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Human Cytomegalovirus Ul97 Kinase Activity Modifies Cell Cycle Checkpoint Regulators

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ABSTRACT

Human cytomegalovirus (CMV) infection results in destructive infections in neonates and immunocompromised individuals. Being the primary congenital infection in the United States, it can often result in permanent neurological deficits in infants. The current therapies for CMV infections all target the viral DNA polymerase and also have dose-limiting toxicities. Isolates resistant to ganciclovir (GCV), the therapy of choice, can sometimes overwhelm immunocompromised hosts. Better therapies for this infection are required.

The CMV UL97 kinase is a key enzyme in the treatment of CMV infection because it phosphorylates GCV. Additionally, maribavir (MBV) specifically inhibits UL97 kinase activity and inhibits viral replication. Recombinant viruses deficient for UL97 kinase activity do not replicate well in vitro. Studies using MBV in wild-type infections complement genetic studies and provide a very powerful tool to confirm results for the recombinant viruses. The aims of this research seek to further expound the functions of CMV UL97 kinase, and have the potential to identify novel antiviral therapies.

Early studies have identified cyclin-dependent kinase (CDK)-like characteristics of this enzyme. UL97 kinase shares many targets with CDK, and our research pinpointed another common target, retinoblastoma protein (RB). Like CDKs, UL97 kinase can hyperphosphorylate and inactivate RB, which is a unique mechanism of RB regulation by

a virus. Studies in viruses utilizing homologous proteins to pUL97 suggest that it not only phosphorylates other CDK targets, but also dysregulates CDKs themselves.

We hypothesized that CMV UL97 kinase is a major modifier of host cell cycle checkpoint regulators to promote viral replication by regulating both CDKs and CDK targets. Mutational analyses of pUL97 indicated that the amino-terminal putative RB-binding motif was important for RB hyperphosphorylation, and disruption of this domain rendered the virus hypersensitive to MBV. We also characterized changes in G_2-M checkpoint regulation induced by UL97 kinase activity, and showed that the kinase was involved in the upregulation of CDK1 and other mitotic regulators within the infected cell.

DEDICATION

Without the love, support, patience, and guidance of my mother, I would not be writing this dissertation today. I love you, and owe you so much more than I could ever repay.

As a daughter who is lucky enough to have two fathers, I am truly blessed. Again, a thousand thanks for shaping me into the person I am today. I love you.

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To my adopted Birmingham family: you chubies are awesome. I love you.

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ACV	acyclovir
AIDS	Acquired Immunodeficiency Syndrome
AP-1	activator protein-1
APC/C	anaphase promotic complex/cyclosome
ATCC	American Type Culture Collection
BAC	bacterial artificial chromosome
BDCRB	2-bromo-5,6-dichloro-(1-ß-D-ribofuranosyl)benzimidazole
CAK	CDK-activating kinase
CDC	Centers for Disease Control and Prevention
CDK	cyclin-dependent kinase
CDV	cidofovir
CHPK	conserved herpesvirus-encoded protein kinases
CKI	cyclin kinase inhibitor
(H)CMV	human cytomegalovirus
CNS	central nervous system
dGTP	deoxyguanosine triphosphate
EBV	Epstein-Barr virus
ER	endoplasmic reticulum
FAM	6-carboxyfluorescein
FBS	fetal bovine serum

FITC	Fluorescein isothiocyanate
FSG	fish skin gelatin
Fzr1	fizzy-related protein 1; Cdh1
G	growth phase (cell cycle)
g(X)	glycoprotein (X = letter of specific glycoprotein)
GCV	ganciclovir
HAT	histone acetyltransferase
HB5	CMV BAC strain derived from AD169
HDAC	histone deacetylase
HEL	human embryonic lung fibroblast
HFF	human foreskin fibroblast
HHV	human herpesvirus
HIV-1	human immunodeficiency virus
hpi	hour(s) post infection
HPV	human papillomavirus
HRP	horseradish peroxidase
HSV	herpes simplex virus
HTLV-I	human T-lymphotropic virus type I
HvU_L	herpesvirus unique long region
IE	immediate early protein
IFA	immunofluorescence analysis
IL	interleukin
K355	invariant lysine in UL97 kinase essential for its phosphorylative activity

kb	kilobase
kDa	kilodalton
KSHV	Kaposi's sarcoma-associated herpesvirus
LTa	large T antigen
M	mitosis phase (cell cycle)
MBV	maribavir
MC	mitotic catastrophe
MEM	minimal Eagle's medium
MOI	multiplicity of infection
MPF	maturation/mitosis promoting factor
MTOC	microtubule-organizing center
NF-Y	nuclear transcription factor - Y
NIAID	National Institute of Allergy and Infectious Diseases
NLS	nuclear localization sequence
OA	okadaic acid
OARE	okadaic acid response element
ORF	open reading frame
oriLyt	CMV lytic replication origin
pac	cleavage/packaging site
PBS	phosphate-buffered saline
PDGFRα	platelet-derived growth factor receptor α
PFA	foscarnet
P-FA	paraformaldehyde

PFU	plaque-forming units
PML	promyelocytic leukemia protein
PORT	portal protein, UL104
PP	protein phosphatase
qRT-PCR	quantitative real-time reverse-transcriptase PCR
RB	retinoblastoma protein
RCΔ97	AD169 virus with UL97 ORF deleted
RC314	AD169 virus with a K355M mutation in pUL97
S	synthesis phase (cell cycle)
SEAP	secreted alkaline phosphatase
Ser	serine
ser/thr	serine/threonine
SV40	simian vacuolating virus 40
Ta	ambient temperature
TAMRA	6 - Carboxytetramethylrhodamine
TER	terminase
Thr	threonine
TORCHES	toxoplasma gondii, rubella, cytomegalovirus, herpes simplex virus, and syphilis infections
TPA	12-O-tetradecanoylphorbol-13-acetate
TRE	TPA response element
TRITC	tetramethylrhodamine isothiocyanate
Tyr	tyrosine

U_L	unique long coding region
UL102	CMV helicase-primase linker protein
UL105	CMV DNA helicase
UL44	CMV DNA polymerase processivity factor
UL51	TER-associated protein, Terminase-binding protein, DNA packaging
UL54	CMV DNA polymerase
UL57	single-stranded DNA binding protein
UL70	CMV DNA primase
UL77	portal capping protein
UL84	CMV nuclear phosphoprotein
U_S	unique short coding region
VACV	valacyclovir
VGCV	valganciclovir
VZV	varicella zoster virus
wt	wild-type
α	immediate early herpesvirus genes
β	delayed early herpesvirus genes
γ	late herpesvirus genes

INTRODUCTION

HERPESVIRIDAE

Herpesviruses are a family of large, double-stranded DNA viruses that belong to the order *Herpesvirales* and can infect nearly every species. All herpesviruses have a common virion structure composed of a densely packed nucleic acid core surrounded by an icosahedral capsid. This capsid is surrounded by a glycoprotein lipid envelope with a layer of viral and cellular proteins in between the capsid and envelope called the tegument. The entire virion is anywhere from 100-300 nanometers, depending on the particular virus. There are many viral proteins that are highly conserved between species, and the open reading frames (ORFs) within the unique regions of the viral genome are flanked by direct or inverted repeats. Recombination can occur between these repeats, and is a consequence of concatemeric DNA replication intermediates that are resolved to genome length during cleavage and packaging of genomic DNA (65).

Viral infection initiates by the virion tethering with low-affinity to cell surface receptors and then forming a closer association with the cell membrane using higher affinity receptors, which initiates viral fusion of the envelope to the cell membrane. Glycoproteins exposed on the viral envelope initiate this attachment, and herpesviruses utilize a broad range of cell surface receptors, allowing them to bind and enter into an extensive repertoire of cell types. Viral glycoproteins mediate the fusion of the viral envelope with the cellular membrane and releases tegument proteins and the nucleocapsid into the cytoplasm. Tegument proteins, such as kinases, phosphatases,

nucleases, and transcriptional activators, are delivered to the host cell and can initiate immediate changes in cellular and viral metabolism, as well as blocking innate antiviral responses (94). The nucleocapsid is transported along the microtubule network to the host nucleus, where it delivers the linear viral DNA through the nuclear pore. The viral DNA immediately circularizes, and replication occurs strictly within the confines of the host nucleus using a set of viral replication enzymes. While the virus utilizes its own DNA polymerase, it commandeers many host proteins to assist in transcription and translation. Herpesviruses undergoing an active lytic infection regulate gene expression in a temporal cascade; specific subsets of genes are transcribed sequentially at biologically relevant stages in infection. Immediate early (α), delayed early (β) and late (γ) transcripts, generally encode for protein products for transcription of viral genes, replication of viral DNA, and structural proteins, respectively. While genomic replication, transcription, and encapsidation takes place in the nucleus, mature capsids egress the nucleus and acquire an envelope in a complex process of envelopment, de-envelopment, and re-envelopment as the capsid transverses into and through the Golgi to acquire a mature set of glycosylated viral membrane proteins. Once complete, enveloped virions are released either through exocytosis or lysis of the host cell (116).

Herpesviruses establish characteristic lifelong infections and initiate latent infections in a specific subset of host cells through the expression of a subset of viral gene products unique to each virus. Latent infections are maintained by the ability of the virus to remain dormant or undetectable within cells that either maintain a certain level of immune privilege or within cells that directly modulate a host's immune response to infection (107).

Phylogenetic analysis of the family *Herpesviridae* within the order *Herpesvirales* has identified three distinct subfamilies based on gene products and sequence similarity (40, 106). *Alphaherpesvirinae,* which consist of the genera *Iltovirus, Mardivirus, Simplexvirus,* and *Varicellovirus*, have variable host and cellular tropism with the ability to replicate quickly (less than 24 hours) and spread rapidly in culture. Although this virus subfamily utilizes neural cells, mainly sensory ganglia, to establish a latent infection, lytic infection is most common in epidermal cells. The subfamily *Betaherpesvirinae* has four assigned genera – *Cytomegalovirus*, *Muromegalovirus*, *Proboscivirus*, and *Roseolovirus*. Viruses in this subfamily are biologically very different from the *Alphaherpesvirinae*. These viruses exhibit an extremely limited host cell range, slow replication kinetics (≥72 hours), enlarged infected cells, and lymphotropic latency. *Gammaherpesvirinae* typically replicate in T and B lymphocytes and can cause cancers such as Kaposi's sarcoma and Burkitt's lymphoma. The genera include *Lymphocryptovirus*, *Rhadinovirus*, *Macavirus*, and *Percavirus*.

Viruses from each subfamily can cause clinical disease in humans and are referred to as human herpesviruses (HHV). *Alphaherpesvirinae* tend to cause ulcerative lesions in the epidermis. Herpes simplex virus (HSV) causes vesicular lesions in and around mucosa; HSV-1 (HHV-1) is mainly associated with oral cold sores or fever blisters while HSV-2 (HHV-2) more commonly infects the anogenital mucosa (189). HSV-2 is one of the most commonly sexually transmitted infections worldwide, and 15 to 40% of individuals are seropositive for the infection, with a frequency that varies depending on gender, ethnicity, age, sexual preference and geographic location (28). In primary or secondary immunocompromised individuals, HSV-1 is the major cause of viral acute

nonepidemic focal encephalitis (43). HSV-2 infection in the genital mucosa induces the infiltration of lymphocytes that are the main target cells of human immunodeficiency virus-1 (HIV-1) and appears to be one of the mechanisms for statistically significant increased susceptibility of acquisition of HIV-1 in HSV-infected individuals (197). Varicella zoster virus (VZV, HHV-3) is the causative agent for varicella (chickenpox), a vesicular, highly-infectious rash often seen in children. After primary infection, VZV remains dormant in neural ganglia, sometimes reactivating in the elderly or immunocompromised patient as a "shingles" infection (118).

Human *Betaherpesvirinae* infections include human cytomegalovirus (CMV; HHV-5), human herpesvirus 6 variants (HHV-6A and HHV-6B) and human herpesvirus 7 (HHV-7). CMV infection is ubiquitous throughout the population worldwide and accounts for about 10% of the cases of viral mononucleosis. Severe viremia affects immunocompromised individuals, and CMV is one of the acquired immunodeficiency syndrome (AIDS)-defining infections. CMV infection is associated with severe neurological defects in congenitally infected neonates and can result in hearing loss and mental disabilities (21). Roseola is a common disease associated with a disseminated rash and is caused by HHV-6B. This rash is preceded by a high fever and is mainly seen in infants and toddlers (194).

The only human herpesviruses subfamily that has proven oncogenic potential is the *Gammaherpesvirinae*. Kaposi's sarcoma-associated herpesvirus (KSHV, HHV-8), as its name implies, is the causative agent behind Kaposi's sarcoma, which is a rare cancer marked by dark, papular, highly-vascularized nodules that are found anywhere on the body. The dermatological "patch lesions" resulting from this cancer is another AIDS-

defining illness, and is usually a more severe disease in HIV-coinfected patients (54). HHV-8 triggers Kaposi's sarcoma and primary effusion lymphoma through the expression of numerous homologs of cellular oncogenes, one of which is a constitutively active viral G-protein coupled receptor that mimics the interleukin-8 (IL-8) receptor (165). This virus also has viral versions of cyclin D, IRF1, Bcl-2, and IL-6 and also modifies the normal cellular ubiquitination system, which is important for regulation of signal transduction, protein trafficking and degradation, and immunomodulatory responses (53). Epstein-Barr virus (EBV, HHV-4) is the etiological agent behind infectious mononucleosis and is associated with Burkitt's lymphoma and nasopharyngeal carcinoma. Preferentially infecting B cells of a host, this common virus can remain latent for years until an immunocompromising event allows for reactivation and potential establishment of lymphoid and epithelial cancers (193). EBV also has links to other maladies, such as systemic lupus erythematosus, rheumatoid arthritis, Sjögren's syndrome, and multiple sclerosis (123). Although the exact mechanism of viral transformation remains unclear, evidence supports that the viral latency protein LMP2A allows infected cells to subvert the normal p53 pathway and result in unchecked cellular growth (11).

Chemotherapeutic treatments for human herpesvirus infections mainly inhibit viral DNA synthesis. Acyclovir (ACV) and valacyclovir (VACV) are nucleoside analogs phosphorylated by thymidine kinases from HSV and VZV and are incorporated into the growing viral DNA strand with high specificity by the viral DNA polymerases. Ganciclovir (GCV) is the drug of choice for CMV infections; however, GCV and the other approved therapies for CMV infection - foscarnet (PFA), cidofovir (CDV) and the

Structure	Compound ID (Brand Name)	Abbreviation	Mechanisms of Action
	maribavir	MBV	Inhibits UL97 kinase activity
	foscarnet (Foscavir®)	PFA	Inhibits DNA polymerase
	ganciclovir (Cytovene®, Cymevene®, Vitrasert®, Zirgan®)	GCV	Phosphorylated by pUL97 and inhibits DNA polymerase
	2-bromo-5,6-dichloro-1-β-D-ribofuranosyl benzimidazole	BDCRB	Inhibits concatameric viral DNA cleavage and packaging
	cidofovir (Vistide ®)	CDV	Inhibits DNA polymerase
	valganciclovir (Valcyte ®)	VGCV	Phosphorylated by pUL97 (Prodrug of GCV)

TABLE 1. Compounds with antiviral activity against CMV.

GCV prodrug valganciclovir (VGCV,)- are all associated with significant toxicities (Table 1) (104). Gammaherpesviruses are not commonly treated with antivirals, but either ACV or GCV can be used to treat symptoms of lytic infections, such as oral hairy

leukoplakia; immunostimulatory therapeutics are usually employed to combat more severe cases (110, 177).

While there are vaccines in various stages of development for all herpesviruses, as of this writing, only VZV has a commercially available vaccine. The VZV vaccine is available in two different potencies and has been approved to prevent chickenpox and shingles (120). HSV-2 vaccines, which utilize either live attenuated virus or inactivated virus with enhanced surface glycoprotein expression, have not generally been shown to be effective in clinical trials. The most promising of which, called *Simplirix*™ in the Herpesvac Trial for Women, did not prove efficacious in Phase III clinical trials, and further development was subsequently terminated (44). Vaccines against KSHV have been limited due to a lower affected population and lack of appropriate animal model (148). Therapeutic and preventative vaccines are in various stages of development for CMV, HSV and EBV (9, 38). Microbicides that would prevent transmission of a broad-spectrum of viruses are also under development (122).

HUMAN CYTOMEGALOVIRUS

Replication

In a clinical setting, CMV can infect almost every cell type, preferring ductal epithelial cells while rarely infecting fibroblasts. Interestingly, highly passaged laboratory strains like AD169 have altered tropism that allows them to most efficiently proliferate in primary fibroblasts, the host cell of choice (114). However, these isolates exhibit restricted replication in primary macrophages, endothelial cells, epithelial cells

and dendritic cells. Fully transformed fibroblasts and most other transformed cell lines are not permissive to infection by CMV, so the replication of this virus has been best characterized in primary fibroblast cells.

Mature virions consist of three main structural features: an icosadeltahedral nucleocapsid containing the genome, the tegument layer, and an outer glycoprotein-containing lipid envelope. The capsid is comprised of five core proteins that self-assemble into the capsid structure (MCP, TRI1, TRI2, SCP, and PORT) (115). The tegument consists of approximately 27 viral proteins, non-coding viral RNAs, and various cellular proteins that surround the capsid (51). The nucleocapsid and tegument are surrounded by a lipid membrane envelope that is rich with viral glycoproteins and subsets of an endoplasmic reticulum (ER)-Golgi intermediate compartment obtained from the host (22).

The CMV 230 kilobase (kb) genome consists of unique long (U_L) and unique short (U_S) sequences flanked by a set of inverted repeats. The repeat sequences contain critical *cis*-acting sequences, such as the cleavage and packaging signals required for the site-specific cleavage of genomic DNA as it is packaged in the capsid. A result of the cleavage and packaging of unit length genomes from concatameric and branched DNA intermediates is that these U_L and U_S regions can invert relative to one another, resulting in 4 potential isomeric forms of the genome (113). AD169 is the prototypic and highly passaged laboratory strain that was isolated from adenoid tissue (142). This isolate is missing approximately 15 kb of sequence containing about 19 ORFs that are found in low-passage clinical isolates (29, 98). Genes expressed from this region are involved in cell tropism and their deletion improves the replication of this strain in primary

fibroblasts in a laboratory setting (133). Merlin, a low passage clinical isolate, replicates well in the laboratory and is more representative of the coding capacity of clinical isolates. This virus has 167 known ORFs that are thought to be legitimate CMV genes (47).

Like other members of the herpesvirus family, CMV expresses its genes in a temporally regulated cascade, with three classes of transcripts including theα, β and γ mRNAs. α gene products are expressed from a very strong promoter enhancer element and do not require viral gene products for their expression. These gene products induce the expression of β genes that generally direct the replication of the viral genome. After viral DNA synthesis commences, γ genes are expressed and include structural proteins (162). The replication cycle for CMV takes 72 hours to produce mature, infectious virus; α genes are turned on immediately after viral infection, while β and γ genes are turned on approximately 8 and 48 hours post infection (hpi), respectively (113).

The CMV nucleocapsid enters the cell within 5 minutes of attachment and virion membrane-bound glycoproteins are essential for tethering, docking, signaling, and fusion events to allow viral entry. CMV glycoprotein complex II, which consists of the abundant glycoproteins M and N (gM/gN), are important for the tethering of the virus to the cellular membrane by binding to heparan sulfate proteoglycans (74). The complex of proteins consisting of glycoproteins H and L (along with gO, this triplex was classically referred to as the glycoprotein complex III) paired with UL128, UL130, and UL131A interact with integrins and facilitate viral fusion with the cellular membrane as well as initiating signaling events in the cell (124, 180). The last important virus/receptor interaction for docking, signaling, and entry appears to be with two complexes of

glycoprotein B (gB), which make up glycoprotein complex I, and platelet-derived growth factor receptor α (PDGFRα) on the host cell membrane (157). The CMV virion is not endocytosed but appears to directly fuse with the cellular membrane (39). The simple attachment of the virion to the cell initiates a signaling cascade network that promotes transport of the nucleocapsid to the nucleus. The viral genetic material is delivered directly to the nucleus, where the nucleocapsid attaches to the nuclear pore via transport along the cytoskeletal network within the cell (46). After delivery, the viral genome immediately circularizes within the nucleus and transcription of α genes commences.

CMV DNA replication occurs in the nucleus, with the virus using a combination of cellular and viral proteins to facilitate this process. The α gene products, mainly represented by the major immediate early (IE) proteins, utilize splicing to create variants (IE-1 and IE-2) which are essential for transcriptional activation of β and γ gene products (60, 66, 102). These genes have a strong enhancer region, and do not require de novo viral protein synthesis, and silencing of this enhancer results in the virus entering a latent state (154).

The major and minor IE proteins stimulate production of a core set of seven DNA herpesviruses synthesis enzymes that are essential for viral DNA synthesis and lytic infection, and include a DNA polymerase (UL54), DNA polymerase processivity factor (UL44), trimeric helicase-primase unit with a linking subunit (UL105, UL70, and UL102, respectively), ssDNA binding protein (UL57), and a nuclear-localizing phosphoprotein (UL84) (4). With CMV, the phosphoprotein UL84 needs to complex with IE-2 in order to bind to the highly conserved lytic replication origin (oriLyt), which initiates DNA replication (132, 190). Initiation of replication is poorly understood, although it is

assumed that the complex at the oriLyt separates the DNA strands, unwinds the viral DNA, and initiates the formation of replication forks (15). The protein pUL69 shuttles the intron-less viral mRNA out of the cytoplasm through a CRM1-independent pathway, and the virus utilizes host machinery to translate its mRNA (92).

