CASPASES AND CASPASE REGULATORS IN LEPIDOPTERA AND DIPTERA

by

WILLIAM BARTON BRYANT

B.S., Middle Tennessee State University, 2002

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

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Abstract

Apoptosis is an extremely conserved process among metazoans. This dissertation will describe apoptotic regulation in two orders of insects, *Lepidoptera* and *Diptera*. In the lepidopteran host *Trichoplusia ni*, we describe phenotypes of infection with the baculovirus *Ac*MNPV lacking the caspase inhibitor gene P35. In the lepidopteran host *Spodoptera frugiperda*, infection with this mutant virus results in apoptosis, which dramatically hinders spread of the virus in the host. In *T. ni*, however, infection with this mutant virus is similar to wild-type with normal spread, but the end result of liquefaction does not occur. Experiments indicated that infection of *T. ni* cells with the P35 mutant virus (P35Δ) resulted in caspase activation, and the P35Δ virus lacked the ability to inhibit these active caspases. With the P35Δ virus a slower entry phenotype was observed, but when the P35Δ virus was grown in the presence of a caspase inhibitor the entry phenotype was rescued. This indicated that caspases have detrimental effects on budded virions, and illustrated that P35 is needed to make robust virions. With regards to *Diptera*, apoptosis-regulatory genes were annotated in the yellow fever mosquito, *Aedes aegypti*. The genes annotated included multiple caspases and caspase regulators. Phylogenetic relationships were determined among the caspases from *Anopheles gambiae*, *Ae. aegypti* and *Drosophila melanogaster*, expression patterns were determined for all the annotated genes in *Ae. aegypti*, and one of the genes, an IAP antagonist named IMP, was functionally characterized. Further characterization of the phylogenetic relationships of caspases from fifteen dipteran species was performed by obtaining gene

models for caspases of recently sequenced genomes for twelve *Drosophila* species and three mosquito species. Furthermore, several *Drosophila* and mosquito species were found to contain caspase genes with substitutions in critical active site residues. These genes were proposed to encode caspase-like decoy molecules. While these have been found in humans and nematodes, this is the first report for these molecules in insects. One of the caspase-like decoy molecules was found to increase the activity of its paralog caspase.

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Dedication

I dedicate this work to my daughter Elanor Grace Bryant. She is one of the main reasons I am doing a PhD to provide a better life for her. Also whenever I am down and depressed about work, her smile helps me get through my times of trouble.

CHAPTER 1 - Introduction

History

Apoptosis is a process of programmed cell death that is very conserved among metazoans. Characteristic hallmarks of apoptotic death include membrane blebbing, chromatin condensation, dismantling contents of the cell and packaging of the dead cell into apoptotic bodies, which are later engulfed by neighboring phagocytes. Other forms of cell death include necrosis and autophagy and are reviewed in (Edinger et al., 2004). Necrosis does not include any metabolic reactions and is thought of as a passive form of cell death in times of stress. Necrosis comes about from physical injury to the cell. Another form of cell death, autophagy, has also been shown to be involved in cell death, but autophagy is first thought of as a survival mechanism when nutrients are limited. The term autophagy means to literally "to eat oneself" and is a very conserved strategy across taxa. Autophagy results in the formation of a double membrane vacuole termed the autophagosome. This complex is used to recycle nutrients and recently has been shown to function in innate immunity by protecting against intracellular pathogens (reviewed in Delgado et al., 2009). Genes involved in this were initially found in studies of yeast and most of the genes are conserved in multi-cellular organisms (Melendez et al., 2008). However, mammalian studies link autophagy to apoptosis. One study in particular illustrated that when particular autophagy genes were knocked-down it was found that these genes were needed for caspase-mediated death (Yu et al., 2004). Other studies have illustrated certain mammalian apoptotic regulators to cross-talk between autophagy and apoptosis (reviewed in Levine et al., 2008). While it is currently thought that necrosis is

not associated with apoptosis, studies on autophagy are suggesting interactions between apoptosis and autophagic pathways.

The term apoptosis came from pathologists describing certain tissue sections. Descriptions from these tissue sections coined the term ‘apoptosis’ which literally means falling of leaves from trees (Kerr et al., 1972). While these observations were reported over the years it was not known what genes were responsible for apoptosis until work done in *Caenorhabditis elegans.* Work done in the lab of H. Robert Horvitz illustrated the *ced* (cell death abnormal) genes as part of the main players in the basic mechanics of programmed cell death (Ellis et al., 1986). Work done with *ced-3* and *ced-4* illustrated that these genes are clearly responsible for cell death in a cell-autonomous manner (Yuan et al., 1990). In *Drosophila melanogaster* multiple studies have illustrated multiple other proteins that are involved in the basic mechanics of cell death. The historic finding of a gene called *reaper* in the locus H99 really opened the door to how cell death is initiated in development in the fruit fly (White et al., 1994). Another *Drosophila* gene called *thread,* was found in a suppressor screen to *reaper*; which suggested these proteins interact genetically (Hay et al., 1995). These three major studies (*ced-3-4, reaper* and *thread*), ranging from nematodes to fruit flies, launched the basic mechanics of apoptosis and illuminated the core of the apoptotic pathway (elaborated on more below).

Core apoptotic pathway

The core pathway of apoptosis consists of certain cysteine proteases called caspases and proteins that regulate these enzymes. These proteases, once activated, are responsible for cleaving multiple targets in the cell and these enzymes have to be able to cleave their substrates for proper dismantling of the cell. Proper regulation of caspases is

what makes up the core apoptotic pathway. These core regulatory proteins are: inhibitor of apoptosis proteins (IAP, *thread* or DIAP1; bind and inhibit active caspases), IAP antagonists (or RHG proteins; bind and inhibit IAPs) and oligomer proteins (Apaf-1 or Ark; bind initiator caspases to initiate the apoptotic pathway). Obviously multiple other proteins (and ones that should not be ruled out) include genes in the Bcl-2 (B-cell lymphoma) family. In mammals, proteins in this family have been shown to be both pro and anti-apoptotic. In *Drosophila*, the literature on these proteins is contradictory and a real clear role has not been resolved for these proteins. However in *C. elegans* there is a clear role for a Bcl-2 like protein called CED-9. In addition to the confusion of the role of *Drosophila* Bcl-2 genes in the regulation of caspases, *C. elegans* does not have a clear functional ortholog for the IAP gene. There are IAP genes in *C. elegans*, but not ones involved in regulating caspases. Even though IAP genes have been found from *Drosophila* to mammals to have clear roles in regulating apoptosis, the regulation of caspases from worms to fruit flies is a little different. Even though there is this difference in caspase regulation among model organisms, the genes involved in this regulation, for the most part, are widely conserved (reviewed in Hay et al., 2006).

In dealing with caspase regulation one needs to understand the two types of caspases found in multiple organisms. *C. elegans* has only one caspase (CED-3) that is involved in the core apoptotic pathway, but in other organisms there are two types of caspases called initiator and effector. *ced-3* is thought to play dual roles of both initiator and effector. In other systems with both types of caspases, they are initially expressed as zymogens. These zymogens have to be cleaved in order to be active enzymes. Initiator caspases are at the beginning of the apoptotic pathway and activation occurs by formation

of an apoptosome (described below). This changes the initiator caspase from an inactive zymogen to an active enzyme. Once initiator caspases are activated by the apoptosome, they cleave and activate effector caspases. Once the effector caspases are activated, they go on to dismantle the cell by cleaving certain cellular substrates. The identities of some of these cellular substrates are known, but many remain to be identified. More details about caspase mechanisms can be found in the **Dipteran** section and in Chapter 4.

In order for proper formation of an apoptosome an adaptor protein is needed. These adaptor molecules in different organisms are known by different names; in mammals it is called Apaf-1 (apoptosome activating factor), in *C. elegans* it is *ced-4*, and in *Drosophila* it is Ark (apaf-1 related killer) (Bao et al., 2007). This adaptor molecule in combination with the initiator caspase forms an apoptosome by oligomerization and in doing so this activates the catalytically inactive zymogen to an active caspase. There is, however, a lot of debate of other needed players in formation of the apoptosome in different organisms. In mammals it has been shown that in addition to Apaf-1 and caspase-9 (an initiator caspase), there is involvement of cytochrome c and ATP, while in *Drosophila*, studies suggest the apoptosome in flies only to include Ark and Dronc (an initiator caspase). *Drosophila* is similar to *C. elegans*, because studies suggest that only *ced-4* and *ced-3* are needed for the nematode apoptosome. Regardless of which model organism one studies, the need for the apoptosome formation is vital to initiation of the apoptotic pathway (Bao et al., 2007).

Once activated, caspases are regulated by IAPs. As stated above *C. elegans* does not employ IAP proteins to regulate active caspases, but *Drosophila* and mammals do use these proteins (mammals actually employ both Bcl-2 family proteins and IAPs to regulate

caspases). IAP genes were first found in the insect virus Cydia pomonetta granulosis virus (CpGV) (Crook et al., 1993) and later a cellular homolog (*thread* or *DIAP1*) was found in *Drosophila* (Hay et al., 1995) (explained in more detail in the **Dipteran** section). IAP proteins bind to active caspases and inhibit them from cleaving substrates, but in order for IAPs to be able to inhibit active caspases they must be activated by cleavage to expose the caspase-binding motif. In addition to directly binding caspases, IAPs also regulate caspases by ubiquitinating the caspases and tagging them for destruction through the proteosome. In addition to DIAP1 tagging active caspases for destruction, it has also been shown to ubiquitinate itself, suggestive of pro-apoptotic regulation of DIAP1, because down-regulation of DIAP1 leads to cell death. DIAP1 can also ubiquitinate other substrates such as RHG-family proteins (reviewed in Hay et al., 2006). Work has also recently illustrated that DIAP1 can inactivate effector caspases through ubiquitination (Ditzel et al., 2008). A recent deubiquitinase has also shown to have a role in regulating death (Ribaya et al., 2009). But besides the relatively small number of known substrates that DIAP1 does ubiquitinate to regulate cell death, many more cellular substrates await further identification. This is yet another poorly studied aspect of caspase regulation, which deserves more attention.

IAP proteins are regulated by proteins called IAP antagonists (or RHG genes). The first RHG gene was found in *Drosophila* in an embryogenesis screen and was called *reaper* (White et al., 1994). Since then only a handful of these proteins have been found. Their names include reaper, HID, grim, sickle and Jafrac-2. Reaper, HID and grim were the first ones found, which termed the name for this family of proteins, RHG genes. Reaper, HID, grim and skl are located near each other in a region defined by a

chromosomal deletion, called H99, in *Drosophila melanogaster* (White et al., 1994; Srinivasula et al., 2002). RHG genes have been difficulte to identify in other organisms due to the extremely low similarity between these types of proteins even within the same organism. RHG genes only share one or two small regions that are responsible for initiating apoptosis. These proteins induce apoptosis by binding and antagonizing IAP proteins from binding activated caspases (more biochemical detail in **Dipteran** section). Once IAP antagonists bind IAPs and liberate them from active caspases, the caspases are then able to dismantle the cell by cleaving cellular substrates. Just like caspases and IAPs, RHG proteins have to be cleaved to be activated. After removal of the N-terminal methionine (presumably by the enzyme methionine aminopeptidase) they are able to release IAPs from active caspases and this results in death. Like IAP proteins, which have the ability to ubiquitinate proteins, RHG proteins have been shown to ubiquitinate DIAP1, however unlike DIAP1 binding and ubiquitinating active caspases and itself, RHG proteins are thought to ubiquitinate DIAP1 in *trans*, probably through *SkpA*, *morgue* or *fat facets* (Hays et al., 2002; Ryoo et al., 2002; Wing et al., 2002). Just like the ubiquitin targets of DIAP1, this aspect of caspase regulation also deserves more attention.

What an insect virus has taught us about apoptosis

While all the basic mechanics of apoptosis have mostly been discovered in *C. elegans* and *D. melanogaster*, studies done in lepidopteran insects pale in comparison. However this does not rule lepidopteran biology out of the spotlight on apoptotic regulation. Studies done on the viral protein P35 (from *Ac*MNPV) have aided tremendously in better understanding of apoptosis in *lepidoptera* and even more so in *Drosophila*. P35 is commonly used in *Drosophila* studies to ask if a process is caspase-

dependent or caspase-independent. P35 can bind and inhibit caspases directly as a suicide substrate inhibitor and can inhibit a range of caspases from nematodes to mammals (Means et al., 2008). The identification of the function of this viral protein illustrated that viruses come into contact with apoptosis as an innate immune response in nature (Clem et al., 1991). In a screen to find more apoptotic regulators, IAP (inhibitor of apoptosis) proteins were found (Crook et al., 1993). Orthologs of P35 have been found in other baculoviruses and recently an ortholog was found outside of the baculovirus family in an entomopox virus (Means et al., 2007), but to date no clear cellular homologs of P35 have been found.

Apoptosis in the Insect Order *Lepidoptera*

The study of apoptosis in *Lepidoptera* is limited when compared to *Drosophila*. There are only a few known caspases and IAPs in the order *Lepidoptera*. Lepidopteran insects are not known for studies of apoptosis at the cellular level alone. Studies of significance stem from observing particular host:virus combinations. One combination in particular is *Ac*MNPV and *Spodoptera frugiperda*. This combination was one of the first to illustrate that apoptosis can be an innate immune response to virus infection. However the fact that apoptosis occurs upon virus infection was not known until studies done with an *Ac*MNPV mutant called "the annihilator". This mutant virus was found to be lacking the gene called P35 (Clem et al., 1991). Another viral inhibitor of apoptosis was found in a granulovirus (a different type of baculovirus) in a screen for genes that rescued the death phenotype of *Ac*MNPV P35Δ infection in *Spodoptera* cells (Crook et al., 1993).

The gene, termed IAP, was later found to have homologs in many eukaryotes and has been proven to be an important player in regulating caspases in multiple systems ranging from fruit flies to mammals (reviewed in Vaux et al., 2005).

IAP

The only lepidopteran IAP genes that have been characterized are found in *S. frugiperda*, *Spodoptera littoralis*, *T. ni*, and *Bombyx mori*. The IAP gene from *S. frugiperda* was found to be essential for viability because once knocked down by RNAi, the cells spontaneously die by apoptosis (Muro et al., 2002). This phenotype was also shared with S2 cells from *D. melanogaster* illustrating conservation of apoptotic mechanims from *Diptera* to *Lepidoptera* (Muro et al., 2002). The *S. littoralis* IAP transcript was shown to correlate with midgut apoptosis *in vivo*. When midgut death was occurring due to metamorphosis of larvae to pupae, lower amounts of the SlIAP transcript were found. The authors suggested this decrease in SlIAP transcript correlated to the increase of apoptosis in the midgut (Vilaplana et al., 2007). This phenotype mimics the cellular studies of *Sf*-IAP-1 that when depleted, apoptosis occurs. IAP genes from *T. ni* (Liao et al., 2002) and *B. mori* (Huang et al., 2001) have been shown to inhibit human caspases or inhibit insults that activate caspases. BmIAP1 has also recently been shown to interact with the first lepidopteran IAP-antagonist (Bryant et al., 2009) called Ibm-1. This work illustrates the conservation of IAP and IAP antagonist interactions in the insect order *Lepidotera* (Bryant et al., 2009).

Caspases

Only effector caspases from *Lepidoptera* have been functionally characterized. These include caspases from *S. littoralis*, *S. frugiperda*, and *T. ni*. Studies on both

effector caspases from *S. littoralis* and *S. frugiperda* have illustrated conserved cleavage sites for activation, which are TETD*G for cleavage site one and DEGD*A for the second cleavage site (* indicates where cleavage occurs in the peptide sequence) (Liu et al., 2005; Liu et al., 2006). The genome for *B. mori* has been annotated and both initiator and effector caspases were found (Tanaka et al., 2008). However in this report the researchers claim a clear Dronc ortholog, but phylogenetic analysis does not come to the same conclusion. There seems to be only a Dredd ortholog and multiple effector caspases, but no clear Dronc ortholog (data not shown). Work has also suggested that *S. frugiperda* has an initiator caspase. When IETD-fmk (an initiator caspase inhibitor) was added to cells this inhibited the processing of the effector caspase *Sf*-caspase-1 (Manji et al., 2001). Other work has shown that a homolog to P35 (called P49) can also inhibit this caspase (Du et al., 1999), but the identity of this elusive initiator caspase (termed *Sf*-caspase-X) in *S. frugiperda* has still not been found. One claim to fame for lepidopteran studies of apoptosis is the solving the structure of the first invertebrate effector caspase, which was from *S. frugiperda*. Due to this achievement other crystal structures for *D. melanogaster* caspases soon followed. The solved crystal structure of *Sf*-caspase-1 illustrated that *Sf*-caspase-1 has similarities in folding when compared to human caspases 1, 3, 7, 8, and 9. The only difference was the N-terminus of the large subunit was oriented differently than the N-terminus of the large subunit of the human caspases. The authors suggested this might have implications for differences in insect caspase regulation or activation (Forsyth et al., 2004).

*Ac*MNPV P35

P35 from *Ac*MNPV was initially characterized at the transcriptional level and found to be an early gene in virus infection in SF-21 (*S. frugiperda*) cells (Friesen et al., 1987). It was not until careful characterization of a mutant *Ac*MNPV called the "annihilator", which caused apoptosis in SF-21 cells, that the role of P35 in virus infection was found. The mutation in the annihilator mutant was mapped to a region in the viral genome that contained the entire *p35* gene and part of the neighboring *p94* gene. A 754-bp deletion in the *p35* gene resulted in truncation of this gene. To confirm the annihilator phenotype was due to this truncation at the *p35* locus and not another mutation somewhere else in the viral genome, another *Ac*MNPV was constructed where P35 was inactivated via insertion of the *E. coli* lacZ gene in the *p35* locus. This virus proved to have identical phenotypes as the annihilator virus, which included membrane blebbing and DNA laddering (Clem et al., 1991).

Follow up studies with this host:virus combination illustrated that cell death led to other phenotypes, namely reduced replication, reduced spread of infection in the larvae, and lower amounts of gene expression of late and very late genes (Clem et al., 1993; Hershberger et al., 1994). Infection also resulted in extremely lower amounts of virus replication (3-log difference) and a higher dose of mutant virus needed to kill larvae. While this phenotype was found with *S. frugiperda*, mutant *Ac*MNPVΔP35 infection in *T. ni* resulted in no apoptosis, no difference in replication, similar levels of late and very late gene expression, and very similar dose needed to kill larvae when compared to wild type. The only phenotype that the two different infected hosts shared was a lack of liquefaction, which is the final step in baculovirus infection (Clem et al., 1993).

*Ac*MNPV infection normally results in liquefying the host, a process called liquefaction, which enables the virus contents to be dispersed to the environment to other unfortunate larvae. Viral genes shown to be involved in this process are the viral genes v-cath and v-chitinase (Hawtin et al., 1997).

