gC and gB mediate the initial interaction with cells, recognizing heparan sulfate proteoglycans (HSPGs) on the cell surface; gC is not essential for entry inasmuch as gB can substitute for gC (75). Simultaneous mutations of the HSPG-binding sites of gC and gB abolish high-affinity interaction with HSPGs and decrease the efficiency of entry (54, 115). This initial heparin-sensitive attachment to cells is relatively weak, and is followed by more stable association to enable entry; the latter step apparently requires gD. Three cellular receptors can bind gD and mediate HSV entry independently: herpesvirus entry mediator (HVEM), nectins, or 3-O-sulfotransferase-modified heparan sulfate (3-O-HS). These molecules do not act as co-
receptors for entry, and the existence of a co-receptor was not known until a recent
discovery that association of immunoglobulin-like type 2 receptor (PILRα) with gB
was required for entry even in the presence of HVEM (178).

The subsequent fusion of the viral envelope with the cell plasma membrane takes
place at physiological pH and temperature in most cells (229), and requires the con-
certed action of three additional viral glycoproteins: gB, gH, and gL. Although the
precise fusion mechanism is still elusive, the current model is that a homotrimer of gB
and heterodimer of gH/gL comprise the core fusion machinery. The conformational
switch in gD upon receptor binding brings gB and gH/gL together, and activates their
fusion activities. Both gB and gH are fusogenic; they can act sequentially or they can
form a complex (3, 200).

As for the endocytic entry of HSV, the same glycoproteins (gB, gD, gH/gL) are
required (142). A gD receptor is absolutely needed, but it is unclear whether it requires
the same receptors as those involved in the direct fusion pathway. The mode of endo-
cytic uptake is cell-type specific and is not always clathrin-dependent. Actin rear-
rangement and RhoA GTPases are likely to be involved (26). In some cells, low pH is
another critical trigger of fusion machinery in addition to receptor mediated activation
(141).

**Translocation of the capsid to the nucleus**

After viral fusion, the tegument is the first to be exposed to the intracellular envi-
ronment. Most of the outer tegument proteins have been shown to dissociate from the
capsid upon viral entry. This dissociation is likely induced by phosphorylation through
virion associated and/or cellular kinases (127). Some of the released proteins provide
important functions both early and late in infection. For example, the virion host shut-
off (VHS, UL41) protein remains in the cytoplasm to selectively degrade mRNA early
in infection (52), whereas protein VP16 (UL48) is transported into the nucleus inde-
pendently of the capsid to act as immediate-early gene trans-inducing factor (αTIF),
and later interacts with and blocks the pUL41 RNase activity (11, 190).

As the majority of the tegument disassembles, the capsid then needs to be trans-
ported to the nucleus for genome replication and transcription. Diffusion alone may
eventually allow delivery of the capsid to its destination. But the slow rate is clearly
not compatible with the need for the capsid to travel long distances in human neurons.
In this case, translocation becomes an active transport event. Studies have shown that
dynein, a microtubule (MT)-based motor, is bound to capsids after entry, and these
results prompted the theory that the incoming capsid is transported along the cellular
microtubule network in a retrograde direction towards the nucleus (192). Other sup-
porting evidence includes the observation that movement of incoming viral particles
was blocked by microtubule depolymerizing drugs (97, 206), or by over-expression of
dynamitin, a protein disrupting the dynein complex (46). However, the HSV receptors
for MT motors are not yet known. A series of non-essential tegument proteins and a
potential dynein binding capsid protein, VP26 (UL35) (47), were tested for roles in
incoming viral retrograde transport, but did not seem to actively participate. The
tegment proteins pUL36 and pUL37 now emerge as candidates based on the fact that
both proteins are associated with capsids as they traverse the cytosol to the nucleus
(71, 111). Moreover pUL36, but not pUL37, is required for the anterograde transport
of capsids along microtubules during the egress phase of infection (112).

When the capsid arrives in the nucleus, it docks at the nuclear pore complex
(NPC). The attachment is importin-β dependent (144). Electron microscopy (EM)
analyses support a mechanism in which the HSV-1 genome is rapidly and efficiently
ejected from capsids bound to the nuclear pore complex, and empty capsids dissociate
from the NPC after release of the genome (10, 113, 192). Experiments have also
shown that the tegument protein VP1/2 (UL36) is critical in liberating the HSV-1 genome from the nucleocapsid. A temperature-sensitive mutation in the UL36 gene caused capsids to accumulate at nuclear pores, but they did not release their DNA at the non-permissive temperature. After a shift to the permissive temperature, the DNA was released successfully into the nucleus (10). Recently, the VP1/2 protein was shown to be proteolytically cleaved only after capsid binding to the NPC, and inhibition of this cleavage by protease inhibitors prevented the release of viral DNA (88). It is therefore proposed that the proteolytic cleavage of VP1/2 induced by binding to the NPC translates into structural changes in the capsid pentons on a particular vertex, which might be identical to the portal of DNA entry during capsid maturation and genome packaging (138).

The transfer of the viral genome through the NPC channel also requires metabolic energy (144). A study using atomic force microscopy (AFM) analyzed interactions between HSV-1 capsid and the NPC; it revealed that the HSV-1 genome was delivered to the nucleus through the NPC central channel as a highly condensed, rod-like structure, and that translocation of the viral genome is associated with a remarkable widening of the NPC central channel (181).

**Viral gene expression**

Once viral DNA enters the host nucleus, it circularizes quickly without viral protein synthesis and prior to DNA replication (64, 81, 149). The DNA is circularized through joining of the genomic termini (199) by cellular DNA ligase IV and its cofactor XRCC4. This conclusion came from the observations that RNAi-mediated depletion of either protein largely inhibited the formation of circular genomes and caused significant reduction of viral DNA synthesis (131).
The viral genome is faced with an important "decision": whether to proceed to productive infection or to establish a latent infection (21). In the latent state, the viral genome is retained quiescently in the circular form as an episome associated with host nucleosomes; gene expression is repressed except for some non-encoding transcripts. In contrast, the lytic cycle is characterized by a progressive cascade of gene expression and DNA replication. In this case, the circular genome serves as a template for initial “$\theta$” type replication followed by rolling circle replication (104).

Viral gene expression during the productive infection is highly coordinated. In general, each viral gene has its own promoter and transcription is catalyzed by RNA polymerase II (pol II) of the infected host cell (30). All genes have been classified into three categories according to their transcriptional kinetics: $\alpha$ (immediate early, IE), $\beta$ (early, E), and $\gamma$ (late, L) genes.

$\alpha$ genes are the first to be expressed, and are expressed optimally at approximately 2 to 4 hours post infection. Their promoters harbor multiple binding sites for cellular transcriptional factors, such as a TATA element, CAAT elements, and SP1 binding sites (223). Their transcription does not require other de novo synthesized viral proteins, but are distinctively regulated by the tegument protein VP16 ($U_{L}48$), which is translocated into the nucleus upon release of the tegument after viral entry. In the nucleus, VP16 forms a complex with host transcription factors Oct-1 and HCF (Host Cell Factor). Oct-1 specifically binds to the characteristic element TAATGArAT in the upstream region of all $\alpha$ gene promoters, and enables VP16 to exert its transcriptional activation functions through promoting the formation of pol II preinitiation complexes on the promoters (114).

$\alpha$ gene products include ICP0 ($R_{L}2$), ICP4 ($R_{S}1$), ICP22 ($U_{S}1$), ICP27 ($U_{L}54$), ICP47 ($U_{S}12$) and $U_{S}1.5$. Excluding ICP47, the other five $\alpha$ proteins have been implicated in stimulating $\beta$ and $\gamma$ gene expression. In particular, ICP4 is required for all
post-α gene expression (27, 45), and its effect is exerted at the transcriptional level (68). ICP4 is also responsible for down regulation of α products including itself and ICP0. In this case, specific consensus binding sites appear to be responsible for ICP4 mediated repression (66, 98). ICP0 is a nonspecific transactivator of α, β, and γ genes (170). It promotes viral infection and gene expression, especially at a low multiplicity of infection (MOI) where its absence leads to a 100 fold decrease in virus yield (187, 198). The shut-off of α genes involves auto down regulation (e.g ICP4), and also repression mediated by β and γ products (169).

β genes produce proteins involved in viral DNA replication (219), such as a single stranded DNA binding protein ICP8 (UL29) and viral DNA polymerase pUL30. The transcription of β genes starts at 3 hours post infection and reaches peak rates between 4 to 8 hours post infection (169). Its activation requires at least the presence of functional ICP4 but not viral DNA synthesis (223). Soon after the production of β proteins, viral DNA replication ensues.

Efficient levels of γ gene transcription require α and β products including ICP4, ICP22, ICP27, and ICP8, and also the synthesis of viral DNA. γ genes predominantly encode proteins comprising the virion particle, and have been subdivided into two groups based on timing of expression and their dependence on viral DNA replication: The expression of γ1 (leaky-late) genes does not require accumulation of viral DNA synthesis whereas the expression of γ2 (true late) genes is entirely dependent on DNA replication (219). Typical γ1 products include the major capsid protein VP5, gB, and gD; and typical γ2 proteins include gC, pUL41 (VHS), and pUL36.

DNA replication

Once β proteins are expressed, several proteins localize to the nucleus where they assemble on the parental viral genome to promote DNA replication. The initial round
of replication starts on the circular viral DNA molecule, creating a “θ” structure. At some point, the replication pattern switches to a rolling circle mechanism and produces head-to-tail concatemers of progeny DNA (82). Seven viral proteins are necessary for viral DNA replication. These are the viral DNA polymerase (pUL30) (154) and its processivity factor (pUL42) (29), an origin binding protein (pUL9), the single stranded DNA binding protein (ICP8), and the helicase-primase complex of three proteins, pUL5, pUL8, and pUL52 (22, 231). Host factors also participate in the replication event. For example, the host DNA polymerase α-primase, DNA ligase, and topoisomerase II are certainly required.

There are three replication origins in the viral genome: oriS, a 45 bp palindromic sequence located in “c” sequences of the genome and present in two copies; and oriL, also a palindromic sequence of 144 bp mapped between the UL29 and UL30 genes (34, 108, 224). However, only one origin is required for replication to occur (79, 151). The reason for the presence of three redundant replication origins is unclear. It may reflect the evolutionary history of the virus (169), and the oriL may be important for replication and reactivation from latency in certain types of tissue (7).

The basic model for HSV genome replication proceeds as follows. First, UL9 protein binds to the origin sequences and unwinds the viral DNA by its helicase activity. ICP8 is then recruited by pUL9 and binds to the single stranded DNA of the unwound portion. Next, the remaining five proteins (pUL5, pUL8, pUL52, pUL30, and pUL42) are targeted to the origin by pUL9 and ICP8. They assemble into a helicase–primase complex and viral DNA polymerase complex to initiate “θ” form replication. The reaction takes place at one or more origins and proceeds bidirectionally. Through an unknown mechanism, replication switches from the “θ” form to a rolling circle form, and UL9 protein is no longer required for rolling circle replication; such replication is not origin dependent. The rolling circle replication generates long head-to-tail con-
catameric DNA complexes, which become cleaved into individual units during packaging of viral DNA into progeny capsids (170).

It is well known that the parental HSV genome and associated replication proteins initially localize to sites adjacent to intranuclear structures containing components of nuclear domain 10 (ND-10) (110, 213). It has been reported that the viral DNA replication origins promoted the localization of viral genome to the ND-10 domains (193), suggesting viral DNA replication proteins may play a role in targeting viral DNA to the ND-10 structures. Alternatively, it has been shown by a more recent study that ND-10 proteins localized to viral genomes, rather than vice versa (53). Thus, viral DNA and replication proteins initially localize to other intranuclear sites and induce redistribution of pre-existing ND-10 proteins leading to formation of novel ND-10 bodies located at sites containing viral genomes.

HSV genome replication initiates at punctate ICP8 foci near the ND-10-like structures (116), and these foci are named “pre-replicative sites”. As DNA replication progresses, the pre-replicative sites expand and coalesce to form replication compartments (33, 193, 203) that eventually fill the nucleus. At this point, replication takes place in “replication compartments” that consist of accumulating DNA molecules and replication complexes (158). It has been reported that this compartmentalization of viral proteins is necessary for efficient viral DNA replication as well as late gene transcription (121).

**Capsid assembly**

After the onset of viral DNA replication, the γ genes are transcribed, including those that encode viral capsid proteins. In the nucleus, these proteins assemble into closed shells with an internal scaffold; viral DNA is then inserted into the capsids and the internal scaffold is expelled giving rise to a mature nucleocapsid. In the infected
nucleus, there are four types of capsids with morphological differences that can be distinguished by electron microscopy (EM): procapsids and type A-, B-, and C-capsids (134). Although the procapsid contains an internal scaffold, it is morphologically distinct from all other capsids because of its porous and roughly spherical appearance (134, 208). A-, B- and C-capsids share a common angularized icosahedral structure of approximately 120 nm in diameter. A-capsids contain only an icosahedral shell without the scaffold. B-capsids contain a spherical internal scaffold but no DNA. The C-capsid is the mature nucleocapsid; it contains DNA and lacks the scaffold (16, 179, 241).

The procapsid has been widely accepted as the precursor of all capsid types; its formation requires interactions of the major capsid protein VP5 (UL19), triplex proteins VP19C (UL38) and VP23 (UL18), and a scaffold protein VP22a (UL26.5) (39, 135, 136). Although the final assembly occurs in the nucleus, some initial interactions take place in the cytoplasm (140, 165). VP5, VP23, and the hexon outer tip protein VP26 are not capable of nuclear localization on their own. However, VP5 can be transported into the nucleus by the triplex protein VP19C and/or by the uncleaved precursor form of scaffold protein VP22a, pre-VP22a. VP23 nuclear localization is VP19C dependent, while VP26 nuclear import requires the presence of both VP5 and VP19C or pre-VP22a (140, 165). Once in the nucleus, VP5/pre-VP22a complexes come together as a result of self assembly of pre-VP22a. The triplex proteins VP19C and VP23 are then added to form a partial capsid. As hexons and pentons are added, the structure assembles into a round procapsid (134). In the procapsid, the internal scaffold is composed of many copies of the pre-VP22a protein encoded by UL26.5 gene, and another unprocessed protein encoded by UL26. The UL26.5 transcript initiates within the UL26 gene and is in the same coding frame. As a result, the UL26 protein shares its C-terminal sequence with pUL26.5 (i.e. pre-VP22a). It is this C-terminal domain that mediates the interactions with VP5 and leads to incorporation of the scaf-
fold proteins into the capsid structure during assembly (37, 78, 221). Uniquely, the pUL26 possesses an N-terminus with intrinsic protease activity. Upon activation, it cleaves itself at two sites and produces two functional units, a serine protease VP24 and a scaffold protein VP21; it also cleaves pre-VP22a into VP22a (148, 222). The procapsid undergoes a structural transformation and becomes polyhedral around the time that the internal scaffold proteins are cleaved (25, 183). The protease activity is required for capsid assembly (63), and its role is presumably to release the capsid shell from constraints that preclude maturation into more angularized capsid forms. A dodecamer of UL6 proteins is also assembled onto the procapsid through interactions with pUL26.5 to form a portal structure that is required for DNA packaging (133, 139, 147).

In the current view, A-, B- and C-capsids are considered to be three different outcomes of the procapsid maturation pathway. A-capsids arise when DNA is not inserted or not retained but the internal scaffold is lost or degraded. B-capsids arise as DNA is not inserted and the internal scaffold is trapped within the capsid. C-capsids are the only capsids to contain properly packaged with DNA; their internal scaffold is released and the DNA is sealed inside by the conformational changes in the capsid shell.

**DNA packaging**

Encapsidation of HSV DNA is a process in which cleavage of unit length viral genomes from concatamers is tightly coupled to their packaging into preformed capsids. It has been observed that viral DNA cleavage does not occur in cells infected with viruses that fail to produce capsids, suggesting that capsids contain essential parts of the cleavage/packaging machinery (36).

The cleavage and packaging machinery is complex and requires seven viral proteins pUL6, pUL15, pUL17, pUL25, pUL28, pUL32, and pUL33 (5, 170). They interact
with the capsid either during capsid assembly or in the course of DNA packaging (5). As described above, viral DNA is believed to enter the capsid through a ring-shaped portal formed by 12 copies of UL6 protein located at one vertex of the capsid (137).

By analogy with bacteriophage DNA packaging systems, the terminase complex comprising pUL15, pUL28 and pUL33 drives the HSV genome into the capsid with energy provided by ATPase activity. The terminase also functions to cleave the concatemeric DNA to yield a monomeric genome. Supporting evidence for this proposition includes sequence similarity between pUL15 and the large subunit of bacteriophage T4 terminase, the ability of pUL28 to bind to the viral DNA packaging signals, and the interaction of pUL15 and pUL28 with the portal protein pUL6 (2, 32, 225, 237). Within the complex, direct interactions have been characterized between pUL15 and pUL28, and also between pUL28 and pUL33, but not between pUL15 and pUL33 (1, 12, 83, 95, 96, 233). A recent study suggested that the three proteins assemble in the cytoplasm and are transported into the nucleus by the nuclear localization signal (NLS) of pUL15 (234).

The final step of DNA packaging is “capsid completion”. This term refers to the formation of a stable DNA-containing capsid. Earlier studies have indicated that the UL25 protein of HSV-1 is not required for cleavage of newly replicated viral DNA but is necessary for stable encapsidation. It was suggested that pUL25 may function as a head completion protein or plug which seals the portal channel after DNA packaging (120). pUL25 has also been reported to interact with the capsid shell and viral DNA, further suggesting a role in anchoring of the genome to the capsid (143). The addition of pUL25 to the capsid is proposed to occur in response to the release of the internal capsid proteins (182).

The functions of pUL17 and pUL32 are less well defined. The infection of UL17 or UL33 null HSV-1 mutant shows a common phenotype: newly replicated concate-
meric DNA is not cleaved into unit-length genomes, and, accordingly, in infected cell nuclei no C capsids are detectable while scaffold-containing B capsids accumulate and mislocalize (101, 176, 202). These observations have suggested pUL17 and pUL32 are involved in properly targeting capsids to viral DNA replication compartments for encapsidation. More light has been cast recently on pUL17 with the finding that pUL17 appears to bind to pUL25 on C-capsids, thus implying a role in stabilizing the capsid-DNA structure (204, 210).

The cleavage of viral DNA also needs cis-acting elements in the viral genome. Two signal segments designated $pac_1$ and $pac_2$ in the “a” sequence define the cleavage sites (35, 191, 216). It is proposed that the packaging complex binds to the DNA concatemer and scans for the first $pac_2$ site in proper orientation. Cleavage occurs at a fixed distance from $pac_2$, generating the L end of one genome that is inserted into the capsid. As the DNA engagement continues, the complex keeps scanning the following DNA until a directly repeated junction is encountered. Then a second cleavage is executed to produces a monomeric genome.

**Viral egress**

After the viral genome is packaged, nucleocapsids exit the nucleus, become enveloped virions in cytoplasm, and eventually gain access to the extracellular space. Enveloped capsids have been observed in the perinuclear space between the inner and outer nuclear membrane (INM and ONM, respectively), and this led to two proposed models of viral egress. The first model (Fig 1.2), also called the “double envelopment” pathway, proposed that nucleocapsids are enveloped at the INM, become de-enveloped by fusion of the virion envelope with the ONM, and then undergo secondary envelopment at cytoplasmic membranes. These virions are transported in vesicles to the plasma membrane and are released into the extracellular space through fusion.
between the vesicle membrane and plasma membrane (51, 123). In the second model, known as the “single nuclear envelopment” pathway, the capsids acquire the initial envelope at the INM, enter into a vesicle at the ONM, and retain their primary and final envelope as they leave the cell through the secretory pathway (19, 86). In this model, virion glycoproteins are modified in transit to the plasma membrane and perinuclear virions should contain the entire complement of tegument and envelope proteins present on mature extra-cellular virions. However, this model is inconsistent with multiple lines of evidence. First, according to electron microscopic examinations, the primary envelope and primary tegument in perinuclear virions clearly differ in ultrastructure from the final envelope and tegument in cytoplasmic and extracellular virions (67, 70). Second, biochemical studies revealed compositional differences between perinuclear virions and extracellular virions. In an HSV-1 mutant, when gD was retained in the endoplasmic reticulum (ER), gD was detected on the perinuclear virions but was absent from extracellular virions (188). Moreover, pUL34 and pUL31 are components of perinuclear virions, but are missing in extracellular ones (163), whereas the major tegument protein pUL49 is associated with cytoplasmic and extracellular virions but not with perinuclear particles (94). Moreover, the phospholipid composition differs between the envelope of extracellular HSV-1 virions and the host cell nuclear membrane (214). All of these differences can only be explained by the two-step envelopment model.

Alternatively, a third model was suggested by a recent report. Wild et al. observed that, in bovine herpesvirus-1 infected cells, nuclear pores were dilated to a size readily allowing capsids to pass through (228); and so they proposed that virus exits the cell via a “dual pathway single envelopment” model (105). In this model, a minor
Figure 1.2 Diagram of herpesvirus egress pathway [reprinted from reference (125)]. In the nucleus capsids assemble around a scaffold (1 – 3), then DNA is packaged (4) into preformed capsids. DNA-filled capsids (5) contact the inner nuclear membrane at the sites where the nuclear lamina has been locally dismantled (6), bud into perinuclear space resulting in enveloped primary virions (7). The primary envelope fuses with the outer nuclear membrane (8), releasing the nucleocapsid into the cytosol where capsid-proximal tegument proteins assemble onto the capsid (9). Concurrently at the trans-Golgi apparatus, glycoproteins and outer tegument proteins assemble together (10). Two subassemblies join to complete the final envelopment (11) and the mature virion is then transported in a vesicle (12) to the plasma membrane for release (13). (10a – 13a) Formation and release of capsid-free particles.
population of capsids egress via a “single nuclear envelopment” pathway early in infection, whereas the majority exit the nucleus through enlarged nuclear pores, become enveloped in the cytoplasm and are released by secretory vesicles. At face value, this model could also be compatible with the above results supporting the “double envelopment” model. Nevertheless, it faces challenges that have not been resolved at this time. For instance, an HSV-1 defective in Us3 kinase induced accumulation of enveloped virions in the perinuclear space and slowed viral production, indicating at least capsid budding into the perinuclear space is not a minor egress pathway (174).

Nuclear egress

HSV nuclear egress is a complex process, involving interactions of viral proteins and cellular components. Nucleocapsids initiate the event by budding at the inner nuclear membrane (INM), acquire INM-derived envelope (specified as the primary envelope), and translocate into the perinuclear space. De-envelopment occurs when the primary envelope fuses with the outer nuclear membrane (ONM); capsids are then released into cytoplasm.

