Tegument Protein Subcellular Localization of Human Cytomegalovirus

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Tegument Protein Subcellular Localization of Human Cytomegalovirus

By

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A Proposal Submitted to the Honors Council

For Honors in Biology (Cell Biology/Biochemistry Program)

12 May 2011

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Program Director: Dr. Kathleen Page
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Table of Contents

Abstract...........................................................................................................................................1

Introduction....................................................................................................................................3

Human Cytomegalovirus Significance.........................................................................................3

Human Cytomegalovirus Structure and Life Cycle......................................................................5

Tegument Structure and Function...............................................................................................7

Tegument Protein pp65......................................................................................................................8

Tegument Protein pp71.....................................................................................................................10

Tegument Proteins pp150 and pp28...............................................................................................11

Tegument Protein Subcellular Localization..................................................................................12

Fluorescence Proteins as Localization Tags..................................................................................14

Experimental Outline and Objectives............................................................................................15

Hypothesis......................................................................................................................................17

Materials and Methods..................................................................................................................19

Cell Culture.....................................................................................................................................19

Transfection of Plasmid DNA into Cells.......................................................................................19

HCMV Live-Cell Tegument Protein Transfection......................................................................20

Confocal Microscopy....................................................................................................................21

Results..........................................................................................................................................22

Tegument Protein Subcellular Localization in Paraformaldehyde Fixed Cells with
DAPI Stain..........................................................................................................................................22
Tegument Protein Subcellular Localization in Live-Cell Imaged Cells without a Nuclear Stain.................................................................32

Tegument Protein Subcellular Localization in Live-Cell Imaged Cells with Hoechst 33342 Stain.................................................................37

Discussion........................................................................................................47

Bibliography.....................................................................................................54

Appendix..........................................................................................................61
List of Tables

Table 1 Characterization of the fluorescent molecules used to tag HCMV tegument proteins pp65, pp71, pp150, and pp28…………………………………………………………………………..15
List of Figures

Structure of Human Cytomegalovirus Virion…………………………………………..…6
    Figure 1 A cartoon depicting the structure of the human cytomegalovirus virion…………………………………………………………………………………………6

Tegument Protein Subcellular Localization in Paraformaldehyde Fixed Cells with DAPI Stain……………………………………………………………………………………………25
    Figure 2 Subcellular localization of CFP-pp65, GFP-pp150, and RFP-pp71 in HeLa cells fixed with paraformaldehyde and stained with the nuclear stain DAPI………………………………………………………………………………………………25
    Figure 3 Subcellular localization composite of CFP-pp65, GFP-pp150, and RFP-pp71 in HeLa cells fixed with paraformaldehyde and stained with the nuclear stain DAPI…………………………………………………………………………………………………26
    Figure 4 Subcellular localization of CFP-pp65 and RFP-pp71 in HeLa cells fixed with paraformaldehyde and stained with the nuclear stain DAPI…………………………………………………………………………………………………27
    Figure 5 Subcellular localization composite of CFP-pp65 and RFP-pp71 in HeLa cells fixed with paraformaldehyde and stained with the nuclear stain DAPI…………………………………………………………………………………………………27
    Figure 6 Subcellular localization of CFP-pp65 and GFP-pp150 in HeLa cells fixed with paraformaldehyde and stained with the nuclear stain DAPI…………………………………………………………………………………………………28
Figure 7 Subcellular localization composite of CFP-pp65 and GFP-pp150 in HeLa cells fixed with paraformaldehyde and stained with the nuclear stain DAPI…………………………………………………………………………………………..28

Figure 8 Subcellular localization of GFP-pp150 and RFP-pp71 in HeLa cells fixed with paraformaldehyde and stained with the nuclear stain DAPI…………………………………………………………………………………………..29

Figure 9 Subcellular localization composite of GFP-pp150 and RFP-pp71 in HeLa cells fixed with paraformaldehyde and stained with the nuclear stain DAPI…………………………………………………………………………………………..29

Figure 10 Subcellular localization of CFP-pp65, GFP-pp28, and RFP-pp71 in HeLa cells fixed with paraformaldehyde and stained with the nuclear stain DAPI…………………………………………………………………………………………..30

Figure 11 Subcellular localization composite of CFP-pp65, GFP-pp28, and RFP-pp71 in HeLa cells fixed with paraformaldehyde and stained with the nuclear stain DAPI…………………………………………………………………………………………..31

Tegument Protein Subcellular Localization in Live-Cell Imaged Cells without a Nuclear Stain………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………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Figure 14 Subcellular localization of CFP-pp65, GFP-pp150, and RFP-pp71 when transfected individually in HeLa cells using live-cell imaging with no nuclear stain.................................................................36

Figure 15 Subcellular localization of GFP-pp28 in HeLa cells using live-cell imaging with no nuclear stain.........................................................36

Tegument Protein Subcellular Localization in Live-Cell Imaged Cells with Hoechst 33342 Stain.........................................................................................39

Figure 16 Subcellular localization of CFP-pp65, GFP-pp150, and RFP-pp71 in HeLa cells using live-cell imaging stained with the nuclear stain Hoechst 33342.................................................................39

Figure 17 Subcellular localization composite of CFP-pp65, GFP-pp150, and RFP-pp71 in HeLa cells using live-cell imaging stained with the nuclear stain Hoechst 33342.........................................................40

Figure 18 Subcellular localization of CFP-pp65, GFP-pp150, and RFP-pp71 in HeLa cells using live-cell imaging stained with the nuclear stain Hoechst 33342.................................................................41

Figure 19 Subcellular localization composite of CFP-pp65, GFP-pp150, and RFP-pp71 in HeLa cells using live-cell imaging stained with the nuclear stain Hoechst 33342.........................................................42

Figure 20 Subcellular localization of RFP-pp71 in HeLa cells using live-cell imaging stained with the nuclear stain Hoechst 33342.................................43
Figure 21 Subcellular localization composite of RFP-pp71 in HeLa cells using live-cell imaging stained with the nuclear stain Hoechst 33342.

Figure 22 Subcellular localization of CFP-pp65 in HeLa cells using live-cell imaging stained with the nuclear stain Hoechst 33342.

Figure 23 Subcellular localization composite of CFP-pp65 in HeLa cells using live-cell imaging stained with the nuclear stain Hoechst 33342.

Figure 24 Subcellular localization of GFP-pp150 in HeLa cells using live-cell imaging stained with the nuclear stain Hoechst 33342.

Figure 25 Subcellular localization composite of GFP-pp150 in HeLa cells using live-cell imaging stained with the nuclear stain Hoechst 33342.

Figure 26 Subcellular localization of GFP-pp28 in HeLa cells using live-cell imaging stained with the nuclear stain Hoechst 33342.