P35 has been shown to inhibit apoptosis in multiple systems ranging from nematodes to mammals. P35 was shown to inhibit the human protease ICE (Bump et al., 1995), which was actually the first caspase isolated. This conservation of apoptotic regulation in the different systems led to utilizing P35 in multiple other systems, which are outlined below. In *C. elegans*, *p35* was shown to rescue a *ced-9* mutant (Sugimoto et al., 1994). *ced-9* is a homolog to the mammalian protein *bcl-2*. The fact that *p35* could substitute for *ced-9* suggested that the death induced by virus infection must be a conserved mechanism compared to death of cells in development. In *Drosophila*, P35 has also been shown to rescue the rough eye phenotype of *reaper* when expressed in the fly eye. Normally when the gene *reaper* is over-expressed with an eye-specific promoter, death occurs in the eye due to the protein reaper activating caspases resulting in the rough eye phenotype. However when the protein P35 is co-expressed with reaper, this eye death phenotype is rescued due to the ability of P35 to inhibit the activated caspases (Hay et al., 1995; Wing et al., 2001). P35 has also been expressed in tomato (Lincoln et al., 2002) and tobacco plants (Wang et al., 2008). This overexpression of P35 led to tolerance to harsh treatments of H_20_2 and methanol and also delayed leaf senescence when starved in the dark, and phenotypes of high NaCl tolerance in tobacoo plants. In tomato plants, P35 over-expression leads to toxin and pathogen resistance. This illustration of the ability of

P35 to inhibit death in multiple systems, even plants, illustrates the wonderful conservation of apoptotic regulation throughout evolution.

Even though P35 has been shown to work in systems as diverse as nematodes and plants, there are studies that are ambiguous as to what role P35 has in other systems. In one system, *Ac*MNPV infection of *S. littoralis* cells results in an apoptotic abortion of infection. Why is *Ac*MNPV not able to infect this Lepidopteran species while it can infect other Lepidoptera like *T. ni*? Since *Ac*MNPV was illustrated to utilize P35 to inhibit apoptosis this is where researchers started. *S. littoralis* cells infected with *Ac*MNPV exhibited poor expression of P35 when compared to *S. frugiperda* cells infected with *Ac*MNPV. To circumvent this poor expression phenotype, a recombinant virus that overexpresses P35 was made. While infection with this P35 overexpression virus was able to inhibit death induced by *Ac*MNPV infection and actinomycinD-induced apoptosis, it did not have significant difference in replication when compared to wild type virus. The group suggested that in this system, apoptosis was not the only barrier for *Ac*MNPV infection in the nonpermissive host *S. littoralis* (Gershburg et al., 1997). Later research showed that reduced expression of the viral protein IE-0 made *S. littoralis* cells more susceptible to *Ac*MNPV infection, and it was suggested that IE-0 played a role in the regulation of infection (Lu et al., 2003). Another interesting phenomenon is *Ac*MNPV infection in another nonpermissive host the gypsy moth, *Lymantria dispar*. This host:virus combination leads to global translation arrest, however when P35 is absent there is no translation arrest. However when other apoptosis inhibiting proteins are used (Opiap, Cpiap, and P49) in the *p35* deleted virus background, global translation arrest is restored, but when infection is supplemented with peptide caspase inhibitors the

phenotype is not rescued. The authors suggested that translation arrest in *Ac*MNPV-infected Ld652Y cells is due to some other function of P35 and IAP besides inhibiting apoptosis (Thiem et al., 2004). So why do IAP and P35 genes lead to translation arrest while the caspase inhibitors did not rescue the translation arrest phenotype? If the only function for these proteins are to inhibit caspases then the chemical caspase inhibitors should have rescued the phenotype, but they did not. These virus:host combinations of *Ac*MNPV-*S. littoralis* and *Ac*MNPV-*L. dispar* infection resulsts are difficult to interpret. At best it suggests that different hosts require different factors besides inhibiting apoptosis for successful infection with *Ac*MNPV.

Apoptosis in the Insect Order *Diptera*

Studies of apoptosis in insects are monopolized by studies done in the model organism *D. melanogaster*. This section will go over the core pathway, gene by gene where most of the studies will come from *D. melanogaster* with a few studies from mosquitoes. Prior to the work reported in Chapter 3, not much was known about apoptosis in mosquitoes.

With the finding of reaper, DIAP1 (also called thread) and caspases, the pathway was outlined in two different systems (yeast and flies) and the pathway still holds ground today. One system involved experiments done in yeast because yeast are not thought to have these types of genes present in their genome (Wang et al., 1999) while the other system was done in flies (Goyal et al., 2000). This latter system utilized overexpression of genes in the fly eye with eye-specific promoters. Phenotypes of the eye, called the

rough eye phenotype driven by over expression of genes that initiate death, were used to determine the order of the pathway by epistatic analysis. Using both of these systems the core apoptotic pathway was found. The RHG proteins (reaper, HID and grim) inhibit DIAP1, DIAP1 inhibits caspases, and active caspases result in death of the cell. This type of regulation of controlling caspases is outlined in Figure 1.1A, which illustrates the core apoptotic pathway in insects. Also in Figure 1.1, for each gene, the species are shown which were known to have a copy of that gene before these studies were begun in 2004. Due to the explosive number of sequenced and annotated genomes in other insects, these genes have now been found in multiple other species, including insect vectors for disease. Even the IAP antagonist genes, which are extremely hard to identify due to low sequence similarity, were found in multiple species due to work by Lei Zhou (Zhou et al., 2005a; Bryant et al., 2009). This expansion of knowledge of the insect core apoptotic pathway in other insects besides *D. melanogaster* is illustrated in Figure 1.1B.

ARK

Ark is the adaptor molecule that is needed to activate the apoptotic pathway. In 1999, three independent studies were done that illustrated the role of Ark in apoptosis (Kanuka et al., 1999; Rodriguez et al., 1999; Zhou et al., 1999). Since the finding of this gene in *Drosophila* was done in three different labs, the name for this gene differs (DARK, HAC-1 and Dapaf-1). For the scope of this review we will use Ark, which is the name used in Flybase (http://flybase.org/). Ark possess three domains, the CARD (caspase recruitment domain), NB-ARC (nucleotide-binding adaptor shared by Apaf-1, R-gene products and CED-4 domain) and WDR (WD-40 repeats). Over-expression of Ark in SL2 (*D. melanogaster*) cells induced slight death. The same study also illustrated

that Ark interacts with Dredd, loss-of-function mutants resulted in melanized tumors, and Ark was needed for reaper, grim and hid-induced death in the fly eye (Rodriguez et al., 1999). Two follow-up studies illustrated that Ark mutants resulted in reduction of apoptosis in embryos, extra neurons in embryos, induction of Ark transcripts due to irradiation (Zhou et al., 1999), two isoforms of Ark, and finally larger organs in Ark mutants due to lack of appropriate death (Kanuka et al., 1999). These studies found the Apaf-1 homolog in insects and illustrated that this oligomerizing molecule is needed to induce death. Ark was later found to have no role in midgut death in larval to pupal development, while Ark is needed for cell death of embryos, larval tissues, removal of larval salivary glands and radiation-induced death (Mills et al., 2006). Earlier work suggested the death of larval midguts is due to autophagic processes instead of apoptotic events (Lee et al., 2002). The latest addition to Ark biology is that Ark can lower the protein levels of Dronc and vice versa. This suppression done by both proteins depends on the catalytic activity of Dronc and a cleavage site in Ark. Even though Dronc can lower Ark levels this does not result in death (Shapiro et al., 2008).

DIAP1 (and other IAPs)

DIAP1 and DIAP2 were found in an enhancer screen of reaper-induced death of the fly eye. Both of the IAPs were able to block normal cell death, which resulted in extra cells in the ommatidia. They were also shown to block cell death induced by reaper or HID in the fly eye (Hay et al., 1995). DIAP1 is vital for viable cells. This was illustrated in cell culture by knocking down transcripts by RNAi, which resulted in spontaneous cell-death (Muro et al., 2002) and shown in the fly eye (Wang et al., 1999). This phenotype was also conserved in the lepidopteran *S. frugiperda* (as discussed in the

Lepidopteran section) (Muro et al., 2002). This phenotype could be inhibited if Ark or Dronc transcripts were also knocked down, which correlates with the pathway illustrated in Figure 1.1 A and B.

IAP proteins usually contain two types of domains (exceptions to the rule are discussed later). They contain the baculovirus IAP repeats (BIRs) at the N-terminus of the protein and RING domains at the C-terminus. There are some IAP proteins which do not have RING domains (also discussed later). The RING domain in DIAP1 is essential for regulating cell death by acting as an E3 ubiquitin ligase and promoting ubiquitination of particular cellular substrates, which include ubiquitination of Dronc and itself. This was illustrated when a mutant of the DIAP1 RING domain inactivated the ubiquitin ligase activity, which enhanced the overexpression phenotype of reaper in the eye. Western blots for ubiquitin were also done on combinations of Dronc and DIAP1 to illustrate this point as well (Wilson et al., 2002).

DIAP1 has to be cleaved via caspases in order for the protein to have the ability to inhibit death efficiently. It has been shown that cleavage occurs at D20 and E205. For cleavage at D20 it is suggested by biochemical analysis that after cleavage at this site, the protein is able to bind DrICE (a *Drosophila* effector caspase). If cleavage does not occur then the BIR1 region is auto-inhibited and is unable to bind DrICE (Yan et al., 2004). For cleavage at E205, it is suggested that cleavage at this point occurs normally in non-apoptotic cells (Yan et al., 2004). Also mutants for both of these cleavage sites show an inability to successfully inhibit reaper-induced death (Muro et al., 2005). Another mechanism of regulation of DIAP1 is the N-end rule pathway (Ditzel et al., 2003). Work on this suggests that after cleavage, the protein is able to go thru N-end degradation and

that this type of regulation for this protein is essential for DIAP1 to inhibit death. A mutant of DIAP1, called Asn-DIAP1, represents an already cleaved DIAP1 that efficiently alleviated reaper-induced death and actually inhibited apoptosis better than wild-type DIAP1. This idea for DIAP1 being regulated by the N-end rule was further illustrated when the protein Arg-RNA protein transferase (*ate-1*) was knocked-down by RNAi. This caused an increase in DIAP1 protein levels. Further analysis was shown with heterozygous mutants for *ate-1* and *dm-ntan1* (both proteins thought to be involved in N-end degradation) that enhanced reaper-induced death in the fly eye, while mutants for these proteins suppressed death induced by HID (another IAP antagonist) (Ditzel et al., 2003). This suggests that HID and reaper act in different ways of inducing death, and it has been shown that these two proteins induce death by different mechanisms (elaborated on later in the **IAP antagonist** section) and that possibly the N-end rule does not apply to all types of death. This conclusion is also backed when the D20 and E205 mutants were analyzed for their ability to inhibit death induced by either reaper or UV. The phenotypes for the cleavage site mutants were more pronounced with reaper-induced death than UV-induced death (Muro et al., 2005). This illustrates that different death stimuli result in different pathways for death in *Drosophila*.

Other insect IAPs have also been shown to require cleavage before the first BIR1 domain in order to be active. A study analyzed IAP genes ranging from the honeybee *Apis mellifera* to lepidopteran insects *B. mori*, *T. ni, S. frugiperda* to *Diptera* including other species of *Drosophila* and the vectors for disease *Aedes aegypti, Aedes triseriatus, Aedes albopictus,* and *Anopheles gambiae* (Tenev et al., 2007). The study suggests all insect IAPs require 'priming' in order for proper function of inhibiting caspases. This

priming is cleavage at D20. While this is interesting, they failed to mention that *An. gambiae* has another IAP gene that is extremely similar to AgIAP3. Both IAPs from this mosquito have two BIRs and a RING domain, and phylogenetic analysis suggests a recent duplication within this insect, but it is not addressed whether the duplicated IAP gene in *An. gambiae* behaves in the same manner as the paralog.

Other IAP genes in *D. melanogaster* are DIAP2, dBRUCE and deterin. While DIAP1 monopolizes apoptotic regulation, it is thought that DIAP2 is involved in innate immunity. Silencing of DIAP2 results in the loss of the ability of *Drosophila* to induce *cecropin* and *Mmp1* transcripts, which are needed to mount an effective immune response against gram negative bacteria (Gesellchen et al., 2005). Another study illustrated mutants of DIAP2 lack the ability to induce *dipt*, *AttA*, *CecA1*, *Def, Dro,* and *Mtk* when challenged with gram negative bacteria. Due to the inability to induce these transcripts, the mutants died quicker than wild type when challenged with the gram negative bacteria *Erwinia carotovora* subsp. *carotovora* 15 (Leulier et al., 2006a). One study suggests that with higher amounts of DIAP2, there are higher immune responses, since DIAP2 promotes cytoplasmic cleavage and nuclear transfer of Relish (NF-κB homolog) and that the RING domain of DIAP2 was needed for these functions (Huh et al., 2007). This is similar to DIAP1 needing a functional RING domain to inhibit death (as discussed earlier). In addition to the role of DIAP2 in innate immunity, it has also been shown that DIAP2 mutants have increased caspase activity when irradiated, DIAP2 overexpression in the fly-eye suppresses RNAi of DIAP1 and DIAP2 interacts with DrICE (Ribeiro et al., 2007). The other two IAP genes, deterin and dBRUCE, are rarely studied. Deterin has been suggested to be an inibitor of death, but when over-expressed it only weakly inhibits

death induced by reaper (Jones et al., 2000). dBRUCE has been shown to inhibit cell death induced by reaper and grim but can not block activity of HID, Dronc or Debcl overexpression (Vernooy et al., 2002).

IAP genes from mosquitoes have been studied in *Ae. triseriatus/albopictus/aegypti*, and *Culex pipiens/tarsalis*. Studies with *Ae. triseriatus* has illustrated developmental expression and a lack of reaction to virus infection with La Crosse virus (Blitvich et al., 2002). Follow up studies illustrated a more interesting phenomenon that showed that these IAP1 genes have alternative 5' untranslated regions (UTRs). DIAP1 from *D. melanogaster* also has an alternatively spliced 5' UTRs (Beck et al., 2007b). Interestingly enough, phylogenetic analysis of these alternative 5' UTRs did not result in grouping among species. Instead the UTRs grouped in a species specific maner (data not shown). In *Ae. aegypti*, another 5' UTR in addition to the ones reported is present (data not shown). This extra 5' UTR was actually one of the isoforms but unspliced and the unspliced isoform was actually expressed at higher levels than the spliced form (data not shown). These alternative isoforms of IAP1 genes suggests yet another form of interesting regulation of IAP genes in *Diptera* and this phenomenon has yet to be analyzed. The different isoforms have different expression patterns in different tissues. One could easily envision that IAP1 might be needed more in certain tissues. This phenomenon deserves more attention.

Caspases

Caspases are the central executioners of apoptosis. The regulation of these enzymes is what makes up the core apoptotic pathway. Initially expressed as inactive zymogens, activation of these enzymes is a main focus of research. A recent review of

insect caspases outlines the major studies in *Drosophila* and other insects (Cooper et al., 2009). Here I will review the main effector and initiator caspases that have clear roles in apoptosis and briefly mention ones that do not, and also discuss non-apoptotic functions of particular caspases.

Dredd was the first caspase found in *D. melanogaster*. It was found that *dredd* mRNA was maternally loaded into oocytes and found in apoptotic nurse cells. Mutants of *dredd* were also shown to be able to suppress reaper and grim-induced death in the fly eye (Chen et al., 1998). Dredd was later found to be mostly involved in innate immunity, where it is required to mount an immune response when *Drosophila* is infected with gram-negative bacteria (Leulier et al., 2000). The identification of Dronc soon followed the discovery of Dredd with studies showing developmental expression in the fly, overexpression of Dronc causing death in NIH 3T3 fibroblasts and substrate preferences (VDVAD) (Dorstyn et al., 1999a). Soon studies of Dronc activation followed. Dronc was shown to cleave itself at a Glu residue (E352) and this was shown to be important for activation (Hawkins et al., 2000). Dronc is needed for reaper, HID and grim-induced death, because a dominant negative mutant of Dronc (where the cysteine is replaced with a serine) inhibited death in the fly eye when the RHG genes were overexpressed. Dronc was also found to induce death in the fly eye when overexpressed and was dose dependent. Interestingly, P35 was not able to rescue this death phenotype (Hawkins et al., 2000). This is contradictory to overexpression of Dronc in NIH 3T3 cells which led to death, but this death was inhibited by P35 (Dorstyn et al., 1999a). Besides cleavage at E352, cleavage also occurs at D135. It is proposed that cleavage at E352 is a result of autoprocessing, while cleavage at D135 is a result of cleavage from DrICE (Muro et al.,

2004). Processing at E352 was found to be essential for Dronc activity while cleavage at D135 had little effect. Further studies illustrated that an uncleavable Dronc zymogen acted in a dominant negative manner in the fly eye (Yan et al., 2006). Mutants for E352 were overexpressed in the fly eye along with reaper, HID or grim. These mutants inhibited death induced by the RHG genes. This same study also illustrated by gel filtration that the auto-cleaved forms resulted in a homodimer, while the uncleaved Dronc zymogen resulted in a monomer. A crystal structure was also solved for Dronc in its zymogen form, which illustrated an unproductive active site, suggestive of the fact that Dronc needs to be cleaved at E352 in order to homodimerize and be an active enzyme (Yan et al., 2006). While these studies seemed to seal the fate of Dronc activation, recent research suggests other mechanisms of activation. One study suggests that Dronc is activated by dimerization, instead of cleavage and this study also suggests that Dronc tolerates Asp or Glu residues but 'prefers' Asp residues (Snipas et al., 2008). Another study used purified recombinant proteins that had substitutions at E352, however these mutations did not abolish Dronc activation (Dorstyn et al., 2008). These last two papers suggest the jury is still out on the precise mechanism of activation for this important initiator caspase.

DrICE was found by degenerate PCR (Fraser et al., 1997a) and is now thought of as the main effector caspase in *Drosophila*. When overexpressed in *Drosophila* cells, the cells were sensitized to apoptotic stimuli like cyclohexamide. Also processing of this protein was found, in addition to determining that an N-terminal truncated version of DrICE resulted in death when overexpressed as compared to wild type (Fraser et al., 1997a). Genetic data places DrICE as the main effector caspase in apoptosis while the

other effector caspases play "supportive roles". This stems from studies done in the fly eye. Overexpression of HID in the eye results in the rough eye phenotype. When DrICE, decay or dcp-1 were knocked down by RNAi, DrICE rescued the phenotype more efficiently than dcp-1 or decay (Leulier et al., 2006b). Null mutants of the main effector caspase DrICE were shown to have defects in embryonic development, formation of adult eyes, arista, wings, and subtle defects in destruction of larval tissues. DrICE null mutants were also resistant to the stresses of irradiation and inhibition of protein synthesis (Muro et al., 2006). Other effector caspases do not have such drastic phenotypes. Dcp-1 has been suggested to be dispensable for cell death, but is thought to be required for stress-induced death in the ovary (Laundrie et al., 2003) suggestive of different modes of death in the ovary. When decay was isolated, functional studies were done in mammalian cells. The study also illustrated developmental expression patterns and substrate preferences of DEVD and VDVAD (Dorstyn et al., 1999b). Damm has been the subject of only one study which illustrates expression in development, substrate preference for VDVAD, a weak rough eye overexpression phenotype and very small amount of apoptotic cells when overexpressed in mammalian cells (Harvey et al., 2001).

