A GLIMPSE INTO THE ROLE AND MECHANISM OF UL135 IN HCMV APOPTOSIS

By

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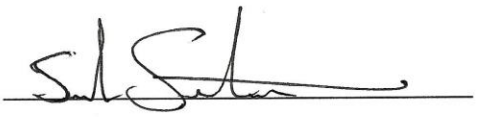
Signed: 

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Abstract

Human cytomegalovirus (HCMV) exists indefinitely in infected individuals through a latent infection that is poorly characterized in hematopoetic cells. We have previously demonstrated that the UL133-UL138 locus within the ULb' region encodes proteins that are integral membrane proteins that are co-localized to the Golgi and are involved in the regulation of viral replication. UL135 is of interest in that it is predicted to activate viral replication. Given preliminary results, exogenous expression of UL135 outside of the context of infection results in apoptosis in fibroblasts through caspase pathways. UL135 possesses five SH3 domain binding sites; we hypothesize that UL135 functions through its interaction with SH3 domain-containing proteins. To confirm the importance of the SH3 domain binding sites, I mutated three of the five SH3 domain binding sites both individually and collectively. Preliminary studies show that UL135 exhibits decreased ability to induce apoptosis when the SH3 domain binding sites are mutated, which suggests that UL135 is indeed acting to activate viral replication by suppressing UL138, a protein previously identified as necessary in establishing a latent infection. Future directions include repetition of these preliminary experiments in addition to creating viruses with these mutants in order to analyze their effect on latency during infection.

Introduction

Human Cytomegalovirus

Human cytomegalovirus is a ubiquitous betaherpesvirus that persists in 60-90% of the world's population (1, 17, 23). The acquisition of HCMV infections often occur earlier in life, particularly in children from areas in which the population is predominantly of lower socioeconomic background, where the seroprevalence tends to be much higher, as opposed to populations from well-developed countries (8, 17, 23). HCMV seropositivity appears to be linked to a variety of factors including female gender, age, in addition to low immunity (8). HCMV infections are typically asymptomatic and usually do not harm the host (12, 33); however, in the case individuals with suppressed immune function, HCMV can cause serious illness similar to mononucleosis caused by Epstein-Barr virus (25, 42). The transmission of HCMV occurs through the body fluids from infected individuals, including sexual activity, saliva, horizontal transmission amongst children, and breast milk (2, 3).

HCMV infection is the leading cause of infectious disease related birth defects and affects 1 in 100 live births in the United States (25). In fact, congenital HCMV infections can lead to serious and permanent disabilities and are the leading infectious cause of deafness (11). During pregnancy, primary HCMV infection in an HCMV seronegative mother carries a 30-40% risk of intrauterine transmission; birth defect likelihood increases for fetuses infected during the first half of gestation (37). Primarily, it is within the first trimester of gestation that fetuses possess a 20-30% risk (much higher than in other trimesters) of developing the most severe manifestations of HCM0056 symptom, such as hearing loss, mental retardation, and seizures (26).

As a member of the beta herpesvirus family, HCMV has linear double-stranded DNA of 230 kilobases and has the largest genome in comparison to all other human herpesviral genomes; also, like other herpesviruses, HCMV can establish a persistent latent infection for the duration of the lifetime of their host (6, 7). The HCMV genome is packaged within an icosahedral protein capsid (T=16) surrounded by the tegument, a mixture of viral proteins and mRNAs (Figure. 1) (18). The nucleocapsid and tegument are both encased in a lipid bilayer containing many viral glycoproteins, which assist the virus in attachment and entry into the cell (15, 22). The HCMV genome is divided into unique long (UL) and unique short (US) that are both flanked by inverted repeat regions, which are involved in the packaging and cleavage of the viral genome, in addition to genome isomer formation(25).

Though the mechanisms of viral attachment and entry are unclear, as many as five glycoproteins are involved in its mediation in various cell types (4, 25). After membrane fusion, the nucleocapsid and tegument proteins are released into the cytoplasm of the cell, triggering an innate immune response in the host cell and initiation of viral replication (25). Viral capsids are delivered to the nucleus via the microtubule network and nuclear pore docking (25). After replication and capsid assembly in the nucleus, HCMV, like all other herpesviruses, kills the cell once replication has been completed (30) but persist in their respective hosts by way of a lifelong latent infection, through various mechanisms (6).

