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MECHANISMS LEADING TO TRANSLATION ARREST IN *AUTOGRAPHA CALIFORNICA* MULTINUCLEOCAPSID NUCLEOPOLYHEDROVIRUS-INFECTED *LYMANTRIA DISPAR* CELLS

presented by

Christy Mecey

has been accepted towards fulfillment
of the requirements for the

Doctoral degree in Genetics

Major Professor's Signature

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# MECHANISMS LEADING TO TRANSLATION ARREST IN *AUTOGRAPHA CALIFORNICA* MULTINUCLEOCAPSID NUCLEOPOLYHEDROVIRUS-INFECTED *LYMANTRIA DISPAR* 652Y CELLS

By

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## ABSTRACT

MECHANISMS LEADING TO TRANSLATION ARREST IN *AUTOGRAPHA CALIFORNICA* MULTINUCLEOCAPSID NUCLEOPOLYHEDROVIRUS-INFECTED *LYMANTRIA DISPAR* 652Y CELLS

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Infection of the *Lymantria dispar* 652Y (Ld652Y) cell line with the baculovirus *Autographa californica* Multinucleocapsid nucleopolyhedrovirus (AcMNPV) results in global translation arrest, initiation of apoptosis in the absence of a functional, virus-encoded, apoptotic suppressor and a non-productive infection. While AcMNPV enters Ld652Y cells and transcription of virus genes occurs, virus progeny are not produced. Expression of a single gene, *host range factor-1* (*hrf-1*), isolated from *Lymantria dispar* Multinucleocapsid Nucleopolyhedrovirus (LdMNPV), precludes translation arrest in AcMNPV-infected Ld652Y cells further allowing permissive replication of the virus. HRF-1 also enables replication of *Hyphantria cunea* NPV, *Bombyx mori* NPV and *Spodoptera exigua* NPV in Ld652Y cells indicating that *hrf-1* is an essential host range factor for baculovirus infection in Ld652Y cells. Database and motif searches conducted with the *hrf-1* sequence provide no indication to its function. The goal of this study was to determine the mechanism of translation arrest in AcMNPV-infected Ld652Y cells to further identify possible functions for HRF-1. We show that eIF2α phosphorylation at serine 51 occurs in AcMNPV-infected Ld652Y cells at 12 h.p.i. correlating to the onset of translation arrest. Ld652Y cells infected with vAchrf-1 show a reduced level of

eIF2α phosphorylation for the full-length peIF2α$^{ser51}$ protein at 6, 12, 24 and 48 h.p.i. as compared to mock-infected cells. PK2, the AcMNPV eIF2α kinase inhibitor, was expressed in AcMNPV-infected Ld652Y cells but did not prevent eIF2α phosphorylation. An apparent proteolytic cleavage of peIF2α$^{ser51}$ was observed beginning at 12 h.p.i.; this cleavage event was investigated further. In Sf21 cells, a permissive cell line for AcMNPV replication, peIF2α$^{ser51}$ was cleaved in both AcMNPV and vAchrf-1-infected cells indicating that HRF-1 was not directly responsible for the cleavage. Cell permeable protease inhibitors did not block cleavage of peIF2α$^{ser51}$. However, the proteasome inhibitor epoxomicin did reduce phosphorylation of eIF2α at serine 51, block cleavage of peIF2α$^{ser51}$ and inhibit virus replication in AcMNPV and vAchrf-1-infected Sf21 cells and vAchrf-1-infected Ld652Y cells. The proteasome inhibitor MG132 mimicked the effects of epoxomicin in vAchrf-1-infected Ld652Y cells. However, treatment with MG132 in AcMNPV and vAchrf-1-infected Sf21 cells resulted in reduced virus titers and no block of peIF2α$^{ser51}$ cleavage. This study suggests a role for the proteasome in enabling baculovirus replication. A cross-reactive antibody to the human eIF2 kinase, PKR, showed enhanced expression of a protein in vAchrf-1-infected cells at 12-24 h.p.i. Mass spectrometry analysis identified this protein as a 90 kDa heat shock protein (HSP90). Inhibition of HSP90 with geldanamycin resulted in a block of occluded virus production in baculovirus-infected Sf21 and Ld652Y cells at 48 h.p.i. Cleavage of occurs in productively-infected cells in the presence of geldanamycin indicating that cleavage is not dependent on HSP90 activity. The data suggest a possible role for HSP90 during the very late stages of baculovirus infection.

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I would like to dedicate this effort to the family members that we have lost in the past year that are missed and remembered: Lee McIntosh, Florence Allison and my 34 year old sister, Jamie Dixon. There is not a day that passes that I don't wish I could trade places with my sister and return her to her lost family.

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## KEY TO SYMBOLS OR ABBREVIATIONS

| | |
|---|---|
| **4E-BP** | eIF4E binding protein |
| **AA** | amino acid |
| **AcMNPV** | *Autographa californica* Multicapsid nuclear Polyhedrosis Virus |
| **AN** | alkaline nuclease |
| **ATF** | activating transcription factor |
| **ATP** | adenosine triphosphate |
| **BeK** | *Bombyx mori* eIF2α kinase |
| **BiP** | glucose regulated protein 78 |
| **BmN** | *Bombyx mori* N cell line |
| **BmNPV** | *Bombyx mori* Multi Nucleocapsid Polyhedrovirus |
| **BV** | budded virus |
| **Cf-203** | *Choristoneura fumiferana* 203 cell line |
| **CfMNPV** | *Choristoneura fumiferana* Multi Nucleocapsid Polyhedrovirus |
| **COP9 / CSN** | COP9 signalosome |
| **Dm** | *Drosophila melanogaster* |
| **DNA** | deoxyribonucleic acid |
| **Dnapol** | DNA polymerase |
| **dsRNA** | double-stranded RNA |
| **EDEM** | ER degradation-enhancing α-mannosidase-like protein |
| **EDTA** | (ethylenedinitrilo) tetraacetic acid |

| | |
|---|---|
| **eIF** | eukaryotic initiation factor |
| **ER** | endoplasmic reticulum |
| **ERAD** | endoplasmic reticulum-associated degradation |
| **ERSE** | endoplasmic reticulum stress responsive element |
| **GCN** | general control non-derepressible |
| **GDP** | guanosine diphosphate |
| **GTP** | guanosine triphosphate |
| **HCF** | host cell factor |
| **HCMV** | human cytomegalovirus |
| **HCV** | hepatitis C virus |
| **HHV** | human herpes virus |
| **HIV** | human immunodeficiency virus |
| **HPI** | hours post-infection |
| **HPPV** | human papillomavirus |
| **HPT** | hours post-treatment |
| **HR** | homologous repeat |
| **HRF** | host range factor |
| **HRI** | heme-regulated inhibitor of protein synthesis |
| **HSC** | heat shock cognate protein |
| **HSP** | heat shock protein |
| **HSV** | herpes simplex virus |
| **IAP** | inhibitor of apoptosis |

| | |
|---|---|
| **IE** | immediate-early gene |
| **IRE** | inositol-requiring enzyme |
| **IRES** | internal ribosomal entry site |
| **JEV** | Japanese encephalitis virus |
| **JNK** | c-Jun kinase |
| **LEF** | late expression factor |
| **Ld652Y** | *Lymantria dispar* 652Y cell line |
| **LdMNPV** | *Lymantria dispar* Multi Nucleocapsid Polyhedrovirus |
| **Met** | methionine |
| **MOI** | multiplicity of infection |
| **mRNA** | messenger RNA |
| **mTOR** | mammalian target of rapamycin |
| **NF-κB** | nuclear factor-kappa B |
| **ODV** | occlusion derived virus |
| **OpMNPV** | *Orygia pseudotsugata* Multi Nucleocapsid Polyhedrovirus |
| **OV** | occluded virus |
| **P35** | baculovirus 35 kD apoptotic suppressor protein |
| **P39** | baculovirus 39 kD capsid protein |
| **PABP** | poly-A binding protein |
| **PERK/PEK** | RNA-dependent-like endoplasmic reticulum kinase |
| **PK2** | AcMNPV inhibitor of eIF2α kinases |
| **RNA** | ribonucleic acid |

| | |
|---|---|
| **RNAi** | RNA interference |
| **RNA Pol. II** | RNA polymerase II |
| **SeMNPV** | *Spodoptera exigua* Multi Nucleocapsid Polyhedrovirus |
| **Sf9** | *Spodoptera frugiperda* 9 cell line |
| **Sf21** | *Spodoptera frugiperda* 21 cell line |
| **Tn368** | *Trichoplusia ni* 368 cell line |
| **TNF** | tumor necrosis factor |
| **TRAF** | TNF-receptor kinase |
| **tRNA** | transfer RNA |
| **UPR** | unfolded protein response |
| **UTR** | untranslated region |
| **vAcΔp35** | AcMNPV strain with the apoptotic suppressor, p35, deleted |
| **VLF** | very late expression factor |
| **vRNA Pol** | viral RNA polymerase |
| **XBP** | X-box binding protein |

## Rationale for Study

In 2001, annual international insecticide sales totaled over 9 billion dollars (Donaldson et al., 2004). Chemical insecticides are often effective but can act broadly and harm non-target species. Many chemical insecticides have deleterious effects on the environment and have been removed from the commercial market or relicensed to limit use (Donaldson et al., 2004). Biopesticides and biological control agents are living organisms or products from living organisms used to suppress pest populations. Biopesticides offer an alternative to chemical-based insecticides and generally affect a narrower range of target species (Thiem, 1997). Baculoviruses primarily infect Lepidopteran insects during the larval stage of development and are a biological control agent used commercially to control soybean pests. Narrow host range is the primary limiting factor to the further utilization and subsequent development of baculoviruses as biopesticides (Maeda, 1995; Moscardi, 1989; Moscardi, 1999; Thiem, 1997).

Unlike bacterial and fungal host-pathogen systems, the molecular mechanisms that govern invertebrate host-specificity to virus infection are not understood. In cell culture, many baculoviruses are able to enter non-host invertebrate cells, virus replication is initiated but then terminated during the late stages of infection (Du and Thiem, 1997a; Lu and Miller, 1996). The Gypsy Moth, *Lymantria dispar*, is a non-permissive host to *Autographa californica* Multinucleocapsid Nucleopolyhedrovirus (AcMNPV), the type virus for the Baculoviridae family (McClintock, Dougherty, and Weiner, 1986). Virus replication begins in Lymantria dispar 652Y (Ld652Y) cells that are infected with AcMNPV but global translation arrest is initiated by 9 h.p.i. correlating to the late stages of infection and no virus progeny are produced (Fig. 1A) (Guzo et al., 1992). Expression

of a single gene, *host range factor-1* (HRF-1), isolated from *Lymantria dispar* Multinucleocapsid nucleopolyhedrovirus (LdMNPV), blocks translation arrest and enables the production of virus progeny in AcMNPV-infected Ld652Y cells and *L. dispar* larvae (Fig. 1B) (Chen et al., 1998; Du and Thiem, 1997a; Du and Thiem, 1997b; Thiem et al., 1996). Homology and motif searches conducted with the *hrf-1* sequence provide no indications to its function.

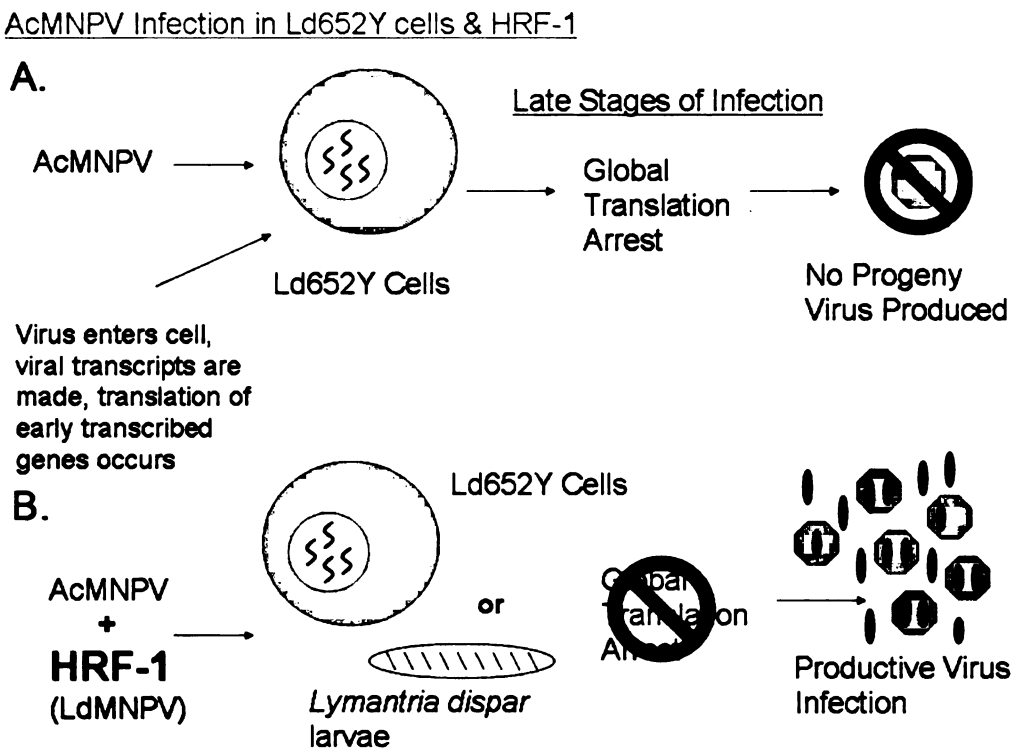



**Figure 1. Global translation arrests and virus replication is blocked in AcMNPV-infected Ld652Y cells (A). Expression of the LdMNPV gene, *host range factor-1* (HRF-1), precludes translation arrest in AcMNPV-infected Ld652Y cells and *Lymantria* dispar larvae further enabling the production of virus progeny (B). (Image Presented in Color)**

It has not been determined whether global translation arrest is the direct mechanism leading to a block in the production of virus progeny in AcMNPV-infected Ld652Y cells

or simply a correlative event resulting from infection. Global translation arrest may be a host-mediated innate immune response to control virus replication or a host-induced response to stress. In mammalian systems, viruses use a variety of mechanisms to block translation of host-encoded genes as a means of ensuring preferential translation of viral transcripts (Jang et al., 1988; Joachims, Van Breugel, and Lloyd, 1999; Kerekatte et al., 1999; Pelletier and Sonenberg, 1988). This is unlikely in baculovirus-infected cells as host macromolecular synthesis appears to be controlled at the transcriptional level (Nobiron, O'Reilly, and Olszewski, 2003; Ooi and Miller, 1988).

In this study, my goal was to identify the mechanism(s) that leads to translation arrest in AcMNPV-infected Ld652Y cells. I hypothesized that identifying this mechanism might provide some indication to the function of HRF-1. Furthermore, we hoped to gain insight into invertebrate host responses that are initiated during non-permissive infections to further understand the components that determine baculovirus host range.

# Chapter 1
## Literature Review

## Introduction

### 1.1 Baculovirus Pathogenesis

Baculoviruses are large, double-stranded DNA viruses that primarily infect lepdiopteran insects during the larval growth stage. *Autographa californica* Multicapsid Nuclear Polyhedrosis virus (AcMNPV) is the type virus for the family Baculoviridae. Baculoviruses have a biphasic replication cycle which results in rod-shaped virions being packaged into a budded and occluded form (Theilmann et al., 2004). Budded virus, produced from a primary infection, is enveloped at the plasma membrane and infects neighboring cells by adsorptive endocytosis (Blissard and Wenz, 1992; Volkman and Goldsmith, 1988) resulting in a systemic infection. Synthesis of budded virus is reduced by 24 hours post-infection (h.p.i.) and there is a switch to occluded virus production (Volkman and Knudson, 1986). The occluded form of the virus (OV) is environmentally stable and consists of multiple, enveloped nucleocapsids that are packaged into a polyhedrin protein-rich matrix. Virions condensed in a polyhedrin matrix are wrapped in a virus-derived, carbohydrate-rich, calyx which forms a unilamellar structure with spike-like projections, forming the occlusion body. Caterpillars ingest the occluded form of the virus, the polyhedra dissolve rapidly within the alkaline pH of the midgut (Summers, 1971; Summers and Arnott, 1969) and the virions pass through the peritrophic membrane of the insect. The virus enters cells via fusion of virion envelope with the microvillar membrane (Granados, Lawler, and Burand, 1981). The virus then travels along the microvillus aided by microtubules (Charlton and Volkman, 1993; Granados, Lawler, and Burand, 1981) and enters the nucleus where it uncoats at the nuclear pore or just inside the nuclear membrane (Bilimoria, 1991) (For review of the baculovirus infection cycle

see Fig. 2) (Blissard and Rohrmann, 1990). Advanced infection of the larvae results in complete cellular destruction and a condition known as "melted" (Federici, 1997).

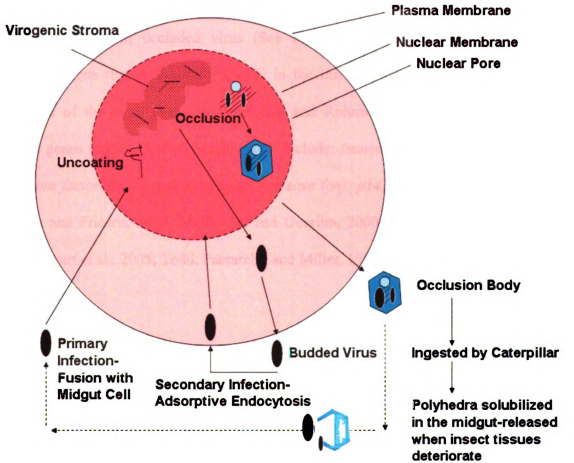



**Figure 2. Schematic diagram of the baculovirus infection cycle adapted from Blissard and Rohrmann 1990 (Image Presented in Color)**

## 1.2 Baculovirus Replication

Within the nucleus of the midgut epithelial cells the infection process begins with immediate and early gene transcription of the virus followed by DNA replication, late gene expression, the production and release of budded virus, very late gene expression and the synthesis of occluded virus (See Fig. 3). Baculoviruses encode a DNA polymerase gene that is transcribed early in the infection process and is essential to replication of the virus (Vanarsdall, Okano, and Rohrmann, 2005). Additional viral-encoded genes required for virus replication include: *immediate-early gene-1* (*ie1*), *late expression factor (lef) 1* and *3*, *alkaline nuclease (an)*, *p143* and *p35* (Ito et al., 2004; LaCount and Friesen, 1997; McDougal and Guarino, 2000; Mikhailov and Rohrmann, 2002; Stewart et al., 2005; Todd, Passarelli, and Miller, 1995).

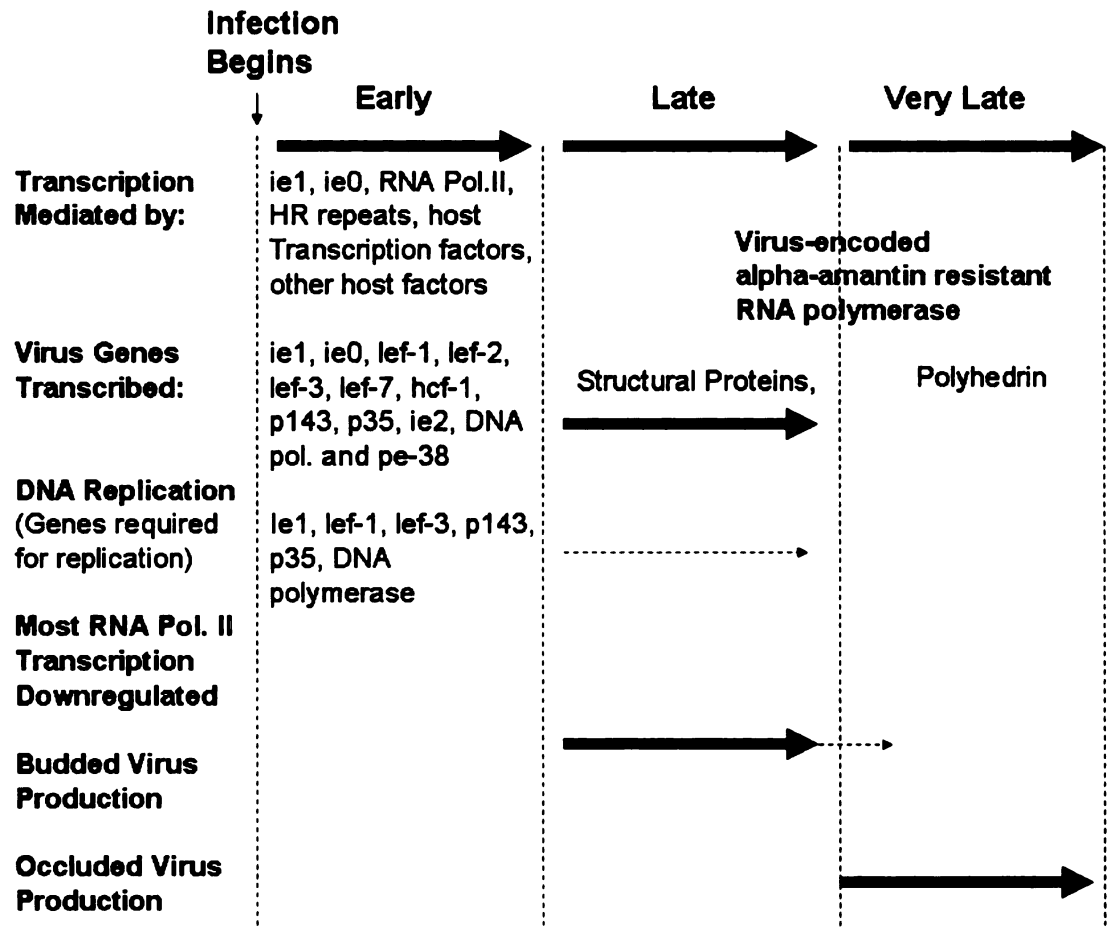



**Figure 3. Schematic diagram of transcriptional regulation that occurs during the baculovirus replication cycle adapted from Rohrman 1992**

Transcription of early expressed baculovirus genes is regulated by host RNA polymerase II and IE1. IE1 has a splicing variant known as IE0. Individually either IE1 or IE0 can support virus replication but both are required to achieve a wildtype infection (Stewart et al., 2005). Homologous repeat (HR) sequences that serve as transcriptional enhancer regions can be found in eight locations of the AcMNPV genome (Rasmussen et al., 1996) and HR regions have been identified in all sequenced baculoviruses. IE1 regulates early virus transcription in *cis* by binding a 28-mer palindromic site in the HR

regions and host transcription factors also bind within the HR region (Landais et al., 2005). LEF-1 is a DNA primase (Mikhailov and Rohrmann, 2002) and p143 a DNA helicase (McDougal and Guarino, 2000) that associates with LEF-3 (Evans et al., 1999), a single-stranded DNA binding protein, for nuclear transport and localization (Chen and Carstens, 2005). It has been proposed that the HR sites also serve as origins of DNA replication, indeed HR sites are essential for virus DNA replication (Leisy et al., 1995). Immunoprecipitation followed by crosslinking has shown that IE-1, P143 and LEF-3 bind viral chromatin at closely linked sites, suggesting that replication complexes form at the homologous repeat regions (Ito et al., 2004). Baculoviruses also encode an early expressed alkaline nuclease (AN) which is essential for virus replication. AN has 5'-to-3' endonuclease activity on single-stranded DNA and 5'-to-3' exonuclease activity when associated with LEF-3. The data suggests that AN plays a role in DNA recombination and the resolution of replication intermediates (Mikhailov, Okano, and Rohrmann, 2004). The apoptotic suppressor, *p35* (Clem, Fechheimer, and Miller, 1991) directly binds caspases, blocking substrate proteolysis. P35 functions at early and late timepoints post-infection (LaCount and Friesen, 1997). Baculovirus IE2, LEF-7, HCF-1 and P38 stimulate viral replication but are not essential to a productive infection (Miller, 1997).

The late stage events of baculovirus infection are defined as those that occur after DNA replication of the virus. The production of budded virus temporally correlates to a time immediately following DNA replication and the production of occluded virus is the result of the late stages of infection in cells infected with baculovirus (Friesen and Miller, 1986; Rice and Miller, 1986). Characterized late genes are transcribed at six to twenty-four hours post-infection (h.p.i.), the corresponding gene products are primarily structural

in nature and include the VP39 capsid protein (Thiem and Miller, 1989) and P6.9 core protein (Wilson et al., 1987). Very late genes are transcribed in a burst of expression from eighteen to seventy-two h.p.i. and include the polyhedrin occlusion body protein (Rohrmann, 1986) and p10, which effects nuclear disintegration upon exit of the occluded virus (Rohrmann, 1992).

Regulation of the late stages of infection is primarily transcriptional. The segue of early-to-late gene transcription is coupled to a decrease in host mRNA levels at six to eight h.p.i. (Nobiron, O'Reilly, and Olszewski, 2003; Ooi and Miller, 1988) and the induction of a virus-encoded (Guarino et al., 1998), alpha-amantin resistant RNA polymerase (vRNApol) (Beniya et al., 1996; Fuchs, 1983; Grula, 1981a; Grula, 1981b; Huh and Weaver, 1990; Yang, Stetler, and Weaver, 1991). By 18 h.p.i. transcription is exclusive to vRNApol (Fuchs, 1983; Huh and Weaver, 1990) (For a review of baculovirus temporal gene regulation, see Fig. 2). The viral RNA polymerase specifically binds the virus template at a consensus sequence of TAAG found in the promoters of late and very late genes (Morris and Miller, 1994; Ooi, Rankin, and Miller, 1989; Rankin, Ooi, and Miller, 1988; Thiem and Miller, 1989; Thiem and Miller, 1990). However, host and virus transcription factors, including *IE1, IE0* and host GATA factors, bind the virus DNA template in concert with the vRNApol (Chen, Tsai, and Chen, 2001; Hefferon, 2004; Krappa et al., 1992; Landais et al., 2005) and the HR enhancer elements play a critical role in gene regulation even at late times during the infection process (Hefferon, 2004). Twenty viral early or constitutively expressed genes are required for late and very late baculovirus gene expression, these include: *ie-1*, *ie-2*, *dnapol*, *p143*, *p47*, *p35*, *39K* and the late expression factors (LEFs) 1-12 (Hefferon, 2004; Li, Harwood, and

Rohrmann, 1999; Lu and Miller, 1995b). The baculovirus-encoded LEF-8 and LEF-9 are homologous to subunits of RNA polymerase II. LEF-4 has guanyltransferase activity and can be purified as a component of the vRNApol complex (Guarino, Jin, and Dong, 1998). The very late expression factor 1 (VLF-1) is required for transcription from the polyhedrin promoter (McLachlin and Miller, 1994; Mistretta and Guarino, 2005).