Non-apoptotic roles have been suggested for caspases in *Drosophila*. It has been illustrated that induction of the cell death pathway can induce cell proliferation (Huh et al., 2004). This study was done in wing discs, and when HID was used to activate the cell death pathway in the presence of P35 (to inhibit effector caspases from dismantling the cell), there was proliferation in the wing disc as visualized by phosphohistone H3 staining. While HID overexpression in the presence of P35 induced proliferation, loss of DIAP1 in the presence of P35 was not sufficient to induce proliferation in the wing disc,

suggestive of different mechanisms of death. Dronc is also needed in this process, because in a Dronc mutant background, proliferation did not occur when HID was overexpressed in the wing disc. This stimulation of cell death signaling in the wing disc (with P35 to inhibit death) results in the activation of the mitogen wingless (Huh et al., 2004). DrICE has also been suggested to be involved in spermatid individualization, which is a nonapoptotic process. Spermatids that are going through development go through an atrophy-like process where the majority of the cytoplasm and organelles are eliminated as the developing spermatids elongate. The testes of DrICE deletion mutants were analyzed and a partial failure in individualization of the cysts was observed, suggestive of an involvement of DrICE in spermatid individualization (Muro et al., 2006). As suggested above, dcp-1 is involved in oogenesis, and other studies have also suggested that the initiator caspases Strica (also called Dream) and Dronc are involved in oogenesis. Null mutants of Strica or Dronc did not have an ovary defective phenotype, but double mutants for these initiator caspases resulted in defective mid-stage egg chambers that resemble phenotypes found with dcp-1 mutants (Baum et al., 2007). This study, along with the dcp-1 study, illustrate a novel pathway in the cell death of the ovary.

Of the mosquito caspases, only two caspases have been functionally characterized and they are *Ae. aegypti* Dronc and Dredd. The *Aedes* Dronc study illustrated transcript levels, substrate preferences of recombinant proteins, and induction of transcripts due to the hormone ecdysone (Cooper et al., 2007b). The *Aedes* Dredd study illustrated transcript expression, caspase substrate preference with recombinant proteins and increases in *Aedes* Dredd transcript due to UV insults (Cooper et al., 2007a). Orthologs of

these two caspases are found in other dipteran genomes with no duplications, suggestive of purifying selection for these genes in the insect order *Diptera* (discussed more in Chapter 4).

IAP antagonists

IAP antagonists were actually the first genes found in the core apoptotic pathway of Drosophila. The gene that was found first is called reaper. In H99 mutants, where reaper is found, virtually all programmed death that occurs in development was blocked. These embryos were also very resistant to x-irradiation and development-induced death (White et al., 1994). When reaper was analyzed by itself, it was found not to be required for most developmental death, however the central nervous system of reaper null flies was greatly hindered (Peterson et al., 2002). Grim and HID were discovered next in the same region H99 close to reaper and were also found to be important in embryogenesis (Grether et al., 1995; Chen et al., 1996). Soon after discovery of IAPs, interaction studies with IAPs and IAP antagonists were done. The Miller lab was the first to illustrate that IAPs interact with the IAP antagonists reaper (Vucic et al., 1997), HID and grim (Vucic et al., 1998) in lepidopteran cells. The studies also illustrated interaction between viral IAPs, Cp-IAP and Op-IAP, with IAP antagonists as well. Again the viral IAPs were the first IAPs found (Crook et al., 1993; Birnbaum et al., 1994). Structural analysis of DIAP1 and grim and HID peptides revealed that when the peptides of HID bind to DIAP1 fragments, there is an induced formation of an additional alpha-helix which aides in stabilizing the peptide to DIAP1. This study also illustrated that the first four amino acids of HID and grim (after cleavage of the methionine) bind the surface groove on BIR2 and the next three amino acids interacted but only through hydrophobic interactions (Wu et

al., 2001). IAP antagonists do not share much sequence similarity. The one motif they all share is the IAP binding motif (IBM), while the other motif is the GH3 motif, which is found in reaper and grim. IBM motifs are the main motif that these proteins use to induce death, while GH3 motifs have been shown to have apoptotic activity in the absence of the IBM (Claveria et al., 2002). Two other IAP antagonists from *Drosophila* have been suggested to be involved in death as well. Sickle (Skl) was illustrated to have an IBM but doesn't seem to have a GH3 motif. In overexpression studies in the fly eye, sickle synergized with grim to induce death. Cell culture studies illustrated that death by overexpression was dependent on the IBM and could be inhibited by P35 (Srinivasula et al., 2002). Another IAP antagonist, Jafrac2, is different from the other IAP antagonists. Jafrac2 is a thioredoxin peroxidase which normally resides in the endoplasmic reticulum, but once apoptosis is induced this protein is released into the cytosol where it can interact with DIAP1 and promote death (Tenev et al., 2002).

Of these IAP antagonists, reaper HID and grim contain have been studied the most. It seems these proteins induce death by both conserved and different mechanisms. HID, reaper and grim have been shown to cause degradation of DIAP1 protein. Reaper and HID were shown to cause DIAP1 degradation by stimulating ubiquitination and destruction through the proteosome (Holley et al., 2002; Yoo et al., 2002), while grim down-regulates DIAP1 levels but this did not depend on the ability of DIAP1 to function as a ubiquitin-protein ligase. Grim was suggested to degrade DIAP1 by inhibiting protein translation (Yoo et al., 2002). In addition to ubiquitination of DIAP1 in *trans*, reaper has also shown to decrease DIAP1 levels by inhibiting protein translation (Holley et al., 2002). In retaliation, it is shown that DIAP1 ubiquitinates these IAP antagonists as well.

This mechanism was shown to be dependent on their interaction because reaper without an IBM was not a substrate for this ubiquitin-mediated degradation. This study also illustrated Lys-deficient reaper was more stable than wildtype and induced death more efficiently. This correlates with the idea that DIAP1 ubiuquitinates reaper for destruction through the proteosome (Olson et al., 2003).

Biochemical analysis has aided tremendously in our understanding of how DIAP1 regulates caspases and how IAP antagonists regulate DIAP1. DIAP1 contains two BIRs and each inhibits different types of caspases. Studies illustrated that BIR1 from DIAP1 interacts with the main effector caspase DrICE (Yan et al., 2004), while BIR2 from DIAP1 interacts with Dronc (Chai et al., 2003). These studies also illustrated that the IAP antagonist HID could compete with either BIR1 and DrICE or BIR2 and Dronc binding and liberate these BIR regions from the caspases. In addition to HID, it has been shown that other IAP antagonists can compete with caspases for binding to DIAP1. Reaper and grim were shown to efficiently interfere with the combination DrICE and DIAP1. Other IAP antagonists were tested for their ability to bind the two different BIRs of DIAP1. BIR1 could bind reaper and grim but interactions with HID and skl were not as strong, while BIR2 could bind all IAP antagonists (reaper, grim, HID, skl, and Jafrac2) (Zachariou et al., 2003).

As discussed earlier, reaper and grim can inhibit protein translation. The mechanism behind this has been analyzed for reaper. Reaper has the ability to bind directly to the 40s ribosomal subunit which affects the late initiation events of translation (Colon-Ramos et al., 2006). This study illustrated reaper as the first known cellular ribosomal binding factor which allows selection of mRNAs with internal ribosomal entry

site (IRES) elements, leaving cap-dependent transcripts untranslated. This makes sense because in mammals it has been shown that translation ties in with apoptosis. Many cellular transcripts contain IRES elements in their 5' UTR (reviewed in (Holcik et al., 2005). Examples of genes involved in apoptosis in mammals with these IRES elements are XIAP and Apaf-1. In *Drosophila*, studies involving this type of regulation of apoptosis have only been done with IAP antagonists. The 5' UTRs of reaper, HID and grim have been demonstrated to have IRES capability, while the 5' UTR from skl does not posses this capability (Hernandez et al., 2004; Vazquez-Pianzola et al., 2007). Therefore, when reaper selects for cap-independent transcripts like itself, HID and grim, this would enhance the translation of these pro-apoptotic transcripts and decrease translation of genes with cap-dependent transcripts that would keep the cell alive. This is a very interesting phenomenon that deserves more attention and could tie in with the alternatively spliced 5' UTRs of DIAP1 as well.

Reaper, grim, HID and sickle orthologs have been found in other *Drosophila* species (Zhou, 2005). Interestingly it was found that there were clear orthologs for these genes in other species of the genus *Drosophila*. The sequence of the reaper IBM was found to be AVAF, while the IBM sequence is AIAY for grim, AVPF for HID and AIPF for skl. This conservation of IBM sequences is suggestive of ancestral genes instead of duplications of these genes within each *Drosophila* species (Zhou, 2005). Mosquitoes also have an IAP antagonist gene. Michelob_x (Mx) was the first IAP-antagonist found outside of *Drosophila* (Zhou et al., 2005a). This study found this gene in the mosquitoes *An. gambiae*, *Ae. aegypti*, and *Ae. albopictus*. It was illustrated that Mx could interact with DIAP1 and that it is up-regulated due to UV treatment. Unlike reaper, Mx without

an IBM did not have an ability to induce death, suggestive that Mx does not have a GH3 motif (Zhou et al., 2005a). Another mosquito IAP antagonist IMP (IAP antagonist Michelob_x-like Protein), was identified using Mx as a query and searching the *Ae. aegypti* genome and is characterized in Chapter 3. IMP was used as a query and an ortholog was found in the west nile virus vector *Culex p. quinquefasciatus*, which can be found at **http://cegg.unige.ch/Insecta/immunodb/**. However an IMP ortholog was not found in *An. gambiae*.

This dissertation will span studies from *Lepidoptera* to *Diptera*. With the lepidopteran story I focus on the interactions between mutant and wild type *Ac*MNPV with *T. ni* and determine that a caspase regulatory gene found in *Ac*MNPV is utilized to make progeny virions in the host *T. ni*. With the dipteran side of this dissertation, I illustrate the annotation and systematic analysis of apoptotic players in mosquitoes. I also annotate and systematically analyze caspases from fifteen different dipteran genomes. This latter analysis illuminated a facet of apoptosis that has yet to be found in insect studies of apoptosis. Studies on P35 have aided in better understanding of the apoptotic pathway in insects, and in Chapter 2, we illustrate for the first time that P35 is used to prevent caspases from causing damage to budded virions in the lepidopteran host *T. ni*. In *Diptera*, before 2004, apoptosis studies were dominated by studies in *Drosophila*, but after 2004 some medically relevant insect vector mosquito genomes were sequenced and annotated and the known apoptotic players have been found by basic bioinformatic methods. Chapter 3 addresses the annotation of the core apoptotic pathway genes (using known *D. melanogaster* genes as a model) in the yellow fever virus vector *Ae. aegypti*, and illustrates the expansion and retention of these genes. While clear orthology was

found for most initiator caspases, the orthology of effector caspases was difficult to assign due to apparent gene expansions and losses in the mosquitoes. We initially thought this might just be unique to mosquitoes until we took advantage of the eleven other *Drosophila* genomes that have been more recently sequenced. In Chapter 4 we analyzed caspase phylogeny using these genomes (along with the third mosquito *Culex p. quinsquefasciatus*). As in Chapter 3, we saw unclear orthology for particular caspases, even in certain *Drosophila* species. The best example of this was the Damm/Dream clade. This was seen more in the species that are farther away from *Drosophila melanogaster* phylogenetically. While there was unclear orthology for certain effector caspases, the best-studied initiator caspases (Dronc and Dredd) had clear 1:1 orthology in all the Diptera analyzed. Another observation that came from this analysis was the discovery of caspase-like decoy molecules. These are types of caspases that recently duplicated in the organism and have mutations in important catalytic residues responsible for caspase activity. In humans these types of duplicated caspases regulate the ancestral genes from which they were duplicated. Examples include caspase-1 and -8 where the duplicated versions of these genes regulate the activity of the original protein. This is the first time this has been illustrated in insects (elaborated more in Chapter 4). Thanks to the recent sequencing of a number of insect genomes, the wealth of knowledge from these genome analyses will lead to multiple doors to discovery of not only apoptotic regulation but multiple other pathways important in vector biology.

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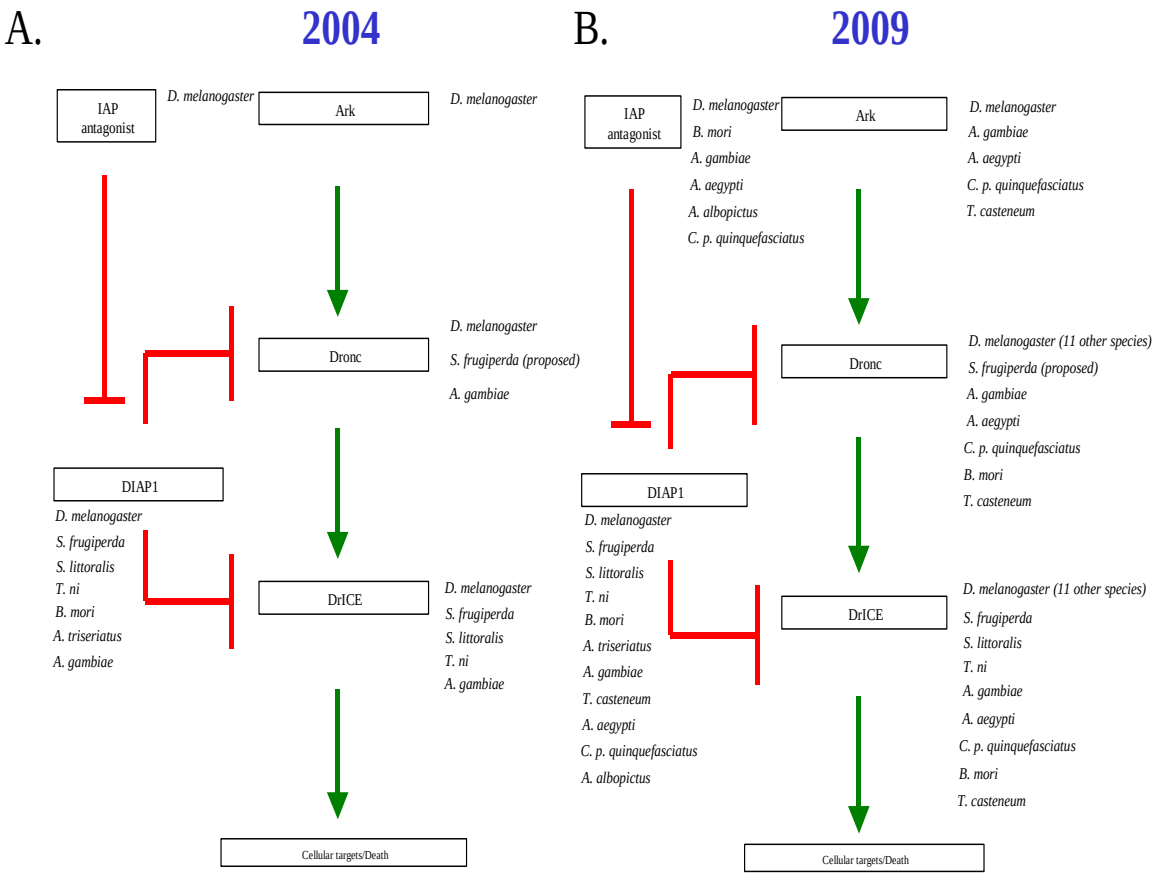

Figure 1.1 Insect core apoptotic pathway

CHAPTER 2 - The caspase inhibitor P35 is required for the production of robust baculovirus virions in *Trichoplusia ni* TN-368 cells

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Abstract

Apoptosis can protect lepidopteran insects against baculovirus infection by limiting viral replication. Baculoviruses counter this response by expressing anti-apoptotic proteins such as the caspase inhibitor P35, which is expressed by several baculoviruses, including *Autographa californica* M nucleopolyhedrovirus (AcMNPV). Mutants of AcMNPV that lack the *p35* gene induce apoptosis in *Spodoptera frugiperda* cells, and replication of these mutants is severely curtailed in *S. frugiperda* cell lines and larvae. However, cells from another lepidopteran species, *Trichoplusia ni*, do not undergo apoptosis when infected with AcMNPV mutants lacking *p35*, and *p35* mutant and wild type viruses replicate to equivalent levels in the *T. ni* cell line TN-368 and have equivalent infectivity in *T. ni* larvae by either oral or intrahemocoelic injection. This has led to the conclusion that *p35* is not required for AcMNPV replication in *T. ni*. However, in this study we found that stocks of *p35* mutant virus produced in TN-368 cells had defects in virion stability and infectivity. TN-368 cells infected with *p35* mutant AcMNPV exhibited caspase activity, despite a lack of apoptosis, and propagation of the mutant virus in the presence of a chemical caspase inhibitor restored the normal infection phenotype to the progeny virus. These results suggest that caspases can directly or indirectly damage baculovirus virions, and reveal a novel aspect of the role of apoptosis in anti-viral defense.

Introduction

The *Baculoviridae* are a family of large DNA viruses that are restricted to infection of arthropods, with most known baculoviruses infecting caterpillars, the larval stage of lepidopteran insects (for general reviews on baculoviruses, see (Okano et al., 2006; Friesen, 2007)). Similar to other types of large DNA viruses, baculoviruses encode many gene products which are involved in viral replication and in modulating host functions. For example, the genome of *Autographa californica* M nucleopolyhedrovirus (AcMNPV), the best-studied baculovirus to date, is approximately 134 kbp in length and is predicted to encode more than 150 genes (Ayers *et al.*, 1994). During their replication cycle baculoviruses produce two distinct types of virions, known as the budded and occlusion-derived virus forms (BV and ODV). Nucleocapsids, which are assembled in the nucleus, initially migrate to the cell periphery and bud through the plasma membrane, resulting in extracellular enveloped BV. These BV particles are infectious in most cell types, and are responsible for spread of infection within the infected insect. Later in infection, ODV are produced when the nucleocapsids are retained in the nucleus of the infected cell, acquire envelopes, and become embedded in large protein crystals known as occlusion bodies. These ODV-containing occlusion bodies are highly stable in the environment and are responsible for horizontal spread of the virus to other susceptible hosts by oral infection. Caterpillars become infected by feeding on occlusion bodies, which dissolve in the caterpillar midgut and release ODV that infect epithelial cells lining the midgut. The envelopes of BV and ODV differ in protein composition, such that ODV are specialized for infection of midgut epithelial cells, while BV can attach and enter almost any kind of cell, including mammalian cells.

The *p35* gene has been shown to be a host range-determining gene in AcMNPV due to its ability to inhibit apoptosis (Clem *et al.*, 1991). AcMNPV mutants lacking *p35* cause apoptosis during infection of *Spodoptera frugiperda* (the fall armyworm) cells and replicate poorly in cell lines from this species, such as the SF-21 cell line or its clonal derivative, Sf9. Such mutants also have defects in late gene expression in SF-21 cells (Hershberger et al., 1992; Clem et al., 1993). In addition, *p35* mutants exhibit greatly reduced infectivity, time to death, and occlusion body production in *S. frugiperda* larvae compared to wild type virus, and these defects correlate well with apoptosis (Clem et al., 1993; Clem et al., 1994). However, cells from *Trichoplusia ni* (the cabbage looper), such as TN-368 cells, are relatively resistant to a variety of apoptotic stimuli (Koval et al., 1988; Clem et al., 1991; Bonner et al., 1994), and mutant viruses lacking *p35* do not cause apoptosis in TN-368 cells. Furthermore, production of BV and occlusion bodies, as well as the overall timing of viral gene expression and DNA replication, have been reported to be similar to cells infected with wild type AcMNPV (Hershberger et al., 1992; Clem et al., 1993). Viruses lacking *p35* also exhibit normal infectivity, time to death, and occlusion body yields when infecting *T. ni* larvae by either the oral or intrahemocoelic routes. There is an observable difference, however, between the phenotypes of *p35* mutant and wild type AcMNPV in infected *T. ni* larvae; both *T. ni* and *S. frugiperda* larvae infected with *p35* mutant viruses do not undergo liquefaction, a process of host degradation often seen during the final stages of baculovirus infection in lepidopteran larvae (Clem et al., 1993; Clem et al., 1994). This observation suggests that P35 plays some type of role in infection of *T. ni*.