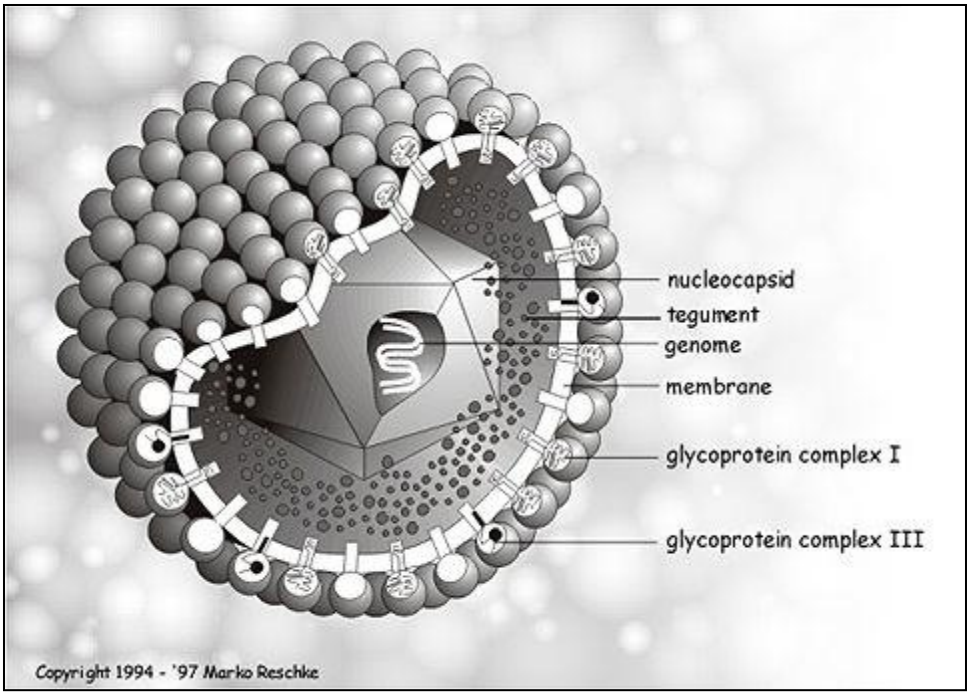

Figure. 1. **Human cytomegalovirus virion**. An HCMV vision has a linear double stranded DNA genome that is protected by an icosahedral nucleocapsid, which are both surrounded by tegument proteins. The virion envelope is derived from the Golgi membranes and is embedded with viral glycoproteins that assist in viral entry and assembly.

Upon entry to the nucleus, viral genes are expressed in a highly regulated cascade in order to instigate viral genome replication and production of viral proteins . Based on expression kinetics during productive infection, there are three groups in which the HCMV genes are classified (25). The first genes that are expressed during a productive HCMV infection are the immediate early (IE) genes, which are expressed immediately upon infection and are key in trans-activating various classes of genes (28) in addition to inhibiting apoptosis by blocking innate defenses to infection, such as p53(43); these are followed by the early (E) genes, which are necessary to replicate viral DNA and late genes, and the late genes, which are vital in mediating virion assembly (25). Though the process of virion morphogenesis and maturation are not well-understood, it is known that the tegument proteins and other viral nonstructural

proteins are involved in a two-step envelopment and egress process after the nucleocapsids have been packaged with the replicated DNA (9). The virions are then released from the plasma membrane via exocytosis (25).

HCMV Latency

During latency, a state of infection with little or no viral gene expression, HCMV is able to persist long-term within its host without viral replication, but is still able to reactivate and resume replication if presented with a stimulus (25). Although the actual mechanisms of HCMV latency are poorly understood, extensive research is being done in order to further understand latency, viral mechanisms and the cellular reservoir of HCMV. HCMV infections have typically been studied in fibroblasts, though this only provides insight into productive infection, as fibroblasts cannot support a latent infection. Analysis of HCMV infection in primary human cell types relevant to viral latency is underrepresented and important in defining mechanisms of latency. A considerable amount of work has been done in order to study HCMV latent infections, leading our laboratory to develop a model in primitive hematopoetic progenitor cells that is phenotypically similar to conditions that occur during latency.

In early research, latent viral genomes were detected in infected monocytes (34), in addition to granulocyte-macrophage progenitor cells following infection (19, 20). HCMV DNA was also shown to reside in the CD14+ monocyte fraction in the peripheral blood of seropositive subjects (39). It has also been found that endogenous HCMV has also been detected in CD34+ bone marrow progenitor cells (24). From these previous studies, it appears that cell-type specific differences play a role in the latent infection.

