FUNCTIONAL ANALYSIS OF THE HUMAN CYTOMEGALOVIRUS UL82 GENE PRODUCT PP71 DURING VIRUS REPLICATION

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DEDICATION

To my husband, Ryan Hagemeier, for encouraging me to do my best both in the lab and at home. I cannot imagine these past few years without your love and encouragement in my life.

To my parents, Paul and Brenda Cantrell, for teaching me to reach for my dreams and strive for the impossible while providing their unconditional love and support. It is through your example that I strive to excel in both my career and my life so that I will become a well-rounded individual that you are proud to call your daughter.

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FUNCTIONAL ANALYSIS OF THE HUMAN CYTOMEGALOVIRUS UL82 GENE PRODUCT PP71 PROTEIN DURING VIRUS REPLICATION

by

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Human cytomegalovirus (HCMV) is a β-herpesvirus that infects the majority of the human population. Although primary infection is usually asymptomatic in immunocompetent individuals, HCMV infection can lead to severe disease in immunosuppressed individuals including neonates, transplant recipients, and AIDS patients. The HCMV UL82 gene encodes for the tegument protein pp71. It has previously been shown that pp71 is required for efficient virus replication at low multiplicities of infection and that it is a regulator of immediate-early gene expression. However, the

mechanism whereby pp71 regulates IE gene expression and/or virus replication has not been elucidated. pp71 has also been shown to bind a number of cellular proteins including Rb familiy member proteins and the cellular protein hDaxx. This dissertation focused on determining if pp71's interaction with either of these proteins is important for viral replication and immediate-early gene expression in the context of a viral infection. We demonstrate that pp71's ability to target Rb family member proteins for degradation is not required for efficient viral replication. However, pp71's ability to interact with hDaxx is required for efficient viral replication and immediate-early gene expression. hDaxx has been identified as a transcriptional regulatory protein and is thought to regulate transcription through its interaction with histone deacetylases and core histones. This dissertation further defines the mechanism by which pp71 enhances viral replication by demonstrating that hDaxx functions to repress HCMV replication and IE gene expression. Importantly, we also demonstrated that the severe growth defect associated with the pp71 deletion mutant could be fully restored following infection of hDaxx knock-down cells. Experiments examining the mechanism by which pp71 relieves hDaxx mediated repression suggest that pp71 interacts with hDaxx to block histone deacetylase activity and therefore promotes the association of acetylated histones with viral immediate-early promoters. Taken together, these results demonstrate that pp71's interaction with hDaxx is critical for efficient virus replication and supports the hypothesis that pp71's interaction with hDaxx is important to "kick-start" the HCMV replication cycle by preventing host cell-mediated repression of viral immediate-early gene expression.

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PRIOR PUBLICATIONS

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2. **Cantrell, S. R.**, and W. A. Bresnahan. 2005. Interaction between the human cytomegalovirus UL82 gene product (pp71) and hDaxx regulates immediate-early gene expression and viral replication. *Journal of Virology*. 79(12):7792-802.

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aa ..... amino acid
AIDS .....acquired immune deficiency syndrome
AP .....assembly protein
AP-1 ..... activator protein 1
ASV .....avian sarcoma virus
ATF ..... activating transcription factor
BAC ..... bacterial artificial chromosome
CBP ..... CREB-binding protein
Cdk ..... cyclin dependent kinase
CDV ..... cidofovir
C/EBP..... CCAAT/enhancer binding protein
ChIP ..... chromatin immunoprecipitation
CID ..... cytomegalic inclusion disease
CMV .....cytomegalovirus
CNS .....central nervous system
CPE .....cytopathic effect
CREB ..... cAMP response element binding protein
CTL .....Cytotoxic T lymphocytes
DMAP1 ..... DNA methyltransferase 1-associated protein
DMEM .....Dulbecco's modification of Eagle's medium
DMSO..... dimethyl sulfoxide
DNA ..... deoxyribonucleic acid
DNMT1 ..... DNA metyltransferase enzyme 1
Dpi ..... days postinfection
E .....early
EBV ..... Epstein Barr virus
EGFP ..... enhanced green fluorescent protein
EGFR ..... epidermal growth factor receptor
ERF ..... Ets2 repressor factor
FADD .....Fas-associated death domain
GAS .....growth arrest specific
GCV .....ganciclovir
gB .....glycoprotein B
Gfi-1 ..... growth factor independence-1
GFP ..... green fluorescent protein
GPCMV ..... guinea pig cytomegalovirus
h .....hours
HAART ..... highly active antiretroviral therapy
HAT .....histone acetyltransferase

HCMV ..... human cytomegalovirus
HCT ..... *Helminthosporium carbonum* toxin
HDAC ..... histone deacetylase
hDaxx ..... human death domain-associated protein 6
HFF ..... human foreskin fibroblast
HHV ..... human herpesvirus
HIPK ..... homeodomain-interacting protein kinase
HIV ..... human immunodeficiency virus
HP1 ..... heterochromatin protein 1
Hpi ..... hours postinfection
HSCT ..... hematopoietic stem cell transplant
HSPG ..... heparin sulfate proteoglycan
HSV ..... herpes simplex virus
ICAM-1 ..... intercellular adhesion molecule-1
IE ..... immediate-early
Ig ..... immunoglobulin
IRL ..... internal repeat long
IRS ..... internal repeat short
JNK ..... c-Jun NH2-terminal kinase
KAN ..... kanamycin
Kb ..... kilobase
kDa ..... kilodalton
KSHV ..... Kaposi's sarcoma associated virus
L ..... late
MCP ..... major capsid protein
mC-BP ..... minor capsid binding protein
MCMV ..... murine cytomegalovirus
mCP ..... minor capsid protein
MHC ..... major histocompatibility complex
MIEP ..... major immediate-early promoter
MRF ..... modulator recognition factor
MOI ..... multiplicity of infection
ND10 ..... nuclear domain 10
NF-1 ..... nuclear factor 1
NFκB ..... nuclear factor kappa B
NK ..... natural killer
NLS ..... nuclear localization signal
ORF ..... open reading frame
PAGE ..... polyacrylamide gel
PAH ..... paired amphipathic helices
PBS ..... phosphate buffer saline

P/CAF ..... p300//CBP associated factor
PCR ..... polymerase chain reaction
PEST ..... praline, glutamate, serine, and threonine
PFA ..... foscarnet
PFU ..... plaque forming units
PML ..... promyelocytic leukemia
POD ..... promyelocytic oncogenic domain
pp ..... phosphoprotein
Rb ..... retinoblastoma protein
RCMV ..... rhesus cytomegalovirus
RNA ..... ribonucleic acid
RIPA ..... RadioImmunoPrecipitation Assay
rpm ..... revolutions per minute
s ..... seconds
SDS ..... sodium dodecyl sulfate
SCP ..... smallest capsid protein
shRNA ..... short hairpin ribonucleic acid
SNHL ..... sensorineural hearing loss
SOT ..... sold organ transplant
SSC ..... saline sodium citrate
SUMO ..... small ubiquitin-related modifier
Tel12 ..... telomerase life extended fibroblast clone 12
TGF ..... transforming growth factor
TLR ..... toll-like receptor
TRL ..... terminal repeat long
TRS ..... terminal repeat short
TSA ..... trichostatin A
U_L ..... unique long
U_S ..... unique short
VP16 ..... virion protein 16
VPA ..... valproic acid
VZV ..... varicella zoster virus
X-gal 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
YY1 ..... yin yang-1

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

Human cytomegalovirus

Epidemiology

Human cytomegalovirus (HCMV) is a ubiquitous human pathogen that infects a large percentage of the population. In developed countries, approximately 30-70% of the population is infected by adulthood, while up to 90% of the population is infected in underdeveloped countries (76). HCMV is primarily asymptomatic in healthy individuals but can lead to severe disease in neonates, infants, and immunocompromised persons, including acquired immune deficiency syndrome (AIDS) patients and transplant recipients. In the early 1950's, HCMV was discovered to be the cause of a severe congenital syndrome known as "generalized cytomegalic inclusion disease" (244). HCMV was later associated with immunocompromised patients when allograft transplants were introduced, and has since become a well known opportunistic pathogen (95, 188, 203). There is current speculation that HCMV may also contribute to human malignancies based on its ability to interfere with the cell cycle, cellular stress responses, and apoptosis following infection (8, 38, 39, 70, 71, 80, 110, 147, 152, 208, 231, 247, 253)

Human cytomegalovirus taxonomy

HCMV, also known as Human herpesvirus 5 (HHV-5), belongs to the *Herpesviridae* family of viruses. Viruses in the *Herpesviridae* family

are classified into the *Alpha*-, *Beta*-, or *Gammaherpesvirinae* subfamilies based on their biological characteristics. HCMV is the prototypic β-herpesvirus based on its long reproductive cycle and formation of cytomegalia or enlarged cells during infection. Other β-herpesviruses include Human herpesviruses 6 and 7 (Roseolovirus). Although viruses in the *Betaherpesvirinae* subfamily have a restricted host range, the *Alphaherpesvirinae* family members have a more variable host range and establish latent infections in sensory ganglia. Members of this subfamily, including Herpes simplex viruses 1 and 2 (HSV-1,-2) and Varicella-zoster virus (VZV), have a relatively short reproductive cycle compared to viruses of the other *Herpesviridae* subfamilies. The *Gammaherpesvirinae* subfamily includes Epstein Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV). These viruses are classified based on their limited host range and usually are specific to replication in T and B lymphocytes and undergo latency in lymphoid tissue.

Clinical disease

HCMV transmission is spread easily through horizontal and vertical infection. Virus infection can occur when an individual comes in direct contact with infectious bodily fluids including saliva, urine, tears, semen, and cervical secretions. Two populations at high risk for infection are children in day care centers and sexually active young adults (3, 170). HCMV can also be transmitted transplacentally from mother to fetus. Transplacental transmission is unique to HCMV and does not occur during infection with any of the other herpesviruses.

Upon transmission, HCMV initially establishes an acute primary infection. In immunocompetent individuals both cell-mediated and humoral immune responses are upregulated in response to infection.

Cytotoxic T lymphocytes (CTL) and natural killer cells are important for targeting infected cells (124). Healthy individuals also generate immunoglobulin M (IgM) antibodies against HCMV for 3-4 months after infection followed by the lifelong production of IgG antibodies. Once the adaptive immune response can be detected following a primary infection, viremia can continue for months in adults (186, 248) or as long as several years in children (169). Following the initial primary infection, the virus enters a state of latency. Virus replication does not occur during latent infection, however latent virus can be reactivated and initiate lytic replication. In some cases, low levels of virus replication occurs establishing a chronic or persistent productive infection. HCMV transmission can occur during chronic infection, reactivation of latent virus, and the transfer of latent infected cells during transplantation.

During natural infection, HCMV is thought to replicate in a variety of epithelial tissues. Fibroblasts, epithelial cells, endothelial cells, smooth muscle cells, macrophages, hepatocytes, mesenchymal cells, and granulocytes have all been shown to support productive virus replication (17, 108, 176, 205, 206). Although the virus is primarily cultured in fibroblasts, *in vivo* the virus appears to predominately replicate in endothelial cells and spread via leukocytes and vascular endothelial cells during primary infection (187, 207, 229, 241).

Following primary infection, HCMV undergoes a life-long latent infection by maintaining its genome in select cells. In a state of latency, the virus infected cells do not show cytopathic effects (CPE), do not produce infectious virus and are not detected or destroyed by the host immune system. Latent HCMV infection can be detected in endothelial cells (205) granulocytes, monocytes, and dendritic cells (88, 201, 213). It is hypothesized that circulating monocytes disseminate latent HCMV

infection and the virus becomes activated upon the differentiation of latently infected monocytes into macrophages (167). Periods of latent infection interrupted by viral reactivation allow for viral persistence throughout an individual's life (31). Viral persistence of HCMV often leads to serious disease in individuals who become immunocompromised.

HCMV disease in immunocompetent individuals

Primary HCMV infection usually remains subclinical in healthy individuals. However, in some cases, immunocompetent individuals present with mononucleosis symptoms including fever, sore throat and malaise. Studies have shown that approximately 8% of mononucleosis cases are HCMV accountable (160). Clinical symptoms can persist for weeks and are very similar to the manifestations presented during EBV-induced disease.

Congenital HCMV infection

Approximately 1% of children are born with congenital HCMV infection in developed countries (76). HCMV can spread from the infected mother by three routes: transplacental, intrapartum, or through breast milk. Transplacental infection can occur during reactivation of latent virus infection or during primary infection. Although the transplacental route of infection is most commonly associated with HCMV morbidity, the intrapartum and breast milk routes of transmission are most common (167). The intrapartum route of infection occurs when the mother sheds virus from the vagina or cervix and infects the infant during child birth. Although most cases of congenital HCMV infection appear asymptomatic, between 5-10% of infected infants suffer from cytomegalic inclusion disease (CID) and develop symptoms involving the reticuloendothelial

system (jaundice, hepatitis, petechiae) and the central nervous system (microcephaly, poor feeding, intracranial calcification) (87). Although many of the symptoms clear, the disease can require long-term hospital care and approximately 10% of symptomatic newborns die. Most of the symptomatic newborns will have lifelong complications due to organ and central nervous system damage and suffer from a combination of mental retardation, hearing loss, impaired vision or cerebral palsy (15, 171, 184). Additionally, congenital HCMV infection is thought to be the leading cause of sensorineural hearing loss (SNHL) and infection related brain damage in children (72).

HCMV infection in transplant patients

HCMV infection can lead to serious disease and mortality in immunosuppressed individuals receiving hematopoietic stem cell transplants (HSCT) or solid organ transplants (SOT). Infection can occur through the reactivation of latent HCMV, reinfection of the patient or by primary infection from the donor cells. HCMV disease manifestations following transplant include fever, muscular rash, malaise, arthralgias, and leucopenia. Some patients will develop severe diseases including pneumonitis, and hepatic dysfunction. Primary disease following infection is often associated with impaired graft function. Due to the severity and high percentage of transplant recipients affected by HCMV, most patients are treated prophylactically to reduce the onset and longitivity of HCMV infection.

HCMV infection in AIDS patients

Like transplant recipients, AIDS patients are at high risk for developing HCMV disease due to their immunocompromised state.

HCMV infection occurs as an opportunistic infection in patients with human immunodeficiency virus (HIV) and is often associated with clinical progression of HIV infection. The most common HCMV disease manifestation for AIDS patients is retinitis (31, 76). HCMV retinitis is usually due to reactivation of latent virus and is characterized by necrotizing retinitis that can lead to detachment of the retina and blindness. In addition to retinitis, HCMV replication in the central nervous system (CNS) of AIDS patients can produce some of the symptoms associated with congenital infection (124). For example, HCMV infection in children with HIV-1 infection has been associated with encephalopathy and death in addition to disease progression (74, 119). Since the development of highly active antiretroviral therapy (HAART), HCMV disease in patients with HIV has been greatly reduced (76).

HCMV therapy

HCMV infections are difficult to treat because the limited number of available drugs are far from ideal. The three drugs commonly prescribed to treat HCMV infection include ganciclovir (GCV), foscarnet (PFA) and cidofovir (CDV). Ganciclovir was the first agent approved for HCMV disease treatment and continues to be the primary drug used to treat and prevent infection. The drug ganciclovir is a synthetic analogue of 2'-deoxy-guanosine and must first be monophosphorylated by the HCMV phosphotransferase (UL97) (129) and then diphosphorylated by cellular kinases to become activated. Upon activation, ganciclovir competitively inhibits the HCMV deoxyribonucleic acid (DNA) polymerase (UL54) by preventing HCMV polymerase incorporation of dGTP and thereby preventing viral DNA elongation.

Ganciclovir, cidofivir and foscarnet limit treatment because they all target the HCMV DNA polymerase. The drug foscarnet inhibits the pyrophosphate binding site of the DNA polymerase in a noncompetitive manner and does not require activation. Cidofivir inhibits the HCMV DNA polymerase in a competitive manner. Resistance to HCMV DNA polymerase inhibitors is emerging, as evidenced by reports of AIDS patients infected with ganciclovir-resistant strains of HCMV (210). Therefore, due to increasing reports of drug resistance, foscarnet and cidofivir are usually only prescribed in cases where the patient is resistant to ganciclovir or suffers from severe side effects.

Additional limitations for HCMV treatment include the limited oral bioavailability and dose-limiting toxicities. Ganciclovir, foscarnet and cidofivir are all administered by intravenous infusion to treat productive HCMV infections. Ganciclovir is available for oral administration but is only recommended for prophylactic therapy. A more recent valine ester prodrug of ganciclovir called valganciclovir can be administered orally and is being frequently administered for prophylaxis and preemptive therapy in organ transplant recipients (202, 204). Although valganciclovir treatment is promising, ganciclovir, foscarnet and cidofivir all have dose-limiting toxicities. Ganciclovir treatment has been associated with adverse hematological effects including neutropenia. Foscarnet and cidofovir may lead to nephrotoxicity at high doses. Therefore, to reduce the side-effects associated with HCMV therapy, antiviral drugs are dose-limited and therefore have a low potency.

Although the drugs ganciclovir, foscarnet and cidofivir have their limitations, they have been shown to effectively treat HCMV disease. Oral ganciclovir or valganciclovir are often prescribed prophylactically in both AIDS and solid organ transplant patients to reduce the onset of HCMV

infection and disease. Foscarnet is often used as a prophylactic in bone marrow transplant patients. In addition to prophylactic treatment, ganciclovir, foscarnet and cidofivir are frequently administered to patients suffering from an established HCMV infection. In AIDS patients with acute HCMV retinitis, intravenous administration of ganciclovir, foscarnet or cidofivir have been demonstrated to slow progression of retinitis or bring the retinitis into remission (167). Intravenous administration of antiviral drugs in AIDS patients has also been used to treat gastrointestinal, neurological, or respiratory diseases associated with HCMV infection. Although intravenous administration of antiviral drugs reduces the effects of HCMV disease in AIDS and transplant patients, there is currently not an effective antiviral therapy for treating newborns suffering from congenital cytomegalovirus infection.

Considering the limitations of the current antiviral drugs used to prevent and treat HCMV disease, there is a large need for an effective vaccine. The first attempt at vaccination involved clinical trials using the attenuated HCMV Towne strain. The Towne strain was originally isolated from congenitally infected infants and became attenuated after multiple passages in human fibroblasts (178). Although some clinical trials yielded positive results in preventing re-infection or reactivation in CMV positive patients (179), other trials showed no difference in vaccinated versus unvaccinated women with children in daycare centers (4). After progress ceased in developing a Towne strain vaccine, investigators began making efforts utilizing recombinant subunits of the major HCMV envelope glycoprotein gB for vaccine generation. Studies using a guinea pig model of CMV showed that admistration of a gB subunit vaccine greatly decreased intrauterine transmission of guinea pig CMV, showing promise for vaccine development (198). The gB subunit vaccine is currently being

tested in clinical trials (168). Other vaccine candidates include DNA vaccines (199), recombinant vaccines, and synthetic peptides containing a T helper epitope and a lipid tail (9). Although there currently is not an effective vaccine for HCMV prevention, the Institute of Medicine has ranked developing a HCMV vaccine at the highest priority (76). Further advances in understanding the human antiviral response to HCMV infection and continued vaccine trials are necessary to develop an effective vaccine that will reduce the effects of HCMV disease.

Molecular virology

In the mid 1950's, HCMV was isolated for tissue culture use, enabling the study and diagnostics of HCMV *in vitro* (49, 193, 212). Human fibroblasts are the tissue culture of choice because HCMV propagates to higher titers in fibroblasts compared to other permissive cell systems. Infection of fibroblasts leads to distinctive cytopathic effects (CPE) including cell rounding, formation of large infected cells known as cytomegalia and the formation of nuclear inclusions. Progeny virus accumulates in the cytoplasm of infected cells and can be detected in the tissue culture media (167).

Cytomegalovirus infects a variety of animals as well as humans. Like HCMV, animal cytomegaloviruses are species specific. Mouse (MCMV), guinea pig (GPCMV) and rhesus (RCMV) cytomegaloviruses are used as animal models to examine viral pathogenesis and the function of viral genes. Although many of the essential genes are conserved between the various CMVs, there are significant differences between unique genes and replication strategies. Additionally, not all of the CMV's are capable of crossing the placenta to establish congenital infection. Due

to these differences, the results from animal CMV studies cannot be used to make conclusions regarding HCMV replication and pathogenesis.

Virion structure

The HCMV virion is structurally similar to other herpesviruses (Fig 1-1). The virion is approximately 200nm in diameter and contains a host-derived lipid envelope studded with viral glycoproteins. A DNA core containing the HCMV linear double-stranded DNA genome is protected within an icosahedral nucleocapsid. Between the nucleocapsid and the envelope is a proteinaceous region called the tegument that is packaged with viral proteins.

Envelope

The envelope of the HCMV virion is derived from the host cell at both nuclear and cytoplasmic intracellular membranes. It is composed of a host lipid bilayer studded with viral glycoproteins. Although HCMV encodes for as many as 60 putative glycoproteins, there are approximately eight glycoproteins that are incorporated into the envelope (78, 214). The main constituent is the abundant and highly conserved UL55 encoded glycoprotein B (gB) which is thought to be required for viral entry (32, 78). gB is a heparin sulfate proteoglycan-binding glycoprotein that is important for virus binding to the cells and initiating viral entry. gB is found on membranes of infected cells and has been shown to function in cell-to-cell virus transmission and fusion of adjacent cells (21, 158). The gB protein is proteolytically processed and covalently linked to form gB dimers. In addition to gB dimers, the virion envelope is also composed of two additional complexes which are important for cell entry and cell-to-cell transmission. One complex consists of the glycoproteins gH (UL75),

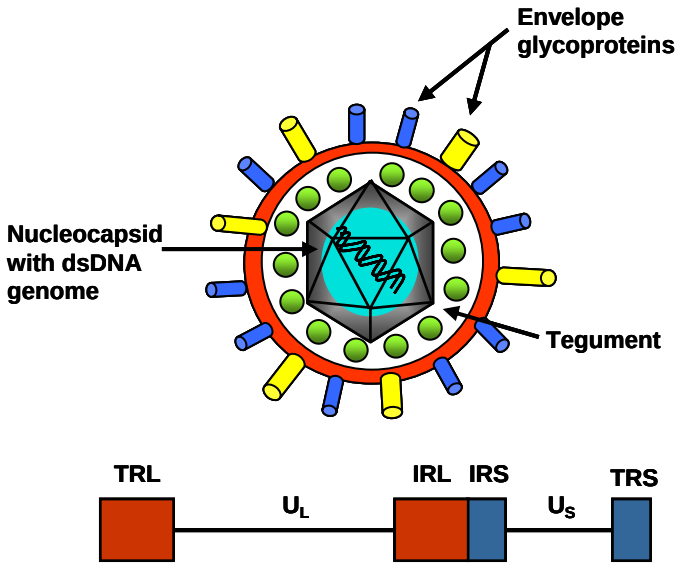

Figure 1-1. HCMV virion and genome organization. The HCMV virion is enveloped and contains a double-stranded DNA genome within an icosahedral nucleocapsid. Between the nucleocapsid and envelope is a proteinaceous layer called the tegument region and is packaged with viral proteins. The HCMV genome is organized into unique long (UL) and unique short (US) regions flanked by internal (IRL and IRS) and terminal repeats (TRL and TRS).

gL (UL115), and gO (UL74) and the other complex is composed of gM (UL10) and gN (UL49.5).

Glycoproteins are not solely studied for their function in viral entry and spread; they are also studied for their immunogenic properties. Many of the glycoproteins have been shown to activate toll-like receptor (TLRs) mediated induction of cytokines (47). In fact, gB continues to be highly studied as possible subunit vaccine candidate due to its abundance, the large number of gB neutralizing antibodies detected in human sera, and the fact that soluble gB can activate the innate immune response in the absence of other viral components.

Tegument

The tegument, termed by Roizman and Furlong (190), describes the region of the HCMV virion between the capsid and the viral envelope. There are at least 25 viral proteins packaged within the tegument (14, 152, 218, 228). Most of the tegument proteins are encoded by conserved open reading frames (ORFs) and are both phosphorylated and immunogenic. The most abundant tegument proteins include UL83 (pp65), UL99 (pp28), UL69, UL48, TRS1, IRS1, UL32 (pp150), and UL82 (pp71) (218). The function of many HCMV tegument proteins has yet to be elucidated but many of the proteins appear to play important roles at very early times postinfection towards establishing a productive infection. Tegument proteins are deposited into the cell cytoplasm and can immediately function in the absence of *de novo* protein synthesis. Some of the tegument proteins are targeted to the nucleus and function as regulators of viral and cellular gene expression. For example, the tegument protein pp71 (UL82) that will be discussed throughout this dissertation is targeted to the cell nucleus upon infection where it is required to initiate efficient

immediate-early gene expression and viral replication (30, 96, 99, 130). Tegument proteins may also be involved in disassembling virus particles, as well as facilitating the maturation of progeny virions (124). In addition to proteins being packaged within the tegument, viral ribonucleic acids (RNA's) appear to be packaged in the tegument and delivered to the host cell during HCMV infection (29). The significance of these viral RNA's is currently unknown.

Capsid

The HCMV capsid is 130 nm in diameter and is composed of hexameric and pentameric capsomeres to form a T=16 icosahedral lattice (34, 45, 227). Seven viral proteins make up the capsid including the major capsid protein (MCP) and the minor capsid protein (mCP). The MCP is highly conserved among the herpesviruses and is the major constituent of the hexamer and pentamer capsid subunits. The mCP and minor-capsid binding protein (mC-BP) form triplexes that connect the hexamer and pentamer capsomeres. Additional proteins associated with the capsid include the smallest capsid protein (SCP) and three assembling/assembly (AP)-related proteins.

Genome

The HCMV DNA genome is linear and double stranded. At approximately 230 kilobases (kb) in length, the genome encodes over 200 ORFs depending on the viral strain (155). The HCMV genome is arranged into two unique regions, unique long (U_L) and unique short (U_S) which are flanked by internal repeats (IR) and terminal repeats (TR) (Fig. 1-1). The ORF location on the genome is often important in the

nomenclature of viral genes. For example the UL82 gene is located in the unique long region of the viral genome.

AD169 and Towne are the HCMV laboratory strains commonly used in tissue culture studies. The highly studied clinical strains include Toledo, TR, FIX and PH (58, 84, 89, 155, 156). In comparison to the AD169 laboratory strain, the clinical strains are deleted for one of the inverted repeats and replaced with an additional 15 kbps in the UL region. This region adds 19 ORFs that are missing from the AD169 genome (41). Approximately 25% of the ORFs conserved throughout the herpesviruses are involved in viral DNA metabolism and replication. The remaining conserved ORFs are thought to function in the structural organization and maturation of the virion. At least 50 of the HCMV ORFs are dispensable for virus replication in fibroblasts (124, 152).

Virus Particles

There are three types of HCMV virus particles produced during the propagation of virus stocks in tissue culture: virions, noninfectious enveloped particles and dense bodies (79, 101, 197). Virions are infectious particles that contain both the nucleocapsid encased DNA core and an envelope (79). Noninfectious enveloped particles lack the electron-dense DNA core and appear to be enveloped B capsid precursors (79, 101, 211, 243). Dense bodies lack the nucleocapsid and viral DNA yet are composed of tegument proteins enclosed in a cytoplasmic membrane derived envelope (50, 101, 117, 123, 197). Both the noninfectious enveloped particles and dense bodies contribute to the high particle-to-PFU ratio (≥100) in virus stocks (215).

HCMV viral replication

HCMV replication occurs in a similar manner to other herpesviruses (Fig. 1-2) (152). During HCMV infection, the glycoproteins on the virion envelope bind and attach to receptors on the cell membrane. Upon fusion, the virus penetrates the cell membrane where the tegument proteins and nucleocapsid are deposited into the cell cytoplasm. The capsid is then targeted to the nuclear pore where the HCMV DNA genome is released into the nucleus. In the nucleus, a cascade of viral gene expression is initiated beginning with immediate-early (IE) gene transcription. The viral DNA is replicated and packaged into newly formed capsids. The DNA-containing nucleocapsid is then targeted to the cytoplasm where it undergoes tegumentation and becomes enveloped in trans-Golgi vesicles (146). Finally, the vesicle encased progeny virion is transported to the plasma membrane where the virion is released from the infected cell (145).