Viral DNA replication proceeds through a complex of high molecular weight, branched, concatameric intermediates that must be resolved into individual genomes during cleavage/packaging (51). Unit length genomes are then encapsidated by means of a highly conserved *cis* –acting element called a *pac* site interacting with core proteins. The terminase that cleaves DNA consists of UL56 and UL89 and appears to interact with the core PORT protein (portal protein or UL104) (45, 147). This heterodimer is likely assisted by the TER-associated protein (UL51), the portal capping protein (UL77), and UL97 kinase activity (16, 187).

Although virion morphogenesis is incompletely understood, there is some evidence that tegument proteins are added to mature nucleocapsids prior to egress from the nucleus. Other tegument proteins are acquired in subsequent rounds of envelopment and de-envelopment as the capsids mature and complete envelopment. The tegument proteins are also structural, and participate in assembly complex formation outside of the nucleus to support the formation of the final virion structure. Due to homologous studies in other HHVs, it seems as though the CMV gene products from UL50 and UL53 ORFs form a nuclear egress portal or complex on the interior of the nuclear membrane, and the UL97 kinase activity is also important in assisting this nuclear egress (81, 119). The nucleocapsid is de-enveloped and then re-enveloped in the cytoplasm; the last envelopment occurs within an intermediate compartment between the ER and Golgi

apparatus (68, 111). As a result of the final envelopment occurring in the cytoplasm of the cell, the tegument contains both viral and cellular products, such as actin and RNAs (168, 176). Once the mature second membrane is formed, the virion is transported in vesicles to the cell membrane and exocytosed.

Latency

All herpesviruses initiate a lifelong infection within their host and exhibit both latent and acute lytic infectious states. However, with CMV it can be difficult to distinguish latency from a persistent, undetectable low-level of replication (21). Viral replication could be so low (and slow in the case of CMV), it would allow the host immune system to keep severe viral pathology in check and prevent widespread formation of lesions or organ damage. CMV establishes latent infections in CD34+ progenitor cells or cells of myeloid lineage. In these cells, the silencing of immediate early (IE) proteins prevents the expression of other genes required for lytic infection (153). Differentiation of these cells, specifically to a mature dendritic cell, appears to stimulate the expression of IE1 and allow for reactivation of viral replication (138).

Epidemiology

CMV is a ubiquitous and often silent infection whose prevalence tends to follow socioeconomic status. CMV has extremely limited tropism and only infects humans. Like other herpesviruses, once a host is infected, the person is latently infected for life,

with primitive monocytes being the main latent viral reservoir (21, 137). Approximately 59% of the United States population over 6 years of age has the infection, with 60-99% of people infected worldwide. CMV is spread via direct contact through all types of bodily fluids and is most commonly spread either through horizontal transmission during sexual contact or amongst young children in childcare settings (51).

Vertical transmission of CMV, especially with a primary maternal infection during early gestation, results in the most debilitating forms of disease. According to the Centers for Disease Control and Prevention (CDC), CMV is the most common congenital infection in the United States. About 4% of women in the United States experience a primary CMV infection while pregnant and about 1/3 of these women will transmit the virus to their fetus. Approximately 300,000 infants are born with CMV every year in the U.S. and about 8,000 children suffer some sort of permanent disability, such as neurosensory hearing loss, microcephaly, seizures and chorioretinitis (18, 27). Despite the prevalence of this infection, few women are aware of the risks associated with CMV in neonates, and prior maternal immunity only slightly lowers the risk of infection (27). CMV belongs to the category of TORCHES infections (TOxoplasma gondii, Rubella, Cytomegalovirus, HErpes simplex virus, and Syphilis); these are infections that are mainly subclinical in the mother yet result in permanent and often fatal damage in the developing infant (161). Sensorineural hearing loss occurs in 50% of infants who exhibit overt symptoms of infection; however, an estimated 90% of infants born with CMV are asymptomatic and 2 out of 25 of these neonates develop permanent auditory damage (24).

Presentation

Clinically, CMV disease is usually asymptomatic in the immunocompetent host; CMV infection accounts for about 10% of all cases of infectious mononucleosis. The average length of CMV mononucleosis is 8 weeks; patients commonly present with malaise, fatigue, myalgia, and a fever lasting longer than 10 days. It is extremely rare for individuals with normal immune systems to develop a severe and debilitating CMV infection (70).

CMV disease in immunocompromised hosts often results as a reactivation of latent virus after an immunocompromising event, such as chemotherapy, solid organ transplantation, or onset of another immunocompromising disease or infection. Disease in these instances is normally enhanced by CMV; evidence of CMV disease can either be "CMV syndrome", an acute condition that mimics mononucleosis and also included rashes and leukocytopenias, or specific organ involvement, such as splenomegaly (136). Global studies have indicated that the cost of solid organ transplants is greatly increased due to CMV infection as it translates to longer hospital stays, increased risk of secondary opportunistic infections and higher rates of rejection (170). CMV infections decrease the likelihood of both patient and graft survival after organ transplantation, and infection occurs in 1/3 of organ transplant recipients, even with prophylactic treatment (27, 173).

Symptomatic congenital CMV infection most often consists of some sort of central nervous system (CNS) abnormalities such as microcephaly, seizures, periventricular calcification, hearing impairment, vision impairment, or mental retardation. Congenital CMV disease can also present as petechiae or purpura, small

gestational size, jaundice or hepatosplenomegaly (18, 51). Unfortunately, due to the CNS deficits, the long-term outlook for congenital CMV disease is often more severe. Out of the live births with active CMV infections, about 10% of these infants will have overt clinical infections. Lethality is about 30% in these infants and is associated with pneumonia and hepatic disease (140).

CMV has also been linked as a causative agent in many other diseases, such as atherosclerosis and cancers, but these associations remain controversial (59, 112). While not yet proven to be oncogenic, CMV does share some characteristics with other known oncogenic DNA viruses, such as the modification of cell-cycle regulators, the increase in both RNA and DNA synthesis, and induction of ornithine decarboxylase (36). CMV antigens are also shown immunohistochemically to be associated with a wide variety and grade of tumors (112).

CMV TREATMENT

Treatment for CMV is mainly in the form of nucleoside drugs (GCV, its valine ester prodrug VGCV and CDV) and the pyrophosphate analog PFA, which all target the CMV viral DNA polymerase (12). GCV and CDV are incorporated by the viral DNA polymerase into the growing viral DNA strand and inhibit the replication of viral DNA. GCV requires an initial phosphorylation by UL97 kinase to the level of the monophosphate; further phosphorylation by cellular enzymes results in the formation of the triphosphate metabolite that competes with cellular deoxyguanosine triphosphate (dGTP) for incorporation by the DNA polymerase into viral DNA. CDV is a nucleoside

monophosphate analog that is phosphorylated by cellular enzymes to the level of the diphosphate that is a substrate for CMV DNA polymerase (41, 166, 188). PFA selectively interacts with the pyrophosphate binding site of pUL54 and inhibits the hydrolysis of dNTP precursors in DNA synthesis (41, 188). Neither CDV nor PFA require phosphorylation by UL97 kinase. While ACV is sometimes used in the prophylactic treatment of CMV, GCV or VGCV are the drugs of choice as they have a broader spectrum of activity and more potent antiviral activity, especially in solid organ transplant recipients (108).

Antiviral therapies are typically indicated for severely ill and immunocompromised individuals or for prophylactic treatment in transplant recipients. Resistance frequently arises in individuals after a few months of antiviral treatment, often due to a combination of non-compliance and incomplete suppression of viral replication in those with high viral loads (150). Since all of these CMV antiviral therapies target the same viral protein, instances of resistant strains with cross-resistance to drugs often arise. Both PFA and CDV must be administered intravenously, and all of these antiviral therapies have dose-limiting toxicities; GCV induces neutropenia, while PFA and CDV are nephrotoxic (150).

CMV Therapeutics in Development

Compounds in development have focused on other aspects of viral replication, from IE proteins to host cellular components utilized by the virus to promote replication. Fomivirsen (ISIS2922) targets IE proteins (127), and indolocarbazole compounds

(nonglycoside indolocarbazole [NGIC]-1 and Gö6976) target pUL97 (105, 198). However, all of these drugs have poor oral bioavailability and pharmacokinetics *in vivo*. Quinazoline compounds, such as gefitinib (Iressa^{TM}) and Ax7396 (RGB-315389) also have activity against both pUL97 and the murine homolog pM97, although there is debate as to whether the mechanism of action of these homologs are the same since pM97 is not affected by other antiviral compounds specific for pUL97 such as maribavir (149, 151, 178). Alkoxyalkylesters of CDV appear to be promising prodrugs of CDV that target the DNA polymerase, specifically the compound hexadecylocypropyl-CDV (HDP-CDV, CMX001), that recently entered into initial clinical trials for treatment of CMV and smallpox (93). Approved drugs that target the putative cellular receptor PDGFR-α and their associated kinase activity have efficacy against CMV infection, and more specific receptor inhibitors are also under investigation for potential CMV antiviral actions (77, 128, 157).

Recently, a vaccine that utilizes recombinant gB from the CMV envelope with a MF59 adjuvant has been shown to be partially efficacious in Phase II clinical trials; 18 out of 225 women (8%) acquired CMV after vaccination versus 31 out of 216 women (14.4%) only receiving placebo (126). Plasmid DNA vaccines are also in clinical trials; these vaccines are priming the immune system against gB and two other viral proteins, pp65 and IE1. One limitation of all of these trials is that the proteins used for viral stimulation are derived from the laboratory Towne strain of CMV, which has slightly different immunogenic potential from the clinical strains of CMV (47).

Maribavir

A benzimidazole compound called maribavir (MBV, 1263W94) is an orally bioavailable candidate therapy for CMV infection that does not appear to be nephrotoxic or hematotoxic. Early clinical studies showed significant decreases in viral load in patients treated with MBV, and the drug was also effective against GCV-resistant viral strains (173). The compound has an excellent safety profile, and the major side effect reported by patients was dysgeusia, or taste disturbance (97). However, MBV failed Phase III clinical trials in transplant patients, as its efficacy was not superior to placebo, and further development is uncertain (48). Even though the optimal dose and duration of therapy has yet to be elucidated, it would be prudent to further review into therapeutic use of this antiviral as subsequent studies have supported its efficacy in the clinical setting (6, 164).

Notwithstanding the difficulties in clinical development, the use of MBV in a laboratory setting has been instrumental in elucidating of the functions of UL97 kinase in CMV replication. Infected cells treated with MBV exhibit a phenotype that closely mimics that of both the UL97-kinase-null recombinant virus (AD169-pUL97^{K355M}; RC314) and the recombinant virus with a large deletion in this gene (AD169-ΔpUL97; RCΔ97) (57). MBV suppresses growth differently in different cell types, and its antiviral activity is enhanced by the addition of CDK inhibitors and provided clues to the mechanisms of UL97 kinase (32). Resistant laboratory isolates to MBV contain mutations in pUL97 and pUL27, and aside from mildly affecting the EBV kinase

BGLF4, MBV does not appear to target other CHPKs or other protein kinases (181). MBV-resistant strains and isolates map mutations in pUL97 around the nucleoside binding domains and phosphate transfer domains (codons 353, 397, 409, and 411), while mutations in pUL27 are more numerous and diverse and are thought to help the virus to adapt to a loss of UL97 kinase activity (31). While pUL27 appears to be a paralog of pUL97, its function appears to oppose that of pUL97. In the absence of UL97 kinase activity, pUL27 appears to actually further hinder viral replication, and does so through a mechanism of Tip60 (Tat interactive protein) acetyltransferase protein degradation and results in a CDK-related G_1 cell cycle arrest (139).

CMV UL97 KINASE

Large DNA viruses like herpesviruses and poxviruses have been shown to encode functional kinases (55, 135, 184), and the family *Herpesviridae* encodes unique viral kinases that are found in all members of the virus family. Sequence analysis of all of the reported herpesvirus families has produced three main classes of kinases homologous to HSV-1: UL13, Us3, and thymidine kinase (83). CMV lacks a Us3 and thymidine kinase, but expresses pp65, which has been reported to have kinase activity with functional properties distinct from any of the three main kinase classes (23). The UL13 kinases, also referred to as herpesvirus unique long region (HvU_L) kinases or CHPKs (conserved herpesvirus-encoded protein kinases), are found in every human herpesvirus and display partial complementary function (141).

The HvU_L serine/threonine (ser/thr) protein kinase family includes the kinases HSV *UL13*, VZV *ORF47*, CMV *UL97*, EBV *BGLF4*, HHV-6 *U69*, HHV-7 *U69*, and HHV-8 *ORF36* (129). While these proteins are important for the life cycles of their respective viruses, and deletion of them often results in severe replication and structural deficits, none are absolutely essential for viral replication (131). These ser/thr kinases all have similar characteristics; they auto and trans-phosphorylate proteins on serines and threonines, are tegument components that localize to the host nucleus, can functionally complement each other to some degree, and can use either ATP or GTP as a phosphate donor (75).

UL97 kinase is the ser/thr kinase encoded from the CMV ORF *UL97*. It is an 80 kilodalton (kDa) minor tegument protein that is expressed with delayed early kinetics from a large transcriptional subunit conserved throughout the herpesvirus genomes (186). Two isoforms of this protein have been reported, with both showing nuclear localization utilizing different nuclear localization signals (NLS) (183). This protein kinase displays the canonical protein kinase domains mainly in its carboxyl-terminal domain; the amino-terminal domain is not absolutely essential for its activity but shares limited homology with chimpanzee and rhesus CMV (Fig. 1) (129). There are also domains within the protein that appear to be important for retinoblastoma protein (RB)-binding and nuclear localization (30, 104, 134). Clinical and laboratory mutations that are passaged under the presence of GCV often disrupt the canonical kinase domains, which are essential for ATP-binding and phosphotransfer activities of the kinase (33).

Many compounds effective against CMV infections are either activated by UL97 kinase (GCV, cyclopropavir) or directly interfere with UL97 kinase activity (MBV,

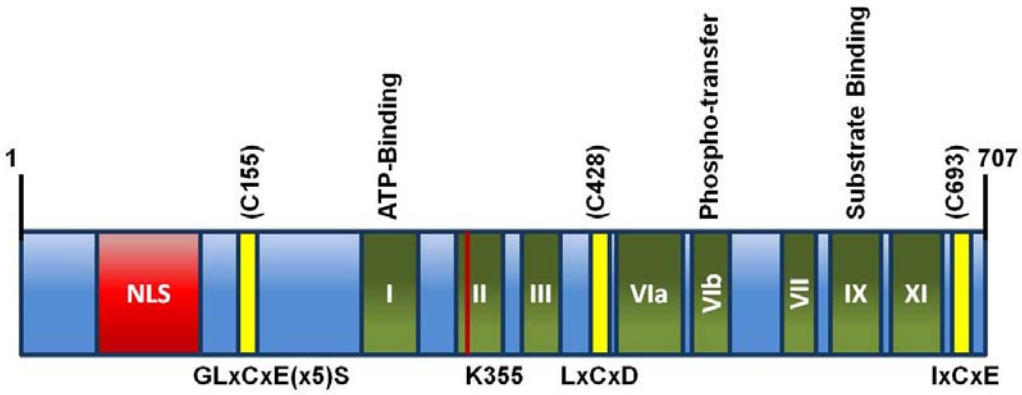

FIGURE 1. Functional Domains of UL97 kinase. The yellow domains are the putative RB-binding domains and contain their sequence below. The cysteine (C) codons depicted have been mutated to identify potential RB-interacting functions. The green domains labeled with roman numerals are the canonical protein kinase domains. The invariant lysine (K355) is highlighted in red. NLS – nuclear localization sequence. All sequences are taken from the UL97 open reading frame from the laboratory isolate AD169 (Protein ID: P16788, Gene ID: X17403).

indolocarbazoles, quinazolines) (129). UL97 kinase phosphorylates the nucleoside analog GCV to its monophosphate form without phosphorylating other natural deoxynucleosides; cellular kinases further phosphorylate it into its triphosphate form. The triphosphate metabolite of GCV inhibits the viral DNA polymerase, severely attenuating viral DNA production (63). MBV is a benzimidazole L-riboside that shows specific activity against CMV and EBV but not HSV-1, HSV-2, VZV, HHV-6, HHV-7, or KSHV (1). MBV is an excellent pharmacological tool to study UL97 kinase activity in vitro because of its extraordinary specificity for the UL97 kinase. Wild-type (wt)-infected cells treated with MBV exhibit a phenotype that mimics both the UL97-kinase deficient (RC314) and deleted (RCΔ97) CMV recombinant viruses (13, 57, 134).

Studies with MBV and with kinase-deficient recombinant viruses (RCΔ97, RC314) have provided great insight as to the function of this viral protein kinase. In the

absence of UL97 kinase activity, there is a modest decrease in viral DNA accumulation. Late gene expression is reduced, as is breakdown of the nuclear lamina and egress of mature capsids from the infected cell nucleus (131, 187). Some reports have identified defects in viral DNA encapsidation; while the kinase does not appear to directly affect cleavage of concatemeric DNA, it seems to be essential for the formation of cytoplasmic assembly complexes (58, 81, 187).

The HvU_L ser/thr kinases share many common targets, both cellular and viral, and rat UL97 or EBV BGLF4 can complement the activity of pUL97 (141). UL97 kinase tends to phosphorylate cyclin-dependent kinase (CDK)-related cellular targets, such as RB (69, 134), EF-1δ (75), p32 (103), Histone H2B (7) and lamin A/C (62). Some viral targets of pUL97 are the DNA polymerase processivity factor, ppUL44 (82), the major tegument protein, pp65 (130), and its own serines (Ser^2, Ser^3, Ser^{11}, Ser^{13} and Ser^{133}) and threonines (Thr^{16}, Thr^{18}, Thr^{134} and Thr^{177}) (129). UL97 kinase also has viral targets, such as pUL69, that are also phosphorylated by CDKs (169). Even though it phosphorylates CDK targets which have a common consensus sequence (S/TPXK/R), it has not been possible to define a consensus phosphorylation sequence for this enzyme.

Since many of the cellular targets of pUL97 are cell checkpoint regulators, it has been suggested that the kinase helps to push the infected cells into certain stages of the cell cycle to aid in the viral replication process. For instance, hyperphosphorylation of RB by pUL97 would allow the cell to enter into synthesis (S) phase, producing cellular proteins and precursors important for DNA replication that the virus can utilize to support its replication (69, 134). CMV also induces a mitotic-like state; this “pseudomitosis” shows an increase in mitotic regulators, an increase in nuclear size and a breakdown of

the nuclear lamina. Destruction of the laminar integrity is thought to provide more space for viral DNA replication and allows for mature viral capsid egress from the nucleus (67). Lamin A/C is phosphorylated by UL97 kinase and recruited to the lamin B receptor by p32, both of which drive the disruption of nuclear lamina integrity (62, 103). UL97 kinase also appears to block certain steps important in normal cellular defense.

The kinase activity of pUL97 also inhibits the formation of aggresomes, which are an innate immune response to viral infection; aggresomes sequester viral proteins and prevent their function. While other viruses appear to utilize these aggresomes as the site of viral DNA/RNA production (185), CMV disrupts their formation and is thought to promote morphogenesis (134). UL97 kinase activity also prevents aggregation of polyglutamine proteins that amass in many neurodegenerative diseases such as Huntington's Disease and Spinocerebellar Ataxia-3 (171).

CELL CYCLE

The cell cycle describes how eukaryotic cells grow, double their genetic material, and divide, with tightly regulated checkpoints between each phase of the cell cycle that prevent aberrant growth (49). There are two main phases a cell undergoes to produce daughter cells: mitosis (M), where a cell splits its chromosomes between two daughter cells and physically divides, and interphase, which consists of growth or "gap" phases (G_1 and G_2) separated by a DNA doubling phase called the S phase (163). Quiescent cells or cells that have temporarily stopped dividing can be considered in a G_0 stage or a resting stage. Activation of each phase of the cell cycle is dependent upon a series of

CDKs and their cyclin regulators to push the cell through various tightly-regulated checkpoints (Fig.2). Cyclins regulate CDKs and allow CDKs to phosphorylate target proteins to orchestrate entry into a different phase of the cell cycle (14).