Furthermore, in the absence of immediate early gene expression, monocytes containing viral genomes were able to be reactivated in order to produce lytic gene expression, and thus the release of infectious virus (29, 35, 36, 40). These results have allowed latency models to be developed using CD34+ and CD14+ cells that are derived from cord blood, fetal liver, and bone marrow.

Much of HCMV research, until quite recently, has used lab adapted viral strains that undergo serial passage in fibroblasts; this results in those strains being non-pathogenic and varies genetically in comparison to the wild-type clinically isolates of HCMV. In comparing the sequences of clinical strains to the laboratory adapted strains, clinical strains of HCMV contain a unique 15-kb region of the genome, called the ULb' region, which contains ORFs UL133 through UL152, that is necessary in establishing HCMV latency(5). Upon further research, individual ORFs within the ULb' region important to latency were identified(13). Specifically, recombinant viruses lacking UL138 failed to establish a latent infection in CD34+ HPC that were infected in vitro. Therefore, it is suggested that UL138 is necessary for the latent infection, yet is not sufficient for latency, since other viral factors are likely to contribute.

The UL133-UL138 Locus

It was previously discovered that pUL138 is encoded on three 3' coterminal transcripts of 3.6-, 2.7-, and 1.4-kb that are produced during both productive and latent infection. These transcripts are polycistronic, encoding three upstream proteins: pUL133, pUL135, and pUL136, in addition to pUL138 (14) (Figure 2). While pUL138 expression is expressed from each transcript, it is encoded at the most 3' end of each

transcript, presenting a challenge for translation using canonical mechanisms. An IRES (internal ribosomal entry site) upstream of UL138 was identified that allows translation of pUL138 by alternative mechanisms. The IRES is stress-inducible, indicating that the polycistronic transcripts of varying lengths could signify a viral mechanism that ensures pUL138 expression(14). Because the transcripts encode multiple proteins, the locus may coordinate expression of these proteins. pUL133, pUL135, pUL136, and pUL138 have not been previously characterized since, as ULb' proteins, they are dispensable for replication in standard fibroblast culture models. pUL138, pUL133, pUL135, and pUL136 all localize to the Golgi apparatus and are integral membrane proteins, possessing N-terminal transmembrane domains that orients the C-terminal domain on the cytosolic side of Golgi membranes (27, 41).

Viruses lacking the entire UL133-UL138 locus replicates like the wild-type virus in fibroblasts, which is expected given that the locus is dispensable for replication in fibroblasts. Considering this, it was unexpected to find that viruses lacking UL135 exhibited a severe growth defect (unpublished results, Umashankar, Rak, and Goodrum). This suggests that UL135 promotes virus replication, while other genes in the locus effectively suppress viral replication in its absence. Yet, disruption of pUL138, but not pUL133 or pUL136, restored replication of the UL135-null virus. These findings suggest that the UL133-UL138 locus encodes for proteins that could possess opposing functions that can positively or negatively modulate HCMV replication, depending on the context (Figure 2).

