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The Role of Viral Protein pUL21a in Human Cytomegalovirus Infection

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THE ROLE OF VIRAL PROTEIN pUL21a IN HUMAN CYTOMEGALOVIRUS INFECTION

by

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ABSTRACT OF THE DISSERTATION

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The double-stranded DNA virus human cytomegalovirus (HCMV) is a ubiquitous human pathogen that causes severe disease in immunocompromised individuals and neonates. The biology of HCMV remains largely undefined and functions of many of the roughly 200 putative HCMV genes are still unknown. One of these genes, UL21a, was shown to be critical for efficient viral replication in two large-scale mutagenesis studies. UL21a encodes for a putative protein that is 123 amino acids and has no homology to any known proteins. The gene products of UL21a, its role during infection, and its function(s) remain undefined.

Here, we characterized UL21a and demonstrated its role in HCMV infection. We identified a single UL21a transcript which was expressed with early gene kinetics. UL21a encoded a protein termed pUL21a which localized to the cytoplasm and underwent proteasome-dependent degradation. UL21a was specifically required for efficient viral replication, and the growth of a UL21a deletion virus was similar to that of a stop codon mutant, suggesting it is pUL21a which facilitates viral replication.

To identify the role of pUL21a during virus infection, we analyzed fibroblasts infected with equal amounts of wild-type and UL21a deletion virus for defects in

multiple steps of the viral lifecycle. The UL21a deletion virus entered cells and initiated viral gene expression efficiently; however, it synthesized viral DNA poorly and accumulated several immediate-early (IE) transcripts at reduced levels at late times of infection. The reduction in IE transcripts was dependent on the reduction in viral DNA synthesis, showing that multiple IE transcripts are dependent on viral DNA synthesis for their full expression. Finally, using complementing cells we show that it is the *de novo* synthesis of pUL21a which facilitates viral DNA synthesis.

To determine the function(s) of pUL21a, we identified proteins that specifically interacted with pUL21a. We found that pUL21a interacts with the Anaphase-Promoting Complex (APC), a multi-subunit E3 ubiquitin ligase which targets multiple proteins for proteasome-dependent degradation. The APC is critical for progression through mitosis and the regulation of cellular DNA synthesis. We have found that pUL21a specifically binds to the APC, and is required for the accumulation of APC substrates, degradation of APC4 and APC5, and dissociation of the APC during HCMV infection. Finally, shRNA knockdown of the APC activator Cdh1 and to a lesser degree APC8, significantly restored late gene expression of the UL21a mutant virus, suggesting the APC has antiviral activity for HCMV. Thus, we propose that one mechanism for pUL21a to promote viral replication is to regulate the function of the APC.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

APC	anaphase-promoting complex
ATP	adenosine triphosphate
ARM	arginine-rich motif
BAC	bacterial artificial chromosome
CAV	chicken anemia virus
CDK	cyclin-dependent kinase
CHX	cycloheximide
DB	dense bodies
EBV	Epstein-Barr virus
EGFR	epidermal growth factor receptor
GCV	gancyclovir
GFP	green fluorescent protein
HCMV	human cytomegalovirus
HFFs	human foreskin fibroblasts
HPV	human papillomavirus
HS	heparan sulfate
HSV	herpes simplex virus
HTLV	human T-lymphotropic virus
IE	immediate-early
IFN	interferon
IP	immunoprecipitation
JNK	Jun N-terminal kinase
KSHV	Kaposi’s Sarcoma-associated herpesvirus

MCMV	murine cytomegalovirus
MCP	major capsid protein
MHC	major histocompatibility
MOI	multiplicity of infection
NK	natural killer cell
NIEP	non-infectious enveloped particle
ORF	open reading frame
PAA	phosphonoacetic acid
PACR	poxviral APC/cyclosome regulator
PDGFR-α	platelet-derived growth factor receptor-α
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase isoform 3
PFU	plaque forming unit
PLK-1	polo-like kinase-1
PP2A	protein phosphatase 2A
qPCR	quantitative polymerase chain reaction
RACE	rapid amplification of cDNA ends
Rb	retinoblastoma tumor suppressor protein
RRM2	ribonucleotide reductase subunit M2
RT	reverse transcriptase
shRNA	short hairpin RNA
siRNA	small interfering RNA
$TCID_{50}$	tissue culture infectious dose
TK	thymidine kinase
TPR	tetratricopeptide repeat

UL	unique-long
US	unique-short
VZV	Varicella-Zoster virus
Y2H	Yeast-2-Hybrid

Chapter I

Introduction

Herpesviruses

Overview

Herpesviruses are a large class of viruses and are widespread throughout nature. Most mammalian species have at least one known herpesvirus pathogen. There are eight herpesvirsues which infect humans. These viruses are: herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), varicella-zoster virus (VZV), Epstein-Barr virus (EBV), human cytomegalovirus (HCMV), human herpesviruses 6A, 6B, and 7, and Kaposi's sarcoma-associated herpesvirus (KSHV) (95).

The relatedness between all herpesviruses suggests that they evolved from a common ancestor, which likely lived many millions of years ago. In fact, herpesviruses even share some features with bacteriophages, suggesting they may have evolved from bacteriophages, a lineage which may have begun at the early stages of parasitism, nearly a billion years ago.

Herpesviruses share at least four significant biological properties. They all encode several proteins involved in nucleotide metabolism, DNA synthesis, and protein processing. The synthesis of viral DNAs and capsid assembly occurs in the nucleus with final envelopment of the virus occurring in the cytoplasm. Productive infection results in the destruction of the host cell, and all herpesviruses studied to date are able to establish latency in their natural host species. While herpesviruses have many similarities, there are also several difference which distinguish them. Herpesviruses can vary widely in the length of their replicative cycle, host cell tropism, and cell types in which they establish latency, and disease manifestations. Based on these differences, the herpesviruses are classified into three main subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae*, and

Gammaherpesvirinae. *Alphaherpesvirinae* have a short replication cycle, a broad species and cell tropism,, and establish a latent infection in sensory ganglia. *Betaherpesvirinae* have a relatively long replication cycle, a broad cell tropism but strict species tropism, and they establish latency in secretory glands, lymphorecticular cells, kidneys, and other tissues. *Gammaherpesvirinae* have a short replication cycle, replicate in epithelial and fibroblast cells, and establish latency and can promote tumorigenesis of T or B lymphocytes.

Structure/Genome

All Herpesviruses share a common structure. The virion consists of a core, the capsid, a tegument layer, and an envelope. The core contains the viral DNA within a proteinaceous spindle. The capsids for all herpesiviruses are very similar, with a 100 nm diameter and a triangulation number of 16, and 960 copies of the capsid protein per capsid. The tegument, the structure found between the capsid and the envelope, is thought to have an ordered structure despite the lack of distinctive features. The tegument proteins are added both in the nucleus following capsid formation and in the cytoplasm prior to envelopment, and these proteins are often involved in helping the virus establish a productive infection during the initial stages of the life cycle. The envelope is a membrane layer around the virus, likely derived from the trans-Golgi or endosome, and contains large numbers of glycoproteins.

Herpesviruses contain linear, double-stranded DNA genomes ranging in size from 120-250 kb. The genome circularizes upon entry into the nuclei of infected cells. Herpesvirus genomes contain terminal and internal repeats in a variety of different

arrangements. In specific viruses like HSV or HCMV, multiple isomers of the genome can be present due to inversion of the repeats. Herpesviruses can encode between 70 and 200 genes, but these numbers are likely underestimates, and do not take into account small RNAs or protein products of less than 100 amino acids.

Lifecycle

Herpesviruses begin their lifecycle with glycoprotein attachment at the surface of the cell. Some viruses enter by endocytosis while others fuse at the plasma membrane. Regardless of their route of entry, all herpesviruses traffic to the nucleus where the genome is released, circularizes, and begins transcription of gene products. Gene expression is a classic regulatory cascade, with viral genes falling into separate groups with respect to their order of expression. There are three major expression kinetic classes for herpesvirus genes: immediate early, early, and late. Immediate-early (IE) genes are expressed within a few hours of infection and primarily act as transactivators for the production of early genes, which are required for DNA replication. Late genes encode structural proteins important for encapsidation and packaging of the virion. There are two sub-classes of late genes, leaky late and true late. Leaky late genes are augmented by viral DNA synthesis while true late genes are completely dependent on viral DNA synthesis for their expression.

Following capsid assembly in the nucleus, the virus goes through an envelopment/deenvelopment step to transit through the nuclear membrane and egress into the cytoplasm. Once in the cytoplasm, the capsid acquires its full complement of tegument proteins and traffics to the assembly center where its envelope is acquired.

Once its envelope is acquired, the virus traffics to the membrane where it is subsequently released to begin the cycle anew.

As mentioned previously, all herpesviruses establish latency as an alternative lifestyle. During latency, the viral genome is maintained as an episome, and only a few viral gene products are expressed. Latent genomes are able to reactivate and begin a productive infection upon certain stress stimuli.

HCMV

Clinical Relevance

Human cytomegalovirus (HCMV), the prototypical betaherpesvirus, is a ubiquitous pathogen that infects the majority of the world's population. HCMV is usually asymptomatic in immunocompetent individuals, except in rare cases where it causes mononucleosis. However, HCMV can cause severe disease and death in immunocompromised individuals such as AIDS patients and transplant recipients. Importantly, HCMV is the most common viral cause of birth defects leading to mental retardation, blindness, and hearing loss (64). In addition, HCMV infection is also a possible risk factor in the development of vascular diseases such as atherosclerosis, transplant vascular sclerosis, and coronary restenosis after angioplasty surgery (24, 50, 59, 65, 115, 120, 148). The economic burden to the U.S. health care system for this virus is estimated at approximately 4 billion dollars annually, with a majority of the costs attributed to long-term sequelae experienced by individuals who acquire congenital HCMV disease (33). A comprehensive understanding of how HCMV interacts with the

host to establish both acute and latent infections will be critical for developing an effective vaccine and novel therapeutics to combat HCMV disease (43, 102).

HCMV Pathogenesis

HCMV is transmitted by direct contact with bodily fluids of infected individuals. The virus replicates in the mucosal epithelium at the site of inoculation. Hematopoietic cells then disseminate the virus during a systemic viral infection to a wide range of cell types, including epithelial, endothelial, fibroblast, macrophage, and dendritic cells. Virus produced from these cells contributes to viral shedding following primary infection which can last for several months in adults to several years in young children (64).

The immune response to HCMV is strong, broad, and long lasting. The immune response lasts for many years and a large percentage of memory T cells in older individuals can be devoted towards CMV antigens (122). Interestingly, despite the strength of the immune response, pre-existing immunity does not prevent reinfection, but does limit acute disease in both immunocompetent and immunocompromised individuals (62, 133). The ability of CMV to continue viral shedding for several months in the face of such a strong immune response is likely due to the many gene products encoded by CMV to modulate both innate and adaptive immune responses (62).

Natural Killer (NK) cells are likely the first immune cells that limit viral replication. Mice with NK cell deficiencies are highly susceptible to murine CMV (MCMV) infection (101). In humans, a patient with NK cell deficiency suffered from severe infection by HCMV, which suggests this response is also important to control HCMV (64). Furthermore, multiple HCMV gene products, including UL16, UL40,

UL140, UL141, and UL142 all play a role in NK cell inhibition using several different mechanisms (62, 140). Cell-mediated immunity and specifically CD8+ cytotoxic T cells are also critical for control of HCMV. Adoptive transfer of CD8+ T cells specific for CMV antigens into both mice and humans can protect from CMV disease (29, 46, 92). HCMV also encodes numerous gene products designed to interfere with MHC I expression and translocation to the cell surface, such as US2, US3, US6, and US11 (62). Antibody response also plays a role during infection, likely by preventing transmission between individuals, as most virus in the host remains cell-associated except in secretions such as saliva and breast milk. Passive immunization with a high titer HCMV-specific immunge globulin can prevent transplacental transmission during pregnancy (72).

Like all herpesviruses, HCMV establishes latency and has the ability to reactivate from latency following immunosuppression; which profoundly affects its ability to establish and cause disease. Latent virus is found primarily in hematopoietic cells which are able to repress viral gene expression. Further differentiation into cells such as macrophages and dendritic cells results in reactivation, suggesting there are differentiation-specific factors that restrict viral gene regulation (88, 90, 91, 107, 111, 112). Study of HCMV latency has been hampered by the lack of a small animal model and thus investigators have relied on cell culture based systems of experimental latency. However, the MCMV surrogate model recapitulates the general process of latency and reactivation and thus has provided unique insight into these processes. However, the viral genes which mediate this process remain mostly elusive (79).

HCMV Virion

The HCMV virion is typical but somewhat larger than other herpesviruses (200-300 nm) (reviewed in (64)). The envelope contains ~20 glycoproteins including the conserved gB, gH:gL, and gM:gN complexes, as well as several G-coupled protein receptors. It also contains a wide array of tegument proteins, most of which are phosphorylated and highly immunogenic. The most abundant tegument proteins are the pp65 and pp71 proteins. pp65 helps block interferon responses and pp71 acts as a viral transactivator (VTA) (4, 8, 34). HCMV has a class E genome, which contains both unique long (UL) and unique short (US) segments flanked by terminal and internal repeats. HCMV is the largest known human virus in terms of genome size, with a genome of ~240 kb, and encodes at least 166 ORFs, several microRNAs, and likely several other unknown gene products (12, 16, 18, 26, 67, 68). Interestingly, HCMV produces an abundant number of both dense bodies (DB) and non-infectious particles (NIEP), in excess of several hundred more DB/NIEP than infectious virus. Dense bodies are largely composed of the tegument protein pp65 surrounded by a cellular membrane while NIEP's are virions which are largely devoid of viral DNA. HCMV is the only herpesvirus to produce DB's and it is unclear why the virus produces so many DB's and NIEP's.

Viral Replication *in vitro*

HCMV has a relatively slow replication cycle, taking 48-72 hours to begin producing infectious virus in cell culture. Commonly used laboratory strains AD169 and Towne, grow well in fibroblasts but are unable to replicate in additional cell types due to

mutations in the UL128-131 genes and large deletions in the ULb' region. Clinical isolates of the virus retain the ability to grow in endothelial, epithelial, macrophage, and dendritic cells *in vitro*. However, except for U373MG cells, fully transformed cells are non-permissive for HCMV replication due to unknown reasons.

HCMV binds to heparan sulfate (HS) on the cell surface but additional receptors remain controversial. It was originally proposed that HCMV uses Epidermal Growth Factor Receptor (EGFR) as a receptor, but this has recently been disputed (19, 39, 137). More recently, cellular integrins and platelet-derived growth factor-α (PDGFR-α) have been proposed to serve as a receptor for the virus (21, 114, 136). Regardless, it has been shown that gB binds to HS and, along with gH:gL, plays a role in fusion (14). HCMV enters the cellular cytoplasm by fusion with a cellular membrane; however, where this occurs depends on the cell type (14). HCMV enters fibroblasts by fusion at the cell membrane, while in epithelial and endothelial cells the virus is internalized via endocytosis before fusion occurs in the cytoplasm (97). Translocation of the virion to the nucleus occurs by a relatively uncharacterized process, but likely involves transport on microtubules followed by uncoating and release of viral DNA at nuclear pores (74). Based on studies of the HSV-1 homolog UL36, it is likely that the conserved UL48 and UL47 proteins play an essential role in this process (5, 17, 143).

Upon entry into the nucleus, the viral genome is repressed by promyelocytic leukemia protein (PML bodies) and requires the tegument protein pp71 to alleviate this repression (98, 99, 124, 125). pp71 targets the PML protein Daxx for ubiquitin-independent, proteaseome-dependent degradation which activates viral immediate-early (IE) gene transcription (37, 99). In fact, co-expression of pp71 along with bacterial

artificial chromosome (BAC) transfection dramatically increases the ability to produce infectious virus from BAC transfection (see below) (4). IE genes do not require viral protein synthesis for transcription and thus are identified by treatment of infected cells with the protein synthesis inhibitor cycloheximide. The virus expresses its IE genes by 2 to 4 h after viral entry and these genes persist throughout the infection. The primary proteins encoded by the major immediate-early (MIE) transcript are IE1-72 and IE2-86, which are produced by alternative splicing (117-119). These IE proteins are critical for the establishment of a productive infection and must be down-regulated for the virus to establish latency. The IE2-86 protein is essential for viral replication, while IE1-72 is required at a low multiplicity of infection (MOI) (22, 25, 31, 57, 63, 100). Both proteins transactivate viral promoters and also modulate the cellular environment to be conducive for viral infection. Additional IE genes, which include TRS1, UL37x1, and US3, help HCMV to overcome innate and adaptive cellular antiviral responses (3, 13, 23, 27, 28, 41, 87).

Transcription of early genes shortly follows IE gene expression, appearing at between 4 and 12 hpi. True early gene transcription requires IE protein expression but not viral DNA replication, and thus can be classified by sensitivity to cycloheximide but resistance to DNA synthesis inhibitors such as phosphonoacetic acid (PAA). Early genes encode DNA replication enzymes, such as UL44 (processivity factor) and UL54 (viral DNA polymerase), as well as viral regulatory proteins that alter host cells creating a favorable environment for replication. DNA replication begins at 14-16 hours post infection and requires bi-directional transcription at the origin of lytic replication (OriLyt) and at least 6 proteins specifically involved in replication. These proteins

include the viral polymerase (UL54), processivity factor (UL44), ssDNA binding protein (UL57), and a trimeric helicase-primase complex (UL70/UL102/UL105). Other genes known to be directly involved in viral DNA synthesis include UL84, UL112-113, UL98 (nuclease), UL114 (uracil DNA glycosidase), and IE2-86 (75). Viral DNA replication then proceeds using a rolling-circle mechanism.

Late genes are expressed following the onset of viral DNA replication, and many of them encode structural proteins, such as the major capsid protein (MCP) and pp28, which are required for assembly and maturation of the virion (106). Other late-gene products, such as pp71, are tegument proteins which can antagonize intrinsic cellular defenses and help progeny virus initiate IE gene expression during subsequent infection (98, 99). Capsid assembly and egress are similar to other herpesviruses, albeit the mechanisms and genes used to achieve the same goal are slightly different.

HCMV Regulation of Apoptosis

Apoptosis can function as an innate antiviral defense and is especially problematic for viruses with long replication cycles, such as CMV. Cellular events leading to apoptosis are classified into 3 different pathways: the mitochondria-mediated intrinsic pathway, the extra-cellular ligand-mediated extrinsic pathway, and the endoplasmic reticulum (ER)-mediated pathway. These pathways lead to the activation of caspase cascades, ultimately culminating in the activation of caspase-3 which leads to cleavage of substrates such as PARP, which ultimately causes cell death. HCMV encodes several factors which block the ability of the cell to initiate apoptosis. During infection, pUL36 (vICA) blocks FAS-mediated apoptosis by inhibiting proteolytic

activation of caspase 8 (108). pUL37x1 (vMIA) prevents activation of caspase 9 by sequestering Bax, and its interaction with GADD45 family members appears to play an important role in this function (3, 23, 87, 110). pUL38 blocks ER stress induced apoptosis by preventing Jun N-terminal kinase (JNK) phosphorylation and by increasing expression of ATF4; however, the mechanism pUL38 uses to achieve this functions remains unknown (127, 141). Interestingly, the 2.7 kb RNA encoded by HCMV can bind to a mitochondrial enzyme complex and block Rotenone-induced cell death (89). In overexpression, IE1 and IE2 block apoptosis by activating the Akt-mediated signaling pathway (145, 149). The ability of HCMV to block apoptosis is clearly important for replication, as growth defects of both UL37x1 and UL38 mutant viruses can be significantly restored by the addition of caspase inhibitors (87, 127).

HCMV Regulation of the Cell Cycle

HCMV also has the ability to regulate cell cycle progression in order to create an environment conducive to its own replication. HCMV has been shown to stimulate cells from G_o but prevent cellular DNA synthesis from occurring, ultimately coercing the cell into maintaining itself at the G_1/S boundary of the cell cycle (84). The prevailing theory is that this stage of the cell cycle will provide substantial nutrients and nucleotides for the virus to replicate, but those resources will not be diverted to helping the cell replicate its own genome. However, this has not been experimentally proven, and preventing cellular DNA synthesis may also promote viral replication in other ways, such as preventing a robust DNA damage response.

One of the major cell cycle control proteins targeted by HCMV is the retinoblastoma tumor-suppressor protein (Rb), which binds to E2F transcription factors in its unphosphorylated form, thus inhibiting expression of genes required for DNA replication. HCMV uses at least two mechanisms to inhibit Rb and stimulate cell cycle progression from G_0 to G_1. First, the pp71 protein targets unphosphorylated Rb for ubiquitin-independent, proteasome-dependent degradation (42). To eliminate any residual Rb activity, the UL97 kinase phosphorylates Rb during infection, rendering it inactive (36).

HCMV also inhibits the anaphase-promoting complex (APC), a large E3 ubiquitin-ligase important for progression through mitosis and proper entry into S phase (130, 132, 139). As HCMV does not infect cells in the G_2/M phase of the cell cycle, it has been thought that the inhibition of the APC would advance cellular progression into S phase prematurely. However, it has not been confirmed that APC inhibition helps the virus promote S phase nor is it known whether it plays an important role during virus infection.

HCMV also uses multiple mechanisms to prevent cellular DNA synthesis. IE2-86 inhibits cyclin A transcription and induces p16 expression, which is capable of preventing Cdk activity (69, 73, 78, 104, 138). pUL69 blocks cellular DNA synthesis by an undefined mechanism, and pUL117 blocks MCM loading and accumulation during infection, which prevents licensing of the replication origins (30, 55, 84). Importantly, all three of these proteins are necessary for the virus to prevent cellular DNA replication, as the absence of any one protein results in a virus unable to prevent cellular DNA synthesis.

BAC Genetic System

Development of Herpes/HCMV BACs

In the late 90's, many herpesviruses were cloned as bacterial artificial chromosomes (BACs) (reviewed in (1)). Prior to this technology, the only way to make mutant viruses was to use homologous recombination in mammalian cells. Due to the large genome size and slow-replication kinetics, this method of creating recombinant viruses was especially difficult in the β-Herpesviruses. To circumvent this problem, researchers inserted the genomes of these viruses into F-plasmids in *Escherichia coli* (*E. coli*) which could be delivered into a suitable mammalian cell type in order to create infectious virus. This method created a system where viral recombinants could be created in the absence of viral growth, thus one can create a viral mutant independently of its viral fitness. Furthermore, the viral genome could be characterized before reconstitution of viral progeny, reducing the likelihood of spurious point mutations or deletions occurring in the process of producing recombinant virus.

One disadvantage to the original BAC system was that a set of non-essential viral genes had to be deleted to accommodate the large BAC vector sequence (~10 kb) and maintain the viral genome within its packaging limit. It has been shown that simply inserting the BAC vector sequence into the viral genome without removing any viral sequence can result in an attenuated virus (144). To eliminate the need to delete viral sequences, BACs were then created which had the BAC vector sequence flanked by loxP sites and also expressed an intron-containing Cre recombinase (109, 144). In *E. Coli*, the Cre recombinase will not be functional due to the intron, which allows maintenance of the BAC vector sequence. Upon delivery into mammalian cells the Cre recombinase is

expressed, the BAC vector sequence is cleaved, and virus is produced that is full length except for the addition of a small loxP site.

In HCMV, several strains have been cloned as BACs including the laboratory strains Towne and AD169, and several clinical isolates including TR, TB40, and Merlin (7, 57, 68, 116). The ability to create recombinant clinical BACs will be critical to the future study of HCMV, as the laboratory strains contain large deletions and many point mutations and thus does not behave precisely like virus acquired directly from patients.

HCMV Functional Profiling

Not long after the advent of the BAC, several groups set out to create a complete library of ORF/gene mutations in HCMV and screen these mutants for their growth in fibroblasts. The functions of many of the 166 HCMV ORFs remains unknown, and thus these mutant libraries represented a significant step in the ability to understand the biology of this virus at the molecular level. Complete mutant libraries were created in both the Towne and AD169 strains (17, 143). These studies identified whether each gene of these HCMV strains was essential (no growth of mutant virus), nonessential (mutant virus grows like wild-type virus), or augmenting (mutant virus has >10 fold growth defect) for growth on fibroblasts. The conclusions of these two studies largely agreed with each other; and occasional differences have been attributed to the location of the mutation (i.e. UL35, (54)) . In the AD169 strain, it was found that 41 genes were essential, 88 non-essential, and 27 augmenting for replication in fibroblasts (143). Some of these results have been confirmed in subsequent studies of individual viral genes. In the Towne strain the nonessential genes were further screened for growth in endothelial

and epithelial cells and a subset were found to be tropism factors for these cell types (17). However, the Towne strain does not grow well in these cell types. Therefore, it will be critical to confirm these results in clinical strains which grow more readily in epithelial and endothelial cells.

UL21a

The original annotation of the HCMV genome identified an open reading frame 3' of UL20 and 5' of UL21.5 as UL21 (11). Subsequently, Davison et. al. determined that the UL21 ORF had no homology in the closely related chimpanzee cytomegalovirus. Instead, gene UL21a, which encodes an alternative reading frame that starts 185 bp downstream of the start codon and stops 29 bp downstream of the stop codon of the putative UL21 ORF, was highly conserved in CCMV and therefore added to the annotation of the HCMV genome (15). UL21a is completely conserved among all sequenced isolates of HCMV, including AD169, Towne, Fix, TR, and Merlin (15-17, 67, 68). UL21a appears to be specific to primate CMVs, as homologs can be found in chimpanzee and rhesus CMVs, but not in the more distantly related MCMV (93). Despite this, no experimental evidence exists to suggest HCMV expresses UL21, UL21a, or both.

UL21a is predicted to encode for a 123 amino acid protein, ~15 kDa in mass. It shares no apparent homology with any viral or cellular proteins in the database, and thus its function is likely unique to CMV biology. Both large-scale mutagenesis screenings classified the previously uncharacterized UL21a locus as augmenting for viral replication in fibroblasts. In AD169, two independent mutations resulted in viruses with a ~50 fold

growth defect (143). In the Towne strain, a complete deletion of the entire UL21 ORF (encompassing most of UL21a) had a ~20,000 fold defect (17). It is likely that several factors, most notably the strain of virus, is responsible for the discrepancy in these two reports. Nonetheless, both studies suggest an important role for the UL21a locus in the lifecycle of HCMV. Further experimental evidence will be required to confirm the existence of the UL21a protein, its role in the viral lifecycle, and its function(s).

Anaphase Promoting Complex

Cellular proliferation and cell cycle progression depends on the specific protein degradation of critical regulators. One class of critical regulators of the cell cycle, cyclins (activating subunit of cyclin-dependent kinases), were found to be regulated by protein degradation. The protein complex required for its degradation was identified in the mid-90's and termed the anaphase-promoting complex/cyclosome, or APC (reviewed in (129)). Without the APC, cells cannot separate sister chromatids during anaphase, exit mitosis, or properly enter S phase.

Many viruses modulate the cell cycle to their advantage, in order to provide the proper resources for the virus to replicate their own genomes (32). As viral genomes are limited in their protein coding capacity, viruses need to target cellular processes where they can exert a maximal effect with relatively few activities. In fact, the study of viral proteins led to the identification of p53 and Rb as critical proteins in the control of the cell cycle. As the APC is another critical node in cell cycle regulation, it is not surprising that in the past 10 years several viral proteins have been identified as regulators of this

complex. Here we will review what is known about the viral proteins that regulate the APC and discuss the questions which need to be addressed in future research.

Background

The anaphase promoting complex (APC) is a large, multi-subunit E3 ubiquitin ligase which targets multiple proteins for ubiquitin-dependent proteasome degradation (reviewed in (77)). E3 ubiquitin ligases are a large class of proteins which catalyze the final step in the ubiquitin transfer reaction, which is the transfer of an ubiquitin moiety from an E2 ubiquitin ligase to a target protein. They serve as a platform for the binding of an E2-ubiquitin and the target protein to facilitate the ability of a lysine residue of the target protein to attack the thioester bond that links the carboxyl terminus of ubiquitin to the active-site cysteine of the E2. Most substrates of the APC are involved in cell cycle regulation, making the APC an essential regulator of the cell cycle. The APC performs two important functions during the cell cycle, it promotes the separation of sister chromatids during anaphase and then functions to prevent a premature entry into S phase when cellular DNA is duplicated (77). As the APC is essential for cell cycle progression, it has become a novel target for anti-cancer therapeutics (147).

The human APC is a 1-1.5 MDa complex, containing 11 subunits along with two co-activators, which function at different stages of the cell cycle. The APC is a cullin-RING E3 ubiquitin ligase, and APC2 and APC11 contain the cullin and RING domains, respectively (123, 135). Another protein in this sub-complex is Doc1 (APC10) which is thought to help bind substrates and bring substrate specificity to the complex (9, 10). On the opposite side of the complex is another subcomplex, which contains several proteins

that carry multiple copies of the tetratricopeptide repeat (TPR) which are thought to facilitate protein-protein interactions and help facilitate substrate binding and specificity (APC3/6/7/8) (128). These two subcomplexes are held together by a bridge composed of APC1/4/5. It is unknown why the APC is so large and is composed of so many subunits, although it is likely that its size gives it large flexibility in its ability to target multiple proteins for ubiquitin-conjugation.

The APC contains two separate co-activator proteins which function at different times during the cell cycle. Cdc20 is the co-activator for the APC from metaphase through anaphase while Cdh1 takes over at the end of mitosis and is active up until S phase. Having two separate activator proteins allows the complex to have distinct functions at different stages of the cell cycle. It is unknown how the activator proteins function, but it is thought to provide binding interactions with the substrates (48).

The interaction of Cdc20 and Cdh1 with the APC is highly regulated, and this regulation allows them to function at different points in the cell cycle (47, 49, 96, 105, 134, 146). Cdc20 can only associate with the APC when it has been phosphorylated by several mitotic kinases. In contrast, Cdh1 is inactivated by phosphorylation, and thus does not become active until cyclin-dependent kinase (CDK) activity has been diminished late in mitosis. The Cdc20 complex (APC^{Cdc20}) is also regulated by the spindle-assembly checkpoint (SAC), which prevents the APC from initiating anaphase until the spindle poles align (38). The APC can also be negatively regulated by the E2F responsive protein early mitotic inhibitor-1 (Emi1) (35). Finally, there is a strong feedback mechanism within the APC, as the APC^{Cdh1} complex is capable of targeting Cdc20 and Cdh1, as well as its own E2 enzyme, UbcH10, for proteasome-dependent

degradation (51, 83, 85, 103, 142). The loss of CDK activity as well as targeted degradation of Cdc20 by APC^{Cdh1} are thought to allow the transition from APC^{Cdc20} to APC^{Cdh1} during the late stages of mitosis, while Cdh1 and UbcH10 degradation as well as Emi1 induction and increasing CDK activity are thought to diminish APC^{Cdh1} activity and restore the APC to its inactive form during late G1/S phase.

The APC targets at least 30 proteins for polyubiquitination and subsequent proteasomal degradation. There are at least 2 well-known recognition motifs which target a protein to become ubiquitinated by the APC, although several others have recently been discovered (2, 40, 52). The primary motifs recognized by the APC are the D-box (RXXLXXXXN/D/E) and the KEN domain (20, 80). Some of the more well-known substrates include the A and B – type cyclins and securin. The degradation of the cyclins during mitosis reduces CDK activity which allows for disassembly of the mitotic spindle, chromosome decondensation, reformation of the nuclear envelope, and the formation of a cytokinetic groove (44, 70, 121). Securin is a mitotic regulator that binds and inhibits separase, an enzyme which cleaves sister-chromatids, allowing for their separation. Degradation of securin by APC^{Cdc20} activates separase and allows anaphase to initiate (reviewed in (71)). In addition, the APC has many targets involved in a variety of functions including but not limited to: mitotic kinases (Aurora kinase A and B, polo-like kinase (PLK-1)), proteins involved in the pre-replication complex (Cdc6 and geminin), nucleotide biosynthesis (thymidine kinase (TK), ribonucleotide reductase (RRM2), and deoxythymidylate kinase), glycolysis (6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase isoform 3 (PFKFB3)), and glutaminolysis (glutaminase 1) (56). However, the role of APC degradation of many of these proteins in cell-cycle regulation

has not been formally determined. In addition, not all substrates of the APC are degraded with equal kinetics. Some are ubiquitinated rapidly, while others take much longer to obtain a large enough number of ubiquitin modifications to be targeted to the proteasome (86). Furthermore, APC targeting of some substrates (i.e. CDC6) is prevented by post-translational modifications (113).

Viral Modulation of the APC

As several viruses, especially DNA viruses, have been known to manipulate the cell cycle, it is not surprising that several viral proteins have been shown to modulate the function of the APC. Those viral proteins that have been shown to modulate the APC include: Adenovirus E4orf4, Human T-lymphotropic virus (HTLV) tax, Human Papillomavirus (HPV) E2, Chicken Anemia Virus (CAV) apoptin, Orf virus PACR (poxvirus APC/cyclosome regulator), HCMV pUL97, and an additional undefined HCMV protein.

Adenovirus E4orf4. E4orf4 has been shown to exert a G2/M phase block in the cell cycle and causes apoptosis in overexpression. These functions are dependent on its interaction with protein phosphatase 2A (PP2A) (reviewed in (94)). It has been published that E4orf4 can both inhibit and activate the APC through its interaction with PP2A (45, 66). This contradictory data may be explained by the fact that PP2A may be able to activate one form of the complex while inhibiting the other, as the APC^{Cdc20} complex is activated by phosphorylation and the APC^{Cdh1} complex is inhibited by it. It was shown that E4orf4 can bind to the APC but both the binding site and the mechanism it may use to regulate the APC remains unknown. While these studies have shown altered

regulation of APC substrates in yeast expressing E4orf4, neither unequivocally demonstrated that this was due to increased or decreased APC activity, nor did they compare the activity of the APC in E4orf4 cells using an *in vitro* assay. E4orf4 has not been shown to regulate the APC in mammalian cells or during virus infection. Furthermore, E4orf4 is non-essential for growth of Adenovirus in human cancer or primary cells in tissue culture, so it is unclear how the regulation of the APC by E4orf4 may impact viral replication (60). Thus there are still many unanswered questions about the ability of E4orf4 to regulate the APC.

HTLV Tax. HTLV is the etiologic agent of T cell leukemia/lymphoma and many pathological findings have shown that mitotic aberrations accompany HTLV-1 viral replication and may promote cancer development. In fact, HTLV-1 transformed T cells are delayed in their progression through the $S/G_2/M$ phases of the cell cycle. Liu et. al. showed that this delay correlated with an increase in several APC substrates, and they further showed that the APC^{Cdc20} is prematurely activated in Tax-expressing cells (53). Tax bound to the APC^{Cdc20}, but its mechanism of action remains unknown. Tax-expressing cells display mitotic aberrations which may be due to increased APC activity; however this has not been experimentally proven and may be difficult to show as Tax has several other functions. A Tax mutant which maintains all functions except its ability to activate the APC will be critical to analyzing the contribution of APC activation to mitotic aberrations induced by Tax. It would also help define the contribution of this function in virus induced tumor formation as this area has not yet been explored.

HPV E2. Human Papillomaviruses are small DNA viruses which cause cervical cancers. The different strains of HPV can be divided into a low-risk and a high risk

group for developing cervical cancer. Bellanger et. al. showed that HPV E2 proteins from high-risk but not the low-risk group were capable of inducing a mitotic block and subsequent apoptosis (6). Interestingly, those cells which overcame this block showed large genomic instability. They went on to show that the high-risk E2 proteins bound to the APC and promoted the accumulation of APC substrates. It is not known whether the ability to inhibit the APC by E2 leads to the genomic instability or whether it could be attributed to other functions of this protein. Also, E2 is required for viral DNA synthesis, and thus APC regulation may be important for the ability of the virus to replicate its genome (76). As with the Tax protein, a mutant E2 protein which maintains all functions except its ability to inhibit the APC will be critical for further study.

CAV apoptin. Like E4orf4, apoptin induces a strong G2/M arrest and apoptosis (126). Teodoro et. al. identified APC1 as a binding partner of apoptin using Co-IP-mass spectrometry, although it is not clear if this is its direct binding partner or if its binds another protein in this complex. As APC1 was the only protein they identified in their pull-down, it is likely that this may be the direct binding partner. This group further showed that apoptin expression caused an increase in APC substrates and showed dramatic dissociation of the APC in the presence of apoptin. They mapped the binding domain to the C-terminal 40 amino acids, which was also sufficient to induce apoptosis. This suggests that the ability of apoptin to induce apoptosis may be an effect of its ability to inhibit the APC. Finally they showed that the addition of APC1 siRNA's induces a G_2/M arrest and apoptosis in a manner very similar to apoptin. It will be of interest to identify the mechanism apoptin uses to dissociate the APC. Apoptin null CAV is defective in DNA synthesis, and a point mutant which synthesizes viral DNA normally

does not produce viral particles (81). It would of interest to determine if inhibiting the APC with siRNAs or a specific inhibitor could restore any level of DNA replication or particle formation of these mutants.

Orf virus PACR. Recently, Mo et. al. identified a poxviral protein with significant sequence homology to APC11 (61). The protein, which they named PACR (poxviral APC/C regulator) is only present in the parapoxvirus family. It binds APC2 in a manner that mimics APC11, but it lacks the catalytic residues, and thus it lacks any *in vitro* ubiquitin ligase activity. Interestingly, swapping the catalytic domains of APC11 and PACR also switched their ubiquitin ligase activity *in vitro*. PACR overexpression also exhibited a G2/M arrest with an increase in APC substrates, suggesting that PACR was able to inhibit the APC by mimicking the function of APC11. The deletion of PACR from the Orf virus genome results in a virus with a growth defect of nearly 2 logs. It is unclear whether this defect is due to its ability to regulate the APC or whether it may have additional functions which are responsible for this defect.