**1.3 Baculovirus Host Range**

Baculoviruses have been commercially developed as eukaryotic protein expression vectors and as biopesticides. Hundreds of different baculoviruses have been isolated from hundreds of species of infected lepidopteran larvae and it is predicted that thousands more exist (Federici, 1997; Theilmann et al., 2004). A small percentage of characterized baculoviruses have the potential to be useful in controlling insect pests (Moscardi, 1999; Yearian, 1982). The use of baculoviruses as insect control agents has been successful for controlling a lepidopteran pest of soybeans (Moscardi, 1989). The limited commercial success is due, in part, to the narrow host range of baculoviruses. AcMNPV has the broadest host range and can permissively infect six Lepidopteran species and semi-permissively infect another twenty-six (Federici, 1997). Permissive hosts are denoted as those being highly susceptible to a particular virus infection while semi-permissive are those that are less susceptible (Bishop, 1995). Environmentally, the limited host range of baculoviruses is beneficial because effects on non-target species are minimal to non-existent. Economically, the limited host range of baculoviruses is detrimental because agricultural producers require a single broad-range insecticide to maintain cost effectiveness. Substantial research has been devoted to expanding host range and/or enhancing insecticidal properties through the development of recombinant

baculoviruses that express host range factors, insect regulatory proteins and/or general insecticidal toxins (Black, 1997; Maeda, 1995).

In mammalian cells, virus host range is frequently determined by the interaction of a viral protein with a host-encoded receptor. This does not appear to be true for baculoviruses. In a number of non-permissive and semi-permissive baculovirus infections the virus enters the cell nucleus, viral DNA replication begins but infection is blocked during the late stages of infection (Miller, 1997; Simon et al., 2004; Thiem, 1997; Yanase, Yasunaga, and Kawarabata, 1998). As an example, AcMNPV infects, has some late gene expression but does not productively replicate in cell lines from *Bombyx mori*, *Choristoneura fumiferana*, *Mamestra brassicae* and *Lymantria dispar*. AcMNPV infects *Drosophila melanogaster* cell lines but late gene expression does not occur (Morris and Miller, 1992; Morris and Miller, 1993). AcMNPV can enter and evoke an immune response in human cells (Abe et al., 2005) but viral DNA is not transcribed because viral promoters are not recognized by host machinery (Thiem, 1997). Host immune response also limits virus infection in mammals. In contrast, little is known about immune response to virus infection in invertebrates.

### 1.3.1. Invertebrate Innate Immunity to Virus Infection

Due to a lack of a molecularly well-characterized virus pathogen and invertebrate host model, the pathways leading to induction of invertebrate innate immune response in response to virus infection have not been clearly elucidated. Recent evidence shows that Toll pathways may contribute to antiviral response in *Drosophila melanogaster*. Adult *Drosophila dif* mutants lacking a Toll pathway-activated NF-κB transcription factor were found to have a decreased tolerance to *Drosophila* X virus (Zambon et al., 2005).

Microarray data from the midgut of *Aedes aegypti* vectoring the Sindbis virus indicate a role for Toll and c-Jun amino terminal cascades in the infection process (Sanders et al., 2005). The RNAi pathway in *Anopheles gambiae* can be silenced with dsRNA of the *Argonaute2* gene. These Ago2 knock-down mosquitos are also more vulnerable to infection with O'nyong-nyong virus, an RNA alphavirus, as compared to untreated animals, which leads to the conclusion that RNAi is an integral antiviral response in *A. gambiae* (Keene et al., 2004).

*Heliothis virescens* larvae are a fully permissive host to AcMNPV while *Helicoverpa zea* larvae, a closely-related species, are semi-permissive, showing a 1000-fold difference in susceptibility (Allen, 1969; Vail, 1978; Vail, 1982). However, larvae of both species that were orally fed AcMNPV ODV show no difference in their susceptibility during primary infection of the midgut cells or secondary infection of the tracheal cells. However, by 48 h.p.i in *Helicoverpa zea,* hemocytes encapsulate melanized infection foci in the trachea and clear the virus. *Helicoverpa zea* hemocytes are resistant to AcMNPV infection while hemocytes of *Heliothis virescens* are not. Encapsulation of infection foci does not occur in AcMNPV-infected *Heliothis virescens*. Originally, virus encapsulation and clearing were believed to be an innate immune response to baculovirus infection in Lepidoptera, however, further study indicates that swelling and tracheal damage occur in response to AcMNPV infection in *Helicoverpa zea,* making melanization and encapsulation a probable response to wounding that occurs from virus-damaged tissues (Trudeau, Washburn, and Volkman, 2001; Washburn, 1996).

### 1.3.2. Developmental Resistance to Baculovirus

There appears to be some level of developmental resistance to baculovirus infection. As larvae age they become more resistant to oral-ingested baculovirus infection. This phenomena appears to be the result of midgut cell sloughing during the molting process. Larvae in the temporal late stages of instar development, immediately preceeding or during a molt, are less likely to establish a productive AcMNPV infection than those at the early instar stage. This is in contrast to *H. virescens* or *Trichoplusia ni* larvae that are hemocoel-injected with one plaque forming unit of AcMNPV BV, which results in 59-89% mortality. It would appear that infected midgut cells are shed during the molting process before a systemic baculovirus infection can be established, thus "clearing" the virus from the insect. This decreased susceptibility is mimicked in *H. virescens* larvae that are fed on cotton where a plant-encoded foliar peroxidase initiates midgut sloughing in the insect (Trudeau, Washburn, and Volkman, 2001; Washburn, Kirkpatrick, and Volkman, 1995; Washburn, 1996).

### 1.3.3. Species-Mediated Resistance to Baculovirus

Based on the current data, many different mechanisms appear to mediate or contribute to the species-specific host range of baculoviruses. In cell culture, host range mechanisms that have been investigated in detail commonly show impaired baculovirus replication and a block during the late stages of infection (Miller, 1997; Thiem, 1997).

#### 1.3.3.1 *Host Cell Factor-1* (HCF-1)

In Sf21 and TN368 cells, both of which are permissive to AcMNPV infection, transient expression assays were conducted that combined a late viral promoter, a reporter gene and expression of eighteen AcMNPV LEFs. In Sf21 cells, expression of the

eighteen characterized LEFs was sufficient to initiate transcription of the reporter gene. However, in Tn368 cells transcription from a late and very late viral promoter required the expression of an additional baculovirus gene, *host cell factor-1* (*hcf-1*) (Lu and Miller, 1995a), which is not homologous with mammalian *hcf-1*. Infection with AcMNPV *hcf-1* deletion mutants had no effect on virulence, virus DNA replication or temporal gene expression in Sf21 cells. However, in *Trichoplusia ni* 368 (Tn368) ovarian cells infected with *hcf-1* null mutants, DNA replication and late gene expression of the virus did not occur and an arrest of host and viral protein synthesis occurred by 18 h.p.i. (Lu and Miller, 1996). Infection with *hcf-1* null mutants in Hi-5 cells, a cell line derived from *Trichoplusia ni* eggs, and in *T. ni* larvae resulted in a reduced virulence of the virus. A 50-fold decrease in infectivity was observed for the budded form of the mutant virus. However, there was no reduction in oral infectivity for larvae that were fed polyhedra lacking the *hcf-1* gene. Thus, HCF-1 has species and tissue-specific effects (Lu and Miller, 1996). HCF-1 has been characterized as an early protein that localizes to the nucleus in infected Tn368 cells and has been shown to interact with itself in coprecipitation and yeast two-hybrid studies (Hefferon, 2004).

**1.3.3.2. p143**

*Bombyx mori* N (BmN) cells are permissive to *Bombyx mori* Multi Nucleocapsid Polyhedrovirus (BmNPV) but Tn368, and the *Spodoptera frugiperda* Sf21 and CLS-79 cell lines are non-permissive to BmNPV infection. Conversely, BmN cells are non-permissive to AcMNPV infection (Summers, 1978). Co-infection of BmNPV and AcMNPV yielded a variant that was capable of replicating in all three cell types that were non-permissive to BmNPV infection (Kondo and Maeda, 1991). Using construction of

recombinant BmNPV variants carrying AcMNPV genome fragments, the gene responsible for the expanded host range was identified as p143-a putative DNA helicase (Maeda, Kamita, and Kondo, 1993). The region within p143 associated with expanding the host range of AcMNPV was narrowed to 3 amino acids that allowed replication of AcMNPV in *B. mori* larvae (Croizier et al., 1994). Eventually a single amino acid (AA) was identified as being sufficient to expand the host range of AcMNPV (Kamita and Maeda, 1997). At a high multiplicity of infection (MOI), AcMNPV variants carrying this single AA substitution were capable of infecting Sf9 and BmN cell lines (Kamita and Maeda, 1996). Since the original host range studies involving p143 were conducted, p143 has been confirmed as an in-vitro DNA helicase (McDougal and Guarino, 2000) with ATP-binding (McDougal and Guarino, 2001) activity that can be found associated with DNA in crosslinking studies (Ito et al., 2004). Suggestions as to how a DNA helicase might alter host range have not been offered. However, it is worth noting that the DNA polymerase from *Lymantria dispar* MNPV (LdMNPV) can functionally replace that of AcMNPV in transient DNA replication assays in Sf21 cells; however, p143 from LdMNPV can not replace AcMNPV p143 in the same assay (Ahrens and Rohrmann, 1996). The host specificity of the helicase indicates a potential role for its interaction with host-encoded proteins within the DNA replication complex of the virus.

**1.3.3.3. Apoptotic Suppressors and Host Range**

There are multiple hypotheses to explain why apoptosis occurs in virus-infected cells. The predominant hypothesis for invertebrates is that apoptosis results in a premature cell lysis further preventing virus replication and subsequent infection of neighboring host cells. Paradoxically, apoptosis may also function as a means for virus

dissemination and has thus, co-evolved with permissive virus infections (Friesen and Miller, 1996). It is important to consider that invertebrates are not equipped with an adaptive immune system and thus, must rely on innate immune responses to guard against pathogen infection. While mammals also rely on innate immunity for protection from pathogens, they are also equipped with immunological memory resulting in antibody-based recognition of epitopes from invading pathogens.

The apoptotic suppressor, p35, is non-essential for AcMNPV replication in Tn368 cells (Clem, Fechheimer, and Miller, 1991; Clem and Miller, 1993; Clem and Miller, 1994). Virus replication occurs in *Trichoplusia ni* larvae infected with AcMNPV p35 deleted (vAcΔp35) virus but there is a reduction in the production of occluded virus, with less melting at the end of infection as compared to a wildtype infection (Clem and Miller, 1993; Clem and Miller, 1994). *Spodoptera frugiperda* 21 (Sf21) cells that are infected with vAcΔp35 show severely impaired virus replication and a non-efficient shut-off of host protein synthesis (Clem and Miller, 1993; Hershberger, Dickson, and Friesen, 1992). In Sf21 cells infected with vAcΔp35, p35 can be replaced with an *inhibitor of apoptosis* (*iap*) gene from other baculoviruses and wildtype infection is restored (Birnbaum, Clem, and Miller, 1994). *Spodoptera frugiperda* larvae that are infected with vAcΔp35 show a 25-fold decrease in rate of infection as compared to wildtype AcMNPV (Clem and Miller, 1993; Clem and Miller, 1994). *Spodoptera littoralis*-2 (Sl-2) (Chejanovsky and Gershburg, 1995) and *Choristoneura fumiferana*-203 (Cf-203) cells (Palli et al., 1996) infected with AcMNPV undergo apoptosis, resulting in a decrease in budded virus production and no occluded virus production. Both cell types are permissive to other baculovirus infections. P35 may be insufficient to block apoptotic response in these cell

lines, as transcriptional analysis revealed that p35 expression is delayed in AcMNPV-infected Cf-203 cells (Palli et al., 1996). Thus, baculovirus apoptotic suppressors are also species and tissue-specific regulators of virus host range.

#### 1.3.3.4. Host Range Factor-1 (*hrf-1*)

AcMNPV enters but does not productively infect the *Lymantria dispar* 652Y (Ld652Y) cell line (McClintock, Dougherty, and Weiner, 1986). Global translation arrest begins in AcMNPV-infected Ld652Y cells by 8 h.p.i. correlating with the onset of DNA replication and the late stages of virus infection. All temporal classes of AcMNPV mRNA are synthesized and transported to the cytoplasm in infected Ld652Y cells and these mRNAs can be translated *in vitro* (Guzo et al., 1992). Expression of single gene, *host range factor-1* (*hrf-1*), isolated from *Lymantria dispar* Multinucleocapsid Nucleopolyhedrovirus (LdMNPV) prevents translation arrest in AcMNPV-infected Ld652Y cells and enables permissive replication of the virus in cell culture and infected larvae (Chen et al., 1998; Du and Thiem, 1997b; Thiem et al., 1996). HRF-1 also expands the host range of the nonpermissive *Hyphantria cunea* Multinucleocapsid Nucleopolyhedrovirus (HcMNPV) and the semi-permissive BmNPV and *Spodoptera exigua* Multinucleocapsid Nucleopolyhedrovirus (SeMNPV) (Ishikawa et al., 2004). These data suggest that HRF-1 is an essential host range factor for baculovirus infection of Ld652Y cells. Motif and homology searches provide no indications as to the functional role of HRF-1. A truncated conceptual protein of 78 amino acids with homology to HRF-1 exists in the genome of *Orygia pseudotsugata* Multinucleocapsid Nucleopolyhedrovirus (OpMNPV) but does not complement for LdMNPV HRF-1 function in AcMNPV-infected Ld652Y cells (Ikeda, Reimbold, and Thiem, 2005). HRF-

1 contains a domain that is predicted to be highly acidic, mutations in this domain result in varied changes in HRF-1 enabled AcMNPV replication in Ld652Y cells suggesting a role for the acidic domain in HRF-1 function (Ikeda, Reimbold, and Thiem, 2005).

In the absence of p35 and HRF-1, translation arrest and apoptosis are still initiated during the early stages of infection in AcMNPV-infected Ld652Y cells, apoptosis is enhanced and global translation arrest occurs at a time that correlates to the onset of DNA replication and the late stages of infection (Thiem and Chejanovsky, 2004). Blocking DNA replication and the late phases of virus replication with aphidicolin precludes global translation arrest in AcMNPV-infected Ld652Y cells, thus associating the arrest of protein synthesis with the late stages of virus infection (Thiem and Chejanovsky, 2004). Events correlating to the late stages of infection include the onset of DNA replication of the virus, the expression of late viral proteins and the shut-off of host RNA Polymerase II-mediated transcription (Fig. 4) (Friesen and Miller, 1986; Thiem and Miller, 1989). Global translation arrest and apoptosis appear to be linked in the response because infecting Ld652Y cells with an AcMNPV mutant carrying a non-functional version of the apoptotic suppressor p35 does not result in global translation arrest (Thiem and Chejanovsky, 2004). The global translation arrest infection phenotype is restored when the same AcMNPV mutant expresses Opiap, Cpiap or p49, apoptotic suppressors isolated from other baculoviruses that have different modes of action as compared to p35 (Thiem and Chejanovsky, 2004). Treatment of vAcΔp35-infected Ld652Y cells with peptide caspase inhibitors does not result in global translation arrest and supports high levels of budded virus production, suggesting that apoptosis must be initiated for global translation arrest to occur (Thiem and Chejanovsky, 2004).

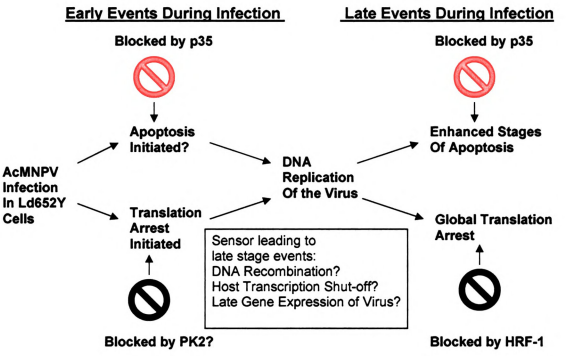



**Figure 4. Events that occur during AcMNPV-infection of Ld652Y cells (Image Presented in Color)**

### 1.3.3.5. Other Baculovirus Proteins Associated with Virus Host Range

The late expression factor, LEF-7, has homology to the herpes-virus UL29 family of single-stranded binding proteins (Lu and Miller, 1995b). LEF-7 has been identified as being an enhancer of viral DNA replication in Sf-21 cells (Chen and Thiem, 1997; Lu and Miller, 1995a) and a contributor to homologous recombination in plasmid transfected insect cells when in the presence of a full complement of viral proteins (Crouch and Passarelli, 2002). Viral replication was reduced to ten percent of wildtype AcMNPV levels in Sf21 and SE1c cells infected with a *lef-7* deletion mutant. The same mutation had no effect on virus replication in Tn368 cells indicating a species-specific role for host range effects of LEF-7 (Chen and Thiem, 1997).

When larvae of *Spodoptera frugiperda and Trichoplusia ni* are fed occluded AcMNPV which lack the immediate early gene, *ie2*, virus infectivity is reduced 1000 to 100 fold, respectively. Changes in infectivity are attributed to a lack of virions packed in the ODV of the mutant virus. However, based on this change oral infectivity, IE2 has been denoted as a possible determinant of virus host range. Larvae infected via intrahemocoelic injection with the same mutant had infection rates similar to wildtype AcMNPV infections (Prikhod'ko et al., 1999). Like LEF-7, the immediate early gene, *ie2*, of BmNPV and AcMNPV also enhances virus DNA replication of BmNPV in BmN cells (Gomi et al., 1997) and AcMNPV in Sf21 cells (Lu and Miller, 1995a), respectively. IE2 has been shown to transactivate IE1 in AcMNPV-infected Sf21 cells (Yoo and Guarino, 1994a; Yoo and Guarino, 1994b), to block cell cycle progression in cell lines from *Spodoptera frugiperda* and *Trichoplusia ni* (Prikhod'ko and Miller, 1998) and IE2 from BmNPV has E3 ubiquitin ligase activity (Imai et al., 2003). The diverse functional activity of IE2 in different virus-host relationships make it difficult to assess its possible role in determining virus host range.

### 1.4. Translational Modifications in Response to Stress

Global translation is reduced in most, if not all, types of cellular stress (Holcik and Sonenberg, 2005). Control of translation provides the cell with the ability to immediately respond to a broad range of stress-induced signals. There is increasing evidence, based on disproportionate amounts of mRNA as compared to coordinate protein levels, which indicate an essential role for translational regulation and protein stability in mediating the cellular response to stress (Gygi et al., 1999; Holcik and Sonenberg, 2005; Rajasekhar and Holland, 2004; Rajasekhar et al., 2003). Translation

can be separated into three distinct phases: initiation, elongation and termination (Mathews, 2000). The majority of examples that currently exist show translational control occurring at the initiation stage as a cessation of translation (Holcik and Sonenberg, 2005).

### 1.4.1. Translation Initiation

The initiation of translation is a complex process coordinated by numerous protein subunits that join at the 5' and 3' ends of an mRNA transcript. In the cytoplasm, ternary complexes are formed which consist of a GTP molecule, a methionine initiator tRNA and the eukaryotic initiation factor 2 complex. The ternary structure joins with the 43S pre-initiation complex which consists of the 40S ribosomal subunit and eIF3, eIF1, eIF1A and eIF5. Binding of the pre-initiation complex to the 5' cap structure is believed to require bridging interactions between eIF3 and the eIF4F protein complex which is bound through eIF4E, the cap binding protein, at the $m^7$GpppN cap structure. Other subunits within the eIF4F complex include eIF4A, the dead-box RNA helicase, and eIF4G which acts as a scaffold protein between eIF4E, eIF4A and eIF3. The subunit eIF4G also interacts with the poly-A binding protein (PABP) and this interaction between proteins bound at the 5' and 3' end of the mRNA transcript, respectively, results in a circularization of the template (See Fig. 5). After recruitment to the mRNA, it is believed that the 43S initiation complex scans the template for an AUG translation initiation codon (Pestova and Kolupaeva, 2002). The methionine initiator tRNA binds to the AUG start site and the 43S ribosomal subunit is joined by the 60S ribosomal subunit to form the 80S ribosome. Ribosome formation is followed by the release of initiation factors for cellular recycling (See Fig. 4) (Gebauer and Hentze, 2004).

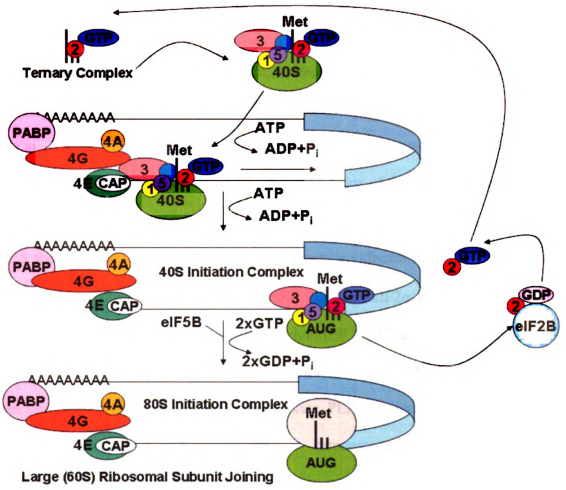



**Figure 5. Schematic diagram of the initiation of protein synthesis in eukaryotes adapted from Gebauer and Hentze 2004 (Image Presented in Color)**

### 1.4.2. Initiation Stage Translational Modifications in Response to Stress

Translation arrest occurring at the initiation stage generally results from post-translational modifications of eukaryotic initiation factors (eIFs). Common post-translational modifications of eIFs include phosphorylation and/or proteolytic cleavage.

Modifications lead to blocked interactions with other components of the translation initiation machinery and result in translation attenuation.

### 1.4.2.1. Phosphorylation of eIF2α

During translation initiation, as the methionine initiation tRNA joins the AUG start codon, eIF2-bound-GTP undergoes hydrolysis yielding a GDP molecule. In order to once again form an active ternary complex, eIF2-bound-GDP must be recharged to GTP by the nucleotide exchange factor eIF2B. Phosphorylation of the alpha subunit of eIF2 at serine 51 results in the sequestering of eIF2B which is available in limited quantities (See Fig. 5 and 6). There are four characterized mammalian kinases of $eIF2\alpha^{ser51}$: the amino acid regulated GCN2, the heme-regulated HRI, PERK-an eIF2α kinase that is activated in response to stress in the endoplasmic reticulum (ER) and the virus-activated, double-stranded RNA-dependent protein kinase (PKR). Homologues of GCN2 and PERK have been characterized for invertebrates. Invertebrate PERK is functionally similar to its mammalian homologue and is activated in response to ER stress but is also developmentally regulated (Sood et al., 2000). A novel eIF2α kinase, BeK, was characterized from the Lepidoptera, *Bombyx mori*. BeK exerts kinase activity in transformed *Drosophila* Schneider cells as a result of heat shock and osmotic stress. BeK is not activated in response to bacterial infection or induced ER or oxidative stress (Prasad et al., 2003) (For overview see Fig. 6).

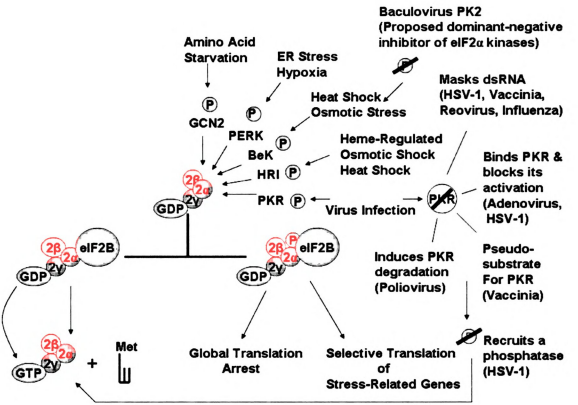



**Figure 6. Schematic diagram of eIF2α phosphorylation and viral strategies for overcoming kinase-induced translation arrest adapted from Holcik and Sonenberg 2005 (Image Presented in Color)**

### 1.4.2.2. 4E-BP and eIF4E

The cap-binding protein, eIF4E, is shaped like a bowl. Its concave surface directly interacts with the m7GpppN structure at the 5' end of the mRNA and its convex surface interacts with the scaffold protein eIF4G. In proliferating cells, the 4E-binding protein (4E-BP) is phosphorylated and unable to bind eIF4E. At times of stress and apoptotic induction, 4E-BP becomes a target for phosphatases or is targeted to the proteasome. Hypophosphorylated 4E-BP competes for binding of eIF4E with eIF4G and eventually the cap binding protein is sequestered, attenuating translation. Translation resumes when

mTOR-activated kinases phosphorylate 4E-BP, causing the release of eIF4E (Gebauer and Hentze, 2004; Holcik and Sonenberg, 2005).

#### 1.4.2.3. Proteolytic Cleavage of eIF4GI, eIF4GII and Other Initiation Factors

Caspase-mediated proteolytic cleavage of several eukaryotic initiation factors can occur in response to the induction of apoptotic programs in a variety of mammalian cells. Different apoptotic pathways can be initiated as a result of ER stress, DNA damage or specific ligand-receptor binding. However, all defined apoptotic pathways cascade to the eventual production of low-level, effector caspases such as caspase 3 or 7. Caspase-3 mediated cleavage has been described for eIF2α, eIF4E, PABP and eIF4GI/eIF4GII (Holcik and Sonenberg, 2005; Morley, Coldwell, and Clemens, 2005). This apoptotic-induced cleavage of translation initiation factors generally leads to a global attenuation of cap-dependent translation.

Caspase-mediated cleavage of eIF2α may be functionally significant as the truncated form appears to no longer be dependent on GDP exchange by eIF2B (Marissen et al., 2000). The truncated form of eIF2α, when over-expressed, was able to overcome translational repression mediated by PKR (Satoh et al., 1999). Cleavage of eukaryotic initiation factors has been proposed as a means for the cell to overcome translational repression by favoring internal ribosomal entry site (IRES) or alternate scanning-based translation of stress responsive genes such as GCN4 in yeast and ATF4 in human cells. Caspase-driven cleavage of eIF4GI and eIF4GII has been reported for virus and stress-induced cell lines (Belsham, McInerney, and Ross-Smith, 2000; Clemens, Bushell, and Morley, 1998; Gradi et al., 1998; Svitkin et al., 1999). In BJAB cells treated with cycloheximide, eIF4GI is cleaved and is incorporated into a long-term, stable eIF4F

complex prior to eventual degradation. This complex may support cap-dependent translation (Bushell et al., 2000).