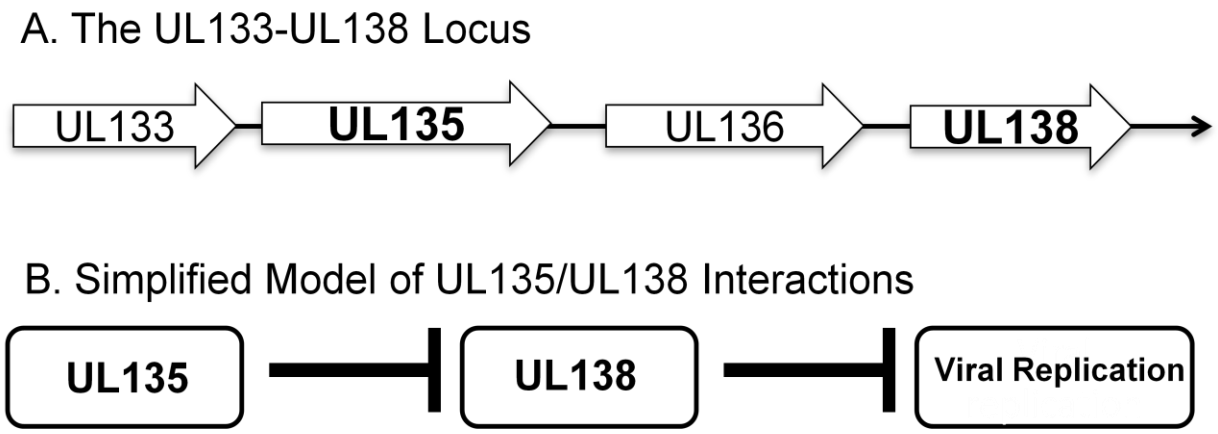

Figure 2. **The UL133-UL138 Locus.** A. The UL133-UL138 locus is a polycistronic locus that encodes four proteins that are expressed differentially on three transcripts. B. Through unknown interactions with cellular proteins, UL135 promotes reactivation or prevents latency by opposing the function of UL138, which is a known latency determinant.

In viewing the exogenous expression of each of the genes in the UL133-UL138 locus outside the context of infection in fibroblasts, exogenous expression of UL135 results in apoptosis; this, in conjunction with the fact that UL135 is required for replication has raised considerable interest in the function of UL135 and the mechanism by which UL135 is causing apoptosis. Preliminary results show that in the presence of both UL135 and caspase inhibitors, the apoptosis phenotype is lost, which has created a primary objective of confirming the presence of caspases (unpublished results, Umashankar, Rak, and Goodrum).

Given that many viral proteins function through their interactions with cellular proteins, a yeast-two hybrid screen, using UL135 as bait, was used to identify cellular interactions that may provide clues as to how pUL135 functions in infection. We identified the Ableson interacting protein 1 (Abi-1) as a prominent interactor (43 of 62 total clones) (unpublished results, Umashankar, Rak, and Goodrum). Abi-1 is a cellular protein that contains multiple polyproline motifs (PxxP, including an src homology 3

(SH3) domain, which is key in binding to Abl proteins, since loss of the SH3 domain results in the loss of binding activity. The SH3 domain is also important in the formation of an epidermal growth factor-inducible complex, in addition to interacting with SOS1, EPS8, Ras, and Rac (10, 31, 32, 38). The SH3 domain proves to be an intriguing characteristic of Abi-1, since many of the aforementioned interactions appear to exist due to the SH3 domain as a binding site. Furthermore, UL135 possesses five SH3 domain binding sites, including an Abi-1 consensus SH3 binding site (Figure 3). Due to this possibility, the main objective is to characterize and understand what exact motifs are playing vital roles in the apoptosis phenotype. This idea that the expression of UL135 kills cells and interacts with Abi-1 presents the possibility that there could be a balancing interaction between UL135 and UL138 that results in UL135 overcoming suppression or promoting replication when UL138 is being expressed.