Viral entry

HCMV can efficiently attach and penetrate a number of permissive and nonpermissive cell types. The ability of HCMV to enter a wide range of cells suggests that the virus can use multiple cellular receptors or cell surface molecules to attach and penetrate the cell (47). Recent data also indicates that HCMV can enter the cell via endocytosis in epithelial and endothelial cells (195). Currently no one has identified a single cellular receptor responsible for HCMV entry. However, several coreceptors have been identified providing a hypothesized model for viral entry (47). Upon infection, gM/gN or gB bind to heparin sulfate proteoglycans (HSPG) through a low- affinity tethering interaction (48) that is stabilized by gB

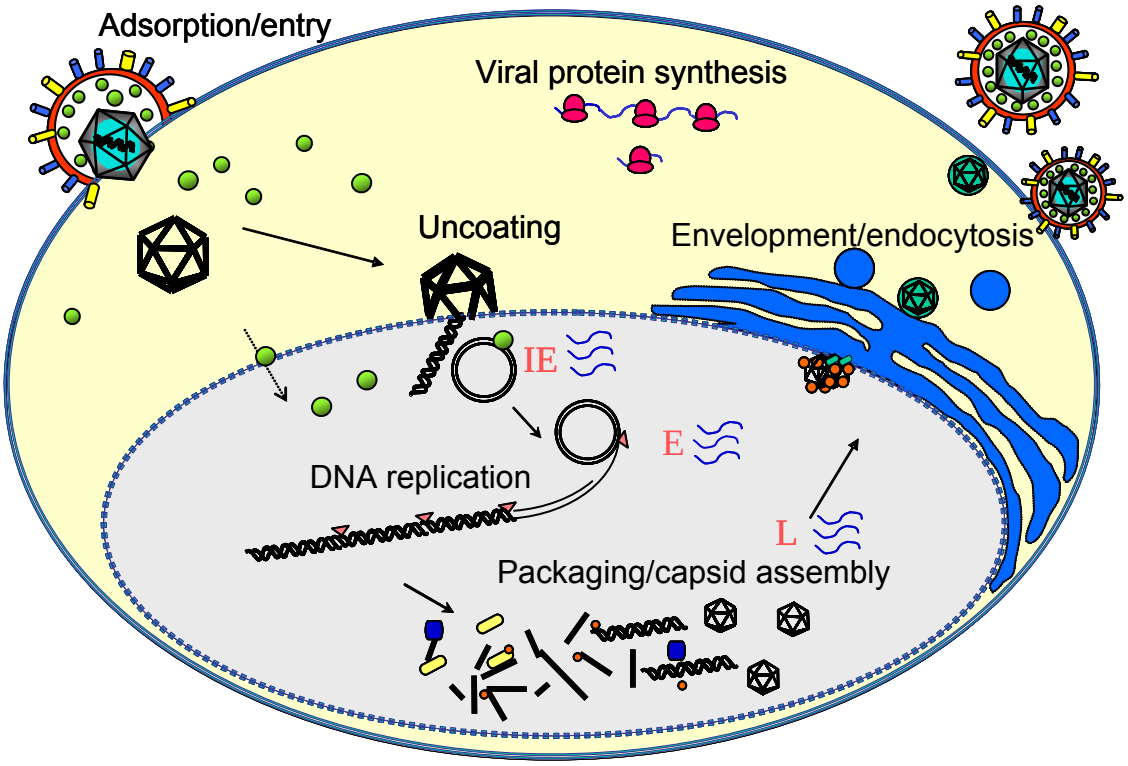

FIGURE 1-2. HCMV replication cycle. A schematic representation of the HCMV replication cycle. Viral glycoproteins on the virion envelope attach and fuse with the receptors on the cell membrane. Upon penetration, the tegument proteins and nucleocapsid are deposited into the cytoplasm of the cell. The nucleocapsid and some of the tegument proteins are targeted to the nucleus. The nucleocapsid is uncoated and the viral genomic DNA enters the nucleus through a nuclear pore. Transcription of the viral genome is initiated and the immediate-early (IE) genes are transcribed in the absence of *de novo* protein synthesis. Following IE protein synthesis, DNA replication occurs followed by synthesis of the virion structural components. Upon assembly and packaging of the progeny virions, the virion undergoes egress through *trans*-Golgi vesicles and is released from the infected cell at the plasma membrane.

docking to the epidermal growth factor receptor (EGFR) (233). Fusion with the cell membrane also requires an interaction between the gH/gL/gO complex with an unidentified receptor (225). Cellular integrins also serve as coreceptors for HCMV glycoproteins and are thought to promote viral internalization (18, 66, 232). Upon binding between viral glycoprotein complexes and cellular receptors, fusion between the virion and the cell membrane is initiated and viral components, including tegument proteins and the nucleocapsid, are internalized into the cell cytoplasm (47).

DNA replication

Upon infection, the HCMV DNA genome is deposited into the nucleus where it circularizes. The DNA begins to replicate from a single origin of replication (7, 90, 138) and is thought to use a directional rolling-circle mechanism similar to other herpesviruses (121, 143). Viral proteins force the host cell cycle into arrest at G1 so that cellular replication proteins are produced and can be used during viral DNA synthesis (27, 56, 106, 133). Viral DNA replication then occurs in viral replication compartments (172). Following DNA replication, the HCMV genome undergoes inversion which results in progeny genomes. These genomes are then directed for cleavage and packaging by pac1 and pac2 sequence elements.

Viral gene expression

Like all herpesviruses, HCMV transcription is temporally regulated in a coordinated cascade which consists of immediate-early (IE), early (E), and late (L) gene expression (Figure 1-3) (152, 234, 235). Immediate-early genes are transcribed first and generally encode for critical regulatory proteins that function in part to control expression of viral early

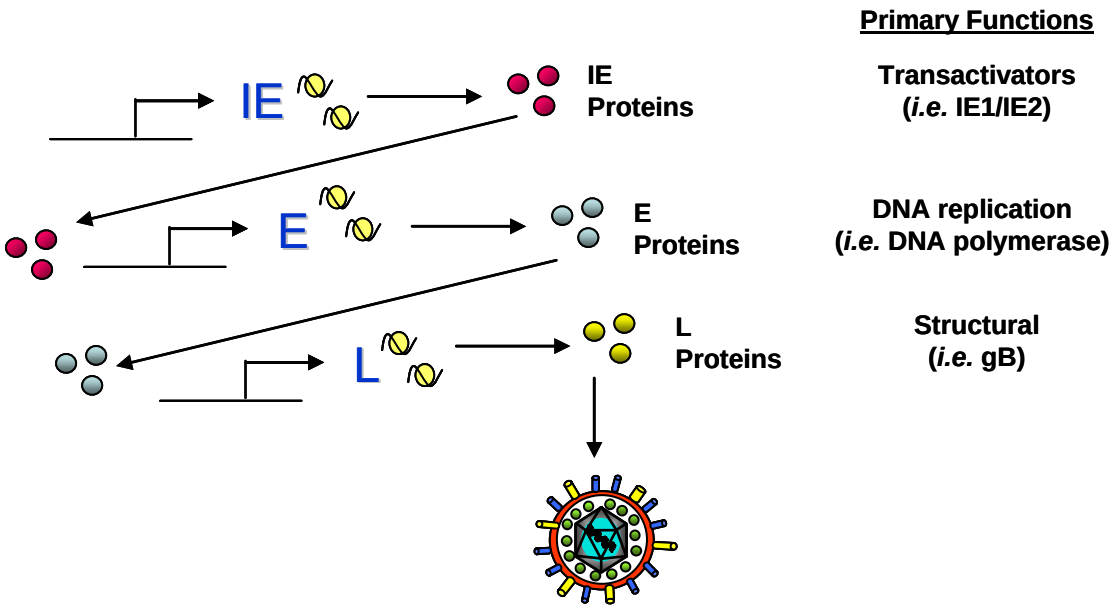

FIGURE 1-3. Viral cascade of gene expression. HCMV gene expression is coordinately regulated through a cascade of events. Immediate-early (IE) gene transcription occurs immediately following infection and does not require de novo protein synthesis. The IE proteins primarily function as transactivators of gene expression and are involved in upregulating early (E) gene expression. Many of the E proteins function during viral DNA replication and include the HCMV DNA polymerase and the DNA processivity factor. The final step of the cascade is late (L) gene expression. Most of the late genes are structural components that make up the virion.

and late genes. By definition, IE genes do not require *de novo* protein synthesis for their transcription (152, 191). However, certain virion tegument proteins which are delivered to the host cell from the infectious virion have been shown to play an important role in controlling efficient IE gene expression (30, 44, 96, 99, 130, 238). Specifically, the tegument proteins pp71 (30, 96, 99, 130) and ppUL69 (238, 239) are involved in regulating the expression of a number of IE genes.

The major IE genes IE1 and IE2 are regulated by the major immediate-early promoter (MIEP). The MIEP is considered to be one of the strongest identified transcriptional enhancers and is used throughout molecular biology (23). It contains binding sites for a variety of host transcription factors including Sp1, activator protein 1 (AP1), p53, nuclear facto kappa B (NF-κB), cAMP response element binding protein/activating transcription factor (CREB/ATF), nuclear factor 1 (NF-1), ELK-1, CCAAT/enhancer binding protein (C/EBP), growth factor independence 1 (Gfi-1), the retinoic acid receptor, and the serum response factor (144, 163, 164). Many of these host transcription factors are important for activating transcription from the MIEP immediately following infection. Expression from the MIEP is negatively regulated by the IE2 protein later during infection (135).

The IE proteins IE1 and IE2 are important for transcriptional regulation and virus replication. IE1 (p72) is expressed first and is important for viral replication. An IE1 deletion mutant virus displays a defective growth phenotype at low multiplicities of infection (MOI) but grows to wild-type levels when cells are infected at a high multiplicity (85, 153). IE1 has been shown to function in further activation of the MIEP (153) and the activation of E and L gene expression (85). The other major IE protein IE2 (p86) is also involved in activating E and L gene expression

and is important in regulating the switch from the IE to the E and L stages of viral gene expression. Although IE2 alone can function as a transactivator, IE2 transactivation is more efficient in the presence of IE1 (152). A temperature sensitive IE2 mutant cannot replicate when cells are infected even at a high multiplicity, suggesting IE2 is required for productive virus replication (91).

Efficient IE gene expression is critical for progression through the cascade of viral gene expression. IE proteins are involved in transactivating early (E) gene expression. Many of the E gene products are involved in DNA replication including the viral DNA polymerase (UL54) and the polymerase processivity factor (UL44). Late (L) gene expression occurs following viral DNA replication. The late proteins are generally structural components of the virion including viral glycoproteins and tegument proteins. Considering that immediate-early, early, and late gene expression are all required to produce infectious progeny, the establishment of IE gene expression is imperative for viral replication. This dissertation discusses in detail how the HCMV pp71 protein functions to enhance IE gene expression and thereby initiate the viral cascade of gene expression leading to a productive virus infection.

Packaging/Egress

Once viral gene expression is established and the HCMV genome has undergone replication, the newly synthesized viral DNA is packaged into a preformed capsid. Formation of these nucleocapsids can be visualized as nuclear inclusions seen at late times postinfection (69). Maturation begins when viral structural proteins form a network around the nucleocapsid adjacent to the viral replication compartment (69, 172). The mature DNA-containing nucleocapsid undergoes primary envelopment at

the inner nuclear membrane which is modified with viral glycoproteins (183, 216). Fusion of the primary envelope with the outer nuclear membrane occurs and the capsid is released into the cytoplasm (146). The capsid then undergoes tegumentation and is targeted to a secondary envelopment site at *trans*-Golgi vesicles (145). The vesicle containing the progeny virion is transported to the plasma membrane where the vesicle and plasma membranes fuse and the mature virions are released from the infected cell (145, 146).

UL82 (pp71)

The UL82 gene encodes for the pp71 (phosphoprotein 71) tegument protein, named for its molecular size upon electrophoresis and the fact that it is phosphorylated during HCMV infection (Fig. 1-4) (165, 189, 194). During infection, pp71 is delivered from the tegument of the virion and targeted to the cell nucleus. The function of pp71 during infection has not been completely elucidated. Previous studies demonstrated that pp71 activates immediate-early gene expression (30, 96, 99, 130). It was also discovered that cotransfection of a plasmid encoding for pp71 enhanced the infectivity of viral DNA, increasing plaque production following transfection (13). Through the use of a UL82 (pp71) deletion mutant, it was demonstrated that pp71 is required for efficient viral replication when cells are infected at a low multiplicity (30). Using the same mutant, it was also shown that pp71 delivered to the host cell from the virus particle plays an important role in regulating IE gene expression during a productive infection (30).

Based on pp71's ability to activate immediate-early gene expression (30, 96, 99, 130) and enhance the infectivity of viral DNA (13), pp71 is thought to be the functional homolog of the HSV-1 virion protein

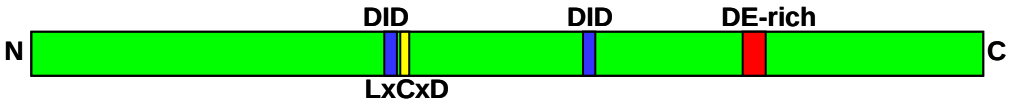

FIGURE 1-4. Schematic representation of pp71. The UL82 encoded protein pp71 contains two hDaxx interacting domains (DID), an LxCxD motif (shown in yellow) involved in targeting Rb family member proteins for degradation, and an acid-rich region (DE-rich).

16 (VP16) protein. VP16 is packaged in the tegument of the HSV-1 virion and is involved in the transcriptional regulation of viral gene expression (16). Similar to the UL82 deletion mutant virus, an HSV-1 virus deleted for the VP16 open reading frame displays a multiplicity dependent growth phenotype (1, 219). Although there remains controversy over whether pp71 and VP16 are functional homologs, preliminary data from our laboratory demonstrates that pp71 can complement a VP16 null mutant (data not shown), suggesting that VP16 is the HSV functional homolog to pp71.

Other functions and interactions had also been attributed to pp71 prior to the work presented in this dissertation. Using *in vitro* overexpression assays, Kalejta *et. al*. demonstrated that pp71 is able to interact with and degrade retinoblastoma (Rb) tumor suppressor family member proteins, resulting in quiescent cells entering the cell cycle (109, 110, 112). In addition to interacting with Rb family member proteins, pp71 has also been shown to interact with the cellular protein hDaxx (96, 105). pp71's ability to interact with hDaxx was demonstrated to be important for transactivation of the MIEP and for pp71 localization to ND10 domains (96). The initial studies identifying pp71 associations with Rb family members and hDaxx were conducted by transiently overexpressing proteins in cells nonpermissive for HCMV infection. Although pp71 has been shown to interact with these cellular proteins, it remained uncertain if these interactions were important for IE gene expression and viral replication. Therefore, the goal of this dissertation was to determine if pp71's ability to target Rb family proteins for degradation or to interact with hDaxx were important for viral replication and IE gene expression in the context of a viral infection. Based on our results, we also wanted to further define the mechanism by which pp71's ability to interact with either

of these cellular proteins enhanced viral replication and immediate-early gene expression.

Interaction with Rb family member proteins

Using *in vitro* overexpression assays, Kalejta *et. al.* demonstrated that pp71 is able to interact with and degrade retinoblastoma (Rb) family member proteins, resulting in quiescent cells entering the cell cycle (109, 110, 112). The Rb family member proteins (pRb, p107 and p130) are tumor suppressor proteins that function to block cell cycle progression during the G0 and G1 phases of the cell cycle (Fig. 1-5). The mechanism Rb proteins utilize to inhibit cell cycle progression is by targeting the E2F family of transcription factors (60, 162). Hypophosphorylated Rb represses gene transcription by binding to E2F proteins through the Rb pocket domain or by directly binding to the promoters of E2F regulated genes in a complex with E2F (2, 67, 92, 182, 254). Additionally, when E2F is bound in a complex with Rb, progression of the cell cycle into S phase is inhibited (107). For cell cycle progression through G1 to occur, the Rb proteins must become phosphorylated, undergo a conformational change and release the E2F transcription factors. During normal cell cycle progression, cyclin D proteins bind to Rb proteins through an LXCXE motif, recruit cyclin dependent kinases (cdk) 4 and 6, and phosphorylate pRb and p130 (60). Later during G1, cyclins E and A coupled to cdk2 further phosphorylate the Rb proteins (52), ultimately leading to the physical dissociation of E2F transcription factors. Upon release, the E2F transcription factors upregulate the expression of genes necessary for cell cycle progression including cyclins E, A and B or DNA replication factors (77).

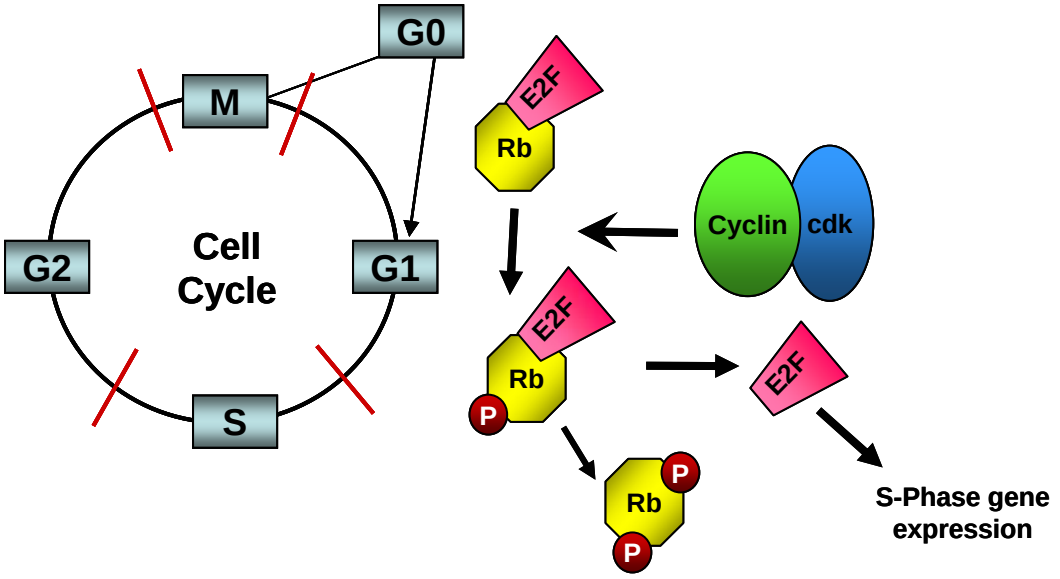

FIGURE 1-5. Rb and the cell cycle. Rb regulates cell cycle progression by interacting with E2F transcription factors. Upon phosphorylation of Rb family member proteins, E2F transcription factors are released and upregulate S-phase gene expression.

Studies by Kalejta *et. al.* indicated that pp71 attacks hypophosphorylated Rb tumor suppressor family members through an LXCXD motif within pp71, targeting the tumor suppressor proteins for proteasome-dependent, ubiquitin-independent degradation (110, 112). This LXCXD motif is similar to the LXCXE motif used by the common DNA tumor virus proteins including adenovirus E1A, SV40 T antigen and papillomavirus E7 to mediate binding with Rb family member proteins. When Kalejta *et. al.'s* group substituted the cysteine within the LXCXD motif with a glycine at residue 219, they abolished pp71's ability to degrade Rb family members and blocked its ability to induce quiescent cells into the cell cycle (110, 112). Interestingly, pp71's effect on Rb family member degradation and the host cell cycle was not linked to its *in vitro* transactivation capabilities as the UL82-C219G mutant activated the major immediate-early promoter similar to wild-type pp71 in a luciferase assay (111). Although none of these studies were conducted in the context of a virus infection, it was hypothesized that pp71's ability to target Rb family member proteins for degradation and progress cells through G0 would provide an advantage for virus replication (110-112).

Interaction with hDaxx

pp71 has also been shown to interact with the cellular protein hDaxx (96, 105). The significance of pp71's interaction with the human death-domain associated protein hDaxx is not fully understood but it is thought to assist in upregulating viral gene transcription at subnuclear sites (96, 105). During HCMV infection, pp71 colocalizes with hDaxx at specific nuclear domains 10 (ND10), (96, 105, 137) which are sites of active viral gene transcription (59, 102, 104, 139, 141, 142). Two hDaxx binding domains were mapped to amino acids (aa) 206-213 and 324-331

of pp71 (96). Transfection studies revealed that removal of either of these binding domains blocked pp71's interaction with hDaxx and prevented pp71 localization to ND10 domains (96). Blocking pp71's ability to interact with hDaxx also abolished pp71's ability to transactivate the major immediate-early promoter in transient reporter assays (96). Additionally, cotransfection of plasmids expressing hDaxx and pp71 synergistically enhanced transcription from the MIEP, suggesting hDaxx functions as a positive regulator of viral gene (96). Considering pp71's ability to interact with hDaxx is important for the regulation of viral gene transcription *in vitro*, it was hypothesized that the interaction between pp71 and hDaxx would provide an advantage for viral gene expression and virus replication in the context of a viral infection (96).

hDaxx

The human death-domain-associated protein hDaxx is a multifunctional cellular protein and has been recognized as a regulator of both apoptosis and gene expression. hDaxx was originally identified as a pro-apoptotic protein which could bind to the death domain of the transmembrane death receptor FAS in the cytoplasm and thereby enhance FAS-induced apoptosis (226, 245). hDaxx is ubiquitously expressed in mammals and is highly conserved with 69% identity between human and mouse (116). Early studies established that hDaxx is required for embryonic development as Daxx null mice were found to be embryonic lethal (150).

Structurally, hDaxx contains two N-terminal paired amphipathic helices (PAHs), a coiled-coil domain, a nuclear localization signal (NLS), three PEST (proline, glutamate, serine and threonine rich) domains, a glutamic acid-rich region and a C-terminal serine/proline/threonine-rich

region (Fig. 1-6). Numerous Daxx interacting proteins have been identified *in vitro*. Most of these proteins have been shown to interact primarily at the amino terminus of hDaxx, while the pp71 protein was shown to interact through two domains on hDaxx; a strong binding site in the glucine-rich region, and a weak binding site in the amino terminus (96). Therefore, pp71 may not need to compete with cellular proteins to bind hDaxx.

hDaxx was initially identified as an inducer of apoptosis. Early studies indicated that Daxx functioned as a pro-apoptotic protein that interacted with both the Fas and tumor growth factor β (TGFβ) II receptors to initiate apoptosis (173, 245). These studies suggest hDaxx functions as a pro-apoptotic protein acting downstream of FAS by activating the c-Jun NH_2-terminal kinase (JNK) pathway independent of the Fas-associated death domain (FADD) protein recruited to activate caspases in the Fas pathway (43, 173, 245). Although these early studies provide extensive data suggesting hDaxx functions to promote apoptosis, there is also evidence indicative of an anti-apoptotic role for hDaxx. For example, disruption of the Daxx gene in mice leads to extensive apoptosis in Daxx null embryos (150). Also, recent studies using small interfering RNA directed against Daxx have reported increases in apoptosis (151) and increased sensitivity to Fas, UV or TNFα induced apoptosis (46). Extensive research will be required to further address the controversial role for Daxx during apoptosis.

hDaxx's role in regulating gene expression is also unclear. Although hDaxx has been associated with transcriptional activation (19, 43), hDaxx is primarily thought to function as a transcriptional repressor (46, 86, 98, 126, 127, 151, 226). Structurally, hDaxx contains the two

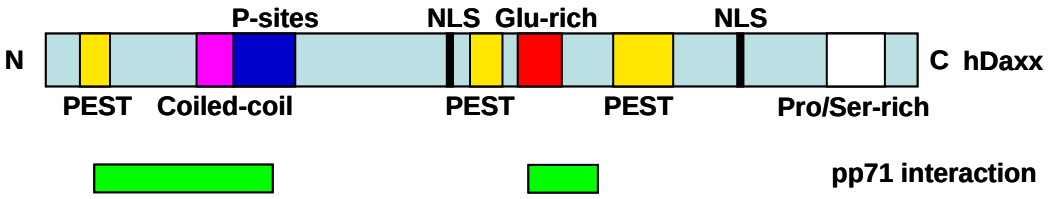

FIGURE 1-6. Schematic representation of hDaxx. The hDaxx protein contains 3 (PEST) domains, a coiled-coil domain, 2 amphipathic helices (P-sites), 2 nuclear localization signals (NLS), a glutamic acid-rich region (Glu-rich), and a proline/serine rich region (Pro/Ser-rich). The hDaxx protein binds to pp71 through a strong binding site at the N-terminus and a weaker binding site in the glucine-rich region.

amphipathic helices similar to the cellular corepressor Sin3a (10), suggesting hDaxx may act as a transcriptional corepressor (98). Studies using siRNA directed against hDaxx have demonstrated that hDaxx can repress NFκB, E2F-1, Pax3, and Ets-1 mediated transactivation (151). hDaxx has also been shown to repress p53 (83, 114), p73 (114) and Smad4 (42) transcriptional activity in luciferase assays. Although the mechanism by which hDaxx represses transcription remains to be defined, it is hypothesized that hDaxx acts as a corepressor by interacting with a number of cellular proteins that are involved in regulating gene expression including DNA methyltransferase 1 (DNMT1) (150), Dek, core histones and histone deacetylases (HDAC) (97).

The role of hDaxx during virus infection has recently begun to be examined. For example, hDaxx has been shown to bind the avian sarcoma virus (ASV) integrase protein and represses ASV transcription (86). In terms of HCMV infection, hDaxx is thought to function as a regulator of viral IE gene expression. However, the mechanism by which hDaxx regulates HCMV IE gene expression was unclear. Transient transfection assays have demonstrated that hDaxx expression activated the HCMV MIEP and that cotransfection of pp71 with hDaxx had a synergistic effect on the activation of the MIEP (96). In addition, HCMV infection of Daxx null mouse cells led to a 2-fold reduction in the number of immediate-early 2 (IE2) protein expressing cells (105). Taken together these results suggest that hDaxx functions as a positive regulator of the MIEP and IE gene expression. However, preliminary studies by Reeves *et. al.* suggest that overexpression of hDaxx represses HCMV replication (185). Therefore, given the conflicting data and multifunctional nature of hDaxx, it was unclear if hDaxx functions as an activator or repressor during HCMV infection.

ND10 domains

During HCMV infection, pp71 associates with hDaxx at subcellular structures termed nuclear domain 10 (ND10). These ND10 domains, also known as nuclear dots, promyelocytic leukemia (PML) nuclear bodies or promyelocytic oncogenic domains (PODs) were originally described in electron microscopy studies as “dense granular bodies and pale round structures, at times showing a complex laminar pattern” (54, 94). ND10 domains are comprised of several cellular proteins including the major ND10-associated proteins PML and Sp100. Depending on cell type, there are usually 5-20 ND10 domains that are approximately 0.5μm in diameter and appear as punctuate foci in the nucleus. ND10 domains have been associated with splicing domains or speckles that correspond to interchromatinic granule clusters during immunofluorescence analysis (177). Although most cell types contain ND10 domains, there are certain cell types which appear to lack ND10 domains including certain neuronal cells and cells derived from germinal centers of the spleen, lymph nodes or Peyer’s patches (51, 68, 75, 122). The formation and integrity of ND10 domains require the PML protein (103) while other ND10-associated proteins including SP100 and hDaxx are not required for ND10 domain formation.

The role of ND10 domains during viral infection began to be studied when the observation was made that HSV-1 infection resulted in the destruction of ND10 domains (140). The HSV-1 regulatory protein ICP0 was shown to interact with the PML protein and induce proteasome-dependent degradation of PML, leading to the disruption of ND10 domains (24, 63, 140). HSV-1 mutant viruses defective for ICP0 expression were unable to induce the degradation of PML and ND10 domains remained intact (140). During HCMV infection, viral interactions with ND10 domains

begin when the pp71 tegument protein accumulates at ND10 domains through an interaction with hDaxx (96, 105, 137). This interaction is thought to be important for initiating IE gene expression (96). Once IE gene expression is upregulated, the IE1 protein binds to PML (6). In contrast to the function of the ICP0 protein during HSV-1 infection, IE1 does not appear to target the PML protein for degradation. However, IE1 appears to sequester the PML protein and therefore initiate dispersion of ND10 domains at early times postinfection (5, 104, 113, 118, 237).

In addition to viral induced disruption of ND10 domains, it has also been demonstrated that many DNA viral genomes and replication centers are localized adjacent to ND10 domains during infection. Initial studies examining DNA viral genome deposition in the cell reported that HSV-1 and adenovirus genomes and/or viral replication centers were localized adjacent to ND10 domains (102). It was later shown that HCMV genomes also localize to ND10 domains (220). It was originally thought that formation of viral replication centers juxtaposed to ND10 domains provide an ideal environment for transcription of the viral genome to occur. Therefore, the arrangement of IE transcripts, IE proteins and adjacent ND10 domains have been described as the immediate transcript environment (104). The same phenomenon has been identified during infection with SV40 virus, polyomavirus, parvovirus AAV, and other herpesviruses (73, 102).