During apoptosis in organisms ranging from nematodes to mammals, a family of cysteine proteases called caspases become activated by proteolytic cleavage (reviewed in (Riedl et al., 2004)). Two main types of caspases exist, known as initiator and effector caspases. Following an apoptotic signal, initiator caspases become activated by autolytic cleavage. Activated initiator caspases cleave and activate effector caspases, which in turn cleave a number of cellular proteins, directly leading to apoptosis. AcMNPV infection causes caspase activation, but wild type AcMNPV is able to inhibit caspase activity, and thereby apoptosis, by virtue of expressing P35, which is a highly effective inhibitor of many caspases, especially effector caspases (Clem, 2005). AcMNPV mutants that lack *p35* cannot inhibit caspase activity in *S. frugiperda* cells, and high levels of caspase activity ensue, resulting in apoptosis of the infected cells. On the other hand, cells derived from *T. ni*, such as TN-368 cells, do not undergo apoptosis following infection by *p35* mutant AcMNPV.

Despite the fact that *p35* mutant AcMNPV does not cause apoptosis in *T. ni* cells, we found that BV stocks of this mutant virus propagated in TN-368 cells had defects in virion stability and infectivity. The infection phenotype was rescued when virus was grown in the presence of a caspase inhibitor, suggesting that caspase activity can have detrimental effects on virions and affect the next round of virus replication. These results reveal a novel consequence of the insect apoptotic response to baculovirus infection.

Methods

Viruses and cells

Construction of the viruses vHSGFP/P35del (referred to here as P35Δ) and vP35delRev (referred to here as Rev) was described previously (Clarke et al., 2003). Both viruses express eGFP from a *Drosophila* hsp70 promoter inserted near the polyhedrin locus, and Rev was constructed from P35Δ by inserting *p35* back into its native locus. Viruses were propagated in TN-368 cells, and virus titers were determined in TN-368 cells by end-point dilution assay using eGFP as a marker for infected cells (O'Reilly *et al.*, 1992). Freshly titered stocks were used in all experiments. In experiments where caspase inhibitor was used, 100μM zVAD-fmk (MP Biomedicals) was added to the cells at the time of infection and was present throughout infection. TN-368 and SF-21 cells were cultured in TC-100 medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals). To induce apoptosis in SF-21 cells, the cells were exposed to UV light by placing the culture dish on a transilluminator for 5 min.

Caspase activity assay

Caspase activity was determined as previously described (Muro *et al.*, 2004) by pre-incubating lysate with 40 μM *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (Ac-DEVD-afc, MP Biomedicals) for 15 min at 37°C, and then measurements (excitation 405 nm, emission 535 nm) were obtained at 15 min intervals to determine enzyme kinetics. All enzyme activity assays were done in triplicate and the results are presented as mean ± SE.

Cell cycle analysis

Cell cycle analysis was determined by flow cytometry using a FACSCalibur cytometer (Becton Dickinson) following a modified protocol (Braunagel et al., 1998; Karpf et al., 1998). Infections were done with 5 x 10^5 cells at an MOI of 5 pfu/cell. At designated times (24 and 48 hpi) cells were harvested and washed twice with PBS by centrifuging at 1000 rpm in a microcentrifuge for 5 min. Cells were resuspended and fixed in 70% ethanol and incubated at -20°C overnight. The next day the cells were washed in PBS and then resuspended in DNA stain buffer (0.05 mg/ml RNase A, 0.1 % Triton X-100, 50 μg/ml propidium iodide (Invitrogen), 0.1 mM EDTA pH 7.5, with PBS as diluent). Cells were incubated in this buffer for at least 30 min before analysis. Ten thousand events were collected for each data point and data was obtained on the slow cycle of the cytometer and did not exceed 150 events/second. Doublets were excluded from analysis by analyzing FL-2A (area) versus FL-2W (width), and the cell cycle was determined from this gated population of cells. AcMNPV-infected cells are arrested at the G2/M phase and also increase in area, which can be observed by an increase in FL-2A. The percentage of cells in the G2/M phase were plotted as mean ± SE.

Endosome exit assay

Viral binding and entry kinetics were determined by inhibiting endosome acidification at designated time points following a previously published protocol (Hefferon *et al.*, 1999). Briefly, 5 x 10^5 cells were plated overnight, and on the following day the cells and virus were incubated separately at 4°C for 45 min. Infections were then done at an MOI of 5 pfu/cell by incubating the chilled virus and cells together for 1 h at 4°C. After this pre-binding step, unbound virus was removed and warm (37^{o}C) media

was added to the cells and the cells were immediately placed in a 27^{o}C incubator. At 0, 30, 60, 120, and 540 min post incubation, the media was removed and media containing 25 mM ammonium chloride was added to the cells. At 24 hpi the proportion of eGFP-positive cells was determined by flow cytometry on a FACSCalibur cytometer. Each data point represents the mean value ± SE of 10,000 events for data collected from three independent infections.

Results

P35Δ budded virions are less stable and initiate infection more slowly than control BV

Normally AcMNPV BV stocks are highly stable, and can be stored for years without losing significant titer, as long as they are protected from light (Jarvis et al., 1994). However, in the past we had often observed anecdotally that titers of *p35* mutant BV stocks seemed to decrease much faster than normal. To directly test this, we used a *p35* mutant virus, P35Δ, and a control revertant virus, Rev. BV stocks of these viruses that had been produced in TN-368 cells were incubated at 30^{o}C in the dark for two months, and their titers were measured both immediately before and after the incubation period. We found that the titer of the P35Δ virus had decreased 192-fold after the two month incubation, while the titer of Rev decreased only 5-fold during this time period, suggesting that expression of P35 is somehow important for the stability of AcMNPV BV.

In addition, while working with the P35Δ and Rev viruses, we also consistently noted what appeared to be a lower level of enhanced green fluorescent protein (eGFP)

expression in TN-368 cells infected with P35Δ as compared to Rev, even when using freshly propagated and titered virus stocks (both viruses express eGFP from a hsp70 promoter (Clarke et al., 2003)). This prompted us to more closely compare the kinetics of infection with P35Δ versus Rev. We first quantified eGFP expression in TN-368 cells infected with the two viruses at 24 hpi using flow cytometry analysis (Fig. 2.1A), and we found that the mean fluorescence intensity (MFI) of TN-368 cells infected with P35Δ was indeed significantly lower than cells infected with Rev. Since the hsp70 promoter behaves as an immediate early promoter, and since these two viruses differ only by the presence of an intact *p35* gene, this suggested that P35 is important for promoting some aspect of infection at an early stage in the replication cycle.

As an additional measure of the kinetics of infection, we used an assay that takes advantage of the fact that AcMNPV infection causes cell cycle arrest in the G2/M stage (Braunagel *et al.*, 1998). TN-368 cells were infected with either Rev or P35Δ at an MOI of 5 pfu/cell, and the cells were subjected to cell cycle analysis by flow cytometry at 24 and 48 hpi. As shown in Fig. 2.1B, a larger proportion of cells infected with Rev were in G2/M than cells infected with P35Δ virus at 24 hpi. By 48 hpi, however, the proportion of cells in G2/M was similar with both viruses (Fig. 2.1B). These results indicated that the entire process of infection was delayed with the P35Δ virus, and not simply expression of eGFP. The fact that there was no difference in cell cycle arrest by 48 hpi indicated that the delay in P35Δ infection was only transient, and occurred at a relatively early stage of infection, prior to G2/M arrest.

To address this, we compared binding and entry of the two viruses. AcMNPV BV particles enter cells through receptor-mediated endocytosis, and the kinetics of entry

can be studied using ammonium chloride, which blocks endosome acidification and virus escape from the endosome (Hefferon *et al.*, 1999). TN-368 cells were incubated with P35Δ or Rev at an MOI of 5 pfu/cell at 4^{o}C for 1 h to allow binding, and then warmed to 27 ^{o}C. Ammonium chloride was then added at 0, 0.5, 1, 2, or 9 hpi, and the infected cells were then incubated at 27^{o}C until 24 hpi, at which time they were assayed for eGFP expression by flow cytometry. If virus had not yet escaped the endosome at the time of ammonium chloride addition, then eGFP expression would not be observed. It should be noted that this assay cannot discriminate between defects in virus binding versus entry. An obvious delay was observed in the binding/entry of P35Δ as compared to Rev (Fig. 2.2). Even when ammonium chloride was added at 9 hpi, only around 20% of the P35Δ-infected cells exhibited eGFP expression at 24 hpi, while Rev had successfully entered approximately 45% of the cells by 9 hpi.

P35Δ-infected TN-368 cells contain active caspases

Since the only well-described function of P35 is its ability to inhibit caspases, we hypothesized that perhaps caspases were being activated in P35Δ-infected TN-368 cells, and that these caspases were somehow damaging the P35Δ virions either during or after their assembly in cells. Although TN-368 cells infected with *p35* mutant viruses do not become apoptotic (Clem et al., 1991; Hershberger et al., 1992; Griffiths et al., 1999), to our knowledge it has not previously been determined whether caspases are activated in TN-368 cells upon infection with viruses lacking *p35*. Kelly *et al.* (2006) observed caspase activation in TN-368 cells infected with a virus known as AcdefrT, but this virus

contains mutations in both *p35* and in the *fp-25* gene, and causes an unusual phenotype in TN-368 cells which somewhat resembles apoptosis.

To measure caspase activity, TN-368 cells were infected with either P35Δ or Rev, and cells were harvested at 24 and 48 hpi and assayed for the ability to cleave the fluorogenic caspase substrate AcDEVD-afc, which is mainly recognized by effector caspases (Fig. 2.3A). Cells infected with Rev showed no detectable caspase activity at either time point, and actually had lower caspase activity than mock-infected cells, presumably due to the expression of P35 (Fig. 2.3A). Cells infected with P35Δ, on the other hand, exhibited higher amounts of caspase activity than mock-infected cells, and the amount of activity increased from 24 to 48 hpi (Fig. 2.3A). Thus, even though P35Δ-infected TN-368 cells do not exhibit morphological signs of apoptosis, some amount of caspase activation still occurs in these cells.

To gain a better appreciation of the relative level of caspase activation in P35Δ-infected TN-368 cells, we wanted to compare this level of caspase activity to that in cells that were actually going through apoptosis. Since TN-368 cells are highly resistant to apoptosis, we used SF-21 cells, which are sensitive to a variety of apoptotic stimuli. SF-21 cells were exposed to ultraviolet light (UV) and harvested at 3 and 5 hrs post-exposure. By 3 hrs post-UV exposure, caspases are activated in SF-21 cells and the cells begin to show morphological characteristics of apoptosis such as plasma membrane blebbing (data not shown and (Manji et al., 2001)). We then compared the level of caspase activity in UV-treated SF-21 cell lysates to lysates harvested from the same number of P35Δ or Rev virus-infected TN-368 cells at 48 hpi (Fig. 2.3B). Because the amount of caspase activity in the UV-treated SF-21 cells was so high, we used only one-

third the amount of lysate used in Fig. 2.3A for all of the samples. SF-21 cells treated with UV had much higher caspase activity than P35Δ-infected TN-368 cells; the activity for the UV-treated SF-21 cells reached a plateau after only a short time, indicating that the substrate in the reaction had been exhausted. It is difficult to make a direct comparison between the different cell lines, but the amount of caspase activity in P35Δ-infected TN-368 cells appears to be considerably lower than that in apoptotic SF-21 cells. Interestingly, untreated SF-21 cells had lower levels of caspase activity than mock-infected TN-368 cells (Fig. 2.3B), suggesting that TN-368 cells may contain a low level of constitutive caspase activity.

Inhibition of caspases in the cells producing P35Δ rescues the entry defect in the next round of replication

To test whether the observed caspase activity in P35Δ-infected cells was involved in the entry defect of P35Δ, we used the chemical pan-caspase inhibitor, zVAD-fmk. We considered two possibilities for when and where the activation of caspases could be having a detrimental effect on P35Δ infectivity: either in the cells used to produce the P35Δ virus stocks (the producer cells), in which case the virions would already be defective at the start of the next round of infection, or during the actual process of infection of the cells used to measure infectivity (the target cells). To test the first possibility, viruses were propagated in the presence of zVAD-fmk to inhibit caspase activation in the producer cells, and these viruses were then analyzed for their infection kinetics. When eGFP expression was analyzed using viruses produced in the presence of zVAD-fmk, we found that the reduction in eGFP expression seen for P35Δ (Fig. 2.1A)

was rescued (Fig. 2.4A). In addition, the P35Δ virus propagated in the presence of zVAD-fmk also had a normal binding/entry phenotype when used to infect TN-368 cells (Fig. 2.4B).

To test the second possibility, P35Δ or Rev stocks that had been produced in cells without caspase inhibitor were used to infect TN-368 cells in the presence of zVAD-fmk and cell cycle analysis was carried out to measure G2/M arrest (Fig. 2.5). The results were very similar to infections done in the absence of zVAD-fmk (Fig. 2.1B); that is, there was a delay in cell cycle arrest at 24 hpi in P35Δ-infected cells compared to Rev, which was no longer evident by 48 hpi. Together, these results indicate that caspases are responsible for the decreased infectivity of P35Δ BV, and that the detrimental effect of caspases on the infectivity of P35Δ BV occurs prior to the next round of replication.

Discussion

Apoptosis has been shown to serve as an anti-viral defense for many viruses because of its negative effects on the process of virus replication. This is the first report, that we are aware of, that illustrates that caspase activity can also have detrimental effects on progeny virus particles and affect their subsequent infectivity in other cells. These effects thus represent an additional, previously unrecognized, facet of the anti-viral apoptotic response. Our results indicate that activated caspases in the cells that produce the progeny virus are responsible for the infectivity defect of P35Δ virions, since entry was restored to normal by treating the cells used to produce the virus stocks with the caspase inhibitor zVAD-fmk. The relative instability of P35Δ virus stocks further

indicates that caspases can either directly or indirectly have negative effects on virions and their ability to initiate infection. Activated caspases could affect virions either during or after their assembly, by either cleaving a cellular substrate that the virus needs for efficient packaging, or by directly cleaving viral proteins. Another possibility is that viral DNA may be damaged by the nucleases that are activated by caspases and digest cellular DNA during apoptosis. We consider this unlikely, however, since *p35* mutant-infected TN-368 cells do not undergo apoptosis or exhibit DNA degradation (Clem *et al.*, 1991), and viral DNA is not degraded even when SF-21 cells are infected with *p35* mutant AcMNPV (Clem RJ and Miller LK, unpublished results).

This is the first report demonstrating that caspase activation, albeit at a low level, occurs in TN-368 cells infected with *p35* mutant AcMNPV. This result was somewhat surprising, since TN-368 cells are resistant to most apoptotic stimuli. It has been shown that caspases are activated in TN-368 cells infected with a virus containing mutations in both *p35* and *fp-25*, but such cells die by a process that appears to have some of the characteristics of apoptosis (Kelly *et al.*, 2006). The mechanism responsible for the resistance of TN-368 cells to apoptosis is unknown, but interestingly, our results indicate that uninfected TN-368 cells have a low level of caspase activity, which is not seen in SF-21 cells. *Mamestra brassicae* and *Panolis flammea* cell lines also have been shown to support replication of *p35* mutant AcMNPV (Griffiths *et al.*, 1999). Given our results, it would be interesting to determine if these cell lines also activate caspases in response to infection.

It has been previously concluded that no significant differences are observed between *p35* mutant and wild type AcMNPV infection in TN-368 cells or *T. ni* larvae

(other than the lack of larval liquefaction). However, when one closely examines the previously published data, our results are actually consistent with results from some of these previous studies both at the organism level and at the cellular level. Data published by Clarke and Clem (2003) suggest a possible slower spread of the P35Δ virus than Rev in *T. ni* larvae, as there was a somewhat lower proportion of hemocytes infected with P35Δ as compared to the Rev virus following intrahemocoelic infection. This difference was seen at 24 and 48 hpi, although the difference was not statistically significant at 24 hpi, and by 72 hpi, the proportions of infected hemocytes were similar between the two viruses. At the cellular level, Clem and Miller (1993) reported differences in virus replication and viral gene expression in SF-21 cells infected with two different *p35* mutants and wild type virus, but no differences were reported in TN-368 cells. However, even though it was not addressed in the report, it appears that there were also slightly lower levels of transcripts for the late *vp39* and the very late *polh* genes in TN-368 cells infected with *p35* mutant virus compared to wild type, although transcript levels of an early gene appeared similar. However, Hershberger *et al.* (1992) reported similar levels of late and very late gene expression for *p35* mutant and control viruses in TN-368 cells. The basis for the differences between these reports is unknown, but Hershberger *et al.* (1992) also noticed a difference in infectivity between viruses containing or lacking *p35*. These authors reported that following infection with *p35* mutant virus at an MOI of 5 pfu/cell, a large proportion of SF-21 cells did not exhibit cytopathic effects, unlike control viruses, which caused cytopathic effects in nearly all cells at the same MOI. Since the same effect was seen when virus was propagated in TN-368 or SF-21 cells, the authors concluded it was not due to apoptosis, and postulated that P35 had a direct role in

infectivity. The same group later showed that P35 protein is associated with purified BV particles, suggesting that P35 might be a component of the virion, necessary either for virus stability (as a structural protein) or for promoting infection in some way, such as inhibiting caspases during the early stages of infection (Hershberger *et al.*, 1994). Our results, however, appear to rule out these possibilities, at least for TN-368 cells. We observed that zVAD-fmk treatment of the cells used to propagate the virus rescued the infectivity defect of progeny P35Δ virus, while the infectivity of P35Δ was not rescued by addition of the inhibitor during subsequent infection, indicating that inhibition of caspases is not necessary during the early stages of infection in TN-368 cells. The situation could be different in SF-21 cells, however, which have higher levels of caspase activity following P35Δ infection.

We suggest that the detrimental effect of caspases on BV infectivity may also occur during replication of *p35* mutant AcMNPV in *T. ni* larvae, and that this could potentially explain the lack of liquefaction in these larvae. As progeny virus is produced in the tissues of *T. ni* larvae, defects in its ability to infect neighboring cells would result in less efficient spread of infection throughout the organism and possibly lower levels of expression of viral genes such as chitinase and cathepsin, which are known to be required for liquefaction (Ohkawa et al., 1994; Slack et al., 1995; Hawtin et al., 1997).

The ability of P35 to protect progeny virions from caspase-induced damage is somewhat analogous to the role of the HIV-1 protein Vif in inhibiting the cellular enzyme APOBEC3G. Vif is packaged in the HIV-1 capsid, and during the subsequent round of replication, binds to APOBEC3G, a cytosine-deaminase that introduces mutations into the viral genome, and targets it for destruction by ubiquitination (Harris et al., 2004).