### 1.4.3. Virus Strategies for Overcoming Host-Mediated Translation Arrest

Viruses are dependent on host-encoded translation machinery to synthesize viral proteins, thus viruses have evolved a number of mechanisms to overcome host-induced translation arrest. Most characterized viral mechanisms for overcoming translation arrest at the initiation stage affect PKR-induced eIF2α phosphorylation. In mammalian cells, PKR exists as a dimer in the cytoplasm. Recognition and subsequent interaction with dsRNA species induces a conformational change in PKR which results in its autophosphorylation and activation as an eIF2α kinase (Kaufman, 2000). Upon infection and cell entry, dsRNA can result from both the transcription of RNA viruses and the dual template transcription from DNA viruses (Schneider and Mohr, 2003). Viruses have developed a number of clever strategies to overcome translational inhibition initiated by PKR. Adenovirus and Epstein-Barr virus produce the abundant and highly structured RNA species $VARNA_1$ (Schneider, 2000) and EBER RNAs (Clemens, 1993), respectively that compete with dsRNA for binding of PKR. Herpes Simplex 1 (HSV-1)-Us11 (Khoo, Perez, and Mohr, 2002; Mulvey et al., 1999), Vaccinia virus-E3L (Beattie et al., 1995), Reovirus-σ3 (Lloyd and Shatkin, 1992) and Influenza-NS1 (Salvatore et al., 2002) are all proteins that bind and mask dsRNA, thus preventing its recognition by PKR. VARNA1, Us11 (Cassady and Gross, 2002; Peters et al., 2002) and the HIV TAR RNA (Gunnery et al., 1990; Maitra et al., 1994) all directly bind PKR to prevent autophosphorylation and activation. Vaccinia virus K3L acts as a pseudo-substrate for PKR (Carroll et al., 1993) and there is some evidence that indicates that poliovirus is

capable of directing cellular proteases to degrade PKR (Gale, Tan, and Katze, 2000). Additionally, the γ34.5 protein of HSV-1 recruits a phosphatase to peIF2α$^{ser51}$ which prevents the sequestering of eIF2B allowing for GDP hydrolysis and continued translation (He, Gross, and Roizman, 1997).

A PKR homologue has not been identified in insects. Double-stranded RNA interference has been shown to be very effective in completely silencing virus and host-encoded gene expression in invertebrate cell lines (Means, Muro, and Clem, 2003), without initiating translation arrest, so, a classical PKR protein may not exist in invertebrates. However, PKR is integral to antiviral response in mammalian cell lines and RNA interference can be used to silence gene expression in those same cells (Cioca, Aoki, and Kiyosawa, 2003; Spankuch and Strebhardt, 2005). Both baculovirus infection and dsRNA used in an attempt to silence hemolin gene expression in Chinese Oak Silk Moth resulted in an induction of hemolin expression, indicating the existence of a lepidopteran molecular response pathway to dsRNA (Hirai et al., 2004). It is possible that other eIF2α kinases, previously characterized for invertebrates, serve as surrogates to PKR function or that another uncharacterized dsRNA response mechanism exists for Lepidoptera. Interestingly, baculoviruses encode PK2, a protein with kinase subdomain homology to PKR and all other eIF2α kinases. It is predicted that PK2 acts as a dominant-negative inhibitor of eIF2α kinases (Li and Miller, 1995), suggesting that eIF2α phosphorylation may be induced in response to baculovirus infection and that the virus has evolved a mechanism(s) to overcome its effects. Indeed, eIF2α is protected from phosphorylation at late times post-infection in SF9 cells infected with AcMNPV (Dever et al., 1998) (Fig. 6).

### 1.4.4. Preferential Translation of Viral Transcripts

In addition to circumventing the effects of host-induced translation arrest programs, viruses have developed a number of mechanisms to ensure preferential translation of viral transcripts over host-encoded mRNA, thus pirating away the host's translational apparatus. Picornaviruses, such as poliovirus, encode a 2A protease which cleaves the N-terminus of eIF4G, thus abolishing the ability of eIF4G to interact with PABP (which is also cleaved by 2A) and eIF4E, which eliminates cap-dependent translation of host RNAs (Joachims, Van Breugel, and Lloyd, 1999; Kerekatte et al., 1999). Picornaviruses enable translation of virus RNA with internal ribosomal entry sites (IRES) and bootlegging of virus-modified, host translation factors (Jang et al., 1988; Pelletier and Sonenberg, 1988). Modification and secondary structure of the 5' UTR of many virus transcripts is essential to ensuring preferential translation. IRES have been identified for all picornaviruses, HIV, HCV and HHV8 (Hellen and Sarnow, 2001). It is interesting to note that IRES sites have been identified for stress-responsive mammalian genes, thus suggesting a mechanism for cell recovery once translation programs have been attenuated (Hellen and Sarnow, 2001). Adenovirus late expressed genes carry a tripartite leader sequence in the 5' UTR that is essential for translation (Schneider, 2000) and the 5' UTR of influenza virus attracts a cellular protein, GRSF-1, which stimulates translation in-vitro (Park, Wilusz, and Katze, 1999). Other novel virus-mediated, translation initiation factor modifications exist but all result in a shutdown of host translation (Aragon et al., 2000; Feigenblum and Schneider, 1993; Piron et al., 1999).

### 1.5. Stress Response Pathways and Virus Infection

Hosts have evolved a number of molecular strategies to respond to the threat of virus infection and other environmental and pathogenic stress inducers. Stress-activated pathways share many components with characterized innate immunity pathways. Likewise, viruses have evolved many mechanisms to overcome these strategies and in some cases, viruses use host-induced defenses to benefit their own replication. Notably, apoptosis and global translation arrest can occur in response to the UPR via endoplasmic reticulum stress which can be induced in response to virus infection.

#### 1.5.1. The Unfolded Protein Response (UPR)

Baculovirus host range systems have been characterized in cell culture. Information gained from cell culture systems is limited because a response initiated in a single type of cell may differ from other cell types from the same organism. Cells from certain tissues may accommodate viral entry and in a living insect system, virus may be cleared or an immune response launched prior to entry of the virus into the cell. However, in characterized, cell culture-based systems, baculovirus host range appears to be controlled during the late stages of infection. Viruses enter cells that are non-permissive to virus replication, early genes are transcribed, DNA replication begins but neither budded nor occluded virus is produced. This is true not only for AcMNPV in Ld652Y cells but in other baculovirus host range systems as well (Thiem, 1997). Host cell transcription is not down-regulated until the late stages of infection. In AcMNPV-infected Ld652Y cells, global translation arrest and enhanced apoptosis do not occur until the late stages of infection. Thus, it is logical to presume that an increasing number of virus-encoded transcripts on the endoplasmic reticulum (ER) and cytosol of the host

places a measurable, increased load on the host's translational machinery and protein folding apparatus. An abundance of unfolded proteins in the ER can result in ER stress, leading to the initiation of the unfolded protein response (UPR). The UPR has been characterized in mammalian cell lines and yeast (Rutkowski and Kaufman, 2004). Homologous components of the UPR have been identified and characterized for invertebrates. The UPR response pathway has characterized in *C. elegans* and is important in developmental regulation of the nematode (Shen et al., 2001). PERK, the ER stress activated eIF2α kinase has been identified and characterized in *Drosophila melanogaster* but not Lepiodptera (Olsen et al., 1998; Sood et al., 2000). Additionally, the transcriptional activator ATFC has been identified as being activated in response to UPR stress in *Bombyx mori* (Goo et al., 2004). Activation of the UPR results in a decrease of global translation, an increase in the synthesis of chaperone proteins and protein degradation coupled to endoplasmic reticulum-associated degradation (ERAD) (Meusser et al., 2005; Rutkowski and Kaufman, 2004).

The endoplasmic reticulum is the site of synthesis, folding and post-translational modification of cell-surface and secretory proteins and proteins that comprise the secretory pathway. Folding of proteins destined for intracellular organelles and the cell surface occurs in the ER and is accomplished by cellular chaperone proteins. The most abundant cellular chaperone in the ER is the glucose-regulated, ER chaperone protein BiP, which is also known as the heat shock 70 protein cognate 3 precursor (HSC70). Three proximal sensors of accumulated unfolded proteins, PERK, IRE1 and ATF6, exist at the lumen of the ER (Bertolotti et al., 2000; Harding, Zhang, and Ron, 1999; Liu, Schroder, and Kaufman, 2000). Long-term activation of PERK also results in cell cycle

arrest (Brewer and Diehl, 2000; Harding et al., 2000b; Harding et al., 2003; Okada et al., 2002). Subsequent to PERK activation, ATF4 is preferentially translated despite eIF2α phosphorylation and serves to activate genes important to stress recovery (Harding et al., 2003). ATF6 is cleaved from the ER in response to stress and is transported to the Golgi body for processing, it is then transported to the nucleus where it acts as a transcriptional activator for genes which alleviate ER stress, including chaperone proteins and XBP1 (Ye et al., 2000). At a step following PERK and ATF6 induction, the IREI pathway is initiated resulting in differential splicing of XBP1 which serves as a transcriptional activator for genes associated with protein degradation (For overview see Fig. 7) (Calfon et al., 2002; Lee et al., 2002; Rutkowski and Kaufman, 2004; Shen et al., 2001; Yoshida et al., 2003; Yoshida et al., 2001).

Extended activation of the unfolded protein response results in the induction of two reversible, interactive, proapoptotic pathways. An intrinsic apoptotic pathway, similar to that stimulated in response to DNA damage, can be initiated in response to the UPR, which results in the mitochondrial release of cytochrome c, leading to caspase 9 activation and the eventual activation of effector caspases. The UPR also activates caspase 9 in Apaf-$1^{-7-}$ cells where caspase 9 cannot be activated with the release of cytochrome c (Rao et al., 2002). In these cells, UPR-induced apoptosis is initiated via a disruption of IRE1 with TRAF2 at the ER membrane, which results in the release of caspase 12 by TRAF2. Caspase 12 subsequently activates caspase 9 and the apoptotic cascade commences (Nakagawa et al., 2000; Yoneda et al., 2001). An extrinsic pathway, as modeled by the association of cell surface receptors in response to ligation of tumor necrosis factor, further stimulates a cascade of effector caspases via caspase 8 activation

and results in apoptosis. Both intrinsic and extrinsic type pathways seem to be required for apoptosis to occur in response to the UPR. It is worthy to note that while apoptotic pathways have been characterized in *C. elegans*, which has the core components of the intrinsic pathway, the nematode lacks the homologous components of the extrinsic pathway. Additionally, the intrinsic pathway in nematodes does not require cytochrome c for activation of Ced-3 (caspase 9) (Richardson and Kumar, 2002; Zheng et al., 1999). Damaged proteins that are retained in the ER during the UPR are eventually targeted for degradation through the endoplasmic reticulum associated degradation (ERAD) pathway. Misfolded and denatured proteins are translocated from the ER to the cytoplasm through special pores in the ER that are linked to ER-bound ubiquitin ligases which, in vitro, act specifically on denatured proteins. Ubiquitin-conjugated proteins are then targeted to the proteasome for degradation (For Review see Meusser et al., 2005).

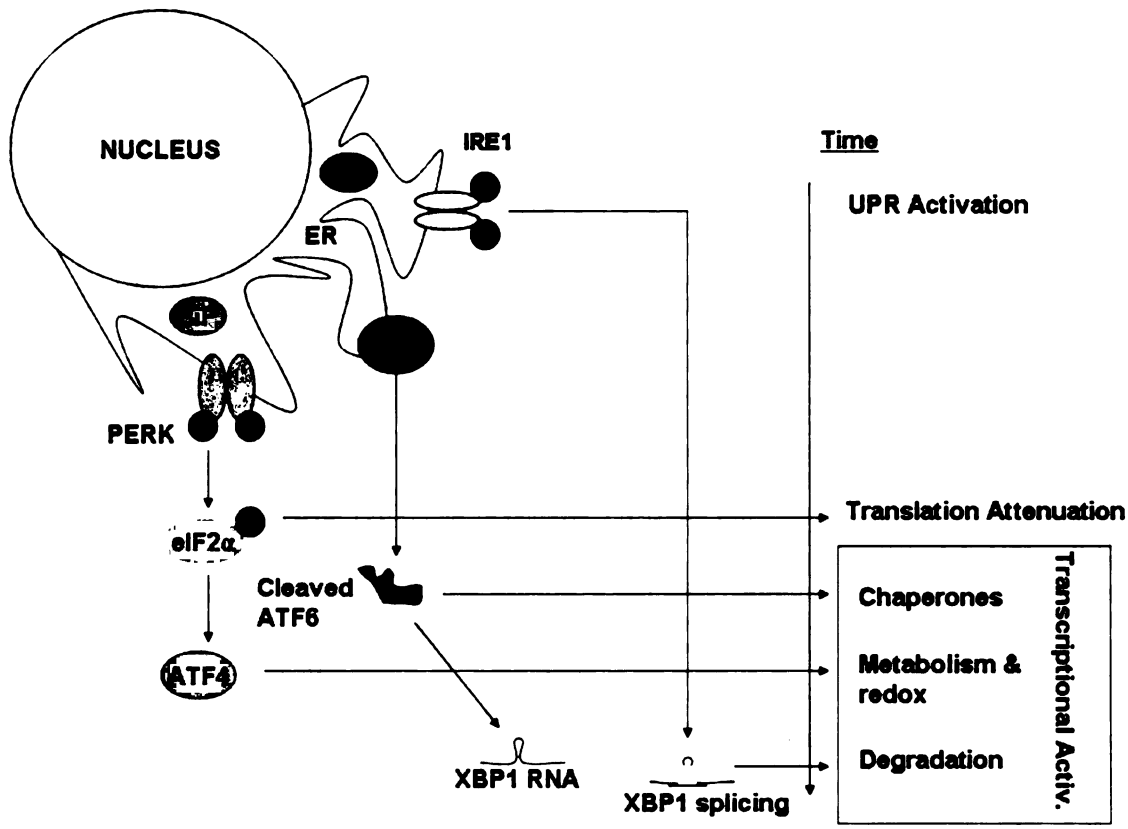



**Figure 7. The unfolded protein response (UPR) in eukaryotes adapted from Rutkowski and Kaufman 2004 (Image Presented in Color)**

### 1.5.1.1. The UPR and Virus Infection

Recent research shows that permissive virus replication in human cell lines results in an accumulation of viral proteins in the ER lumen (Cheng, Feng, and He, 2005; Su, Liao, and Lin, 2002). The emerging data shows that the UPR is activated in response to many different types of infections, and that viruses have either evolved to suppress the pathway or to command components of the UPR to aid in virus replication. Herpes Simplex Virus (HSV) activates PERK and regulates its dephosphorylation with the viral-encoded protein $\gamma_1$34.5 (Cheng, Feng, and He, 2005) and PERK contributes to cellular

resistance to Vesicular Stomatitis Virus (VSV) as evidenced by enhanced virus replication in PERK$^-$ cell lines (Baltzis et al., 2004). Hepatitis C Virus (HCV) activates the IRE1-XBP1 pathway of the UPR which directs both refolding and degradation of unfolded proteins in the ER lumen. While expression of XBP1 is increased in cells carrying HCV subgenomic replicons, its transactivating activity is blocked, resulting in no transcriptional induction of the ER degradation-enhancing α-mannosidase-like protein (EDEM) which is required for the degradation of misfolded proteins (Tardif et al., 2004). Japanese Encephalitis Virus (JEV) induces the UPR in human fibroblast and neuronal cells, resulting in eventual apoptotic cell death. Strangely, apoptotic-resistant K562 cells show no UPR induction following JEV infection, suggesting a regulatory feedback loop for the virus-induced UPR (Su, Liao, and Lin, 2002). Perhaps the most intriguing example of the UPR and virus infection is Human Cytomegalovirus (HCMV) which selectively induces and suppresses individual mechanisms of the UPR. HCMV induces the UPR and PERK but phosphorylation of eIF2α is limited and translation does not attenuate. In contrast, ATF4, which normally lies downstream of PERK-mediated eIF2α phosphorylation, is transcriptionally activated, allowing for transcriptional activation of stress recovery genes. In HCMV infected cells, ATF6 activation is suppressed but synthesis of chaperone proteins is increased. Additionally, splicing of XBP-1 occurs, indicating a prolonged UPR stress response, but transcriptional activation of EDEM is suppressed (Isler, Skalet, and Alwine, 2005).

#### 1.5.1.1.1. Host-Encoded Molecular Chaperone Requirements for Virus Infection

As previously mentioned, molecular chaperone proteins are transcriptionally up-regulated as a part of the UPR to relieve the ER stress induced from an abundance of

unfolded proteins retained within the ER lumen. Additional translational regulation of molecular chaperones may also exist. Several groups have documented the importance of host-encoded chaperone proteins in the virus infection process. Heat shock protein 90 (HSP90) is required to enable the reverse transcriptase activity of Hepatitis B Virus (Hu and Seeger, 1996). HSP90 also facilitates virus replication of Vaccinia Virus in human cell lines (Hung, Chung, and Chang, 2002) and Flock House Virus in *Drosophila* cells (Kampmueller and Miller, 2005). HSC70, also known as BiP, is shown to be transcriptionally activated in AcMNPV-infected Sf21 cells at a time when most host-encoded transcription is being repressed (Nobiron, O'Reilly, and Olszewski, 2003). It follows that if the UPR stress response is integral to many different types of virus infection, expression of the ER luminal chaperone, BiP, may also play a role in baculovirus infection.

### 1.6. Proteasome Activation and Virus Infection

An increasing number of reports indicate that proteasome-mediated degradation is a key mechanism utilized by viruses to enable permissive replication cycles. Likewise, proteasome-mediated degradation is key to orchestrating other cellular functions such as DNA repair, cell-cycle progression, signal transduction and immune response. In eukaryotes, multi-subunit proteasomes reside in the nucleus and cytosol. Proteins are targeted to the proteasome for degradation via ubiquitination by cellular localized E3 ubiqitin ligases. Ubiquitination leads to translocation of the targeted protein to the proteasome where deubiquitination occurs prior to entry into the 26S active proteasome (Jentsch and Schlenker, 1995). Proteasome-mediated degradation generates peptide fragments of 7-8 amino acids in length (Lowe et al., 1995). The COP9 signalosome

provides another level of complexity to proteasome-mediated degradation. COP9 is integral to many signaling pathways including pathogen response (Azevedo et al., 2002; Liu et al., 2002), in a wide variety of organisms. Recent evidence suggests that the COP9 signalosome may serve as platform for kinases, kinase substrates and the proteasome thus regulating the degradation of phosphorylated proteins (Harari-Steinberg and Chamovitz, 2004).

Viruses can alter the cell cycle to enable virus replication. The E6 protein of human papillomavirus (HPV) binds the p53 tumor suppressor protein, targeting it for proteasome degradation (Stewart et al., 2004) and pp71 of HCMV acts in a similar fashion on the human retinoblastoma protein (Kalejta, Bechtel, and Shenk, 2003). In both cases, the result is stimulation of DNA synthesis. Viruses also direct innate immunity pathways through proteasome activation. HCV and Paramyxovirus X direct proteasome-dependent degradation of the IFN-signaling protein, STAT1, thus disabling host innate control of the virus (Lin et al., 2005; Nishio et al., 2005). HCMV directs ubiquitination and degradation of IκB via the proteasome thus allowing activation of the Nf-κB pathway and virus replication (Evers, Wang, and Huang, 2004). The RTA immediate-early (IE) transcription factor acts as a E3 ubiquitin ligase that targets IFN7 for proteasomal degradation, which further blocks induction of the interferon response in human cells (Yu, Wang, and Hayward, 2005) . Of other interest is the requirement for proteasome activation in coordinating Vmw110 modification and the switch from latent to lytic HSV infection (Everett, Orr, and Preston, 1998). Additionally, proteasome inhibition results in the accumulation of Murine Coronavirus in the endosome and denser lysosome of DBT

cells, indicating a role for the proteasome in mediating viral entry into the cytosol (Yu and Lai, 2005).

### 1.6.1. The COP9 Signalosome, Protein Degradation and Kinase Signaling

The COP9 signalosome (CSN) is structurally similar to the regulatory lid of the 26S proteasome and the eukaryotic initiation factor 3 (eIF3) (Kim et al., 2001). The CSN has been identified and functionally analyzed in fungi (Busch et al., 2003), fission yeast (Mundt, Liu, and Carr, 2002; Mundt et al., 1999), mammals (Seeger et al., 1998; Wei and Deng, 1998), plants and *Drosophila*. This complex has been shown to play a key role in regulating the development of the latter two organisms (Freilich et al., 1999; Peng, Serino, and Deng, 2001a; Peng, Serino, and Deng, 2001b). COP9 has also been identified as playing a role in regulating cellular response to viral and fungal pathogens (Azevedo et al., 2002; Liu et al., 2002), responding to environmental stress and directing growth hormone signaling cascades in plants (Schwechheimer et al., 2001). The signalosome functions through the two distinct but not mutually exclusive pathways of kinase signaling and ubiquitin-dependent, proteasome-mediated degradation (Harari-Steinberg and Chamovitz, 2004). The CSN acts as a target for kinases and also coordinates kinase activity. It also has deneddylation and deubiquitination activities. The complex coordinates ubiquitin conjugation of key components of regulatory signaling cascades. CSN has kinase activity against IκB, p105 and c-JUN (Seeger et al., 1998) and the CSN-associated proteins p53, $p27^{kip}$ and ICSBP are phosphorylated by CSN-associated kinases (Bech-Otschir, Seeger, and Dubiel, 2002). C-JUN is normally activated in response to environmental stress, radiation and growth factors resulting in an increase in AP-1 mediated transcription (Seeger et al., 1998). CSN-dependent JNK phosphorylation of c-

JUN stabilizes the transcription factor and protects it from proteasome-dependent degradation. In contrast, the CSN maintains constant, proteasome-mediated degradation of the p53 tumor suppressor protein, maintaining a low level of the protein until a variety of cell stress can result in stabilization of p53 and activation of the cell cycle (Sharpless and DePinho, 2002). Both c-JUN and p53 can be sumolated, which impacts proteasome-mediated degradation (Schwechheimer and Deng, 2001). One possible model poses that the CSN serves as a master docking station that coordinates the stabilization or proteasome-mediated degradation of phosphorylated proteins through their interacting kinases (Harari-Steinberg and Chamovitz, 2004).

# Chapter 2

## In baculovirus-infected Lepidoptera cells, proteasome inhibitors reduce virus production and eIF2α phosphorylation at serine 51

## 2.1. Introduction

Baculoviruses infect lepidopteran larvae. Permissive baculovirus replication results in the production of occluded and budded virus. Caterpillars ingest occluded virus that is embedded in polyhedral occlusion bodies. Occluded viruses are released and infect midgut cells. Infected midgut cells produce budded virus, which then spreads to other tissues. *Autographa californica* Multinucleocapsid Nucleopolyhedrovirus (AcMNPV), the type virus for the family *Baculoviridae* (Theilmann et al., 2004), enters but does not productively infect the *Lymantria dispar* 652Y (Ld652Y) cell line (McClintock, Dougherty, and Weiner, 1986). Translation arrests in AcMNPV-infected Ld652Y cells beginning at approximately eight hours post-infection (h.p.i.) and correlates with the onset of virus DNA replication (Goodwin, Tompkins, and McCawley, 1978; Guzo et al., 1992). Translation is rescued by the expression of a single gene, *host range factor-1* (*hrf-1*) (Du and Thiem, 1997a; Thiem et al., 1996), which was isolated from *Lymantria dispar* Multinucleocapsid Nucleopolyhedrovirus (LdMNPV), a baculovirus that productively infects Ld652Y cells. Homology and motif searches conducted with the *hrf-1* sequence do not provide any indications of its function.

Translational arrest is induced in response to a variety of stress signals and is commonly controlled at initiation. A key mechanism mediating translation arrest is phosphorylation of eIF2α at serine 51. Phosphorylated eIF2$\alpha^{ser51}$ binds and sequesters the guanosine nucleotide exchange factor eIF2B thus preventing the formation of the active ternary complex required for translation initiation (Hershey and Mathews, 1996). To counter viral infection, mammalian cells utilize the Double-stranded RNA-dependent Protein Kinase (PKR) which forms a dimer upon binding double-stranded RNA and is

activated by autophosphorylation. Activated PKR then phosphorylates eIF2α at serine 51 (Williams, 1999). Virus infection can also lead to eIF2α phosphorylation mediated by the endoplasmic reticulum (ER) resident kinase (PERK or PEK) through the unfolded protein response (UPR). ER stress occurs presumably as the result of large scale host synthesis of virus proteins (Isler, Skalet, and Alwine, 2005). A PKR homolog has not been identified in invertebrates. However, other eIF2α kinase homologs have been identified and characterized from invertebrates including the amino acid regulated GCN2 (Olsen et al., 1998; Santoyo et al., 1997), ER stress-regulated PERK/PEK (Sood et al., 2000) and *Bombyx mori* eIF2α kinase (BeK), a kinase specific to lepidopteran species that is activated in response to heat shock and osmotic stress in transformed *Drosophila* Schneider cells (Prasad et al., 2003).

Other host cell mechanisms that regulate protein synthesis in response to virus infection include stress granule formation, cleavage of mRNAs and tRNAs, and specific proteolytic cleavage of translation initiation factors (Clemens, 2005; Marissen et al., 2000; Ohlmann et al., 2002; Satoh et al., 1999; Tee and Proud, 2002). Currently, host cell mechanism(s) responsible for proteolysis of translation initiation factors are poorly understood. However, the translation initiation factors eIF2α, 4E-BP, eIFGI and eIFGII have been shown to be specifically cleaved by different caspases in response to the induction of apoptosis (Clemens et al., 2000; Marissen et al., 2000; Satoh et al., 1999; Tee and Proud, 2002). The host translation factor, eIF4GI, is proteolytically cleaved in a different pattern in picornavirus-infected cells by viral proteases (Lamphear et al., 1993) and dissected into multiple fragments in-vitro by an HIV protease (Ohlmann et al., 2002).