Primary envelopment at the INM

It is believed that DNA packaging is a prerequisite for nuclear egress of capsids. In wild type virus infections, a preference has been noted for primary envelopment of C-capsids over other non-DNA-containing capsid forms. The mechanism underlying this preferential envelopment of C capsids is unclear. In an early study, the conserved capsid-associated protein pUL25 was suggested to play a role in this process (197); more recently, the pUL25 homolog in pseudorabies virus was found to be necessary for the nuclear egress of capsids (92). However, no capsid nor capsid-associated protein has been identified so far that physically interacts with the egress complex (pUL31/pUL34) to drive primary envelopment. DNA-filled capsids move from in-
tranuclear assembly sites to contact the inner nuclear membrane prior to primary envelopment. This intranuclear transport of HSV-1 capsids appears to be actin dependent, inasmuch as it could be inhibited by latrunculin B (60). This is additionally supported by the finding of actin filaments in the host cell nucleus induced by HSV-1 infection (58).

Two HSV-1 proteins, pUL31 and pUL34 are required to initiate the primary envelopment at the INM (163). Analysis of U1.L31 and U1.L34 deletion mutants has indicated that the absence of either protein results in a drastic impairment in primary envelopment with capsids trapped within the nucleus (24, 62, 94, 163, 173). The U1.L34 or U1.L31 null viruses failed to replicate on most non-complementing cell lines. An exception is that rabbit skin cells were permissive to U1.L31 null virus to a limited extent (107).

pUL31 and pUL34 are highly conserved in the herpesviridae family. pUL34 is predicted to be a type II integral membrane protein with a 22 amino acid transmembrane domain at the C-terminus (157, 173, 184), while pUL31 is a nuclear phosphoprotein (23). In wild type virus infected cells, pUL31 and pUL34 co-localize on the nuclear rim. In the absence of pUL34, pUL31 is exclusively intranuclear, whereas without pUL31, pUL34 redistributes to the ER, although the protein appears to possess an intrinsic nuclear targeting signal (94, 163). Physical interaction between the two proteins has been demonstrated; and a 50 amino acid region of pUL34 is known as an essential domain interacting with pUL31 (106). It is very clear that the complex formation of two proteins is important for proper positioning of both partners at the inner nuclear membrane, which is a prerequisite for primary envelopment (163). Both proteins are incorporated into the perinuclear virions, but are likely lost at the ONM through de-envelopment (164).
The pUL31/pUL34 complex is considered to act as a membrane receptor for capsids to recognize, bind, and then bud into the perinuclear space. However, it is unclear whether additional viral or cellular proteins are required in this process. In an in vitro system, reconstruction of nuclear egress required cytosol, whereas infected cytosol was not necessary (160). This data strongly implied involvement of host factors. In another study, co-expression of pUL31 and pUL34 induced vesicles to form from the INM in the absence of viral infection; those vesicles resembled primary envelopes without a nucleocapsid (91). This also argues for the contribution of cellular factors in the budding reaction. Such factors might be recruited from their natural cytoplasmic location, or were previously unrecognized in the nucleus.

Alteration of the nuclear lamina has been observed after HSV infection and may be required so that the inner nuclear membrane is accessible to capsids (103, 162, 180). Multiple lines of evidence indicate that pUL31 and pUL34 play a role in lamina modification. Over expression of pUL31 alone was sufficient to relocalize lamin A/C from the nuclear rim into nucleoplasmic aggregates, whereas over-expression of pUL34 causes some lamin A/C redistribution into the cytoplasm (162). More importantly, both pUL31 and pUL34 are able to directly bind lamins A/C and/or B, suggesting that pUL31 and pUL34 modify the conformation of the nuclear lamina in infected cells, possibly by direct interaction with lamin proteins (69, 162). Given that the nuclear lamina potentially excludes nucleocapsids from envelopment sites at the inner nuclear membrane, the lamina alteration may reflect a role of the pUL31/pUL34 protein complex in perturbing the lamina to promote nucleocapsid egress from the nucleus. Lamins A/C and B appear to participate in viral infection in different ways; their respective roles are covered in more detail in Chapter III.

In addition, the complex can also recruit protein kinases to modify the lamina. Cellular protein kinase C (PKC) is enriched on the nuclear rim in a pUL31/pUL34-
dependent manner, which in turn phosphorylates lamins A/C and/or B (130, 146). This could result in local dismantling of the lamina network and the underlying chromatin layer (185), enabling the capsids to access the inner nuclear membrane. Viral encoded kinases are also involved in this process. The UL13 kinase of human cytomegalovirus (HCMV) is targeted to the lamin B receptor via interaction with cellular protein p32, causing redistribution of lamins A/C and B (115). In a pUL34-dependent manner, HSV-1 US3 kinase hyperphosphorylates emerin, a lamin-interacting nuclear membrane protein, and induces localizational changes of this protein (103). This kinase has also been found to phosphorylate lamin A/C during infection, as reported in Chapter II. Transient expression of pUS3 alone is sufficient to disrupt the nuclear lamina (15).

The US3 kinase also assists pUL31/pUL34 in proper positioning, and is needed for efficient nuclear egress. Deletion or deactivation of the kinase induces aggregation of pUL31/pUL34 on the nuclear rim in infected cells, and results in a delay in viral production, but has little effect on the final peak yield of infectious virus (164, 174). Electron microscopic examinations of cells infected by a US3-defective HSV mutant have revealed perinuclear virions that accumulate in invaginations of the inner nuclear membrane (164, 174). These structures are assumed to form as a result of delayed perinuclear virion de-envelopment, and they appear to cause restriction of nucleocapsid envelopment to certain sites within the inner nuclear membrane. Therefore, US3 kinase activity modulates pUL31/pUL34 distribution in the nuclear membrane as well as the fusion of the primary envelope with the outer nuclear membrane.

pUS3 is a PKA-like serine/threonine kinase (14, 155). Interestingly, both pUL31 and pUL34 are phosphorylated by pUS3 in vivo (89, 156). Although the catalytic relationship between pUS3 and pUL34 has been examined in details, the phosphorylation of pUL34 is not demonstrably important to nuclear egress (174). Instead, I show in
Chapter IV that pU_{L31} is a functionally important downstream effector of the U_{S3} kinase.

**De-envelopment at the ONM**

Several glycoproteins have been identified as components of the perinuclear virion, including gD, gB and gM (6, 188, 195, 207). It is reasonable to assume that the fusion of the primary envelope with the ONM is conducted by the same set of glycoproteins involved in entry and other fusion events. In earlier studies, however, deletion of these major glycoproteins required in other membrane fusion did not affect the de-envelopment process (18, 70, 84). The core fusion machinery of the perinuclear virion has been elusive. Recently, an HSV-1 mutant lacking both gB and gH produced a large number of perinuclear virions, indicating a failure of de-envelopment, whereas a single deletion of either gene had no detectable effect on the de-envelopment process (56). This observation indicates that fusion relevant to nuclear egress requires both gB and gH, and these proteins function in a redundant manner. It also suggests that fusion during nuclear egress is mechanistically different from that of viral entry, because in the latter, both gB and gH are required and are not functionally redundant.

**Tegumentation in the cytoplasm**

After translocation into the cytoplasm, capsids undergo tegumentation and secondary envelopment through a highly ordered network of protein-protein interactions. The tegument proteins interact with the capsid on one side and with the viral envelope proteins on the other in order to link the structural components to the final envelope of the HSV-1 virion and to secure the integrity of the virus particle (123). It has become clear that final tegumentation can initiate at two different sites, the capsid and the future envelope, resulting in two “subassemblies” that combine to produce a mature virion (125).
The first layer of tegument around the capsid is composed of pUL36 and pUL37. pUL36 has been shown to interact with the major capsid protein VP5 (139, 238), whereas pUL37 is likely incorporated via interaction with pUL36 (90). pUL36 and pUL37 are the only tegument components conserved in the Herpesviridae family. The absence of pUL36 and pUL37 abolishes virus maturation (38, 40). The level of these proteins in the virion is strictly controlled, whereas stoichiometry of outer tegument proteins can vary extensively (126). As mentioned in a previous section, pUL36 and pUL37 remain associated with incoming capsids while outer tegument proteins disperse after viral entry (71, 111), and are involved in intracytoplasmic transport of capsids during entry and exit (112, 230). Besides these conserved components, other non-conserved proteins might also be components of the inner tegument. For instance, pUS3 is also encompassed in the capsid-proximal tegument.

Concurrently, at the final envelopment site in the trans-Golgi network, another subset of tegument proteins including pUL46, pUL47 and pUL49, are assembled together with viral glycoproteins (211). Viral glycoproteins are recruited to cytoplasmic envelopment sites by an envelope protein gM, which is also thought to retain other glycoproteins at the envelopment site, or to retrieve them from the cell surface (31). Tegument proteins may also be recruited by a small myristoylated protein pUL11, which has intrinsic targeting properties to the Golgi apparatus (17, 109).

The capsids coated with pUL36 and pUL37 are then transported to the cytoplasmic envelopment site where the remaining tegument and envelope glycoproteins await them. Although it is not yet clear which proteins form the bridge between the two subassemblies, some experiments indicate that pUL48 (VP16) may play a role in this process (62, 218). pUL48 has been shown to interact with tegument proteins pUL49 and pUL41 (VHS) (189). Mutant forms of pUL41 that do not bind pUL48 fail to be-
come incorporated into the virion (159). Cross-linking studies have indicated interactions between pUL48 and gB, gD, and gH (242).

**Secondary Envelopment and release**

Following tegumentation, HSV-1 capsids bud into cytoplasmic vesicles derived from the trans-Golgi Network (TGN) or endosomes by wrapping the membranous cisterna around the capsid to form a mature herpesvirus particle within a cellular vesicle. This vesicle is then transported to the plasma membrane. After fusion of the vesicle and plasma membrane, the nascent virion is released into the extracellular space.

Studies of viral mutants have shed light on the roles of certain viral envelope proteins in this event. As revealed by EM, HSV-1 mutants lacking either Ul20 or gK produced dramatic accumulations of un-enveloped and aberrantly enveloped capsids in the cytoplasm with a marked absence of extra-cellular virion (61, 84). A triple mutant lacking gD, gE, and gI had a severe defect in the final envelopment step. It is proposed that gD and the gE/gI heterodimeric complex act in a redundant fashion to anchor the virion envelope onto tegumented capsids (54). In contrast, it is also possible that deletion of multiple glycoproteins indirectly disrupts the integrity of many different protein-protein interactions required for the final envelopment step and that the resulting phenotype results from the compound effects of an abnormal glycoprotein profile.

Secondary envelopment does not require the presence of capsids. Capsid-free particles can be formed by enclosing tegument proteins in an envelope in a process mimicking normal capsid envelopment (119, 166). These structures are called light (L) particles. These particles form in abundance in cells infected with particular mutant viruses, in which normal capsid assembly and tegument formation is inhibited (119, 166). This demonstrates that capsids are not required to trigger secondary envelopment. It has been proposed that normal tegumentation requires pUL36 and pUL37 in-
interactions with the capsid and that pUL49 may be required for proper interactions with viral glycoproteins in the final envelopment step (123). In the absence of capsids, tegument assembly could be mediated through UL49 anchoring, resulting in the formation of L particles.

The release of nascent virions following final envelopment mimics the cellular secretory pathway. Viral proteins are apparently involved, although their functions in this particular step remain elusive. It is also known that the late stages in viral egress may differ depending on the cell type (85, 125). For example, in polarized epithelial cells, wild type virions are sorted predominantly to cell junctions, whereas gE/gI null virions are released nonspecifically into the extracellular space (44).

**Cell-cell spread**

Dissemination of HSV occurs via both the release of progeny viruses into the extracellular environment and direct cell-to-cell spread. In the process of cell-cell spread, nascent virions are targeted to cell junctions that contact neighboring cells. These virions are then released and bound immediately by the adjacent cell for internalization (85). In the host, HSV disseminate mostly by spreading from cell to cell. This strategy is expected to enhance viral spread and enable viruses to avoid host immune defenses.

The glycoproteins that are required for HSV cell-cell spread are gB, gD, gH/gL and gE/gI (85). The requirements for gD and its receptor as well as gB and gH/gL parallel those involved in entry of cell free virions. gE and gI, although not required for entry of cell free virions, are important in promoting HSV cell-cell spread (150, 175). Viruses lacking gE or gI cause no disease in rodents and produce smaller plaques in culture compared to wild type virus (43). Subsequent studies showed that gE/gI is involved in routing virions to the basolateral surfaces of polarized cells (55, 87). It is proposed that gE/gI is concentrated at cell junctions through interaction with
an as-yet-unidentified extracellular ligand, and so diverts viral trafficking from apical to basolateral membranes (150). Moreover, the enrichment of nectins at junctions of neurons and epithelial cells, coupled with the ability of gD to bind them, suggests an alternative mechanism by which virions are directed towards the neuronal terminal (85).

In addition, cells infected by HSV-1 or PRV can form extended protrusions to contact neighboring cells and deliver virions to the contacted cell (57, 100, 215). These F actin-associated structures can be induced by US3 kinase in PRV and can be reproduced by inhibition of the Rho-associated kinase (ROCK) when pUS3 is absent (57).

**Brief outline of dissertation research**

The goal of the work herein was to shed some light on the machinery of HSV nuclear egress. With the proposal that viral US3 kinase phosphotylates viral and/or cellular factors to regulate the capsid primary envelopment at the INM and subsequent deenvelopment at the ONM, several putative substrates of pUS3 were identified and the functional relevance of their catalytic relationships was evaluated. In Chapter II, the distribution of lamin A/C was observed to be modified by pUS3 kinase activity, and lamin A/C was identified as a substrate of the kinase. Therefore I hypothesized that lamin A/C might be the factor required for pUS3 functions. However, this hypothesis was not favored by the data shown in Chapter III, where I examined the respective roles of lamin A/C and lamin B1 during viral infection. Finally, in Chapter IV, I determined that pUL31 is a major substrate mediating pUS3 regulatory effects, and found the phosphorylation of pUL31 to be a dynamic event during nuclear egress.
CHAPTER II

US3 OF HERPES SIMPLEX VIRUS TYPE I ENCODES A
PROMISCUOUS PROTEIN KINASE THAT PHOSPHORYLATES AND
ALTERS LOCALIZATION OF LAMIN A/C IN INFECTED CELLS*

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Abstract

The herpes simplex virus type 1 (HSV-1) US3 gene encodes a serine/threonine kinase that, when inactivated, causes capsids to aggregate aberrantly between the inner and outer nuclear membranes (INM and ONM, respectively) within evaginations/extensions of the perinuclear space. In both Hep2 cells and an engineered cell line derived from Hep2 cells expressing lamin A/C fused to enhanced green fluorescent protein (eGFP-lamin A/C), lamin A/C localized mostly in a reticular pattern with small regions of the INM devoid of eGFP-lamin A/C when they were either mock infected or infected with wild-type HSV-1(F). Cells infected with HSV-1(F) also contained some larger diffuse regions lacking lamin A/C. Proteins UL31 and UL34, markers of potential envelopment sites at the INM and perinuclear virions, localized within the regions devoid of lamin A/C and also in regions containing lamin A/C. Similar to previous observations with Vero cells (S. L. Bjerke and R. J. Roller, Virology 347:261-276, 2006), the proteins UL34 and UL31 localized exclusively in very discrete regions of the nuclear lamina lacking lamin A/C in the absence of US3 kinase activity. To determine how US3 alters lamin A/C distribution, US3 was purified and shown to phosphorylate lamin A/C at multiple sites in vitro, despite the presence of only one putative US3 kinase consensus site in the lamin A/C sequence. US3 kinase activity was also sufficient to invoke partial solubilization of lamin A/C from permeabilized Hep2 cell nuclei in an ATP-dependent manner. Two-dimensional electrophoretic analyses of lamin A/C revealed that lamin A/C is phosphorylated in HSV-infected cells, and the full spectrum of phosphorylation requires US3 kinase activity. These data suggest that US3 kinase activity regulates HSV-1 capsid nuclear egress at least in part by phosphorylation of lamin A/C.
Introduction

Like orthologues in other members of the subfamily Alphaherpesvirinae, the US3 gene of herpes simplex virus type 1 (HSV-1) encodes a serine/threonine kinase (60, 160). These proteins have been implicated in nuclear egress, prevention of apoptosis, and modulation of the actin cytoskeleton to promote the cell-to-cell spread of virions (55, 63, 85, 94, 107, 170, 176, 183, 220). The current study focuses on the role of the US3 gene-encoded kinase activity in nuclear egress of nucleocapsids and virions.

Although models of HSV virion egress differ as to the extent of its contribution, all models propose that at some point during the course of infection with wild-type herpesviruses, nucleocapsids assemble in the nucleoplasm and bud through the inner nuclear membrane (INM) and into the perinuclear space (87, 199, 226). This compartment is delimited by the INM and outer nuclear membrane (ONM) and is continuous with the lumen of the endoplasmic reticulum. To become enveloped, capsids must bypass the nuclear lamina, a fibrous meshwork lining the nucleoplasmic face of the INM. The nuclear lamina provides structural rigidity to the nucleus and is essential for transcription and DNA replication (73, 189). The lamina contains a series of type 5 intermediate filaments composed of lamins A, B1, B2, and C; types A and C are products of RNA splice variants from the Lmna transcript, whereas types B1 and B2 are derived from other genes (57, 76, 77). Like all intermediate filaments, lamins comprise globular head and tail domains that flank a rod domain (59). The rod domains of two lamins intertwine to form protomers, whereas regions bordering the rod/head and rod/tail domains likely interact with other lamin protomers to form longer filaments (162). The globular domains interact with a variety of proteins in the lamina and INM.

One remarkable feature of the lamina is its dynamic nature. The lamina expands by the addition of protomers during interphase, is completely disassembled prior to
mitosis, and is partially disrupted during apoptosis. Phosphorylation likely plays a role in lamin dynamics in all phases of the cell cycle. The disassembly of the lamina during mitosis is associated with phosphorylation of lamin A/C by cdc2 kinase at Ser390 and Ser392 and during apoptosis by protein kinase C (PKC) delta (26, 30, 46, 150, 151). Protein kinase C can phosphorylate lamin A/C at Ser572 in vitro (48).

The architecture of the nuclear lamina is altered from its normal state during HSV-1 infection (13, 147, 168, 184, 190). Depending on the cell line and time after infection, these changes include (i) limited displacement and conformational changes of lamin A/C (13, 168, 190), (ii) redistribution of lamin B to a perinuclear region (147, 184), and (iii) increased mobility and mislocalization of lamin B receptor, one of several integral membrane proteins that anchor the nuclear lamina to the INM (184, 189). In attempts to understand the mechanism(s) by which the lamina becomes displaced, it was found that PKC of the alpha and delta subfamilies are recruited to the nuclear rim of HSV-1-infected cells and that lamin B becomes hyperphosphorylated during HSV infection, in part due to phosphorylation by PKC (146). Given these observations, it was postulated that HSV coopts cellular mechanisms to disrupt the nuclear lamina and thereby promotes egress of nucleocapsids (146). The state of phosphorylation of lamin A/C in HSV-infected cells is unknown, in contrast to that of lamin B. Previous analyses using one-dimensional gel electrophoresis indicated that HSV infection did not drastically alter the electrophoretic mobility of lamin A/C from human foreskin fibroblasts or from Hep2 cells, suggesting that phosphorylation is not extensive (164, 168).

As detected by immunoelectron microscopy, nascent virions located in the perinuclear space contain the UL3 kinase and at least two of its substrates, the proteins encoded by the UL31 and UL34 genes (as products pUL31 and pUL34, respectively) (90, 156, 161, 170). The proteins pUL31 and pUL34 are required for efficient envelopment of nucleocapsids at the INM (22, 110, 169, 175). The observations that proteins pUL31,
pUL31, and pUL34 are located within perinuclear virions and at the INM suggest that these proteins are incorporated into the virion during budding at the INM (164). Interestingly, all of the HSV-1-induced alterations in lamin A and B distribution/conformation require expression of pUL31 and pUL34 (13, 147, 168, 191). How pUL31 and pUL34 alter the structure of the nuclear lamina is not known, but the alteration likely involves multiple mechanisms. For example, both pUL31 and pUL34 are required for the recruitment of protein kinase C alpha and delta to the nuclear rim, and both can interact with lamin A/C in vitro (147, 168). Thus, UL31/UL34 might interrupt the nuclear lamina by mechanical interference with lamin-lamin interactions, by a disruption of lamin folding, by recruitment of lamina-disrupting kinases, or by all three mechanisms.

Deletion of US3 delays the onset of production of infectious virus and reduces peak infectious titers by approximately 10- to 30-fold with Hep2 cells (170, 176). The absence of US3 or its kinase activity does not preclude budding at the INM, nor incorporation of the pUL31/pUL34 into perinuclear virions, but causes these virions to aggregate aberrantly within the perinuclear space (94, 170, 176, 183, 220). These observations with both the HSV and the pseudorabies herpesvirus (PRV) systems have led to the conclusion that US3 is involved in the exit of virions from the perinuclear space into the cytoplasm (94, 170). On the other hand, if the exit from the perinuclear space was the sole contribution of US3 to nuclear egress, one might expect that pUS3(−) perinuclear virions would distribute evenly throughout the perinuclear space, but this is not the case. Rather, virions are tightly packed together within discrete invaginations/extensions of the nuclear membrane (NM). How US3 kinase activity precludes this aberrant aggregation of perinuclear virions is unknown.

In vitro biochemical studies characterized a US3 kinase minimal consensus sequence as (R)n-X-(S/T)-Y-Y, where n is >2, S/T is the target site where either serine
or threonine is phosphorylated, X can be absent or any amino acid but preferably Arg, Ala, Val, Pro, or Ser, and Y is similar to X except that it cannot be an absent amino acid, proline, or an acidic residue (105, 158, 159). The optimal consensus sequence is similar except that X is not absent and n is 3. Both pUL34 and pUL31 have one or more sequences matching this consensus motif (117). Of the known US3 kinase substrates localizing at the NM, the lack of phosphorylation of pUL34 is not likely responsible for the aberrant NM morphology in US3 mutant-infected cells (174), whereas the role of pUL31 phosphorylation has not yet been tested. The current studies were initiated under the hypothesis that substrates in addition to pUL31 and pUL34 may be involved in capsid and virion egress from the NM.