The first growth phase, G_1, initiates interphase and takes place between the cytokinetic end of mitosis and the onset of the S phase. The length of this phase depends on the cell type and other environmental factors and allows the cell to begin biosynthesis of regulators and proteins necessary for entry into and progression through S phase (20). If sufficient growth signals are not received or initiated past a pre-designated restriction point in G_1, sometimes called the G_1-S checkpoint, the cell will exit the cell cycle and enter G_0. Once a cell passes this restriction point, it is committed to enter into S. One of the main regulators of entry into the S phase through the G_1-S checkpoint is the tumor-suppressor protein RB, which is a 110 kDa nuclear phosphoprotein (56). RB does not have an enzymatic function but rather sequesters and binds to multiple proteins depending on its phosphorylated state (80). In its active, hypophosphorylated form, RB is bound to the transactivation domain of the transcription factor E2F; the RB-E2F complex is bound to the E2F promoters but is transcriptionally inactive (196). The RB-E2F complex also recruits histone deacetylases (HDACs) to the DNA promoters, further repressing transcription (52). Hyperphosphorylation of RB by CDK/cyclin complexes results in a conformational change that inactivates and releases the protein from E2F, allowing for active transcription of E2F targets. The initial phosphorylation of RB is done by CDK4 or CDK6 coupled with cyclin D; RB is further phosphorylated in a positive-feedback loop by CDK2/cyclin E and remains hyperphosphorylated throughout

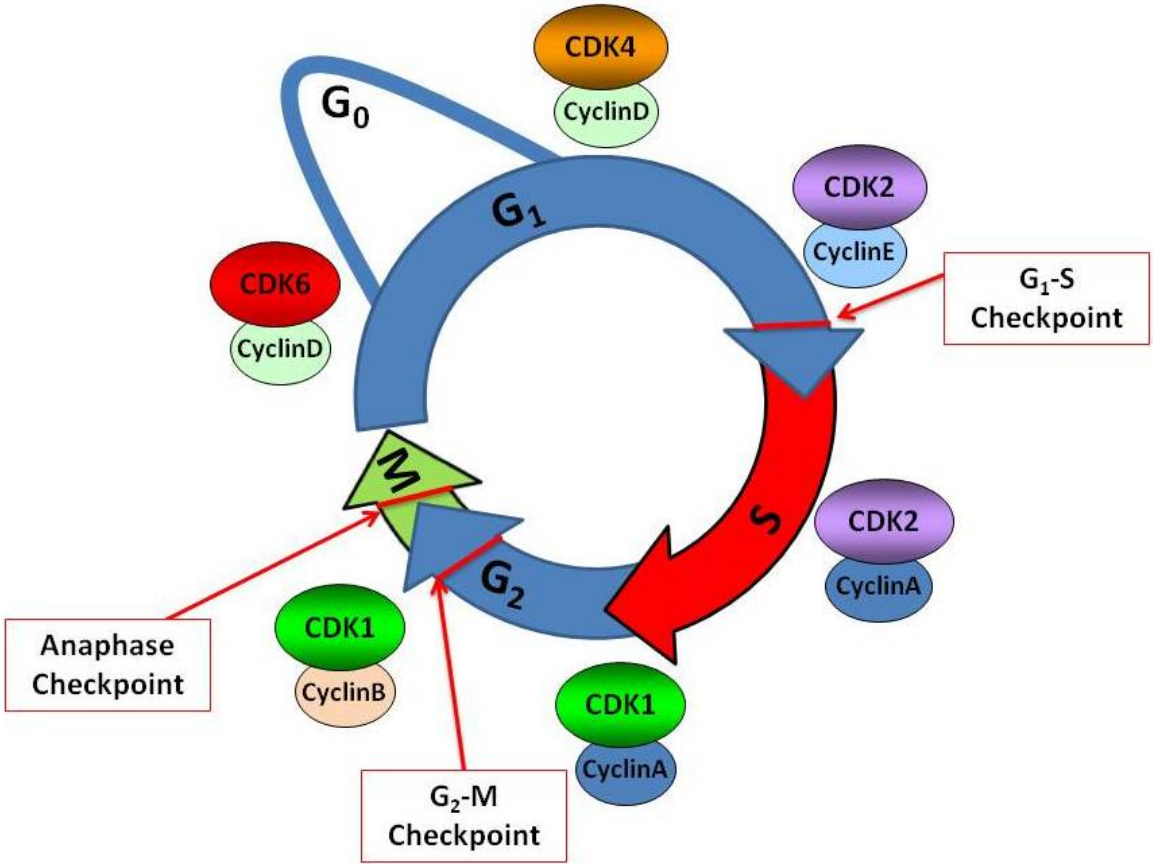

FIGURE 2. The Cell Cycle, Cyclins, and CDKs. Cells grow and divide according to the cell cycle. Mitosis, which is represented by the green arrow, divides the cell into two daughter cells. Interphase, which encompasses the remainder of the cell cycle, is marked by two growth phases (G_1 and G_2, blue arrows) divided by a DNA synthesis phase (S, red arrow). The G_0 state can happen in either growth phase, and is a "resting" state for the cell where it is metabolically inactive. The CDKs and their regulatory cyclins are shown at each phase for which they are important. The three main checkpoints are delineated by red lines.

S, G_2, and M phases (86). This release of transcriptional regulation allows for the up-regulation of other cyclins such as cyclin A and B, which support further CDK activation and cell entry into M phase.

After a cell doubles its DNA, it enters another growth phase called G_2 before it prepares to divide and produce daughter cells in M; to enter into M the cell must pass through the G_2-M checkpoint (91). Another check the cell has in G_2 analyzes the quality of the DNA that was produced in S. If the DNA is damaged, the cell will either repair the

DNA or enter into apoptosis (160). If a cell with damaged DNA enters into mitosis, apoptosis or uncontrolled oncogenic cell proliferation may occur (64).

During mid-G_2 phase, if the cell has replicated normally, cyclin B levels will peak and start to activate CDK1. Full activation of the cyclin B/CDK1 complex, also known as the maturation or mitosis promoting factor (MPF), is regulated by multiple factors. T-loop phosphorylation at tyrosine 161 and the interplay between Wee1/Myt1 inhibitory kinases and cdc25 activating phosphatases are the main phosphorylative regulatory mechanisms of CDK1.

Once a cell enters mitosis, there are five main sub-steps within mitosis defined by localization of the cellular environment and sister chromatids (96). The first stage is prophase, during which the chromatin starts to condense, the nucleolus disappears, two centrioles form and spindle fibers formed from microtubules start to extend outwards from each centriole. In metaphase, the nuclear membrane completely dissolves, the spindle fibers attach to each chromatid bundle at the centromere creating kinetochores, and the spindle fibers align the chromosomes along the midline of the cell. In this stage, there is one last checkpoint known as the spindle assembly checkpoint, the anaphase checkpoint, or the mitotic spindle checkpoint. If all of the chromosomes have aligned correctly along the midline mitotic plate, the kinetochores will separate the sister chromatids using the kinetochores (121). During anaphase, the sister chromatids are separated, and the kinetochores move each chromosome copy along the length of the spindle fibers to the opposite side of the cell. Cyclin B expression starts decreasing at the end of metaphase due to the anaphase promoting complex/cyclosome (APC/C) ubiquitinating cyclin B and eventually terminating MPF activity (26). New nuclear

membranes start to form around the separated chromosomes during telophase; the chromosomes and spindle fibers start to disperse and the cell begins to contract in the middle. Cytokinesis is the final event, with the actual division of the cell to produce two identical daughter cells, both of which are now back at the beginning of the cell cycle in interphase (G_1).

Normally, the cell closely regulates the cell cycle checkpoints, and will only allow a cell to enter a stage, especially mitosis, only if it is absolutely ready to complete the process. It is often assumed that once a checkpoint is passed, the cell is crossing a "point-of-no-return", and there is little option for a cell aside from cell death or cell-cycle arrest if a cell enters S or M when it is unready to complete the process. However, there have been exceptions noted to this rule, mainly in the form of endocycling, mitotic catastrophe (MC) and pseudomitosis.

Endocycling is when a cell undergoes multiple rounds of DNA replication without a cytokinetic division. To allow a cell to relicense DNA replication, G_2-M regulators accumulate, especially CDK1, but in an inactive form. Endocycling is essential for Drosophila development, yet very few mammalian cells normally undergo this type of replication (89). The creation of polyploid cells in mammals is limited to gametic lines such as trophoblast giant cells, and normally results in an enlarged cell with a giant nucleus, similar to the morphology seen in CMV infection (175).

A mitotically-induced programmed cell death that results from cells inappropriately entering into mitosis is known as MC. Although MC is considered a "prestage" event that occurs before entry into necrotic or apoptotic cell death, certain studies show that cells entering MC can be stalled in a limbo "pseudomitotic" state from

which cells can be rescued and remain viable (25). MC is characterized by microtubule defects, aberrant nuclear morphology (especially formation of small nuclear envelopes around individual clusters of mis-segregated chromosomes), premature chromatin condensation, incomplete DNA synthesis, and enlarged "giant" cells (174); regardless, this pseudomitotic cellular state is amenable to viral maturation and egress since it allows for nuclear membrane disintegration.

Pseudomitosis, which has mainly been described in the context of herpesvirus infections, involves the combination of MC and endocycling states, such as multiple rounds of DNA replication, an enlarged cell and nucleus, multiple spindle poles, irregular condensation, and mislocalized chromosomal DNA (67). Published reports indicate that CMV-infected cells upregulate certain G_2-M regulators such as CDK1 and cyclin B1 and mitotic markers such as Eg-5 to enter pseudomitosis (159). This pseudomitotic state induced by the virus most likely required to form precursors for viral DNA replication as well as egress from the nucleus, which are vital steps for mature virion production.

Cyclin-Dependent Kinases

CDKs are ser/thr kinases that are involved in the regulation of both the cell cycle and transcription (95). Their activity requires association with a regulatory subunit, called a cyclin. Cyclins, as their name implies, change in activity and concentration throughout the cell cycle while CDKs are constitutively expressed at relatively stable levels. Upon sequence analysis of the human genome, there are approximately 26 genes that encode CDKs or CDK-like proteins; 11 of these are well-defined kinases with

overlapping functions (99). However, other CDKs cannot compensate for the loss of CDK1 but they can compensate for the loss of CDK2, CDK4, or CDK6 (145-146).

CDKs can be regulated by four main mechanisms: association with their cyclin regulatory subunit, inhibition by a cyclin kinase inhibitor (CKI), phosphorylation at a highly conserved threonine residue, and phosphorylation on a threonine and/or a tyrosine near the catalytic core (85). CKIs come in two main families that are separated by homology and mechanism of action. INK4 CKIs will prevent the CDK from associating with its regulatory cyclin, while Cip/Kip inhibitors can bind directly to the CDK/cyclin functional unit (10). For a CDK to become active, two events must happen to relieve steric hindrance of its active site. A CDK is first phosphorylated by a CDK Activating Kinase (CAK) at a conserved threonine residue in the T-loop which allows the large, flexible loop to bend. This, in turn, allows for proper conformation of the CDK to bind with its cyclin, which then fully alters the CDK into a fully active conformation (49). Kinases such as Wee1 and Myt1 can phosphorylate and inactivate CDKs at adjacent threonine and tyrosine residues when they are bound to cyclins; however, cdc25 phosphatases can relieve this inhibitory phosphorylation (50).

There are checkpoints at each phase in the cell cycle that are either prevented or supported by the action of the CDK/cyclin complexes. CDK4 and CDK6 will associate with cyclin D during G_1 and promote entry into S phase by phosphorylating and inactivating many pocket proteins, including RB, RBL1 (p107) and RBL2 (p130). This phosphorylation relieves suppression of S-phase gene expression by allowing the liberation of E2F from RB; this initial release of repression allows for up-regulation of E-

type cyclins (E1 and E2), which activate CDK2, causing further inactivation of pocket proteins and full progression into S phase (146).

For entry into mitosis, CDK1 requires the cyclins A and B; A-type cyclins are necessary for onset of mitosis at the end of G_2 while cyclin B facilitates the progression through mitosis. CDK1 forms a heterodimer with cyclin B to create the MPF; this phosphorylates multiple targets and allows the cell to enter mitosis. CDK1 localizes mainly to the cytoplasm but is found in the nucleus during mitosis after the breakdown of nuclear lamina (117, 146). MPF specifically promotes mitotic events such as chromatin condensation, mitotic spindle formation, lamin network breakdown and Golgi apparatus degradation (91).

The MPF is regulated by a series of checks and balances. In G_2, the MPF is mainly regulated by phosphorylation. The tumor suppressor p53 and DNA damage sensory kinases PK, ATM, and ATR will block activation of the MPF by various mechanisms, one of which is preventing phosphorylation and activation of cdc25B (109). Phosphorylation at threonine 14 (Thr^{14}) and tyrosine 15 (Tyr^{15}) on CDK1 by either Wee1 or Myt1 kinases is inhibitory and overrides the CAK activating phosphorylation at Thr^{161} in the T loop. While both Wee1 and Myt1 have similar functions, Wee1 is predominantly nuclear while Myt1 is cytoplasmic. Both are phosphorylated by MPF and inactivated; while Myt1 kinase activity is inhibited by phosphoryation, phosphorylation of Wee1 inhibits and degrades it (182). In turn, the cdc25 phosphatases will remove the inhibitory phosphates at Thr^{14} and Tyr^{15}. Cdc25B initiates the activation of MPF, and in a positive feedback loop, MPF will phosphorylate cdc25C and activate further phosphatase activity (19). Phosphorylation of cyclin B by either CDK1, MAPK, or Plk1

will also promote activity by hiding a nuclear export signal and enhancing nuclear import (179, 191). MPF will start localizing at centrosomes during late G_2 and help promote centrosome maturation and microtubule-organizing center (MTOC) assembly. The activity of the MPF drastically decreases at the end of metaphase due to the APC/C promoting the ubiquitination of cyclin B and destruction of the MPF (26)

CMV products are able to mimic and interact with many cellular CDKs. In one study, CDK9 was shown to localize with CMV viral DNA, while CDK7 is found within viral replication centers (167). The HvU_L kinases share many of the same cellular and viral targets as CDKs, and even phosphorylate the targets at the same ser/thr residues (75). HSV-1 UL13 appears to phosphorylate and activate CDK1 itself in order to assist late gene expression (2-3). CDK1 phosphorylates IE62 in VZV and is incorporated directly into virions (87). Mimicking the function of CDKs is also important for disrupting innate antiviral activities within the cell, as aggresomes are able to form when the UL97 kinase has its putative RB-binding domains mutated or its kinase activity inhibited (134).

Viral Infection and the Host Cell Cycle

Viruses have the well-documented ability to alter the cell cycle. These obligate intracellular parasites require a host to replicate themselves and exhort the host environment to produce products or bypass cellular checkpoints to promote viral replication. Viral induction of aberrant cell cycle regulation often results in cancer; Hepatitis B and C have been linked to hepatocellular carcinoma while retroviruses, like

HIV-1 or Human T-lymphotropic virus Type I (HTLV-I) can activate oncogenes by insertion into the host DNA genome (51). The gammaherpesviruses have proven oncogenic capabilities; EBV causes Burkitt's lymphoma due to its ability to transform and immortalize B cells (125) while KSHV triggers Kaposi's sarcoma and primary effusion lymphoma by encoding numerous homologs of cellular oncogenes (165).

Viruses commonly modulate the regulation of cell cycle checkpoints. They can force the infected cell past the G_1-S checkpoint, especially through manipulation of the tumor suppressor RB. Small DNA viruses encode the following oncoproteins that are known to interact with RB: large T antigen (LTa) from simian vacuolating virus 40 (SV40), E1A from adenovirus and E7 from papillomavirus (42, 100) LTa and E1A prevent RB association with E2F; E7 not only prevents association with E2F but also marks RB for degradation.

CMV also dysregulates the entry into S phase. IE1-72, IE2-86 and pp71 all interact with and inactivate RB-family pocket proteins (144, 156). UL97 kinase directly hyperphosphorylates RB in order to inactivate it, mimicking the function of CDK4 and CDK6; this method of inactivation differs from other viral RB-associated proteins which instead prevent RB's function by physically sequestering it from E2F (134). UL97 kinase is required not only for the phosphorylation, but also stabilization of RB (Fig. 3) (134). Subsequent studies have highlighted that the kinase directly phosphorylates specific residues on RB, including Ser^{780}, Ser^{807}, and Thr^{821} , and this phosphorylation is independent of cellular CDKs (69). Viruses with point mutations that either disrupt the cysteine residue in the putative LxCxE amino terminal motif or disrupt the invariant

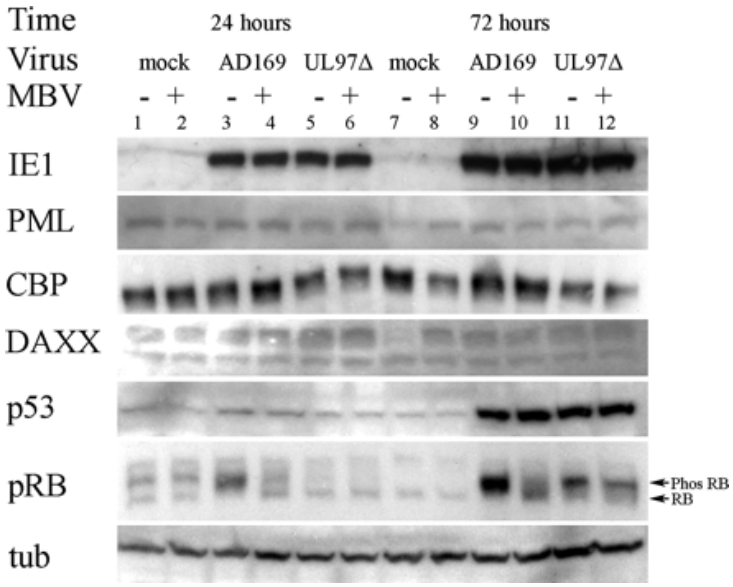

FIGURE 3. UL97 kinase activity is required for the hyperphosphorylation of RB in infected cells. HFF cells were mock infected or infected at an MOI of 2 PFU/cell with AD169 or a UL97 null virus (UL97Δ), both with (+) and without (–) the addition of MBV as shown. Cell lysates were harvested at 24 and 72 h following infection, separated on polyacrylamide gels, and transferred to polyvinylidene difluoride membranes. Shown are immunoblots, with monoclonal antibodies to the proteins indicated to the left of the figure. The accumulation of hyperphosphorylated forms of RB was reduced when the UL97 kinase was deleted or when its activity was inhibited with MBV. Note: From "Human cytomegalovirus UL97 kinase activity is required for the hyperphosphorylation of retinoblastoma protein and inhibits the formation of nuclear aggresomes" by Prichard MN, Sztul E, Daily SL, Perry AL, Frederick SL, Gill RB,et. al. J Virol. 2008 May;82(10):5054-67. Copyright 2008 by American Society for Microbiology. Reprinted with permission.

lysine motif K355 are unable to stabilize and phosphorylate RB (Fig.4). Normally, UL97 kinase prevents the host from forming nuclear aggresomes, an innate antiviral response that sequesters viral proteins and prevents viral replication. When UL97 kinase activity is inhibited, these aggresomes form normally and is likely a direct effect of multiple units of promyelocytic leukemia protein (PML), a protein known for aggregating and sequestering proteins in the nucleus, binding to hypophosphorylated RB and sequestering other cellular and viral proteins (Fig. 5). Consensus sequence analysis of the pUL97 LxCxE domain shows remarkable similarity amongst other viral proteins known to

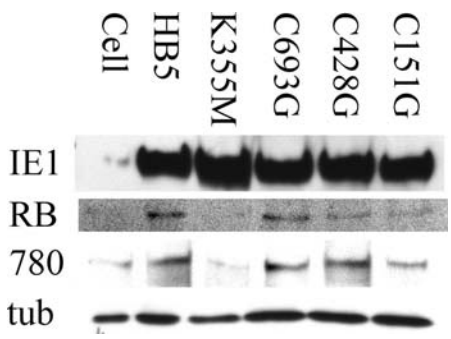

FIGURE 4. Recombinant viruses with point mutations in either the LxCxE RB binding motif or the kinase motif are impaired in their abilities to stabilize and phosphorylate RB. HFF cells were infected (at an MOI of 2 PFU/cell) with the wt virus HB5 or with recombinant viruses containing the point mutations in pUL97, as shown. Cell lysates were harvested at 24 h following infection, separated on polyacrylamide gels, and transferred to nitrocellulose membranes. Shown are immunoblots, with the antibodies to the proteins indicated to the left of the figure. The accumulation of RB occurred in cells infected with HB5, but was reduced in cells infected with the K355M mutant and the C151G mutant. The phosphorylation of RB on serine 780 was determined with specific antisera. Cells infected with HB5 contained increased levels of RB phosphorylated on serine 780. Cells infected with viruses containing point mutations that abrogated UL97 kinase activity (K355M) or disrupted the LxCxE motif (C151G) exhibited reduced levels of RB phosphorylated on serine 780. The expression of IE1 confirmed that cells were infected, and tubulin (tub) was included as a loading control. Note: From "Human cytomegalovirus UL97 kinase activity is required for the hyperphosphorylation of retinoblastoma protein and inhibits the formation of nuclear aggresomes" by Prichard MN, Sztul E, Daily SL, Perry AL, Frederick SL, Gill RB,et. al. J Virol. 2008 May;82(10):5054-67. Copyright 2008 by American Society for Microbiology. Reprinted with permission.

interact with RB, such as adenovirus E1a, papillomavirus E7, and SV40 large T antigen and other homologous herpesvirus kinases (U69 from HHV-6 and HHV-7 and chimpanzee CMV pUL97.) (Fig. 6)

Likewise, viruses also promote dysregulation of the G_2-M checkpoint to induce a cellular state that further promotes viral replication and egress. HIV-1 peripheral $CD4^+$ and $CD8^+$ T-cells have a marked increase in cyclin B while the HIV proteins Env and Vpr both induce inhibitory phosphorylation of CDK1 and a G_2-M cell cycle arrest that results in apoptosis (26, 78). E4 protein of HPV induces over expression of Wee1,