HCMV UL97. HCMV has been shown to modulate the function of the APC during infection (131, 132, 139). One of the mechanisms it uses to regulate the APC is to promote the phosphorylation of Cdh1. It was recently shown by Tran et. al. that the UL97 protein kinase was directly responsible for Cdh1 phosphorylation during infection. UL97 is a CDK mimic which phosphorylates Rb (36, 82), and thus it is not surprising that it phosphorylates Cdh1 as Cdh1 is a normal target of CDKs. In a UL97 deletion mutant virus, which does not cause phosphorylation of Cdh1 upon infection, APC substrate accumulation was delayed compared to wild-type virus. However, by 24-36 hpi, the accumulation of APC substrates was nearly identical between wild-type and

mutant virus, suggesting that HCMV must have other mechanisms to inhibit the APC (131). It is unknown whether the inhibition of the APC by UL97 plays a role in the ability of the virus to replicate or cause disease.

Undefined HCMV Protein. In addition to UL97, it is clear that HCMV contains at least one other factor which can regulate the APC. As shown by Tran et. al., in the absence of UL97 APC substrates still accumulate to nearly the same levels as in wild-type virus, and the complex dissociates as had been shown previously (131). They further investigated the levels of numerous APC subunits and found that APC4 and APC5 were degraded in a proteasome-dependent manner during infection. The degradation of APC4 and APC5 likely lead to the dissociation of the complex as the APC dissociation is prevented by proteasome inhibitors. Finally, it was shown that this phenotype was likely due to an early viral protein as the phenotype was seen by 6 hpi and viral tegument and IE protein expression were insufficient to target APC4 and APC5 for degradation. The identification of this factor and whether its function is critical for viral replication will be important questions to address in the future.

Why Target the APC?

What downstream effects of the APC may be important for viral replication? The APC both promotes mitosis using the Cdc20 activator and helps maintain cells in G1 using the Cdh1 activator. It is largely assumed that most of the viruses that target the APC target the Cdh1 complex to promote S phase and a proliferative state. In fact, HCMV only infects cells in G1 phase, it is therefore unlikely that it would target the mitotic version of the APC. However, this has not been experimentally verified. The

APC targets at least 30-40 proteins for ubiquitination and degradation, and thus inhibiting this complex would stabilize the levels of numerous proteins, any one of which may be important for viral replication and pathogenesis. Furthermore, viral proteins may be targets of the APC. It has been shown that the bovine papillomavirus replicative helicase E1 is targeted by the APC and several HCMV proteins contain the consensus D-box which is recognized by the APC (58, 131). However, none of these HCMV proteins have been shown to be regulated by proteasome-dependent proteolysis.

It is interesting to note that in both poxviruses and herpesviruses families, the only viruses which have been found to modulate the APC (Parapoxviruses and HCMV) are those that do not encode for their own thymidine kinase (TK) and ribonucleotide reductase (RR) enzymes (61, 139). Both these enzymes are critical for the production of deoxyribonucleotides. It seems plausible that APC regulation is one mechanism used by viruses to accumulate a substantial concentration of nucleotides so they can efficiently replicate their own genomes, but this has not been experimentally verified. It is also possible that viruses regulate the APC to enhance the accumulation of other substrate proteins such as geminin, cyclins A and B, PFKFB3, and glutaminase, to name a few. It will be intriguing to see if any APC substrates appear in future siRNA screens for proteins required for efficient replication of the viruses which regulate the APC.

In sum, multiple viral proteins, mostly from DNA viruses, have now been shown to modulate the function of the APC. The limitation to these studies is that, except for UL97, none of them have been shown to have this function during viral infection. Future studies should be focused on identifying the mechanisms these proteins use to regulate the APC, whether or not they perform these functions in the context of a viral infection,

and ultimately determine how the APC and its substrates may affect viral replication and pathogenesis. Further study of these proteins promises not only to shed light on mechanisms viruses use to promote replication, but will also be important to understand the biology of the APC. Research on the mechanisms used by viral proteins to disrupt the APC may lead to novel ideas for targeting the APC for anti-cancer therapy.

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Chapter II

Human Cytomegalovirus Gene UL21a Encodes a Short-lived Cytoplasmic Protein and Facilitates Virus Replication in Fibroblasts

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ABSTRACT

The human cytomegalovirus (HCMV) gene UL21a was recently annotated by its conservation in chimpanzee cytomegalovirus. Two large-scale mutagenic analyses showed that mutations in overlapping UL21a/UL21 resulted in a severe defect of virus growth in fibroblasts. Here we characterized UL21a and demonstrated its role in HCMV infection. We mapped a UL21a-specific transcript of ~600 bp that was expressed with early kinetics. UL21a encoded pUL21a, a protein of ~15 kDa, which was expressed and localized predominantly to the cytoplasm during HCMV infection or when expressed alone. Interestingly, pUL21a was drastically stabilized in the presence of proteasome inhibitor MG132, but its instability was independent of a functional ubiquitin-mediated pathway, suggesting that pUL21a underwent proteasome-dependent, ubiquitin-independent degradation. A UL21a-deletion virus was attenuated in primary human newborn foreskin fibroblasts (HFFs) and embryonic lung fibroblasts (MRC-5) while a marker-rescued virus and mutant viruses lacking the neighboring or overlapping genes UL20, UL21, or UL21.5-UL23 replicated at wild-type levels. The growth defect of UL21a-deficient virus in MRC-5 cells was more pronounced than that in HFFs. At high multiplicity of infection, the UL21a-deletion virus synthesized viral proteins with wild-type kinetics but had a 2-3 fold defect in viral DNA replication. More importantly, although pUL21a was not detected in the virion, progeny virions produced by the mutant virus were ~10 times less infectious than wild-type virus, suggesting that UL21a is required for HCMV to establish efficient productive infection. We conclude that UL21a encodes a short-lived cytoplasmic protein and facilitates HCMV replication in fibroblasts.

INTRODUCTION

Human cytomegalovirus (HCMV), the prototypic β-herpesviruses, is a ubiquitous pathogen that infects the majority of the world's population. HCMV is usually asymptomatic in immunocompetent individuals, except in rare cases where it causes mononucleosis. However, HCMV can cause severe disease and death in immunocompromised individuals such as AIDS patients and transplant recipients. Importantly, HCMV is the most common viral cause of birth defects leading to mental retardation, blindness, and hearing loss (5). In addition, HCMV infection is also a possible risk factor in the development of vascular diseases such as atherosclerosis, transplant vascular sclerosis, and coronary restenosis after angioplasty surgery (17, 21, 23, 26, 34, 35, 46).

HCMV contains a 240-kb double-stranded DNA genome that encodes at least 166 putative open reading frames (ORFs) and several miRNAs (8, 12, 13, 15, 18, 28, 29). With the advent of the infectious bacterial artificial chromosome (BAC) clone-based genetic system for HCMV (3, 44), the functions of many HCMV genes have started to be elucidated. Genome-scale mutagenesis approaches have been used to delineate the functions of genes encoded by HCMV (14, 43). These systematic studies have identified a subset of candidate viral genes that are important for HCMV to establish infection in tissue culture models of primary human cells including fibroblasts. Nonetheless, products of more than half of the annotated viral genes have not been experimentally identified and characterized (25).

Little is known about the gene products produced from the viral genomic region where UL21a resides. UL21.5 is the only gene within this region that has been characterized in detail. UL21.5 encodes a late transcript that is 400-500 bp in length, spliced, and incorporated into virions (4, 32) (Fig. 2.2A). The protein product of UL21.5, pUL21.5, is a soluble receptor decoy for CC chemokines, selectively binds to RANTES, and prevents binding with its cognate receptors (27, 38). UL23 is a member of the US22 gene family and encodes a tegument protein (1). In addition, HCMV also encodes a miRNA UL22A-1 with unknown targets that is expressed with early gene kinetics from a locus adjacent to UL21.5 (15, 18). However, no gene products emanating from UL21 or UL21a have been identified.

The original annotation of the HCMV genome recognized the putative gene UL21 that was predicted to encode the largest ORF between neighboring genes UL20 and UL21.5 (8). Subsequently, an elegant comparative analysis by Davison and coworkers showed that the UL21 ORF had no homologue in the closely related chimpanzee cytomegalovirus (CCMV) (12). Instead, gene UL21a, which encodes an alternative reading frame that starts 185 bp downstream of the start codon and stops 29 bp downstream of the stop codon of the putative UL21 ORF, was highly conserved in CCMV and therefore added to the annotation of the HCMV genome (12) (Fig. 2.1 and data not shown). Furthermore, UL21a is entirely conserved among all HCMV strains that have been sequenced, including AD169, Towne, Toledo, PH, Merlin, and TR (12-14, 28, 29). However, no experimental evidence has been documented as to whether HCMV expresses UL21a, UL21, or both.

UL21a appears specific to primate CMVs as its homologues are found in CCMV and Rhesus CMV (RhCMV) (33), but are not present in the more distantly related viruses such as murine CMV (MCMV) (data not shown). UL21a is predicted to encode a protein of 123 amino acids (aa) with the calculated molecular weight of 14.3 kDa. It shares no apparent homology with any known cellular or viral proteins in the database, suggesting that the function of this protein is likely to be unique to CMV biology. Two large-scale mutagenic analyses started to reveal the importance of this previously uncharacterized UL21a/UL21 locus in HCMV infection (14, 43). In the AD169 strain, two independent insertional mutations within UL21a resulted in a growth defect of ~50 fold in human foreskin fibroblasts (HFFs) (43). In the Towne strain, a substitution mutation targeting UL21 simultaneously deleted the overlapping UL21a, and a more severe growth defect of > 20,000 fold was observed with this mutant virus (14). It is possible that several factors, such as the nature of the mutations, the difference of virus strains, and the assays for growth analysis, are responsible for the different degrees of growth attenuation seen in mutant AD169 and Towne viruses. Nonetheless, these studies suggest an important role of the UL21a/UL21 locus for the virus to establish efficient infection.

In this study, we mapped the transcript arising from the UL21a locus and characterized its protein product, termed pUL21a. Analysis of recombinant HCMV virus lacking UL21a, UL21, or its neighboring genes indicates that UL21a, but not UL21 or other neighboring genes, is required for HCMV growth in primary human fibroblasts. Although we could not detect pUL21a in the virion, the UL21a-deletion virus produced progeny that are ~10 times less infectious than wild-type virus, suggesting that UL21a is required for HCMV to initiate efficient productive infection.

MATERIALS AND METHODS

Plasmids and antibodies. pYD-C235 is a pLPCX-derived retroviral vector (Clontech) that expresses a DsRed gene driven by an internal ribosome entry site 2 (IRES2) (36). pYD-C423, pYD-C428, and pYD-C429 were created by amplifying the UL21a, UL99 (encoding pp28), and UL44 coding sequences and cloning the PCR products upstream of the IRES2 of pYD-C235, respectively. pYD-C255 contained a *GalK*/*kanamycin* dual-expression cassette that was used for the first step of linear recombination (31) (see below).

To generate a polyclonal antibody to pUL21a, the entire UL21a coding sequence was cloned upstream of a six-His tag in the expression vector pET-22b (Novagen). The His-tagged fusion protein was produced in *Eschericia coli*, purified using Ni-NTA agarose beads (Qiagen), and used as an immunogen to generate the rabbit antisera (Covance). The rabbit antisera were subsequently affinity-purified using the UL21a recombinant protein (Covance), resulting in the high titer, highly specific anti-pUL21a polyclonal antibody that was tested for its specific interaction with the immunogen or the virus-produced pUL21a by immunoblot assay. Additional primary antibodies used in this study included: anti-β-actin (clone AC15; Abcam); anti-p53 (clone OP-03, Calbiochem); anti-UL44 (clone 10D8; Virusys); anti-MCP (major capsid protein) (generous gift from Wade Gibson, John Hopkins University); anti-IE1, anti-pp65, anti-pUL69, and anti-pp28 (36) (generous gifts from Thomas Shenk, Princeton University).

Cells and viruses. Primary human newborn foreskin fibroblasts (HFFs), embryonic lung fibroblasts (MRC-5), Hela cells, and mouse ts20 cells (10) (a generous gift from Harvey

Ozer, UMDNJ-New Jersey Medical School) were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, non-essential amino acids, and penicillin/streptomycin. For transient expression, Hela cells were transfected using lipofectamine (Invitrogen) and HFFs were electroporated using Amaxa technologies (Lonza) with plasmids expressing the proteins of interest according to the manufacturers' instructions.

Various BAC-HCMV clones were constructed to reconstitute recombinant HCMV viruses. The BAC-HCMV clone pAD-GFP was used as the parental clone to produce wild-type virus (AD*wt*). pAD-GFP carries the full-length genome of HCMV strain AD169 and contains a simian virus 40 (SV40) early promoter-driven GFP gene in place of the viral US4-US6 region (36, 44). All other recombinant BAC clones were constructed using a linear recombination protocol in the bacterial strain SW102 (31). A *GalK/kanamycin* dual marker cassette was amplified by PCR from pYD-C255 with a pair of 70-bp primers that had 5'-terminal 50-bp sequences homologous to the viral sequences of targeted sites (See Table 2.1 for all primers). The marker cassette was subsequently recombined into pAD-GFP at the locus of interest by linear recombination to generate substitution or insertional mutant BAC-HCMV clones. Resulting transformants were selected on kanamycin-containing LB plates to identify clones carrying the marker cassette. One of the substitution mutant BAC-HCMV clones, pAD*sub*UL21a, carried the marker cassette in place of UL21a. To create a UL21a marker rescue BAC-HCMV clone (pAD*rev*UL21a), the UL21a sequence was amplified by PCR and subsequently recombined into pAD*sub*UL21a to replace the marker cassette by linear recombination. The resulting recombinants were selected on 2-deoxy-galactose (DOG)-containing

minimal media plates for the loss of *GalK/kanamycin*. To create a UL21a clean-deletion BAC-HCMV clone (pAD*dl*UL21a), a pair of overlapping 70-bp primers were generated that together spanned 50 bps both upstream and downstream of the UL21a ORF. The PCR fragment generated from these primers was recombined into pAD*sub*UL21a to replace the marker cassette by linear recombination. To insert the GFP-S tag at the N-terminus of the UL21a ORF in the viral genome, the *GalK/kanamycin* marker was amplified by PCR and inserted immediately downstream of the UL21a start codon by linear recombination in pAD/Cre, a BAC clone carrying the full-length AD169 genome without GFP, resulting in the clone AD*in*GalK/kan-UL21a. The GFP-S tag sequence was amplified from the plasmid pIC113 (9) and recombined into the viral genome of AD*in*GalK/kan-UL21a, in frame at the N terminus of the UL21a ORF, to replace the marker sequence by linear recombination, resulting in AD*in*GFP-UL21a. All recombinant BACs were verified by restriction digestion, Southern blot, PCR, and direct sequencing analysis.

To reconstitute virus, 2-4 µg of the BAC-HCMV DNA and 1 µg of the pp71-expression plasmid were transfected into MRC-5 fibroblasts by electroporation (44). Culture medium was changed 24 hours later, and virus was harvested by collecting cell-free culture supernatant when the entire monolayer of infected cells was lysed. Alternatively, virus was also produced by collecting cell-free culture supernatant from HFFs infected at a multiplicity of infection (MOI) of 0.005. Virus-containing culture supernatants were then purified by ultra-centrifugation through 20% D-sorbitol cushion at an average rcf of 53,000 x g for 1 hr, resuspended in DMEM with 10% fetal calf serum, and saved as viral stocks. HCMV virus titers were determined in duplicate by plaque

assay (31), and their DNA content was determined by realtime-quantitative PCR (see below).

Analysis of viral growth kinetics. HFFs or MRC-5 cells were seeded in 12-well plates overnight to produce a subconfluent monolayer. Cells were then inoculated with recombinant HCMV viruses for 1 hr at an MOI of 0.001 for multi-step growth analysis or 1 for single-step growth analysis. Inoculum was removed, infected monolayers were rinsed with PBS, and fresh medium was replenished. At various times postinfection, cell-free virus was collected by harvesting medium from infected cultures. In addition, infected cells were pelleted, lysed by a freeze-thaw cycle followed by sonication, cell debris was cleared by low-speed centrifugation, and supernatants were saved as cell-associated virus samples. Titers of each virus sample were determined by plaque assay. Moreover, plaque size was measured using Image J software (NIH), and the *P* value associated with Student's paired *t*-test with a two-tailed distribution was calculated by scoring at least 50 plaques per infection.

Analysis of DNA, RNA, and proteins. To prepare virion DNA for quantification by realtime-quantitative PCR, 100 µl of cell-free virus (or 10µl of purified virus) was treated with DNase I (30 U, Roche) at 37°C for 30 min to remove contaminating DNA, followed by incubation at 75°C for 20 min to stop the reaction. The samples were then incubated overnight at 55°C in lysis buffer (400 mM NaCl, 10 mM Tris [pH 8.0], 10 mM EDTA, 0.1 mg/ml proteinase K, 0.2% sodium dodecyl sulfate [SDS]), and DNA was extracted with phenol-chloroform and treated with 20 µg/ml RNase A for 1 hr at 37°C. DNA was extracted again with phenol-chloroform,

precipitated with ethanol, and re-suspended in nuclease-free water (Ambion). To prepare intra-cellular DNA from infected cells for quantification, HCMV-infected HFFs were collected at various times postinfection, re-suspended in lysis buffer, and incubated at 55°C overnight. DNA was extracted with phenol-chloroform, treated with 100 µg/ml RNase A for 1 h at 37°C, extracted again with phenol-chloroform, precipitated with ethanol, and re-suspended in nuclease-free water.

Viral DNA was quantified by realtime-quantitative PCR as previously described (31) using a TaqMan probe (Applied Biosystems) and primers specific for the HCMV UL54 gene (30). Cellular DNA was quantified with SYBR Green PCR Master Mix (Clonetech) and a primer pair specific for the human β-actin gene (5'-CTC CAT CCT GGC CTC GCT GT-3' and 5'-GCT GTC ACC TTC ACC GTT CC-3'). The accumulation of viral DNA was normalized by dividing UL54 gene equivalents by β-actin equivalents. The accumulation of wild-type viral DNA at 2 hours postinfection (2 hpi) was arbitrarily set as 1.

mRNA transcripts expressed during HCMV infection were analyzed by Northern blotting and Rapid Amplification of cDNA Ends (RACE) as previously described (31, 44). For Northern blot analysis, single-stranded DNA probes were prepared by using PCR-generated templates and a Strip-EZ PCR kit (Ambion) according to the manufacturer's instructions. Primer pairs used to generate the templates were as follows: 5'-ATG GGA GGT AGC CCT GTT CC-3' and 5'-TTA AAA CTG GTC CCA ATG TTC TT-3' (for the UL21a probe); 5'-GTA GCC TAC ACT TTG GCC ACC-3' and 5'-TTA CTG GTC AGC CTT GCT TCT A-3' (for the UL123 probe); 5'-GCG CGC CAG

TAC TTT AAC ACA G-3' and 5'-TCA CGA GTT AAA TAA CAT GGA TTG-3' (for the UL86 probe). Primers used to generate the ^{32}P-labeled strand-specific anti-sense probes were as follows: 5'-TCGTCCAGCAGTAGCACCAGCGGATTGG-3' (for the UL21a probe), 5'-TTA CTG GTC AGC CTT GCT TCT A-3' (for the UL123 probe), and 5'-TCA CGA GTT AAA TAA CAT GGA TTG-3' (for the UL86 probe). RACE analysis was performed by using SMART^{TM} PCR cDNA Synthesis Kit (Clontech). Gene-specific primers 5'-TCG TCC AGC AGT AGC ACC AGC GGA TTG G-3' and 5'-ATC TGT GCG CAT GGA CTT TCG GGC TCG C-3' were used for 5' and 3' RACE, respectively (Fig. 1A).

To analyze protein accumulation by immunoblotting, total cell extracts were prepared by lysing PBS-washed infected cells in sodium dodecyl sulfate (SDS)-containing sample buffer. Virion proteins were prepared by purifying virions by ultra-centrifugation through sorbitol cushion and re-suspending in SDS-containing sample buffer. Proteins were resolved by electrophoresis on a SDS-containing polyacrylamide gel, transferred to PVDF membranes, hybridized with primary antibodies, reacted with HRP-conjugated secondary antibodies, and visualized by SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) (42).

To analyze intra-cellular protein localization by immunofluorescence, cells grown on glass coverslips were fixed in 2% paraformaldehyde, permeabilized with 0.1% Triton X-100 for 5 min, incubated with the primary antibody, and subsequently labeled with the secondary antibody. Labeled cells were counterstained with TO-PRO-3 (Molecular Probes) to visualize the nuclei and then mounted on slides with Prolong Gold antifade

reagent (Molecular Probes). Images were captured using Zeiss LSM Image software with a Zeiss LSM 510 META confocal laser scanning microscope (31).

Proteasome inhibition assays. Protease inhibitors Z-Leu-Leu-Leu-CHO (MG132), N-acetyl-Leu-Leu-Met (ALLM), and N-acetyl-Leu-Leu-Nle-CHO (ALLN) (Calbiochem) were resuspended in DMSO (Sigma) at the concentration of 10mM as stock solutions. In over-expression experiments, protease inhibitors were added to culture medium 48 hours after Hela cells were transfected with expression plasmids, and cell lysates were collected 24 hours later for immunoblot analysis. In HCMV infection experiments, MG132 was added to culture medium of infected HFFs 10 hours before cell lysates were collected.

RESULTS

UL21a encodes a single transcript with early gene expression kinetics.

No products have been previously identified from the UL21/UL21a gene locus. As UL21a is highly conserved at the amino acid level among primate CMVs we hypothesized that this gene encoded a protein product.

We carried out Northern blot analysis to assess potential transcripts arising from the UL21a gene locus during HCMV infection (Fig. 2.1B). Using a UL21a-specific anti-sense probe, we detected a single specific transcript of ~600 bp during wild-type virus infection. The accumulation of this transcript was evident as early as 8 hours postinfection (hpi) and became more abundant by 24 hpi. The transcript was absent in infection of AD*sub*UL21a, the recombinant virus in which the entire UL21a putative coding sequence was replaced by a marker sequence (Fig. 2.5A and data not shown),

indicating its UL21a-specific origin. The expression kinetics of the transcript was determined by inhibition assays for protein translation and DNA replication. Cycloheximide (CHX), a protein synthesis inhibitor, increased the accumulation of the immediate-early transcript of UL123 whereas it significantly reduced the accumulation of the UL21a transcript at 8 and 24 hpi, ruling out UL21a as an immediate-early gene. Phosphonoacetic acid (PAA), a DNA synthesis inhibitor, blocked the accumulation of the 8-kb transcript of the late gene UL86 (7) but did not decrease production of the UL21a transcript at 48 hpi, ruling out UL21a as a late or leaky late gene. Together with its temporal expression pattern, these results indicate that UL21a is transcribed with early gene kinetics.

To map the ends of the UL21a transcript, we performed both 5'- and 3'- RACE analysis of HCMV-infected HFFs using two UL21a-specific primers that would produce overlapping RACE products (Fig. 2.1A). Both RACE reactions produced a single band, providing additional evidence that UL21a encodes a single transcript (Fig. 2.21C). Sequence analysis of RACE products indicated that the UL21a transcript was unspliced and initiated 23 bp upstream of the start codon. All of the three 3'-RACE products terminated at different locations within 30-75 bp downstream of the stop codon, suggesting that the transcript can be polyadenylated at multiple sites within this region (Fig. 1A). Notably, 5'-RACE analysis did not detect a product that could encode the N-terminal portion of the putative UL21 ORF (Fig. 2.1A), even after extending the reaction for an additional 10 cycles (data not shown). Therefore, we mapped the UL21a transcript but found no evidence for the presence of a UL21-specific transcript.

pUL21a is expressed but targeted for degradation in a proteasome-dependent manner during HCMV infection.

To directly detect the protein products encoded by UL21a, we created a rabbit polyclonal antibody against a recombinant peptide derived from the UL21a coding sequence. The anti-sera were affinity-purified with the UL21a recombinant peptide to generate a specific polyclonal antibody. The antibody was sensitive as it could detect the recombinant protein at the level of 10^{-8} nanomoles when used in immunoblot analysis (data not shown). Importantly, this antibody detected a single protein, termed pUL21a, with an apparent molecular weight of ~15 kDa in the lysates of cells infected with wild-type virus but not in mock-infected or cells infected with UL21a-deletion virus (Fig. 2.2A). Therefore, pUL21a was encoded by UL21a, was expressed at the predicted size, and was lost in the deletion mutant virus. The accumulation of pUL21a became visible 10 hpi, peaked at 24-48 hpi, and then gradually decreased. Despite the strong sensitivity of the antibody, the signal detected in infected cell lysates was weak, even at 24 hpi, suggesting low levels of pUL21a accumulated during HCMV infection.

We were intrigued by the apparent low levels of pUL21a because the accumulation of the UL21a transcript appeared abundant during infection (Fig. 2.1B), and thus hypothesized that pUL21a was targeted for degradation. We tested this hypothesis by expressing pUL21a in the presence or absence of protease inhibitors. MG132 is a specific inhibitor of the proteasome, ALLN is a calpain inhibitor that also acts as a weak inhibitor of the proteasome, and ALLM is a calpain inhibitor that does not inhibit the proteasome at the concentrations used in this study (22). When proteins were expressed from the HCMV IE promoter in Hela cells in a transient expression

experiment, the proteasome inhibitor MG132 drastically enhanced the accumulation of pUL21a whereas the calpain inhibitor ALLM had a minimal effect (Fig. 2.2B). ALLN increased the accumulation of pUL21a to some extent, consistent with its weak inhibitory activity to the proteasome. As a control, all three inhibitors had a minimal effect on the accumulation of another HCMV protein, pp28. The degradation of pUL21a could be markedly inhibited by MG132 at a concentration as low as 0.5 μM (Fig. 2.2C). At the concentration of 2.5 μM, treatment with MG132 for as little as 5 hours had a clear effect in the rescue of pUL21a accumulation (Fig. 2.2C). Interestingly, when pUL21a accumulation was stabilized, a second, faster-migrating protein band was also detected by the α-pUL21a antibody, suggesting that an additional form of pUL21a might be produced. These results indicate that the proteasome-mediated degradation of pUL21a is independent of other viral factors. Furthermore, such degradation appears to be pUL21a-specific as the viral protein pp28 was stable independent of proteasome inhibition.

We then tested whether pUL21a was also subject to rapid degradation in the context of viral infection. HFFs were infected with AD*wt* or AD*sub*UL21a, treated with MG132 or DMSO control at 38 hpi, and analyzed for the accumulation of viral proteins at 48 hpi by immunoblot analysis (Fig. 2.2D). Again, while MG132 had a marginal effect on the accumulation of HCMV proteins IE1 and pUL44, it drastically increased the accumulation of pUL21a during HCMV infection. Therefore pUL21a is targeted for proteasome-dependent degradation during HCMV infection.

Degradation of pUL21a is unaltered in cells lacking a functional ubiquitin-conjugation system.

The most common mechanism by which proteins are targeted to the 26S proteasome is via polyubiquitination at internal lysine residues, and in some cases, ubiquitination can occur at cysteine, serine, and threonine residues (6, 40), or at the N terminus of the protein (2, 11). The UL21a ORF contains no lysines or cysteines, and furthermore, we could not detect pUL21a-ubiquitin conjugates in cells treated with MG132 (data not shown). We hypothesized that pUL21a degradation was ubiquitin-independent, a mechanism that has been recently reported for a limited number of proteins (24, 45). To test this hypothesis, we transiently transfected pUL21a-expressing vector into mouse ts20 cells that carried a temperature-sensitive E1 ubiquitin-activating enzyme (10), and examined the accumulation of pUL21a at both permissive (35 ^{o}C) and restrictive (39^{o}C) temperatures. In these cells, the ubiquitin pathway was disabled at the restrictive temperature, thus stabilizing proteins, such as p53, that were targeted for the ubiquitin-mediated degradation (10). In this experiment, the ubiquitin pathway was active at 35°C, reducing p53 to the undetectable level, and as anticipated, MG132 treatment stabilized p53 (Fig. 2.3). At 39^{o}C, p53 was stabilized and MG132 treatment had no effect on its accumulation, indicating that the ubiquitin-conjugation pathway was indeed disabled at this restrictive temperature. In contrast, the pUL21a level was equally low at both temperatures but the protein level was greatly enhanced by MG132, suggesting that the degradation of pUL21a is proteasome-dependent and ubiquitin-independent.

pUL21a specifically localizes to the cytoplasm.

Next we sought to determine the intra-cellular localization of pUL21a by indirect immunofluorescence. To determine the localization pattern of pUL21a in the absence of

any other viral proteins, Hela or HFF cells were transfected with the plasmid expressing both a CMV IE promoter-driven viral protein (i.e., pUL21a, pp28, or pUL44) and IRES-driven dsRed. The over-expressed pUL21a was detected throughout the cytoplasm by the α-pUL21a antibody but was almost completely excluded from the nucleus of dsRed-positive cells (Fig. 2.4A and data not shown). The antibody was specific to pUL21a as it did not cross-react with other over-expressed viral proteins (i.e., pp28 and pUL44). We then analyzed the localization of pUL21a during virus infection. Unfortunately, the low level of pUL21a accumulation during infection relative to that in over-expression (data not shown) and perhaps also the low sensitivity of α-pUL21a antibody to the non-denatured protein prevented us from detecting the native pUL21a during infection by immunofluorescence analysis, even in the presence of MG132 (Fig. 2.4B and data not shown). To circumvent this issue, we created a recombinant HCMV virus, AD*in*GFP-UL21a, which accumulated a functional, GFP-S tagged variant of pUL21a at much higher levels than the native protein during infection by an unknown mechanism (Fig. 2.6B). Similar to what was observed in over-expression experiments, the tagged UL21a protein was detected predominantly in the cytoplasm by the α-pUL21a antibody during the entire course of AD*in*GFP-UL21a infection (Fig. 2.4B). In a few infected and transfected cells, some pUL21a-specific staining was also observed in the nucleus (data not shown). pUL21a is only 123-aa long with the apparent molecular weight of ~15 kDa, a size that would allow it to pass through nuclear pores freely. The low level of pUL21a staining in the nucleus of these cells might represent the background level of passive diffusion of the protein through the nuclear pores. Nonetheless, even in these cells, the distribution of pUL21a in the cytoplasm was much more abundant than that in the

nucleus. In both over-expression and infection experiments, pUL21a distribution appeared to be diffuse with no apparent co-localization with cytoplasmic organelles.

UL21a facilitates HCMV replication in fibroblasts.

Previously, two genome-wide mutagenic analyses tentatively classified overlapping UL21a/UL21 as augmenting genes because mutations in this locus resulted in attenuated growth of HCMV in fibroblasts (14, 43). As the transcript and the protein product arising from UL21a have now been identified, we created mutant viruses where UL21a, UL21, and its neighboring genes were individually deleted, and determined whether the UL21a gene was directly required for growth of the virus.

We constructed three deletion mutant BAC-HCMV clones (pAD*sub*UL20, pAD*sub*UL21a, and pAD*sub*UL21.5-UL23) using the linear recombination-based BAC mutagenesis approach (Fig. 2.5A) (see Materials and Methods). Each of these clones was derived from the parental BAC clone (pAD-GFP) that carries the genome of the HCMV AD169 strain, expresses an SV40 early promoter-driven GFP gene, and reconstitutes a wild-type control virus AD*wt* (31, 44). The mutant BAC clones were used to generate mutant viruses, namely AD*sub* UL20, AD*sub*UL21a, and AD*sub*UL21.5-UL23, respectively, upon transfection into human fibroblasts. In pAD*sub*UL21a or pAD*sub*UL20, the entire coding sequence of UL21a or UL20 was replaced by a *GalK/kanamycin* marker cassette. In pAD*sub*UL21.5-UL23, the viral genomic sequence encoding the C-terminal half of the UL21.5 ORF (i.e., aa 64-103), the miRNA UL22A-1, and the C-terminal half of the UL23 ORF (i.e., aa 121-284) was replaced by the marker cassette. In addition, the UL21a gene in AD*sub*UL21a was subsequently repaired,

producing the marker-rescued BAC clone, pAD*rev*UL21a, that was used to generate the marker-rescued virus, AD*rev*UL21a (Fig. 2.5A). All recombinant BAC clones were examined for integrity and desired alterations using EcoR I endonuclease digestion, Southern blotting, PCR, and direct sequencing analysis (data not shown). All other recombinant BAC clones used in this study were examined with the same level of analysis and were shown to carry the intact viral genome and contain the precise modification at the correct locus.

We then tested the roles of neighboring genes UL20, UL21a/UL21, and UL21.5-UL23, in HCMV growth in HFFs. The first indication of the importance of UL21a in HCMV growth was the observation that the UL21a-deletion virus AD*sub*UL21a generated much smaller plaques and produced progeny virus with titers about 30 fold lower than those by wild-type virus (data not shown). On average, the size of plaque produced by AD*sub*UL21a was no more than 40% of those produced by AD*wt* at 12 days postinfection (dpi) (Fig. 2.5B). A multi-step growth analysis demonstrated that two independent isolates of UL21a-deletion virus were attenuated in growth, producing ~30 fold less infectious virus than wild-type virus by 16 dpi (Fig. 2.5C). The marker-rescued virus AD*rev*UL21a replicated indistinguishably from wild-type virus. Furthermore, AD*dl*UL21a, a mutant virus in which UL21a was deleted without the insertion of *GalK/kanamycin* marker sequence, replicated indistinguishably from AD*sub*UL21a virus (data not shown). In contrast, mutant viruses lacking neighboring genes UL20 (AD*sub*UL20) or UL21.5-UL23 (AD*sub*UL21.5-UL23) replicated at wild-type levels, consistent with previous reports (Fig. 2.5C) (14, 38, 43). These results indicated that UL21a-deletion virus is markedly attenuated in growth. This defect is the direct result of

loss of UL21a rather than aberrant expression of neighboring genes resulting from the deletion of UL21a or inadvertent second-site mutations generated during mutagenesis.

Under single-step growth condition, the UL21a-deletion virus was also attenuated, producing 25 fold less cell-free infectious virus than the wild-type virus at 4 dpi (Fig. 2.5C). This attenuation was not the result of a defect in the release of infectious virus from host cells because the accumulation of cell-associated infectious virus was similarly reduced in UL21a-deletion virus (Fig. 2.5C).

Intriguingly, we found that the growth defect of UL21a-deletion virus was even more pronounced in MRC-5 human embryonic lung fibroblasts (Fig. 2.5D). As anticipated, AD*sub*UL20 and AD*sub*UL21.5-UL23 replicated indistinguishably from AD*rev*UL21a in MRC-5 fibroblasts. In contrast, at an MOI of 0.001, AD*sub*UL21a grew at a much reduced rate with delayed kinetics. It produced > 2,500 fold less infectious virus than marker-rescued virus AD*rev*UL21a at 15 dpi, the time when AD*rev*UL21a reached its peak yield. The growth of AD*sub*UL21a did not reach its peak until 20 dpi, producing a titer that was still > 60 fold lower than the peak yield of AD*rev*UL21a (Fig. 2.5D). Thus, the growth of UL21a-deletion virus appeared to be differentially attenuated in HFFs and MRC-5 fibroblasts, which to our knowledge has not previously been reported for any other HCMV mutant viruses.

As UL21a overlaps with the originally annotated putative gene UL21 (8), it was formally possible that the growth defect of pUL21a-deletion virus was the result of the inadvertent deletion of UL21. However, UL21 might not represent a real viral gene because the putative ORF that it encoded was not conserved among primate CMVs, and

because our analysis found no evidence for the presence of UL21-specific transcripts (Fig. 2.1). In order to exclude any possible involvement of UL21 in the phenotype of UL21a-deletion virus, we took advantage of the recombinant HCMV virus, AD*in*GFP-UL21a, in which the insertion of GFP fortuitously disrupted the putative UL21 ORF in a manner similar to AD*sub*UL21a (Fig. 2.6A). Specifically, both viruses would alter the reading frame at amino acid 63 of the UL21 ORF and introduce a stop codon shortly thereafter. Importantly, AD*in*GFP-UL21a replicated at wild-type levels despite the expected disruption of UL21 in this virus (Fig. 2.6C). Collectively, our data provides experimental evidence that the loss of UL21a, not UL21, is directly responsible for the growth defect of AD*sub*UL21a.

UL21-deletion virus produces progeny with reduced infectivity.

To determine the step of the viral replication cycle that is compromised in the absence of UL21a, we first analyzed viral gene expression and DNA replication in the absence of UL21a. At an MOI of 1, UL21a-deletion virus produced representative viral proteins at near wild-type levels (Fig. 2.7A). The mutant virus had a small decrease (2-3 fold) in viral DNA replication at late times postinfection (Fig. 2.7B), correlating to a similar decrease in the release of DNase-resistant virion DNA into the supernatants (Fig. 2.7C). Interestingly, cells infected with the UL21a-deletion virus contained significantly more viral DNA (Fig. 2.7B) and virion proteins (pp65, pp28, and MCP) (Fig. 2.7A) at 2 hpi, a time prior to viral gene expression. Consistently, ~10 times more DNA-containing virions were needed for the mutant virus to achieve the same infectivity as the wild-type virus (Fig. 2.7C). Together, these results suggest that UL21a may have multiple roles;

one is to enhance viral DNA replication and another is to promote the ability of the virus to establish productive infection.

pUL21a is not detected in HCMV virions.