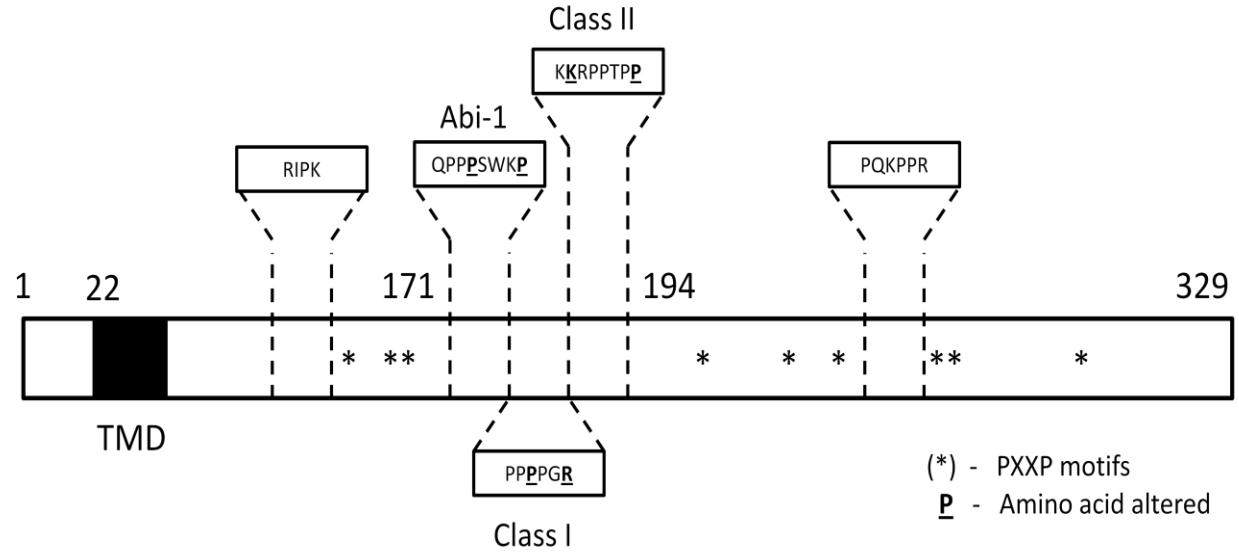

Figure 3. **Map of UL135 SH3 domain binding sites.** UL135 contains many polyproline PxxP motifs throughout its amino acid sequence; there are five consensus SH3 domain binding sites for interaction with SH3 domains of other proteins. The SH3 domain binding sites of interest are depicted above.

Materials and Methods

Cells

Human embryonic lung fibroblasts (MRC5) and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES, 1mM sodium pyruvate, 2mM L-glutamine, 0.1 mM non-essential amino acids, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were cultured at 37°C with 5% CO_2. During the UL135 apoptosis pathway experiment, cells were treated with varying levels of etoposide (50 µM and 100 µM) and pulsed for three hours and recovered after 72 hours in order to induce apoptosis in the cells while being measured alongside cells treated with UL135 simultaneously.

Plasmids

All UL135 mutants were generated using Phusion mutagenesis in order to alter an Abi-1-SH3 consensus binding site, in addition to the Class I and Class II SH3 binding sites. All primers used in the mutagenesis are listed in Table 1. Primers were designed to knockout individual SH3 binding domains in addition to multiple combinations of the binding domains. Reaction mixtures were prepared in 25µl volumes. Each reaction mixture contained 14 µL water, 5.0 µL of 5X buffer, 0.5 µL of deoxyribonucleotide triphosphates, 0.5 µL of each primer, 1.5 µL of DMSO, 2.5 µL of Betaine, 0.25 µL of Phusion polymerase, and 1.0 µL of the template UL135 DNA. PCR cycling conditions were as follows: 1 cycle of 98°C for :30 seconds (denaturation), 25 cycles of 98°C for :15 seconds (denaturation), 25 cycles of 72°C for 2:30 min (annealing), 25 cycles of 72°C for 3:00 min (extension), and finally 1 cycle of 72°Cfor 7:00 min (final extension),

and then cooled to 10°C. The products were visualized using a 1% agarose gel and ran alongside a 1-kb ladder (New England Biolabs) as the standard.

Table 1. Primers used to clone UL135 mutants

Primer Number	Primer sequence (5' to 3')
1	TGCCTTCCAGGATGCCGGCGGCTGATGCAGTACCGTGTCG
2	CCTCCGCCGCCCGGGCG
3	TGGCTTCCAGGATGGCGGCGGC
4	CCTCCGGCGCCCGGGGCCAAGAAGCGGCCGCCTACGCCGCC
5	CCTCCGCCGCCCGGGCGCAAGAGGCGGCCGCCTACGCCGGCGGTCCG GGCCCCCACCACG
6	CCTCCGGCGCCCGGGGCCAAGAGGCGGCCGCCTACGCCGGCGGTCC GGGCCCCCACCACG

Immunoblotting

Protein lysates (30 µg of protein per lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to 0.45 µm PVDF membranes (Immobilon-FL, Millipore, Billerica, MA). Membranes were blocked in Tris-buffered saline (TBS) (25mM Tris [pH 8.0], 137mM NaCl, 3 mM KCL, 1.5 mM $MgCl_2$, pH 8.0) plus 5% nonfat dry milk and 2.5 mg/ml BSA overnight at 4ºC. Blots were then washed three times with TBS-BT for 10 minutes each**.** Blots were incubated with the Cleaved PARP (Asp214) primary antibody in TBS supplemented with 5% BSA and

0.1% Tween-20 (TBS-BT) overnight at 4ºC at a concentration of 1:750. Blots were then washed three times with TBS-BT for 10 minutes each; blots were then incubated with a goat anti-rabbit (H+L) DyLight 800-conjugated secondary antibodies (Pierce) at a concentration of 1:12,000 for ninety minutes. Blots were again washed three times with TBS-BT and then visualized using the Odyssey infrared imaging system (Li-Cor, Lincoln, NE).