**Materials and methods**

**Cells and viruses.** Wild-type HSV-1(F) virus and a US3 deletion mutant virus, R7041, have been described and were obtained from B. Roizman (48, 160). Virus vRR1204, containing a mutation at US3 codon 220 changing lysine to alanine (K220A) in an HSV-1(F) background, was a kind gift of R. J. Roller (174). The viruses were grown and titers were determined on Vero cells as described previously (132).

An enhanced green fluorescent protein (eGFP)-lamin A/C-expressing cell line was made by transfection of DNA containing a cDNA of Lmna fused to eGFP-C1 and encoding neomycin resistance (a kind gift from D. Gilbert, Upstate Medical Center) into human Hep2 cells (80). Individual eGFP-positive cells were sorted based on the intensity of fluorescence determined using an ExCalibur fluorescence-activated cell sorter (FACS), into individual wells of a 96-well plate. The cells were grown in growth medium (Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum and antibiotics) supplemented with 250 µg/ml Geneticin (Invitrogen).
The highest-expression cells failed to expand further, whereas some intermediate expressers and all low expressers expanded into colonies (not shown). A single clone of several, containing intermediate levels of nuclear fluorescence, was further amplified in growth medium supplemented with 50 to 250 µg/ml Geneticin and was used for further studies.

**Construction of recombinant baculoviruses.** Full-length U₅3 and U₅3(K220A) were PCR amplified from viral DNA of HSV-1(F) or the U₅3 mutant virus vRR1204, respectively. The PCRs used primers with a BglIII restriction endonuclease site incorporated into the forward primer (5'-TAA TAG ATC TAT GGC CTG TCG TAA GTT TTG-3') and an EcoRI site in the reverse primer (5'-ATA TGA ATT CTC ATT TCT GTT GAA ACA GCG-3'; restriction sites are in italics). PCR products were then cloned into the BamHI and EcoRI sites of the pGEX4T-1 vector such that they were in frame with the gene encoding glutathione-S-transferase (GST). DNA in the plasmid clones was sequenced, and those clones with the proper GST-U₅3 fusions were used as templates for a second PCR to amplify GST-U₅3 and GST-U₅3(K220A). This second PCR used primer 5'-AGG CAG ATC TAT TCA TGT CCC CTA TAC TAG-3' containing another BglIII site (italicized) and the U₅3 reverse primer. Amplicons were then subcloned into the BglII/EcoRI sites of the pBacPAK8 vector (Clontech). This construct was then transfected into insect Sf9 cells along with BacPAK6 baculoviral DNA (Clontech) according to the instructions of the BacPAK baculovirus expression system, and recombinant baculoviruses expressing the target proteins were plaque purified and amplified to produce viral stocks.

**Antibodies and immunofluorescence.** Polyclonal chicken antibody against pU₁34 was a kind gift from R. J. Roller (163). Chicken anti-lamin A/C polyclonal and rabbit anti-pU₁31 antisera were prepared in our laboratory and were described previ-
ously (168, 169). Lamin B-specific goat polyclonal antibody was purchased from Santa Cruz Biotechnology (catalog number sc-6218).

To characterize the localization of pUL31 and lamin A/C in infected cells, 200 µl of rabbit anti-pUL31 antisera was adsorbed against an acetone powder (177) prepared from approximately 2 x 10^8 Hep2 cells that were infected 24 h previously with a U131 deletion virus (107). The powder and antisera were added to 4 ml phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and were mixed overnight. After centrifugation, the supernatant was passed through a 0.2-µm-pore-sized filter.

Hep2 cells growing on glass coverslips were mock infected or infected with wild-type HSV-1(F), U53 null, or U53(K220A) HSV-1 at a multiplicity of infection (MOI) of 5.0 for 16 h. Cells were fixed with 3% paraformaldehyde for 15 min, followed by treatment for 5 min at −20°C in methanol. The cells were then permeabilized with 0.1% Triton X-100 and reacted with 10% human serum in PBS to block nonspecific immunoreactivity and were subsequently probed with the preadsorbed pUL31 rabbit polyclonal antibody diluted 1:50 in PBS supplemented with 1% BSA. Ten percent BlockHen II (Aves Lab) was used for a second round of blocking before probing with a 1:200 dilution of chicken anti-lamin A/C polyclonal antibody. Bound primary antibodies were recognized by Texas Red-conjugated donkey anti-rabbit and fluorescein isothiocyanate (FITC)-conjugated donkey anti-chicken immunoglobulin (Jackson ImmunoResearch).

In some experiments, the cell line expressing eGFP-lamin A/C was mock infected or infected as described above, fixed in 3% paraformaldehyde, and permeabilized with 0.1% Triton X-100. Epitopes were then blocked by incubation with 10% human serum in PBS for 1 h, followed by blocking with 10% BlockHen II and reaction with pUL34-specific antisera diluted 1:200. After extensive washing, primary antibody was recog-
nized by Texas Red-conjugated donkey anti-chicken immunoglobulin (Jackson ImmunoResearch).

Images were visualized and recorded using either an Olympus or a Zeiss laser scanning confocal microscope equipped with a 488-nm argon or 568-nm krypton laser, respectively, and associated software. Digital images were exported in a tagged image file format (TIFF) to Adobe Photoshop for processing.

**Purification of wild-type and mutant HSV-1 pU53.** Sf9 cells were infected with recombinant baculoviruses expressing GST-pU53 or GST-pU53(K220A) and were lysed 42 hr post infection by brief sonication in ice-cold lysis buffer (50 mM Tris [pH 7.5], 100 mM NaCl, 5 mM MgCl2, 0.1% NP-40, 10% glycerol) containing 1x Complete protease inhibitor cocktail (Roche). The U53 proteins were purified from the lysates by affinity chromatography using glutathione Sepharose beads (Amersham) as previously described (89). Proteins were eluted in 100 mM reduced glutathione according to the manufacturer's protocol and dialyzed into storage buffer (20 mM Tris-HCl [pH 7.5], 25 mM KCl, 0.1 mM EDTA, 2 mM dithiothreitol [DTT], 50% glycerol). Protein concentration was determined by an RC DC protein assay kit (Bio-Rad), and aliquots were frozen at –80°C.

GST fusion proteins were partially purified from Escherichia coli lysates, as described previously (162), and retained on the Sepharose beads without elution for reactions. Protein concentration was estimated by elution and separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by comparison with known amounts of BSA standards stained with Coomassie brilliant blue.

**In vitro kinase assay.** A kinase assay was performed using purified GST-pU53 or GST-pU53(K220A) essentially as described previously, with minor modifications (21). Briefly, 0.1 µg GST-pU53 or at least an equivalent amount of GST-pU53(K220A) was
incubated separately with 1 to 3 µg of partially purified *E. coli*-expressed GST fusion proteins for 30 min at 30°C in 50 µl US3-specific kinase buffer (50 mM Tris [pH 9.0], 20 mM MgCl2, 0.1% NP-40, 1 mM DTT) containing 10 µM ATP, and 10 µCi [γ-32P]ATP (Amersham). The Sepharose beads bearing the fusion proteins were then washed three times with TNE buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA) and in some cases were resuspended in bacteriophage lambda pyrophosphatase (λ-PPase) buffer containing 200 U of enzyme (New England Biolabs) and incubated for another 30 min at 30°C. The proteins on the beads were again washed three times in TNE. All the samples were dissolved in SDS-PAGE sample buffer (10 mM Tris-HCl [pH 8.0], 10 mM β-mercaptoethanol, 20% glycerol, 5% SDS, trace amounts of bromophenol blue) and subjected to electrophoresis in a 12% polyacrylamide gel in the presence of 0.1% SDS. Gels were then stained with Coomassie brilliant blue, dried, and autoradiographed using X-ray film (Pierce).

**Lamin disassembly assay.** A previously described lamin disassembly assay was modified for the current studies (28). Approximately 1 x 10^7 Hep2 cells were homogenized in 5.0 ml nuclear buffer (250 mM sucrose, 5 mM MgCl2, 25 mM KCl, 10 mM Tris [pH 7.4], 1 mM DTT, 1x Complete protease inhibitor cocktail [Roche]) by three strokes of a Dounce homogenizer. Nuclei were pelleted at 800 x g for 10 min in a refrigerated tabletop centrifuge (Eppendorf). The supernatant was discarded, and pelleted nuclei were resuspended in nuclear buffer and then permeabilized by the addition of 0.1% Triton X-100, followed by incubation on ice for 20 min. Nuclei were again pelleted at 800 x g for 10 min, and the supernatant was removed. The pelleted permeabilized nuclei were then washed twice with an excess volume of US3-specific kinase buffer and finally resuspended in 400 µl of this buffer.
For the in vitro disassembly assay, three 80-µl aliquots of resuspended nuclei were incubated with 1 µg GST-pUS3 or GST-pUS3(K220A) in 120 µl kinase buffer in the presence or absence of 1 mM ATP. After 30 min of incubation at 30°C, the reaction mixture tubes were gently vortexed, and 10 µl of the reaction mixtures (samples designated L) were set aside. All subsequent steps were performed at 0 to 4°C. After centrifugation at 2,000 x g for 5 min, supernatants of each sample (30 µl) were collected and were designated S1. The remaining nuclei were centrifuged again at 10,000 x g for 2 min, and equal volumes of the supernatants were collected and designated S2. Proteins in the L, S1, and S2, samples were denatured in SDS-PAGE sample buffer, electrophoretically separated in denaturing polyacrylamide gels, and transferred to nitrocellulose for immunoblotting with lamin-specific antibodies.

**Two-dimensional gel electrophoresis.** To solubilize lamins, 2 x 10^6 infected Hep2 cells were lysed directly on culture dishes into 250 µl of urea rehydration buffer {9 M urea, 4% CHAPS [3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate], 50 mM DTT, 0.2% ampholytes [Bio-Rad]} containing 1x Complete protease inhibitor cocktail (Roche) and a serine/threonine phosphatase inhibitor (10 mM NaF) (99). Approximately 100 µg of protein was subjected to isoelectric focusing in a Bio-Rad Protean isoelectric focusing cell using an 11-cm-long pH 3.0 to 10.0 nonlinear or a pH 5.0 to 8.0 linear immobilized pH gradient ReadyStrip (Bio-Rad). For separation in the second dimension, the strips were loaded on precast Criterion 10% bis-Tris polyacrylamide gels (Bio-Rad), followed by electrophoresis. The separated proteins were then transferred electrically to nitrocellulose for immunoblotting.

In some experiments, cells were first permeabilized in 0.5% CHAPS on ice for 5 min in the presence of Complete protease inhibitor, clarified by centrifugation at 800 x
g for 5 min at 4°C, and incubated with 400 U of λ-PPase in a supplied buffer (New England Biolabs) at 30°C for 30 min. After this reaction, cells were repelleted and lysed in urea rehydration buffer, followed by two-dimensional electrophoresis and immunoblotting.

**Immunoblotting.** Nitrocellulose sheets bearing proteins of interest were blocked in 5% nonfat milk plus 0.2% Tween 20 for at least 2 h. Membranes were then probed with lamin A/C chicken polyclonal antibody and lamin B goat polyclonal antibody if needed. Primary antibodies were detected by horseradish peroxidase-conjugated rabbit anti-chicken (Jackson ImmunoResearch) and/or bovine anti-goat secondary antibodies (Santa Cruz Biotechnology). Bound immunoglobulin was visualized by enhanced chemiluminescence (Pierce) followed by exposure to X-ray film. Signal intensities were quantified and calibrated using a Syngene Chemi-Genius imaging system and associated GeneTools software as needed.

**Results**

**U₅₃ kinase activity modifies nuclear lamin A/C organization and localization of pU₅₁₃₁ and pU₅₁₃₄ during HSV-1 infection.** Because Hep2 cells are generally resistant to HSV-induced cytoskeletal changes, one goal of these studies was to investigate whether the nuclear lamina of Hep2 cells was affected by U₅₃, as was previously reported with Vero cells (15). In considering the design of such experiments, we noted in previous studies that some commercial lamin A/C antibodies are rendered less immunoreactive upon infection with HSV-1 (162). To avoid the potentially misleading effects of antibody staining, we attempted to use eGFP-lamin A/C in transient-expression assays but found that overexpression of such constructs disrupted the nuclear lamina in many cells, even in the absence of infection (not shown). To avoid
these overexpression artifacts, and under the expectation that cells experiencing associated cytotoxic effects should be outgrown by healthy cells expressing the construct, we sought to produce a cell line stably expressing eGFP-lamin A/C. Hep2 cells were therefore transfected with a plasmid conferring G418 resistance and encoding eGFP fused to lamin A/C. Individual cells expressing eGFP were cloned by FACS, followed by amplification in G418-containing medium. Cell lines expressing fluorescence in association with the nuclear rim were selected for further study.

One such cell line was either mock infected or infected with wild-type HSV-1(F) or virus vRR1204 bearing a mutation inactivating US3 kinase activity [designated US3(K220A) in Fig. 2.1] (174). At 16 h postinfection, cells were fixed, permeabilized, and reacted with chicken antibody directed against UL34 protein. Bound chicken antibody was revealed by reaction with Texas Red-conjugated donkey anti-chicken antibody, followed by confocal microscopy.

In general, the shape of nuclei infected with HSV-1(F) was more irregular than that of uninfected cells. As revealed upon collection of confocal images at high magnification, followed by digital enlargement, eGFP-lamin A/C appeared mostly as a series of filaments and dots linking larger solid areas of fluorescence. Overall, the fluorescence appeared in a reticular pattern in the nuclear rim of both the mock-infected and the HSV-1(F)-infected cells. The reticular pattern was most readily noticeable in optical sections of the tops and bottoms of the cells in which a planar view of the nuclear lamina was most readily apparent (Fig. 2.1B). In both mock-infected and HSV-1(F)-infected cells, the reticular filaments were bordered by spaces that lacked eGFP fluorescence. Although the reticular pattern of NM fluorescence in mock-infected and HSV-1-infected cells did not differ significantly, most cells infected with HSV-1(F) contained larger regions near the bottom of the cell that were devoid of fluorescence (Fig. 2.1B, arrow). In general, the appearance of these cells is in contrast to the
Figure 2.1 Confocal analyses of a Hep2-derived cell line stably expressing eGFP-lamin A/C. Cells were either mock infected (Mock) or infected with 5.0 PFU/cell of wild-type HSV-1(F) (designated F) or mutant U53(K220A), lacking pU53 kinase activity. Cells were fixed and permeabilized at 16 h postinfection and stained with polyclonal anti-pUL34 chicken polyclonal antibody, followed by Texas Red-conjugated secondary antibody, and examined with confocal microscopy. (A) Analysis of optical sections taken through the middle of cells. (B) Optical sections taken at the bottom of cells. Regions indicated in white rectangles in panels D, E, F, J, K, and L are digitally magnified in the panel immediately below them (panels G, H, I, L, M and N, respectively). Coincident signals in the merged images (rightmost column) are indicated by a yellow color. An arrow indicates one region of the infected cell containing less lamin A/C than surrounding areas.
Figure 2.1 (Continued)
appearance reported for HSV-infected Hep2 cells expressing eGFP-lamin A/C transiently in which large discrete holes in the eGFP-specific fluorescence were seen (186). Differences in the two studies include the use of transient expression of eGFP-lamin A/C, followed by infection with 50 PFU/cell in the previous study, versus the use of a stably expressing cell line and an MOI of 5 in the current study.

In contrast to the results obtained upon infection with wild-type virus, the pattern of eGFP-lamin A/C fluorescence was markedly different after infection with the kinase-inactivated US3 mutant virus. Specifically, sites within the reticular pattern lacking eGFP fluorescence were substantially larger in diameter and more likely oval to round. In addition, the larger regions of HSV-1(F)-infected cells lacking the most eGFP in the nuclear rim displaying a paucity of lamin A/C were absent. These results indicated that the kinase activity of pUS3 is necessary for the distribution of lamin A/C normally seen in cells infected with wild-type HSV.

As noted previously, pUL34-specific immunostaining was distributed throughout the nuclear rim in Hep2 cells infected with the wild-type virus HSV-1(F) (163). This was readily apparent in optical cross-sections taken through the middle of cells (Fig. 2.1A). Superficially, eGFP-lamin A/C fluorescence and pUL34-specific immunostaining mostly colocalized in these cross-sections. However, high magnification of planar sections near the bottom of the cells revealed regions containing only pUL34 immunostaining or only eGFP-lamin A/C, as well as regions where eGFP-lamin A/C and pUL34 colocalized (Fig. 2.1B). In the larger regions lacking lamin A/C, seen only in cells infected with HSV-1(F), pUL34 immunostaining could also be detected.

In striking contrast to the localization of pUL34 in HSV-1(F)-infected cells, cells infected with the US3 kinase mutant contained pUL34 almost exclusively within punctate foci at the nuclear rim in optical cross-sections taken near the centers of cells (Fig. 2.1A) (13, 170, 176). These regions invariably corresponded to the well-defined
breaches in the reticular distribution of eGFP-lamin A/C noted above. This dramatic change in the distribution of lamin A/C became even more striking in optical sections that focused on the bottom of infected cell nuclei. In such sections (Fig. 2.1B, lower two rows), foci staining intensely with pUL34-specific antibody coincided with regions that entirely lacked detectable eGFP-lamin A/C. We conclude that the kinase activity of pUL3 is necessary for partial colocalization of pUL34 and eGFP-lamin A/C at the nuclear rim and for the wide distribution of pUL34 throughout the nuclear rim. These data are consistent with previous immunostaining studies indicating that US3 kinase activity is necessary for the normal distribution of pUL34 with Vero cells (13, 170, 176).

To ensure that the above-described observations were not idiosyncratic to the eGFP-lamin A/C cell line, Hep2 cells were also mock infected or were infected with the wild-type HSV-1(F), the US3 kinase-dead viral mutant, or a viral mutant containing a deletion of US3. The cells were fixed at 16 h after infection and were immunostained with antibody against pUL31 and a chicken polyclonal antibody directed against lamin A/C that was insensitive to HSV-induced epitope masking (162).

As shown in Fig. 2.2, lamin A/C localized in a reticular pattern in mock-infected cells that superficially resembled that of cells infected with HSV-1(F), except that some regions near the nuclear rim of infected cells did not stain with lamin A/C antibody (e.g., as shown by the arrow in Fig. 2.2D). pUL31 immunostaining also appeared in a reticular pattern, but this pattern did not completely overlap that of lamin A/C immunostaining. Specifically, regions containing only pUL31 immunostaining or only lamin A/C immunostaining and colocalization of these were observed. Thus, the patterns of pUL31 immunostaining were similar to those of pUL34, as indicated above.

More importantly for the purposes of subsequent experiments herein, planar sections of cells infected with either the US3 deletion virus or the kinase-dead mutant
Figure 2.2 Digital confocal images of Hep2 cells immunostained with antibodies to lamin A/C and pUL31. Hep2 cells were mock infected (Mock) or infected with a US3 deletion mutant (dUS3), a mutant lacking US3 kinase activity [US3 (K220A)], or wild-type virus HSV-1(F) (designated F). Sixteen hours after infection, the cells were fixed, permeabilized, and reacted with preadsorbed rabbit polyclonal antiserum against pUL31 or chicken immunoglobulin Y directed against lamin A/C. Bound antibodies were revealed with Texas Red-conjugated anti-rabbit antibody or FITC-conjugated anti-chicken antibodies. Optical sections closest to the glass coverslips are shown. An arrow indicates regions of the nuclear rim that are mostly devoid of lamin A/C immunostaining. White boxes indicate areas of interest that are magnified in an inset in the same panel.
virus revealed well-circumscribed, roughly circular regions that completely lacked lamin A/C immunostaining. These regions invariably contained most of the pUL31 immunostaining in the cell, i.e., there were very few regions in which pUL31 and lamin A/C colocalized except on the edges of these "holes" in the nuclear lamina. Taken together, the results are reminiscent of the above-described study using the eGFP-lamin A/C-expressing cell line and indicate that US3 kinase and its activity are required for (i) partial colocalization of pUL31/pUL34 with lamin A/C, (ii) for the reticular distribution of lamin A/C and pUL31/pUL34 seen in cells infected with wild-type viruses, and (iii) for the large regions devoid of lamin A/C immunostaining as seen in cells infected with HSV-1(F).

**Adaptation of a US3 in vitro kinase assay and identification of lamin A as a substrate.** The above-described observations indicated that the kinase activity encoded by the US3 gene altered lamin A/C distribution. The simplest mechanism to explain this phenomenon would be that US3 phosphorylates lamin A/C directly, thereby altering lamin A/C's interaction with other proteins in the nuclear lamina. Further studies were undertaken to test this possibility.

Inasmuch as preliminary observations indicated that the Thr-10 residue of lamin A/C (within the sequence Arg7-Arg8-Ala9-Thr10-Arg11-Ser12, from GenBank accession number NP_733821) matched the optimal consensus target, experiments were designed to determine whether lamin A/C could be phosphorylated by the US3 kinase. Thus, US3 fused to GST was expressed in insect SF9 cells and was purified on glutathione beads as previously described (89). As a control, the kinase-inactivated mutant US3(K220A) was purified in the same manner. Coomassie blue staining of the proteins indicated at least 90% purity (Fig. 2.3A).
**Figure 2.3 Analysis of *in vitro* kinase activities of purified GST-pU₅₃ and the mutant GST-pU₅₃(K220A).** (A) Denaturing polyacrylamide gel containing purified GST-pU₅₃ and GST-pU₅₃(K220A) and stained with Coomassie brilliant blue. (B) *In vitro* kinase reaction. The fusion proteins indicated at the top of the left panel were mixed with 100 ng GST-pU₅₃. After 30 min at 30°C in the presence of $[\gamma^32P]ATP$, the reaction mixtures were resolved on a denaturing polyacrylamide gel and visualized by Coomassie (CBB) staining (left panel) and autoradiography (right panel). The position of the approximately 90,000 apparent Mr GST-pU₅₃ fusion protein is shown in the right panel. GST-pU₅₃ (amino acids 205 to 275) is a negative control inasmuch as it does not contain the phosphorylation consensus site in pU₅₃. (C) *In vitro* kinase activities of GST-pU₅₃ and GST-pU₅₃(K220A). Purified GST-pU₅₃ (0.1 µg) or 0.5 µg GST-pU₅₃(K220A) was incubated with $[\gamma^32P]ATP$ in the presence or absence of partially purified GST-pU₅₃ as a potential substrate, followed by subsequent incubation in the presence or absence of λ-PPase. The reaction components were denatured, electrophoretically separated, and stained with Coomassie brilliant blue (CBB, bottom panel) or dried and autoradiographed (upper panel). For comparative purposes, the molecular weight standards from the bottom panel were copied and aligned with the top panel, using Adobe Photoshop. Sizes of the standards are indicated to the left of the figure in thousands [K]).
To test their enzymatic activities, 0.1 µg of the purified proteins was reacted with purified GST, or with pUL31 fused to GST, in the presence of [γ-32P]ATP. As an additional negative control, the pUL34 amino acids 205 to 275 region, comprising a region of pUL34 separate from that of the known US3 phosphorylation site, was also subjected to the kinase assay. The reaction mixtures were then electrophoretically separated on a denaturing polyacrylamide gel and subjected to autoradiography (Fig. 2.3B and C). Consistent with a previous report (89), GST-pUS3 phosphorylated itself (Fig. 2.3B and C, lanes 2 and 5), whereas GST-pUS3(K220A) had no such activity (Fig. 2.3C, lane 4). GST-pUS3 also readily phosphorylated the partially purified pUL31-GST fusion protein (Fig. 2.3C, lane 5). In contrast, GST-pUS3 did not phosphorylate GST: GST fused to the UL34 amino acids 205 to 275 or other E. coli proteins within the GST-pUL31 preparation that were readily visible on the Coomassie blue-stained gel (Fig. 2.3B and C, lane 5). GST-pUS3(K220A) did not phosphorylate GST-pUL31 or other proteins in the E. coli lysate (Fig. 2.3C, lane 7). As expected, addition of bacteriophage λ-PPase significantly reduced the phosphorylation of GST-pUS3 (Fig. 2.3C, lane 3) and eliminated the phosphorylation of GST-pUL31 that was mediated by GST-pUS3 (Fig. 2.3C, lane 6).