Targeted ubiquitination leading to proteasome-mediated degradation is essential to many cellular regulatory processes including DNA repair, cell cycle progression and immune response (Duan et al., 2004; Greene et al., 2003; Hoege et al., 2002; Sarmento et al., 2005). It has also been shown that proteasome-mediated degradation of target proteins may be critical to successful replication of some viruses in mammalian systems. For example, RTA, the immediate-early transcription factor of Kaposi's Sarcoma-Associated Herpesvirus (KSHV or HHV8), has E3 ubiquitin ligase activity that targets proteasome-mediated degradation of IRF-7, thus blocking the induction of a host innate immune response (Yu, Wang, and Hayward, 2005). Proteasome activation is also believed to be required for the transition of HSV-1 from a latent to a lytic infection through Vmw110 modification (Everett, Orr, and Preston, 1998). Additionally, the proteasome inhibitor, MG132, has been shown to block replication of human cytomegalovirus (HCMV) at the immediate-early stage of infection (Prosch et al., 2003). In this study we identify a virus-induced, proteasome-dependent, phosphorylation and cleavage of eIF2$\alpha$ that accompanies permissive AcMNPV replication in two invertebrate cell lines. Phosphorylation and cleavage occur during the late stages of the infection cycle. We further show that proteasome inhibition with epoxomicin or MG132 blocked vAchrf-1 replication in Ld652Y cells. Epoxomicin was effective at blocking vAchrf-1 and AcMNPV replication in Sf21 cells but MG132 only reduced budded virus titers in the same productively-infected Sf21 cells.

## 2.2. Materials and Methods

### 2.2.1. Viruses and Cell Lines.

Sf-21 cells (Vaughn et al., 1977) and Ld652Y cells (Goodwin, Tompkins, and McCawley, 1978) were maintained in TC100 medium (JRH BioScience, Lenexa, KS) supplemented with 10% fetal bovine serum and 0.26% tryptose broth. AcMNPV variant L1 (Lee and Miller, 1978), vAcΔPK2 (Li and Miller, 1995) and vAchrf-1 (previously designated vAcLdPD, Du and Thiem, 1997) were propagated in Sf21 cells with titers being determined by plaque assay.

### 2.2.2. Cytoplasmic fraction extraction and polysome profiling.

Polysome profiles were prepared using a protocol adapted from Brecht and Parsons (Brecht and Parsons, 1998). Five 100mm tissue culture plates were seeded with 5 x $10^6$ Ld652Y cells per plate and infected with either AcMNPV L1 or vAchrf-1 at an MOI of 10 PFU per cell. After 1 h incubation, the inoculum was removed and complete medium was added to cells. The cells were then incubated at 27°C until the indicated time. At 9 h.p.i., AcMNPV and vAcHrf-1 infected Ld652Y cells were treated with 100 μg/ml cycloheximide for 5 min and the cells were harvested. The harvested cells were washed with 10 ml of ice-cold phosphate buffered saline (PBS), then suspended in 750 μl of ice-cold polysome buffer A (20mM Tris HCl [pH 7.4], 10 mM NaCl, 3 mM $MgCl_2$, 100 μg/ml cycloheximide, 1 μM DTT and 15 μg RNasin) and equilibrated on ice for 3 min. Cells were lysed by the addition of 250 μl of lysis buffer (polysome buffer A containing 0.2% NP40 and 0.2M sucrose) followed by incubation on ice for 5 min with occasional mixing. The lysates were centrifuged at 1000 x *g* at 4°C for 10 min to remove nuclei and the supernatants were transferred to a new tube containing 40 μl 4M NaCl,

100 µl heparin (10 mg/ml) and 20 µg RNasin. 400 µl of the cytoplasmic extract was then layered on a linear 15-50% sucrose gradient and centrifuged at 230,000 x *g* at 4°C for 1.5 h in a SW40 rotor. Following centrifugation, the gradients were harvested from the top using an ISCO density gradient fractionator while continuously measuring the optical density at 254nm.

**2.2.3. Cell Stress Induction Assays.**

Sf21 cells were seeded in 35 mm cell culture plates at a density of 1 x $10^6$ or Ld652Y cells at a density of 5 x $10^5$ and each plate was overlaid with 3 ml of TC100 medium. Stress inducing compounds were added directly to cell culture media to reach the following final concentrations: 2 µM epoxomicin (Calbiochem #324800), 50 µM hydrogen peroxide, 90 µM methyl methanesulfonate (MMS) (Sigma #M 4016), 1 µg/ml rapamycin (Sigma #R 0395), 1 µM thapsigargin (Sigma #T 9033) and 12 µM tunicamycin (Sigma #T 7765). After treatment, cultures were returned to 27°C incubation. Cells receiving heat shock were cultured at 39°C. Media and cells were collected at 6 and 24 hours post-treatment, centrifuged at 2,600 x *g* for 8 minutes, media was removed and cells were lysed in 100 µl of NP40 buffer containing a protease and phosphatase inhibitor cocktail of 1mM benzamidine, 10 µg/ml aprotonin, 1 mM PMSF, 5 µg/ml leupeptin and 100 µM okadaic acid. Cells were lysed on ice for 30 minutes then debris was collected via refrigerated centrifugation at 17,900 x *g* for 8 minutes. Supernatant was collected and stored at -80°C for future quantification and western blot analysis.

### 2.2.4. Protease and Proteasome Inhibitor Assays.

Cell culture plates (60 mm) were seeded with either Sf21 cells at a density of 2 x $10^6$ or Ld652Y cells at a density of 1 x $10^6$. For aphidicolin treatment, cells are pre-conditioned for one hour prior to infection with aphidicolin added directly to the cell culture media to a final concentration of 5μg/ml. After cell attachment, media was removed and cells were infected at an MOI of 10 with AcMNPV or vAchrf-1 respectively. After a one hour infection, virus was removed and cells were refed with 4 ml of TC100 media per plate. Inhibitors were added directly to cell culture media to reach the following final concentrations: 1 μM aprotonin (Calbiochem #616370), 5 μg per ml of aphidicolin (Sigma #A 0781), 1 μM E64d/EST (Calbiochem #330005), 10 mM EDTA-$Na_2$ (Gibco, #15575-038), 10 μM pepstatin A (Calbiochem #516481) and cells for the proteasome inhibitor assay were treated with 2 μM epoxomicin or 10 μM MG132. Infected cell cultures that were assayed at 48 hours post-infection were re-treated with fresh inhibitors at 24 hours post-infection to counteract possible inhibitor degradation. Media and cells were collected at 24 and 48 hours post-infection, centrifuged at 2,800 x *g* for 8 minutes, media was removed and cells were lysed as described above.

### 2.2.5. Western Blot Analysis.

Protein samples were quantified using the DC Reagent System (Bio-Rad #500-0116), a Lowry-based detergent compatible assay. Protein levels were measured in 96 well ELISA plates at 750 nm using a μQuant microplate reader (Witech AG, Switzerland) and BioTek-$KC_{Junior}$ Analysis software package (Fisher Scientific #BT-5270501). Each sample containing 20 μg of total protein and 10 μl 2X SDS sample buffer was boiled at 98°C for 10 minutes, briefly centrifuged to collect sample and then

loaded into a 15% tris-glycine polyacrylamide gel. Samples were separated for 2 hours at 110 volts using SDS-PAGE (Laemmli, 1970) and then transferred to Immobilon-P, PVDF membrane (Millipore Corp., Bedford, MA) overnight at 4°C in Towbin Buffer (25 mM Tris, 192 mM glycine and 20% methanol) at 40 volts. Membranes were blocked for 5 hours in 5% lowfat milk in 1X TBS-Tween (0.2 M NaCl, 66 mM Tris, 0.1% Tween). Immunodetection was performed by incubating blots overnight with 2% lowfat milk in 1X TBS-Tween and one of the following primary antibodies: anti-eIF2α phosphor-Ser51 serum (Biosource, #44-728Z- lot 0103) diluted to 1:3000. The blot was then washed with 2% milk in 1X TBS-Tween and incubated with horseradish peroxidase conjugated anti-rabbit antibody (Pierce, #31460) diluted to 1:20,000 in 2% lowfat milk in 1X TBS-Tween for 2 hours. Bands were visualized using an ECL detection kit (Amersham-Pharmacia Biotech, #RPN 2209) and exposure on Classic Blue Sensitive autoradiography film (Midwest Scientific). Following detection for peIF2α$^{ser51}$, blots for proteasome inhibitor assays were stripped by agitation at 37°C in Restore Western Blot Stripping Buffer (Pierce, #21059), re-blocked for 3 hours and then incubated overnight in anti-polyhedra serum (M. Ikeda) at a dilution of 1:20,000. Blots were sequentially probed with secondary antibodies and the interaction was detected as described above. Blots for initial eIF2α phosphorylation studies were probed with peIF2α$^{ser51}$ antibodies (Biosource, #44-728Z- lot 0103), then stripped and reprobed with polyclonal antibodies to eIF2α (Santa Cruz Biotechnology, #FL-315) at a dilution of 1:1000 and monoclonal α-tubulin antibodies (Santa Cruz Biotechnology, #TU-02) diluted to 1:1000. Blots probed with antibodies to α-tubulin were probed with horseradish peroxidase-linked anti-mouse

secondary antibodies (Pierce, 31430) at 1:20000. Incubation, stripping and ECL detection were conducted as described above.

**2.2.6. Plaque Assays.**

Cell culture plates (60 mm) were seeded with either Sf21 cells at a density of 2 x $10^6$ or Ld652Y cells at a density of 1 x $10^6$. After cell attachment, media was removed and cells were mock-infected or infected at an MOI of 10 with AcMNPV or vAchrf-1 respectively. After one hour, virus was removed and cells were washed twice with TC100. Cell cultures were refed with 4 ml of TC100 media per plate, epoxomicin was added directly to the media for indicated samples as previously described. Cells and media were collected at indicated times and a standard plaque assay was used to determine virus titers (O'Reilly, Miller, and Luckow, 1994). Plates were scored for $occ^+$ plaques at 4 and 6 days post-infection.

**2.3. Results**

**2.3.1. Translation arrest in AcMNPV-infected Ld652Y cells occurs at the initiation stage.**

In order to determine the stage at which translation arrests in AcMNPV-infected Ld652Y cells, polysome profiles of wild-type AcMNPV-infected cells were compared to those of vAchrf-1-infected cells. vAchrf-1 is a recombinant AcMNPV that expresses the LdMNPV *hrf-1* gene (Du and Thiem, 1997b). At six hours post-infection polysome profiles of AcMNPV and vAchrf-1-infected cells are identical (Fig. 8A). The six hour time point was selected because translation arrest is not observed in AcMNPV-infected Ld652Y cells at this time (Guzo et al., 1992; McClintock, Dougherty, and Weiner, 1986). Figure 8A shows polysome profiles in infected cells prior to translation arrest. Figure

8B shows polysome profiles from AcMNPV and vAchrf-1-infected Ld652Y cells at 9 hours post-infection, during translation arrest. In these profiles a large increase in the size of the monosome peak (arrow Fig. 8B) is evident in AcMNPV-infected cells as compared to the same peak from the vAchrf-1-infected cells (Fig. 8B). The increase in the monosome peak in AcMNPV-infected Ld652Y cells (Fig. 8B) is indicative of an arrest in translation initiation. However, polysomes are still evident in AcMNPV-infected cells (bracket, Fig. 8B). The most likely explanation for this observation is that translation does not arrest uniformly in the population of cells sampled. It is also possible that polysomes persist as a result of proteins that are translated despite translation arrest such as those synthesized during cap-independent translation (Pelletier and Sonenberg, 1988; Pestova et al., 2001). (Polysome profiling experiments were completed by Wade A. Williams, M.S.U. Dept. of Microbiology and Molecular Genetics.)

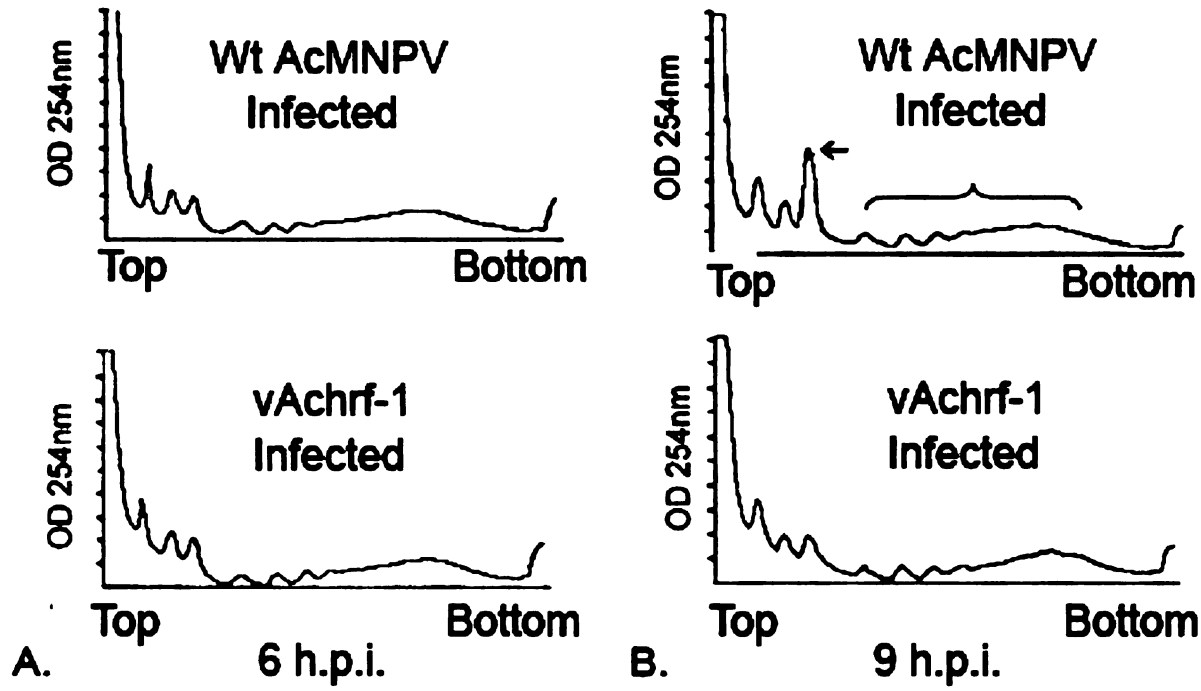



**Figure 8. Translation arrest occurs at translation initiation in AcMNPV-infected Ld652Y cells.** Polysome profiles prepared from AcMNPV and vAcHrf-1-infected (Fig. 8 caption, cont.) Ld652Y cells at 6 h.p.i. (A) and 9h.p.i. (B) post-infection. Arrow in panel B indicates a monosome peak in AcMNPV-infected cells at 9 h.p.i., the bracket indicates polysomes. Infected cells were lysed and nuclei removed. Cytoplasmic components were separated on 15-50% sucrose gradients and analyzed with an ICSO density gradient fractionator. (Polysome profiling experiments were completed by Wade A. Williams, M.S.U. Dept. of Microbiology and Molecular Genetics.)

### 2.3.2. Translation arrest in AcMNPV-infected Ld652Y cells temporally correlates to phosphorylation of eIF2α at serine 51

Translation arrest is induced in response to various stress signals and is commonly controlled at the initiation stage (Schneider and Mohr, 2003). Two well characterized mechanisms are dephosphorylation of eIF4E binding proteins in response to nutritional stress (Attardo, Hansen, and Raikhel, 2005; Attardo et al., 2003; Clemens, 2005; Hansen et al., 2004; Miron et al., 2001) and phosphorylation of eIF2α at serine 51 in response to

amino acid depletion, heme-depletion, endoplasmic reticulum (ER) stress, or virus infection (Chen, 2000; Chen and London, 1995; Harding et al., 2000a; Kaufman, 2000; Mathews, 2000; Williams, 1999). Phosphorylation at serine 51 causes eIF2α to bind to and competitively inhibit the nucleotide exchange factor eIF2B, resulting in translation arrest. Because it is the predominant mechanism mediating translation arrest at initiation, we analyzed the phosphorylation state of eIF2α in mock and virus-infected cells using western blots. For these experiments we infected cells with AcMNPV or with one of two modified AcMNPV, vAchrf-1 and vAcΔpk2. vAcΔpk2 is an AcMNPV deletion mutant which lacks *pk2*, a gene encoding a truncated eIF2α kinase homolog (Li and Miller, 1995). PK2 functions as a dominant-negative inhibitor of the eIF2α kinases, GCN2 and PKR, and presumably other members of this kinase family (Dever, 1998). Western blots were generated using whole cell lysates and probed with polyclonal antibodies raised against human eIF2α or against an eIF2α derived peptide phosphorylated at serine 51 that specifically recognizes phosphorylated eIF2α (peIF2α$^{ser51}$) (Grundmann, Mosch, and Braus, 2001; Kimball, 1999; Kumar et al., 2001; Li and Koromilas, 2001; Pataer et al., 2002; Patel et al., 2000; Soboloff and Berger, 2002). These antibodies were expected to recognize the *L. dispar* proteins, as the alpha subunit of eIF2α is highly conserved. In BLAST comparisons between full length human (Genbank accession no. AAP36281) and lepidopteran, *S. frugiperda* (Genbank accession no. AAO15491) eIF2α, proteins there are 69% identical and 79% positive residues, with 100% identity in the region between residues 35-64, surrounding the phosphorylation site at serine 51. In our experiments, these antibodies recognized proteins of the expected size with no cross-reactivity. At 6 h.p.i., we observe reduced levels of peIF2α$^{ser51}$ in Ld652Y cells infected

with AcMNPV, vAchrf-1 or vAcΔpk2 as compared to mock-infected cells (Fig. 9, lanes 2, 3 and 4 as compared to lane 1) which suggests that peIF2α$^{ser51}$ is dephosphorylated during the early stages of baulovirus infecton. However, an increase in eIF2α$^{ser51}$ phosphorylation is observed in AcMNPV and vAcΔpk2-infected cells relative to mock-infected cells at 12 hours post-infection (Fig. 9, compare lanes 6 and 8 with lane 5). The similar phosphorylation levels of eIF2α$^{ser51}$ in AcMNPV and vAcΔpk2-infected cells indicate that PK2 does not inhibit eIF2α phosphorylation in AcMNPV infected Ld652Y cells at 12 h.p.i. (Fig. 9, lanes 6 and 8). However, levels of peIF2α$^{ser51}$ are apparently reduced in Achrf-1-infected Ld652Y cells at 12 post-infection (Fig. 9, lane 7). At 24 h.p.i., phosphorylation levels of eIF2α in AcMNPV-infected Ld652Y cells are similar to mock infected cells (Fig. 9, lanes 6 and 10). This reduction in the eIF2α phosphorylation state in AcMNPV-infected Ld652Y cells at 24 h.p.i. may be due to an overall decrease in eIF2α levels resulting from translation arrest.

The antibodies against peIF2α$^{ser51}$ recognized a second band of ~26 kDa only in vAchrf-1-infected Ld652Y cells (Fig. 9, lane 7 and 11). The ~26 kDa band was not observed at 6 h.p.i. (Fig. 9, lane 3). The ~26 kDa band apparently represents a proteolytic fragment of peIF2α$^{ser51}$. When gels for western blots were run for shorter times and probed for eIF2α, a band of ~11 kDa was detected (Fig. 10, lane 3). The sum of the ~26 kDa band detected on blots probed with antibodies to peIF2α$^{ser51}$ (Fig. 9, lanes 7 and 11, Fig. 10, lane 6) plus the 11 kDa band detected with antibodies to eIF2α total ~37 kDa, the band recognized by both antibodies, which is the predicted molecular mass for *S. frugiperda* eIF2α. These data support the idea that peIF2α$^{ser51}$ is proteolytically cleaved in Ld652Y cells infected with vAchrf-1. In vAchrf-1-infected Ld652Y cells,

phosphorylation of eIF2α may serve as a signal for cleavage of the protein. Since eIF2α is phosphorylated but not cleaved in AcMNPV-infected Ld652Y cells, it appears that cleavage is directly or indirectly enabled by HRF-1.

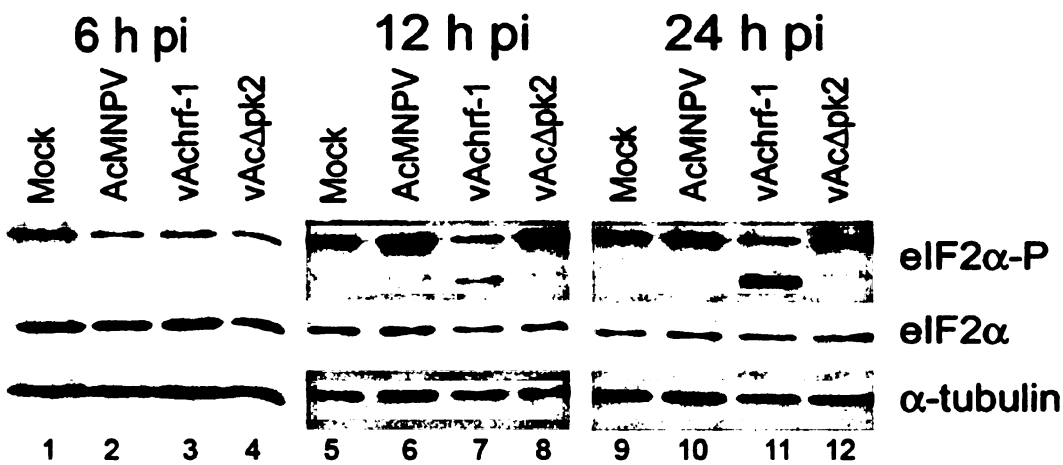



**Figure 9. Phosphorylation of full length, eukaryotic initiation factor 2α (eIF2α) at serine 51 is reduced in vAchrf-1-infected Ld652Y cells as compared to AcMNPV-infected, temporally correlating to the onset of translation arrest in AcMNPV-infected Ld652Y cells.** (Fig. 9 caption, cont.) Levels of total eIF2α were assayed by using antibodies that specifically recognize the phosphorylated and total endogenous forms of the protein. Total cell lysates were prepared from Ld652Y cells that were mock-infected or infected with AcMNPV, vAchrf-1 or vAcΔpk2. Infected cells were harvested at 6, 12 h and 24 h post-infection. Proteins from whole cell lysates were separated on 15% SDS-PAGE gels and analyzed with immunoblotting. The same blot for each time point was stripped and reprobed sequentially with antibodies to phosphorylated eIF2α$^{ser51}$, eIF2α, and α-tubulin. Samples from the 6 h.p.i. timepoint were collected from a separate infection timecourse than the 12 and 24 h.p.i. samples.

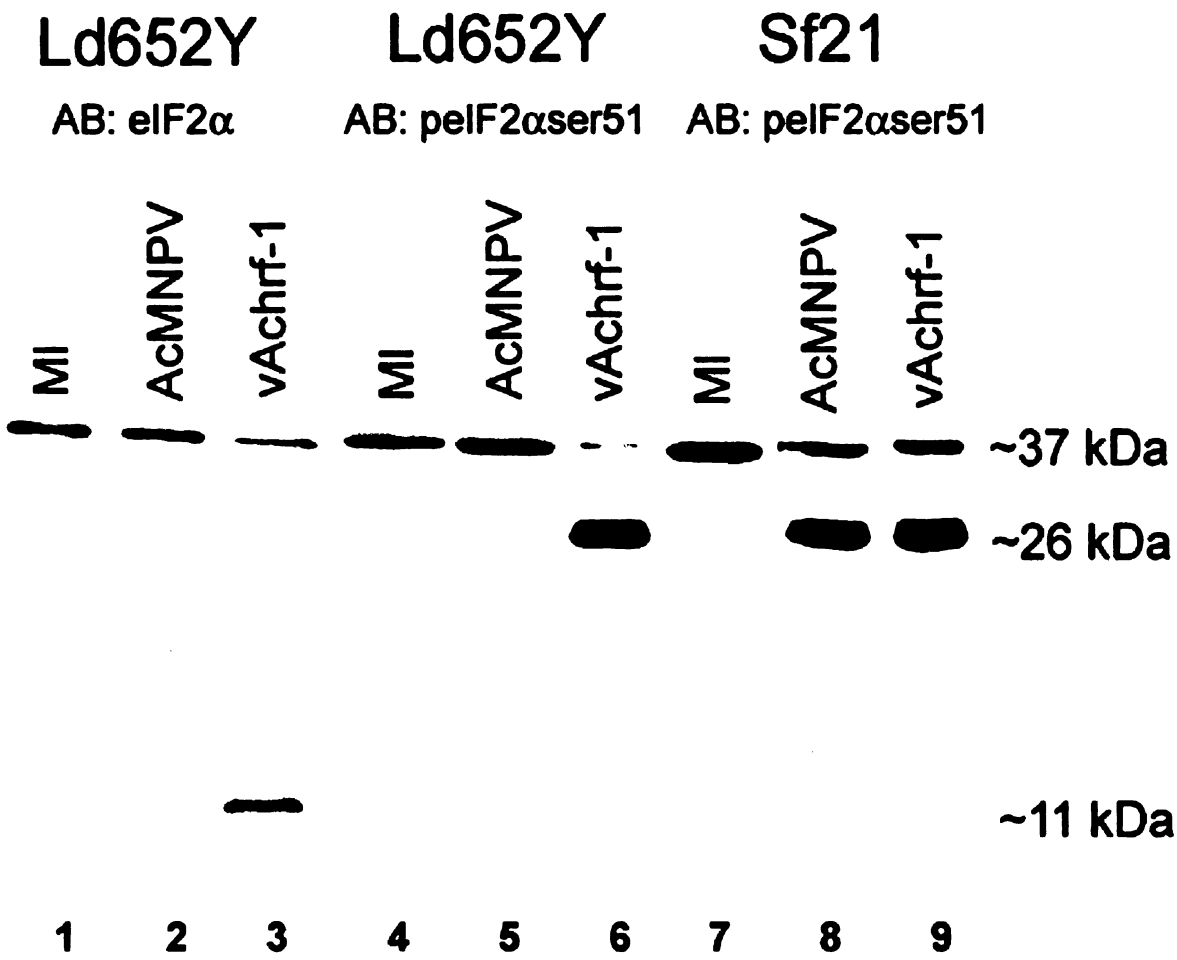



**Figure 10. Phosphorylated eIF2α$^{ser51}$ is proteolytically cleaved in cell lines that are productively infected with AcMNPV or vAchrf-1.** Proteolytic cleavage of eIF2α was assayed at 24 h.p.i. in mock, AcMNPV and vAchrf-1-infected Ld652Y and Sf21 cells with antibodies that specifically recognize the phosphorylated or total endogenous forms of the protein. Proteins from whole cell lysates were separated on 15% SDS-PAGE gels and analyzed with immunoblotting. The same blot for Ld652Y cells was probed with antibodies to peIF2α$^{ser51}$ (AB: peIF2α$^{ser51}$) (lanes 4-6) then stripped and reprobed with antibodies to eIF2α (AB: eIF2α) (lanes 1-3). Cell lines and antibodies used are shown above the panels and the molecular sizes of immune reactive bands are indicated to the right of the panels.