Like Vif, P35 inhibits cellular enzymes that can potentially cause damage to virions. Whether P35 needs to be packaged in the virion to carry out this aspect of its function, and whether the damage is due to direct cleavage of viral structural proteins, or to an indirect effect on virus assembly, remain open questions.

In this report we have shown that caspases are activated upon AcMNPV infection in TN-368 cells from the host *T. ni*, even though these cells do not die by apoptosis. Based on prior studies, it had been thought that P35 is not necessary for replication in *T. ni* cells and larvae, but our results indicate that even a low level of caspase activity can have detrimental effects on the infectivity of baculovirus virions. This potentially represents a previously unappreciated consequence of the anti-apoptotic response against viruses. It will be interesting to determine whether other types of viruses are also negatively impacted by caspase activation during apoptosis of their host cells.

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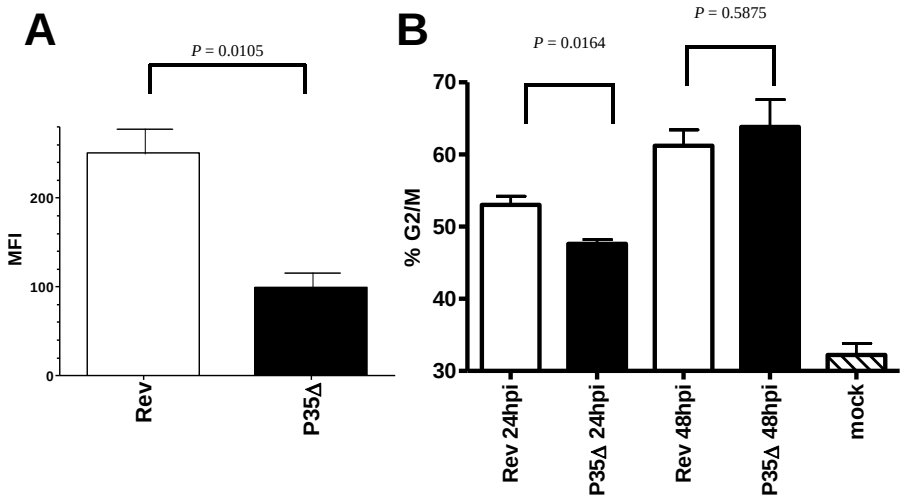

Figure 2.1 The timing of P35Δ infection is slower than Rev control virus.
(A) eGFP expression, measured as mean fluorescence intensity (MFI), in TN-368 cells infected with P35Δ or Rev at an MOI of 5 pfu/cell. MFI was determined at 24 hpi. (B) Cell cycle arrest of TN-368 cells infected with P35Δ or Rev. Cells were infected at an MOI of 5 pfu/cell with either P35Δ or Rev and harvested at 24 and 48 hpi. In both panels, the data shown are the combined results of three independent experiments and are illustrated as mean ± SE. The data were analyzed by Student's *t*-test and *P* values are indicated.

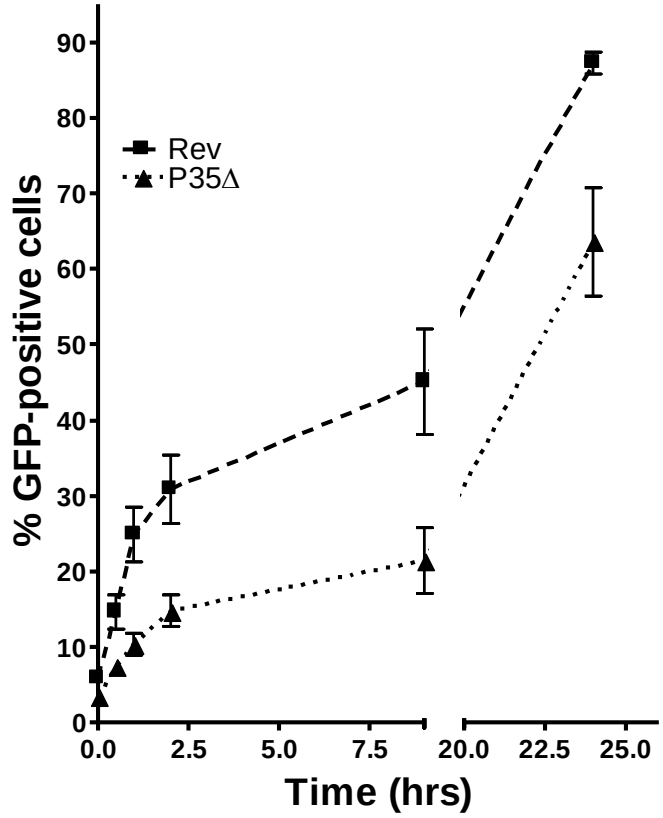

Figure 2.2 Endosome exit assay of P35Δ or Rev in TN-368 cells.
TN-368 cells were infected at an MOI of 5 pfu/cell and ammonium chloride was added at 0, 0.5, 1, 2, and 9 hpi to block endosome acidification. At 24 hpi, the proportion of infected cells (as measured by eGFP expression) was determined. The 24 h time point represents cells that were not treated with ammonium chloride. The data shown are the combined results of three independent experiments and are illustrated as mean ± SE. The two data sets were found to be statistically different as analyzed by two-way ANOVA (P<0.0001).

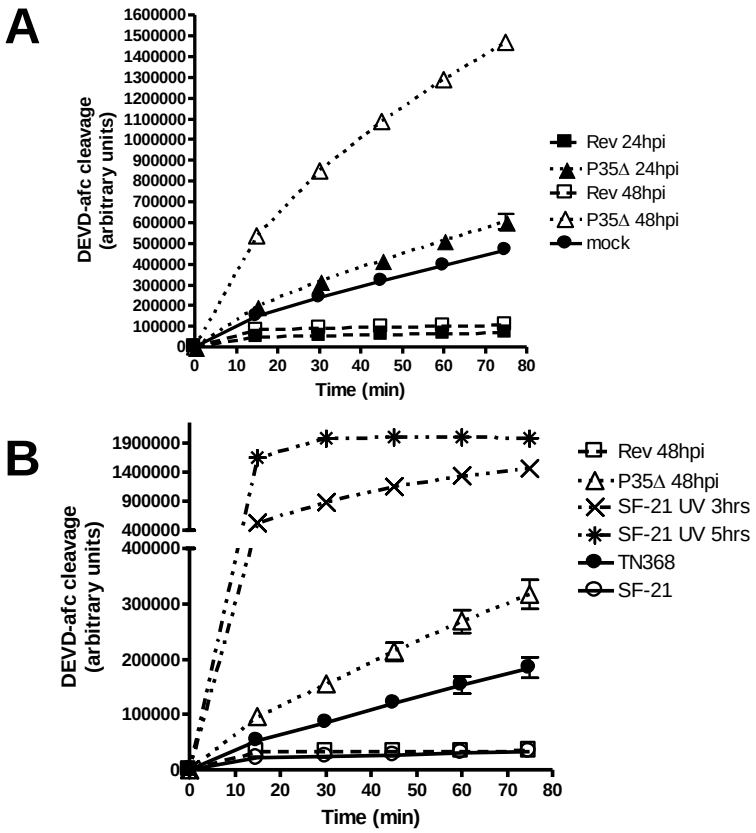

Figure 2.3 Caspase activity in infected TN-368 cells.
(A) TN-368 cells were infected with P35Δ or Rev at an MOI of 5 pfu/cell and harvested at 24 and 48 hpi. Viral-infected and mock-infected lysates were incubated with the fluorogenic caspase substrate Ac-DEVD-AFC to measure effector caspase activity. Following a 15 min pre-incubation with substrate, fluorescence was measured every 15 min for 75 min. (B) Comparison of P35Δ-induced caspase activity in TN-368 cells to caspase activity in SF-21 cells treated with UV to induce apoptosis and harvested at 3 or 5 hrs post-UV treatment. One-third the amount of lysate was used in (B) compared to (A). For both panels, the data shown were obtained from three independent experiments and are plotted as mean ± SE. Data were analyzed by two-way ANOVA (P<0.0001).

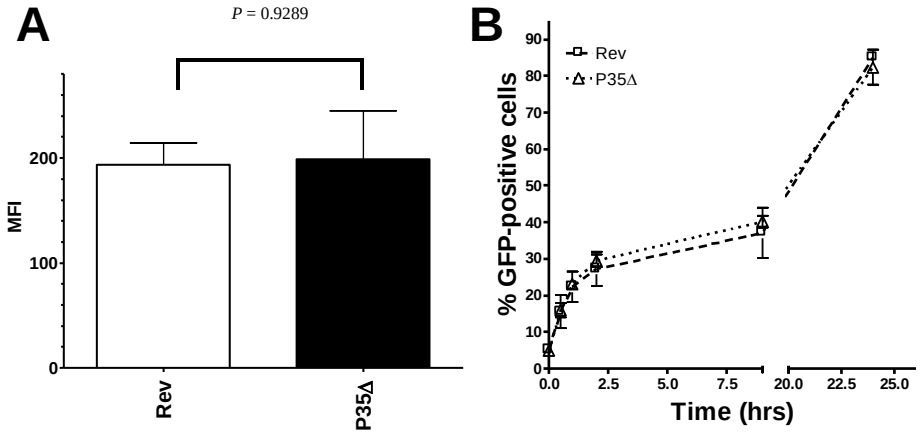

Figure 2.4 Treatment of the cells used to propagate the virus stocks with caspase inhibitor zVAD-fmk rescues the infectivity of P35Δ.
Virus stocks propagated in the presence of zVAD-fmk were used to infect TN-368 cells at an MOI of 5 pfu/cell. (A) eGFP fluorescence was determined at 24 hpi. (B) Ammonium chloride was added at 0, 0.5, 1, 2, and 9 hpi and the proportion of infected cells (as measured by eGFP expression) was determined at 24 hpi. The cells in the 24 h time point were not treated with ammonium chloride. In both panels A and B, the data shown are the combined results from three independent experiments and are illustrated as mean ± SE. The data were not significantly different as determined by Student's *t*-test in panel (A) or two-way ANOVA in panel (B) ($P>0.0001$).

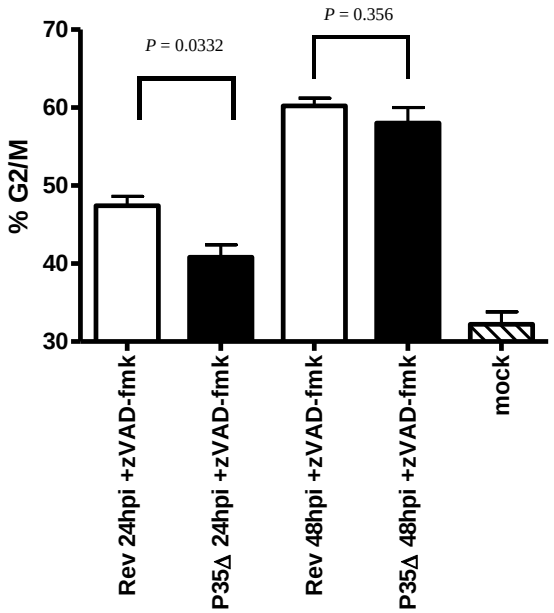

Figure 2.5 The presence of zVAD-fmk at the time of infection does not restore the infectivity of P35Δ.

Viruses propagated without zVAD-fmk were used to infect TN-368 cells in the presence of zVAD-fmk, harvested at 24 and 48 hpi, and subjected to cell cycle analysis. The data represent the combined results from three independent experiments and are illustrated as mean ± SE. The data were analyzed by Student's *t*-test and *P* values are shown.

CHAPTER 3 - Annotation and expression profiling of apoptosis-related genes in the yellow fever mosquito, *Aedes aegypti*

This chapter has been previously published in Insect Biochemistry and Molecular Biology by ELSEVIER (Bryant B., Blair C.D., Olson K.E., Clem R.J. Annotation and expression profiling of apoptosis-related genes in the yellow fever mosquito, Aedes aegypti. 2008. Insect Biochemistry and Molecular Biology 38: 331-345).

Abstract

Apoptosis has been extensively studied in *Drosophila* by both biochemical and genetic approaches, but there is a lack of knowledge about the mechanisms of apoptosis regulation in other insects. In mosquitoes, apoptosis occurs during *Plasmodium* and arbovirus infection in the midgut, suggesting that apoptosis plays a role in mosquito innate immunity. We searched the *Aedes aegypti* genome for apoptosis-related genes using *Drosophila* and *Anopheles gambiae* protein sequences as queries. In this study we have identified eleven caspases, three inhibitor of apoptosis (IAP) proteins, a previously unreported IAP antagonist, and orthologs of *Drosophila* Ark, Dnr1, and BG4 (also called dFadd). While most of these genes have been previously annotated, we have improved the annotation of several of them, and we also report the discovery of four previously unannotated apoptosis-related genes. We examined the developmental expression profile of these genes in *Ae. aegypti* larvae, pupae and adults, and we also studied the function of a novel IAP antagonist, IMP. Expression of IMP in mosquito cells caused apoptosis, indicating that it is a functional pro-death protein. Further characterization of these genes will help elucidate the molecular mechanisms of apoptosis regulation in *Ae. aegypti*.

Introduction

Apoptosis is a key pathway involved in normal processes such as development, tissue homeostasis, and DNA damage responses, as well as pathological processes including cancer, ischemia, neurological diseases, and defense against pathogens like viruses (Vaux et al., 1999; Opferman et al., 2003; James et al., 2004; Clem, 2005). The process of apoptosis is largely carried out by a family of cysteine proteases called caspases. These enzymes are expressed as zymogens and are activated by multiple stimuli. There are two types of caspases, initiator and effector, which carry out different functions in apoptosis. A death insult first results in activation of one or more members of the initiator class, which then cleave and activate members of the effector class. The effector caspases cleave many cellular targets and dismantle the cell. All caspases consist of a prodomain and large and small catalytic domains, which are freed from each other by cleavage at aspartate residues. The large and small catalytic domains form a dimer, and two of these heterodimers associate to form the active caspase molecule. Initiator caspases contain long prodomains, which are involved in interaction with adaptor proteins, while effector caspases contain short prodomains.

Most of what is known about the molecular mechanisms of apoptosis in insects comes from study of the fruitfly *Drosophila melanogaster* (reviewed in (Hay et al., 2006). There are seven caspases encoded in the *Drosophila* genome, including the three initiator caspases Nc (also known as Dronc), Dredd, and dream (also known as Strica) and the four effector caspases Ice (also known as Drice), Dcp-1, decay, and Damm (also known as Daydream). Among the initiator caspases found in *Drosophila*, Nc appears to be the most important in carrying out apoptosis, while Dredd is important in the immune

response, and dream is relatively uncharacterized. Among the effector caspases, Ice appears to be the most important for apoptosis, with Dcp-1 playing a supportive role.

Activation of initiator caspases by intrinsic signals involves the formation of a large protein complex called the apoptosome. Homologs of an adaptor protein that is an integral part of the apoptosome have been found in the nematode *Caenorhabditis elegans* (CED-4), in mammals (APAF-1), and in *Drosophila* (Ark). In mammals, cytochrome c binding to APAF-1 is required for apoptosome formation, but cytochrome c does not appear to be required for apoptosome formation in *Drosophila* (Zimmermann et al., 2002; Means et al., 2006; Yu et al., 2006; Bao et al., 2007).

Multiple gene products regulate caspases, either positively or negatively. Among the most important negative caspase inhibitors are the IAP (Inhibitor of Apoptosis) proteins. IAP proteins were first discovered in baculoviruses (Crook et al., 1993), but are now known to exist in cellular genomes ranging from yeast to mammals, where they play important roles in regulating apoptosis and cell division (Vaux et al., 2005). In *Drosophila*, the IAP protein that is most important in regulating apoptosis is thread (also known as DIAP1). Thread was first identified in an enhancer screen for apoptosis-regulating genes (Hay et al., 1995). Overexpression of thread inhibits apoptosis, while loss of thread leads to spontaneous apoptosis, both in the developing fly embryo and in cultured *Drosophila* cells (Hay et al., 2006). Thread has the ability to directly bind and inhibit effector caspases. It also can bind to Nc and causes its degradation via the ubiquitin-proteasome pathway (Wilson et al., 2002).

In addition to IAPs, a second type of negative caspase inhibitor was recently reported in *Drosophila*. The Dnr1 (defense repressor 1) protein was first identified in a

cell-based screen for innate immunity, and was found to inhibit Dredd activity (Foley et al., 2004). More recently, Dnr1 has been shown to inhibit apoptosis by causing a reduction in the level of Nc protein (Primrose et al., 2007).

In *Drosophila*, apoptosis depends on the expression of a set of proteins collectively referred to as IAP antagonists or RHG proteins (Hay et al., 2004). Four of these proteins, rpr (reaper), grim, W (also known as wrinkled or hid), and skl (sickle), are encoded by genes that are in a chromosomal region called the H99 interval (White et al., 1994; Grether et al., 1995; Chen et al., 1996). The RHG genes *rpr* and *grim* encode small proteins that are transcriptionally upregulated in cells that are destined to die (Kumar et al., 2004a), while the *W* gene encodes a larger protein that is regulated post-translationally by phosphorylation (Bergmann et al., 1998). The protein encoded by *skl* is less well characterized. The RHG proteins each physically interact with thread through a short motif at their amino termini (Vucic et al., 1997; Vucic et al., 1998), and this interaction plays an important role in determining whether a cell lives or dies.

Apoptosis has been established as a component of the innate immune response in baculovirus infections of lepidopteran insects (Clem, 2005). In addition, cross-talk exists between innate immunity pathways and apoptosis pathways in insects. In *Drosophila,* Dredd (Elrod-Erickson et al., 2000; Leulier et al., 2000; Stoven et al., 2000), Iap2 (Gesellchen et al., 2005; Kleino et al., 2005), BG4 (also known as dFADD) (Zhou et al., 2005b) and Dnr1 (Foley et al., 2004) have already been shown to play roles in innate immunity. At one day post infection with Sindbis virus, the midgut of *Aedes aegypti* exhibited an increase in expression of the *Ae. aegypti* ortholog of Dif, which is part of the Toll pathway in *Drosophila* (Sanders et al., 2005). In *Drosophila*, it has been shown that

the protein MyD88 is a component of the Toll pathway, and MyD88 was shown to bind to BG4 (dFADD) and Dredd (Horng et al., 2001). In mammals, FADD plays a role in activation of caspases through the extrinsic pathway (Chinnaiyan et al., 1996).

In mosquitoes, there are reports that arbovirus infection causes pathology resembling apoptosis in the midgut and salivary glands. This pathology has been found in *Ae. aegypti* infected with Semliki Forest virus (Mims et al., 1966), in *Culiseta melanura* infected with eastern equine encephalitis virus (Weaver et al., 1988), and in *Aedes albopictus* infected with Sindbis virus (Bowers et al., 2003). Long-term West Nile virus infection has also been shown to induce cell death in the salivary glands of *Culex pipiens quinquefasciatus* (Girard et al., 2005) and the same group later suggested that this late pathology affected transmission rates (Girard et al., 2007). Recently it has been shown that a lab-derived strain of *Culex pipiens pipiens* was refractory to infection with West Nile virus, and that infection with this virus caused extensive cell death in the midgut epithelial cells of these mosquitoes (Vaidyanathan et al., 2006). Besides viral infection, a number of apoptosis-related genes, as well as other immune response genes, are expressed in hemocytes of *Ae. aegypti* and *Armigeres subalbatus* infected with bacterial pathogens (Bartholomay et al., 2004). In addition, *Plasmodium* can elicit pathology resembling apoptosis in mosquito vectors. *Plasmodium berghei* infection causes apoptosis in midgut cells of *Anopheles stephensi* (Han et al., 2000) and *An. gambiae* (Vlachou et al., 2004), and activation of An-caspase 7 in midgut cells of *An. stephensi* (Abraham et al., 2004). With *P. gallinaceum* and *Ae. aegypti*, ookinete infection of midgut cells has also been reported to activate caspases (Zieler et al., 2000).