Collectively, these data indicate that the US3 gene-encoded kinase activity was functional and specific inasmuch as it induced phosphorylation of two known substrates (pUL31 and pUS3) but not GST or other tested proteins. In addition, the data indicate that the K220A mutation inactivated all detectable US3 kinase activity. Thus, an efficient and specific in vitro kinase assay system was established for testing of additional substrates.

To determine whether lamin A was a target of the US3 kinase, GST fused to lamin A was purified from E. coli and subjected to the in vitro kinase assay. The results, shown in Fig. 2.4, indicated that the lamin A-GST fusion protein was heavily
Figure 2.4 Phosphorylation of lamin A by Us3 kinase in vitro. Full-length lamin A fused to GST was partially purified and reacted with either purified GST-pUs3 or GST-pUs3(K220A) in the presence of $\gamma^{32}$P]ATP, followed by incubation in the presence or absence of $\lambda$-PPase. The reaction components were separated on a polyacrylamide gel and stained with Coomassie brilliant blue (CBB, lower panel) and autoradiographed (upper panel).
phosphorylated in the presence of GST-pUS3 (Fig. 2.4, lane 1) but not in the presence of GST-pUS3(K220A) (Fig. 2.4, lane 3). The phosphorylation of lamin A-GST was eliminated upon incubation with λ-PPase (Fig. 2.4, lane 2). These data indicate that lamin A is an in vitro substrate of the US3 kinase.

**US3 has broader kinase activity than predicted and phosphorylates lamin A/C at multiple sites in vitro.** Because lamin A/C is phosphorylated at different sites depending on the physiological mechanism of lamin disassembly (see the introduction), it was of interest to identify which region of lamin A was phosphorylated by the US3 kinase. To identify the region targeted by the US3 kinase, five subfragments of lamin A fused to GST were purified from lysates of *E. coli* and incubated with GST-pUS3 in the presence of $[^{32}\text{P}]ATP$, followed by denaturing gel electrophoresis, Coomassie blue staining, and autoradiography. The amount of radioactive phosphate in a given protein band was normalized to the amount of protein present in the reaction mixtures and expressed as a percentage of the degree to which full-length lamin A was phosphorylated. The results are shown in Fig. 2.5.

Comparing the radiolabeling of full-length protein with that of truncated lamin A proteins normalized to the amount of protein in individual reaction mixtures revealed that full-length lamin A was phosphorylated to a higher degree than any single subfragment. While only Thr-10 matched the optimal US3 kinase consensus sequence, it was surprising to find that all five subfragments of lamin A were phosphorylated to various extents when reacted with purified GST-pUS3. Although the head (Fig. 2.5, lane H) domain (amino acids 1 to 129) containing the US3 consensus site was phosphorylated to a level that was 14% of that of full-length lamin A/C, the tail 1 (Fig. 2.5, lane T1) domain (amino acids 369 to 519) was even more heavily phosphorylated (to 37% of that of full-length lamin A/C), despite the lack of a pUS3 phosphorylation...
Figure 2.5 (A) Schematic diagram of lamin A primary structure and five subdomains fused to GST. The amino acids (AA) in each peptide subdomain are indicated, assuming that the start methionine codon is 1 [reprinted from reference (162)]. (B) Full-length (FL) lamin A or lamin A fragments fused to GST and reacted with GST-pU₅₃ kinase. The five GST fusion proteins bearing fragments of lamin A as detailed for panel A were purified, mixed with U₅₃-GST and [γ⁻³²P]ATP, and electrophoretically separated and stained with Coomassie brilliant blue (lower panel) and autoradiographed (upper panel). H, head; R1, rod1; R2, rod2; T1, tail 1; T2, tail2; lane C, GST-pU₅₃4 (amino acids 205 to 275).
consensus motif. As expected from previous results, GST-pU134 (amino acids 205 to 275) lacking the Us3 kinase consensus motif was not phosphorylated by pUs3 (Fig. 2.5, lane C).

Taken together, these observations indicate that the full-length lamin A-GST was phosphorylated at multiple sites within lamin A. It follows that, at least in vitro, the previously derived Us3 kinase consensus site is overly restrictive and other sites can also be phosphorylated.

**Us3 induces partial lamin A/C solubilization in purified Hep2 cell nuclei.** Because the phosphorylation of lamins is well known to regulate lamin disassembly, it was of interest to determine whether phosphorylation by Us3 kinase could increase the solubility of lamin A/C. This was of particular interest because the complex structure of the nuclear lamina might preclude the access of pUs3 to lamin A/C or lamin A/C phosphorylation sites. To test whether Us3 kinase activity could modify the nuclear lamina, an in vitro lamina disassembly assay was developed.

Briefly, nuclei from Hep2 cells were isolated, permeabilized, and incubated in Us3 kinase buffer with GST-pUs3 or GST-pUs3(K220A) in the presence or absence of ATP. After the reaction, nuclei were pelleted by centrifugation, and equal amounts of supernatant were collected for analysis. To ensure minimal contamination of the supernatants with insoluble material, nuclei were subjected to a second high-speed centrifugation, and a portion of the supernatants were again collected. Protein in the supernatants and pellets were denatured in SDS, electrophoretically separated, transferred to nitrocellulose, and probed with a chicken polyclonal antibody to lamin A/C.

As shown in Fig. 2.6, the presence of GST-pUs3 and ATP increased the amount of lamin A/C solubilized into the supernatants by approximately twofold compared to that in reaction mixtures containing either GST-pUs3(K220A) plus ATP or GST-pUs3.
Figure 2.6 Immunoblot of total and solubilized lamin A/C from permeabilized Hep2 cell nuclei reacted with wild-type and mutant Us3-encoded kinases. Purified and permeabilized Hep2 cell nuclei were incubated for 30 min in the presence or absence of the indicated fusion proteins and ATP. As a loading control, a sample of the total material (L) was collected immediately after the reactions. S1 was collected after centrifugation at low speed. A second supernatant fraction (S2) was collected after high-speed centrifugation. Proteins in the various fractions were denatured in SDS, electrophoretically separated, transferred to nitrocellulose, and probed with lamin A/C-specific antibody. Bound immunoglobulin was detected by a horseradish peroxidase-conjugated secondary antibody and chemiluminescence. The intensity of the chemiluminescence signals of the lamin A and C bands were quantified using a Syngene Chemi-Genius imaging system and associated GeneTools software, and the amount of chemiluminescence was pooled. The reported percentages represent the intensity of chemiluminescence in that lane relative to that detected in the first lane (sample L of GST-pUs3 plus ATP).
without ATP. This reaction was repeated three times with similar results. We conclude that US3 kinase activity and ATP are sufficient to increase the solubility of lamin A/C from an endogenous preexisting nuclear lamina in vitro.

**Lamin A/C is phosphorylated in HSV-infected cells, and the full spectrum of phosphorylation requires pUS3 kinase activity.** The studies described above were performed in vitro. In order to determine whether US3 phosphorylates lamin A/C in infected cells, two-dimensional electrophoretic analysis of lamin A/C was performed under various conditions. Specifically, Hep2 cells were mock infected or infected with 5.0 PFU/cell of wild-type HSV-1(F) or the US3(K220A) mutant virus and were lysed 16 h postinfection in 9 M urea to solubilize nuclear lamins (99). The proteins were separated by isoelectric focusing in the first dimension and by size in the second dimension. The electrophoretically separated proteins were transferred to nitrocellulose, and the positions of various isoforms of lamin A/C were revealed by immunoblotting with lamin A/C-specific chicken polyclonal antibody. The immunoblots were also probed with a goat antibody directed against lamin B1 and B2 with predicted isoelectric points of 5.11 and 5.29, respectively.

The results (Fig. 2.7) showed that unlike lamin A/C, neither lamin B isoform showed consistent or obvious shifts in isoelectric points under the various conditions tested. The lamin B-specific spots were therefore used as an internal control for alignment of the lamin A/C immunoblots. Upon separation in the first dimension in the pH 3.0 to 10.0 range, lamin A/C from mock-infected cells and cells infected with wild-type HSV-1(F) were broadly distributed throughout a wide pH range. Superficially, the patterns of lamin A/C-specific spots were similar in distribution, but mock-infected cells contained lamin A/C isoforms that were evenly distributed throughout the middle of the range, whereas some individual spots were more defined and easier to discern in
Figure 2.7 Two-dimensional gel electrophoresis and immunoblots of lamin A/C isoforms from mock-infected and HSV-infected cells. Hep2 cells were mock infected or infected with wild-type HSV-1(F) (panels labeled F) or mutant US3(K220A). Proteins were extracted in buffer containing 9 M urea to solubilize lamins and separated by isoelectric focusing in the first dimension using non-linear pH gradients from pH 3.0 to 10.0 (A and B) or linear pH gradients 5.0 to 8.0 (C) and by size on denaturing polyacrylamide gels in the second dimension. The proteins were then transferred to nitrocellulose and probed with polyclonal chicken antibody directed against lamin A/C or a rabbit antibody against lamin B1 and lamin B2. Bound immunoglobulin was detected by appropriate horseradish peroxidase-conjugated secondary antibodies. Chemiluminescence generated by the addition of appropriate substrates was recorded on X-ray film. In some experiments (λ-PPase), proteins were reacted with λ-PPase before electrophoretic separation. For comparative purposes, the three blots in panels A and B were aligned by position of lamin B-specific spots and by distance from the cationic and anionic poles. The positions of the terminal poles on the pH strip of the four immunoblots shown in panel C are also aligned. For orientation, the positions of the lamin A-, B-, and C-specific spots are indicated to the left of the top immunoblot shown in panels A and C.
lamin A/C taken from cells infected with HSV-1(F). This suggested that lamin A/C in 
HSV-1(F)-infected cells are modified differently than in mock-infected cells, but these 
differences are subtle, at least as viewed by two-dimensional gel electrophoresis. In 
contrast, however, US3(K220A)-infected cells lacked many of the more acidic lamin 
A/C species present within the populations of lamin A/C isoforms in HSV-1(F)- 
infected or mock-infected cells. The predicted isoelectric points of lamin A and C are 
6.57 or 6.40, respectively. Separation in the first dimension using a narrower pH range, 
closer to the lamin A/C isoelectric point (pH 5.0 to 8.0) (Fig. 2.7C), confirmed that 
numerous acidic lamin A/C species detected in HSV-1(F)-infected cells were absent 
from cells infected with the US3 kinase mutant.

Because the wide range of distribution of lamin A/C on first dimension might be 
due to posttranslational modifications other than phosphorylation (such as polyade-
nylylation), samples were treated with λ-PPase, followed by two-dimensional electro-
phoretic separation and immunoblotting with lamin A/C antibody. As shown in Fig. 
2.7B and C, phosphatase treatment of lamins from mock-infected cells resulted in a 
dramatic loss of the most acidic species of lamin A/C, indicating that the acidity of 
these species is a consequence of phosphorylation. Moreover, λ-PPase treatment of 
lamin A/C also eliminated some acidic species detected in cells infected with the 
US3(K220A) mutant virus. Thus, US3 kinase activity cannot account for all of the 
lamin A/C phosphorylation in HSV-infected cells.

Taken together, these data indicate that many of the acidic lamin A/C isoforms 
are phosphorylated species whose acidity in infected cells depends on US3 kinase ac-
tivity. We conclude that endogenous lamin A/C is phosphorylated during HSV-1 in-
fec tion and that the full spectrum of phosphorylation requires US3 kinase activity.
Discussion

The effect of US3 on the distribution of lamin A/C in HSV-1-infected cells. Using two different methods, we have shown that US3 kinase activity is necessary for the pattern of lamin A/C distribution in HSV-1-infected Hep2 cells. Whereas lamin A/C and pUL31 and pUL34 immunostaining partially colocalized in a reticular pattern in Hep2 cells infected with wild-type virus, the lack of kinase activity in infected cells caused pUL31 and pUL34 to localize solely within circular regions lacking lamin A/C. These regions were wider in diameter and more circumscribed than most areas of cells infected with wild-type viruses containing pUL31/pUL34 but lacking lamin A/C. Similar conclusions were reached in studies of Vero cells, with the exception that the disruption of the lamina induced by both wild-type and US3(−) viruses was considerably more dramatic than that of uninfected cells, probably as a result of using different cell lines in the two studies (146). Our results are not a consequence of epitope masking because we used an eGFP-lamin A/C-expressing cell line and a polyclonal antibody insensitive to this HSV-induced effect with similar results.

We also noted subtle differences in the distribution of lamin A/C between the lamina of uninfected and HSV-1(F)-infected Hep2 cells. In both cases, the lamina contained small regions that lacked eGFP-lamin A/C. In infected cells, many, but not all, of these contained pUL34 and pUL31, proteins that are incorporated into nascent virions and are required for envelopment of nucleocapsids (169, 170, 175). Unlike the regions in uninfected Hep2 cells, larger regions of the nuclear rim, as viewed in planar sections, seemed to be devoid of lamin A/C in infected cells. It is unclear if these regions also lack lamin B or correspond to regions that communicate with the HSV DNA replication compartment (185). In any event, the concentration of pUL31 and pUL34 in both of the larger regions lacking lamin A/C and small gaps in the reticular
pattern of lamin A/C argue that they both represent potential sites of virion egress. In contrast, the absence of US3 kinase or its activity restricted potential envelopment sites to fewer, very discrete, and round foci that also lacked lamin A/C.

These data suggest that during infection with wild-type virus, some potential envelopment sites (i.e., in the reticular pattern) may be very small or a consequence of localized thinning of the lamina, whereas others, while larger, remain somewhat diffuse. These observations suggest a sophisticated manipulation of the dynamics of nuclear lamins by HSV to promote virus egress while maintaining most laminar structure. This balance depends on US3 kinase activity, inasmuch as the absence of this activity causes very discrete breaches in the lamina as viewed by the distribution of eGFP-lamin A/C. It seems likely that capsids pass through these exaggerated breaches in the lamina and accumulate in the perinuclear space adjacent to these sites. This is supported by the observation that in the absence of US3, perinuclear virions accumulate in evaginations of the NM (170, 176). It would be useful to confirm this by live cell microscopy.

How does US3 kinase activity preclude the presence of discrete "holes" in the nuclear lamina and aggregation of virions in the perinuclear space? We speculate that there are three possibilities. The first possibility is that US3 kinase activity serves to increase lamin turnover and dynamics. Thus, phosphorylation by US3 would remove lamin A/C from some regions where US3 accumulates. Subsequent dephosphorylation of these lamins (not accounted for in these studies) would allow their reincorporation into gaps in the lamina, similar to mechanisms by which the lamina expands during interphase (145). The result is a more dynamic and flexible lamina to facilitate nuclear egress at multiple sites and the absence of most obvious gaps, since these are subsequently filled in. A second, related possibility is that US3's effects on lamin A/C distribution are unrelated to the direct phosphorylation of lamin A/C by US3. In this sce-
nario, US3 kinase would phosphorylate other proteins that would then serve to increase lamin A/C phosphorylation in infected cells, leading to fewer discrete breaches in the lamina. A third possibility is related to the effect of US3 kinase on the localization of pUL31 and pUL34. At least pUL31 likely encodes its own depolymerizing effects on the lamina (168, 190), and its concentration in discrete areas of the nuclear rim in the absence of US3 kinase activity might promote highly localized lamina disruption. The converse of this hypothesis is that US3's kinase activity serves to redistribute the lamina depolymerizing, envelopment machinery (specifically the pUL34/pUL31 complex) more widely throughout the NM. Clearly, further studies are necessary to test specific aspects of these models and to determine whether US3's phosphorylation and displacement of lamin A/C are related to the localization of pUL34/pUL31.

**Lamin A/C phosphorylation in infected cells.** Two-dimensional gel electrophoresis was chosen to characterize lamin A/C in infected and uninfected cells because of its ability to rapidly characterize differences between soluble and insoluble isoforms of lamin A/C. While it might seem surprising that the migration of lamin A/C in uninfected and HSV-1(F)-infected cells was only subtly different, we speculate that some of the phosphorylation of lamin A/C in mock-infected cells is consequential to phosphorylation by cellular kinases like cdc2 kinase in some mitotic cells (150, 151), whereas in HSV-infected cells, lamin A/C is primarily phosphorylated by the US3 kinase (Fig. 2.7). Thus, while collectively the electrophoretic profiles under the two conditions are similar, the mechanisms responsible for lamin A/C phosphorylation in the two circumstances are very different. These interpretations are supported by the observations that (i) HSV-infected cells do not undergo mitosis late in infection (47), (ii) US3 can directly phosphorylate lamin A/C at multiple sites in vitro (Fig. 2.5), and (iii) most highly phosphorylated lamin A/C species in HSV-1(F)-infected cells are not
detected upon infection with the U₅3 kinase mutant (Fig. 2.7). On the other hand, some phosphorylated species of lamin A/C were detected even in the absence of U₅3 kinase activity, suggesting that other kinases also phosphorylate lamin A/C in HSV-1(F)-infected cells.

**Substrates of the U₅3 kinase.** It was surprising that multiple regions of lamin A could be phosphorylated by purified GST-pU₅3, despite the fact that only codon 10 matched the consensus sequence derived from previous analyses of peptide substrates (105, 158, 159). Indeed, some lamin A peptides lacking a bona fide pU₅3 phosphorylation consensus sequence (e.g., amino acids 369 to 519 in the tail domain) were phosphorylated more heavily than the lamin A head domain bearing this motif (Fig. 2.5). In the same reactions, the U₅3 kinase did not phosphorylate a variety of *E. coli* proteins, nor GST, suggesting kinase specificity for lamin A/C. Parenthetically, the consensus phosphorylation site of cyclic AMP-dependent PKA phosphorylation is R-X-S/T or R-R/K-X-S/T, i.e., very similar to and partially overlapping that of pU₅3, although the $K_m$ values of the respective enzymes differ (155). Although it has been speculated that pU₅3 kinase activity functionally overlaps that of PKA in HSV-infected cells (12), the spectrum of U₅3 kinase substrates is likely broader inasmuch as protein kinase A phosphorylates lamin A/C only at N-terminal amino acids 1 to 32 *in vitro* (48).

These data indicate that the U₅3 kinase is more promiscuous than previously thought and suggest the existence of more substrates than previously predicted. Like other promiscuous kinases, it seems likely that the most important factor limiting phosphorylation by pU₅3 would be access to the substrate in the living cell or virion, rather than the presence or absence of the previously reported phosphorylation motif. Given this consideration, it is reasonable to postulate that U₅3 kinase substrates other than lamin A/C could also contribute to alterations in the nuclear lamina of HSV-
infected cells. Candidates include lamin B, various lamin receptors, and the HSV-1 UL31 protein.

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CHAPTER III

EFFECTS OF LAMIN A/C, LAMIN B1, AND VIRAL U₅3 KINASE ACTIVITY ON VIRAL INFECTIVITY, VIRION EGRESS, AND THE TARGETING OF HERPES SIMPLEX VIRUS UL34-ENCODED PROTEIN TO THE INNER NUCLEAR MEMBRANE*

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Abstract

Previous results indicated that the UL34 protein (pUL34) of herpes simplex virus 1 (HSV-1) is targeted to the nuclear membrane and is essential for nuclear egress of nucleocapsids. The normal localization of pUL34 and virions requires the US3-encoded kinase that phosphorylates pUL34 and lamin A/C. Moreover, pUL34 was shown to interact with lamin A in vitro. In the present study, glutathione S-transferase/pUL34 was shown to specifically pull down lamin A and lamin B1 from cellular lysates. To determine the role of these interactions on viral infectivity and pUL34 targeting to the inner nuclear membrane (INM), the localization of pUL34 was determined in Lmna−/− and Lmnb1−/− mouse embryonic fibroblasts (MEFs) by indirect immunofluorescence and immunogold electron microscopy in the presence or absence of US3 kinase activity. While pUL34 INM targeting was not affected by the absence of lamin B1 in MEFs infected with wild-type HSV as viewed by indirect immunofluorescence, it localized in densely staining scallop-shaped distortions of the nuclear membrane in lamin B1 knockout cells infected with a US3 kinase-dead virus. Lamin B1 knockout cells were relatively less permissive for viral replication than wild-type MEFs, with viral titers decreased at least 10-fold. The absence of lamin A (i) caused clustering of pUL34 in the nuclear rim of cells infected with wild-type virus, (ii) produced extensions of the INM bearing pUL34 protein in cells infected with a US3 kinase-dead mutant, (iii) precluded accumulation of virions in the perinuclear space of cells infected with this mutant, and (iv) partially restored replication of this virus. The latter observation suggests that lamin A normally impedes viral infectivity and that US3 kinase activity partially alleviates this impediment. On the other hand, lamin B1 is necessary for optimal viral replication, probably through its well-documented effects on many cellular pathways. Finally, neither lamin A nor B1 was absolutely required for targeting pUL34 to the
INM, suggesting that this targeting is mediated by redundant functions or can be mediated by other proteins.

Introduction

Herpesvirus nucleocapsids are assembled in the nucleus and become enveloped initially at the inner nuclear membrane (INM) in a reaction termed primary envelopment (4). The herpes simplex virus UL31 and UL34 proteins (pUL31 and pUL34, respectively) and their orthologs in other herpesviruses are targeted to the INM and are required for the primary envelopment reaction in many cell lines (70, 113, 169, 170, 181).