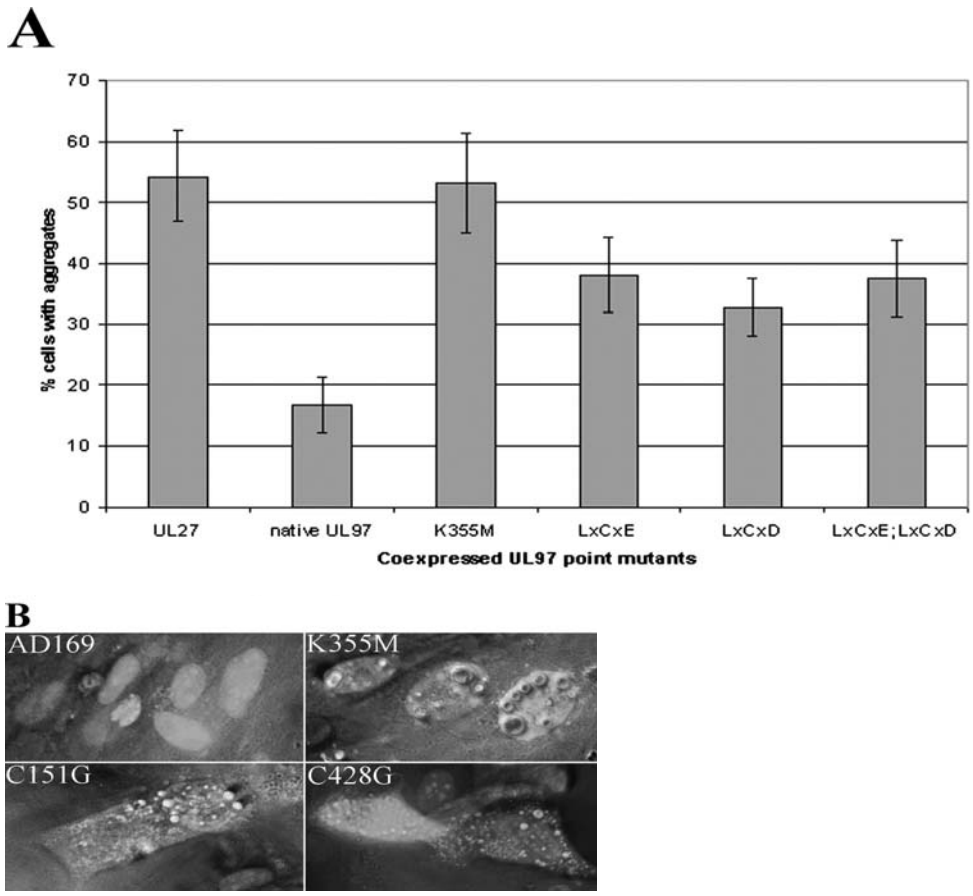

FIGURE 5. RB binding motifs in pUL97 impact the inhibition of aggresome formation. COS7 cells were transfected with plasmids expressing pp65-GFP, plasmids expressing a UL27-negative control, and plasmids expressing UL97, with the point mutations as labeled. (A) The graph depicts the percentage of cells containing visible aggregates. The values shown are the averages of six separate experiments, with the standard deviations (error bars) shown; the exception was the double mutant, for which values were determined twice. (B) Recombinant viruses with mutations in the UL97 kinase domain or the RB binding domains also form large aggregates. Viruses with point mutations in the amino acids shown were used to infect confluent HFF cells and were harvested 8 days following infection. Shown are fluorescent phase-contrast images of infected cells stained with an antibody to pp65 to confirm viral infection. Note: From "Human cytomegalovirus UL97 kinase activity is required for the hyperphosphorylation of retinoblastoma protein and inhibits the formation of nuclear aggresomes" by Prichard MN, Sztul E, Daily SL, Perry AL, Frederick SL, Gill RB,et. al. J Virol. 2008 May;82(10):5054-67. Copyright 2008 by American Society for Microbiology. Reprinted with permission.

possibly through protein phosphatase 2A (PP2A), which is thought to activate Wee1 (79).