In AcMNPV-infected Ld652Y cells, incubation of cell lysates with m$^7$GTP-bound sepharose followed by western blot analysis showed that the cap-binding ability of eIF4E was not impaired (Fig. 26, Supplementary Data). In addition, western blots generated

with antibodies raised against *Drosophila melanogaster* 4E-BP (kindly provided by Mathieu Miron) did not show an increase in 4E-BP protein levels in AcMNPV-infected Ld652Y cells (Fig. 27, Supplementary Data). The results suggest that modification or sequestering of eIF4E is not the primary mechanism leading to global translation arrest in AcMNPV-infected Ld652Y cells.

To show that eIF2α phosphorylation was the mechanism leading to global translation arrest in AcMNPV-infected Ld652Y cells, experiments were conducted to silence endogenous eIF2α expression in invertebrate cell lines using RNAi technology. Silenced Ld652Y cells were infected with a recombinant AcMNPV that constitutively expressed the *Spodoptera frugiperda* eIF2α gene with an alanine substitution at serine 51 (vAcHAeIF2α$^{ala51}$). Isotopic labeling of protein synthesis in Ld652Y cells silenced for eIF2α expression and infected with the vAcHAeIF2α$^{ala51}$ recombinant were inconclusive (data not shown).

### 2.3.3. Proteolytic cleavage of peIF2α$^{ser51}$ occurs in AcMNPV and vAchrf-1-infected Sf21 cells.

Cleavage of peIF2α$^{ser51}$ in vAchrf-1-infected cells suggested a possible mechanism for HRF-1 to overcome translation arrest in AcMNPV-infected Ld652Y cells. One hypothesis was that cleavage of peIF2α$^{ser51}$ could prevent its binding of the guanosine nucleotide exchange factor eIF2B thus allowing protein synthesis to continue. To determine whether cleavage of peIF2α$^{ser51}$ was mediated by HRF-1 we compared peIF2α$^{ser51}$ cleavage in cell lysates of vAchrf-1- and AcMNPV-infected Sf21 cells, using western blots probed with antibodies to peIF2α$^{ser51}$. In contrast to Ld652Y cells, Sf21

cells are permissive for AcMNPV replication. Cleavage of peIF2$\alpha^{ser51}$ occurred in both AcMNPV and vAchrf-1-infected Sf21 cells by 24 h.p.i. (Fig. 10, lanes 8 and 9) and not in mock-infected cells (Fig. 10, lane 7) suggesting that cleavage is not directly-mediated by HRF-1. In the cell lines studied, cleavage of peIF2$\alpha^{ser51}$ occurs only in those that are productively-infected with baculovirus. Cleavage of peIF2$\alpha^{ser51}$ may be directly or indirectly mediated by HRF-1 in Ld652Y cells as a host protein may be functionally homologous to HRF-1 during AcMNPV infection in Sf21 cells.

**2.3.4. PK2 is expressed in AcMNPV-infected Ld652Y cells.**

PK2 was expected to inhibit eIF2α kinases, yet similar levels of phosphorylation were observed in cells infected with AcMNPV and vAcΔpk2 (Fig. 9, lanes 6 and 10 as compared to lanes 8 and 12). To determine if PK2 was expressed in AcMNPV-infected Ld652Y cells, we performed western blot analysis using antibodies to PK2 (R. Clem from L.K. Miller collection). Although PK2 levels decreased over time, it was still present in Ld652Y cells infected with AcMNPV and Achrf-1 at 6, 12 and 24 hours post-infection (Fig. 11, lanes 3, 4, 7, 8, 11 and 12). Thus, it is likely that concentrations of PK2 are too low to block eIF2α kinase activity at later time points. Alternatively, it is possible that the eIF2α kinase activated by AcMNPV infection in Ld652Y cells is not inhibited by PK2. There is a non-specific band of ~31 kD that is evident in all samples at 6 h.p.i. (Fig. 11, lanes 1, 2, 3 and 4) and has previously been observed with this antibody (Li and Miller 1995).

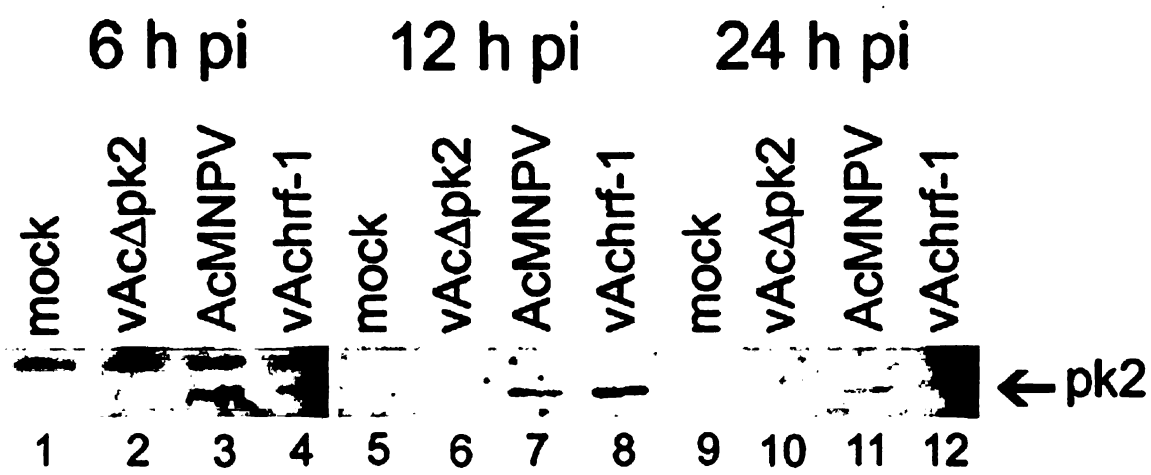



**Figure 11. PK2 is present at late time points post-infection in Ld652Y cells infected with AcMNPV and Achrf-1.** Infected cells were harvested at 6, 12 and 24 hours post-infection, subjected to SDS-PAGE on 15% polyacrylamide gels and immunoblotted with antibodies to PK2.

### 2.3.5. Cleavage of peIF2$\alpha^{ser51}$ in Sf21 and Ld652Y cell lines is not induced by stress-inducing agents.

To test if peIF2$\alpha^{ser51}$ cleavage resulted from a general stress response, we treated Sf21 and Ld652Y cells with stress-inducing agents and assayed for peIF2$\alpha^{ser51}$ cleavage by western blot analysis. To screen for the effects of genotoxic stress, uninfected cells were treated with methyl methanesulfonate. Nutritional stress was induced with rapamycin, oxidative stress with hydrogen peroxide, proteasome inhibition with epoxomicin and heat shock by incubation at 39° C. ER-stress and the unfolded protein response (UPR) were induced using thapsigargin and tunicamycin. Assays were conducted at 6 and 24 hours post-treatment (h.p.t.). Cleavage of peIF2$\alpha^{ser51}$ was not observed in either Ld652Y or Sf21 cells, in response to any of these treatments (Fig. 12). The reduction in eIF2$\alpha$ phosphorylation, in both Ld652Y and Sf21 cells treated with

hydrogen peroxide at 24 h.p.t. (Fig.12A, lane 8 and 12B, lane 8), may be the result of cell damage initiated by reactive oxygen species.

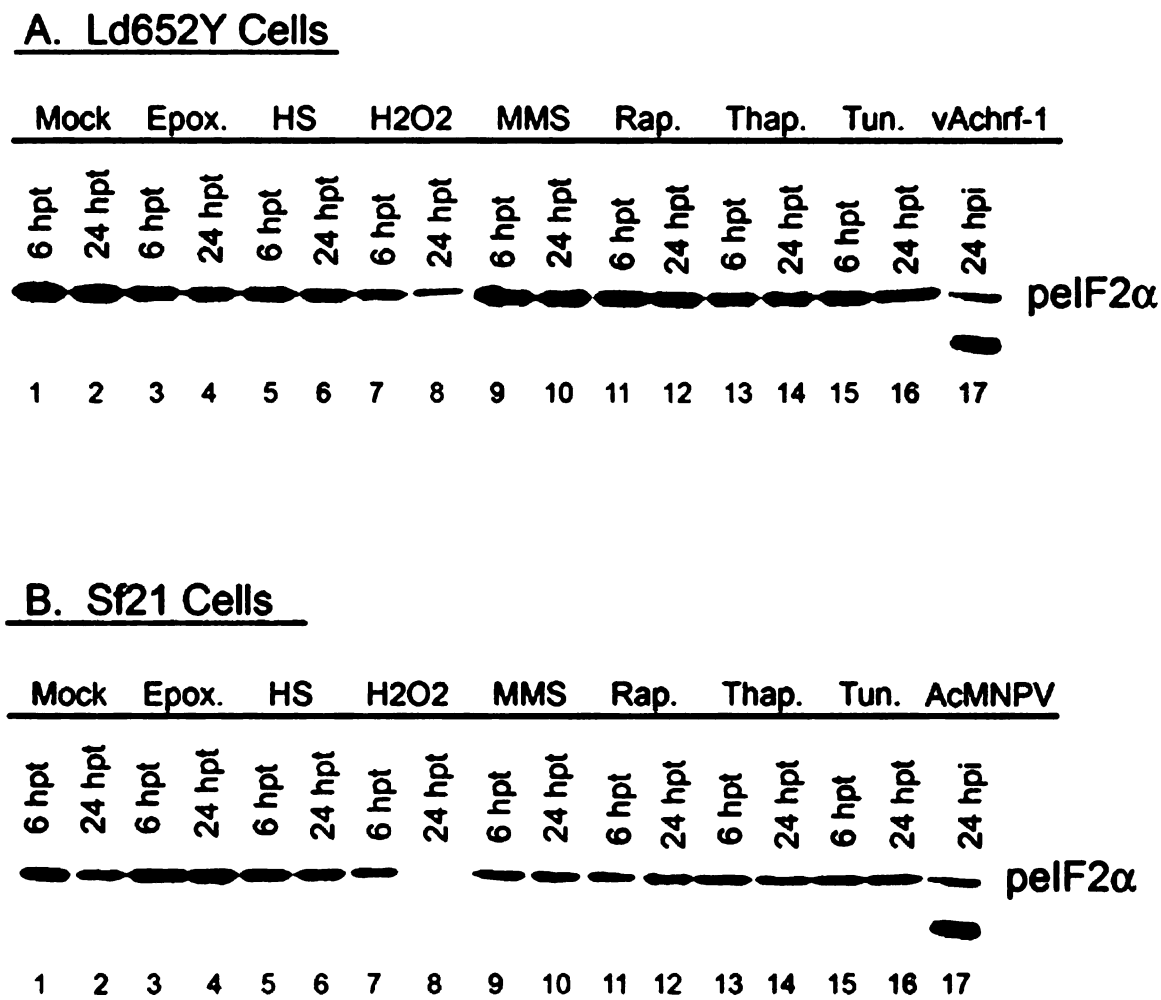



**Figure 12. Proteolytic cleavage of peIF2α$^{ser51}$ does not occur in Sf21 or Ld652Y cells that are treated with stress-inducing agents.** Ld652Y and Sf21 cells were treated with stress inducing agents as indicated above each panel. Cells were harvested at 6 and 24 hours post-treatment. Proteins from whole cell lysates were fractionated on 15% SDS-PAGE gels and analyzed on western blots probed with antibodies against peIF2α$^{ser51}$. Cell lines and treatments are shown above the panels.

### 2.3.6. Cleavage of peIF2α$^{ser51}$ is blocked by aphidicolin but not by protease inhibitors.

Late events in the baculovirus replication cycle enhance apoptosis in AcMNPV-infected Sf21 cells in the absence of the virally encoded apoptotic suppressor, p35 (LaCount and Friesen, 1997), and stimulate translation arrest in AcMNPV-infected Ld652Y cells (Thiem and Chejanovsky, 2004). To test if peIF2α$^{ser51}$ cleavage correlated with the onset of late viral gene expression, we treated vAchrf-1-infected Ld652Y cells with the DNA polymerase inhibitor aphidicolin and scored for peIF2α$^{ser51}$ cleavage at 24 h.p.i. on western blots. Cleavage was not observed and eIF2α phosphorylation was reduced in aphidicolin-treated, vAchrf-1-infected Ld652Y cells and AcMNPV-infected Sf21 cells (Fig. 13, lanes 1 and 6) indicating that phosphorylation and cleavage of peIF2α$^{ser51}$ required a late event in the AcMNPV replication cycle.

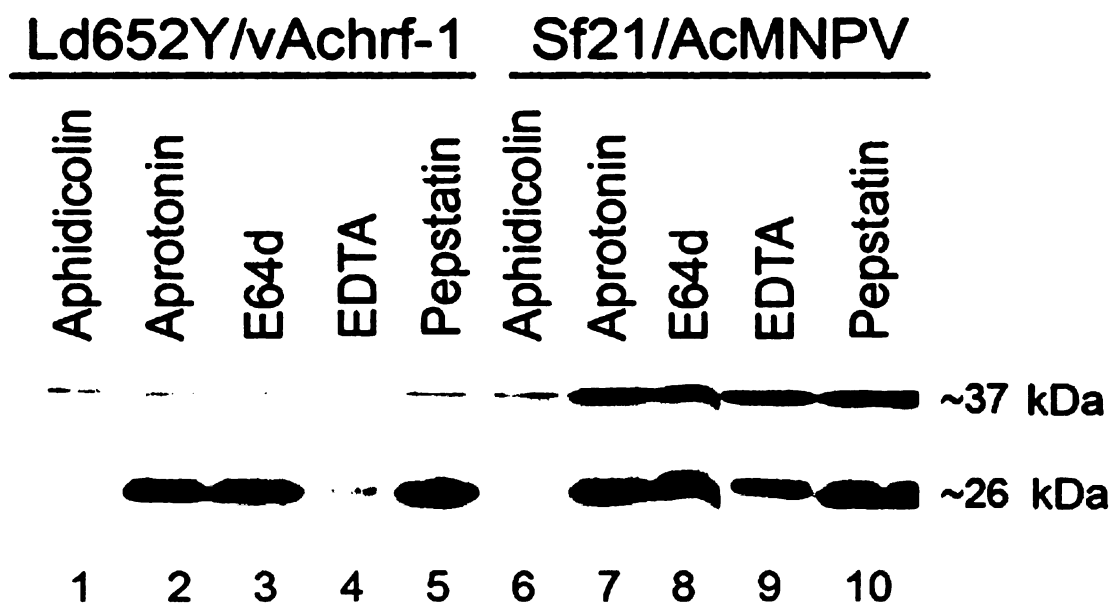



**Figure 13. Phosphorylation and proteolytic cleavage of peIF2α$^{ser51}$ is inhibited in Lepidoptera cells that are productively-infected with baculovirus and treated with aphidicolin. Proteolytic cleavage of peIF2α$^{ser51}$ is not blocked by protease inhibitors.** (Figure 13 caption cont.) Ld652Y or Sf21 cells were mock-infected or infected with either vAchrf-1 or AcMNPV respectively. Cells were harvested at 24 h.p.i.. Proteins from whole cell lysates were fractionated on 15% SDS-PAGE gels and analyzed on western blots probed with antibodies against peIF2α$^{ser51}$. Cell lines and treatments are shown above the panels and the molecular sizes of immune reactive bands are indicated to the right of the panels.

To identify a candidate protease responsible for cleavage of peIF2α$^{ser51}$ in AcMNPV- and Achrf-1-infected Sf21 and vAchrf-1-infected Ld652Y cells, we attempted to block cleavage with membrane-permeable protease inhibitors. Inhibitors were added directly to the cell culture media. We used aprotonin to block serine protease activity, E64d (EST) to inhibit cysteine proteases, (ethylenedinitrilo) tetraacetic acid (EDTA) for metalloproteases and pepstatin to inhibit aspartic proteases. Inhibitor concentrations were based on recommendations from previous studies (Buttle et al., 1992; Gray and Tsai, 1994; Janas, Marks, and LaRusso, 1994; Tamai et al., 1987; Thiem and Chejanovsky,

2004). We note, however, the pepstatin derivative we used has not been analyzed for membrane permeability. None of these inhibitors prevented cleavage of peIF2$\alpha^{ser51}$ (Fig. 13, lanes 2-5 and 7-10). However, phosphorylation levels of full-length peIF2$\alpha^{ser51}$ appear to be reduced in vAchrf-1-infected Ld652Y cells that were also treated with a protease inhibitor (Fig. 13, lanes 2-5). This reduction in peIF2$\alpha^{ser51}$ levels is most evident in vAchrf-1-infected Ld652Y cells that were treated with EDTA (Fig. 13, lane 4). Due to the broad range effects that EDTA imposes on cellular processes, it is difficult to determine if this reduction in peIF2$\alpha^{ser51}$ levels is significant in EDTA-treated Ld652Y cells during vAchrf-1 infection. However, there is still a light ~26 kD band present in EDTA-treated Ld652Y cells infected with vAchrf-1 indicating that proteolytic cleavage of peIF2$\alpha^{ser51}$ still occurs and is not inhibited by EDTA.

We also tested possible roles for caspases and cathepsin caspase in peIF2$\alpha^{ser51}$ cleavage. Cell permeable, caspase and cathepsin inhibitors were added to the media of AcMNPV-infected Sf21 cells or vAchrf-1-infected Ld652Y cells, as previously described (Thiem and Chejanovsky, 2004). In addition, because the AcMNPV apoptotic suppressor p35 may not be expressed at sufficient levels to completely block apoptosis (P. Friesen, personal communication) we examined Sf21 cells infected with a recombinant AcMNPV with enhanced expression of p35 (kindly provided by P. Friesen). Cells were harvested at 24 h.p.i. and lysates analyzed by western blot. Inhibiting either caspase or cathepsin activity did not prevent peIF2$\alpha^{ser51}$ cleavage (data not shown).

### 2.3.7. Proteasome inhibitors reduce phosphorylation of eIF2α at serine 51 and virus production in lepidopteran cells productively-infected with baculovirus.

Protease inhibition did not block cleavage of peIF2α$^{ser51}$ in invertebrate cell lines productively-infected with baculovirus, so, we examined the proteasome as a possible mediator of proteolytic activity against peIF2α$^{ser51}$. The role of proteasome activity and its relation to eIF2α phosphorylation and cleavage were investigated using the proteasome-specific inhibitors epoxomicin and MG132. Epoxomicin blocks trypsin-like (T-L) and chymotrypsin-like (CT-L) activities of the proteasome without inhibiting other proteases. Additionally, epoxomicin blocks the peptidylglutamyl peptide hydrolyzing (PGPH) caspase-like activity of the proteasome. Epoxomicin covalently binds the LMP-7, MECL1 and Z catalytic subunits of the proteasome and that direct interaction is a probable mechanism for its inhibitor activity (Meng et al., 1999; Sin et al., 1999) . MG132 is a peptide aldehyde and a less potent inhibitor of the proteasome. MG132 reduces the degradation of ubiquitin-conjugated proteins by the 26S complex without inhibiting the isopeptidase or ATPase activities of the proteasome (Steinhilb, Turner, and Gaut, 2001). Inhibitors were added directly to infected cell cultures and their effects on eIF2α phosphorylation and cleavage assayed at 24 and 48 h.p.i. as previously described. At 24 and 48 h.p.i., it appears that levels of full-length peIF2α$^{ser51}$ are reduced in both Sf21 and Ld652Y cells that are infected with baculovirus and treated with a proteasome inhibitor (Fig. 14A, lanes 2, 3, 8, 9, 11, 12, 17 and 18; 14B, lanes 2, 3, 8, 9, 11, 12, 17 and 18) as compared to mock-infected samples that are treated with a proteasome inhibitor at the corresponding time points (Fig. 14A, lanes 5, 6, 14 and 15; 14B, lanes 5, 6, 14 and 15). Cleavage of peIF2α$^{ser51}$ was not observed in vAchrf-infected Ld652Y cells

or AcMNPV and vAchrf-1-infected Sf21 cells that were treated with epoxomycin (Fig. 14A, lanes 8 and 17; 14B, lanes 2, 8, 11 and 17). There is a faint band of ~30kDa appearing in infected cells treated with proteasome inhibitors (Fig. 14A, lanes 6, 14, 15 and 17). This faint band may reflect proteolytic degradation of peIF2$\alpha^{ser51}$ via an initiated proteolytic pathway that is not proteasome dependent. In virus-infected Ld652Y and Sf21 cells, MG132 appears to reduce eIF2$\alpha$ phosphorylation levels (Fig. 14A, lanes 9 and 18; 14B, lanes 3, 9, 12 and 18) but cleavage of peIF2$\alpha^{ser51}$ is prevented only in vAchrf-1-infected Ld652Y cells (Fig. 14A, lanes 9 and 18). MG132 treatment did not prevent peIF2$\alpha^{ser51}$ cleavage in vAchrf-1 and AcMNPV-infected Sf21 cells (Fig. 14B, lanes 3, 9, 12 and 18).

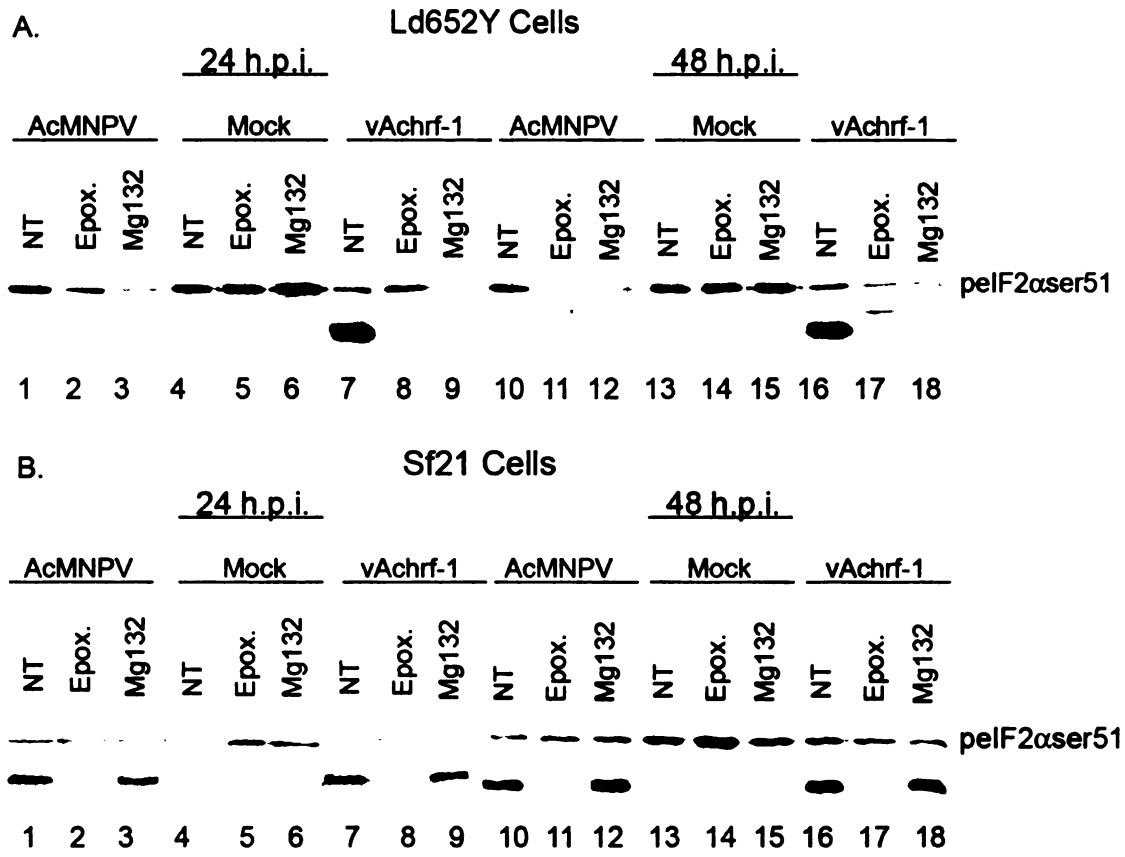



**Figure 14. Proteasome inhibitors, MG132 and epoxomicin, inhibit phosphorylation and proteolytic cleavage of peIF2α$^{ser51}$ in vAchrf-1-infected Ld652Y cells.** Ld652Y or Sf21 cells were treated with 2 μM epoxomicin or 10 μM MG132 from 1 hour preceding infection to time of collection. Cells were mock-infected or infected with either AcMNPV or vAchrf-1 and harvested at 24 h.p.i. and 48 h.p.i. Proteins from whole cell lysates were fractionated on 15% SDS-PAGE gels and analyzed on western blots probed with antibodies against peIF2α$^{ser51}$. Cell lines and treatments are shown above the panels.

Because polyhedra were not observed in epoxomycin-treated cells, we examined whether polyhedrin protein was synthesized. Immunoblots shown in Figure 14 were stripped and reprobed with antibodies to the baculovirus polyhedrin protein. Polyhedra or occlusion bodies are produced in the very late stages of permissive baculovirus infection from the polyhedrin protein. Polyhedrin protein was not observed in either epoxomicin or

MG132-treated vAchrf-1-infected Ld652Y cells at 24 and 48 h.p.i. (Fig. 15A, lanes 8, 9, 17 and 18). Epoxomicin blocked production of the polyhedrin protein in AcMNPV and vAchrf-1-infected Sf21 cells (Fig. 15B, lanes 2, 8, 11, and 17) but MG132 did not (Fig. 15B, lanes 3, 9, 12 and 18). Polyhedrin degradation products are evident on western blots in non-treated, productively-infected Ld652Y and Sf21 cells by 48 h.p.i.