Upon infection of restrictive cells such as Vero and Hep2 with herpes simplex virus (HSV) deletion mutants lacking UL31 or UL34, nucleocapsids remain in the nucleoplasm and infectious titers are reduced at least 1,000-fold (22, 110, 175).

The UL34 protein is a type II integral membrane protein of 30,000 \( M_r \), with a predicted 250-amino-acid (aa) nucleoplasmic domain, a 23-aa transmembrane domain, and 5 aa in the perinuclear space (121, 188). The UL31 protein is a largely hydrophobic phosphoprotein of 32,000 \( M_r \) that requires interaction with pUL34 for targeting to the nucleoplasmic face of the INM (121, 169, 230). While pUL31 and pUL34 are incorporated into nascent virions, they are undetectable in extracellular virions, suggesting they are lost from the nascent virion when its envelope fuses with the outer nuclear membrane (62, 170). This observation is consistent with the most prominent model of virion egress in which the de-enveloped nucleocapsid subsequently gains a new envelope by budding into cytoplasmic membranes (194). In the absence of the activity of a serine kinase encoded by alphaherpesvirus genes, such as HSV-1 US3, or upon deletion of the HSV genes encoding glycoproteins B and H (gB and gH, respectively), virions accumulate aberrantly in the perinuclear space, suggesting that US3, gB, and
gH promote the fusion of the nascent virion envelope with the outer nuclear membrane (54, 94, 170, 176, 220). Interestingly, lamin A, pUL31, and pUL34 are phosphorylated by the US3 kinase, and the kinase activity is required for smooth localization of the UL31/UL34 complex throughout the nuclear rim of infected cells (46, 71, 91, 100). Otherwise, the UL31/UL34 complex accumulates aberrantly in discrete foci located at the nuclear rim. At least some of these foci are adjacent to areas of the perinuclear space in which virions accumulate.

How the pUL31/pUL34 complex is targeted to budding sites at the INM is an important but unresolved question. Host proteins are sufficient for targeting the complex to the INM because transiently expressed pUL31 and pUL34 are cotargeted to the nuclear rim in the absence of other viral proteins (79, 107). A current model proposes that most INM-destined integral membrane proteins in the endoplasmic reticulum use a subset of cytosolic nuclear localization signals to target them past the nuclear pore membrane to the INM via a karyopherin β1-driven pathway (26, 34). The proteins then become anchored to the INM by interaction with relatively immobile elements of the nuclear lamina (50). The nuclear lamina comprises a complex network of proteins that line the entire nucleoplasmic surface of the INM, structurally support the nucleus, and interact with chromatin (72). Intermediate filaments made of proteins called lamins comprise the major structural component of the nuclear lamina (59, 118). Lamins consist of two types, designated A and B. An alternatively spliced version of lamin A produces lamin type C that has a unique carboxyl terminus (59). Lamin B is expressed from two homologous genes, which encode lamin B1 and lamin B2 (76, 77). We previously showed that both pUL31 and pUL34 interact with lamin A produced in rabbit reticulocyte lysates, suggesting that this interaction might play a role in anchoring pUL31/pUL34 to the INM (162).
The main goal in initiating the current work was to test whether lamins are required for targeting of the pUL31/pUL34 complex to the inner nuclear membrane.

**Materials and methods**

**Cells and viruses.** Wild-type virus HSV-1(F) and a mutant HSV-1(F), containing a lysine-to-alanine mutation in US3 codon 220 (K220A), have been described and were obtained from B. Roizman and R. J. Roller, respectively. The viruses were grown and titers were determined on Vero cells as described previously.

Hep2 cell lines engineered to express green fluorescent protein (GFP) fused to the N terminus of lamin A/C have been described previously (128). An immortalized lamin A/C knockout mouse embryonic fibroblast (MEF) cell line lacking the gene encoding lamin A/C \((Lmna^{-/-})\) was a gift from Colin Stewart, NIH (201). To enhance cell adherence, \(Lmna^{-/-}\) cells were propagated on cultureware pretreated with poly-L-lysine before seeding. Control MEFs (3T3) were obtained from Robert Weiss (Cornell University). This treatment precluded adherence of lamin B1 knockout MEFs \((Lmnb1^{-/-})\), and corresponding wild-type MEFs \((Lmnb1^{+/+})\) were a generous gift from Karen Reue and have been described elsewhere (217). Pretreatment with poly-L-lysine precluded adherence of these cells and was not used in their propagation. All MEFs were grown in growth medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics), except that the \(Lmnb1^{-/-}\) MEFs and their control wild-type cells were supplemented with additional nonessential amino acids (Gibco) and Glutamax (Gibco).

**Antibodies and immunofluorescence.** Polyclonal chicken antibody against pUL34 was a kind gift of Richard Roller and has been described elsewhere (173). Purified polyclonal chicken immunoglobulin Y (IgY) directed against lamin A/C was made
in our laboratory and has been published previously (162). Mouse monoclonal antibody directed against lamin A/C and goat polyclonal IgG directed against lamin B were purchased from Santa Cruz Biotechnology (catalog numbers sc-7292 and sc-6216, respectively).

For immunofluorescence, MEFs grown on glass coverslips were infected with 5.0 PFU of wild-type HSV-1(F) or U₃3(K220A) HSV-1 per cell. Thirteen hours later, cells were fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min, quenched with 50 mM NH₄Cl for 15 min, and then permeabilized with 0.1% Triton X-100. Nonspecific immunoreactivity was blocked by reaction with 10% human serum in PBS for 1 hour at room temperature and another 1 hour with 10% BlockHen II (Aves Lab). The cells were then reacted with anti-pUL34 chicken IgY diluted 1:400 and goat polyclonal anti-lamin B antibody diluted 1:100 in PBS plus 1% bovine serum albumin for 1 h at room temperature as needed. Bound primary antibodies were recognized by Texas Red- or fluorescein isothiocyanate-conjugated anti-immunoglobulins (Jackson ImmunoResearch).

**One-step viral growth curves.** Lmna⁻/⁻ and 3T3 MEFs grown in 12-well plates pretreated with poly-L-lysine were infected with 5 PFU of HSV-1(F) or U₃3(K220A) HSV-1 per cell. After 1 h of incubation at 37°C, residual surface infectivity was inactivated with a low-pH wash (40 mM citric acid [pH 3.0], 10 mM KCl, 135 mM NaCl). A similar approach was used for the lamin B knockout cells and 3T3 control MEFS infected in parallel, except that cultureware was not pretreated. At the indicated time points, the cultures were frozen and thawed to lyse the cells, and infectious virus was titrated by plaque assay on Vero cells. Mean values of two independent experiments and corresponding standard deviations were calculated and plotted.
GST pull-down assay and mass spectrometry. Approximately $4 \times 10^8$ Hep2 cells were infected with 5 PFU/cell of wild-type HSV-1(F). At 16 h postinfection the cells were lysed in 30 ml modified radioimmunoprecipitation assay buffer {50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, 0.5% Na-deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1 mM EDTA} containing 1x Complete protease inhibitor cocktail (Roche) and phosphatase inhibitor (10 mM NaF, 10 mM Na$_3$VO$_4$) with gentle rocking for 2 h at 4°C. The lysate was clarified by centrifugation at 10,000 x $g$ for 20 min at 4°C, and the supernatant was reacted with glutathione-Sepharose beads (GE) at 4°C. A glutathione S-transferase (GST)-pUL34 fusion protein was affinity purified from lysates of *Escherichia coli* using glutathione-Sepharose beads as described previously (162). The fusion protein was then covalently cross-linked to the Sepharose beads by reaction with 5 mM bis(sulfosuccinimidyl)suberate (Pierce) according to the manufacturer's protocol. GST identically cross-linked to Sepharose beads served as a control. After overnight incubation of the precleared HSV-infected cell lysate with the protein-Sepharose beads bearing GST or GST-pUL34 at 4°C, the beads were washed two times with ice-cold modified radioimmunoprecipitation assay buffer and then three times with 0.5% Tween 20-PBS. The bound proteins were eluted by boiling for 10 min in SDS-polyacrylamide gel electrophoresis sample buffer (10 mM Tris-HCl [pH 8.0], 10 mM β-mercaptoethanol, 20% glycerol, 5% SDS, trace amounts of bromophenol blue). The eluted proteins were then separated electrophoretically on a 10% polyacrylamide gel (SDS-polyacrylamide gel electrophoresis) and visualized by Sypro ruby staining. Bands overrepresented in the pUL34-GST pull-down relative to that with GST were excised and submitted for mass spectrometric analysis at the Biotechnology Resource Center, Cornell University, where the proteins in the gel were digested by trypsin and the masses of derived peptides determined by liquid chromatography-mass spectrome-
try (LC-MS). Peptides were identified by comparison to the NCBI Human database using MASCOT software (Matrix Science).

In separate experiments, the GST-pU134 fusion protein bound to glutathione-Sepharose beads was reacted with lysates of uninfected Hep2 cells, and proteins bound to the beads were eluted, electrophoretically separated, and identified by LC-MS as described above.

**Immunoblotting.** Nitrocellulose sheets bearing proteins of interest were blocked in 5% nonfat milk plus 0.2% Tween 20 for at least 2 hrs. The membrane was then probed with lamin A/C mouse monoclonal antibody. Primary antibody was detected by horseradish peroxidase-conjugated bovine anti-mouse secondary antibody (Santa Cruz Biotechnology). All bound immunoglobulins were visualized by enhanced chemiluminescence (Pierce) followed by exposure to X-ray film. Signals were quantified using NIH Image software.

**Conventional and immunogold electron microscopy.** Cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) and 0.25% glutaraldehyde (Electron Microscopy Sciences) in 0.1 M sodium phosphate buffer, pH 7.4, for 30 min at room temperature and then 90 min at 4°C. Cells were washed three times for 5 min each with the same buffer and then dehydrated with a graduated series of ethanol concentrations (10%, 30%, 50%, 70%, and 100%) at 4°C and then –20°C. This was followed by stepwise infiltration with LR-White resin (catalogue no. 14381; Electron Microscopy Sciences) over the course of 48 h at –20°C. Samples were dispensed into gel capsules, and the resin was polymerized at 50°C for 18 h. Thin sections (60 to 90 nm thick) were collected on 300-mesh nickel grids (Ted Pella, Inc., Redding, CA) and floated on drops for the following procedures.
For electron microscopic immunostaining, grids were blocked with 10% normal goat serum and 10% human serum in PBS-0.05% Tween (PBST) and 1% fish gelatin for 15 min at room temperature and were incubated on drops of pU L34-specific chicken antibody diluted 1:100 in PBST plus 1% fish gelatin for 3 h in a humidity chamber at room temperature. After incubation, grids were washed by brief passage over a series of 3 drops in a high-salt buffer (phosphate-buffered 750 mM NaCl, 0.05% Tween, and 1% fish gelatin) and then 5 drops of 1x PBST and fish gelatin. The secondary antibody, donkey anti-chicken immunoglobulin conjugated with 12-nm colloidal gold, was diluted 1:100 in PBST-1% fish gelatin and reacted for 1 h in a humidity chamber at room temperature. The grids were then washed as before on 6 successive drops of PBST-1% fish gelatin and then rinsed in a beaker of 200 ml of filtered water. Grids were air dried at room temperature prior to staining with 2% aqueous uranyl acetate for 20 min and then Reynolds lead citrate for 7 min. Stained grids were viewed in a Philips 201 transmission electron microscope. Conventionally rendered negatives of electron microscopic images were scanned by using a Microtek Scanmaker 5 and Scanwizard Pro PPC 1.02 software. Positive images were rendered from digitized negatives with Adobe Photoshop software.

Conventional electron microscopy was performed as above except that the cells were fixed in 2.5% glutaraldehyde in 0.1 M Na-cacodylate pH 7.4, followed by 2% OsO4 and embedded in Epon-Araldyte resin (EM Sciences).

Results

**GST-pU L34 interacts with lamins A and B1 in infected cell lysates.** To identify interaction partners that associated with pU L34, GST or GST fused to the N terminus of full-length pU L34 (GST/pU L34) was affinity purified from *Escherichia coli* cells. Lysates of uninfected Hep2 cells or Hep2 cells infected with wild-type HSV-1(F)
were then reacted with GST or the GST/pUL34 fusion protein bound to glutathione-
Sepharose beads. Proteins were eluted from the beads in SDS sample buffer, electrophoretically separated on an SDS-polyacrylamide gel, and stained with Sypro ruby. Bands specifically emphasized or unique to the GST/pUL34 reactions as opposed to reactions with GST alone were excised and digested with trypsin, and the masses of peptides were determined by LC-MS. Within a protein band from infected cell lysates corresponding to approximately 70,000 apparent $M_r$, the masses of 12 tryptic peptides matched tryptic peptides predicted for human lamin A (Table 3.1). Moreover, a band containing protein of an apparent $M_r$ of 75,000 from uninfected Hep2 cells contained five peptides consistent with human lamin B1. These data indicate that pUL34 interacts with lamin A/C and lamin B1 in cellular lysates. Although we cannot exclude the possibility that this interaction is indirect, the result combined with our previous observations showing that lamin A and pUL34 interact in rabbit reticulocyte lysates argue for a direct interaction (162).

To determine whether lamin A/C and lamin B1 were specifically pulled down by GST/pUL34, the material pulled down by pUL34/GST and GST alone was electrophoretically separated on SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with either mouse monoclonal antibody directed against lamin A/C or goat polyclonal antibody directed against lamin B (see Materials and Methods). As shown in Fig. 3.1, although some lamin A/C and B1 was bound to Sepharose beads bearing GST alone, the presence of GST-pUL34 increased the amount of bound lamin A and B1 approximately by five fold as revealed by densitometry. In contrast, lamin C was not overrepresented in the GST/pUL34 pull down, despite the fact that lamins A and C were present in roughly equal amounts in the infected cell lysate. These data confirm the specificity of the interaction between pUL34 and lamins A and B1 in cellular lysates and suggest that lamin C is not bound by GST/pUL34.
Table 3.1 Mass spectrometric parameters of lamin tryptic peptides from GST/pU1.34 pull-down assays.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Exp-$m/z^a$</th>
<th>Exp-$Mr^b$</th>
<th>Calc-$Mr^c$</th>
<th>Peptide$^d$</th>
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$^a$ Mass-to-charge ratio ($m/z$) of experimental peptide.

$^b$ $Mr$ of experimental tryptic peptide.

$^c$ Calculated $Mr$ of lamin peptide from database.

$^d$ Amino acid sequence of matching peptide.
Figure 3.1 Immunoblots of lamin A/C and lamin B pulled down by GST and GST/pUL34. Full-length pUL34 fused to GST or GST alone on glutathione-Sepharose beads was reacted with HSV-1(F)-infected (A) or uninfected Hep2 cell (B) lysates, respectively. Proteins bound to GST fusion proteins were eluted and electrophoretically separated on SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with either a mouse monoclonal antibody against lamin A/C (A) or goat polyclonal antibody against lamin B (B). Bound immunoglobulin was revealed by reaction with appropriate horseradish peroxidase-conjugated secondary antibodies followed by enhanced chemiluminescence. The signal intensities of protein bands were quantified and compared using Image J software, and the relative intensities of individual bands are indicated below corresponding lanes.
Lamin A/C is required for the proper distribution of pUL34 in infected mouse embryonic fibroblasts. Given the above data suggesting that lamin A and pUL34 interact in infected cells, we wanted to determine whether the pUL34-lamin A interaction was involved in targeting pUL34 to the nuclear membrane. To test this hypothesis, immortalized Lmna−/− MEFs or immortalized MEFs were infected with wild-type HSV-1(F), and the localization of pUL34 was determined by indirect immunofluorescence and confocal microscopy. To assess nuclear shape, the cells were also immunostained with antibody directed against lamin B.

Preliminary experiments indicated that cytopathic effects consequential to HSV infection were accentuated in Lmna−/− MEFs as opposed to wild-type MEFs. Specifically, many Lmna−/− MEFs underwent dramatic rounding upon infection and subsequently detached from the culture dish by 14 h after infection. By 16 h postinfection, the Lmna−/− MEF nuclei were highly distorted in shape, with irregular pUL34 staining in punctate foci that associated with the nuclear rim (data not shown). Because the exaggerated changes in nuclear shape at late times after infection might affect pUL34-specific localization indirectly, the cells were fixed at 13 hours postinfection when effects on nuclear shape were less prominent. The cells were then permeabilized and immunostained with antibodies directed against pUL34 and lamin B. The results are shown in Fig. 3.2.

Optical slices recorded from points midway between the top and adherent surface of the cells revealed that pUL34-specific immunostaining localized at the nuclear rim of Lmna−/− cells, despite the absence of lamin A. On the other hand, the smooth distribution of pUL34 seen in infected MEFs was not observed in cells lacking lamin A/C. Rather, considerable areas of the nuclear rim of Lmna knockout cells were stained with lamin B-specific antibody but lacked detectable pUL34-specific immunostaining. Other regions of the nuclear rim contained areas with intense pUL34 immunostaining,
Figure 3.2 Indirect immunofluorescence localization of pUL34 in HSV-1 infected MEFs containing or lacking lamin A/C. Control MEFs or Lmna−/− MEFs were infected with either wild-type HSV-1(F) (A to F) or Us3 kinase-dead mutant virus (K220A) (G to L). At 13 h postinfection, cells were fixed in paraformaldehyde and immunostained for lamin B (green) and pUL34 (red) and visualized by confocal microscopy. Optical sections for display were taken through a point between the top and bottom of cells. Insets contain magnified regions delimited by white rectangles in the same panel.
and these regions often overlapped lamin B immunostaining. We conclude that while lamin A/C is dispensable for localization of pUL34 at the nuclear rim, its presence is required for the normal smooth localization of pUL34 around the nuclear rim.

Given that lamin A/C was required for the normal distribution of pUL34 throughout the nuclear rim, we next wanted to determine whether lamin A/C was necessary for localization of pUL34 at the INM. To test this possibility, Lmna<sup>−/−</sup> or wild-type MEFs were infected with HSV-1(F), and thin sections of the infected cells were reacted with pUL34-specific IgY followed by reaction with 12-nm colloidal gold-conjugated anti-IgY. As shown in Fig. 3.3, pUL34-specific immunoreactivity was associated with both the inner and outer nuclear membranes of both cell types (Fig. 3.3A to C). We therefore conclude that lamin A/C is not required for targeting pUL34 to the INM.

To determine the effects of lamin A/C on HSV-1 replication, Lmna<sup>−/−</sup> and wild-type MEFs were infected with 5.0 PFU/cell of wild-type HSV-1(F), and the virus infectivity was measured at various times after infection. The results, shown in Fig. 3.4, indicated very little difference in the amount of infectious virus produced from the two cell types at all time points. Thus, lamin A/C neither enhanced nor hindered production of wild-type HSV-1(F), at least at high multiplicities of infection.

**Effects of U<sub>S3</sub> kinase activity on viral infectivity and localization of pUL34 in the presence and absence of lamin A/C.** The U<sub>S3</sub> kinase phosphorylates lamin A/C and pUL34, promotes production of infectious virus, and optimizes nuclear egress of virions. To determine whether the effects of U<sub>S3</sub> kinase activity on nuclear egress and pUL34 localization were dependent on lamin A/C, Lmna<sup>−/+</sup> or wild-type MEFs were infected either with a virus containing a mutation in U<sub>S3</sub> (K220A) that precludes
Figure 3.3. Ultrastructural localization of pUL34 in MEFs or Lmna−/− MEFs infected with HSV-1(F) or US3 kinase-dead virus. (A) Wild-type MEFs were infected with HSV-1(F), and localization of pUL34 was determined by reaction with pUL34-specific IgY followed by reaction with anti-chicken immunoglobulin conjugated to 12-nm colloidal gold. Arrows indicate positions of gold beads indicative of pUL34 localization. (B and C) An experiment similar to that in panel A, except that Lmna−/− MEFs were used. Colloidal gold beads are present on the INM and are indicated by arrows. (D) Immunogold localization of pUL34 in MEFs infected with the US3 kinase-dead virus. Insets show increased magnifications of the boxed areas and presence of colloidal gold beads on the INM and perinuclear virions. (E and F) Conventionally fixed images of MEFs infected with US3 kinase-dead virus showing virions within pouches of nuclear membrane. (G) Immunogold localization of pUL34 in Lmna−/− cells infected with US3 kinase-dead virus. Arrows show round extensions of the nuclear membrane emanating into the cytoplasm. The inset shows a negative image of a higher magnification of the boxed area and contains an extension of the INM heavily decorated with pUL34-specific gold beads. The image is displayed as a negative to more clearly demonstrate the gold beads in white. Despite the presence of pUL34, neither virions nor nucleocapsids accumulated within the extensions of nuclear membrane.
kinase activity or with wild-type HSV-1(F) virus. As shown in Fig. 3.2G to L, and consistent with results from other cell lines, both cell types exhibited a distribution of pUL34 that differed from that of cells infected with the virus expressing active US3 kinase. Specifically, pUL34 localized in punctate regions at the nuclear rim, as opposed to the more diffuse distribution seen in the presence of US3 kinase activity. On the other hand, the position of the pUL34-containing foci in Lmna\(^{-/-}\) cells was most often on the cytoplasmic side of the nuclear rim, whereas these foci were located mostly on the nucleoplasmic side of the nuclear rim in normal MEFs. The latter distribution resembled pUL34 localization in other cell lines examined previously (174). Thus, although lamin A/C is not required for the punctate distribution of pUL34 at the nuclear rim of cells infected with the US3 kinase-dead virus, it is required to retain these pUL34-containing foci within the nucleoplasm.

To more precisely analyze the effects of US3 kinase activity on virion egress in the presence or absence of lamin A/C, thin sections of Lmna knockout MEFs and normal MEFs infected with wild-type and US3 kinase-dead viruses were examined by electron microscopy. As shown in Fig. 3.3D, virions accumulated aberrantly in pockets of nuclear membranes of cells infected with the US3 kinase-dead virus in normal MEFs. In most cases, these virion-containing regions were located internal to the nuclear rim. The appearance of infected lamin A knockout cells differed substantially in three respects. First, membranous structures that appeared to represent extensions of the INM were often observed (Fig. 3.3G and inset). Secondly, the space between the INM and ONM was often exaggerated relative to uninfected cells and cells infected with wild-type HSV-1(F). Third, virions were not observed to accumulate to a large extent in the perinuclear space, even upon infection with the US3 kinase-dead virus. We conclude that lamin A is required for (i) the accumulation of virions in discrete
regions of the perinuclear space normally seen in cells infected with the US3 kinase-dead virus and (ii) the morphology of the INM normally seen in infected cells.