Figure 27 Subcellular localization composite of GFP-pp28 in HeLa cells using live-cell imaging stained with the nuclear stain Hoechst 33342.
Abstract

Human cytomegalovirus (HCMV), a member of the Betaherpesvirinae sub-family of Herpesviridae family, is a widespread pathogen that infects a majority of the world’s population by early adulthood. In individuals whose immune systems are immature or weakened, HCMV is a significant pathogen causing morbidity and mortality. There is no effective vaccine and only limited antiviral treatments against HCMV infection to date. A possible target for novel antiviral treatments is the HCMV proteins that localize to the tegument of the virion, since they play important roles in all stages of the viral life cycle, including, viral entry, gene expression, immune evasion, assembly, and egress. The most likely tegument protein candidates are pp65 (immune evasion), pp71 (gene expression), and pp150 and pp28 (assembly and egress). Although the subcellular localization of these proteins has been identified during HCMV infections in vitro, their localization patterns have not been elucidated when each protein is expressed individually in living cells.

The objective of this research was to determine the subcellular localization of the tegument proteins pp65, pp71, pp150, and pp28 as fusions to one of several fluorescent proteins. Since these tegument proteins play pivotal roles in several stages of the viral life cycle, knowledge of where and the mechanism of how these proteins localize upon release could result in a better understanding of their function during a lytic infection as well as assist in the development of an effective, novel antiviral treatment. The localization patterns were determined via fluorescent confocal microscopy in paraformaldehyde fixed cell and live-cell imaging experiments after transfection of the
fusion protein plasmids. It was shown that the localization of each tegument protein was independent of each other, and that live-cell imaging experiments gave better results than fixed cell imaging experiments. Furthermore, the subcellular localization of pp71 and pp150 (nucleus), and pp28 (cytoplasm) were identical to what occurs in a typical HCMV lytic infection, suggesting a strong correlation between localization and function.

However, pp65, the major tegument protein involved in immune evasion, did not show its characteristic nuclear localization pattern as reported previously. Instead it has been seen to localize to the cytoplasm of the cell. This is interesting for two reasons. One is that the localization patterns of the tegument proteins are highly correlated with their function during the lytic cycle of HCMV infections. If pp65 doesn’t localize to the nucleus, the lytic cycle will not develop. The second is that pp65 is known to have a bipartite nuclear localization signal. Therefore, it was hypothesized that there is another molecule in the HCMV virion necessary to localize pp65 into the nucleus. Potentially, the nuclear localization signal of pp65 is within an inaccessible region to nuclear transport molecules. A mechanism could involve the other molecule within the HCMV virion binding to pp65, inducing a conformational change that results in the nuclear localization signal becoming accessible and recognizable to nuclear transport molecules and its subsequent localization to the nucleus. Thus, a novel antiviral treatment could target the other molecule in the HCMV virion, and prevent pp65 from getting into the nucleus; subsequently inhibiting the HCMV lytic infection. This research has increased our understanding of HCMV lytic infections as well as identifies a potential target for a novel, antiviral treatment.
Introduction

*Human Cytomegalovirus Significance*

Human cytomegalovirus (HCMV) is a member of the *Betaherpesvirinae* subfamily of *Herpesviridae*. It is a widespread pathogen that infects a majority of the world’s population by early adulthood (1). In fact, by the age of 40, between 50 and 85% of adults are infected by HCMV (2). The virus establishes a life-long infection with some cells being latently infected, a state where the virus has the ability to lie dormant within a cell, while others are persistently infected, where the infection cannot be cleared from an organism and there is intermittent shedding of infectious virions (3). Immunocompetent individuals, who can develop a strong immune response, typically display no symptoms of infection (4). However, in individuals whose immune systems are immature or weakened, such as organ transplant and AIDS patients, HCMV is a significant pathogen causing morbidity and mortality (5). Symptoms in these individuals typically consist of spiking fever, leucopenia (decrease in white blood cells), malaise, hepatitis, pneumonia, gastrointestinal disease and/or retinitis (inflammation of the retina) (4). HCMV is also responsible for approximately 8% of infectious mononucleosis cases (6) and is the leading viral cause of birth defects often causing deafness and mental retardation in the fetus if a woman is infected during pregnancy (7).

HCMV has been implicated in playing a role in inflammatory and proliferative diseases, including certain cardiovascular diseases and cancer (8). Epidemiological and
pathological studies have espoused a strong link between HCMV and atherosclerosis (8). Several mechanisms have been proposed in which HCMV could influence the development of atherosclerotic vascular abnormalities (9). A proposed role of HCMV in the pathogenesis of atherosclerosis involves the reactivation of a latent HCMV infection followed by virus-induced enhancement of vascular inflammation and damage through smooth cell proliferation, uptake of low-density lipoproteins by smooth cells, neointimal formation (thickened arterial layer via cell migration and proliferation), and narrowing of the vessel lumen (9).

There is no effective vaccine against HCMV, and drugs that inhibit viral replication exist but are ineffective due to high toxicity, low bioavailability, and the development of drug-resistant virus strains (8,10). The primary antiviral agents used to treat HCMV infections in patients with an impaired immune system are ganciclovir, foscarnet, and cidofovir (9). These drugs have improved the survival and quality of life of immunocompromised individuals suffering from HCMV, but they are far from ideal due to major hematologic, renal, and neutropenia toxicity (9). Therefore, the lack of an effective treatment for HCMV infections, especially in immunocompromised individuals, has resulted in intense study for the identification of proteins and processes that could be targeted by novel antiviral treatments (8).
Human Cytomegalovirus Structure and Life Cycle

HCMV has the prototypical herpesvirus virion structure (Figure 1) and the replication cycle has a well controlled cascade of gene expression (9). The virion has an icosahedral protein capsid that contains the 235-kb double-stranded DNA. The capsid is surrounded by a proteinaceous tegument and an outer lipid envelope (1). Virions gain entry into a cell through a membrane fusion event involving the outer membrane of the cell and glycoproteins on the lipid envelope of virions. Once the fusion of these two membranes occurs, the DNA-containing protein capsid and the tegument proteins are released into the cell (11).

The gene expression pattern follows a similar cascade as used by other herpesviruses as reviewed in Kalejta (2008) (12). During the lytic infection, viral immediate-early genes are expressed (8). The expression of these genes results in the production of viral immediate-early proteins that modulate the host cell environment and stimulate the expression of viral early genes (1). The viral-immediate early genes produce proteins that are responsible for replicating the double-stranded viral genomic DNA; after DNA replication, these immediate-early genes turn on the expression of viral late genes (1). The viral late proteins are mainly structural components of the virion that assist in the assembly and egress of newly formed viral particles (1). Immediate-early genes in HCMV can be silenced in certain cell types upon infection though, which results in a latent infection (13). A latent infection is characterized by the minimization of viral gene expression and the inhibition of the assembly and egress of new viral progeny (11).
Latent infections can reactivate into a lytic infection upon certain environmental cues, which causes disease and allows viral spread (1, 12).

Figure 1 A cartoon depicting the structure of the HCMV virion. (Image obtained from (http://www.virology.net/big_virology/bvdnaherpes.html) courtesy of Dr. Marko Reschke in Marburg, Germany).