Even though numerous reports suggest that cell death might play a role in certain infections of mosquitoes, knowledge of the basic mechanics of apoptosis in mosquito vectors is lacking. There have been reports of *IAP1* genes in *Aedes triseriatus* (Blitvich et al., 2002) and *Ae. albopictus* (Li et al., 2007). A recent study suggests that these genes are regulated by an alternative splicing mechanism (Beck et al., 2007a). The initiator caspases Dredd and Dronc have been reported in *Ae. aegypti* (Cooper et al., 2007a; Cooper et al., 2007b). There has also been a report of an IAP-antagonist related to the rpr protein from *Drosophila* found in mosquitoes called Michelob_x (Zhou et al., 2005a). In addition, a number of caspases and IAP proteins were recently annotated in the *Ae. aegypti* genome (Waterhouse et al., 2007). We independently identified a number of apoptosis-related genes, using the available *Ae. aegypti* genome sequence. In most cases, our results agreed with these previous annotations, but we have made improvements to the annotation of several of these genes, and we have identified four additional apoptosis-related genes that were not previously annotated in *Ae. aegypti*.

Methods

Cells and insect rearing

ATC-10 (*Ae. aegypti*) and C6/36 (*Ae. albopictus*) cells were maintained in L-15 medium (Gibco) supplemented with 20% FBS (ATC-10) or 10% FBS (C6/36) at 25°C. *Ae. aegypti* mosquitoes (RexD strain) were reared at the Arthropod-Borne and Infectious Disease Laboratory at 26-28°C, 80-82% humidity, under a 10h dark/14h light regime. Adults were maintained on sucrose and naive adult females were collected at 4 days post-eclosion.

Database mining

The *Ae. aegypti* genome was searched for apoptosis related genes using known proteins from *Drosophila* (obtained from FlyBase (http://flybase.bio.indiana.edu)) and predicted proteins from the *An. gambiae* genome as queries in BLAST searches. Supercontigs from the *Ae. aegypti* genome were obtained and small portions that were identified from the BLAST search were used as queries to search the EST_others database at NCBI using nucleotide-nucleotide BLAST. ESTs from *Ae. aegypti* identified after the initial BLAST search were then used as queries reiteratively until no new ESTs were obtained. The ESTs were then translated and aligned using Vector NTI to assemble mini-contigs for transcripts. Genscan (http://genes.mit.edu/GENSCAN.html) and fgenesh (http://www.SoftBerry.com) were used to predict genes and complete missing regions of some EST contigs.

Updated annotations for CASPS7, CASPS20, CASPS17, CASPS21, IAP2, DNR1, ARK, and IMP have been submitted to VectorBase (http://www.vectorbase.org) and are visible as part of the "Manual annotation track" in the genome browser. These sequences will be incorporated into the next VectorBase genebuild (Neil Lobo, personal communication).

Protein domain determination

Domains for the proteins were predicted using the programs ExPASY PROSITE (Hulo et al., 2006), SMART (Schultz et al., 1998), and Conserved Domain Database at NCBI (Marchler-Bauer et al., 2005).

Phylogenetic analysis

Amino acid sequences were aligned using the ClustalW algorithm with the default parameters found in MEGA 3.1 (Kumar et al., 2004b). Phylogenetic trees were assembled using MEGA 3.1. Trees were built using neighbor end joining, complete deletion, and p-distance. Other parameters were set at default values.

Plasmid construction

Michelob_x cDNA was obtained as a generous gift from L. Zhou (University of Florida). EST clones DV326893, DV369010, DV323242, DV328064, DV330266 were obtained from D. Severson (University of Notre Dame). IMP and Michelob_x cDNAs were inserted into the expression vector pHSP70PLVI+Rpr-epi (Vucic et al., 1997), replacing the Rpr gene using BglII and SpeI, and the constructs were named pHSMichelob_x-c-EpiHisVI+ and pHSIMP-c-EpiHisVI+. This resulted in HA and His6 tags being fused to the C terminus of the inserted coding sequence. The plasmid pHSP70GFPBsu36I, expressing eGFP (enhanced green fluorescent protein), has been previously described (Clarke et al., 2002). All other genes were cloned into the pCRII vector (Invitrogen) for sequencing purposes. 5' RACE was performed as previously published (Beck et al., 2007a).

Transfections

C6/36 cells were plated at a density of $2x10^6$ cells per well in six-well plates and incubated overnight. The following day, cells were transfected using FuGENE 6 (Roche) following the manufacturer's instructions. A total of 6 μg of DNA with 9μl of FuGENE

6 was used for each transfection. Under these conditions, transfection efficiency was approximately 20%.

Annexin V staining and caspase activity measurements

At 24 hours post transfection, cells were harvested and assayed for Annexin V staining and caspase activity. Cells were stained with Annexin V-PE (BD Pharmingen) following manufacturer's instructions and analyzed by flow cytometry. Caspase activity was measured using N-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (Ac-DEVD-AFC) fluorescent substrate as previously described (Muro et al., 2004). The assays were done in triplicate. The caspase assay results include the results from all three assays, while the Annexin V staining results are from one representative assay.

Expression analysis

RNA was isolated from homogenized tissues of pooled individuals or lysed ATC-10 cells by Trizol (Invitrogen) and treated with Turbo DNA-free DNase (Ambion), following manufacturers instructions, to reduce the possibility of genomic DNA contamination. Approximately 2-3 μg of RNA was used to synthesize cDNA using M-MLV RT (Invitrogen) and an oligo dT-20 primer. The resulting cDNA was analyzed for expression of all eighteen genes (Table 3.1) as well as Michelob_x (GenBank accession number **ABD47742**) and actin 6 (*act-6*, GenBank accession number **DQ124691**). Expression was initially analyzed by semi-quantitative PCR and the amplicons were analyzed by agarose gel electrophoresis to verify their correct size. Real time PCR was performed using the BioRad iCycler Optical Module. Primers were designed by Beacon Designer 3 (Premier Biosoft) and are shown in Table 3.3. Primers were tested by Tm

gradient to determine the optimal Tm for each primer. iQ SYBR Green Supermix was used in all cycle threshold (Ct) determinations following manufacturers instructions (BioRad). To determine the concentration of cDNA needed for expression analysis, dilutions of the cDNA were assayed using *act-6* primers. For each of the five stages or tissues (L1/L2, L3/L4, pupae, female adult without midgut, and female adult midgut), three cDNA batches were made. All genes were assayed in duplicate using each cDNA batch. The PCR efficiency curves and melt curves were analyzed for each gene in each stage. For each cDNA batch that was analyzed, the RNA from each stage was used to determine the background for each pair of primers. Some primer sets gave a background Ct while others yielded no background Ct values. If the Ct from a certain gene was not below the background Ct, then the gene was considered not to be expressed.

Results

By searching the *Ae. aegypti* genome, we found numerous genes with homology to known apoptosis-related genes in *Drosophila*. The majority of these genes have been previously annotated (Waterhouse et al., 2007), but we were able to make improvements to some of these annotations (Table 1). We also identified four additional apoptosis-related genes, including an additional effector-type caspase, orthologs of *Drosophila* Ark and Dnr1, and an additional IAP antagonist, which we have named IMP (IAP-antagonist Micheob_x-like Protein). In keeping with the previously proposed genetic nomenclature (Waterhouse et al., 2007), we have named the new caspase CASPS21. Table 3.2 summarizes the bioinformatics analysis for each gene. If the EST overlap method yielded a full-length predicted transcript (as defined in Materials and Methods), and the

existence of this transcript was verified by RT-PCR, then information about the gene is found in Table 3.2 and it is not discussed further here. Those that did not yield a full-length predicted transcript, however, are discussed further below.

Caspases

In NCBI, part of CASPS7 was represented by EAT35718, and this same fragment was also reported by Waterhouse et al. (Waterhouse et al., 2007). However, we determined that this was not the full-length transcript using the EST DV369010 (obtained from D. Severson). Portions of DV369010 are found on two supercontigs, 659 and 791. The portion represented by EAT35718, which includes the 3' end of the transcript, is found on supercontig 659. Gene predictions, using GenScan, predicted that there were additional transcribed sequences further upstream of the gene in supercontig 791. When DV369010 was sequenced further, we found that the gene prediction program was correct, and this was verifed by RT-PCR from ATC-10 cells. This analysis added an additional 76 amino acids to the N-terminus of CASPS7.

CASPS20 bioinformatics analysis yielded a full-length predicted transcript that is represented in NCBI as EAT33088, but the N-termini of the NCBI-predicted protein and the transcript constructed by overlapping ESTs are different. (Waterhouse et al., 2007) reported the same sequence that is found in NCBI. 5' RACE was done on RNA from early stage (L1/L2) larvae and we found that the overlapping EST prediction for the transcript was correct. However, it is possible that there are alternatively spliced forms of CASPS20 present in other stages or tissues.

CASPS17 does not have a predicted protein in NCBI. Waterhouse et al. (Waterhouse et al., 2007) identified this gene, but their analysis did not include the full

length transcript. CASPS17 is represented by EST DV323242 (obtained from D. Severson). DV323242 has a 3' stop codon, but further sequencing of DV323242 did not reveal an upstream stop codon in the same reading frame as the start codon. We were able to predict a potential 5' end of this transcript using the fgenesh gene prediction program, but we were unable to amplify this predicted transcript from ATC-10 cells, meaning that this transcript will require further verification. However, an interesting observation with this gene was that the 3'UTR (represented by DV323242) contained a repetitive DNA element approximately 35 bp in length that is found on multiple supercontigs and ESTs in *Ae. aegypti*. When this EST is used as a BLAST query against the genome, approximately 100 supercontigs result in hits. The exact nature of this repetitive sequence is unknown at this time.

Phylogenetic analysis of the caspases was done using MEGA 3.1 (Kumar et al., 2004b), utilizing the full-length amino acid sequences to analyze caspases from *Ae. aegypti*, *An. gambiae* and *Drosophila*. It should be noted that the predicted caspases from *An. gambiae* have not been confirmed yet, and many are not full length. The results of the phylogenetic analysis are illustrated in Fig. 3.1A (effector caspases) and B (initiator caspases). *Drosophila* Damm and its mosquito orthologs, which are predicted to be effector caspases based on their short prodomains, actually aligned more closely with the initiator caspases, and so are included in the phylogenetic analysis with initiator caspases.

The effector caspases (Fig. 3.1A) are represented by two clades and an outlier caspase from *Ae. aegypti*, CASPS20. CASPS20 remained an outlier when all of the caspases were included in a single tree (not shown). Clade I includes only one caspase

from *Drosophila*, decay. However, in both mosquito species, and especially in *An. gambiae*, there has been expansion of this gene. *An. gambiae* genes in Clade I include s3, s4, s5, s6, and s11, while *Ae. aegypti* is represented by CASPS19 and CASPS18. Also within Clade I there are additional caspases s1, s2 and s14 from *An. gambiae*. These are the only caspases in the analysis that have either serine or threonine instead of alanine in the active site sequence QAC(R/Q/G)(G/E) (Vernooy et al., 2000). Clade II includes caspases from all three species analyzed, but we were not able to distinguish clear orthologs for the effector caspases Ice and Dcp-1 from *Drosophila*, since Ice and Dcp-1 are more closely related to each other than to any of the mosquito caspases. However, CASPS7 and CASPS8 are the closest *Ae. aegypti* relatives to *Drosophila* Ice and Dcp-1.

Fig. 3.1B illustrates the phylogeny of the initiator caspases for the three dipteran species analyzed. The initiator caspases fall into three clades, Clades III through V. Clade III includes dream and Damm from *Drosophila* and their mosquito orthologs. It appears that *Drosophila* dream and Damm arose from a gene duplication event, which is particularly interesting since they differ in the lengths of their prodomains (dream has a long prodomain, while Damm has a short prodomain). The analysis suggests that these genes have been duplicated within each mosquito lineage. We note that the *An. gambiae* caspases s9 and s12 are extremely similar (98% nucleotide identity, including intron sequences). Thus we conclude these are alleles of the same gene, rather than two individual genes and so we have excluded s12 from our analysis. In contrast to Clade III, Clades IV and V include clear mosquito orthologs for *Drosophila* Nc and Dredd.

The caspase genes decay, Damm and dream all appear to have undergone duplication events in mosquitoes. Fig. 3.2 illustrates the genomic architecture for these

duplications and how they compare in the three Dipteran species. CASPS15 and CASPS21 were found using gene prediction programs on supercontig 182, close to CASPS16 and CASPS17 (Fig. 3.2A). CASPS21 is a new caspase that was not previously identified. In *Ae. aegypti*, the predictions for CASPS16 and CASPS15 yielded introns with sizes of 16 and 11.5 Kbp. This is not surprising given that the *Ae. aegypti* genome contains a large number of transposable elements in the introns of genes, which has led to intron expansion (Nene et al., 2007). Accordingly, when we performed gene prediction analysis for the Damm and dream orthologs in *Ae. aegypti*, we found remnants of transposable elements in the introns of some of these genes. Fig. 3.2B shows the genome arrangements of expanded decay paralogs in mosquitoes.

Caspase regulators

In addition to caspases, we also identified a number of genes that regulate caspases. This group includes proteins that are known in *Drosophila* to inhibit caspase activity or are involved in caspase activation. Fig. 3.3 illustrates the protein domains for the predicted *Ae. aegypti* proteins from the caspase regulator group and their *Drosophila* orthologs.

Among the genes in this category that we identified are members of the IAP family. We found five IAP homologs in the *Ae. aegypti* genome, but we only characterized three. Of the two that we did not characterize, one represents a dBruce ortholog and the other contains a single BIR domain, but determination of its 3' end was problematic due to the presence of transposable elements. The three IAP proteins that we characterized and their domains are illustrated in Fig. 3.3A-C. These *Ae. aegypti* IAPs have a protein architecture similar to that of the *Drosophila* IAPs. The first, which is

designated as IAP1, is the ortholog of thread (th) and is represented in NCBI as ABK1289. The *Ae. aegypti* ortholog of Iap2 from *Drosophila* is represented in NCBI as EAT41756, but previous gene predictions do not appear to represent the full transcript because they do not include an initiating methionine. We employed GenScan and found an apparent initiator methionine in supercontig 214. We were able to amplify the predicted transcript from the ATC-10 cell line using RT-PCR, verifying the gene prediction results. This analysis added 90 amino acids to the N-terminus of *Ae. aegypti* IAP2. IAP5, the ortholog of *Drosophila* det (also called Deterin), is represented in NCBI as EAT33476. GenScan predictions did not result in additional sequences being included in this transcript.

In addition to members of the IAP family, we also identified the *Ae. aegypti* ortholog of the caspase inhibitor Dnr1. *Ae. aegypti* DNR1 has a predicted protein domain architecture similar to that of Dnr1 from *Drosophila*, as illustrated in Fig. 3.3D. The ESTs encoding the *Ae. aegypti* DNR1 did not contain an initiation codon, but did yield 3' stop codons. However the predicted protein in NCBI (EAT48387) has an initiation codon. We were able to verify the NCBI-predicted transcript by RT-PCR from ATC-10 cells.

We also identified genes that encode activators of caspases. The *Ae. aegypti* ortholog for Ark was found by using three domains found in Ark, the CARD (caspase recruitment domain), NB-ARC (nucleotide-binding adaptor shared by Apaf-1, certain R gene products and CED-4), and WD-40 domains, in individual BLAST searches against NCBI predicted proteins for *Ae. aegypti*. Two predicted proteins in NCBI, EAT48065 and EAT48066, contained these domains but were in separate predicted proteins found

relatively close to each other. EAT48065 encodes CARD and NB-ARC domains, while EAT 48066 encodes WD-40 repeat domains. Primers were designed from the 5' end of EAT48065 and 3' end of EAT48066, and we were able to amplify a single continuous transcript from ATC-10 cells, thus confirming that these domains are all part of a single protein. Fig. 3.3E illustrates that *Ae. aegypti* ARK and *Drosophila* Ark are highly similar in their architecture, except for one fewer WD-40 domain in *Ae. aegypti* ARK.

BG4 is also a known activator of caspases in *Drosophila*. The *Ae. aegypti* BG4 ortholog has been previously annotated (Waterhouse et al., 2007). Our bioinformatics analysis yielded a full-length transcript that is represented in NCBI as EAT46931. We were able to verify the full-length *Ae. aegypti* BG4 transcript by RT-PCR from ATC-10 cells.

Another group of proteins involved in caspase activation are the IAP antagonists, including rpr, W (wrinkled), grim and skl in *Drosophila*. These proteins do not share significant similarity, except for a small motif called the IAP Binding Motif (IBM) found at the N-terminus. By using Michelob_x as a query, we identified another protein containing an IBM in *Ae. aegypti*. The EST DV326893 (obtained from D. Severson) contained the transcript with 5' and 3' flanking initiation and stop codons. This gene, which we named IMP, is represented in NCBI as EAT44230.

Because of the low level of similarity between IAP antagonists, we sought to verify that IMP encodes a functional pro-apoptotic gene. The IMP cDNA was cloned into an expression vector and expressed in C6/36 cells by transient transfection. Cells overexpressing IMP were analyzed at 24 h post-transfection for Annexin V staining (a marker for early apoptosis) by flow cytometry (Fig. 3.4A) and for caspase activity by

incubating lysate from transfected cells with the (human) caspase-3 substrate Ac-DEVD-AFC and analyzing AFC fluorescence (Fig. 3.4B). Expression of either Michelob_x or IMP, but not GFP, resulted in higher Annexin V staining (shown by a shift to the right) in a portion of the cells consistent with the level of transfection efficiency routinely observed using these cells (around 20%). There was also a decrease in cell size (shown by a downward shift) upon expression of Michelob_x or IMP, which is also characteristic of apoptotic cells.

In support of the flow cytometry results, we also observed increased effector caspase activity in C6/36 cells expressing Michelob_x or IMP. Lysates from cells expressing either Michelob_x or IMP cleaved significantly higher amounts of the effector caspase substrate Ac-DEVD-AFC during a 60 min incubation period than control cells expressing GFP (Fig. 3.4B). Cells expressing Michelob_x or IMP also exhibited blebbing morphology typical of apoptotic cells (data not shown). Together, these results verify that IMP is a pro-apoptotic protein similar to Michelob_x.

Expression analysis

To examine the expression of these potential regulators of apoptosis throughout the life cycle of *Ae. aegypti*, we employed quantitative reverse transcriptase PCR (RT-PCR). We tested early larvae (pooled L1 and L2 larval stages), late larvae (pooled L3 and L4 larval stages), pupae, and adults. For the adults, we analyzed females only, and analyzed midguts separately from the rest of the adult insect.