As UL21a enhanced the infectivity of HCMV virions, it was possible that pUL21a is a tegument or structural protein. However, a proteomics study failed to detect pUL21a in the HCMV virions (37). To confirm this result, supernatants were harvested from AD*rev*UL21a or AD*sub*UL21a infected cells at 96 hpi, and the virions were partially purified by ultracentrifugation through a sorbital cushion. These virion samples appeared to be largely free of contamination of cellular debris as they did not contain detectable amounts of the viral protein IE1 or the cellular protein Bip (Fig. 2.8). On the other hand, the major capsid protein (MCP) was readily detected, and importantly, pUL69, a protein that is present in the tegument and dense bodies in very low amounts (37, 41), could also be detected in the virion samples of AD*rev*UL21a (Fig. 2.8). Under these conditions, however, we could not detect pUL21a in the virion samples. Thus, pUL21a is unlikely to be a component of the HCMV virion.

DISCUSSION

In this study we report the identification and characterization of the products of HCMV gene UL21a. A single transcript of ~600 bp was transcribed from this gene locus with early gene expression kinetics (Fig. 2.1B). The transcript initiated exactly 23 bp upstream and terminated within 30-75 bp downstream of the predicted UL21a ORF (Fig. 2.1A). Presumably a poly A tail of 100-150 bp was added to the transcript and allowed its final size to reach ~ 600 bp. Supporting our mapping analysis, a putative TATA box

was found 55 bp upstream and a consensus polyA site immediately followed the stop codon of the UL21a ORF. The UL21a-specific polyclonal antibody allowed the detection of a protein, pUL21a, with an apparent molecular weight of ~15 kDa, which was only present in infection with wild-type virus but lost in infection with UL21a-deletion virus (Fig. 2.2A). pUL21a was detected as early as 10 hpi and reached its peak accumulation by 24-48 hpi (Fig. 2.2A), and therefore its expression was consistent with early gene kinetics of UL21a (Fig. 2.1B). Thus, our study provides the first experimental evidence for the presence of viral gene UL21a and its product pUL21a. pUL21a was likely the sole product encoded by the UL21a locus because our sequence analysis found no evidence for the presence of miRNAs, other non-coding RNAs, or small proteins predicted to be encoded by the UL21a transcript (data not shown). In addition, extensive third-base wobble was found in the conserved regions of the UL21a protein homologues in CCMV and RhCMV (data not shown), suggesting that it was the UL21a protein sequence that was maintained by evolutionary selection pressure.

UL21a was recently added to the annotation of the HCMV genome only based on the presence of its homologue but not the originally annotated UL21 in chimpanzee CMV (12). UL21 overlaps with UL21a, and encodes the largest putative ORF (i.e., 175 aa) in this region, an essential criteria used in the original annotation (8). However, both UL21a and UL21 are conserved among all sequenced isolates of HCMV (29), and have equal likelihoods to encode proteins as predicted by a Bio-Dictionary-based Gene Finder algorithm (28). In this study, we mapped the UL21a-specific transcript and identified the protein pUL21a but found no evidence for the presence of a UL21 transcript (Fig. 2.1). Furthermore, a GFP insertion that disrupted the putative UL21 ORF, but preserved the

UL21a ORF, replicated as efficiently as wild-type virus (data not shown), ruling out any potential role of UL21, if it exists, in HCMV growth in fibroblasts. These results lead us to favor the view that UL21a, and not UL21, is expressed and required for efficient HCMV growth in fibroblasts.

UL21a was important for HCMV to establish efficient infection because the deletion virus was attenuated in growth, particularly in MRC-5 fibroblasts as compared to that in HFFs (Fig. 2.5). The basis for such an elevated defect in UL21a-deletion virus growth in MRC-5 cells is unclear but it is likely due to physiological differences between these two cell types. For instance, MRC-5 are embryonic lung fibroblasts that proliferate more rapidly than HFFs and are able to grow up to > 30 passages, whereas HFFs are newborn foreskin fibroblasts that generally undergo senescence much sooner. In addition, an unknown difference between the two cell types, such as differential expression of a specific gene or a set of genes, may also targeted by UL21a and contribute to, at least in part, the difference that we observed. Nonetheless, analysis of the life cycle of UL21a-deletion virus in HFFs under a high MOI condition reveals a minor defect in viral DNA replication at later times (2-3 fold) and a pronounced reduction in infectivity of progeny produced (~10 fold) (Fig. 2.7). Intriguingly, pUL21a does not appeared to be a tegument or structural protein (Fig. 2.8) (37), raising the question of how pUL21a enhances the infectivity of the virion. It is conceivable that pUL21a may help proper tegumentation of virion particles. Alternatively, pUL21a may promote efficient viral gene expression or DNA replication, a function that can be compensated for by the addition of excess virions. We are currently searching for viral or

cellular factors that pUL21a may interact with and investigating the mechanistic basis of its function.

pUL21a underwent proteasome-dependent degradation during infection (Fig. 2.2) despite its important role in HCMV growth (Fig. 2.5). pUL21a degradation was blocked by the treatment of the proteasome inhibitor MG132 and was independent of any other viral factors. The absence of any lysines or cysteines in the pUL21a sequence and the failure to detect pUL21a-ubiquitin conjugates (data not shown) led us to hypothesize that the degradation of pUL21a might be ubiquitin-independent as shown for the parvovirus minute virus NS2 protein and the HCV F protein (24, 45). Our hypothesis was supported by the fact that pUL21a was efficiently degraded in cells that lacked a functional ubiquitin-conjugation system (Fig. 2.3). Interestingly, HCMV contains a virus-encoded deubiquitinating protease within its virion, which may favor ubiquitin-independent protein degradation in infected cells (39). Notably, the HCMV protein pp71 directs cellular proteins Rb and hDaxx for ubiquitin-independent proteasome-dependent degradation shortly after infection (19, 20). How would then pUL21a be targeted to the proteasome? pUL21a is rich in prolines (21/123= 17%) that are dispersed but are more predominant at the C-terminus (data not shown), reminiscent of a relatively unstructured protein. We speculate that pUL21a may be recognized as a mis-folded protein in the cytoplasm and targeted to the proteasome for degradation. In accordance with this hypothesis, an N-terminal GFP tag, which would presumably add significant structure to the protein, dramatically stabilized levels of pUL21a (Fig. 2.6B).

We detected a second, faster-migrating form of the UL21a-specific protein during pUL21a overexpression in the presence of MG132 or during HCMV infection where pUL21a was stabilized by the N-terminal GFP tag (Fig. 2.2 and 2.6B). It is possible that one of the forms of pUL21a results from post-translational modification, such as phosphorylation, as the protein contains multiple serine/threonine residues at its N-terminus. In addition, the faster-migrating form may be derived from alternative translation as the UL21a ORF contains multiple methionines. Finally, the faster-migrating form may represent an intermediate product of proteasome degradation that is stabilized by MG132.

What would be the benefit for the virus to target pUL21a to the proteasome? Perhaps the binding of pUL21a to the proteasome is important to regulate general protein degradation during HCMV infection as proposed for the HCV F protein (45), or perhaps pUL21a interacts with certain antiviral factors and directs them for degradation. This would be reminiscent of the HIV protein Vif that binds to APOBEC3G, leading to poly-ubiquitination and degradation of both proteins (16). Studies are currently under way to test these hypotheses.

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FIGURE LEGENDS

Figure 2.1. UL21a encodes a single unspliced transcript with early gene kinetics. (A) The HCMV genomic region spanning UL20-UL23. The top panel shows the schematic structure of the viral genomic region. Annotated viral open reading frames (ORF) are indicated by open boxed arrows. The HCMV-encoded micro RNA UL22A-1 is indicated by the black boxed arrow. Also shown are the transcripts from this region that have been identified in previous studies or in this study. The bottom panel shows the genomic sequence where UL21a resides with the sense-strand sequence depicted. The mapped start and termination sites of the UL21a transcript are indicated. The UL21a ORF is highlighted in gray. Also shown are the putative TATA box, poly A site (both indicated with lines), the start and stop codons of the putative UL21 ORF (both indicated with boxes), and gene specific primers used for 5' or 3' RACE (both indicated with arrows). (B) Northern blot analysis of transcripts arising from the UL21a/UL21 gene locus. HFFs were either mock-infected or infected with wild-type virus (AD*wt*) or UL21a-deletion virus (AD*sub*UL21a) at an MOI of 1 in the presence or absence of 100μg/ml CHX or 200μg/ml PAA. Cells were harvested at indicated times, total RNA was isolated, and the UL21a-specific transcript or UL123 (encoding IE1) and UL86 (encoding MCP) control transcripts were analyzed by Northern blotting using UL21a, UL123, and UL86 strand-specific anti-sense probes. Also shown is the ethidium bromide-staining of 18S rRNA as the loading control. Molecular size markers (in kb) are indicated. (C) RACE analysis of UL21a-specific transcripts. HFFs were infected with AD*wt* at an MOI of 1, cells were harvested at 40 hpi, total RNA were isolated, cDNA was generated by reverse transcription (RT), and 5' or 3' portions of the sequences of the UL21a-specific

transcripts were amplified by 5' or 3' RACE using universal primer mix (UPM) and UL21a gene specific primers (GSP) (see Fig. 2A and Materials and Methods), respectively. RACE products were analyzed by agarose gel electrophoresis, individual product was cloned, their sequences were determined, and the assembled UL21a transcript was indicated in Fig. 2A. Molecular size markers (in bp) are indicated.

Figure 2.2. pUL21a is expressed but undergoes proteasome-dependent degradation during HCMV infection. (A) Kinetics of pUL21a accumulation in HCMV infection. HFFs were infected with AD*wt* or AD*sub*UL21a at an MOI of 1, cells were collected at indicated times, and total lysates were analyzed by immunoblotting using an affinity-purified rabbit polyclonal antibody raised against a UL21a recombinant protein. (Blotting was performed under a long exposure to reveal the presence of pUL21a at 10 hpi). The antibody to viral protein IE1 or cellular protein actin was used as infection or loading control, respectively. (B) Proteasome inhibitor stabilizes the accumulation of over-expressed pUL21a. Hela cells were transfected with vector expressing pUL21a (pLP-pUL21a) or another HCMV protein pp28 (pLP-pp28), and protease inhibitor as indicated or DMSO control was added at 48 hours to the final concentration of 10 μM. Total cell lysates were collected 24 hours later and analyzed by immunoblotting using the antibody to pUL21a or pp28 (Blotting was performed under a short exposure to reveal differentiated levels of pUL21a in the presence or absence of MG132). (C) Dose- and time-dependent effects of proteasome inhibitor on the accumulation of over-expressed pUL21a. Hela cells transfected with pLP-pUL21a were incubated with MG132 at different concentration as indicated (μM) for 24 hours (left panel) or with 2.5 μM MG132 for different times as indicated (right panel), and cell lysates were prepared for

immunoblot analysis using the α-pUL21a antibody (short exposure). (D) HFFs were infected with indicated HCMV virus at an MOI of 1, MG132 or DMSO control was added at 38 hpi to the final concentration of 10 μM, cells were collected at 48 hpi, and total lysates were analyzed by immunoblotting using the antibody to viral proteins IE1, pUL44, or pUL21a (short exposure).

Figure 2.3. Degradation of pUL21a is unaltered in cells lacking a functional ubiquitin-conjugation system. Mouse ts20 cells were transfected with pLP-pUL21a or the empty vector pLP, and cultured at 35°C. 24 hours later, DMSO control or MG132 was added to indicated cell culture at the final concentration of 10 μM, one set of cells were kept at 35°C, and the second set were shifted to 39°C. Total cell lysates were prepared after 20 hours of drug treatment and analyzed by immunoblotting for accumulation of p53 and pUL21a. The antibody to actin was used as a loading control.

Figure 2.4. pUL21a predominantly localizes to the cytoplasm of both overexpressing and infected cells. (A) Hela cells (left panel) or HFFs (right panel) were transfected with plasmid expressing pUL21a or control viral proteins (pp28 or pUL44), seeded onto coverslips at 48 hours post-transfection, and analyzed for the subcellular localization of pUL21a 24 hours later by immunofluorescence using the α-pUL21a antibody. Cells were also counterstained with TO-PRO3 to visualize the nuclei of the cells. Scale bars (10 μm) are shown. (B) HFFs were infected with AD*sub*UL21a, AD*wt*, or AD*in*GFP-UL21a at an MOI of 0.1, and then analyzed by immunofluorescence using the α-pUL21a antibody at indicated times postinfection.

Figure 2.5. Deletion of UL21a results in attenuated growth of HCMV in fibroblasts. (A) Schematic structure of the viral genomic region in recombinant BAC-HCMV clones where genetic alterations were introduced. Viral ORFs and known miRNAs are indicated by open and black boxed arrows, respectively. The boxes below the first line represent the locations of viral sequences that were replaced by the *GalK/kanamycin* marker sequence in the substitution mutants, as indicated. Note that the UL21a sequence was reintroduced into pAD*sub*UL21a to replace the marker sequence and generate the marker-rescued clone pAD*rev*UL21a. (B) Representative images of plaques (as indicated by virus-driven GFP expression) from HFFs that were infected with wild-type or UL21a-deletion virus, overlaid with agarose, and examined under a fluorescent microscope at 12 dpi. Scale bars (0.1 mm) are shown. Average sizes of 50 plaques from wild type- or mutant- virus infection, and the *P* value by Student's paired *t*-test are also shown. (C-D) Growth kinetic analysis of recombinant HCMV viruses. HFFs (C) or MRC-5 primary human embryonic lung fibroblasts (D) were infected with indicated viruses at an MOI of 0.001 or 1, cell-free and cell-associated viruses were collected at different days postinfection, and their yields were determined by plaque assay.

Figure 2.6. Characterization of GFP-insertional recombinant HCMV virus AD*in*GFP-UL21a. (A) A GFP-S tag was inserted in-frame at the N-terminus of the UL21a ORF by linear recombination-based BAC mutagenesis, resulting in the recombinant virus AD*in*GFP-UL21a. The insertion of the GFP-S tag concurrently disrupted and resulted in the premature termination of the overlapping UL21 ORF. Shown are predicted sequences and positions of amino acids encoded by the putative UL21 gene in AD*wt*, AD*sub*UL21a, and AD*in*GFP-UL21a. Amino acid changes of the UL21 ORF in

AD*sub*UL21a and AD*in*GFP-UL21a are highlighted in gray, and stop codons are indicated by *. (B) HFFs were mock-infected, infected with AD*wt* or AD*in*GFP-UL21a at an MOI of 1, and total cell lysates were prepared at 24 hpi and analyzed by immunoblotting. The blot hybridized to the α-pUL21a antibody was over-exposed in order to visualize the accumulation of both native and GFP-tagged pUL21a. The antibody to viral protein IE1 or cellular protein actin was used as infection or loading control, respectively. (C) HFFs were infected with indicated viruses at an MOI of 0.001, culture medium was collected at different days postinfection, and the yield of cell-free virus produced was quantified by plaque assay.

Figure 2.7. Analysis of the infection cycle of the UL21a-deletion virus. (A) Analysis of viral gene expression. HFFs were infected with AD*wt* or AD*sub*UL21a at an MOI of 1, total cell lysates were collected at indicated times, and were analyzed by immunoblotting with antibodies against IE1, pUL44, pp65, pp28, MCP, and pUL21a. (Note the difference in tegument proteins at 2 hpi.) (B) Analysis of viral DNA replication. HFFs were infected as described above, the total cell-associated DNA was isolated at indicated times, and the accumulation of viral genomes was determined by realtime quantitative PCR. (Note the difference in viral DNA at 2 hpi.) (C) Analysis of the infectivity of progeny virions. HFFs were infected as described as above, cell-free virus was collected at 72 and 96 hpi, and same volumes of viral samples were measured for their viral genome copies by realtime quantitative PCR (top panel) and for their infectivity by plaque assay (middle panel). The viral genome copy normalized by PFU was presented in the bottom panel. The relative genome copy per PFU of wild-type virus was set to 1.

Figure 2.8. pUL21a is not detected in HCMV virions. $5X10^6$ HFFs were infected with AD*rev*UL21a or AD*sub*UL21a at an MOI of 0.2, and total cell lysates and cell-free virions were collected at 96 hpi. Cell-free virions were then partially purified by ultracentrifugation through a sorbitol cushion and quantified for their genome copies by realtime-quantitative PCR. Cell lysates from $3.75x10^5$ cells and virions from $6x10^9$ genome copies were analyzed by immunoblotting for MCP, pUL69, IE1, and Bip. For pUL21a, twice as many virions ($1.2X10^{10}$ genome copies) were used in the experiment.

TABLE 2.1. Primers used to create substitutions and insertions in the HCMV genome

Targeted HCMV gene	Type of genetic alteration	Primer pairs used to introduce alteration[a]	Resulting recombinant HCMV virus
UL21a	Substitution	5'-agccatgcagcgtgcggcgcctctctcatggatccactgtcac cgtcgcg CCTGTTGACAATTAATCATCG-3' 5'-atacagacttttatatgatccttgtacagatgtaaataaaatgttttt at CTCAGCAAAAGTTCGATTTA-3'	AD*sub*UL21a
UL20	Substitution	5'-tggaacggtctttatatatacaaacgccgttatgttcagtgtcc ggcaagCCTGTTGACAATTAATCATCG - 3' 5'-tatggaaaatatgtagtccgtaccgcttggggctcaaagttca aagtccgCTCAGCAAAAGTTCGATTTA-3'	AD*sub*UL20
UL21.5-23	Substitution	5'-gcaagaacgtaactctcagtcagggggggtccaccaccgac ggagacgaaCCTGTTGACAATTAATCATCG-3' 5'-gaccgaccacatctactctgactcgttgacctttgtggccgaga gcatca CTCAGCAAAAGTTCGATTTA-3'	AD*sub*UL21.5-23
UL21a	N-terminal Insertion	5'-agccatgcagcgtgcggcgcctctctcatggatccactgtcac cgtcgcg CCTGTTGACAATTAATCATCG-3' 5'-ggcatgagtccttgagtgacggtggtgagctggggaacagg gctacctcc CTCAGCAAAAGTTCGATTTA-3'	AD*in*GalK/kan-UL21a
UL21a	N-terminal Insertion	5'-agccatgcagcgtgcggcgcctctctcatggatccactgtcac cgtcgcg ATGGTGAGCAAGGGCGAGGAG-3' 5'-ggcatgagtccttgagtgacggtggtgagctggggaacagg gctacctcc CTCGAGACTAGTACCTCCACC-3'	AD*in*GFP-UL21a

[a] Virus specific sequences are in lower case, *GalK-kanamycin* marker specific sequences are in upper case, GFP-S tag specific sequences are underlined and in upper case.

Figure 2.1

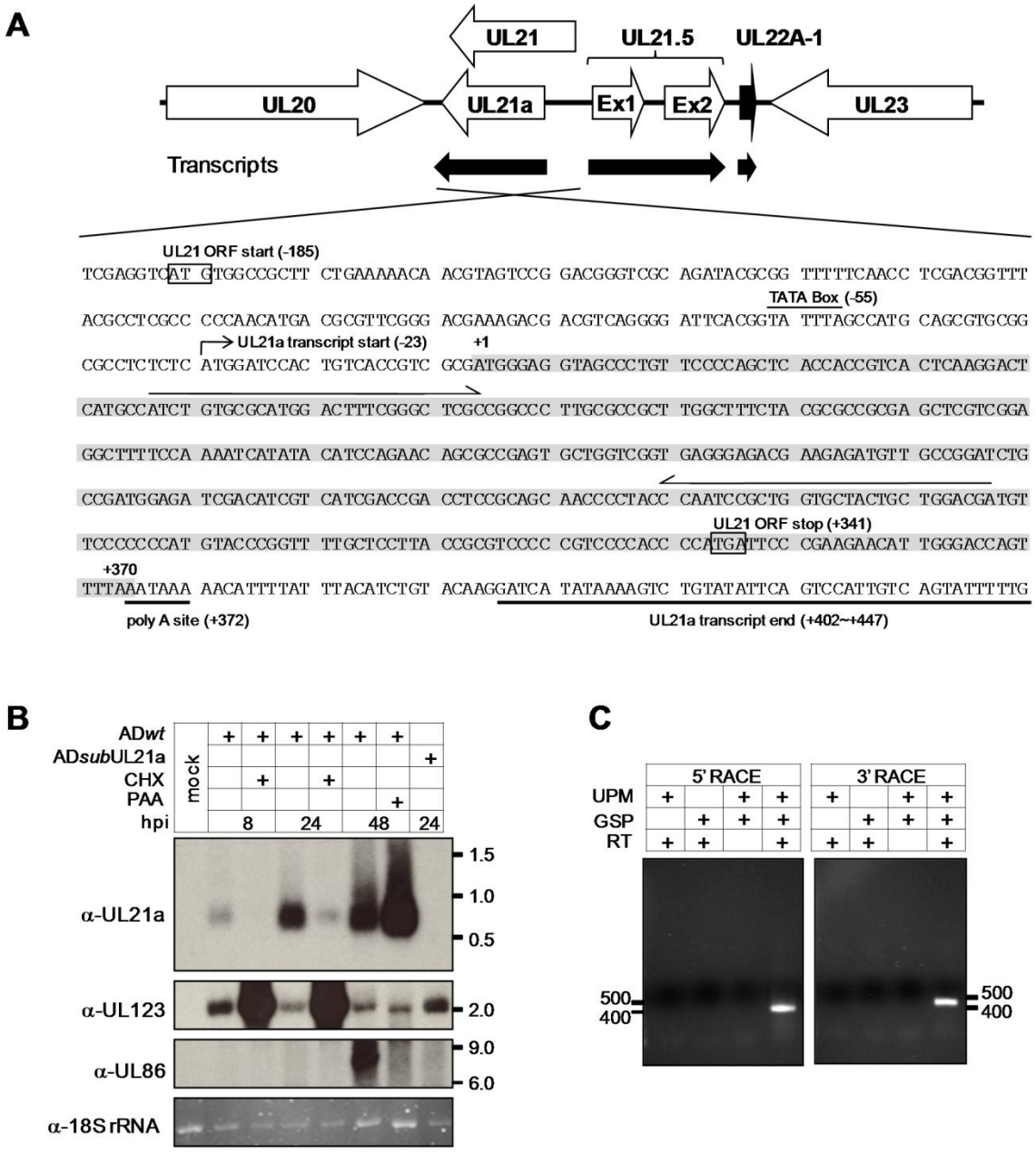
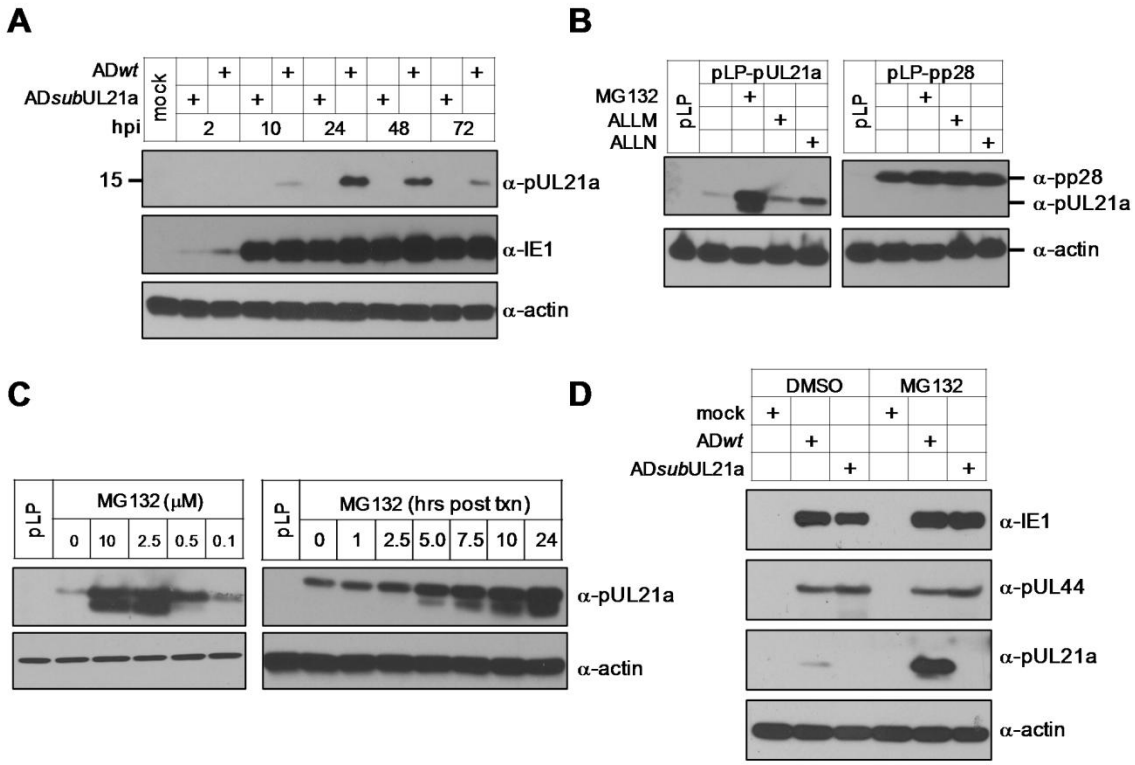

A
UL21
UL21.5
UL22A-1
UL20
UL21a
Ex1
Ex2
UL23
Transcripts
UL21 ORF start (-185)
TCGAGGTCAT GTGGCCGCTT CTGAAAAACA ACGTAGTCCG GACGGGTCGC AGATACGCGG TTTTTCAACC TCGACGGTTT
TATA Box (-55)
ACGCCTCGCC CCCAACATGA CGCGTTCGGG ACGAAAGACG ACGTCAGGGG ATTCACGGTA TTTAGCCATG CAGCGTGCGG
UL21a transcript start (-23)
+1
CGCCTCTCTC ATGGATCCAC TGTCACCGTC GCGATGGGAG GTAGCCCTGT TCCCCAGCTC ACCACCGTCA CTCAAGGACT
CATGCCATCT GTGCGCATGG ACTTTCGGGC TCGCCGGCCC TTGCGCCGCT TGGCTTTCTA CGCGCCGCGA GCTCGTCGGA
GGCTTTTCCA AAATCATATA CATCCAGAAC AGCGCCGAGT GCTGGTCGGT GAGGGAGACG AAGAGATGTT GCCGGATCTG
CCGATGGAGA TCGACATCGT CATCGACCGA CCTCCGCAGC AACCCCTACC CAATCCGCTG GTGCTACTGC TGGACGATGT
UL21 ORF stop (+341)
TCCCCCCCAT GTACCCGGTT TTGCTCCTTA CCGCGTCCCC CGTCCCCACC CCATGATTCC CGAAGAACAT TGGGACCAGT
+370
TTTAAATAAA AACATTTTAT TTACATCTGT ACAAGGATCA TATAAAAGTC TGTATATTCA GTCCATTGTC AGTATTTTTG
poly A site (+372)
UL21a transcript end (+402~+447)
B
ADwt
ADsubUL21a
CHX
PAA
hpi
mock
8
24
48
24
α-UL21a
α-UL123
α-UL86
α-18S rRNA
1.5
1.0
0.5
2.0
9.0
6.0
C
5' RACE
3' RACE
UPM
GSP
RT
500
400
500
400

Figure 2.2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ADwt
ADsubUL21a
mock
hpi
2
10
24
48
72
15
α-pUL21a
α-IE1
α-actin
B
MG132
ALLM
ALLN
pLP
pLP-pUL21a
pLP-pp28
α-pp28
α-pUL21a
α-actin
C
pLP
MG132 (μM)
0
10
2.5
0.5
0.1
MG132 (hrs post txn)
0
1
2.5
5.0
7.5
10
24
α-pUL21a
α-actin
D
DMSO
MG132
mock
ADwt
ADsubUL21a
α-IE1
α-pUL44
α-pUL21a
α-actin

Figure 2.3

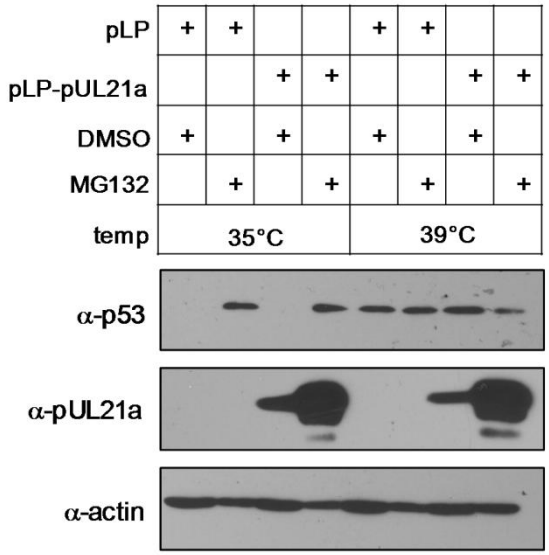


Figure 2.4

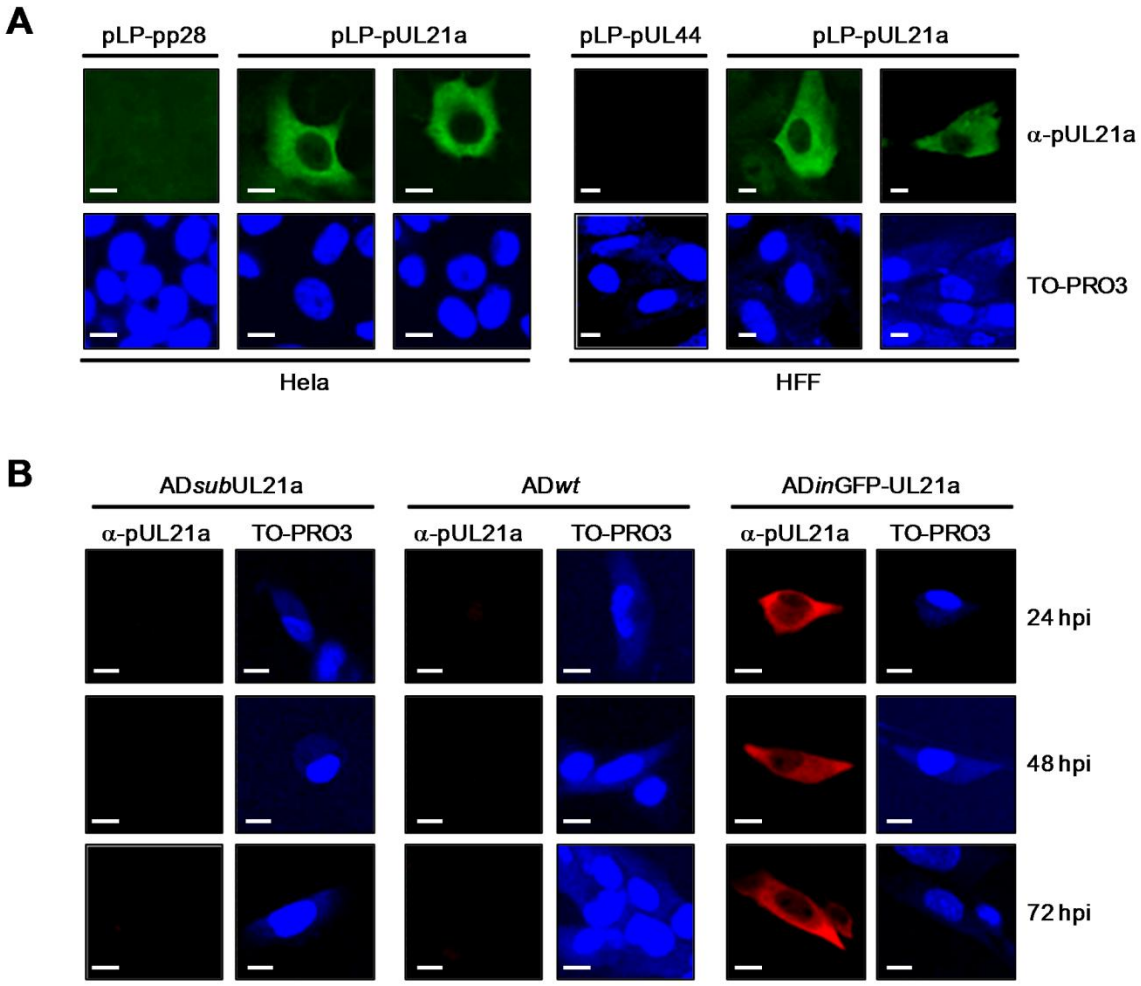

A
pLP-pp28
pLP-pUL21a
pLP-pUL44
pLP-pUL21a
α-pUL21a
TO-PRO3
Hela
HFF
B
ADsubUL21a
ADwt
ADinGFP-UL21a
α-pUL21a
TO-PRO3
α-pUL21a
TO-PRO3
α-pUL21a
TO-PRO3
24 hpi
48 hpi
72 hpi

Figure 2.5

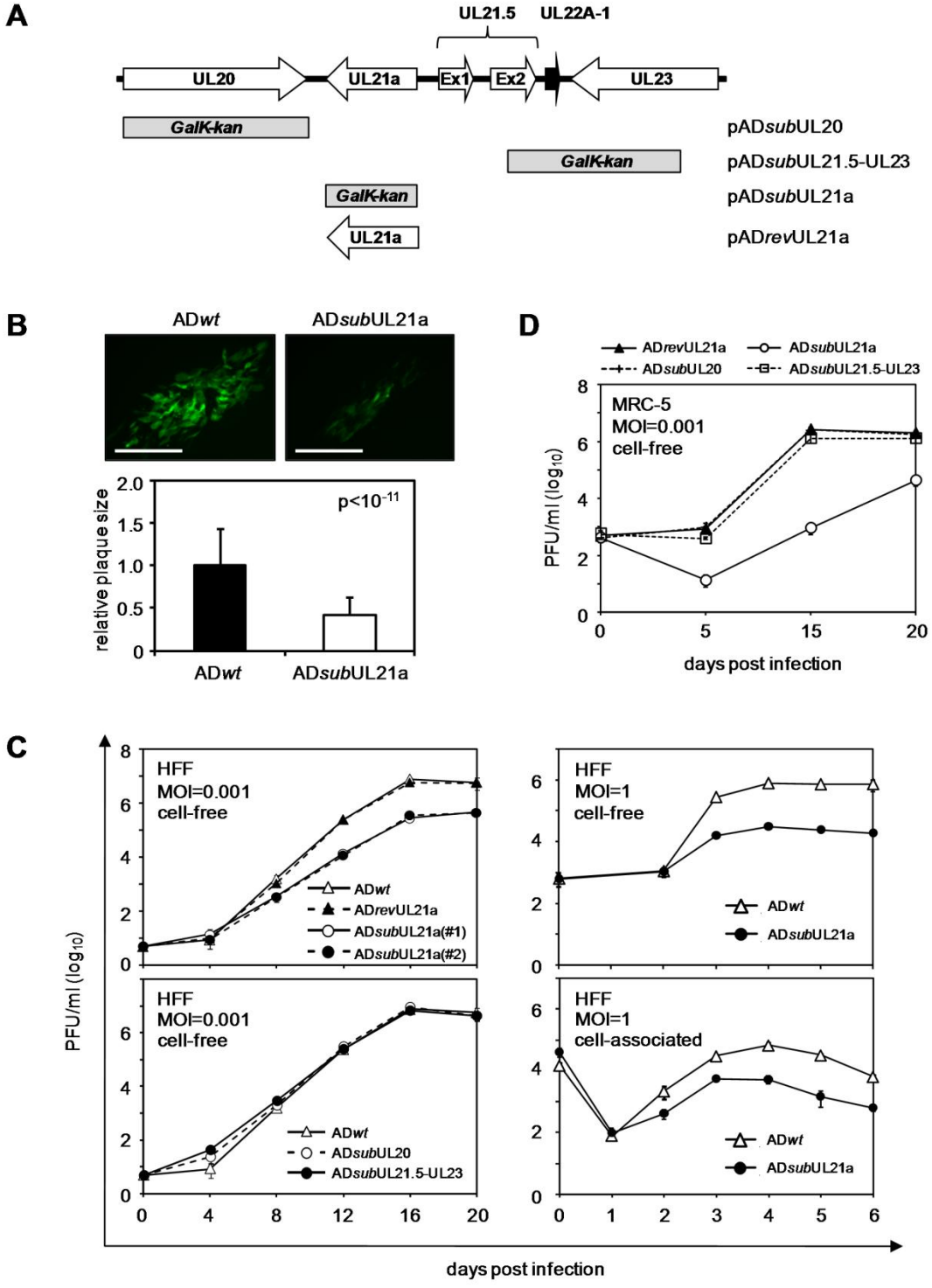

A
UL21.5
UL22A-1
UL20
UL21a
Ex1
Ex2
UL23
GalK-kan
GalK-kan
GalK-kan
UL21a
pADsubUL20
pADsubUL21.5-UL23
pADsubUL21a
pADrevUL21a
B
ADwt
ADsubUL21a
relative plaque size
p<10-11
ADwt
ADsubUL21a
D
ADrevUL21a
ADsubUL21a
ADsubUL20
ADsubUL21.5-UL23
MRC-5
MOI=0.001
cell-free
PFU/ml (log10)
days post infection
C
HFF
MOI=0.001
cell-free
ADwt
ADrevUL21a
ADsubUL21a(#1)
ADsubUL21a(#2)
HFF
MOI=1
cell-free
ADwt
ADsubUL21a
HFF
MOI=0.001
cell-free
ADwt
ADsubUL20
ADsubUL21.5-UL23
HFF
MOI=1
cell-associated
ADwt
ADsubUL21a
PFU/ml (log10)
days post infection