HCMV infected cells also produce noninfectious enveloped particles and dense bodies in addition to infectious virions as reviewed in Kalejta (2008) (12). Noninfectious enveloped particles are similar to infectious virions in that they contain a nearly an identical assortment of envelope, tegument, and capsid proteins, but they lack the double-stranded viral genome within the icosahedral capsid (14). Dense bodies on the other hand are enveloped tegument proteins that lack capsids and are primarily composed of
the viral pp65 protein (14). The significance of the noninfectious enveloped particles and dense bodies is not known in wild-type strains of HCMV (14).

*Tegument Structure and Function*

A possible target for novel antiviral treatments is the HCMV proteins that localize to the tegument. As mentioned, the tegument in HCMV is located between the outer lipid membrane and the icosahedral protein capsid, which contains the viral genomic double-stranded DNA (11). The tegument is generally thought to be unstructured and amorphous in nature although some structuring is seen with the binding of tegument proteins to the protein capsid (15). The tegument proteins comprise more than half of the total proteins found within infectious virions (16). Tegument proteins are phosphorylated, but the significance of this and other posttranslational modifications to these proteins remains largely unexplored (11). A common sequence to direct proteins into the tegument has not been identified through either experimental or bioinformatic approaches (17). The process of assembling the viral tegument upon viral egress and disassembly upon viral entry into cells is largely unknown (17). However as reviewed in Kalejta (2008), phosphorylation, subcellular localization to the assembly site, and interaction with capsids or the cytoplasmic tails of envelope proteins, likely facilitate the incorporation of proteins into the HCMV tegument (12).

As mentioned above, virions gain entry into a cell through a membrane fusion event involving the outer membrane of the cell and the lipid membrane of virions. The
entry of the tegument proteins as well as the DNA-containing protein capsid upon viral entry occurs after the fusion of these two membranes (11). Upon release into the cytoplasm, tegument proteins become functionally active, where they play important roles in all stages of the viral life cycle, including, viral entry, gene expression, immune evasion, assembly, and egress (1, 11). There are several tegument proteins that are of particular interest due to the role (elucidated below) that they play in the HCMV replication cycle, including pp65, pp71, pp150, and pp28.

Tegument Protein pp65

Pp65 is the most abundant tegument protein and the major constituent of extracellular virus particles (18). However, pp65 is not essential for the production of new infectious virus particles as evidenced in strains that lack the pp65 gene (18) which can still replicate in culture. Pp65 is the major tegument protein responsible for modulating/evading the host cell immune response during HCMV infections (19). As reviewed in Kalejta (2008), pp65 is implicated in counteracting both innate and adaptive immune responses during HCMV infections (12). pp65’s role in immune evasion is largely attributable to its targeting of both humoral and cellular immunity as well as serving as the dominant target antigen of cytotoxic T lymphocytes (19). It has been demonstrated that pp65 not only prevents immediate-early proteins from being recognized by components of the immune system, but it also inhibits the synthesis of the various components involved in the host cell’s immune response (20). One of the ways
in which pp65 counteracts adaptive immunity is through its enzymatic kinase activity (21). It was shown that pp65 mediates the phosphorylation of viral immediate-early proteins, which blocks their presentation to the major histocompatibility complex class I molecules (22). The kinase activity of pp65 has also been implicated in causing the degradation of the alpha chain in the major histocompatibility class II cell surface receptor, HLA-DR, via an accumulation of HLA class II molecules in the lysosome (20). Furthermore, several studies have presented evidence that pp65 is involved in mediating a decrease in the expression of major histocompatibility complex class II molecules (20). This is significant in that major histocompatibility complex class I and II molecules are responsible for lymphocyte recognition and antigen presentation with class I molecules presenting to cytotoxic T lymphocytes CD8+ and class II molecules to helper T lymphocytes CD4+ (20).

Another pivotal role that pp65 has in immune evasion during HCMV infections is through the inhibition of natural killer cell cytotoxicity (23). Specifically, it was shown that pp65 can act as an antagonistic ligand that can bind to the NKp30 activating receptor to protect the killing of infected cells as well as interfere with the ability of NKp30 to cross-talk between other natural killer cells and dendritic cells (23,24).

Finally, pp65 has been shown to attenuate the interferon response (25). It is thought that pp65 is involved in down modulating beta interferon and a number of chemokines, which is based on the observation of an elevated expression of interferon genes in infections with a strain lacking the pp65 protein (25).
Thus, the role that pp65 has in immune evasion in HCMV infections is to prevent infected cells from being destroyed by the immune system. Furthermore, it has been shown to protect infected cells from the immune response by binding to components of the immune system, thereby inhibiting their activation (24).

_Tegument Protein pp71_

Pp71, by comparison, plays an important role in the activation of immediate-early gene expression at the start of the lytic replication cycle (26). Although this protein is not absolutely essential, it is necessary for efficient viral replication as reviewed in Kalejta (2008) (12). A proposed mechanism for how pp71 activates viral gene expression is by neutralizing the effects of the cellular Daxx protein, which is recruited to promoters by DNA-binding transcription factors, resulting in the repression of transcription (27). Pp71 can bind to two inherent domains on Daxx and induce its proteasomal degradation (28). Additionally, it was demonstrated that pp71 increases the infectivity of viral genomic DNA when transfected into cultured cells (29).

Recently though, pp71 has also been implicated in immune evasion, similar to pp65, by disrupting the major cell surface expression of components of the immune response (30). Specifically, pp71 appears to target the cell surface receptors of major histocompatibility complex class I proteins by slowing their intracellular transport (30). This limits the ability of infected cells to display viral antigens to the immune system and prevents recognition by cytotoxic T lymphocytes.
Tegument Proteins pp150 and pp28

Pp150 and pp28 are highly immunogenic and play roles in the assembly and egress of virus particles. Both of these tegument proteins play very similar roles, but have some distinct functions. pp150, the second most abundant tegument protein behind pp65, is necessary to incorporate nucleocapsids into virus particles (16). Pp150 is essential for maintaining the stability of the cytoplasmic capsids and directing their movement (1,31).

Pp150 also plays a role in the reorganization of the cytoplasmic assembly compartment during virion assembly (31). The process of virion assembly in HCMV has been reviewed in Kalejta (2008) (12). After the viral genome and late genes are expressed, capsid formation and DNA packaging into the preformed capsids begins to occur in the nucleus. Capsids acquire a primary envelope when they bud through the inner nuclear membrane into the perinuclear space, which they lose upon budding through the outer nuclear membrane into the cytoplasm. The capsids then bud into Golgi apparatus-derived vesicles and obtain their final envelope. When these vesicles fuse with the cell membrane, the enveloped virion is released. Pp28 is largely responsible for the cytoplasmic envelopment of tegument proteins and capsids in HCMV during the assembly and egress process (12, 32).
As illustrated, the tegument proteins play important roles in all stages of the viral life cycle, including viral entry, gene expression, immune evasion, assembly, and egress (11). However, the subcellular localization of the tegument proteins after their release into the cytoplasm at the beginning stage of infection has not been fully elucidated. This is especially true for the localization of the tegument proteins when they are expressed individually or in combinations without the rest of the HCMV virion. Furthermore, the structure of the tegument itself is not known, and the process of assembling the tegument upon viral egress, as well as the disassembly of the tegument upon viral entry into cells, is poorly understood (17). Therefore, the objective of this research was to determine the subcellular localization of the primary tegument proteins pp65, pp71, pp150, and pp28, after transfection of plasmid DNA expressing each protein as a fluorescent protein fusion. Since these tegument proteins play pivotal roles in several stages of the viral life cycle, knowledge of where and the mechanism of how these proteins localize upon release could be fundamental in the development of effective, novel antiviral treatments for this widespread human pathogen, which would have great therapeutic value for immunocompromised individuals.