HIV-1, HTLV-1, EBV, adenovirus, and reovirus all encode proteins that block G_2-M

```
UL97 LxCxD   HDQWKLACID-SYRRAFCTLADAI
UL97 LxCxE   DGYHGLRCRETSAMWSFEYDRDGD
UL97 IxCxE   RRTTSIICEE-------DLDGDCR
pp71 LxCxD   DALEQLACSD-PNTYIHKTETDER
Consensus    DA   LAC D S  WAFDTDADAR

HAdV E1A            SEESKRG--EELDLKCYEECLP-SSDSETEQTGGDGCTEPVVKNEPVL
HPV16 E7            HEYMLDLQPETTDLYCYEQLND-SSEEEDEIDGPAGQAEPDRAHYNIV
SV40 large T        EWEQWWNAFNEENLFCSEEMP--SSDDEATADSQHSTPPKKKRKVEDP
HCMV UL97 LxCxE     SRGSAASGDGYHGLRCRETSAMWSFEYDRDGDVTSVRRALFTGGSDPS
CCMV UL97 LXCXE     SAVSSDGGSSYHGLRCRESSDMWSFEYEGDDGVTSVRGGVVGATNGGG
HHV7  U69 LxCxE     SETDLCPDPSVTDLLCHESLTV-SPKFERDGLSACTEFENFMDTRKIV
HHV6A U69 LxCxE     KHEIACDCSAIEELLCHESLLD-SPMKLSNAHTIFSSNKWKLELEKII
Consensus           S           DL CHESL   S E E DA              IV
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FIGURE 6. RB-binding Motifs in pUL97. The three putative RB-binding domains in pUL97 are aligned above with the RB-binding domain of pp71. Viral proteins with known RB-binding properties were also aligned with the pUL97 amino acid sequence for the LxCxE domain for comparison. All show a conserved serine residue approximately 5 residues from the glutamine. The viral proteins known to have strong interactions with RB have an aspartic acid immediately upstream of the RB-binding domain, while those with weaker interactions tend to have a glycine or other amino acid. Sequences were obtained from CMV (NP_040032.1), chimpanzee CMV UL97 (NP_612729), SV40 large T (NP_043127), human adenovirus E1A (ABK35030.1), human papillomavirus 16 E7, (AAD33253.1), HHV-6A U69 (NP_042962.1), HHV-6B U69 (T44214), HHV-7 U69 (YP_073809.1), and CMV pp71 (NP_040017). Note: From "Human cytomegalovirus UL97 kinase activity is required for the hyperphosphorylation of retinoblastoma protein and inhibits the formation of nuclear aggresomes" by Prichard MN, Sztul E, Daily SL, Perry AL, Frederick SL, Gill RB,et. al. J Virol. 2008 May;82(10):5054-67. Copyright 2008 by American Society for Microbiology. Reprinted with permission.

progression, sometimes through multiple mechanisms, indicating that it is highly advantageous for a virus to prevent progression through mitosis (192).

CMV alters the cell cycle of infected cells in order to promote a milieu that supports viral replication, and also dysregulates the G_2-M transition to allow for viral maturation and egress. IE2-86 was shown to phosphorylate and activate p53, which, during G_2, assesses the integrity of the DNA produced in S phase (144). CMV infection increases the levels of Cyclin B and CDK1 (143) and nuclear lamina breakdown (67). The APC/C is inactivated and disassembled during CMV infection, reportedly due to the degradation of some of its important subunits (172). The UL97 kinase has been shown to

directly phosphorylate the nuclear structural component lamin A/C, which is normally performed by CDK1 in mitosis to disrupt the integrity of the nuclear lamina (62, 103).

Viruses also take advantage of the DNA damage response machinery which assesses the cellular genome, and either promotes DNA repair or apoptosis depending on the severity of the response. Viruses manipulate these stress response pathways, specifically the ATM/Chk1/Chk2/Cdc25C and ATM/p53/p21$^{Cip1/Waf1}$ DNA damage regulators, and defects in these pathways prevent viruses such as herpesviruses and reoviruses from productively replicating (35, 88). This is advantageous for a virus as it stalls the infected cell in G_2-M stages and allows for accumulation of checkpoint proteins. This leads to a broad stress response that, in part, prevents host cellular translation and leads to apoptosis (158). However, CMV is able prevent the cell from completing apoptosis and promote the translation of its own mRNA over cellular mRNA through tight regulation of such mechanisms as phosphorylation and assembly of the translational machinery (72).

FOCUS OF THE DISSERTATION

CMV is a ubiquitous infection that can cause serious infections in neonates and immunocompromised individuals (51). CMV is the primary congenital viral infection in the United States and can often result in death or permanent neurological deficits such as hearing loss, seizure disorders and mental disability (21). The current frontline FDA-approved drugs for treating CMV infection- CDV, PFA and GCV- have major clinical limitations, such as limited oral bioavailability and dose-limiting toxicity. Each of these

drugs also target pUL54, the viral DNA polymerase, and multidrug resistant viral isolates are sometimes seen in immunocompromised hosts following chemotherapy (5, 30, 34). The high incidence of morbidity and mortality from this infection in both neonates and immunocompromised individuals demands the development of new CMV antiviral strategies. Therefore, to combat CMV infection, additional antiviral therapies which utilize a novel mechanism of inhibition must be identified.

We have focused on the role of UL97 kinase in CMV infection because of its importance in the therapy of CMV infections. Although not essential for replication, viruses deficient for pUL97 show a viral yield reduction of over 2 orders of magnitude (131). A benzimidazole compound in clinical drug trials, MBV, specifically inhibits UL97 kinase activity through a direct interaction with the ATP-binding site (1, 13, 30). Since MBV is an extremely selective inhibitor of this kinase, it is an excellent pharmacological tool to study the function of the UL97 kinase activity in vitro. Studies using this drug are complementary to genetic studies and provide a very powerful method to understand the functions of pUL97. The aims of this research seek to further elucidate the functions of CMV pUL97, a novel antiviral target, and these studies promise to provide a better understanding of the role of this kinase in viral infection.

Analyses using MBV have supported those with recombinant viruses and have brought to light that this protein shares CDK targets such as EF-1δ, p32, and lamin A/C (129). We have also demonstrated that pUL97 hyperphosphorylates and inactivates the CDK4 and CDK6 target, RB, and that the LxCxE domain of pUL97 is the likely binding region (134). However, additional functions of pUL97 have yet to be fully identified, and studies in related viruses suggest that pUL97 likely phosphorylates other CDK targets,

including CDKs themselves and their regulators (2). Thus, in addition to having functional similarities to CDK1, UL97 kinase may also alter the expression and localization of CDKs. We hypothesize that CMV UL97 kinase modifies the host cell cycle to promote viral replication, and that it does so by regulating both CDKs and CDK targets.

Specific Aims

Two specific aims were established to help understand the function of the CMV UL97 kinase and its effect on the host cell. Exploration of these aims will improve our understanding of the many targets of UL97 kinase and how its activity promoted viral replication. This information has the potential to be used to develop or enhance therapies for CMV infections.

The first aim sought to further clarify the biological significance of pUL97 interaction with RB. The UL97 kinase appeared to physically interact with RB, and this interaction was important for the replication cycle of CMV. Mutational analyses of pUL97 indicated that the amino-terminal putative RB-binding motif was important for phosphorylation of this tumor suppressor. These findings have contributed to a more thorough understanding of the mechanisms behind pUL97 interaction with the tumor-suppressor protein RB.

The second aim characterized changes in G_2-M checkpoint regulation induced by UL97 kinase activity. CMV-infected cells showed an increase in G_2-M checkpoint regulators, such as CDK1 and Cyclin B1, related to the induction of a pseudomitotic

state. Some of these changes were known to be kinase-specific; pUL97 phosphorylates lamin A/C, which allowed for breakdown of the nuclear structure during CMV infection (62). Interestingly, CDK1 also phosphorylates lamin A/C, and homologs of UL97 kinase were known to interact with CDK1 to support viral replication. Our findings showed that UL97 kinase activity was important for the transcriptional regulation of CDK1, and also affected the overall expression and localization of this mitotic regulator. These studies provided necessary information regarding additional targets of UL97 kinase, as well as illuminated the role of kinase induction of pseudomitosis within the infected cell.

Presented in the next chapter is data summarizing results from the first aim, published in 2009. This paper further elaborates on our earlier findings published in 2008 as to the mechanism of pUL97 interaction with RB. The following chapter discusses major findings from the second aim, including detailing pUL97 distribution throughout an infection as well as UL97 kinase activity upon CDK1.

CONSERVED RETINOBLASTOMA PROTEIN-BINDING MOTIF IN HUMAN CYTOMEGALOVIRUS UL97 KINASE MINIMALLY IMPACTS VIRAL REPLICATION BUT AFFECTS SUSCEPTIBILITY TO MARIBAVIR

by

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ABSTRACT

The UL97 kinase has been shown to phosphorylate and inactivate the retinoblastoma protein (Rb) and has three consensus Rb-binding motifs that might contribute to this activity. Recombinant viruses containing mutations in the Rb-binding motifs generally replicated well in human foreskin fibroblasts with only a slight delay in replication kinetics. Their susceptibility to the specific UL97 kinase inhibitor, maribavir, was also examined. Mutation of the amino terminal motif, which is involved in the inactivation of Rb, also renders the virus hypersensitive to the drug and suggests that the motif may play a role in its mechanism of action.

FINDINGS

Human cytomegalovirus (HCMV) is a ubiquitous virus that can be problematic in immunocompromised populations, including individuals with AIDS or recipients of allograft transplants. It is the most common congenital infection in the United States (6) and sequela include permanent neurological deficits, including hearing loss (5, 11) . Ganciclovir (GCV), foscarnet and cidofovir (CDV) have all been approved for the treatment of HCMV infection, but each drug is associated with dose-limiting toxicities (3). The benzimidazole L-riboside, maribavir (MBV), is currently in Phase III clinical trials for the treatment of HCMV infections and inhibits viral replication by a distinct mechanism involving the direct inhibition of UL97 kinase activity (3-4, 7, 23). While this drug clearly inhibits the enzymatic activity of the UL97 kinase in infected cells, the consequences of its inhibition are complex and incompletely understood as the kinase affects many cellular and viral processes.

The UL97 serine/threonine kinase is expressed early in infection and is found within the tegument of infectious virions (18, 22). Although the kinase is not required for viral replication, null mutants exhibit severe replication deficits (19), which is consistent with the inhibitory effects of MBV (4). This enzyme has been shown to phosphorylate viral proteins including itself, ppUL44 and pp65 (1, 14, 16), as well as the large subunit of RNA polymerase II, eukaryotic elongation factor 1delta, P32 and lamins A/C (2, 15, 17). The tumor suppressor retinoblastoma (Rb) has also been shown to be hyperphosphorylated in cells infected with HCMV (13), and this phosphorylation is dependent on UL97 kinase activity (20). This report also showed that mutations in either the essential lysine (K355) or the conserved LxCxE Rb-binding motif in the amino terminus of pUL97 reduced the inactivation of Rb (20). A separate study showed that the kinase phosphorylated Rb directly and did not require other proteins (12). This activity is intriguing since Rb is also targeted by many viruses, including human papilloma virus, simian virus 40, and adenovirus (10). However, the interaction seems to be finely-tuned between HCMV and the cell and does not appear to result in an oncogenic phenotype exhibited by other viruses which target Rb.

Rb belongs to the family of pocket proteins which prevent the progression of the cell through the G_1/S checkpoint by binding to and suppressing the function of the transcription factor E2F; hyperphosphorylation of Rb causes it to release E2F which activates key steps in the cell cycle (21). The inactivation of Rb by the kinase is presumed to modify cell checkpoint protein expression and induce the expression of cellular proteins required for viral infection, but its impact on viral replication has not been established. The UL97 gene product contains three consensus binding sequences for Rb (12, 20); disruption of the essential lysine or the amino terminal Rb-

binding motif (LxCxE) reduces the Rb phosphorylation seen in HCMV wild type (wt) inoculated cells (20). Therefore, we hypothesized that viruses with disrupted Rb-binding sites in the UL97 kinase might have an impaired replication phenotype or altered susceptibility to antiviral drugs.

Recombinant viruses were engineered with point mutations in the *UL97* open reading frame (ORF) and their construction was reported previously (20). RC314 is a kinase-null virus with a K355M mutation. Recombinants RC295, RC312, and RC316 contain amino acid substitutions C151G (LxCxE), C428G (LxCxD) and C693G (IxCxE), respectively, that disrupt individual putative Rb-binding elements in the amino acid sequence of *UL97*. Another virus was constructed that contains both the C151G and the C428G mutations (RC323). Replication kinetics of each of these viruses were evaluated in low MOI infections of stationary HFF cells in the presence of 2% FBS. Disruption of the essential kinase motif in the *UL97* ORF with a K355M mutation severely impacted the replication of the virus and was indistinguishable from that of RCΔ97 [Figure 1A]. This confirmed that the absence of kinase activity was responsible for the replication deficiency in the deletion mutant (19), and is consistent with results published previously (20). Disruption of individual Rb binding motifs had minimal impact on virus replication in a 14-day time course [Figure 1B]. Growth curves for RC312, RC295, RC316, and RC323 were similar to that of the wt virus (HB5), although there appeared to be a slight delay at 4 and 5 days post infection, with RC295 having the lowest titers. This result was repeatable and was confirmed in a second independent experiment with these viruses (data not shown). The double-mutant virus (RC323) also exhibited a minor replication delay in a separate experiment [Figure 1C]. These data suggest that the mutation of these sites has a minimal effect on viral replication.

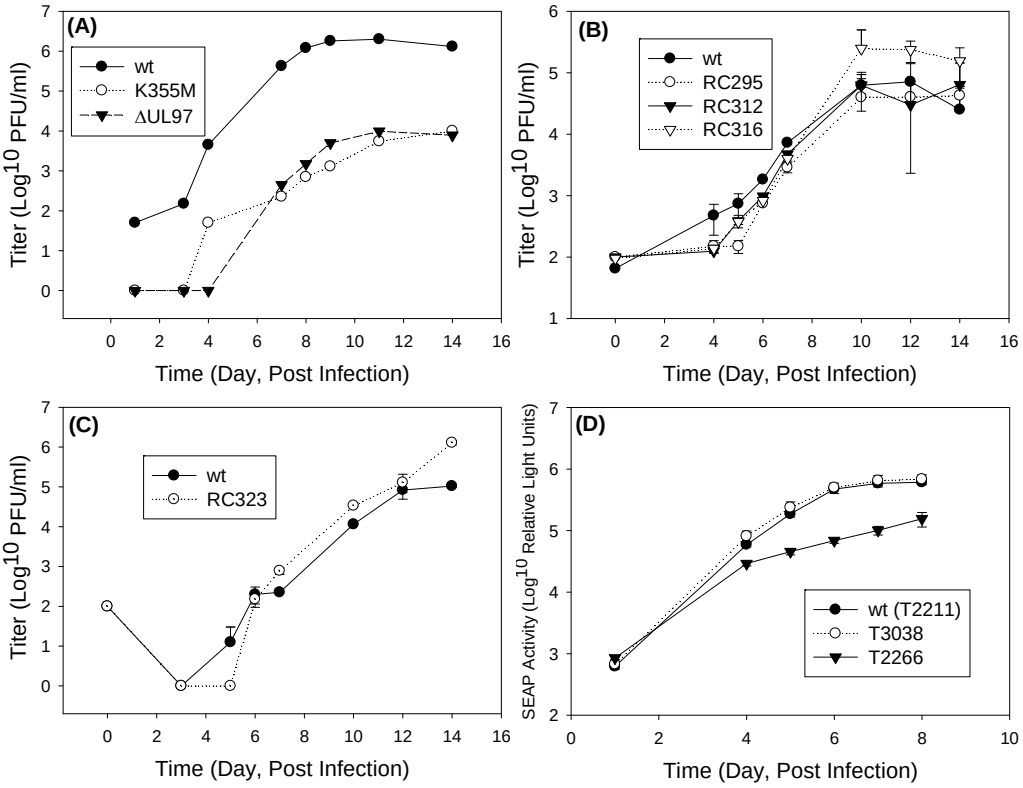

FIGURE 1. Growth curves of HCMV recombinants with mutations in *UL97*. HFF cells were infected at an MOI of 0.01 PFU/cell and titers of the resulting progeny virus in cell lysates were determined at the indicated times. (A) Both a *UL97*-deleted virus (black triangles) and a null mutant with a K355M mutation (open circles) replicate poorly compared to the HB5 parent virus (black circles). (B) Replication kinetics of recombinant viruses with mutations in putative Rb-binding sites were determined and exhibit a slight delay in replication. Shown are the average titers from 2 replicate cultures with error bars representing the standard deviation values. RC295 (open circles) contains a mutation in the LxCxE motif, RC312 (black triangles) has a mutation in the LxCxD motif, RC316 (open triangles) carries a mutation in the IxCxE motif, and titers of the parent virus are shown as black circles. (C) RC323 (open circles) contains mutations in both the LxCxE and the LxCxD motifs and is shown with HB5 as a control (black circles). (D) Growth curves of SEAP-expressing HCMV recombinants with mutations in *UL97* were examined separately; shown is the average SEAP activity of 5 replicates with the error bars representing the standard deviations. Replication of the wild-type virus with a SEAP-expression cassette (black circles) is similar to that of the recombinant virus with a deletion of codon 151 which disrupts the LxCxE motif (open circles), and both replicate much better than the virus with a truncation of the UL97 open reading frame (black triangles).

A second set of viruses was constructed that contained a secreted alkaline phosphatase (SEAP)-expression cassette at US6 (T2211) (9). The LxCxE motif was

disrupted with a C151 deletion (T3038) and pUL97 was truncated at codon 536 to yield a null mutant (T2266). Replication of these viruses was assessed in human embryonic lung (HEL) cells infected at an MOI of 0.01-0.03 PFU/cell, and SEAP supernatant activity was determined with a chemiluminescent substrate. No detectable differences were observed between the wt virus (T2211) and the UL97 ΔC151 virus (T3038) [Figure 1D]. The UL97-truncated virus (T2266) replicated poorly and had growth characteristics similar to those of RC314, and confirmed that defects in kinase activity could be detected in these studies. Results from both experiments indicated that mutation of the LxCxE motif did not severely impact virus replication. The minor delay in virus replication was not apparent in the T3038 and is likely related to assay differences. We conclude that the disruption of each of the Rb binding motifs individually or of the two amino terminal motifs in tandem has only minor effects on viral replication in cell culture. However, we cannot exclude the possibility that the disruption of all three may impact growth or that replication may be more compromised in other systems.

The mutations also had the potential to impact the efficacy of MBV and GCV, so the susceptibility of the mutants was determined by standard plaque reduction assays (24). MBV was obtained from the National Institute of Allergy and Infectious Diseases (NIAID), CDV was a gift from Gilead Sciences and GCV was obtained from University of Alabama, Birmingham Hospital Pharmacy. All of the viruses were equally susceptible to the CDV control and was expected since this nucleotide analog does not require initial phosphorylation by the UL97 kinase [Table 1]. The kinase null virus, RC314, exhibited reduced susceptibility to GCV and MBV and is similar

Virus	AA Mutation	Rb-binding Motif	MBV[a]	CDV	GCV
HB5	wt	wt	0.37±0.03	0.15±0.05	3.6±3
RC314	K355M	wt	14±5	0.1±0.07	38±10
RC295	C151G	LxCxE	0.22±0.08[c]	0.18±0.04	2.8±0.9
RC312	C428G	LxCxD	0.39±0.2	0.22±0.1	4.2±2
RC316	C693G	IxCxE	0.3±0.1	0.18±0.1	3.2±3
RC323	C428G/C151G	LxCxD/LxCxE	0.5±0.2	0.08±0.05	3.5±3
T2211[b]	wt	wt	0.100±0.008	0.23±0.01	1.16±0.18
T3038[b]	ΔC151	LxCxE	0.031±0.002[c]	0.27±0.01	1.03±0.13

TABLE 1. UL97 recombinant sensitivity to Maribavir.

a. Values shown are the concentrations of drugs (μM) that are sufficient to reduce viral replication by 50% (EC_{50}) with standard deviation shown. Each value is the average of at least 3 experiments.
b Values were obtained using a surrogate assay from viruses containing a SEAP expression cassette at US6.
c. Values were reduced significantly from the isogenic control viruses as determined by the Student's T-test ($p < 0.05$).

to results reported previously for RCΔ97 (24). None of the viruses with mutations in the Rb binding motifs exhibited significant differences in their sensitivity to GCV, and confirmed that the mutations did not impact the enzymatic activity of the kinase. Interestingly, the disruption of the amino terminal LxCxE motif in RC295 rendered it modestly hypersensitive to MBV, which suggests that the Rb binding motif might be related to the mechanism of action of MBV. This is consistent with a previous report, which showed only this motif appeared to impact Rb phosphorylation (20). It was not clear, however, why this mutation did not affect MBV susceptibility of the double mutant, but this effect was difficult to measure because of the assay-to-assay variability with this virus.

To confirm these data, the susceptibility of the SEAP-reporter viruses was also evaluated against GCV and MBV using SEAP activity as a surrogate marker of viral replication. Data were obtained by infecting confluent HEL fibroblasts at an MOI of 0.01-0.03 PFU/cell and SEAP activity in the supernatant was determined 6 days

following infection (8). MBV sensitivity data are the average of 8 determinations from 3 independent experiments for the T3038 and T2211 viruses tested simultaneously; the GCV EC_{50} values are consistent with historical data and are shown as the average of triplicate determinations. These data confirmed that the disruption of the amino terminal Rb-binding domain resulted in significantly increased sensitivity to MBV. While the EC_{50} values differ between the plaque reduction assays and the SEAP assays, both assays are consistent in that they both show that disruption of the amino terminal Rb binding motif results in increased susceptibility to MBV. These suggest that the mechanism of action of MBV may involve the LxCxE Rb binding motif and is consistent with the idea that the prevention of Rb inactivation by MBV might be an important aspect of its mechanism of action.

Disruption of the central cysteines in the UL97 Rb-binding motifs does not result in a severe replication deficiency reminiscent of kinase null viruses, but rather results in a very modest delay in viral replication. This might suggest that the inactivation of Rb is not a crucial function in HFF or HEL cells and that it may be important only in vivo. Alternatively, it is possible that the motifs are redundant and all of them must be deleted to impact replicative ability of the virus. Nevertheless, the C151G mutation in the RC295 virus and the C151 deletion in the T3038 virus clearly conferred hypersensitivity to MBV when compared to their isogenic controls. While potential mechanisms for this are unclear, it indicates that mechanism of action of the drug is related to the function of the amino terminal Rb-binding motif at some level. Additional experiments will be required to assess the potential impact of double and triple mutants. Nonetheless, these findings offer insight into a new aspect of MBV

