The Role of the Unfolded Protein Response Regulator Bip in Hcmv Virion Assembly and Egress

Nicholas J. Buchkovich
University of Pennsylvania, buchkovi@mail.med.upenn.edu

Follow this and additional works at: http://repository.upenn.edu/edissertations

Part of the Virology Commons

Recommended Citation
http://repository.upenn.edu/edissertations/241

This paper is posted at ScholarlyCommons. http://repository.upenn.edu/edissertations/241
For more information, please contact libraryrepository@pobox.upenn.edu.
The Role of the Unfolded Protein Response Regulator Bip in Hcmv Virion Assembly and Egress

Abstract
Human cytomegalovirus (HCMV) regulates the unfolded protein response, including specifically inducing the ER chaperone BiP. The increase in BiP protein during infection is the result of promoter activation by the HCMV MIEPs and La autoantigen activation of the internal ribosome entry site of BiP mRNA. To determine the effects of BiP on HCMV replication, BiP was depleted using the SubAB subtilase cytotoxin or short hairpin RNAs. Depletion of BiP had little effect on viral protein synthesis. However, progeny virion formation was significantly inhibited, suggesting that BiP is important for virion formation. Electron microscopic analysis showed that infected cells were resistant to the toxin and showed none of the cytotoxic effects seen in uninfected cells. However, all viral activity in the cytoplasm ceased. During infection, BiP localizes in two cytoplasmic structures, regions of condensed ER near the periphery of the cell and the viral cytoplasmic assembly compartment, where it interacts with the viral proteins pp28 and TRS1. Depletion of BiP causes the assembly compartment to dissociate and the cytoplasm to return to a more normal morphology, indicating that BiP is important for assembly compartment integrity and confirming its role in viral cytoplasmic activity. Furthermore, depletion of BiP and the corresponding disruption of assembly compartment integrity results in the loss of virally-induced nuclear lamina rearrangement and a decrease in lamin phosphorylation. These results are due to an interaction between BiP and pUL50. Thus, BiP depletion affects both nuclear and cytoplasmic viral activity. These data and the following observations support an intricate link between viral nuclear and cytoplasmic activity. The inhibition of the molecular motor dynein results in the loss of both assembly compartment integrity and the nuclear morphology characteristic of an HCMV infection. Furthermore, the nuclear periphery is dramatically altered adjacent to the assembly compartment, where the nuclear lamina is rearranged, the outer nuclear membrane is altered, and the nucleus becomes permeable to large molecules. The perinuclear space is also enlarged, a result of decreasing the SUN proteins during infection. These results support a model for a highly integrated assembly-egress continuum, linking viral nuclear and cytoplasmic activity.

Degree Type
Dissertation

Degree Name
Doctor of Philosophy (PhD)

Graduate Group
Cell & Molecular Biology

First Advisor
James C. Alwine

Keywords
virology, HCMV, viral assembly, BiP, GRP78, ER chaperone

This dissertation is available at ScholarlyCommons: http://repository.upenn.edu/edissertations/241
Subject Categories
Virology

This dissertation is available at ScholarlyCommons: http://repository.upenn.edu/edissertations/241
THE ROLE OF THE UNFOLDED PROTEIN RESPONSE REGULATOR BIP IN HCMV
VIRION ASSEMBLY AND EGRESS
Nicholas J. Buchkovich

A DISSERTATION
In
Cell and Molecular Biology
Presented to the faculties of the University of Pennsylvania in
Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy
2010

Supervisor of Dissertation
James C. Alwine, Professor of Cancer Biology

Graduate Group Chairperson
Daniel S. Kessler, Associate Professor of Cell and Developmental Biology

Dissertation Committee:
Constantinos Koumenis, Associate Professor of Radiation Oncology
Paul Bates, Associate Professor of Microbiology
J. Alan Diehl, Professor of Cancer Biology
Erle S. Robertson, Professor of Microbiology
ACKNOWLEDGEMENTS

I would like to acknowledge my mentor, Dr. James C. Alwine, for allowing me to pursue my graduate work in his laboratory. As a mentor, Dr. Alwine provided me with helpful guidance and suggestions in a way that did not infringe upon my freedom to develop and pursue my ideas. Dr. Alwine allowed me to generate ideas, carry out the necessary experiments to test those ideas, and analyze and interpret the results they generated. He also provided me with ample opportunities to share those results through both publications and attendance at international scientific meetings.

In addition, I would like to acknowledge the members of my thesis committee, Drs. Constantinos Koumenis, J. Alan Diehl, Paul Bates and Erle S. Robertson, for taking their time to provide me with advice and support throughout my graduate training. I also want to acknowledge Dr. Robertson for accepting me onto NIH training grant T32 CA115299, which funded much of my work.

I would like to acknowledge my undergraduate mentor at Brigham Young University, Dr. Byron Murray, for providing me with laboratory training, as well as Drs. Phillip Sass, David Bearss, and their respective companies, for offering me internship opportunities. I would also like to acknowledge the employees at those companies who provided me with basic scientific training and helpful guidance. All of these people were essential in providing a solid foundation for my graduate training.

I would like to acknowledge past and present members of the Alwine lab for their friendship, advice and patience throughout my graduate career. I would especially like to thank Tobi G. Maguire for managing the lab and for providing her assistance and expertise with experiments, particularly with immunofluorescence and growth curve analyses.
Finally, and most importantly, I would like to acknowledge members of my family for their support and especially thank my wife Natalie and son Weston. I want to dedicate this thesis to them, and acknowledge them for their patience, understanding, and willingness to deal with the demands on my time, energy and efforts that graduate school requires.
ABSTRACT

THE ROLE OF THE UNFOLDED PROTEIN RESPONSE REGULATOR BIP IN HCMV VIRION ASSEMBLY AND EGRESS

NICHOLAS J. BUCHKOVICH

ADVISOR: JAMES C. ALWINE, Ph.D.

Human cytomegalovirus (HCMV) regulates the unfolded protein response, including specifically inducing the ER chaperone BiP. The increase in BiP protein during infection is the result of promoter activation by the HCMV MIEPs and La autoantigen activation of the internal ribosome entry site of BiP mRNA. To determine the effects of BiP on HCMV replication, BiP was depleted using the SubAB subtilase cytotoxin or short hairpin RNAs. Depletion of BiP had little effect on viral protein synthesis. However, progeny virion formation was significantly inhibited, suggesting that BiP is important for virion formation. Electron microscopic analysis showed that infected cells were resistant to the toxin and showed none of the cytotoxic effects seen in uninfected cells. However, all viral activity in the cytoplasm ceased. During infection, BiP localizes in two cytoplasmic structures, regions of condensed ER near the periphery of the cell and the viral cytoplasmic assembly compartment, where it interacts with the viral proteins pp28 and TRS1. Depletion of BiP causes the assembly compartment to dissociate and the cytoplasm to return to a more normal morphology, indicating that BiP is important for assembly compartment integrity and confirming its role in viral cytoplasmic activity. Furthermore, depletion of BiP and the corresponding disruption of assembly compartment integrity results in the loss of virally-induced nuclear lamina rearrangement.
and a decrease in lamin phosphorylation. These results are due to an interaction between BiP and pUL50. Thus, BiP depletion affects both nuclear and cytoplasmic viral activity. These data and the following observations support an intricate link between viral nuclear and cytoplasmic activity. The inhibition of the molecular motor dynein results in the loss of both assembly compartment integrity and the nuclear morphology characteristic of an HCMV infection. Furthermore, the nuclear periphery is dramatically altered adjacent to the assembly compartment, where the nuclear lamina is rearranged, the outer nuclear membrane is altered, and the nucleus becomes permeable to large molecules. The perinuclear space is also enlarged, a result of decreasing the SUN proteins during infection. These results support a model for a highly integrated assembly-egress continuum, linking viral nuclear and cytoplasmic activity.
# TABLE OF CONTENTS

## CHAPTER 1: Introduction

- Human cytomegalovirus ................................................................. 2
- Unfolded protein response ............................................................. 11
- Unfolded protein response and viruses .......................................... 18
- Immunoglobulin heavy chain binding protein ............................... 26

## CHAPTER 2: Materials and Methods

- Cell lines, reagents and plasmids .................................................. 37
- Virus preparation and titration ....................................................... 44
- Assay protocols ........................................................................ 48
- Microscopy ................................................................................ 55

## CHAPTER 3: HCMV upregulates the ER chaperone BiP

- HCMV increases BiP protein during infection ............................... 58
- HCMV infection increases BiP mRNA levels ................................. 60
- HCMV activates the BiP gene promoter in an ESRE-independent manner ........ 60
- The BiP promoter is not activated by incoming virions .................. 64
- The HCMV MIEPs activate the BiP promoter ............................... 64
- Knockdown of IE72 by siRNA impairs BiP promoter activation ...... 66
- HCMV activates translation from the BiP IRES ............................ 68

## CHAPTER 4: HCMV requires BiP for viron assembly

- HCMV steady state protein levels are not altered when BiP is depleted .... 73
- BiP is required for infectious virion formation .............................. 75
- BiP is required for the cytoplasmic activity of HCMV .................... 77
- BiP is relocalized to the viral cytoplasmic assembly compartment .......... 82
- A ring of BiP forms concurrently with assembly compartment formation .... 89
BiP is localized to condensed ER in infected cells......................................................89
BiP is required for assembly compartment integrity..................................................92
BiP interacts with the viral proteins pp28 and TRS1...............................................96

CHAPTER 5: HCMV-induced alteration of the nuclear architecture.........................100

BiP interacts with the nuclear egress factor UL50..................................................102
Lamins are not rearranged when BiP is depleted......................................................104
BiP is involved in the phosphorylation of the lamins..........................................107

Dynein is required for assembly compartment integrity and
nuclear enlargement..................................................................................................110

The nuclear periphery is altered in proximity to the assembly compartment...113

HCMV downregulates the SUN-domain containing proteins..............................115

Nuclear membrane permeability is altered during an infection.........................119

CHAPTER 6: Discussion ..........................................................................................123

REFERENCES..............................................................................................................137

APPENDIX: Abbreviations.......................................................................................161
LIST OF TABLE AND FIGURES

Figure 1. Structural elements of HCMV virion ................................................................. 3
Figure 2. HCMV cytoplasmic assembly compartment ....................................................... 9
Figure 3. Unfolded protein response signaling ................................................................. 12
Figure 4. Viral regulation of UPR ................................................................................... 20
Figure 5. BiP protein and promoter organization ............................................................ 29
Figure 6. HCMV temporally regulates BiP protein levels ............................................... 59
Figure 7. HCMV temporally regulates BiP mRNA levels ................................................. 61
Figure 8. HCMV activates the BiP promoter ................................................................. 63
Figure 9. The BiP promoter is not activated by incoming virions ..................................... 65
Figure 10. The HCMV MIEPs activate the BiP promoter .............................................. 67
Figure 11. Depleting IE72 reduces BiP promoter activation and mRNA ....................... 69
Figure 12. HCMV activates translation from the BiP IRES ............................................ 71
Figure 13. Depletion of BiP does not alter steady state levels of viral proteins .......... 74
Figure 14. HCMV requires BiP to produce infectious virions ....................................... 76
Figure 15. Treatment with SubAB abolishes HCMV cytoplasmic activity ..................... 78
Figure 16. Depletion of BiP by shRNA mimics BiP depletion by SubAB toxin ............... 80
Figure 17. Nucleocapsids stall outside of the nucleus in the absence of BiP ............... 81
Figure 18. BiP is localized to two distinct locations during HCMV infection ............. 83
Figure 19. Bip colocalizes with pp28 in perinuclear ring ............................................. 86
Figure 20. BiP cosediments with pp28 and gB in a sucrose gradient ............................ 88
Figure 21. Time course staining of BiP during an HCMV infection ............................. 90
Figure 22. The clumped cytoplasmic regions of BiP are condensed ER .................... 91
Figure 23. BiP is required for maintaining assembly compartment integrity .......... 93
Figure 24. BiP depletion leads to rapid disruption of assembly compartment.................95
Figure 25. BiP associates with pp28 and TRS1 during infection..................................97
Figure 26. Assembly compartment remains localized next to nucleus.........................101
Figure 27. BiP interacts with nuclear egress factor pUL50............................................103
Figure 28. Nuclear lamins are not rearranged with BiP is depleted..............................106
Figure 29. Depletion of BiP alters nuclear lamin phosphorylation..................................109
Figure 30. Dynein is required to maintain assembly compartment integrity..................112
Figure 31. HCMV alters the nuclear periphery next to the assembly compartment......114
Figure 32. HCMV increases the perinuclear space.......................................................116
Figure 33. HCMV downregulates SUN proteins during infection.............................118
Figure 34. HCMV alters nuclear membrane permeability..........................................120
Figure 35. Model of assembly-egress continuum.......................................................135

Table 1. Summary of commercially available BiP antibodies.....................................39
CHAPTER 1: Introduction

Viruses are obligate intracellular parasites that require the assistance of their host cells to undergo processes critical for producing viral progeny: transcription, translation, genome replication and virion assembly. The increased metabolic activity in the cell due to viral activity exhausts vital cellular resources. This leads to the activation of key cellular stress responses; the activation of which restores cellular homeostasis. As a result of stress signaling, key metabolic processes are shut down and quality control mechanisms are activated to conserve resources until the cell can return to more favorable growth conditions. While shutting down key cellular processes is detrimental to viral replication, the induction of quality control machinery can be either detrimental or beneficial. Thus, it is expedient that viruses modulate stress responses to their advantage.

Endoplasmic reticulum (ER) stress is one type of cellular stress induced by many viruses. One of these viruses, human cytomegalovirus (HCMV), encodes several proteins that traverse the ER to be processed and receive post-translational modifications. The additional protein load in the ER activates the ER stress response, also referred to as the unfolded protein response (UPR). Like other stress responses, UPR activation has both beneficial and detrimental effects on viral replication. Studies have shown that HCMV does regulate the UPR to take advantage of these beneficial effects, while inhibiting the detrimental ones (136). For example, while HCMV prevents the induction of the UPR transcriptional regulators, beneficial factors like the ER chaperone immunoglobulin binding protein (BiP), also called glucose regulated protein 78 (GRP78), are still induced. The hypothesis tested herein is that HCMV specifically
induces the ER chaperone BiP independently of the UPR transcription factors because BiP plays an important role during an HCMV infection.

**Human cytomegalovirus**

Human cytomegalovirus, also referred to as human herpesvirus 5 (HHV-5), is a member of the *Herpesviridae* virus family. Members of this family share similar virion structural elements: an icosahedral nucleocapsid that houses its genome, a tegument layer surrounding the nucleocapsid and an outer lipid bilayer envelope studded with virally-encoded glycoproteins (Figure 1) (291). Like other herpesviruses, HCMV undergoes both a lytic and a latent infection.

*Herpesviridae* family members are divided into three subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae*. There are 26 core open reading frames shared among the different families (3). HCMV is the representative member of the subfamily *Betaherpesvirinae*. Other members of the *Betaherpesvirinae* subfamily include HHV-6 and HHV-7. Betaherpesviruses share a core set of 70 to 80 conserved open reading frames (269). Several characteristics common among family members during infection include a long replication cycle, restricted host range and induction of cytomegaly in infected cells (291).

HCMV is universally present in human populations with a large number of seropositive individuals (269). Developed countries show a lower incidence of infection due to an increase of hygiene, leaving a larger number of individuals susceptible to a primary infection (235). During an acute infection HCMV has a broad tropism, infecting a variety of cell types and organs. Infections have been reported in both mononuclear and polymorphonuclear leukocytes, macrophages, epithelial cells, endothelial cells, smooth muscle cells, glial cells, neurons, mesenchymal cells and hepatocytes
Figure 1. Structural elements of HCMV virion

- Tegument Layer
- Glycoprotein Complexes
- Nucleocapsid
- Lipid Bilayer Envelope

~230 kB Genome
Following primary infection, a latent infection occurs preferentially in several cell types, including smooth muscle cells, endothelial cells and myeloid lineage cells (138, 139, 368). A large number of an infected individual’s T-cell repertoire is directed against HCMV-encoded antigens, on average 10% but as high as 40% in some individuals (310, 338).

HCMV infections in children and healthy adults are usually asymptomatic; however, it has been attributed to approximately 8% of all cases of mononucleosis (160). In contrast to an infection in immunocompetent individuals, infection of the immunocompromised is often symptomatic. Clinical manifestations of an infection depend on the site of primary infection, often resulting in pneumonitis, retinitis, myocarditis, hemolytic anemia, hepatitis, encephalopathy and/or peripheral polyradiculopathy and neuropathy (177). Recent literature reviews have also focused on the role of HCMV in atherosclerosis, coronary artery restenosis, transplant vascular sclerosis and cancer (229, 332, 335). In addition to causing symptoms in the immunocompromised, a congenital HCMV infection can have severe effects for newborns. It is considered to be the leading cause of sensorineural deafness and the leading infectious cause of brain damage in children (84, 235). HCMV remains a significant clinical problem in these subsets of patients.

HCMV replication cycle

Characteristic of the family Betaherpesvirinae, HCMV undergoes a long replication cycle that progresses slowly in culture. The cycle begins upon attachment to cellular receptors and is completed upon release of infectious virions from the cell. As is the case for all Herpesviruses, synthesis of viral deoxyribonucleic acid (DNA) and
nucleocapsid assembly occurs in the nucleus and a productive viral infection results in the destruction of the host cell (291).

Viral entry into cells is mediated by attachment and fusion of its envelope glycoproteins to cellular receptors (55). Viral attachment and penetration are pH-independent and occur rapidly in both permissive and non-permissive cell types, suggesting a post-entry mechanism for determining tropism (325). The ability of HCMV to enter such a wide range of host cells suggests that the cellular receptor must be broadly expressed and/or multiple receptors may play redundant roles in HCMV entry (54). Cellular receptors that have been implicated thus far in HCMV attachment and entry are heparin sulfate proteoglycans (HSPG), the integrins αVβ3, α2β1, α6β1 and the epidermal growth factor receptor (EGFR) (79, 373, 374). However, the involvement of EGFR in entry is controversial (134, 374). A weak interaction between the viral glycoprotein complex gM/gN or gB with the HSPGs is responsible for initial viral attachment; however this interaction is not sufficient for entry (54, 55). Viral fusion and entry are mediated by interactions between the viral glycoprotein complex gH/gL/gO or gB and cellular receptors.

HCMV gene expression and DNA replication

After entry, the viral nucleocapsids proceed quickly to the nucleus to begin viral gene expression, which is divided temporally into three stages: immediate early, early and late. Immediate early expression begins one to four hours post infection and does not require the expression of previous viral genes. Viral immediate early proteins prepare the cell for infection by modulating the immune response, inhibiting apoptosis and activating transcription from many viral and cellular promoters (145, 235). Early gene expression requires the immediate early genes and is divided into two categories,
early and delayed early. Early gene products are mainly involved in viral DNA replication (for example, UL44, UL112/113, UL54) and immune evasion (US2, US11, US28) (235). The last viral genes expressed during infection are the late genes, also divided temporally into early late and late genes. Microarray data suggest that the majority of HCMV proteins are late proteins (41). Late proteins are mainly structural proteins and proteins involved in virion assembly and maturation (177). Late gene expression requires the presence of both immediate early and early genes, as well as the initiation of viral genome replication (83).

The HCMV genome is greater than 230 kilobases, the largest of the human herpesviruses. For the most part, HCMV encodes its own replication machinery. This includes a polymerase, single-stranded DNA binding protein, helicase-primase complex and processivity factor; however, unlike other herpesviruses, HCMV does not encode deoxyribonucleotide synthesis enzymes, relying instead on the host cell for nucleotide production (46, 177). Before DNA replication can initiate, the input genome must circularize. This can occur as early as 4 hours post infection (hpi); however, replication does not begin until 16 hpi. Replication initially begins from a single origin and proceeds in a bidirectional manner. Concatamers are eventually formed and replication continues by a rolling circle mechanism (222). Nascent genomes are inverted and cleaved before being packaged into preformed, empty nucleocapsids.

HCMV nucleocapsid formation and nuclear egress

HCMV nucleocapsids are 100 nanometer (nm) icosahedrons consisting of 162 capsomer shells that form a T=16 lattice (36). Nucleocapsids consist of four viral proteins, the major capsid protein (MCP) and the minor capsid protein (mCP), encoded by UL86 and UL85 respectively, UL46 (minor capsid binding protein, mC-BP) and the
The smallest capsid protein (SCP) encoded by UL48.5 (12, 36, 93, 94, 133). The MCP is the major protein in nucleocapsid pentamers and hexamers. mCP and mC-BP form triplexes in a 2:1 ratio that interdigitate the hexamers and pentamers. SCP is found on hexon tips and is believed to direct tegumentation. Additionally, UL80 encodes three viral proteins associated with nucleocapsid assembly, the most abundant being assembly protein (AP). AP is a scaffolding protein that is associated with capsid intermediates and noninfectious enveloped particles, but not mature virions (133).

Once assembled, nucleocapsids must migrate from the nucleus to the cytoplasm to finish virion maturation and egress from the cell. To do this, the nucleocapsids must access the inner nuclear membrane (INM), which requires them to overcome barriers such as heterochromatin and the nuclear lamina that block access to the INM. This involves the viral nuclear egress complex, which consists of two viral proteins, pUL50 and pUL53, that recruit both viral and cellular proteins to the nuclear membrane (38). This complex includes two kinases, the virally encoded kinase UL97 and the cellular kinase protein kinase C (PKC), which are responsible for phosphorylating the nuclear lamina, leading to their rearrangement and providing nucleocapsids access to the INM (107, 231). A similar egress function has been described for homologues of pUL50 and pUL53 in both herpes simplex virus-1 (HSV-1) and in mouse cytomegalovirus (MCMV) (244, 267, 290). An alternative mechanism for phosphorylating the lamins has been suggested in which the viral kinase UL97 is recruited by the cellular protein p32 (218). Phosphorylation of the lamina creates a novel binding motif for the peptidyl-prolyl cis/trans isomerase Pin1, which may promote the conformational modulation of the lamina (232). The rearrangement of the nuclear lamina allows for infoldings of the nuclear membrane (35, 65, 95, 263, 296, 311, 312). The current model of nuclear egress suggests that nucleocapsids egress from these infoldings by acquiring a
temporary envelope from the inner nuclear membrane by budding into the perinuclear space. This membrane is lost upon fusion with the outer nuclear membrane, releasing naked nucleocapsids into the cytoplasm (327).

**HCMV assembly compartment, cytoplasmic maturation and egress**

Once in the cytoplasm, the nucleocapsid acquires its tegument layer and final envelope. This process involves the perinuclear cytoplasmic assembly compartment, visualized by electron microscopy in Figure 2A. This structure is nestled against the concave surface of the enlarged, kidney-like nucleus characteristic of HCMV infection. It is located at the microtubule organizing center (MTOC) (Figure 2B), which is necessary for compartment integrity (302). The assembly compartment consists of a reorganized secretory apparatus, as shown by immunofluorescence in Figure 2C, with an early endosomal core (detected by early endosome antigen 1 (EEA1)), surrounded by consecutive rings of trans-Golgi network (TGN) (p230, TGN46, TGN38, Golgin 97) and Golgi network (mannosidase II, GM130, p115) (68). The ER is found at the periphery of the assembly compartment, as shown by antibodies that detect the KDEL ER-localization signal (Figure 2C). A three-dimensional model of the assembly compartment proposes that the assembly compartment is composed of these organelle-specific vesicles which form nested cylinders that make up ordered layers of the assembly compartment (68). Each layer is proposed to contain a specific set of tegument proteins which are transferred to the developing virions as nucleocapsids successively move from the outside to the center of the assembly compartment.

Defining the exact origin of this compartment has been complicated by the observation of specific organellar markers in and around the compartment, while other markers of the same organelle are not detected. For example, immunofluorescence
Figure 2. HCMV cytoplasmic assembly compartment

(A) Electron microscopic analysis of viral cytoplasmic activity at 96 hpi showing the perinuclear cytoplasmic assembly compartment. (B) Higher magnification of viral assembly compartment depicting viral activity radiating from central microtubule organizing center (MTOC). (C) Immunofluorescence analysis of HCMV assembly compartment at 96 hpi showing concentric rings of cellular organelles. Antibodies against p230, EEA1, and the ER-localization signal KDEL were used to detect respective organelles. The Golgi was detected by expression of Mannosidase II-GFP (MannII).
examination localizes the early endosomal marker EEA1 at the center of the assembly compartment (67, 68); however, Rab4 and Rab5, other early endosomal markers, were not detected (122). This suggests that the virus recruits and relocalizes specific cellular factors to form the assembly compartment.

In addition to the cellular markers, many viral proteins exist in the assembly compartment. These include both tegument proteins (pp28, pp65 and pp150) and glycoproteins (gB, gO, gH, gL, gM, gN and gp65) (302, 343). It is proposed that as the nucleocapsid progresses through the rings of the assembly compartment, it acquires its tegument layer and final envelope before egressing from the cell (68). Although the origin of the final envelopment is still being elucidated, a recent study has shown the presence of both TGN and early endosome markers in the final envelope (40). After acquiring a final envelope, infectious viral particles travel in cellular vesicles to the plasma membrane where they are released into the extracellular space (236). Little is understood about the process of virion transport by cellular vesicles and their release from cells.

**HCMV and the induction of stress responses**

One characteristic of HCMV and the betaherpesviruses, as mentioned above, is a long replication cycle that progresses slowly in cultured cells. During this cycle, beginning as early as viral attachment, the virus alters cellular signaling pathways to optimize conditions for replication. Some of the pathways that the virus modulates are stress response pathways that are activated as a result of increased demand for nutrients, energy, nucleic acids and amino acids during a viral infection (6). These stress responses could both benefit and inhibit viral replication. The virus must evolve to overcome detrimental signaling results while taking advantage of the beneficial ones. An
example of a stress response with both beneficial and detrimental effects is the unfolded protein response.