Results

UL135 SH3 Binding Site Mutants.

We hypothesized that the SH3 domain binding sites are important in analyzing the interactions between UL135 and other cellular interactors, including Abi-1. Using Phusion site-directed mutagenesis, we created mutants of UL135 by disrupting the SH3 domain binding sites; we specifically converted prolines, arganines, and lysines capable of disrupting the binding sites into alanines. These specific disruptions abrogate the docking characteristics of the PXXP domains, causing 90% of the binding activity to be decreased (16). By mutating each of the SH3 domain binding sites of interest, the Abi-1 consensus, Class I, and Class II domains, we utilized the Phusion mutagenesis in order to create knock-outs for each SH3 binding domain independently, and then collectively as well (Figure 4).

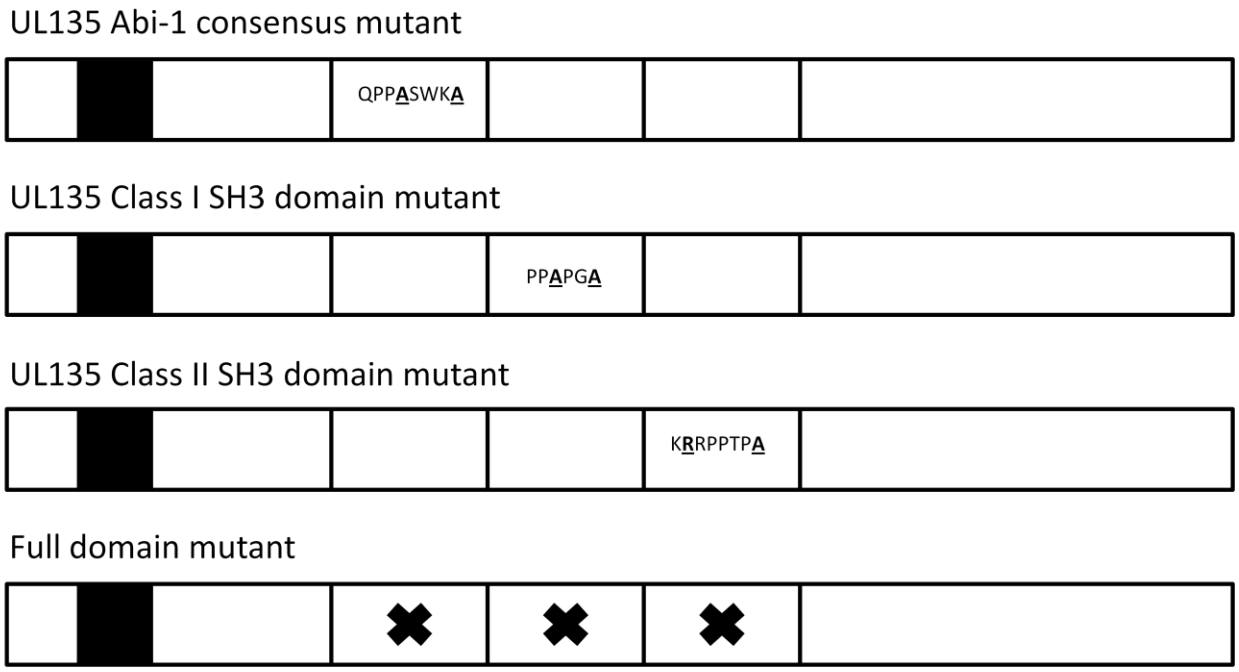

Figure 4. **UL135 SH3 domain binding site mutants**. Each mutant was created in order to view the effect of the knockout in each SH3 domain binding sites of interest. The above mutants were created in order to determine the role of these sites and their cognate interactions in pUL135 function.

Caspase PARP Antibody Optimization

Work in our laboratory demonstrated that transient expression of pUL135 outside the context of infection induce apoptosis, which could be inhibited by a pan caspase inhibitor (M. rak, unpublished results). Apoptosis can be induced through both extrinsic and intrinsic pathways; extrinsic pathways begin outside of the cell from factors such as hormones or toxins that must physically cross the membrane or transduce in order to induce apoptosis, and intrinsic pathways begin when the cell undergoes internal injury from stress such as DNA damage or oncogenes. To determine the pathway of cell death induced by pUL135, we sought to analyze PARP cleavage outside of the context of infection. Optimization of the PARP antibody has currently been completed, showing

bold expression of PARP in HeLa cells under varying conditions of apoptosis induction with the use of etoposide (Figure 5). Future experiments will analyze the expression of PARP during UL135 transduction in fibroblasts.