To determine whether the concentrations of pUL34 as revealed by immunofluorescence reflected localization of pUL34 within the extensions of the INM observed in Lmna knockout cells, we performed immunogold electron microscopy on these cells infected with the US3 kinase-dead virus. Whereas immunoreactivity with pUL34-specific antibody was not detected in any cell line infected with the UL31 null virus (data not shown), extensions of INM were heavily decorated with gold beads reflecting the localization of pUL34 (Fig. 3.3G, inset). Thus, pUL34 localization in nuclear membrane extensions correlated with the punctate distribution of Lmna knockout cells infected with the US3 kinase-dead virus.

To determine the effects of lamin A/C and US3 kinase activity on viral infectivity, Lmna−/− MEFs or wild-type MEFs were infected with US3(K220A) or HSV-1(F) virus at 5.0 PFU/cell. At various times after infection the cells were lysed and the amount of infectious virus was determined by plaque assay on Vero cell monolayers. As shown in Fig. 3.4, the amount of virus produced by US3(K220A) was reduced by at least 10-fold at all times relative to the amount produced by HSV-1(F) regardless of whether the cells produced lamin A/C or not. Perhaps the most striking effect, as noted in previous studies (164, 174), was a delay in the onset of production of infectious virus in the absence of US3 kinase activity, resulting in a reduction in viral titer by more than 1,000-fold at 6 h postinfection. This delay occurred whether or not lamin A/C was produced. These data indicate that US3 kinase activity enhances both early and late production of infectious virus independently of its effects on lamin A/C.

Pertaining more to the purposes of this report was the observation that infection of Lmna−/− cells with the US3(K220A) virus yielded approximately 10-fold more infectious virus at 12 h postinfection relative to the amount produced in wild type MEFs.
Figure 3.4 One-step growth curve of HSV-1 on normal MEFs or *Lmna*+/– MEFs. Cells were infected with either HSV-1(F) or U53(K220A) at 5 PFU per cell. Residual infectivity was inactivated by a low-pH wash at 1 hour postinfection. At the indicated time points, the whole culture was collected, virus was released by freeze-thawing, and infectivity was titrated on Vero cells. Experiments were performed in duplicate. Mean values are plotted, and standard deviations are represented by error bars.
The discrepancy in titer decreased later in infection, such that at 18 and 24 h after infection infectious virus produced by the Lmna knockout cells was increased only five-fold and threefold, respectively, over that obtained from normal MEFs. These data indicate that expression of lamin A/C decreases production of infectious virus and that US3 kinase activity partially relieves the lamin A/C-dependent impediment to viral replication.

Lamin B1 is dispensable for localization of pUL34 to the nuclear rim but is required for normal viral growth. As shown in Table 3.1 and noted above, peptides in the pUL34/GST pull-down assay were consistent with the predicted masses of tryptic peptides derived from four different regions of lamin B1. To determine whether the interaction between pUL34 and lamin B was specific, the proteins pulled down with pUL34/GST or GST were subjected to immunoblotting with lamin B-specific antibody. As shown in Fig. 3.1B, lamin B-specific immunoreactivity was readily detected in the pUL34/GST pull-down assay, confirming the mass spectrometric analysis. Moreover, densitometry revealed that fivefold less immunoreactivity was detected in the material pulled down with GST. Thus, we conclude that the interaction with lamin B1 was specific, although we could not exclude the possibility that the interaction was indirect.

Given the potential interaction between pUL34 and lamin B1, we determined whether lamin B1 plays a role in localization of pUL31 and pUL34 to the nuclear membrane by using a similar strategy as employed in the study of lamin A. Thus, MEFs lacking lamin B1 were obtained and infected with 5.0 PFU HSV-1(F) or US3(K220A) per cell, and the localization of pUL34 was determined by indirect immunofluorescence 13 h later. As shown in Fig. 3.5, and unlike the effects caused by the absence of lamin A/C, the distribution of pUL34-specific immunostaining in the
Figure 3.5 Confocal indirect immunofluorescence determination of the distribution of pUL34 in HSV-1 infected MEFs containing or lacking lamin B1. Wild-type MEFs (A and C) or Lmnb1+/− MEFs (B and D) were infected with HSV-1(F) or Us3(K220A) virus, fixed at 13 h postinfection, and immunostained for pUL34 as described in the legend to Fig. 2. The green channel and transmitted light from optical sections were taken near the middle of infected cells and digitally superimposed for illustrative purposes. The unaltered image was then converted to grayscale in Adobe Photoshop. Bar, 10 µM (upper panels) or 15 µM (lower panels).
lamin B knockout cells was not visibly altered from that seen in wild-type MEFs. On the other hand, HSV-induced cytopathic effects were accentuated in the lamin B1 knockout cells compared with infected normal MEFs. The alteration in cell morphology as a consequence of virus infection was severe enough to pose difficulties in maintaining the lamin B knockout cells on coverslips late after infection (data not shown).

To determine the effects of the US3 kinase on distribution of pUL34 in the absence of lamin B1, the Lmnb1 knockout cells and wild-type MEFs were infected with the US3(K220A) virus and the localization of pUL34 was determined by indirect immunofluorescence. As shown in Fig. 3.5, pUL34 localized in a similar punctate distribution at the nuclear rim of both cell lines. On the other hand, the foci in the lamin B1 knockout cells appeared less numerous than those observed in the wild-type MEFs infected with the US3 kinase-dead virus.

As shown in Fig. 3.6C, D, and E, representative electron microscopic images of many cells examined revealed that, unlike the case in the lamin A knockout cells, lamin B1 knockout cells infected with the US3 kinase-dead virus contained regions of nuclear membrane bearing virions. A feature unique to these cells infected with this virus was the presence of very densely staining scalloped-shaped extensions of what appeared to be the nuclear membrane (Fig. 3.6D and E). These observations taken together with the indirect immunofluorescence studies indicate that lamin B1 is dispensable for targeting of pUL34 to the nuclear rim of infected MEFs, but it is required for normal nuclear membrane morphology of cells infected with the US3 kinase-dead virus.

Immunogold electron microscopy experiments were also performed to determine the localization of pUL34 in the lamin B1 knockout cells. As shown in Fig. 3.6B, gold beads indicative of the localization of pUL34 localized at the nuclear rim of cells
**Figure 3.6 Localization of pU₃4 in Lmnb⁻/⁻ cells infected with HSV-1(F) or Us3(K220A) viruses.** (A) Low magnification of an HSV-1(F)-infected cell. (B) Section of nuclear membrane of cell infected with HSV-1(F). Positions of gold beads indicative of pU₃4 localization are indicated by arrows. (C) Low magnification of lamin B1 knockout MEF cell infected with Us3 kinase-dead virus. (D and E) Higher magnification of cells infected with Us3 kinase-dead virus, showing several gold beads associated with scalloped dense-staining nuclear membrane and perinuclear virions. As a size standard, capsids are 125 nm in diameter.
infected with the wild-type virus. Moreover, pUL34-specific immunoreactivity was noted at the INM of lamin B1 knockout cells infected with the US3 kinase-dead mutant. This immunostaining localized mostly within the densely staining, scallop-shaped regions of the nuclear membrane or was associated with nearby virions (Fig. 3.6D and E). These data therefore indicate that lamin B1 is dispensable for targeting pUL34 to the INM but necessary for the normal morphology of the nuclear membrane in the absence of US3 kinase activity.

We next examined the effects of the absence of lamin B1 on viral infectivity in the presence and absence of the US3 kinase. As shown in Fig. 3.7, the lamin B1 knockout cells were less permissive to HSV replication than normal MEFs, regardless of whether the US3 kinase was active. Specifically, titers at all time points in wild-type MEFs were at least 10-fold greater than those reached in the lamin B1 knockout cells infected with either wild-type virus or the US3 mutant virus. We therefore conclude that lamin B1 contributes substantially to the production of infectious virus, but this does not reflect a role in targeting pUL34 to the INM.

Discussion

Taken together, the data presented herein indicate that targeting of pUL34 to the INM and aggregation in the absence of US3 kinase activity are not dependent on expression of lamin A/C or B1, despite the observation that these proteins were shown to interact with pUL34 in infected cell lysates. On the other hand, the absence of lamin A/C altered the normal smooth distribution of pUL34 as revealed by indirect immunofluorescence, suggesting that it is directly or indirectly involved in pUL34 distribution within the nuclear rim. In contrast, lamin B1 was entirely dispensable for pUL34 targeting to the nuclear rim, as viewed by indirect immunofluorescence, and to the INM, as viewed by immunogold electron microscopy. Thus, lamin A or lamin A-
Figure 3.7 Growth curves of HSV-1(F) or U₃3 kinase-dead virus in MEFs or *Lmnb1⁺/⁻* MEFs. The experiment was performed similarly to that described in the legend to Fig. 3.4.
interacting components of the nuclear lamina are more relevant to pUL34 targeting than lamin B1 or lamin B1-interacting components. The effects of lamin A on pUL34 targeting may reflect effects of lamin A on lamina mechanics and stiffness, functions which are largely independent of lamin B1 (102). Alternatively, data obtained in this study and in previous work in which lamin A, pUL31, and pUL34 were shown to interact in pull-down assays from rabbit reticulocyte lysates suggest that the lamin A-pUL34 interaction is direct and may play a role in pUL34 targeting (162). The data are also consistent with the observation that emerin, a lamin A-interacting protein, can interact with pUL34 and is displaced in HSV-infected cells in a pUL34-dependent manner (103). Whether the emerin-pUL34 interaction is mediated through interactions with lamin A/C will require further studies. A final possibility is that lamin B1 is involved in INM targeting of pUL34, but lamin A confers redundant functions that compensate for the absence of lamin B1.

An untested but widely accepted hypothesis is that the nuclear lamina poses a barrier that herpesviruses must breach to allow nucleocapsids access to the INM for envelopment. Along these lines, previous observations indicated that the lamina is disrupted in HSV-infected cells in a pUL31/pUL34-dependent manner (15, 128, 162, 185, 186). Several studies have identified mechanisms by which pUL31/pUL34 of HSV may accomplish nuclear lamina disruption. Specifically, protein kinase C-δ and -α have been shown to be recruited to the nuclear membrane in a pUL31/pUL34-dependent manner to augment lamin B phosphorylation, whereas US3 has been shown to phosphorylate lamin A directly in vitro and to be required for its optimal phosphorylation in infected cells (128, 146). Moreover, pUL31/pUL34 may have their own lamina-depolymerizing activities inasmuch as overexpression of these proteins in the absence of other proteins is sufficient to disrupt the lamina, and locally high concentrations of pUL31/pUL34 exaggerate adjacent lamina perforations (15, 128, 162, 185). The obser-
vation in this study that lamin A knockout cells are more permissive to replication of a US3 kinase-dead virus at high multiplicities of infection further argues that at least lamin A poses a barrier to replication (and presumably nucleocapsid envelopment) and that US3 helps to overcome this barrier. Thus, disruption of the lamina might indeed reflect an important role for these proteins in promoting virion budding at the INM.

Despite their relative permissiveness to virus replication at high multiplicities of infection, the lamin A knockout cells exhibited altered NM morphology when infected with the US3 kinase-dead virus. Specifically, exaggerated extensions of the INM and increased space between the INM and ONM were noted. Because these effects were precluded by US3 kinase activity, it follows that substrates of US3 kinase other than lamin A are responsible for these phenotypes. Previous studies reported increased spacing between the INM and ONM upon overexpression of UL34, pUL31/pUL34-dependent alterations of the nuclear lamina, and budding from the INM upon coexpression of pUL31 and pUL34 of pseudorabies virus in the absence of capsids (93, 236). Thus, the effects on nuclear membrane morphology induced by the combined absence of US3 kinase activity and lamin A might be consequential to increased concentration of pUL34/pUL31 in certain regions of the INM. This is consistent with the observation by immunogold electron microscopy that pUL34 was specifically detected in the unusual nuclear membrane extensions observed in this study.

Also interesting to us was the absence of virions trapped within the perinuclear space of the Lmna knockout MEFs infected with the US3 kinase-dead virus, despite the fact that this feature was commonplace in similarly infected normal MEFs. Thus, lamin A is somehow required for the observed retention of virions in discrete regions within the perinuclear space. We speculate that this reflects the general permissivity of primary envelopment in the lamin A knockout cells. Thus, capsids may bud through the INM at multiple sites in the absence of lamin A, whereas the positions of envelop-
ment sites are more restricted by the lamin A-imposed barrier in normal MEFs. The absence of U₅3 kinase activity would still delay fusion of the virion envelope and ONM, but the wide dispersal of virions in the perinuclear space of Lmna knockout cells would preclude virion aggregation. Alternatively, we cannot rule out the possibility that the nuclear envelope is generally more fragile in the lamin A knockout cells, thus precluding stable association of virions within the perinuclear space of cells as they are pelleted prior to fixation for electron microscopy.

We also noted that pUL34-containing regions mostly localized external to the nuclear membrane in lamin A knockout cells infected with the U₅3 kinase mutant but internal to the nuclear membrane in MEFs or lamin B1 knockout cells infected with this virus. We speculate that lamin A, by enhancing lamina stiffness, restrains locally high concentrations of the pUL31/pUL34 complex from inducing cytoplasmic protrusions of the nuclear membrane, although we cannot exclude more indirect mechanisms.

In striking contrast to the results with lamin A, Lmnb1 knockout MEFs were less permissive to viral replication at high multiplicities of infection, and this was observed in both the presence and absence of U₅3 kinase activity. The role of lamin B1 in HSV infection is unclear, but it is required for a number of important nuclear functions including transcription, DNA replication, cell signaling, and optimal reassembly of the nucleus after mitosis (196). Thus, disturbance of any of these or related functions might render the cells less permissive by impairing the machinery that the virus ultimately needs to commandeer for optimal infection. This may be one reason why alteration of the nuclear lamina during viral infection is mostly limited to very discrete regions. Such a strategy might limit detrimental effects on the cell while still allowing nucleocapsids to bud through the INM.
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CHAPTER IV

PHOSPHORYLATION OF THE U₅₆.31 PROTEIN OF HERPES SIMPLEX VIRUS 1 BY THE U₅.3 ENCODED KINASE REGULATES LOCALIZATION OF THE NUCLEAR ENVELOPMENT COMPLEX AND EGRESS OF NUCLEOCAPSIDS

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Abstract

Herpes simplex virus 1 (HSV-1) nucleocapsids bud through the inner nuclear membrane (INM) into the perinuclear space to obtain a primary viral envelope. This process requires a protein complex at the INM composed of the UL31 and UL34 gene products. While it is clear that the viral kinase encoded by US3 regulates the localization of pUL31/pUL34 within the INM, the molecular mechanism by which this is accomplished remains enigmatic. Here, we have determined that (i) the N-terminus of pUL31 is indispensable for the protein’s normal function and contains up to 6 serines that are phosphorylated by the US3 kinase during infection. (ii) Phosphorylation at these 6 serines was not essential for productive infection, but was necessary for optimal viral growth kinetics. (iii) In the presence of active US3 kinase, changing the serines to alanine caused the pUL31/pUL34 complex to aggregate at the nuclear rim and caused some virions to accumulate aberrantly in herniations of the nuclear membrane, much as in cells infected with a US3 kinase-dead mutant. (iv) Replacement of the 6 serines of pUL31 with glutamic acid largely restored the smooth distribution of pUL34/pUL31 at the NM, and precluded the accumulation of virions in herniations whether or not US3 kinase was active, but also precluded optimal primary envelopment of nucleocapsids. These observations indicate that phosphorylation of pUL31 by pUS3 represents an important regulatory event in the virion egress pathway that can account for much of pUS3’s role in nuclear egress. The data also suggest that the dynamics of pUL31 phosphorylation modulate both primary envelopment and subsequent fusion of the nascent virion envelope with the outer nuclear membrane.

Introduction

The UL31 and UL34 proteins of herpes simplex virus 1 (HSV-1) form a complex that locates at the inner nuclear membrane (INM) of infected cells (163, 164). This
complex is essential for budding of nucleocapsids through the INM into the perinuclear space (163, 173). pUL34, an integral membrane protein with a 247 amino acid nucleoplasmic domain, binds pUL31 and holds the latter in close approximation to the INM (117, 163, 232, 235). Both proteins become incorporated into nascent virions indicating they directly or indirectly interact with nucleocapsids during the budding event (164). Interestingly, co-expression of the pseudorabies virus homologs of HSV pUL31 and pUL34 are sufficient to induce budding from the INM in the absence of other viral proteins (91).

The most prominent model of nuclear egress proposes that the step following primary envelopment involves fusion of the perinuclear virion envelope with the outer nuclear membrane (ONM), allowing subsequent steps in which the de-enveloped capsid engages budding sites in the Golgi or trans-Golgi network (123, 194). The US3 protein is a promiscuous kinase that phosphorylates pUL31, pUL34, and several other viral and cellular components (13, 14, 20, 89, 103, 128, 129, 152, 153, 156, 174). In the absence of pUS3 kinase activity (i) virions accumulate within the perinuclear space, often causing distensions of this space that herniate into the nucleoplasm (93, 164, 174), (ii) the pUL31/pUL34 complex is mislocalized at the nuclear rim from a smooth pattern to discrete foci that accumulate adjacent to nuclear membrane herniations and (iii) the onset of infectious virus production is delayed (128, 174).

Aberrant accumulations of perinuclear virions similar to those observed in cells infected with US3 kinase-dead viruses have been observed in cells infected with viruses lacking the capacity to produce glycoproteins H and B (gH and gB, respectively) (56). Because these proteins are required for fusion with the plasma membrane or endocytic vesicles during HSV entry (3, 65, 161), it has been speculated that the accumulation of perinuclear virions in the absence of gH and gB reflects a failure in the apparatus that normally mediates fusion between the nascent virion envelope and
ONM. By extension of this hypothesis, pU_S3 might act to trigger this perinuclear fusions event.

The substrate(s) of pU_S3 kinase responsible for the altered localization of the pU_L31/pU_L34 complex and aberrant accumulation of perinuclear virions is unknown. In one study to identify such a substrate, it was determined that precluding phosphorylation of pU_L34 was not responsible for the nuclear egress defects induced by the absence of pU_S3 or its kinase activity (174). The current study was therefore undertaken to investigate the hypothesis that pU_S3-mediated phosphorylation of pU_L31 is critical to regulate nuclear egress. Evidence presented herein indicates that aspects of the U_S3 kinase-dead phenotype, including retention of virions in the perinuclear space, mislocalization of the pU_L31/pU_L34 complex, and delayed onset of virus replication, can be replicated by precluding pU_L31 phosphorylation in the presence or absence of pU_S3 kinase activity. The data also suggest that dynamic phosphorylation of pU_L31 is important for efficient primary envelopment of nucleocapsids.

Materials and methods

**Cells and viruses.** Wild type virus HSV-1(F), U_L31 null virus, and mutant virus containing a Lysine to Alanine mutation in U_S3 codon 220 (K220A), have been described and were obtained from B. Roizman and R. J. Roller, respectively (24, 49, 174). The HSV-1(F) and U_S3(K220A) viruses were propagated and titered on Vero cells, whereas the U_L31 null virus was grown and titered on a U_L31-expressing rabbit skin cells described previously (107).

**Plasmids.** For transient expression, the U_L31 gene was cloned into the pcDNA3 vector (Invitrogen) or into the pBudCE4.1 vector (Invitrogen) with a FLAG epitopic tag on the 3’ end as indicated. Full length U_L31 ORF in pcDNA3 was previously de-
scribed (163). The U\textsubscript{1}31-pcDNA3 then served as a template for other pcDNA3 or pBudCE4.1 constructs using PCR primers listed in Table 4.1. pGEX4T vectors (Amersham) were used for GST fusion expression plasmids. The construction of GST fused to full length U\textsubscript{1}31 was also reported previously (163). Standard cloning methods were used to generate a series of GST fusion subclones. Briefly, U\textsubscript{1}31 sequences were amplified from HSV-1 (F) genomic DNA using the primers listed in Table 4.1. The amplicons were first ligated into the pCR2.1 vector (Invitrogen) using TA cloning, and were flanked by EcoRI restriction sites as a result. The EcoRI fragments containing U\textsubscript{1}31 sequences were subcloned into the EcoRI site of the pGEX4T vector (Amersham) in frame with the GST gene. To generate the point mutant plasmids, U\textsubscript{1}31 were amplified from the corresponding mutant viral BAC DNAs bearing such mutations (described below); these amplicons were then cloned into the pGEX4T vector at the EcoRI and XhoI sites.

**Recombinant viruses and BAC mutagenesis.** A series of recombinant viruses were constructed using en passant mutagenesis of a bacterial artificial chromosome (BAC) containing the entire HSV-1(F) genome as previously described (205). Briefly, a PCR amplicon bearing fragments containing a Kan resistance cassette, Sce-I restriction endonucleae site and desired mutations flanked by homologous regions to the target gene was generated with primer pairs listed in Table 4.2 and pEPkan-S plasmid as template. The resulting amplicons were electroporated into recombination competent GS1783 E. coli, which harbors the target BAC and a SceI endonuclease gene integrated into its genome (a kind gift of Greg Smith). Following Kanamycin resistance screening and restriction length polymorphism (RFLP) confirmation, the Kan\textsuperscript{R} sequence was removed by RED recombination between internal homologous sequences that were generated through SceI cleavage, which was initiated by arabinose-induced
Table 4.1 Vectors and primers used to generate Ul31 subclones (restriction sites used for cloning were underlined and Flag sequence was colored in red).