**Unfolded protein response**

The unfolded protein response (UPR) is a response pathway activated by conditions that perturb ER homeostasis. Three ER transmembrane proteins, inositol-requiring enzyme 1 (IRE1), activating transcription factor 6 (ATF6) and PKR-like ER kinase (PERK) act as ER stress sensors (19, 315). IRE1, ATF6 and PERK all bind to BiP in their inactive states (19, 315). BiP is the master regulator of UPR activation; overexpressing BiP prevents UPR activation during ER stress (238). In addition, an abnormally high level of any protein that binds BiP can activate the UPR, while accumulation of misfolded proteins that do not bind BiP have no effect on UPR activation (100, 249). One model is that unfolded proteins compete with UPR sensors for BiP binding, titrating away BiP and allowing the sensors to become activated (19). However, during ER stress BiP is actively dissociated from ATF6, suggesting that activation of at least one UPR sensor may have additional complexity than the simple competition model predicts (317). When activated, the UPR sensors initiate a complex signaling cascade that optimizes cellular conditions for alleviating ER stress (Figure 3).

**Inositol-requiring enzyme 1 (IRE1)**

IRE1 was first identified in yeast as the sole ER stress sensor and UPR activator (58) and subsequently cloned in mammalian cells as one of three stress sensors (348, 375). It is a transmembrane protein that contains an ER stress-sensing luminal domain and a cytoplasmic domain with both kinase and endoribonuclease activity (199, 348). During ER stress, IRE oligomerizes and recruits X-box binding protein 1 (XBP1)
Figure 3. Unfolded protein response signaling.

During ER stress, BiP binds misfolded proteins and dissociates from the ER stress sensors IRE1, ATF6 and PERK. These sensors become activated and initiate a complex signaling cascade that ultimately results in an inhibition of global translation initiation, cell cycle arrest, ER expansion and the transcription of chaperones, degradation factors, and antioxidant genes. Prolonged UPR signaling results in apoptosis. See text for detailed explanation and abbreviations.
mRNA (9, 164). The cytoplasmic domain of IRE1 then excises a 26-nucleotide intron from the XBP1 mRNA, producing an mRNA that encodes a highly active transcriptional form of XBP1 (37, 404). XBP1 is essential for the induction of the ER-associated degradation (ERAD) factors: ER degradation-enhancing α-mannosidase-like protein (EDEM), HMG-CoA reductase degradation 1 (HRD1), Derlin-2 and Derlin-3, the DnaJ containing proteins ERdj3, ERdj4 and p58^{IPK}, protein disulfide isomerase-5 (PDI-5) and ribosome-associated membrane protein 4 (RAMP4) (146, 181, 253, 403). Other genes can be induced by XBP1, but are not absolutely dependent on it since expression of these genes can be induced in XBP1-/- cells (37, 181), suggesting compensatory mechanisms of induction. These include the molecular chaperones BiP and GRP94, Herp, Armt, AW124040, β-interferon and XBP1 itself. The autoinduction of XBP1 allows for sustained activation of target genes in response to prolonged stress (37, 181, 403).

XPB1 is also involved in ER expansion during stress, inducing the synthesis of the primary phospholipid of the ER membrane, phosphatidylcholine (PtdCho) (330). In addition to splicing XBP1, the endonuclease activity of IRE1 is involved in reducing the incoming load of protein to the ER by cleaving a subset of ER-localized mRNAs (121) and by cleaving the 28S ribosomal RNA to repress translation (137).

IRE1 also activates c-Jun amino-terminal kinases (JNKs), alternatively called stress-activated protein kinases (SAPKs), to help regulate both autophagy and apoptosis (18, 361). Autophagy is an important process for recovery from ER stress; excess ER produced by UPR activation and the misfolded proteins associated with it are degraded by autophagosomes (18). In addition to promoting survival, JNK activation by IRE1 is involved in promoting apoptosis; ER-stress induced apoptosis is reduced in IRE1-deficient cells (361). IRE1’s utilization of JNK for both pro-survival and pro-apoptotic
signaling illustrates the complex balance between survival and death signals generated by UPR activation.

**Activating transcription factor 6 (ATF6)**

The second activator of the UPR is the 90 kilodalton (kD) protein ATF6 (418). ATF6 contains a transmembrane domain with its N-terminus facing the cytoplasm (116). During ER stress, ATF6 is transcriptionally induced in an IRE1 dependent manner (377) and undergoes several stress-dependent alterations. ATF6 becomes underglycosylated (124) and exposes two Golgi-localization signals (GLS) (315). Upon exposure of the GLS, ATF6 migrates to the Golgi (256) where it is sequentially cleaved by Site-1 protease (S1P) and Site-2 protease (S2P), releasing its 50 kD N-terminal cytoplasmic domain (115, 400). This domain translocates to the nucleus and acts as a transcription factor (115). ATF6 interacts with other transcription factors associated with the endoplasmic reticulum stress elements (ERSEs), including CCAAT-binding factor/nuclear factor-Y (CBF/NF-Y) through the CBF/NF-YC subunit (406) and Yin Yang-1 (YY1) (194). ATF6 binds to the CCACG sequence of the ERSE, but only when CBF/NF-Y is bound to the CCAAT sequence nine nucleotides upstream (405). ATF6 is the stress-inducible factor responsible for transcriptional complex assembly at the ERSE.

Similar to XBP-1, ATF6 is involved in transcriptional activation of both molecular chaperones, such as BiP (402), and degradation factors, such as Herp (210). ATF6 is also involved in ER expansion by upregulating PtdCho, although its upregulation of PtdCho occurs differently and independently of PtdCho regulation by XBP1 (25). Another similarity between ATF6 and XBP1 is the upregulation of XBP1 (185, 404). The interconnection between the two signaling arms and redundancy in target activation may allow for temporal regulation of UPR signaling. The cleavage of pre-existing ATF6 can
occur rapidly in response to stress and result in immediate activation of target genes, including XBP1. In contrast, XBP1 requires translating mRNA that must first be transcribed and spliced. Its autoregulation, however, may allow for sustained activation of its targets during prolonged stress.

Other functions have been attributed to ATF6 during ER stress. ATF6 is involved in the regulation of calcium. Maintaining calcium levels are important for protein folding and ER homeostasis. In response to stress, ATF6 upregulates the calcium pump sarco/endoplasmic reticulum calcium ATPase-2 (SERCA2) to help maintain proper calcium levels (346). ATF6 also contributes to apoptosis by upregulating the transcription factor C/EBP-homologous Protein (CHOP, also called growth arrest and DNA damage gene (GADD)153) (209). In addition to activating target genes during ER stress, ATF6 has a repressional function as well. ATF6 represses both cholesterol and lipid synthesis by inhibiting the sterol-regulating element binding protein 2 (SREBP2) (411). Inhibiting these two metabolic pathways conserves energy resources required to withstand ER stress.

PKR-like endoplasmic reticulum kinase (PERK)

The third UPR sensor, PERK, contains an ER luminal domain for sensing stress and an active cytoplasmic kinase domain (111, 199, 319). A major consequence of PERK activation during ER stress is the repression of global translation initiation. PERK phosphorylates eukaryotic initiation factor 2α (eIF2α) at serine 51 (110, 111). eIF2α is part of the heterotrimeric eIF2 complex that forms a ternary complex with guanosine triphosphate (GTP) and methionyl-initiator tRNA (Met-tRNAi). Binding of this ternary complex to the 40S ribosomal subunit promotes translation initiation (190). After initiation, GTP is hydrolyzed to guanosine diphosphate (GDP) and the eIF2-GDP
In order to reinitiate translation, the GDP of the eIF2 complex needs to be exchanged for GTP. Another initiation factor, eIF2B, is responsible for this exchange. When eIF2α is phosphorylated, eIF2B cannot exchange GDP for GTP, and initiation of translation is repressed as the available pool of eIF2-GTP is depleted (220). Prolonged repression of translation initiation would be detrimental to production of UPR transcription factors and ER stress recovery. To terminate PERK phosphorylation at the later stages of UPR induction and promote normal initiation of translation, p58IPK, a negative regulator of PERK, is upregulated (397). Additionally, GADD34 is induced by ER stress and interacts with the protein phospatase 1 complex (PP1) to direct the dephosphorylation of eIF2α and restore normal translational initiation (57, 252).

During ER stress, PERK-dependent repression of translation results in both cell cycle arrest and an increase in ATF4 translation. Cell cycle arrest occurs because of the inhibition of cyclin D1 translation (29). The increase in ATF4 translation is a result of an increase in ribosome activity at a downstream open reading frame (ORF) due to the reduced initiation of translation (109). The decrease in available eIF2-GTP creates a delay in translation initiation that allows the ribosome to read through the inhibitory uORF2 and reinitiate translation at the ATF4 ORF (205, 364). ATF4 is a transcription factor that, similar to ATF6, can activate BiP (207), the ERAD factor Herp (210) and CHOP (78, 109). ATF4 also regulates the UPR through its activation of ATF3 (140), and upregulates genes involved in amino acid import, glutathione biosynthesis and oxidative stress resistance (112).

In addition to translational repression and ATF4 activation, activated PERK also phosphorylates the transcription factor NF-E2-related factor-2 (Nrf2). Phosphorylation of Nrf2 releases it from its cytoplasmic inhibitor Kelch-like ECH-associated protein 1 (Keap1), allowing it to escape ubiquitin-dependent proteolysis and migrate to the
nucleus where it activates transcription from promoters containing antioxidant response elements (61, 62). PERK regulates other signaling pathways as well, including the phosphoinositide-3 kinase (PI3K)/Akt pathway (152), indicating a potential role for preparing cells for recovery from ER stress (257).

**UPR-induced apoptosis**

Prolonged ER stress and sustained activation of the UPR can indicate irreparable conditions, resulting in UPR-induced apoptosis. All three stress sensors activate both pro-survival and pro-apoptotic pathways, although the stimulus that triggers the shift from survival to apoptosis is not well understood and evidence shows that both protective and apoptotic signaling pathways are activated at the same time (120, 297). The balance between survival and apoptosis may depend on a balance between the pro- and anti-apoptotic Bcl2 family members. Several Bcl2 family members and other apoptotic factors are upregulated during UPR signaling, including the pro-apoptotic factors Bim, p53-upregulated modulator of apoptosis (PUMA) and Noxa (192, 280, 289). During ER stress, Bim is protected from proteasomal degradation and becomes phosphorylated by JNKs to promote Bax-mediated apoptosis (186, 279, 280). Another pro-apoptotic factor, CHOP, is a target of ATF4 and ATF6 (209, 257, 376). CHOP is required for UPR-induced apoptosis (420), but the exact mechanisms of CHOP-induced apoptosis are still under investigation. CHOP upregulates pro-apoptotic factors such as death receptor 5 (DR5) and tribbles homolog 3 (TRB3) (255, 396), suggesting that CHOP may induce apoptosis through transcriptional activation. CHOP has also been shown to downregulate the anti-apoptotic factor Bcl-2, resulting in decreased cellular glutathione levels and increased oxidative stress (221). Furthermore, CHOP promotes the translocation of Bax from the cytosol to the mitochondria, a critical step for apoptosis.
Understanding the mechanisms of ER-induced apoptosis and the balance between survival and apoptosis during UPR activation is important, particularly for a viral infection, as premature apoptosis would be an example of a UPR effect detrimental to viral infection.

**Unfolded protein response and viruses**

Successful modulation of the different signaling arms of the UPR is a critical event for the completion of many viral replication cycles, as many viruses activate UPR signaling. Viruses have adopted different regulation strategies for modulating the UPR. For example, many viruses require the cellular machinery for translation and must overcome the UPR-induced repression of translational initiation; some viruses accomplish this by inhibiting phosphorylation of eIF2α, while others have developed translational mechanisms that don’t require eIF2α (Figure 4A) (49, 129, 151, 239, 241, 358, 367). The human immunodeficiency virus type 1 (HIV-1) tat protein activates the UPR by deregulating calcium signaling, an event that has been shown to be critical for the neuropathogenesis of HIV-1 (251). Another viral protein, the latent membrane protein 1 (LMP1) of Epstein-Barr virus (EBV) can activate all three arms of UPR signaling (183). The UPR is also activated during a human t-lymphotrophic virus (HTLV-1) infection, as shown by increased expression of BiP, CHOP, EDEM and XBP1 (168). Viruses such as these that activate the UPR must ensure that UPR signaling does not adversely affect viral replication.

**Virus modulation UPR-induced block in translation initiation**

Activation of the PERK pathway by viruses would result in an inhibition of translation initiation. To circumvent this, viruses have adopted several distinct strategies
HSV-1 encodes several viral proteins to counteract eIF2α phosphorylation. One viral protein, γ134.5, recruits the cellular phosphatase complex PP1 to dephosphorylate eIF2α (49). Another HSV-1 protein, US11, directly inhibits eIF2α phosphorylation late in infection, suggesting the evolution of two temporally distinct mechanisms to control the cellular inhibition of translation during HSV-1 infection (241). Despite these two mechanisms, it was reported that additional viral proteins are required to completely overcome the translational inhibition by phospho-eIF2α during an HSV-1 infection (240). One of these proteins has been identified as the viral glycoprotein gB, which interacts with PERK to resist PERK’s activation (239). Additionally, similar to the HSV-1 γ134.5 protein, the human papillomavirus E6 protein associates with the PP1 complex to dephosphorylate eIF2α (153). The cowpox virus CP77 protein suppresses eIF2α phosphorylation; although the exact mechanism of its suppression is still undetermined (129). Instead of inhibiting eIF2α phosphorylation, the Dengue viruses types 1 and 2 (DEN-1, DEN-2) overcome activation of PERK and eIF2α phosphorylation by upregulating GADD34, which is responsible for dephosphorylating eIF2α (358).

Rather than overcoming the translational inhibition by PERK, some viruses have developed translational mechanisms that allow them to circumvent the need for eIF2α. Two alphaviruses, Semliki Forest virus and Sindbus virus, have developed a translational mechanism that bypasses the requirement of functional eIF2α by using a hairpin downstream of the initiation codon to stall the ribosome at the correct initiation site (367). Evolving a mechanism to overcome the block in translation is important for the progression of viral infection, as demonstrated by the vesicular stomatitis virus (VSV). Cells infected with VSV require the PERK-induced block in translation as a host defense mechanism; VSV replicates to higher titers in PERK-deficient cells (13). The virus is
Figure 4. Viral regulation of UPR

(A) To overcome the inhibition of translation by UPR signaling, viruses must regulate the level of phospho-eIF2α or promote the eIF2α-independent initiation of translation. (B) Viruses that activate the UPR must account for UPR-induced apoptosis. Viruses that benefit from apoptosis can promote the induction of apoptotic factors. Conversely, viruses that need to inhibit apoptosis must prevent these factors. See text for more information on viral regulation of UPR signaling.
able to generate more viral progeny in the absence of PERK, because cells cannot phosphorylate eIF2α and inhibit translation of viral proteins.

**Viruses and UPR-induced apoptosis**

A second result of UPR signaling that would directly affect viral replication is the induction of apoptosis. Although premature apoptosis could potentially halt viral replication, viruses could also use apoptosis as an effective way to spread infectious virions once their replication has been completed. Several viruses adapt the latter strategy and use the UPR to induce apoptosis (Figure 4B). The bovine viral diarrhea virus (BVDV), which uses the ER as the primary site for envelope glycoprotein biogenesis, genomic replication, and particle assembly, activates the UPR and ultimately induces CHOP, leading to viral-induced apoptosis (143). A related virus, BVDV-2, has similarly been shown to induce apoptosis through ER stress signaling (213). Other viruses that induce apoptosis via CHOP and UPR induction include the Japanese encephalitis virus (JEV) (336) and respiratory syncytial virus (22). Similarly, the gPr80env proteins of two related viruses, the Maloney murine leukemia virus (MoMuLV) and mink cell focus-forming murine leukemia virus (MCF MLV), accumulate in the ER and activate the UPR (203, 247). Interestingly, this CHOP-dependent UPR-induced apoptosis by MoMuLV and another mouse retrovirus, FrCasE, is a significant contributor to their neuroimmunodegenerative phenotype (72, 156, 203). This phenotype is lost in a related avirulent mouse retrovirus, F43, that has a different envelope protein, one that does not initiate ER stress (72, 73). Thus, the ability of mouse retroviruses to induce ER-stress dependent apoptosis is required for virulence.

Apoptosis, however, would not be beneficial for all viruses. The African swine fever virus (ASFV) has been shown to inhibit the production of CHOP during infection
(Figure 4B), even under conditions, such as thapsigargin addition, that normally cause ER stress and upregulate CHOP (248). Since ASFV replicates on the cytoplasmic side of the ER and uses the ER cisternae for envelopment, potentially causing ER stress, inhibiting the induction of CHOP prevents premature apoptosis and ensures a productive viral infection. This is not the case for West Nile virus (WNV). The non-structural proteins of WNV induce apoptosis by increasing CHOP protein levels. Inducing apoptosis via CHOP limits the amount of virus produced, as the virus replicates to higher titers in CHOP -/- cells (223). Although it appears that the host cell uses CHOP-induced apoptosis as a defense mechanism, WNV-induced apoptosis plays a key role in viral pathogenesis, resulting in apoptosis of infected neurons and leading to paralysis and encephalitis. Although CHOP-induced apoptosis prevents the virus from replicating to its full titers, WNV uses it as a mechanism for viral dispersal. Interestingly, a closer examination of a WNV infection shows that all three signaling branches of UPR signaling are activated. ATF6 is rapidly targeted for degradation by the proteasomes and although XBP1 is spliced, it is dispensable for infection and has no effect on viral growth (223). WNV has prevented the transcriptional activity of these UPR molecules, most likely to avoid an increase in ERAD. Importantly however, the ER chaperones BiP, PDI, calreticulin, calnexin and GRP94 are all still upregulated (223), illustrating careful modulation of the UPR by WNV to promote beneficial outcomes while inhibiting detrimental ones.

Another virus, Borna disease virus (BDV), induces apoptosis in infected neurons, contributing to the susceptibility of the host to neurodegeneration and neonatal Borna disease. Interestingly, the virus exhibits differential regulation of apoptosis depending on the cell type infected; Purkinje cells undergo apoptosis but astrocytes are resistant. All three UPR sensors are activated during infection in both cell types, resulting in the
induction of the pro-apoptotic factor CHOP; however levels of the pro-survival UPR targets BiP and PDI are increased only in the apoptotic resistant astrocytes (388). This demonstrates cell type specificity in the evolution of the virus that has led to the selective ability to overcome the pro-survival signaling arm of the UPR.

Viral regulation of UPR transcriptional signaling and ER chaperones

Several viruses have been reported to regulate the transcriptional arm of the UPR and induce ER chaperones. BiP and GRP94 are both transcriptionally induced by the rotavirus nonstructural protein 4 (NSP4) (394). BiP is also upregulated by Rous sarcoma virus, paramyxovirus, New Castles disease virus and DEN-2 (53, 272, 334, 379). The induction of BiP by DEN-2 is required for viral antigen production (379). The hepatitis B virus X protein activates both ATF6 and IRE1 (191) and the E5 protein of canine papillomavirus induces splicing of XBP1 mRNA (56). XBP1 is also upregulated by the flaviviruses, JEV and DEN-2 (197). The number of viruses that upregulate ER chaperones either independently of, or through, UPR signaling is indicative of the important role chaperones play during infection.

Viruses adapt UPR molecules to benefit viral replication

Additional roles adapted by UPR proteins during a viral infection may provide further incentive for regulating the UPR. Despite its role in repressing translation, activating PERK would be beneficial for a virus as PERK is involved in the phosphorylation-dependent ubiquitination and degradation of the interferon alpha receptor 1 subunit (IFNAR1), inhibiting type I interferon signaling and antiviral defenses (202). In HTLV-1, XBP1 encoded by both the spliced and unspliced forms of mRNA have been reported to bind and activate transcription from the tax-responsive element of
the long terminal repeat of HTLV-1 (336). Further, BiP appears to play a role in the pathogenesis of HSV-1. The neuroinvasive strains of HSV-1, SP7 and 490, induce BiP levels during infection; however, the attenuated strain KOS does not (216). Another virus, coxsackievirus A9, uses BiP as a coreceptor for entry (353). These data suggest that viruses can induce additional roles for UPR signaling molecules to further benefit viral replication.

Modulation of the UPR by specific viruses

Studies have elucidated regulation of UPR signaling by viruses through a variety of mechanisms. Extensive studies on the hepatitis C virus (HCV) reveal how a single virus selectively modulates the various arms of UPR signaling for the advantage of its replication. The viral envelope E2 protein selectively binds and inhibits two eIF2α kinases, PERK and protein kinase R (PKR), an interaction that is sufficient to overcome the repression of translation (271, 342). E2 also induces the transcription of both BiP and GRP94 (197). This induction is a result of an interaction between E2 and BiP, which may sequester it from the UPR sensors and allow for UPR activation. Along with E1, E2 induces CHOP via a PERK-dependent mechanism and activates XBP1 mRNA splicing by IRE1 (43). IRE1 activation and XBP1 mRNA splicing are also induced by the viral nonstructural protein 4B (NS4B), which interacts with ATF6 and promotes its cleavage and transcriptional activity (195, 350, 416). Expression of the complete HCV genome results in ATF6 cleavage, transcriptional activation of its target gene BiP and activation of translation from BiP’s internal ribosome entry site (IRES) (340). Expression of the HCV genome also activates IRE1, resulting in the splicing of XBP1 mRNA; EDEM is not however induced, suggesting viral regulation of the UPR to prevent the production of degradation factors (339, 416). An interesting observation from studies with HCV is that
the JFH1 strain, the first HCV strain to replicate in culture, exhibits differential regulation of PERK. This suggests both a potential role for PERK in in vitro replication of HCV, as well as strain variations among viruses in UPR regulation (326).

Similar to HCV, two viral proteins of severe acute respiratory syndrome virus (SARS) selectively modulate the UPR. The S protein activates PERK resulting in eIF2\(\alpha\) phosphorylation; however, it does not induce ATF6 transcription or splicing of XBP1. Despite repression of XBP1 and ATF6, transcription of BiP, GRP94 and CHOP is still induced by the S protein (42). BiP and GRP94 are also upregulated by the SARS-CoV protein 8ab, which is likely a result of ATF6 activation since 8ab only activates the ATF6 pathway and not IRE1 or PERK (17). Work on another coronavirus, mouse hepatitis virus (MHV), also shows selective modulation of the UPR. ATF6 is cleaved and XBP1 is spliced, but the target genes ERdj4, EDEM and p58\(^{\text{IPK}}\) are not upregulated (17). Although eIF2\(\alpha\) is highly phosphorylated and GADD34 expression is suppressed, halting cellular translation, preferential translation of viral transcripts occurs in an eIF2\(\alpha\)-independent manner. This is another example of a virus counteracting a detrimental effect of UPR signaling (17).

**HCMV modulation of the UPR**

HCMV also activates and selectively regulates all three arms of UPR signaling. PERK is activated during infection as indicated by an increase in phospho-PERK levels; consequently, levels of phospho-eIF2\(\alpha\) increase as well (136). Interestingly, HCMV modulates this pathway so that the increase in phospho-eIF2\(\alpha\) levels does not result in a repression of translation in infected cells, although the mechanism of this regulation is not yet known. However, since HCMV significantly increases the total level of eIF2\(\alpha\), the ratio of unphosphorylated to phosphorylated eIF2\(\alpha\) is high. This may account for
HCMV’s ability to overcome the inhibition of translation initiation by phospho-eIF2α. This also suggests that the viral infection affects translational mechanisms, since ATF4 expression is increased during infection even though translation is not repressed (136). ATF4 is normally only expressed during the eIF2α-dependent repression in translation. Recently, the HCMV protein UL38 has been shown to affect ATF4 expression (395).

Another ER stress sensor, ATF6, is not cleaved during HCMV infection; although the stress-dependent shift in glycosylation can be detected, suggesting that transport to the Golgi for cleavage may be blocked (136). Additionally, the splicing of XBP1 mRNA is severely reduced during infection, demonstrating that, similar to ATF6 and PERK, IRE1 is also regulated by HCMV. Accordingly, the levels of the XBP1 target gene EDEM are not increased (136). Further regulation of IRE1 by HCMV is demonstrated by the ability of HCMV UL38 to suppress the IRE1-mediated phosphorylation of JNK and its UPR-induced cell death (395). Interestingly, despite the suppression of ATF6 and IRE1, levels of two ER chaperones, BiP and GRP94, are upregulated during infection. Thus, HCMV appears to utilize an alternative method to induce these chaperones, both of which would be beneficial for viral replication. Furthermore, the upregulation of these proteins allows HCMV to utilize them in novel ways to aid in viral replication. During infection, BiP interacts with two HCMV proteins, US2 and US11; this interaction is critical for downregulating the major histocompatibility complex 1 (MHC1), contributing to HCMV’s well-developed immune evasion strategy (117, 259, 349). In this thesis it will be shown that HCMV also induces an alternative function of BiP that is important for virion assembly and egress.

**Immunoglobulin heavy chain binding protein**

A 78 kD protein that was independently identified and given the names glucose
regulated protein 78 (GRP78) (321) and immunoglobulin heavy chain binding protein (BiP) (104) was later shown to be the same protein (243). It is an ER resident member of the heat shock protein 70 (HSP70) molecular chaperone family (320, 410) with 61% homology to its cytosolic family member Hsp70 (243). BiP promotes the proper folding and assembly of peptides by binding aberrantly glycosylated and misfolded proteins (91, 118, 149), participating in complexes with other ER chaperones (173, 225, 226, 341, 414). BiP contains a cleavable leader sequence that directs BiP to translocate to the ER and a C-terminal KDEL sequence that retains BiP’s ER localization (Figure 5A) (243). Although primarily located in the ER, reports have also associated BiP with the plasma membrane (354), nucleus (219), mitochondria (337), and most recently the cytosol (208). It is essential for cell and organismal survival, as mice deficient for BiP exhibit early embryonic lethality (193). In addition to BiP’s role in development, it is required to maintain ER integrity and for stress-induced activation of cellular autophagy (193).