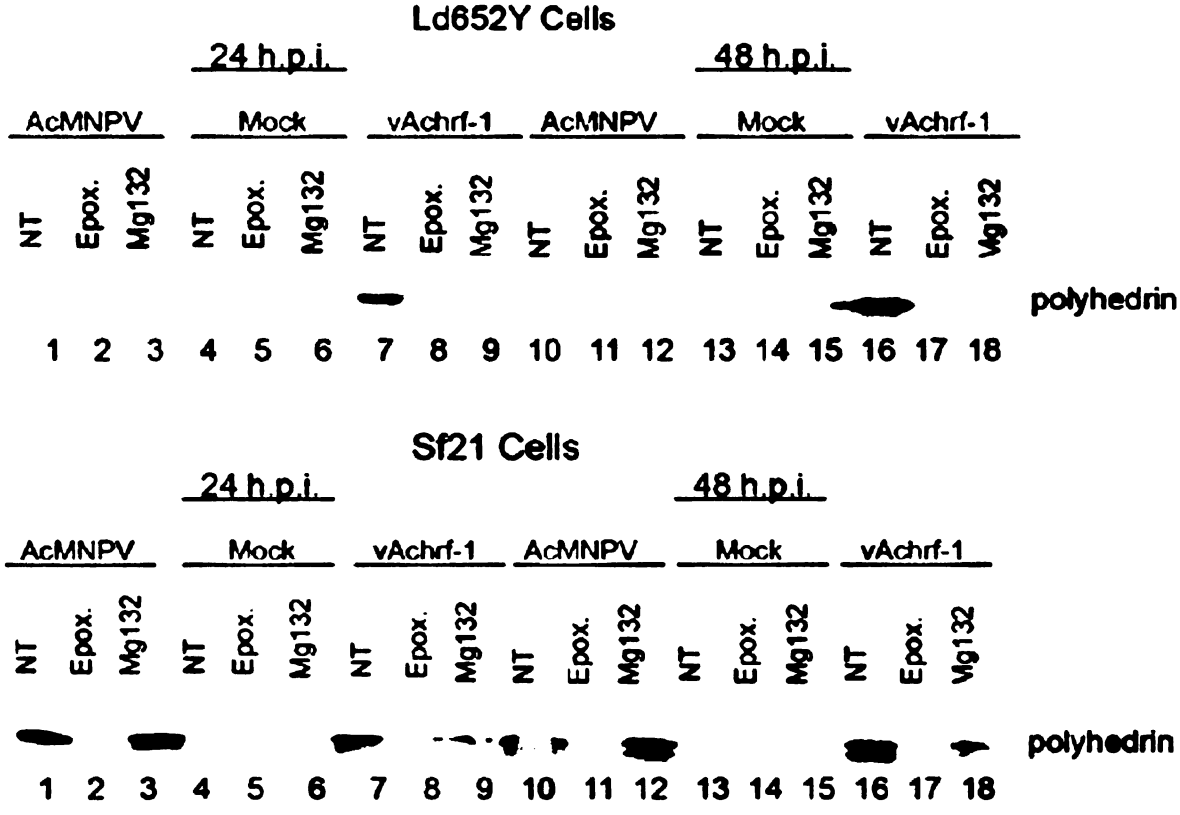



**Figure 15. The very late baculovirus occlusion body protein, polyhedrin, is not produced in permissively-infected cells that are treated with epoxomicin.** Ld652Y or Sf21 cells were pre-treated with 2 μM epoxomicin or 10 μM MG132 from 1 hour preceding infection to time of collection. Cells were mock-infected or infected with either AcMNPV or vAchrf-1 and harvested at 24 h.p.i. and 48 h.p.i.. Proteins from whole cell lysates were fractionated on 15% SDS-PAGE gels and analyzed on western blots probed with antibodies against peIF2$\alpha^{ser51}$, stripped and sequentially probed with polyclonal antibodies against polyhedrin. Cell lines and treatments are shown above the panels and immune reactive bands are indicated to the right of the panels.

To determine if proteasome inhibition blocked virus replication, budded virus (BV) production in the presence or absence of epoxomicin or MG132 was measured over time by plaque assay. Experiments for all samples were plated in duplicate, titers noted in Figure 16 and 17 represent the average titer for both samples. Epoxomicin treatment eliminated or reduced BV production in vAchrf-1-infected Ld652Y cells (Fig. 16) and in Sf21 cells infected with either vAchrf-1 or AcMNPV (Fig. 17). Treatment of vAchrf-1-infected Ld652Y cells with MG132, resulted in a block of budded virus production at 24 h.p.i. (Fig. 16). AcMNPV and vAchrf-1-infected SF21 cells treated with MG132 showed reduced level of budded virus production of at least one order of magnitude as compared to cells that were not treated with MG132 (Fig. 17). The different response observed in Sf21 cells treated with epoxomicin as compared to those treated with MG132 may be a direct measure of the potency of the inhibitor or may specifically relate to the mechanism of the inhibitor. Epoxomicin blocks proteasome activity by directly binding catalytic subunits of the proteasome while MG132 only reduces the degradation of ubiquitin-conjugated proteins, so if MG132 treatment results in a partial block to virus production, it would suggest that complete degradation of one or more proteins is essential to establishing a productive baculovirus infection. Both proteasome inhibitors, epoxomicin and MG132, are effective in blocking the production of virus progeny in vAchrf-1-infected Ld652Y cells. However, there may be a small increase in budded virus production in vAchrf-1-infected Ld652Y cells treated with MG132 by 48 h.p.i. The data suggests a potential difference in proteasome response in permissively-infected Ld652Y cells as compared to Sf21 (Fig. 16 as compared to Fig. 17). These experiments suggest

that the proteasome plays a role in promoting a productive baculovirus infection in cell culture.

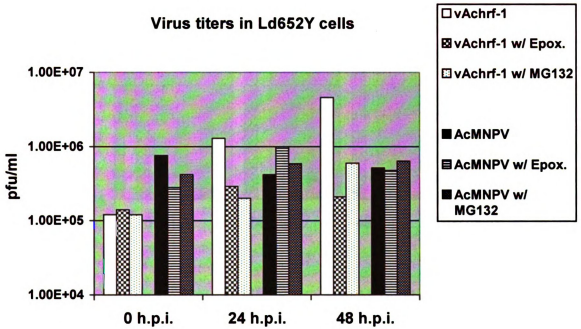



**Figure 16. Proteasome inhibitors prevent budded virus production in vAchrf-1-infected Ld652Y cells.** vAchrf-1 titers are reduced within at least one order of magnitude in cells treated with either epoxomicin or MG132. Ld652Y cells were pre-treated with 2 μM epoxomicin or 10 μM MG132 from 1 hour preceding infection to time of collection. Cells were mock-infected or infected with either AcMNPV or vAchrf-1 and virus was harvested at 24 h.p.i. and 48 h.p.i. Virus was titered on Sf21 cells using a standard plaque assay (O'Reilly, Miller, and Luckow, 1994). Plates were scored for *occ*$^+$ plaques at 4 and 6 days post-infection.

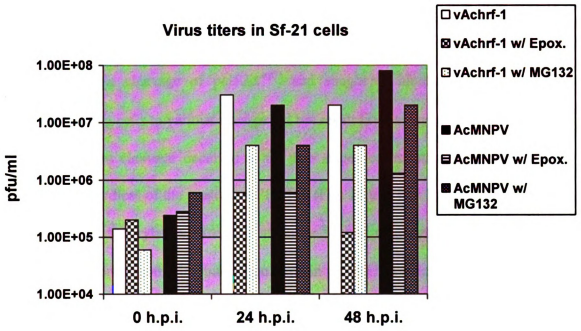



**Figure 17. Epoxomicin prevents AcMNPV or vAchrf-1 budded virus production in Sf21 cells, MG132 reduces budded virus production in those same infected cells.** AcMNPV and vAchrf-1 titers are reduced within at least one order of magnitude in infected Sf21 cells treated with epoxomicin. Sf21 cells were pre-treated with 2 μM epoxomicin or 10 μM MG132 from 1 hour preceding infection to time of collection. Cells were mock-infected or infected with either AcMNPV or vAchrf-1 and virus was harvested at 24 h.p.i. and 48 h.p.i.. Virus was titered on Sf21 cells using a standard plaque assay (O'Reilly, Miller, and Luckow, 1994). Plates were scored for $occ^{+}$ plaques at 4 and 6 days post-infection.

### 2.4. Discussion

Global translation arrest occurs in Ld652Y cells infected with the baculovirus AcMNPV in a non-productive infection. The addition of a single gene, *hrf-1*, isolated from LdMNPV, rescues translation arrest and promotes a productive virus infection. In this study, while searching for mechanisms that lead to translation arrest in AcMNPV-infected Ld652Y cells, we discovered that the eukaryotic initiation factor 2α is phosphorylated at serine 51 ($peIF2\alpha^{ser51}$) in AcMNPV-infected Ld652Y cells (Fig. 9) but

that peIF2α$^{ser51}$ is proteolytically cleaved during the late stages of infection in vAchrf-1-infected Ld652Y cells (Fig. 9 and 10) . In this study, we investigated a possible role for HRF-1 in directing cleavage of peIF2α$^{ser51}$ and sought to identify the responsible protease. Subjecting cells to various stresses did not result in cleavage of peIF2α$^{ser51}$ (Fig. 12). We found that cleavage occurred at the late stages of infection in productively-infected cells, regardless of the presence of HRF-1 (Fig. 10 and 13). In vAchrf-1-infected Ld652Y cells, phosphorylation of eIF2α at serine 51 and cleavage of peIF2α$^{ser51}$ was prevented by the proteasome inhibitors epoxomicin or MG132 (Fig. 14). Production of virus progeny appears to be prevented in vAchrf-1-infected Ld652Y cells treated with epoxomicin or MG132 and in AcMNPV and vAchrf-1-infected Sf21 cells treated with epoxomicin (Fig. 16 and 17).

Cleavage of peIF2α$^{ser51}$ suggested a potential mechanism for HRF-1 to overcome translation arrest in Ld652Y cells. One hypothesis was that cleaved peIF2α$^{ser51}$ was conformationally-altered and no longer able to bind and sequester the nucleotide exchange factor eIF2B, thus preventing translation arrest. The fact that cleavage of peIF2α$^{ser51}$ occurs in Sf21 cells infected with AcMNPV as well as in those infected with vAchrf-1 suggested that HRF-1 is not directly responsible for inducing peIF2α$^{ser51}$ cleavage (Fig. 10). However, this may not be true if HRF-1 is functionally replacing a host-encoded protein available in Sf21 cells that does not exist in Ld652Y cells. This question could be addressed by expressing a truncated eIF2α gene from a recombinant AcMNPV virus, then infecting Ld652Y cells to determine if budded virus production was enabled by the recombinant. Similar experiments could be conducted by transfecting Ld652Y cells with an expression plasmid encoding a truncated form of eIF2α. Earlier

reports which examined the phosphorylation state of eIF2α in response to productive AcMNPV infection in Sf9 cells, did not identify cleavage of the initiation factor (Dever et al., 1998). However, in those studies, isoelectric focusing gels were used to determine the phosphorylation state of eIF2α which would not have easily identified a cleavage product.

Treatment with a range of chemical protease inhibitors had no effect on the cleavage of peIF2α$^{ser51}$ in Sf21 or Ld652Y cells permissively-infected with AcMNPV or vAchrf-1, respectively (Fig. 13). However, the proteasome-specific inhibitor, epoxomicin, prevented phosphorylation of eIF2α and cleavage of peIF2α$^{ser51}$ at 24 and 48 hours post-infection in vAchrf-1 and AcMNPV-infected Sf21 cells and vAchrf-1-infected Ld652Y cells (Fig. 14A, lanes 8 and 17; 14B, lanes 2, 8, 11 and 17). In contrast to MG132-treated Sf21 cells, vAchrf-1-infected, MG132-treated, Ld652Y cells show little to no increase in budded virus titers at 48 h.p.i. over initial inputs (Fig. 10) which may indicate a role for HRF-1 in mediating the proteasome response in Ld652Y cells. The differential effect of MG132 on productive virus infection in Ld652Y cells as compared to Sf21 cells may be significant. MG132 reduces the degradation of ubiquitin-tagged proteins and MG132 prevents vAchrf-1 replication in Ld652Y cells, suggesting that degradation of a ubiquitinated protein, possibly eIF2α, is essential to promoting a productive infection in Ld652Y cells. A reduction in ubiquitin mediated degradation may not be sufficient to block AcMNPV replication in Sf21 cells as AcMNPV may have a mechanism to ensure degradation of certain proteins in that cell type. Epoxomicin blocks proteasomal activity through an irreversible, direct interaction with proteasomal subunits, our data shows that epoxomicin blocks all cleavage of peIF2α$^{ser51}$ and virus replication in

vAchrf-1-infected Ld652Y cells and AcMNPV or vAchrf-1-infected Sf21 cells. In this study, the impairment to budded virus production in productively-infected cells treated with epoxomicin suggests that the virus has evolved to use the proteasome as a part of its replication cycle (Fig. 16 and Fig. 17). To our knowledge, this is the first account of a proteasome-dependent cleavage of a eukaryotic translation initiation factor. Virus-mediated proteasome utilization is not without precedent. Human cytomegalovirus mediates proteasomal destruction of hypophosphorylated host retinoblastoma proteins in a ubiquitin-independent manner (Kalejta and Shenk, 2003) which results in stimulation of cell cycle progression thus allowing virus replication (Kalejta, Bechtel, and Shenk, 2003). Herpes simplex virus I is unable to transition from a latent to lytic state in the presence of proteosome inhibitors and Hepatitis C virus evades the host antiviral interferon response by directing proteasomal degradation of phosphorylated STAT1 (Lin et al., 2005).

It is possible that activation of the proteasome is part of an orchestrated host response to the late stages of virus infection. Proteasome enabled cleavage of peIF2$\alpha^{ser51}$ in Ld652Y and Sf21 cells infected with vAchrf-1 and AcMNPV, respectively, is blocked in the presence of aphidicolin (Fig. 13, lanes 1 and 6). In those same productively-infected cells that are treated with aphidicolin or proteasome inhibitors, eIF2$\alpha$ is not phosphorylated (Fig. 13, lanes 1 and 6, Fig. 14A, lanes 8, 9, 17 and 18; 14B, lanes 2, 3, 8, 9, 11, 12, 17 and 18 ). This indicates that eIF2$\alpha$ phosphorylation is the result of an event or events that occur during the late stages of infection and may be a signal for the initiation of cleavage. Other studies have shown late stages in the baculovirus infection cycle, including viral DNA replication or late viral gene expression (LaCount and Friesen, 1997), to promote apoptosis and translation arrest (Thiem and Chejanovsky,

2004). It is interesting to note the phosphorylation state of eIF2α in productively-infected cells treated with proteasome inhibitors compared to those same treated cells that are not infected. Reduced eIF2α phosphorylation levels occur only in baculovirus infected cells at 6 h.p.i. (Fig. 9, lanes 2, 3 and 4) and in those cells that are baculovirus-infected and treated with proteasome inhibitors (Fig. 14A, lanes 8, 9, 17 and 18 as compared to lanes 5, 6, 14 and 15 and 14B, lanes 2, 3, 8, 9, 11, 12, 17 and 18 as compared to lanes 5, 6, 14 and 15). The data may suggest that baculoviruses orchestrate a response between the proteasome, an eIF2α kinase and its substrate. Such a mechanism has been modeled for the COP9 signalosome (CSN). Models predict that the CSN may serve as a scaffold for ubiquitylation and subsequent degradation of specific phosphorylated substrates (Harari-Steinberg and Chamovitz, 2004). Potentially, the COP9 signalasome could play a regulatory role in responding to baculovirus infection by providing selective degradation of phosphorylated proteins including peIF2α$^{ser51}$. It may be possible to determine if the CSN has an effect on baculovirus infection by using CSN-specific inhibitors in cell culture. The CSN is essential for development in *Drosophila melanogaster* (Freilich et al., 1999) and appears to direct cell differentiation by orchestrating regulatory cascades. CSN is structurally similar to the 19S regulatory lid of the proteasome and to eukaryotic initiation factor 3 (eIF3) (Kim et al., 2001).

Targeting of proteins to the proteasome begins with substrate recognition by an E3 ubiquitin ligase followed by ubiquitination and delivery to the proteasome. E3 ligases provide the specificity for targeting of individual proteins to the proteasome. E3 targeting can occur as a result of endoplasmic reticulum-associated degradation (ERAD), oxidative stress or the unfolded protein response (UPR) (Crews, 2003). Interestingly, all

presently characterized baculoviruses encode a ubiquitin-like protein and several baculovirus proteins including BmMNPV IAP2, IE2 and PE38 have E3 ubiquitin ligase activity (Imai et al., 2003). BMNPV IE2, PE38 and the AcMNPV homolog for IAP2 are all transcribed at early and late stages of infection and are thus, not probable candidates for mediating late infection cleavage of peIF2$\alpha^{ser51}$. However, recent microarray data indicates that these genes are up-regulated during the late stages of infection (Yamagishi et al., 2003) and thus, may differentially ubiquitinate substrates resulting in a possible change in proteasome activity during this stage of the infection cycle. Evolutionary conservation of proteins involved with proteasome targeting and activation suggests that modulation of the proteasome by baculoviruses is important to the replication of the virus. For example, the ubiquitin ligase activity of IAP3 from OpMNPV is capable of ubiquitinating pro-apoptotic proteins from *Drosophila* (Green, Monser, and Clem, 2004). While ubiquitin has been shown to be a structural part of budded AcMNPV virions (Guarino, Smith, and Dong, 1995), virus-encoded ubiquitin does not appear to be required for production of budded virus (Reilly and Guarino, 1996). The *Spodoptera frugiperda* eIF2$\alpha$ sequence (van Oers et al., 2003) has several lysine residues surrounding one of the two possible cleavage sites of peIF2$\alpha^{ser51}$ (GenBank Accession #AF447395) which are candidates for ubiquitylation. Interestingly, one of these is a SUMO-like sequence at amino acid K211 (LKAG) which is preceded by a hydrophobic residue (*ABGENT-SUMOplot™ Prediction*). SUMO is a small ubiquitin-related modifier and shares 18% similarity with ubiquitin, it has been proposed to mediate protein-to-protein interactions by differential ubiquitination of protein substrates. In yeast, proliferating cell nuclear antigen (PCNA) is alternatively modified by SUMO and

ubiquitin in response to DNA damaging agents (Hoege et al., 2002). Sumolation of *Sf*eIF2α could be evaluated using immunoprecipitation with eIF2α antibodies followed by western blots with anti-SUMO antibodies.

Currently, we have no indication as to the number or types of proteins, virus or host-encoded, that are targeted for proteasome-mediated degradation during permissive baculovirus infection. Cleavage of peIF2α$^{ser51}$ may not be a specific event but a by-product of a generalized host response in which many proteins are degraded. However, we observe a single cleavage of peIF2α$^{ser51}$ rather than extensive cleavage into many short fragments that result from characterized proteasomal degradation (Jentsch and Schlenker, 1995). Moreover, the proteasome may not directly degrade peIF2α$^{ser51}$ but may indirectly mediate cleavage of peIF2α$^{ser51}$ through a proteolytic cascade. What remains to be determined is how a generalized or targeted activation of the proteasome aids in the baculovirus replication process. Defining the role that the proteasome and eIF2α phosphorylation plays during baculovirus infection, including its possible connections to HRF-1, will provide unique insights into the field of virus host range in invertebrates.

**Acknowledgements**

The author would like to thank Dr. Paul Friesen for providing vAcIEp35, Dr. Mathieu Miron for providing antibodies to Dm4E-BP and Dr. Motoko Ikeda for providing antibodies to polyhedrin. This work was supported by funding from the United States Department of Education, Graduate Assistance in Areas of National Need (GAANN) Program.

# Chapter 3
# HSP90 Expression is Enhanced in vAchrf-1-infected Ld652Y Cells

## 3.1. Introduction

Baculoviruses are large, double-stranded DNA (dsDNA) viruses that primarily infect lepidopteran species. Baculovirus progeny are produced in a budded or occluded virus form. The occluded form of the virus is environmentally-stable and consists of enveloped virions surrounded by a polyhedrin matrix that is encapsulated in an electron-dense envelope of carbohydrate and protein (Theilmann et al., 2004). The type virus for Baculoviridae is *Autographa californica* Multinucleocapsid nucleopolyhedrovirus (AcMNPV) which permissively infects *Spodoptera frugiperda* 21 (Sf21) cell lines but not *Lymantria dispar* 652Y cells (McClintock, Dougherty, and Weiner, 1986). Ld652Y cells infected with AcMNPV initiate global translation arrest by 9-12 hours post-infection (h.p.i.) resulting in a non-productive infection (Guzo et al., 1992). In the absence of a full length, baculovirus apoptotic suppressor, apoptotic programs are also induced in AcMNPV-infected Ld652Y cells (Du and Thiem, 1997a). Infection with AcMNPV variants that encode a single additional gene, *host range factor-1 (hrf-1)*, isolated from *Lymantria dispar* Multinucleocapsid nucleopolyhedrovirus (LdMNPV), results in a productive infection in Ld652Y cells (Du and Thiem, 1997b). Phosphorylation of eIF2α at serine 51 (peIF2α$^{ser51}$) is the probable mechanism of translation arrest in AcMNPV-infected Ld652Y cells as eIF2α$^{ser51}$ phosphorylation occurs in AcMNPV-infected Ld652Y cells at a time that correlates with the onset of translation arrest (Mecey, William and Thiem-manuscript in progress). Additionally, translation continues at 48 and 72 h.p.i. in Ld652Y cells infected with AcMNPV variants that encode a lepidopteran gene for eIF2α with an alanine substitution at serine 51 under regulation of an immediate-early and late baculovirus promoter (Mecey-unpublished results). The lepidopteran eIF2α

kinase, BeK (Prasad et al., 2003), expression levels appear to be activated at 12 h.p.i. in AcMNPV-infected Ld652Y cells temporally correlating to phosphorylation of eIF2α and translation arrest (Mecey-unpublished results) (Fig. 28, Supplementary Data). Proteolytic cleavage of peIF2α$^{ser51}$ occurs in Ld652Y and Sf21 cells that are permissively-infected with baculovirus but not in Ld652Y cells infected with AcMNPV where virus replication does not occur. Phosphorylation of eIF2α or cleavage of peIF2α$^{ser51}$ appears to be proteasome dependent as proteasome inhibition with epoxomicin results in a block of eIF2α phosphorylation, cleavage and permissive virus replication in AcMNPV and vAchrf-1-infected Sf21 cells and vAchrf-1-infected Ld652Y cells.

The unfolded protein response (UPR) is ancient, with components that are evolutionarily-conserved from yeast to mammals (Rutkowski and Kaufman, 2004). The UPR is activated in response to a variety of cellular stresses which result in an accumulation of unfolded proteins in the endoplasmic reticulum. Hallmarks of the UPR include, but are not limited to: phosphorylation of eIF2α via the PKR-like endoplasmic reticulum (ER) resident protein kinase (PERK), an increased synthesis of chaperone proteins, transcriptional activation of stress-related genes including the GRP78 ER resident chaperone-BiP, and activation of endoplasmic reticulum associated degradation (ERAD) via the proteasome (Rutkowski and Kaufman, 2004). In mammalian cells, BiP binds and regulates the degradation of ERAD substrates (Chillaron and Haas, 2000; Skowronek, Hendershot, and Haas, 1998). Conversely, deletion of protein components in the ERAD pathway can result in UPR activation (Ng, Spear, and Walter, 2000). Proteasome inhibition can also result in eIF2α phosphorylation and translation arrest by the kinase GCN2, an eIF2α kinase that is activated in response to amino acid starvation

(Jiang and Wek, 2005). During the UPR, PERK activation can initiate cell cycle arrest (Brewer and Diehl, 2000). Cis-acting unfolded protein response elements (UPRE) have been identified in the promoters of ER stress-activated genes as being sufficient for gene modulation in response to an induced UPR (Mori et al., 1998; Patil, Li, and Walter, 2004; Yoshida et al., 1998).

In mammalian systems, eIF2α phosphorylation occurring in response to virus infection is generally mediated by the double-stranded RNA (dsRNA)-activated protein kinase, PKR. Viruses have evolved numerous mechanisms to overcome recognition by and the effects of PKR (Gale, Tan, and Katze, 2000; Schneider and Mohr, 2003). Gene silencing with dsRNA is effective when targeted at a variety of genes in a number of insect models, including Lepidoptera (Means, Muro, and Clem, 2003) and a PKR homolog has not been identified in sequenced invertebrate genomes. However, in shrimp, innate antiviral immunity and a antiviral RNAi interference (RNAi)-like mechanism are both induced by dsRNA. Double-stranded RNA and baculovirus infection have been shown to result in activation of the lepidopteran, pathogenesis-related protein, hemolin (Hirai et al., 2004), indicating that a dsRNA response pathway exists for lepidoptera. Due to the abundance of viral proteins produced during a permissive virus infection, the UPR can be initiated during virus infection. The UPR has been shown to be activated by Japanese Encephalitis Virus (JEV) in fibroblast BHK-12 and neuronal cell lines resulting in eventual apoptosis but in apoptosis-resistant K562 cells, infection with JEV does not result in UPR activation (Su, Liao, and Lin, 2002). Human Cytomegalovirus (HCMV) activates the UPR and then modulates its components in infected fibroblast cells (Isler, Skalet, and Alwine, 2005). Herpes Simplex Virus 1

activates PERK in a mechanism that correlates with increased viral protein synthesis (Cheng, Feng, and He, 2005).

Heat shock protein 90 (HSP90) acts in a multi-chaperone complex and is known to interact with proteins involved in signal transduction, transcriptional and cell cycle regulation (Beere, 2001; Pratt and Toft, 2003; Young, Barral, and Ulrich Hartl, 2003). Inhibition of HSP90 with geldanamycin delays replication of Vaccinia Virus in RK13 cells lines and while HSP90 expression is not enhanced in RK13 cells infected with Vaccinia virus, its cellular distribution is altered (Hung, Chung, and Chang, 2002). HSP90 interacts with a number of viral proteins including the reverse transcriptase of Hepatitis B Virus and Simian Virus 40 T antigen (Hu and Seeger, 1996; Miyata and Yahara, 2000). Additionally, HSP90 and HSP70 are components of a receptor complex for dengue virus entry in human cell lines (Reyes-Del Valle et al., 2005). The *Drosophila melanogaster* protein, Dpit47, has been identified as an HSP90 co-chaperone that binds DNA polymerase α, inhibiting its activity (Crevel et al., 2001). HSP90 has over 100 identified client proteins including three eIF2α kinases: GCN2, PKR and the heme-regulated protein kinase (HR1), to which it binds in concert with HSP70 to prevent autophosphorylation (Pratt and Toft, 2003). In response to proteasome inhibition, protein levels of HSP90 and HSP70 have been shown to increase, while mRNA levels of HSP90 remained unchanged, suggesting possible translational regulation of the protein (Awasthi and Wagner, 2005). Heat shock cognate protein 70 (HSC70) is one of few host genes that is transcriptionally activated in response to baculovirus infection, indicating a role for chaperone proteins during permissive infection. Preferential synthesis of viral proteins appears to be regulated at the transcriptional level as an overall decrease of host gene

transcription occurs in response to AcMNPV infection in Sf21 cells (Nobiron, O'Reilly, and Olszewski, 2003). This decreased synthesis of host-encoded mRNA may lead to a quelled requirement for chaperone proteins and a bypass of a cellular initiated UPR stress response. Alternatively, chaperone proteins during productive baculovirus infection, other than HSC70, may be regulated at a translational level thus overcoming the effects of inhibited host transcription.