The overall function of ND10 domains during viral infection remains unclear. It was originally thought that ND10 domains enhanced viral replication and that replication centers formed adjacent to ND10 domains to enhance viral transcription. However, recent studies indicate that during HSV-1 infection, ND10 domain components are recruited toward the viral genome, and new ND10 domain structures form juxtaposed to the

sites of incoming genome (64, 65). These data suggest that ND10 domains are a cellular response to viral infection. It is also now known that ND10 domains are not required for viral infection based on a study demonstrating HCMV replicates more efficiently in PML deficient cells lacking ND10 domains (221). Together, these recent discoveries and the results presented in this dissertation regarding ND10 domains and viral infection provide evidence that ND10 domains function as an intrinsic immune response to HCMV infection.

Chromatin structure regulation of viral gene expression

Many of the ND10 domain associated proteins, including hDaxx, have been shown to function as regulators of transcription. Eukaryotic gene expression is highly regulated by chromatin structure and the recruitment of transcriptional activators or repressors. Nucleosomes consisting of histone proteins and chromosomal DNA make up the chromatin structure and have a “beads on a string” appearance. Chromatin structure and histone modifications play a major factor in transcriptional regulation and packaging DNA. Histone modifications have been well characterized and strongly correlate with the transcriptional state of the associated DNA. Modifications to the histones include acetylation (57), deacetylation (132), methylation (249), sumoylation (200), ubiquitination and phosphorylation (134). These modifications determine the structure of the chromatin and provide a code (217) that determines the binding of regulatory proteins. Therefore, the pattern of modification to histones determines if the recruited proteins will upregulate or repress gene expression from the associated DNA.

Histone acetylation has been correlated with transcriptionally active

DNA (Fig. 1-7) (120, 134). Histone acetyltransferases (HATs) including CREB-binding protein (CBP), p300 and p300/CBP-associated factor (P/CAF), are enzymes that catalyze the acetylation of lysines on the N-terminal regions or “histone tails”. Acetylation of the histone reduces the affinity of the histones for DNA and allows for a looser association between the DNA and histones. This allows proteins involved in transcription to access the genome and initiate gene expression. Histone deacetylases (HDACs) are enzymes that remove the acetyl groups from the histones. Upon removal of the acetyl groups, the positively charged lysine residues form a tight interaction with the negatively charged DNA. This results in compaction of the chromatin and induces a transcriptionally inactive state.

Herpesvirus DNA has been shown to associate with histones during viral infection. Similar to eukaryotic gene expression, modifying the chromatin structure is important for regulating viral gene expression (12). Chromatin structure has also been correlated with latency and cell tropism during HCMV infection. Studies have demonstrated that the MIEP is associated with acetylated histones at early times postinfection and in permissive cell types (157). In non-permissive cells or later times postinfection, the MIEP has been shown to be associated with methylated histones, indicating a transcriptionally inactive state (157). Both histone modification and transcriptional activity appear to be correlated with cell tropism and latency during HCMV infection.

There are many factors that contribute to histone modification and chromatin structure at viral promoters during infection. In the case of the MIEP, corepressors have been shown to bind and repress the MIEP including Ets2 repressor factor (ERF) (11), yin yang-1 (YY1) (131), modulator recognition factor (MRF) (100) and growth factor

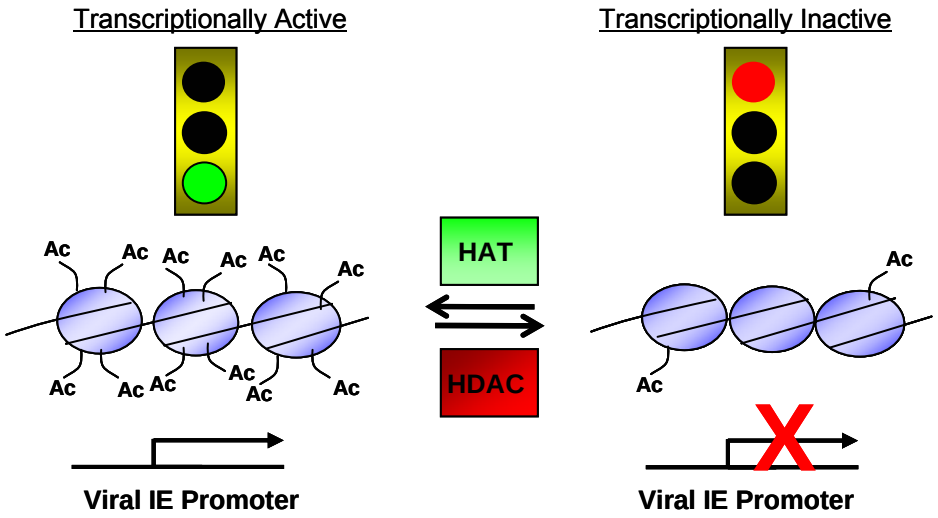

FIGURE 1-7. Histones and transcriptional activity. In a transcriptionally active state, histone acetyltransferases (HAT) acetylate histones associated with the promoter enabling transcription. Histone deacetylases (HDAC) remove acetyl groups associated with the promoter resulting in transcriptional repression.

independence-1 (Gfi-1) (255). Although these factors directly bind to the MIEP, it appears that repression is mediated through interactions with chromatin remodeling cofactors. Both YY1 and ERF interact with HDAC family member proteins and may repress the MIEP by recruiting HDAC proteins to deacetylate histones associated with the MIEP. Additionally, ERF recruits the methyltransferase SUvar to the MIEP and represses transcriptional activity (242). Many of these events involving the repression of viral promoters are thought to be important in regulating latency and reactivation from latency.

Chromatin structure and histone modifications are also important for promoting lytic infection. Multiple viral proteins have been shown to upregulate gene expression from the MIEP by interacting with cellular modifiers. In some instances viral proteins interact with cellular proteins associated with transcriptional repression. During HCMV infection, IE1 binds to HDAC1 to prevent deacetylation of histones associated with the MIEP (161). Other viral proteins recruit HATs to acetylate histones associated with the MIEP and enable transcription (12). For example, the viral protein IE2 interacts with P/CAF and this interaction is thought to enhance activity from target promoters through P/CAFs HAT activity (33). Therefore, the recruitment of HATs or HDACs to the viral promoter regions is important in regulating the chromatin structure and transcriptional activity of HCMV viral gene expression.

Summary

Infection with human cytomegalovirus can lead to severe disease during a primary lytic infection or by establishing a life-long latent infection that can become reactivated. With limited drug availability and lack of an efficient vaccine, research regarding how HCMV interacts with the host to ensure productive viral replication is needed to make advances in HCMV prevention and therapy. This dissertation is based on the hypothesis that the HCMV pp71 protein interacts with a cellular protein to enhance viral immediate-early gene expression and replication during a low multiplicity infection. The research presented in this dissertation outlines our efforts to define the function of the pp71 protein during viral infection and provide insight into a mechanism by which virus overcomes a cellular intrinsic immune response to human cytomegalovirus infection.

CHAPTER TWO
Methodology

Cell culture and virus propagation

Human foreskin fibroblast (HFF) cells, Telomerase life extended fibroblasts (Tel12), Phoenix A cells, U373 MG cells, and stable cell lines were cultured in Dulbecco's modification of Eagle's medium (DMEM, Mediatech) supplemented with 10% (vol/vol) fetal calf serum (Gemini or Hyclone), 100 units/ml penicillin (Sigma), and 100 μg/ml streptomycin (EM Science) in an atmosphere of 5% CO_2 at 37°C.

Recombinant viruses were generated by transfecting BAC DNA (~1 μg) into 5 x 10^6 UL82 complementing cells (Tel UL82 #5) via electroporation (950μF, 260V). Cells were seeded into dishes and infectious virus harvested when 100% cytopathic effect (CPE) was observed. Wild-type and UL82 recombinant viruses generated from bacterial artificial chromosome (BAC) DNA were propagated as described previously (Fig. 2-1) (30). After collecting the infected supernatant, the cells were scraped and sonicated in a waterbath sonicator twice for 45 seconds (s) in a borosilicate tube. The sonicated sample was added back to the supernatant and cell debris pelleted at 3750 revolutions per minute (rpm) for 10 minutes (min). The supernatant was aliquoted and frozen at -80°C. Infectious titers for all viruses were determined by plaque assay on HFF, Tel12, or the UL82 complementing cells (Tel UL82 #5) as described

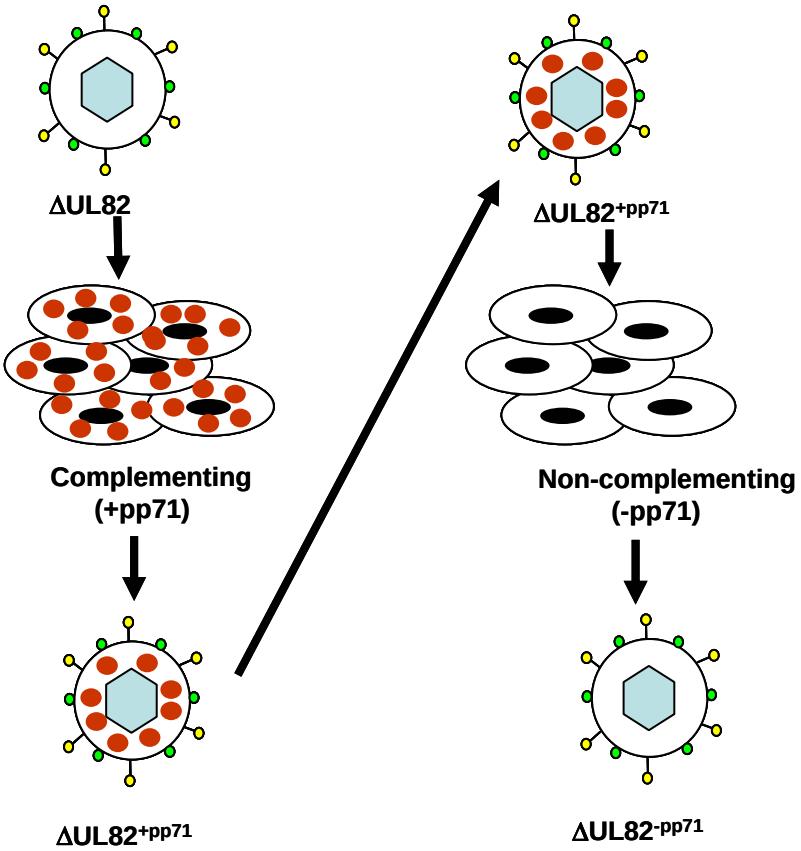

FIGURE 2-1. Generation of UL82 deletion mutant virus stocks. The UL82 deletion mutant virus is first propagated on cells expressing pp71. Virus harvested from the pp71 complementing cells contains pp71 in the virus tegument but lacks UL82 in the viral genome (ΔUL82+pp71). The ΔUL82+pp71 virus is subsequently used to infect non-complementing fibroblasts to generate a high titer stock that is void of pp71 (UL82 in both the tegument and the viral genome (ΔUL82-pp71). (●) represents the pp71 protein.

(30). Mutations or deletions were also confirmed by directly sequencing DNA isolated from viral particles. HFFs or complementing cell lines were seeded onto 6-well dishes and grown to confluency. Cells were infected at serial dilutions in a minimal volume of media for 2h. 3-4 days postinfection (dpi) infected cells were overlayed in DMEM containing 0.75% low-melting point agarose (Invitrogen) and 20% fetal bovine serum. Cells were fixed to the plate with 10% formalin (EM Science) and agarose overlays removed 15-20 dpi. Cells were stained with 0.05% methylene blue and plaques counted under a light microscope.

Plasmid construction

Plasmids pGS284 (209), pCGNpp71 (13), pEGFP-hDaxx (250), and pGEMTKan/LacZ (246) have been described elsewhere.

Oligonucleotides

The oligonucleotides used for site-directed mutagenesis, constructing expression vectors, and sequencing were obtained from Integrated DNA Technologies and are listed in Table 2-1.

Expression vectors

pCGNpp71-C219G was generated using the Stratagene QuikChange Site-Directed Mutagenesis Kit according to the manufacturer's protocol. Briefly, pCGNpp71 was digested with *Xba*I and *Sal*I to drop out a 1.6kB fragment from the UL82 open reading frame. The *Xba*I/*Sal*I fragment was subsequently cloned into *Xba*I/*Sal*I digested pGEM11 (Clontech) to yield pGEMpp71. pGEMpp71, in conjunction with oligonucleotides C219G-S and C219G-AS, were used to generate the

Primer Name	Sequence
C219G-S	GGAGCAGCTGGCCGGTTCGGACCCTAACACG
C219G-AS	CGTGTTAGGGTCCGAACCGGCCAGCTGCTCC
SC48	GCGGAAGCTTTGGTCGCCTGC
SC49	GAATTCGATGGCGCCGGCGCGAAAGG
SC50	GAATTCGAGCAGCTGGCCTGTTCGG
SC51	GCATGCCGTAGTGCGGCGTGCTGCACG
SC52	GAATTCGATGTTTTCCGGGAAAAAGATGG
SC53	GAATTCCCGCTACCCGATCGTGTGCG
ADVCGN-5'	CTCTGGATCCGGTACCATGGCTTCTAGCTACCTTATG
ADVCGN-3'	GCGGCGCCAAACTCACCCTGAAGTTCTC
LF-5'	CAACTAGTCGGCGTGACGGAGCGCGAGTC
LF-3'	GATTAATTAACCTAGGGGGCGGGATGGGGGGAGGGTCAGG
RF-5'	CTCTTAATTAACGGTCCGTGCCCGCGCCACGACC
RF-3'	GTTACGTATCTACCGCCGCTTTTACG
Stop82-199-AS	GGCGCCGGCGCGTTAGGCGAACTGGC
Stop82-199-S	GCCAGTTCGCCTAACGCGCCGGCGCC
Stop-82-223-S	GGACCCTAACACGTAGATCCACTAAACGGAGACGG
Stop82-223-AS	CCGTCTCCGTTTAGTGGATCTACGTGTTAGGGTCC
Stop82-318-AS	CGCCCGGGATGTTTTACGGCTAAAAGATGGTGTCGTG
Stop82-318-S	CACGACACCATC TTTAGCCGTAAAACATCCCGGGC
Stop82-410-AS	CGGTGGGTGGTTATCAGGGGGACTGCGG
Stop82-410-S	CCGCAGTCCCCCTGATAACCACCCACCG
shDaxx-S	GATCCCCGGAGTTGGATCTCTCAGAATTCAAGAGATTCTGAGAGATC CAACTCCTTTTTA
shDaxx-AS	AGCTTAAAAAGGAGTTGGATCTCTCAGAATCTCTTGAATTCTGAGAGA TCCAACTCCGGG
shScramble-S	GATCCCCGCGCGCTTTGTAGGATTCGTTCAAGAGACGAATCCTACAA AGCGCGCTTTTTA
shScramble-AS	AGCTTAAAAAGCGCGCTTTGTAGGATTCGTCTCTTGAACGAATCCTAC AAAGCGCGCGGG

Table 2-1. Cloning oligonucleotides. Primers used for site-directed mutagenesis and plasmid construction are listed with the oligonucleotide sequence. All oligonucleotides were obtained from Integrated DNA Technologies.

plasmid pGEMpp71-C219G which contains the cysteine to glycine point mutation at position 219. The *Hind*III/*Sac*II fragment from pGEMpp71-219G containing the mutation was then ligated into *Hind*III/*Sac*II digested pCGNpp71 to yield pCGNpp71-C219G.

pp71-hDaxx binding mutant constructs pCGNpp71Δ206-213 and pCGNpp71Δ324-331 were generated by using primer pairs SC48/SC49 and SC48/SC52 to amplify the N-terminus and primer pairs SC50/SC51 and SC51/SC53 to amplify the C-terminus respectively of UL82 using pCGNpp71 as template. Fragments were TA cloned into pGEMT-Easy (Clontech) and the corresponding N- and C-terminal regions were ligated together via the *Eco*RI restriction sites within the primers to yield plasmids pGEMTEpp71Δ206-213 and pGEMTEpp71Δ324-331. These plasmids were digested with *Hind*III and *Kpn*I and the fragments containing the deletions were ligated into *Hind*III/*Kpn*I digested pCGNpp71.

The UL82 stop codon mutant constructs were generated using the Stratagene QuikChange Site-Directed Mutagenesis Kit according to the manufacturer's protocol. pCGNpp71 was used as a template and the oligonucleotides designated in parentheses were used to generate pCGNpp71-199Stop (Stop82-199-AS, Stop82-188-S), pCGNpp71-223Stop (Stop82-223-AS, Stop82-223-S), pCGNpp71-318Stop (Stop82-318-AS, Stop82-318-S), and pCGNpp71-410Stop (Stop82-410-AS, Stop82-410-S). These plasmids were mutated to contain stop codons at pp71 amino acids 199, 223, 338, or 410 respectively. All mutant plasmids were confirmed by DNA sequencing.

Retrovirus vectors

pRevTREpp71HA was generated by using primers ADVCGN-5' and ADVCGN-3' to polymerase chain reaction (PCR) amplify the UL82 open reading frame and N-terminal HA tag, using pCGNpp71 as a template. The *Bam*HI digested PCR product was then ligated into *Bam*HI digested pRevTRE vector (Clontech). pRevTREpp71Δ206-213 and pRevTREpp71Δ324-331 were generated by excising a *Rsr*II/*Not*I fragment from pCGNpp71Δ206-213 and pCGNpp71Δ324-331 respectively and ligating the fragments into pRevTREpp71HA that was digested with *Rsr*II and partially digested with *Not*I.

Shuttle vectors for allelic exchange

To construct the pGS284ΔUL82-2A shuttle vector, UL82 flanking regions corresponding to nucleotides 121,748-119,164 and 117,566-115,926 of the HCMV AD169 strain genome were amplified using primer pairs RF-5'/RF-3' and LF-5'/LF-3' respectively and cloned into the pGEMT-Easy vector (Clontech) to yield pUL82Flanks. pGEMTEKan/LacZ (246) was digested with *Pac*I to excise the kanamycin(Kan)/LacZ cassette which was then cloned into *Pac*I digested pUL82Flanks to yield pΔUL82Kan/LacZ. pΔUL82Kan/LacZ was digested with *Sph*I and partially digested with *Nsi*I (which drops out the UL82 flanks and Kan/LacZ cassette) and was cloned into *Nsi*I and *Sph*I digested pGS284 to yield pGS284ΔUL82-2A. pGS284ΔUL82-2A contains a Kan/LacZ cassette in place of the UL82 coding region from nucleotides 119,171 to 117,566. Shuttling vectors used for allelic exchange to generate the various recombinant viruses were generated by digesting pCGNpp71, pCGNpp71-C219G, pCGNpp71Δ206-213, pCGNpp71Δ324-331, pCGNpp71-199Stop,

pCGNpp71-223Stop, pCGNpp71-318Stop, and pCGNpp71-410Stop with *Xba*I and *Rsr*II. The corresponding fragments were ligated into pGS284ΔUL82-2A digested with *Avr*II and *Rsr*II to generate pGS284UL82, pGS284C219G, pGS284UL82Δ206-213, pGS284UL82Δ324-331, pGS284UL82-199Stop, pGS295UL82-223Stop, pGS285UL82-318Stop, and pGS284UL82-410Stop respectively. All constructs used in these studies were verified by DNA sequencing.

Short hairpin RNA retrovirus vectors

Oligonucleotides encoding short hairpin RNA sequences against hDaxx (Chen) or a scramble sequence (Oligoengine) were designed and inserted into the pSuperRetroPuro plasmid (Oligoengine) according to the manufacturer's protocol. Oligonucleotides shDaxx(S) and shDaxx(AS) or shScramble(S) and shScramble(AS) were annealed and ligated into pSuperRetroPuro vector linearized with *HindIII* and *Bgl*II restriction enzymes to generate pSuperDaxx and pSuperScramble respectively. Insertion of the oligonucleotides was confirmed by DNA sequence analysis.

Allelic exchange and BAC generation

Allelic exchange methods that have been previously described (209, 246) were used to generate the HCMV BACs. Cultures for both the BAC construct and the pGS284 shuttle vector were grown overnight and equal volumes of each culture were transferred to a 10mM MgSO4 solution and run through a sterile 10.45 μM HA filter (Millipore). The filter was incubated on a LB agar (Miller formula) plate for 6 hours, transferred into LB broth (Miller formula) without antibiotics, and cultured overnight at

37ºC. The following day 5 μl of the overnight culture was used to inoculate 10 mls of LB broth containing the appropriate antibiotics and incubated at 37°C. After 6 hours, dilutions of the culture were plated onto LB plates containing 5% sucrose, the appropriate antibiotics, and a solution of XGal (EM Science) and IPTG (Calbiochem) for blue/white screening of the LacZ cassette when necessary. Colonies were replica plated onto LB agar plates containing different antibiotics and grown overnight at 37°C. Colonies which were resistant or sensitive to the appropriate antibiotics were screened by restriction digest. The following antibiotics were used during the screening process: ampicillin (100μg/ml, EM Science), carbenecillin (100μg/ml, Gemini), kanamycin (50μg/ml, EM Science), and chloramphenicol (15μg/ml, EM Science).

pADCREGFP BAC was generated via allelic exchange using the HCMV AD169 BAC clone pAD/CRE (246) and the shuttling vector pGS284-UL21.5-GFP-Puro (See Fig. 3-1). The pADΔUL82 BAC was generated by standard allelic exchange procedures using the pADCREGFP BAC and pGS284ΔUL82-2A shuttle vector. pADUL82Rev, pADUL82-C219G, pADUL82Δ206-213, pADUL82Δ324-331, pADUL82-199Stop, pADUL82-223-Stop, pADUL82-318Stop, and pADUL82-410Stop BACs were generated by allelic exchange using the pADΔUL82 BAC and the pGS284UL82, pGS284UL82-C219G, pGS284UL82Δ206-213, pGS284UL82Δ324-331, pGS284UL82-199Stop, pGS284UL82-223Stop, pGS284UL82-318Stop, and pGS284UL82-410Stop shuttle vectors respectively. All HCMV BACs were screened by restriction digest, Southern blot analysis, and direct DNA sequencing to confirm proper recombination and incorporation of the mutations.

Retrovirus production and generation of stable cell lines

Retrovirus stocks were prepared as described previously (115)**.** Briefly**,** 10μg of the various pRevTRE plasmids were transfected into Phoenix-A cells (provided by Garry Nolan) via the calcium phosphate method (Promega). 48 hours (h) after transfection, supernatant containing the retrovirus was collected and cell debris was removed via centrifugation (3,000 x g for 10 min). Polybrene (4μg/ml) was added to the retrovirus stock and the solution was used to infect cells for 12 h.

UL82 expressing cell lines

Telomerase 12 15-1 neo cells were generated by transfecting Telomerase 12 cells (28) with *Sca*I linearized pUHD15-1neo plasmid (82). 48 h after transfection, cells were cultured in the presence of G418 (400μg/ml). Individual clones were then picked, expanded, and screened for their ability to be regulated by tetracycline. One clone (Telomerase 12 15-1neo #3) was used for the generation of all subsequent cell lines. Cell lines expressing pp71, pp71Δ206-213, or pp71Δ324-331 were generated by transducing Telomerase 12 15-1neo #3 cells with the replication defective retroviruses REVTREpp71, REVTREpp71Δ206-213, or REVTREpp71Δ324-331 which express their respective pp71 proteins. Cells were selected in hygromycin (150μg/ml, Mediatech) and G418 (400μg/ml, Mediatech). Individual clones were then picked, expanded, and screened for expression of pp71 by Western blot analysis.

hDaxx knock-down and scramble cell lines

U373 cells were transduced with the replication deficient retroviruses pSuperhDaxx or pSuperScramble using the calcium

phosphate transduction method previously described. 48h postinfection cells were selected in puromycin (1μg/ml). Individual clones were then picked, expanded, and screened for expression of hDaxx by Western blot analysis.

hDaxx and GFP overexpressing cell lines

U373 cells were transfected with linearized pEGFP-N1 or pEGFP-hDaxx plasmids via electroporation (950μF, 260V). Cells were selected with G418 (500μg/ml) and individual clones were isolated. Clones were visually screened for GFP expression by fluorescent microscopy and screened for hDaxx expression by Western blot analysis.

Southern blot analysis

HCMV BAC DNA was digested with restriction enzymes and fragmented DNA was separated on a 0.8% agarose gel. DNA was then transferred to an Optitran BA-S nitrocellulose membrane using a Turboblotter according to the manufacturer's protocol for neutral transfer of DNA (Schleicher & Schuell). The membrane was then probed for the designated sequence using ^{32}P-dCTP labeled PCR products in ULTRAhyb (Ambion) at 42°C. Membranes were then washed twice in Low Stringency wash buffer (2X saline sodium citrate (SSC) buffer [1XSSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% SDS) for 5 min followed by two 20 minute washes in Ultra High Stringency wash buffer (0.1X SSC, 0.1% SDS) at 42°C. Fragments were detected by autoradiography.

Antibodies

The following antibodies were obtained from commercial sources: α-pp65 (1205-S; Rumbaugh-Goodwin Institute); α-hDAXX (M-112; Santa Cruz, or D7810; Sigma); α-PML (PG-M3; Santa Cruz) α-tubulin (TU-02; Santa Cruz); α-HA (16B12; Babco); α-IE1/2 (MAB810; Chemicon, or 1203; Rumbaugh-Goodwin Institute); α-Acetyl H4 (06-866; Upstate). pp71 antibodies were a generous gift from T. Shenk and have been previously described (110).

Western blotting and immunoprecipitation analysis

Western blots and immunoprecipitations were conducted as previously described (26). Briefly, cells were harvested by trypsinization, collected by centrifugation, and lysed in RIPA (RadioImmunoPrecipitation Assay) buffer (50mM Tris-HCL, 1% NP-40, 0.25% Na-deoxycholate). Cellular debris was removed by centrifugation and the supernatant fluids reserved. The protein concentration was determined by the method of Bradford (25). Equal amounts (40μg) of protein were resolved by electrophoresis in the presence of sodium dodecyl sulfate (SDS) on 7.5-10% polyacrylamide gels (SDS-PAGE). Proteins were transferred to a nitrocellulose membrane (Optitran; Schleicher & Schuell) and probed with primary and secondary antibody. Immunoreactive proteins were detected by the ECL chemiluminescent system (Amersham).

For immunoprecipitation experiments, cells were infected with the indicated viruses and harvested at the indicated time points. Cells were lysed in NP-40 buffer (50mM TRIS pH 7.4, 150mM NaCl, 0.5% NP-40, 0.75% IPEGAL) containing protease inhibitor cocktail (Roche) for 20 min at 4°C. Lysates were cleared by centrifugation and protein concentrations

determined by Bradford assay. 100μg protein was incubated with the appropriate antibody for 2 h at 4°C. Antibody complexes were recovered on Protein A/G-agarose beads (Santa Cruz), washed three times in NP-40 buffer for 5 min and boiled in 2X SDS-sample buffer (62.5mM Tris-HCl pH 6.8, 2.5% SDS, 20% Glycerol, 1% β-Mercaptoethanol). Proteins were then separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed as described previously.

Immunofluorescence analysis

HFF cells were seeded onto chamber slides (Falcon) or onto glass coverslips and infected the following day with the indicated viruses. Cells were washed twice with phosphate buffered saline (PBS) and subsequently fixed with 4% paraformaldehyde or 50:50 acetone:methanol solution for 20 min at the designated time postinfection. Cells were permeabilized with PBST (PBS+ 0.05% Tween 20, 0.1% TritonX-100) for 25 min at room temperature, and incubated with blocking solution (PBST+ 0.5% BSA, 1% Goat Serum) for an additional 30 min. Cells were then incubated with primary antibody for 1 h at room temperature, washed three times in PBST and incubated with Alexa-488 or Alexa-546 (Invitrogen) conjugated secondary antibody for 1 h. Slides were washed in ddH_2O and nuclei stained with Hoechst 33258 (Sigma) for 5 min. Slides were sealed with coverslips and cells visualized using a Zeiss Axioplan 2 microscope and images taken with a SPOT camera (Diagnostic Instruments).

Viral DNA purification and infectivity assay

HCMV ADCREGFP viral DNA was isolated as described previously (13). Briefly, confluent monolayers of HFF cells were infected with

ADCREGFP virus at a multiplicity of 2 plaque forming units (PFU)/cell. Supernatants were collected approximately 6 days postinfection and virions were pelleted through a 20% sorbitol cushion by ultracentrifugation in a SW28 rotor at 20,000 rpm for 1 h at room temperature. Infectious viral DNA was isolated, quantified, and used for infectivity assays. Viral DNA infectivity assays were done as previously described (13). Briefly, 5 x 10^6 HFF cells were transfected with 1μg viral DNA, and 5μg plasmid DNA via electroporation. Transfected cells were plated into 6-well dishes and overlayed (0.75% agarose DMEM solution) 3 days posttransfection. Plaques were then fixed, stained, and counted 16 days posttransfection.