**Structure and function of BiP**

Structurally, the HSP70 family members consist of an N-terminal adenosine triphosphate (ATP)-ase domain (44) and a C-terminal peptide or substrate binding domain (372) (Figure 5A). The substrate binding domain of BiP recognizes stretches of seven amino acids with a preference for aliphatic and aromatic amino acids, although it can tolerate polar and charged residues (24, 82). The crystal structure of the ATPase domain of BiP has recently been published, and closely resembles the ATPase domain of other Hsp70 family members (389). Structural studies on HSP70 family members revealed a mechanism in which ATP hydrolysis in the ATPase domain causes a significant conformational change in the substrate binding domain (80, 141, 419). This conformational change increases the affinity of the substrate binding domain for peptides,
as substrates have a higher affinity for the adenosine di-phosphate (ADP)-bound state of HSP70 family members (262, 305). Accordingly, peptide binding to the substrate binding domain increases ATP hydrolysis rates of the ATPase domain (103). This suggests a mechanism in which peptides bind HSP70 family members in the ATP-bound state, stimulate ATP hydrolysis and then become “locked” to the chaperone until ADP is exchanged for ATP and the substrate is released (81, 101, 243, 261). The exchange of ADP for ATP on HSP70 family members is done by nucleotide exchange factors. Two ER-localized co-chaperones, BiP-associated protein (BAP) (52) and GRP170 (382), have been identified as nucleotide exchange factors for BiP. In addition to the nucleotide exchange factors, other co-chaperones known as DnaJ domain containing proteins can bind and stimulate BiP’s ATPase activity through their J domains. These include the ER-localized DnaJ Homologues 1-5 (ERdj1 or Mtj1/HTJ1 (166), ERdj2 or hSec63 (329), Erdj3 or HEDJ (407), ERdj4 (318), ERdj5 or JPD1 (63, 126)) and p58IPK (298). Conversely, the ATPase activity of BiP is negatively regulated by the FK506-binding protein 23 (FKBP23) (414).

In addition to its role in protein folding and assembly, BiP is also involved in protein translocation; although studies suggest that the mammalian BiP may not play as big as role in protein translocation as its yeast homologue Kar2 (71, 159, 250). One study suggests that mammalian BiP can be lowered by 10,000 fold without adversely affecting protein translocation (97). Despite this report, a definitive role for mammalian BiP has been established in protein translocation. BiP binds incoming and newly translocated proteins, in part to ensure unidirectional import of the nascent polypeptides (356). BiP also seals both the luminal end of actively translocating pores and inactive translocons to maintain the ER membrane permeability barrier (105, 108). This is an
Figure 5. BiP protein and promoter organization

(A) Schematic of BiP showing domain organization, localization signals and SubAB cleavage site. Ribbon diagrams of the ATPase domain of BiP and the peptide binding domain of the BiP homologue DNAK are shown above. Ribbon diagrams of BiP ATPase domain and DNAK peptide binding domain taken from http://www.thesgc.org/structures/structure_description/3IUC/ and http://people.cryst.bbk.ac.uk/~ubcg16z/hsplec.html, respectively. (B) Organization of the BiP promoter with ERSE’s, ATF/CREB site, TATA box and five CCAAT elements. The transcriptional complex that forms in response to ER stress and ATF6 cleavage is shown assembled at the ERSE.
ATP hydrolysis-dependent mechanism, similar to the mechanism used by BiP to bind and release unfolded peptides (4).

BiP also plays a role in the reverse process of ER translocation, ERAD, and the targeting of misfolded proteins for export out of the ER. BiP is one component of an ER quality control scaffold involved in ERAD (127). Depletion of BiP increased the half-life of the alpha chain of the T-cell receptor, a model for ERAD, confirming a role for BiP in ERAD (179). BiP’s role in ERAD is dependent on both its concentration and an intact peptide binding domain (144), and requires the co-chaperone function of several of the ERdj proteins (76, 362). Despite utilizing many of the same proteins as ER protein translocation, BiP-mediated export is mechanistically distinct from import (31). Since ERAD targets are determined by a protein’s confirmation, not primary sequence information, BiP must maintain a delicate balance of maintaining proteins in a foldable state and targeting them for degradation (161, 328).

**Transcriptional control of BiP**

BiP is ubiquitously expressed as an abundant ER luminal protein in all cells during unstressed conditions. Basal expression of BiP, in at least some cell lines, is dependent on growth factors like IL-3 and CSF-1 (28). The BiP promoter is constitutively depleted of nucleosomes (88) and contains five CCAAT elements, a TATA box and two potential binding sites for SP1 (347) (Figure 5B). There are also three ERSEs with the consensus sequence CCAAT(N9)CCACG, which are critical for the stress-induction of BiP (402). Many transcription factors involved in activating the BiP promoter have been identified, including YY1 (196), CBF/NF-Y(294), CAAT-binding transcription factor-nuclear factor 1 (CTF-NF1) (390), transcription factor II-I (TFII-I) (268), cAMP response element binding protein 1 (CREB1), ATF1, ATF4 (207), ATF6
(402), XBP-1 (404), GATA4 (215), c-Myb (285), Sp1, Sp3 and Sp4 (1). In many cases these appear to be cell type and stress-dependent. GATA4 interacts with YY1 to induce BiP transcription only in the embryonic heart (215) and c-Myb is associated with BiP’s promoter only in cancer cells (285). In contrast to activation of the BiP promoter, the cellular E2 transcription factor 1 (E2F1) represses BiP transcription (283).

Several transcription factors are involved in the stress-induction of BiP. The BiP promoter contains an upstream ATF/cAMP response element (CRE) site that is activated during stress by ATF4 in conjunction with CREB1 and ATF1 (207). Downstream of this site, CBF/NF-Y constitutively binds to the proximal CCAAT element (295) and interacts with ATF6 during stress (406). ATF6 also interacts with TFII-I (268), which during stress gets phosphorylated by the c-Src tyrosine kinase (123) and binds the promoter in the intermediate nine nucleotides of the ERSE (293). This complex has also been shown to bind Sp1, Sp3 and Sp4 (1). YY1 also acts as a cofactor for ATF6 (15) and interacts with protein arginine methyltransferase 1 (PRMT1) to enhance the BiP promoter during stress (15). Another chromatin modifier, the histone deacetylase p300, is also present at the BiP promoter during ER stress (15). The stress-induced complex that forms during ER stress is depicted in Figure 5B.

A number of conditions and treatments have been reported to increase BiP levels. Many of these perturb protein processing and increase the presence of malfolded proteins in the ER (165), ultimately activating the UPR and increasing BiP. Alterations in protein glycosylation induce BiP, including glucose deprivation (276) and treatment with glucosamine, 2-deoxyglucose, castanospermine (380) or tunicamycin (258). Perturbing calcium levels by adding calcium free media (175) and calcium modulators ionomycin (391), A23187 (383) and thapsigargin (277) also upregulate BiP. Inhibiting protein transport by treatment with brefeldin A or the trimeric G-protein inhibitor Aif4− (148, 277)
also increase BiP levels. Other inducers include insulin (148), β-mercaptoethanol, low pH (385), nitric oxide (162), the amino acid analogue azetidine (380), homocysteine (306) and exposure to anaerobic conditions (306). BiP is also upregulated by a variety of drug treatments including cisplatin (201), iodoacetamide (201), the non-steroidal anti-inflammatory indomethacin (392), methylseleninic acid (47), valproate (26, 314), lithium (314), and the antidiabetics rosiglitazone and troglitazone (214). The variety of stimuli that increase BiP production is indicative of the important role BiP plays in restoring ER homeostasis and protecting cells from damage.

**Post-transcriptional control of BiP**

In addition to transcriptional mechanisms for inducing BiP, evidence also suggests post-transcriptional regulation. In some cell types brefeldin A induction of BiP occurs at the transcriptional level, while in others it is post-transcriptional (200). Similarly, both treatment with dexamethasone (174) and expression of a mutant mouse mammary tumor viral glycoprotein p74 (357) increase BiP protein levels without increasing mRNA levels. Furthermore, artificially increasing BiP levels during unstressed conditions does not result in BiP protein production, suggesting a stress-dependent regulation of BiP translation (102). During ER stress induction, BiP translation efficiency increases independently of transcript levels (102). A similar observation has also been reported for BiP in plants (344) and chicken HSP70, in which protein levels are increased by regulating protein elongation (345). Human HSP70 is also regulated post-transcriptionally; however, it appears to be due to an increase in mRNA half-life (345). One feature that may allow for the unique translational regulation of BiP mRNA is the IRES in its 5’ untranslated region (UTR) that allows for internal ribosome binding (211). This element is regulated by NS1-associated protein 1 (NSAP1), La autoantigen (also
called Sjogren syndrome antigen B, SSB), p50 and p95 (51, 158, 398). The multiple methods of upregulating BiP demonstrate that maintaining levels of BiP is critical for cell homeostasis.

BiP is also regulated post-translationally by both phosphorylation and ribosylation (39, 383). BiP can be phosphorylated on serine and threonine residues in the peptide binding domain, by either autophosphorylation or an unidentified ER kinase (89, 189). This phosphorylation does not occur on BiP that is associating with misfolded proteins, thus phosphorylation may be inhibitory to BiP’s chaperone function (119). The ribosylation of BiP is reversible and is not present on the majority of BiP in stressed cells (119, 187, 188). These results suggest a role for post-translational modifications in regulating the function of BiP. This was confirmed by a study identifying the different cellular states of BiP. BiP actively functions as a chaperone in the monomeric form, while unassociated BiP exists as either an unmodified monomer or in an aggregate modified by both phosphorylation and ribosylation (85).

Another post-translational regulator of BiP is calcium (Ca\(^{2+}\)). Ca\(^{2+}\) has been linked to both the induction and proper function of BiP. BiP’s amino terminal nucleotide binding domain is enriched for glutamic and aspartic acid, creating an acidic, Ca\(^{2+}\) -binding domain (182). Accordingly, BiP was verified as an ER-resident Ca\(^{2+}\) -binding protein (212) and as participating in ER Ca\(^{2+}\) storage (198). The role of BiP as a meaningful player in ER Ca\(^{2+}\) storage has since been disputed and it has instead been shown that Ca\(^{2+}\) binding to BiP alters its affinities for ATP and ADP (176). This observation could explain the necessity of Ca\(^{2+}\) for proper protein folding (204). However, too much Ca\(^{2+}\) is detrimental to protein folding as high concentrations of Ca\(^{2+}\) ions inhibits BiP’s ATPase activity (150). Other roles connecting BiP with Ca\(^{2+}\) include
regulation of GRP94 phosphorylation (284) and Ca\textsuperscript{2+} signaling from ER to mitochondria through an interaction between BiP and the sigma-1 receptor (114).

**BiP’s role in disease**

Ongoing studies of BiP have implicated it in a variety of disease. Recent studies have shown elevated levels of BiP in many different cancers (20, 66, 322, 371, 393), although the effects of elevated BiP vary greatly. BiP can provide some cancerous cells with a survival advantage (34, 64, 86, 87, 234, 286, 287, 313, 401), while in others be pro-apoptotic (34). Depending on the cancer type, elevated BiP levels can represent either a negative (66, 96, 184, 233, 275, 413) or a positive (130, 178, 304, 360) prognosis. BiP is strongly associated with drug resistance in cancer cells (14, 45, 75, 154, 163, 228, 275, 281, 287, 299, 316, 355, 370, 409, 412, 421), but also shows promise as a tool for cancer therapy (10, 48, 90, 157). Other diseases associated with BiP and ER stress include two neurodegeneration diseases, Alzheimer’s disease (125, 172, 408, 415) and Marinesco-Sjogren neurodegenerative disorder (8, 307). BiP also plays a protective role in atherosclerosis (381, 384). Due to BiP’s critical role in maintaining cellular homeostasis, deregulating BiP may sensitize cells to disease.

BiP’s critical role in maintaining cellular homeostasis and promoting cell survival makes it a target for several natural compounds. Prunustatin A isolated from *Streptomyces violaceoniger* 4521-SVS3 downregulates BiP expression (359), and both the soy protein genistein and the macrocyclic compound versipelostatin inhibit the transcription of BiP (266, 417). Epigallocatechin gallate (EGCG) from green tea and the plant signaling molecule salicylic acid, from which aspirin is derived, both bind BiP and inactivate it by altering its ATPase activity (69, 77). Another naturally produced molecule, the cholera toxin, upregulates BiP and other ERAD factors to aid in its
retrotranslocation from the ER to the cytoplasm (74). The cholera toxin is only one AB₅ toxin that regulates BiP.

**Subtilase cytotoxin**

The AB₅ toxins consist of the cholera toxin, pertusis toxin, and the shiga toxins and related *Escherichia coli* heat labile enterotoxins. The AB₅ toxins are named for their subunits, a single A subunit and a pentamer of B subunits. Generally, the B subunits are involved in entry and the A subunit is responsible for toxicity by disrupting a key cellular process. A recently identified AB₅ toxin, subtilase cytotoxin (SubAB), selectively cleaves and inactivates BiP (270). It consists of a 35 kD subtilase-like serine protease A subunit and five 13 kD B subunits. SubAB cleaves BiP quickly in culture, within 1 to 2 hours post treatment. An inactive derivative of SubAB is made by mutating a serine to alanine in a key catalytic triad of the A subunit at amino acid 272 (SubA₂₇²B). Alternatively, to produce a toxin-resistant BiP that retains chaperone activity, amino acid 416 at the toxin cleavage site can be mutated from a leucine to aspartic acid (270). These properties of SubAB make it a good experimental tool for inactivating BiP.

The experiments described in this dissertation investigate the regulation of BiP and its role during an HCMV infection. They first examine the kinetics of BiP expression at both the mRNA and protein level and the mechanisms used by HCMV to regulate BiP. They also aim to identify a role for BiP in infection, including any effects it might have on a viral infection and the processes required to produce infectious virions. The localization of BiP during infection and any infection-specific interactions between BiP and viral or cellular proteins is also investigated. Interestingly, although depleting BiP did not alter the steady state levels of viral proteins as might be expected for a molecular
chaperone, it did halt the production of infectious virions. The results show that BiP is required to maintain viral cytoplasmic activity and has an important role in the intricate relationship that occurs between the cytoplasm and nucleus during infection.
CHAPTER 2: Materials and Methods

Cell lines, reagents and plasmids

**Cells and cell culture:** Lifetime extended-human foreskin fibroblasts (LE-HFF) (27) were cultured at 37 degrees (°) Celsius (C) and 5% carbon dioxide (CO2) in Dulbecco's Modified Eagle Medium with 10% fetal calf serum, 100 units/ milliliter (ml) penicillin, 100 micrograms (µg)/ml streptomycin and 2 millimolar (mM) GlutaMAX (DMEM10). All cell culture reagents were Gibco brand, purchased from Invitrogen (Carlsbad, CA). For experimentation, fibroblasts were used between passages 3 and 13. 293T cells are a variation of the HEK293 cell line that express polyomavirus large T antigen and were used for lentivirus production (99). 293T cells were cultured as described previously for LE-HFF.

To generate stable cell lines expressing luciferase under control of either the wildtype BiP promoter or the BiP promoter with mutations in the ERSEs, LE-HFF were electroporated with plasmids containing the respective promoters in the pGL4.17 plasmid backbone (cat# E6721, Promega, Madison, WI). Electroporation was performed using program U-023 of the Nucleofector Device (cat# AAD-1001, Lonza, Basal, Switzerland ) following the manufacturer’s instructions. Briefly, the cells were released from the culture plate with trypsin (cat# 25300-054, Invitrogen) and centrifuged at 1200 revolutions per minute (rpm) (~250 x g) and 4°C in a Sorvall RT7 table top centrifuge (Thermo Scientific, Waltham, MA). The cell pellet was resuspended in DMEM10, alliquotted into microcentrifuge tubes (1 X 10^6 cells per tube) and centrifuged at 1000 rpm (61 x g) and 4°C in a Beckman-Coulter Microfuge 22R Centrifuge with a S241.5 rotor (Brea, CA). The cell pellets were resuspended in 100 microliters (µl) Basic Nucleofector Solution for Mammalian Fibroblasts from the Basic Nucleofector Kit for
Primary Mammalian Fibroblasts (cat# VPI-1002, Lonza) and transferred to a cuvette to be electroporated. After electroporation, the cells were transferred to 1 ml DMEM10 and plated in pre-warmed media. Stably electroporated cells were selected by serially passaging the electroporated cells in the presence of 1 milligram (mg)/ml G418 (cat# 61-234-RF, Mediatech Inc.). After selection, cells were propagated in media containing 200 µg/ml G418 and then maintained in normal DMEM10.

**Reagents:** Thapsigargin (cat# 586005) and non-glycosidic indolocarbazole-1 (NGIC-1) (cat# 481500) were purchased from EMD Chemicals (Darmstadt, Germany). Thapsigargin was used at a concentration of 2 µg/ml. NGIC-1 was used at a concentration of 0.5 micromolar (µM). SubAB and SubA_{277}B (270) were generously provided by James and Adrian Paton (University of Adelaide) at stock concentrations of 1.49 µg/ml and 0.97 µg/ml, respectively, and diluted in DMEM10 to a working dilution of 100 nanograms (ng)/ml. Tetramethylrhodamine (TRITC)-conjugated dextran with an average molecular weight of 155 kD was purchased from Sigma-Aldrich (cat# T1287) and used at a concentration of 10 mg/ml. The control siRNA against green fluorescent protein (GFP) was purchased from Qiagen (cat# 1022064, Germantown, MD). The sense (GGUUAUCAGUGUAAUGAAG) and antisense (CUUCAUUACACUGAUAACC) siRNA sequences that have been previously shown to target IE72 (131) were custom generated by Thermo Scientific (formerly Dharmacon, Lafayette, CO). The siRNA against La autoantigen (cat# D-006877-01-0005) and the scrambled control (cat# D-001210-03-05) was also purchased from Thermo Scientific.

**Antibodies:** All antibodies against BiP are commercially available and are summarized in Table 1. The two antibodies against TRS1, SD2 and ZH3, were generously provided
<table>
<thead>
<tr>
<th>Name</th>
<th>Type</th>
<th>Source</th>
<th>Cat#</th>
<th>Epitope (AAs)</th>
<th>Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>BiPA</td>
<td>Rabbit Poly</td>
<td>Abgent (AP1335a)</td>
<td>1-654</td>
<td>IF</td>
<td></td>
</tr>
<tr>
<td>BiPB</td>
<td>Mouse Mono</td>
<td>BD Bioscience (610978)</td>
<td>525-628</td>
<td>W</td>
<td></td>
</tr>
<tr>
<td>BiPC</td>
<td>Goat Poly</td>
<td>Santa Cruz (C-20, sc-1051)</td>
<td>20AAs within 600-654</td>
<td>IF, W</td>
<td></td>
</tr>
<tr>
<td>BiPD</td>
<td>Mouse Mono</td>
<td>R&amp;D Systems (MAB4846)</td>
<td>1-654</td>
<td>IF</td>
<td></td>
</tr>
<tr>
<td>BiPH</td>
<td>Rabbit Poly</td>
<td>Santa Cruz (H-129, sc-13968)</td>
<td>525-654</td>
<td>IF</td>
<td></td>
</tr>
<tr>
<td>BiPN</td>
<td>Goat Poly</td>
<td>Santa Cruz (N-20, sc-1050)</td>
<td>20AAs within 1-50</td>
<td>IP</td>
<td></td>
</tr>
<tr>
<td>BiPR</td>
<td>Rabbit Poly</td>
<td>Sigma (GL-19, G8918)</td>
<td>636-654</td>
<td>IF</td>
<td></td>
</tr>
<tr>
<td>BiPS</td>
<td>Rabbit Poly</td>
<td>Sigma (ET-21, G9043)</td>
<td>71-91</td>
<td>IF</td>
<td></td>
</tr>
<tr>
<td>BiPY</td>
<td>Rabbit Poly</td>
<td>Abcam (ab21685)</td>
<td>600-654</td>
<td>IF, IP</td>
<td></td>
</tr>
<tr>
<td>KDEL</td>
<td>Mouse Mono</td>
<td>MBL (SR-827F)</td>
<td>KDEL (649-654)</td>
<td>IF, IP</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1. Summary of commercially available BiP antibodies.**

AAs, amino acids; Mono, monoclonal; Poly, polyclonal; IF, immunofluorescence; IP, immunoprecipitation; W, Western
by Thomas Shenk (Princeton University) (292). The rabbit polyclonal anti-ex2/3 antibody, which recognizes the common exons 2 and 3 of the HCMV major immediate early proteins (MIEPs), was previously produced for this laboratory by Cocalico Biologicals (Reamstown, PA) (113). An antibody that recognizes the HCMV viral protein produced from the UL50 ORF was produced in rabbits and affinity purified using a peptide containing amino acids 152-170 (GPENEGERENLLRELYAKK) of pUL50 by Open Biosystems (Huntsville, AL) for this laboratory. The anti-phospho lamin A/C serine 22 antibody was purchased from Cell Signaling Technology (cat# 2026, Beverly, MA). The monoclonal antibody against pp28 used for immunofluorescence (IF) and immunoprecipitation (IP) assays was from Abcam (cat# ab6502). The pp28 monoclonal antibody used for Western blot analysis was from Santa Cruz Biotechnology (5C3, cat# sc-56975, Santa Cruz, CA). Other antibodies purchased from Santa Cruz include the polyclonal antibodies against phosphofructokinase-1 (PFK-1) (K-15, cat# sc-31712), PDI (H-160, sc-20132), lamin B (C-20, sc-6216) and lamin A/C (N-18, cat# sc-6215), the monoclonal antibody against UL44 (p52) (CH13, cat# sc-69745), and the normal rabbit IgG (cat# sc-2027). Antibodies against p230 (cat# 611280) and EEA1 (cat# 610456) were purchased from BD Biosciences (San Jose, CA). The SUN1 (cat# HPA008346) and SUN2 (cat# HPA001209) antibodies were from Sigma-Aldrich (St. Louis, MO). The antibody against the HCMV protein pp65 (c9100-25) was from US Biological (Swampscott, MA). The antibody against SSB/La autoantigen (cat# H00006741-M01) was purchased from Novus Biologicals (Littleton, CO). The anti-gB monoclonal antibody was purchased from East Coast Bio (cat# CA005, North Berwick, ME) and the anti-actin monoclonal antibody (cat# MAB1501) was from Chemicon International (Temecula, CA).

All horseradish-conjugated secondary antibodies for Western analysis were purchased from Thermo Scientific (formerly Pierce Biotechnologies, Rockford, IL): goat
anti-rabbit IgG (H+L) pre-adsorbed with human serum (cat# 31462), goat alpha-mouse IgG (H+L) pre-adsorbed with human, horse and bovine serum (cat# 31432), and rabbit anti-goat IgG (H+L) (cat# 31402). All secondary antibodies used for immunofluorescence were purchased from either Santa Cruz: pre-adsorbed donkey anti-rabbit IgG TRITC-conjugated (cat# sc-2095) or FITC-conjugated (cat# sc-2090), pre-adsorbed isotype-balanced donkey anti-mouse IgG TRITC-conjugated (cat# sc-2300) or FITC-conjugated (cat# sc-2099), pre-adsorbed donkey anti-goat IgG TRITC-conjugated (cat# sc-2094) or FITC-conjugated (cat# sc-2024); or Invitrogen: donkey anti-goat IgG (H+L) Alexa Fluor 594 pre-adsorbed with rabbit, rat, mouse and human serum (cat# A-11058), donkey anti-mouse IgG (H+L) Alex Fluor 594 (cat# A-21203), donkey anti-rabbit IgG (H+L) Alexa Fluor 594 (cat# A-21207), donkey anti-goat IgG (H+L) Alexa Fluor 647 pre-adsorbed with rabbit, rat, mouse and human serum (cat# A-21447), donkey anti-mouse IgG (H+L) Alex Fluor 647 (cat# A-31571) and donkey anti-rabbit IgG (H+L) Alexa Flour 647 (cat# A-31573)

**Plasmids:** The luciferase reporter plasmids with the wildtype and mutant BiP promoters in the pGL3 backbone were a gift from Kazutoshi Mori (Kyoto Research Park) (402). For stable cell line generation described above, promoter sequences were cut from the pGL3 backbone using the restriction sites Kpn1 (cat# R0142S) and HindIII (cat# R0104S) and inserted into pGL4.17. All restriction enzymes used for cloning were purchased from New England Biolabs (Ipswich, MA). The pDL-N dual-luciferase plasmid has been described previously and was a gift to the lab (366). The BiP 5' UTR region containing the BiP IRES was PCR amplified from the pSVCAT/BiP/Luc plasmid (211) provided generously by Maria Hatzoglou using the following forward (CGCTGCAGAGGTCGACGCCGG) and reverse (CGGGTACCCTTGCCAGCCAGTTG...
G) primers. The PCR product was cloned into pDL-N using the restriction enzymes KpnI and PstI (cat# R0140).

Plasmids expressing the viral proteins IRS1 (292), TRS1 (292), pp65 (12) and pp71 (12) were gifts from Thomas Shenk. Plasmids pRL43a (274) and pSVH (70), containing HCMV MIEP genomic DNA, were obtained from Gary Hayward (Johns Hopkins University) and Richard Stenberg (Eastern Virginia Medical School), respectively. The MIEP genomic DNA expressing plasmid pCD-MIE was made by Yongjun Yu in a two step cloning process in which the genomic region of pRL43a was inserted into pcDNA3. First, a 1.4 kb XhoI/SalI blunt fragment from pRL43a replaced the XhoI/BsmI piece in pcDNA3; then, a 3.8 kb NdeI/XhoI fragment from pRL43a replaced Ndel/XhoI piece in the plasmid made in the first step. The IE1 and IE2 cDNA expressing plasmids pRSV72 (92), pRSV86 (92), pIE72 (333) and pIE86 (333) were all obtained from Richard Stenberg. Plasmids expressing the viral protein products UL69 and UL84 were created by Yongjun Yu by cloning the respective PCR products into pcDNA3 using the restriction sites BamHI (cat# R0136S) and XhoI (cat# R0146S) or EcoR1 (cat# R0101S) and EcoR5 (cat# 10667145001, Roche), respectively. All PCR products were verified by sequencing at the University of Pennsylvania DNA Sequencing Facility.

The GFP expressing plasmid pMAX-GFP is from Lonza and provided as part of the Basic Nucleofector Kit for Primary Mammalian Fibroblasts. The CC1-mCherry plasmid was generously provided by Erika Holzbaur (University of Pennsylvania). Plasmids expressing the proteins tagged with fluorescent markers used for live-cell analysis, pp28-red fluorescent monomeric protein (DsRed) (308) and lamin A-GFP were gifts from Bill Britt (University of Alabama-Birmingham) and Robert Kalejta (University of Wisconsin), respectively.
Plasmids encoding short hairpin RNAs (shRNAs) against BiP (cat# RHS3979-9569448, 449, 451, Open Biosystems) and GFP (plasmid# 12273, Addgene, Cambridge, MA) in the lentiviral packaging plasmid pLKO.1-puro were purchased from their respective companies. The third generation lentiviral packaging plasmids pMDL-RRE, pRSV-Rev and pCMV-VSVg necessary for lentiviral production were a gift from Eric Brown (University of Pennsylvania).