Figure 2.6

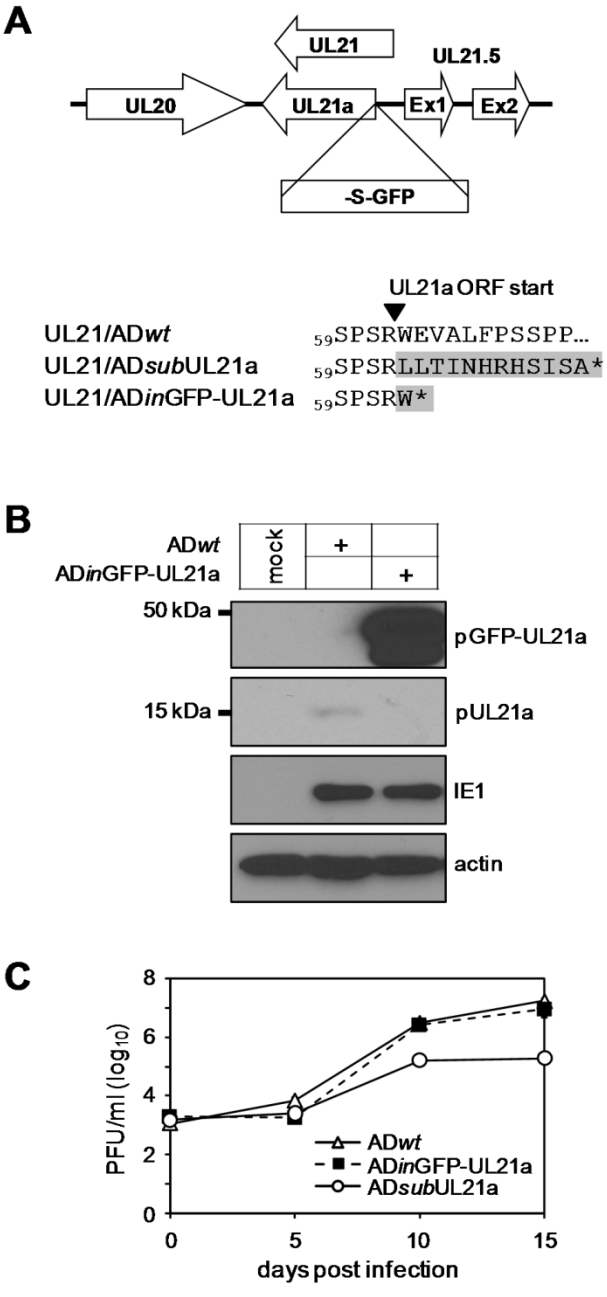

A
UL21
UL21.5
UL20
UL21a
Ex1
Ex2
-S-GFP
UL21a ORF start
UL21/ADwt 59SPSRWEVALFPSSPP...
UL21/ADsubUL21a 59SPSRLLTINHRHSISA*
UL21/ADinGFP-UL21a 59SPSRW*
B
ADwt
ADinGFP-UL21a
mock
+
+
50 kDa
pGFP-UL21a
15 kDa
pUL21a
IE1
actin
C
PFU/ml (log10)
8
6
4
2
0
0
5
10
15
days post infection
ADwt
ADinGFP-UL21a
ADsubUL21a

Figure 2.7

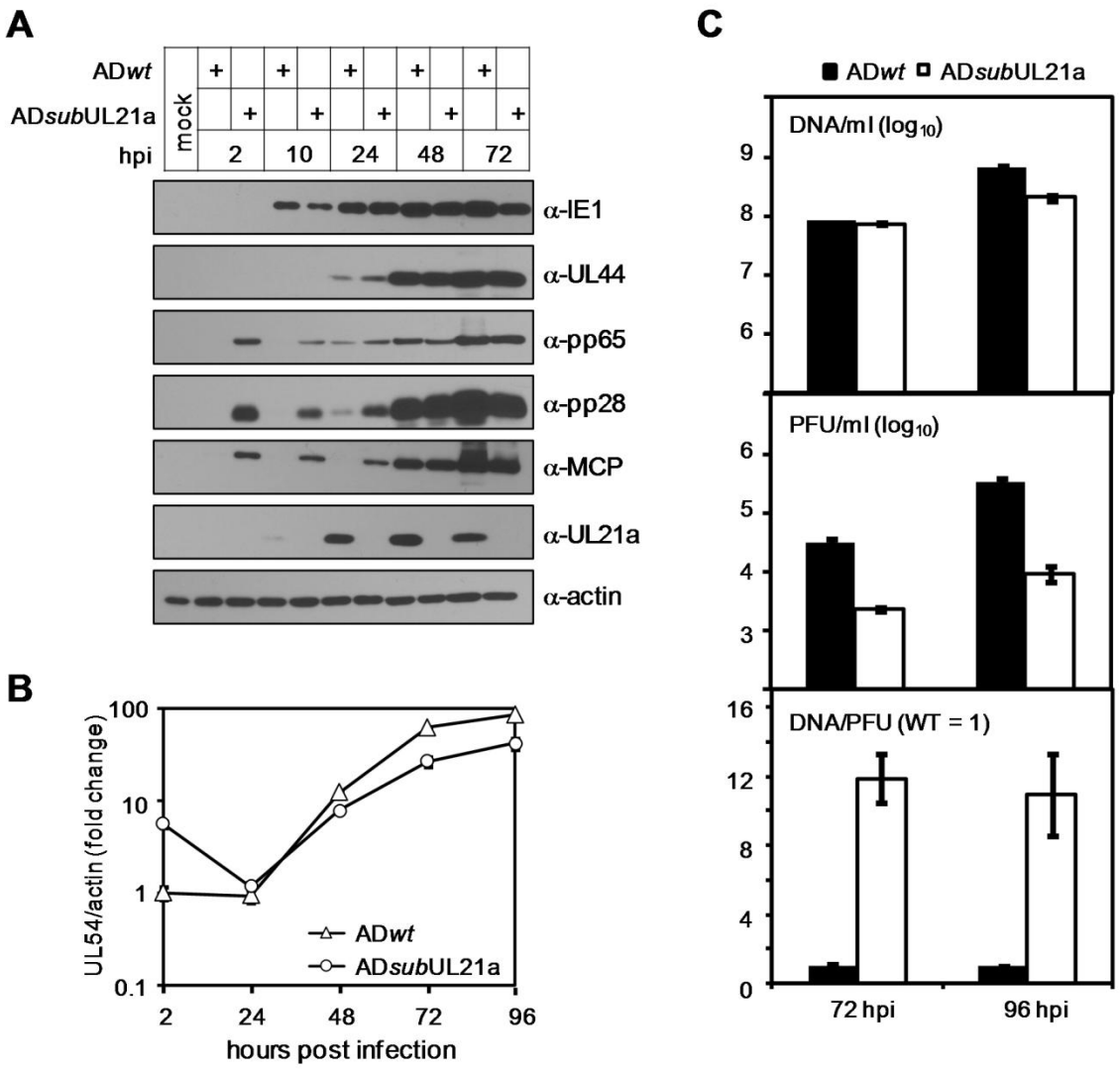
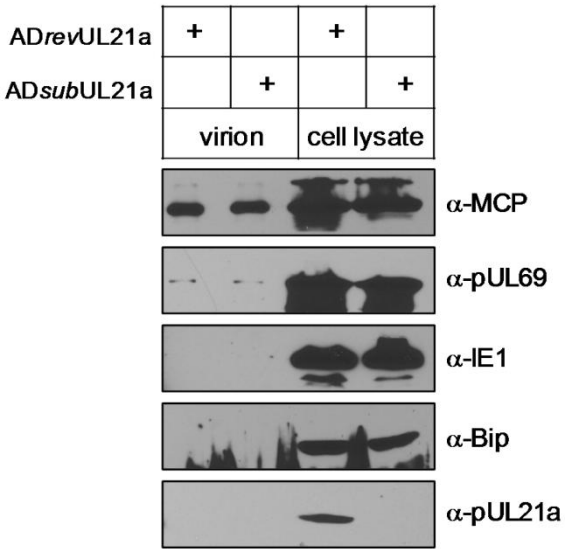

A
ADwt
ADsubUL21a
hpi
mock
2
10
24
48
72
α-IE1
α-UL44
α-pp65
α-pp28
α-MCP
α-UL21a
α-actin
B
UL54/actin (fold change)
100
10
1
0.1
2
24
48
72
96
hours post infection
ADwt
ADsubUL21a
C
ADwt
ADsubUL21a
DNA/ml (log10)
PFU/ml (log10)
DNA/PFU (WT = 1)
72 hpi
96 hpi

Figure 2.8


ADrevUL21a
ADsubUL21a
+
+
+
+
virion
cell lysate
α-MCP
α-pUL69
α-IE1
α-Bip
α-pUL21a

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Chapter III

Human cytomegalovirus early protein pUL21a promotes efficient viral DNA synthesis and the late accumulation of immediate-early transcripts

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ABSTRACT

We have previously reported that a newly annotated gene of human cytomegalovirus (HCMV), UL21a, encodes an early viral protein termed pUL21a. Most notably, the virions of UL21a-deletion virus had markedly reduced infectivity, indicating that UL21a is required to establish an efficient productive infection. In this report, we infected fibroblasts with equal numbers of DNA-containing viral particles and identified where in the viral life cycle pUL21a acted. The UL21a-deletion virus entered cells and initiated viral gene expression efficiently; however, it synthesized viral DNA poorly and accumulated several immediate-early (IE) transcripts at reduced levels at late times of infection. The defect in viral DNA synthesis preceded that in gene expression, and inhibition of viral DNA synthesis reduced the late accumulation of IE transcripts in both wild-type and mutant virus infected cells to equivalent levels. This suggests that reduced viral DNA synthesis is the cause of reduced IE gene expression in the absence of UL21a. The growth of UL21a-deletion virus was similar to that of recombinant HCMV where pUL21a expression was abrogated by stop-codon mutations, and the defect was rescued in pUL21a-expressing fibroblasts. pUL21a expression *in trans* was sufficient to restore viral DNA synthesis and gene expression of mutant virus produced from normal fibroblasts whereas mutant virus produced from complementing cells still exhibited the defect in normal fibroblasts. Thus, pUL21a does not promote the functionality of HCMV virions; rather, its *de novo* synthesis facilitates viral DNA synthesis, which is necessary for the late accumulation of IE transcripts and establishment of a productive infection.

INTRODUCTION

Human cytomegalovirus (HCMV), the prototypic β-herpesviruses, is a ubiquitous pathogen that infects 50-90% of the world's population. Upon primary infection, HCMV establishes a lifelong latent or persistent/recurrent infection within its host. Though asymptomatic in most immunocompetent individuals, HCMV can cause severe disease and death in immunocompromised individuals, including AIDS patients and organ transplant recipients. HCMV is also the most common viral cause of birth defects, leading to hearing loss, blindness, and mental retardation in congenitally and perinatally infected infants (32). The economic burden to the US health care system for this virus is estimated at approximately four billion dollars annually with a majority of the costs attributed to long-term sequelae experienced by individuals who acquire congenital HCMV disease (19). A comprehensive understanding of how HCMV interacts with the host to establish both acute and latent infections will be critical for developing an effective vaccine and novel therapeutics to combat HCMV disease (24, 59).

HCMV expresses its genes in a highly regulated temporal cascade during a productive infection (32). The virus first expresses its immediate-early genes, which appear 2-4 hours after viral entry and persist throughout the infection. The products of the major immediate-early (MIE) transcript are critical for the establishment of a productive infection, and must be downregulated for the virus to establish latency (32). The primary proteins encoded by the MIE transcript are IE1-72 and IE2-86, which are produced by alternative splicing (62, 64, 66). IE1-72 is abundantly expressed during the first few hours of infection. Its abundance then only undergoes a limited increase throughout the remainder of the infection (63). In contrast, the accumulation of IE2-86 is

low during the first 12 hours of infection but it increases considerably between 24 and 72 hpi (62, 64). The reason for this differential MIE expression is unclear, but a specific inhibitor of the cyclin-dependent kinases (CDK) causes a shift in the ratio of IE1/IE2 during the first 12 hours of infection (55), suggesting that CDK activity may play a role in this regulation. The IE2-86 protein is essential for viral replication while IE1-72 is only required at a low MOI (11, 14, 18, 29, 31, 58). Both proteins transactivate viral promoters and also modulate the cellular environment to be more conducive for viral infection. Additional IE genes, which include TRS1, UL37x1, and US3, help HCMV to overcome innate and adaptive cellular antiviral responses (1, 4, 5, 13, 17, 23, 44).

Transcription of early genes shortly follows IE gene expression, appearing between 4 and 12 hpi. These genes encode DNA replication enzymes, such as UL44 (processivity factor) and UL54 (viral DNA polymerase), as well as viral regulatory proteins that alter host cells for a cellular environment conducive to viral replication. Late genes are expressed following the onset of viral DNA replication and many of them encode structural proteins, such as the major capsid protein (MCP) and pp28, which are required for assembly and maturation of the virion. Other late genes, such as pp71, are tegument proteins which can antagonize intrinsic cellular defenses and help progeny virus initiate IE gene expression during subsequent infection (20, 51, 52).

HCMV contains a 240-kb double-stranded DNA genome that encodes at least 166 putative open reading frames (ORFs) and several miRNAs (3, 7, 9, 15, 34, 35). With the advent of the infectious bacterial artificial chromosome (BAC) clone-based genetic system for HCMV (2, 73), the functions of HCMV genes have started to be elucidated. Genome-scale mutagenesis approaches have identified a subset of candidate viral genes

that are important for HCMV to establish infection in tissue culture models of primary human cells including fibroblasts (8, 72). More detailed investigations have begun to uncover the functions of selected viral genes. We have recently identified a 15-kDa viral protein, pUL21a, which is expressed with early kinetics from the newly annotated HCMV gene UL21a (10). pUL21a predominantly localizes to the cytoplasm and undergoes rapid degradation in a ubiquitin-independent, proteasome-dependent manner (10). Analysis of mutant HCMV virus lacking the UL21a coding sequence reveals a minor defect in viral DNA replication (~2-3 fold) and a more pronounced reduction in infectivity of progeny viruses (~10 fold), suggesting that UL21a is required for the establishment of a productive infection (10). It is possible that UL21a promotes the proper tegumentation of the HCMV virion, enabling the virus to effectively initiate productive infection. Alternatively, it may act at the later stages of infection, such as viral gene expression or DNA replication, to drive the progression of the viral life cycle.

In this study, we sought to determine how UL21a drives HCMV infection by analyzing fibroblasts infected with equal numbers of DNA-containing virions of recombinant viruses. We report that UL21a is not required for HCMV to establish the initial events of productive infection, but it facilitates efficient viral DNA synthesis and the late accumulation of several viral IE transcripts. Moreover, we present evidence that DNA synthesis is the cause and not a consequence of the late accumulation of the IE transcripts. We show that pUL21a, the protein product of UL21a, carries out the function of this gene locus. pUL21a expression *in trans* restores viral DNA synthesis and lytic gene expression of mutant virus that is originally produced from normal fibroblasts. However, mutant virus produced from complementing cells still exhibited reduced lytic

gene expression in normal fibroblasts. Collectively, our results indicate that HCMV virions function normally independent of pUL21a, but the *de novo* synthesis of this protein facilitates viral DNA synthesis, which is necessary for the efficient late accumulation of IE transcripts and the establishment of a productive infection.

MATERIALS AND METHODS

Plasmids and antibodies. pYD-C160 and pYD-C476 are retroviral vectors derived from pRetro-EBNA. pYD-C160, which carries the coding sequence of the green fluorescent protein (GFP), was previously described (69), and pYD-C476 was created by PCR amplifying the coding sequence of the GFP-UL21a fusion protein from pAD*in*GFP-UL21a (10) and inserting it into the multiple cloning site of pRetro-EBNA.

The primary antibodies used in the present study included anti-β actin (AC-15, Abcam); anti-IE2 (mAB8140, Chemicon); anti-acetyl histone H3 (DAM1422332, Upstate); anti-Bip/Grp 78 (H-129, Santa Cruz); anti-UL21a (10); anti-UL38 (69); and anti-IE1, anti-pp28 (60), and anti-TRS1 (49) (generous gifts from Thomas Shenk, Princeton University).

Cells and Viruses. Primary embryonic lung fibroblasts (MRC-5) and human newborn foreskin fibroblasts (HFFs) were propagated with Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, non-essential amino acids, and penicillin-streptomycin. To create MRC-5 cells expressing GFP (MRC-5-GFP) or the GFP/UL21a fusion protein (MRC-5-GFP/UL21a), retrovirus stocks were prepared by transfecting the retroviral vector pYD-C160 or pYD-C476 into Phoenix-Ampho cells (25) using lipofectamine (Invitrogen) according to the manufacturers' instructions.

MRC-5 cells were transduced with retrovirus three times and then let recover for 72 hours to generate a pool of cells expressing the protein of interest.

Five BAC-HCMV clones were used in the present study to reconstitute recombinant HCMV viruses. pAD-GFP carried the GFP-tagged genome of the HCMV AD169 strain and was used to produce wild-type virus AD*wt* (73). pAD*sub*UL21a carried a GalK/kanamycin dual mutagenic cassette in place of the entire UL21a coding sequence as previously described (10). pAD*pm*pUL21a carried stop-codon mutations at Gln^{8} and Thr^{39} engineered to specifically disrupt pUL21a expression (Fig. 3.6). It was constructed by replacing the GalK/kanamycin cassette in pAD*sub*UL21a with the PCR fragment of the UL21a gene that contained the desired mutations via linear recombination as previously described (43). These mutations were first generated by using a QuickChange XL kit (Stratagene) with two primer pairs containing the desired mutations and the template plasmid pYD-C417 carrying the HCMV UL21a sequence, resulting in the plasmid pYD-C478 carrying these mutations. The primer pairs are as follows: 1st pair (Q8X), 5'-GGTAGCCCTGTTCCCTAGCTCACCACCGTCACT-3' and 5'-AGTGACGGTGGTGAGCTAGGGAACAGGGCTACC-3'; 2nd pair (R39X), 5'-GCCTCCGACGAGCTCACGGCGCTAGAAAGC-3'and 5'-GCTTTCTACGCGCCGTGAGCTCGTCGGAGGC-3'. pAD*rev*pUL21a was a marker-rescued clone and was created by replacing the mutant UL21a gene in pAD*pm*pUL21a with a wild-type copy of UL21a via two-step linear recombination as previously described (43). pTR is a BAC clone carrying the genome of a clinical HCMV isolate TR that was originally isolated from the ocular vitreous fluid of a patient with advanced HIV with CMV retinitis (61) and was used to reconstitute HCMV TR virus (35). All

recombinant BACs were verified by restriction digestion, PCR analysis, and direct sequencing.

To reconstitute virus, 2 µg of the BAC-HCMV DNA and 1 µg of the pp71-expressing plasmid were transfected into MRC-5 cells by electroporation. Culture medium was changed 24 hours later, and virus stock was prepared by harvesting cell-free culture supernatant when the entire monolayer of cells was lysed. Alternatively, virus stocks were produced by collecting cell-free culture medium from infection of HFFs at the multiplicity of infection (MOI) of 0.05. Virus-containing culture supernatants were then purified by ultracentrifugation through a 20% D-sorbitol cushion at an average relative centrifugal force of 64,000 x *g* for 1 hour, resuspended in DMEM with 10% fetal calf serum, and saved as viral stocks. HCMV virus titers were determined in duplicate in HFFs by Tissue Culture Infectious Dose 50 ($TCID_{50}$) assay and their DNA content was determined by real-time quantitative PCR (qPCR) (see below).

Cellular fractionation. Nuclear and cytoplasmic fractions were derived from infected cells at 2 hours post-infection as previously described (27). The cell pellet was resuspended in RSB (10 mM NaCl, 3 mM MgCl2, 10 mM Tris-HCl [pH 7.4]), and 20X detergent (10% sodium deoxycholate and 20% Tween 40 in RSB) was added to a 1X final concentration. Samples were then vortexed and incubated on ice for 10 min. Nuclei were pelleted by centrifugation at 1,000 x g for 3 min at 4°C. The cytoplasmic (supernatant) and nuclear (pellet) fractions were then processed for total DNA and proteins as stated below.

Analysis of virion and intracellular DNA. Virion DNA was prepared as previously described (10). Briefly, to prepare virion DNA, 100 µl of cell-free virus (or 10 µl of

purified virus) was treated with DNase I (30 U; Roche) at 37°C for 30 min, followed by incubation at 75°C for 20 min to stop the reaction. The samples were then incubated at 55°C overnight in lysis buffer (400 mM NaCl, 10 mM Tris [pH 8.0], 10 mM EDTA, 0.1 mg of proteinase K/ml, 0.2% sodium dodecyl sulfate [SDS]). Virion DNA was extracted with phenol-chloroform and treated with RNase A at 20 μg/ml at 37°C for 1 hour. DNA was extracted again with phenol-chloroform, precipitated with ethanol, and resuspended in nuclease-free water (Ambion). To prepare intracellular DNA, HCMV-infected MRC-5 cells were collected at various times post-infection, resuspended in lysis buffer, and incubated at 55°C overnight. DNA was extracted with phenol-chloroform, treated with RNase A, extracted again with phenol-chloroform, precipitated with ethanol, and resuspended in nuclease-free water. Viral DNA was quantified by qPCR as previously described by using a TaqMan probe (Applied Biosystems) and primers specific for the HCMV UL54 gene (41). Cellular DNA was quantified with SYBR green PCR Master Mix (Clonetech) and a primer pair specific for the human β-actin gene as previously described (43). The accumulation of viral DNA was normalized by dividing UL54 gene equivalents by β-actin equivalents.

Protein analysis. Protein accumulation and subcellular localization were determined by immunoblotting and immunofluorescence, respectively. For immunoblotting, total cell extracts were prepared by lysing phosphate-buffered saline-washed infected cells in SDS-containing sample buffer. Proteins were resolved by electrophoresis on a SDS-containing polyacrylamide gel, transferred to a polyvinylidene difluoride membranes, hybridized with a primary antibody, reacted with the horseradish peroxidase-conjugated secondary antibody, and visualized by using SuperSignal West Pico chemiluminescent substrate

(Thermo Scientific). For immunofluorescence, cells grown on glass coverslips were fixed in 2% paraformaldehyde, permeabilized with 0.1% Triton X-100 for 5 min, incubated with a primary antibody, and subsequently labeled with the AlexaFluor 488 secondary antibody (Invitrogen). Labeled cells were counterstained with TO-PRO-3 (Molecular Probes) to visualize the nuclei and then mounted on slides with Prolong Gold antifade reagent (Molecular Probes). Images were captured using Zeiss LSM Image software with a Zeiss LSM 510 META confocal laser scanning microscope.

Reverse transcription coupled-quantitative PCR analysis. Total RNA was extracted by using the TRIzol reagent (Invitrogen) and treated with the Turbo DNA-free reagent (Ambion) to remove genomic DNA contaminants. cDNA was reverse transcribed from total RNA with random hexamer primers using the High Capacity cDNA reverse transcription kit (Applied Biosystems). cDNA was quantified by qPCR using a Taqman probe and primers specific for the viral gene IE1 or UL37. Alternatively, cDNA was quantified using SYBR Advantage qPCR Premix (Clontech) and primers for the viral genes TRS1, IE2, exon 3 of the major immediate-early (MIE) transcript, or the human GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene. Primers and probes specific for IE1 and IE2 spanned the splice junction of these genes as previously reported (70). All of the primer pairs and Taqman probes are listed in Table 3.1. cDNA from infected cells was used to generate a standard curve for each gene examined. The standard curve was then used to calculate the relative amount of specific RNA present in a sample. The amounts of TRS1, UL37, IE2-86, IE1-72, and the MIE transcript were normalized using GAPDH as an internal control.

RESULTS

UL21a-deletion virus has a more pronounced growth defect in MRC-5 cells than in HFFs. We have previously shown that UL21a is required for efficient HCMV replication in fibroblasts, and interestingly, the defect of the UL21a-deletion virus is more pronounced in human embryonic lung fibroblasts (MRC-5) than that in human foreskin fibroblast cells (HFFs) in a multiple-step growth curve (10). In agreement, we found that the UL21a-deletion virus also had a greater growth defect in MRC-5 cells compared to that in HFFs in a single-step growth curve (Fig. 3.1A). In HFFs, the growth of mutant virus was 11- and 23- fold lower than that of wild-type virus at 72 and 96 hours postinfection (hpi). In MRC-5 cells, the growth reduction of the mutant virus relative to wild-type virus increased to 32- and 72-fold, respectively. Because of this enhanced growth defect, we used MRC-5 cells in the present study to investigate the role of UL21a during HCMV infection. Importantly, similar to that observed in HFFs (10), the mutant progeny virus produced in MRC-5 had markedly reduced infectivity; the relative ratio of virion DNA-to-infectious particles of the mutant virus was 5 to 10 fold greater than that of wild-type virus (Fig. 3.1B). In order to determine why the UL21a-deletion virus failed to establish an efficient productive infection, we carried out all of the infection experiments with recombinant viruses using equal input genome number equivalents in this study.

HCMV enters host cells efficiently independent of UL21a. We systematically dissected the lifecycle of UL21a-deletion virus to define where the defect resided. We first infected MRC-5 cells with wild-type or UL21a-deletion virus at equal input genome number equivalents, and analyzed the ability of the mutant virus to enter cells by

determining translocation of viral protein and DNA to the nucleus at 2 hpi (Fig. 3.2). Both immunoblot and immunofluoresence analyses showed that the viral phosphoprotein pp65 translocated to the nucleus in cells infected with the mutant virus as efficiently as that with wild-type virus (Figs. 3.2A and 2B). Moreover, most of the viral protein pp28 stayed in the cytoplasm, as has been previously reported (Figs. 3.2A and B) (28, 54). The amount of cytoplasmic pp28 and nuclear pp65 in mutant virus infection was equivalent to that in wild-type virus infection. In addition, real-time quantitative PCR (qPCR) analysis of nuclear fractions of infected cells indicated that equivalent amounts of virion DNA had trafficked to the nucleus of cells infected with wild-type and UL21a-deletion virus (Fig. 3.2C). We conclude that the UL21a-deletion virus is competent for viral entry.

UL21a is dispensable for HCMV to initiate immediate-early (IE) gene expression but it is required for efficient viral DNA synthesis and the late accumulation of viral IE transcripts. We next compared the profiles of viral gene expression between wild-type and UL21a-deletion virus infection. We chose to perform the experiment at both low and high MOIs to ensure an infection condition where the level of viral replication was proportional to the quantity of input virus used. UL21a-deletion virus expressed IE1-72 and IE2-86 at levels no less than wild-type at 8 (data not shown) and 24 hpi (Fig. 3.3A). Moreover, the early HCMV protein pUL38 was also efficiently expressed at 24 hpi during mutant virus infection (Fig. 3.3A). These results suggest that UL21a-deletion virus was not only competent for viral entry, but was also able to initiate viral gene expression.

Intriguingly, while the UL21a-deletion virus continued to accumulate IE1-72 and pUL38 similar to wild-type virus throughout the infection, there was a marked reduction

in IE2-86 accumulation starting at 48 hpi (Fig. 3.3A). This defect coincided with a reduced accumulation of the late protein pp28. The reduction in the late accumulation of IE2-86 was not due to an inefficient second round of mutant virus infection, because this reduction was also clear at high MOI infection (Fig. 3.3A). We next sought to determine whether the defect in IE2-86 protein accumulation was the result of the reduced transcript accumulation. We measured the accumulation of the IE2-86 transcript at both low and high MOI infections (Fig. 3.3B) by reverse transcription coupled-qPCR (RT-qPCR) (Fig. 3.3B) (70). All transcripts detected were specific and were not the result of genomic DNA contamination as mock cells and reactions done in the absence of reverse transcriptase failed to produce any products (data not shown). Importantly, the IE2 primer pair used in this experiment only amplified the IE2-86 transcript but not any other known late transcripts from this region, such as IE2-60 or IE2-40 (42, 71), as the 5' primer is located in exon 3, which is upstream of the start site of these two late transcripts. Consistent with the protein expression profile (Fig. 3.3A), the levels of the IE2 transcript in mutant virus and wild-type virus infection were nearly equivalent at 24 hpi (Fig. 3.3B). However, by 48 hpi the IE2 transcript accumulation in cells infected with UL21a-deletion virus was reduced, and at 72 hpi, it was ~4 fold lower than that with wild-type virus (Fig. 3.3B). The total MIE transcript was also reduced by ~3 fold at 72 hpi, suggesting that decreased IE2 expression was mostly due to the reduced accumulation of the MIE transcript at late times of infection (Fig. 3.3B). Consistent with protein analysis, there was no significant difference in the accumulation of the IE1 transcript between wild-type and mutant virus infection, particularly at high MOI. This raises the possibility that at late times the IE2 transcript is the major product of MIE

transcripts (56). Moreover, the reduced accumulation of late IE transcripts in mutant virus infected cells was not limited to the MIE transcript. Other IE transcripts, including UL37 and TRS1, were also significantly reduced at late times in UL21a-deletion virus infected cells (Fig. 3.3B).

We then analyzed viral DNA synthesis in wild-type and UL21a-deletion virus infected cells at both equal input infectious particles and equal input genome number equivalents (Fig. 3.3C). When cells were infected with equal input infectious particles, the UL21a-deletion virus started with much higher levels of input viral DNA, consistent with its elevated virion DNA-to-infectious particle ratio (Fig. 3.1B), but by 72 hpi the two viruses had similar levels of viral DNA. When cells were infected with equal input genome number equivalents, viral DNA levels in both virus infection were similar at 2 and 24 hpi. However, viral DNA synthesis in cells infected with mutant virus was 5-fold lower than that with wild-type virus at 48 hpi. By 72 hpi, the defect in viral DNA synthesis was ~20 fold during mutant virus infection. We conclude that the UL21a-deletion virus is defective in viral DNA synthesis and the late accumulation of viral IE transcripts.

The defect in viral DNA synthesis precedes that in the late accumulation of IE transcripts during UL21a-deletion virus infection. IE2-86 is essential for viral early gene expression and DNA synthesis (18, 29, 57). However, the UL21a-deletion virus was able to express IE and early proteins, including IE2-86, at normal levels up to 24 hpi following initiation of viral DNA synthesis. It has been shown that IE2-86 undergoes a significant increase in transcript and protein accumulation between 24 and 72 hpi, which is dependent on DNA synthesis (62, 68). This raises the question of

whether the reduced IE2-86 expression in UL21a-deletion virus infected cells is the cause or a consequence of defective viral DNA synthesis. To address this question, we first examined the temporal relationship between the defect in viral DNA synthesis and that in MIE transcript accumulation. In this experiment, a reduction in viral DNA synthesis was clearly evident at 32 hpi, and that reduction reached 4-fold by 40 hpi in mutant virus infection (Fig. 3.4A). In contrast, the difference of MIE expression was minimal between wild-type and mutant virus infection, particularly at 32 hpi (Fig. 3.4B). This result led us to hypothesize that the lack of pUL21a results in reduced viral DNA synthesis, which is the cause of the reduced late accumulation of IE transcripts.

Inhibition of viral DNA synthesis reduces the late accumulation of IE transcripts in wild-type and UL21a-deletion virus infected cells to equivalent levels. To directly test the hypothesis that the late accumulation of IE transcripts is dependent on UL21a-mediated viral DNA synthesis during HCMV infection, we infected MRC-5 cells with wild-type and UL21a-deletion virus at a high MOI in the presence or absence of the viral DNA synthesis inhibitor phosphonoacetic acid (PAA). The accumulation of IE2-86 and the late protein pp28 were reduced to undetectable levels in both wild-type and mutant virus infected cells in the presence of PAA, particularly at 48 and 72 hpi (Fig. 3.5A). Conversely, the expression levels of IE1-72 and early proteins pUL38 and pUL21a were minimally affected. Therefore, the efficient accumulation of IE2-86 protein at late times is dependent on viral DNA synthesis. This effect of viral DNA synthesis on late IE2 expression occurred primarily at the transcriptional level as PAA reduced the accumulation of IE2 transcript by ~4 and ~9-fold during wild-type virus infection at 24 and 72 hpi, respectively (Fig. 3.5B). Importantly, IE2 was not the only IE

gene whose transcript accumulation was affected by inhibition of viral DNA synthesis. This effect was global as PAA also markedly reduced transcript accumulation of two other IE genes, UL37 and TRS1. As the effect of PAA on IE expression was evident as early as 24 hpi at high MOI infection, it was unlikely the result of reduced secondary infection. Moreover, PAA reduced all the IE transcripts examined to equivalent levels in wild-type and UL21a-deletion virus infection, even at 72 hpi, when the difference in IE expression is most notable between these two virus infections (Fig. 3.5B). Collectively, these results suggest that the reduced late IE transcript accumulation in the absence of UL21a is a consequence of reduced viral DNA synthesis.

Finally, we analyzed viral gene expression in the presence of an additional viral DNA synthesis inhibitor, gancyclovir (GCV), to rule out any potential off-target effects of PAA. In addition, we also examined the gene expression profile of an HCMV clinical isolate to determine whether viral DNA synthesis-dependent late IE gene expression was limited to the laboratory strains of HCMV. MRC-5 cells were infected with either AD*wt* (Fig. 3.5C, left panel) or the BAC-derived clinical HCMV isolate TR (Fig. 5C, right panel) and treated with PAA or GCV. As expected, neither drug reduced the levels of the early proteins pUL38 or pUL21a in either viral strain. Importantly, both PAA and GCV significantly reduced the level of IE2-86 and the late protein pp28 in cells infected with AD*wt*, indicating that reduced IE2-86 levels were not due to any potential off-target effects of PAA (Fig. 3.5C). During TR infection, GCV had a minimal effect on viral protein expression at all times examined. This is consistent with the fact that TR was isolated from drug-resistant patient with advanced HIV with CMV retinitis (35, 61), though this has not been previously shown in tissue culture. Nonetheless, PAA reduced

the accumulation of IE2-86 and pp28 at 72 hpi in TR virus infected cells, indicating that DNA synthesis-dependent late IE gene expression was not strain specific.

The defect of UL21a-deletion virus is due to the loss of the protein product pUL21a. Previously, using various recombinant viruses, we were able to show that the defect of the UL21a-deletion virus was due to the loss of the UL21a gene but not the overlapping UL21 ORF or altered regulation of the neighboring genes (10). However, it is formally possible that the defect of the mutant virus was due to other unidentified functional products or DNA sequence within the UL21a gene rather than the identified protein product, pUL21a. To rule out this possibility, we created a recombinant virus, AD*pm*pUL21a, where we introduced two stop-codon point mutations at residues Gln^{8} and Arg^{39} of the UL21a ORF to abrogate the expression of pUL21a (Fig. 6A). In addition, we also created a specific marker-rescued virus for this mutant, AD*rev*pUL21a (Fig. 3.6A). We included two stop-codon mutations as the virus was able to produce a truncated protein when only one stop-codon mutation was inserted at Gln^{8} (data not shown). AD*pm*pUL21a did not express pUL21a (Fig. 3.6C) and grew indistinguishably from AD*sub*UL21a in a low MOI growth curve in MRC-5 cells, while the growth of AD*rev*pUL21a was nearly identical to that of AD*rev*UL21a, which was previously shown to grow like wild-type (Fig. 3.6B) (10). Additionally, when infecting with equal input genome number equivalents, AD*pm*pUL21a produced markedly reduced amounts of IE2-86 protein compared to the marker-rescued virus in a manner indistinguishable from the UL21a-deletion virus (Fig. 3.6C). We conclude that the phenotype of the UL21a-deletion virus is due to the loss of pUL21a and not the loss of any other unidentified functional gene products or DNA sequence.

***De novo* expression of pUL21a is necessary and sufficient for efficient viral gene expression.** pUL21a may facilitate viral DNA synthesis directly or it may be required for the proper composition of the virion, enabling it to initiate efficient viral DNA synthesis. To differentiate these potential mechanisms, we first attempted to create an inducible expression system to express pUL21a. Multiple approaches were used, including expressing pUL21a within the virus from a tetracycline-inducible promoter or tagging pUL21a with a destabilization domain, ddFKBP (12, 50). However, neither system was able to efficiently regulate the production of pUL21a, possibly due to the highly unstable nature of the protein (data not shown) (10). Instead, we developed a complementation system in which we expressed a functional GFP/UL21a fusion protein (10) in MRC-5 cells by retroviral transduction. The GFP/UL21a fusion protein was abundantly expressed in these cells and appeared as two bands (Fig. 3.8B), possibly due to partial degradation or posttranslational modification as we previously reported (10). Overexpression of the GFP/UL21a protein (pGFP/UL21a) complemented growth of the UL21a-deletion virus by greater than 200-fold (Fig. 3.7A). A difference of 10-20 fold in titer of progeny virus remained between AD*wt* and AD*sub*UL21a at 12-18 dpi when they were grown in pGFP/UL21a-expressing cells. One likely reason for this difference is that we titered progeny virus in normal fibroblasts in which the mutant virus had reduced infectivity (Fig. 3.1B). To test this hypothesis, the titers of wild-type and mutant virus were determined on cells expressing GFP/UL21a or control cells expressing GFP only. When virus collected from pGFP/UL21a-expressing cells at 12 dpi was titered on non complementing GFP-expressing cells, there was a 10-fold difference in titer between AD*wt* and AD*sub*UL21a, similar to that on untransduced cells (Figs. 3.7A-B). When

these viruses were titered on GFP/UL21a cells, there was no difference between AD*wt* and AD*sub*UL21a (Fig. 3.7B). Thus, we restored the ability of the mutant virus to replicate using pGFP/UL21a-expressing complementing cells.