When HCMV virions fuse with the membrane of host cells, some tegument proteins remain in the cytoplasm, while others migrate to the nucleus of the cell (33). Other tegument proteins will remain tightly associated to the nucleocapsids, and mediate their delivery to the nuclear pore complex via the microtubule assembly as reviewed in
Several tegument proteins though will have a specific localization within the cell depending on the stage of the lytic cycle.

In the early stage of infection, pp65 tends to independently migrate to the nucleoli of the cell (34). The localization of pp65 to the nucleoli suggests a functional relationship between the localization of pp65 and the development of the lytic cycle of HCMV (34). However, pp65 begins to migrate to the cytoplasm 48 hours into the lytic cycle with nuclear pore complex becoming devoid of the protein (35). This migration appears to be mediated by cyclin-dependent kinase activity and a Crm1 exporter (36).

pp71, by comparison, has a similar localization pattern as pp65 during the lytic cycle of HCMV infections. The subcellular localization of pp71 upon viral entry is to the nucleus of the host cell (33). This localization appears to be essential for the initiation of either a lytic or latent infection (37). During the later stages of infection, some pp71 appears to localize subcellularly to both the nucleus and the cytoplasm (38).

Unlike the subcellular localization of pp65 and pp71, which appears to be dependent on the stage of the lytic life cycle of HCMV, the localization of pp150 has not been well-defined. Some studies have suggested that the subcellular localization of pp150 initially when it associates with the viral nucleocapsids is to the nucleus, while other studies suggest that it is to the cytoplasm (39,40). The subcellular localization of pp28 on the other hand appears to be to specific cytoplasmic compartments (41). This localization appears to be essential for the production of viral progeny, since it is localized at the site of final envelopment (41).
Fluorescent Proteins as Localization Tags

The development of fluorescent protein molecules to act as localization tags for subcellular components has revolutionized the biomedical sciences (42). The first instance occurred when *Aequorea victoria* jellyfish wild-type green fluorescent protein (GFP) was used to highlight sensory neurons in the nematode (43). Fluorescence is such a powerful tool in that it allows one to distinguish and identify cellular components that are either too small or lack little contrast with the background with traditional microscopy techniques. The wild-type green fluorescent protein molecule was modified to yield different variants due to its complex emission spectrum (44). Nonetheless, extensive research has occurred in recent years to produce new and improved fluorescent tags that are brighter, cover a broad spectral range, and also exhibit enhanced photostability, reduced oligomerization, pH insensitivity, and faster maturation rates (42).

Due to the success of various fluorescent protein molecules being used as tags to assess the subcellular localization and of many cellular and viral proteins, several distinct fluorescent protein molecules were utilized in this experiment to identify the subcellular localization of HCMV tegument proteins pp65, pp71, pp150, and pp28, respectively (44). The fluorescent tegument protein fusions were constructed previously by inserting the open reading frame of each tegument protein into a plasmid containing a fluorescence gene so that the two protein coding regions are in-frame and produce a fusion protein (Pizzorno et al, unpublished data). After the plasmid and tegument proteins undergo restriction digests, the resultant sticky ends fuse to form the fluorescently labeled
tegument protein plasmid. The fluorescent tegument protein fusions are identified by transforming them into bacterial cells on a plate containing a specific antibiotic. Since the plasmid contains the antibiotic resistance gene, the colonies that are transformed with the fusion plasmid are able to survive on the plate. The fusion plasmids are then isolated from the bacterial colonies that were transformed.

In total, three variant fluorescent proteins (Cyan-blue, RFP (DsRed2)-red, GFP-green) were used to tag and label the tegument proteins. Pp65 was tagged with Cyan, pp71 with RFP, and pp150 and pp28 with GFP. The characterization of each fluorescent protein molecule can be seen in Table 1. Additionally, the location of the fluorescent tags on each protein can be seen in the appendix.

Table 1 Characterization of the fluorescent molecules used to tag HCMV tegument proteins pp65, pp71, pp150, and pp28.

<table>
<thead>
<tr>
<th>Fluorescent Molecule</th>
<th>Tegument Protein(s) Labeled</th>
<th>Excitation Peak (nm)</th>
<th>Emission Peak (nm)</th>
<th>Brightness</th>
<th>Photostability</th>
<th>pKa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyan</td>
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*Experimental Outline and Objectives*

As mentioned, the objectives of this study were to identify the subcellular localization of HCMV tegument proteins pp65, pp71, pp150, and pp28 to understand
how these proteins interact with the cell and function during the lytic cycle of HCMV infections. Additionally, identification of the HCMV tegument protein localization patterns could assist in the development of a better targeted effective, novel antiviral treatment of HCMV infections, especially in immunocompromised individuals. This was accomplished through the use of cell culture and microscopy techniques. The tegument proteins of interest were first fused to a fluorescence protein (Cyan-blue, RFP-red, GFP-green), as described to in the previous section, that will emit/fluoresce when exposed to a certain wavelength of light. The DNA that encodes these modified tegument proteins were then transfected into an established human cell lines (HeLa cells). Confocal microscopy, which is an imaging technique that is used to increase the optical resolution and contrast of photographs obtained through a microscope, was then utilized to determine the subcellular localization of each tegument protein within the transfected cells by noting where the Cyan, RFP, or GFP molecule fluoresces to reconstruct three-dimensional structures of the obtained images. Additionally, the images obtained from the fluorescence tags were compared and overlaid with images obtained by concurrently staining with DAPI or Hoechst 33342, which are fluorescent stains that have a high affinity for DNA. Since the DNA is located within the nucleus of cells, this allowed for a definitive identification of where these tegument proteins localize subcellularly in transfected cells.

There were three distinct series of steps in determining the localization of the tegument proteins. The localization of the tegument proteins were first identified in fixed cells that were transfected with plasmids containing the tegument proteins of interest.
Since the localization of each protein may be affected by the presence or absence of other tegument proteins, a series of transfections occurred, including the tegument proteins by themselves and a combination of the different tegument proteins of interest. Since we have three different fluorescence tags, we were able to observe the localization of three tegument proteins in a single cell. The localization patterns in the fixed cells were then compared to the same series of transfections in live cells to acquire more accurate observations of the localization patterns as well as to rule out the possibility that artifacts of the fixation process may have affected the subcellular localization patterns of the tegument proteins.