For plasmid DNA replication, the plasmids were transformed into 50 µl Subcloning Efficiency DH5α chemically competent cells (Invitrogen, cat# 18265-017). The plasmid DNA was incubated with the competent cells on ice for 30 min, followed by a 1 minute heat shock at 42°C. After two minute incubation on ice, 450 µl Luria-Bertani, Miller broth (LB) (cat# 244620, BD Biosciences) was added and the cultures were incubated for 90 minutes at 37°C. The cultures were then plated on LB agar (cat# 244520, BD Biosciences) plates containing the appropriate antibiotic, 30 µg/ml Kanamycin or 50 µg/ml Ampicillin, and the plates were incubated at 37°C overnight. One colony from each plate was inoculated in 100 ml LB media containing the appropriate antibiotic and shaken overnight at 37°C and ~225 rpm. Plasmids were extracted from bacteria using Qiagen Plasmid MaxiKit (cat# 12163, Qiagen) according to the manufacturer’s instructions. Briefly, overnight cultures were pelleted by centrifugation at 10,000 rpm (15,180 x g) and 4°C in a SLA-1500 rotor and Sorvall RC-5B Refrigerated Superspeed Centrifuge. The pellets were resuspended and lysed, and the cellular debris was precipitated using Qiagen buffers 1, 2 and 3. The precipitated material was cleared from the lysate by subsequent centrifugation steps at 13,000 rpm (20,199 x g) and 4°C in a SS-34 rotor for 30 and 15 minutes. The supernatant containing the plasmid DNA was applied to a Qiagen column, and the bound DNA was washed and eluted with Qiagen buffers QC and QF. The eluted plasmid DNA was
precipitated in isopropanol and washed with 70% ethanol. After the precipitation and wash steps, DNA was pelleted by centrifugation in a SS-34 rotor at 11,500 rpm (15,806 x g) for 30 and 10 minutes, respectively. The final DNA pellet was dried and resuspended in distilled deionized water (ddH2O) and stored at -20°C. The concentration of the plasmid DNA was acquired by reading absorbance at 260 and 280 nanometers on a DU800 spectrophotometer (Beckman-Coulter).

Virus preparation and titration

**HCMV preparation and stocks:** All experiments were done with the Towne strain of HCMV (cat# VR-977, American Type Culture Collection (ATCC), Manassas, VA). Some experiments were done with a Towne strain of HCMV expressing GFP under the control of the SV40 promoter. The GFP insert replaces a region of non-essential genes from US1–US11 (217). For experiments with HCMV, LE-HFF were infected at a multiplicity of infection (MOI) of 3 plaque forming units (pfu)/cell. For infection, the virus was thawed in a 37°C water bath, sonicated 10 times with a one second pulse in a Bransonic 200 Ultrasonic Cleaner (Branson, Danbury, CT) and vortexed. The virus was diluted in an appropriate amount of DMEM10 and added to plates containing LE-HFF, which were then incubated at 37°C. After the absorption phase of infection, (~2 hpi), the plates were refed with fresh DMEM10 and returned to 37°C. For experiments with UV-inactivated HCMV, virus was exposed to short wavelength, 254 nanometer, ultraviolet (UV) light administered in a UV Stratalinker 1800 (Stratagene, La Jolla, CA) for 5 minutes in a 35-millimeter uncovered tissue culture dish. Cells were then infected as described above.

HCMV was propagated in LE-HFF plated in 15-centimeter (cm) plates and infected in 10 ml DMEM10 at a very low MOI, 0.01 - 0.1 pfu/cell. After appearance of cytopathic effects (CPE) in 80-90% of cells, an additional 10 ml DMEM10 was added to
Virus was harvested 3-4 days post media addition by scraping cells into the media and pooling the media/cell mixture from each plate for centrifugation at 4000 rpm (3313 x g) in a Sorvall RT7 table top centrifuge for 30 min at 4°C. After centrifugation, the supernatants were pooled and stored at 4°C. The cell pellets were resuspended in a small amount of DMEM10, distributed in 500 µl aliquots to microcentrifuge tubes, sonicated 10 times with a 1 second pulse, vortexed 15 seconds and spun down at 13,000 rpm (10,268 x g) for 2 minutes in a Microfuge 22R Centrifuge with a S241.5 rotor. The supernatant was added to the previously pooled supernatant and the cell pellets were resuspended in 500 µl DMEM10. The sonication, vortex and centrifugation steps were repeated and the supernatant was again added to the previously pooled supernatants. The pooled media was then alliquotted into polyclear ultracentrifuge tubes (cat# 7052, Seton, Los Gatos, CA). A 7 ml D-sorbitol cushion (20% sorbitol, 50mM Tris-HCl (hydrochloric acid), pH 7.2, 1mM MgCl₂, 100 µg/ml Bacitracin (cat# B0125, Sigma-Aldrich)) was applied beneath the medium using a sterile 10 cm steel needle and syringe. The medium was then centrifuged, after being weighed and counterbalanced, at 19,000 rpm (47,700 x g) for 1 hour at room temperature (RT) using a SW28 rotor and L8-80M ultracentrifuge. The media and sorbitol cushion were removed from the pellet by vacuum aspiration and the virus was resuspended in DMEM and alliquotted into cryovirals to be flash frozen in liquid nitrogen and stored at -80°C.

**HCMV titrations and growth curves:** Virus titers were determined using the 50% tissue culture infectious dose (TCID₅₀) method (288). Serial dilutions of the virus to be titered were made as follows: 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶, 10⁻⁷ and 10⁻⁸. 100 µl of the dilutions were added to LE-HFF plated in 1 row (12 wells) of a 96-well plate. Each titration was performed in triplicate plates. Each plate contained a row of no virus control.
After 1 week, the cells were refed media by adding 100 µl DMEM10 to each well of the plates. At 14 days, viral titers were read by monitoring the presence of either CPE for the non-GFP Towne virus or expression of GFP for the Towne-GFP virus. Each well with CPE or GFP was scored as positive. The two dilutions after the dilution that scored 12 positive wells were used to calculate the titer using the TCID<sub>50</sub> chart (288).

Titration of virus samples for growth curves was performed using the TCID<sub>50</sub> method as described above. For growth curve samples, cells were washed 3 times in DMEM10 following the absorption phase of infection and 2 ml DMEM10 was added to each sample. At this time, the input virus sample (~2 hpi) was harvested as described below with the exception that the initial volume was only 1 ml, and this sample was harvested before washing with DMEM10. The other viral samples were harvested at the indicated times post infection by transferring 1.5 ml of medium to a conical vial on ice. The cells were then scraped into the remaining 500 µl of medium and transferred to a microcentrifuge tube to be sonicated 10 times with a 1 second pulse, vortexed 15 seconds and microcentrifuged at 13,000 rpm (16,060 x g) for 1 min at RT in a Sorvall Pico Biofuge (Thermo). The supernatant was added to the previously collected medium and the sample was frozen at -80°C. For BiP depletion growth curves, SubA<sub>A272B</sub> was added at 2 hpi and SubAB was added at either 2 hpi or 12 hours before harvest.

**Lentiviral production and harvest:** For lentiviral production, four 10-cm plates of 293T cells were transfected 24 hours after plating using the following protocol. For each plate to be transfected, 20 µg of either shBiP or shGFP pLKO.1 plasmids, 10 µg pMDL-RRE, 5 µg pRSV-Rev and 6 µg pCMV-VSVg were combined in a microcentrifuge tube and brought to a volume of 500 µl in sterile ddH<sub>2</sub>O. Two tubes were prepared for each shRNA type. To each tube, 500 µl of 2X HBS solution (0.27 molar (M) sodium chloride
(NaCl), 0.01 M potassium chloride (KCl), 0.001 M sodium phosphate (Na$_2$HPO$_4$), 0.042M 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES), and 0.011 M glucose, pH 7.05) and 50 µl of 2.5 M calcium chloride (CaCl$_2$) was added. After a 25 minute incubation at RT, the transfection mix was added to 5 ml fresh medium on each plate and the plates were placed at 37°C. After 6 hours, the medium was replaced with 7.5 ml fresh DMEM10 and the plates were returned to 37°C. At 48 hours post transfection, the medium was collected from each plate and replaced with 7.5 ml fresh DMEM10. The plates were returned to 37°C. The collected medium from plates transfected with the same shRNA were combined and stored at 4°C. At 72 hours post transfection, the cells were scraped into 7.5 ml of medium. Cells from the plates transfected with the same shRNA were combined and spun down at 3000 rpm (1864 x g) for 5 min at 4°C in a Sorvall RT7 table top centrifuge. The supernatant was combined with the 15 ml of media collected the previous day. The cell pellet was resuspended in 4 ml serum-free medium and alliquotted into 4 microcentrifuge tubes to be sonicated 10 times with a 1 second pulse. After sonication the cells were centrifuged at 13,000 rpm (10,268 x g) for 5 minutes at 4°C in a Microfuge 22R Centrifuge with a S241.5 rotor. The supernatant was combined to the 30 ml media already collected and the pooled media was filtered through a 0.45 µM Steriflip vacuum-driven filtration system (cat# SE1M003M00, Millipore, Billerica, MA). After filtration, the media was weighed, counterbalanced and centrifuged for 2 hours at 4°C, 26,000 rpm (89,454 x g) in a SW-28 rotor, L8-80M ultracentrifuge. The supernatant was removed and the pellet was resuspended in 2.5 ml DMEM10. The virus was then alliquotted into 10 cryovials in 250 µl amounts, snap frozen in liquid nitrogen and stored at -80°C. Virus was used without being titrated.
**Lentiviral infection:** For knockdown of BiP using shRNA, 250 µl of BiP shRNA expressing lentivirus was added to 2.25 ml DMEM10 containing 8 µg/ml polybrene (cat# 107689, Sigma-Aldrich). Two 6-cm plates with LE-HFF were infected with 1 ml of medium containing the BiP shRNA lentivirus. As a control, 1 plate was infected with 1 ml GFP shRNA lentivirus. One plate was left untreated and was not infected with lentivirus. The plates were incubated at 37°C for 6 hours before replacing the medium with 4 ml of fresh DMEM10. At 24 hpi, all 4 plates were infected with HCMV at an MOI of 3. At 96 hours post HCMV infection (120 hours post lentiviral infection), the plates were either prepared for electron microscopy or harvested for Western analysis.

**Assay protocols**

**Protein harvests and quantification:** LE-HFF were plated in either 6-cm plates or 6-well dishes and infected with HCMV-Towne-GFP at an MOI of 3. For BiP depletion studies, mock- or HCMV-infected LE-HFF were treated with SubA_{A272B} at 2 hpi or with SubAB at either 2 hpi or 12 hours before harvest. For lamin phosphorylation studies, cells were treated with NGIC-1 or SubAB at 2 and 72 hpi. Mock- and HCMV-infected samples were harvested for protein at the indicated times post infection by washing one time in 1 ml chilled phosphate buffered saline (PBS) (140mM NaCl, 2.7 mM potassium chloride, 1.5 mM potassium phosphate monobasic, 8 mM sodium phosphate dibasic). Following the wash, 200 – 300 µl radioimmunoprecipitation assay (RIPA) buffer (1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.15 M NaCl, 10 mM sodium phosphate [ph 7.2], 2 mM ethylenediaminetetraacetic acid (EDTA), 50 mM sodium fluoride (NaF), 1 mM phenylmethylsulfonfonyl fluoride (PMSF), 1.7 µg/ml aprotinin, 0.2 mM sodium orthovanadate, 1 µg/ml leupeptin) was added and cells were rocked 5 minutes at 4°C. Cells were then scraped into the lysis buffer, transferred to a
microcentrifuge tube and centrifuged at 13,000 rpm (10,268 x g) for 10 minutes at 4°C in Microfuge 22R Centrifuge with a S241.5 rotor. The supernatant was transferred to a new tube and frozen at -80°C.

Protein lysates were quantified by adding 5 µl protein sample to 995 µl ddH2O and 250 µl Bio-Rad Protein Assay Dye Reagent Concentrate (cat# 500-0006, BioRad, Hercules, CA) and light absorbance at 595 nm was read on a DU800 spectrophotometer. Bovine serum albumin (BSA) dilutions of 2.5 mg, 5 mg, 7.5 mg, 10 mg, 15 mg and 20 mg were used as protein standards.

**Western analysis:** Proteins were prepared for Western analysis by adding 15-30 µg protein to 3X SDS loading buffer (187.5 mM Tris-HCl, pH 6.8, 6% SDS, 30% glycerol, 0.03% bromophenol blue, 467 mM β-mercaptoethanol). Samples were boiled for 5 minutes, centrifuged at 13,000 rpm (16,060 x g) for 1 minute at RT in Sorvall Pico Biofuge (Thermo), and loaded onto polyacrylamide gels. Gels were either 8% or 10% polyacrylamide, depending on the size of proteins to be investigated. Molecular weight standards (cat# P7708S, New England Biolabs) were also run to aid in identification of protein band size. Gels were run in 25 mM Tris, 192 mM glycine, 0.1% SDS (1X running buffer) until the desired separation among bands was obtained, as monitored by the molecular weight standard. Following electrophoresis, gels were transferred to nitrocellulose membranes (cat# 162-0115, Bio-Rad) under constant current in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). Membranes were blocked in 5% non-fat dry milk in tris-buffered saline-Tween 20 (TBST) (20 mM Tris pH 7.5, 150 mM NaCl, 1% Tween 20) with gentle rocking at RT. Primary antibodies were diluted in 2% BSA-TBST using optimized dilutions based on manufacturer’s recommendations, or experience, and rocked gently, usually overnight at 4°C. Following primary antibody
incubation, membranes were washed 3 times in TBST. Secondary antibodies were
diluted 1:5000 in 5% non-fat dry milk-TBST and rocked gently at RT. Membranes were
washed 3 times in TBST before addition of Lumi-Light Western Blotting Substrate (cat#
12 015 200 001, Roche, Indianapolis, IN) for 5 min. For bands with low signal,
membranes were incubated for 1 min with a 1:5 dilution of SuperSignal Femto Substrate
(cat# 34094, Thermo). Membranes were then exposed to either X-Omat AR-5 non-
interleaved film (cat# 165 1454, Kodak, Rochester, NY) or BlueLite Autorad Film (cat#
F-9024-8X10, ISC BioExpress, Kaysville, UT).

**Quantitative PCR (polymerase chain reaction):** LE-HFF were plated in 6-cm dishes
and infected with HCMV-Towne or HCMV-Towne-GFP at an MOI of 3. At the indicated
times post infection, RNA was isolated from fibroblasts using the RNeasy Mini Kit (cat#
74104, Qiagen) according to manufacturer’s instructions as follows. The cells were
harvested in Buffer RLT provided by Qiagen, homogenized by passing 5 times through a
20-guage needle with a 1-ml syringe and applied to a Qiagen column after addition of
70% ethanol. After washing with Qiagen buffers RW1 and RPE, RNA was eluted in 50
µl ddH₂O and quantified by reading absorbance at 280 and 260 nanometers on a DU800
spectrophotometer.

cDNA was generated from 1-2 µg RNA with Superscript First-Strand Synthesis
System for RT-PCR (reverse transcriptase-PCR) (cat# 11904-018, Invitrogen) using the
manufacturer’s instructions. The RNA was incubated with deoxynucleotide
triphosphates (dNTPs) and random hexamers for 5 minutes at 65°C and then cooled 1
minute on ice. Following addition of Qiagen’s reverse transcriptase buffer, magnesium
chloride, dithiothreitol and an RNase inhibitor, samples were incubated at RT for 2
minutes. Superscript II reverse transcriptase was added and the samples were
incubated at RT for 10 minutes, 42°C for 50 minutes and 70°C for 15 minutes. The samples were cooled on ice before addition of RNase H and incubation at 37°C for 20 minutes. cDNA was stored at -20°C.

Quantitative-PCR was performed with a 7900 HT system (Applied Biosystems, Foster City, CA) using Taqman Universal PCR Master Mix (cat# 4304437) and gene expression assays for BiP (Hs00607129_gH) and GAPDH (Hs00266705_g1) from Applied Biosystems. Reactions were incubated for 2 minutes at 50°C and 10 minutes at 95°C and then cycled 40 times between 15 seconds at 95°C and 1 minute at 60°C. PCR results were analyzed using Sequence Detection Systems software version 2.3 (Applied Biosystems).

**Luciferase assays:** Luciferase reporter plasmids were introduced into LE-HFF by electroporation using one of two methods, with 260 volts and 950 µFarad administered by Bio-Rad Gene Pulser II or by using program U-023 of the Nucleofector Device as described previously. For luciferase experiments with HCMV samples, cells were infected 24 hours post electroporation by Gene Pulser II and harvested at the indicated time points. For experiments not involving an HCMV infection, cells containing transfected plasmids were harvested 48 hours post transfection. To harvest samples, cells were washed one time in PBS before addition of 200 µl 1X reporter lysis buffer, part of the Luciferase Assay System (cat# E4030, Promega) or 1X Renilla luciferase assay lysis buffer, part of the Renilla Luciferase Assay System (cat# E2810, Promega). Cells were scraped into lysis buffer, transferred to a microcentrifuge tube and immediately frozen at -80°C. Cells were then thawed, centrifuged at 13,000 rpm (10,268 x g) for 10 minutes, 4°C in a Microfuge 22R Centrifuge with a S241.5 rotor and quantified as described above under protein quantification. Luciferase assays were performed by
adding equal amounts of protein, brought to a constant volume with the respective lysis buffer, to Luciferase Assay Substrate from either Promega's Luciferase Assay System or Renilla Luciferase Assay System. Samples were read for 10 seconds on Lumat LB 9501 Luminometer (PerkinElmer, Waltham, MA) and results were graphed in Microsoft Excel (Redmond, WA).

**Assembly compartment isolation:** LE-HFF plated in 15-cm dishes were either infected with HCMV-Towne-GFP at an MOI of 3 or left uninfected. At 60 hpi, one mock and one infected plate were treated with either SubAB or SubA_{A272}B. Plates were harvested at 72 hpi by removing all but approximately 10 ml of medium. The cells were scraped into the remaining medium, transferred to a 15-ml conical vial and spun down at 1100 rpm (~225 x g) at 4°C in Sorvall RT7 table top centrifuge. After centrifugation, the medium was removed and the cell pellet was washed 2 times in 1 ml PBS, using the same centrifugation conditions as above to pellet cells after each wash. After the second wash, the cell pellet was resuspended in 1 ml Tris-buffered isotonic saline pH 7.4 containing 5 mM EDTA and 0.25 M sucrose and passed 20 times through a 27-gauge needle. The samples were then centrifuged for 10 minutes at 4°C at 3500 rpm (744 x g) in a Microfuge 22R Centrifuge with a S241.5 rotor. The supernatant was applied to a sucrose step-gradient of 0.5 ml 2 M, 1.5 ml 1.6 M, 2.5 ml 1.4 M, 3.5 ml 1.2 M and 1.5 ml 0.8 M sucrose. The gradients were weighed, counterbalanced and centrifuged for 3 hours at 25,000 rpm (77,175 x g) and 4°C in a SW-41Ti rotor, L8-80M ultracentrifuge. Fractions were collected using Piston Gradient Fractionator (BioComp, Fredericton, Canada) at 0.3 rpm speed and 2.4 cm distance. About 30 fractions were collected from each sample and frozen at -80°C.
**Immunoprecipitation (IP) reactions:** Mock- or HCMV-infected LE-HFF were washed one time in 1X PBS at 96 hpi before addition of 1% Triton-X buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 1% Triton-X 100, 10% glycerol, 1 mM EDTA, 10 mM tetrasodium pyrophosphate, 100 mM NaF, 17.5 mM β-glycerophosphate, 1 mM PMSF, 8.5 µg/ml aprotinin, 1.25 µg/ml pepstatin (cat# 11359053001, Roche)). Cells were then rocked 5 minutes at 4°C, scraped into the Triton-X buffer and transferred to a microcentrifuge tube. After centrifugation at 13,000 rpm (10,268 x g) in a S241.5 rotor, Microfuge 22R Centrifuge, the supernatant was transferred to a new microcentrifuge tube, flash frozen in liquid nitrogen and stored at -80°C. Protein concentrations were determined as previously described.

For IP reactions with pp28, TRS1, KDEL, BiPY and UL50 antibodies, 20 µl of Dynabeads protein G (cat# 100-03D, Invitrogen) per sample were washed 2 times in wash and bind buffer (0.1 M sodium phosphate buffer, 0.1% Tween 20 pH 8.2). After each wash step and throughout the protocol, beads were separated from the supernatant by incubation on MagnaRack (cat# CS15000, Invitrogen) for at least 1 minute. Generally, 2 µg of antibody was then added per 20 µl of washed Dynabeads. Because the concentration of the TRS1 antibodies was unknown, 10 µl of each TRS1 antibody was combined with one tube of washed Dynabeads. The samples were brought to an equal volume with wash and bind buffer and then mixed gently on a nutator at RT for 30 minutes. Following three washes in 1X PBS, 150 µg of either mock or HCMV protein lysate was added and the samples were gently mixed on a nutator at RT for 30 minutes. The samples were washed 3 times in 1X PBS before addition of 20 µl 1X SDS loading buffer with 2X β-mercaptoethanol and incubation at 70°C for 10 minutes. After collecting the liquid by a quick centrifugation, the samples were separated on MagnaRack and transferred to a new tube. For input samples, 10 µl of
mock and HCMV lysates were added to 5 µl 3X SDS loading buffer. The above IP protocol varied depending on the antibodies and antibody lots used.

For immunoprecipitation reactions involving pUL50, the beads/antibody complex was cross-linked after incubation using the following protocol. The antibody conjugated beads were washed twice in conjugation buffer (20 mM sodium phosphate, 150 mM NaCl, pH 7.8) before addition of 5 mM BS$_3$ crosslinker (Bis(sulfosuccinimidyl) suberate). After incubation with gentle mixing on a nutator at RT for 30 minutes, the BS$_3$ was quenched by addition of 1 M Tris-HCl, pH 7.5, and left on nutator for 15 minutes. The beads were then washed 3 times in PBS-0.5% Tween 20 before addition of protein lysates and continuation with the IP protocol.

For analysis of IP, input and IP samples were either subjected to Western analysis as described above or analyzed by mass spectrometry. For mass spectrometry analysis, the gel was rinsed two times in ddH$_2$O following gel electrophoresis, and then fixed and stained in Coomassie Brilliant Blue (cat# 161-0400, Bio-Rad) diluted in 45% methanol and 10% acetic acid overnight at RT with gentle rocking. The gel was first destained at RT with gentle shaking in two washes of 45% methanol and 10% acetic acid and then changed to 12% methanol and 10% acetic acid, with periodic changes of the destain solution. Bands of interest were then excised from the gel and subjected to nano-reverse phase high performance liquid chromatography tandem mass spectrometry for identification. Peptide results were run against the National Center for Biotechnology Information non-redundant database.

**Dextran staining:** Mock- and HCMV-infected LE-HFF were loaded with dextran at 48 hpi. Cells were removed from the plate by trypsinization and pelleted at 1200 rpm (~250 x g) for 5 min at 4°C. The cell pellet was washed one time in PBS and one time in
hypertonic buffer (0.5 M Sucrose, 10% polyethylene glycol 1000, 20 mM HEPES pH 7.2) (246). The cells were then incubated 10 min at 37°C in hypertonic buffer containing 10 mg/ml TRITC-dextran 155 kD. After addition of 80 volumes of DMEM10, the cells were incubated an additional 10 minutes at 37°C. The cells were then washed two times in DMEM10 and plated on coverslips. At 72 and 96 hpi, the cells were stained with 4’6’-diamidino-2-phenylindole (DAPI), observed by microscopy on a Nikon Eclipse E600 and analyzed as described under the *Indirect Immunofluorescence* section.

**Microscopy**

*Electron microscopy (EM):* LE-HFF were plated in 6-cm plates and either infected at an MOI of 3 with HCMV-Towne-GFP or left uninfected. For BiP depletion samples, SubAB and SubA<sub>A272</sub>B were added to LE-HFF at 84 hpi and prepared for EM analysis 12 hours post treatment at 96 hpi. A description of BiP depletion using shRNA is described previously. These samples were prepared for EM analysis at 96 hours post HCMV infection (120 hours post lentiviral infection). To harvest samples for EM, cells were washed 1 time with PBS before addition of EM fixative (2.5% glutaraldehyde, 2% paraformaldehyde in sodium cacodylate). Cells were prepared for EM analysis by the Penn Biomedical Imaging Core. Cells were pelleted in microcentrifuge tubes and then postfixed with 1% aqueous osmium tetroxide (OsO<sub>4</sub>) for 1 hour. The cell pellets were dehydrated with ethanol and propylene oxide, embedded in epoxy resin, and polymerized at 65°C for 28 hours. Ultrathin (~80 nm thick) sections were cut with a diamond knife, mounted on single-slot grids, stained with uranyl acetate and bismuth, and examined with an FEI Tecnai T12 transmission electron microscope.
**Indirect Immunofluorescence (IF):** Coverslips containing either mock-infected LE-HFF or LE-HFF infected with HCMV at an MOI of 3 were prepared for IF at the indicated times post infection. For BiP depletion experiments, SubAB and SubA<sub>272B</sub> were added 12 hours before harvest. For dynein studies, cells were electroporated at 24 hpi using Nucleofector device as described above and harvested at 96 hpi. Cells were prepared for IF analysis by washing three times in 1 ml 1X PBS before fixing in 1 ml 4% paraformaldehyde at RT for 20 minutes. Cells were then washed three times in 1 ml PBS and stored in PBS at 4°C until used for staining.

Cells were prepared for incubation with antibodies by permeabilizing in PBS containing 0.5% Triton X-100 and blocking in PBS containing 5% BSA and 0.5% Tween 20. Alternatively, cells were blocked with PBS containing 10% human or goat serum (cat# H4522 & G6761, Sigma-Aldrich). Cells were incubated with primary antibodies diluted in blocking buffer; optimal antibody dilutions were determined based on manufacturer’s recommendations. Following primary antibody incubation, cells were washed three times for 5 minutes in PBS and then incubated with secondary antibody diluted 1:100 in blocking buffer for 30 minutes at RT. Following three 5 minute washes in 1 ml PBS, cells were rinsed in ddH<sub>2</sub>O and mounted on slides (cat# 12-550-343, Fisher Scientific, Pittsburgh, Pa) with Vectashield mounting medium containing DAPI (cat# H-1200, Vector Laboratories, Burlingame, CA). Slides were examined on a Nikon Eclipse E600 or Leica DM 6000 Confocal microscope. Micrographs were acquired, processed by deconvolution, and analyzed using Image-Pro 6.3 and Autoquant X2 software (MediaCybernetics, Bethesda, MD) or Leica Confocal Software (Leica Microsystems, Wetzlar, Germany).