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>UL31 codons</th>
<th>Vectors</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ul31(dN)-pCDNA3</td>
<td>AA 45-306</td>
<td>pcDNA3</td>
<td>5’-ATACTCGAGATGCCGCTCAG G-3’ 5’-TATACTCGAGCTACGGCGAGGAAAC-3’</td>
</tr>
<tr>
<td>Ul31-Flag</td>
<td>AA 1-306</td>
<td>pBudCE 4.1</td>
<td>5’-ATACTCGAGACCTATGTATGA CACCGACC-3’ 5’-ATAAGATCTTTACTTATCCTGT CATCC TTGTAAATCCGGCGAGGA AACTC-3’</td>
</tr>
<tr>
<td>Ul31(dN)-Flag</td>
<td>AA 45-306</td>
<td>pBudCE 4.1</td>
<td>5’-ATACTCGAGACCTATGTATGA CACCGACC-3’ 5’-ATAAGATCTTTACTTATCCTGT CATCC TTGTAAATCCGGCGAGGA AACTC-3’</td>
</tr>
<tr>
<td>GST-Ul31 (1-50aa)</td>
<td>AA 1-50</td>
<td>pCR2.1, pGEX4T-2</td>
<td>5’-ATGTATGACACCGACCCCCAT C-3’ 5’-TTTGCGGGCGTGAGGC-3’</td>
</tr>
<tr>
<td>GST-Ul31 (52-165aa)</td>
<td>AA 52-165</td>
<td>pCR2.1, pGEX4T-1</td>
<td>5’-TCTCGAGATGGAGGAGCTGTG TTTAC-3’ 5’-TCTAGGCAACACGACC-3’</td>
</tr>
<tr>
<td>GST-Ul31 (159-265aa)</td>
<td>AA 159-265</td>
<td>pCR2.1, pGEX4T-1</td>
<td>5’-GCTCGAGATGGTGGCTGCCTGCT GTG-3’ 5’-CCTAGTCCGGGGGTGTGTCTCTGT GTT-3’</td>
</tr>
<tr>
<td>GST-Ul31 (255-306)</td>
<td>AA 259-306</td>
<td>pCR2.1, pGEX4T-1</td>
<td>5’-CCTCGAGATGGCAACCGAG AACC-3’ 5’-CCTACGGCGAGGAAAACCTC-3’</td>
</tr>
<tr>
<td>GST-Ul31(SA3)</td>
<td>AA 1-306</td>
<td>pGEX4T-1</td>
<td>5’-TAGAATCCATGTATGACACCC ACCCCCACC-3’ 5’-TATACTCGAGCTACGGCGAGGAAAC-3’</td>
</tr>
<tr>
<td>GST-Ul31(SA6)</td>
<td>AA 1-306</td>
<td>pGEX4T-1</td>
<td>5’-TAGAATCCATGTATGACACCC ACCCCCACC-3’ 5’-TATACTCGAGCTACGGCGAGGAAAC-3’</td>
</tr>
</tbody>
</table>
Table 4.2 Primers for BAC mutagenesis (introduced mutations were colored in red).

<table>
<thead>
<tr>
<th>BAC</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>UL31(dN)</td>
<td>5’-ATC TCG CTC CTG TCC CTG GAG CAC ACC CTG TGT ACC TAT GCC GCC TCA CGC CCG CAA ACA TAG GGA TAA CAG GGT-3’</td>
</tr>
<tr>
<td></td>
<td>5’-GCT CGT GTA AAC ACA GCT CCT GTT TGC GGG CTG GAG GCG GCA TAG GTA CAC AGG GTG TGC GCC AGT GTT ACA ACC-3’</td>
</tr>
<tr>
<td>UL31(SA3)</td>
<td>5’-TCG ATC TCG CTC CTG TCC CTG GAG CAC ACC CTG TGT ACC TAT GTA TGA CAC CGA CCC CAT CGC CGC GGC GCC CGG CCC TAT CAC GCC AAG GAG CGC CGG GGG GCG CGC TCC TCT GCC TGG GCC GCC GTA GGG ATA ACA GGG T-3’</td>
</tr>
<tr>
<td></td>
<td>5’-GGC CCC GAT AGC GCT GGC GCT CGT GTA AAC ACA GCT CCT GTT TGC GGG CTG GAG GCG GCA GTG C CT TCC GGG C GAC GCA CCA CGC CCA GAG TCC CGC CGG CCG CAG AGG AGC G CG CCC GCC GGC GCT CCT TGC CAG TGT TAC AAC C-3’</td>
</tr>
<tr>
<td>UL31(SA6)</td>
<td>5’-GCC CGG GCC CTA TCA CGG CAA GGA GCG CCG GCG GCC GCG CCC TGC TGC GGC CGG CGG GAC TCT GGG CGT GTA AAC ACA GCT CCT GTT TGC GGG CTG GAG GCG GCA GTG CCT TCC GGG CCC GAC GCA CCA CCC GAC GCA CCA CGC CCA GAG TCC CGC CGG CCG TGC GAG TCG TAC AAG C-3’</td>
</tr>
<tr>
<td>UL31(SAR)</td>
<td>5’-GGG CCT CCC GGA AGA GCC TGC CGC CTC ACG CCC GCA AAC AGG AGC TGT GTT TAC ACG AGC TAG GGA TAA CAG GGT-3’</td>
</tr>
<tr>
<td></td>
<td>5’-GCT CGT GTA AAC ACA GCT CCT GTT TGC GGG CTG GAG GCG GCA TAG GTA CAC AGG GTG TGC GCC AGT GTT ACA ACC-3’</td>
</tr>
</tbody>
</table>

Amplicon generated using above primers were PCR fused with a second amplicon generated from wild type HSV-1 (F) genomic DNA with primer pair 5’-tgcatagcgttgctgcc-3’ and 5’-tttgcgggcgtgagc-3’ to obtain the 5’ extension required for homologous recombination.
<table>
<thead>
<tr>
<th>BAC</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>U₅31(SAE)</strong></td>
<td>5'-CCC GGG CCC TAT CAC GGC AAG GAG CGC CGG CGG GCG GCG GAA GAA GCG GCC GCC CTA GGG ATA ACA GGG T-3’</td>
</tr>
<tr>
<td></td>
<td>5’-GTG TAA ACA CAG CTC CTG TTT GCG GGC GTG AGG CGG CAG TTC CTT CCG GGC CCG AC CAC CAC GCC CAG AGT CCC GCC GGC CGC CAG TGT TAC TAC AAC C-3’</td>
</tr>
<tr>
<td><strong>U₅31(SE6)</strong></td>
<td>5’-TCG ATC TCG CTC TCT TCC CTG GAG CAC ACC CTG TGT ACC TAT GTA TGA CAC CGA CCC CAT CGC CGC GGC GAA CGG CCC GGG CCC TAT CAC GGC AAG GAG CGC CGG CGG GAA CGC GAC GAC GGC GCA GTT CCT TTC C CGG CCC CTG CTT CTT GCC GGC GCC GCT CCT TGC CAG TGT TAC AAC C-3’</td>
</tr>
<tr>
<td></td>
<td>5’-GCC CCC GAT AGC GCT GGC GCT CGT GTA AAC ACA GCT CCT GTT TGC GGG GTG GCG GCA GTT CCT TCC GGT CGG CCC GAC GCA CCA CGC CCA GAG TCC CGC CGG CCG CTT CTT GCC GGC GCC GCT CCT TGC CAG TGT TAC AAC C-3’</td>
</tr>
<tr>
<td><strong>U₅31(SE6)/U₅3(K220A)</strong></td>
<td>5’-TGA CAG CAG CCA TCC AGA TTA CCC CCA ACG GGT AAT CGT GCC GCC GGG GTG GTA CAC GAG CAT AGG GAT AAC AGG GTA ATC GAT TT-3’</td>
</tr>
<tr>
<td></td>
<td>5’-CAG TCG CGC CTC GTG GCT CGT GGT GTA CCA CCC CGC GCC CAC GAT TAC CCG TTG GGG GTG CCA GTG TTA CAA CCA ATT AAC C-3’</td>
</tr>
</tbody>
</table>
SceI expression in GS1783 *E. coli*. Positive clones were verified through Kanamycin selection and further by RFLP and DNA sequencing (not shown). Rabbit skin cells were transfected with the BAC and a plasmid encoding FRT recombinase to remove the BAC sequences as described previously (107). Viral plaques were propagated into viral stocks using Vero cells.

All the BACs were designated according to their genotypes. Before obtaining the UL31(SA6) BAC, the sequence of the UL31 gene from 3 to 132 nucleotides was first deleted from wild type HSV-1 (F) BAC, producing an intermediate BAC designated UL31(dN). The deleted region bearing designed mutations was then inserted, thus changing codons 11, 24 and 40 from serine into alanine. The resulting BAC was named UL31(SA3), which then served as the parental BAC to generate UL31(SA6) BAC by changing serine codons 26, 27 and 43 to alanine. The repair BAC UL31(SAR) was created by repeating the deletion step starting with the UL31(SA6) BAC, thus regenerating the intermediate UL31(dN) genotype, followed by insertion of wild type sequences. In a separate experiment, this UL31(dN) BAC served as the recipient of mutations changing codons 11, 24, 26, 27, 40 and 43 to those encoding glutamic acid. This BAC was designated BAC UL31(SE6). The UL31(SAE) BAC was derived from the BAC UL31(SA6) by mutating alanine codons 26, 27 and 43 into glutamic acid codons. Incorporation of mutations substituting lysine codon 220 of US3 with alanine in the UL31(SE6) BAC led to the double mutant BAC UL31(SE6)/US3(K220A).

**In vitro US3 kinase assays.** A kinase assay was performed using purified GST-pUS3 essentially as described previously (128). Briefly, 0.1 μg GST-pUS3 was incubated separately with approximately 1 μg of pUL31-GST fusion proteins partially purified from *E. coli* and bound to glutathione Sepharose beads. The kinase reaction was performed for 30 minutes at 30°C in 50 μl pUS3 specific kinase buffer (50mM Tris
[pH9.0], 20mM MgCl2, 0.1% NP40, 1mM DTT) containing 10 μM ATP and 10 µCi [γ-32P] ATP (Amersham). The Sepharose beads bearing the fusion proteins were then washed three times with TNE buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA). The proteins were eluted in SDS-PAGE sample buffer (10 mM Tris-HCl [pH 8.0], 10 mM β-mercaptoethanol, 20% glycerol, 5% SDS, trace amounts of bromophenol blue) and subjected to electrophoresis in a 12% polyacrylamide gel in the presence of 0.1% SDS. Gels were then stained with Coomassie brilliant blue, dried and autoradiographed using X-ray film (Pierce).

**Transient complementation assay.** Hep2 cells were seeded at 90% confluence in 12 well plates and were transfected with 1.6 μg plasmid DNA using lipofectamine (Invitrogen). After 20 hr incubation at 37°C, the cells were infected with 5.0 PFU per cell of the U₃13 null virus. At 24 hpi, whole cell cultures were lysed for viral titering on U₃13 expressing rabbit skin cells. Experiments were performed in duplicate. Means and standard deviations were calculated and plotted.

**One-step viral growth assay.** 100% confluent Vero cells grown in 12-well plates were infected with 5.0 PFU per cell of the indicated viruses. After 1 hr incubation at 37°C, residual surface infectivity was inactivated with a low-pH wash (40mM Citric Acid [pH 3.0], 10mM KCl, 135mM NaCl). At the indicated time points, the cultures were frozen and thawed to lyse the cells, and infectious virus was titrated by plaque assay on Vero cells. The mean values of two independent experiments and corresponding standard deviations were calculated and plotted.

**Antibodies and Immunofluorescence assay.** Polyclonal chicken antibody against pU₃4 was a kind gift from R. J. Roller. Rabbit anti-pU₃1 antisera were pre-
pared in our laboratory and was described previously (163). M2 mouse monoclonal antibody against the Flag epitopic tag was purchased from Sigma.

Hep2 cells growing on glass coverslips were mock infected or were infected with the indicated viruses at an MOI of 5 PFU/cell for 16 hrs. Cells were fixed with 3% PFA for 15 min. In experiments where the pre-adsorbed pUL31-specific rabbit polyclonal antibody was to be used, the cells were treated for another 15 min at −20°C in methanol. The cells were then permeabilized with 0.1% Triton-X 100 and reacted with 10% human serum in phospho-buffered saline (PBS) to block nonspecific immunoreactivity, and subsequently probed with the pre-adsorbed pUL31 antiserum diluted 1:50 in PBS supplemented with 1% BSA. Thereafter, 10% BlockHen II (Aves Lab) was used for a second round of blocking before probing with a 1:400 dilution of chicken anti-pUL34 polyclonal antibody. Bound primary antibodies were recognized by corresponding secondary antibodies conjugated with FITC or Texas Red, respectively (Jackson ImmunoResearch).

In some experiments, cells were transfected with equal amounts of U131-encoding plasmid DNA for 20 hrs prior to infection using lipofectamine (Invitrogen). Infected cells were processed for indirect immunofluorescence in the same way except that no methanol fixation was used, and the M2 antibody directed against the Flag epitope was diluted 1:100. Images were visualized and recorded using an Olympus confocal microscope. Digital images were processed with Adobe Photoshop.

**Electron microscopy.** Cells were first fixed with 2.5% glutaraldehyde in 0.1 M Na-cacodylate pH 7.4 (Electron Microscopy Sciences) for 30 min at room temperature and then 90 min at 4°C. After three 5 minute washes with the same buffer, the cells were fixed with 2% OsO4 in the same buffer and rinsed again. Cells were then dehydrated at 4°C using a graduated series of ethanol concentrations (20%, 50%, 70%,
100%), and subsequent acetone/ethanol mixture (1:1), and finally, 100% acetone. This
was followed by stepwise infiltration with Epon-Araldyte resin (EM Sciences) over
the course of 64 hrs. Samples were dispensed into beem capsules, and the resin was
polymerized at 80°C overnight. Thin sections (60 to 90 nm thick) were collected on
300-mesh copper grids and viewed in a Philips 201 transmission electron microscope.
Conventionally rendered negatives of electron microscopic images were scanned by
using Microtek Scanmaker 5 and Scanwizard Pro PPC 1.02 software. Positive images
were rendered from digitized negatives with Adobe Photoshop software.

Results

The N-terminal domain of pUL31 is functionally important to nuclear egress.
The HSV-1 U_L31 protein is highly conserved among members of the herpesviridae.
Interestingly, the first 50 amino acids of the N-terminal of U_L31 protein share little
homology at the primary sequence level with other herpesvirus homologs. The clus-
tered charged residues and hydrophilic nature distinguish this region from the remain-
der of HSV-1 pUL31, which is mostly hydrophobic (not shown). To investigate the
functional relevance of this charged region of pUL31, a truncated U_L31 gene U_L31(dN)
lacking the region from codon 2 to 44 was transfected into Hep2 cells and its ability to
support replication of the U_L31 null HSV-1 mutant was determined by plaque assay.
Plasmids bearing the full length U_L31 open reading frame and vector DNA (pCDNA3)
served as positive and negative controls, respectively. Viral infectivity at 24 hr post
infection was titrated on U_L31 complementing cells.

As shown in Fig. 4.1A, expression of full length pUL31 increased the yield of in-
fec tious virus more than 100 fold above the background levels produced by transfec-
tion of vector DNA. In contrast to this result, the plasmid encoding N-terminally trun-
cated pUL31 produced only a 5-fold enhancement of viral yield over background. This
Figure 4.1 Functional analysis of the N-terminus of pUL31. (A) Ability of pUL31 mutants to support replication of a U_L31 null virus. Hep2 cells were transfected with a pcDNA3 vector plasmid or plasmids bearing full length U_L31(FL) or the N-terminally deleted U_L31(dN) used in panel A. The cells were then infected with the U_L31 null virus. Infectivity at 24 hpi was determined by freeze/thawing the infected cells, followed by titration on U_L31 complementing cell lines. Experiments were performed in duplicate. Each histogram represents the mean value, and the mean +/- standard deviations are indicated by error bars. (B) pUL31 localization and ability to target pUL34 to the nuclear rim. Hep2 cells were transfected with plasmids encoding Flag-tagged full length U_L31 [U_L31(FL); a to c] or U_L31 lacking the first 43 amino acid codons [U_L31(dN)] (d to f). The cells were then infected with a U_L31 null virus. Cells were stained with M2 antibody directed against the Flag tag (green) and pUL34 (red), and images were captured by confocal microscopy.
result indicated that the N-terminal domain is important for full function of the HSV-1 Ul31 protein.

To determine the role of the pUl31 N-terminus in targeting the pUl31/pUl34 complex to the nuclear rim, Hep2 cells were transfected with plasmids encoding either full length pUl31, or the N-terminally truncated protein fused to a Flag epitopic tag. The cells were subsequently infected with the Ul31 deletion virus and were fixed and immunostained with the FLAG-specific antibody and polyclonal pUl34-specific IgY. As shown in Fig. 4.1B, the full length pUl31-Flag-specific immunostaining colocalized with pUl34-specific immunostaining at the nuclear rim. In contrast to this result, the cells expressing pUl31(dN), and subsequently infected with the Ul31 deletion virus contained Flag-specific immunostaining (denoting the location of truncated pUl31) mostly in the cytoplasm. Under these conditions, pUl34 specific immunostaining co-localized with pUL31 in the cytoplasm adjacent to the nucleus. The pattern of pUl34-specific immunostaining was reminiscent of the pattern of pUl34 distribution in cells infected with Ul31 null viruses (107, 163), and was similar whether or not pUl31-Flag was detected in a particular cell. These observations imply that the N-terminus of Ul31 protein is required for strict localization of the pUl31/pUl34 complex to the nuclear rim.

The pUl31 N-terminal domain harbors multiple phosphorylation sites of the viral Us3 kinase. The N-terminal sequence of pUl31 is arginine and serine rich, which is reminiscent of Us3 serine/threonine kinase phosphorylation motifs (155). Because it was already known that pUl31 was a substrate of pUs3, we hypothesized that Us3 kinase phosphorylated pUl31 at least some of the putative Us3 phosphorylation motifs located at its N-terminus. pUs3 phosphorylation sites on pUl31 were therefore mapped as follows. The Ul31 codons 1-50, 51-165, 155-165, and 255-306 (Fig. 4.2A)
were fused to the gene encoding GST, and the corresponding fusion proteins were purified from *E. coli* by GST affinity chromatography. The purified proteins were then incubated with recombinant baculovirus-expressed US3 kinase in buffer containing [γ-32P]-ATP. Reaction components were then separated on an SDS polyacrylamide gel, and the gel was stained with Coomassie brilliant blue, dried and exposed to X-ray film. As shown in Fig. 4.2B, equal amounts of all four fragments were present in the reactions (upper panel), but only the N-terminal 50 amino acids of pUL31 were labeled with 32P (lower panel).

Analysis of the primary sequence of this region indicated that 6 serine residues were in a context potentially consistent with pUS3 phosphorylation sites (Fig. 4.2A). Of the 6 residues, Serines 11, 24, and 40 completely matched the predicted pUS3 consensus motif (RnXS/T [n>=2]) (155), while the other 3 Serines (S26, 27, and 43) could not be excluded since the kinase was found to be more promiscuous than originally predicted (128). With this in mind, we systematically substituted these serines with alanines and tested the corresponding mutants for their capacity to be phosphorylated by pUS3 *in vitro*. The constructs were designated pUL31(SA3) (bearing S11, S24, and S40 changed to alanine codons) and pUL31(SA6) which bears all 6 serines (S11, 24, 25, 26, 40, 43A) changed to alanines. Mixing GST fusion protein bearing these mutations with active US3 kinase and [32P-ATP] revealed that wild type pUL31 was heavily phosphorylated by US3 kinase, whereas mutating all 6 serines completely abolished phosphorylation. In contrast, the SA3 mutations decreased, but did not eliminate US3-dependent phosphorylation (Fig. 4.2C). The results revealed that the pUL31 N-terminus contains multiple sites that US3 kinase can phosphorylate *in vitro*, and suggests that the protein is phosphorylated by the US3 kinase during infection as indicated previously (89).
**Figure 4.2 Mapping pU_l31 phosphorylation sites of pU_s3 kinase.** (A) Schematic illustration of full length U_l31 protein and its sub-fragments used in panel B, and the primary sequence showing potential phosphorylation sites (underlined). A series of constructs with point mutations used in subsequent experiments are listed. (B) *In vitro* kinase assay with sub-fragments of pU_l31. GST fusion proteins purified from E.coli were incubated with purified U_s3 kinase assay with sub-fragments of pU_l31. GST fusion proteins purified from *E. coli* were incubated with purified U_s3 kinase and \([\gamma-P^{32}]ATP\). The reaction products were electrophoretically separated on a denaturing gel and were stained by Coomassie brilliant blue, dried and exposed to X-ray film. (C) Wild type and mutant pU_l31 reacted with purified U_s3 kinase *in vitro*. Reactions were performed as in panel B. Reactants were separated on a denaturing gel, visualized by CBB staining, and dried. The presence or absence of phosphorylation was determined by autoradiography of the same gel.
Precluding phosphorylation of the pUL31 N-terminus causes a delay in viral replication. We next proceeded to determine whether the N-terminus of pUL31 was phosphorylated by US3 during viral infection. Recombinant viruses bearing the 6 Serine to Alanine mutations in UL31 and its corresponding genetically repaired virus UL31(SAR) were generated through BAC mutagenesis. Cells were infected with 5.0 PFU/cell of the wild type virus HSV-1(F), kinase dead virus US3(K220A), UL31 mutant virus UL31(SA6), or the repaired virus UL31(SAR). At 16 hpi, the cells were harvested, and electrophoretically separated proteins were subjected to immunoblotting with pUL31-specific antiserum.

The results, shown in Fig. 4.3, indicated that, (i) consistent with previous reports (89), phosphatase (PPase) treatment caused an increase in the electrophoretic mobility of the pUL31-specific band of HSV-1(F) (lanes 1 and 2). The migration of this band was similar to that of non-treated pUL31 from cells infected with US3(K220A) (lane 3), indicating that pUS3 played an important role in phosphorylation of pUL31 during infection directly or indirectly. (ii) The electrophoretic mobility of pUL31 from US3(K220A) infected cells was also accelerated by PPase addition (lane 3 and 4), indicating that while pUS3 is important for pUL31 phosphorylation, other kinases also play a role. (iii) Substitution of all 6 serines by alanines increased the electrophoretic migration of UL31 protein. PPase treatment did not cause a mobility change of this mutant (lane 5 and 6) indicating that this was the only phosphorylation site of pUL31, at least as detected by one dimensional electrophoresis. In contrast, the mobility of pUL31 expressed by the genetically repaired virus UL31(SAR) (lane 7 and 8), was greatly increased by PPase treatment, confirming that the pUL31 phosphorylation was successfully blocked by the SA6 mutations. (iv) All of the non-mutated pUL31 proteins (lanes 1, 3, 7) produced wider bands than their PPase treated counterparts (lanes 2, 4, 8), implying the existence of heterogeneously phosphorylated pUL31 species.
Figure 4.3 Immunoblot to determine the phosphorylation state of pUL31 during infection with wild type and mutant viruses. Hep2 cells were infected with wild type HSV-1(F) (lanes 1, 2), U53(K220A) (lanes 3, 4), U131(SA6) (lanes 5, 6), or U131(SAR) (lane 7, 8) viruses for 16hrs. The lysates were treated or untreated with bacteriophage lambda phosphatase as indicated. Cell lysates were electrophoretically resolved on denaturing polyacrylamide gels, electrically transferred to nitrocellulose, and probed with antibody to pUL31.
during infection. (v) Unexpectedly, the PPase treated U_L31 proteins of HSV-1(F) and U_L31(SAR) viruses (lane 2, 8) migrated slightly slower than the pU_L31 of U_S3(K220A) virus. This finding suggested either that not all phosphorylation is precluded by PPase treatment, or that active U_S3 kinase induces post-translational modifications other than phosphorylation on pU_L31 in infected cells.