To test the hypothesis that *de novo* expression of pUL21a facilitates efficient viral replication, we performed two distinct experiments. First, we infected MRC-5 cells expressing either GFP or GFP/UL21a with wild-type or UL21a-deletion virus that was produced from normal MRC-5 cells. The expression of pGFP/UL21a but not GFP restored the ability of the UL21a-deletion virus to efficiently synthesize viral DNA and produce IE2-86 and pp28, particularly at late times post infection (Figs. 3.8A-B).

In the second experiment, we infected normal MRC-5 cells with wild-type or UL21a-deletion virus that was produced from GFP or pGFP/UL21a-expressing cells. We found that even when UL21a-deletion virus was produced on complementing cells, it still expressed IE2-86 and pp28 at markedly reduced levels similar to the mutant virus that was produced on GFP control cells (Fig. 3.8C). Therefore, pUL21a is unlikely to be required for the functionality of HCMV virions. Together, these two sets of experiments suggest that the *de novo* expression of pUL21a is both necessary and sufficient for efficient viral lytic replication.

Collectively, our data indicate that *de novo* pUL21a expression facilitates HCMV DNA synthesis, leading to efficient late expression of viral IE genes.

DISCUSSION

In a previous study, we have found that the HCMV gene UL21a encodes a small 15-kDa protein, pUL21a, which predominantly localizes to the cytoplasm (10). The

UL21a-deletion virus has an elevated virion particle-to-infectious virus ratio, suggesting that UL21a is required for HCMV to establish an efficient productive infection (10). In the present study we determined why the mutant virus was unable to establish a productive infection by performing infection analysis using equal numbers of DNA-containing virion particles instead of infectious particles. The UL21a-deletion virus entered host cells and initiated its gene expression program like the wild-type virus. However, by 48-72 hpi the mutant virus failed to synthesize its DNA efficiently and expressed its IE genes at significantly reduced levels, even though the IE1-72 transcript and protein were not affected (Fig. 3.3).

Analysis of the UL21a mutant virus infection also provided evidence to clarify the relationship of viral DNA synthesis and late accumulation of viral IE gene products. The immediate-early expression of the viral IE2-86 protein is required for early gene expression and viral DNA synthesis (18, 29, 57). However, a couple of earlier studies have also suggested that the late accumulation of the IE2-86 protein and transcript is dependent on viral DNA synthesis (62, 68). Stenberg and coworkers showed that the late IE2-86 accumulation was eliminated in a temperature sensitive mutant of HCMV which could not synthesize viral DNA at the restrictive temperature (62). Nonetheless, this mutation in the viral genome has not been mapped, leaving open the possibility that an additional defect prior to DNA synthesis controls the late IE2-86 accumulation. Tenney and coworkers showed that the late accumulation of the IE2-86 specific transcript was sensitive to the viral DNA synthesis inhibitor, phosphonoformic acid (PFA) (67). Nonetheless, it was unclear whether viral DNA synthesis promotes the late expression of only IE2 or the entire IE gene class. In our study, the reduced levels of IE proteins,

including IE2-86, seen in UL21a mutant virus were not detected until after 24 hpi, a time when viral DNA synthesis has already initiated. In fact, we could detect reduced DNA synthesis before reduced IE transcript accumulation in mutant virus infection (Fig. 3.4). Importantly, PAA reduced the expression of IE genes in wild-type and UL21a-deletion virus infected cells to equivalent levels (Fig. 3.5). Collectively, these data strongly suggests that pUL21a facilitates viral DNA synthesis, which then enhances expression of IE genes, including IE2, at late times during infection.

Inhibition of viral DNA synthesis also reduced the expression of additional IE genes, such as total MIE, TRS1, and UL37, but it had a much smaller or negligible effect on expression of IE1 and the true early gene UL38 (Fig. 3.5). Our result on TRS1 expression is consistent with previous reports that the accumulation of TRS1 transcript and protein dramatically increases between 24 and 48 hpi (30, 36). Our result on UL37 expression is somewhat different from an earlier report showing no detectable reduction of UL37 transcript levels in the presence of PFA (67). The reason for this discrepancy is unclear but it is possibly due to different assays used in these studies. We used the more sensitive RT-qPCR method instead of Northern blot for transcript analysis. Moreover, we used GAPDH instead of total RNA as an internal control as the GAPDH transcript remains constant throughout infection (33, 47) and is a widely used control for qPCR analysis of HCMV infection (22, 53). Nonetheless, to our knowledge, this is the first report showing that the efficient transcript accumulation of multiple IE genes, in addition to IE2, is dependent on viral DNA synthesis.

Why does inhibition of viral DNA synthesis by PAA treatment or infection in the absence of pUL21a have such a drastic effect on IE2 expression, but have little-to-

negligible effect on IE1 expression (Fig. 3.5B)? It has clearly been shown by several groups that IE1 and IE2 proteins accumulate with different kinetics during the viral lifecycle. Despite being produced from the same pre-mRNA, IE1 largely accumulates during the very early stages of infection whereas IE2 primarily accumulates between 24 and 72 hpi, and its accumulation is dependent on DNA synthesis (62, 68) (Fig. 3.5B). These data suggest that the onset of viral DNA synthesis triggers a viral event that results in a switch from producing IE1 to IE2 transcript. This could be due to alternative splicing or altered stability of one of the MIE transcripts. Interestingly, it has been reported that CDK inhibitors reverse the accumulation of IE1 and IE2 at early stages of infection (55), suggesting that CDK activity may be promoting IE1 accumulation early during infection. CDKs appeared to accomplish this by regulating splicing, as the inhibitors had the similar effect on another spliced IE transcript, UL37, but not on unspliced viral genes (55). It is intriguing to speculate that viral DNA synthesis may somehow alter one or more CDK activities, which results in a change of the splicing pattern of the MIE transcript, and the switch from IE1 to IE2 production.

How does pUL21a augment viral DNA synthesis? Our analysis of the mutant virus infection in complementing cells indicates that the *de novo* expression of pUL21a is necessary and sufficient for the establishment of a productive infection (Fig. 3.8). Moreover, the mutant virus was competent for entry and initiation of gene expression (Figs. 3.2 and 3.3). These results, together with the fact that pUL21a is undetectable in the HCMV virion nor does it localize to cytoplasmic viral assembly centers (10), argue strongly against its potential involvement in virion assembly. pUL21a has a diffuse cytopasmic distribution in both infected and overexpressing cells and shares no

homology to any known DNA replication proteins. Furthermore, it is not expressed from genomic loci required for transient complementation of HCMV oriLyt dependent replication (39), suggesting that this protein is unlikely to act directly as a core DNA replication enzyme to synthesize viral DNA. pUL21a may play other roles, for instance, by increasing concentrations of dNTPs within infected cells, enhancing accumulation of viral and cellular DNA replication enzymes required for viral DNA synthesis, shuttling critical factors in or out of the nucleus, or by blocking cellular antiviral defenses against viral DNA synthesis. We are currently testing these potential roles for pUL21a, identifying viral or cellular factors that it may interact with, and investigating the mechanistic basis of its function.

Little is known about regulation of immediate-early gene expression by viral DNA synthesis in herpesvirus infection. It is well understood that the expression of subsets of early (β2, delayed-early) and late (γ1, early-late) viral genes are augmented by viral DNA replication during lytic infection of herpesviruses (48). Early evidence suggested that expression of the IE2 gene could be also augmented by viral DNA synthesis in HCMV infection (62, 68). In addition, the expression of the IE1 protein in MCMV infection and the expression of an IE protein ICP-27 during HSV-1 infection of trigeminal ganglion and rat superior cervical ganglia neurons have also been shown to be enhanced by viral DNA synthesis (26, 38, 45). For HSV-1, the studies led to the hypothesis that DNA synthesis represents a critical decision point in the choice between a latent and lytic infection. In neurons, HSV-1 expresses limited IE genes during the initial phase of the infection. If robust viral DNA synthesis follows, the accumulation of the IE genes is dramatically increased, allowing the virus to establish a lytic infection.

Conversely, such as in the case of a TK mutant or in the presence of PAA, viral DNA synthesis is inhibited, no additional IE genes are produced, and the virus will establish a latent infection (26, 38). It is intriguing to speculate that DNA synthesis may also be critical for HCMV to establish a latent or lytic infection in appropriate cell types, such as in CD34+ cells or undifferentiated THP-1 cells. When these cells were treated with histone deacetylase (HDAC) inhibitors, HCMV initiated IE1-72 expression and progressed to early gene expression, but the infection aborted prior to late gene expression (51, 53). Thus, in addition to a block at IE gene expression, HCMV perhaps is also unable to initiate efficient viral DNA replication in these cells, both of which may contribute to the establishment of its latency.

Finally, it will be important to identify the function(s) of the late accumulating IE proteins, particularly the IE2-86 protein. It is clear that expression of IE2-86 at immediate early times is essential for early viral gene expression and viral DNA synthesis in HCMV infection (18, 29, 57). Almost paradoxically, the greatest increase in IE2-86 accumulation comes at late times of infection, and it is not entirely clear what, if any, role this late accumulating IE2-86 protein plays during viral replication. It has been shown that IE2-86 is involved in auto-repression of the MIEP during the late stages of infection by its interactions with histone modifying enzymes (46). Mutation within the *cis* repression sequence (*crs*) in the MIEP increases the abundance of both IE transcripts and protein during late times of infection (46). However, mutations within the *crs* also resulted in defects in the early events of viral infection, including reduced MIE gene expression and viral DNA replication, thus complicating the analysis defining the precise role of this IE auto-repression during HCMV infection (21). Another potential function

for the newly synthesized IE2-86 is to transactivate the late promoters of HCMV. Late promoters remain largely associated with repressive chromatin marks and only begin to become acetylated starting at 24 hpi (6, 16). Binding of IE2-86 to HDACs 1, 2, 3, and multiple histone methyltransferases (HMTs) during the late stages of infection may activate transcription from late promoters (37, 40, 46). However, it is also possible that IE2-86 transactivates late promoters through its interactions with many other viral and cellular partners, or that the late accumulating IE2-86 is responsible for one of the many other functions ascribed to this protein (65). In any event, it is sensible for the virus to couple DNA synthesis with expression of transactivator proteins that are required for late gene expression. This will ensure that late proteins, such as capsid and tegument proteins, will accumulate along with viral DNA synthesis, enhancing the production of infectious viral particles and facilitating the ability of the virus to spread to new host cells.

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FIGURE LEGEND

Figure 3.1. UL21a-deletion virus has reduced infectivity. (A) Growth of UL21a-deletion virus in HFF and MRC-5 fibroblasts. HFFs or MRC-5 fibroblasts were infected with wild-type virus (AD*wt*) or UL21a-deletion virus (AD*sub*UL21a) at an MOI of 5, cell-free viruses were collected at 72 and 96 hours postinfection (hpi), and their yields were determined by $TCID_{50}$ assay. Data are presented as the fold reduction in growth of AD*sub*UL21a relative to AD*wt* in each cell type. (B) Relative infectivity of progeny virions. MRC-5 cells were infected and cell-free virus was collected as described in (A). The same volumes of viral samples were measured for their viral genome copies by real-time quantitative PCR (qPCR) and for their infectivity by $TCID_{50}$ assay. The result is presented as the viral genome copy number per $TCID_{50}$ unit. The relative genome copy per $TCID_{50}$ unit of wild-type virus at 72 hpi was set to 1. Shown is a representative of at least 2 independent experiments.

Figure 3.2. UL21a-deletion virus is competent for viral entry. (A-B) Translocation of tegument proteins pp65 and pp28 delivered into infected cells. MRC-5 cells were infected with AD*wt* and AD*sub*UL21a at an input genome number equivalent to 1 or 10 $TCID_{50}$ units of wild-type virus/cell, collected at 2 hpi, and analyzed by immunoblotting (A) or immunofluorescence (B), respectively. For immunoblotting, total lysate ("T"), nuclear ("N"), and cytoplasmic ("C") fractions were prepared and analyzed as indicated. Acetyl-Histone H3 and Bip were used as nuclear and cytoplasmic fractionation controls, respectively. For immunofluorescence, the staining patterns of nuclei and viral antigens were shown as blue and green, respectively. Scale bar = 10 μm. (C) Nuclear translocation of virion DNA. Intracellular DNA was isolated from both total and nuclear

fractions at 2 hpi. The quantity of viral genomes in each fraction was determined by qPCR using primers specific for viral UL54 gene and viral genome copies were normalized to cellular genomes using β-actin primers. The percentage of viral DNA translocated into the nucleus was determined by dividing viral genome equivalents in the nuclear fraction by those in the total cell lysate. Shown is a representative of at least 2 independent experiments.

Figure 3.3. HCMV accumulates less IE2-86 protein and IE transcripts at late times, and synthesizes viral DNA at reduced levels in the absence of UL21a. (A) Analysis of viral protein accumulation. MRC-5 cells were infected with AD*wt* or AD*sub*UL21a at an input genome number equivalent to 0.25 (left panel) or 3 (right panel) $TCID_{50}$ units of wild-type virus/cell. Cells were collected at indicated times, and total cell lysates were analyzed by immunoblotting with indicated antibodies. (B) Analysis of viral transcript accumulation. MRC-5 cells were infected with AD*wt* or AD*sub*UL21a at an input genome number equivalent to 0.25 (left panel) or 3 (right panel) $TCID_{50}$ units of wild-type virus/cell. Total RNA was collected at indicated times, amounts of selected viral IE transcripts were measured by reverse transcription-coupled quantitative PCR (RT-qPCR), and normalized to that of GAPDH. Also shown are the genomic structure of the MIE locus and the locations of RT-PCR primers for analyzing IE1, IE2 and total IE transcripts. The normalized amount of viral transcript at 24 hpi during wild-type virus infection was set to 1. (C) Analysis of viral DNA synthesis. MRC-5 cells were infected with AD*wt* or AD*sub*UL21a at either an input genome number equivalent to 0.25 $TCID_{50}$ units of wild-type virus/cell or an MOI of 0.05. Total intracellular DNA was collected, viral DNA was measured by qPCR, and the amount was normalized to cellular genome

copies with primers to β-actin. The normalized amount of viral DNA at 2 hpi during wild-type virus infection was set to 1. Shown is a representative of at least 2 independent experiments.

Figure 3.4. Defects in viral DNA synthesis precede defects in the late accumulation of viral IE transcripts in UL21a-deletion virus infected cells. (A) Analysis of viral DNA synthesis. MRC-5 cells were infected with AD*wt* or AD*sub*UL21a at an input genome number equivalent to 0.25 $TCID_{50}$ units of wild-type virus/cell. Total intracellular DNA was collected at indicated times and analyzed by qPCR as described in legend to Fig. 3C. The normalized amount of viral DNA at 24 hpi during wild-type virus infection was set to 1. (B) Analysis of viral transcript accumulation. MRC-5 cells were infected as described in (A), total RNA was collected, and amounts of MIE transcript were analyzed by RT-qPCR as described in legend to Fig. 3B. The normalized amount of viral transcript at 24 hpi during wild-type virus infection was set to 1. Shown is a representative of at least 2 independent experiments. n.s., $P>0.05$; *, $P<0.05$ by Student's *t* test.

Figure 3.5. Inhibition of viral DNA synthesis reduces the late expression of IE genes in wild-type and UL21a-deletion virus infected cells to equivalent levels. (A) Analysis of viral protein accumulation in the presence of PAA. MRC-5 cells were infected with AD*wt* or AD*sub*UL21a at an input genome number equivalent to 3 $TCID_{50}$ units of wild-type virus/cell in the presence or absence of PAA (100 μg/ml). Cells were collected at indicated times, and total cell lysate was analyzed by immunoblotting with indicated antibodies. (B) Analysis of viral transcript accumulation. MRC-5 cells were infected in the presence ("+") or absence ("-") of PAA as in (A), total RNA was collected and

selected IE transcripts were analyzed by RT-qPCR and normalized to that of GAPDH. The normalized amount of viral transcript at 24 hpi during wild-type virus infection was set to 1. n.s., $P>0.05$; *, $P<0.05$ by Student's *t* test. (C) Viral protein accumulation in presence of PAA or gancyclovir (GCV) during infection of HCMV strains AD169 and TR. MRC-5 cells were infected with AD*wt* (left panel) or TR (right panel) at an MOI of 2 with addition of no drug, PAA (100 µg/ml), or GCV (30 µg/ml). Cells were harvested at indicated times and analyzed by immunoblotting with indicated antibodies. Shown is a representative of at least 2 independent experiments.

Figure 3.6. The growth defect of UL21a-deletion virus is due to the loss of the protein product pUL21a. (A) Diagram of the UL21a locus in UL21a-deletion virus (AD*sub*UL21a), point mutant virus in which 2 stop-codon mutations were introduced into the UL21a ORF (AD*pm*pUL21a), and its marker-rescued virus (AD*rev*pUL21a). (B) Growth kinetic analysis of recombinant HCMV viruses AD*pm*pUL21a and AD*rev*pUL21a. MRC-5 cells were infected with indicated viruses at an MOI of 0.01, cell-free viruses were collected at different days post infection, and their yields were determined by $TCID_{50}$. (C) Analysis of viral protein accumulation. MRC-5 cells were infected with AD*pm*pUL21a or AD*rev*pUL21a at an input genome number equivalent to 0.25 $TCID_{50}$ units of wild-type virus/cell, cells were collected at indicated times, and total lysate was analyzed by immunoblotting. Shown is a representative of at least 2 independent experiments.

Figure 3.7. Expression of pUL21a *in trans* complements the growth of UL21a-deletion virus. MRC-5 cells were transduced with retrovirus expressing GFP (MRC5-GFP) or expressing the GFP/UL21a fusion protein (MRC5-GFP/UL21a), and then infected with

AD*wt* and AD*sub*UL21a at an MOI of 0.2. Cell-free viruses were collected at different days postinfection, and their yields were determined by $TCID_{50}$ in HFFs (A). In addition, AD*wt* and AD*sub*UL21a collected at 12 dpi from MRC5-GFP/UL21a cells were also titered on both MRC5-GFP and MRC5-GFP/UL21a cells to compare their titers measured on complementing and non-complementing cells (B).

Figure 3.8. *De novo* expression of pUL21a is necessary and sufficient for efficient viral DNA synthesis and late accumulation of viral proteins. Viral DNA synthesis (A) and protein accumulation (B) in GFP- or GFP/UL21a- expressing cells infected with recombinant virus that is generated in non-complementing cells. MRC5-GFP cells or MRC5-GFP/UL21a cells were infected with AD*wt* and AD*sub*UL21a virus at an input genome number equivalent to 2 $TCID_{50}$ units of wild-type virus/cell. Infected cells were collected at indicated times and analyzed for viral DNA by qPCR (A) and viral proteins by immunoblotting (B). (C) Viral protein accumulation in normal MRC-5 cells infected with recombinant virus that is generated in GFP- or GFP/UL21a- expressing cells. Normal MRC-5 cells were infected with AD*wt* or AD*sub*UL21a virus that was produced from either GFP ("MRC5-GFP") or GFP/UL21a ("MRC5-GFP/UL21a") expressing cells at an input genome number equivalent to 2 $TCID_{50}$ units of wild-type virus/cell. Infected cells were collected at indicated times and analyzed for viral proteins by immunoblotting. Shown is a representative of at least 2 independent experiments.

TABLE 3.1. Primers and probes used for RT-qPCR

Transcript	qPCR Type	Primers	6FAM-TAMRA Taqman Probe
UL122-123 pre-mRNA	Sybr Green	5'-tctgccaggacatctttctcg-3’ 5’-ggagacccgctgtttccag-3’	Not applicable
UL123 (IE1)	Taqman	5’-caagtgaccgaggattgcaa-3’ 5’-caccatgtccactcgaacctt-3’	5’-tcctggcagaactcgtcaaacaga-3’
UL122 (IE2)	Sybr Green	5’-tgaccgaggattgcaacga-3’ 5’-cggcatgattgacagcctg -3’	Not applicable
UL37	Taqman	5’-ccaaggcgggagaggat-3’ 5’-ctggccttttggtactttagct-3’	5’-ttcaaggcgttttcgctggatcc-3’
TRS1	Sybr Green	5’-ctgtgcaaatgtggaagatacct-3’ 5’-gtccagtcccagagcttgag-3’	Not applicable

Figure 3.1

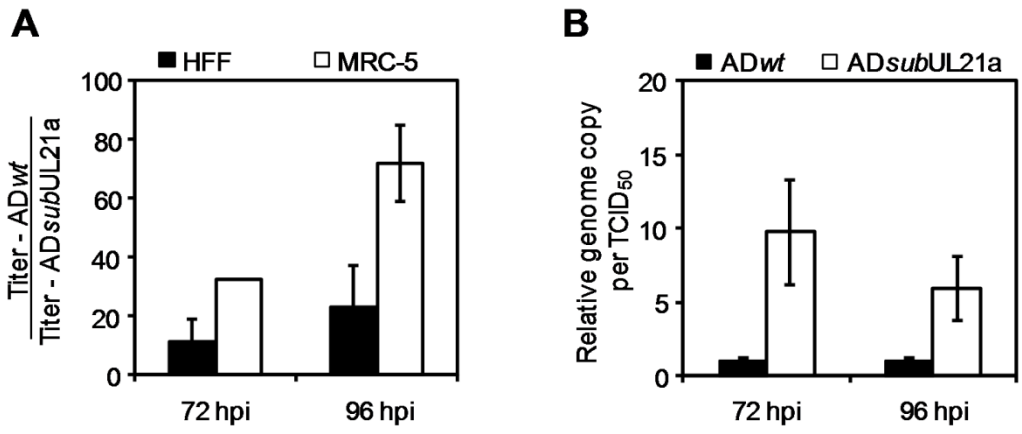

A
HFF
MRC-5
Titer - ADwt / Titer - ADsubUL21a
100
80
60
40
20
0
72 hpi
96 hpi
B
ADwt
ADsubUL21a
Relative genome copy per TCID50
20
15
10
5
0
72 hpi
96 hpi

Figure 3.2

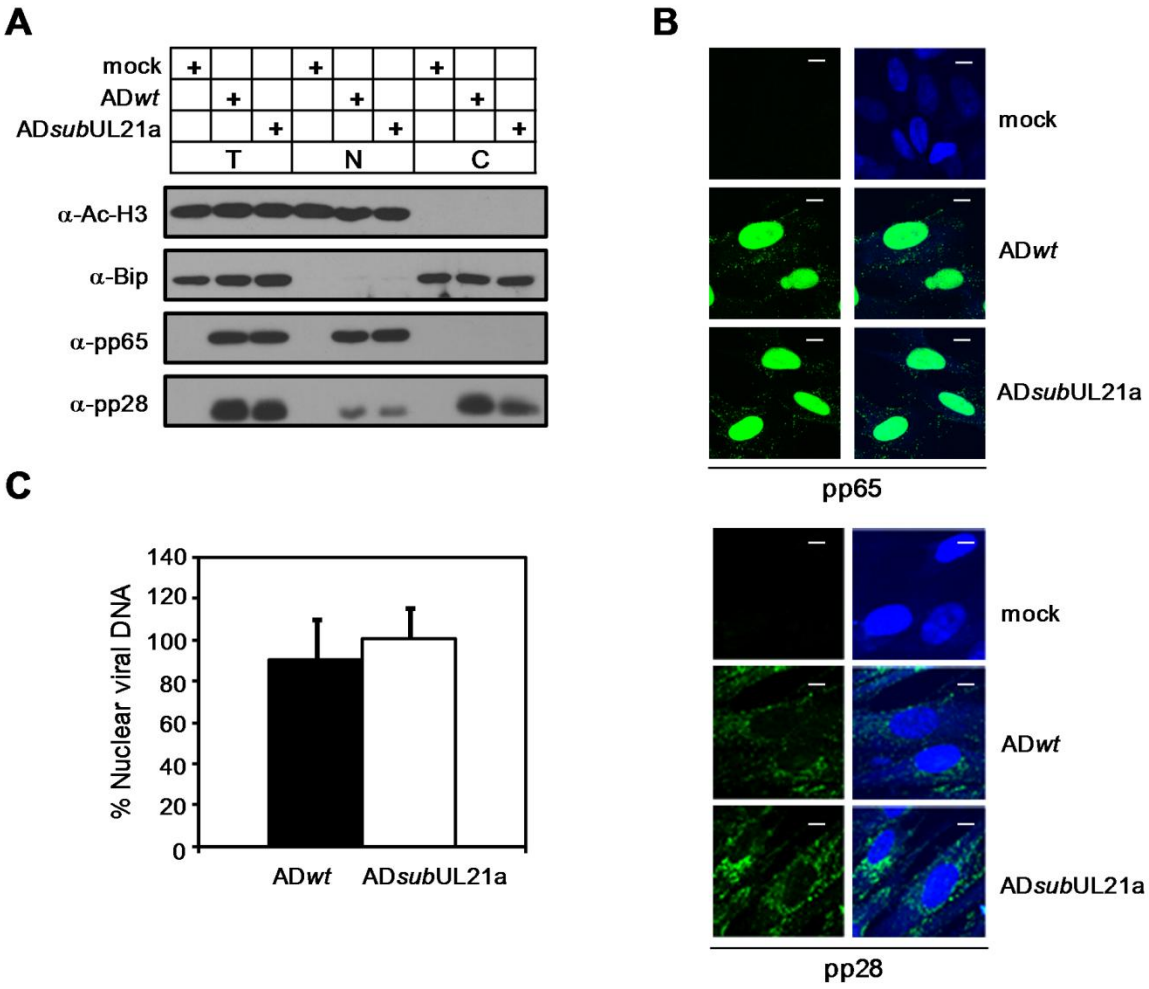

A
mock
ADwt
ADsubUL21a
T
N
C
α-Ac-H3
α-Bip
α-pp65
α-pp28
B
mock
ADwt
ADsubUL21a
pp65
mock
ADwt
ADsubUL21a
pp28
C
% Nuclear viral DNA
140
120
100
80
60
40
20
0
ADwt
ADsubUL21a

Figure 3.3

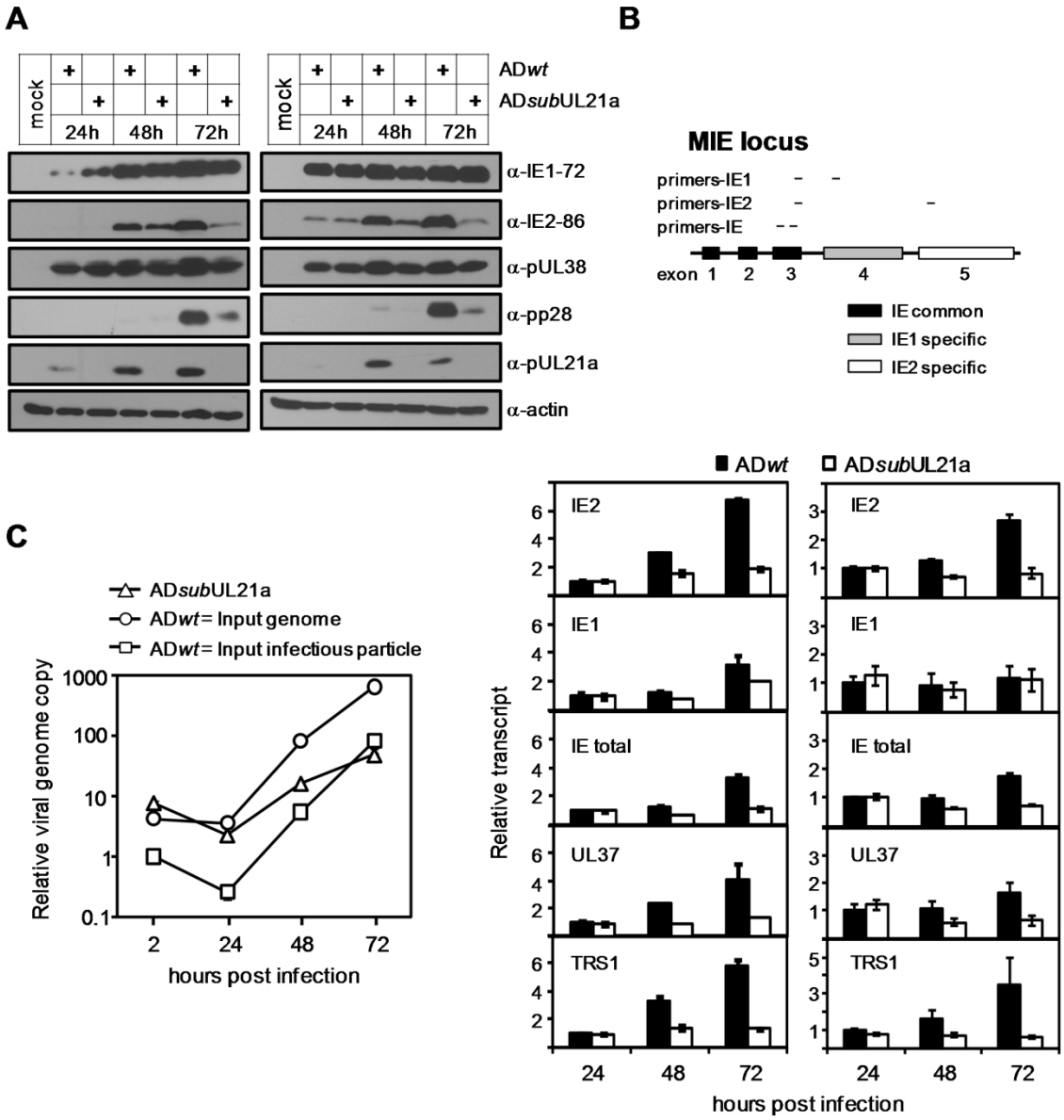

A
mock
24h
48h
72h
ADwt
ADsubUL21a
α-IE1-72
α-IE2-86
α-pUL38
α-pp28
α-pUL21a
α-actin
B
MIE locus
primers-IE1
primers-IE2
primers-IE
exon 1 2 3 4 5
IE common
IE1 specific
IE2 specific
C
ADsubUL21a
ADwt= Input genome
ADwt= Input infectious particle
Relative viral genome copy
hours post infection
ADwt
ADsubUL21a
IE2
IE1
IE total
UL37
TRS1
Relative transcript
hours post infection

Figure 3.4

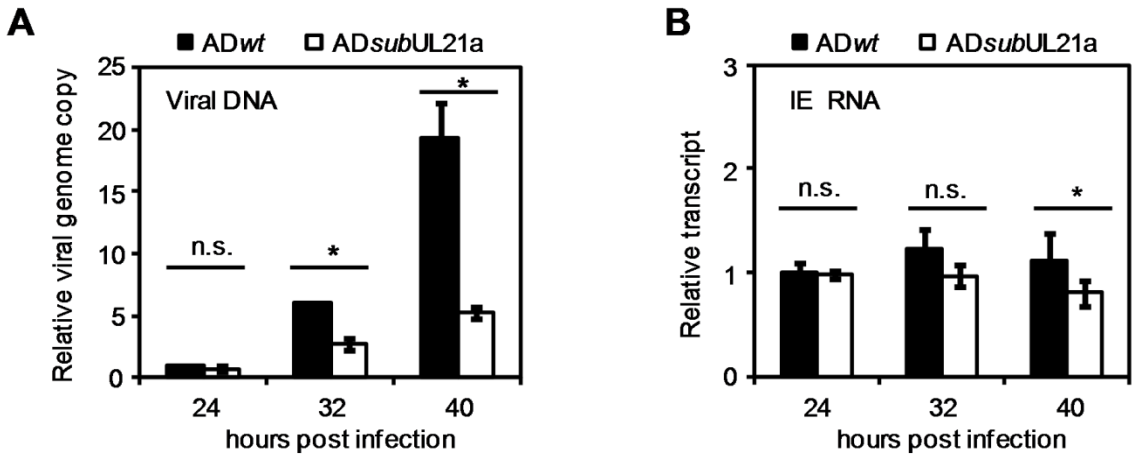

A
ADwt
ADsubUL21a
Viral DNA
Relative viral genome copy
25
20
15
10
5
0
n.s.
*
*
24
32
40
hours post infection
B
ADwt
ADsubUL21a
IE RNA
Relative transcript
3
2
1
0
n.s.
n.s.
*
24
32
40
hours post infection

Figure 3.5

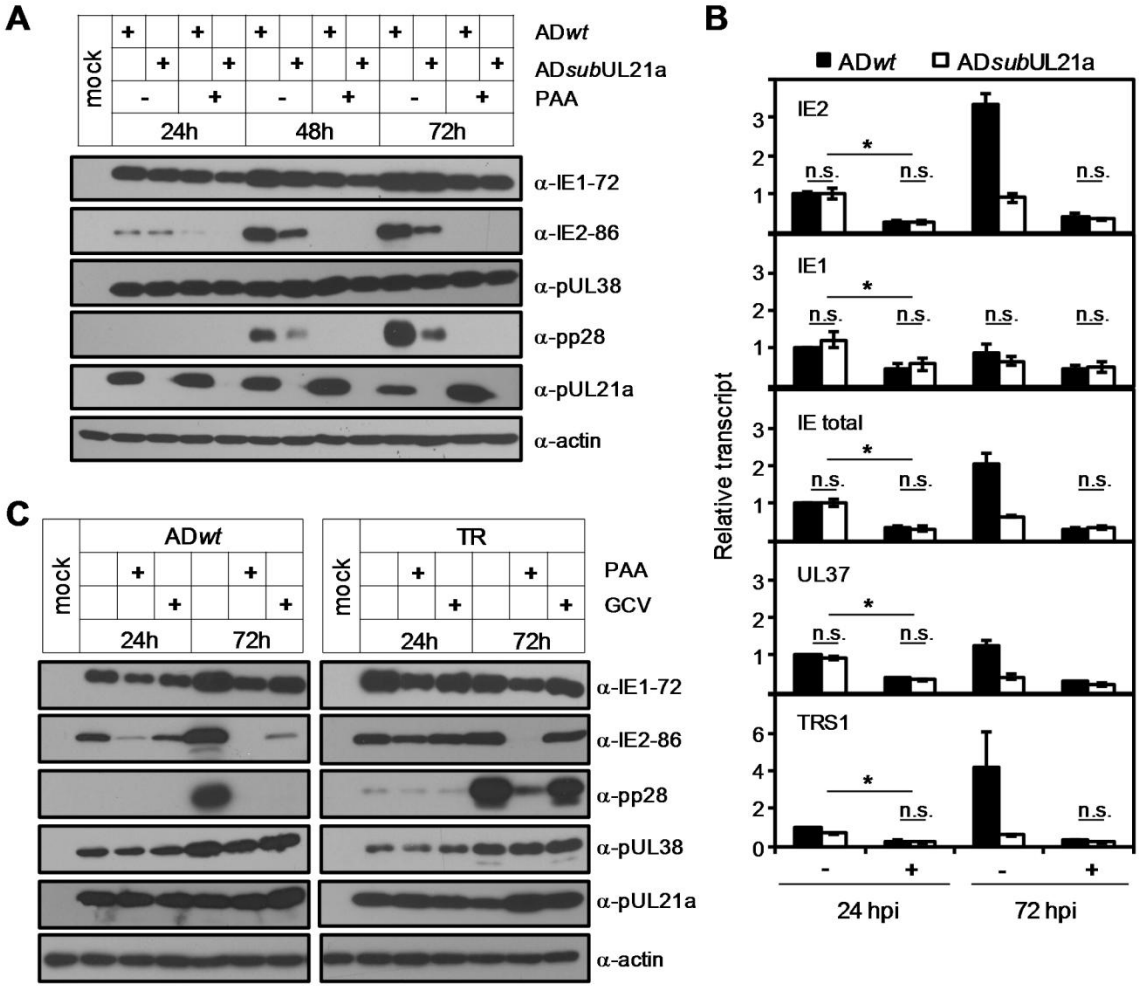

A
mock
ADwt
ADsubUL21a
PAA
24h
48h
72h
α-IE1-72
α-IE2-86
α-pUL38
α-pp28
α-pUL21a
α-actin
B
ADwt
ADsubUL21a
IE2
IE1
IE total
UL37
TRS1
Relative transcript
n.s.
*
24 hpi
72 hpi
C
ADwt
TR
PAA
GCV
24h
72h
α-IE1-72
α-IE2-86
α-pp28
α-pUL38
α-pUL21a
α-actin