For live-cell microscopy, HCMV-infected cells were electroporated with plasmids expressing lamin A fused to GFP and pp28 fused to DsRed at 2 hpi. Following
electroporation, cells were plated in Lab-Tek II chambered coverglass slides (cat# 155379, Nalgene). Images were acquired on an Olympus IX71 inverted microscope and analyzed using Volocity (PerkinElmer). For BiP depletion experiments, samples were treated with 100 ng/ml SubAB or SubA_{272}B immediately before analysis.
CHAPTER 3: HCMV upregulates the ER chaperone BiP

As mentioned in Chapter 1, the levels of the ER chaperones BiP and GRP94 are increased during HCMV infection. The experiments in this chapter detail the kinetics of BiP induction during infection at the level of both protein and mRNA. Furthermore, during infection the activation of two of the UPR transcription factors responsible for inducing ER chaperones in response to stress, XBP1 and ATF6, is inhibited (136). Although ATF4, another UPR transcription factor that induces transcription of BiP, is expressed during infection, its activation appears to succeed that of BiP (136). These observations suggest that HCMV must use an alternative method for inducing ER chaperones such as BiP. The experiments in this chapter will investigate the mechanism of HCMV-mediated expression of BiP.

**HCMV increases BiP protein during infection**

To determine the kinetics with which BiP protein is increased during an HCMV infection, LE-HFF grown to confluence were infected with HCMV Towne strain at an MOI of 3. Cell lysates were collected from infected cells at 4, 8, 12, 24, 36, 48, 60, 72 and 96 hpi. Cell lysates were also prepared from mock-infected cells left untreated or treated for 48 hours with thapsigargin, which induces UPR activation and BiP expression. The lysates were analyzed by Western analysis using antibodies that detect epitopes at both the N- and C-termini of BiP. Both antibodies show that BiP protein levels are significantly increased as early as 36 hpi, peak at 60-72 hpi, and decrease by 96 hpi (Figure 6). The increase in BiP approaches the level seen after the UPR has been robustly activated by treatment with thapsigargin for 48 hours. To coordinate the increase in BiP with the progression of the infection, antibodies against the following viral
Figure 6. HCMV temporally regulates BiP protein levels

Proteins were harvested from mock- and HCMV-infected LE-HFF at the indicated times post infection or from uninfected cells treated for 24 h with thapsigargin (Thaps). Thirty micrograms of extracted protein from each sample were analyzed by Western analysis. BiP was detected using antibodies directed to the C-terminus (BiP-C) and N-terminus (BiP-N). Antibodies directed against the MIEPs (IE86 and IE72), an early protein p52 (UL44), and a late protein p28 (UL99) were used to monitor viral protein expression. Actin was used as a loading control. Copyright © American Society for Microbiology, Journal of Virology, 82, 2008, 32, 10.1128/JVI.01881-07.
proteins were used: the HCMV MIEPs (IE72 and IE86), an early protein, p52, and a late protein, p28 (Figure 6). The peak expression of BiP correlates with the expression of proteins during the late phase of infection, a time of abundant viral protein synthesis, many of which traverse the ER.

**HCMV infection increases BiP mRNA levels**

To determine if the HCMV-induced increase in BiP protein involves increased BiP mRNA levels, total mRNA was harvested from mock-infected fibroblasts and fibroblasts infected with HCMV at 4, 8, 12, 24, 36, 48, 60, 72 and 96 hpi. Quantitative-PCR showed an increase in BiP mRNA as early as 8 hpi. This increase was sustained throughout infection, peaking at a level three times greater than mock between 36 and 72 hpi before decreasing slightly at the later times during infection (Figure 7A). The unexplained decline in BiP RNA at 24 hpi is quite repeatable. The peak level of BiP mRNA during infection corresponds with the peak in BiP protein. However, the peak level of BiP mRNA was much less than after treatment with the UPR inducer thapsigargin for 24 hours, which resulted in greater than 100 fold induction (Figure 7B). These data suggest that although increased BiP protein levels in HCMV-infected cells correspond to an increase in BiP mRNA, other mechanisms may be involved as well.

**HCMV activates the BiP gene promoter in an ERSE-independent manner**

During UPR activation, increased BiP transcription results from activation of the BiP gene’s ERSE-containing promoter. Thus, activation of the BiP promoter during infection was investigated using luciferase reporter plasmids, where the luciferase gene was under the control of either the wild type BiP promoter (wt-BiP-Luc) or a mutant BiP promoter (mut-BiP-luc) which lacked the three ERSEs (402). After electroporation of the reporter plasmids,
Figure 7. HCMV temporally regulates BiP mRNA levels

(A) cDNA was generated from total RNA harvested from mock (M)- or HCMV-infected LE-HFF at the indicated times post infection and subjected to quantitative-PCR analysis using primers specific for BiP and GAPDH. BiP mRNA was normalized to GAPDH mRNA. (B) RNA was harvested from untreated (-) or thapsigargin (Thaps)-treated (+) LE-HFF and analyzed as described in A. Fold activation in A & B is relative to the mock sample, which was set to 1. Graphs show results from one of multiple experiments performed in triplicate. Error bars represent standard deviation of the triplicate samples from represented experiment.
LE-HFF were infected with HCMV. Increased luciferase levels from the WT-BiP-Luc plasmid is detected between 8 and 12 hpi; by 24 hpi luciferase levels are greatly increased (Figure 8A). The levels of luciferase produced from the mut-BiP-Luc promoter are similar to those from the WT-BiP-Luc promoter, suggesting that HCMV-induced activation of the BiP promoter does not require the ERSEs. Importantly, treating electroporated cells with thapsigargin, a robust activator of the UPR, increased luciferase levels in cells expressing the wt-BiP-Luc plasmid, but not the mut-BiP-Luc reporter (Figure 8A). This confirms that under the electroporation conditions, both reporter plasmids respond as expected to ER stress. It is important to note the discrepancy between the fold activation in BiP mRNA and promoter activation, as measured by luciferase assay, in thapsigargin treated samples. While the luciferase assay, which is dependent on the protein level of luciferase, is subject to the thapsigargin-induced UPR inhibition of translation initiation, the production and detection of BiP mRNA is not affected by this block. The data suggest that during HCMV infection the BiP gene promoter is activated by a viral-mediated mechanism that does not require the endoplasmic reticulum stress elements.

In the HCMV field there is a dogmatic belief that plasmid-borne promoters are readily and promiscuously activated by the HCMV MIEPs. Despite evidence refuting this belief, it persists (206). Thus to ensure the specificity of BiP promoter activation by HCMV, stable cell lines expressing the luciferase reporter from either the wildtype or ERSE-mutant BiP promoters were generated. Similar to the transient electroporation results, when LE-HFF stably expressing the luciferase plasmids were infected, an increase in luciferase is detected from both the wildtype and mutant promoters. Similar to the transient electroporation results, activation occurs between 8 and 12 hpi (Figure 8B). Treatment with the UPR inducer thapsigargin confirmed inducibility of the wildtype (WT) BiP promoter, but not the mutant promoter in response to ER stress (Figure 8B). This data confirms that the
Figure 8. HCMV activates the BiP promoter

(A) LE-HFF were electroporated with plasmids expressing luciferase under control of the WT BiP promoter (WT-BiP-Luc) or a promoter with mutations in the three ERSEs (mut-BiP-Luc). The cells were either infected with HCMV 24 hours post electroporation or treated with thapsigargin (Thaps) for 24 hours. Proteins were harvested at the indicated times post infection for luciferase analysis. Fold activation was determined by normalizing luciferase levels in infected/treated samples to levels in mock samples. (B) LE-HFF stably expressing WT-BiP-Luc or mut-BiP-Luc were mock-infected, HCMV-infected or treated with Thaps and analyzed for luciferase expression as described in A. Graphs show results from one of multiple experiments performed in triplicate. Error bars represent standard deviation of the triplicate samples from represented experiment.
BiP promoter is activated during infection and that the activation occurs independently of the ERSEs.

**The BiP promoter is not activated by incoming virions**

The increase in BiP mRNA levels by 8 hpi suggests that activation may be mediated by either proteins that enter with the virions, for example tegument proteins, or by the synthesis of immediate early viral proteins. To test if the BiP promoter is activated by incoming viral proteins, LE-HFF stably expressing luciferase under control of the BiP promoter were infected with either HCMV or UV-irradiated HCMV. UV irradiation damages the HCMV genome such that the virus can enter cells, release tegument proteins and initiate signaling pathways that prepare the cell for infection; however no gene expression occurs from the damaged DNA. Proteins were harvested for luciferase assays at 4, 8 and 24 hpi. UV-inactivation of HCMV was confirmed by detecting the HCMV MIEPs in the normal HCMV, but not UV-irradiated lysates at 24 hpi (Figure 9). Luciferase levels are increased in cells infected with normal HCMV at 24 hpi, indicative of the BiP promoter activation described above. In contrast, luciferase levels are not increased in cells infected with UV-inactivated HCMV (Figure 9). Western analysis confirmed successful entry of virus, showing equivalent levels of pp65, a prominent tegument protein, in cell extracts from both normal and UV-irradiated virus infections extracted at 4 hpi (Figure 9).

**The HCMV MIEPs activate the BiP promoter**

The above data show that the BiP promoter is activated prior to 24 hpi by newly synthesized viral immediate early proteins. To test this, plasmids expressing an array of HCMV immediate early and early proteins, MIEPs, TRS1, IRS1, pp71, pp65, pUL69 and
Figure 9. The BiP promoter is not activated by incoming virions

LE-HFF stably expressing the WT BiP promoter were infected with normal HCMV or HCMV irradiated with UV light (UV-HCMV). Protein samples were harvested for luciferase analysis at the indicated times post infection. Fold activation was determined by normalizing luciferase levels in infected samples to levels in mock samples. Graph shows results from one of multiple experiments performed in triplicate. Error bars represent standard deviation of the triplicate samples from represented experiment. The level of the viral MIEPs and tegument protein pp65 were detected by Western analysis in lysates from normal HCMV and UV-HCMV samples. Actin was used as a loading control.
pUL84, were co-electroporated with the WT-BiP-Luc reporter plasmid into LE-HFF. Figure 10A shows that only the MIEPs significantly activated the BiP promoter. The most abundant MIEPs are the 72 kD IE72 (also called IE1) and the 86 kD IE86 (also called IE2); therefore, plasmids expressing these proteins individually were co-electroporated with either WT-BiP-Luc or mut-BiP-Luc. Figure 10B shows that increased luciferase activity was detected by the introduction of either IE72 or IE86. Similar analysis using the mut-BiP-Luc reporter showed that promoter activation by IE72 and IE86 does not depend on the ERSEs, although there is some reduction in activation by IE86. This may be a result of the mutations in the CAAT elements of the ERSE since previous analysis has shown that IE86 has a preference for CAAT sites (206). In sum, the data suggest that the HCMV MIEPs can activate the BiP promoter by a mechanism that does not require the ERSEs.

Since the HCMV MIEPs may be non-specifically activating the reporter plasmids, MIEP activation of the BiP promoter was tested in the stable cell line expressing luciferase under control of the WT BiP promoter. Two plasmids expressing MIEP genomic DNA, as well as plasmids expressing the cDNA of either IE72 or IE86 were tested. All increased luciferase levels, suggesting activation of the BiP promoter (Figure 10C). A plasmid expressing GFP did not increase luciferase levels, suggesting that activation of the BiP promoter was specific for the viral MIEPs. Western analysis of the electroporated lysates confirmed the presence of the MIEP proteins (Figure 10). The above data suggest that the HCMV MIEPs activate the BiP promoter independently of the ERSEs.

**Knockdown of IE72 by siRNA impairs BiP promoter activation**

To confirm a role for the MIEPs in inducing BiP promoter activation during infection, one of the MIEPs, IE72, was depleted using an IE72-specific siRNA. The stable cell line expressing luciferase under control of the BiP promoter was electroporated
Figure 10. The HCMV MIEPs activate the BiP promoter

(A) A vector control plasmid or plasmids expressing the following viral proteins: MIEPs, TRS1, IRS1, pp71, pp65, UL69 and UL84, were co-electroporated into LE-HFF with WT-BiP-Luc and harvested 48 hours post electroporation for luciferase analysis. Fold activation was determined by normalizing luciferase levels to those of the vector control, set at 1. (B) WT-BiP-Luc and mut-BiP-Luc were co-electroporated with plasmids expressing genomic MIEP DNA or cDNAs for IE72 and IE86 and harvested 48 hours post electroporation for luciferase analysis as in A. (C) LE-HFF stably expressing luciferase under control of the WT BiP promoter were electroporated with plasmids expressing genomic MIEP DNA or cDNAs expressing IE72, IE86 and GFP. Protein lysates were harvested 48 hours post electroporation for luciferase and Western analyses. Graphs show results from one of multiple experiments performed in triplicate. Error bars represent standard deviation of the triplicate samples from represented experiment. Actin was used as a loading control for Western analysis.
with siRNA against IE72 or GFP. Western analysis shows that IE72 was significantly, but not completely, depleted in cells containing the siRNA targeting IE72 (Figure 11A). Analysis of luciferase levels from protein lysates shows the increase in luciferase levels reported earlier that occurs in HCMV-infected cells. This increase was reduced in cells with a depleted level of IE72, when compared to cells treated with the control siRNA against GFP (Figure 11A), confirming a role for IE72 in BiP promoter activation.

To further investigate a role for IE72 in activating BiP, mRNA was harvested from mock- or HCMV-infected cells treated with siRNA against IE72 or GFP. Similar to the luciferase results, the level of BiP mRNA is reduced in cells treated with an siRNA against IE72 (Figure 11B). The reduction in BiP mRNA is not as large as the reduction seen in the luciferase assay, but the IE72 depletion is less, as shown by Western analysis (Figure 11B). The above data confirm a role for IE72 and the MIEPs in inducing BiP transcription during infection.

**HCMV activates translation from the BiP IRES**

The level of BiP mRNA is increased during HCMV infection; however the increase does not correlate with the significant increase in BiP protein. This suggests that HCMV may utilize additional methods for increasing BiP protein. BiP mRNA contains an IRES in the 5’ UTR, a feature HCMV may utilize for increasing BiP protein during infection. To investigate the activation of the BiP IRES by HCMV, a dual luciferase plasmid was generated that expresses *Renilla* luciferase under control of the HCMV promoter and firefly luciferase under control of first 221 nucleotides of the BiP transcript, the 5’ UTR of BiP, which contains the BiP IRES (Figure 12A). A stable stem-loop structure inserted after the *Renilla* luciferase ORF reduces activation of firefly luciferase by ribosomal scanning (366).
Figure 11. Depleting IE72 reduces BiP promoter activation and mRNA

(A) Mock- and HCMV-infected LE-HFF stably expressing luciferase under control of the BiP promoter were electroporated with siRNA against IE72 or GFP at 2 hpi. Protein samples were harvested for Western and luciferase analyses. Fold activation was relative to Mock samples. (B) RNA was harvested from HCMV-infected LE-HFF and analyzed by quantitative-PCR using primers against BiP and GAPDH. BiP mRNA was normalized to GAPDH mRNA. Fold increase is relative to Mock sample. Graphs show results from one of multiple experiments performed in triplicate. Error bars represent standard deviation of the triplicate samples from represented experiment. Protein was harvested concurrently with RNA for western analysis to monitor knockdown of IE72. For western analysis, actin was used as a loading control.
LE-HFF were electroporated with the BiP IRES dual-luciferase plasmid, or a vector control plasmid that did not contain the BiP IRES. The cells were mock-infected or infected with HCMV 24 hours post electroporation. Protein was harvested for luciferase analysis at 8 and 24 hours post infection. Figure 12B shows the levels of both Renilla and firefly luciferase expressed from either the BiP IRES or control plasmid. Luciferase levels were normalized to mock levels, which were set at 1. The level of Renilla luciferase, representing transcript level, was comparable between the two plasmid samples at all time points. In contrast, although the level of firefly luciferase was comparable between the two samples at 8 hpi, the level was significantly greater in the presence of the BiP IRES when compared to the vector control at 24 hpi. This is represented graphically in Figure 12C, which shows the ratio of firefly to Renilla luciferase for both plasmids in either mock-infected samples or HCMV-infected samples at 8 and 24 hpi. This suggests that BiP IRES activity is activated during an HCMV infection. The activation of IRES activity by 24 hpi correlates with the increase in BiP protein, which also begins to increase by 24 hpi (Figure 6).

As mentioned in Chapter 1, several cellular proteins bind and regulate the BiP IRES. One cellular protein of interest was La autoantigen, also called SSB, due to its activation during HCMV infection. Western analysis shows that SSB is increased beginning at 12 hpi, peaks at 24 hpi, and declines slightly thereafter before maintaining a constant level during the late times of infection (Figure 12D). The increase in SSB from 12-24 hpi correlates with both the onset of BiP IRES activity and the increase in BiP protein, implicating it as a potential regulator of BiP IRES activity during infection.

To investigate the role of SSB in regulating BiP IRES activity during HCMV infection, mock- or HCMV-infected LE-HFF were electroporated with the BiP IRES dual-luciferase plasmid and either an siRNA targeting SSB, or a scrambled (SCR) non-specific siRNA. Proteins were harvested for luciferase assays from the mock- and
Figure 12. HCMV activates translation from the BiP IRES

(A) Schematic of dual-luciferase plasmid generated by inserting the 221 nucleotide 5’UTR of BiP (BiP IRES) into pDL-N (vector) (B & C) LE-HFF expressing plasmids with Renilla luciferase under control of the HCMV promoter and firefly luciferase from either the BiP IRES or its vector control were mock- and HCMV-infected. Protein lysates were harvested at 8 and 24 hpi. Results are graphed as luciferase values at each time point set relative to Mock samples (B) and as the ratio of firefly to Renilla luciferase for each sample (C). (D) Protein from mock (M)- and HCMV-infected LE-HFF were harvested at the indicated times post infection. Western analysis was performed using antibodies against SSB and actin (loading control). (E) Mock- and HCMV-infected LE-HFF were electroporated with the dual-luciferase BiP IRES plasmid and siRNA against a control scrambled sequence (SCR) or SSB. Protein was harvested for luciferase and Western analysis at the indicated times post infection. Results are graphed as the ratio of firefly to Renilla luciferase for each sample. Western analysis confirms knockdown of SSB and a reduced level of BiP protein. Actin was used as a loading control. Graphs in A, C, & E show results from one of multiple experiments performed in triplicate. Error bars represent standard deviation of the triplicate samples from represented experiment.
HCMV-infected samples at 8 and 24 hpi. The ratio between *Renilla* and firefly luciferase is comparable between the mock-infected and 8 hpi samples (Figure 12E). In contrast, the ratio of *Renilla* to firefly luciferase is lower in the samples with reduced levels of SSB, when compared to the SCR control samples. Western analysis confirms both the knockdown of SSB and a reduction in BiP protein in the SSB siRNA treated samples (Figure 12E). Thus, the cellular protein SSB is at least one factor that activates the BiP IRES during infection.

The above data show that the ER chaperone BiP is induced during HCMV infection at both the level of transcription and translation. HCMV must specifically upregulate BiP, as cellular transcription factors normally responsible for BiP induction are not induced during infection. These results show that HCMV accomplishes this by activating transcription from the BiP promoter and by increasing BiP IRES activity. HCMV utilizes a unique mechanism to activate the BiP promoter which does not require the ERSEs or the transcription factors that are normally induced to activate transcription from the ERSEs. For translational regulation, HCMV enhances the utilization of the BiP IRES. At least one mechanism to accomplish this involves the upregulation of SSB/La autoantigen, which is induced early in infection.
CHAPTER 4: HCMV requires BiP for virion assembly.

The data and indicated figures from this chapter are published in the Journal of Virology in the following journal articles “Human Cytomegalovirus Specifically Controls the Levels of the Endoplasmic Reticulum Chaperone BiP/GRP78, Which is Required for Virion Assembly” (33) and “The Endoplasmic Reticulum Chaperone BiP/GRP78 Is Important in the Structure and Function of the Human Cytomegalovirus Assembly Compartment” (32).

Since HCMV specifically upregulates BiP during infection, the virus may require it to complete a successful replication cycle. BiP levels peak at a period of intense viral protein production and the onset of late protein synthesis and virion assembly; many late proteins traverse the ER to receive post-translation modifications. Thus, BiP may function in virion protein folding and assembly. To investigate the role of BiP during an HCMV infection, BiP was depleted using shRNA against BiP or the SubAB cytotoxin. The quick cleavage of BiP by SubAB provides a means to obtain a snapshot of BiP’s function at various times during infection.

**HCMV steady state protein levels are not altered when BiP is depleted**

LE-HFF were infected with HCMV and samples were harvested for protein analysis at 24, 48, 60, 72 and 96 hpi. At 2 hpi, 100 ng/ml SubAB or SubA_{A272B} were added and samples were harvested at 48, 72 and 96 hpi. Since it was feared that the viral infection might not get established in the absence of BiP and that extended BiP depletion would be deleterious to cells, a second strategy was utilized in which SubAB
Figure 13. Depletion of BiP does not alter steady state levels of viral proteins.

Proteins were harvested from HCMV-infected LE-HFF that were either left untreated (No Treatment) or treated with SubAB (WT Toxin) or SubA272B (Mut Toxin). The times (hours post infection) of toxin addition and protein harvest are given. Proteins were analyzed by Western analysis using antibodies that detect BiP or the viral proteins IE72, IE86, p52 and pp28 to monitor viral protein expression. Actin was used as a loading control. BCP, the BiP C-terminal cleavage product. Copyright © American Society for Microbiology, Journal of Virology, 82, 2008, 33, 10.1128/JVI.01881-07.
was added for a 12 hour period before harvest. Protein lysates following this treatment were harvested at 48, 60, 72 and 96 hpi.

Western analysis of the protein lysates showed a normal infection in the untreated samples, as shown by an increase in BiP and the onset of viral immediate-early (IE1 and IE2), early (p52) and late proteins (pp28) (Figure 13). Treatment with SubA_{A272}B did not alter steady state levels of BiP or of viral proteins. In contrast, BiP was cleaved in all samples treated with SubAB, whether treated at 2 hpi or for 12 hours before harvest, as shown by the disappearance of full-length BiP and the appearance of the 28 kD cleavage product (Figure 13). As anticipated for samples treated with SubAB at 2 hpi, both the levels of the major immediate early proteins and the early protein p52 were significantly reduced. The late protein pp28 was not detected, suggesting that the infection did not proceed to the late phase in the absence of BiP. Interestingly, treatment with SubAB for 12 hour periods did not significantly reduce the steady state levels of the MIEPs, p52 or pp28 (Figure 13). Therefore, depletion of BiP during an established infection does not significantly affect the steady state levels of viral proteins.

**BiP is required for infectious virion formation**

Although BiP depletion did not alter viral protein steady state levels in an established infection, HCMV may induce and require BiP to produce infectious virions. To test this hypothesis, a viral growth curve was generated where both cell-free and cell-associated virus were collected from infected samples that were either untreated, or treated with SubAB or SubA_{A272}B as described above. In the untreated samples, production of infectious virions initiated between 24 and 48 hpi. Viral titers peaked at 72 hpi before reaching a plateau at the later times during infection (Figure 14A). As expected, treatment with SubA_{A272}B did not alter infectious virion production. In contrast,
Figure 14. HCMV requires BiP to produce infectious virions

(A) LE-HFF were infected with HCMV, and infectious virions were harvested at the indicated times post infection (Normal Growth Curve). Virus was also harvested at 48, 72, and 96 hpi from infected LE-HFF that had been treated with either SubAB (Toxin) or SubA<sub>A272B</sub> (Mutant Toxin) at 2 hpi. Viral titers were determined using the TCID<sub>50</sub> method.

(B) Growth curves were performed as described for panel A, except that SubAB was added 12 h before the harvesting of virus at 48, 60, 72, or 96 hpi. Copyright © American Society for Microbiology, Journal of Virology, 82, 2008, 34, 10.1128/JVI.01881-07.
no infectious virions were produced at any time during infection when SubAB was added at 2 hpi, consistent with the Western analysis that showed late protein synthesis did not occur. BiP depletion is toxic to cells and the inability of cells to produce infectious virions may be a consequence of cells not being healthy enough to support a viral infection. To circumvent this, a different treatment strategy was used in which SubAB was added 12 hours before harvest. This approach allowed the infection to get established before BiP depletion. Interestingly, infectious viral titers were decreased after addition of SubAB for 12 hours at any point during an infection (Figure 14B). This result suggests a role for BiP in the production of infectious virions.

**BiP is required for the cytoplasmic activity of HCMV**

Since the data indicate that BiP plays a role in virion formation without affecting the steady state levels of viral proteins, electron microscopy was performed to further analyze BiP’s role during an HCMV infection. Mock- and HCMV-infected LE-HFF were harvested at 96 hpi after treatment with 100 ng/ml SubAB or SubA\textsubscript{A272}B for 12 hours. A fibroblast treated with SubA\textsubscript{A272}B appeared normal and was indistinguishable from an untreated mock fibroblast (Figure 15A). In contrast, an uninfected fibroblast treated with SubAB showed obvious cytotoxic effects, indicated by the formation of secondary lysosomes and autophagic vesicles (Figure 15B&C). This is predictable since BiP depletion has been shown to be deleterious for cells (270). Similar to what was observed with SubA\textsubscript{A272}B treatment of mock cells, treatment with SubA\textsubscript{A272}B had no effect on HCMV-infected cells (Figure 15D). The infected fibroblasts treated with SubA\textsubscript{A272}B resembled the infected fibroblasts that were untreated. An abundance of viral cytoplasmic activity was apparent as demonstrated by tegument bodies and maturing virions. Virions that had completed cytoplasmic maturation and had egressed from the
Figure 15. Treatment with SubAB abolishes HCMV cytoplasmic activity

Mock-infected (A to C) and HCMV-infected (D to F) LE-HFF were treated with either SubA\textsubscript{A272B} (mutant toxin) (A and D) or SubAB (WT toxin) (B, C, E, & F) at 84 hpi and were harvested at 96 hpi for analysis by EM. White arrows in E & F point to nucleocapsids in the nucleus. Copyright © American Society for Microbiology, Journal of Virology, 82, 2008, 35, 10.1128/JVI.01881-07.
cell were present in the extracellular space. Nucleocapsids were also present in the nucleus.