Here we investigate regulation of the chaperone proteins HSP90 and HSC70 during AcMNPV and vAchrf-1 infection of Ld652Y cells. We show an increase in levels of a protein that interacts and immunoprecipitates with antibodies raised against human PKR that mass spectrometry identifies as HSP90. Inhibition of HSP90 with geldanamycin results in a block of occluded virus production at 48 h.p.i. in productively-infected cells and a significant decrease in polyhedrin protein levels.

## 3.2. Materials and Methods

### 3.2.1. Viruses and cell lines

Sf-21 cells (Vaughn et al., 1977) and Ld652Y cells (Goodwin, Tompkins, and McCawley, 1978) were maintained in TC100 medium (JRH BioScience, Lenexa, KS) supplemented with 10% fetal bovine serum and 0.26% tryptose broth. AcMNPV variant L1 (Lee and Miller, 1978) and vAchrf-1 (previously designated vAcLdPD, Du and Thiem, 1997) were propagated in Sf21 cells with titers being determined by plaque assay.

### 3.2.2. Immunoprecipitation

Cell culture plates (100 mm) were seeded with Ld652Y cells at 2 x $10^6$, cells were allowed to adhere to the plate surface for at least one hour. Ld652Y cells were infected with vAchrf-1 at an MOI of 10, after a one hour infection, virus was removed and cells were refed with TC-100. At 12 h.p.i., cells were collected, media was removed and infected cells were lysed in 900 ml of RIPA buffer containing a protease and phosphatase inhibitor cocktail. Lysates were passed through a 21 gauge needle to shear DNA and were incubated on ice for 60 minutes. Soluble cellular proteins were then separated from debris by micrcentrifuging for 10 minutes at 10,000xg in 4°C. The supernatant was transferred to a sterile eppendorf tube, 25 µg of normal rabbit IgG and 20µl of suspended (25% v/v) Protein-A-Agarose conjugate (Santa Cruz Biotechnology-#sc-2001) were added to the supernatant. Whole cell lysate was pre-cleared by rocking at 4°C for 30 minutes then beads were pelleted at 1,450 x *g* for 30 seconds. The supernatant was transferred to a sterile eppendorf tube and combined with 10 µg of anti-PKR agarose conjugate (Santa Cruz Biotechnology-sc #1702AC) which was then rocked and incubated overnight at 4°C. The agarose bound antibody and interactive proteins were collected via

centrifugation at 1,450 x *g* for 30 seconds in 4°C. The pellet was rinsed three times with RIPA buffer, buffer was discarded after the final wash and the pellet was resuspended in 30 µl of 2X SDS sample buffer. Samples were boiled at 100°C and separated using SDS-PAGE (Laemmli, 1970) on 12.5% polyacrylamide gels. Proteins were visualized using Coomassie brilliant blue, destained and excised directly from the gel. The gel band was excised and hydrolyzed with trypsin according to Shevchenko et al. (1996).

### 3.2.3. MALDI-TOF-MS

0.5 µl of the digested peptide was mixed with 0.5 µl of saturated of α-cyano-4-hydroxycinnamic acid in 60% acetonitrile/0.1% trifluoracetic acid. Mass spectra were obtained on a Voyager-DE-STR MALDI-TOF mass spectrometer (formerly PerSeptive Biosystems, Inc., Framingham, MA, now Applied Biosystems) in the linear mode. Peak lists of the tryptic peptide masses were generated and searched against public databases using the program Protein Prospector from the University of California, San Francisco (http://prospector.ucsf.edu).

### 3.2.4. LC-ESI-MS

Further analysis of the digests and sequence confirmation were performed using a capillary liquid chromatography system (Waters Corp., Milford, MA) coupled to an LCQ-Deca ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) equipped with a nanospray ionization source. Digests were trapped on a Cap Trap (Michrom BioRsources, Inc., Auburn, CA) and subsequently separated on a 5 cm x 75 µm ID picofrit column packed with 5 µm ProteoPep C18 material (New Objective, Woburn, MA). Peptides were eluted with a gradient of 2-95% acetonitrile in 0.1% formic acid.

Mass spectra of fragmented ions were analyzed using the SEQUEST and MASCOT programs.

All mass spectrometry analyses were conducted at the Michigan State University Mass Spectrometry Facility.

**3.2.5. Western Blot Analysis**

Protein samples were quantified using the DC Reagent System (Bio-Rad #500-0116), a Lowry-based detergent compatible assay. Protein levels were measured in 96 well ELISA plates at 750 nm using a uQuant microplate reader (Witech AG, Switzerland) and BioTek-$KC_{Junior}$ Analysis software package (Fischer Scientific #BT-5270501). Each sample containing 20 ug of total protein and 10 ul 2X SDS sample buffer was boiled at 98° C for 10 minutes, briefly centrifuged to collect sample and then loaded into a 15% tris-glycine polyacrylamide gel. Samples were separated for 2 hours at 110 volts using SDS-PAGE (Laemmli, 1970) and then transferred to Immobilon-P, PVDF membrane (Millipore Corp., Bedford, MA) overnight at 4° C in Towbin Buffer (25 mM Tris, 192 mM glycine and 20% ethanol) at 40 volts. Membranes were blocked for 5 hours in 5% lowfat milk in 1X TBS-Tween (0.2 M NaCl, 66 mM Tris, 0.1% Tween). Immunodetection was performed by incubating blots overnight with 2% lowfat milk in 1X TBS-Tween and one of the following primary antibodies: anti-eIF2α phosphor-Ser51 serum (Biosource, #44-726G) diluted to 1:3000, anti-KDEL monoclonal antibody (Calbiochem #420400) diluted to 1:300 or anti-polyhedrin polyclonal (M. Ikeda) diluted to 1:30,000. The blot was then washed with 2% milk in 1X TBS-Tween and incubated with horseradish peroxidase conjugated anti-rabbit antibody (Pierce, #31460) or anti-mouse antibody (Pierce, #31430) diluted to 1:10,000 in 2% lowfat milk in 1X TBS-

Tween for 2 hours. Bands were visualized using an ECL detection kit (Amersham-Pharmacia Biotech, #RPN 2209) and exposure on Classic Blue Sensitive autoradiography film (Midwest Scientific).

### 3.3. Results

#### 3.3.1. HSP90 is activated in Ld652Y cells productively-infected with Achrf-1

In searching for an eIF2α kinase that was activated in response to AcMNPV and vAchrf-1 infection in Ld652Y cells, western blots showed an increasing level of a protein at 12 and 24 h.p.i. that interacted with antibodies raised against human PKR, specifically in vAchrf-1-infected cells (Fig. 18, Lanes 3 and 7, as compared to Lanes 1, 2, 4-6, 8). Immunoprecipitation with PKR antibodies and cell lysates from vAchrf-1-infected Ld652Y cells combined with subsequent MALDI-TOF mass spectrometry analysis identified four independent peptide matches to *Spodoptera frugiperda* 90 kDa heat shock protein (HSP83) (See Table 1). One lane was excised from the IP gel for western blot transfer to correlate band for excision with the immunoreactive band (Fig. 29, Supplementary Data). Additional western blots utilizing a commercial HSP90 control protein and PKR antibodies confirmed that the antibody was cross-reactive (Fig. 18, Lane 10). HSP90 is ubiquitous throughout eukaryotic cells and is identified in many mass spectrometry analyses. However, considering that the anti-PKR antibody recognized the HSP90 control protein, it is likely that the actual protein that was immunoprecipitated by anti-PKR was HSP90.

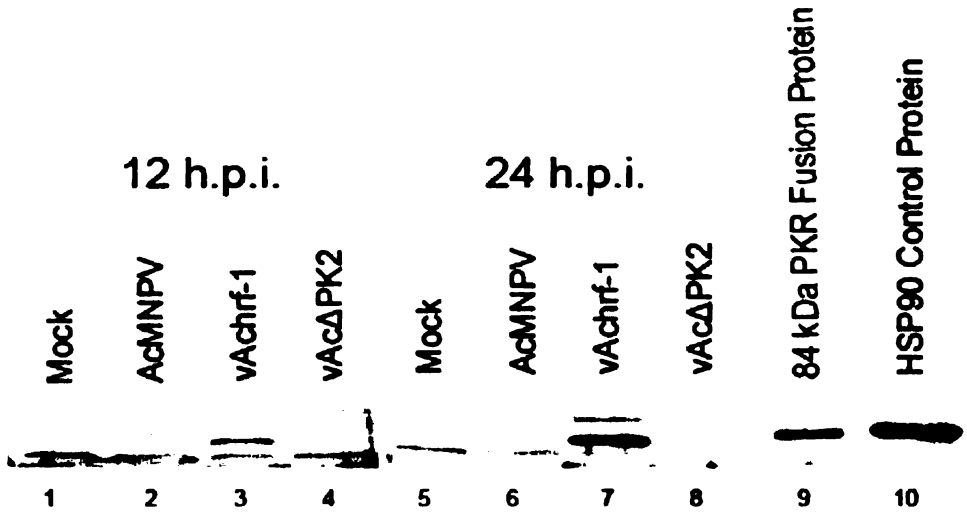



**Figure 18. HSP90 levels increase in productively-infected vAchrf-1 infected Ld652Y cells from 12 to 24 h.p.i.** Western blots showing increasing levels of an ~80kD protein in vAchrf-1 infected Ld652Ycells from 12 h.p.i. (lane 3) to 24 h.p.i. (lane 7). Western blots were probed with anti-PKR polyclonal antibody which interacts with an 84 kDa PKR fusion protein (lane 9), antibody also interacts with HSP90 control protein of approximately the same size (lane 10).

| TIC | gi Ref. | Xcorr | Sequence |
|---|---|---|---|
| 9.9e5 | *S. Frugiperda* 90 kDa Heat Shock Protein gi 12005809 | 4.11 | (K)HLEINPDHSIVETLR |
| 7.7e6 | gi 12005809 | 3.93 | (K)GVVDSEDLPLNISR |
| 3.9e6 | gi 12005809 | 3.30 | (K)GVVDSEDLPLNISR |
| 3.4e6 | gi 12005809 | 3.29 | (K)HLEINPDHSIVETLR |
| 1.6e6 | gi 12005809 | 2.96 | (K)HFSVEGQLEFR |
| 9.5e5 | gi 12005809 | 2.94 | (K)HFSVEGQLEFR |
| 2.6e6 | gi 12005809 | 2.86 | (K)SLTNDWEDHLAVK |

TIC: Total Ion Chromatogram
Xcorr: Cross-correlative value with other peptides in database after MS/MS analysis
gi Ref: Genbank general accession number

**Table 1.** ***Sequest*** summary of mass spectrometry analysis for immunoprecipitation with anti-PKR antibodies in vAchrf-1-infected Ld652Y cells at 12 h.p.i.

### 3.3.2. Inhibition of HSP90 with geldanamycin inhibits polyhedrin protein synthesis and occlusion body production in permissively-infected cells at 48 h.p.i. but has little effect on budded virus prodcution

It was interesting to note a de-novo synthesis of HSP90 in vAchrf-1-infected Ld652Y cells beginning at a post-infection time that correlates to the onset of translation arrest in AcMNPV-infected Ld652Y cells. While heat shock proteins are ubiquitous throughout the cell, increased synthesis of heat shock proteins is a hallmark of the unfolded protein response that is activated in response to virus infections in systems other than invertebrates. HSP90 activity can be directly inhibited by the antibiotic

geldanamycin which blocks the ATP binding site of HSP90. HSP90 chaperone function is dependent upon its ATPase activity. To define a role for HSP90 during permissive baculovirus infection we added 1 μM geldanamycin directly to cell culture media immediately following the infection of Sf21 cells with AcMNPV and vAchrf-1. Treated cells were examined at 3, 12, 24 and 48 h.p.i. using a Nikon TMS inverted phase-contrast microscope, 48 hour infected cultures were re-treated with geldanamycin at 24 h.p.i. to guard against effects attributable to degradation of the inhibitor. Surprisingly, virus occlusion bodies were not seen in permissively infected cells treated with geldanamycin at 24 h.p.i. and were found at low levels at 48 h.p.i. (Fig. 19, 2B compared to 2A and 3B compared to 3A). Similar results were found in vAchrf-1-infected Ld652Y cells that were treated with geldanamycin (data not shown). To determine whether HSP90 inhibition was completely blocking synthesis of late viral proteins, cell lysates were collected at 24 and 48 from Sf21 cells permissively-infected with AcMNPV and Ld652Y cells infected with AcMNPV and vAchrf-1. Lysates were screened using western blots with antibodies raised against the late baculovirus proteins polyhedrin and p39. Blots showed that geldanamycin neither enabled AcMNPV replication in Ld652Y cells (Fig. 20, Lanes 4 and 10) nor blocked late viral protein synthesis in permissively-infected cells at 48 h.p.i. (Fig. 20, Lanes 12 and 14). However, in both permissively-infected Sf21 cells at 48 h.p.i. and Ld652Y cells at 24 and 48 h.p.i., polyhedrin protein synthesis was reduced in those cells treated with geldanamycin (Fig. 20, Lane 14 as compared to Lane 13 and Fig. 3, Lanes 6 and 12 as compared to 5 and 11). The other bands found on Fig. 20, lanes 5, 11 and 12 are consistently produced on western blots from productively-infected invertebrate cells at the very late stages of infection when probed with anti-polyhedrin

antibodies. On these blots, by 48 h.p.i., many bands can be seen, ranging in size from 1-26 kDa, possibly representing degraded fragments of the polyhedrin protein. In mock-infected samples and at earlier times post-infection, the anti-polyhedrin antibody is not cross-reactive. Similar results were observed when the same blots were stripped and reprobed with p39 antibodies (data not shown). To determine whether HSP90 inhibition had any effect on budded virus production, infected cells were treated with geldanamycin as previously described and titered via standard plaque assay. Plaque assays showed that geldanamycin may have a minor inhibitory or slowing effect on permissive virus replication in infected Ld652Y and Sf21 cells (Figure 21).

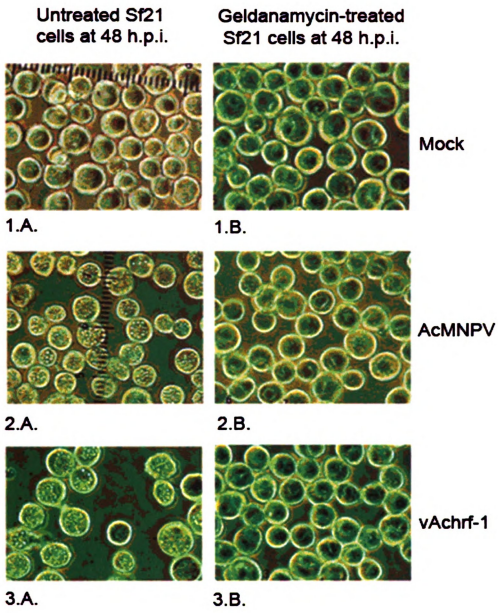



**Figure 19. Geldanamycin delays or prevents occlusion body formation in lepidopteran cells permissively-infected with baculovirus.** Sf21 cells that are permissively-infected with either AcMNPV (panel 2A) or vAchrf-1 (panel 3A) normally show production of occlusion bodies by 48 h.p.i. When Sf21 cells are treated with 1 μM geldanamycin, occlusion bodies are not visible at 48 h.p.i. in AcMNPV (panel 2B) or vAchrf-1 (panel 3B) –infected cells. **(Image Presented in Color)**

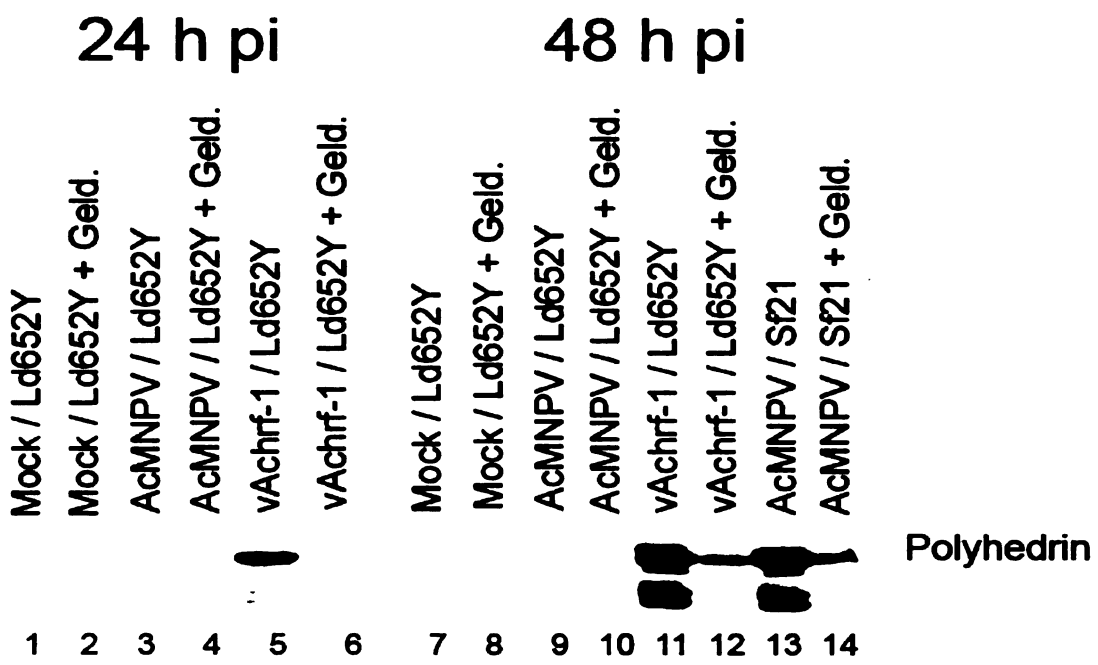
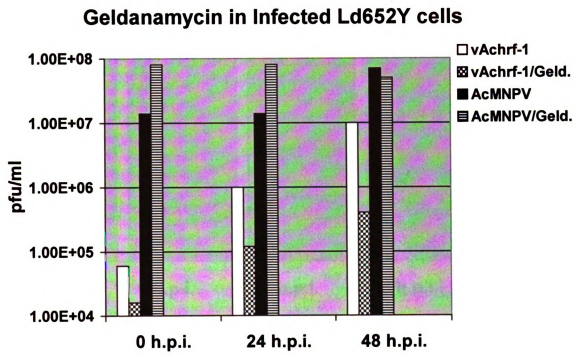



**Figure 20. In cells treated with the HSP90 inhibitor, geldanamycin, polyhedrin protein levels are reduced in Sf21 and Ld652Y cells productively infected with baculovirus.** Polyhedrin protein levels were assayed by using anti-polyhedrin antibodies Total cell lysates were prepared from Ld652Y cells that were mock-infected or infected with AcMNPV or vAchrf-1 or AcMNPV-infected Sf21 cells. Infected cells were harvested at 24 and 48 hours post-infection. Proteins from whole cell lysates were separated on 15% SDS-PAGE gels and analyzed with immunoblotting.

**A.**


Geldanamycin in Infected Ld652Y cells
vAchrf-1
vAchrf-1/Geld.
AcMNPV
AcMNPV/Geld.
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
pfu/ml
0 h.p.i.
24 h.p.i.
48 h.p.i.

**B.**

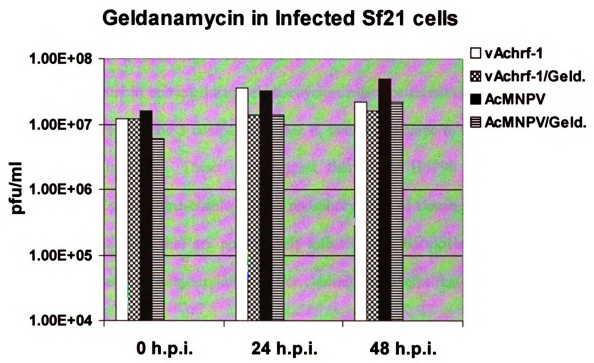



**Figure 21. Geldanamycin reduces budded virus production in vAchrf-1-infected Ld652Y cells.** A. vAchrf-1 titers are reduced within at least one order of magnitude in infected Ld652Y cells treated with geldanamycin. B. Geldanamycin does not reduce vAchrf-1 or AcMNPV budded virus production in Sf21 cells. Ld652Y or Sf21 cells were pre-treated with 1 μM geldanamycin from 1 hour preceding infection to time of collection. Cells were mock-infected or infected with either AcMNPV or vAchrf-1 and virus was harvested at 24 h.p.i. and 48 h.p.i. Virus was titered on Sf21 cells using a standard plaque assay (O'Reilly, Miller, and Luckow, 1994). Plates were scored for *occ*$^{+}$ plaques at 4 and 6 days post-infection.

### 3.3.3. Proteolytic cleavage of peIF2α$^{ser51}$ precedes and is independent of HSP90 activation in permissively-infected Ld652Y cells

We previously reported that a proteasome-dependent, proteolytic cleavage of peIF2α$^{ser51}$ occurs by 24 h.p.i. in invertebrate cells permissively-infected with baculovirus (Mecey-

manuscript in progress). To determine if that cleavage was dependent upon an increased synthesis of HSP90; mock, AcMNPV and vAchrf-1-infected Ld652Y and Sf21 cell lysates were collected at 24 and 48 h.p.i. and western blots were conducted to assay for peIF2$\alpha^{ser51}$ cleavage in geldanamycin-treated cells. Western blots show that cleavage of peIF2$\alpha^{ser51}$ occurs in untreated and geldanamycin-treated, permissively-infected cells by 24 h.p.i. (Figure 22, lane 3 compared to lane 6, lane 8 compared to lane 11 and lane 9 compared to lane 12). These data suggest that either HSP90 activation occurs downstream of proteasome-enabled eIF2$\alpha$ phosphorylation or cleavage of peIF2$\alpha^{ser51}$ in invertebrate cell lines that are permissively-infected with baculovirus or that HSP90 activation and eIF2$\alpha$ phosphorylation and/or cleavage are events that occur independent of one another.

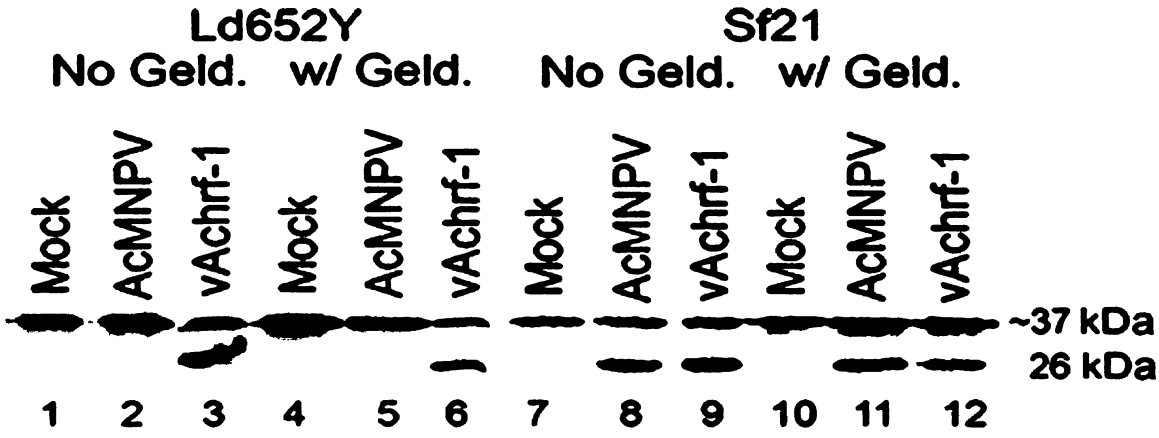



**Figure 22. Proteolytic cleavage of peIF2$\alpha^{ser51}$ occurs in Ld652Y and Sf21 cells permissively-infected with vAchrf-1 and AcMNPV in the presence of geldanamycin.** Western blots showing proteolytic cleavage of peIF2$\alpha^{ser51}$ in Ld652Y cells infected with AcMNPV and vAchrf-1 that were untreated (lanes 2 and 3) or in the presence of geldanamycin (lanes 5 and 6) at 24 h.p.i. Proteolytic cleavage of peIF2$\alpha^{ser51}$ also occurs in Sf21 cells infected with AcMNPV or vAchrf-1 that were untreated (lanes 8 and 9) or treated with geldanamycin (lanes 11 and 12).

### 3.4. Discussion

Infection of the *Lymantria dispar* 652Y cell line with the baculovirus AcMNPV results in global translation arrest and a non-productive infection (Guzo et al., 1992; McClintock, Dougherty, and Weiner, 1986). Expression of a single gene, *host range factor-1*, isolated from LdMNPV precludes translation arrest in AcMNPV-infected Ld652Y cells and results in virus replication (Chen et al., 1998; Du and Thiem, 1997b; Thiem et al., 1996). Apoptosis is enhanced and global translation arrest is initiated during the late stages of AcMNPV infection in Ld652Y cells (Thiem and Chejanovsky, 2004). During a productive infection, events correlating to the late phases of virus replication are DNA replication of the virus, expression of late viral genes and a virus-mediated shut-off of host transcription.

In this study we identify an increased expression of the chaperone protein HSP90 that occurs in cells permissively-infected with vAchrf-1 during the late stages of infection (Fig. 18). Further, we determine that geldanamycin-mediated inhibition of HSP90 delays or inhibits the production of the very late baculovirus polyhedrin protein and reduces or eliminates the production of occluded virus in productively-infected cells at 48 h.p.i., a time when occluded virus is normally present (Fig. 19 and 21). Inhibition of HSP90 with geldanamycin has no inhibitory effect on the proteasome-dependent cleavage of peIF2$\alpha^{ser51}$, indicating that HSP90 activation occurs independently or downstream of eIF2$\alpha$ phosphorylation at serine 51 (Fig. 22).