activity and additional studies with the inhibitor together with genetic studies will help define the function of the kinase in viral infection.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHOR'S CONTRIBUTIONS

RBG participated in virus production and data analysis and drafted the manuscript. SLF carried out virus production. CLH performed drug sensitivity studies and growth curve analyses. SC participated in construction and analyses of SEAP virus experiments. MNP participated in experimental design and implementation and assisted in drafting the manuscript. All authors read and approved the final manuscript.

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HUMAN CYTOMEGALOVIRUS UL97 KINASE ACTIVITY ALTERS REGULATION OF G_2/M CHECKPOINT ELEMENTS

by

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ABSTRACT

The UL97 protein kinase is a serine/threonine kinase expressed by human cytomegalovirus (CMV). An investigation of the subcellular localization of pUL97 in infected cells indicated that early in infection pUL97 localized to focal sites in the nucleus that transitioned into subnuclear compartments and eventually throughout the entire nucleus. When UL97 kinase activity was abrogated with a K355M mutation or pharmacologically inhibited with maribavir, the expansion and redistribution of pUL97 foci within the nucleus was delayed. Nuclear reorganization did not occur, and assembly complexes in the cytoplasm failed to form normally. Since UL97 kinase and its homologs appear to be functionally related with CDK1, a known regulator of nuclear structural organization, we investigated the effects of the UL97 kinase on CDK1. Expression of CDK1 in infected cells appeared to be induced by UL97 kinase activity at the transcriptional level and was not tied to other viral life cycle events, such as viral DNA replication or virion assembly. These results suggest that in addition to phosphorylating CDK1 targets, the UL97 kinase modifies G_2-M cell cycle checkpoint regulators, specifically CDK1, to promote viral replication.

INTRODUCTION

Human Cytomegalovirus (CMV) is a ubiquitous virus and infections can be severe in neonates and immunocompromised individuals. CMV is a double-stranded DNA virus within the family *Herpesviridae* and belongs to the subfamily *betaherpesviridae*, which is characterized by slow replication and high species-

specificity. Like other herpesviruses, once a host is infected with CMV, the virus establishes a lifelong infection, with primitive monocytes being a persistent viral reservoir (8, 47). An estimated 60-99% of people are infected worldwide with CMV; in the United States, approximately 59% of the population over 6 years of age is seropositive. CMV is acquired via direct contact with bodily fluids and is most commonly spread through horizontal transmission (17). CMV accounts for about 10% of all cases of infectious mononucleosis, and the virus has potential links to other diseases, such as atherosclerosis and cancer (14, 21). CMV has not yet been proven to be directly oncogenic, yet viral antigens are expressed in a wide variety and grade of tumors (37). This virus also shares functional properties found in other known oncogenic viruses, such as the ability to modify cell-cycle regulators and to increase both RNA and DNA synthesis (13).

Approved therapies for CMV infections target the viral DNA polymerase and include ganciclovir and its valine ester, valganciclovir, cidofovir (CDV), and foscarnet (6). Because each of these antiviral agents is associated with dose-limiting toxicities, new antivirals with improved efficacy and reduced toxicity need to be developed (15, 53). One new antiviral under development is maribavir (MBV), which disrupts viral replication through the specific inhibition of the CMV UL97 serine/threonine (ser/thr) kinase (1, 7, 12, 41). Although not essential for replication, viruses deficient in UL97 kinase activity exhibit a reduction in viral yields of over two orders of magnitude (19, 43). Since MBV is a highly specific inhibitor of this kinase, it is an excellent pharmacological tool to study the function of the UL97 kinase activity in vitro. Infected cells treated with MBV exhibit a phenotype that resembles UL97 kinase deficient viruses,

including a recombinant virus (RC314) with a mutation in the invariant lysine (K355M) essential for enzymatic activity, as well as the deletion mutant (RCΔ97) (19, 45). Thus, studies with MBV complement those with recombinant viruses.

As we expound the functions of UL97 kinase, it is clear that this enzyme plays an important role in many aspects of infection. UL97 kinase has been reported to be expressed with delayed early/late (β/γ) kinetics, although it might also function earlier in infection on cellular and viral metabolism since it is released into the infected cell as a tegument component (56). In the absence of UL97 kinase activity, there is a modest decrease in viral DNA synthesis and late gene expression (27, 43, 58). The degradation of the nuclear lamina and egress of mature capsids from the infected cell nucleus is also defective (22, 27, 36, 58). The UL97 kinase is also required to prevent the formation of nuclear aggresomes, which are an innate immune response thought to sequester viral proteins and prevent their proper function (29, 45). Some viral targets of UL97 kinase are the ppUL44 DNA polymerase processivity factor (28), the pp65 major tegument protein (11, 25, 42), as well as autophosphorylation on serines and threonines (41).

Many cellular targets of UL97 kinase are also cyclin-dependent kinase (CDK)-related targets, including the CTD of RNA pol II, retinoblastoma protein (RB)(24, 45), EF-1δ (26), p32 (36), Histone H2B (5) and lamin A/C (22). CDKs are ser/thr kinases involved in the tightly-regulated checkpoints of the cell cycle; their activity requires association with a regulatory subunit, called a cyclin (34). The UL97 kinase mimics many actions of CDKs without need of a cyclin regulatory subunit. Hyperphosphorylation of RB, which is performed by the cyclin/CDK combinations of either cyclin D and CDK4 or CDK6 and cyclin E/CDK2, allows the cell to enter into S

phase. This phosphorylation is also performed by UL97 kinase, thus promoting production of S phase cellular proteins important for DNA replication which are commandeered by the virus to support viral replication (24, 45).

Cells require the maturation/mitosis promoting factor (MPF) to enter mitosis, which is comprised of a heterodimer of cyclin B and CDK1. MPF specifically promotes mitotic events such as chromatin condensation, mitotic spindle formation, lamin network breakdown, and Golgi apparatus degradation (32). CMV induces a pseudomitotic state where many of these same events occur without an intervening cytokinesis, including increased levels of Cyclin B and CDK1 (16, 51) and nuclear lamina breakdown (23). UL97 kinase, like its viral homologs BGLF4 and UL13, phosphorylates lamin A/C which is recruited to the lamin B receptor by p32, both of which contribute to the disruption of the nuclear lamina to allow viral capsids to exit the nucleus (9, 22, 31, 36). The HSV-1 homologue of pUL97, UL13, was also shown to phosphorylate CDK1, so it is possible that pUL97 might alter the expression or function of this CDK to further induce this pseudomitotic state (2).

Since pUL97 kinase mimics many of the functions of CDK1, including the redistributions of lamins, and the HSV-1 UL13 kinase directly interacts CDK1, we characterized both the subnuclear localization of pUL97 during infection and assessed its effects on CDK1. Initially, the subcellular localization of pUL97 in infected cells was characterized to help understand where it might be functioning in infected cells. Small foci containing pUL97 appeared very early in viral infection and were subsequently reorganized within the nucleus, appeared to surround the replication compartments and supported nuclear deformation. This reorganization was dependent upon both kinase

activity and viral DNA synthesis. UL97 kinase activity also appeared to alter CDK1 expression, presumably to promote viral replication and assembly.

MATERIALS AND METHODS

Cells, viruses, plasmids and compounds

Primary human foreskin fibroblast (HFF) cells were produced as described previously (48) and HEL299 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cells were routinely propagated in monolayer cultures in minimum Eagle's medium (MEM) with Earle's salts supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 µg/ml penicillin G, and 25 µg/ml gentamicin. The CMV strain AD169 was obtained from ATCC. Virus stocks were prepared, and titers were determined as described previously (44). The construction and characterization of a kinase-inactive pUL97 mutant (RC314) was described previously (19). MBV and 2-Bromo-5,6-dichloro-1-(β-d-ribofuranosyl)benzimidazole (BDCRB) were kindly provided by John Drach (University of Michigan, Ann Arbor, MI). CDV was a gift from Mick Hitchcock (Gilead Sciences, Foster City, CA). Construction of the plasmids expressing the V5 -epitope-tagged version of pUL97 and the catalytically inactive K355M mutant were described previously (42).

Antibodies

The CMV-specific antibodies IE1-72/IE2-86 (IE1/IE2; 8B1.2), and pp65 (12D10) were obtained from Chemicon (Temecula, CA), and the ppUL44 (28-21) monoclonal antibody was kindly provided by Bill Britt (University of Alabama at Birmingham, Birmingham, AL). The antibody specific for the carboxyl terminus of pUL97 (EEDLDGDCRQLFPE) was produced using a synthetic peptide. The resulting monoclonal antibody (4-1) specifically stained CMV infected cells and did not recognize epitopes in uninfected cells or in cells infected with the UL97 deletion mutant. This antibody also recognizes both variants of pUL97 (57). The phospho-CDK1 (Tyr^{15}) (10A11) was obtained from Cell Signaling Technology (Boston, MA). The antibodies specific for CDK1 p34 (B-6), CDK1 p34 (C-19), and PML (PG-M3) were acquired from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Actin (AC-40) was from Sigma-Aldrich (St. Louis, MO). The monoclonal antibody for the V5 epitope tag (Invitrogen, Carlsbad, CA) and fluorescently labeled antibodies specific for mouse (isotype-specific), goat, and rabbit immunoglobulins were obtained from SouthernBiotech (Birmingham, AL). Horseradish peroxidase (HRP)-conjugated secondary antibodies for western studies were obtained from Pierce/Thermo Scientific (Rockford, IL).

Transient transfection

Plasmid DNA was transiently transfected into cells to express either the UL97 kinase (pMP92) or the kinase-inactivated mutant protein (pMP307). Using a kit designed for primary cells, HEL299 cells were transfected with 3 μg of each plasmid according to

the Lonza Nucleofector Kit protocol for normal human adult dermal fibroblasts (Walkersville, MD). MBV was added one hour post-transfection to noted wells at a concentration of 20 μM. Cells were incubated at 37°C, 5% CO_2 for 36 h before fixation for IFA analysis. Transfection of pUL97-expressing plasmids and visualization of overall protein expression in COS7 cells was performed on coverslips as reported previously (42, 45).

Indirect immunofluorescence Assay (IFA)

Confluent monolayers of low-passage primary HFF cells were prepared 2 days prior to infection. All infections were performed in media containing 2% FBS at a multiplicity of infection (MOI) of 1 plaque forming unit per cell (PFU/cell) unless otherwise noted. Inocula were aspirated, the monolayers washed and fresh media was added with the addition of MBV, CDV, or BDCRB where indicated. To visualize protein expression and localization in transiently transfected cells or infected cells, cell monolayers were washed twice with phosphate-buffered saline (PBS) and fixed with freshly-made 4% PF-A (paraformaldehyde) in PBS for one hour at ambient temperature (Ta). Fixed cells were then permeabilized for 30 minutes using 0.2% triton-X-100 and blocked in 0.4% fish skin gelatin (FSG) for 1 hour at Ta. Primary antibodies were diluted according to manufacturers' recommendations in 0.4% FSG in PBS and incubated for one hour at Ta. Coverslips or slides were washed gently with PBS and incubated with fluorescein isothiocyanate (FITC), Texas Red, or tetramethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibody for one hour at Ta. Coverslips were mounted

using SlowFade Antifade Reagent with DAPI (4',6-diamidino-2-phenylindole; Invitrogen, Carlsbad, CA) and visualized using 400x magnification on an Olympus BX41 Microscope. Slides for confocal analysis were prepared as above, except nuclear staining was performed with TO-PRO®-3 iodide (642/661, Molecular Probes/Invitrogen), mounted using SlowFade Antifade reagent and were visualized under oil immersion at 600x magnification using the Olympus BX51 Confocal Microscope and Fluoview Software (v.5.0, Olympus, Center Valley, PA.). To evaluate the effect of pUL97 on CDK1, at least three fields from coverslips were photographed and cells that co-expressed each protein were counted. Cells were scored as positive for pUL97 and CDK1 if they exhibited visible staining.

Quantitative real time RT-PCR

Monolayers of infected HFF cells in 96-well plates were harvested at 24 and 72 h following infection using SV 96 Total RNA Isolation System (Promega; Madison, WI) and cDNA was produced using High Capacity RNA-to-cDNA Kit from Applied Biosystems (Foster City, CA). Primer and probes specific for pUL97, RPL13α, CDK1, Wee1, Fzr1, and Cyclin B1 were designed using the Primer Express® software v3.0 (Applied Biosystems). Primers were purchased from Integrated DNA Technologies (Coralville, IA), and the TaqMan probes (5'FAM/3'TAMRA [6-carboxyfluorescein/tetramethylrhodamine]) were obtained from Applied Biosystems (Table 1). Reactions containing 250 ng of cDNA were performed using an ABI 7300 Real-Time PCR System using TaqMan Universal PCR Master Mix (Applied

Gene Target	NCBI Gene ID/ Nucleotide ID	Forward/ Reverse Primers	Probe (5'FAM/3'TAMRA)
Wee1	7465 NM_003390.3	CAA GAA AGC ACA GAT GGC AAA AG GCC ATC CGG TCA GTG AAG AG	TGCAGCTGAGGAAAG
IE1*	3077513 NC_006273.2	AAG CGG CCT CTG ATA ACC AAG GAG CAG ACT CTC AGA GGA TCG	N/A
IE2.3*	3077513 NC_006273.2	GAG CCC GAC TTT ACC ATC CA CAG CCG GCG GTA TCG A	N/A
UL97	3077517 NC_006273.2	CCT GAG TTC CGT CAG CAC AA GAA CGC ATG CGG AAA AAG TC	CACCGTGCTTGGAC
CycB1	891 NM_031966.2	CCC TGC TGC AAC CTC CAA TTT GTT ACC AAT GTC CCC AAG AG	CGGACTGAGGCCAAGA
RPL13	6137 NM_000977.2	TGG TGT GTT TCG TGG GAA CA AGG AGG AAG TCA CAG CAG TGA AG	TGGGCCTGGGATGG
FZR1	51343 NM_001136198.1	CTT CCC AAA GGG CGA GAA C CAT CCG TCG CTC TGA GTA CAG A	TGGACGGTCCCGGCT
CDK1	983 NM_001786.3	GGC TTC AAA GCT GGC TCT TG GCA GCG GCA GCT ACA ACA A	AAATTGAGCGGAGAGCG

TABLE 1. qRT-PCR primer sequences.

Biosystems.). Each condition was run in triplicate and statistical analyses of the data were performed using a paired Student's T-test. The fold regulation was determined using the method described by Pfaffl et. al. (40) using the cellular transcript for RPL13α to normalize the data. Infections were confirmed using primers specific for IE1-72 and IE2-86 using Power SYBR Green real-time PCR Master Mix technologies (Applied Biosystems).

Polyacrylamide gels and western blotting

Cells were infected as noted in IFA methods. At specific times following infection, cells were washed twice with ice-cold PBS and cold RIPA buffer was added to the wells. Cells underwent three -80°C freeze/thaw cycle to aid in cellular and nuclear membrane lysis. Cells were scraped from each well and run through a 25g sterile needle to aid further in membrane lysis. Extracts were spun down at 8,000 RPM, 4°C for 10

minutes and the supernatant was subjected to BCA Protein Assay Kit (Pierce/Thermo Scientific) to determine total protein concentration. 25 μg of each sample was loaded on a 7.5% Tris-glycine SDS-Polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane using the Bio-Rad semi-dry transfer method (Hercules, CA). Blocking was performed for 1 hour at 25°C in Pierce StartingBlock™ T20 blocking buffer and membranes were incubated overnight at 4°C in 5mL of blocking buffer containing antibody diluted according to the manufacturer's recommendation. Membranes were washed five times with gentle shaking in PBS+ 0.1% tween-20 and secondary antibodies conjugated to HRP were diluted in 5 mL of blocking buffer and incubated for 2 h at 4°C. HRP was detected using SuperSignal® West Dura Extended Duration Substrate (Pierce/Thermo Scientific).

RESULTS

Localization of pUL97 in infected cells

A monoclonal antibody specific for the carboxyl terminal 14 amino acids of pUL97 was used to follow the localization patterns of the UL97 kinase throughout the course of infection. The subcellular localization of pUL97 was determined at 1 h following infection to assess the potential delivery of this protein from the virion tegument into the cell. At this point in time, the abundant pp65 tegument protein was easily detectable and was predominantly localized to the nucleus as reported previously (52) (Fig. 1, middle column, red on merge). The delivery of pUL97 to the cells was also detected at this point in time (Fig. 1, left column, green on merge). Punctate staining of

pUL97 was noted in the cytoplasm, and localization to the nucleus was not as rapid as pp65. No staining was observed in mock-infected cells using the pp65 or pUL97 monoclonal antibodies at any point in time (data not shown).

At 8, 24, 48, and 72 h following infection, pUL97 localized predominantly to the nuclei of infected cells (Fig. 1). Distinct intranuclear foci (approximately 5 per cell) were observed at 8 h after infection. Staining for pUL97 at later stages of infection localized to subnuclear compartments that resembled replication compartments. Deformation of the nucleus into a "kidney bean" shape was also noted starting at around 48 h consistent with the reorganization of the nuclear lamina. By 96 h after infection, more diffuse pan-nuclear pUL97 staining was observed and was also present in the center of the nascent assembly complexes in the cytoplasm (Fig. 1, green arrow). Intracellular localization of pp65 was included as a control and exhibited the pattern of staining reported previously, beginning with nuclear localization within replication compartments followed by the quantitative flushing from the nucleus around 48 h following infection (4, 42, 49-50, 52). Late in infection pp65 localized just outside of the nucleus, initiating the formation of assembly complexes. At 96 h following infection, viral DNA was also detectable within the assembly complex (Fig.1 inset, merge, yellow arrow). This pUL97 localization pattern is consistent with packaging as part of the tegument in the mature virus.

When the kinase activity of pUL97 was inhibited with MBV starting at 1 h following infection, the subcellular localization of both pp65 and pUL97 was significantly altered. The localization of both pUL97 and pp65 was similar to the wt virus at 8 h following infection, yet progression to the larger subnuclear compartments was not observed (Fig. 2). Later in infection, staining appeared to progress throughout the

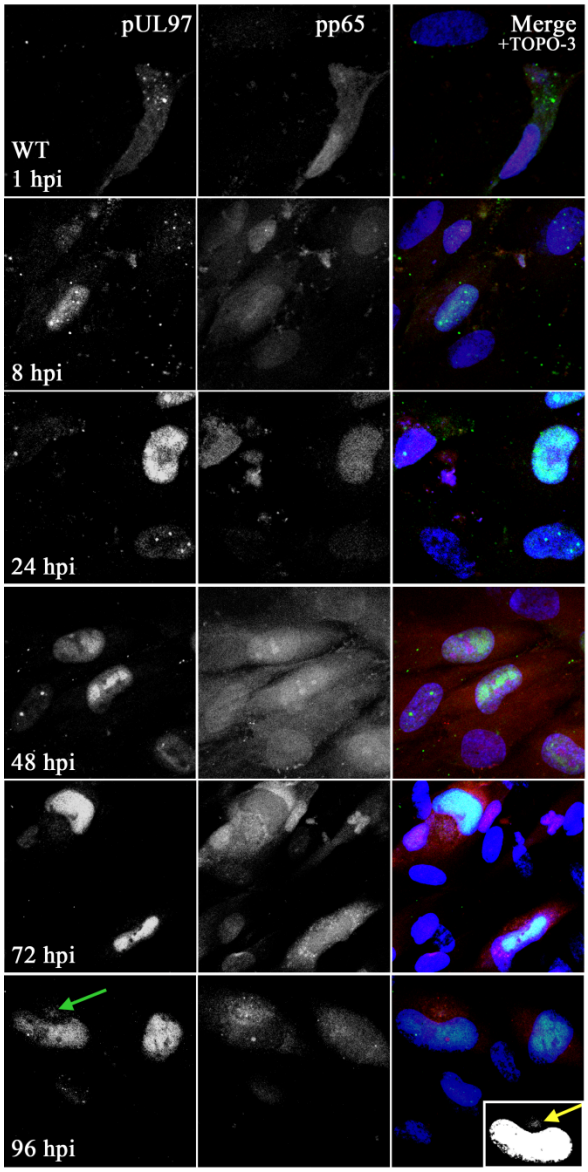

FIGURE 1. Timecourse of pUL97 localization and expression during infection. Confluent low-passage HFFs were infected for one hour with AD169 at a MOI of approximately 1 PFU/cell and then washed and replaced with low-serum media. Cells were fixed at 1, 8, 24, 48, 72 and 96 hours post-infection (hpi). Using confocal imaging, the cells were screened at 600x resolution for the presence of pUL97 (left column, green merge) or pp65 (middle column, red merge), with the nucleus visible in the merged image (TOPO-3, right column, blue merge.) The arrows at 96 hours (h) are highlighting pUL97 (green arrow) and DNA (yellow arrow, merge inset) packaging within the assembly complex.

nucleus as observed in the wt virus, but pUL97 also localized to many small foci that clustered near the periphery of the nucleus. While pp65 localization initially appeared to

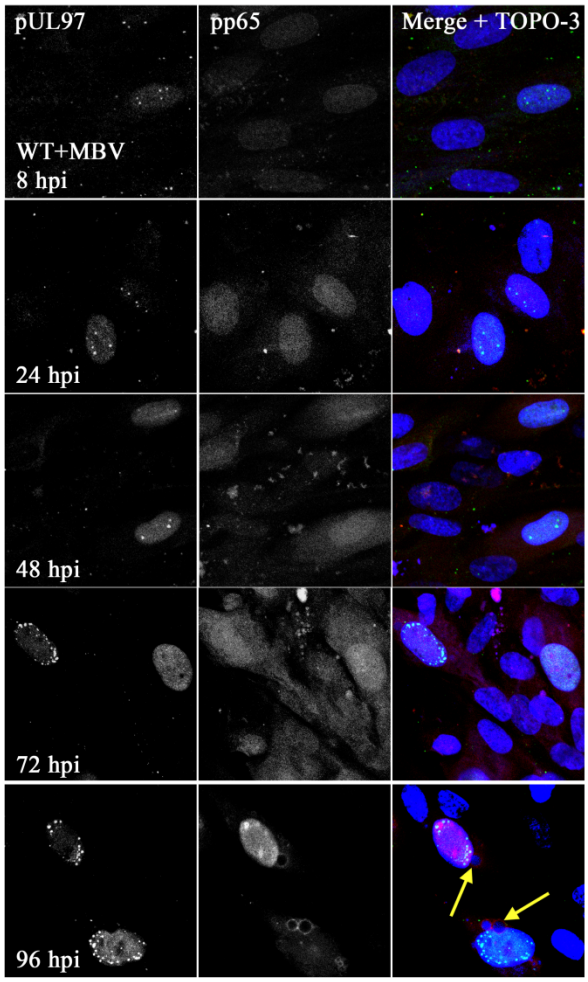

FIGURE 2. Timecourse of pUL97 localization and expression in a MBV-treated WT infection. Cells were infected as those in Figure 1 with CMV AD169 and treated with 20 μM of maribavir (WT+MBV), were fixed at 8, 24, 48, 72 and 96 hpi and stained for pUL97 (left column, green merge) or pp65 (middle column, red merge) with the nucleus visible in the merged image (TOPO-3, right column, blue merge.) Yellow arrows on 96 hpi merge image are highlighting the abnormal circular blebs outside of the nucleus that are lined by pp65.

be normal, by 48 h there was a noticeable lack of nuclear focal localization and the flushing of this protein to the cytoplasm was not observed. At 72 h, pp65 started to flush into the cytoplasm, but there were defects in both nuclear deformation and assembly complex formation. Well formed assembly complexes were not apparent by 96 h, but rather large, abnormal circular blebs were observed in the cytoplasm near the nuclei (yellow arrows on merge, Figure 2). These blebs were surrounded by pp65, and pUL97

did not appear to be present in the cytoplasm. Generally, levels of both viral proteins were below those in cells infected with the wt virus.