Figure 3.6

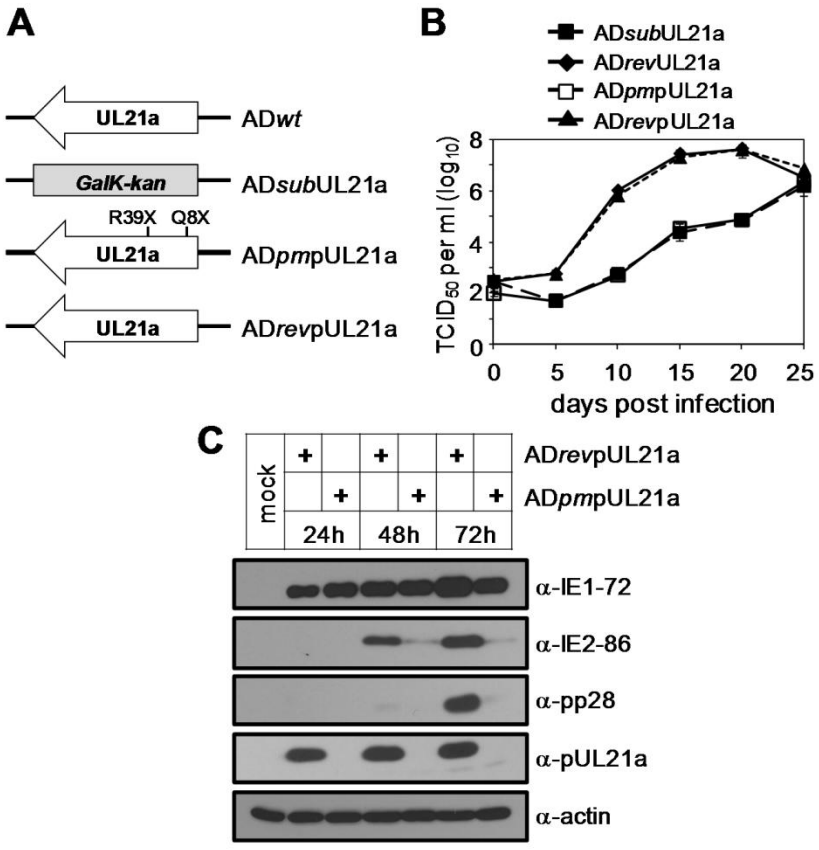

A
UL21a
ADwt
GalK-kan
ADsubUL21a
R39X
Q8X
UL21a
ADpmpUL21a
UL21a
ADrevpUL21a
B
ADsubUL21a
ADrevUL21a
ADpmpUL21a
ADrevpUL21a
TCID50 per ml (log10)
0
2
4
6
8
0
5
10
15
20
25
days post infection
C
mock
+
+
+
+
+
+
ADrevpUL21a
ADpmpUL21a
24h
48h
72h
α-IE1-72
α-IE2-86
α-pp28
α-pUL21a
α-actin

Figure 3.7

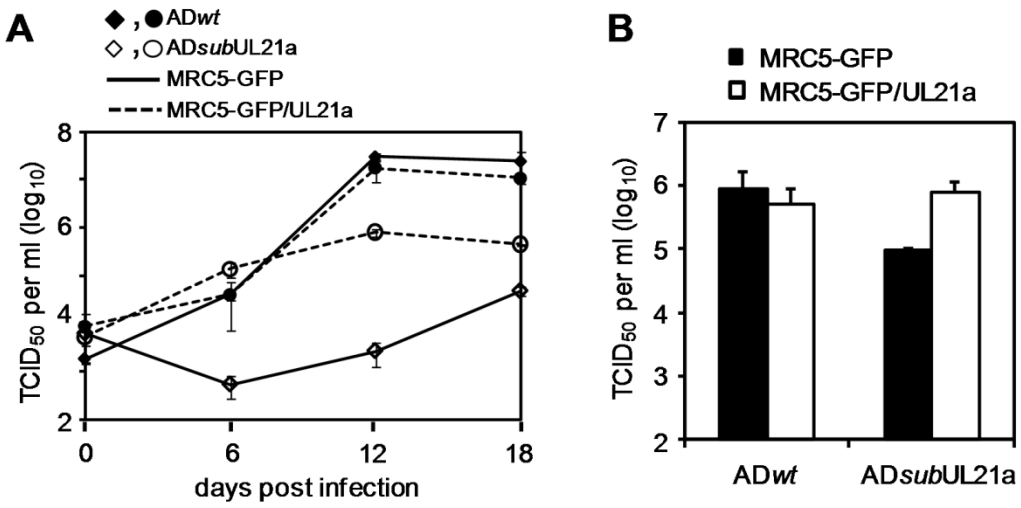
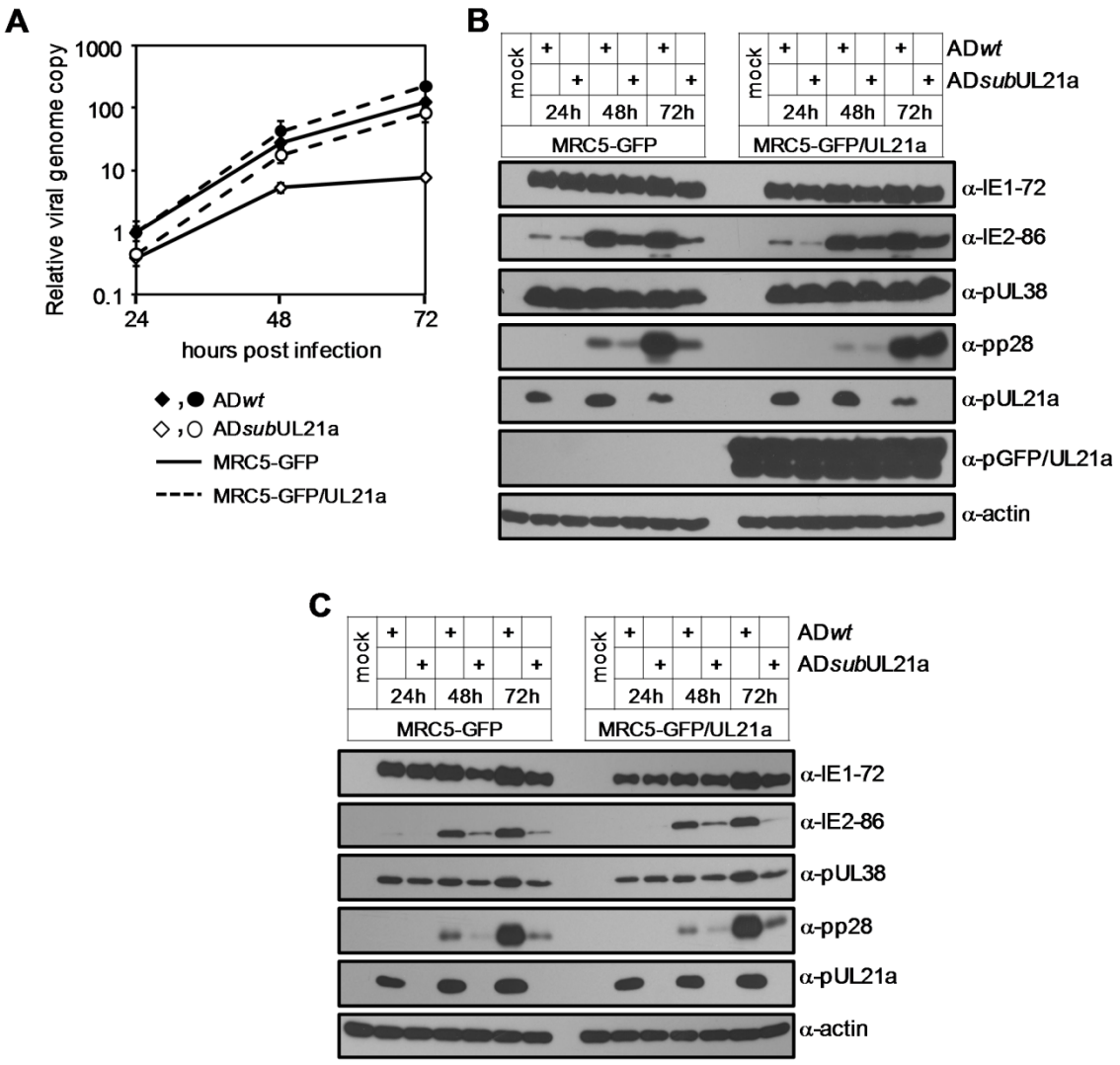

A
◆ ,● ADwt
◇ ,○ ADsubUL21a
MRC5-GFP
MRC5-GFP/UL21a
TCID50 per ml (log10)
8
6
4
2
0
6
12
18
days post infection
B
MRC5-GFP
MRC5-GFP/UL21a
TCID50 per ml (log10)
7
6
5
4
3
2
ADwt
ADsubUL21a

Figure 3.8


A
Relative viral genome copy
1000
100
10
1
0.1
24
48
72
hours post infection
◆ ,● ADwt
◇ ,○ ADsubUL21a
MRC5-GFP
MRC5-GFP/UL21a
B
mock
24h
48h
72h
MRC5-GFP
MRC5-GFP/UL21a
ADwt
ADsubUL21a
α-IE1-72
α-IE2-86
α-pUL38
α-pp28
α-pUL21a
α-pGFP/UL21a
α-actin
C
mock
24h
48h
72h
MRC5-GFP
MRC5-GFP/UL21a
ADwt
ADsubUL21a
α-IE1-72
α-IE2-86
α-pUL38
α-pp28
α-pUL21a
α-actin

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Chapter IV

HCMV pUL21a Regulates the Anaphase Promoting Complex and Targets APC4 and APC5 for Proteasome-Dependent Degradation

ABSTRACT

HCMV encodes several proteins which manipulate important cell cycle regulators in order facilitate viral replication. Recently, HCMV was shown to regulate the anaphase-promoting complex/cyclosome (APC), a multi-subunit E3 ubiquitin ligase which is critical for cell-cycle progression. HCMV regulates the APC in part by targeting two subunits, APC4 and APC5, for proteasome-dependent degradation and causing dissociation of the complex. However, the viral protein which mediates this activity has not been identified. Here we used a focused proteomics approach and found that the HCMV protein pUL21a interacted with the APC during infection and mapped the interaction domain. Importantly, we found that pUL21a was required for the degradation of APC4 and APC5, dissociation of the APC, and accumulation of APC substrates during infection. Finally, knockdown of APC components Cdh1 and APC8 was able to significantly restore late protein expression in the absence of pUL21a, suggesting that the APC^{Cdh1} has anti-viral activity during HCMV infection. Thus, pUL21a appears to promote viral replication, at least in part, by regulating the function of the APC.

INTRODUCTION

Protein degradation plays a role in many different cellular processes, including cell cycle progression, innate immunity, antigen presentation, and the turnover of denatured, unfolded, or oxidized proteins. Most protein degradation is carried out by the ubiquitin-proteasome system (UPS). Ubiquitin is conjugated to target proteins using a cascade of ubiquitin conjugating enzymes, which attaches a poly-ubiquitin chain to a lysine residue of the target protein. These ubiquitin molecules target the protein to the 26S proteasome where it is degraded. As the UPS is a powerful tool for regulating protein levels, it is no surprise that many viruses have co-opted the UPS for their own benefit. On one hand, many viruses target cellular proteins for degradation either by encoding their own E3 ubiquitin ligase, or by targeting proteins to a cellular E3 ubiquitin ligase, thus limiting their usefulness in fighting off infection. Classic examples of viral E3 ligases include HSV-1 ICP0 (3, 7) and the KSHV proteins K3 and K5 (reviewed in (34)), while numerous proteins, including HIV-1 vpr and vif (reviewed in (4, 22)), paramyxovirus V (6), HPV E6 and E7 (reviewed in (2)), and HCMV pp71 (14) and UL27 (28) proteins, target cellular proteins for proteasome-dependent degradation. In fact, pharmacological inhibition of the proteasome often restricts multiple stages of the virus lifecycle, suggesting viruses rely on the UPS for replication (5, 10, 27, 33, 35). On the other hand, it is likely that viruses must also inhibit cellular E3 ligases in order to efficiently replicate as ubiquitination regulates many important cellular processes centreal to virus infection. The SV40 large T antigen inhibits the SCF^{fbw7} ubiquitin ligase which increases cyclin E levels (36), and influenza virus NS1 inhibits TRIM 25 mediated ubiquitination of RIG-I, thereby attenuating interferon production (11).

The anaphase-promoting complex/cyclosome (APC) is a large cullin-ring E3 ubiquitin ligase which is highly conserved across all eukaryotes (reviewed in (23)). It consists of at least 11 subunits and 2 co-activator proteins which act at different stages of the cell cycle. It is composed of 3 separate subcomplexes, including the cullin-ring ligase domain (APC2/APC11/APC10), the specificity arm (APC3/6/7/8), and the bridge (APC1/4/5). The primary functions of the APC are to promote progression through mitosis and to help cells maintain G_1/G_0 until the cell is ready for DNA synthesis. The APC targets >40 proteins for polyubiquitination by interacting with specific elements such as the D-box, KEN-box, and others. Many of these targets, such as the A- and B-type cyclins, are important cell cycle regulators. Due to its essential role in cell cycle progression, the APC has become a novel target for anti-cancer therapeutics (38).

Many viruses modulate the host cell cycle to establish optimal conditions for viral replication. As such, several viruses appear to target the APC, possibly to direct the cell from G_0 or G_1 into S phase, an environment which is potentially more suitable for viral replication. Viral proteins from adenoviruses, chicken anemia virus (CAV), human papillomaviruses (HPV), human T-lymphotropic virus (HTLV), parapoxviruses, and human cytomegalovirus (HCMV) have been shown to alter the function of the APC (1, 15, 16, 19, 21, 29, 31). However, the mechanisms these viral proteins use to disarm the APC are largely unknown, and most have only been studied in overexpression. To date, it has not been definitively shown that the APC can act in an anti- or pro-viral fashion.

HCMV, the prototypic β-herpesvirus, is a globally important opportunistic pathogen which causes severe diseases in immunocompromised individuals and is the leading viral cause of birth defects. This virus stimulates cell cycle progression of

quiescent cells but also blocks host DNA synthesis, arresting host cells in a pseudo-G1/S phase (26). HCMV promotes cell cycle progression at least in part by targeting Rb for proteasome-dependent degradation and inactivating phosphorylation (13, 14). HCMV has recently been shown to target the APC using multiple mechanisms, which may be another means by which the virus stimulates cell cycle progression from G_0/G_1 to a pseudo-G1/S phase (31, 32, 37). The HCMV UL97 kinase can phosphorylate Cdh1, which should render it mostly inactive. However, UL97 mutant virus showed only slightly increased levels of APC activity during the early stages of infection, and by 24 -48 hpi the complex became inactive, suggesting that HCMV can inhibit the APC even in the absence of Cdh1 phosphorylation. It was also shown that HCMV causes dissociation of the APC during infection. This dissociation is likely mediated by the proteasome-dependent degradation of two APC subunits, APC 4 and APC5, observed as early as 6-8 hours after infection. Using a variety of drug treatments, it was shown that the dissociation of the APC and degradation of APC4 and APC5 were due to an unknown early viral protein (31).

Our previous studies of the HCMV protein pUL21a have shown that it is an early protein which is specifically required for efficient viral DNA synthesis (8, 9). Here, using a focused proteomics approach, we found that pUL21a interacted with the APC and mapped the APC binding domain of pUL21a to its C-terminus. Additionally, pUL21a was required for APC4 and APC5 degradation, APC dissociation, and APC substrate accumulation during infection. Finally, shRNA knockdown of the APC significantly restored viral protein expression in the absence of pUL21a, showing for the first time that the APC can act as an inhibitor of viral replication.

MATERIALS AND METHODS

Plasmids and Reagents. pYD-C235 is a pLPCX-derived retroviral vector (Clontech) that expresses a DsRed gene driven by an internal ribosome entry site 2 (IRES2). pYD-C474 was created by PCR amplifying the coding sequence of the pGFP-UL21a fusion protein from pAD*gfp*UL21a and ligating it into the multiple cloning site of pYD-C235. pYD-C580 was created by PCR amplifying a version of UL21a carrying two stop-codon mutations at the N-terminus and inserting it in place of wild-type UL21a in pYD-C474 (8). All truncation mutants of pGFP-UL21a were derived in pYD-C235 while point mutants were derived in pYD-C474. All primers used to create these mutants are listed in Table 1. All point mutants were created using a QuickChange XL kit (Stratagene). pYD-C160 and pYD-C175 are pEBNA derived retroviral vectors expressing GFP and UL21a, respectively. pYD-C648 and pYD-C649 are pLKO lentiviral vectors containing the GFP-TetR and CMV-TetO_2 constructs, respectively (generous gifts from Roger Everett, University of Glasgow Centre for Viral Research) (7). YD-C663 was created by PCR amplifying the UL21a sequence from pYD-C175 and ligating it into the multiple cloning site of YD-C649. All pLKO derived shRNA lentiviral vectors were purchased from the Washington University Children's Discovery Institute/Genome Center.

The primary antibodies used in the present study included anti-β actin (AC-15, Abcam); anti-HA (HA.11, Covance); anti-GFP (3E6 and A6455, Invitrogen); anti-APC3 (AF3.1, Santa Cruz and 610454, BD); anti-APC8 (6114, Biolegend); anti-APC4 (A301-176A, Bethyl laboratories); anti-APC5 (A301-026A, Bethyl laboratories); anti-geminin (sc-13015, Santa Cruz); anti-thymidine kinase (sc-56967, Santa Cruz); anti-Cdh1 (DH01, Calbiochem); anti-cyclin B1 (ms868 P1, Thermo-Scientific); anti-UL21a (9); anti-IE2

(mAB8140, Chemicon); and anti-IE1 and anti-pp28 (generous gifts from Thomas Shenk, Princeton University) (30). Phosphonoacetic acid (PAA), MG132, tetracycline, and polyethylenimine (PEI) were purchased from Sigma-Aldrich. Lipofectamine 2000 and Protein A conjugated Dynal® beads were purchased from Invitrogen.

Cells and Viruses. Primary embryonic lung fibroblasts (MRC-5), human newborn foreskin fibroblasts (HFFs), 293T, and Hela cells were propagated with Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, non-essential amino acids, and penicillin-streptomycin. 293T cells were transfected using either lipofectamine or PEI according to the manufacturers' instructions. For shRNA knockdown, pLKO derived shRNA plasmids were transfected into 293T cells along with packaging plasmids. Lentivirus was collected at 48 and 72 hours and then applied to MRC-5 cells. Those lentiviruses giving the strongest knockdown at 48 hours post-transfection were chosen for further study. The shRNA sequence for Cdh1 knockdown was 5'-CCAGTCAGAACCGGAAAGCCA-3' and the shRNA sequence for APC8 knockdown was 5'-GCAGGAGGTAATATGCTATAA-3'. To create GFP-TetR stable Hela cells, GFP-TetR lentivirus was produced as described for shRNA lentivirus. The virus was then added to Hela cells which were sorted for GFP expression 48 hours later. GFP positive cells were collected, grown in the presence of G418 (500 µg/ml), and frozen as a stable cell line. TetO constructs were then transduced into this cell line, selected with puromycin, and tested for the ability to be regulated by tetracycline (1 µg/ml).

Various recombinant HCMV AD169 viruses were reconstituted from transfection of corresponding BAC-HCMV clones as described. Viral stocks were prepared by ultra-

centrifugation of infected culture supernatant through 20% D-sorbitol cushion and re-suspending pelleted virus in serum-free medium. BAC-HCMV clones used in the present study include: pAD-GFP, which carried the GFP-tagged genome of the HCMV AD169 strain and was used to produce wild-type virus AD*gfp* (30); pAD*gfp*UL21a, which carries an N-terminally GFP-tagged version of pUL21a as previously described (9); pAD*sub*UL21a, which carries a GalK/kanamycin dual mutagenic cassette in place of the entire UL21a coding sequence as previously described (9); pAD*in*UL21a^{stop}, which carries a mutant version of UL21a containing two stop-codons in the N-terminus of UL21a as previously described (8); finally, pAD*gfp*UL21a$^{PR\text{-}AA}$, pAD*gfp*UL21a$^{PH\text{-}AA}$, pAD*in*UL21a$^{PR\text{-}AA}$, and pAD*in*UL21a$^{PH\text{-}AA}$, were constructed by replacing the GalK/kanamycin cassette in pAD*sub*UL21a with a PCR fragment of the GFP tagged or native UL21a gene that contained the desired mutations via linear recombination. GFP-tagged proteins were introduced in pAD*sub*UL21a within an HCMV AD169 BAC which does not contain GFP. All BACs were confirmed by restriction digestion, PCR, and sequencing. HCMV virus titers were determined in duplicate in HFFs by Tissue Culture Infectious Dose 50 ($TCID_{50}$) assays and DNA content was determined by real-time quantitative PCR (qPCR) as described previously (9).

HCMV Infection. MRC-5 cells synchronized by contact inhibition were reseeded at sub-confluency with serum-containing medium for 24 hours. Cells were then inoculated with recombinant HCMV viruses for 1 hr at equal virion DNA content with a wild-type MOI of 3, unless otherwise indicated. Then, inoculum was removed, infected monolayers were rinsed with PBS, and fresh medium was replenished. When needed, PAA (100 µg/ml) was added immediately following infection and MG132 (10 µM) was added 14 hours

prior to harvest. For viral growth analysis, cell-free virus was collected by harvesting medium from infected cultures and viral titers were determined by $TCID_{50}$. Cells were analyzed for protein or RNA as described below. For shRNA knockdown experiments, MRC-5 cells were transduced with lentivirus 24 hours following reseeding. They were then incubated in fresh media for 48 hours and infected as described above.

Immunoprecipitation (IP). Frozen cell pellets were lysed in NP-40 lysis buffer (0.5% NP-40, 50 mM Tris-Cl pH 8.0, 125 mM NaCl, supplemented with protease and phosphatase inhibitors) using an end-over-end rotator at 4˚C for 30 minutes. Lysates were centrifuged at 16,000 x g for 15 min, and supernatants were collected. Lysates were then incubated with protein A dynal-beads coupled to 1 µg anti-HA (HA.11, Covance), 1 µg anti-GFP (3E6, Invitrogen) or 2 µg anti-APC3 (AF3.1, Santa Cruz) monoclonal antibodies for 1 hour at 4˚C. Beads were washed with PBS and eluted in reducing sample buffer by boiling for 5 minutes. Cell lysates (pre-IP) were also collected and boiled in reducing sample buffer.

Protein Analysis. Protein accumulation was determined by immunoblotting. Total cell or pre-IP extracts were collected, washed, and lysed in SDS-containing sample buffer containing protease and phosphatase inhibitors. Proteins were resolved by electrophoresis on a SDS-containing polyacrylamide gel, transferred to a polyvinylidene difluoride (PVDF) membrane, hybridized with a primary antibody, reacted with the horseradish peroxidase-conjugated secondary antibody, and visualized using SuperSignal West Pico chemiluminescent substrate (Thermo Scientific).

Reverse Transcription Coupled-Quantitative PCR Analysis (RT-qPCR). Total RNA was extracted withTRIzol (Invitrogen) and treated with Turbo DNA-free reagent (Ambion) to remove genomic DNA contaminants. cDNA was reverse transcribed from total RNA with random hexamer primers using the High Capacity cDNA reverse transcription kit (Applied Biosystems). cDNA was quantified using SYBR Advantage qPCR Premix (Clontech) and primers for the cellular genes geminin, APC4, APC5, and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as an internal control. Primers are as follows: geminin forward (F) 5'-GCCTTCTGCATCTGGATCTCTT-3' and reverse (R) 5'-CGATGTTTCCTTTTGGACAAGC-3' (31); APC4 F 5'-ATTCTCGTCCTTGGAGGAAGCTCT-3' and R 5'-TTCTGGCCATCCGAGTTACTTCAG-3' (31); APC5 F 5'-GTGCCATGTTCTTAGTGGCCAAGT-3' and R 5'-GATGCGCTCTTTGCAGTCAACCTT-3' (31); GAPDH F 5'-CTGTTGCTGTAGCCAAATTCGT-3' and R 5'-ACCCACTCCTCCACCTTTGAC-3' (8). cDNA from infected cells was used to generate a standard curve for each gene examined. The standard curve was then used to calculate the relative amount of specific RNA present in a sample.

RESULTS

pUL21a interacts with the anaphase-promoting complex (APC)

HCMV gene UL21a encodes a 15 kDa protein expressed with early gene kinetics which facilitates efficient viral DNA synthesis (8, 9). However, the protein contains no identifiable domains or significant homology with any known protein, thus its function(s) remains unknown. To identify potential functions for this protein we took a proteomics approach to identify its interacting partners. We used a recombinant virus which expresses a GFP-tagged UL21a in its normal locus to identify its binding partners. This virus grows with wild-type kinetics, and importantly, the pGFP-UL21a fusion protein appears to be more stable and thus is present at much higher levels than native pUL21a, making it feasible to detect interacting proteins (9). We infected cells with HCMV expressing either free GFP (AD*gfp*) or pGFP-UL21a fusion protein (AD*gfp*UL21a) at an MOI of 5, collected cells at 48 hpi, and immunoprecipitated (IP'd) lysates using a rapid one-step immunoaffinity purification on magnetic beads coated with protein A and coupled to GFP antibody. The eluted fraction was resolved by electrophoresis, silver stained, and UL21a-specific bands were identified by mass spectrometry. At least 7 specific bands were detected upon silver staining the eluted protein fraction (Fig. 4.1A), and the two bands depicted with arrows were identified as APC subunits APC3 and APC8, both of which ran at the expected molecular weights of ~97 and ~65 kDa, respectively (Fig. 4.1A and data not shown). To confirm these interactions, we collected HCMV infected cells and immunoprecipitated lysates with both the GFP antibody and APC3 antibody and analyzed eluted proteins by immunoblot (Fig. 4.1B). Free GFP and pGFP-UL21a fusion proteins were expressed at similar levels, but only pGFP-UL21a

pulled down APC3 and APC8 during infection. The fact that pGFP-UL21a appears to pull-down multiple subunits of the APC strongly suggests that it binds the complex, and not just a free subunit. In the reciprocal IP, APC3 pulled down APC8 and GFP-UL21a, but could not pull-down free GFP. These interactions are specific; neither GFP nor APC3 antibody could pull down cellular PCNA, and HA antibody could not pull down any of the proteins detected here (data not shown).

To show that these interactions were not an artifact of the GFP tag, IPs were performed on both AD*gfp* (wild-type) and AD*sub*UL21a (UL21a mutant) virus in the presence or absence of proteasome inhibitor, MG132 (Fig. 4.1C). MG132 dramatically stabilizes the levels of pUL21a, thus increasing the likelihood that we could detect an APC-pUL21a interaction (9). In the presence of MG132, APC3 pulled down native pUL21a, but not PCNA or the viral processivity factor UL44. Addition of MG132 is necessary as pUL21a is highly unstable (Fig. 4.1C). Otherwise it could not accumulate to levels that would readily allow defection of its interaction proteins.

To test if pUL21a was sufficient to bind the APC in the absence of other viral proteins, we transfected *gfp*UL21a^{wt} and *gfp*UL21a^{stop} (UL21a containing two stop-codons at the N-terminus of the protein) expressing plasmids into 293T cells and performed the Co-IP-Western blot as described previously (Fig. 4.1D). pGFP-UL21a^{wt} and pGFP-UL21a^{stop} fusion proteins were expressed at equal levels, but only pGFP-UL21a^{wt} was able to pull-down both APC3 and APC8. Furthermore, APC3 was able to pull-down pGFP-UL21a^{wt} but not pGFP-UL21a^{stop}, confirming the interaction. We conclude that pUL21a interacts with the APC during infection and in overexpression.

The C-terminus of pUL21a is necessary and sufficient for APC binding

To dissect the mechanism for pUL21a to interact with the APC, we first identified its interaction domain. Based on sequence alignment of pUL21a with its homologues in chimpanzee CMV (CCMV) and Rhesus CMV (RhCMV), we divided the 123-amino acid UL21a coding sequence into three regions (N, M, and C) (Fig. 4.2A). The N-terminus contains a highly conserved arginine-rich motif (ARM). The middle region has limited conservation, and the C-terminus contains several conserved amino acids along with a highly hydrophobic stretch of amino acids (LVLLL). Based on this analysis, we created individual truncation mutants fused to GFP, overexpressed them in 293T cells and analyzed their ability to immunoprecipitate the APC (Fig. 4.2B-C). All truncation mutants were expressed at similar levels, and could be efficiently pulled-down with the GFP antibody (Fig. 4.2C and data not shown). As shown previously, the full-length pGFP-UL21a fusion protein was able to pull-down both APC3 and APC8, while a stop-codon fusion protein did not. Only the C-terminal fragment of pUL21a could consistently pull-down both APC3 and APC8. To show the C-terminus is also necessary for binding, we made a fusion protein with a combination of the N and M fragments of pUL21a, which was also unable to pull-down the APC. Thus the C-terminus of pUL21a was both necessary and sufficient for APC binding.

To identify residues critical for APC binding, we made alanine substitution mutants of conserved residues within the C-terminus of pGFP-UL21a. We created a total of 5 different mutations covering 12 conserved residues, with each mutation spanning 2-3 consecutive conserved residues (i.e. PL-AA) (Fig. 4.2A). As a control we made an alanine mutation of the non-conserved proline-histidine residues at amino acids 111-112

of the HCMV protein (Fig.2A). Once again, all mutants were stable and were efficiently pulled-down with the GFP antibody (Fig. 4.3A and data not shown). None of the first four mutants, including those that disrupt the hydrophobic stretch of amino acids, had a noticeable effect on APC binding. However, the ability of the most C-terminal conserved mutant, PR109-110AA to pull down APC 3 and APC 8 was markedly compromised. Importantly, mutation of the adjoining non-conserved residues, PH111-112AA, had no affect on pUL21a binding to the APC. As proline-histidine have nearly identical biological properties as proline-arginine, it is unlikely that the PR-AA mutant would disrupt the overall structure of the protein while the PH-AA mutant would not. We confirmed that these residues were important for APC binding in the context of infection by inserting both the GFP-tagged and native forms of these proteins into recombinant AD169 BACs (AD*gfp*UL21a^{PR-AA}, AD*gfp*UL21a^{PH-AA}, AD*in*UL21a^{PR-AA}, and AD*in*UL21a^{PH-AA}) and assayed their ability to interact with the APC during infection. The GFP-UL21a^{PH-AA} mutant protein interacted with APC3 and APC8 and was pulled-down with APC3 antibody while the GFP-UL21a^{PR-AA} mutant was not (Fig. 4.3B). Finally, in the presence of MG132, APC3 antibody pulled-down pUL21a^{PH-AA} but not pUL21a^{PR-AA} (Fig. 4.3C).

pUL21a is required for APC dissociation and proteasome-dependent degradation of APC4 and APC5

It has previously been shown that HCMV induces APC dissociation and APC4 and APC5 proteasome-dependent degradation, beginning as early as 6-8 hpi (31). To test whether pUL21a may be required for these activities, we infected cells with various recombinant HCMV viruses and analyzed cell lysates for APC subunit accumulation and

APC stability. As previously reported, we saw dramatic reduction in total APC4 and APC5 protein levels during wild-type infection at 24 hpi. However, when infected with UL21a mutant virus, no difference was observed in APC4 and APC5 accumulation when compared to mock-infected cells (Fig. 4.4A, left panel). To rule out the possibility that the absence of APC4/APC5 degradation during UL21a mutant virus infection was due to the lack of late gene expression (8), we analyzed APC4/APC5 accumulation in the presence of the viral DNA synthesis inhibitor PAA. In the presence of PAA late genes were not expressed (data not shown), but APC4 and APC5 were still degraded during wild-type but not UL21a mutant virus infection although total levels of APC4 were increased by PAA (Fig. 4.4A, right panel). The loss of APC4 and APC5 was due to proteasome-dependent degradation as the addition of MG132 restored expression in wild-type but did not affect expression in UL21a mutant virus infected cells (Fig. 4.4B). In addition, cells were infected with point mutant recombinant viruses created in Fig. 4.3C and analyzed for APC4/APC5 accumulation (Fig. 4.4B). Like UL21a mutant virus, AD*in*UL21a$^{PR\text{-}AA}$ was unable to reduce accumulation of these subunits, while AD*in*UL21a$^{PH\text{-}AA}$ recombinant virus was able to target these proteins for proteasome-dependent degradation similar to wild-type virus. These data correlate with the ability of these point mutants to bind the APC. No appreciable difference was observed in APC4 and APC5 transcript levels between mock, wild-type, and UL21a mutant virus infections, further suggesting the difference is posttranslational and likely due to proteasome-dependent degradation of these subunits (Fig. 4.4C).

To test whether expression of pUL21a was sufficient to reduce the protein levels of APC4 and APC5, pUL21a was overexpressed in 293T cells and total cell lysates

analyzed for APC4/APC5 protein accumulation. The expression of pUL21a but not GFP was sufficient to significantly reduce the levels of APC4 and APC5, but not APC3 in 293T cells (Fig. 4.4D, left panel). As an alternative method to address this question, we developed an inducible expression system where lentivirus carrying UL21a under a CMV-TetO promoter was transduced into Hela cells stably expressing the TetR gene. Expression of pUL21a could be detected 24 h after the addition of tetracycline but not in its absence, suggesting very tight regulation of this promoter (Fig. 4.4D, right panel). The addition of tetracycline significantly reduced the levels of APC4 and APC5, but not APC3 in these cells, once again showing that pUL21a is sufficient to reduce APC4 and APC5 protein levels (Fig. 4.4D, right panel).

We next tested whether pUL21a was also required for APC dissociation during infection. Cells were infected with wild-type or UL21a mutant virus and lysates were collected at 20 hpi and IP'd with APC3 antibody. The ability of multiple APC subunits to complex with APC3 was assayed by western blot (Fig. 4.5A). APC3 was pulled down in roughly equal proportions compared to total protein levels between mock, wild-type, and UL21a mutant virus infection. APC4 and APC5 were pulled down with much reduced efficiency during wild-type virus infection, consistent with their proteasome degradation. Finally, we analyzed the APC subunit APC10/Doc1 for its ability to remain in the APC complex during infection. APC10 is proposed to bind APC substrates along with the co-activator proteins. It is primarily bound to the APC2/APC11 subunits on the opposite side of the complex, but its location in the inner cavity of the APC allows it to also contact APC3 and APC16. Upon wild-type virus infection, APC10 is no longer pulled down by APC3 antibody as in the mock-infection. In the absence of pUL21a, the APC10 subunit

remains in the APC complex and is present upon pulldown with APC3 (Fig. 4.5A, right panel). The integrity of the APC during wild-type virus infection is largely restored upon addition of MG132, although total protein levels are reduced likely due to increased cell death (Fig. 4.5A, right panel and data not shown). These data are recapitulated using point mutant recombinant viruses AD*in*UL21a$^{PR\text{-}AA}$ and AD*in*UL21a$^{PH\text{-}AA}$ which act like UL21a mutant and wild-type virus, respectively (Fig. 4.5B).

APC activity is regulated by pUL21a

To determine if pUL21a-dependent APC dissociation resulted in reduced APC activity, we first analyzed the accumulation of multiple APC substrates during wild-type and UL21a mutant virus infection (Fig. 4.6A). Both geminin and the APC co-activator protein Cdh1 (which is also a substrate of the APC) were dramatically stabilized by wild-type virus infection compared to mock by at least 24 hpi, as previously described. The accumulation of these proteins was significantly reduced during UL21a mutant virus infection, suggesting increased APC activity. This phenotype was maintained and even increased throughout the infection. There was no difference in geminin transcript accumulation between wild-type and mutant virus, suggesting these differences in protein levels are not due to transcript regulation (Fig. 4.6B). Thymidine kinase (TK) was also consistently reduced during UL21a mutant virus infection, but this was not apparent until later times of infection (48-72 hpi). Interestingly, some substrates such as cyclin B1, were not reduced and in some experiments even increased during UL21a mutant virus infection. It is possible that the level of APC inhibition by UL97-mediated Cdh1 phosphorylation is sufficient for certain APC substrates. Alternatively the cyclin B1 transcript may be mis-regulated during UL21a mutant virus infection. The addition of

PAA had no effect on substrate accumulation, confirming that this phenotype is not due to the lack of late gene expression during UL21a mutant virus infection (Fig. 4.6C). Addition of MG132 largely restored substrate accumulations during UL21a mutant virus infection, proving that the difference is due to increased proteasome-dependent degradation (Fig. 4.6D). Once again, these phenotypes were recapitulated using the point mutant viruses AD*in*UL21a$^{PR\text{-}AA}$ and AD*in*UL21a$^{PH\text{-}AA}$ (Fig. 4.6D).

To show that reduced APC substrate accumulation during UL21a mutant virus infection is due to increased APC activity, we used shRNA knockdown of multiple APC components to deplete APC activity during virus infection. Potential shRNAs were tested against APC8 and Cdh1. For these experiments, we transduced cells with shRNA expressing lentivirus, then let the cells incubate 48 h. We then infected cells with wild-type, UL21a mutant, PR-AA, and PH-AA recombinant viruses as previously described, collected cells at 72 hpi, and analyzed protein accumulations by Western blot. Both shRNAs efficiently knocked down their respective targets (Fig. 4.7A and data not shown). Cells infected with control shRNA lentivirus displayed the expected difference in APC substrates Cdh1 and geminin between wild-type and UL21a mutant virus infection. Knockdown of APC8 restored geminin and Cdh1 levels in UL21a mutant virus infected cells to that of cells infected with wild-type virus. Likewise, Cdh1 knockdown restored geminin levels in UL21a mutant virus infected cells (Fig. 4.7A). Once again, these results were recapitulated using the point mutant viruses AD*in*UL21a$^{PR\text{-}AA}$ and AD*in*UL21a$^{PH\text{-}AA}$ (Fig. 4.7B).

Knockdown or inhibition of the APC is sufficient to restore UL21a mutant virus late protein expression

To determine whether APC inhibition by UL21a is beneficial for viral replication, we used both proteasome inhibition and shRNA knockdown of APC components and determined if it could restore viral late gene expression in UL21a mutant virus. We previously showed that the UL21a mutant virus is defective in viral DNA synthesis and the late expression of both IE2-86 and pp28, so we used these proteins as markers for viral replication (8). Inhibition of the proteasome with MG132 even for only 14 hours (58-72 hpi) significantly restored expression of pp28 and IE2-86 (Fig. 4.8A). Additionally, knockdown of Cdh1 and to a lesser extent APC8 was clearly able to increase pp28 and IE2-86 expression (Fig. 4.8B). This data suggests that Cdh1 and likely the APC^{Cdh1} complex is limiting the ability of the UL21a mutant virus to replicate.

The failure to regulate the APC alone is not sufficient to negatively affect HCMV growth in human fibroblasts.