**Hypothesis**

It was hypothesized that each of the tegument proteins, pp65, pp71, pp150, and pp28, would display subcellular localization patterns similar to what is seen in natural HCMV infections in both fixed and live-cell imaging. Furthermore, it was expected that the subcellular localization of each HCMV tegument protein would have display a strong correlation between its known function during natural HCMV infections. It was hypothesized that pp65 would localize subcellularly to the nucleus as seen in the early stages of a natural HCMV infection. However, it was expected that very little pp65 would be found in the cytoplasm, since only the initial stage of the viral life cycle was mimicked during the transfection procedure. Pp71 was also hypothesized to localize subcellularly to the nucleus as seen in natural HCMV infections due to its necessity in
efficient viral replication. Pp150 on the other was expected to be in either the nucleus, cytoplasm, or both due to its poorly defined localization pattern within natural HCMV infections. Nonetheless, it was felt that pp150 would localize more to the nucleus than the cytoplasm. Finally, it was anticipated that pp28 would localize subcellularly to various cytoplasmic compartments within the cell as seen in natural HCMV infections due to its essential role in the production of viral progeny at the site of final envelopment, which occurs in the cytoplasm of the cell. It was also hypothesized that the localization patterns of some of the tegument proteins could be affected via co-transfection with other tegument proteins, since previous data in the laboratory has suggested that the localization pattern of pp150 is dependent upon whether or not pp71 is present.
Materials and Methods

Cell Culture

HeLa cells were grown in 1x Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 10% fetal calf serum (FCS), 100x Glutamax (Invitrogen), and the antibiotics penicillin and streptomycin (PS). They were maintained by subculturing at 1:20 ratio unless otherwise noted after reaching 80-85% confluency. The cells were incubated at 37°C and 5% carbon dioxide.

Transfection of Plasmid DNA into Cells

Prior to transfection, 5x10^4 HeLa cells were cultured in each well of a 24 well culture vessel containing sterile glass coverslips in 1 mL DMEM/10% FCS/PS(Invitrogen). The HeLa cells were transfected 24 hours later using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s protocol. Tegument protein DNA (0.8 μg) per well was diluted in 50 μL of DMEM without FCS and antibiotic and mixed. The plasmid DNA that was transfected expressed either pp65, pp71, pp150, or pp28 fused to a fluorescent protein. Each plasmid was transfected alone or in combination with one or two additional tegument protein plasmids.

Lipofectamine™ 2000 (2.0 μL) per well was diluted in 50 μL of DMEM without FCS and antibiotic and incubated for 5 minutes at room temperature. After the five
minute incubation period the diluted tegument protein DNA and Lipofectamine™ 2000 were mixed and incubated for 20 minutes at room temperature. Following the 20 minute incubation period, the 100 μL DNA and Lipofectamine™ 2000 complexes were added to each well and mixed. The transfected HeLa cells were then incubated at 37°C with 5% carbon dioxide for 12 hours. After the incubation period, the growth media in each well was changed, which was followed by another 24 hour incubation period.

Following the 24 hour incubation period, the growth media was removed and the cells were washed twice in 1x phosphate buffered saline (PBS). The HeLa cells were then fixed with 4% paraformaldehyde in 1x PBS for 10 minutes at room temperature. The paraformaldehyde was then removed, and the cells were washed three times in 1x Phosphate Buffered Saline Tween-20 (1x PBS, 0.1% Triton-X 100, 0.05% Tween 20). The cells were washed an additional time in 1x PBS. After the wash, the cells were placed in 1x PBS.

The coverslips in each well were removed and dipped three times in dH2O. They were then placed on a slide in a drop of the DAPI (Vector Laboratories) mounting media and stored at room temperature in the dark overnight. The slides were then stored at -20°C until imaged with a Leica SP5 confocal microscope.

**HCMV Live-Cell Tegument Protein Transfection**

Prior to transfection, 5x10⁴ cells were transferred into glass bottom culture dishes with coverslips with a thickness of 0.16-0.19 mm in 1 mL DMEM/10% FCS/PS
(Invitrogen). The HeLa cells were then transfected 24 hours later using Lipofectamine™ 2000 as described above. The transfected HeLa cells were then incubated at 37°C with 5% carbon dioxide for 24 hours.

Following the 24 hour incubation period, 0.9 mL of the HeLa cell growth media was removed. The HeLa cells were then either imaged with a Leica SP5 confocal microscope or incubated with 1 μg/mL of the nuclear stain Hoechst 33342 (Thermo Scientific) for five minutes at room temperature. The cells that were incubated for five minutes with the Hoechst 33342 nuclear stain were then imaged with a Leica SP5 confocal microscope.

Confocal Microscopy

Fixed cell- and live-cell imaging was performed using a Leica SP5 confocal laser scanning microscope. Confocal image scans were processed using the LAS AF software.
Results

Tegument Protein Subcellular Localization in Paraformaldehyde Fixed Cells with DAPI Stain

The subcellular localization of HCMV tegument proteins pp65, pp71, pp150, and pp28 was first determined in HeLa cells that had been fixed with paraformaldehyde. Plasmids expressing various versions of the tegument proteins fused to a fluorescent protein had been constructed previously (pp65-CFP, pp71-RFP, pp150 and pp28-GFP) and the size of the fusion protein has been confirmed using Western blot (M. Pizzorno, personal communication). These expression plasmids were transfected into HeLa cells and the cells were fixed 36 hours post-transfection with 4% paraformaldehyde. The fixed cells were then mounted with a DAPI (nuclear stain) containing mounting medium, which binds to DNA and acts as a nuclear stain, and imaged with confocal microscopy.

The subcellular localization of pp65, pp71, and pp150 when expressed together in HeLa cells that were then fixed with paraformaldehyde can be seen in Figure 2. Based on the location of the nucleus as identified by the DAPI nuclear stain in Figure 2A, it appears that both pp150 and pp71 (Figure 2C/D respectively) appear to localize to the nucleus of the cell. Furthermore, it appears that pp150 and pp71 have very similar if almost identical localization patterns, which could suggest co-localization of the proteins to the nucleus of the cell. Pp65 by comparison displays a unique pattern of subcellular localization in that it has a sparse localization pattern (Figure 2B). It appears that pp65 is
potentially localized to the nucleus although it appears that its localization is just outside
the nucleus in the cytoplasm.

Likewise, the composite image in Figure 3 consisting of the overlaid channels of
Figure 2 supports the initial analysis that pp150 and pp71 appear to localize to the
nucleus. It is more difficult to determine if in fact pp65 localizes to the cytoplasm in the
composite image, since the nuclear signal is very strong, while the pp65 signal is weak
and sparse. Furthermore, the nuclear signal may be confounded by DAPI’s potential
affinity for RNA. However, if one focuses only on the fluorescently labeled tegument
proteins in Figure 2 B-D, pp65 does appear to localize to the cytoplasm of the cell.