To confirm that the changes in MBV treated cells were related to reduced kinase activity, the localization of pUL97 was also characterized in cells infected with the kinase-null mutant, RC314 (Fig. 3). The poor replication of this recombinant virus precluded the production of high titers of virus, so cellular debris was apparent in all images, particularly those early in infection. Nonetheless, the subcellular localization of pUL97 and pp65 appeared to be similar to that observed in the presence of MBV. However, we were unable to detect delivery of either pUL97 or pp65 to infected cells immediately following infection, possibly suggesting defects in the virions of the RC314 virus (data not shown). At 8 h following infection, pUL97 localized to foci in the nucleus that resembled those in MBV treated cells infected with the wt virus. Very low levels of pp65 were observed in nuclei at 24 h although the typical focal localization of pUL97 in the nucleus was apparent. Like cells treated with MBV, these foci persisted through 48 h and progression to pan-nuclear staining was delayed. Again, as with MBV treatment, pp65 levels continued to rise, but remained distinctly nuclear at 48 h. At 72 h, both cytoplasmic and nuclear distribution of pp65 was observed. Interestingly, by 96 h, a few infected cells exhibited strong nuclear localization of pp65. Like cells treated with MBV, it appeared as though the pp65 lined the cytoplasmic blebs (Figure 3, 96 hpi merge, yellow arrow). These data confirmed observations with MBV and suggested that the observed defects in the localization of pUL97, lack of assembly complex formation and reformation of the infected nuclei were related to the absence of kinase activity.

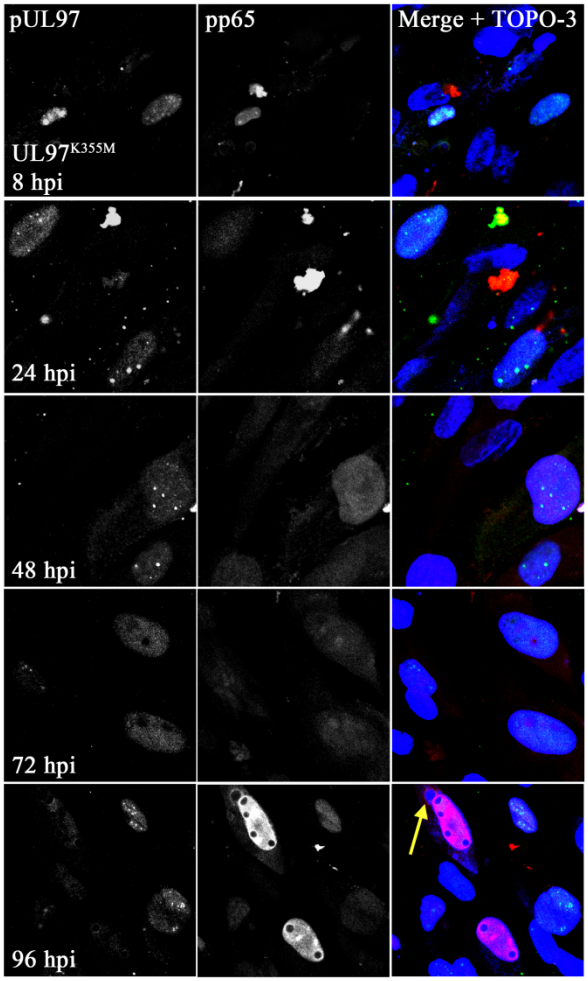

FIGURE 3. Timecourse of pUL97 localization and expression in an AD169-UL97^{K355M} (RC314) infection. Cells were infected with kinase-inactivated virus (RC314 - UL97^{K355M}), were fixed at 8, 24, 48, 72 and 96 hpi and stained for pUL97 (left column, green merge) or pp65 (middle column, red merge) with the nucleus visible in the merged image (TOPO-3, right column, blue merge.) The yellow arrow on 96 hpi merge image is highlighting an abnormal circular bleb outside of the nucleus that is lined by pp65.

Since infected cells treated with MBV and those infected with the RC314 virus exhibit modest reduction of viral DNA, the subcellular localization of pUL97 and pp65 was also characterized in infected cells treated with CDV. When viral DNA synthesis was blocked with CDV, prominent foci containing pUL97 formed but never transitioned into larger subcellular compartments or pan-nuclear staining (Fig. 4). There was also a marked decrease of pp65 expression throughout the time course and was expected

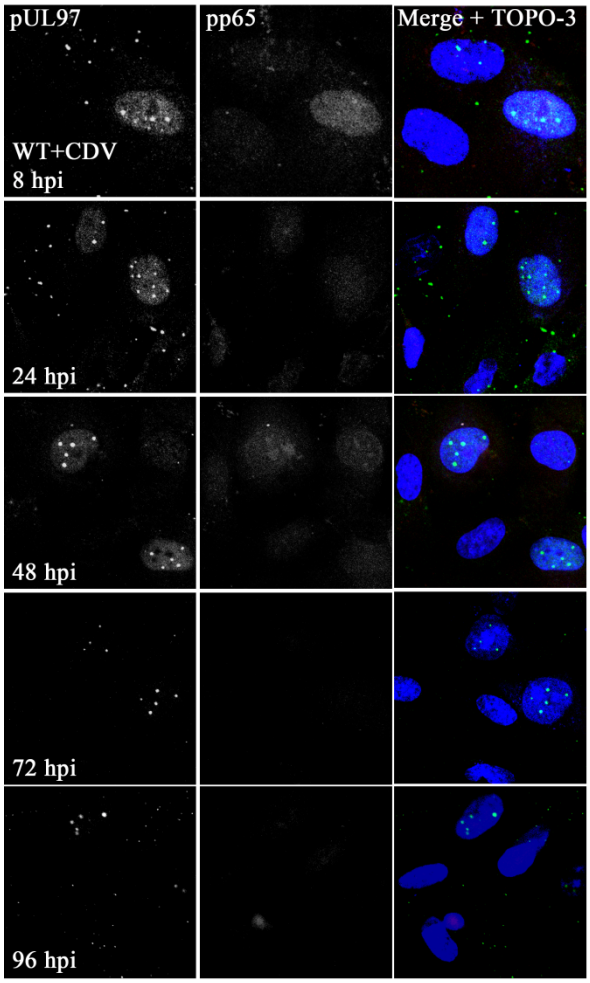

FIGURE 4. Timecourse of pUL97 localization and expression in CDV-treated CMV infection. Cells were infected as those in Figure 1 with CMV AD169 virus and treated with 10 μM of cidofovir (WT+CDV), were fixed at 8, 24, 48, 72 and 96 hpi and stained for pUL97 (left column, green merge) or pp65 (middle column, red merge) with the nucleus visible in the merged image (TOPO-3, right column, blue merge.)

because its β/γ expression kinetics. It was interesting that the pUL97 foci still formed in the absence of viral DNA replication, indicating that the foci may have formed from pUL97 delivered from the tegument. The pUL97 foci were apparent at 8 h and there were, on estimation, around 5 foci per infected cell by 24 h. This number appeared to remain stable, yet by 96 h, there was a notable reduction in the size of these foci. Again,

there was a lack of viral replication, no detectable increase of pp65, no assembly complex formation, and no noticeable deformation of infected cell nuclei.

Viral replication compartments do not contain pUL97

The localization of pUL97 in infected nuclei resembled that of replication compartments, so co-localization studies were done using a monoclonal antibody to ppUL44 as a marker for CMV replication compartments (28). In cells infected with the wt virus, ppUL44 localized to replication compartments within nuclei by 48 h following infection (Fig. 5). Subcellular compartments containing pUL97 were also apparent at this time; however, they were distinct from the replication compartments. As noted earlier, formation of subcellular compartments containing pUL97 was delayed with the K355M deletion virus, and in cells treated either with MBV or CDV. However, the foci still did not localize within replication compartments. We conclude that immediately following infection the localization of pUL97 is focal. As replication progresses pUL97 staining is observed in subcellular compartments which are distinct from replication compartments.

UL97 kinase activity affects the levels of CDK1 in infected cells

Nuclear remodeling is noticeably reduced in the absence of UL97 kinase activity, which is expected in the absence of laminar phosphorylation by this enzyme. However, it is also possible that pUL97 could be working in conjunction with CDK1, since the HSV-1 homolog to pUL97 interacts with CDK1, which is the cellular kinase that

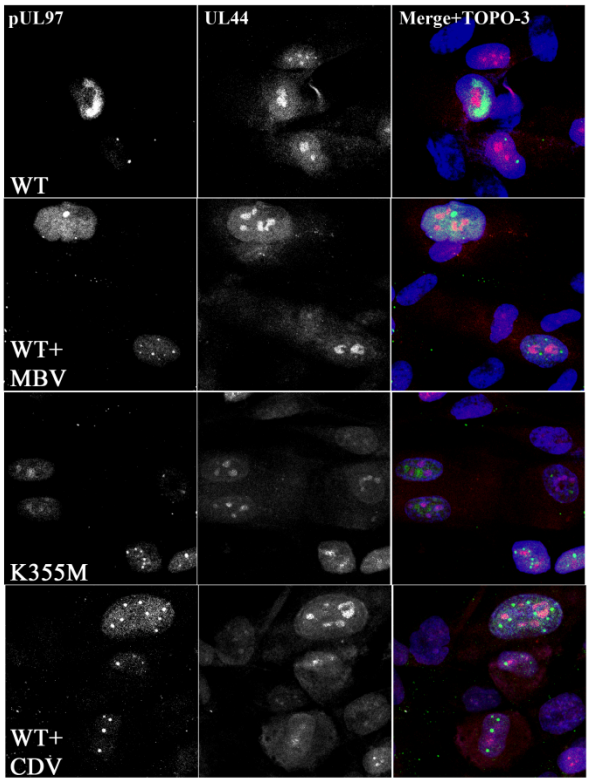

FIGURE 5. pUL97 foci do not localize with viral replication compartments. The DNA polymerase processivity factor, ppUL44, is a marker for viral replication compartments. Confluent HFFs were allowed to remain stationary for two days before infection. Monolayers were infected at an MOI of 1 PFU/cell, washed twice, and replaced with low-serum media. 48 h after infection, cells were fixed and pUL97 (left column, green merge) and ppUL44 (middle column, red merge) were detected using monoclonal antibodies, using TOPO-3 (right column, blue merge) as a nuclear marker. The confocal, oil-immersion images are shown above (600x magnification.)

phosphorylates lamin A/C during mitosis (3). To see if UL97 kinase activity is connected to CDK1, we first examined CDK1 expression in infected HFF cells in the presence and absence of UL97 kinase activity (Fig. 6). CDK1 expression is induced by CMV infection, which supports previous findings (51), and there was negligible CDK1 in the mock-infected cells, even in the presence of antiviral compounds. Western blots using a rabbit polyclonal antibody against CDK1 revealed a modest decrease in levels of CDK1 in the presence of 20 μM MBV and in cells infected with the kinase null virus at

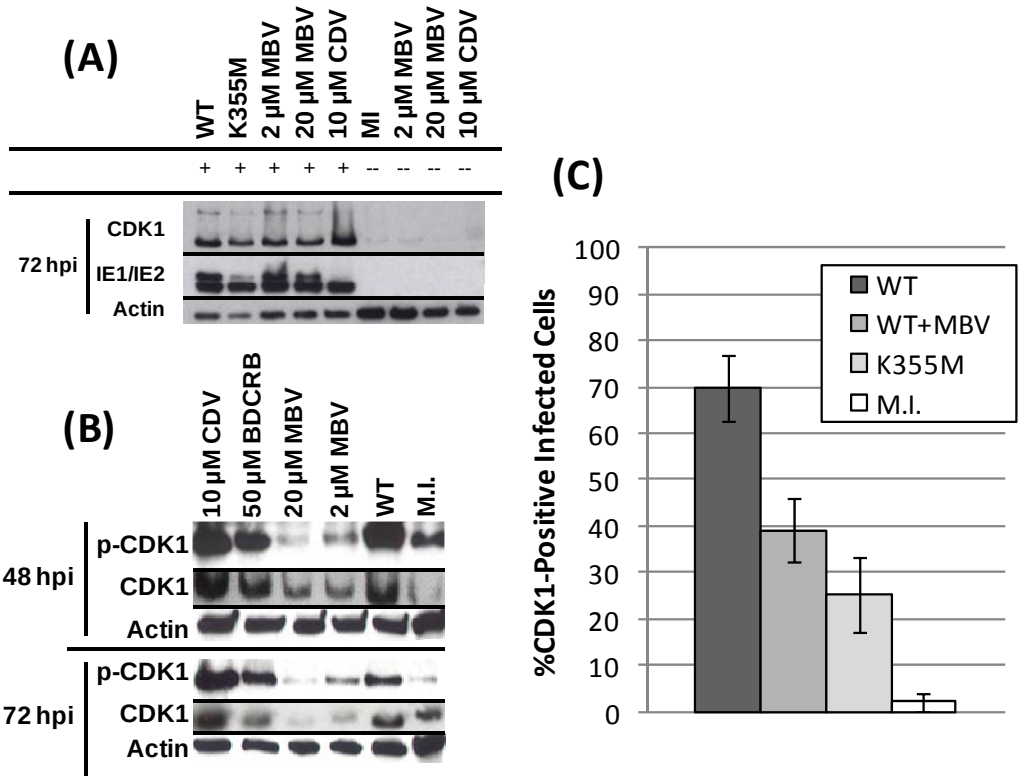

FIGURE 6. CDK1 expression in CMV-infected HFFs is affected by UL97 kinase activity. Confluent HFFs were infected with CMV for 1 h; cells were then washed and replaced with fresh, low-serum media. Cell lysates were harvested using RIPA buffer containing protease/phosphatase inhibitors at the timepoints noted (either 48 or 72 hpi) and 25 µg of protein for each infectious condition was probed for CDK1 expression. (a) HFFs were infected with 0.5 PFU/cell of either CMV AD169 (WT) or the RC314 kinase-null virus (K355M). As noted, infected and mock-infected (MI) cells were treated with MBV (2 or 20 µM) or CDV (10 µM) after washing. Cell lysates were probed using polyclonal CDK1 antibody (p34/C-19), IE1/IE2 antibody as an infectious control. (b) HFFs infected with CMV AD169 at a MOI of 1 PFU/cell were harvested at 48 and 72 hpi. Cells were either mock-infected (M.I.), treated at 1 hpi with either 10 µM of CDV (CDV), 50 µM of BDCRB (BDCRB), varying doses of MBV, or left untreated (WT). Cell lysates were probed for CDK1 (p34/B-6) and phospho-CDK1(10A11). Actin was used as a loading control for all westerns. (c) Confluent HFFs were infected on coverslips as noted in Materials and Methods. Cells were fixed and visually analyzed for the presence of pUL97, CDK1, and DAPI at 72 hpi. Values shown represent the average of data from at least three separate experiments with standard deviations. All values are significant when compared to WT using the Student's T-test evaluation: WT/WT+MBV $p< (0.0008)$; WT/K355M $p< (0.0002)$; WT/M.I. $p<(0.0001)$; WT+MBV/MI $p<(0.0001)$; K355M/M.I. $p< (0.0017)$; WT+MBV/K355M $p< (0.04)$. BDCRB: 2-bromo-5,6-dichloro-1-(ß-D-ribofuranosyl) benzimidazole; CDV: cidofovir.

72 h following infection. This appeared to be unrelated to viral replication since no reduction was seen in the presence of CDV (Fig. 6A).

To confirm these results, the experiment was repeated at an MOI of 1 PFU/cell and monoclonal antibodies specific for CDK1 (P0H1) or a monoclonal antibody specific for CDK1 phosphorylated at Tyr^{15} (10A11) were used to detected the presence of CDK1 in the cellular lysates. It was not possible to include RC314 as a control since the low titers of this virus precluded its use in this experiment. However, we added an additional inhibitor, BDCRB (2-bromo-5,6-dichloro-1-(β-D-ribofuranosyl) benzimidazole), which is structurally related to MBV, but prevents cleavage of viral concatameric DNA for packaging into the virion. At the higher MOI, inhibition of UL97 kinase activity with MBV clearly reduced both CDK1 expression and phosphorylation at 48 and 72 h following infection and was dose dependent (Fig. 6B). Reductions in CDK1 levels were only observed with MBV treatment and did not occur when DNA synthesis was inhibited with CDV or when packaging was inhibited with BDCRB.

To further confirm the induction of CDK1 by UL97 kinase activity in viral infection noted in our western blots, CDK1 expression was examined by IFA. The percentage of infected cells, as determined by pUL97 staining, that also had detectable amounts of CDK1 is shown in Figure 6C. Mock-infected cells were included in each experiment, and the low level of CDK1 detected (only 3% of cells were scored positive for CDK1 expression) further supported the resting, quiescent state of the cells before infection. There is also a statistically significant difference in the kinase-active versus inactive infectious conditions, further supporting that the kinase activity is important for CDK1 over-expression. The Student's T test revealed the difference between mock-

infected (M.I.) and WT and M.I. and WT+MBV to be extremely significant with p values less than 0.0001, while the differences between WT+MBV and WT [p(0.0008)], WT and RC314 [p(0.0002)], and RC314 and M.I. [p(0.0017)] were all very significant. While analyzing the IFA coverslips, it was notable that levels of CDK1 in wt infected cells were dramatically upregulated by 72 h. In infected HFFs either treated with MBV or infected with RC314, even though they had infected cells that ranked positive for expressing CDK1, the CDK1 levels appeared lower and remained distinctly nuclear compared to wt-infected cells (data not shown). Infected cells treated with CDV mirrored the CDK1 expression in wt-infected cells, which again supports this upregulation being due to an early and kinase-specific event. In summary, the UL97 kinase activity does appear to affect the overall levels of CDK1 in CMV infection.

Transient expression of UL97 kinase increases CDK1 expression

To confirm the link between UL97 kinase activity and CDK1 expression, the effect of UL97 kinase expression in transient assays was examined, and representative images are shown for each condition (Fig. 7A.) CDK1 was upregulated and co-localized with pUL97 in HEL299 cells transiently expressing the viral protein. However, when transfected cells were treated with MBV, or when cells were transfected with the plasmid expressing the kinase-null mutant, CDK1 was not detected. These data support the earlier findings, and further confirm that an increase in CDK1 levels is directly related to UL97 kinase activity. To confirm the HEL299 data, we performed a transfection of COS7 cells seeded on coverslips and counted the proportion of cells that expressed both

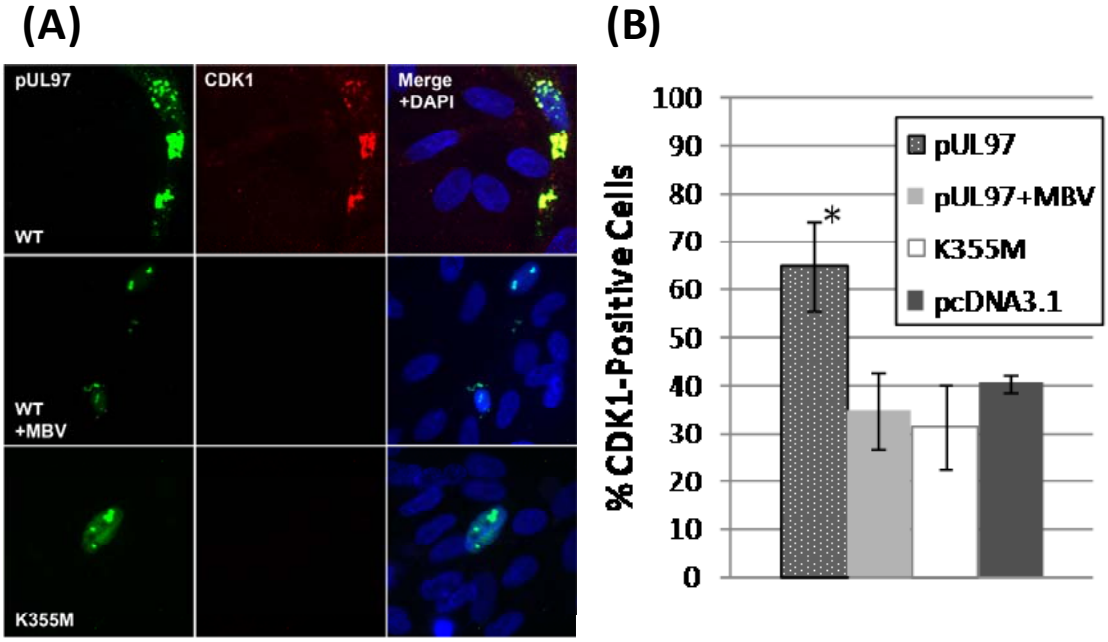

FIGURE 7. Transiently expressed pUL97 increases the expression of CDK1. (a) Human embryonic lung fibroblasts (HEL299) cells were transfected with 5µg total plasmid DNA using Amaxa technology. Plasmids expressed either wild-type UL97 (pMP92; WT) or UL97 with the K355M deletion (pMP307; K355M). One subset of transfected pMP92 cells were treated with 20 µM of maribavir (WT+MBV) after transfection. Cells were fixed and stained at 36 h after transfection and probed for pUL97, CDK1, and DAPI before photographing the fluorescence at 400x resolution. Cells transfected with 5 µg of the empty vector did not exhibit any significant changes in CDK1 expression (data not shown). (b) COS7 cells were plated on coverslips in 24-well plates and transfected with 5 µg pMP92 (pUL97), pMP307 (K355M) or pcDNA3.1 vector control. 24 h after transfection, media was replaced and fresh media was added, some of which was supplemented with 20 µM of maribavir (pUL97+MBV). Cells were fixed, stained, and photographed at 400x resolution 36 h after transfection and counted for the presence of DAPI, pUL97, and CDK1. Shown here are the averages of 3 separate experiments with standard deviations for the % of cells expressing CDK1. (*) indicates significance using Student's T statistic at $p = 0.02$.

pUL97 and CDK1. These data indicated that there was a statistically significant increase in the percentage of cells with detectable CDK1 expression ($P < 0.02$). In the absence of UL97 kinase activity, cells expressing pUL97 did not induce increased levels of CDK1 (Fig. 7B). Overall, these data indicated that the transient expression of UL97 kinase activity increased the expression of CDK1 in HEL299 and COS7 cells.

UL97 kinase activity is required for the CMV-induced transcriptional upregulation of CDK1

Since CDK1 is normally at extremely low yet static levels in the cell, we wanted to determine if the increase in protein expression was related to transcriptional upregulation. Quantitative real time reverse-transcriptase PCR studies (qRT-PCR) of CDK1, Wee1, Cyclin B1, and Fzr1 mRNA levels were also evaluated. Cyclin B1, Fzr1 (Cdh1) and Wee1 were included as they are known regulators of CDK1. At 24 and 72 h following infection, CDK1 mRNA levels were significantly increased by CMV infection, and inhibition of UL97 kinase activity with MBV down-regulated transcription of CDK1 (Table 2). This decrease was kinase specific because it was not observed in the presence of CDV. Interestingly, at 24 h following infection, Wee1 was also upregulated in infected cells. This upregulation, though almost seven times lower than the wild-type infection at the same timepoint, was still significant when the kinase activity was disrupted. No significant differences were observed in Wee1 transcript levels at 72 h. Inhibition of kinase activity had a modest effect on Fzr1 and Cyclin B1 upregulation at 24 h following infection. While Fzr1 did not exhibit any significant differences in regulation at 72 h, Cyclin B1 showed a significant increase in the number of transcripts at 72 h. Interestingly, inhibition of the kinase actually promoted transcription of Cyclin B1 at this timepoint. These data taken together indicate that UL97 kinase activity is associated with CDK1 over-expression, and normal kinase activity promotes transcriptional upregulation of CDK1 throughout the course of infection. In addition, kinase activity may also impact the regulation of other CDK1 regulators.

		Fold Regulation (WT vs. MI)			
		CDK1	Wee1	Fzr 1	Cyclin B1
24 hpi	WT	4.3**	49.3***	2.1	1.3
	20 µM MBV	-1.3	7.2*	1.0	1.0
	10 µM CDV	4.0*	95.2***	2.7**	1.8
72 hpi	WT	4.2***	1.9	1.3	2.5*
	20 µM MBV	-3.3	-1.1	1.9	3.7***
	10 µM CDV	4.2*	1.3	1.7	3.3*

* Significant at p(0.05)
** Significant at p(0.02)
*** Significant at p(0.01)

TABLE 2. Fold Regulation of G_2-M checkpoint mRNA transcripts in CMV-infected HFFs compared to mock-infected HFFs.

DISCUSSION

The UL97 kinase has become a therapeutic target of interest because it is required for the phosphorylation of GCV and because of its influence on viral replication. Both MBV and a new monoclonal antibody specific for pUL97 were used to further dissect the properties of this viral kinase. Results from these studies indicated that (i) pUL97 is quickly delivered from the tegument to foci in the cytoplasm that translocate to the nucleus by 8 h after infection; (ii) both UL97 kinase activity and viral DNA replication are required for the nuclear foci to transition into subnuclear compartments that are distinct from replication compartments; (iii) UL97 kinase activity was required for the formation of assembly complexes and the remodeling of the nucleus; (iv) in infected cells UL97 kinase activity during CMV infection promotes the upregulation of a G_2-M regulator, CDK1, at the transcriptional level; and (v) UL97 kinase activity is sufficient to upregulate CDK1 expression.

The subcellular localization of pUL97 throughout an infected cell and nuclear remodeling depends upon the activity of this viral kinase. While pUL97 and pp65 are

delivered into the infected cell from the tegument, pUL97 does not localize to the nucleus as quickly as pp65. Foci containing pUL97 are observed in the cytoplasm at 1 h following infection but by 8 h they are exclusively localized to the nucleus. While these foci seem to expand to subnuclear compartments as the infection progresses, they are distinct from viral replication compartments as defined by ppUL44 staining. Late in infection, CMV assembly complexes containing pp65, form in the nook of bean-shaped nuclei late in viral infection. The UL97 kinase, along with viral DNA, localizes in the core of these cytoplasmic complexes, consistent with its reported incorporation in virions as a tegument protein.

Inhibition of UL97 kinase activity alters the subcellular distribution of this protein. When the UL97 kinase activity is inhibited with MBV the enlargement of foci containing pUL97 is delayed. This also occurs in cells infected with the kinase null recombinant virus. Thus the redistribution of pUL97 to subcellular compartments is kinase dependent. Clearly, redistribution of foci containing pUL97 is also dependent upon viral DNA replication as evidenced by treatment with CDV. The identity of subnuclear compartments containing pUL97 has not yet been elucidated, but they are distinct from PML bodies and replication compartments (data not shown, Fig. 5). In size and number, small nuclear foci are similar to Cajal bodies, which would represent areas of high transcriptional activity (38). Identification of the specific sub-nuclear host structure associated with the foci could easily be confirmed with antibodies specific to these structures, such as coilin (Cajal bodies), EBP2 (nucleoli), or SC-35 (nuclear speckles). It is also possible that other viral proteins are involved in subcellular

localization and nuclear remodeling activity of pUL97 since the same localization patterns are not observed in cells transiently expressing pUL97.

The formation of assembly complexes late in infection is also disrupted in the absence of UL97 kinase activity. Under these conditions, assembly complexes are not observed and pp65 localizes to the periphery of round blebs in the cytoplasm just outside the nuclear membrane. While these blebs appear to contain nucleic acid material, pUL97 does not appear to be incorporated into these abnormal structures. These blebs are similar to those structures described by Goldberg et. al.; in the absence of kinase activity, they observe pp65-rich abnormal cytoplasmic aggregates with deformed intracytoplasmic membranes that replace the normal design of the assembly complex (20). Inhibition of UL97 kinase also prevents the enlargement and "kidney bean" distortion of the nuclei and is consistent with a previous report by Hamirally et. al.(22). This structural defect could be due to lack of laminar phosphorylation by UL97 kinase, which would prevent nuclear membrane reorganization and eventual egress of nucleocapsids from the nuclei. This defect could also be due, in part, to the cellular kinase CDK1, which is upregulated in CMV infection and also phosphorylates lamins.

The UL97 kinase has well-documented interactions with many cellular and viral proteins, like pUL69, that are also targets of CDKs (46, 55). Levels of G_2-M checkpoint regulators, such as Cyclin B1 and CDK1, are increased in CMV-infected cells, and the HSV-1 UL97 homolog, UL13, promotes stabilization and activity of CDK1 (2, 51). Since we know that (i) both CDK1 and pUL97 phosphorylate nuclear structural lamins inducing nuclear structural reorganization; (ii) CDK1 levels are elevated in CMV-infected cells and (iii) pUL97 homologues interact with CDK1, we thought it prudent to

determine if there were other links between pUL97 and CDK1. Interestingly, the upregulation of CDK1 in infected cells noted in previous studies appears to be dependent on UL97 kinase activity (Fig. 6). This regulation seems to be mediated at the level of transcription since UL97 kinase activity increases the accumulation of CDK1 mRNA (Table 2). The transient expression of pUL97 in uninfected cells also appears to induce the over expression of CDK1 and is consistent with the idea that UL97 kinase activity is required for this induction (Fig. 7). While increased levels of CDK1 transcripts are observed in the presence of UL97 kinase activity and are consistent with increased levels of CDK1, we cannot distinguish between increased transcription rates, increased transcript stability, or reduced degradation of CDK1 as potential causes of CDK1 accumulation.

Transcriptional induction of CDK1 normally occurs when cells are stimulated to enter the cell cycle from quiescence (G_o), as healthy, dividing cells regulate static, low levels of CDK1 through post-translational modifications (39, 59). Since HFF cells were allowed to rest for two days after reaching confluency, they were in a more G_0-like state and could be stimulated to enter the cell cycle by CMV infection, possibly by mechanisms similar to those induced by the shellfish toxin okadaic acid (OA). Many cell checkpoint regulators, including CDK1, have a promoter element called the OA response element (OARE), or a CCAAT box; when quiescent cells are treated with OA, there is an inhibition of protein phosphatase activity (PP1 and PP2a) and increased transcription from promoters containing these elements. The transcriptional activator NF-Y, which is normally bound to an OARE, is released from inhibition via MPF phosphorylation (18, 33, 35). UL97 kinase could be mimicking the MPF, which phosphorylates the NF-Y^A