To determine whether APC regulation was also necessary for efficient virus growth, we performed growth curve analysis with an HCMV pUL21a APC binding mutant, AD*in*UL21a$^{PR\text{-}AA}$. We have shown in multiple assays that this mutant is not only unable to bind the APC, but is also defective in targeting APC4/APC5 for degradation and in regulating the APC. However, in a multi-step growth curve, this mutant virus grew with equivalent kinetics to AD*wt* and AD*in*UL21a$^{PH\text{-}AA}$ recombinant viruses, while AD*in*UL21a^{stop} had a severe growth defect (Fig. 4.8C). This data suggests that the failure to regulate the APC alone is not sufficient to negatively affect HCMV growth in human fibroblasts, and that pUL21a likely has additional functions to promote growth of HCMV in human fibroblasts.

DISCUSSION

In warfare, bridge destruction is a common way to limit the ability of an enemy to obtain supplies, maintain communication, and mobilize. Here we describe a viral protein whose function is to destroy an important molecular bridge, likely aiding the virus in its attempt to overtake the host cell. We found that the HCMV protein pUL21a interacts with the anaphase-promoting complex (APC), regulates it, and promotes its dissociation, likely by targeting components of the APC bridge (APC4/APC5) for proteasome-dependent degradation. In fact, when cells infected with wild-type virus were treated with the proteasome inhibitor MG132, APC4 and APC5 were stabilized, and the APC remained intact (Fig. 4.5). Under these conditions, pUL21a was clearly expressed, suggesting that the lack of dissociation was not due to the lack of pUL21a expression. Thus, the evidence strongly suggests that proteasome-dependent degradation of APC4 and APC5 causes the dissociation of the APC during wild-type virus infection.

How does pUL21a target these proteins for proteasome-dependent degradation? It is unlikely that pUL21a itself would act as an E3 ligase as it contains no domain or motif which could have E3 ligase activity. We hypothesize that pUL21a either directly binds to APC4/APC5 and targets them to the proteasome directly, or it recruits a cellular E3 ligase to directly ubiquitinate these proteins. To identify the mechanism, we are currently working to identify the direct binding partner for pUL21a, and also determining whether APC4 and APC5 are ubiquitinated during wild-type virus infection.

Several other viral proteins have been shown to regulate the APC using a variety of mechanisms. Some, like HPV E2, appear to bind and inhibit the Cdh1 activator protein

(1), while the Orf virus protein PACR appears to function as an APC11 mimic, but without the enzymatic activity of a cullin-RING ligase (19, 20). The viral protein whose mechanism appears to be most closely related to pUL21a is the CAV protein apoptin, which likely binds the APC at the bridge and causes its dissociation (29). However, it is presently unclear whether apoptin targets APC subunits for proteasome-dependent degradation or if it simply dissociates the complex by preventing the association of two specific subunits within the bridge of the APC. The fact that two independent viral proteins have devised similar mechanisms to disrupt the APC suggests that targeting the bridge of the APC may be an efficient means to disable the activity of this complex.

Most viral proteins which regulate the APC have only been studied in overexpression. Recently, the UL97 kinase of HCMV was shown to phosphorylate Cdh1 and partially inhibit the complex during infection (31). APC substrate accumulation was slightly delayed during a UL97 mutant virus infection, however all substrates eventually accumulated at wild-type levels. It is unknown whether the phosphorylation of Cdh1 by UL97 has an effect on viral replication. Deletion of PACR, the parapoxvirus APC11 mimic, or apoptin from CAV, resulted in viruses with a substantial growth defect in tissue culture (19, 24). However, it has not been clarified whether PACR or apoptin inhibit the APC during infection. Furthermore, it is unknown whether the defects of these mutant viruses are due to an increase in APC activity. Here, we showed that a defect in late viral gene expression could be partially restored by targeted knockdown of the APC (Fig.4.8B). While it is likely that the APC restricts viral gene expression by inhibiting viral DNA synthesis as this is the specific defect of the UL21a mutant virus, we cannot rule out the possibility that both pp28 and IE2-86 are substrates of the APC. Regardless,

our study is the first to show that knockdown of APC components can result in increased viral gene expression, suggesting that the APC has antiviral activities for HCMV which can be overcome by expression of pUL21a. Despite this, mutation of the APC binding domain in pUL21a did not affect the ability of HCMV to replicate in tissue culture like a complete deletion of pUL21a does. This data suggests that pUL21a must have an additional function which is critical for replication of the virus (see below).

What would be the benefit for the virus to disable the APC? The APC targets >40 proteins for proteasome-dependent degradation, and any of these proteins could be important for viral replication. Alternatively, the virus may inhibit the APC to allow entry of the cell into a different stage of the cell cycle where activity of other proteins (i.e. CDKs) may be crucial for viral replication. It is interesting to note that in both poxvirus and herpesvirus families, the only viruses which have been found to modulate the APC (Parapoxviruses and HCMV) are those that do not encode for their own thymidine kinase (TK) and ribonucleotide reductase subunit M2 (RRM2) enzymes. Both of these enzymes are critical for the production of deoxyribonucleotides. This correlates well with the fact that the UL21a deletion virus has a large defect in viral DNA synthesis (8). It seems plausible that APC regulation is one mechanism used by HCMV and other viruses to accumulate a substantial concentration of nucleotides so they can efficiently replicate their own genomes. This hypothesis has previously been proposed but has not been experimentally verified to date (19, 37). However, the APC also targets proteins involved in cellular DNA replication, glycolysis/glutaminolysis, and multiple kinases/cyclins, all of which could impact viral replication (17). Also, viral proteins have been shown to be targets of the APC (18), and several HCMV proteins contain a putative D-box,

suggesting the APC may be able to degrade viral proteins as well (31). Future work will need to address which substrates of the APC may be important for viral replication.

Based on these results and observations, we propose a model whereby pUL21a disrupts the APC in order to increase the accumulation of at least one APC substrate (Fig. 4.9). The increased substrate protein(s) will help the cell modulate its nucleotide pool/energy level/kinase activity, which in turn enhances the ability of the virus to synthesize its DNA and produce late viral proteins. However, based on the fact that a PR-AA point mutant cannot regulate the APC but still grows with wild-type kinetics, it is likely that pUL21a also has at least one additional function. This function may be redundant with APC inhibition, based on the data showing that knockdown of Cdh1 or treatment with MG132 significantly restored late viral gene expression during mutant virus infection. In dividing cells, only one of these two functions may be necessary for efficient viral DNA replication; however, perhaps in terminally differentiated cells such as neurons, both functions are necessary for efficient viral DNA replication as nucleotide or energy pools in these cells would be much lower. It will be of interest to test the ability of UL21a mutant viruses to grow in these cell types.

Study of the mechanism pUL21a uses to disarm the APC may have broad impacts in cancer and neuronal disease. Due to its essential role in cell cycle progression, the APC is a novel anti-cancer target (12). Recently drugs have been developed targeting this complex and may be useful therapeutics (38). In fact, our preliminary data shows that overexpression of pUL21a can prevent transformed cell lines from growing (data not shown). Thus, study of pUL21a may shed light on novel mechanisms to target the APC

for drug development, provide useful information on how the APC is assembled, and give further impetus to target the APC for anti-cancer therapies.

Finally, several studies have recently highlighted the importance of APC^{Cdh1} in neuronal development (reviewed in (25)). Congenital HCMV disease leads to neuronal defects and severe complications such as blindness, hearing loss, and mental retardation. HCMV infects neuronal precursor cells, differentiated neurons, and astroglia, and deregulation of neuronal cells during HCMV infection is likely linked to the ability of this virus to cause neuronal defects. It is interesting to speculate that inhibition of the APC by pUL21a may be one of the factors leading to neuronal disease in congenitally infected infants. Thus, study of pUL21a and the regulation of the APC during HCMV infection may uncover new insights into the molecular basis of HCMV pathogenesis.

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FIGURE LEGENDS

Figure 4.1. pUL21a interacts with the APC. (A) Identification of pUL21a interacting partners. MRC-5 cells were infected with AD*gfp* or AD*gfp*-UL21a at an MOI of 5, collected at 48 hpi, and immunoprecipitated (IP'd) with GFP antibody. Eluted proteins were run on an SDS-containing polyacrylamide gel and silver stained. The bands indicated with an arrow were identified by mass spectrometry as APC3 (100 kDa) and APC8 (65 kDa). (B-C) pUL21a immunoprecipitates the APC during infection. (B) MRC-5 cells were infected as described in A and lysates were IP'd with both GFP and APC3 antibodies. Cell lysates and IP'd proteins were analyzed by immunoblotting with indicated antibodies. GFP blots were cropped to save space but are from the same lane and exposed film. (C) MRC-5 cells were infected with AD*gfp* or AD*sub*UL21a at an input genome number equivalent to 3 $TCID_{50}$ units of wild-type virus/cell (as described in Materials and Methods) in the presence (+) or absence (-) of 10μM MG132. Cells were collected at 24 hpi and lysates were IP'd with APC3 antibody. Cell lysates and IP'd proteins were analyzed by immunoblotting with indicated antibodies. (D) pUL21a interacts with the APC when overexpressed in 293T cells. 293T cells were transfected with GFP-UL21a or GFP-UL21a^{stop} expressing plasmids. The GFP-UL21a^{stop} protein contains 2 stop-codons at the N-terminus of UL21a and thus does not express pUL21a. Cells were collected at 72 hours post-transfection (hpt) and cell lysates were IP'd as in B. Cell lysates and IP'd proteins were analyzed by immunoblotting with indicated antibodies. PCNA and pUL44 were used as cellular and viral negative controls, respectively. * indicates non-specific cross-reacting bands.

Figure 4.2. The C-terminus of pUL21a is both necessary and sufficient for APC binding. (A) Aminio acid alignment of UL21a proteins from human, chimpanzee, and rhesus CMVs. Boxes above aligned proteins divide the protein into N-terminal, Middle, and C-terminal regions. Red boxes indicate points mutants created and used in part D-F. (B) Diagrams of truncation mutants created. (C) The C-terminus of pUL21a binds to APC. GFP fusion truncation mutants were transfected into 293T cells and cells were collected at 72 hpt and IP'd with GFP antibody. Cell lysates and IP'd proteins were analyzed by immunoblotting with indicated antibodies.

Figure 4.3. Identification of residues critical for APC binding. (A) UL21a point mutant proteins indicated in Fig. 4.2A were tested for APC binding as described in Fig. 4.2C. (B and C) GFP fusion (B) and native (C) UL21a$^{PH\text{-}AA}$ and UL21a$^{PR\text{-}AA}$ point mutant proteins were introduced into the AD169 genome and analyzed for APC binding during infection. Cells were infected with indicated virus at an input genome number equivalent to 3 $TCID_{50}$ units of wild-type virus/cell in the presence (+) or absence (-) of 10μM MG132. Cells were collected at 20 hpi and lysates were IP'd with APC3 antibody. Cell lysates and IP'd proteins were analyzed by immunoblotting with indicated antibodies.

Figure 4.4. pUL21a targets APC4 and APC5 for proteasome-dependent degradation. (A) pUL21a is required for reduced accumulation of APC4 and APC5 during HCMV infection. MRC-5 cells were infected in the presence or absence of PAA with AD*gfp* or AD*sub*UL21a. They were then collected at 24 hpi, and total cell lysates were analyzed by immunoblotting with indicated antibodies. pUL21a, IE1-72, and actin antibodies were used as infection and loading controls, respectively. (B) pUL21a APC binding is required for APC4 and APC5 proteasome-dependent degradation during HCMV infection. MRC-5

cells were infected in the presence or absence of MG132 (added at 6 hpi) with AD*gfp*, AD*sub*UL21a, AD*in*UL21a^{PH-AA}, or AD*in*UL21a^{PR-AA}. They were collected at 20 hpi and analyzed as in part A. (C) HCMV infection does not alter APC4 or APC5 transcript levels. MRC-5 cells were infected with AD*gfp* or AD*sub*UL21a, total RNA was collected at indicated times, and amounts of APC4/APC5 transcripts were measured by reverse transcription-coupled quantitative PCR (RT-qPCR) and normalized to that of GAPDH. (D) pUL21a overexpression results in reduced accumulation of APC4/APC5. (Left panel) pUL21a or GFP were overexpressed in 293T cells, puromycin was added at 24 h to select for expressing cells, and cells were collected at 72 hpt. (Right panel) GFP-TetR expressing Hela cells were transduced with lentivirus expressing pUL21a under a CMV promoter with a Tet operator. Cells were selected with puromycin for 72 h then treated with tetracycline for 24 h. In both cases, cell lysates were extracted with NP-40 buffer and were analyzed by immunoblotting with indicated antibodies.

Figure 4.5. pUL21a is required for APC dissociation during HCMV infection. (A-B) MRC-5 cells were infected in the presence or absence of 10μM MG132 (added at 6 hpi) with either AD*gfp* and AD*sub*UL21a (A) or AD*in*UL21a^{PH-AA} and AD*in*UL21a^{PR-AA} (B) and collected at 20 hpi. Cell lysates were IP'd with APC3 antibody, then both cell lysates and IP'd proteins were analyzed by immunoblotting with indicated antibodies.

Figure 4.6. pUL21a inhibits the proteasome-dependent degradation of multiple APC substrates during infection. (A) pUL21a is required for the accumulation of APC substrate proteins. MRC-5 cells were infected with AD*gfp* or AD*sub*UL21a and were collected at indicated times. Total cell lysates were analyzed by immunoblotting with indicated antibodies. (B) pUL21a is not required for geminin transcript accumulation.

MRC-5 cells were infected with AD*gfp* or AD*sub*UL21a and total RNA was collected at indicated times. The amount of geminin transcript was measured by RT-qPCR and normalized to that of GAPDH. (C) Reduced accumulation of APC substrates in UL21a mutant virus is not due to a defect in late gene expression. MRC-5 cells were infected in the presence or absence of PAA with AD*gfp* or AD*sub*UL21a and were collected at 72 hpi. Total cell lysates were analyzed by immunoblotting with indicated antibodies. (D) Proteasome-dependent degradation of APC substrates is dependent on UL21a APC binding. MRC-5 cells were infected in the presence or absence of MG132 (added at 6 hpi) with AD*gfp*, AD*sub*UL21a, AD*in*UL21a$^{PH\text{-}AA}$, or AD*in*UL21a$^{PR\text{-}AA}$. Cells were collected at 20 hpi, and total cell lysates were analyzed by immunoblotting with indicated antibodies.

Figure 4.7. APC knockdown restores APC substrate accumulation during UL21a mutant virus infection. (A-B) MRC-5 cells were transduced with the indicated shRNA expressing lentivirus (see Materials and Methods for shRNA sequence) and allowed to sit for 48 hours. The cells were then infected with either AD*gfp* and AD*sub*UL21a (A) or AD*in*UL21a$^{PH\text{-}AA}$ and AD*in*UL21a$^{PR\text{-}AA}$ (B) and were collected at 72 hpi. Total cell lysates were analyzed by immunoblotting with indicated antibodies.

Figure 4.8. APC inhibition is sufficient to restore UL21a mutant virus late gene expression, but is not required for viral growth in fibroblasts. (A) Proteasome inhibition restores viral late protein expression of UL21a mutant virus. MRC-5 cells were infected in the presence or absence of MG132 (added at 58 hpi) with AD*gfp* and AD*sub*UL21a. They were collected at 72 hpi and analyzed for indicated viral late protein expression by immunoblotting with pp28 and IE2-86 antibodies. (B) APC knockdown restores viral late

protein expression of UL21a mutant virus. Cells lysates from the experiment in Fig. 4.6A were analyzed for indicated viral late protein expression by immunoblotting with pp28 and IE2-86 antibodies. (C) pUL21a APC binding mutants grow with wild-type kinetics in human fibroblast cells. MRC-5 cells were infected at an MOI of 0.01 $TCID_{50}$/cell with AD*gfp* (WT), AD*in*UL21a^{stop} (STOP), AD*in*UL21a$^{PR\text{-}AA}$ (PR-AA), and AD*in*UL21a$^{PH\text{-}AA}$ (PH-AA). Viral titers at indicated days post-infection were determined by $TCID_{50}$ assay.

Figure 4.9. Model of pUL21a functions during HCMV infection. pUL21a inhibits the APC which in turn increases the accumulation of multiple APC substrates, at least one of which may be important for viral replication. The increased APC substrates may lead to increased dNTP pools, energy production, or CDK activity, which ultimately results in increased viral DNA replication. pUL21a also likely has a second function which may be redundant with APC inhibition, as abrogation of its ability to inhibit the APC does not results in reduced replication of HCMV in human fibroblasts.

TABLE 4.1. Primers used to create UL21a truncation/point mutants

UL21a Construct	Type of genetic alteration	Primer pairs used to introduce alteration[a]	Plasmid Name
UL21a N 1-47	Truncation	5'-gtgactcgag ATGGGAGGTAGCCCTGTTCC-3' 5'-ggaattctta ATTTTGGAAAAGCCTCCGAC-3'	pLP-UL21a-N (1-47)
UL21a M 46-87	Truncation	5'-gtgactcgag CAAAATCATATACATCCAGAA-3' 5'-ggaattctta ATTGGGTAGGGGTTGCTGCGG-3'	pLP-UL21a-M (46-87)
UL21a C 84-122	Truncation	5'-gtgactcgag CCCCTACCCAATCCGCTGGTG-3' 5'-ggaattc TTAAAACTGGTCCCAATGTTCTT-3'	pLP-UL21a-C (84-123)
UL21a NM 1-87	Truncation	5'-gtgactcgag ATGGGAGGTAGCCCTGTTCC-3' 5'- ggaattc TTAATTGGGTAGGGGTTGCTGCGG -3'	pLP-UL21a-NM (1-87)
UL21a PL84-85AA	Point mutant	5'-CGACCTCCGCAGCAAGCC GCACCCAATCCGCTGGTG-3' 5'-CACCAGCGGATTGGGTGC GGCTTGCTGCGGAGGTCG-3'	pLP-UL21a^{PL-AA}
UL21a PLV88-90AAA	Point mutant	5'-GCAACCCCTACCCAATGCGG CGGCGCTACTGCTGGACGATG-3' 5'-CATCGTCCAGCAGTAGCGCC GCCGCATTGGGTAGGGGTTGC-3'	pLP-UL21a$^{PLV-AAA}$
UL21a LLL91-93AAA	Point mutant	5'-CTACCCAATCCGCTGGTGGCAG CGGCGGACGATGTTCCCCCCCAT-3' 5'-ATGGGGGGGAACATCGTCCGCC GCTGCCACCAGCGGATTGGGTAG-3'	pLP-UL21a$^{LLL-AAA}$
UL21a DD94-95AA	Point mutant	5'-CTGGTGCTACTGCTGGCC GCTGTTCCCCCCCATGTA-3' 5'-TACATGGGGGGGAACAGC GGCCAGCAGTAGCACCAG-3'	pLP-UL21a^{DD-AA}
UL21a PR109-110AA	Point mutant	5'-GCTCCTTACCGCGTCGCC GCTCCCCACCCCATGATT-3' 5'-AATCATGGGGTGGGGAGC GGCGACGCGGTAAGGAGC-3'	pLP-UL21a^{PR-AA}
UL21a PH111-112AA	Point mutant	5'-TACCGCGTCCCCCGTGCC GCCCCCATGATTCCCGAA-3' 5'-TTCGGGAATCATGGGGGC GGCACGGGGGACGCGGTA-3'	pLP-UL21a^{PH-AA}

[a] Restriction enzyme sites are in lowercase, UL21a specific sequences are in upper case, base pair changes used to create mutations are underlined and in upper case.

Figure 4.1

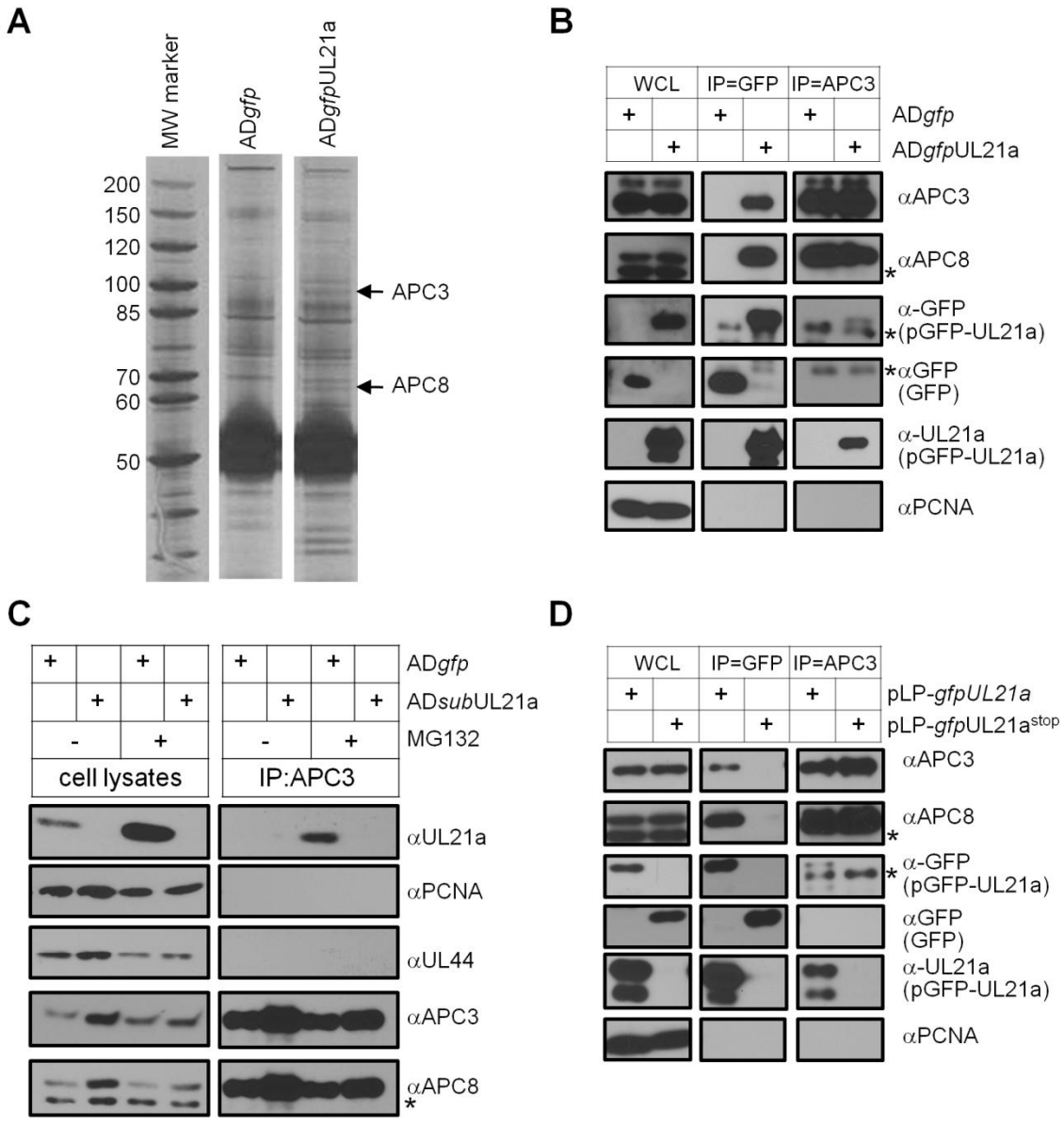

A
MW marker
ADgfp
ADgfpUL21a
200
150
120
100
85
70
60
50
APC3
APC8
B
WCL
IP=GFP
IP=APC3
ADgfp
ADgfpUL21a
αAPC3
αAPC8
*
α-GFP
*(pGFP-UL21a)
*αGFP
(GFP)
α-UL21a
(pGFP-UL21a)
αPCNA
C
ADgfp
ADsubUL21a
MG132
cell lysates
IP:APC3
αUL21a
αPCNA
αUL44
αAPC3
αAPC8
*
D
WCL
IP=GFP
IP=APC3
pLP-gfpUL21a
pLP-gfpUL21a^stop
αAPC3
αAPC8
*
α-GFP
(pGFP-UL21a)
αGFP
(GFP)
α-UL21a
(pGFP-UL21a)
αPCNA

Figure 4.2

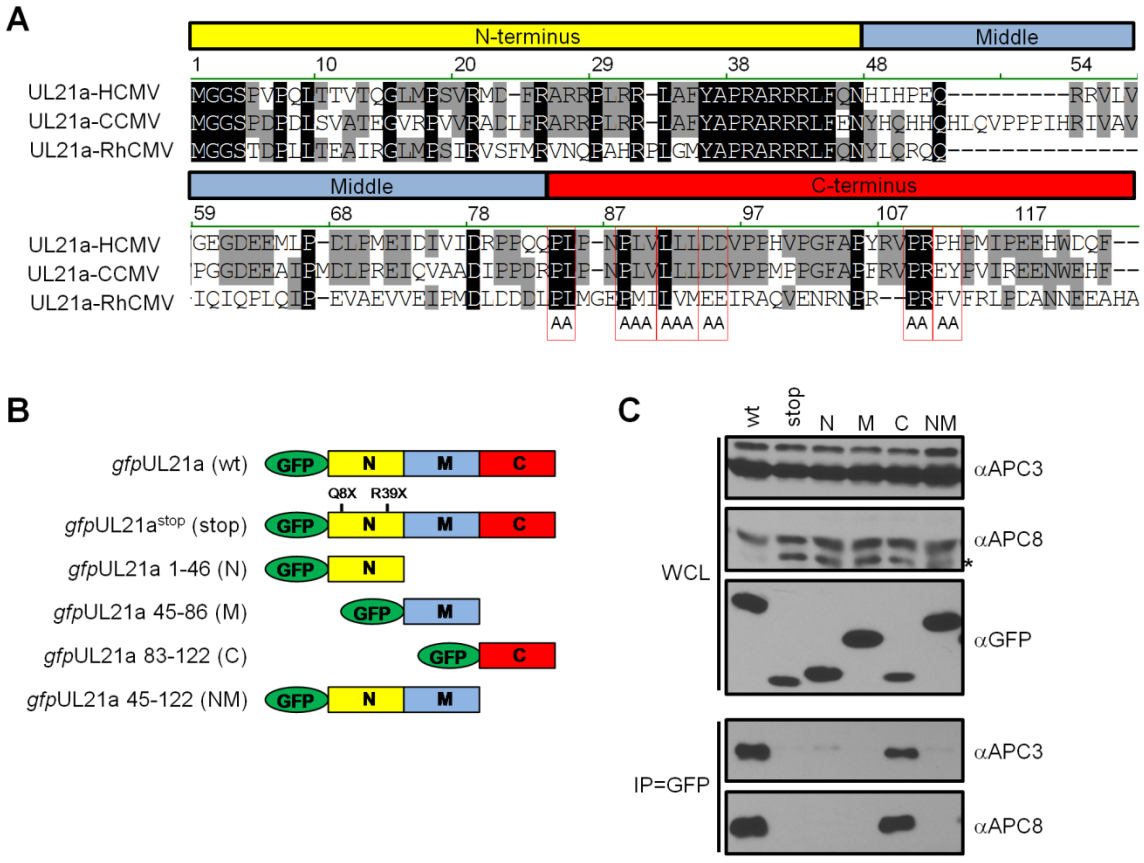

A
N-terminus
Middle
Middle
C-terminus
UL21a-HCMV
UL21a-CCMV
UL21a-RhCMV
B
gfpUL21a (wt)
gfpUL21a^stop (stop)
gfpUL21a 1-46 (N)
gfpUL21a 45-86 (M)
gfpUL21a 83-122 (C)
gfpUL21a 45-122 (NM)
GFP
N
M
C
Q8X
R39X
C
wt
stop
N
M
C
NM
αAPC3
αAPC8
αGFP
WCL
IP=GFP

Figure 4.3

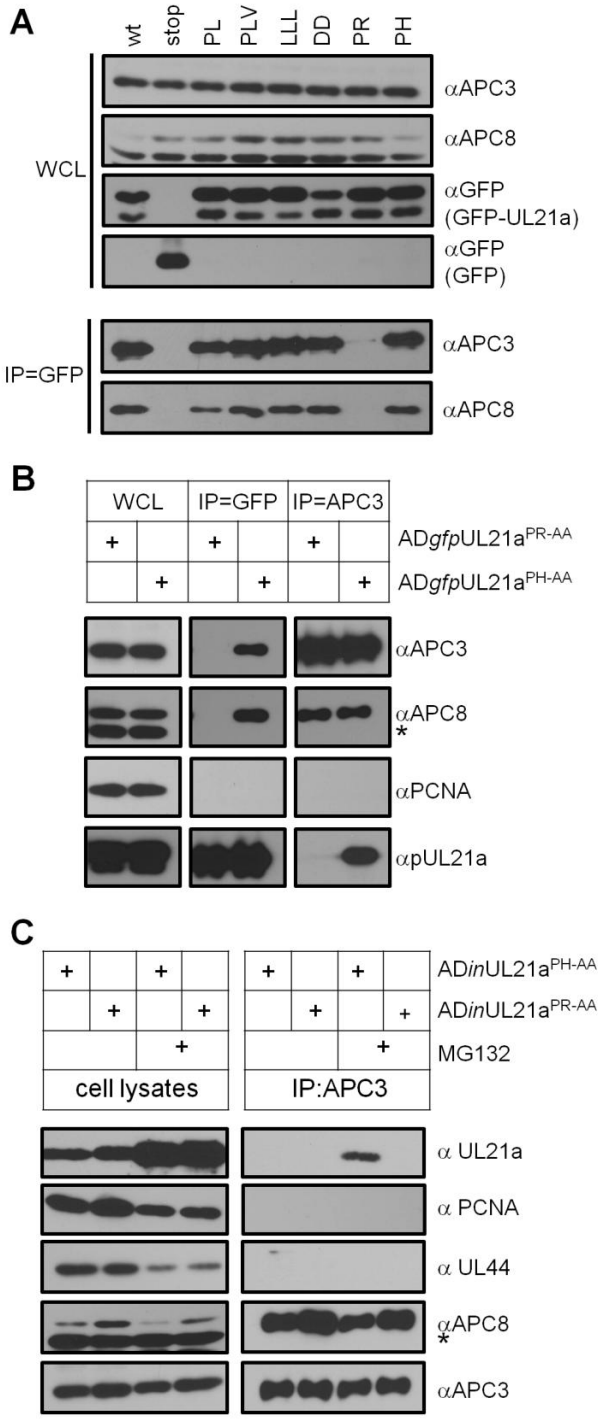

A
wt
stop
PL
PLV
LLL
DD
PR
PH
WCL
αAPC3
αAPC8
αGFP (GFP-UL21a)
αGFP (GFP)
IP=GFP
αAPC3
αAPC8
B
WCL
IP=GFP
IP=APC3
ADgfpUL21aPR-AA
ADgfpUL21aPH-AA
αAPC3
αAPC8 *
αPCNA
αpUL21a
C
ADinUL21aPH-AA
ADinUL21aPR-AA
MG132
cell lysates
IP:APC3
α UL21a
α PCNA
α UL44
αAPC8 *
αAPC3

Figure 4.4

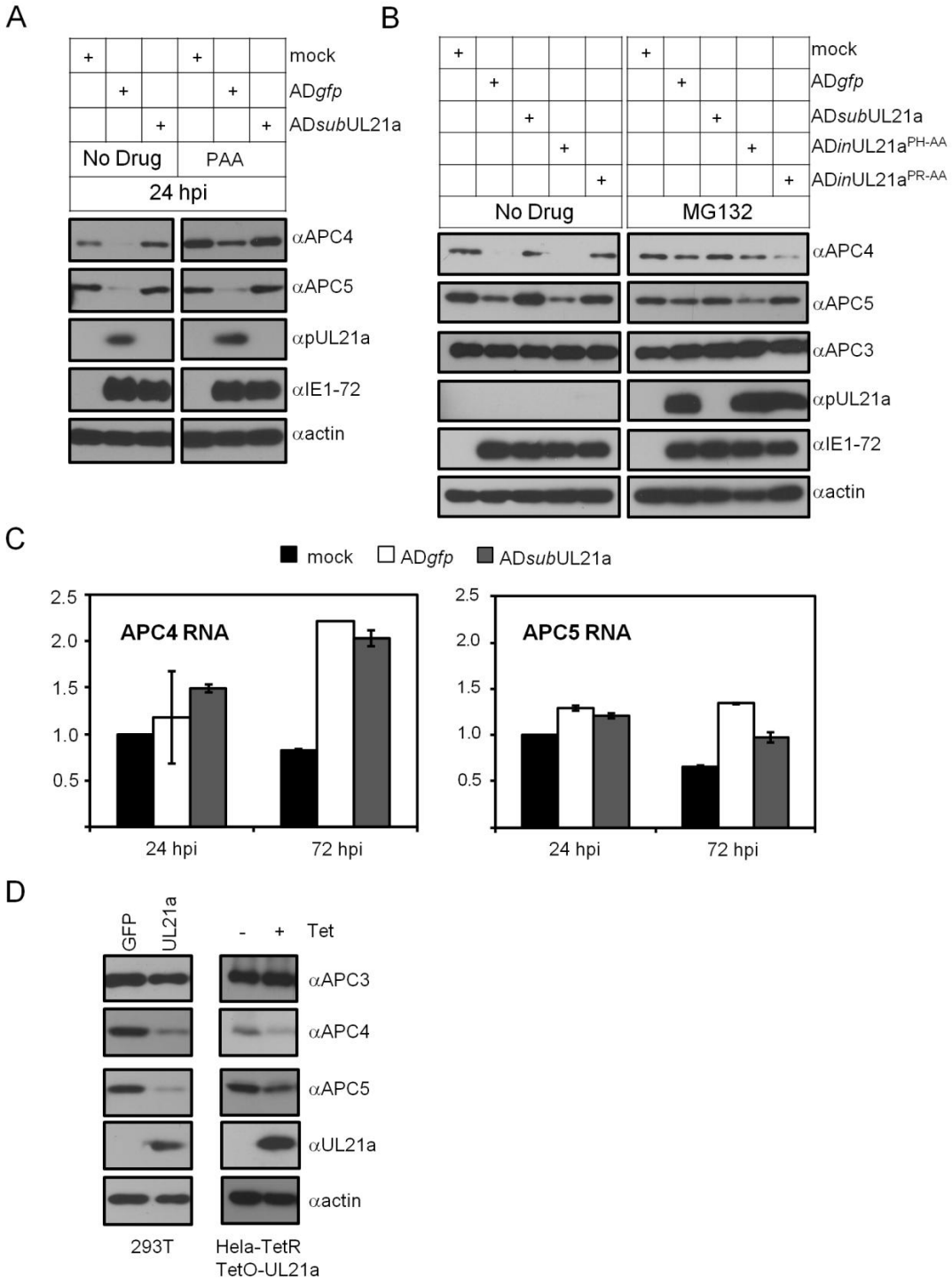

A
mock
ADgfp
ADsubUL21a
No Drug
PAA
24 hpi
αAPC4
αAPC5
αpUL21a
αIE1-72
αactin
B
mock
ADgfp
ADsubUL21a
ADinUL21aPH-AA
ADinUL21aPR-AA
No Drug
MG132
αAPC4
αAPC5
αAPC3
αpUL21a
αIE1-72
αactin
C
mock
ADgfp
ADsubUL21a
APC4 RNA
APC5 RNA
2.5
2.0
1.5
1.0
0.5
24 hpi
72 hpi
D
GFP
UL21a
- + Tet
αAPC3
αAPC4
αAPC5
αUL21a
αactin
293T
Hela-TetR
TetO-UL21a

Figure 4.5

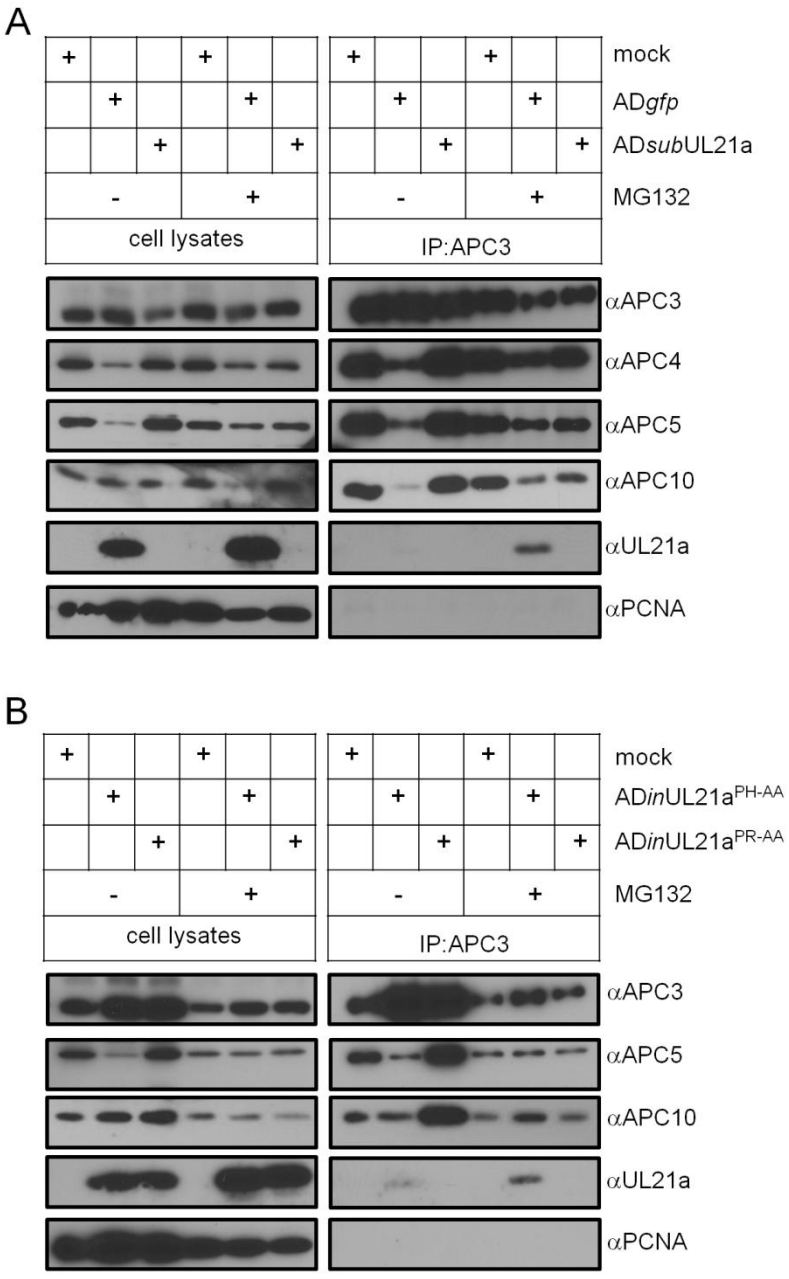

A
mock
ADgfp
ADsubUL21a
MG132
cell lysates
IP:APC3
αAPC3
αAPC4
αAPC5
αAPC10
αUL21a
αPCNA
B
mock
ADinUL21aPH-AA
ADinUL21aPR-AA
MG132
cell lysates
IP:APC3
αAPC3
αAPC5
αAPC10
αUL21a
αPCNA