This localization pattern of the tegument proteins pp65, pp150, and pp71 when all
three are expressed together (Figures 2 and 3) is supported when HeLa cells were
transfected with two of the three tegument proteins plasmids (Figures 4-9) as well as in
HeLa cells transfected with only a single tegument protein plasmid (data not shown).
This also refutes any possibility of one of the tegument proteins affecting the subcellular
localization of another (See Discussion). It can be seen that pp150 and pp71 definitively
localize to the nucleus with very similar patterns of localization. Nonetheless, the
localization pattern of pp65 is still quite complicated although it still does appear that
pp65 could potentially localize to the cytoplasm, since it generally is located near the
very edge of the nucleus. It is somewhat surprising that the nucleus doesn’t have a
distinct structure in a majority of these images although it could be attributable to
artifacts of the fixation or procedure or DAPI mounting media. Another area of concern
is that the interior of the nucleus remains unstained in several of the images although this
is also likely attributable to artifacts in the staining procedure (See Discussion for further explanation).

The subcellular localization of pp28, when co-expressed with pp65 and pp71, was determined by confocal microscopy in transfected and paraformaldehyde fixed HeLa cells (Figure 10). Similar to the previous images (Figure 2 and 3); this image also suffers from likely artifacts from the fixation procedure. DAPI could also potentially bind with a lower affinity to RNA in the cytoplasm of the cell, making the location of the nucleus more difficult to discern. It appears that there are actually two different nuclei based on the localization of pp65 (Figure 10B). It appears that a lot of the signal in the DAPI, pp28, and pp71 confocal scans (Figure 10 A,C,D respectively) could be affected by artifacts of the DAPI staining procedure. Potentially this could have resulted from the cell lysing during the fixation process. Nonetheless, it is difficult to say where pp28 and pp71 localize in Figure 10. The composite image in Figure 11 supports the difficulty in determining the subcellular localization of the tegument proteins in Figure 10. Similar results were noted when pp28 was transfected alone in HeLa cells (data not shown).
Figure 2 Subcellular localization of CFP-pp65, GFP-pp150, and RFP-pp71 in HeLa cells fixed with paraformaldehyde and stained with the nuclear stain DAPI; A- DAPI Stain; B- CFP-pp65; C- GFP-pp150; D- RFP-pp71.
Figure 3 Subcellular localization composite of CFP-pp65, GFP-pp150, and RFP-pp71 in HeLa cells fixed with paraformaldehyde and stained with the nuclear stain DAPI.
Figure 4 Subcellular localization of CFP-pp65 and RFP-pp71 in HeLa cells fixed with paraformaldehyde and stained with the nuclear stain DAPI; A- DAPI Stain; B- CFP-pp65; C- RFP-pp71.

Figure 5 Subcellular localization composite of CFP-pp65 and RFP-pp71 in HeLa cells fixed with paraformaldehyde and stained with the nuclear stain DAPI.
Figure 6 Subcellular localization of CFP-pp65 and GFP-pp150 in HeLa cells fixed with paraformaldehyde and stained with the nuclear stain DAPI; A- DAPI Stain; B- CFP-pp65; C- GFP-pp150.

Figure 7 Subcellular localization composite of CFP-pp65 and GFP-pp150 in HeLa cells fixed with paraformaldehyde and stained with the nuclear stain DAPI.
Figure 8 Subcellular localization of GFP-pp150 and RFP-pp71 in HeLa cells fixed with paraformaldehyde and stained with the nuclear stain DAPI; A- DAPI Stain; B- GFP-pp150; C- RFP-pp71.

Figure 9 Subcellular localization composite of GFP-pp150 and RFP-pp71 in HeLa cells fixed with paraformaldehyde and stained with the nuclear stain DAPI.
Figure 10 Subcellular localization of CFP-pp65, GFP-pp28, and RFP-pp71 in HeLa cells fixed with paraformaldehyde and stained with the nuclear stain DAPI; A- DAPI Stain; B- CFP-pp65; C- GFP-pp28; D- RFP-pp71.
Figure 11 Subcellular localization composite of CFP-pp65, GFP-pp28, and RFP-pp71 in HeLa cells fixed with paraformaldehyde and stained with the nuclear stain DAPI.
Tegument Protein Subcellular Localization in Live-Cell Imaged Cells without a Nuclear Stain

Based on the confocal scan images of the paraformaldehyde fixed cells that were expressing each of the tegument proteins (pp65, pp71, pp150, and pp28), it was difficult to definitively determine where the tegument proteins in the analysis were localizing within HeLa cells. Therefore, live-cell confocal imaging scans were taken 24 hours post-transfection to acquire better confocal images and negate any artifacts that could have occurred during the fixation procedure or from the DAPI stain. Initially, live-cell imaging was performed with the absence of any nuclear stain to act as a control and account for any effects that a DNA stain may have on the subcellular localization of the tegument proteins.

Figure 12 is a representative live-cell confocal scan of HeLa cells that were transfected with pp65, pp150, and pp71 plasmids together. Even without the presence of a nuclear stain, the subcellular localization patterns of these tegument proteins can be determined. Pp65 (Figure 12A) appears to localize to the cytoplasm of the cell outlining the nucleus of the cell, which was somewhat inferred from the experiments using paraformaldehyde fixed HeLa cells. It can also be seen that pp150 (Figure 12B) and pp71 (Figure 12C) appear to localize to the nucleus as confirmed in the paraformaldehyde fixed HeLa cells. However, unlike in the fixed cells, the subcellular localization patterns of pp150 and pp71 are more distinct from each other likely due to the lack of fixation artifacts. When the individual images in Figure 12 are merged
(Figure 13), it further supports the subcellular localization of the tegument proteins pp65, pp150, and pp71. It appears that pp65 is displaying a localization pattern to the cytoplasm while pp150 and pp71 display a localization pattern to the nucleus of the HeLa cell.

The subcellular localization patterns of pp65, pp150, and pp71 when expressed together (Figures 12 and 13) are identical to those observed when the tegument proteins are expressed individually in HeLa cells in the absence of a nuclear stain (Figure 14). Furthermore, HeLa cells transfected with the pp28 expression plasmid by itself points to the subcellular localization of pp28 occurring within the cytoplasm of the cell surrounding the nucleus (Figure 15). This signifies that the fixation artifacts in the paraformaldehyde fixed HeLa cells could have impaired the true localization pattern of pp28 from being observed. The subcellular localization of pp28 is further examined below.
Figure 12 Subcellular localization of CFP-pp65, GFP-pp150, and RFP-pp71 in HeLa cells using live-cell imaging with no nuclear stain; A- CFP-pp65; B- GFP-pp150; C- RFP-pp71.
Figure 13 Subcellular localization of CFP-pp65, GFP-pp150, and RFP-pp71 in HeLa cells using live-cell imaging with no nuclear stain.
Figure 14 Subcellular localization of CFP-pp65, GFP-pp150, and RFP-pp71 when transfected individually in HeLa cells using live-cell imaging with no nuclear stain; A- CFP-pp65; B- GFP-pp150; C- RFP-pp71.