Fig. 3.5 illustrates the quantitative RT-PCR results for the genes across the five stages and tissues, with the exception of CASPS15. Although we did not detect expression of CASPS15 in any of the samples we assayed, there are ESTs corresponding

to CASPS15 in NCBI, indicating that it is an expressed gene. The RT-PCR results were normalized by comparison to *act-6* expression and are shown as $2^{-\Delta Ct}$. We observed that expression was highest in the adult female midgut for a majority of the genes, with exceptions being ARK, Dronc, CASPS8 and CASPS20, which showed the highest expression in pupae. The larval stages tended to have the lowest expression for most genes. Caspases were expressed at the highest levels in the midgut. ARK, CASPS17, CASPS21, CASPS20, CASPS19 and CASPS18 did not show Ct values above background in the adult body minus the midgut, while BG4, CASPS17, CASPS21, Dredd, CASPS19 and CASPS18 did not show Ct values above background in pupae. Michelob_x was the only gene that did not show expression above background levels in early larvae (L1/L2). In these cases where Ct values were not above background, it is still possible that these genes are expressed in these stages or tissues, but at low levels. These results suggest that different components of the apoptotic machinery are expressed at varying levels in different developmental stages and tissues in *Ae. aegypti*.

Discussion

This study reports the annotation of a number of potential apoptosis related genes in *Ae. aegypti.* To date, our understanding of apoptosis in insects is based almost entirely on studies from *Drosophila*. This study represents the most thorough attempt to date to identify apoptosis related genes in any other insect. With the exception of IAP1 (Beck et al., 2007a), Dredd (Cooper et al., 2007a), Dronc (Cooper et al., 2007b), and Michelob_x (Zhou et al., 2005a), the genes reported here have not been previously verified by cDNA analysis. *In silico* analysis helps tremendously in annotating genes, but the importance of

verifying cDNA sequences is illustrated by the differences we observed between predicted proteins and cDNA comparisons with ARK, CASPS7, CASPS20 and IAP2. We also identified one additional caspase (CASPS21) that has not been previously annotated. The genes identified in this study hold promise for improving our understanding of apoptotic regulation in the important disease vector *Ae. aegypti*. In addition to elucidating the mechanics of apoptotic regulation, identification of these genes promises to aide in improving our understanding of innate immunity in the yellow fever mosquito. Several of the genes examined in this study are thought to play important roles in *Drosophila* immunity, including Dredd, Iap2, BG4, and Dnr1. It is likely that these genes are also important in *Ae. aegypti* immunity, but further study is needed to determine their exact roles in this process.

Apparent gene duplications have occurred with several caspase genes in both *Ae. aegypti* and *An. gambiae*. At this time it is not clear why mosquitoes possess more caspase genes than *Drosophila*. One possibility is that there is more of a need for caspases in regulating the innate immune responses in mosquitoes, since they may be exposed to more potential pathogens because of their hemophagic life style. For *Ae. aegypti*, these gene duplications are seen in Clade I (CASPS19 and CASPS18) and Clade III (CASPS16, CASPS15, CASPS17 and CASPS21). In Clade I, the *Drosophila* gene decay is expanded in both mosquito species analyzed. In *An. gambiae* there are eight homologs of this gene while *Ae. aegypti* has two homologs. The function of decay has not been studied in detail in *Drosophila* (Dorstyn et al., 1999b), but there may be additional selective pressures for these caspases in mosquitoes. An interesting observation is that CASPS18 has a serine instead of a cysteine in its active site. This

makes it unlikely that CASPS18 encodes a functional caspase. Similar levels of expression were observed for both CASPS19 and CASPS18 throughout the life cycle of *Ae. aegypti*. Thus it is possible that CASPS18 regulates CASPS19 in a dominant-negative manner, similar to what has been shown in humans with caspase-1 being regulated by Pseudo-ICE and ICEBERG (Druilhe et al., 2001).

Clade III includes duplications for both Damm and dream in the mosquitoes. The phylogeny of Damm and dream is interesting, since this appears to be a case where gene duplication occurred and one of the duplicated genes later either acquired or lost a long prodomain sequence. While dream appears to be an initiator caspase, whether Damm is an effector or initiator caspase is not entirely clear. The phylogeny results would suggest that Damm may be an unusual type of initiator caspase, although it is also possible that Damm may not be correctly annotated in any of these insect species. Damm and dream have also not been extensively studied in *Drosophila* (Doumanis et al., 2001; Harvey et al., 2001), and so studies of these duplicated genes in mosquitoes should not only lead to a better understanding of apoptosis and innate immunity in mosquitoes, but also may help in gaining a better understanding of the role of decay, Damm and dream in *Drosophila*.

When IMP is used as a BLAST query, a representative for this gene does not appear in other mosquitoes. In *Drosophila*, the RHG genes share very little similarity, so this is not surprising. However, when Michelob_x from *Ae. aegypti* is used as a query, one can easily find *An. gambiae* and *Ae. albopictus* orthologs of Michelob_x. Thus it appears that IMP is not as well conserved as Michelob_x in mosquitoes. Our results indicate that IMP is a pro-apoptotic gene that likely functions as an IAP antagonist, similar to Michelob_x. In *Drosophila*, there are at least four IAP antagonists (rpr, grim,

W, and skl). Thus it is likely that there are additional IAP antagonists in the genomes of the mosquitoes, but their level of similarity is too low to detect by traditional BLAST searches.

Fig. 3.6 illustrates the differences between mosquitoes and *Drosophila* in the numbers of genes involved in the core apoptotic pathway, based on the pathway that has been established in *Drosophila*. In *Drosophila,* Nc is the main initiator caspase that activates Ice and Dcp-1, which ultimately leads to death. Thread has been shown to inhibit both Nc and Ice, and IAP antagonist interactions with IAP proteins have also been studied extensively (Chai et al., 2003; Yan et al., 2004). According to this simplified model, thread is required to prevent the accumulation of active Nc, which appears to be constitutively activated in an ARK-dependent manner. IAP antagonists interrupt the interaction between Nc and thread. This interaction frees Nc, which is then able to activate Ice, resulting in apoptosis (Hay et al., 2006). Fig. 3.6C illustrates the high amount of divergence among the IAP antagonists in different insects. However, even though these genes are extremely poorly conserved except for their IBM motifs, the genes still cluster in a species-specific manner. In Fig. 3.6D we illustrate the expansion that has occurred for the effector caspases in mosquitoes. Another interesting phenomenon is that *An. gambiae* has two paralogs of thread, while *Ae. aegypti* has only one version of this gene, as does *Drosophila*. While the effector caspases and IAP1 genes have duplications in mosquitoes, other genes that make up the core apoptotic pathway have not gone through a duplication event, such as the initiator caspase Nc and the adaptor protein ARK.

For a productive infection and transmission cycle to occur, pathogens such as arboviruses and *Plasmodium* must overcome many barriers in the mosquito vector. These pathogens must establish infection of the midgut epithelium and replicate in these cells, escape from this barrier by passing through the basal lamina, replicate efficiently in other organs, and finally infect the salivary glands and penetrate into the lumen of the salivary glands for transmission (Black et al., 2002). If any one of these barriers is not crossed, the pathogen cannot establish infection and be transmitted from mosquito to a vertebrate host. Interestingly, the midgut showed higher expression for many of the presumptive apoptotic regulating genes as compared to the other tissues or stages. Whether this apparent high level of expression is due to differences in *act-6* expression between tissues or stages is not known at this time (since transcript levels were normalized against *act-6*). In addition, it must be kept in mind that the level of protein may not correspond to the level of transcript. Especially intriguing is that expression of IAP1 was much higher in midgut than in any other tissue or stage analyzed. Based on the known crucial role of thread and SfIAP in preventing spontaneous apoptosis in *Drosophila* and *Spodoptera frugiperda* cells, respectively (Igaki et al., 2002; Muro et al., 2002; Zimmermann et al., 2002), it is tempting to speculate that *Ae. aegypti* IAP1 might have a similar role in regulating apoptosis. This needs to be determined, but if correct, then high levels of IAP1 expression may function to protect midgut cells from spontaneous apoptosis. It has been shown in *An. stephensi* (Han et al., 2000; Abraham et al., 2004), *An. gambiae* (Vlachou et al., 2004), and *Ae. aegypti* (Zieler et al., 2000) that apoptosis occurs during the establishment of infection in the midgut by different *Plasmodium spp*. It has also been demonstrated in *An. gambiae* by microarray analysis

that transcript levels of the thread homologs AgIAP3 and AgIAP4 are up-regulated during midgut invasion by *P. berghei* and down-regulated after invasion (Vlachou et al., 2005). This suggests that, similar to thread and SfIAP, down regulation of AgIAP3 and AgIAP4 by *Plasmodium* infection may play a role in triggering apoptosis in midgut cells. Depending on the pathogen, regulation of IAP1 levels could possibly play a role in establishment of infection of *Ae. aegypti* midgut by *Plasmodium* and/or arboviruses.

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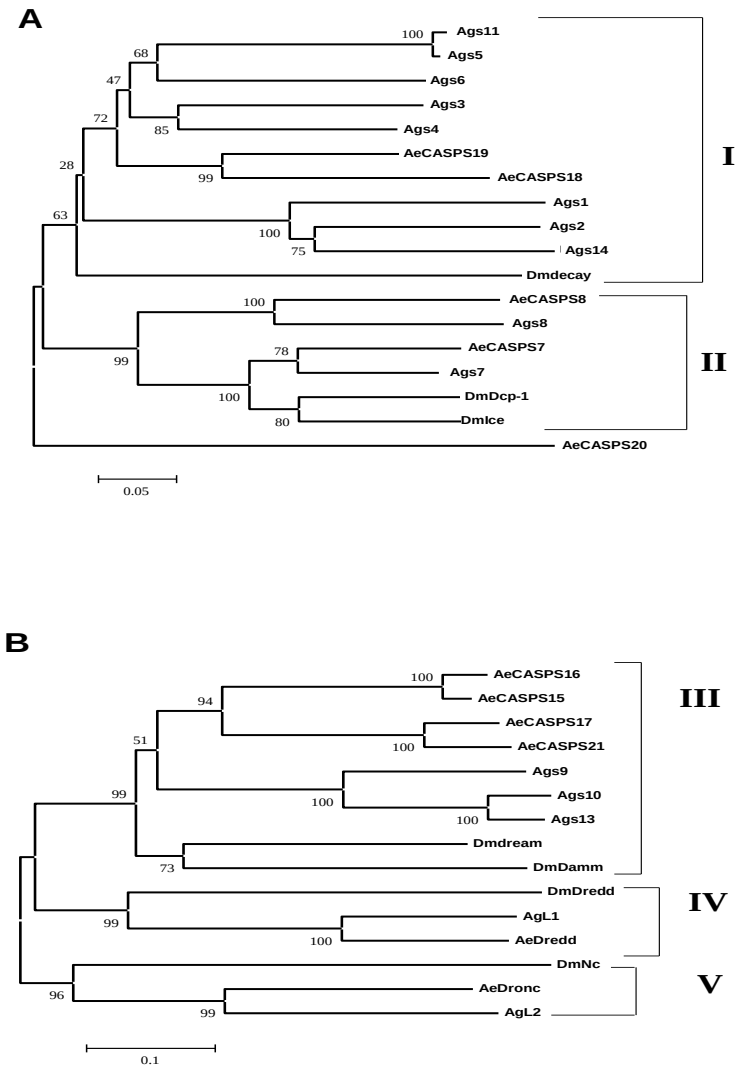

Figure 3.1 Phylogenetic analysis of caspases in *Drosophila*, *An. gambiae*, and *Ae. aegypti*.
Full length amino acid sequences were used to build phylogenetic trees using MEGA 3.1. Panel A includes effector caspases, while panel B includes initiator caspases and caspases related to Damm, which is predicted to be an effector caspase based on its short prodomain, but which groups with initiator caspases. Clades were determined by branching patterns and are represented as vertical lines on the right. Bootstrap values are shown.

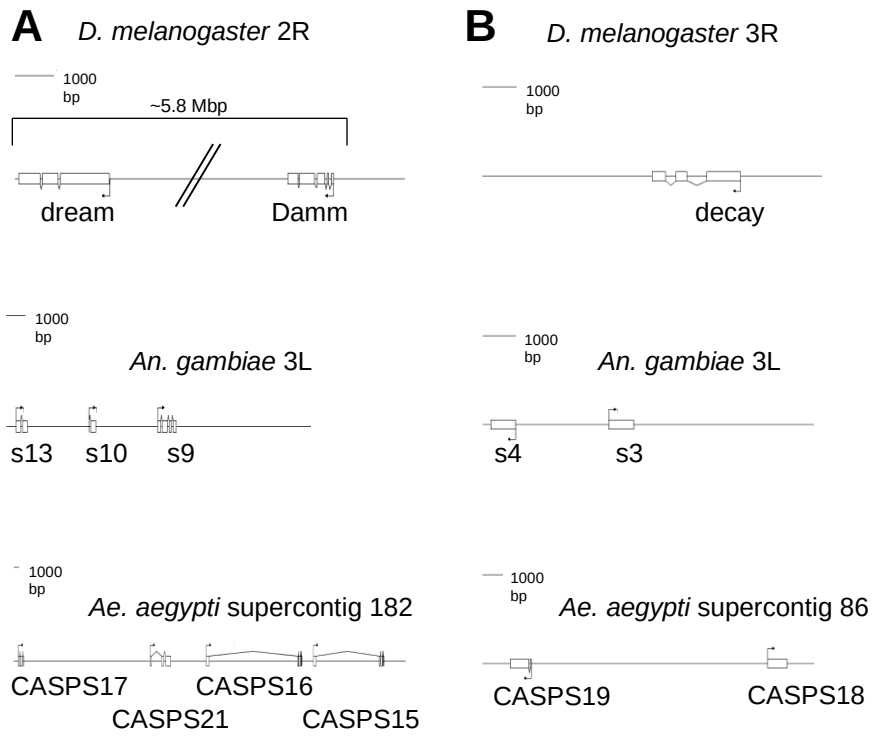

Figure 3.2 Genome architecture of caspase genes within three dipteran species
A, Genome organization of Damm and dream in *Drosophila* and their paralogs in *An. gambiae* and *Ae. aegypti*. **B**, Genome organization of Decay in *Drosophila* and its paralogs in *An. gambiae* and *Ae. aegypti*. Genome information was obtained from websites for each organism, as explained in Materials and Methods. The illustrations were produced using GenePalette.

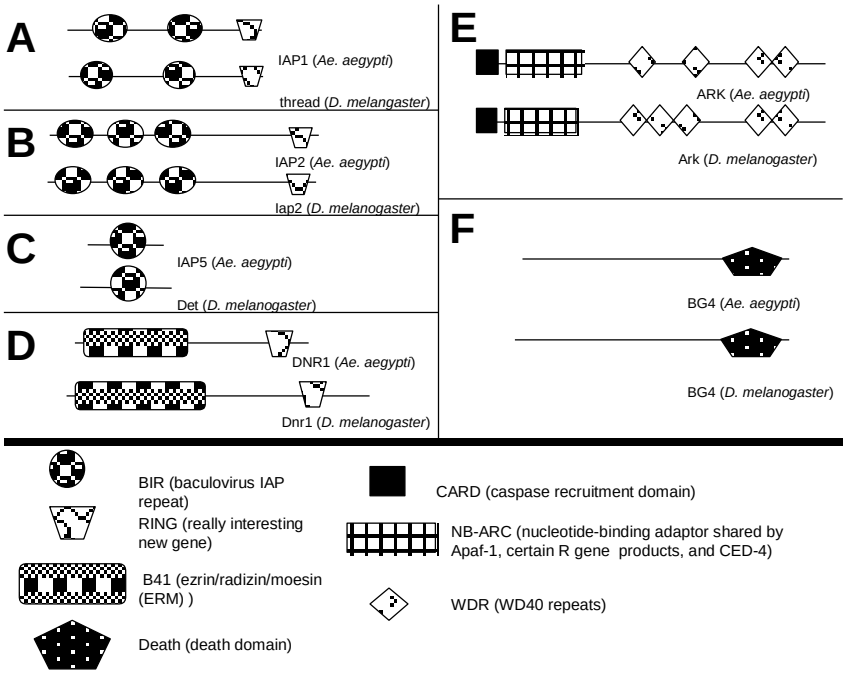

Figure 3.3 Predicted domain architecture for some of the proteins identified in this study.
Protein domains were predicted using SMART. Proteins for *Drosophila* were obtained from NCBI and FlyBase.

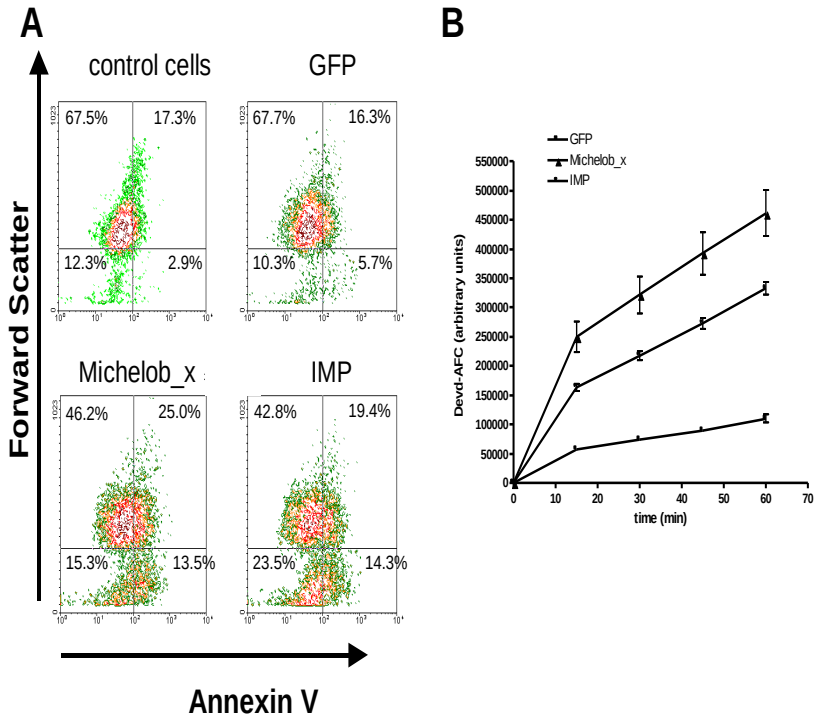

Figure 3.4 Expression of IMP or Michelob_x causes apoptosis in C6/36 cells.
A, AnnexinV staining (X-axis) versus forward scatter (Y-axis) of untransfected (control) cells or cells transfected with constructs expressing GFP, Michelob_x, or IMP, as analyzed by flow cytometry. Percentages are given for each quadrant. Each graph represents analysis of 10,000 cells. **B,** caspase activity in lysates from cells expressing GFP, Michelob_x, or IMP as determined by liberated AFC fluorescence over 60 min incubation with the caspase substrate Ac-DEVD-AFC. The data shown represent the combined results from three independent transfections.