In contrast, treatment with SubAB abolished all of the cytoplasmic and extracellular viral activity, suggesting the viral cytoplasmic events involved with virion formation cease when BiP is depleted (Figure 15E&F). This is in accordance with the growth curve data which shows that infectious virions are not produced when BiP is depleted. However, nucleocapsids were present in the nucleus, indicating that a viral infection had initiated. Interestingly, the infected cells also did not show the cytotoxic effects of SubAB treatment seen in mock-infected cells. Specifically, the cytoplasm was relatively quiet and secondary lysosomes and autophagic vesicles were not present. This suggests that the alterations made to the cellular physiology by the viral infection protect the cell against the cytotoxic effects of the toxin.

To verify that the above results are in fact a phenotype of BiP depletion and not a result of a yet unidentified effect of SubAB, an alternative strategy was used to deplete BiP during infection. BiP expression was silenced using a shRNA introduced into fibroblasts using a lentiviral vector. shRNAs silence gene expression by forming a tight hairpin that is processed by the RNA interference machinery into a 21-nucleotide RNA that recognizes target mRNAs (260). A set of 3 shRNAs targeting BiP were tested for BiP knockdown. LE-HFF were infected with lentivirus expressing the BiP shRNA that exhibited the best BiP knockdown, or a control shRNA against green fluorescent protein (GFP). The cells were infected with HCMV 24 hours post lentiviral infection. Due to the long half life of BiP, the peak of BiP knockdown, 10-20% of normal levels, was not achieved until 48 to 72 hours after infection with HCMV. This allowed the viral infection to get established before BiP depletion, in contrast to cells treated with SubAB in which BiP was depleted almost immediately. Electron microscopic analysis revealed that
Figure 16. Depletion of BiP by shRNA mimics BiP depletion by SubAB toxin.

LE-HFF were infected with HCMV 24 hours after infection with lentivirus vectors expressing shRNA against GFP (control shRNA, A & B) or BiP (C to F). The cells were harvested at 96 hours post-HCMV infection for analysis by EM. White arrows indicate the presence of nucleocapsids in the nucleus. Copyright © American Society for Microbiology, Journal of Virology, 82, 2008, 36, 10.1128/JVI.01881-07.
Figure 17.  Nucleocapsids stall outside of the nucleus in the absence of BiP

LE-HFF were infected with HCMV, treated with either SubAB (WT toxin) (A, B, & C) or SubA\textsubscript{A272}B (Mut. toxin) (D & E) at 84 hpi, and harvested at 96 hpi for analysis by EM. White arrows in panels A to C show accumulation of virion particles outside of the nuclear membrane. White arrows in panels D and E indicate the nuclear membrane. Copyright © American Society for Microbiology, Journal of Virology, 82, 2008, 37, 10.1128/JVI.01881-07.
HCMV-infected cells depleted of BiP by shRNA had a similar phenotype to cells depleted of BiP by SubAB (Figure 16C,D,F). The cells had reduced cytoplasmic activity and nucleocapsids were present in the nucleus. Importantly, cells infected with the control GFP shRNA showed normal viral cytoplasmic activity (Figure 16A&B). The similar phenotype between the BiP shRNA cells and the SubAB treated cells confirms that depleting BiP inhibits viral cytoplasmic activity.

The above results suggest a role for BiP in either nuclear egress or the cytoplasmic events of tegumentation, envelopment and egress. Closer examination of the EM sections revealed that in some SubAB-treated cells, depending on how the samples were sectioned, cytoplasmic nucleocapsids were lined up just outside the outer nuclear membrane (ONM) (Figure 17). These nucleocapsids appear to have stalled in the maturation and egress process. This heavy concentration of nucleocapsids on one side of the nucleus is not detected at any time in cells treated with SubA_{A272}\text{B}. Therefore, after egressing from the nucleus in the absence of BiP, and upon the cessation of viral cytoplasmic activity, nucleocapsids are stalled outside the nuclear membrane and all further cytoplasmic viral progression is halted.

**BiP is relocalized to the viral cytoplasmic assembly compartment**

To further investigate BiP’s role during an infection, the localization of BiP was examined using indirect immunofluorescence microscopy. Localization of BiP was performed with seven different commercially available antibodies (Table 1). Each antibody detected BiP in its normal ER localization in mock-infected cells. Staining of HCMV-infected LE-HFF revealed two distinct BiP localizations (Figure 18). Antibodies directed against epitopes generated from full-length BiP (BiPA), the N-terminus of BiP (BiPS) or a large portion of the C-terminus (BiPH, BiPY), detected BiP in a perinuclear,
Mock- or HCMV-infected LE-HFF were prepared for immunofluorescence microscopy at 96 hpi. BiP (red) was detected using the following antibodies described in Table 1: BiPA (A), BiPS (S), BiPH (H), BiPY (Y), BiPC (C), KDEL (BiPK, K) and BiPR (R). Normal rabbit IgG (IgG) was used as a control. Nuclei were stained with DAPI (blue). Copyright © American Society for Microbiology, Journal of Virology, 83, 2009, 11423, 10.1128/JVI.00762-09.
cytoplasmic compartment. The location of the compartment next to the kidney-shaped nucleus suggests that this could be the viral cytoplasmic assembly compartment described in Chapter 1 (302). In contrast, antibodies with epitopes at the C-terminus (BiPH and BiPY) also detected BiP in a second localization, often found at the periphery of the cells. These regions looked condensed or clumped compared to the cytoplasmic localization of BiP in mock-infected cells. Three additional antibodies, BiPC, BiPR and BiPK, detected these BiP-containing structures, but did not detect the perinuclear structure. All three of these BiP antibodies were directed against epitopes to the extreme C-terminus. The failure of these antibodies to detect BiP in the perinuclear compartment suggests that the extreme C-terminus of BiP is occluded in this compartment. Since the ER-retention signal is located at the C-terminus, BiP’s relocalization from the ER to this structure may occur by blocking this retention signal and consequently the epitope for these antibodies.

Using antibodies to detect the localization of proteins in HCMV-infected cells can be problematic since the virus encodes Fc receptors that interact with antibodies, particularly of rabbit origin (7). The antibodies that detect BiP in the perinuclear region (BiPA, BiPS, BiPH, BiPY) are all rabbit polyclonal antibodies, raising concern that the antibodies are interacting with the Fc receptors and incorrectly localizing BiP to the perinuclear structure. However, one rabbit polyclonal antibody, BiPR, did not detect the perinuclear structure. Furthermore, using equivalent staining conditions and exposure times, control normal rabbit IgG also did not detect the perinuclear structure (Figure 18). The structure could however be detected by increasing the exposure time and gain when examining the normal IgG staining. However, these controls may not be sufficient as HCMV Fc receptors have a higher affinity for some IgG subclasses than others (245). The differences in subclasses of the rabbit IgG’s could account for the differences in
staining. To remove the variability of rabbit antibody preparations, a mouse monoclonal antibody generated against full length BiP (BiPD) was used to stain mock and infected cells. The antibody was not recommended by the manufacturer for immunofluorescence studies, and accordingly, in mock-infected cells, BiP could not be detected in its typical ER localization (Figure 19A). In infected cells, however, staining in a perinuclear structure was detected. The staining was notably different from that seen with the rabbit antibodies (Figure 18 & Figure 19A). BiPD stained a perinuclear ring with a dot in the middle. This ring was similar to the rings of organelles that constitute the assembly compartment described in Chapter 1.

Although the results of staining with both BiPD and the rabbit antibodies suggest that BiP is associated with a perinuclear structure, the localization in this perinuclear structured differed between the rabbit antibodies and BiPD. To resolve this discrepancy, it was necessary to use the rabbit antibodies under a condition where the Fc receptors were blocked before addition of the antibodies. The previous experiments with the rabbit antibodies had been done with bovine serum albumin (BSA) as the blocking reagent, which would not be sufficient to block the HCMV Fc receptors. Previous studies of the perinuclear assembly compartment region have used goat serum for blocking HCMV-infected samples (67, 68, 302). However, goat IgG has been reported to have very little affinity for HCMV Fc receptors (7) and blocking with goat serum did not alter the staining of BiP in the perinuclear structure using the rabbit antibodies (Figure 19B). Since human serum binds well to the HCMV Fc receptors, it was utilized for blocking HCMV-infected samples. Interestingly, when blocked with HCMV-negative human serum, the rabbit antibody BiPS stained BiP in a perinuclear ring with a dot in the middle; the same staining pattern that was observed with the mouse monoclonal antibody BiPD. Blocking with human serum did not change the staining pattern of BiPR, which detected BiP in the
Figure 19. BiP colocalizes with pp28 in perinuclear ring

(A) Mock- and HCMV-infected LE-HFF were prepared for immunofluorescence at 96 hpi and stained for BiPD (red), a mouse monoclonal antibody. (B) HCMV-infected LE-HFF were prepared for immunofluorescence by blocking with 10% normal goat serum in PBS and stained for BiP (red) using BiPH (Table 1). (C) HCMV-infected cells were prepared at 96 hpi and stained for BiP (red) using BiPS and BiPR antibodies (Table 1) and either pp28 or gB (green). Colocalization of BiP and pp28 is indicated in yellow. Nuclei shown in panels A and B were stained with DAPI (blue). Comp., composite. Adapted from Figure 2 of reference 32.
condensed cytoplasmic regions at the periphery of the cell but not in any perinuclear region (Figure 19C). This result was similar to what was observed in cells blocked with BSA. Thus, blocking with human serum allows for the use of rabbit antibodies in immunofluorescence of HCMV-infected cells. These observations confirm that BiP is localized in two distinct regions, one of which is a perinuclear ring resembling a layer of the viral cytoplasmic assembly compartment.

To determine if the perinuclear ring of BiP was in fact part of the assembly compartment, infected cells were co-stained for BiP and viral proteins that localize to the assembly compartment, pp28 and gB. BiP did not colocalize with gB, which stained the space between the peripheral BiP ring and the central dot (Figure 19C). The peripheral ring of BiP did, however, colocalize with pp28 (Figure 19C). Not surprisingly, pp28 did not colocalize with the condensed cytoplasmic structures recognized by BiPD. These results show that the peripheral ring of BiP is in fact part of the assembly compartment, and that BiP colocalizes with the viral tegument protein pp28 in this ring.

To confirm the microscopic evidence of BiP’s localization to the assembly compartment, a non-microscopic approach was taken. A previous study reported that the assembly compartment could be isolated by sucrose gradient centrifugation (302). Western analysis showed a significant portion of BiP sedimented with both pp28 and gB, confirming the results obtained by microscopy (Figure 20). A control protein not reported to be associated with the assembly compartment, the cellular glycolytic enzyme phosphofructosekinase-1 (PFK-1), does not sediment with BiP and viral assembly compartment markers. These results provide further evidence that BiP is associated with the assembly compartment of HCMV.
Figure 20. BiP cosediments with pp28 and gB in a sucrose gradient

Lysates were prepared from infected cells treated with SubA\textsubscript{A272B} (Mutant Toxin) and sedimented on a discontinuous sucrose gradient as previously described (302). The gradient was fractionated from the top (300 µl/ fraction) and analyzed by Western analysis using antibodies against BiP, pp28, gB, TRS1 and PFK-1. AC, assembly compartment. Adapted from Figure 3 of reference 32.
**A ring of BiP forms concurrently with assembly compartment formation**

The above observations detected BiP in the assembly compartment late in infection at 96 hpi. To determine at what time during infection BiP enters the assembly compartment, coverslips containing LE-HFF were infected with HCMV and fixed in paraformaldehyde at 24, 36, 48, 60, 72 and 96 hpi. In addition to staining for BiP, coverslips were co-stained with pp28 to monitor assembly compartment formation throughout infection. Figure 21 shows that early in infection (24-36 hpi), BiP localization remains unaltered when compared to staining in mock-infected cells. At 48 hpi, large amounts of pp28 are detected outside the nucleus at what presumably will become the site of the assembly compartment (Figure 21). Although BiP is not yet detected in this region, the BiP-containing clumps at the cell periphery are now detectable (Figure 21). By 60 hpi, pp28 has coalesced into a round perinuclear structure. A distinct ring of BiP is beginning to form around the pp28 structure. At 72 and 96 hpi, the previously described ring of BiP with a central dot is present.

**BiP is localized to condensed ER in infected cells**

In addition to the ring around the assembly compartment, BiP is detected in condensed cytoplasmic structures recognized by BiPD. During an HCMV infection, BiP performs important functions in the ER; one of these is the downregulation of MHC1 through its interaction with the viral proteins US2 and US11 (117, 349). The cytoplasmic clumps of BiP at the periphery of the cell could be condensed ER, as normal organelles have been reported to be condensed at the cell periphery (254). This is likely a result of the abundance of viral activity in the cytoplasm; the cytoplasmic clumps don’t appear until after late protein synthesis and accumulation in the cytoplasm (Figure 21). To test if BiP colocalized with another ER marker in the condensed cytoplasmic region, HCMV-
Figure 21. Time course staining of BiP during an HCMV infection

At the indicated times post infection, cells were prepared for immunofluorescence microscopy and co-stained for pp28 (green) and BiP (red) using an antibody that sees BiP in the condensed cytoplasmic regions (BiPY, top panels) or the cytoplasmic assembly compartment (BiPS, bottom panels). Nuclei were stained with DAPI (blue). Comp, composite
Figure 22. The clumped cytoplasmic regions of BiP are condensed ER

HCMV-infected LE-HFF on coverslips were treated with SubA_{272}B (Mut Toxin) or SubAB (Toxin) at 60 hpi and fixed for immunofluorescence microscopy at 72 hpi. Coverslips were stained for BiP (red) and PDI (green). Nuclei were stained with DAPI (blue). Copyright © American Society for Microbiology, Journal of Virology, 83, 2009, 11425, 10.1128/JVI.00762-09.
infected coverslips treated with SubAB or SubA_{A272}B for 12 hours were prepared for immunofluorescence at 72 hpi. Detection of BiP using the BiPC antibody, which detects only the cytoplasmic clumps and not the perinuclear region, colocalized with protein disulfide isomerase (PDI), an established ER marker, in the condensed cytoplasmic regions (Figure 22). This suggests that these regions are condensed ER. Furthermore, the clumps disappeared in the SubAB treated cells, consistent with the EM data that viral cytoplasmic activity is abolished after toxin treatment and that the cytoplasm returns to a more normal appearance.

**BiP is required for assembly compartment integrity**

Our data show that BiP is induced by HCMV, relocalized to the viral cytoplasmic assembly compartment and required for the maintenance of viral cytoplasmic activity. These observations suggest that BiP may play an important role in the maintenance and/or formation of the assembly compartment. To test this, HCMV-infected cells that were either untreated, or treated with 100 ng/ml SubAB or SubA_{A272}B for 12 hours, were stained for the viral assembly compartment markers pp28 and gB. Intact assembly compartments were present in untreated cells or cells treated with SubA_{A272}B, as demonstrated by both viral protein markers (Figure 23A). In contrast, the integrity of the assembly compartment was disrupted in cells depleted of BiP by SubAB. This result suggests that BiP is important for maintaining the viral cytoplasmic assembly compartment.

To confirm BiP’s role in maintaining assembly compartment integrity, lysates from HCMV-infected cells treated with SubAB were subjected to the same sucrose gradient sedimentation described previously for SubA_{A272}B. The presence of BiP was not detected in any gradient fraction, indicating the successful depletion of BiP.
Figure 23. BiP is required for maintaining assembly compartment integrity

(A) At 60 hpi, HCMV-infected cells were treated with SubAB (Toxin), SubA_{A272} (Mutant Toxin) or left untreated (No Toxin). At 72 hpi, the cells were prepared for immunofluorescence microscopy, and assembly compartment integrity was examined by staining for gB or pp28 (green). Nuclei were stained with DAPI (blue). (B) Lysates from HCMV-infected LE-HFF treated with SubAB were subjected to sucrose gradient centrifugation and analyzed by Western analysis using antibodies against BiP, gB, pp28 and TRS1. AC, assembly compartment. Adapted from Figures 3 & 4 of reference 32.
Additionally, less gB and pp28 sedimented in the assembly compartment region, consistent with a disrupted assembly compartment structure. These observations suggest that BiP is necessary for maintaining the integrity of the assembly compartment, consistent with the observation that depleting BiP abolishes viral cytoplasmic activity and virion formation.

In the above experiments, assembly compartment integrity was analyzed after 12 hours of SubAB treatment. However, cleavage of BiP by SubAB in Vero cells occurs very rapidly, and can be detected as early as 20 minutes post SubAB treatment (270). To monitor the integrity of the assembly compartment after BiP depletion by SubAB, HCMV-infected LE-HFF were electroporated with plasmids expressing lamin A fused to GFP and pp28 fused to DsRed. At 96 hpi, samples were treated with 100 ng/ml SubAB or SubA_{272B}. Assembly compartment integrity was monitored by live-cell imaging using an Olympus IX71 inverted microscope. Micrographs were acquired every 10 minutes for 220 minutes.

In cells treated with SubA_{272B}, the assembly compartment remained intact throughout the entire period of observation (Figure 24). In contrast, the integrity of the assembly compartment in cells treated with SubAB was rapidly lost. At 30 minutes post SubAB treatment, pp28 was detected in a perinuclear location, although it was not in the compact assembly compartment observed in SubA_{272B} treated cells, indicating that the assembly compartment integrity was already compromised. By 220 minutes post treatment, pp28 had spread throughout the cytoplasm and a perinuclear region resembling an assembly compartment was no longer detectable. Interestingly, as assembly compartment integrity was lost, the HCMV-induced curvature to the nucleus, visualized by lamin A, was also lost and the nucleus appeared to return to a more normal morphology. The rapid onset of assembly compartment disruption represented
HCMV-infected LE-HFF were electroporated with plasmids expressing lamin A (green) and pp28 (red) tagged with the fluorescent markers GFP and DsRed, respectively. Micrographs were acquired on an Olympus IX71 inverted microscope every 10 minutes for 220 minutes.
in the above sample was variable among cells, which is likely dependent on the variation in the uptake of SubAB from cell to cell. However, in all cells monitored, assembly compartment disruption began within the observed 220 minute window. These data show that HCMV assembly compartment integrity is rapidly lost upon depletion of BiP and confirms a role for BiP in maintaining assembly compartment integrity.

**BiP interacts with the viral proteins pp28 and TRS1**

BiP is present in the viral cytoplasmic assembly compartment where it colocalizes with pp28 and is important for maintaining assembly compartment integrity. To determine if BiP and pp28 interact in the assembly compartment, immunoprecipitation assays were performed. Lysates from mock- and HCMV-infected LE-HFF were prepared at 96 hpi. Immunoprecipitations were done with antibodies against BiP, using BiPY or BiPK, and pp28. Western analysis showed an interaction between pp28 and BiP, detected by both the BiPY and pp28 immunoprecipitations (Figure 25A&B). The interaction between BiP and pp28 was also detected by others (Nat Moorman and Tom Shenk, personal communication). Interestingly, an antibody directed against BiP’s KDEL ER-localization signal, BiPK, did not precipitate pp28. Since BiPK does not detect BiP in the assembly compartment or coprecipitate pp28, the KDEL ER-localization sequence may be blocked when BiP is in the assembly compartment ring. This could potentially occur through an interaction with a viral protein, such as pp28. It is reasonable to propose that this signal becomes blocked during infection, allowing BiP to leave the ER and progress further down the secretory pathway into the assembly compartment.

A second viral protein also coimmunoprecipitated with BiP. An interaction between BiP and TRS1 was first detected by mass spectrometry and then confirmed by
Figure 25. BiP associates with pp28 and TRS1 during infection.

(A) Lysates from mock (M)- and HCMV (V)-infected LE-HFF were prepared at 96 hpi for immunoprecipitation with BiPY, an antibody that detects BiP in the assembly compartment, and the KDEL antibody, which does not detect BiP in the assembly compartment. Following immunoprecipitation, reactions were analyzed by Western analysis using antibodies against BiP, TRS1, and pp28. Western analysis of 20% of the input is also shown (Input). (B and C) Similar lysates from mock- and HCMV-infected cells were immunoprecipitated with anti-pp28, and analyzed by Western analysis for BiP (B) or TRS1 (C). A cross-reaction with light chains by the pp28 antibody is shown (LC) in panels B and C. Copyright © American Society for Microbiology, Journal of Virology, 83, 2009, 11426, 10.1128/JVI.00762-09.
Western analysis (Figure 25A). This interaction was interesting because both immunofluorescence and electron microscopy data show that cells infected with a TRS1-deficient HCMV and infected cells depleted of BiP show a similar intracellular phenotype (2, 23). Specifically, TRS1-deficient HCMV-infected fibroblasts have a disrupted assembly compartment and a lack of viral cytoplasmic activity. In sucrose gradient fractions, a small fraction of TRS1 is present in the assembly compartment region (Figure 20). Since TRS1 performs several functions during infection (2, 50, 106, 265, 292, 331), it is possible that only a fraction is dedicated to function with BiP. Alternatively, since the amount of TRS1 sedimenting with the assembly compartment fraction is less than what would be expected from the immunoprecipitation data, the various components of the assembly compartment may have different affinities and some may have been dissociated during purification and sedimentation. A suitable TRS1 antibody for immunoprecipitation was not available to confirm the interaction between BiP and TRS1, but TRS1 did coimmunoprecipitate with a pp28 antibody (Figure 25C). Overall, these data provide evidence for complexes containing BiP, pp28 and TRS1 in the assembly compartment. This complex is likely involved in maintaining assembly compartment integrity and viral cytoplasmic activity.

The experiments in this chapter show that the ER chaperone BiP, which is upregulated by HCMV, is required for a productive HCMV infection. Although BiP depletion does not affect the steady state levels of viral protein, infectious virions are not produced. In addition to its normal ER localization, BiP is also localized to the viral cytoplasmic assembly compartment, a key center of viral cytoplasmic activity. The integrity of this compartment is rapidly disrupted upon depletion of BiP, suggesting a key role for BiP in the cytoplasmic progression of immature virions. This is confirmed by EM
analysis, which shows that viral cytoplasmic activity ceases in the absence of BiP. The role of BiP in the assembly compartment is likely dependent on its interaction with the viral proteins pp28 and TRS1; the depletion of which exhibit similar phenotypes to BiP depletion. These experiments demonstrate a key role for BiP during HCMV virion maturation.
CHAPTER 5: HCMV-induced alteration of the nuclear architecture

The data from this chapter were submitted for publication to the Journal of Virology.

The assembly and egress of HCMV virions requires significant morphological alterations of both nuclear and cytoplasmic architecture. The relationship between the curvature and enlargement of the nucleus and the assembly compartment is unknown. However, they may be intricately connected since perturbation of the perinuclear placement of the assembly compartment results in the nucleus regaining normal shape (11, 107, 278). These same conditions abolish cytoplasmic viral activity. The previous experiments have shown that BiP is required for HCMV viral cytoplasmic activity and assembly compartment integrity. During BiP depletion, nucleocapsids either remain in the nucleus or are stalled just outside of the nucleus (Figure 17). This provides another example where perturbation of viral cytoplasmic activity affects activity in and around the nucleus. These observations suggest that factors involved with viral activity in the cytoplasm must communicate with factors involved in viral nuclear activity, such as the nuclear egress complex, setting up a highly interconnected assembly-egress continuum.

Evidence for an interconnected assembly-egress continuum and factors that link the assembly compartment to the nucleus was provided by live-cell imaging. Two hours after infection with HCMV, LE-HFF were electroporated with plasmids expressing lamin A fused to GFP and the viral protein pp28 fused to DsRed. At 54 hpi, samples were subjected to live-cell analysis using an Olympus IX71 inverted microscope. Pictures were taken every 15 minutes over a 20 hour time period. From 54 to 60 hpi, pp28 is found in a perinuclear region representing the nascent assembly compartment which is shared
Figure 26. Assembly compartment remains localized next to nucleus

HCMV-infected LE-HFF were electroporated with plasmids expressing lamin A (green) and pp28 (red) tagged with the fluorescent markers GFP and DsRed, respectively. Micrographs were acquired on an Olympus IX71 inverted microscope every 15 minutes for 20 hours.
between two nuclei. At 60 hpi, the nuclei begin to migrate apart from each other. The
distance between the nuclei gradually increases until 74 hpi, the last observed time point.
Interestingly, as the nuclei migrate apart from each other, the assembly compartment
maintains a constant association with each nucleus. This results in an elongated or
stretched assembly compartment (Figure 26). The constant association between the
assembly compartment with each nucleus provides further evidence of an intricate
relationship between factors involved in viral cytoplasmic and nuclear activity. Since BiP is
located in an outer ring of the assembly compartment, it is possible that BiP may be a factor
involved in the crosstalk between the assembly compartment and nucleus during an HCMV
infection.

**BiP interacts with the nuclear egress factor pUL50**

To investigate if BiP interacts with a component of the nuclear egress complex,
an immunoprecipitation reaction was performed using an antibody against the N-
terminus of BiP, which detects BiP in the assembly compartment. BiP was precipitated
from mock- and HCMV-infected lysates and the precipitated proteins were separated by
polyacrylamide gel electrophoresis. Protein bands were cut from the gel and identified
by liquid chromatography tandem mass spectrometry. One protein identified was the
viral protein expressed by the HCMV UL50 ORF (Figure 27A). pUL50 is an inner
nuclear membrane protein responsible for recruiting other nuclear egress factors, which
form a complex to phosphorylate and rearrange the nuclear lamins in preparation for
nuclear egress, as described previously in Chapter 1.