Histone deacetylase inhibitors

To examine the effect of histone deacetylase inhibitors on virus replication, fibroblasts were pretreated one day prior to infection with 500nM trichostatin A (TSA, Sigma-Aldrich) or 100nM *Helminthosporium carbonum toxin* (HC Toxin, Sigma-Aldrich). Infections were carried out in the presence of the appropriate inhibitor and the media was replaced with fresh inhibitor every 4 days postinfection.

Chromatin Immunoprecipitation (ChIP) Assays

ChIP assays were conducted as previously described (240) (Fig. 2-1). Confluent monolayers of the designated cells were infected with ADCREGFP or ADCREGFPΔUL82 virus at a multiplicity of 0.1 or 0.2 PFU/cell. Following a one hour adsorption period, cells were washed once with DMEM and fresh DMEM (20% FBS) was added. At various times postinfection, cells were crosslinked with 18.5% fresh formaldehyde (1% final concentration) for 10 minutes at room temperature and the reaction was quenched by adding 1M glycine (125mM final concentration)

for 5 minutes at room temperature. Cells were washed twice with PBS and scraped in PBS. The cells were pelleted for 5 minutes at 1500 rpm and the PBS was aspirated. Cell pellets were then lysed in sonication buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl pH 8, Roche protease inhibitor cocktail) and placed at -80°C. Cells were thawed and sonicated in the cuphorn of a Misonix 3000 sonicator for 18 cycles of 5 seconds on output level 4 with 15 seconds in between cycles. Sonicated samples were centrifuged for 10 minutes at 4°C to remove cellular debris. Supernatant was then precleared with A/G agarose beads (Santa Cruz) prepared in triton lysis buffer (50mM Tris-HCl pH 7.4, 150mM Nacl, 1% Triton X-100, 1% BSA, 10ug/ml salmon sperm DNA) for 1 hour. Precleared lysates were then incubated with 5-10μg antibody overnight at 4°C. Antibody complexes were recovered by incubating with the prepared A/G agarose beads for 4 hours at 4°C. The beads were then washed for five minutes in low salt wash buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH 8, 150mM NaCl), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH 8, 500mM NaCl), LiCl detergent (0.25M LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid, 1mM EDTA, 10mM Tris-HCl pH 8), and TE (10mM Tris-HCl pH 8, 1mM EDTA pH 8). Complexes were eluted by incubating the beads twice for 20 minutes in elution buffer (0.1M NaHCO3, 1% SDS) at room temperature and pelleting the beads for 5 minutes at 3.5 rpm. Eluted complexes and 10% unprecipitated input samples were reverse crosslinked by adding 5M NaCl to a final concentration of 200mM and incubated at 65°C overnight. Samples were treated with 10μg RNase for 30 minutes at 37°C and protein complexes degraded by adding 0.5M EDTA (10mM final concentration), 1M Tris-HCl pH 7.5 (40mM final concentration), 20ug

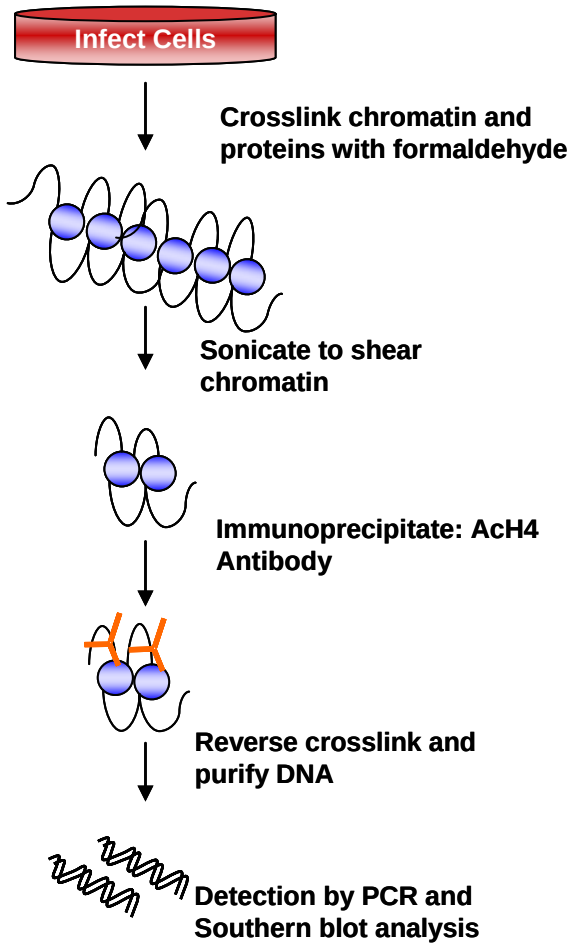

FIGURE 2-2. Chromatin Immunoprecipitation (ChIP) Analysis.

proteinase K and incubating at 45°C for 1-2 hours. DNA was then phenol/chloroform extracted and precipitated with 100% ethanol, sodium acetate, and glycogen. Pellets were air dried and resuspended in TE.

Precipitated DNA was PCR amplified for the MIEP (MIEP-Sin-For and MIEP-Sin-Rev) and the GAPDH promoter (GAPDHP-842 and GAPDHP-1199) (Table 2-2). PCR products were run on a 1.5% agarose gel and transferred to a nitrocellulose membrane using the turboblotter method according to the manufacturer's protocol. Southern blot analysis was carried out as previously described using probe for inner fragements of the MIEP (MIEP-Sblot-S and MIEP-Sblot-F) and the GAPDH promoter (GAPDHPro-F and GAPDHPro-R). Results were analyzed by autoradiography and quantitated using Image J software. Oligonucleotides used for PCR amplification and Southern blot analysis are listed in Table 2-2.

Primer Name	Sequence
GAPDHPro-F	TACTAGCGGTTTTACGGGCG
GAPDHPro-R	TCGAACAGGAGGAGCAGAGAGCGA
GAPDHP-842	CGGGCCAATCTCAGTCCCTTC
GAPDHP-1199	CGTCTTCACCTGGCGACGC
MIEP-Sin-For	TGGGACTTTCCTACTTGG
MIEP-Sin-Rev	CCAGGCGATCTCACGGTT
MIEP-Sblot-S	ATTACCATGGTGATGCGGTT
MIEP-Sblot-AS	GGCGGAGTTGTTACGACAT

Table 2-2. Oligonucleotides used for ChIP analysis. Oligonucleotides and corresponding sequences used for PCR amplification and Southern blot analysis during ChIP analysis. Oligonucleotides were obtained from Integrated DNA Technologies.

CHAPTER THREE
RESULTS

PP71'S INTERACTION WITH HDAXX IS REQUIRED FOR EFFICIENT VIRUS REPLICATION

Introduction

The function of pp71 has not been completely elucidated. Through the use of a UL82 (pp71) deletion mutant, we have demonstrated that pp71 is required for efficient viral replication when cells are infected at a low multiplicity (30). Using the same mutant, we have also demonstrated that pp71 delivered to the host cell from the virus particle plays an important role in regulating IE gene expression during a productive infection (30). Other functions and interactions attributed to pp71 have recently been described. Using *in vitro* overexpression assays, Kalejta *et. al.* demonstrated that pp71 is able to interact with and degrade retinoblastoma (Rb) family member proteins, resulting in quiescent cells entering the cell cycle (109, 110, 112). These studies indicated that pp71 attacks hypophosphorylated Rb tumor suppressor family members through an LXCXD motif within pp71, targeting the tumor suppressor protein for proteasome-dependent, ubiquitin-independent degradation (110, 112). They also demonstrated that substitution of the cysteine with a glycine within the LXCXD motif at pp71 residue 219 abolished pp71's ability to degrade Rb family members and blocked it's ability to induce quiescent cells into the cell cycle (110, 112). Interestingly, pp71's effect on

Rb family member degradation and the host cell cycle was not linked to its *in vitro* transactivation capabilities (111).

pp71 has also been shown to interact with the cellular protein hDaxx (96, 105). The significance of pp71's interaction with hDaxx is not fully understood but it is thought to assist in upregulating viral gene transcription at subnuclear sites (96, 105). During HCMV infection, pp71 colocalizes with hDaxx at specific nuclear domains (ND10) (96, 105, 137) which are sites of active viral gene transcription (59, 102, 104, 139, 141, 142). Two hDaxx binding domains were mapped to amino acids 206-213 and 324-331 of pp71 (96). Transfection studies revealed that removal of either of these binding domains blocked pp71's interaction with hDaxx, prevented pp71 ND10 localization, and abolished pp71's ability to transactivate the major immediate-early promoter in transient reporter assays (96).

Despite the identification of cellular pp71 binding partners, the significance of these interactions has not been determined in the context of a productive viral infection where these proteins are expressed at physiological levels and in the presence of the full complement of viral proteins. Therefore, this study utilizes HCMV UL82 mutants to identify which pp71 interactions are required to mediate IE gene expression and are necessary to overcome the growth defect associated with the UL82 deletion mutant. Using viral mutants, we demonstrate that the LXCXD motif within pp71, which is required to degrade Rb family members and induce quiescent cells into the cell cycle, is not required to enhance the infectivity of viral DNA, control viral replication, or regulate IE gene expression. However, pp71 mutants that are unable to bind hDaxx are severely attenuated for virus replication. We also demonstrate that the interaction of pp71 and hDaxx plays an important role in controlling IE

gene expression and this interaction is involved in regulating pp71's ability to enhance the infectivity of viral DNA.

Results

Generation of the UL82 deletion mutant

Previous studies demonstrated that pp71 is required for efficient viral replication and IE gene expression during infection at a low multiplicity (30). These studies utilized a UL82 deletion mutant termed AD*sub*UL82 that was generated by homologous recombination within human fibroblasts. The generation of large deletion mutants using this method is well established, however it is not conducive to the generation of point mutants or small deletion mutants (22). Therefore, in order to rapidly create multiple UL82 mutants we utilized a bacterial artificial chromosome (BAC) that contains the HCMV genome to generate our UL82 viral mutants. First we generated a wild-type construct of the HCMV AD169 laboratory strain that could be used to generate a UL82 deletion mutant. pADCREGFP BAC was generated via allelic exchange using the HCMV AD169 BAC clone pAD/CRE (246) and the shuttling vector pGS284-UL21.5-GFP-Puro (Fig. 3-1). This recombination results in the HCMV sequence from base pair 27,105 to 27,611 being substituted with a marker cassette containing the green fluorescent protein (GFP) under control of a SV40 promoter, followed by an internal ribosomal entry site and a puromycin resistance gene. This substitution removes the UL21.5 open reading frame of HCMV and replaces it with a GFP/puromycin cassette allowing for the visualization of virus infected cells by fluorescent microscopy. The UL21.5 ORF is non-essential for HCMV replication and we have demonstrated that virus generated from this BAC termed

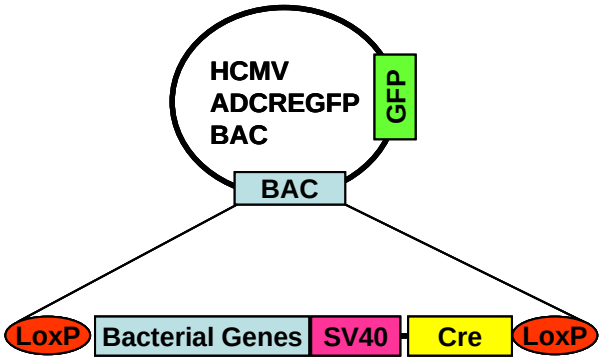

FIGURE 3-1 HCMV pADCREGFP BAC. Schematic representation of the HCMV ADCREGFP BAC used to generate the UL82 mutants.

ADCREGFP replicates to wild-type levels and has no observable growth defect (data not shown) (230).

We next generated a UL82 deletion mutant that could subsequently be used to reinsert a variety of mutated UL82 open reading frames (Fig. 3-2A). The UL82 deletion mutant was generated by replacing the UL82 open reading frame within the pADCREGFP BAC with a kanamycin resistance and LacZ cassette using standard allelic exchange protocols (209, 246). This UL82 deletion mutant BAC has the same UL82 sequence deleted as AD*sub*UL82 and is termed pADΔUL82. Southern blot analysis of the pADΔUL82 DNA digested with *BamH*I confirmed that pADΔUL82 lacks the UL82 coding region and that the marker cassette had recombined properly within the viral genome (Fig. 3-2B). A revertant BAC, termed pADUL82Rev was also generated using the pADΔUL82 BAC and the pGS284UL82 shuttling construct to demonstrate our ability to reincorporate either the wild-type sequence or mutated UL82 sequence. Southern blot analysis of pADUL82Rev DNA digested with *BamH*I revealed that the pADUL82Rev had undergone proper recombination and now contains the UL82 coding sequence. Stocks of wild-type ADCREGFP, ADΔUL82, and ADUL82Rev viruses were then generated as previously described (30). To confirm that ADΔUL82 was unable to express pp71, HFF were infected with wild-type, ADΔUL82, or ADUL82Rev viruses at a high multiplicity of 2 plaque forming units (PFU)/cell and harvested for Western blot analysis 72 h postinfection (Fig. 3-2C). pp71 was expressed in cells infected with WT virus and the UL82 revertant virus but was not expressed in cells infected with the UL82 deletion mutant. UL83 (pp65) was expressed at similar levels in cells infected with any of the viruses.

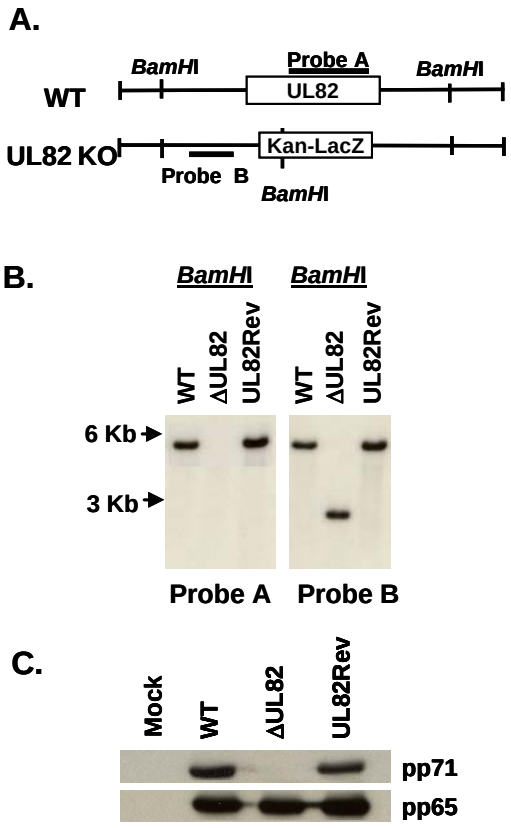

FIG. 3-2. Generation of the UL82 deletion mutant BAC and recombinant virus. (A) Schematic representation of WT and the UL82 deletion mutant genomes. (B) Southern blot analysis of WT pADCREGFP, pADΔUL82, and pADUL82Rev BACs digested with *BamH*I restriction enzyme and probed for either UL82(Probe A) or the UL82 right flanking region (Probe B-UL83). (C) Western blot analysis of pp71 expression at 72 h postinfection in HFF cells mock (M) infected or infected with wild-type (WT), ADΔUL82, or ADUL82Rev virus. As a control, the expression of pp65 was also measured.

Viral growth curves were then conducted comparing replication of wild-type ADCREGFP, AD∆UL82, and ADUL82Rev viruses at a multiplicity of 0.01 or 4 PFU/cell (Fig. 3-3). Identical to AD*sub*UL82 (28, 30), AD∆UL82 failed to efficiently replicate on HFF cells when infected at a low multiplicity. However the growth defect was overcome when cells were infected at a high multiplicity, thus confirming the multiplicity dependent UL82 growth phenotype of AD∆UL82. The pAD∆UL82 BAC was subsequently used to generate the other UL82 mutants in a similar fashion to that described for the UL82 revertant.

Mutation of the pp71 LXCXD domain does not affect virus replication

pp71 has been shown to bind to and induce degradation of hypophosphorylated Rb family member proteins (pRb, p107, and p130) in transfected cells (110, 112). The ability of pp71 to cause degradation of these proteins was mapped to a LXCXD motif within pp71 (112). When the cysteine residue at position 219 within this motif was replaced with a glycine residue, pp71 lost its ability to degrade Rb family member proteins and drive quiescent cells into the cell cycle (110, 112). To determine if pp71's ability to degrade Rb family members and induce quiescent cells is required for efficient virus replication, the C219G mutation was incorporated into the HCMV genome using the pAD∆UL82 BAC and pGS284C219G shuttle vector via allelic exchange. Recombinants were screened by restriction enzyme analysis, polymerase chain reaction (PCR), and direct sequencing of pADUL82-C219G BAC DNA (data not shown). Southern blot analysis of the pADUL82-C219G mutant DNA digested with *BamH*I revealed that the pADUL82-C219G mutant had undergone proper recombination with the UL82 deletion mutant and now

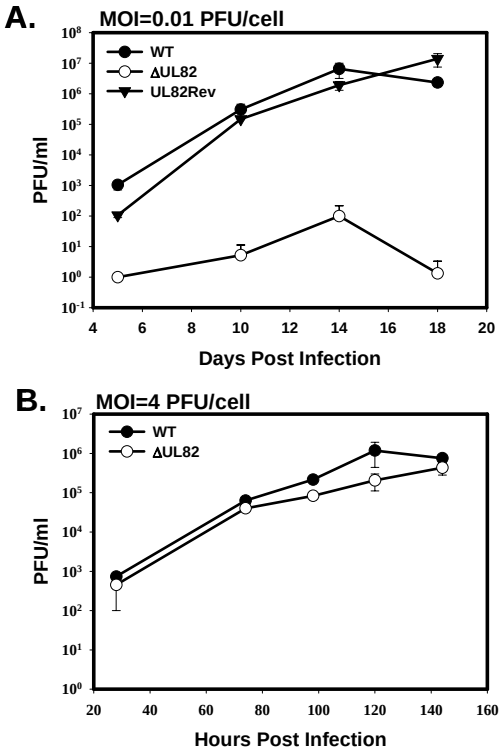

FIGURE 3-3. Growth kinetics of the UL82 deletion mutant virus. HFF cells were infected at a multiplicity of 0.01 PFU/cell (A) or 4 PFU/cell (B) with WT ADCREGFP (●), ADΔUL82 (○), or ADUL82Rev (▼) virus. Cultures were harvested at the indicated times postinfection and infectious virus was quantified by plaque assay on UL82 complementing cells.

contains the UL82 coding sequence (Fig. 3-4B).

pADUL82-C219G BAC DNA was then transfected into both UL82 complementing and non-complementing HFF cells. ADUL82-C219G recombinant virus was isolated from both cell types. ADUL82-C219G viral stocks were generated on non-complementing cells and assayed for viral growth. Growth curve analysis was conducted by infecting HFF cells with either wild-type ADCREGFP, ADUL82-C219G, or AD∆UL82 virus at a multiplicity of 0.01 PFU/cell. Virus was harvested at various times postinfection and infectious virus was quantified by plaque assay. As shown in Figure 3-5B, the ADUL82-C219G mutant virus replicated to wild-type levels whereas replication of the AD∆UL82 deletion mutant was severely attenuated showing a greater than 4 log reduction in virus production. To confirm the expression of pp71 from the ADUL82-C219G virus, HFF cells were infected at a multiplicity of 2 PFU/cell and harvested 72 h after infection for Western blot analysis. As shown in Figure 3-5C, cells infected with the ADUL82-C219G virus expressed pp71 at wild-type levels. Expression levels of the tegument protein pp65 were also examined and served as a control. ADUL82-C219G viral DNA was also isolated and sequenced to confirm that the C219G mutation was retained within the viral genome (data not shown). This data demonstrates pp71's LXCXD motif is not necessary for efficient virus replication, suggesting that pp71's ability to degrade Rb family member proteins and induce quiescent cells into the cell cycle is not required for efficient virus replication and that these functions of pp71 are not responsible for the growth defect associated with the UL82 deletion mutant.

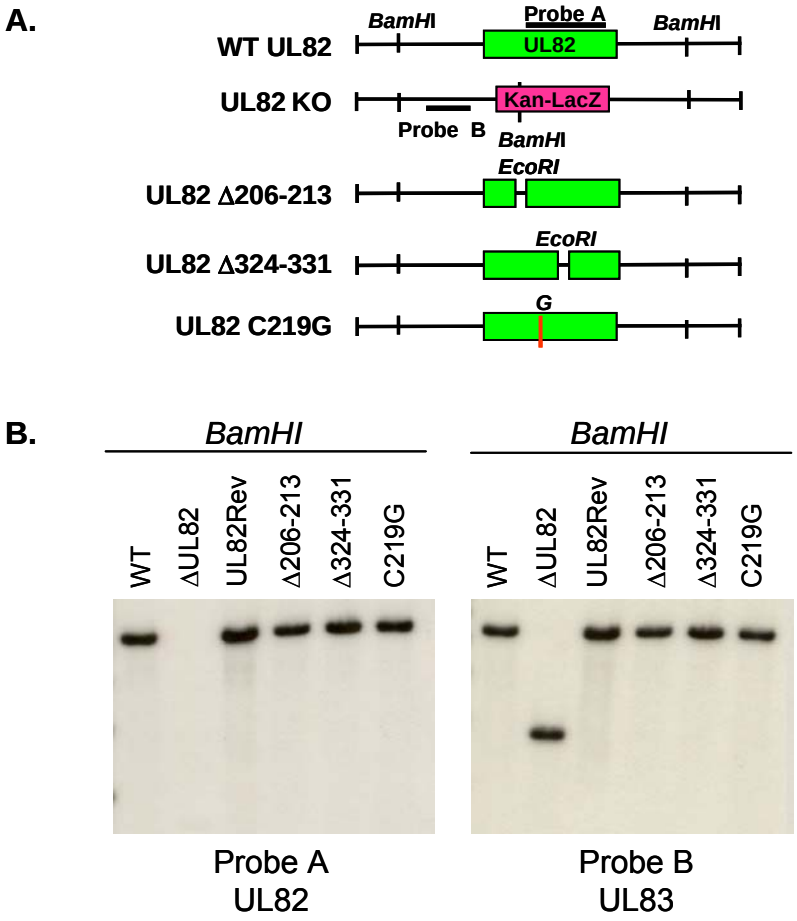

FIGURE 3-4. Southern blot analysis of UL82 mutant BACs. (A) Schematic representation of the UL82 mutant viruses. (B) Southern blot analysis of WT pADCREGFP, pADΔUL82, pADUL82Rev, pADUL82D206-21, pADUL82D324-331, and pADUL82C219G BACs digested with *BamH*I restriction enzyme and probed for either UL82 (Probe A) or the UL82 right flanking region (Probe B-UL83).

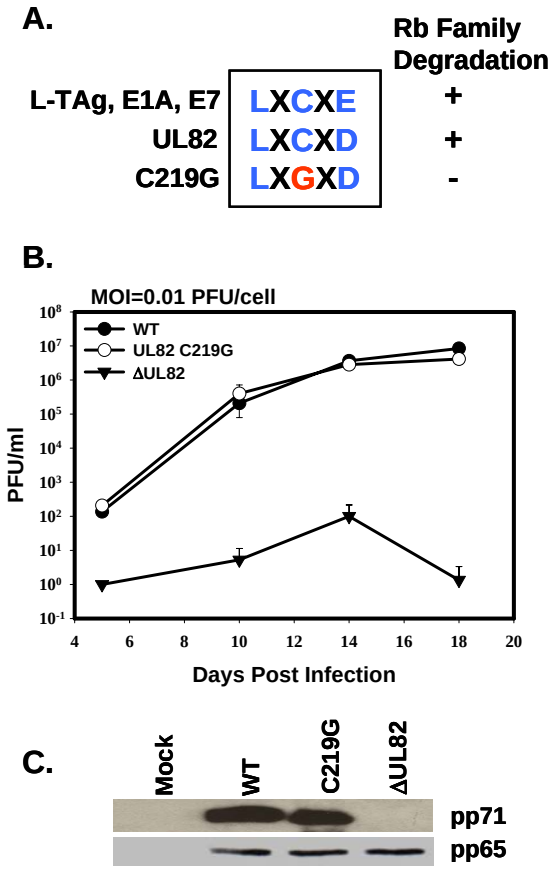

FIGURE 3-5. Growth kinetics of ADUL82-C219G mutant virus. (A) Schematic of the UL82-C219G mutation. (B) HFF cells were infected (0.01 PFU/cell) with WT ADCREGFP (●), ADUL82-C219G (○), or ADΔUL82 (▼) virus. Cultures were harvested at the indicated times postinfection and infectious virus was quantified by plaque assay on UL82 complementing cells. (C) Western blot analysis of pp71 expression at 72 h postinfection in HFF cells mock (M) infected or infected with wild-type (WT), ADUL82-C219G, or ADΔUL82 virus at a multiplicity of 2 PFU/cell. As a control, the expression of pp65 was also measured.

Interaction between pp71 and hDaxx regulates efficient virus replication

Using yeast two-hybrid and transient overexpression assays, it has been shown that pp71 can interact with the cellular protein hDaxx (96, 105). In these studies, the authors identified two separate eight amino acid regions within pp71 (206-213 and 324-331) that are required for the interaction between pp71 and hDaxx (96) (Fig. 3-4A). Deletion of either region independently abolished pp71's interaction with hDaxx and inhibited pp71's ability to transactivate the HCMV major immediate-early promoter in transient transfection assays (96). To determine the importance of pp71's interaction with hDaxx during a productive infection, we incorporated the two small deletions (Δ206-213 or Δ324-331) into the viral genome via allelic exchange using the ADΔUL82 BAC and shuttle vectors pGS284Δ206-213 and pGS284Δ324-331. DNA from all mutant BACs was directly sequenced to confirm proper recombination and incorporation of the deletions (data not shown). Southern blot analysis of the UL82 mutant's DNA digested with *BamH*I revealed that the hDaxx binding mutants had undergone proper recombination with the UL82 deletion mutant and now contain the UL82 coding sequence (Fig. 3-4B). In an attempt to recover mutant virus, BAC DNA was transfected into both UL82 complementing and non-complementing HFF cells. We were unable to recover infectious virus from the non-complementing cells. However, we were able to propagate the two hDaxx binding mutants on UL82 complementing cells. The failure to propagate the two hDaxx binding mutants on non-complementing cells following transfection suggested that pp71's interaction with hDaxx is required for efficient virus replication on HFF cells. To confirm this, growth curves of wild-type virus,

the hDaxx binding mutant viruses (ADUL82Δ206-213 and ADUL82Δ324-331), and the UL82 deletion virus (ADΔUL82) were analyzed at a low multiplicity of 0.01 PFU/cell on non-complementing HFF cells. As shown in Figure 3-6A, the ADΔUL82 deletion virus and the two hDaxx binding mutants failed to efficiently replicate on non-complementing cells. In fact, the growth curves for the two hDaxx binding mutants paralleled that of the UL82 deletion mutant demonstrating a greater than four log reduction in infectious virus when compared with wild-type virus. However, when non-complementing cells were infected at a high multiplicity of 4 PFU/cell the growth defect was abolished and the UL82 mutants replicated to wild-type levels (Fig. 3-6B), demonstrating the multiplicity-dependent growth phenotype of the UL82 mutants. To confirm that the growth defect associated with these viruses was due to the mutations within UL82 and not elsewhere in the genome, UL82 complementing cells were infected at a multiplicity of 0.01 PFU/cell for growth curve analysis with wild-type, ADΔUL82, ADUL82Δ206-213, or ADUL82Δ324-331 virus. As shown in Figure 3-6C, all viruses replicated to wild-type levels on complementing cells, demonstrating that the growth defect of the UL82 deletion mutant and the two hDaxx binding mutants is a direct result of the mutations within UL82 and not a secondary mutation elsewhere in the genome. pp71 expression and subcellular localization were also examined following infection with wild-type, ADΔUL82, ADUL82Δ206-213, and ADUL82Δ324-331 virus. HFF cells were infected at a multiplicity of 2 PFU/cell and lysates were harvested 72 hours after infection for Western blot analysis. As shown in Figure 3-7A, the two UL82 hDaxx binding mutants express pp71, but at somewhat reduced levels when compared to wild-type virus. However, the subcellular localization of the mutant pp71

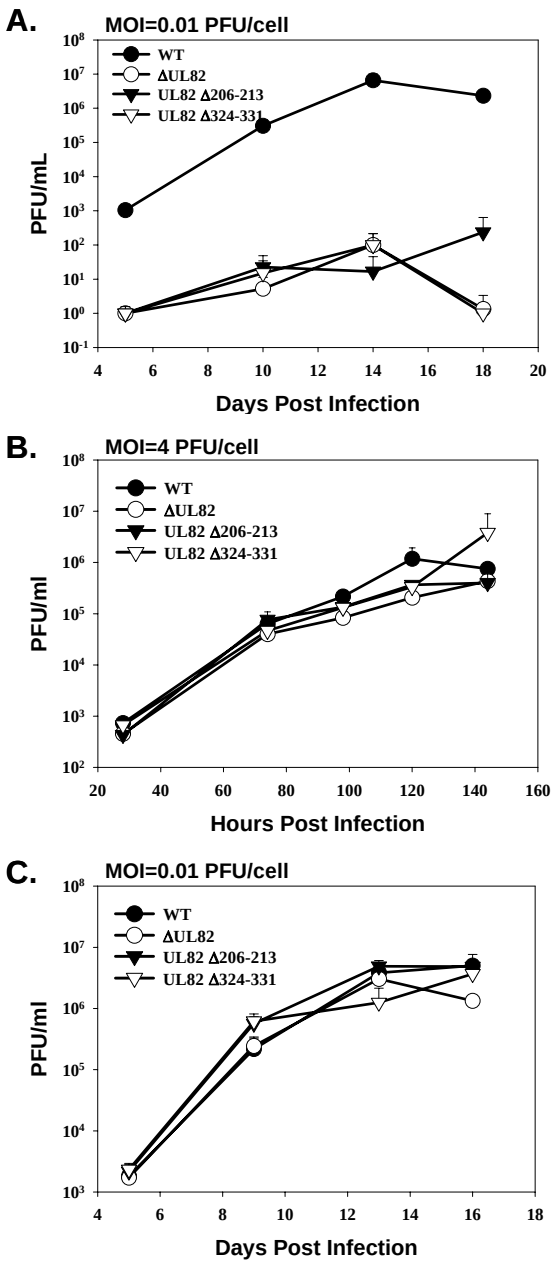

FIGURE 3-6. Growth kinetics of wild-type and UL82-hDaxx binding mutants. HFF cells were infected with (A) 0.01 PFU/cell or (B) 4 PFU/cell of WT (●), ADΔUL82 (○), ADUL82Δ206-213 (▼), or ADUL82Δ324-331 (▽) virus. Cultures were harvested at the indicated times postinfection and infectious virus was quantified by plaque assay on UL82 complementing cells. (C) UL82 complementing cells were infected (0.01 PFU/cell) with WT (●), ADΔUL82 (○), ADUL82Δ206-213 (▼), or ADUL82Δ324-331 (▽) virus. Cultures were harvested at the indicated times postinfection and infectious virus was quantified by plaque assay on UL82 complementing cells.