Other groups have shown that chaperone proteins may be required during permissive baculovirus infection. HSC70, also known as BiP, was shown to be transcriptionally up-regulated in Sf9 cells permissively-infected with AcMNPV (Nobiron, O'Reilly, and Olszewski, 2003). HSP90 was not identified in the same study as being transcriptionally activated; however, the same group shows the majority of host transcription being shut-off between 12 and 18 h.p.i. during a permissive infection. The shut-off of host transcription suggests a potential role for translational regulation of chaperone transcripts during a permissive infection. Indeed, HSP90 levels increase from 12 to 24 h.p.i. in Ld652Y cells that are permissively-infected with vAchrf-1 and HSP90 appears to play a role in the production of late viral proteins and occluded virus (Fig. 19, 20 and 21).

In the non-productive infection of AcMNPV in Ld652Y cells, the virus enters the nucleus and executes the early stages of virus replication. During these early stages of infection, host and virus transcription and translation continue (Guzo et al., 1992). Previously, we have shown that eIF2α phosphorylation at serine 51 occurs during AcMNPV infection of Ld652Y cells at a time that correlates to the onset of global translation arrest. Additionally, we have shown that both eIF2α phosphorylation at serine 51 and cleavage of peIF2αser51 are enabled by the proteasome (Mecey-manuscript in progress). The additional load of viral transcripts produced during the early stages of infection may result in stress on the protein processing capacity of the endoplasmic reticulum and its localized chaperone machinery cumulating in the induction of an unfolded protein response.

The unfolded protein response (UPR) has been characterized in *C. elegans* and plays a regulatory role in the development of the nematode (Shen et al., 2001). Many homologous components of the mammalian UPR pathway have been identified and characterized in other invertebrates (Olsen et al., 1998; Santoyo et al., 1997) including the lepidopteran, *Bombyx mori* (Goo et al., 2004). Hallmarks of the unfolded protein response include eIF2α phosphorylation at serine 51 resulting in a global arrest of cap-dependent protein synthesis and a diverted pathway resulting in either apoptosis and cell destruction, or cell recovery as mediated by an increased synthesis of chaperone proteins and ERAD degradation of unfolded/unprocessed proteins (Ma and Hendershot, 2004; Rutkowski and Kaufman, 2004). The proteasome has recently been identified as a key regulator in the UPR by facilitating ERAD degradation (Ng, Spear, and Walter, 2000).

Although our host range system has many of the hallmarks of a UPR stress response pathway, one must consider that this pathway has not been fully characterized for Lepidopteran insects and that the comparisons made are based on information obtained from mammalian systems. Different stress response pathways such as those resulting from DNA or oxidative damage may also be induced in response to infection. Investigation into kinase signaling leading to eIF2α phosphorylation in AcMNPV-infected Ld652Y cells may help to define initiated pathways during non-permissive infection. Additonal information should also be gathered with regards to the expression of other chaperone proteins during baculovirus infection.

# Chapter 4
## Summary and Future Directions

## 4.1. Research Summary

**a.)** Research conducted by other laboratory members show that translation arrests gradually in AcMNPV-infected Ld652Y cells with a complete arrest of protein synthesis occurring by 13-14 h.p.i. (work conducted by X. Du). In those same infected cells, polysome profiles show a monosome peak occurring at 9 h.p.i. indicating that translation arrests at the initiation stage (work conducted by W.A. Williams). Possible effects occurring from the inhibition of translation elongation were not investigated in this study.

**b.)** Phosphorylation of eIF2α at serine 51 occurs in Ld652Y cells that are infected with AcMNPV at 12 h.p.i. In Ld652Y cells that are infected with vAchrf-1, eIF2α appears to be protected from phosphorylation as compared to mock and AcMNPV-infected cells. Expression of the proposed AcMNPV-encoded, dominant-negative inhibitor of eIF2α kinases, PK2, continued throughout the infection but PK2 was insufficient at blocking phosphorylation of eIF2α in AcMNPV-infected Ld652Y cells at late times post-infection.

**c.)** Proteolytic cleavage of peIF2$\alpha^{ser51}$ appears to occur in Ld652Y cells permissively-infected with vAchrf-1 and Sf21 cells permissively-infected with AcMNPV and vAchrf-1. Cleavage of peIF2$\alpha^{ser51}$ was not induced by stress-inducing agents and was not blocked with cell permeable protease inhibitors.

**d.)** Treatment with the proteasome inhibitor epoxomicin in Sf21 and Ld652Y cell lines infected with AcMNPV and vAchrf-1 or vAchrf-1, respectively, resulted in prevention of eIF2α phosphorylation at serine 51, a block of peIF2$\alpha^{ser51}$ cleavage and no virus replication. The proteasome inhibitor, MG132, yielded similar results in vAchrf-1-infected Ld652Y cells but only reduced virus titers while not blocking peIF2$\alpha^{ser51}$

cleavage in infected Sf21 cells. The conflicting result observed for both proteasome inhibitors may result from a varying potency of each inhibitor or its specific mode of action. Both results indicate that the proteasome is involved during baculovirus infection.

**e.)** Heat shock protein 90 expression is enhanced in Ld652Y cells that are permissively-infected with vAchrf-1. Inhibition of HSP90 with geldanamycin resulted in a delay or reduction of very late stage baculovirus protein expression and a block of occlusion body production at 48 h.p.i. Cleavage of peIF2$\alpha^{ser51}$ occurs independently of enhanced HSP90 expression. These results suggest that enhanced expression of HSP90 accompanies productive baculovirus infection and is integral to the production of occluded virus.

## 4.2. Future Directions

The current data indicates that proteasome-enabled cleavage of peIF2$\alpha^{ser51}$ accompanies productive virus infection in both Ld652Y and Sf21 cells infected with vAchrf-1 and AcMNPV and vAchrf-1, respectively. Additional data produced in this study demonstrates that the inhibition of the proteasome with epoxomicin or MG132 blocks phosphorylation of eIF2α at serine 51 and inhibits or reduces the production of virus progeny in those same infected, lepidopteran cell lines (See Fig. 23). The primary questions that remain are: is baculovirus replication enabled by the proteolytic cleavage of peIF2$\alpha^{ser51}$ and, if so, does HRF-1 participate in that process?

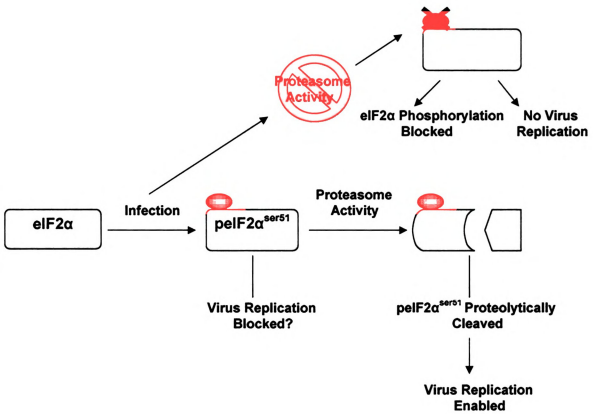



**Figure 23. Proposed model of post-translational modification of eIF2α in response to baculovirus infection.** The current data shows eIF2α becoming phosphorylated in response to AcMNPV-infection in Ld652Y cells. In Sf21 and Ld652Y cells that are productively-infected with AcMNPV and vAchrf-1 or vAchrf-1, respectively, a (Fig. 23 caption, cont.) proteasome-enabled cleavage of peIF2$\alpha^{ser51}$ occurs by 24 h.p.i. In those same infected cells, when treated with proteasome inhibitors, eIF2α is protected from phosphorylation at serine 51 and virus replication is inhibited. **(Image Presented in Color)**

Indeed, if cleavage of peIF2$\alpha^{ser51}$ enables baculovirus replication by preventing global translation arrest, then one can envision two scenarios as being mechanistically significant. The first and most probable mechanism involves disabling the inhibitory capabilities of peIF2$\alpha^{ser51}$ on eIF2B, the second involves the proteolytic degradation of peIF2$\alpha^{ser51}$ coupled to an increased synthesis of eIF2$\alpha$. The nucleotide exchange factor eIF2B is a limited factor in the cell, phosphorylation of eIF2$\alpha$ at serine 51 results in the binding and sequestering of eIF2B. This impoundment of eIF2B prevents the exchange of GDP to GTP required for the formation of an active eIF2 ternary complex resulting in translation arrest at the initiation stage (See Fig. 24A and 24B). Proteolytic cleavage of peIF2$\alpha^{ser51}$ may render two partial eIF2$\alpha$ fragments that are incapable of inhibiting eIF2B but capable of forming a functional ternary complex with other subunits (See Fig. 24C). One method to test this hypothesis is to express the predicted partial eIF2$\alpha$ fragments individually from a recombinant AcMNPV in Ld652Y cells and then conduct plaque assays to determine budded virus titers. Alternatively, engineered plasmid constructs with truncated forms of eIF2$\alpha$ could be transfected into Ld652Y cells followed by AcMNPV infection and plaque assay. While Figure 24C does not reflect it, functional ternary complex formation could result from interaction with either partial eIF2$\alpha$ component so, both would have to be expressed and evaluated. If budded virus titers increased as compared to those produced at 0 hours post-infection, one could conclude that AcMNPV replication had been enabled by the expression of a truncated eIF2$\alpha$ sequence. It is possible that phosphorylation of eIF2$\alpha$ at serine 51 occurs as a general response to stress in lepidopteran insects. While experiments utilizing general stress induction agents in

both Ld652Y and Sf21 cells did not result in cleavage of peIF2α$^{ser51}$, levels of full-length peIF2α$^{ser51}$ may show differential regulation in response to stress (Fig. 12). More experiments need to be conducted to determine the effects of stress on eIF2α phosphorylation in lepidopteran cells. Proteasome-enabled degradation of peIF2α$^{ser51}$ may occur as a means to overcome translation arrest while levels of eIF2α recover. If budded virus titers do not increase in Ld652Y cells infected with recombinant AcMNPV expressing either eIF2α truncated protein (vAccleIF2α), protein synthesis levels should be evaluated to determine if global translation arrest occurs. If translation arrest is prevented in vAccleIF2α-infected Ld652Y cells and a productive infection is not established, it would indicate that global translation arrest is not the mechanism preventing AcMNPV replication in that cell line. Other groups have shown that expression of a C-terminal, truncated form of eIF2α, was not sequestered by eIF2B in mammalian SAOS-2 cells. Cells expressing this mutant were able to overcome translational repression induced by PKR even though the truncated eIF2α was phosphorylated by the kinase (Satoh, et. al. 1999).

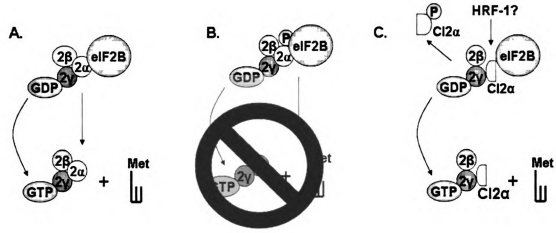



**Figure 24. The interaction of eIF2α with eIF2B.** A.) The nucleotide exchange factor eIF2B coordinates the exchange of GDP to GTP to restore functional ternary complex formation required for the initiation of protein synthesis. B.) When eIF2α is phosphorylated at serine 51 it binds eIF2B preventing nucleotide exchange, active ternary complexes can not form and global translation arrest is initiated. C.) A proposed model of how a cleaved fragment of peIF2α$^{ser51}$ may interact with eIF2B and still function in an active ternary complex to initiate protein synthesis. **(Image Presented in Color)**

The second proposed mechanism involves the proteasome-enabled cleavage of peIF2α$^{ser51}$ as a means to remove the phosphorylated protein from the cell as the cell recovers from a particular stress. Eukaryotic cells are dependent on eIF2α as a required component of the initiation of protein synthesis so, this proteolytic degradation would have to be accompanied by an increased synthesis of eIF2α which is best evaluated at the transcriptional level using northern blots or real-time PCR. Current data do not suggest that eIF2α levels increase in productively-infected cells but that is difficult to determine by observing protein levels as host transcription is being shut-off. In either model as it relates to vAchrf-1-infected Ld652Y cells, a viral protein may mediate the proteasome-enabled cleavage of peIF2α$^{ser51}$ to initiate recovery from stress resulting from virus

infection, thus restoring protein synthesis and possibly enabling permissive virus infection.

As a part of an initiated stress response pathway, proteasome activity can lead to to eIF2α phosphorylation at serine 51 (Jiang and Wek, 2005). One can envision a scenario where once proteasome-dependent eIF2α phosphorylation is initiated in baculovirus-infected cells, a viral protein, like HRF-1, may mediate the degradation of peIF2α$^{ser51}$ by directing proteasome activity (Fig. 25). The question of HRF-1 involvement in enabling baculovirus replication in *Lymantria dispar* and its correlation to the proteolytic cleavage of peIF2α$^{ser51}$ are best answered by searching for proteins that directly interact with HRF-1. Yeast two-hybrid analysis or immunoprecipitation studies coupled to mass spectrometry analysis may identify HRF-1 interacting proteins and further provide indications to the level of function for HRF-1. Another protein(s) that functions in a similar manner to HRF-1 may be encoded by the *Spodoptera frugiperda* genome thus facilitating AcMNPV replication in Sf21 cells. This would offer an explanation of why cleavage of peIF2α$^{ser51}$ occurs in Sf21 cells during infection with AcMNPV. Alternatively, Ld652Y cells may physiologically differ from Sf21 cells to such an extreme, that a HRF-1-type protein is not required to promote AcMNPV replication in Sf21 cells.

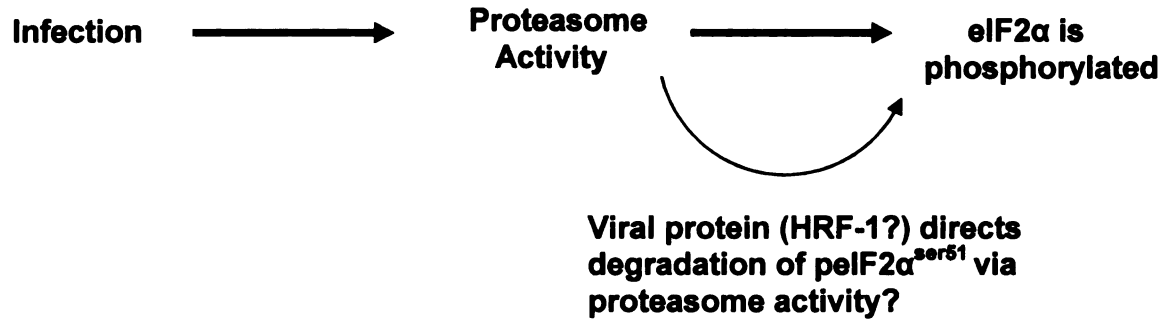



**Figure 25. Proposed model for HRF-1 function in vAchrf-1-infected Ld652Y cells.** AcMNPV-infection of Ld652Y cells results in eIF2α phosphorylation at serine 51 and global translation arrest. This study shows that proteasome inhibition blocks the phosphorylation of eIF2α, cleavage of peIF2α$^{ser51}$ and blocks the production of virus progeny in baculovirus-infected, invertebrate cell lines. In the proposed model, HRF-1 would act downstream of proteasome activation to coordinate cleavage of peIF2α$^{ser51}$.

# Appendix A
## Supplementary Data

**eIF4E is available to bind cap substrates in AcMNPV-infected Ld652Y cells.**

Protein synthesis can be attenuated through the formation of an inhibitory eIF4E-4E binding protein (4E-BP) complex. To exclude the possibility that mechanisms other than eIF2α phosphorylation might be contributing to translation arrest we isolated total cell lysates from mock, AcMNPV and Achrf-1-infected Ld652Y cells and precipitated against $m^7$G-bound sepharose (Amersham-Pharmacia). Western blots of precipitated proteins were probed with Lepidopteran eIF4E antibodies. Western blots show equal amounts of eIF4E bound to the $m^7$G cap in mock, AcMNPV and Achrf-1 infected cells at 3 and 20 hpi (Fig. 15, lanes 1, 2, 3, 4, 5 and 6). This indicates that eIF4E is not prevented from binding the cap and by inference, that it was not sequestered by 4E-BP.

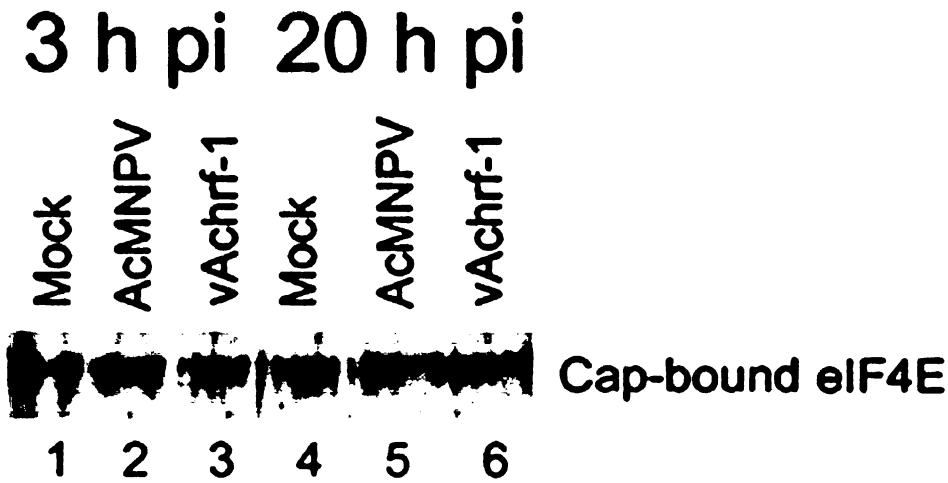



**Figure 26. AcMNPV infection in Ld652Y cells does not impair the m7G cap-binding ability of eIF4E.** Total cell lysates from mock-, AcMNPV-, and Achrf-1-infected Ld652Y cells were immunoprecipitated with m7GTP-bound sepharose. Proteins were boiled off sepharose beads, subjected to SDS-PAGE and western blotting with lepidopteran eIF4E antibodies.

**4E-BP is not over-expressed in AcMNPV-infected Ld652Y cells.**

To further investigate the possibility of 4E-BP contributing to global translation arrest in AcMNPV-infected Ld652Y cells, we probed western blots of total cell lysates isolated from Mock, AcMNPV and Achrf-1-infected cells at 3, 12 and 20 hpi with antibodies raised against *Drosophila melanogaster* 4E-BP (kindly provided by Mathieu Miron). There was no detectable increase in 4E-BP levels in infected cells over mock-infected (Fig. 16, lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12) suggesting that, unlike translation arrest induced in *Drosophila* in response to bacterial infection and wounding (Bernal and Kimbrell, 2000), over-expression of 4E-BP does not contribute to translation arrest in AcMNPV-infected Ld652Y cells. These experiments do not rule out possible changes in 4E-BP phosphorylation. However, the combined eIF4E and 4E-BP data indicates that translation arrest is not controlled through inhibition of eIF4E.

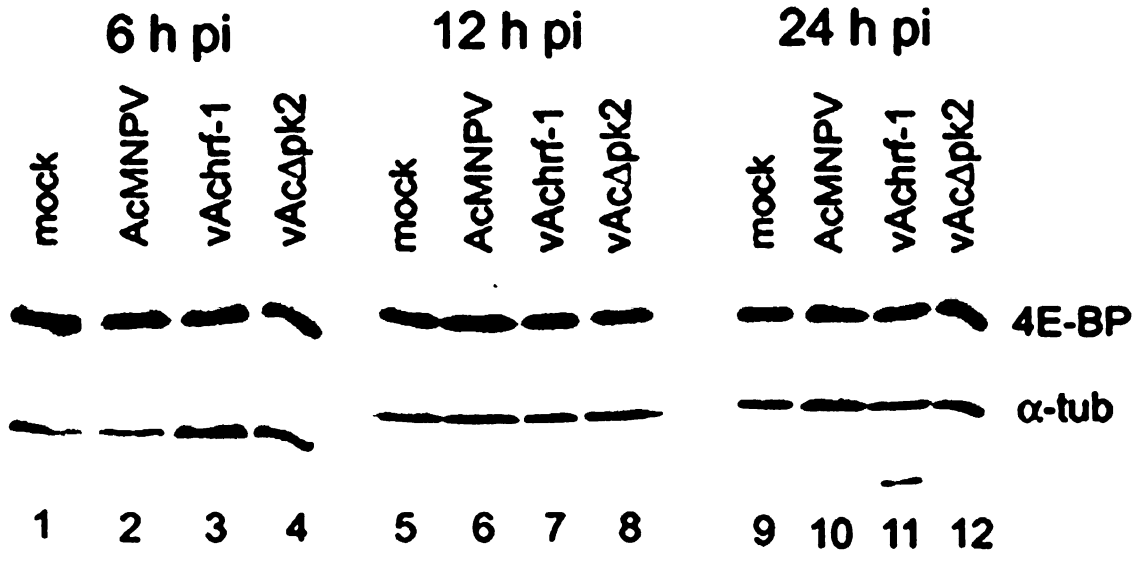



**Figure 27. 4E-BP is not up-regulated in response to AcMNPV-infection in Ld652Y cells.** Total cell lysates were prepared from Ld652Y cells that were mock-infected or (Caption continued from p. ) infected with AcMNPV, vAchrf-1 or vAcΔpk2. Lysates were collected at 12 h and 24 h post-infection. The same blot for each time point was stripped and reprobed sequentially with antibodies to Drosophila 4E-BP and α-tubulin.

**The lepidoteran eIF2α kinase, BeK, is activated in AcMNPV-infected Ld652Y cells.**

A novel lepidopteran eIF2α kinase, BeK, was identified in *Bombyx mori* and characterized as being activated in response to heat shock and osmotic stress in transformed *Drosophila* S2 cells but not in response to immune, endoplasmic reticulum or oxidative stress. The same group showed that PK2 was capable of reducing BeK activity suggesting that BeK may act in an anti-viral response (Prasad et al., 2003). Using western blots and antibodies raised against BeK (P. Brey), we screened infected Ld652Y cells for BeK induction. Western blots of infected cell lysates show expression of a 65 kDa protein that interacts with anti-BeK antibodies at 12 hours post-infection in AcMNPV-infected Ld652Y cells (Fig. 14), a time that correlates to eIF2α phosphorylation in AcMNPV-infected Ld652Y cells. Western blots produced from

mock-infected Ld652Y cells or those infected with vAchrf-1 from 3-24 hours post-infection are devoid of any antibody interaction when probed with anti-BeK antibodies (Fig. 14). An additional band of ~40 kDa is also seen in AcMNPV-infected Ld652Y cells at 12 h.p.i. Results from this western blot indicates that BeK is expressed at a time that correlates to eIF2α phosphorylation and global translation arrest in AcMNPV-infected Ld652Y cells.

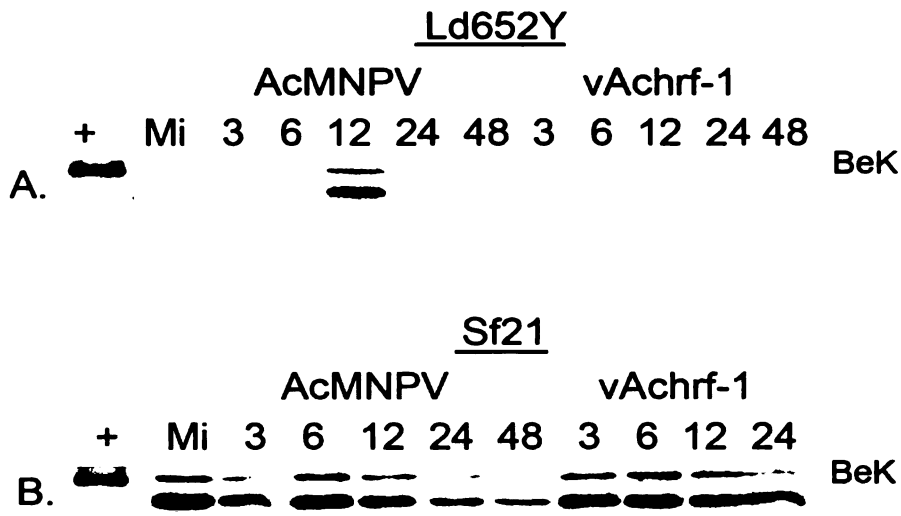



**Figure 28. The lepidopteran kinase, BeK, is expressed in AcMNPV-infected Ld652Y cells at a time that correlates to the onset of global translation arrest.** Total cell lysates were collected at 3, 6, 12, 24 and 48 hours post-infection, subjected to SDS-PAGE and immunoblotted with polyclonal antibodies to BeK.

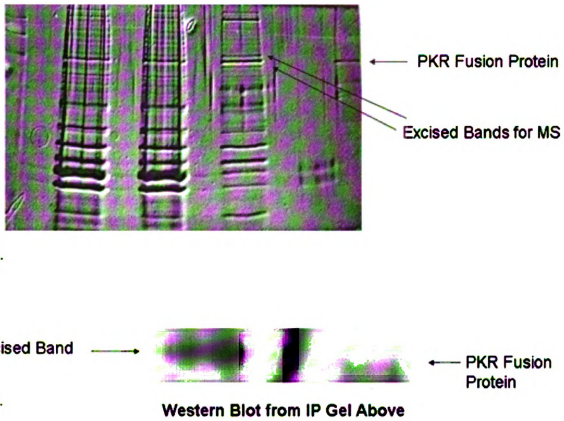



**Figure 29. Polyacrylamide gel of proteins immunoprecipitated with human PKR antibodies in vAchrf-1-infected Ld652Y cells.** A. Arrows indicate the bands that were excised for mass spectrometry analysis and co-migration of the PKR control protein. Infected cell lysates were immunoprecipitated against sepharose bound antibody overnight, then boiled and separated using SDS-PAGE, gel was stained with Coomassie blue stain and destained for 5 hours. B. One lane from the IP gel was excised and transferred to western blot along with the PKR control protein lane. Western blots were incubated with anti-PKR antibodies; HRP-linked, anti-rabbit secondary antibodies and bands were detected using ECL detection kit.

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