We then conducted one-step viral growth assays to gauge the infectivity of the U_L31(SA6) virus. Cells were infected with 5 PFU/cell of U_L31(SA6), the restored virus U_L31(SAR), wild type HSV-1(F), or U_S3(K220A) at an MOI of 5 and viral yields at the indicated time points were titrated on Vero cells. As shown in Fig. 4.4, the U_L31(SA6) and U_S3(K220A) viruses displayed a similar pattern of viral growth over time. Of note was a nearly 10-fold decrease in titer at 12 hpi compared to that of the wild type virus; on the other hand, both recombinant viruses produced titers similar to HSV-1(F) at 24 hpi. Impaired viral growth was eliminated by repairing the SA6 mutation inasmuch as the virus U_L31(SAR) replicated as efficiently as HSV-1(F). We conclude from these data that precluding phosphorylation of pU_L31 mimics the growth defect of the U_S3 kinase dead virus.

**Precluding phosphorylation of the pU_L31 N-terminus mimics the effects of U_S3 kinase deficiency in pU_L31/pU_L34 complex localization and perinuclear virion accumulation.** Although the absence of U_S3 kinase activity causes a defect in capsid nuclear egress, including mislocalization of pU_L34 at the nuclear rim and accumulation of primary enveloped virions in the perinuclear space, the responsible kinase substrate has not been identified. Because the growth kinetics of U_L31(SA6) virus and U_S3(K220A) were very similar, we compared the localization of pU_L34 and virions in cells infected with the U_L31(SA6) and U_S3 kinase dead viruses.

Hep2 cells were infected with either wild type HSV-1(F), U_S3(K220A),
Figure 4.4 One-step growth curves of wild type and mutant viruses. Hep-2 cells were infected with either wild type HSV-1(F), U₈3(K220A), U₇131(SA6), or U₁₃11(SAR) viruses at an MOI of 5. After adsorption for one hour, residual infectivity was inactivated by a low-pH wash. At the indicated time points, pooled intracellular and extracellular viral infectivity was titrated on Vero cells. Experiments were done in duplicate. Mean values are plotted and deviations are represented by error bars.
Ul31(SA6), or the Ul31(SAR) virus for 16 hours, at which time the cells were immunostained with antibodies against pUl31 and pUl34 and visualized by confocal microscopy (Fig. 4.5). Consistent with previous reports, the pUl31 and Ul34 proteins colocalized smoothly along the nuclear envelope in cells infected with HSV-1(F). Deactivation of the Us3 kinase as in cells infected with the Us3(K220A) virus induced discrete foci of pUl31 and pUl34 at the nuclear rim. In cells infected with the Ul31(SA6) virus, the focal distribution of pUl31/pUl34 appeared very similar to that of cells infected with Us3(K220A). The aberrant distribution of pUl31/pUl34 was restored to the wild type distribution in cells infected with the genetically repaired virus Ul31(SAR).

To examine the effects of pUl31 phosphorylation on the distribution of virions, cells were infected with wild type HSV-1(F), Us3(K220A), Ul31(SA6), or the Ul31(SAR) viruses, embedded, and thin sections were stained and examined by electron microscopy. As shown in Fig. 4.6, Ul31(SA6) viral infection produced nuclear membrane invaginations containing multiple primary enveloped virions. The distribution of these virions resembled that of cells infected with Us3(K220A) virus implying an egress defect, but was dissimilar to the appearance of cells infected with HSV-1(F) or the restored virus Ul31(SAR) (not shown).

We conclude that phosphorylation of pUl31 is necessary for proper localization of the pUl31/pUl34 complex in the nuclear rim, and for optimal egress of virions from the perinuclear space.

pUl31/pUl34 distribution is dependent on the acidity of the pUl31 N-terminus. In preliminary studies, localization of pUl31/pUl34 in cells infected with a virus [designated Ul31(SA3)] expressing pUl31 with point mutations S11A, S24A and S40A was undistinguishable from that in cells infected with wild type virus (not
Figure 4.5 Confocal immunofluorescence staining of pU₃1₃ and pU₃1₃₄ in HSV-1 infected Hep2 cells. Cells were infected with either the wild type HSV-1(F) [designated (F)], U₅₃(K220A), U₃1(SA6), or U₄₃1(SAR) viruses. After 16 hours the cells were fixed in paraformaldehyde and methanol, permeabilized, immunostained for pU₃1₃ (green) and pU₃₄ (red), and visualized by confocal microscopy. Optical sections were taken through the middle of cells.
<table>
<thead>
<tr>
<th></th>
<th>pUL31</th>
<th>pUL34</th>
<th>Merge</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>F</strong></td>
<td><img src="image" alt="a" /></td>
<td><img src="image" alt="b" /></td>
<td><img src="image" alt="c" /></td>
</tr>
<tr>
<td><strong>Us3(K220A)</strong></td>
<td><img src="image" alt="d" /></td>
<td><img src="image" alt="e" /></td>
<td><img src="image" alt="f" /></td>
</tr>
<tr>
<td><strong>UL31(SA6)</strong></td>
<td><img src="image" alt="g" /></td>
<td><img src="image" alt="h" /></td>
<td><img src="image" alt="i" /></td>
</tr>
<tr>
<td><strong>UL31(SAR)</strong></td>
<td><img src="image" alt="j" /></td>
<td><img src="image" alt="k" /></td>
<td><img src="image" alt="l" /></td>
</tr>
</tbody>
</table>
Figure 4.6 Electron microscopy of Hep2 cells infected with HSV mutants. Cells were infected for 16 hours with the indicated viruses, fixed, embedded, sectioned, stained, and examined in a transmission electron microscope. Images show primary enveloped virion accumulation in Hep2 cells infected by Us3(K220A) viruses or UL31(SA6) viruses. (N, nucleus; C, cytoplasm). The scale in μM is indicated.
shown). This observation suggested that phosphorylation at sites S25, S26 and/or S43 were sufficient to enable pUL31 to function properly. With this in mind, two pseudo-phosphorylated pUL31 expressing viruses were generated, one termed UL31(SAE) encoded residues 11, 24 and 40 as alanines while residues 25, 26 and 43 were changed to glutamic acid, and UL31(SE6), which had all six residues changed to glutamic acid (diagrammed in Fig. 4.2A).

Immunoflourescence analysis of the pUL31 and pUL34 distribution in cells infected with these viruses revealed phenotypic differences (Fig 4.7). Specifically, UL31(SAE) still produced punctate pUL31/pUL34-specific staining like the UL31(SA6) or US3(K220A) viruses, whereas these proteins were distributed smoothly throughout the nuclear rim in cells infected with UL31(SE6). Taken together with the distribution patterns of pUL31/pUL34 in cells infected with of UL31(SA3) and UL31(SA6) viruses, the data indicated that acidity of the pUL31 N-terminus was a critical factor mediating pUL31/pUL34 localization.

In order to confirm the rescuing effect of the acidity of pUL31 in pUL31/pUL34 complex localization, and to see if effecters of US3 other than UL31 play a role, a novel virus was generated in which the mutation abolishing US3 kinase activity was introduced into the UL31(SE6) virus, resulting in a double mutant virus UL31(SE6)/US3(K220A). Analysis of the distribution of the pUL31/pUL34 complex in cells infected with this mutant indicated that the complex localized in a smooth distribution at the nuclear rim in contrast to what would be expected in cells infected with US3(K220A). Thus the rescuing phenotype of completely pseudo-phosphorylated pUL31 was dominant over the inactive US3 kinase phenotype. These observations suggested that pUL31 is the major substrate of US3 kinase responsible for regulating the localization of pUL31/pUL34 at the nuclear rim.
Figure 4.7 pUL31 and pUL34 localization in Hep2 Cells infected with pseudo-phosphorylated UL31 viruses. Hep2 cells were infected with the indicated viruses and were fixed at 16 hours after infection in paraformaldehyde and methanol. The cells were then immunostained for pUL31 (green) and pUL34 (red) and visualized by confocal microscopy. Optical sections were taken through the middle of cells.
**Pseudo-phosphorylated pUL31 inhibits primary envelopment of nucleocapsids.** Based on the immunofluorescence data above, we presumed that unlike the US3 kinase-dead virus, (i) the UL31(SE6) virus and UL31(SE6)/US3(K220A) viruses would not induce herniations of nuclear membrane containing perinuclear virions, and (ii) would replicate as efficiently as wild type HSV-1. To test the first prediction, cells were infected with these viruses and examined by electron microscopy 16 hours later. Consistent with the first hypothesis, no excessive perinuclear virion accumulation was observed in cells infected with either virus over that seen in cells infected with wild type HSV-1(F). Inconsistent with the second hypothesis, however, very few viral particles were detected in the cytoplasm or on the cell surface of cells infected with the UL31(SE6) or UL31(SE6)/US3(K220A) viruses. This was the case despite ample numbers of capsids within nuclei of these cells. Counting a representative 9 cell sections indicated that whereas the ratio of cytoplasmic to intranuclear capsids was approximately 1.55 in cells infected with HSV-1(F), this ratio was only 0.11 in cells infected with the mutant virus UL31(SE6) virus and 0.02 for the UL31(SE6)/US3(K220A) double mutant (Table 4.3). These data suggest that the mutant virions were enveloped at the inner nuclear membrane less efficiently than their wild type counterparts.

To further investigate the phenotype of the UL31(SE6) and UL31(SE6)/US3(K220A) viruses, cells were infected with these viruses at 5.0 PFU/cell and the amount of infectious virus was determined at various times after infection. As shown in Fig. 4.8, the UL31(SE6) and UL31(SE6)/US3(K220A) viruses produced less infectious virus throughout the infection compared to HSV-1(F). The most marked discrepancy was observed at 12 hpi, when the amount of infectious UL31(SE6) virus was reduced approximately 10 fold compared to that of HSV-1(F), whereas the UL31(SE6)/US3(K220A) virus titer was reduced more than 20 fold. As shown previously, the US3(K220A) virus also displayed a 10 fold lower titer at 12 hpi compared to
Table 4.3 Subcellular distribution of viral particles in Hep2 cells infected for 16 hrs (examined by EM).

<table>
<thead>
<tr>
<th>Virus</th>
<th>Total intracellular viral particle numbers</th>
<th>Cells counted</th>
<th>Average Ratio (C/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nuclear (N)</td>
<td>Cytoplasmic (C)</td>
<td>Perinuclear (PN)</td>
</tr>
<tr>
<td>HSV-1 (F)</td>
<td>73</td>
<td>113</td>
<td>10</td>
</tr>
<tr>
<td>Ul31(SE6)</td>
<td>186</td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>Ul31(SE6)/Us3(K220A)</td>
<td>129</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>
Figure 4.8 One-step growth curves of pseudo-phosphorylated UL31 viruses. Hep-2 cells were infected with either wild type HSV-1(F), US3(K220A), UL31(SE6), or UL31(SE6)/US3(K220A) at an MOI of 5 PFU/cell. Residual viruses were inactivated by a low-pH wash at 1 hpi. At the indicated time points, virus in the whole culture was collected and titrated on Vero cells. Experiments were done in duplicate. Mean values are plotted and simple standard deviations are represented by error bars.
HSV-1(F) but eventually produced infectious virus titers approaching those of the wild type virus. In contrast to this result, the two UL31 mutants reached their peak titers at 18 hpi, and never reached infectious titers approaching those of wild type HSV-1(F). These data indicate that the nuclear egress defects of the UL31 mutants are more persistent than that of the US3(K220A) virus, suggesting a defect in primary envelopment that does not diminish as infection proceeds. We also noted that the double mutant UL31(SE6)/US3(K220A) replicated slightly less efficiently than either of the viruses with single mutations, indicating that the pseudo-phosphorylated pUL31 is insufficient to complement fully the contribution of US3 kinase activity to viral infectivity.

**Discussion**

Normally, nucleocapsid budding through the inner nuclear membrane and fusion of the nascent virion envelope with the outer nuclear membrane is highly efficient. This conclusion comes from the observation that during wild type virus infection, there is rarely extensive virion accumulation in the perinuclear space. In cells infected with US3 kinase-dead viruses however, the accumulation of perinuclear virions in herniations of the perinuclear space is commonplace. This aberrant virion accumulation likely reflects an imbalance between the rate of delivery into localized regions of the perinuclear space and the rate of exit from these regions. Taken from this perspective, the US3 kinase might serve to decrease the numbers of virions in particular perinuclear regions because its activity disperses the nuclear envelopment complex widely throughout the nuclear membrane. Thus, the rate of exit from the nucleoplasm is similar to wild type virus, but the number of envelopment sites is increased, and perinuclear virions do not accumulate in particular regions. On the other side of the inner nuclear membrane, US3 may promote fusion of the perinuclear virion envelope with the outer nuclear membrane.
Perhaps the most important finding of the current work is that most of the US3 nuclear egress phenotype, including mislocalization of the pUL31/pUL34 complex and virion accumulation in herniations of the perinuclear space, can be mimicked by precluding phosphorylation of pUL31 at its N-terminus. Thus, US3 mediates much of its effects in nuclear egress through phosphorylation of pUL31. Aggregation of pUL31/pUL34 may restrict sites of budding causing nucleocapsids to continue budding into herniations already containing multiple perinuclear virions, thereby overwhelming the fusion machinery. There is otherwise no evidence that the rate of budding into the perinuclear space is impaired by the absence of phosphorylation of pUL31.

Our data also indicate that the pseudo-phosphorylated pUL31(SE6) is defective in primary envelopment of nucleocapsids at the INM, and is insufficient to complement the full function of US3 kinase. Thus, some functions of US3 kinase activity important to viral replication are mediated by substrates other than pUL31. We speculate that these other substrates may include an impaired or mis-triggered fusion apparatus that mediates fusion between the virion envelope and ONM (possibly mediated through gB and gH). It is unclear whether the pUL31(SE6) mutation interferes with the fusion event because the paucity of perinuclear virions in cells infected with this mutation precludes assessment of this possibility.

Together, the data suggest that the function of pUL31 is tightly and dynamically controlled by phosphorylation. Based on all the data presented herein, we hypothesize that there is a mixed population of both unphosphorylated and phosphorylated pUL31 (Fig. 4.9). At primary envelopment sites in the inner nuclear membrane, the unphosphorylated form is involved in initiation of the budding reaction, possibly by direct or indirect interaction with the nucleocapsid or other egress components, whereas the phosphorylated form coexists and is required to prevent pUL31/pUL34 aggregation. Once in the perinuclear space, pre-existing or newly phosphorylated pUL31 is needed...
Figure 4.9 Proposed model of dynamic phosphorylation of pUL31 during HSV nuclear egress. In HSV infected cells, phosphorylated pUL31 are scattered through the inner nuclear membrane (INM) via interaction with pUL34. DNA-filled capsids (1) contact the INM at the sites where unphosphorylated pUL31 are concentrated to initiate the primary envelopment reaction (2). Capsids translocate into the perinuclear space (PNS) with an envelope containing fusion proteins (3). In the PNS, phosphorylated forms of pUL31 induce conformational changes in the fusion proteins (4). These alterations trigger the fusion of the envelope with the outer nuclear membrane (ONM) and subsequent release of capsids into the cytoplasm (5).
to promote the fusion of the perinuclear virion with the ONM, probably by altering protein-protein interactions to activate the fusion proteins gH and gB.

Finally, we note that in our previous study of cellular lamin A/C, we reasoned that one possibility to explain the punctate distribution of pUL31/pUL34 in cells infected with US3 kinase-dead viruses was that this aberrant distribution reflected mobility constraints caused by a hypophosphorylated, and therefore less permeable, nuclear lamina. The current observation that the UL31/UL34 proteins are of punctate distribution in cells infected with the UL31(SA6) mutant argue against this possibility because these cells contain active pUS3, which would be expected to phosphorylate lamin A/C. Thus phosphorylation of lamin A/C is separable from functions required for the proper localization of pUL31/pUL34.
CHAPTER V

SUMMARY AND FUTURE STUDIES

Summary

The research goal of my study is to understand the machinery mediating the nuclear egress of HSV-1 capsids. The work initiated with the observation that in cells infected with a mutant HSV defective in US3 kinase, enveloped virions accumulate in invaginations of the inner nuclear membrane (INM), and viral production is delayed compared to wild type HSV-1(F) virus. The kinase therefore is postulated to regulate the capsid egress from the nucleus. This process involves primary envelopment at the INM and de-envelopment at the outer nuclear membrane (ONM) and requires the UL31 and UL34 proteins. Several substrates of pUS3 have been identified and investigated for their role in this process, but had yet to be implicated.

In order to determine the US3 substrate(s) responsible, an in vitro assay system using baculovirus-expressed pUS3 was established. As shown in Chapter II, nuclear lamin A/C was identified as a potential substrate and could be dissolved partially in vitro by US3 kinase activity. This was further confirmed by an in vivo assay showing that lamin A/C was hyper-phosphorylated during HSV infection and a full spectrum of phosphorylation required pUS3 activity. The nuclear lamina was found to be dramatically perforated when US3 kinase was deactivated; and the essential nuclear egress complex pUL31/pUL34 aggregated near those lamina perforations. Given that phosphorylation is a common strategy to rearrange lamina structure, I hypothesized that the lack of pUS3 activity caused a decrease in the phosphorylation of lamin A/C, which in turn made the lamina less flexible and hence restricted the pUL31/pUL34 distribution. However, an alternative possibility was that pUL31/pUL34 aggregation might mechanically perforate the lamina.
The nuclear lamina has long been considered a potential barrier for viral capsids to access primary envelopment sites at the inner nuclear membrane; together with the above hypothesis, I proceeded to investigate the roles of certain types of lamins during HSV-1 infection, using lamin knockout MEFs. In chapter III, pUL34 positioning and general permissivity of wild type and US3 kinase dead viral infection was examined in Lmna⁻/⁻ or Lmnb1⁻/⁻ MEFs. I found the two lamin types contributed differently to viral infection. Lamin A/C was dispensable for the infections. In particular, the replication of US3 kinase dead virus was augmented and produced no perinuclear virion accumulation. The localization of pUL34 was slightly altered upon the loss of lamin A/C in both viral infections, but it still aggregated in discrete foci in the absence of US3 kinase activity. In contrast, lamin B1 was required for efficient replication of both viruses. US3 mutant virus still induced perinuclear virion herniations, although pUL34 aggregated in a less extensive fashion. These findings led to the conclusions that (i) lamin A/C and lamin B1 have different roles during HSV-1 infection; (ii) pUL31/pUL34 aggregates independently of lamin proteins, although lamins do play a direct or indirect role modifying the localization of the complex.

The substrate mediating pUS3 activity was finally discovered, as described in chapter IV. pUL31 was recognized as the major downstream effector of pUS3 regulatory pathway by using a phosphorylation-deficient UL31 mutant that reproduced the defect seen in cells infected with the US3 mutant virus, even in the presence of the active US3 kinase. The mimicked defects included pUL31/pUL34 aggregation, perinuclear virion herniations, and slower viral replication kinetics. I went on to further confirm and characterize the functional significance of the pUS3/pUL31 catalytic relationship and found some interesting results. When pUL31 was constantly phosphorylated in an artificial manner, pUL31/pUL34 was properly positioned on the nuclear rim but the primary envelopment of capsids was impaired. Introducing a mutation ablating
US3 kinase activity did not alter the distribution of the pUL31/pUL34 complex or induce nuclear membrane herniations, but the viral replication was more decreased than with either mutation alone. These data imply that phosphorylation of pUL31 may be a dynamic event during infection and also that pUS3 has to phosphorylate substrate(s) other than pUL31 to exert its full contribution to HSV-1 replication. Hence, I propose that there is a mixed population of pUL31. The unphosphorylated form is present to initiate the primary envelopment process by interacting with capsids or other egress components, while the phosphorylated form is required to prevent pUL31/pUL34 complex from aggregating. At the subsequent de-envelopment stage, phosphorylation of pUL31 and other substrates are needed to promote the fusion of the nascent viral envelope with the ONM.

**Future studies**

(i) The proposed model of pUL31 phosphorylation does not address whether there are two static or interchanging populations of pUL31 and what is their relative ratio. Hence it will be interesting to determine when cells are co-infected by the phospho-deficient UL31(SA6) and pseudo-phosphorylated UL31(SE6) viruses, how these two static pUL31 populations will interact. An approach allowing precise switch-off and -on of the US3 kinase activity will be very useful to characterize the process in more spatial and temporal details. To achieve this goal, solving pUS3 structure and generating a specific inhibitor to the kinase would be useful.

(ii) The core question remaining unanswered is the specific mechanism by which the pUL31/pUL34 complex initiates primary envelopment of capsids. Co-expressing the pUL31/pUL34 homologs of pseudorabies virus is sufficient to induce vesicles derived from the INM in the absence of other viral proteins (91). Therefore, it is necessary to test if pUL34 alone is capable of such a function inasmuch as pUL34 can be
properly targeted to the INM in rabbit skin cells in the absence of pUL31, and pUL31 is less essential for viral replication in this particular cell line (107).

Since the research strategies are hampered by difficulties involved in working with nuclei, it will be very useful if we could reconstruct primary envelopment machinery on the plasma membrane. pUL34 is a type II membrane protein and may be delivered to the cell surface via genetic engineering or by interaction with synthesized liposomes. pUL31 is easier to manipulate because its NLS is in the poorly conserved N-terminal domain that appears to be dispensable for interaction with pUL34 (unpublished data). If the system works, and budding vesicles are induced from plasma membrane, it means the process does not need nuclear components, and the vesicles can be harvested for composition analysis to identify additional cellular factors.

Alternatively, leaving pUL31/pUL34 on the nuclear rim, we may take advantage of the aggregates induced by the U33 mutation, with the assumption that the essential egress components are enriched in these pUL31/pUL34 containing microdomains. In this case, cross-linking and in vivo labeling strategies may allow us to isolate functional candidates.

(iii) It is equally important to determine the structure of pUL31 and pUL34. Primary sequence analysis only inefficiently predicts any obvious structural or functional domains in the two proteins. In particular, it is indicated in Chapter IV that the phosphorylation at pUL31 N-terminus may induce significant conformational changes in the protein that is required for efficient primary envelopment and de-envelopment. Therefore, structural analysis will greatly help elucidate the nature of the protein functions.
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