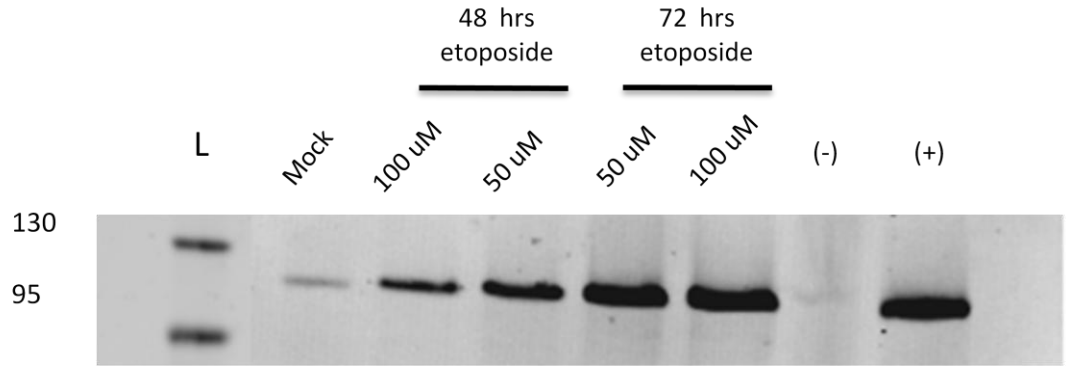

Figure 5. **Optimization of Cleaved PARP Antibody.** HeLa cells were pulsed with differing levels of etoposide for varied amounts of time in order to confirm the presence of the cleaved PARP protein and to determine level of expression from cells.

Discussion

HCMV is a ubiquitous herpesvirus that persists indefinitely in the host through a latent infection that is poorly understood. Our laboratory discovered a novel genetic locus in the ULb' region in the virus that encodes four proteins from a polycistronic locus: UL133, UL135, UL136, and UL138. While some of these proteins, namely UL133 and UL138, function to suppress viral replication from latency, UL135 functions to promote virus replication, potentially contributing to reactivation. This locus coordinates the activity of both positive- and negative-acting regulators of HCMV replication, and it may represent a molecular switch modulating entry into and exit out of latency. My work focused on the function of UL135 and the mechanism by which it causes apoptosis in fibroblasts.

From our preliminary studies, UL135 has the potential to function as a key factor in reactivation from latency, possibly by opposing the action of UL138. We hypothesize that UL135 functions through its interactions with cellular proteins and that the SH3 domain binding sites are critical for these interactions. In support of this hypothesis, infected cell protein 0 (ICP0) in herpes simplex virus type 1 (HSV1) is important for reactivation from latency and contains several SH3 domain binding sites that are important to its function (21). SH3 domain binding sites are important to protein function because they allow interaction with proteins typically involved in various cellular signaling pathways (16). To investigate the function and cellular interactions of UL135, we mutated three SH3 domain binding sites within UL135 to understand the roles of these sites and identify which sites are specifically important to the function of UL135 and its apoptotic function. By disrupting the sites at which these proteins dock, we predict that the interactions between UL135 and the proteins that possess the SH3 domains will be down-regulated, which would result in a loss of the apoptotic phenotype.

Preliminary studies with the UL135 SH3 domain binding site mutants have suggested that when UL135 is expressed through lentiviral transduction, the apoptotic phenotype is lost. This indicates that these SH3 domains could indeed be vital to function of UL135. Future studies will include identifying what other proteins are interacting with UL135 and analyzing those interactions when the SH3 domain binding sites are disrupted. Once particular interactions are known, another future study would be to build viruses with the disrupted SH3 domain binding sites in order to analyze the

effect of the mutations during latency, with the prediction that perhaps these viruses would reactivate from latency.

Abelson interacting protein 1 (Abi-1) was identified to interact with UL135 through a yeast two hybrid screen. This interaction was further confirmed through immunoprecipitation. Interestingly, UL135 contains a SH3 domains binding consensus sequence for interaction with Abi-1, which could imply that it is the site at which they interact. Future studies will determine the ability of the UL135 SH3 mutant proteins to interact with Abi-1 and will help determine the role of this interaction during infection.

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