Confirmation of an interaction between BiP and pUL50 was complicated by the
lack of availability of an antibody against pUL50 suitable for IP and Western analyses.
To overcome this, a polyclonal antibody against pUL50 was generated in rabbits and
Figure 27. BiP interacts with nuclear egress factor pUL50

(A) Mass spectrometry results identifying a potential interaction between BiP and pUL50. Protein score is based on the calculated probability that the observed match between the experimental data and the database sequence is a random event. A score of 70 with at least 2 peptides is considered significant. (B) Proteins were harvested from mock (M)- and HCMV-infected LE-HFF at the indicated times post infection and analyzed by Western analysis using the newly generated antibody that detects pUL50. Actin was used as a loading control. (C) Mock (M)- and HCMV-infected lysates harvested at 96 hpi were subjected to immunoprecipitation (IP) analysis using antibodies that detect BiP or pUL50 and analyzed by Western analysis. Input represents 20% of protein lysates used for IP analysis.
affinity-purified using a peptide containing amino acids 152-170 of pUL50 (GPENEGEYNLLRELYAKK). The antibody was tested in Western analysis on protein lysates from mock- and HCMV-infected samples harvested at 4, 8, 12, 24, 36, 48, 60, 72 and 96 hpi. A 43 kD band is detected beginning at 24 hpi and increases in intensity throughout infection (Figure 27B). This band is consistent with the predicted molecular weight of pUL50. The induction of pUL50 by 24 hpi suggests that UL50 is an expressed temporally in infection as an HCMV early gene.

To confirm an interaction between BiP and pUL50, mock- and HCMV-infected protein lysates were subjected to immunoprecipitation and analyzed by Western analysis. Immunoprecipitation was performed using the newly generated antibody against pUL50 and an antibody against BiP, BiPY. Western analysis confirms the interaction between BiP and pUL50 (Figure 27C). BiP coprecipitates with the pUL50 antibody in the HCMV-infected lysate, but was not present in the mock-infected precipitate. Conversely, pUL50 coprecipitated with the BiPY antibody in HCMV-infected lysates. The interaction between BiP and pUL50 has since been detected by another laboratory (Don Coen, Harvard University, personal communication). BiP’s interaction with both pUL50 of the nuclear egress complex and pp28 of the assembly compartment suggests that it could be a potential candidate for a factor that is involved in intricately connecting viral cytoplasmic and nuclear activity.

**Lamins are not rearranged when BiP is depleted**

During infection, nuclear egress is believed to occur at sites of nuclear membrane infoldings (35, 65, 95, 263, 296, 311, 312). A major function of pUL50 and the nuclear egress complex is to rearrange the rigid nuclear lamina during infection, which gives the nuclear membrane the flexibility necessary to form membrane infoldings
and allow nucleocapsids to gain access to the inner nuclear membrane for egress (38). To visualize this lamin rearrangement, HCMV-infected LE-HFF on coverslips were prepared for immunofluorescence by using antibodies that detect lamin B and pp28. Slides were examined by confocal microscopy on a Leica DM 6000 Confocal microscope. Figure 28A shows the maximum projection Z-axis stack of the rearrangement of the nuclear lamins from the nuclear rim to a lamin structure set back into an interior plane of the nucleus. Importantly, the lamin rearrangement does not occur uniformly around the nuclear rim, but rather occurs only at the point of the nucleus in immediate proximity to the assembly compartment, visualized by staining for pp28. The panel on the far right is slightly enlarged and rotated forward and to the left to provide an alternate view of the lamin layers and emphasize their position set back from the nuclear periphery.

Since BiP interacts with pUL50, BiP may be involved in the rearrangement of the nuclear lamina. To test if lamin rearrangement occurs when BiP is depleted, HCMV-infected LE-HFF on coverslips were treated at 84 hpi with 100 ng/ml SubAB, 100 ng/ml SubA272B or left untreated. Samples were prepared for immunofluorescence at 96 hpi and lamin rearrangement was visualized by two-dimensional widefield fluorescence microscopy using a Nikon Eclipse E600. In mock-infected cells, lamin B staining is unaltered by treatment with SubAB or SubA272B (Figure 28B). In HCMV-infected cells, rearrangement of lamin B is detected in cells left untreated or treated with SubA272B. Furthermore, an intact assembly compartment is present in both of these samples as visualized by staining for pp28 (Figure 28B). In contrast, the assembly compartment integrity in cells treated with SubAB is disrupted, as described previously. Interestingly, the rearrangement of the nuclear lamina is also lost in SubAB treated cells. Thus, the presence of either BiP and/or an intact assembly compartment is required to maintain
Figure 28. Nuclear lamins are not rearranged with BiP is depleted

(A) HCMV-infected LE-HFF were prepared for immunofluorescence at 96 hpi and stained for pp28 (green) and lamin B (red). Slides were examined by confocal microscopy. The far right panel was enlarged and rotated as indicated by the white arrow. (B) Mock- or HCMV-infected LE-HFF were treated with SubAB (Toxin), SubA$_{272}$B (Mut.Toxin) or left untreated (No Toxin) at 84 hpi and prepared for immunofluorescence analysis at 96 hpi by staining with pp28 (green) and lamin b (red).
lamin rearrangement. This provides further evidence for a relationship between viral nuclear and cytoplasmic activity.

**BiP is involved in the phosphorylation of the lamins**

The rearrangement of the nuclear lamina in HCMV-infected cells is largely thought to be the result of lamin phosphorylation by two kinases, the viral kinase pUL97 and the cellular kinase PKC. Recently, serine 22 of lamin A and C has been shown to be phosphorylated during infection by the viral kinase pUL97 (107). This residue and the equivalent residue on lamin B, serine 16, are also important for lamin breakdown during mitosis (273, 378). To investigate the phosphorylation of lamins during infection, mock- and HCMV-infected protein lysates harvested at 4, 8, 12, 24, 36, 48, 60, 72 and 96 hpi were subjected to Western analysis using antibodies that detect total lamin A and C or lamin A and C phosphorylated on serine 22. Lamin A and C was used for the phosphorylation studies instead of lamin B, which was used in immunofluorescence, since a reliable antibody against phosphorylated serine 16 of lamin B is not available. A detailed time course shows that lamin A and C levels remain constant during infection (Figure 29A). This agrees with published data that show lamin levels late in infection are not increased when compared to mock-infected cells (38). In contrast, phosphorylation of serine 22 on lamin A and C is drastically increased during infection. This increase begins early, at 4 hpi, peaks at 24 hpi and shows a slow decrease until 96 hpi. Despite decreasing after 24 hpi, the level of phosphorylated lamin A and C late in infection is greatly increased compared to the mock-infected sample (Figure 29A). The early increase in phosphorylation of lamins A and C was surprising, especially considering the induction of pUL50 as an early protein (Figure 27B) and pUL53 as a late protein (65). One possibility is that a nuclear egress complex-independent
phosphorylation of lamin A and lamin C precedes the pUL50/pUL53 dependent lamin phosphorylation. Alternatively, pUL50, pUL53 and pUL97 have all been detected in purified virions and may function upon entry with incoming virions (65, 363).

Since BiP interacts with pUL50 and is required for lamin rearrangement, BiP may be involved in the phosphorylation of the lamins. To test BiP’s role in lamin phosphorylation, mock- and HCMV-infected samples were treated with 0.5 µM NGIC-1, 100 ng/ml SubAB or left untreated at 2 hpi and harvested for protein at 24 hpi. To study lamin phosphorylation at later times during infection, HCMV-infected samples were treated as described above at 72 hpi and harvested for protein at 96 hpi (Figure 13). NGIC-1 has been shown to inhibit both kinases implicated in lamin phosphorylation during infection, PKC and pUL97.

Western analysis using an antibody that detects phospho-serine 22 of lamin A and C shows the increase in the phosphorylation of serine 22 described previously at both 24 and 96 hpi (Figure 29B). As expected, treatment with the kinase inhibitor NGIC-1 significantly reduces this phosphorylation at both time points. Interestingly, the depletion of BiP with SubAB does not reduce phosphorylation of serine 22 at 24 hpi. This is not surprising as immunofluorescence of BiP at this point in infection shows that BiP remains in its typical ER localization (Figure 21). Late proteins, which include viral assembly compartment markers like pp28 or the nuclear egress factor pUL53, have not yet been synthesized. In contrast, the phosphorylation of serine 22 of lamins A and C is reduced in both the NGIC-1 and SubAB treated samples harvested at 96 hpi. Thus, depletion of BiP late in infection reduces phosphorylation of lamins A and C. At this time point during infection, BiP interacts with both pp28 and pUL50, is located in the assembly compartment and is required to maintain viral cytoplasmic activity. As is the
Figure 29. Depletion of BiP alters nuclear lamin phosphorylation

(A) Proteins were harvested from mock- and HCMV-infected LE-HFF at the indicated times postinfection and analyzed by Western blot analysis using antibodies that detect total lamin A and C and lamin A and C phosphorylated at serine 22. Actin was used as a loading control. (B) Proteins were harvested from mock- and HCMV-infected samples treated with SubAB (Toxin) or NGIC-1 and analyzed by Western analysis using antibodies that detect total lamin A and C, lamin A and C phosphorylated at serine 22, BiP and pUL50. Antibodies directed against the MIEPs, an early protein p52, and a late protein pp28 were used to monitor viral protein expression. Actin was used as a loading control. * Lighter exposures were used in these panels to maintain band integrity.
case with lamin rearrangement, lamin phosphorylation is dependent on BiP and/or an intact assembly compartment.

**Dynein is required for assembly compartment integrity and nuclear enlargement**

The early phosphorylation of the nuclear lamins during infection without the rearrangement of the nuclear lamina suggests that nuclear lamin phosphorylation alone is not sufficient for the rearrangement of the lamins during HCMV infection. Studies investigating the disassembly of the nuclear lamina during mitosis have similarly shown that lamin phosphorylation is not sufficient for nuclear lamina breakdown, but that breakdown of the nuclear envelope requires the molecular motor dynein. Dynein attaches to the nuclear membrane and moves toward the centromere, generating tension on the nucleus and folds in the inner nuclear membrane (16, 301). Thus, a modified version of nuclear envelope breakdown may be adapted by HCMV in which dynein is recruited to bind the nucleus and move toward the microtubule organizing center, located at the center of the assembly compartment, creating tension on the nucleus and causing the concave nuclear shape and membrane infoldings from which nucleocapsids egress. Intriguingly, dynein interacts with the HSV-1 protein UL34, the homologue of pUL50 (399). Furthermore, dynein is involved in protein trafficking and maintenance of the secretory system and may play a role in assembly compartment formation and/or maintenance. The previously described roles for dynein make it an intriguing factor to investigate in the relationship between viral nuclear and cytoplasmic activity.

In order to perform many of its cellular functions, dynein requires an interaction with dynactin via dynactin’s p150Glued subunit (147, 365). Overexpression of a p150Glued construct containing amino acids 217-548, termed CC1, affects a variety of motility
functions. CC1 lacks two N-terminal microtubule binding domains, but contains the binding domain for the dynactin intermediate chain. It is therefore believed that CC1 binds dynein directly and acts as a competitive inhibitor for the interaction between dynein and intact dynactin (282). To test if dynein has a role in the HCMV-induced nuclear alterations or in assembly compartment maintenance, HCMV-infected fibroblasts were electroporated with CC1 fused to the mCherry fluorescent tag at 24 hpi. Cells were prepared for immunofluorescence at late times during infection, 72 or 96 hpi. Antibodies against pp28 and gB were used to visualize the assembly compartment and nuclei were visualized with DAPI. In cells not expressing CC1, intact assembly compartments are present next to enlarged nuclei as shown with both pp28 and gB, typical of HCMV-infected cells (Figure 30). In contrast, cells expressing CC1-mCherry do not have an intact assembly compartment. Both gB and pp28 are dispersed throughout the cytoplasm in small speckles (Figure 30). Thus, dynein is required to maintain assembly compartment integrity. Furthermore, the nuclei of cells expressing CC1-mCherry are round and not enlarged; indicating that dynein and/or an intact assembly compartment is required for the viral-induced alteration to nuclear morphology during infection. Similar to what was observed in cells depleted of BiP, the loss of assembly compartment integrity was accompanied by the loss of viral-induced nuclear alterations.

Interestingly, one cell can be observed in Figure 30 in which expression of CC1-mCherry appears to be expressed at a low level. This cell contains an enlarged nucleus, even in the presence of CC1-mCherry. Furthermore, gB is not dispersed throughout the cytoplasm in clumps as seen in other cells, but is kept in proximity to the nucleus, although not in the typical assembly compartment ring. Since CC1-mCherry acts as a competitive inhibitor, it is reasonable to speculate that the lower expression of the inhibitor allows for some functional dynein to be present. The functional dynein
Figure 30. Dynein is required to maintain assembly compartment integrity

HCMV-infected LE-HFF electroporated with CC1-mCherry (red) were prepared for immunofluorescence at 72 or 96 hpi. White arrows show an uninfected cell, an infected cell expressing CC1 and an infected cell not expressing CC1, as indicated. Assembly compartment integrity was monitored using antibodies that detect pp28 or gB (green). Nuclei were stained with DAPI (blue).
complexes appear to keep gB in a perinuclear location, however they are not sufficient for maintaining the ring structure of the assembly compartment. Interestingly, the residual perinuclear viral activity appears to be sufficient to maintain the relationship between viral and cytoplasmic activity, resulting in an enlarged nucleus and allowing for viral-induced nuclear morphological changes to occur.

The nuclear periphery is altered in proximity to the assembly compartment

To further investigate the relationship between viral cytoplasmic and nuclear activity, mock- and HCMV-infected LE-HFF were prepared for electron microscopy at 96 hpi. The nucleus of the mock-infected cell shows a relatively uniform periphery around the nucleus, with the nuclear membrane backed by a darker region that is likely to represent the lamina and heterochromatin (Figure 31A). The periphery of the infected cell nucleus is however more varied. The darker region at the nuclear periphery is present around most of the nucleus. However, this dark region is absent from the membrane area adjacent to the assembly compartment, which appears thinner and likely represents an area of only nuclear membrane (Figure 31B). These data suggest that the nuclear periphery adjacent to the assembly compartment is significantly altered, probably by removal of heterochromatin and rearrangement of the lamina as seen by immunofluorescence (Figure 28A). Such thinning of the nuclear periphery adjacent to the assembly compartment provides further evidence of an intricate relationship between viral cytoplasmic activity and virus-induced nuclear alterations.

Closer examination of the nuclear membrane reveals additional differences between mock- and HCMV-infected nuclei. The inner and outer nuclear membranes in mock-infected cells are distinct and the perinuclear space between them is relatively uniform (Figure 31C). In infected cells, the membrane appears to lose its integrity. The
Figure 31. HCMV alters the nuclear periphery next to the assembly compartment

Mock - (A, C) and HCMV-infected LE-HFF (B, D, E) were prepared for EM analysis at 96 hpi. (A) A mock-infected cell showing the nucleus with a uniform nuclear periphery. (B) HCMV-infected cell showing an altered nuclear periphery (white arrow) in proximity to the assembly compartment (AC). (C) High magnification micrograph of a mock-infected cell highlighting the inner (INM) and outer (ONM) nuclear membrane (white arrows). (D) The ONM and INM (thick white arrows) of an HCMV-infected cell with the region of indistinct ONM (area between thin white arrows) (E) The perinuclear space of HCMV-infected cells is inconsistent and exhibits areas with large gaps between the INM and ONM (INM and ONM shown by white arrows).
outer nuclear membrane becomes indistinct and seems to disappear with the appearance of vesicles and nucleocapsids that have recently exited the nucleus, as seen in Figure 31D between the two thin white arrows. Additionally, the space between the inner and outer nuclear membranes is variable, with occasional large bulges (Figure 31E).

To document the increase in the perinuclear space, the distance between the inner and outer nuclear membranes in mock- and HCMV-infected samples was digitally measured in high magnification micrographs of the nuclear membrane. Using the micrographs in Figure 32, thirty measurements were obtained from regions of the nuclei where the inner and outer nuclear membranes could be readily detected. It is important to note that in infected cells, the regions containing an intact outer nuclear membrane may be minimally altered by infection and the increase in the perinuclear space may be underestimated. In mock-infected cells, the perinuclear space was measured to be 32 nm with a standard deviation of 7 nm (Figure 32). This space is increased in HCMV-infected cells to 44 nm with a standard deviation of 8 nm. The observed alterations in the nuclear membrane of infected cells and the increase in the perinuclear space may be necessary to provide the nuclear membrane with the flexibility required to form the membrane infoldings for nucleocapsid egress.

**HCMV downregulates the SUN-domain containing proteins**

In order to increase the perinuclear space, HCMV must regulate the cellular machinery involved in maintaining a constant distance between the inner and outer nuclear membranes. The Sad1/UNC84 homology (SUN) and Klarischt, Anc-1, Syne homology (KASH) domain containing proteins are involved in maintaining the perinuclear space by keeping the outer nuclear membrane in place around the inner nuclear
Figure 32. HCMV increases the perinuclear space

Mock- or HCMV-infected LE-HFF were prepared for EM analysis at 96 hpi. Thirty measurements of the perinuclear space were digitally taken on high magnification micrographs of the nuclear membrane.
membrane. In mammalian cells, two nuclear envelope SUN-domain proteins have been identified, SUN1 and SUN2. Both SUN proteins are inner nuclear membrane proteins that form homo- and heterodimers (59). The C-terminal domains are located in the perinuclear space and interact with the KASH-domain of the nesprin family of proteins. The nesprin proteins, recently reviewed by Wilhelmsen et al. (387), are very large proteins, up to a megadalton in size, that extend from the outer nuclear membrane into the cytoplasm where they interact with the cytoskeleton directly or through adaptor proteins via their N-terminal domains. Nesprins require the interaction with the SUN proteins to maintain outer nuclear membrane localization (59). Depletion of the SUN proteins is sufficient to alter the perinuclear space, creating large gaps between the inner and outer nuclear membranes (59). In the context of an HCMV infection, removal of the SUN proteins may be required to allow for the increase in perinuclear space and the formation of the nuclear membrane infoldings necessary for nucleocapsid egress.

To monitor the level of SUN proteins in HCMV-infected cells, protein was harvested from mock- or HCMV-infected LE-HFF at 4, 8, 12, 24, 36, 48, 60, 72 and 96 hpi and subjected to Western analysis using antibodies against SUN1 or SUN2. Figure 33A shows that during infection, SUN2 levels remain constant throughout the early times during infection until 24 hpi. By 36 hpi, SUN2 levels begin to decrease and continue until almost no SUN2 remains at 96 hpi. The decrease in SUN2 correlates with the onset of late protein synthesis and the beginning stages of assembly compartment formation (Figure 21). Western analysis of SUN1 shows that similar to SUN2, the level of SUN1 protein is also decreased by HCMV. SUN1 is not decreased as much as SUN2 and the decrease in SUN1 begins later in infection, between 48 and 60 hpi (Figure 33A). Therefore, HCMV decreases both SUN1 and SUN2 levels during infection and this
Figure 33. HCMV downregulates SUN proteins during infection

(A) Proteins were harvested from mock (M)- and HCMV-infected LE-HFF at the indicated times postinfection and analyzed by Western blot analysis using antibodies that detect SUN1 and SUN2. Actin was used as a loading control. (B) Mock- and HCMV-infected LE-HFF were prepared for immunofluorescence at 96 hpi and examined for SUN2 localization (green).
decrease correlates temporally with the formation of the assembly compartment and presumably, nucleocapsid egress.

Immunofluorescence analysis of SUN2 in mock- or HCMV-infected LE-HFF at 96 hpi also shows a reduction in SUN2 staining, confirming a decrease in SUN2 levels during infection. In mock-infected cells, SUN2 staining is present at the nuclear periphery in all cells. In HCMV-infected cells, this staining is lost in most cells (Figure 33B). Interestingly, in HCMV-infected cells that retain SUN2 staining, SUN2 is found at the site of lamin rearrangement. This suggests that when present, SUN2 may be rearranged with the nuclear lamina, removing it from the nuclear periphery and the inner nuclear membrane. Therefore, HCMV regulation of SUN1 and SUN2 correlates with the increase in the perinuclear space during infection.

**Nuclear membrane permeability is altered during an infection**

HCMV alters the nuclear periphery proximal to the assembly compartment by rearranging the rigid lamina layer and increasing the perinuclear space. These alterations, which may be necessary for nucleocapsid egress, would alter the rigidity of the nucleus and may affect nuclear properties such as permeability. To study nuclear permeability during infection, dextran labeled with TRITC was introduced into mock- or HCMV-infected cells. Dextran is a complex, branched polysaccharide made of glucose molecules. Dextran can be used for a variety of biomedical applications, including monitoring the permeability of cellular structures. Permeability is monitored by tracking the location of dextran polysaccharides of varying sizes under different cellular conditions. Dextran polysaccharides of 155 kD are excluded from the nucleus and were used to study the permeability of the nuclear membrane during infection.
Figure 34. HCMV alters nuclear membrane permeability

(A) Mock- and (B) HCMV-infected LE-HFF were loaded with dextran-TRITC (green) at 48 hpi. Cells were examined for the presence of nuclear dextran at 72 and 96 hpi. (C) At 96 hpi, z-axis slices were taken of an infected cell. Z-axis slices from top to bottom are shown left to right. The z-axis nuclei were stained with DAPI (blue).
Mock- or HCMV-infected cells were loaded with 155 kD dextran at 48 hpi. The status of nuclear dextran was investigated at both 72 and 96 hpi. In mock-infected cells, dextran is clearly excluded from the nucleus (Figure 34A). In HCMV infected cells, dextran also appears to be excluded from the majority of the nucleus. However, nuclear dextran can be detected in the region of lamin rearrangement next to the assembly compartment (Figure 34B). Thus, dextran can enter the nucleus proximal to the assembly compartment, suggesting the permeability of the nucleus is altered in this region.

It is not clear from the 2D pictures whether the dextran is really within the nucleus or concentrated above or below it. To clarify this, a Z-axis series was taken through an HCMV-infected cell showing an assembly compartment. Figure 34C shows four slices moving through the cell from top to bottom (left to right). In all cases the dextran is in the nucleus, not just at the top or the bottom. These data suggest that the nuclear membrane adjacent to the assembly compartment is modified such that the dextran can penetrate into the nucleoplasm. The alteration of nuclear permeability only in the region of the assembly compartment provides another piece of evidence linking the viral cytoplasmic activity to viral nuclear activity. Additionally, a viral-induced change in nuclear permeability may explain how a cytoplasmic factor, such as BiP, can interact with the nuclear egress complex to participate in the highly interconnected assembly-egress continuum.

The experiments in this chapter provide evidence that BiP and an intact assembly compartment are required for nuclear lamin phosphorylation and rearrangement, providing evidence that viral cytoplasmic and nuclear activity are intricately linked. The data also suggest a stable and elastic interaction between the
assembly compartment and the nucleus. Furthermore, alterations of the nuclear membrane in infected cells occur in the area adjacent to the assembly compartment. These alterations include rearrangement of the nuclear lamina, alteration and disruption of the outer nuclear membrane, and an increase in nuclear permeability to large molecules. In addition, the perinuclear space is also increased in infected cells, which is likely the result of the reduction in SUN proteins during infection. In summary, the data of this chapter provide evidence for an assembly-egress continuum, providing a means for nucleocapsids to egress the nucleus, access the assembly compartment and become mature virions.
CHAPTER 6: Discussion

Several characteristics of the human cytomegalovirus replication cycle make it conducive to activating cellular stress responses of its host cell. With its prolonged replication cycle, HCMV must keep its host functioning for an extended period of time. As the virus replicates, it must keep metabolic processes active under stress conditions that would normally shut them down, such as the exhaustion of key cellular resources and the overloading of cellular organelles. Thus, HCMV modulation of cellular stress signaling pathways is a critical step for successfully producing viral progeny. Growing evidence shows that HCMV infection modulates many cellular stress signaling pathways (50, 106, 169-171, 237, 369).

By circumventing the effects of cellular stress signaling, the virus maintains needed cellular metabolic and synthetic functions and avoids apoptosis. These data suggest that a major goal of the virus is to make the host cell ignore signs of severe stress. This is demonstrated in this thesis, where complete depletion of BiP during HCMV infection has very little adverse effect on HCMV-infected cells, though it is very deleterious to uninfected cells. Just as the viral infection can circumvent the inhibition of viral protein synthesis by stresses that activate the UPR, such as thapsigargin or hypoxia (135, 171), it can also maintain protein synthesis after the depletion of BiP by the SubAB toxin. In uninfected cells, hypoxia, thapsigargin and BiP depletion activate the unfolded protein response. Activation of the UPR effector PERK and the corresponding phosphorylation of eIF2α results in an inhibition of global translation. However, although both PERK and eIF2α are phosphorylated during infection, translation is not inhibited (136). HCMV can overcome the UPR-induced inhibition of translation.
The maintenance of translation is not the only aspect of UPR signaling modulated by HCMV. As mentioned earlier, although IRE1 is activated and ATF6 translocates to the Golgi during infection, cleavage of ATF6 and the splicing of XBP1 does not occur (136). These two transcription factors induce a wide range of factors that under normal conditions restore cellular homeostasis, but in the context of a viral infection could be detrimental. HCMV counteracts the detrimental effects of these targets by inhibiting the production of the transcription factors responsible for their induction. The virus must therefore selectively induce any factor with beneficial effects that it requires for infection. Accordingly, the ER chaperones BiP and GRP94 are both induced during infection (136). The experiments described in this thesis have focused on the specific viral induction of BiP, and the role that it plays in an HCMV infection.

In the studies presented here, the expression of the ER chaperone BiP is closely regulated during HCMV infection. This results in a temporally precise increase and decrease in BiP levels, which increase by 36 hpi, peak by 60 to 72 hpi, and decrease thereafter. During ER stress, the increased level of BiP protein correlates with increased transcription from the BiP promoter and a corresponding increase in BiP mRNA. During HCMV infection, the level of BiP mRNA is also increased. The increase in BiP mRNA occurs early in infection, beginning as early has 4 hpi and peaking from 12 to 60 hpi. The data suggest that the increase in BiP protein is, at least in part, due to an increase in BiP mRNA.

Since increases in BiP mRNA occur by both transcriptional and post-transcriptional mechanisms (200), the activity of the BiP promoter was investigated during infection. Activation of the BiP promoter occurs early in infection, between 4 and 8 hpi. This early activation of the BiP promoter corresponds with the increase in BiP mRNA. HCMV’s activation of the BiP promoter early in infection implicates either proteins that enter with
virions or the immediate early proteins. Data presented herein show that newly synthesized HCMV MIEPs, and not incoming virion proteins, activate the BiP promoter. Furthermore, this activation is independent of the ERSEs normally required for the stress induction of the BiP promoter.