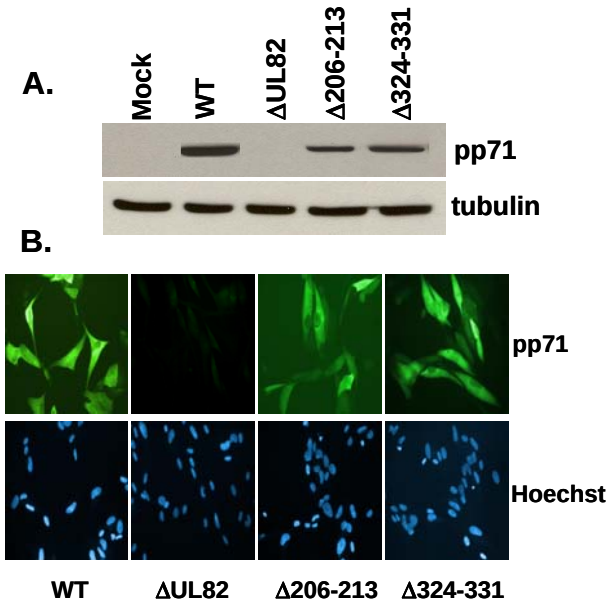

FIGURE 3-7. Expression of pp71 from UL82 mutants. (A) HFF cells were infected with WT, ADΔUL82, ADUL82Δ206-213, or ADUL82Δ324-331 virus at a multiplicity of 2 PFU/cell. Lysates were prepared 72 h postinfection and assayed for pp71 expression by Western blotting. α-tubulin was included as an internal loading control. (B) HFF cells were infected at a multiplicity of 1.0 PFU/cell with WT, ADΔUL82, ADUL82Δ206-213, or ADUL82Δ324-331 virus. Cells were fixed 72 h postinfection and immunostained with monoclonal antibody against pp71. Cell nuclei were detected with Hoechst stain.

expressed from the two hDaxx binding mutants during infection of HFF cells displayed a similar cellular staining pattern to that observed with wild-type pp71 (Fig. 3-7B).

pp71-hDaxx binding mutant proteins are unable to complement the UL82 deletion mutant growth defect

To eliminate the possibility that the lower levels of pp71 expressed from the ADUL82Δ206-213 and ADUL82Δ324-331 viruses were responsible for the growth phenotype, we generated stable cell lines that express the pp71 mutant proteins which are unable to bind hDaxx. Wild-type or pp71-hDaxx binding mutant cDNAs were inserted into replication deficient retrovirus constructs and used to generate stable cell lines expressing wild-type pp71, pp71Δ206-213, or pp71Δ324-331. Stable clones were screened for pp71 expression by Western blot analysis and assayed for their ability to complement the growth defect of the ADΔUL82 deletion mutant. HFF cells, UL82 complementing cells, pp71Δ206-213, or pp71Δ324-331 expressing cells were infected with wild-type or ADΔUL82 virus at a multiplicity of 0.01PFU/cell. Virus was harvested 15 days postinfection and infectious virus was quantified by plaque assay on UL82 complementing cells. We tested 8 clones each that expressed either pp71Δ206-213 or pp71Δ324-331 and none of the clones were capable of complementing the growth defect of the UL82 deletion mutant. However, all clones supported wild-type virus replication and the UL82 deletion mutant replicated to wild-type levels on the cell lines expressing wild-type pp71. Representative expression levels and complementation results for two clones of each cell type are shown in Figures 3-8A & B. All of the pp71Δ206-213 and pp71Δ324-331 cell clones expressed protein levels

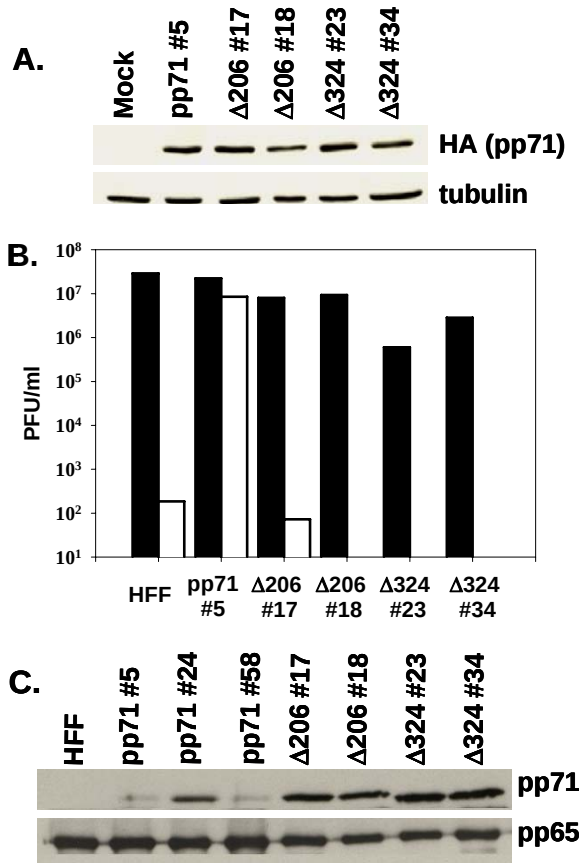

FIGURE 3-8. Cell lines expressing pp71-hDaxx mutant proteins fail to complement the UL82 deletion mutant growth defect. (A) Cell lysates from stable HFF cell lines expressing WT pp71 or pp71 which is unable to interact with hDaxx were examined for pp71 expression by Western blot analysis. Two clones of each pp71-hDaxx binding mutant and the pp71 complementing cells (pp71 #5) were examined. α-tubulin was included as an internal loading control. (B) HFF cells or cells expressing the various pp71 mutant proteins were infected (0.01 PFU/cell) with WT (black bars) or ADΔUL82 (open bars) virus. Cultures were harvested and infectious virus was quantified by plaque assay on UL82 complementing cells. (C) HFF cells or cells expressing the WT or various pp71 mutant proteins were infected (4 PFU/cell) with ADΔUL82 virus. 96 h postinfection, virus was collected and purified via ultracentrifugation. Virus particles were lysed and examined for tegument incorporation of pp71 by Western blot analysis. As a control, the tegument protein pp65 was also examined.

equal to or greater than the wild-type pp71 complementing cell line but were unable to complement the UL82 deletion mutant growth defect. These results suggest that the decreased pp71 expression levels observed with the hDaxx mutants is not responsible for the growth phenotype associated with the two hDaxx binding mutants.

It is also possible that the pp71 protein with the hDaxx binding domain deleted may be defective for pp71 tegument incorporation, leading to the growth phenotype observed. To rule out this possibility, stable cell lines expressing either wild-type pp71 or the pp71-hDaxx binding mutant proteins (pp71Δ206-213 or pp71Δ324-331) were infected with the ADΔUL82 virus at a multiplicity of 4 PFU/cell. 96 h postinfection virus particles were harvested and purified by ultracentrifugation. Virions were then lysed and virion proteins were separated by SDS-PAGE, transferred to membranes, and examined for pp71 incorporation by Western blot analysis. As shown in Figure 3-8C, pp71 expressed from the pp71Δ206-213 or pp71Δ324-331 cell lines incorporated into virions at the same or greater efficiency when compared to wild-type pp71 expressed from the UL82 complementing cell lines. Additionally, levels of pp65 were examined as an internal control for tegument incorporation. These results demonstrate that the growth phenotype associated with the pp71-hDaxx binding mutants is not due to a block in pp71's ability to incorporate into the tegument of the virus particle.

pp71 expressed from ADUL82Δ206-213 or ADUL82Δ324-331 is unable to interact with cellular hDaxx and does not colocalize to ND10 domains

We next examined the expression of hDaxx following infection and confirmed that pp71 expressed from the ADUL82Δ206-213 and ADUL82Δ324-331 mutants could no longer bind hDaxx. HFF cells were infected with wild-type virus at a multiplicity of 3 PFU/cell, and lysates harvested at various times postinfection and examined for hDaxx expression by Western blot analysis. As shown in Figure 3-9A, hDaxx is expressed throughout HCMV infection with its expression increasing between 12 and 24 h postinfection. The kinetics of pp71's interaction with hDaxx was then examined by immunoprecipitation assays. Cell lysates from HFF cells infected with wild-type virus at a multiplicity of 2 PFU/cell were harvested at various times postinfection and incubated with hDaxx antibody. Immune complexes were collected, washed, separated by SDS-PAGE, and transferred to membranes. Membranes were then probed for pp71 expression by Western blotting. As shown in Figure 3-9B, pp71 bound to hDaxx could be detected as early as 4 h postinfection and became more abundant at late time points when pp71 is highly expressed. The interaction between pp71 and hDaxx was present at all times assayed during infection. However, at 8 and 12 h postinfection we consistently observed a slight decrease in pp71 bound to hDaxx. Additionally, cell lysates were examined by Western blot analysis to demonstrate pp71 expression levels throughout the course of infection. To confirm that pp71 expressed from the ADUL82Δ206-213 and ADUL82Δ324-331 viruses was unable to interact with hDaxx, HFF cells were infected with either wild-type, ADΔUL82, ADUL82Δ206-213, or ADUL82Δ324-331 virus at a

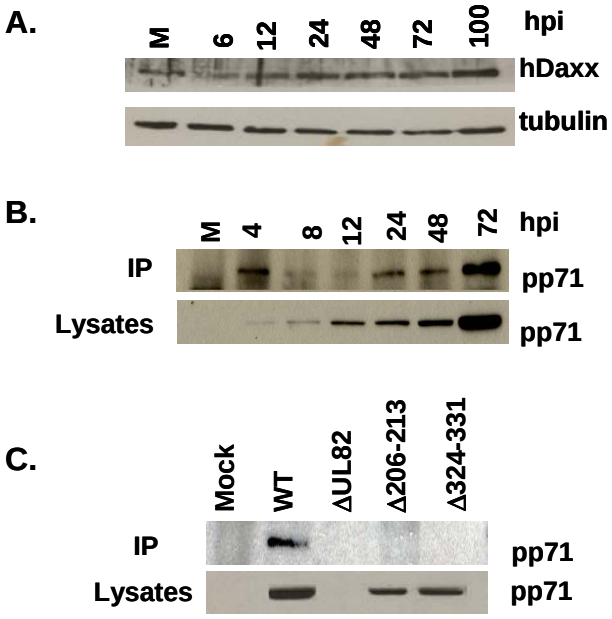

FIGURE 3-9. pp71 interaction with hDaxx during virus infections. (A) HFF cells were infected with WT virus at a multiplicity of 3 PFU/cell. Lysates were prepared at the indicated times postinfection and assayed for hDaxx expression by Western blotting. (B) HFF cells were infected with WT virus at a multiplicity of 2 PFU/cell. Lysates were prepared at the indicated times postinfection and incubated with antibody against hDaxx. Immune complexes were collected, separated by SDS-PAGE, transferred to membranes, and probed for pp71 via Western blotting. Lysates were also examined for pp71 expression by Western blot analysis. (C) HFF cells were infected with WT, ADΔUL82, ADUL82Δ206-213, or ADUL82Δ324-331 virus (1 PFU/cell). Cell lysates were prepared 72 h postinfection and incubated with hDaxx antibody. Immune complexes were collected, separated by SDS-PAGE, transferred to membranes, and probed for pp71 via Western blotting. Cell lysates were also examined for pp71 expression by Western blot analysis.

multiplicity of 1 PFU/cell. Cell lysates were prepared at 72 h postinfection and assayed for the ability of pp71 to interact with hDaxx. As shown in Figure 3-9C, pp71 expressed from wild-type HCMV was able to interact with hDaxx. However, we were unable to detect an interaction of pp71 with hDaxx from lysates infected with AD∆UL82, ADUL82∆206-213, or ADUL82∆324-331 viruses.

Previous reports have demonstrated that when expressed from plasmids, pp71 that contains either the ∆206-213 or ∆324-331 deletion is unable to colocalize to ND10 domains following transient transfection (96). To confirm that pp71 expressed from the viral hDaxx binding mutants ADUL82∆206-213 and ADUL82∆324-331 were unable to localize to ND10 domains, HFF cells were infected with either wild-type ADCREGFP, AD∆UL82, ADUL82∆206-213 or ADUL82∆324-331 at a high multiplicity in the presence of cycloheximide and fixed for immunofluorescence analysis 2 h postinfection. ND10 domains were detected by staining for the promyelocytic leukemia (PML) protein. As shown in Figure 3-10, pp71 expressed from WT virus colocalized to ND10 domains. However, pp71 expressed from ADUL82∆206-213 or ADUL82∆324-331 did not colocalize to ND10 domains and displayed a diffuse nuclear staining pattern. These results demonstrate that the pp71 delivered from the virion of the hDaxx mutant viruses is unable to colocalize to ND10 domains.

pp71 interaction with hDaxx regulates efficient IE gene expression

We have previously reported that UL82 is required for efficient IE gene expression when cells are infected at a low multiplicity (30). Therefore, we examined if pp71's interaction with hDaxx was involved in

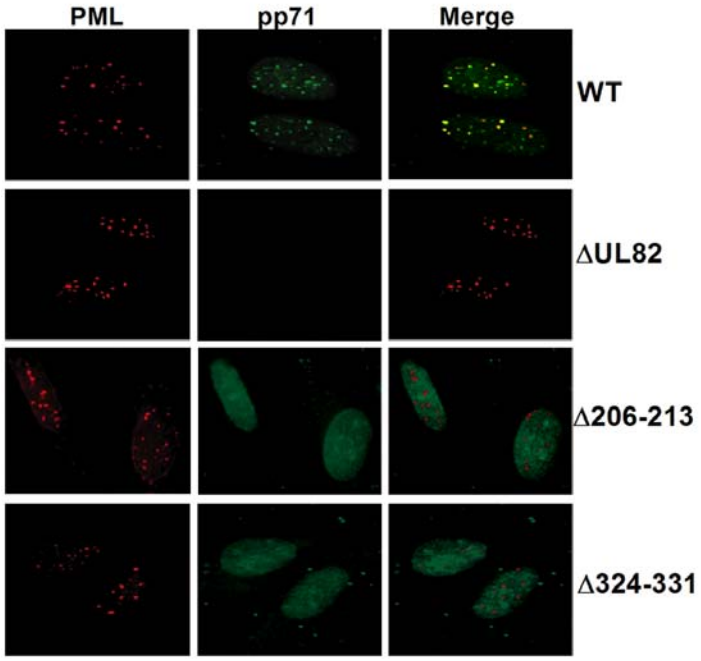

FIGURE 3-10. pp71-hDaxx binding mutants are unable to localize to ND10 domains. HFF cells were infected at a multiplicity of 10 PFU/cell with WT, ADΔUL82, ADUL82Δ206-213, or ADUL82Δ324-331 virus in the presence of 100μg/ml cycloheximide. Cells were fixed 2 h postinfection and immunostained with monoclonal antibody against pp71 (green) and polyclonal antibody against PML (red) to detect ND10 domains.

regulating efficient IE gene expression. HFF cells were infected at a multiplicity of 0.1 PFU/cell with wild-type, ADΔUL82, ADUL82Δ206-213, ADUL82Δ324-331, or ADUL82-C219G virus. Lysates were harvested at various times postinfection and assayed for IE1 and IE2 protein expression by Western blot analysis. As shown in Figure 3-11A, IE1 expression was detectable by 6 h postinfection and IE2 was detectable between 12 and 24 h following infection with wild-type virus. However, IE1 expression was dramatically reduced and was delayed by 12-24 hours in cells infected with the hDaxx binding mutants or the UL82 deletion mutant. IE2 expression was also dramatically reduced and only slightly above the limits of detection after infection with the UL82 mutant viruses (Fig. 3-11A). However, IE1 and IE2 levels were unaltered when cells were infected with the ADUL82-C219G virus demonstrating this mutation has no effect on IE gene expression. When the experiment was repeated on UL82 complementing cells, the UL82 mutants displayed wild-type IE gene expression levels and kinetics (Fig. 3-11B). Since the UL82 growth defect is multiplicity dependent, we wanted to determine if the effect on IE gene expression was also multiplicity dependent. To test this, non-complementing HFF cells were infected with either wild-type, ADΔUL82, ADUL82Δ206-213, ADUL82Δ324-331, or ADUL82-C219G virus at a multiplicity of 2 PFU/cell. Cell lysates were harvested at various times postinfection and assayed for IE1 and IE2 expression by Western blotting. As shown in Figure 3-11C, IE1 and IE2 expression levels and kinetics were the same regardless of the virus used to infect the cells. Taken together, these results indicate that like the growth phenotype of the UL82 deletion mutant, the ability of pp71 to regulate IE gene expression is multiplicity dependent and involves an interaction with hDaxx.

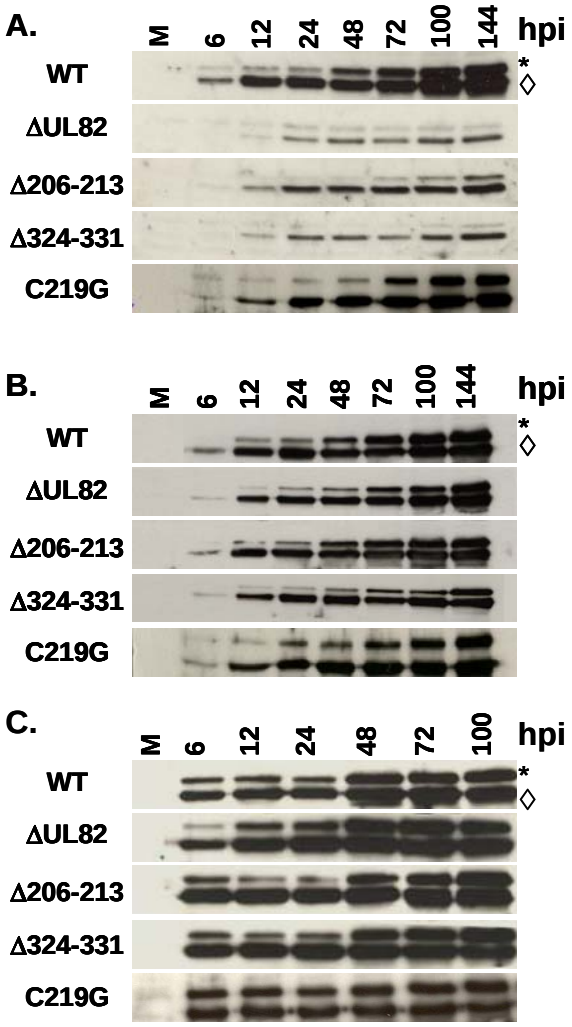

FIGURE 3-11. IE gene expression following infection with UL82 mutant viruses. HFF cells were infected at a multiplicity of 0.1 PFU/cell (A) or 2.0 PFU/cell (C) with WT, ADΔUL82, ADUL82Δ206-213, ADUL82Δ324-331, or ADUL82-C219G virus. Cell lysates were prepared at the indicated times postinfection and assayed for IE1 (◊) and IE2 (*) expression by Western blotting. (B) UL82 complementing cells were infected (0.1 PFU/cell) with WT, ADΔUL82, ADUL82Δ206-213, ADUL82Δ324-331, or ADUL82-C219G virus. Cell lysates were prepared at the indicated times postinfection and examined for IE1 (◊) and IE2 (*) expression by Western blotting.

pp71-hDaxx binding mutants are unable to enhance the infectivity of viral DNA

pp71 enhances the infectivity of viral DNA when co-transfected into HFF cells (13). To determine if pp71's interaction with hDaxx is also involved in enhancing the infectivity of viral DNA, plasmids which express the various pp71 mutants were co-transfected into HFF cells with purified wild-type viral DNA and assayed for infectious virus production by plaque assay. As shown in Figure 3-12A, cells transfected with empty vector, pp71Δ206-213, or pp71Δ324-331 plasmids produced approximately 30 plaques per microgram of transfected viral DNA. However, co-transfection of either wild-type pp71 or pp71C219G plasmid enhanced the infectivity of viral DNA and resulted in a 5 to 7 fold increase in plaque production. Western blot analysis was conducted on an equal aliquot of the transfected cells to confirm equivalent transfection efficiencies and expression levels of pp71 (Fig. 3-12B). These data demonstrate that pp71's interaction with hDaxx is also involved in enhancing the infectivity of viral DNA.

Discussion

During HCMV infection, pp71 is delivered from the virion to the host cell nucleus and is involved in regulating HCMV IE gene expression (30, 44, 93, 96, 99, 104, 130, 238). Using a UL82 deletion virus we have previously demonstrated that pp71 is required for efficient IE gene expression and virus replication when cells are infected at a low input multiplicity (30). Although several functions and interactions have been attributed to pp71, the function(s) and/or interaction(s) involved in efficient IE gene expression and virus replication have not been elucidated. More importantly, this has not been studied in the context of a HCMV infection.

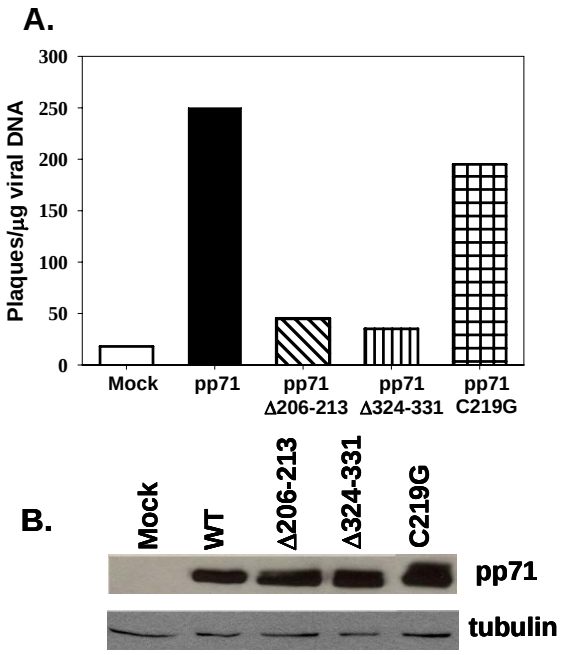

FIGURE 3-12. Effect of pp71 mutant proteins on viral DNA infectivity. (A) HFF cells were transfected with WT ADCREGFP viral DNA (1μg) and 5μg pCGN vector (open bar), pCGNpp71 (black bar), pCGNpp71Δ206-213 (diagonal lines), pCGNpp71Δ324-331 (vertical lines), or pCGNpp71-C219G (hatched bar). Cells were plated in 6-well dishes and overlayed with agarose. Plaques were fixed, stained, and counted 15 days post transfection. (B) Cell lysates from one well of each transfection described in A was harvested 36 h post transfection and assayed for pp71 expression by Western blot analysis. α-tubulin was included as an internal loading control.

In this study, we utilized UL82 mutant viruses to identify an interaction that is critical for pp71's ability to enhance the infectivity of viral DNA, regulate IE gene expression and promote viral replication.

The pp71 LXCXD motif is not required for viral replication

Rb family member proteins which include pRb, p107, and p130 function to regulate cell cycle progression out of G0 and through the G1 phase of the cell cycle (236). pp71 has previously been shown to target hypophosphorylated Rb family member proteins for degradation in a proteasome-dependent and ubiquitin-independent fashion which allows quiescent G0 cells to enter the cell cycle and progress through G1 (109-112). These studies also demonstrated that substitution of the cysteine residue within the pp71 LXCXD motif with a glycine residue blocked the degradation of hypophosphorylated Rb family members and the induction of quiescent cells into the cell cycle (110, 112). To determine if these functions of pp71 were required for efficient IE gene expression and virus replication we generated a UL82 mutant that incorporated the cysteine to glycine substitution within the pp71 LXCXD motif. Growth curve analysis demonstrated that the ADUL82-C219G mutant virus replicated with wild-type kinetics and produced infectious virus at wild-type levels regardless of the multiplicity used to infect cells (Fig. 3-5). Additionally, the UL82-C219G mutation did not attenuate expression of IE1 or IE2 (Fig. 3-11), and expression of UL82-C219G was able to enhance the infectivity of viral DNA to wild-type levels (Fig. 3-12) (110). These results strongly suggest that pp71's ability to degrade hypophosphorylated Rb family members and induce quiescent cells into the cell cycle are not required for efficient virus replication in human fibroblasts and are not responsible for the growth phenotype associated with the UL82 deletion mutant.

pp71 interaction with hDaxx regulates efficient HCMV replication

In addition to pp71's involvement with the cell cycle, pp71 has also been reported to interact with the cellular protein hDaxx during HCMV infection (96, 105). hDaxx was first identified as a proapoptotic protein (245) and later shown to act as a transcriptional regulator of gene expression (43, 126, 127, 139, 150, 151). Although hDaxx has been associated with transcriptional activation, it is primarily thought to function as a transcriptional repressor. hDaxx represses Pax3 (98), and Ets-1 associated transcription (127) through its interaction with histone deacetylases (97). hDaxx has also been shown to regulate Fas-mediated apoptosis through a transcriptional repression mechanism (127, 226). hDaxx colocalizes with promyelocytic leukemia protein (PML) within the nucleus at ND10 domains, a site of active gene transcription and viral genome deposition (59, 96, 102-105, 139, 141) During HCMV infection, pp71 interacts with hDaxx and localizes to ND10 domains (96, 105, 137). Transient assays have demonstrated that disruption of the pp71-hDaxx interaction inhibits pp71 localization to ND10 domains and attenuates activation of the major immediate-early promoter (MIEP). Based on these results, the authors suggest that pp71's interaction with hDaxx is important for viral IE gene expression and viral replication (96, 105). To test this hypothesis we generated UL82 viral mutants that have the hDaxx binding domains deleted and assayed them for their effect on IE gene expression and viral replication. Growth curve analysis and kinetics of IE1 and IE2 expression with the hDaxx binding mutants were indistinguishable from those obtained with the UL82 deletion virus (Fig. 3-6 and 3-11). Replication of the hDaxx binding mutants and UL82 deletion mutant were severely attenuated when cells were infected at a multiplicity of 0.01

PFU/cell. However, this growth defect was overcome if cells were infected at a multiplicity of 4 PFU/cell or if UL82 complementing cells were infected (Fig. 3-6). Similar results were obtained when we examined the kinetics and expression levels of IE1 and IE2 following infection with the mutants. The UL82 deletion mutant and hDaxx binding mutants showed a dramatic decrease in the abundance of IE1 and IE2 and also demonstrated a significant delay in their expression when compared to wild-type virus (Fig. 3-11). Although these results do not eliminate the possibility that the mutant viruses interfere with an undetermined function of pp71, the results suggest that pp71's ability to interact with hDaxx is required for efficient IE gene expression and virus replication.

pp71's interaction with hDaxx is involved in enhancing the infectivity of viral DNA

pp71 has been shown to enhance the infectivity of viral DNA (13). Since loss of the pp71-hDaxx interaction results in repressed and delayed IE gene expression and a multiplicity dependent growth defect, we examined if the pp71-hDaxx interaction was involved in pp71's ability to enhance viral DNA infectivity. Co-transfection of plasmids expressing the pp71-hDaxx binding mutants and viral DNA failed to enhance plaque production (Fig. 3-12A), demonstrating the interaction between pp71 and hDaxx is involved in controlling this function of pp71. It has been suggested that pp71 increases the infectivity of viral DNA by allowing for the efficient expression of IE genes and thus serves to "kick-start" the viral replication cycle (13, 96, 104). Previous reports have shown that co-transfection of IE1 and IE2 expression plasmids with viral DNA in the absence of pp71 were unable to increase the number of infectious plaques, suggesting that pp71's regulation of IE1 and IE2 alone is

insufficient to enhance the infectivity of viral DNA (13). Therefore, it is likely that the regulation of all or some combination of IE genes by pp71 is required to enhance the infectivity of viral DNA and initiate the viral cascade of gene regulation.