Figure 15 Subcellular localization of GFP-pp28 in HeLa cells using live-cell imaging with no nuclear stain.
Tegument Protein Subcellular Localization in Live-Cell Imaged Cells with Hoechst 33342 Stain

Once the subcellular localization of the HCMV proteins (pp65, pp71, pp150, and pp28) was determined in the absence of a nuclear stain, a similar procedure was performed with the inclusion of a nuclear stain to verify earlier results and definitively conclude the subcellular localization patterns of these tegument proteins. Thus, live-cell confocal imaging scans were taken 24 hours post-transfection of HeLa cells with the tegument-fluorescent protein fusion plasmids. Just prior to the confocal imaging, the transfected HeLa cells were incubated the nuclear Hoechst 33342 stain. Hoechst 33342 stain was used instead of DAPI, because it is well suited for live-cell imaging. Furthermore, it can cross cellular membranes easier due to its greater lipophilic nature than DAPI.

The subcellular localization of pp65, pp150, and pp71 when expressed in HeLa cells as fluorescent protein fusion and then stained with the Hoechst 33342 nuclear stain were observed via live-cell confocal microscopy (Figure 16). Based on the location of the nucleus labeled with the Hoechst 33342 stain, it can be seen that the preliminary results obtained in the absence of the Hoechst 33342 stain are confirmed. Pp65 (Figure 16B) localizes to the cytoplasm of the cell, while pp150 and pp71 (Figure 16 C/D respectively) localize to the nucleus of the host cell. Furthermore, it can be seen that the Hoechst 33342 stain did not affect the subcellular localization of either of the tegument proteins when compared to Figures 12 and 13 lacking the nuclear stain. The localization
of these tegument proteins is even more vivid in the composite image in Figure 17. Further support to these localization patterns of the tegument proteins can be seen in Figures 18 and 19 with Figure 18 displaying each individual confocal scan channel and Figure 19 corresponding to the composite image.

The localization patterns observed in Figures 16-19 are further supported by Figures 20-25 in which each expression plasmid was transfected into HeLa cells individually, which were then incubated with the Hoechst 33342 nuclear stain. It can be seen in Figures 20-25 that pp71 and pp150 do in fact localize to the nucleus, while pp65 localizes to the cytoplasm of the cell after transfection.

Additionally, the subcellular localization of pp28 was determined when expressed by itself in HeLa cells that were incubated with the Hoechst 33342 nuclear stain (Figure 26 and 27). The confocal microscopy images confirm the results obtained without the nuclear stain (Figure 15) in that pp28 is found in the cytoplasm of the host cell. Pp28 was not expressed along with other any of the tegument proteins with the nuclear stain, because it was felt that the localization of it would not change as seen when pp65, pp150, and pp71 were expressed into HeLa cells together.
Figure 16 Subcellular localization of CFP-pp65, GFP-pp150, and RFP-pp71 in HeLa cells using live-cell imaging stained with the nuclear stain Hoechst 33342; A- Hoechst 33342 Stain; B- CFP-pp65; C- GFP-pp150; D- RFP-pp71.
Figure 17 Subcellular localization composite of CFP-pp65, GFP-pp150, and RFP-pp71 in HeLa cells using live-cell imaging stained with the nuclear stain Hoechst 33342.
Figure 18 Subcellular localization of CFP-pp65, GFP-pp150, and RFP-pp71 in HeLa cells using live-cell imaging stained with the nuclear stain Hoechst 33342; A- Hoechst 33342 Stain; B- CFP-pp65; C- GFP-pp150; D- RFP-pp71.
Figure 19 Subcellular localization composite of CFP-pp65, GFP-pp150, and RFP-pp71 in HeLa cells using live-cell imaging stained with the nuclear stain Hoechst 33342.
Figure 20 Subcellular localization of RFP-pp71 in HeLa cells using live-cell imaging stained with the nuclear stain Hoechst 33342; A- Hoechst 33342 Stain; B- RFP-pp71.

Figure 21 Subcellular localization composite of RFP-pp71 in HeLa cells using live-cell imaging stained with the nuclear stain Hoechst 33342.
Figure 22 Subcellular localization of CFP-pp65 in HeLa cells using live-cell imaging stained with the nuclear stain Hoechst 33342; A- Hoechst 33342 Stain; B- CFP-pp65.

Figure 23 Subcellular localization composite of CFP-pp65 in HeLa cells using live-cell imaging stained with the nuclear stain Hoechst 33342.
Figure 24 Subcellular localization of GFP-pp150 in HeLa cells using live-cell imaging stained with the nuclear stain Hoechst 33342; A- Hoechst 33342 Stain; B- GFP-pp150.

Figure 25 Subcellular localization composite of GFP-pp150 in HeLa cells using live-cell imaging stained with the nuclear stain Hoechst 33342.
Figure 26 Subcellular localization of GFP-pp28 in HeLa cells using live-cell imaging stained with the nuclear stain Hoechst 33342; A- Hoechst 33342 Stain; B- GFP-pp28.

Figure 27 Subcellular localization composite of GFP-pp28 in HeLa cells using live-cell imaging stained with the nuclear stain Hoechst 33342.
Discussion

The goal of this study was to determine the subcellular localization of several of the major tegument proteins (pp65, pp71, pp150, and pp28) of the HCMV when expressed in HeLa cells as fluorescent protein fusions, which may mimic the early stage of viral infection before the virus begins replication. It was demonstrated that pp65 and pp28 both localize to the cytoplasm, while pp150 and pp28 both display a nuclear localization within the host cell.

It was observed in paraformaldehyde fixed HeLa cells that the subcellular localization patterns identified and their interpretations were supported definitively in the live-cell imaging experiments, which presented a clear image of where each tegument proteins localizes within the cell. This was especially true of pp150 and pp71, which are clearly seen to localize to the nucleus. However, it was more difficult to determine the localization patterns of pp65 and pp28. For example, Figures 10 and 11 indicate that pp65 and pp28 could potentially localize to the nucleus of the host cell. However, the location of the nucleus as identified in Figure 10A with the DAPI stain is not well defined. Furthermore, it appears that there could be two adjacent cells next to each other that could have potentially been lysed during the fixation protocol with paraformaldehyde based on the signals in Figure 10. It is not possible to definitively state the localization of either tegument protein pp65 or pp28 in the paraformaldehyde fixed HeLa cell confocal scan images. Thus, the paraformaldehyde fixed HeLa cells were likely altered in the fixation protocol, and some of the fluorescence signal could be attributable to fixation.
artifacts. Another possibility in not being able to definitively identify the localization patterns of either tegument protein in the paraformaldehyde fixation experiment is due to the quality of the DAPI mounting medium that was used. The DAPI mounting medium had passed its expiration date, which could potentially have affected its ability to bind to nuclear DNA. An excellent example of this can be seen in Figure 10A and Figure 11.