Figure 4.6

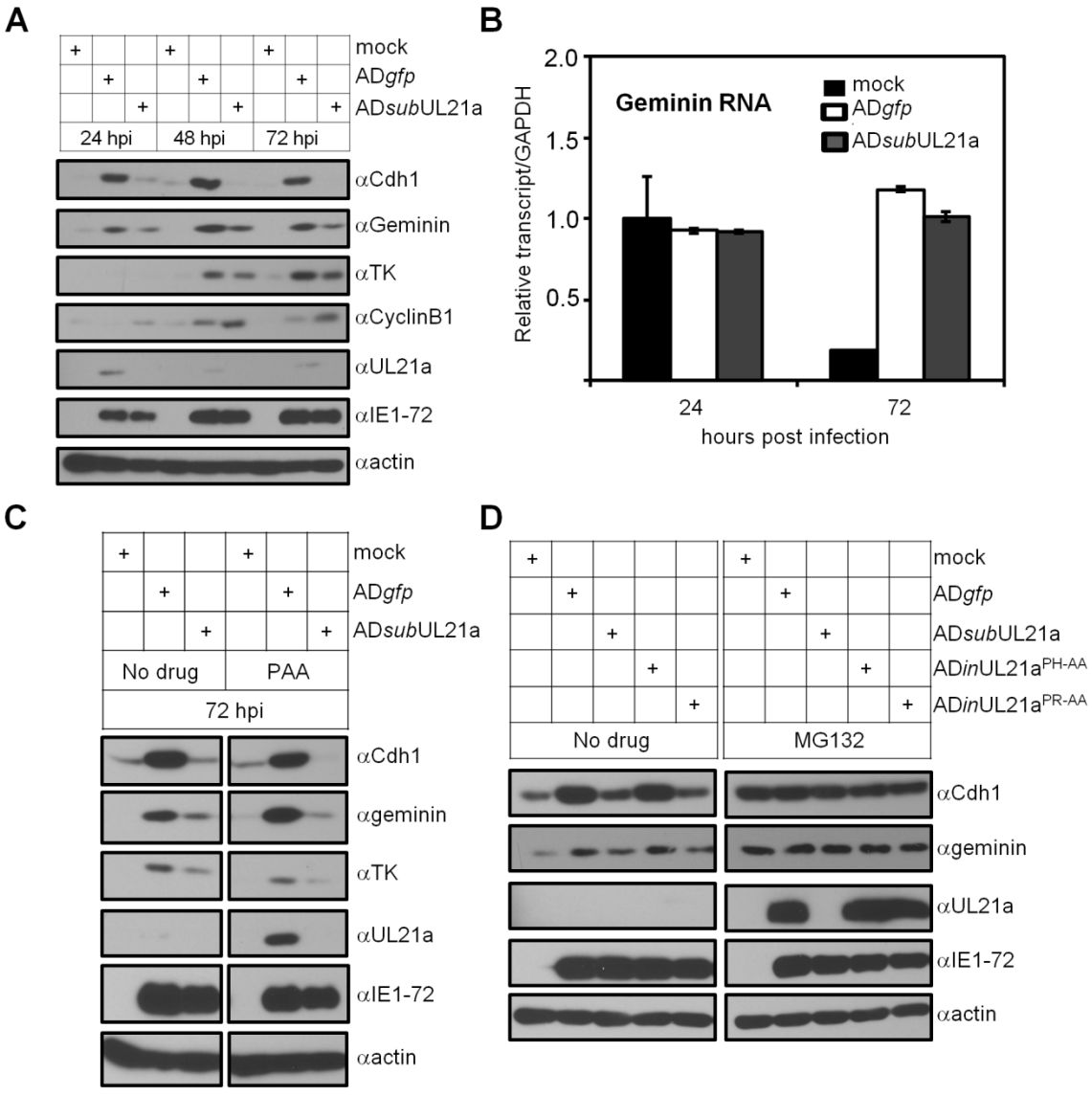

A
mock
ADgfp
ADsubUL21a
24 hpi
48 hpi
72 hpi
αCdh1
αGeminin
αTK
αCyclinB1
αUL21a
αIE1-72
αactin
B
Geminin RNA
mock
ADgfp
ADsubUL21a
Relative transcript/GAPDH
2.0
1.5
1.0
0.5
24
72
hours post infection
C
mock
ADgfp
ADsubUL21a
No drug
PAA
72 hpi
αCdh1
αgeminin
αTK
αUL21a
αIE1-72
αactin
D
mock
ADgfp
ADsubUL21a
ADinUL21aPH-AA
ADinUL21aPR-AA
No drug
MG132
αCdh1
αgeminin
αUL21a
αIE1-72
αactin

Figure 4.7

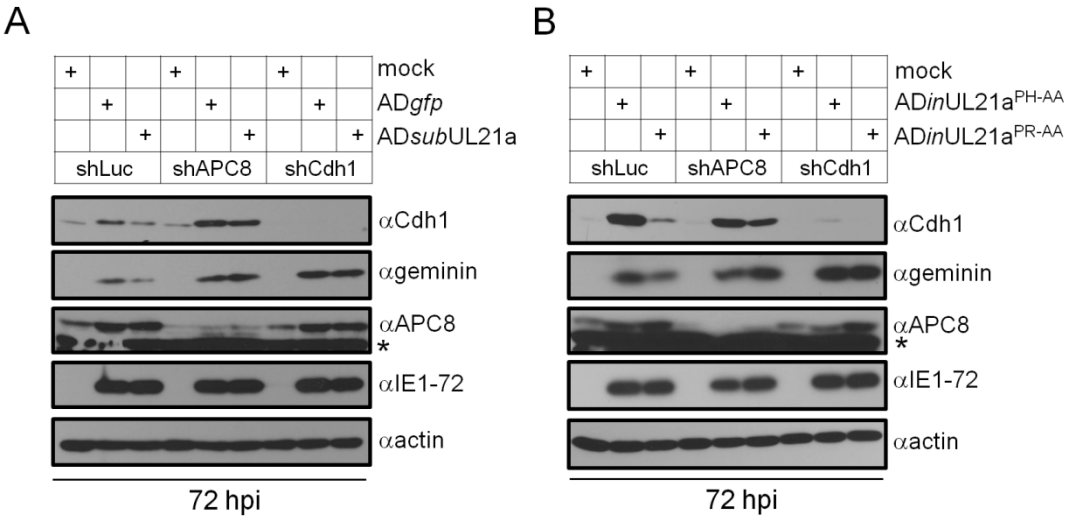

A
B
mock
ADgfp
ADsubUL21a
ADinUL21aPH-AA
ADinUL21aPR-AA
shLuc
shAPC8
shCdh1
αCdh1
αgeminin
αAPC8
*
αIE1-72
αactin
72 hpi

Figure 4.8

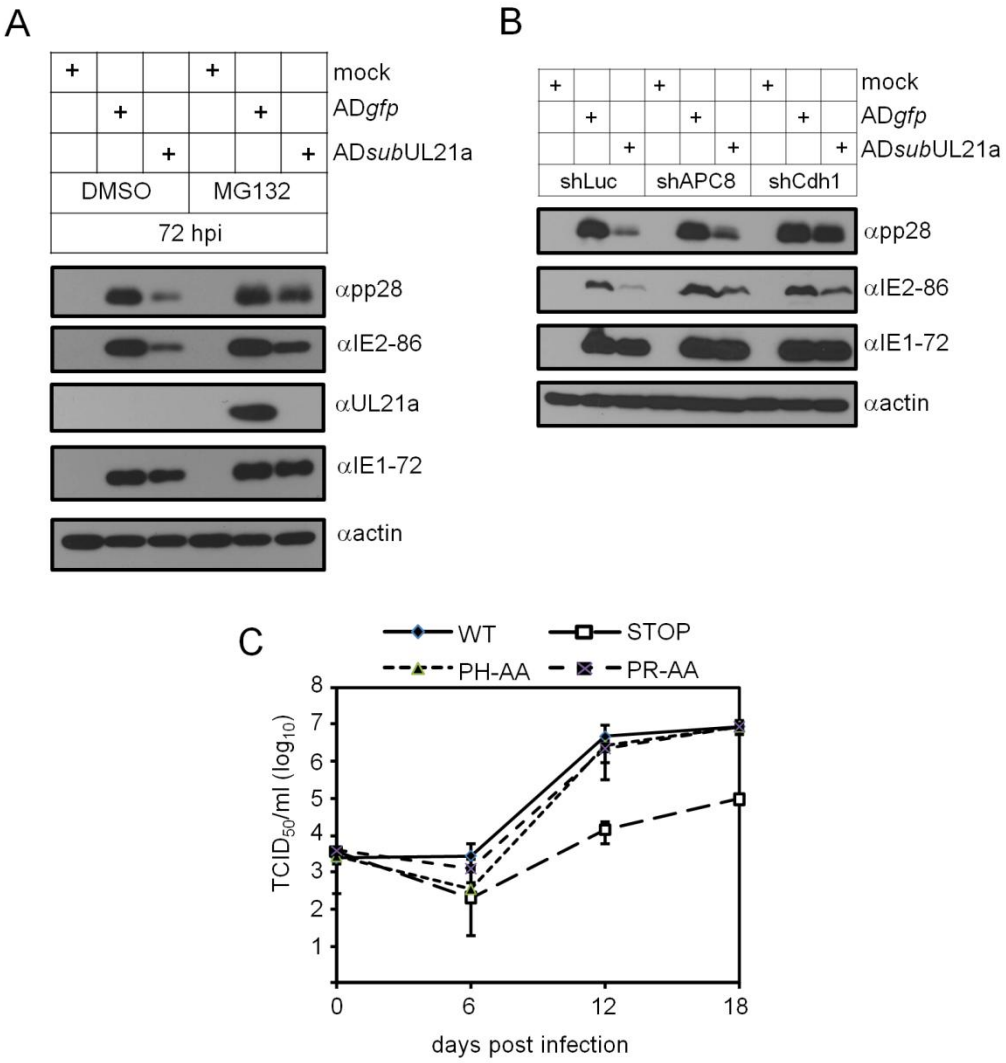
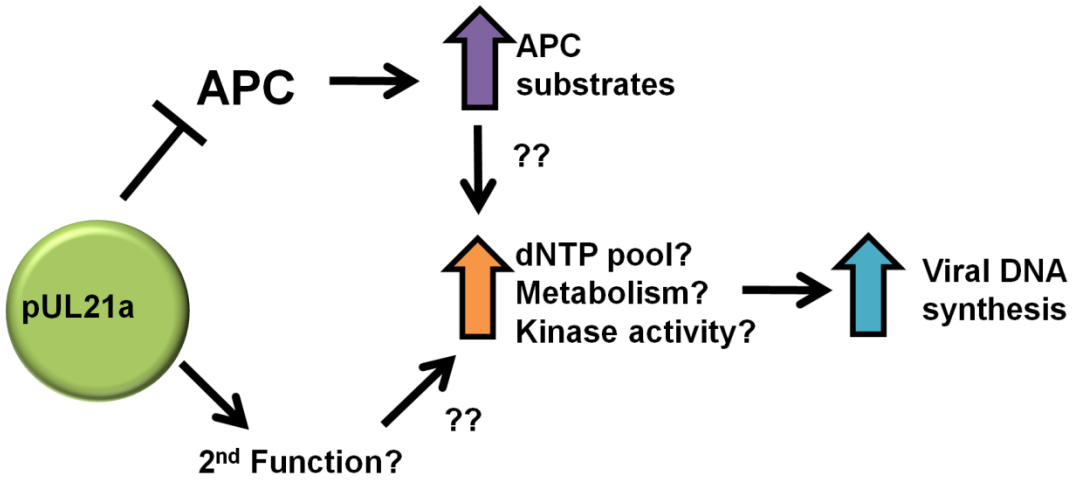

A
mock
ADgfp
ADsubUL21a
DMSO
MG132
72 hpi
αpp28
αIE2-86
αUL21a
αIE1-72
αactin
B
mock
ADgfp
ADsubUL21a
shLuc
shAPC8
shCdh1
αpp28
αIE2-86
αIE1-72
αactin
C
WT
STOP
PH-AA
PR-AA
TCID50/ml (log10)
days post infection

Figure 4.9


APC
APC substrates
??
pUL21a
dNTP pool?
Metabolism?
Kinase activity?
Viral DNA synthesis
??
2nd Function?

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Chapter V

Summary and Future Directions

Summary

Two large-scale mutagenesis screens identified the previously uncharacterized HCMV gene UL21a as being important but not essential for virus replication (4, 26). However, no experimental evidence had been documented as to whether HCMV expresses UL21a. Here, we began the study of UL21a and extensively characterized its transcript and protein products. We found that UL21a encodes an early transcript and an ~15 kDa protein product (6). The UL21a protein, termed pUL21a, was found to be degraded in a proteasome-dependent, but ubiquitin-independent manner (6). It localized to the cytoplasm, but was not found in the HCMV virion (6). Using multiple HCMV recombinant viruses and a complementing cell line, we further confirmed that pUL21a, and not the neighboring genes or other gene products within UL21a, was important for viral replication (5, 6). The UL21a mutant virus possessed a cell-type specific defect, as the mutant had as much as a 1000-fold defect on MRC-5 cells whereas the defect never got higher than 30-fold on HFFs (5, 6). The UL21a mutant virus had a high particle-to-PFU ratio, and when normalized to equal viral genome copies, it had a dramatic defect in viral DNA synthesis, but not early gene expression (5). Then, using a complementing cell line, we showed that it was *de novo* synthesis of pUL21a and not an indirect effect on the virion which caused this defect in DNA synthesis (5).

The current dogma in HCMV suggests that IE genes are expressed early and remain at similar levels throughout the infection cycle. However, we found that most, if not all, IE genes are regulated by viral DNA synthesis (5). There were some indications in the literature in the late 80's and early 90's that this was the case, as researchers found that the accumulation of the IE2-86 transcript and protein was inhibited by

phosphonoacetic acid (PAA) (20, 21). However, this data has largely been forgotten (14). Our study was the first to extensively analyze multiple IE transcripts in the presence or absence of PAA. All transcripts analyzed, including IE1-72, IE2-86, UL37x1, and TRS1, were inhibited by PAA at least 2-fold (5). Furthermore, the protein products of all but IE1-72 were profoundly affected as well. The lack of a strong effect on IE1-72 protein expression is likely why this regulation has gone unnoticed as most researchers use IE1-72 protein expression as a marker for IE gene expression.

UL21a contains no identifiable domains or significant homology with any known proteins. Even though we had extensively characterized the UL21a gene, there was no indication to its actual function(s). To identify potential functions for this protein we took a proteomics approach to identify its interacting partners. We found that pUL21a interacts with the anaphase-promoting complex (APC), a large E3 ubiquitin-ligase involved primarily in cell-cycle progression. We further mapped the APC binding domain to the C-terminus of pUL21a and identified two amino acids (PR108-109) which were critical for this interaction. HCMV inhibits the APC by both phosphorylating the co-activator Cdh1, and by targeting two subunits, APC4 and APC5, for proteasome-dependent degradation (22, 23). The UL97 kinase was shown to phosphorylate Cdh1, but the gene targeting APC4 and APC5 for degradation remained unknown (22). Here we showed that pUL21a was both necessary and sufficient for APC4 and APC5 degradation and was required for APC complex dissociation during infection. The degradation of APC4 and APC5 was likely the cause of the dissociation, as proteasome inhibition could restore the integrity of the APC. The degradation of APC4 and APC5 was dependent on the ability of pUL21a to bind to the APC, as the PR108-109AA point mutant could not

induce their degradation and acted in all assays like a UL21a deletion virus. In the absence of UL21a, increased APC activity could be detected by the increased degradation of its substrates. The level of APC substrate accumulation during UL21a mutant virus infection could be restored to wild-type levels by shRNA knockdown of APC components, showing that decreased accumulation of these substrates was due to APC activity. Most importantly, we showed that knockdown of APC8 and the co-activator Cdh1 and proteasome inhibition could at least partially restore late viral gene expression during UL21a mutant virus infection. This indicates for the first time that Cdh1, and likely APC^{Cdh1}, may have anti-viral functions.

Implications

The work described here has many implications for HCMV and virology research. We are the first to identify and characterize the UL21a ORF, and have identified its role during the viral lifecycle. We showed that the protein is degraded in a proteasome-dependent, but ubiquitin-independent manner. Ubiquitin-independent degradation is somewhat rare but has been reported for a few viral proteins, including the parvovirus minute virus NS2 protein (11), the HCV F protein (27), and the MCMV m140 and m141 proteins (1). Interestingly, pp71 targets the cellular proteins Rb and Daxx for proteasome-dependent ubiquitin-independent degradation (7, 8). It is unknown why the virus appears to favor ubiquitin-independent mechanisms for proteasome degradation, but it could be because the viral encodes its own deubiquitinase which may reduce the effectiveness of ubiquitination (24).

We found that UL21a was required for viral DNA synthesis, which was not unexpected as most early genes are important for viral DNA synthesis. However, the additional finding that IE genes are regulated by viral DNA synthesis was intriguing as the dogma in herpesvirus biology is that IE genes are expressed at high levels early and stay elevated throughout infection. However, our study was not the first to identify this type of regulation. Two previous studies had shown that IE2-86 protein and transcript were inhibited by PAA (20, 21), and others have shown that the MCMV IE-1 and HSV-1 IE expression in trigeminal ganglion can be inhibited by PAA (9, 15, 19). Currently, it is unknown whether the late accumulation of IE proteins, such as IE2-86, has any function in promoting replication of the virus. Our lab has recently identified a novel late gene regulator, UL79, in HCMV (17). In the absence of UL79, viral DNA replication and IE2-86 production are normal, but no late genes are expressed. Perhaps IE2-86 or other IE genes are increased at late times in order to activate expression of UL79 and other late gene regulators, ultimately promoting viral replication. Clearly, further experiments are needed to clarify this issue.

The most significant work of this thesis is the identification of pUL21a as a novel regulator of the APC. We have clearly shown that pUL21a is both necessary and sufficient for APC4 and APC5 proteasome-dependent degradation. During HCMV infection, this likely leads to the dissociation of the complex, as APC4, APC5, and APC10 were not pulled down with an APC3 antibody during wild-type infection as efficiently as during mock or UL21a mutant virus infection. Proteasome inhibitors restored the integrity of the complex without dramatically affecting pUL21a expression, which strongly implicates APC4 and APC5 degradation as the cause of the dissociation.

However, this cannot be formally proven without the ability to overexpress non-degradable forms of APC4 and APC5, which may prove difficult.

pUL21a is only one of a handful of viral proteins which have been shown to regulate the APC (13). Only pUL21a and the UL97 protein kinase of HCMV have been shown to inhibit this complex during virus infection. The fact that two viral proteins are needed to inhibit this complex during HCMV infection strongly implies that the complex is detrimental to virus replication. Knockdown of APC8 and especially Cdh1, was able to restore some late viral protein expression in a UL21a mutant virus. This is further supported by the observation that the proteasome inhibitor MG132 also significantly restored late viral protein expression in a UL21a mutant virus. This data shows for the first time that Cdh1, and likely the entire APC complex, may act in an antiviral manner during HCMV infection. Further work will be needed to clarify the role of the APC during infection in fibroblasts and other cell types infected by HCMV.

Finally, it is clear that pUL21a must have additional functions other than to inhibit the APC. A pUL21a point mutant (PR108-109AA) which was unable to bind and regulate the APC grew with wild-type virus kinetics, unlike a complete deletion virus. The fact that knockdown of multiple APC subunits and the use of proteasome inhibitors significantly restored late gene expression in a UL21a mutant implies that its functions may be redundant (See Fig. 4.9). Perhaps in fibroblasts only one of these two functions is sufficient to promote efficient replication, but in a terminally differentiated cell, such as a neuron, both functions may be needed. It is currently unknown what this additional

function(s) may be, but pUL21a does have an Arginine Rich Motif (ARM) which in other viral proteins has been shown to bind RNA (3).

Future Directions

There are many different future experiments which may follow this work. The most pressing issues will be to clarify the role of the APC during virus infection of multiple cell types, identify the APC substrates which may be important for viral replication, determine the mechanism pUL21a uses to target APC4 and APC5 for proteasome-dependent degradation, and continue identification of additional interacting partners to discover novel functions for pUL21a.

I. Clarify the role of APC inhibition during HCMV infection

We have shown that knockdown of APC components restores some late viral gene expression during UL21a mutant virus infection. However, this may not be the most effective means to inhibit this complex due to residual expression of these proteins and the possibility that they may be multi-functional. Recently, a novel pharmacologic inhibitor of the APC was described (proTAME), and we have obtained this compound. In the near future we will test whether this compound can restore viral DNA synthesis, late gene expression, and virus production during UL21a mutant virus infection. We will also create the UL21a$^{PR\text{-}AA}$ point mutant virus in an HCMV clinical strain, TR, which will allow us to identify cell types where pUL21a inhibition of the APC may be required for viral replication. We will test the growth of this virus in multiple cell types, including endothelial cells, epithelial cells, macrophages, and neuronal cells, then confirm that wild-type pUL21a but not pUL21a$^{PR\text{-}AA}$ can regulate the APC in these cell types.

Why does the virus inhibit the APC? The APC targets > 40 cellular proteins for ubiquitination and subsequent proteasome degradation, thereby limiting their function. It is likely that one or more of these proteins are important for viral replication. Interestingly, two substrates of the APC are thymidine kinase (TK) and ribonucleotide reductase M2 (RRM2), which are critical enzymes for nucleotide biosynthesis. All α- and γ-herpesviruses encode their own versions of these enzymes while HCMV and other β-herpesviruses do not (25). Thus HCMV must rely on these cellular enzymes for nucleotide biosynthesis, and it is possible that APC inhibition is at least one mechanism it uses to achieve this goal. In fact, a similar phenomenon has been described in poxviruses (12). To test this hypothesis we plan on overexpressing TK and RRM2 from HSV-2 alone or in combination and testing whether one or both can restore viral DNA synthesis in the UL21a mutant virus. We will use the HSV enzymes because they do not contain an APC recognition domain and thus are not targeted for proteasome degradation. We realize the APC targets multiple substrates for degradation, and many have the capability to affect viral replication. If TK and RRM2 are not found to be important for viral growth, we may systematically knockdown each known substrate of the APC and see which ones are important for viral replication. One problem to this experiment is the possibility that multiple APC substrates may be redundant in their functions, and thus knocking down one may not produce a phenotype. Another issue is that not all APC substrates have been described, and new ones are published nearly every month, so it is likely we may leave out the critical regulators because they have not been discovered.

II. Determine the mechanism pUL21a uses to target APC4/APC5 for degradation

To fully understand how pUL21a inhibits the APC, it will be important to know how pUL21a targets APC components for degradation. The first major step to answering this question will be to identify the direct binding partner for pUL21a. We have shown that pUL21a binds to the APC complex, but it unknown whether it binds directly to one of the subunits or if it binds through an adapter protein. We hypothesize that pUL21a either directly binds to APC4 and APC5 or to the directly adjacent subunits (APC8 and APC1). We are currently setting up a yeast-2-hybrid (Y2H) assay to test whether these proteins can bind directly to pUL21a. If neither of these proteins works, we will continue to clone additional APC subunits into the Y2H vectors. Once we have identified a candidate, we will create recombinant proteins and determine whether these proteins bind *in vitro*.

The second part of this project will be to determine whether APC4 and APC5 are ubiquitinated during infection. Previous published data suggested that E1 enzymes were necessary for APC4/APC5 degradation; however this data is somewhat controversial, as there was no way to determine whether the inhibitor was affecting the expression of pUL21a (22). Regardless, it has not been directly shown that they are ubiquitinated. If they are ubiquitinated, it would be important to identify the E3 ubiquitin ligase responsible. It is highly unlikely that pUL21a could act as an E3 ubiquitin ligase since it contains none of the sequence domains necessary for this enzymatic activity, thus it would likely recruit a cellular E3 ligase. Genetic or biochemical experiments would be used to identify this E3 ubiquitin ligase.

Alternatively, pUL21a may target these proteins directly as pUL21a is also degraded in a proteasome-dependent manner. If pUL21a can directly target APC4/APC5

for degradation, we will need to define the biochemistry of this activity. The GFP-tagged UL21a protein is able to bind the APC, but it cannot target these proteins for degradation, suggesting that binding is not sufficient (data not shown). Targeted mutagenesis of conserved residues within pUL21a should be able to identify additional sequence important for this activity. In fact, we have recently created a mutant in the arginine rich motif (ARM) of pUL21a which grows like a null virus, suggesting this region is clearly important for one of its activities. It will be important to characterize this mutant and determine whether it can regulate the APC. Alternatively, this region may be important for other functions of this protein (see below).

III. Characterize the cell death inhibitory activity of pUL21a

In addition to the data in chapters 2-4, we have data which shows pUL21a is required to prevent cell death during HCMV infection (Fig. 5.1). This cell death was likely due to increased apoptosis as increased levels of cleaved caspase-3 and PARP could be detected, which could be inhibited by caspase inhibitors (Fig. 5.2). It is unknown whether the increased levels of apoptosis restrict viral growth. It has also not yet been determined whether the increased apoptosis is caused by increased APC activity. To test this, we can infect with our APC binding deficient mutant, AD*in*UL21a$^{PR\text{-}AA}$, and test whether it causes increased cell death. Alternatively we could knockdown or inhibit the APC and determine whether cell survival can be restored. If cell death is dependent on APC activity, we could identify those substrates which can promote cell survival using similar methods outlined in part I. If cell death is not dependent on APC activity, it will be important to identify a second function for pUL21a and determine whether this function promotes cell survival.

IV. Identify additional pUL21a function(s)

As mentioned previously, it is highly likely that pUL21a is a multi-functional protein. To identify its additional function(s) we are continuing to use Co-IP to identify additional binding partners of pUL21a. Also, as stated above, we have created a point mutant with the ARM removed. This virus grows like UL21a deletion virus suggesting this region is critical for pUL21a function(s). ARMs in other viral proteins have been shown to mediate RNA binding, and in certain cases may help viruses escape RNA sensors in the cytoplasm of its host cell. It may be of interest to test whether this region confers RNA binding ability upon pUL21a and whether it is required to prevent a robust interferon response.

V. Determine the requirement of Cdh1 for viral replication

Surprisingly, while knockdown of Cdh1 is capable of restoring viral late gene expression to the UL21a mutant virus, it also has a strong negative effect on production of infectious virus. In our preliminary studies, knockdown of Cdh1 reduces HCMV titers in a single-step growth curve by as much as 2 logs, while knockdown of APC8 had a much less dramatic effect (data not shown). This suggests that Cdh1 may have APC independent function(s) which are critical for viral replication, but also could be due to a different level of knockdown of each protein. It will be important to confirm these results using additional methods (additional shRNAs, APC inhibitors) and to fully characterize the ability of Cdh1 to inhibit viral replication. This data may have important consequences both for HCMV and cell biology as Cdh1 has never been shown to have APC independent functions.

Concluding Remarks

We have now extensively characterized the UL21a gene, described its role during HCMV infection, and have identified at least one of its functions, to regulate the anaphase-promoting complex. This work will provide significant new insight into the mechanisms HCMV uses to facilitate its intra-cellular replication and may provide novel targets for anti-viral therapies.

The APC has been shown to be critical for neuronal development (18), and HCMV is largely known for its ability to cause neuronal defects in congenitally infected infants. HCMV infects many types of neuronal cells *in vitro*, including differentiated neurons, astrocytes, and neuronal precursor cells (2, 10, 16). It's possible that the ability of HCMV to inhibit the APC plays a role in the ability of the virus to infect neuronal cells and cause neuronal defects. Further mechanistic study of pUL21a and its ability to inhibit the APC may lead to novel insights into the molecular pathogenesis of HCMV.

The APC is a critical mediator of cell cycle progression, as inhibitors of the APC prevent cells from progressing past the G2/M phase of the cell cycle. Further mechanistic study should provide unique insights into the biology of the APC and may lead us to new methods to target the APC for anti-cancer therapeutics. Over the last 50 years viruses have provided a number of unique tools which have allowed for groundbreaking discoveries in cell biology, such as the discovery of prominent cell cycle regulators such as p53 and Rb. Study of pUL21a will shed new light on cell biology, cell cycle regulation, and will highlight additional ways HCMV has continued to evolve and interact with its host in order to facilitate its own replication.

FIGURE LEGENDS

Figure 5.1. UL21a is required for cell survival during HCMV infection. (A-B) UL21a mutant virus induces cell death. (A) MRC-5 cells were infected with AD*wt* or AD*sub*UL21a at an equivalent MOI of 2 $TCID_{50}$/cell (left two columns) or equivalent genome copies (AD*wt* MOI equivalent to 10) (right two columns). Bright-field and fluorescent microscopy was performed and pictures were taken with a Leica microscope at 96 hpi. (B) MRC-5 cells were infected as in A and cell death was quantified using an ATP assay (Promega) at the indicated times post-infection. (C) UL21a mutant virus induced cell death requires viral gene expression but not DNA replication. MRC-5 cells were infected as in B in the presence of PAA (100 μg/ml) or with UV-inactivated virus. Cell death was quantified as in B at 120 hpi. (D) Expression of pGFP-UL21a can prevent UL21a mutant virus induced cell death. MRC-5 cells were transduced 3X with pGFP-UL21a expressing retrovirus, allowed to rest for 72 h, then infected with equivalent genome copies as in part A and cell death was measured at indicated times post infection using the ATP assay.

Figure 5.2. UL21a is required to prevent caspase activation during HCMV infection. (A-B) UL21a mutant virus induces cleavage of caspase-3 and PARP which is dependent on caspase activity. (A) MRC-5 cells were infected as in Fig. 5.1D and whole cell lysates were collected at indicated times and analyzed by immunoblotting with indicated antibodies. (B) MRC-5 cells were infected as in A and treated with DMSO or ZVAD-FMK (10μM). Whole cell lysates were collected at indicated times and analyzed by immunoblotting with indicated antibodies.

Figure 5.1

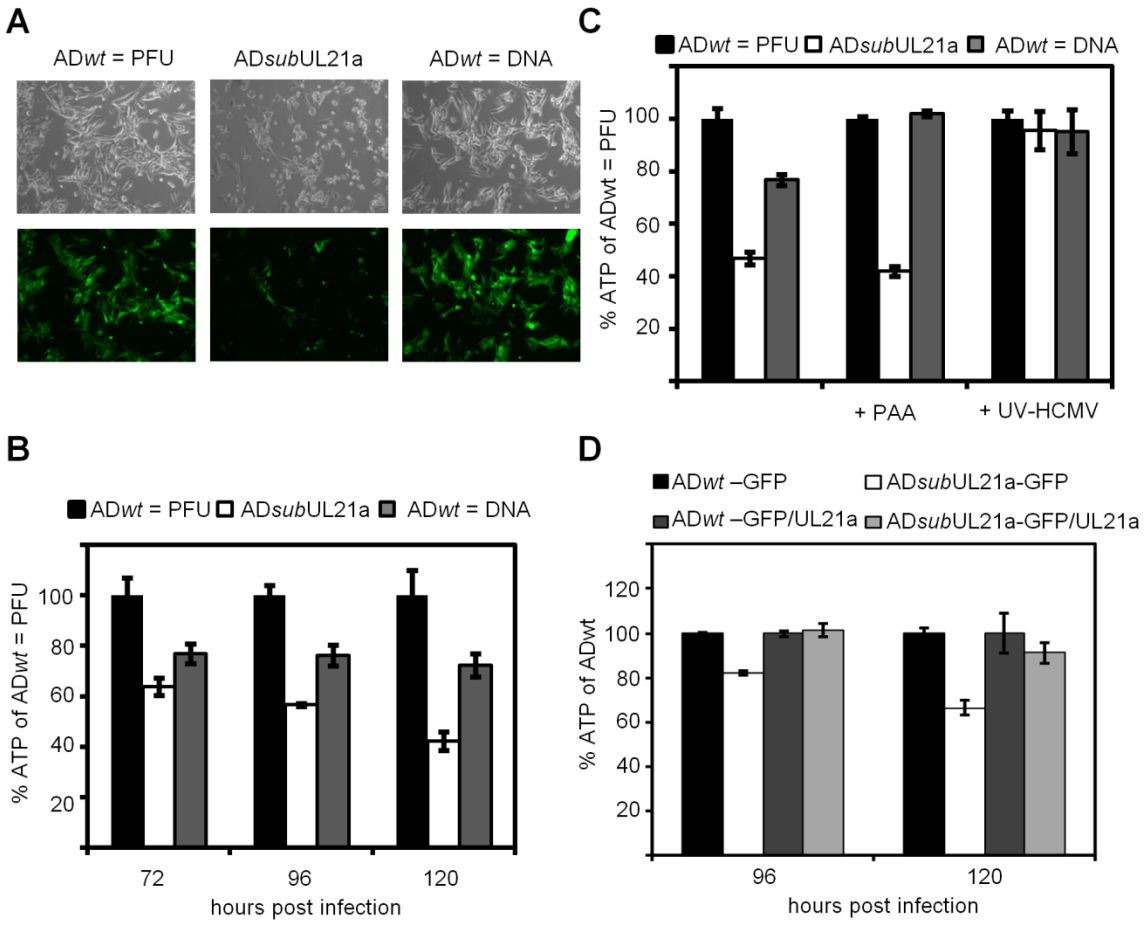
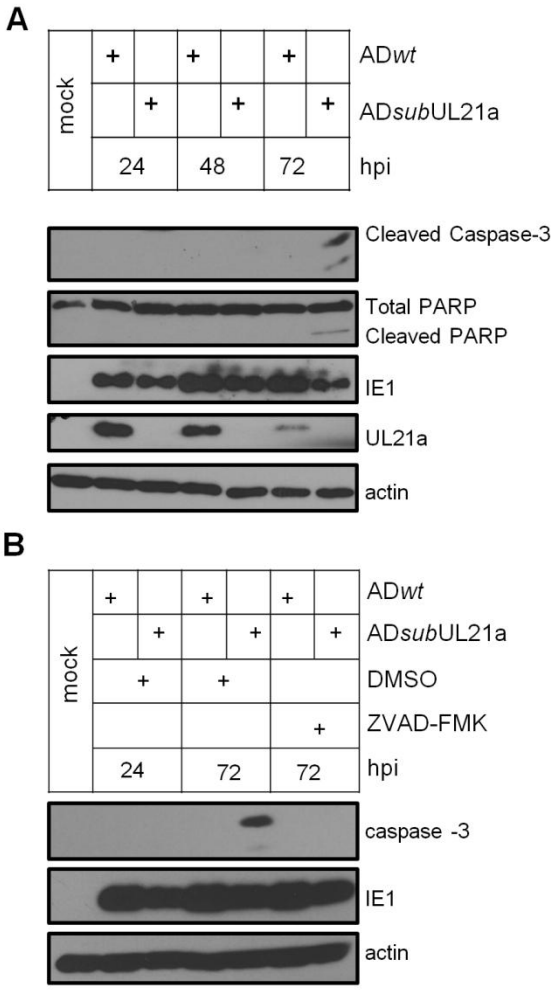

A
ADwt = PFU
ADsubUL21a
ADwt = DNA
B
ADwt = PFU
ADsubUL21a
ADwt = DNA
% ATP of ADwt = PFU
100
80
60
40
20
72
96
120
hours post infection
C
ADwt = PFU
ADsubUL21a
ADwt = DNA
% ATP of ADwt = PFU
100
80
60
40
20
+ PAA
+ UV-HCMV
D
ADwt –GFP
ADsubUL21a-GFP
ADwt –GFP/UL21a
ADsubUL21a-GFP/UL21a
% ATP of ADwt
120
100
80
60
40
20
96
120
hours post infection

Figure 5.2


A
mock
+
+
+
+
+
+
ADwt
ADsubUL21a
24
48
72
hpi
Cleaved Caspase-3
Total PARP
Cleaved PARP
IE1
UL21a
actin
B
mock
+
+
+
+
+
+
ADwt
ADsubUL21a
+
+
DMSO
+
ZVAD-FMK
24
72
72
hpi
caspase -3
IE1
actin

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