The mechanism by which the interaction between pp71 and hDaxx enables efficient virus replication, IE gene expression, and DNA infectivity has yet to be determined. pp71 and hDaxx have been shown to synergistically transactivate viral gene expression in transient assays, suggesting a model in which pp71 interacts with hDaxx at ND10 domains to transactivate IE genes (96). This model is consistent with studies indicating ND10 sequestered hDaxx is inhibited in its ability to mediate transcriptional repression (126). However, other studies suggest hDaxx retains its ability to mediate transcriptional repression from ND10 domains (81, 98). Therefore, it has also been suggested the pp71-hDaxx interaction may serve to down-regulate cellular gene transcription and provide an advantageous site for viral gene expression (105). Although our results clearly demonstrate that the pp71-hDaxx interaction is involved in efficient IE gene expression and viral replication they do not favor one model over the other.

CHAPTER FOUR
RESULTS

PP71 RELIEVES HDAXX MEDIATED REPRESSION OF HCMV REPLICATION

Introduction

The mechanism by which pp71 regulates IE gene expression is currently unclear. pp71 has been shown to interact with several cellular proteins including hDaxx (96, 105). During HCMV infection, pp71 and hDaxx colocalize at specific nuclear structures called ND10 domains (37, 96, 105). Previous reports have demonstrated that the genomes of HCMV and other herpesvirus localize to ND10 domains immediately after infection and that ND10 domains represent sites of active viral gene transcription (59, 96, 102-105, 139, 141, 192). Abolishing pp71's ability to interact with hDaxx blocked pp71 localization to ND10 domains (37, 96, 105) and inhibited pp71's ability to transactivate the major immediate early promoter (MIEP) in transient reporter assays (96). We have also demonstrated that pp71 mutant viruses lacking either of two hDaxx binding domains (96) were severely inhibited for viral replication and IE gene expression at low multiplicities of infection (37). These data suggest that pp71's interaction with the cellular protein hDaxx is important for regulating IE gene expression and viral replication.

hDaxx has been recognized as a regulator of both apoptosis and gene expression (149). The mechanism by which hDaxx regulates these two processes is controversial and not completely understood. hDaxx was originally identified as a pro-apoptotic protein which could enhance FAS-induced apoptosis (226, 245). However, other reports using siRNA directed against hDaxx have demonstrated that hDaxx functions as an anti-apoptotic protein following certain stimuli (46, 150, 151). hDaxx's role in regulating gene expression is also unclear. Although hDaxx has been associated with transcriptional activation, hDaxx is primarily thought to function as a transcriptional repressor (46, 86, 98, 126, 127, 151, 226). Studies using siRNA directed against hDaxx have demonstrated that hDaxx can repress NFκB, E2F-1, Pax3, and Ets-1 mediated transactivation (151). Additionally, hDaxx has been shown to bind the avian sarcoma virus (ASV) integrase protein and represses ASV transcription (86).

The mechanism by which hDaxx regulates HCMV IE gene expression is currently unclear. Transient transfection assays have demonstrated that hDaxx expression activated the HCMV MIEP and that cotransfection of pp71 with hDaxx had a synergistic effect on the activation of the MIEP (96). In addition, HCMV infection of Daxx null mouse cells led to a two-fold reduction in the number of immediate-early 2 (IE2) protein expressing cells (105). Taken together these results suggest that hDaxx functions as a positive regulator of the MIEP and IE gene expression. However, preliminary studies by Reeves *et. al.* suggest that overexpression of hDaxx represses HCMV replication (185). Therefore, given the conflicting data and multifunctional nature of hDaxx, it is unclear if hDaxx functions as an activator or repressor during HCMV infection. In this study HCMV permissive cell lines that either overexpress hDaxx or

are depleted for hDaxx expression are used to determine whether hDaxx functions as an activator or repressor of HCMV IE gene expression and replication.

Results

hDaxx represses wild-type virus replication

If hDaxx functions to positively regulate viral transcription, wild-type virus may replicate more efficiently in cells overexpressing hDaxx. However, if hDaxx functions as a repressor, wild-type virus replication and IE gene expression may be inhibited in cells overexpressing hDaxx. To test these predictions, cell lines overexpressing hDaxx were generated. U373 cells were transfected via electroporation with a plasmid that expresses a hDaxx-green fluorescent protein (GFP) fusion and a neomycin resistance gene (250). As a control, cells were also transfected with a GFP plasmid that does not express hDaxx. Cells were selected with G418 (500μg/ml) and stable clones were isolated. As shown in Figure 4-1A, hDaxx overexpression in cell clones was confirmed by Western blot analysis using either an hDaxx or GFP antibody. Endogenous hDaxx levels were constant in all clones and can be differentiated by its faster migration when compared to the GFP-hDaxx fusion protein. To determine the effect of hDaxx overexpression on wild-type viral replication, GFP control and hDaxx overexpressing cells were infected with wild-type virus at a MOI of 5.0, 1.0 or 0.2 PFU/cell. Virus was harvested at 5 or 7 days postinfection, and quantified by plaque assay on HFF cells. As shown in Figure 4-1B, wild-type virus replication was inhibited on hDaxx overexpressing cells in a multiplicity-dependent manner. When hDaxx overexpressing cells were infected at a multiplicity of 1.0 or 0.2 PFU/cell, wild-type virus replication was inhibited by greater

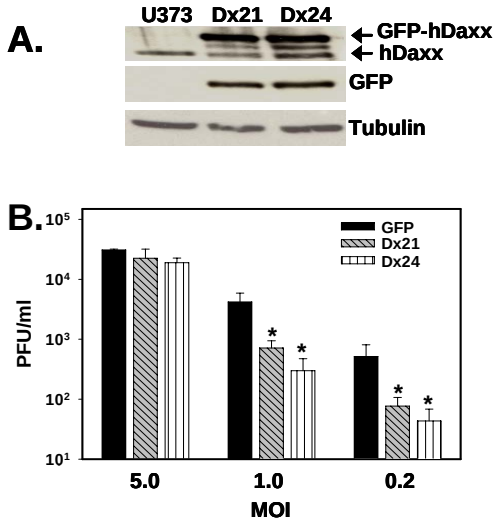

FIGURE 4-1. Overexpression of hDaxx represses viral replication. (A) Western blot analysis examining expression of hDaxx and GFP in control U373 cells and GFP-hDaxx overexpressing U373 clones Dx21 and Dx24. Tubulin was included as an internal loading control. (B) Control U373-GFP (black bar) cells or hDaxx overexpressing cells Dx21 (diagonal lines) or Dx24 (vertical lines) were infected with wild-type virus at a MOI of 5.0, 1.0 or 0.2 PFU/cell. Infectious virus was harvested at 5 days postinfection for cells infected at a M.O.I. of 5.0 PFU/cell or 7 days postinfection for cells infected at a M.O.I. of 1.0 or 0.2 PFU/cell and quantified by plaque assay on HFF cells. Asterisks (*) indicate a significant difference in viral titers (p-value < 0.05) between virus produced on hDaxx overexpressing cells compared to control U373-GFP cells. Error bars indicate standard deviations derived from three independent experiments.

than 80% when compared with control cells. To confirm that overexpression of hDaxx does not inhibit viral entry, control or hDaxx overexpressing cells were seeded onto coverslips and infected with wild-type virus at a MOI=1.0 PFU/cell (Fig. 4-2). Cells were fixed 5 hours postinfection (hpi) and immunostained for the tegument protein pp65. pp65 was present in both the control and hDaxx overexpressing cells, demonstrating that overexpression of hDaxx does not inhibit viral entry or tegument delivery.

IE gene expression was also examined following infection of hDaxx overexpressing cells. Control or hDaxx overexpressing cells were infected with wild-type virus at a MOI of 0.2 (Fig. 4-3A) or 1.0 (Fig.4-3B) PFU/cell. Cell lysates were harvested at various times postinfection and assayed for IE1 and IE2 protein expression by Western blotting. As shown in Figure 4-3A and B, expression of IE1 and IE2 was markedly delayed and reduced following infection of hDaxx overexpressing cells when compared to control cells. Together, these results demonstrate that overexpression of hDaxx does not enhance HCMV replication and suggests that hDaxx functions to repress both HCMV viral replication and IE gene expression.

hDaxx depletion enhances HCMV infection

If hDaxx is functioning as a repressor during viral infection, we hypothesized that wild-type virus and possibly the UL82 deletion mutant virus would replicate more efficiently on hDaxx knock-down cells. To test this, U373 hDaxx knock-down cell lines were generated using replication-deficient retroviruses encoding short hairpin RNA (shRNA) sequences against hDaxx. A shRNA sequence directed against hDaxx (46) or a control scrambled (Oligoengine) shRNA sequence was inserted into the pSuperRetroPuro vector (Oligoengine) according to the manufacturer's

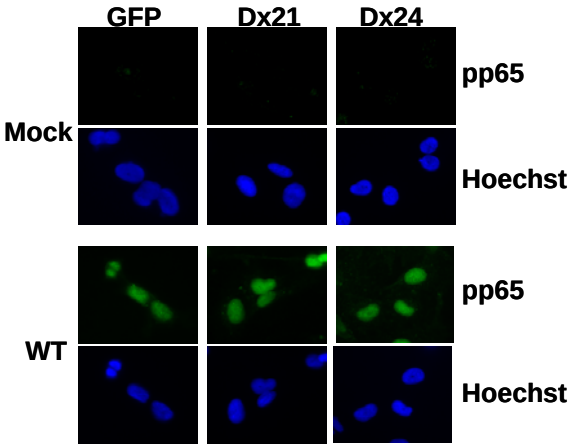

FIGURE 4-2. hDaxx expression does not alter the efficiency of viral entry. (A) Control (GFP) or hDaxx overexpressing cells (Dx21 and Dx24) were infected with wild-type virus at a multiplicity of 1.0 PFU/cell. Cells were fixed 5 hours postinfection and immunostained for pp65. Cell nuclei were detected with Hoechst staining.

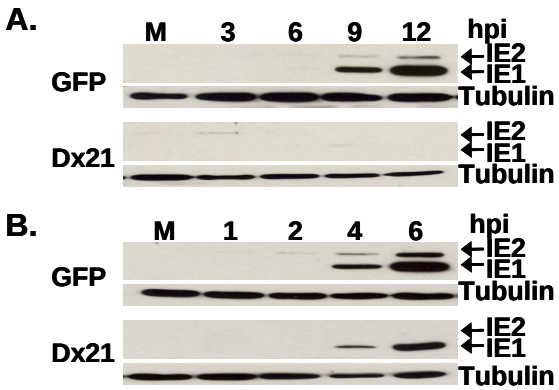

FIGURE 4-3. Overexpression of hDaxx inhibits IE gene expression. Control GFP or hDaxx overexpressing cells (Dx21) were infected with wild-type (WT) virus at a MOI of 0.2 (A) or 1.0 (B) PFU/cell. Cell lysates were prepared at the indicated hour postinfection (hpi) and assayed for IE1 and IE2 expression by Western blotting. Tubulin was included as an internal loading control.

protocol. Infectious retrovirus was then generated and U373 cells transduced as previously described (37, 115). Transduced cells were selected with puromycin (1μg/ml) and individual stable clones were isolated. Western blot analysis was then conducted to examine the hDaxx levels in individual clones. Figure 4-4A shows a representative blot of hDaxx expression in control cells and hDaxx knock-down cells. These results demonstrate that cells expressing the shRNA directed against hDaxx expressed dramatically reduced levels of hDaxx when compared with control cells. The control cells and hDaxx knock-down cells were then infected at a MOI of 0.2 PFU/cell with either wild-type virus or the UL82 deletion mutant virus termed ADΔUL82. Since both viruses contain a GFP gene within the viral genome, we can easily identify infected cells by looking for GFP expression. As shown in Figure 4-4B, there were only a few GFP positive cells present when control cells were infected with the ADΔUL82 virus (Fig. 4-4B panel b) demonstrating the growth defect of the UL82 deletion virus. However, when the hDaxx knock-down cells were infected with the UL82 deletion mutant we observed a dramatic increase in the number of GFP positive cells (compare Fig. 4-4B panels b and d). In addition, when hDaxx knock-down cells were infected with the UL82 deletion virus we observed an approximately equal number of GFP positive cells to those observed following wild-type infection of control cells (compare Fig. 4-4B panels a and d).

Replication of wild-type virus and the UL82 deletion virus were then quantified by plaque assay following infection of either control cells or hDaxx knock-down cells. Control cells or three individual clones of hDaxx knock-down cells were infected with wild-type or the UL82 deletion virus at a MOI of 0.2 (Fig. 4-4C) or 1.0 (Fig. 4-4D) PFU/cell. Infectious virus was

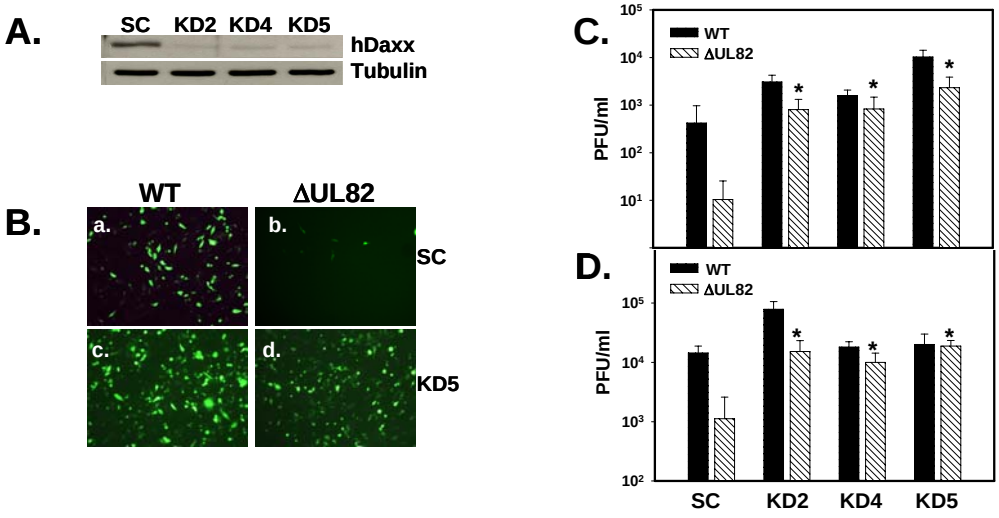

FIGURE 4-4. Infection of hDaxx knockdown cells abolish the UL82 deletion mutant growth phenotype. (A) Western blot analysis of hDaxx expression in control cells expressing a scramble shRNA (SC) and hDaxx knockdown cell lines KD2, KD4, and KD5. Tubulin was included as an internal loading control. (B) Control (SC) and hDaxx knock-down cells (KD5) were infected with wild-type virus (WT) or the UL82 deletion mutant (ΔUL82) virus at a MOI of 0.2 PFU/cell and examined 5 days postinfection for GFP positive cells. Control (SC) and hDaxx knock-down cells (KD2, KD4, KD5) were infected with either wild-type (black bars) or ΔUL82 (diagonal lines) virus at a MOI of 0.2 (C) or 1.0 (D) PFU/cell. Virus was harvested at 7 and 5 days postinfection respectively and infectious virus was quantified by plaque assay on UL82 complementing cells. Asterisks (*) indicate a significant difference in viral titers (p-value < 0.005) between virus produced on hDaxx knock-down cells compared to control cells. Error bars indicate standard deviation derived from three independent experiments.

harvested at 7 and 5 days postinfection respectively and quantified by plaque assay on UL82 complementing cells (37). UL82 deletion virus production was dramatically reduced (> 92% inhibition) at both multiplicities when compared with wild-type virus production on control cells. However, when hDaxx knock-down cells were infected with the UL82 deletion virus, the growth defect associated with the virus was completely abolished and virus production was restored to wild-type levels (Fig. 4-4C & D). In addition, when infected at a multiplicity of 0.2 PFU/cell, wild-type virus replication was also significantly enhanced on hDaxx knock-down cells. Immunostaining for the pp65 tegument protein in control and hDaxx knock-down cells was done to confirm that viral entry is not affected by decreased expression of hDaxx (Fig. 4-5).

In addition to viral replication, IE gene expression was examined following infection of hDaxx knock-down cells with either wild-type or the UL82 deletion virus. Control or hDaxx knock-down cells were infected with wild-type or UL82 deletion virus at a MOI of 0.2 (Fig. 4-6A) or 1.0 (Fig. 4-6B) PFU/cell and cell lysates harvested at various time points following infection. Western blot analysis was conducted to examine the expression of the immediate-early proteins IE1 and IE2 following infection. As shown in Figure 3A and B IE gene expression was inhibited following infection of control cells with the UL82 deletion mutant compared to wild-type virus. However, when hDaxx knock-down cells were infected with the UL82 deletion virus, IE gene expression was restored to near wild-type levels (Fig. 4-6A and B). Taken together, these results demonstrate that hDaxx is not required for HCMV replication and that hDaxx functions to repress IE gene expression and HCMV replication. Since knocking down hDaxx expression abolished the UL82 growth phenotype and restored IE gene expression, our results also suggest the function of pp71 required for

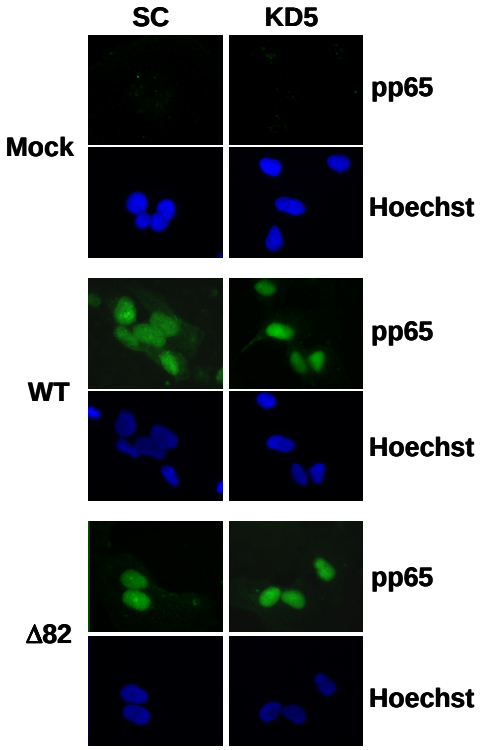

FIGURE 4-5. Knocking down hDaxx expression does not alter the efficiency of viral entry. Control (SC) or hDaxx knock-down (KD5) cells were infected with wild-type or the UL82 deletion mutant virus at a multiplicity of 1.0 PFU/cell. Cells were fixed 5 hours postinfection and immunostained for pp65. Cell nuclei were detected with Hoechst staining.

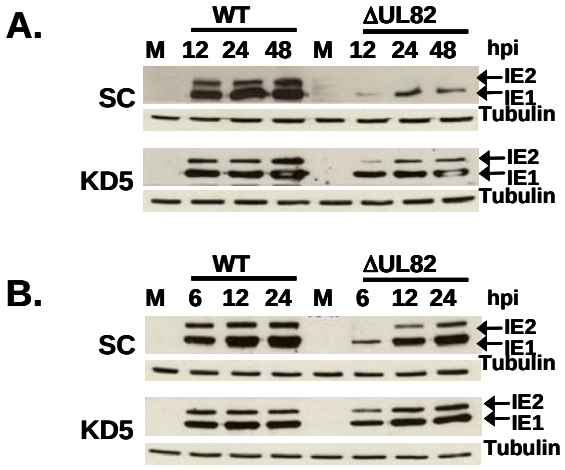

FIGURE 4-6. IE gene expression following infection of hDaxx knock-down cells. Control (SC) or hDaxx knock-down cells (KD5) were infected with either wild-type (WT) or the UL82 deletion mutant (ΔUL82) virus at a MOI of 0.2 (A) or 1.0 (B) PFU/cell. Cell lysates were prepared at the indicated hours postinfection (hpi) and assayed for IE1 and IE2 expression by Western blotting. Tubulin was included as an internal loading control.

efficient IE gene expression and viral replication is to relieve hDaxx mediated repression of IE gene expression.

Histone deacetylase inhibitors complement the UL82 deletion mutant

We next wanted to examine the mechanism by which pp71's interaction with hDaxx enhances viral replication and IE gene expression. It was previously shown that hDaxx interacts with core histones and histone deacetylases (HDACs) (97). Therefore we hypothesized that pp71 interacts with hDaxx and blocks the histone deacetylase activity of HDAC's that are associated with hDaxx; therefore, acetylation of histones associated with the major immediate-early promoter (MIEP) can occur which results in enhanced IE gene transcription (Fig. 4-7). To test this hypothesis, we pretreated fibroblasts with the histone deacetylase inhibitors trichostatin A (TSA), *Helminthosporium carbonum toxin* (HC toxin), or mock treated with dimethyl sulfoxide (DMSO). Treated cells were infected with either wild-type or the UL82 deletion mutant virus at a MOI=0.2 PFU/cell in the presence or absence of inhibitor and examined for viral GFP expression. As shown in Figure 4-8, there were only a few GFP positive cells present when mock treated cells were infected with the AD∆UL82 virus (Fig. 4-8 panel D) demonstrating the growth defect of the UL82 deletion virus. However, when cells pretreated with TSA or HC toxin were infected with the UL82 deletion mutant we observed a dramatic increase in the number of GFP positive cells (compare Fig. 4-8 panels D-F). There was also an increase in the number of GFP expressing cells when cells pretreated with HDAC inhibitors were infected with wild-type virus. Replication of wild-type virus and the UL82 deletion virus were then quantified by plaque assay following infection of cells pretreated with HDAC inhibitors. Cells mock treated with DMSO or pretreated overnight

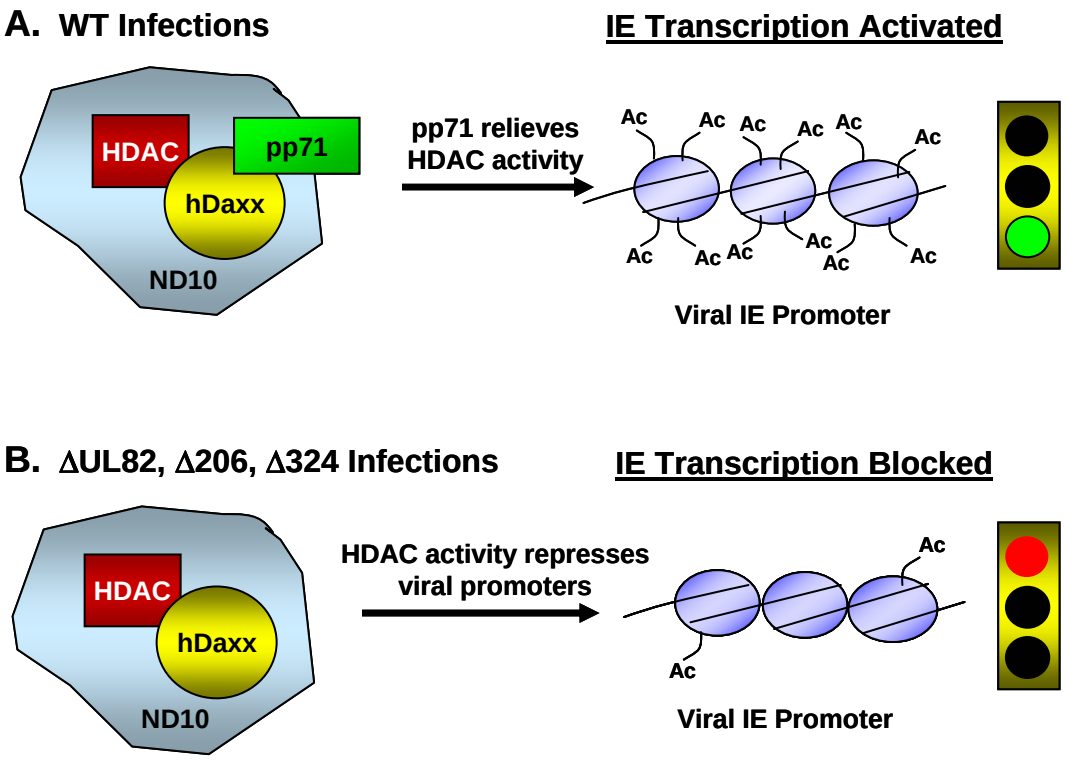

FIGURE 4-7. Hypothesized model for pp71 relief of hDaxx repression. The working hypothesis is that during wild-type infection (A), pp71 interacts with hDaxx to block histone deacetylase activity (HDAC). This allows for the acetylation of histones associated with viral immediate-early (IE) promoters and transcription is activated. (B) In the absence of pp71 or if pp71 is unable to bind hDaxx, it is hypothesized that HDACs deacetylate histones associated with viral IE promoters and block transcription.

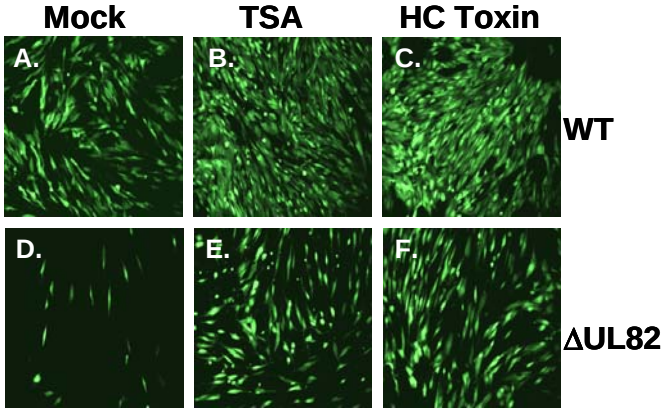

FIGURE 4-8. HDAC inhibitors enhance HCMV virus infection. HFF cells were pretreated with Trichostatin A (TSA, 500nM) or HC Toxin (100nM) overnight and infected with wild-type (WT) or ΔUL82 virus at MOI=0.2 PFU/cell and examined for GFP expression 5 days postinfection.

with HDAC inhibitors (500nM TSA or 100nM HC toxin) were infected with wild-type or the UL82 deletion mutant virus at a multiplicity of 0.2 PFU/cell or 1 PFU/cell (Fig. 4-9). Virus was harvested at various times postinfection and quantitated by plaque assay on UL82 complementing cells. At both multiplicities, there was a significant increase (p-value < 0.05) in virus replication on cells pretreated with HDAC inhibitors compared to untreated cells. These data suggest that pretreatment of fibroblasts with histone deacetylase inhibitors can partially complement the UL82 associated growth defect and that pp71 may function to block histone deacetylase activity.