subunit to induce a conformational change that promotes p300 and histone acetyl transferase binding, and allows for upregulation of genes with OAREs in their promoter regions (10). Data also supports the role of NF-Y in the activation of certain promoter regions in HSV-1, so pUL97 could not only be affecting certain cellular proteins with OAREs in their promoter regions, but viral proteins as well (30). While UL97 kinase activity might be sufficient to induce expression of CDK1, the exact mechanism as to how the kinase does so is unknown.

It is unclear as to why the increase in CDK1 levels would be beneficial to the virus. It has been suggested that instead of interacting with a cyclin, CDK1 interacts with the DNA polymerase processivity factor to facilitate replication (54). This would certainly suggest a role for initiating CDK1 induction by the virus, and validates further inquiry into this mechanism. Another possibility is that the UL97 kinase could be working to promote a unique pseudomitotic state through upregulation of mitotic regulators such as CDK1 and Wee1. This activity would further promote the disruption of nuclear integrity to allow nucleocapsid egress. As additional functions of this viral kinase are elucidated, it is apparent that this kinase impacts many aspects of CMV replication and is consistent with the pleiotropic effects observed in the absence of its enzymatic activity.

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CONCLUSIONS

Discussion

Human cytomegalovirus, like other viruses, has adapted ways of altering cell cycle checkpoint regulators of the infected host cell. While other viruses encode proteins that interfere with the function of RB through steric inhibition and ubiquitination, UL97 kinase phosphorylates RB directly to promote dissociation from E2F and progression through S phase of the cell cycle. The putative RB-binding site near the carboxyl terminal domain appears very important for this function. Not only does UL97 kinase activity induce a progression through S, but it also helps to induce a pseudomitotic state, as evidenced by nuclear remodeling and increased expression of G_2-M regulators. UL97 kinase activity during CMV infection promotes the upregulation of CDK1 and other mitotic regulators at the transcriptional level and affects the post-translational modification at CDK1 Tyr^{15}. All of these activities function to support viral DNA production and maturation of CMV virions.

Initial studies in our lab established the importance of the UL97 kinase for phosphorylating RB in CMV infected cells, and these studies also suggested a link between RB binding domains and aggresome formation. Subsequent studies also indicated that the LxCxE domain near the amino-terminal domain of pUL97 altered the sensitivity of the virus to MBV treatment, whereas mutations in the other two putative RB-binding domains did not impact them significantly. This also suggests a potential role of this domain in the antiviral function of MBV, possibly related to altered

enzymatic activity or association with other proteins. However, the replication phenotype associated with mutations in the central cysteine of this domain was very close to that of the wt virus, aside from an initial lag in replication. This might reflect a delay in replication associated with reduced phosphorylation of RB; an effect that the virus can overcome later in the infectious cycle through other mechanisms, possibly through degradation by pp71 activity (73) or phosphorylation by IE-1 (37). Regardless, it is apparent that the disruption of normal RB activity is important for CMV replication, as it has developed multiple mechanisms to ensure the infected cell exits G_0/G_1 and enters into S phase. The role of UL97 kinase in this critical process is certainly consistent with severe replication deficits in the absence of its enzymatic activity.

The subcellular distribution of pUL97 was characterized throughout the course of infection to help understand the potential roles of the kinase on viral maturation. This protein was clearly delivered from the tegument to the host cell and localized in foci: initially within the cytoplasm, followed by a transition to the nucleus. In cells infected with the wt virus, these foci enlarged and coalesced into subnuclear compartments, yet remained distinct from viral replication compartments. At later times in infection, pUL97 localized to the cytoplasmic assembly complexes along with viral DNA. However, in the absence of kinase activity, the transition from nuclear foci into larger subnuclear compartments was delayed. When viral DNA replication was blocked using CDV, these foci never increased in size or number, suggesting a role for other late viral proteins in this process.

Upon investigating the structural morphology of the infected cell, it was apparent that the kinase activity was important for supporting nuclear reorganization, as well as

pp65 egress and formation of cytoplasmic assembly compartments, reinforcing previous reports (58, 62). However, when we utilized an inhibitor of viral DNA replication, it was also apparent that delayed early and late gene products also assisted these morphological changes. Regardless, active UL97 kinase was at least partially responsible for inducing adjustments of nuclear morphology during CMV infection.

Since it has already been reported that CDK1 and UL97 kinase reorganized nuclear lamins through phosphorylation and that homologs to UL97 were interacting with CDK1, we investigated the effects of UL97 kinase activity on CDK1. In accordance with earlier data, CDK1 levels were increased in CMV-infected cells. Interestingly, this increase appeared to be dependent upon UL97 kinase activity (Fig. 1). This increase appeared to take place at the level of transcriptional upregulation, and was also noted for other G_2- M regulators such as Wee1 and Cyclin B1. It is unclear whether this was a result of increased transcript production, stabilization of mRNA transcripts, or blockage of protein degradation, but protein levels for CDK1 in infected cells are higher than those in uninfected cells and in infected cells lacking kinase activity. These data are consistent with increased transcript levels.

Of note is the increase in CDK1 levels in CDV-treated cells infected with the wt virus. While there was not extensive kidney bean nuclear remodeling as in a wt infection, suggesting that morphological change is due to kinase activity and other β/γ viral proteins, many of the infected cells had extremely large, round nuclei (data not shown). A potential role for the nuclear CDK1 can be inferred from data showing HSV-1 DNA polymerase processivity factor, UL42, mimics a cyclin and CDK1/UL42 interaction is essential for viral gene expression (90, 155). With the UL97 kinase still

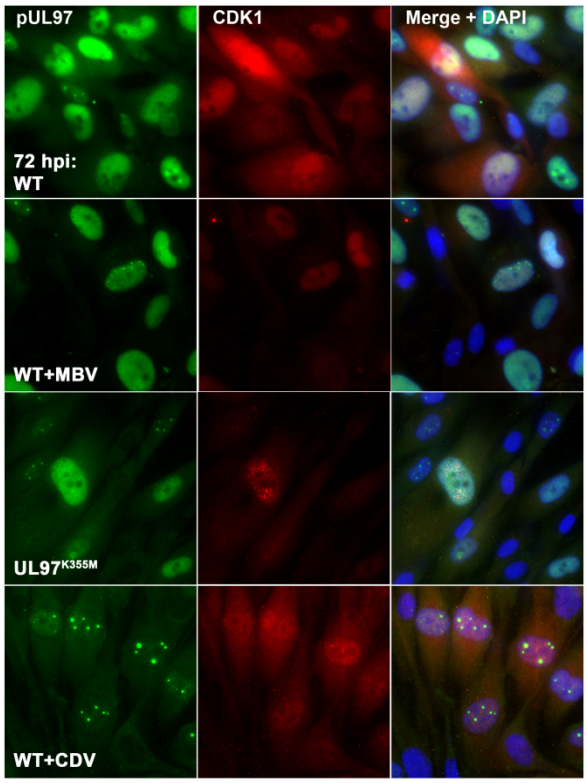

FIGURE 1. CDK1 expression in infected HFF cells. Confluent HFFs in an 8-well chamber slide were infected with CMV strain AD169 (WT) or the UL97 kinase-inactivated AD169 strain ($UL97^{K355M}$) at an MOI of 1 PFU/cell for 1 hour before washing and replacing with low-serum media. If applicable, cells were treated with 20 µM of Maribavir (WT + MBV) or 10 µM of cidofovir (WT+CDV) at 1 hour post-infection (hpi). Cells were fixed, permeabilized, and blocked at 72 hpi before staining for either pUL97 (green) or CDK1 (red). Cells were mounted in DAPI-containing media and fluorescence was visualized and photographed at 400x resolution. Mock-infected controls (with and without compounds) did not show any appreciable staining of either pUL97 or CDK1 at any timepoint (data not shown).

active in the presence of CDV, there could be extensive upregulation of CDK1, thus resulting in massive protein accumulation in the absence of viral replication. This would certainly suggest a role for initiating CDK1 induction by the virus, and validates further inquiry into this mechanism.

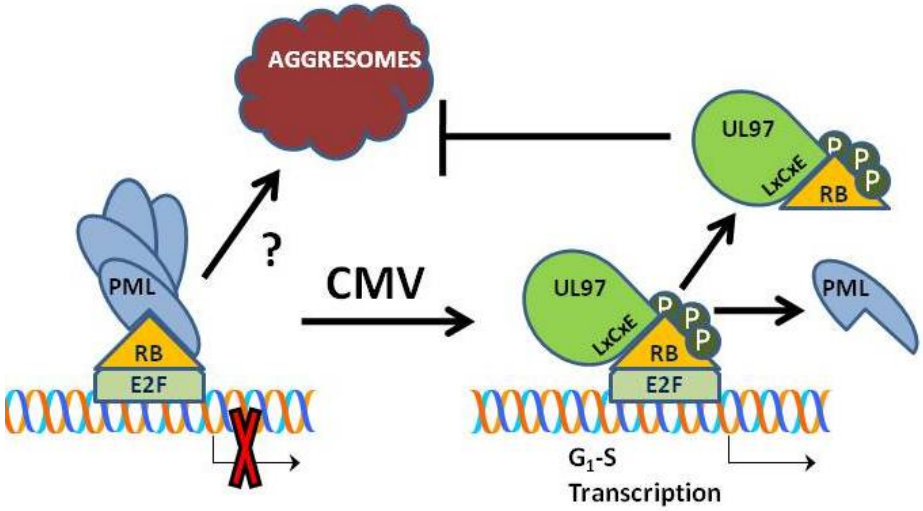

FIGURE 2. Model of the effect of pUL97 on RB phosphorylation and aggresome formation. In uninfected, resting cells, RB interacts with E2F and PML and promotes the formation of PML domains and aggresomes. In cells infected with CMV, UL97 kinase phosphorylates RB, which leads to the dissociation of RB and PML, impairs the ability of the cell to sequester viral proteins in aggresomes, and may cause degradation of RB via pp71 via unknown mechanisms. After RB dissociates from E2F, this transcriptional regulator can upregulate mRNA production of proteins important for S phase.

Model of Cell Cycle Alteration by UL97 Kinase Activity

The mechanism as to how UL97 kinase alters the G_1-S phase regulator RB has been partially elucidated (Fig.2). UL97 kinase hyperphosphorylates RB at residues 780, 807, and 821 (69, 134). In the absence of kinase activity, aggresomes are formed and viral replication is severely impaired. The amino terminal binding domain within pUL97 seems to be required to support the activity of the kinase, as we also observe aggresome formation upon mutation of this domain and an increase in MBV sensitivity.

The mechanism behind CDK1 regulation is less clear; one way CMV could be promoting an increase in certain mitotic regulators is through direct upregulation at promoter elements. UL97 kinase may help overcome cellular serine/threonine phosphatases, such

as PP1 and $PP2A_C$ that normally inactivate cellular transcription and translation during times of stress (61). CDK1 and other mitotic regulators contain an okadaic acid response element (OARE) in their promoters that are bound by the transcriptional activator nuclear transcription factor-Y (NF-Y). In times of stress or in early interphase, NF-Y is bound and repressed by p53 and HDACs. When PP1 and PP2a are inhibited, NF-Y is phosphorylated and changes conformation, p53 and HDAC release, histone acetyltransferases (HAT) and co-activators p300 promote translation, and transcription of G_2-M regulators is increased (17).

It is possible that UL97 kinase phosphorylates the transcriptional regulator (and known CDK1 and HSV-1 target) NF-Y to promote the upregulation of CDK1 (84). Normally, the MPF (composed of Cyclin B1 and CDK1) will phosphorylate a NF-Y subunit and induce binding of co-activators to upregulate G_2-M proteins; UL97 kinase could be mimicking this action (Fig. 3). However, it remains to be determined if UL97 activity also prevents the degradation of CDK1, and if pUL97 is working in concert with other viral proteins or the MPF to promote CDK1 upregulation. All of these regulatory actions may be initiated very early, as pUL97 is clearly delivered from the tegument, localized to the nucleus in discrete foci that might have been interacting with cellular transcriptional machinery since certain cellular mitotic mRNAs were clearly upregulated very early upon infection.

Unlike CDK1, Wee1 does not have an OARE in its promoter, but does have an activator protein-1 (AP-1) motif in its promoter. AP-1 is a transcriptional activator that is a heterodimers of the proto-oncogenes c-Fos and c-Jun that bind to and activate 12-*O*-

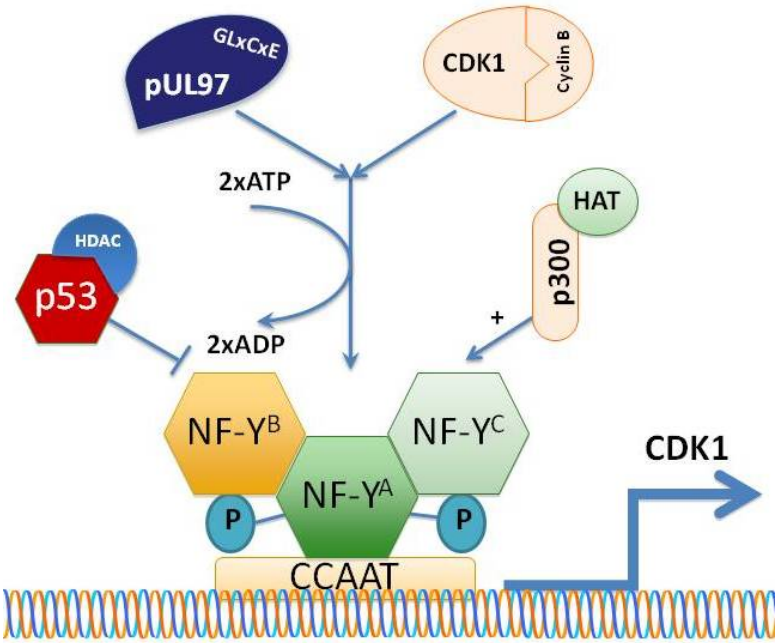

FIGURE 3. Model of CDK1 upregulation by pUL97. The transcriptional activator NF-Y, which is composed of 3 subunits (A, B, and C), is bound to an okadaic acid response element (OARE) in the promoter. The transcriptional repressor p53 binds to NF-Y and prevents transcription; the MPF (CDK1/Cyclin B) can phosphorylate the A subunit, cause a conformational change, and promote dissociation of p53 and co-repressors such as HDACs. Co-activators such as p300 and HATs can now bind, promoting upregulation of proteins important for the G_2-M transition. We hypothesize that the UL97 kinase is mimicking the action of MPF to account for the upregulation of CDK1 witnessed in our studies.

tetradecanoylphorbol-13-acetate (TPA) response elements (TRE) (152). AP-1 induction by CMV has been previously described, and is important for IE protein induction (71).The role that UL97 kinase plays in Wee1 induction is unclear, but it could be phosphorylating one of the AP-1 subunits such as c-Jun, as other tegument proteins such as pp71 have been stated to induce transcription from AP-1 promoter elements (71).

Upregulation of Wee1 could be supporting the pseudomitotic state, as it has been noted that when there is an abnormal increase in Wee1, cells enter into an abnormal mitosis or polyploid state (76). While in a previous report, Wee1 is proteasomally degraded early in infection; however, initial western data in our lab supports an increase

in Wee1 levels within infected cells (143). While we cannot conclude whether Wee1 is transcriptionally active or if it is causing the noted inhibitory Tyr^{15} phosphorylation of CDK1, we can surmise that the upregulation of Wee1 is partially tied to UL97 kinase activity.

Viruses such as CMV may have an increase in these G_2-M regulators because it is either a side effect of viral protein induction. The viruses intentionally bypass DNA regulatory checkpoints, or they have a need for an increase in some of these proteins. Viral tegument proteins such as pUL69, which is a transcriptional transactivator, an unspliced mRNA exporter, and known target of UL97 kinase activity, could also be assisting in the transcriptional upregulation (8, 92).

Future Directions

The mechanisms used by UL97 kinase to regulate CDKs and CDK targets are not completely understood. To further elucidate the pUL97 interaction with RB, a mutational analysis of the glycine and serine residues which flank the amino-terminal RB-binding domain LxCxE will be required (Fig. 4). There is excellent experimental evidence to support the important role this domain plays in the function of pUL97, which is reinforced by conservation of these residues throughout viral species (131). Generation of pUL97-expressing plasmids and recombinant viruses will allow determination of the importance of glycine (G^{148}) and serine (S^{159}) residues in the activity of pUL97, including (but not limited to) the phosphorylation of RB, the stability of the pUL97-RB interaction, and the effect on CMV and cellular replication. In human papillomavirus, a

443 GGC TTG CGC TGC CGC GAA ACT TCG GCC ATG TGG TCG TTC GAG TAC GAT 490
148 G L R C R E T S A M W S F E Y D 163

FIGURE 4. LxCxE RB-Binding Domain in pUL97. The nucleotide (top) and corresponding amino acid sequence (bottom) is given for the putatively most-relevant RB-binding domain in pUL97. Highlighted in green is the canonical LxCxE binding domain, while the upstream glycine (G) that affects oncogenic potential is shown in red text. The conserved serine (S) that lies in a near-canonical CDK-binding site is highlighted in red.

glycine (G) or an aspartic acid (D) immediately upstream of the LxCxE motif in the E7 protein constitutes the difference between low-risk and high-risk cellular proliferation, respectively (195). Conversion of G^{148} to D^{148} will expand the knowledge regarding pUL97 activity on the interactions, expression and stabilization of RB. Transfection of Saos-2 cells, which are unable to phosphorylate RB, could confirm that any changes in RB phosphorylation are due to effects of pUL97. Also, since S^{159} lies within a putative CDK-recognition sequence, it will be important to determine if this serine is potentially targeted by CDKs or pUL97 itself.

UL97 kinase activity affects the mRNA and protein levels of CDK1. It would be important to characterize the kinase effect upon other RB-specific CDKs, specifically CDK2, -4, and -6. RB dysregulation plays a critical role in neonatal development and cell growth, and it is vital to dissect how CMV dysregulates RB as this is one of the likely mechanisms behind CMV generating permanent sequelae within neonates (86).

To further confirm that CMV induces pseudomitosis in infected cells at late stages of infection, it will be vital to investigate protein levels of other known mitotic/G_2-M regulatory markers, such as Cyclin B1, Wee1, Myt1, and cdc25B and C. Previous reports suggest that the increased levels of cyclin B1 and Wee1 in CMV-infected cells are due to

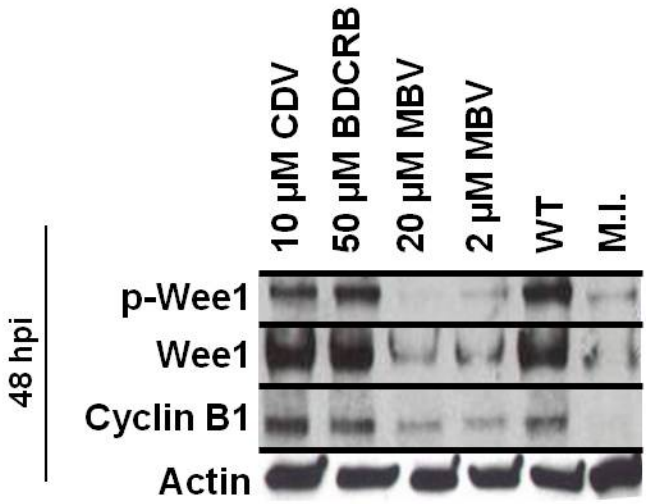

FIGURE 5. Western Analysis of Wee1 and Cyclin B1 in infected cells**.** Confluent HFFs were infected at an MOI of 1 PFU/cell with CMV for 1 h; cells were then washed and replaced with fresh, low-serum media. Cell lysates were harvested using RIPA buffer containing protease/phosphatase inhibitors at 48 hpi and 25 µg of protein for each infectious condition was probed for Wee1, phosphor-Wee1, and Cyclin B1 expression. Cells were either mock-infected (M.I.), treated at 1 hpi with either 10 µM of CDV (CDV), 50 µM of BDCRB (BDCRB), varying doses of MBV, or left untreated (WT). Cell lysates were probed for Cyclin B1 (V152), Wee1 (4936) and phospho-Wee1(D47G5). Actin was used as a loading control for all westerns.

changes in regulation at both synthesis and degradation (143). Although our extensive CDK1 data and preliminary Cyclin B1 and Wee1 western supported our mRNA data (Fig. 5), these preliminary westerns did not show any distinct changes in levels or phosphorylation states of other G_2-M regulators like Myt1 or cdc25C (data not shown). Cyclin B1 and Wee1 levels appear to be increased and dependent upon UL97 kinase activity (Fig. 6), yet interestingly Cyclin B1 and CDK1 did not appear to strictly co-localize in CMV infection, with a lot of CDK1 found within the nucleus (Fig. 7). Even though it appears that CMV, through the action of UL97 kinase, increases levels of many mitotic regulatory proteins in infected cells, especially Wee1, Cyclin B1 and CDK1, from

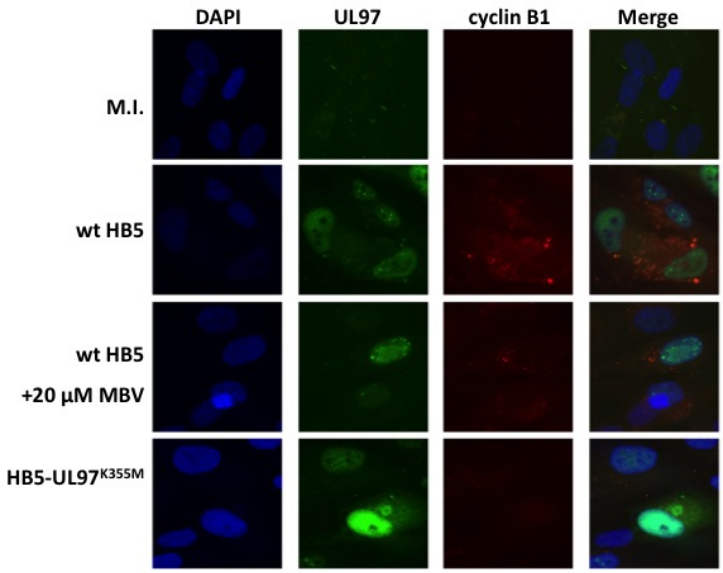

FIGURE 6. Cyclin B1 localization in infected cells. Confluent HFFs on coverslips were infected with CMV bacterial artificial chromosome (BAC) AD169-derived strain HB5 (wt HB5) or the UL97 kinase-inactivated HB5 strain (HB5-UL97^{K355M}) at an MOI of 1 PFU/cell for 1 hour before washing and replacing with low-serum media. If applicable, cells were treated with 20 µM of Maribavir (WT + MBV) at 1 hour post-infection (hpi). Cells were fixed, permeabilized, and blocked at 72 hpi before staining for either pUL97 (green) or Cyclin B1 (red). Cells were mounted in DAPI-containing media and fluorescence was visualized and photographed at 400x resolution. M.I.: Mock-infected control.

our studies it is unclear as to the mechanism behind the increase and if these proteins are fully functional. Our initial Cyclin B1/CDK1 colocalization data reveals a modest localization of these two proteins, mainly in the cytoplasm around the perimeter of the nucleus (Fig.7).

Future studies will determine if UL97 kinase activity affects other G_2-M checkpoint proteins noted in this study, specifically CDK1, Wee1, and Cyclin B1. It will also be necessary to clarify if UL97 kinase is phosphorylating CDK1 directly and if pUL97 is stabilizing constitutively expressed mRNA transcripts. Previous reports state that the increased levels of cyclin B1 and Wee1 in CMV-infected cells is due to changes

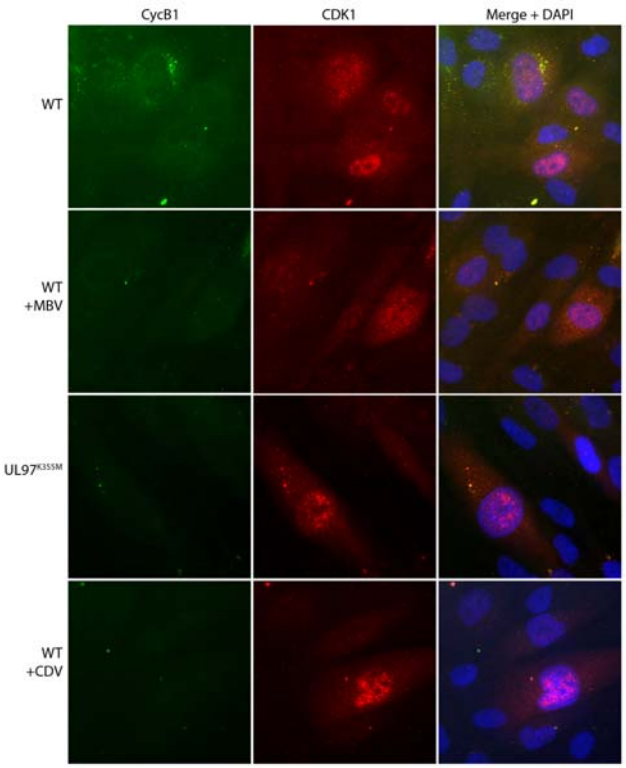

FIGURE 7. Localization of Cyclin B1 and CDK1 in infected HFF cells. Confluent HFFs in an 8-well chamber slide were infected with CMV strain AD169 (WT) or the UL97 kinase-inactivated AD169 strain ($UL97^{K355M}$) at an MOI of 1 PFU/cell for 1 hour before washing and replacing with low-serum media. If applicable, cells were treated with 20 µM of Maribavir (WT + MBV) or 10 µM of cidofovir (WT+CDV) at 1 hour post-infection (hpi). Cells were fixed, permeabilized, and blocked at 72 hpi before staining for either Cyclin B1 (green) or CDK1 (red). Cells were mounted in DAPI-containing media and fluorescence was visualized and photographed at 400x resolution. Mock-infected controls (with and without compounds) did not show any appreciable staining of either Cyclin B1 or CDK1 (data not shown).

in regulation at both synthesis and degradation (143); our future studies must evaluate whether UL97 kinase affects degradation of CDK1 and other related proteins. Since there is a chance that the CDK1 might be interacting with a viral regulator, it will be vital to determine if Cyclin B1 and CDK1 are associating with one another to form an active MPF, and if UL97 kinase activity plays a role in this association.

Another goal is to elucidate the mechanism by which pUL97 increases CDK1 levels in infected cells. In order to test the hypothesis that pUL97 is phosphorylating the

transcriptional regulator NF-Y, it will be essential to probe for pUL97 and NF-Y interactions, as well as regulation at known OARE/NF-Y promoter elements. NF-Y is a trimeric protein, composed of NF-Y^A, NF-Y^B and NF-Y^C. NF-Y^A is the regulatory subunit of the protein and is actively bound to CCAAT boxes in promoter elements known as okadaic acid response elements (OARE) (101). Regulation of NF-Y often occurs through p300 interaction; use of a novel specific p300 inhibitor would further flesh out the mechanism by which NF-Y is regulated by CMV.

CMV dysregulates the G_2-M transition in order to facilitate viral maturation; dissection of the mechanism by which the virus promotes a pseudomitotic state will open up avenues for development of new antivirals.

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