Although the increase in BiP mRNA is sustained throughout infection, the three fold activation is minimal compared to the increase that occurs in response to ER stress. Treatment with thapsigargin results in a greater than 100 fold increase in BiP mRNA when compared to unstressed cells. This result suggests that HCMV may utilize additional mechanisms to obtain the robust increase in BiP protein. BiP protein levels can be increased by altering the stability of the BiP protein or by increasing the translation efficiency of BiP mRNA (102, 180, 357). The translational efficiency of BiP mRNA is regulated by the presence of an IRES, located in the 5’ UTR of the BiP mRNA (211).

A recent study has shown that a related virus, HSV, activates the BiP IRES during infection (300). Similarly, the studies presented herein show that HCMV also activates translation form the BiP IRES. Activation occurs between 8 and 24 hpi, the same time during infection that the increase in BiP protein is first detected. Several cellular proteins have been shown to activate the BiP IRES. Of interest is SSB, also called La autoantigen, which activates the IRES during both poliovirus and HCV infections (5, 224). Analysis of SSB protein during infection revealed that SSB is induced early in infection, peaking at 24 hpi, the same time that the BiP IRES is robustly activated. Accordingly, knockdown of SSB reduces BiP IRES activity and protein level at 24 hpi, confirming a role for SSB in activating the BiP IRES during HCMV infection.

The specific regulation of BiP by HCMV suggests that BiP may play a critical role during an HCMV infection. The peak of BiP protein levels in infected cells corresponds to a period of intense virion structural protein and glycoprotein synthesis/processing and virion
EM analysis of infected cells depleted of BiP using the SubAB toxin shows that viral activity in the cytoplasm ceases after only 12 hours. A similar result is seen in cells depleted of BiP using an shRNA. This is significant because it indicates that the toxin's effects on viral replication are due to the depletion of BiP and not to an uncharacterized effect of the toxin. It is also important to point out the differences between these two depletion approaches. The toxin depletes very rapidly and completely due to its cleavage efficiency. With the shRNA, depletion of BiP is slow and not as complete; the lowest levels of BiP occur 48 to 72 hours after lentivirus infection. Thus, HCMV, which is added 24 hours after the lentivirus vector, has sufficient levels of BiP to initiate the infection before the levels drop to the point where the effects on cytoplasmic egress can be seen. The fact that the same effect can be seen after only a few hours of toxin treatment indicates how quickly the loss of BiP can be manifested. This is particularly evident in live cell analysis of cells treated with toxin, in which the effects of BiP depletion is observed as early as 30 minutes after addition of toxin.

In addition to the lack of viral activity in the cytoplasm of BiP-depleted cells, nucleocapsids were also accumulated just outside of the outer nuclear membrane. This would suggest that in the absence of BiP, the nucleocapsids can depart from the nucleus, although we cannot rule out the possibility that this process may be debilitated. However, the accumulation outside of the outer nuclear membrane suggests that without BiP, movement away from the outer nuclear membrane is inhibited; correspondingly, the cytoplasmic processes of tegumentation, envelopment, and egress do not occur. These data suggest that HCMV precisely controls the expression of BiP, which aids in a number of functions needed by the virus, including cytoplasmic viral egress.

During infection, BiP is relocalized to two distinct cytoplasmic localizations. One pool is located in condensed structures in the periphery of the cytoplasm which also contain
other ER markers, thus appearing to originate from the ER. It is likely that this pool of BiP is performing its known ER functions as well as its virus-induced ER function of interacting with HCMV proteins US2 and US11, which mediates the degradation of major histocompatibility complex class I (117, 349).

The second structure containing BiP is the assembly compartment, which is a complex perinuclear structure. The assembly compartment has been reported to be a modified compartment made of many vesicles derived from the cellular secretory system, early endosomes, or the trans-Golgi network. It is not believed to be derived from the ER, ER-Golgi intermediate compartment, cis- or medial Golgi, or lysosomes. Components of the secretory apparatus become reoriented in the assembly compartment in conjunction with virion maturation (60, 302, 309, 351). In agreement with its role in virion maturation, the assembly compartment also contains many different virally encoded tegument, envelope, and nonstructural proteins (60, 122, 303, 309, 343). Microscopically, the assembly compartment has a cylindrical appearance with a height that is similar to that of the nucleus (68); its perinuclear location and the adjacent enlarged, kidney-shaped nucleus gives HCMV-infected cells a distinct morphology (311). It has been suggested that the many vesicles that make up the assembly compartment each hold tegument proteins which can be deposited onto nucleocapsids as they move through the space between the vesicles (68).

BiP’s localization in these two cytoplasmic compartments is detectable by different antibodies. Antibodies to the C-terminal end of BiP cannot detect or immunoprecipitate BiP localized in the assembly compartment. This is especially true for antibodies to the KDEL sequence at the very C-terminus of the protein, suggesting that C-terminal epitopes are blocked when BiP is in the assembly compartment. This is supported by the immunoprecipitation reactions in which antibodies against the C-terminus of BiP do not
coprecipitate pp28. Such blockage of the KDEL ER localization signal by a viral protein would explain how BiP can be diverted from its normal ER localization to the assembly compartment.

The data show that BiP is located in a ring at the outer edge of the cylindrical assembly compartment where it colocalizes with pp28, but not with gB. gB is located in an inner ring of the assembly compartment. The outer ring of BiP fits well with the current model that suggests that the assembly compartment is made up of concentric rings of vesicles, each with specific tegument proteins that are sequentially picked up by nucleocapsids as they move from the outside to the center of the assembly compartment (68). In this scenario, BiP's protein binding and chaperone functions could aid in depositing tegument proteins on nucleocapsids, a mechanism of action that is supported by the finding that BiP is in the tegument layer of purified virions (363).

The essential role that BiP plays once relocalized to the assembly compartment may explain the EM data which shows that viral cytopathic effects in the cytoplasm of infected cells disappears after depletion of BiP using either the SubAB toxin or short hairpin RNAs. A similar situation occurs in infections with HCMV containing a mutation in the TRS1 gene (2, 23), suggesting that BiP and TRS1 may have shared or coordinate functions. The TRS1 gene product has been reported to have several functions during HCMV infection. Transient expression analyses suggest that it is a transcriptional activator, and other studies have suggested that it promotes oriLyt-dependent DNA replication (264, 265, 292, 331). Additionally, as discussed earlier, both TRS1 and its homolog IRS1 prevent the phosphorylation of eIF2α (50, 106). More importantly for the BiP study is the suggestion that TRS1 has a role in virion assembly (2, 23). Our observations that BiP and TRS1 associate in the assembly compartment, and that a fraction of TRS1 copurifies with
the assembly compartment, suggest that there is a functional relationship between BiP and TRS1 which is needed for assembly compartment integrity.

In addition to an interaction with TRS1, BiP also interacts with pp28 in the assembly compartment. The pp28 protein (UL99 ORF) is a very abundant constituent of the tegument layer (227, 363); it is a true late protein that is myristoylated and phosphorylated (142, 155, 303, 323). It is essential for the production of infectious virions, and mutation of the UL99 open reading frame resulted in nonenveloped and noninfectious cytoplasmic particles (30, 142, 323). This phenotype is similar to what was seen in cells depleted of BiP. Thus, the association of BiP and pp28 appears to contribute to the same steps in virion maturation. Studies have demonstrated higher-molecular-weight forms of pp28 in infected cells, and both FRET assays and subcellular fractionation of infected cells suggested that pp28 multimerization occurs in the assembly compartment (308). Given BiP’s chaperone function, it is possible that the close association between BiP and pp28 contributes to the multimerization of pp28.

It has been suggested that the formation of the assembly compartment causes the condensation of other cytoplasmic structures (254). These studies noted that the normal organelles become located toward the periphery of the cell relative to the assembly compartment. This is similar to what we report by examining BiP’s ER localization in infected cells. Thus, there is a connection between the formation of the assembly compartment and the BiP-containing condensed ER structures. Although the mechanisms of assembly compartment formation and cytoplasmic ER condensation are unclear, our data suggest that the integrity of these structures is dependent on BiP, since BiP depletion causes both structures to dissociate. Furthermore, the HCMV-induced nuclear morphological changes characteristic of infection also disappeared upon BiP depletion, suggesting that events that occur in the cytoplasm are intricately linked to events that alter
the nucleus. All of this appears to be highly interconnected as part of an assembly-egress continuum that provides the means for nuclear nucleocapsids to egress the nucleus, become fully tegumented and move on into the cytoplasm for envelopment and cellular egress. The intricate relationship between viral activity in the cytoplasm and nucleus is demonstrated by live cell imaging, in which a single assembly compartment becomes distorted in order to remain in its association with two nuclei as the distance between the nuclei increases. The close association of the different aspects of the continuum is further supported by both data presented herein and existing data, suggesting that disruption of any one aspect of the continuum disrupts the entire process (2, 11, 167).

The process of assembly and egress of HCMV virions requires significant morphological alterations of the nuclear and cytoplasmic architecture. The manifestations of this include enlargement of the nucleus, formation of the kidney shaped nuclei, alteration of the nuclear membrane, rearrangement of the nuclear lamina and formation of the cytoplasmic assembly compartment. For example, BiP is integral in the formation and integrity of the assembly compartment and its depletion not only causes assembly compartment disruption, but also disrupts nuclear remodeling as well as the cytoplasmic phenotype of an HCMV infection. This signifies that the viral induced morphological changes are reversible. Results presented in this thesis show that the depletion of BiP and the disruption of the assembly compartment reverse the characteristic rearrangement of the nuclear lamina in infected cells. Under BiP depleted conditions, not only is the nuclear lamina rearrangement lost, but the nuclei regain a more normal shape and size. Similarly, disrupting nuclear egress by inhibiting the activity of pUL97 and PKC results in a loss of assembly compartment integrity (11). Thus, as suggested above, disruption of one aspect of the assembly-egress continuum disrupts the entire process, restoring infected cells to a more normal morphology.
HCMV-mediated lamina rearrangement requires phosphorylation of the lamins in a manner that may mimic nuclear lamina rearrangement following metaphase. However, HCMV appears to modify this process through the utilization of a viral specific kinase, UL97, in a viral specific nuclear egress complex which includes the HCMV proteins pUL50 and pUL53, plus the cellular proteins p32, lamin B receptor and PKC (231). pUL50 is anchored in the INM and believed to associate with the nuclear lamina (230). pUL53 binds pUL50, and it is proposed that these proteins attract the kinases which phosphorylate the lamins for rearrangement (38, 218, 230).

The connection between BiP and the lamina rearrangement process manifests later in infection, after the formation of the assembly compartment, when the depletion of BiP results in the loss of lamin A and C phosphorylation on serine 22 and the loss of viral specific lamina rearrangement. These data suggest that BiP and the integrity of the assembly compartment positively affect lamin phosphorylation and rearrangement. These events correlate with an interaction between BiP and pUL50; thus the effects of BiP on lamin phosphorylation, and lamina rearrangement, appear to be due to a direct affect on the function of the pUL50 containing nuclear egress complex. These data also show that BiP has an integral role in both viral cytoplasmic and nuclear activity and implicate it as a critical factor in the assembly-egress continuum.

An interaction between BiP in the assembly compartment and pUL50 anchored in the inner nuclear membrane is complicated by the outer nuclear membrane lying between the two. However, results suggest several alterations of the nuclear membranes that may facilitate the interaction. First, EM data indicate that the nuclear periphery near the assembly compartment is very thin compared to other regions of infected nuclei, and compared to mock-infected nuclei. In addition, the data suggest that some regions of the outer nuclear membrane are disrupted during infection. Such changes would favor
increased access of BiP in the assembly compartment to pUL50 in the inner nuclear membrane.

The findings that the nuclear membrane in the region of the assembly compartment is significantly altered, and potentially disrupted, are supported by the observation that 155,000 molecular weight dextran, loaded into mock- and HCMV-infected cells, appears inside the nuclei of infected cells, whereas it does not enter uninfected nuclei. The dextran detected in infected cell nuclei was especially concentrated at sites adjacent to the assembly compartment. This result suggests that the nuclear membrane adjacent to the assembly compartment is more permeable to large molecules. Such permeability also suggests a means for the BiP-pUL50 interaction. Overall, the data suggest that the nuclear membrane, especially in the region of the assembly compartment, is significantly altered and more permeable to large molecules, features that would support interactions between assembly compartment proteins, for example BiP, and nuclear proteins such as pUL50.

In addition to the above alteration of the nuclear membrane, the distance between the inner and outer nuclear membranes increases in infected cells. This correlates with a decrease in the levels of SUN-domain proteins during the course of an HCMV infection. The loss of SUN-domain proteins has been shown to result in the separation of the inner and outer nuclear membranes (59). Specifically, dimers of SUN-domain proteins, anchored in the inner nuclear membrane, interact in the perinuclear space with KASH-domain proteins which are anchored in the outer nuclear membrane. This interaction tethers the inner and outer nuclear membranes and maintains a relatively uniform distance between the two (59). The loss of SUN-domain proteins relieves the tethering and allows the inner and outer nuclear membranes to disassociate from one another and function separately.
One result of this is that large nucleocapsids in the process of nuclear egress can push the two membranes apart and fit between them, in the perinuclear space.

One of the most intriguing questions about the assembly-egress continuum is how the virus mediates the enlargement of the nucleus and its change to a kidney shape wrapped around the assembly compartment. Studies of nuclear envelope breakdown (NEBD) suggest that the cellular motor dynein attaches to the nuclear membrane and moves toward the centromere, generating tension on the nucleus and creating folds in the nuclear envelope (16, 301); the stress eventually causes tearing and breakdown of the nuclear membrane.

Data presented in this thesis show that in cells with impaired dynein function, the nuclear shape and size is normal. Furthermore, inhibition of dynein causes the assembly compartment components to disperse throughout the cytoplasm. This confirms recent data suggesting that dynein is integral in assembly compartment formation (132), and provides further evidence that viral activity in the cytoplasm is intricately linked to viral-induced morphological changes. Thus, the virus may use a modified version of NEBD, in which dynein binds assembly compartment components and the nuclear membrane and moves toward the microtubule organizing center that will form the center of the assembly compartment. This pulling of the nuclear membrane toward the MTOC can account for the concave nuclear shape formed around the assembly compartment and may promote tight association between the assembly compartment and the nucleus. During normal NEBD, the stress of dynein’s pulling results in tearing of the nuclear membrane and nuclear breakdown. In infected cells nuclear breakdown does not occur, but the nuclei do enlarge. A potential explanation for this is that nuclear enlargement is facilitated by the addition of new nuclear membrane to relieve the stress. This is a feasible possibility given the recent observation of a dramatic increase in fatty acid synthesis for membrane formation that
occurs in HCMV infected cells (242). In addition, it is very likely that the dynein-mediated reformation of the nucleus and the rearrangement of the nuclear lamina are interrelated. In an HSV-1 infection, an interaction between dynein and UL34, the HSV-1 homolog of UL50, has been reported (399). While an interaction between pUL50 and dynein has yet to be reported with HCMV, these observations suggest the intriguing possibility of an integrated mechanism involving BiP, pUL50 and dynein in assembly compartment formation and nuclear restructuring.

Assimilating the data presented herein with current models in the literature creates the following model, represented in Figure 35. The nuclear periphery in mock-infected cells (Figure 35A) consists of a uniform inner and outer nuclear membrane, held in place by the interaction in the perinuclear space between SUN- and KASH-domain containing proteins. The SUN proteins, located in the inner nuclear membrane, interact with the nuclear lamins, which form the rigid nuclear lamina just inside of the nuclear membrane. The nuclear periphery in HCMV-infected cells is drastically altered. Based on the model of Das et al. (68), the assembly compartment is shown as concentric rings made up of specific organellar components and virion structural components, for example tegument proteins. BiP is located in an outer assembly compartment ring, adjacent to the nucleus. The nuclear membrane near the assembly compartment is modified, with disruption of the outer nuclear membrane and both increased permeability and separation between the inner and outer nuclear membranes. All of these changes would increase the access of the proteins in the assembly compartment to nuclear proteins. This may account for the ability of BiP and pUL50 to interact. The model shows the nuclear egress complex as recruited by pUL50 and pUL53 (Figure 35B, #1). The model also shows the rearrangement of the nuclear lamina in the region of the assembly compartment (Figure 35B, #2). This rearrangement, as well as the prerequisite phosphorylation of the lamins, requires BiP and
Figure 35. Model of assembly-egress continuum

(A) Nuclear periphery in mock-infected cells. (B) Assembly-egress continuum in HCMV-infected cells. The model is based on the data presented and previous models (68). See text for detailed explanation of assembly-egress events. INM, inner nuclear membrane; ONM, outer nuclear membrane; MTOC, microtubule organizing center; LBR, lamin B receptor; PKC, protein kinase C
an intact assembly compartment. The data suggest that the interaction between BiP and pUL50 mediates signaling between the assembly compartment and the nucleus to direct lamina rearrangement adjacent to the assembly compartment. The model also shows the dynein-mediated formation of the assembly compartment and remodeling of nuclear shape (Figure 35B, #3). This appears to be facilitated by microtubules radiating from the microtubule organizing center within the assembly compartment.

All of this coordinately forms the assembly-egress continuum which provides a means for nucleocapsids to navigate through the lamina (Figure 35B, #4) and access the nuclear membranes near the assembly compartment. The nucleocapsid then egresses from the nucleus into the perinuclear space (Figure 35B, #5). As suggested by the model of Das et al. (68) the nucleocapsids move through the layers of the assembly compartment (Figure 35B, #6) and emerge tegumented (Figure 35B, #7) and possibly enveloped. Perturbation of any of the above described events in the assembly-egress continuum disrupts the entire process and restores the viral-induced nuclear and cytoplasmic morphological changes to a more normal appearance. Thus, the individual steps that constitute the assembly-egress continuum during HCMV infection require that all other factors of the assembly-egress continuum remain intact in order to maintain the intricate relationship between viral cytoplasmic and nuclear activity, and support virion maturation and egress.
REFERENCES


Hayashi, T., and T. P. Su. 2007. Sigma-1 receptor chaperones at the ER-mitochondrion interface regulate Ca(2+) signaling and cell survival. Cell 131:596-610.


Protein synthesis and endoplasmic reticulum stress can be modulated by the hepatitis C virus envelope protein E2 through the eukaryotic initiation factor 2alpha kinase PERK. J Virol 77:3578-85.
274. Pizzorno, M. C., P. O'Hare, L. Sha, R. L. LaFemina, and G. S. Hayward. 1988. trans-
276. Pouyssegur, J., R. P. Shiou, and I. Pastan. 1977. Induction of two transformation-
279. Putcha, G. V., S. Le, S. Frank, C. G. Besirli, K. Clark, B. Chu, S. Alix, R. J. Youle, A. 


APPENDIX: Abbreviations

° degrees
µg microgram
µl microliter
µM micromolar
ADP adenosine diphosphate
AP assembly protein
ASFV African swine fever virus
ATCC American Type Culture Collection
ATF3 activating transcription factor 3
ATF4 activating transcription factor 4
ATF6 activating transcription factor 6
ATP adenosine triphosphate
BAP BiP-associated protein
BDV Borna disease virus
BiP immunoglobulin binding protein
BS³ bis(sulfosuccinimidyl) suberate
BSA bovine serum albumin
BVDV Bovine viral diarrhea virus
C Celsius
Ca²⁺ calcium
CaCl₂ calcium chloride
CHOP C/EBP-homologous protein
cm centimeter
CO₂ carbon dioxide
CPE cytopathic effects
CRE ATF/cAMP response element
CREB1 cAMP response element binding protein 1
CTF-NF1 CAAT-binding transcription factor-nuclear factor 1
DAPI 4',6'-diamidino-2-phenylindole
ddH₂O distilled deionized water
DEN-1 Dengue virus type 1
DEN-2 Dengue virus type 2
DMEM10 Dulbecco's Modified Eagle Medium
supplemented with 10% serum, glutamax and antibiotics
DNA deoxyribonucleic acid
dNTP deoxynucleotide triphosphate
DR5 death receptor 5
DsRed red fluorescent monomeric protein
E2F1 E2 transcription factor 1
EDEM ER degradation-enhancing α-mannosidase-like
EDTA ethylenediaminetetraacetic acid
EEA1 early endosome antigen 1
EGCG epigallocatechin gallate
EGFR epidermal growth factor receptor
eIF2α eukaryotic initiation factor 2α
EM electron microscopy
ER endoplasmic reticulum
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERAD</td>
<td>ER-associated degradation</td>
<td>IFNAR1</td>
<td>interferon alpha receptor 1</td>
</tr>
<tr>
<td>ERSE</td>
<td>endoplasmic reticulum stress element</td>
<td>INM</td>
<td>inner nuclear membrane</td>
</tr>
<tr>
<td>FKBP23</td>
<td>FK506-binding protein 23</td>
<td>IRE1</td>
<td>inositol-requiring enzyme 1</td>
</tr>
<tr>
<td>GADD34</td>
<td>growth arrest and DNA damage gene 34</td>
<td>IRES</td>
<td>internal ribosome entry site</td>
</tr>
<tr>
<td>GADD153</td>
<td>growth arrest and DNA damage gene 153</td>
<td>JEV</td>
<td>Japanese encephalitis virus</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
<td>kD</td>
<td>kilodalton</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
<td>Keap1</td>
<td>Kelch-like ECH-associated protein 1</td>
</tr>
<tr>
<td>GLS</td>
<td>Golgi-localization signal</td>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>GRP78</td>
<td>glucose regulated protein 78</td>
<td>LE-HFF</td>
<td>lifetime extended-human foreskin fibroblasts</td>
</tr>
<tr>
<td>GRP170</td>
<td>glucose regulated protein 170</td>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
<td>MCF</td>
<td>mink cell focus-forming murine leukemia viruses</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
<td>MCP</td>
<td>major capsid protein</td>
</tr>
<tr>
<td>HCMV</td>
<td>human cytomegalovirus</td>
<td>MCP-BP</td>
<td>minor capsid protein-binding protein</td>
</tr>
<tr>
<td>HCV</td>
<td>hepatitis C virus</td>
<td>Met-tRNAi</td>
<td>methionyl-initiator tRNA</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
<td>MCMV</td>
<td>mouse cytomegalovirus</td>
</tr>
<tr>
<td>HHV-5</td>
<td>human herpesvirus 5</td>
<td>MHC1</td>
<td>major histocompatibility complex 1</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
<td>MHV</td>
<td>mouse hepatitis virus</td>
</tr>
<tr>
<td>hrpi</td>
<td>hours post infection</td>
<td>MIEP</td>
<td>major immediate early protein</td>
</tr>
<tr>
<td>HRD1</td>
<td>HMG-CoA reductase degradation 1</td>
<td>ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>HSP70</td>
<td>heat shock protein 70</td>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>HSPG</td>
<td>heparin sulfate proteoglycan</td>
<td>ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>HSV-1</td>
<td>herpes simplex-1</td>
<td>mlM</td>
<td>millimolar</td>
</tr>
<tr>
<td>HTLV-1</td>
<td>human t-lymphotrophic virus</td>
<td>mlM</td>
<td>millimolar</td>
</tr>
<tr>
<td>IF</td>
<td>immunofluorescence</td>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>IFNAR1</td>
<td>interferon alpha receptor 1</td>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Mo-MuLV</td>
<td>Moloney murine leukemia virus</td>
<td>PRMT1</td>
<td>protein arginine methyltransferase 1</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
<td>PtdCho</td>
<td>phosphatidylcholine</td>
</tr>
<tr>
<td>MTOC</td>
<td>microtubule organizing center</td>
<td>PUMA</td>
<td>p53-upregulated modulator of apoptosis</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>sodium phosphate</td>
<td>RAMP4</td>
<td>ribosome-associated membrane</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>NaF</td>
<td>sodium fluoride</td>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>NEBD</td>
<td>nuclear envelope breakdown</td>
<td>RIPA</td>
<td>radioimmunoprecipitation assay</td>
</tr>
<tr>
<td>NF-Y</td>
<td>nuclear factor Y</td>
<td>ng</td>
<td>nanograms</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>Nrf2</td>
<td>NF-E2-related factor-2</td>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>NS4B</td>
<td>non-structural protein 4B</td>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>NSAP1</td>
<td>NS1-associated protein 1</td>
<td>PDI</td>
<td>protein disulfide isomerase</td>
</tr>
<tr>
<td>NSP4</td>
<td>non-structural protein 4</td>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>ONM</td>
<td>outer nuclear membrane</td>
<td>PDI</td>
<td>protein disulfide isomerase</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
<td>PI3K</td>
<td>phosphoinositide-3 kinase</td>
</tr>
<tr>
<td>OsO₄</td>
<td>osmium tetroxide</td>
<td>PFK-1</td>
<td>phosphofructosekinase-1</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
<td>PP1</td>
<td>protein phosphatase 1</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
<td>PI3K</td>
<td>phosphoinositide-3 kinase</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PDI</td>
<td>protein disulfide isomerase</td>
<td>PKR</td>
<td>protein kinase R</td>
</tr>
<tr>
<td>PERK</td>
<td>PKR-like ER kinase</td>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PFK-1</td>
<td>phosphofructosekinase-1</td>
<td>PP1</td>
<td>protein phosphatase 1</td>
</tr>
<tr>
<td>pfu</td>
<td>plaque forming unit</td>
<td>PUMA</td>
<td>p53-upregulated modulator of apoptosis</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositide-3 kinase</td>
<td>PUMA</td>
<td>p53-upregulated modulator of apoptosis</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
<td>PKR</td>
<td>protein kinase R</td>
</tr>
<tr>
<td>PKR</td>
<td>protein kinase R</td>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
<td>PP1</td>
<td>protein phosphatase 1</td>
</tr>
<tr>
<td>PP1</td>
<td>protein phosphatase 1</td>
<td>PUMA</td>
<td>p53-upregulated modulator of apoptosis</td>
</tr>
<tr>
<td>PRMT1</td>
<td>protein arginine methyltransferase 1</td>
<td>PtdCho</td>
<td>phosphatidylcholine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------</td>
<td>--------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>TGN</td>
<td>trans-Golgi network</td>
<td>VSV</td>
<td>Vesicular stomatitis virus</td>
</tr>
<tr>
<td>TRB3</td>
<td>tribbles homolog 3</td>
<td>WNV</td>
<td>West Nile virus</td>
</tr>
<tr>
<td>TRITC</td>
<td>tetramethylrhodamine</td>
<td>WT</td>
<td>wildtype</td>
</tr>
<tr>
<td>UPR</td>
<td>unfolded protein response</td>
<td>XBP1</td>
<td>X-box binding protein 1</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
<td>YY1</td>
<td>Yin Yang-1</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>