Acetylated histones associated with the MIEP

Since the HDAC inhibitors partially complemented the UL82 deletion mutant virus, we next wanted to compare the association of acetylated histone with the major immediate-early promoter (MIEP) during infection with wild-type or the UL82 deletion mutant viruses. HFF cells were infected with either wild-type or the UL82 deletion mutant virus at a multiplicity of 0.1 PFU/cell and harvested for chromatin immunoprecipitation (ChIP) analysis at various times postinfection (Fig. 4-10). To harvest the samples, cells were crosslinked with formaldehyde, pelleted and resuspended in sonication buffer. The samples were sonicated and incubated with antibody against acetylated histone H4 or a rabbit serum (RS) control. Sample bound antibody was captured on Protein A/G conjugated agarose beads, reverse crosslinked overnight, and treated with RNase. Protein was degraded with proteinase K and the remaining DNA was phenol/chloroform extracted. Isolated DNA was then used as template for PCR to detect the MIEP and the GAPDH promoter. Southern blot analysis was conducted on the PCR products and results

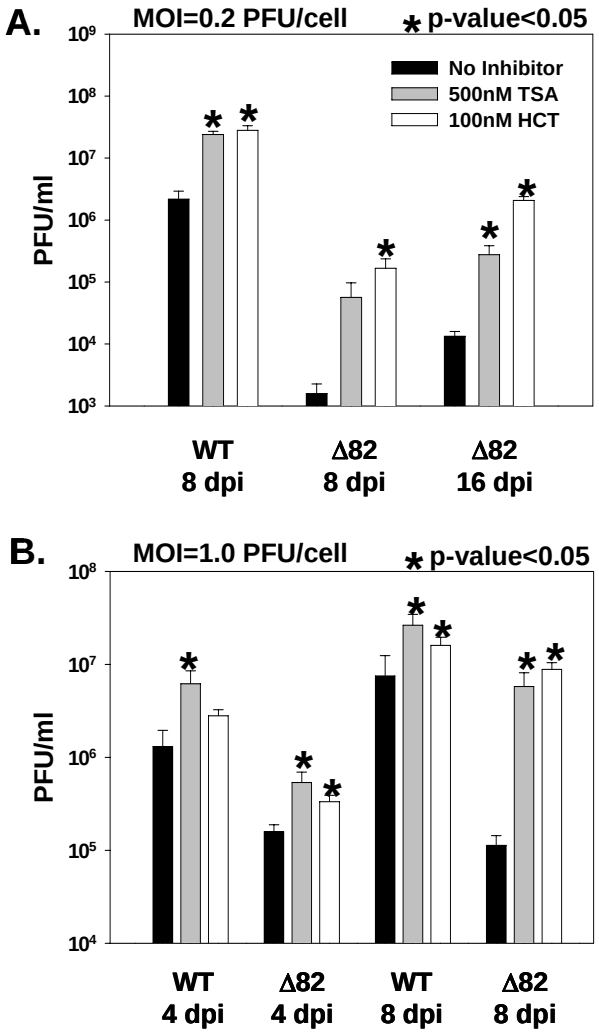

FIGURE. 4-9. Histone deacetylase (HDAC) inhibitors enhance DUL82 viral replication. HFF cells were infected in the presence or absence of HDAC inhibitors TSA(500nM) or HC Toxin (100nM) with ΔUL82 at a multiplicity of 0.2 (A) or 1 (B) PFU/cell. At various times postinfection, virus was harvested and quantified by plaque assay on UL82 complementing cells. Asterisks (*) indicate a significant difference in viral titers (p-value < 0.05) between virus produced on cells pretreated with inhibitor compared to untreated cells. Error bars indicate standard deviation derived from three independent experiments.

quantitated by Image J analysis. Samples were normalized to input viral DNA and ΔUL82 values set to 1 for each time point. As shown in Figure 4-10A, there was less acetylated histone H4 associated with the MIEP following infection with the UL82 deletion mutant virus when compared to the wild-type virus. When the data were quantitated, there was up to 7-fold increase in the amount of acetylated histone H4 associated with the MIEP following wild-type infection compared to infection with the UL82 deletion mutant (Fig. 4-10B). The GAPDH promoter was also examined as a control to demonstrate that equal amounts of cell lysates were used for each immunoprecipitation (Fig. 4-10A). .

Since the UL82 deletion mutant virus replicated more efficiently on the hDaxx knock-down cells, we next wanted to determine if there were increased levels of acetylated histone H4 associated with the MIEP when hDaxx knock-down cells were infected with the UL82 deletion mutant virus. Control scramble (SC) or hDaxx knock-down cells (KD2 and KD5) were infected with the UL82 deletion mutant virus at a MOI=0.2 PFU/cell and harvested for ChIP analysis 24 h postinfection. ChIP analysis was conducted as previously described using antibody against acetylated histone H4 or rabbit serum (RS) as a control. PCR and Southern blot analysis was conducted on the isolated DNA for the MIEP (Fig. 4-11). As shown in figure 4-11, there was an increase in acetylated H4 associated with the MIEP when hDaxx knock-down cells were infected with the UL82 deletion mutant virus when compared with control infected cells. The GAPDH promoter was also examined as a control to demonstrate that equal amounts of cell lysates were used for each immunoprecipitation (Fig. 4-11).

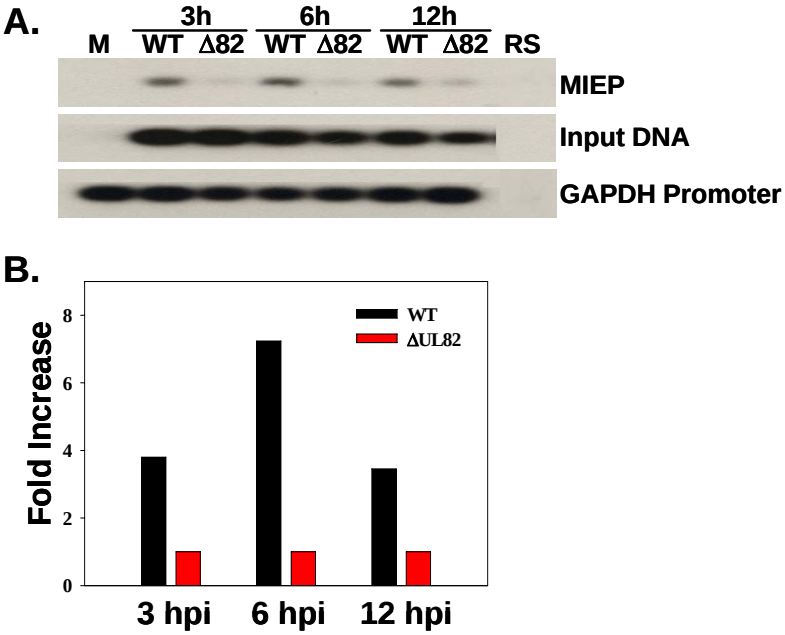

FIGURE 4-10. Acetylated histone H4 association with the MIEP during WT and ΔUL82 infection. (A) HFF cells were infected with either wild-type (WT) or ΔUL82 (Δ82) viruses at a MOI=0.1 PFU/cell and harvested for chromatin immunoprecipitation (ChIP) analysis. Samples were immunoprecipitated with an antibody against acetylated histone H4 or rabbit serum (RS) at various times postinfection. Southern blot analysis was conducted on the isolated DNA for the MIEP and the GAPDH promoter. (B) Data from the MIEP were quantified by ImageJ analysis and graphed as the fold increase in acetylated H4 associated with the MIEP during wild-type infection compared to ΔUL82. Samples were normalized to input viral DNA and ΔUL82 values set to 1 for each time point.

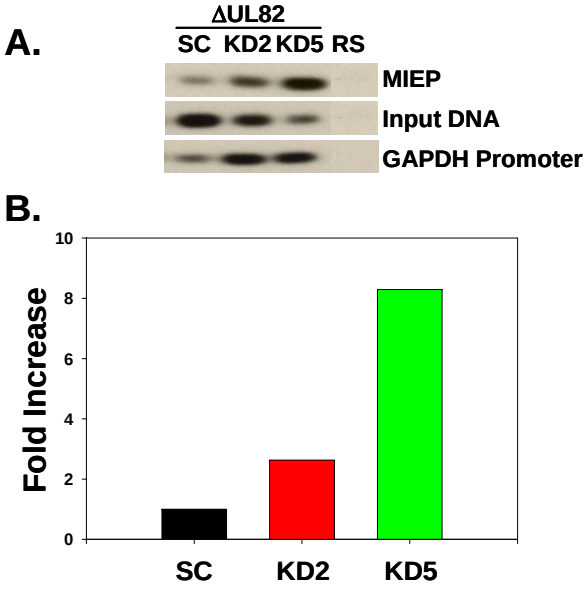

FIGURE 4-11. Increased acetylation of IE promoters during infection on hDaxx knock-down cells. Control (black) or hDaxx knock-down cells KD2 (red) and KD5 (green) were infected with wild-type or the UL82 deletion mutant virus at a MOI=0.2 PFU/cell and harvested for ChIP analysis at 12 hpi. ChIP analysis was performed using antibody against acetylated histone 4 or rabbit serum (RS). (A) DNA precipitated with the acetylated H4 antibody was PCR amplified with primers against the MIEP and GAPDH and analyzed by Southern blot analysis. (B) Graphical presentation of the fold increase of acetylated H4 associated with the MIEP during ΔUL82 infection of the hDaxx knock-down cells compared to control scramble cells. Samples were quantified by ImageJ analysis and normalized to Input DNA.

Discussion

We previously demonstrated that pp71's ability to interact with hDaxx was important for enhancing HCMV IE gene expression and viral replication (Chapter 3). The mechanism by which this interaction enhanced viral infection remained to be elucidated. It was also unknown if hDaxx served as a positive or negative regulator of viral gene expression during HCMV infection. Therefore, the studies presented in this chapter were initiated to determine the function of hDaxx during infection and to further examine the mechanism by which pp71 and hDaxx regulate viral IE gene expression and replication.

hDaxx represses HCMV infection

Previous studies showed that overexpression of hDaxx enhanced MIEP activity during transient transfection assays suggesting hDaxx may function to promote HCMV transcription (96). However, since hDaxx is primarily considered to function as a transcriptional repressor and has been suggested to repress viral infection (97, 185), we wanted to examine the effect of hDaxx expression on IE gene expression and HCMV replication in the context of a viral infection. Using stable cell lines that overexpress hDaxx we demonstrated that wild-type virus replication and IE gene expression are severely inhibited on hDaxx overexpressing cells in a multiplicity dependent manner (Fig. 4-1, Fig. 4-3). Using shRNA directed against hDaxx we also demonstrated that wild-type virus replication is enhanced in hDaxx knock-down cells (Fig. 4-4, Fig. 4-6). Finally and most importantly, we have demonstrated that we can abolish the UL82 deletion mutant associated defects in viral replication and IE gene expression by infecting hDaxx knock-down cells (Fig. 4-4, Fig. 4-6). Taken together these results indicate that hDaxx functions as a repressor

during HCMV infection and pp71 is responsible for relieving this repression.

HDAC inhibitors enhance HCMV infection

The mechanism by which the interaction between pp71 and hDaxx enables efficient virus replication, IE gene expression, and DNA infectivity has yet to be determined. Previous studies demonstrated that hDaxx interacts with core histones and histone deacetylase 2 (HDAC2) (97). Therefore, it was hypothesized that pp71 interacts with hDaxx to relieve histone deacetylase activity and thereby enable the acetylation of histones associated with IE promoters. To test this hypothesis, fibroblasts were infected in the presence or absence of histone deacetylase inhibitors. Wild-type virus replicated to higher levels in the presence of the HDAC inhibitors (Fig. 4-8 and Fig. 4-9). Additionally, the UL82 deletion mutant was partially complemented in the presence of the HDAC inhibitors and viral replication was significantly enhanced. Since the UL82 deletion mutant spread in the presence of HDAC inhibitors, these data support the hypothesis that pp71 functions to relieve histone deacetylase activity.

Increased levels of acetylated histones associated with the MIEP in the presence of pp71

To further examine the hypothesis that pp71 functions to relieve histone deacetylase activity, ChIP analysis was conducted to look at the association of acetylated histone H4 with the MIEP during infection with wild-type or the UL82 deletion mutant viruses. At all the examined times during infection, there was at least a three fold increase in acetylated histone H4 associated with the MIEP during wild-type infection compared to infection with the UL82 deletion mutant (Fig. 4-10). There was also an

increase in the association of acetylated histone H4 with the MIEP following infection of hDaxx knock-down cells with the UL82 deletion mutant virus (Fig. 4-11). These data support the hypothesis that pp71 functions to relieve histone deacetylase activity and allow for the acetylation of histones associated with the MIEP during infection.

Although these data support our hypothesis, they do not completely define the mechanism by which pp71s interaction with hDaxx leads to the relief of histone deacetylase activity during virus infection. There are several possible scenarios for how pp71 blocks hDaxx mediated repression of HCMV replication and IE gene expression based on our results and current literature findings. These potential mechanisms will be further discussed in Chapter 5.

CHAPTER FIVE
DISCUSSION AND FUTURE DIRECTIONS

UL82 (pp71)

The goal of this dissertation was to elucidate the function(s) of the human cytomegalovirus UL82 encoded tegument protein pp71 that is required for efficient virus replication and immediate-early gene expression. Prior to the work presented in this dissertation, several functions had been attributed to pp71. Studies overexpressing the pp71 protein demonstrated that pp71 activates immediate-early gene expression (30, 96, 99, 130) and that cotransfection of a plasmid encoding for pp71 enhances the infectivity of viral DNA, increasing plaque production following transfection (13). It was later shown through the use of a UL82 (pp71) deletion mutant virus that pp71 is required for efficient viral replication when cells are infected at a low multiplicity (30). Using the same mutant, it was also demonstrated that pp71 delivered to the host cell from the virus particle plays an important role in regulating IE gene expression during a productive infection (30). Based on these results it was hypothesized that pp71 functions to “kick-start” the infection by enhancing immediate-early gene expression and viral replication.

pp71 has been shown to interact with several cellular proteins. Using *in vitro* overexpression assays, Kalejta *et. al*. demonstrated that pp71 is able to interact with and degrade retinoblastoma (Rb) family member proteins, resulting in quiescent cells entering the cell cycle (109,

110, 112). In addition to interacting with Rb family member proteins, pp71 has also been shown to interact with the cellular protein hDaxx in transient transfection studies (96). pp71's ability to interact with hDaxx was demonstrated to be important for pp71 localization to ND10 domains and for transactivation of the MIEP (96). Although several functions had been attributed to pp71, the function of pp71 required for enhancing viral replication and immediate-early gene expression remained unknown. Therefore, the first aim of this dissertation was to determine if any of the previously demonstrated interactions with pp71 were important for immediate-early gene expression and viral replication in the context of a viral infection. Upon determining which interactions with pp71 are involved in controlling efficient viral replication, the second aim of this dissertation was to define the mechanism by which pp71 functions to enhance viral replication and immediate-early gene expression.

pp71 and Rb family member proteins

We first examined pp71's interaction with the Rb tumor suppressor family member proteins. pp71 has previously been shown to target hypophosphorylated Rb family member proteins for degradation in a proteasome-dependent, ubiquitin-independent fashion, which allows quiescent G0 cells to enter the cell cycle and progress through G1 (109-112). These studies also demonstrated that substitution of the cysteine residue within the pp71 LXCXD motif with a glycine residue blocked the degradation of hypophosphorylated Rb family members and the induction of quiescent cells into the cell cycle (110, 112). Interestingly, pp71's effect on Rb family member degradation and the host cell cycle was not linked to it's *in vitro* transactivation capabilities (111). Based on these studies it was hypothesized that pp71's ability to target Rb family member proteins

for degradation and progress cells through G1 would provide an advantage for virus replication (110-112). To determine if pp71 mediated degradation of Rb family member proteins was required for efficient IE gene expression and virus replication, we generated a UL82 mutant virus termed ADUL82-C219G which incorporated the cysteine to glycine substitution within the pp71 LXCXD motif. As described in Chapter 3, the ADUL82-C219G mutant replicated with wild-type kinetics (Fig. 3-3) and did not display attenuated IE gene expression (Fig. 3-11). Additionally, expression of the UL82-C219G protein was able to enhance the infectivity of viral DNA to wild-type levels (Fig. 3-12) (110). Taken together these results strongly suggest that pp71's ability to degrade hypophosphorylated Rb family members and induce quiescent cells into the cell cycle are not required for efficient virus replication in human fibroblasts and are not responsible for the growth phenotype associated with the UL82 deletion mutant.

Even though pp71 degradation of Rb family members and induction of quiescent cells into the cell cycle is not required for efficient viral replication, IE gene expression, or viral DNA infectivity, it does not mean HCMV's ability to affect these processes is not important for efficient virus replication. Other viral proteins involved in these processes may compensate for the loss of functions associated with the C219G mutant. For example, IE2 has also been shown to drive quiescent G0 cells into the cell cycle (39, 40) while IE1 can bind to (180) and phosphorylate (166) Rb family members, indicating redundant mechanisms for HCMV regulation of the cell cycle. Consistent with this hypothesis, data from our laboratory has demonstrated the UL82 deletion mutant virus retains the ability to inhibit the expression of the G0 marker GAS-1 (Growth Arrest Specific Gene-1), and to induce expression of the late G1 marker cyclin E and its

associated kinase activity (data not shown). This suggests other viral proteins in the absence of pp71 are capable of modulating G0 to G1 cell cycle progression and that pp71's ability to target Rb family member proteins for degradation through the LXCXD motif is not responsible for the growth phenotype associated with the UL82 deletion mutant.

The results presented in this dissertation using the ADUL82-C219G mutant exemplify the need to examine viral protein function in the context of a viral infection. The initial studies examining pp71 interactions with Rb family member proteins suggested that pp71's ability to target Rb family member proteins for degradation through an LXCXD motif would provide an advantage for viral replication. However, the results presented in this dissertation demonstrate that mutation of the LXCXD motif does not attenuate immediate-early gene expression or viral replication. Additionally, an interaction between pp71 and Rb family members has never been demonstrated in the context of a viral infection. The initial studies transiently overexpressed both pp71 and the Rb family member proteins pRb, p107, and p130 in cells which are nonpermissive for HCMV infection (112). Our attempts to immunoprecipitate pp71 with Rb family member proteins during wild-type infection in permissive fibroblasts have been unsuccessful. It is currently unknown if our unsuccessful attempts are due to technical error, or if there are certain conditions that allow for pp71 to interact with Rb during transient overexpression studies. While the initial transient transfection based studies implied a function for pp71 and cell cycle regulation that would benefit viral replication, our studies demonstrate that this function of pp71 is not required for immediate-early gene expression or viral replication. Therefore, our results demonstrate why experiments should be conducted in the context of a viral infection

when examining the functional role of viral proteins during virus replication.

pp71 and hDaxx

Upon determining that pp71's ability to target Rb family member proteins for degradation through an LXCXD motif was not required for efficient viral replication and IE gene expression, pp71's ability to interact with hDaxx was examined. hDaxx is primarily thought to function as a transcriptional repressor and colocalizes with PML within the nucleus at ND10 domains, a site of active gene transcription and viral genome deposition (59, 96, 102-105, 139, 141, 142). During HCMV infection, pp71 interacts with hDaxx and localizes to ND10 domains (96, 105, 137). Transient assays have demonstrated that disruption of the pp71-hDaxx interaction inhibits pp71 localization to ND10 domains and attenuates activation of the major immediate-early promoter (96). These results suggest pp71's interaction with hDaxx is important for viral IE gene expression and viral replication (96, 105). To test this hypothesis we generated UL82 viral mutants that have the hDaxx binding domains deleted and assayed them for their effect on IE gene expression and viral replication. As described in Chapter 3, growth curve analysis and the kinetics of IE1 and IE2 expression with the hDaxx binding mutants were indistinguishable from those obtained with the UL82 deletion virus (Fig. 3-6 and 3-11). Replication of the hDaxx binding mutants and the UL82 deletion mutant were severely attenuated when cells were infected at a multiplicity of 0.01 PFU/cell. The UL82 deletion mutant and hDaxx binding mutant viruses also showed a dramatic decrease in the abundance of IE1 and IE2 and demonstrated a significant delay in their expression when compared to wild-type virus (Fig. 3-11). Cotransfection of plasmids

expressing the pp71-hDaxx binding mutants and viral DNA failed to enhance plaque production (Fig. 3-12A), demonstrating the interaction between pp71 and hDaxx is involved in controlling this function of pp71. Although these results do not eliminate the possibility that the mutant viruses interfere with an undetermined function of pp71, the results suggest that pp71's ability to interact with hDaxx is required for efficient IE gene expression and virus replication.

In addition to identifying the function of pp71 that was important for enhancing HCMV infection, we also wanted to further define the mechanism by which the interaction between pp71 and hDaxx enables efficient virus replication, IE gene expression, and DNA infectivity. pp71 and hDaxx have been shown to synergistically transactivate viral gene expression in transient assays, suggesting a model in which pp71 interacts with hDaxx at ND10 domains to transactivate IE genes (96). This model is consistent with studies indicating ND10 sequestered hDaxx is inhibited in its ability to mediate transcriptional repression (126). However, other studies suggest hDaxx retains its ability to mediate transcriptional repression from ND10 domains (81, 98). It has also been suggested that the pp71-hDaxx interaction may serve to down-regulate cellular gene transcription and provide an advantageous site for viral gene expression (105). Although our results clearly demonstrate that the pp71-hDaxx interaction is involved in efficient IE gene expression and viral replication, they did not favor one model over the other.

Since hDaxx has been recognized as both a positive and negative regulator of transcription but has been suggested to repress viral infection (86, 185), we wanted to examine the effect of hDaxx expression on IE gene expression and HCMV replication in the context of a viral infection. We predicted that if hDaxx functions to positively regulate viral IE gene

expression and replication, then wild-type virus would be enhanced on cells overexpressing hDaxx. However, if hDaxx functions to negatively regulate viral IE gene expression and replication, then wild-type virus would be inhibited on cells overexpressing hDaxx. Based on these predictions, stable cell lines that overexpress hDaxx were generated and infected with wild-type virus. As demonstrated in Chapter 4, wild-type virus replication and IE gene expression were severely inhibited on hDaxx overexpressing cells in a multiplicity dependent manner, suggesting hDaxx functions to repress HCMV replication. (Fig. 4-1B, Fig. 4-3). Based on these results, we predicted wild-type virus and possibly the UL82 deletion mutant virus would be enhanced on cells knocked down for hDaxx. Our prediction was correct. Wild-type virus replication and IE gene expression were enhanced in hDaxx knock-down cells (Fig. 4-4C-D, Fig. 4-6). Most importantly, we demonstrated the UL82 deletion mutant associated defects in viral replication and IE gene expression are abolished by infecting hDaxx knock-down cells (Fig. 4-4, Fig. 4-6). Taken together these results indicate that hDaxx functions as a repressor during HCMV infection and pp71 is responsible for relieving this repression.

After we reported pp71 interacts with hDaxx to relieve hDaxx mediated repression of HCMV IE gene expression and replication, other groups confirmed our findings. Several reports demonstrated that hDaxx attenuates IE gene expression during wild-type infection (196, 240). Additionally, ChIP analysis demonstrated that overexpression of hDaxx led to increased levels of methylated histone associated with the MIEP during wild-type HCMV infection, suggesting a transcriptionally repressed state (240). However, when cells were knocked-down for hDaxx expression and infected with wild-type virus, there were fewer methylated histones and more acetylated histones associated with the MIEP, thereby

suggesting a transcriptionally active status for the MIEP in the absence of hDaxx (240). hDaxx has also been shown to function as a repressor of other viruses. For example, hDaxx has been shown to repress avian sarcoma virus transcription (86). While there are currently no reports of other herpesvirus proteins interacting with hDaxx to enhance viral replication, it is becoming increasingly clear that DNA viruses target proteins within ND10 domains to provide an environment advantageous for viral replication and gene expression.

Intrinsic immunity against HCMV: hDaxx and ND10 domains

We have clearly demonstrated the importance of pp71's ability to interact with hDaxx at ND10 domains for viral replication and IE gene expression. Although there have been numerous studies regarding ND10 domains, the overall function of ND10 domains during viral infection remains controversial. ND10 domains are composed of a number of proteins including transcription factors, chromatin remodeling proteins and interferon stimulated proteins. It is currently unknown why a variety of proteins involved in different cellular processes are deposited or recruited to ND10 domains. Individual ND10 protein functions do not necessarily correlate with functions associated with the ND10 domain. Changes to protein content within ND10 domains in response to different stimuli including interferon, viral infection, and heat shock make understanding the function of these nuclear depots even more challenging.

ND10 domains have been referred to as nuclear depots in which a variety of proteins reside until released following a stress response or external stimuli (159). Therefore, ND10 would function as a response mechanism to viral infection and ND10 associated proteins would be released to inhibit viral replication. Considering the many negative affects

of ND10 proteins on viral infection, this would explain why many DNA viruses disrupt ND10 domain organization. Although there are many questions that remain unanswered regarding these ND10 domains during viral infection, it has been hypothesized that ND10 domains serve as an intrinsic immunity against viral evasion (196, 220).

hDaxx is recruited to ND10 domains by PML, the primary constituent of ND10 domains (103). In the case of PML null mice, ND10 proteins are dispersed throughout the cell and ND10 domains are not observed (103, 251, 252). Both PML and hDaxx have been shown to function in a negative manner during HCMV infection. Cells knocked down for either PML or hDaxx showed increases in IE gene expression during wild-type HCMV infection (36, 181, 196, 221, 240). It is likely that the results seen in PML knock-down cells are the result of hDaxx being dispersed throughout the nucleus, thereby preventing hDaxx localization to ND10 domains which results in the repression of IE gene expression. Although it is unknown if PML and hDaxx function independently to repress HCMV infection, the fact that HCMV can replicate in PML deficient cells lacking ND10 domains suggests that ND10 domains are not necessary for a productive HCMV infection (221). Additionally, since both PML and hDaxx function as negative regulators of IE gene expression, it is thought that ND10 domains may function as an intrinsic immune defense mechanism that the virus must overcome to enable efficient virus replication.

Although ND10 domains do not appear to be required for HCMV infection, it is uncertain why replication compartments form adjacent to ND10 domains. Based on HSV-1 studies, it does not appear that viral gene transcription and protein expression are required for the viral genome to associate with ND10 domains (102, 141). There are also

studies which suggest ND10 domains form adjacent to the genome following infection with HSV-1 (64). Based on these results and studies indicating HCMV replicates more efficiently in cells lacking ND10 domains, the localization of viral replication compartments adjacent to ND10 domains may not provide an advantage for viral DNA replication. However, this does not eliminate the possibility that the virus may disrupt ND10 domains not only to avoid the negative affects presented by ND10 proteins but to also gain access to specific ND10 associated proteins that may be advantageous for viral replication. If ND10 proteins enhance viral gene transcription, genome localization adjacent to ND10 domains may be important in initiating IE gene expression. While it is uncertain if replication compartment localization adjacent to ND10 domains is advantageous, it is becoming increasingly clear that ND10 domains serve as an intrinsic innate immune response to viral infection which must be counteracted by the virus in order to establish a productive infection.

Mechanism of action: pp71 relief of hDaxx repression

Although our studies demonstrated pp71's ability to interact with hDaxx at ND10 domains is required to prevent hDaxx repression of HCMV viral replication and IE gene expression, the mechanism by which pp71 relieves this repression remained unclear. hDaxx has been shown to interact with a number of ND10 domain associated proteins that are involved in regulating gene expression including the Pax-3 (98) and Ets-1 (127) transcription factors, DNA methyltransferase 1 (150), Dek, core histones, and histone deacetylases (HDAC) (97). During HCMV infection, HDACs have been shown to repress the MIEP (144, 157). Treatment of cells nonpermissive for HCMV infection with HDAC inhibitors renders the cells permissive (144, 157). Additionally, IE1 has been shown to

antagonize histone deacetylation by interacting with HDAC1, resulting in increased association of acetylated histones with the MIEP (161). Based on these previous studies, it is apparent that HDACs play an important role in regulating the MIEP. Therefore, we hypothesized that pp71 interacts with hDaxx to block HDAC activity and allow for the acetylation of histones associated with viral IE promoters.

To test the hypothesis that pp71 interacts with hDaxx to relieve HDAC activity, fibroblasts were infected with either wild-type or the UL82 deletion mutant viruses in the presence or absence of the HDAC inhibitors TSA or HC Toxin. Wild-type virus replicated to higher levels in the presence of the HDAC inhibitors compared to DMSO control treated cells. Importantly, the UL82 deletion mutant virus replicated to significantly greater levels in the presence of TSA or HC toxin (Fig. 4-9), spreading at slightly slower levels than the wild-type virus (data not shown). Therefore, the UL82 deletion mutant virus appeared to be partially complemented in the presence of HDAC inhibitors, suggesting that pp71 plays a role in relieving HDAC mediated repression during HCMV infection.