However, the localization pattern of the tegument proteins (pp65, pp71, pp150, and pp28) were fully elucidated in the live-cell imaging experiments in both the absence of a nuclear counterstain (Figures 12 to 15) and in the presence of a DNA stain (Figures 16 to 27). In the clearest images (Figure 16, 17, 26 and 27) it can be noted that both pp150 and pp71 localize to the nucleus of the host cell, while pp65 and pp28 localize to the cytoplasm of the host cell. Additionally, it can be seen that none of the tegument proteins appears to affect the subcellular localization of another. Similarly, neither tegument protein is essential for the subcellular localization pattern of another. This contradicts the results of earlier experiments using the DAPI nuclear stain with paraformaldehyde fixed cells in the Pizzorno laboratory, which suggested that the localization of pp150 is dependent on whether or not pp71 is present (M. Pizzorno, personal communication). It was seen in these previous experiments that pp150 localized to the nucleus only when co-transfected with pp71. The observation that pp150 localized to the nucleus only in the presence of pp71 could have been due to the fixation procedure or the potential for DAPI to bind at a lower affinity to RNA in the cytoplasm. Nonetheless, the live-cell imaging experiment appears to be a more accurate way of determining the localization of these proteins than using cells fixed with
paraformaldehyde, since the localization patterns are not confounded by fixation or staining artifacts that the paraformaldehyde or DAPI stain may cause. Additionally, the live-cell confocal scans yielded a better representative figure of the entire cell and nucleus, which subsequently allowed for easier analysis and interpretation.

There are several significant implications that arise from the identification of the subcellular localization patterns of the HCMV tegument proteins in these experiments. The first is that the localization patterns of pp71, pp150, and pp28 is similar to what is seen in natural HCMV infections in both fixed and live-cell imaging experiments (33,39,40,41). This also supports a strong correlation to the localization pattern of these tegument proteins and their known function during natural HCMV infections as hypothesized. Pp71 localized subcellularly to the nucleus as seen in natural HCMV infections due to its role in transactivating transcription of the major immediate-early gene (26). Pp150 localized subcellularly to the nucleus, since it is necessary to incorporate nucleocapsids into virus particles but is not required in nucleocapsid assembly itself (16). Pp28, by comparison, localized subcellularly to various cytoplasmic compartments within the cell as seen in natural HCMV infections due to its essential role in the production of viral progeny at the site of final envelopment, which occurs in the cytoplasm of the cell (32). Furthermore, since the subcellular localization patterns of these three tegument proteins portrayed similar patterns of localization as what is seen in normal HCMV infections with the entire virion being present, it can be concluded that no other viral protein, such as another tegument protein or one of the capsid proteins, is necessary for the correct subcellular localization of these three tegument proteins.
The most significant piece of information that can be drawn from this experiment, though, concerns that subcellular localization pattern that was observed for pp65. In the early stage of a normal HCMV infection, pp65 tends to independently migrate to the nucleoli of the cell (34). This localization to the nucleoli suggests a functional relationship between the localization of pp65 and the development of the lytic cycle of HCMV (34). However, pp65 begins to migrate to the cytoplasm 48 hours into the lytic cycle with nuclear pore complex becoming devoid of the protein (35). In accordance with previous experiments, it was hypothesized that pp65 would subcellularly localize to the nucleus of the cell, since the localization pattern of the tegument proteins were being assessed 36 hours post-transfection for the paraformaldehyde fixation experiment and 24 hours post-transfection for the live-cell imaging experiments. However, it can clearly be seen that pp65, particularly in Figures 16-19, localizes to the cytoplasm of the transfected HeLa cells.

The observed localization pattern of pp65, which is the major tegument protein involved in immune evasion of the host cell, in this experiment in transfected HeLa cells could result in a breakthrough in the development of a better targeted and effective, novel antiviral treatment of HCMV infections, especially in immunocompromised individuals. This localization pattern suggests that something else in the HCMV virion is crucial for pp65 to localize to the nucleus of a host cell in the early stages of infection. Moreover, the localization of pp65 to the nucleoli in the early stages of infection suggests a functional relationship between the localization of pp65 and the development of the lytic cycle of HCMV (24). This implies that if a novel, antiviral treatment could target the
other molecule in the HCMV virion that is required to assist pp65 to get into the nucleus of the host cell, HCMV infections and its devastating effects, especially in immunocompromised individuals, could be alleviated. This new data suggests that a potential effective, novel, antiviral treatment for HCMV infections could be synthesized from inhibiting the localization of pp65 to the nucleus of the cell in the early stages of infection.

The results that pp65 does not localize to the nucleus are interesting, since it is known that pp65 has a bipartite nuclear localization signal (45). This implies that the mechanism by which the other molecule within the HCMV virion that is necessary for pp65 nuclear localization during the initial stages of the lytic infection is to expose the pp65 nuclear localization signal. It is known that proteins or other molecules with nuclear localization signals do not localize to the nucleus until the signal is exposed and recognized by an importin protein. Thus, the other molecule within the HVMC virion may bind to pp65, which yields a conformational change in pp65, thereby, exposing its nuclear localization signal to importin proteins during the early stages of the lytic cycle.

There are other possibilities as to why pp65 subcellularly localized to the cytoplasm instead of the nucleus in HeLa cells. One of which relates to the fact that nucleolar accumulation of pp65 is prominent only in G1 and G1/S phases of the cell-cycle, while it is very poor in S and G2/M phases of the cell-cycle (24). It is highly unlikely that the HeLa cells would not enter the G1 and G1/S phases of the cell-cycle post-transfection. Another possibility is that HeLa cells are not a differentiated (permissive) cell type. Typically, only HCMV is able to produce a lytic infection in
differentiated cell types. It is known in undifferentiated cell types that transcription from the major immediate early promoter is repressed by differentiation-dependent transcriptional repressors and co-repressors, which impart on the major immediate early promoter an overall repressive chromatin structure (46). This likely had no effect on the observed subcellular localization of pp65 to the cytoplasm, since no major immediate-early promoter was present.

There are several studies that could be conducted to build upon the information obtained here. One could perform a time-lapse study for a normal HCMV infection with only the tegument proteins being transfected into both undifferentiated and differentiated cells. Additionally, tegument proteins along with virus could be transfected into both undifferentiated and differentiated cells for a comparison between cell type and how the localization patterns of the tegument proteins are similar/different to transfections involving only tegument proteins. Additionally, initial steps could be used to identify any HCMV molecule that is essential for the localization subcellularly of pp65 to the nucleus of the cell in the early stages of viral infection. Once this molecule is identified, a novel, antiviral treatment could be synthesized to target it and inhibit the localization of pp65 to the nucleus in the early stages of infection, which is essential for the establishment of the lytic phase of the viral life cycle in HCMV.

The subcellular localization of several HCMV tegument proteins mimicking the early stages of a normal viral infection was determined. It can be seen that pp150 and pp71 localize subcellularly to the nucleus post-transfection, while pp28 localizes subcellularly to the cytoplasm post-transfection. These results agree with what is seen in
a normal HCMV infection, and suggests a strong correlation between localization and function. Pp65 by comparison was identified to localize subcellularly to the cytoplasm, which is not what occurs normally in the early stages of a HCMV infection. This suggests that a molecule within the HCMV virion is essential to getting pp65 into the nucleus of the host cell post-transfection. Thus, this molecule could potentially be targeted for the development and synthesis of an effective novel, antiviral treatment of HCMV infections that cause significant morbidity and mortality in immunocompromised individuals.
Bibliography


Appendix

Fluorescently labeled HCMV tegument protein constructs.

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