To further examine our hypothesis that pp71 functions to relieve HDAC repression, ChIP analysis was conducted to examine the levels of acetylated histone H4 associated with the MIEP during HCMV infection. Since the UL82 deletion mutant virus replicated more efficiently in the presence of HDAC inhibitors, we hypothesized there would be greater levels of acetylated histone H4 associated with the MIEP in the presence of pp71. As predicted, increased levels of acetylated histone H4 were associated with the MIEP during wild-type infection compared to the UL82 deletion mutant virus (Fig. 4-10). In fact, at 6h postinfection, there was approximately seven times the amount of acetylated histone H4 associated with the MIEP in the presence of pp71.

In addition to examining the association of acetylated histones with the MIEP in the presence and absence of pp71, we also wanted to examine the levels of acetylated histone H4 associated with the MIEP during infection with the UL82 deletion mutant in hDaxx knock-down cells. It was previously reported that there is an increase in acetylated histone H4 during wild-type infection of hDaxx knock-down cells compared to control scramble cells (240). The combination of prior results and our data demonstrating the UL82 deletion mutant virus replicates to wild-type levels in hDaxx knock-down cells, led us to hypothesize there would be an increase in the amount of acetylated histone H4 associated with the MIEP during ΔUL82 infection of hDaxx knock-down cells compared to the control scramble cells. As predicted, ChIP analysis of control and hDaxx knock-down cells revealed an increase in acetylated histone H4 associated with the MIEP during infection with the UL82 deletion mutant when hDaxx was depleted (Fig. 4-11). These results suggest the UL82 deletion mutant growth phenotype can be overcome in the absence of hDaxx due to the increased association of acetylated histones with the MIEP. These results also support our hypothesis that the primary function of pp71 during HCMV replication is to interact with hDaxx and block histone deacetylase activity, allowing for increased expression of immediate-early proteins.

Previous studies have also examined the association of methylated histones with the MIEP during HCMV infection. The association of methylated histones with gene promoters is correlated with transcriptional repression. During HCMV infection in nonpermissive cells, there was reported to be an increase in methylated histones associated with the MIEP (144, 157). It was also reported that there were fewer methylated histones associated with the MIEP during wild-type infection of hDaxx

knock-down cells (240). While our data only examines the association of acetylated histones, we predict there are likely to be more methylated histones associated with the MIEP in the absence of pp71. We also predict there are fewer methylated histones associated with the MIEP during infection of hDaxx knock-down cells with the UL82 deletion mutant virus. Further ChIP analyses using antibody against methylated histone H4 are required to confirm the role of pp71 and hDaxx on the methylation of histones associated with viral IE promoters.

While our data suggests pp71 relieves HDAC activity during infection, it does not define the mechanism by which HDAC activity is inhibited. TSA and HC toxin are both broad spectrum deacetylase inhibitors that disrupt the activity of multiple HDAC proteins. Currently, hDaxx has only been demonstrated to interact with HDAC2 (97). Therefore, to further define the mechanism by which pp71 interacts with hDaxx to relieve HDAC activity, the specificity of HDAC activity needs to be elucidated. Immunoprecipitation analyses need to be conducted to determine if hDaxx can interact with other HDAC proteins. shRNAs can then be used to target and deplete individual HDACs identified as hDaxx binding partners. If the UL82 deletion mutant virus is complemented in the absence of an individual HDAC, it suggests pp71 interacts with hDaxx to relieve specific HDAC activity and that hDaxx utilizes specific histone deacetylases to repress viral gene expression. These experiments may prove to be difficult since there are at least 12 identified histone deacetylases (53).

Potential models for hDaxx repression of HCMV

Based on the results demonstrated in this dissertation, it is likely hDaxx functions to repress HCMV infection through its ability to regulate

HDAC activity. However, the data presented does not address the mechanism by which pp71 may relieve HDAC activity. One proposed mechanism suggests pp71 targets hDaxx for degradation at early times postinfection to relieve HDAC activity (196). Another possible model is that pp71's interaction with hDaxx induces changes to hDaxx associated complexes at ND10 domains which prevent HDAC activity at the MIEP. A third model is that pp71 association with hDaxx results in a modification to hDaxx, HDACs, or other transcriptional regulators found at ND10 domains. Further research is required to determine if pp71 utilizes any of these models or a combination of the mechanisms presented within these models to regulated hDaxx mediated repression during HCMV replication and IE gene expression.

Model 1: pp71 disrupts hDaxx interactions with cellular proteins

hDaxx has been shown to interact with a number of cellular proteins that are involved in regulating gene expression including the Pax-3 (98) and Ets-1 (127) transcription factors, DNA methyltransferase 1 (DNMT1) (150), Dek, core histones and histone deacetylases (HDAC) (97). Therefore, pp71 binding to hDaxx may alter hDaxx's ability to interact with one or more of these proteins or alter the activity associated with these hDaxx complexes (Fig. 5-1). Further experiments are necessary to examine hDaxx interactions with cellular proteins in both the presence and absence of pp71. For example, one experiment would be to demonstrate if hDaxx is able to interact with HDAC2 at early times following wild-type infection. We demonstrated in this dissertation that the UL82 deletion mutant virus is partially complemented in the presence of histone deacetylase inhibitors, suggesting pp71 functions to relieve HDAC activity. Therefore, it is possible that pp71's interaction with hDaxx blocks

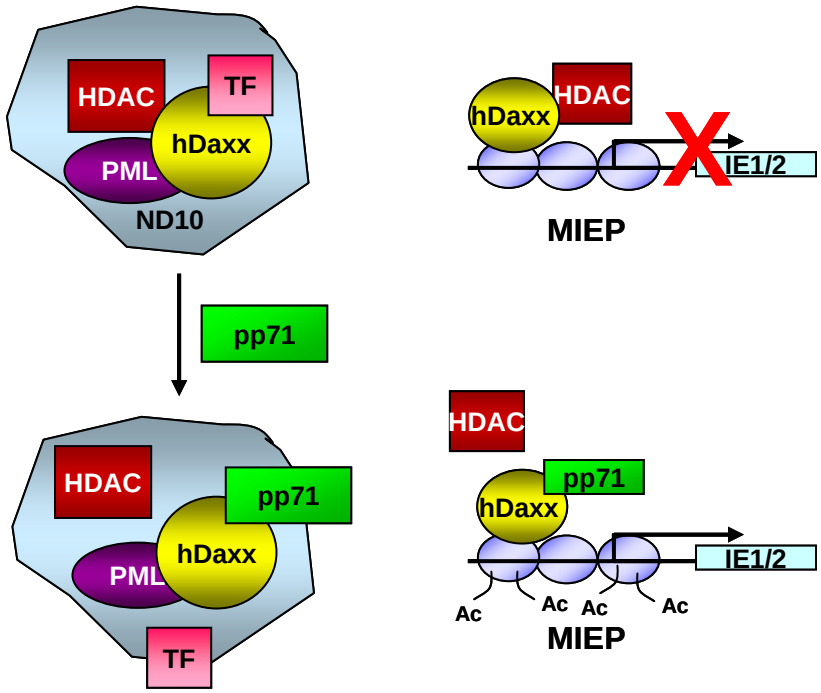

FIGURE 5-1. Model 1: pp71 disrupts hDaxx interactions with cellular proteins. In this model, pp71 binds to hDaxx and disrupts hDaxx interactions with other cellular proteins at ND10 and blocks repression of the MIEP. For example, pp71 binding hDaxx prevents HDAC binding with hDaxx and therefore blocks HDAC mediated repression of the MIEP. Definitions: HDAC=histone deacetylase, TF=transcription factors, PML=promyelocytic leukemia protein, MIEP=major immediate-early promoter, Ac=acetyl group, WT=wild-type.

hDaxx's ability to interact with HDAC2. Using this model we predict interfering with hDaxx's ability to interact with HDAC2 would relieve HDAC activity and thereby allow for the acetylation of histones associated with the MIEP. Currently, our attempts to immunoprecipitate hDaxx with HDAC2 in uninfected or HCMV infected cells have been unsuccessful. Our attempts to examine HDAC2 activity in cells infected with wild-type or the UL82 deletion mutant virus have also been unsuccessful. It is important to first confirm that hDaxx interacts with HDAC2 or other histone deacetylases in the absence of viral infection. Once an interaction between HDAC2 or other histone deacetylases can be demonstrated during mock infection, immunoprecipitation analysis will need to be conducted during infection with both wild-type and the UL82 deletion mutant viruses to determine if the presence of pp71 prevents the association of hDaxx with HDAC2 or another HDAC. Additionally, cells infected with pp71 expressing adenoviruses may also be harvested for immunoprecipitation experiments to determine if pp71 disrupts hDaxx interactions with histone deacetylases or other ND10 domain proteins.

While studies presented in this dissertation suggest hDaxx's interaction with HDACs may play a critical role in repressing viral gene expression, we cannot overlook the functions of other hDaxx interacting proteins during infection. hDaxx also interacts with other transcriptional regulatory proteins including transcription factors, DNMT1 and the DNA methyltransferase 1 associated protein (DMAP1) (154), which may function during viral gene expression. To determine if pp71 alters hDaxx association with cellular proteins, cell lysates can be collected from wild-type or the UL82 deletion mutant infected fibroblasts radiolabeled with ^{35}S-methionine. The harvested samples can then be immunoprecipitated with antibody against hDaxx, separated by SDS-PAGE, and pulled down

proteins visualized by autoradiography. The immunoprecipitation results can then be compared to determine if certain proteins are pulled down with hDaxx in the presence or absence of pp71. The protein can then be identified either by mass spectrophotometry or by repeating the immunoprecipitation using specific antibodies against hDaxx associated proteins. For example, a protein the size of DNMT1 may be pulled down with hDaxx in the absence of pp71 but not in the presence of pp71, indicating pp71 may interfere with the association of hDaxx and DNMT1. If pp71 disrupts this interaction, methylation of histones associated with the MIEP may be blocked. If the activity of methyl transferases is blocked, we hypothesize there would be fewer methylated histones and potentially an increase in acetylated histones associated with the MIEP in the presence of pp71. To examine this possibility, lysates from cells infected with wild-type or the UL82 deletion mutant viruses need to be immunoprecipitated with antibody against hDaxx or DNMT1, followed by western blot analysis for DNMT1 or hDaxx respectively to determine if pp71 blocks the association of hDaxx with DNMT1. A compilation of immunoprecipitation assays are necessary to determine if pp71 alters hDaxx associated complexes during HCMV infection to enhance IE gene expression.

Model 2: pp71 targets hDaxx for degradation

Saffert *et. al.* demonstrated pp71 targeting of hDaxx for degradation at early times postinfection (196). It has been proposed that pp71 targets hDaxx for degradation, preventing histone deacetylation at IE promoters (Fig. 5-2). Although the degradation of hDaxx at early times during infection would inhibit hDaxx mediated repression, there are a number of

issues surrounding this model, suggesting hDaxx degradation alone is not sufficient to enhance viral replication and IE gene expression.

Results from our laboratory do not reveal a complete loss of hDaxx at early times postinfection, raising concern that pp71 relieves hDaxx mediated repression by targeting hDaxx for degradation (Fig. 3-9). If hDaxx can still be detected by immunoblot analysis, it is unclear whether the remaining hDaxx can efficiently function to repress viral gene expression. The timing of hDaxx degradation during infection is also questionable in regards to this model. Published data showed hDaxx levels decreased immediately following infection and then returned to normal 12-24 hours postinfection. It has been proposed that the accumulation of hDaxx at late times following infection is either the result of hDaxx upregulation by the cellular interferon response or the inhibition of pp71 mediated degradation of hDaxx (196). Our data shows pp71 interacts with hDaxx at late times postinfection, which raises the question as to why pp71 targets hDaxx for degradation at early times postinfection and not at late times postinfection. Additionally, we see IE1/IE2 gene expression prior to hDaxx degradation, suggesting hDaxx degradation alone is not sufficient to prevent hDaxx mediated repression of IE gene expression.

This model presents a simple mechanism by which pp71 targets hDaxx for degradation, thereby relieving hDaxx mediated repression. More studies need to be conducted to potentially address the mechanism by which pp71 targets hDaxx for degradation and what prevents hDaxx degradation at later times during infection. Although data in this dissertation demonstrates depleting hDaxx enhances viral replication, it remains unclear if hDaxx targets HDACs to viral promoters or if there are other ND10 domain associated factors involved.

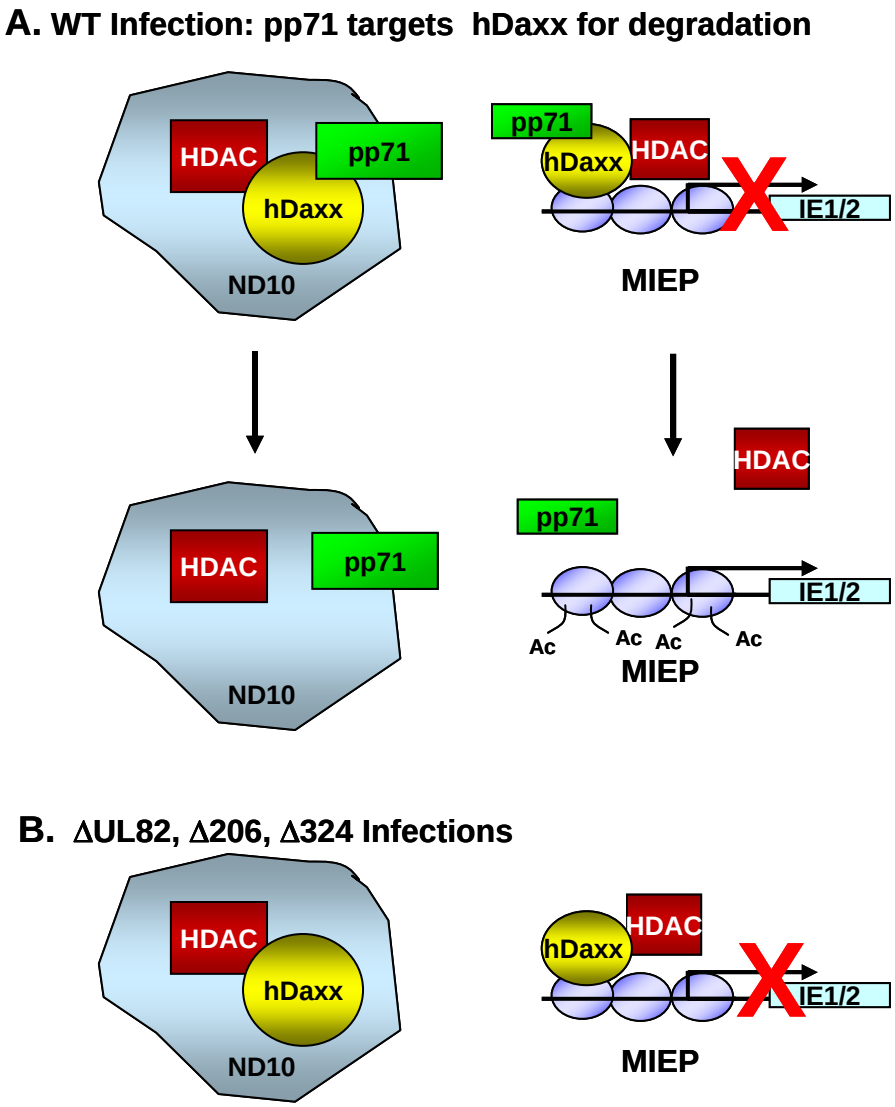

FIGURE 5-2. Model 2: pp71 targets hDaxx for degradation. (A) During wild-type infection, pp71 binds to hDaxx and targets hDaxx for degradation. In the absence of hDaxx, the MIEP is transcriptionally active. (B) During infection with the UL82 deletion mutant virus or the pp71-hDaxx binding mutant viruses Δ206 or Δ324, pp71 does not bind hDaxx and does not target hDaxx for degradation. Without hDaxx degradation, the MIEP is transcriptionally repressed, possibly through hDaxx's ability to interact with HDACs. Definitions: HDAC=histone deacetylase, MIEP=major immediate-early promoter, Ac=acetyl group, WT=wild-type.

Model 3: pp71 interaction with hDaxx alters protein modifications

Protein modifications, including sumoylation and phosphorylation, have been shown to be important for regulating the dynamics of ND10 domains. Sumoylation, the covalent attachment of the 11 kilodalton (kDa) small ubiquitin-related modifier 1 (SUMO) to lysine residues, has been shown to alter protein interactions, cellular localization, stability, and enzymatic activity (20). Sumoylation occurs by a mechanism similar to ubiquitination but does not necessarily result in protein degradation. The ND10 domain proteins PML, Sp100, hDaxx and heterochromatin protein 1 (HP1) have all been shown to be modified by SUMO-1. Current studies suggest the removal of the SUMO adducts from PML by homeodomain-interacting kinase PKM (HIPK-2) results in ND10 domain disruption (62). In addition to sumoylation, phosphorylation is an important post-translational modification on ND10 associated proteins. For example, HIPK-1 has been shown to bind and phosphorylate hDaxx on serine residues, inhibiting hDaxx's function as a transcriptional repressor and relocalizing hDaxx from ND10 domains (61). The activity of HDAC1 and HDAC2 are also regulated by phosphorylation with hyperphosphorylation leading to significant increases in deacetylase activity (53). Considering the significant role of post-translational modifications in regulating the transcription and the dynamics of ND10 domains, it is not surprising that viruses manipulate these processes to benefit virus replication.

It has recently been demonstrated that several DNA viruses interfere with protein sumoylation to favor virus propagation. For example, the HCMV IE1 protein has been shown to bind sumoylated PML at ND10 domains and prevent or remove SUMO modifications leading to ND10 domain disruption (125). Based on previous evidence of HCMV interfering with sumoylation of host proteins, it is reasonable to propose a model in

which pp71 affects post-translational modifications of hDaxx or other ND10 proteins (Fig. 5-3). For example, pp71 binding to hDaxx may induce the phosphorylation of serine residues on hDaxx and inhibit hDaxx mediated repression. hDaxx's ability to interact with SUMO has recently been demonstrated as important for hDaxx suppression of transcription factors (128). Therefore, if pp71 inhibits hDaxx sumoylation, transcriptional repression would be blocked. To determine if pp71 alters hDaxx phosphorylation or sumoylation, cell lysates from wild-type or UL82 deletion mutant virus infected cells can be immunoprecipitated with antibody against phosphoserine or SUMO and western blot analysis conducted using antibodies against hDaxx. If pp71 alters hDaxx posttranslational modifications, we would expect to see a change in hDaxx modifications during wild-type infection but not during infection with the UL82 deletion mutant virus when compared with mock infected cell lysates.

All of these models present different mechanisms by which pp71 interacts with hDaxx to enhance IE gene expression and viral replication. Further studies are necessary to determine if any of the mechanisms presented within these models are utilized to enhance HCMV infection. It is likely that pp71 functions through a combination of the proposed models. For example, pp71 binding to hDaxx may alter the sumoylation status of hDaxx which leads to complex dissociation between hDaxx and transcription factors. It is also possible that pp71 and hDaxx utilize a completely different mechanism than the ones proposed in this dissertation. Additional research is necessary to define the mechanism by which pp71 relieves hDaxx mediated repression. These findings will potentially provide significant insights into the mechanisms employed by

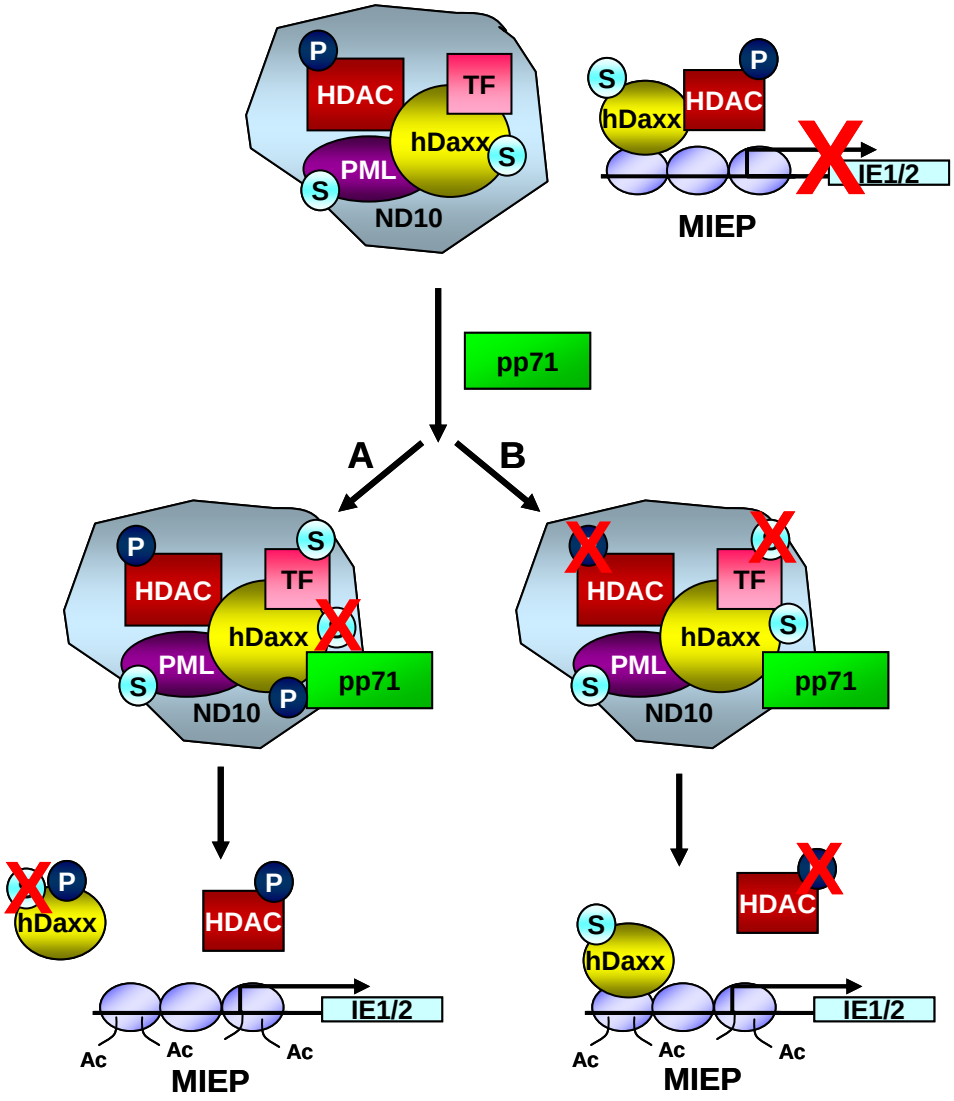

FIGURE 5-3. Model 3: pp71 interaction with hDaxx alters protein modifications. This model predicts that pp71 may lead to altered phosphorylation (P) or sumoylation (S) of hDaxx, HDACs or other ND10 proteins. Possibility A: pp71 alters the phosphorylation or sumoylation status of hDaxx blocking hDaxx mediated repression and HDAC activity at the MIEP, thereby enhancing transcription. Possibility B: pp71 interaction with hDaxx alters the phosphorylation of HDAC and blocks histone deacetylase activity, thereby enhancing transcription from the MIEP. Definitions: HDAC=histone deacetylase, MIEP=major immediate-early promoter, Ac=acetyl group, S=SUMO, P=phosphorylated, WT=wild-type, PML=promyelocytic leukemia protein .

HCMV to overcome the intrinsic immunity of ND10 domains and optimize viral infection.

Insight to theraputics

HDAC inhibitor therapy and HCMV infection

Histone deacetylases have recently become the target for treating a variety of diseases including epilepsy, cancer and AIDS. Histone deacetylase inhibitors are being used both in practice and clinical trials to block HDAC activity. The mechanism by which the HDAC inhibitors fight disease varies. In the case of epilepsy, valproic acid (VPA) is administered and is hypothesized to act as an anticonvulsant through several mechanisms (174). In cancer cells, HDAC inhibitors are ideal drugs because they induce apoptosis and inhibit growth of transformed cells while remaining relatively nontoxic to normal cells (35, 136, 175). For AIDS patients, HDAC inhibitors induce reactivation of HIV latent infected cells (55). It has been proposed that activation of HIV infection in combination with highly active antiretroviral therapy (HAART) will significantly decrease the number of HIV infected cells and potentially eradicate infection. While the use of HDAC inhibitors for various diseases is promising, it raises concern regarding the potential impact of HCMV disease in patients undergoing HDAC inhibitor therapy.

Work within this dissertation and studies from other laboratories have demonstrated that HDAC inhibitors enhance HCMV replication by upregulating viral gene expression. HDAC inhibitors have also been shown to allow for HCMV replication in normally nonpermissive cells (12, 157, 185, 201). Considering the effects of HDAC inhibitors on HCMV replication and tropism, the use of HDAC inhibitors to treat both cancer and AIDS raises many concerns regarding HCMV infection and disease.

In particular, there is evidence that AIDS patients receiving VPA are at increased risk for HCMV retinitis (148). Further studies need to be conducted to understand the affects of HDAC inhibitor therapy on HCMV pathogenesis in immunocompromised individuals. Therefore, it is imperative that patients undergoing therapy with histone deacetylase inhibitors be monitored for HCMV disease and treated accordingly.

pp71 and HCMV therapy

The studies presented throughout this dissertation clearly demonstrate that the HCMV pp71 protein functions to block the repression of the cellular protein hDaxx during viral infection. These data not only present insight into a mechanism employed by HCMV to enhance IE gene expression and replication, but also provides insight for potential HCMV prevention and therapy. As more cases of HCMV drug resistance emerge, the need for additional drug targets becomes necessary. Pharmaceutical companies could potentially generate drugs targeting the interaction between pp71 and hDaxx to limit HCMV gene expression and replication. Additionally, a drug could potentially be designed to target infected cells, upregulate hDaxx expression and ultimately repress viral gene expression and replication.

In addition to using the data presented in this dissertation for the design of HCMV drug therapies, our data suggests the UL82 deletion mutant may serve as a potential vaccine candidate for HCMV prevention. The UL82 deletion mutant virus is able to efficiently enter cells and is expected to activate the innate immune response through interactions with cell surface receptors. However, viral gene expression is disrupted during infection with the UL82 deletion mutant virus when cells are infected at a low multiplicity. Therefore, the expression of viral proteins that function to

counteract the host anti-viral response would be blocked. For example, IE2 expression is severely inhibited during infection with the UL82 deletion mutant virus. IE2 has been shown to antagonize the host innate immune response by attenuating IFNβ and blocking chemokine expression (222-224). Without IE2 expression, it is predicted that an increased number of CTL and natural killer (NK) cells will be recruited to the infected cell, initiating a more robust immune response. Due to the growth phenotype associated with the UL82 deletion mutant virus, viral replication would be limited if administered at a low input multiplicity. Therefore it is expected that the virus infected cells would initiate a robust anti-viral response and ultimately be cleared by the host.

Closing Statement

Herpesviruses use a variety of methods to counteract host responses and enhance viral infection. There is a common theme emerging amongst the DNA viruses whereby viral proteins target ND10 domains. While the function of ND10 domains during viral infection remains uncertain, it is clear that several viruses target ND10 domains to enhance viral gene expression. We report in this dissertation that the ND10 domain protein hDaxx functions as a repressor of HCMV immediate-early gene expression and viral replication. Additionally, we are the first to demonstrate that the viral encoded pp71 tegument protein targets the cellular protein hDaxx at ND10 domains to block hDaxx mediated repression of viral immediate-early gene expression. This step is required during low input multiplicities of infection and is important to "kick-start" the infection by upregulating immediate-early gene expression. Our preliminary data indicates pp71 interacts with hDaxx to relieve histone deacetylase activity and therefore allow for transcription from immediate-

early promoters. This dissertation has outlined our attempts to further define the mechanism by which the HCMV pp71 protein interacts with the host to overcome the intrinsic innate immunity of ND10 domains and provide an ideal environment for viral gene expression and replication.

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VITAE

Stacy Renee Hagemeier (Cantrell) was born in Charleston, West Virginia, on August 10, 1979, the daughter of Brenda Joyce Cantrell and Paul Eugene Cantrell. She attended Capital High School and entered West Virginia University in Morgantown, West Virginia where she graduated Magna cum laude with two Bachelor of Arts degrees in Biochemisty , Chemistry and Biology. While Stacy was doing her undergraduate studies, she worked as an intern for the Environmental Department at Columbia Natural Resources and conducted undergraduate research at the National Institute for Occupational Health and Safety (NIOSH). In August, 2001 Stacy entered the Graduate School of Biomedical Sciences at the University of Texas Health Science Center at Dallas. She joined the laboratory of Wade A. Bresnahan in 2002 and moved to Minneapolis, MN in 2004 to complete her research under Dr. Bresnahan's supervision at the University of Minnesota. Stacy was married to Ryan W. Hagemeier on August 5, 2006.

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