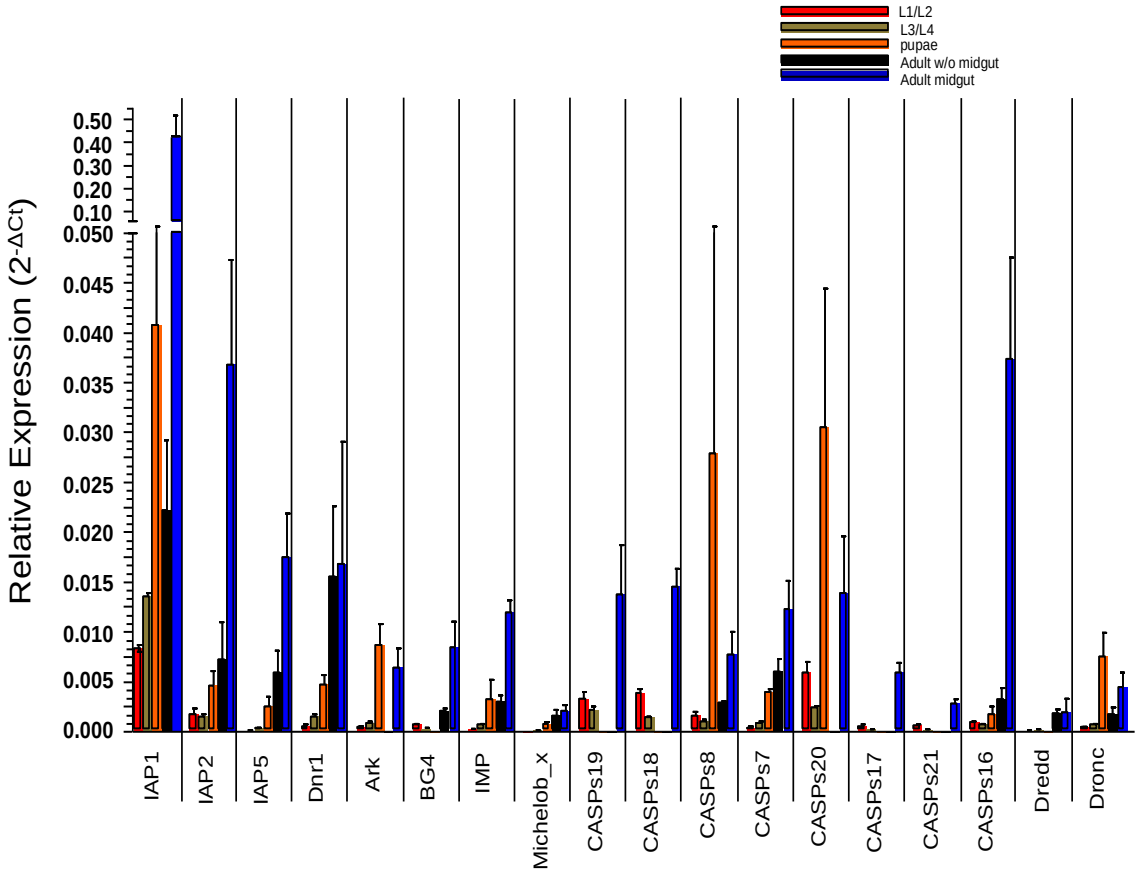

Figure 3.5 Quantitative analysis of transcript levels for the annotated apoptosis regulatory genes in *Ae. aegypti* throughout the life cycle of the organism.
The results of real time RT-PCR analysis in early larvae (L1/L2), late larvae (L3/L4), pupae, adult females without midgut, or adult female midgut are shown. Each data point represents the average $2^{-\Delta Ct}$ (+/- SE) obtained from three batches of cDNA made from each stage or tissue.

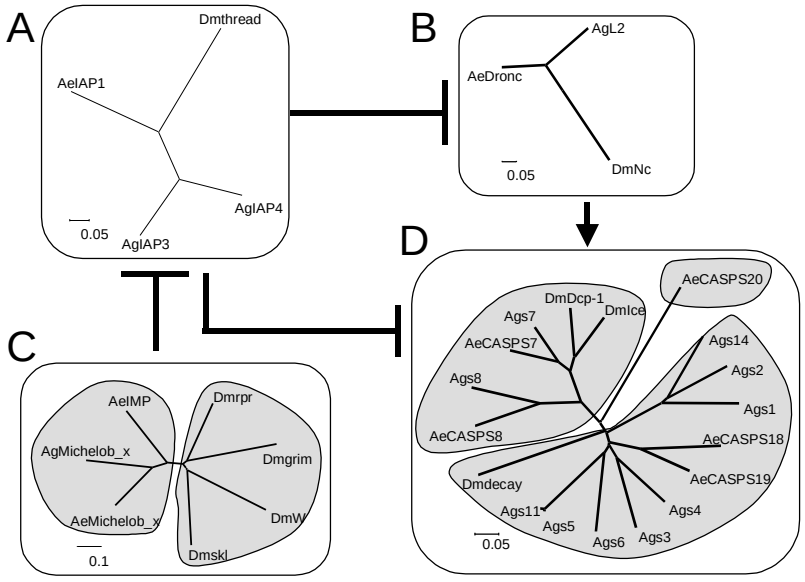

Figure 3.6 Illustration of the conservation of core apoptosis regulatory genes within *Drosophila, An. gambiae* and *Ae. aegypti*.
The unrooted trees were built using neighbor end joining, complete deletion, p-distance with full amino acid sequences. The pathway illustrated is based on information obtained from studies using *Drosophila*. In *Drosophila*, thread inhibits Nc and Ice, while thread itself is inhibited by the IBM-containing proteins rpr, W, and grim. The initiator caspase Nc is responsible for cleaving and activating effector caspases including Ice and Dcp-1. Nc is activated by Ark, which for simplicity is not shown but which is also conserved in *Ae. aegypti*.

Table 3.1 *Ae. aegypti* apoptotic regulatory genes characterized in this study. Shown are the NCBI predicted proteins (if any), supercontigs the genes reside on, percent amino acid similarity and identity to the closest relative in *Drosophila*, and whether the confirmed sequence differs from that predicted in NCBI.

Gene	NCBI protein(s)	Supercontig(s)	%Similarity/Identity	Different from predicted?
IAP1	ABK01289	368	51.7/39.4 (th)	No
IAP2	EAT41756	214	46.8/34.3 (IAP2)	Yes
IAP5	EAT33476	1049	50.6/41 (det)	No
DNR1	EAT48387	11	45.9/35.1 (Dnr1)	No
ARK	EAT48065, EAT48066	18	30.4/18.8 (Ark)	Yes
BG4	EAT46931	46	35.1/21 (BG4)	No
IMP	EAT44230	117	10.9/7 (rpr)	No
CASPS19	EAT45302	86	53.8/37.8 (decay)	No
CASPS18	EAT45303	86	47.9/37.4 (decay)	No
CASPS8	EAT33369	1085	47.7/38.3 (Ice)	No
CASPS7	EAT35718	791, 659	61.1/52.5 (Ice)	Yes
CASPS20	EAT33088	1207	36.1/28.1 (Ice)	Yes
Dredd	EAT33580	1019	46.6/31.3 (Dredd)	No
Dronc	EAT36368	589	42.4/30.3 (Nc)	No
CASPS17	none	182	35.4/25.3 (Damm)	Yes
CASPS21	none	182	36.9/25 (Damm)	Yes
CASPS16	EAT42502	182	32.9/23.3 (dream)	No
CASPS15	EAT42503	182	34.2/24 (dream)	No

Table 3.2 Bioinformatic analysis overview for the genes characterized in this study
Shown are the overlapping ESTs used to determine the transcript (if any), whether the ESTs yielded a full-length transcript, whether the transcript could be verified by RT-PCR from the ATC-10 cell line, and whether gene prediction programs were employed for each gene.

Gene	ESTs used	ESTs full-length?	Amplify from ATC-10 cell line?	Gene prediction used?
IAP1	NA[a]	NA	Yes	No
IAP2	dv383781 eb090402	No	Yes	Yes/GenScan
IAP5	dv364268	No	Yes	Yes/GenScan
DNR1	dv237984 eg007533 eb089278 dw221134 dw205154	No	Yes	No
ARK	NA	NA	Yes	No
BG4	dv416615 dv416614 dv383790	Yes	Yes	No
IMP	dv326893	Yes	ND[b]	No
CASPS19	dv395012 dv330266	Yes	No	No
CASPS18	NA	NA	ND	No
CASPS8	dv250139 dv250137	Yes	Yes	No
CASPS7	dv369010	No	Yes	Yes/GenScan
CASPS20	dw202685 ee999223	Yes	ND	No
Dredd	dv382387 dv382385 dw190212	Yes	Yes	No
Dronc	dw220447 dw207394 dv343751 dv356662	Yes	Yes	No
CASPS17	dv323242	No	No	Yes/fgenesh
CASPS21	NA	NA	ND	Yes/fgenesh
CASPS16	dv241054 dv332892 dv328064	Yes	No	No
CASPS15	NA	NA	ND	Yes/fgenesh

[a]NA, not applicable

[b]ND, not determined

Table 3.3 Primers utilized for expression analysis of presumptive apoptotic regulators of *Ae. aegypti*.

Gene	Forward	Reverse
IAP1	CTGAAACTAATGAAGGGCGAAGC	TTGAGATGACTGAAGCGAGGATG
IAP2	CCATTATCGTCGCCGTCTACC	CTTTCAGTCGTTTGTTCTCCTCTTC
IAP5	ACGACAAGGAGGACGAAGAC	TCCAGCAGGTTAAGCATTTCC
DNR1	GAAGATACTGAACTCGGCAAACG	CGGCAGGCGGTAATATGTCC
ARK	TGTCTAGCGTTTCGGTCTTGAG	GCGTTGGTTAGCCTGGATAATAATC
BG4	ACTTTGCCTGCTCAATTTCTTTCTC	GATACGCTGTTCTCCCTGTTGG
Michelob_x	CAACAGCAAAATCAGAACCAAATCAG	GCACAGCAGACATCGGGAAC
IMP	GCTGGACTGAGAACGCCTTC	ACGACTGATGAGAACAACAACAAC
CASPS19	CTCGCCGTGTGACATCATAAC	AAGCAAGGAAGTTCTCGTTTCTC
CASPS18	CTGTCTTGTGGTAGTTGTGATGTC	CGGATGCTTGTGATTCTTCTTCTC
CASPS8	TGGCAAAAGCAAACAGGAAGTC	GGGATGAAGGCGAAGTAATATACG
CASPS7	TTGGCAGAACGCACCGAAAC	CGAAAGTCAGCAGGGTCAGTAG
CASPS20	GCGGATTGCCTGATGGTATTC	ATGCTTGGACTATGAACAACTTCG
Dredd	AGAAGTATGTAATATCGTGGAAGAATGC	AGAACAGTGATGCGGCTCAAC
Dronc	CAACTTTCCAACTGCCTATAAATTGC	CTCCACCGTATCGTTATTGTTCTTAG
CASPS17	TGCCATTGATGAGAAGAGAATTTGAG	GCCTACTTGTCCCGTGTTACC
CASPS21	CGATTGTAATAAAACGGTTCCTAGTCC	CTATTGACATTTCTGGCATCTCTCTTAG
CASPS16	TCCGCTATCTTCATATTGTATCCTTTG	GACCCGCCACTGTATCTCTG
CASPS15	CCTAACTTGGGTTTGACGATTGC	AATGTCCGCTATCTTCATATTGTATCC
Act-6	AAGGCTAACCGTGAGAAGATGAC	GATTGGGACAGTGTGGGAGAC

CHAPTER 4 - Evolution of caspases and caspase-like decoy molecules in the insect order *Diptera*

Bryant B., Ungerer M.C., Waterhouse R.W., Clem R.J. Evolution of caspases and caspase-like decoy molecules in the insect order *Diptera*. This work has been submitted.

Abstract

Apoptosis is a highly conserved process among metazoans, and is involved in diverse processes such as development, tissue homeostasis, and immunity. Caspases, a family of cysteine proteases, play a critical role in carrying out apoptosis, as well as other cellular processes. We analyzed the evolutionary relationships between caspase genes from fifteen dipteran insect genomes by phylogenetic analysis and found that the caspases fell into six clades, with three clades each of initiator and effector caspases. Two of the initiator caspase clades were conserved as single copy genes in all of the genomes analyzed, but the other four caspase clades included multiple gene duplication/losses, which were found even among certain *Drosophila* species. Interestingly, in several dipteran species we found examples of recently duplicated caspase paralogs with a lack of critical residues involved in catalysis. Similar types of duplicated caspases, also known as caspase-like decoy molecules, have been previously found in mammals and nematodes, where they have been shown to regulate caspase activity both negatively and positively. We examined the activity of one of these caspase-like decoy molecules, and found that it increased the activity of its paralog. This is the first report of caspase-like decoy molecules in insects and the first functional data indicating that these invertebrate caspase-like decoy molecules can positively regulate their paralogous caspases.

Introduction

Caspases are a conserved family of cysteine proteases, which play important roles in apoptosis and in other cellular processes. These enzymes are initially expressed as zymogens that are later cleaved to release large and small subunits, as well as a prodomain of varying length. The large and small subunits heterodimerize to form the active site, and two of these heterodimers then further dimerize to form the active caspase holoenzyme. There are two types of caspases, initiator and effector, and usually the length of the prodomain determines the type of caspase, with initiator caspases having longer prodomains than effector caspases. Initiator caspases are the first to be activated following an apoptotic stimulus. The activation of initiator caspases is dependent on adaptor molecules, which bind to the prodomain and are thought to promote dimerization and autocatalysis (reviewed in Bao et al., 2007). Once an initiator caspase is activated, it cleaves and activates effector caspases, which then cleave numerous substrates and dismantle the cell.

The genome of the model insect *Drosophila melanogaster* contains seven caspases, including the initiator caspases Dronc, Dredd, and Strica, and the effector caspases Damm, DrICE, Dcp-1, and Decay. Mechanisms of caspase activation in *Drosophila* are reviewed elsewhere (Riedl et al., 2004; Shi, 2004; Hay et al., 2006) with a recent review of insect caspases which reviews insect caspases other than *Drosophila* (Cooper et al., 2009). Of the caspases found in *D. melanogaster*, Dronc and DrICE seem to be the most important players in apoptosis (Fraser et al., 1997b; Quinn et al., 2000; Chew et al., 2004; Daish et al., 2004; Xu et al., 2005; Muro et al., 2006). Dredd is important in innate immunity (Leulier et al., 2000), while Strica seems to be involved in

ovary death along with Dronc in a redundant manner (Baum et al., 2007), and the function of Damm is unclear (Harvey et al., 2001).

While these studies outline Dronc and DrICE as the main caspases involved in death, there have also been reports of these caspases having non-apoptotic roles. One in particular is proliferation induced by overexpression of reaper in the presence of the baculovirus protein P35 to inhibit cell death (Huh et al., 2004). This study illustrated that stimulation of cell death in the absence of death results in proliferation and ectopic expression of Wingless, a mitogen in the wing, and Dronc is needed for this process. DrICE has been illustrated to participate in the non-apoptotic process of spermatid differentiation (Muro et al., 2006), and Dcp-1 has been shown to be involved in autophagic (Hou et al., 2008) and stress-induced death (Laundrie et al., 2003) of ovaries.

In mosquitoes, caspases from mosquitoes have been shown to be transcriptionally regulated by chloroquine in *Anopheles gambiae* (Abrantes et al., 2008) and Dengue virus infection in *Aedes aegypti* (Xi et al., 2008), but to date only two mosquito caspases have been examined experimentally. *Ae. aegypti* Dronc was shown to be up-regulated by ecdysone (Cooper et al., 2007b) while *Ae. aegypti* Dredd was shown to be up-regulated by UV insults (Cooper et al., 2007a).

Recently we analyzed the phylogeny of caspases from *D. melanogaster*, *Ae. aegypti*, and *An. gambiae* (Waterhouse et al., 2007; Bryant et al., 2008). From this analysis we found several interesting trends: while we were able to find clear orthology for the main initiator caspases Dronc and Dredd, clear orthologs to the effector caspases were problematic due to gene duplications/losses in mosquitoes. For the Damm/Dream and DrICE/Dcp1 clades, the caspases had duplicated within each individual mosquito

species after the divergence of flies from mosquitoes, which made assigning orthologs impossible. There was also a single effector caspase from *Ae. aegypti*, CASPs20, that was an ancestral outgroup without orthologs in either *Drosophila* or *Anopheles*, suggestive of a gene-loss in all these species.

We and others have postulated that this trend of gene loss/duplications might be especially prevalent in mosquitoes, but this was based on only one fruit fly and two mosquito species, on opposite ends of the insect order *Diptera*. Here we have taken advantage of the recent genome sequencing of eleven additional *Drosophila* species (Clark et al., 2007) and a third mosquito species, *Culex pipiens quinquefasciatus* and analyzed the caspases in these genomes. The results revealed multiple gene duplications even among the *Drosophila* lineage. We found clear orthology in all fifteen genomes only for the initiator caspases Dronc and Dredd. All of the other caspase clades exhibited extensive gene losses and/or duplications. In addition, our analysis revealed the presence of several caspase-like decoy molecules in both mosquitoes and *Drosophila* species. This is the first report of caspase-like decoy molecules in insects, which are not present in the genome of the model organism *D. melanogaster*.

Methods

Database mining

Caspases from *D. melanogaster* were used as queries against the other eleven available *Drosophila* species at Flybase (http://flybase.org/) to obtain gene models for caspases from the eleven genomes. *An. gambiae* and *Ae. aegytpi* caspase gene models

were obtained previously (Bryant et al., 2008). The *Culex p. quinquefasciatus* genome was mined for caspases at the BROAD institute (http://www.broad.mit.edu/annotation/genome/culex_pipiens/Home.html). The most up to date gene models for mosquitoes were obtained at http://cegg.unige.ch/Insecta/immunodb/. Gene models were then checked by determining domains at SMART (http://smart.embl-heidelberg.de/) and ExPASY Prosite (http://ca.expasy.org/prosite/). ESTs were used when gene models were questionable and other gene models were corrected using the trace archives at NCBI.

Phylogenetic analysis

Amino acid sequences from the region surrounding the active site were used for alignment. Alignment was performed using Clustal W using MEGA3.1 (Kumar et al., 2004b). This alignment file was then used to build NJ trees using PAUP software (Swofford, 2002).

Overexpression analysis

cDNA from adult *Ae. aegypti* RexD mosquitoes was used as a template to isolate the full length cDNAs of *AeCASPs18*, *AeCASPs19* and *AeIAP1*. Decay from *D. melanogaster* was obtained as an EST from Flybase (AT03047) (http://flybase.org/). Caspases were cloned into the expression plasmid pHSP70PLVI+*Rpr*-epi (Vucic et al., 1997) by replacing *Rpr* with each caspase, yielding pHSP70PLVI+*AeCASPs19*-epi, pHSP70PLVI+*AeCASPs18*-epi, and pHSP70PLVI+*Decay*-epi where epi indicates the presence of a C-terminal HA tag. *AeIAP1* was cloned into pHSP70PLVI+epi-*Opiap* by replacing *Opiap* with *AeIAP1,* yielding pHSP70PLVI+epi-*AeIAP1*. Transfections were done in C6/36 cells as previously described in (Bryant et al., 2008). After transfection,

cells were analyzed for caspase activity by incubating lysate from transfected cells with human caspase-3 substrate (DEVD-afc) for 15 minutes, after which consecutive measurements were obtained as previously described (Bryant et al., 2008).

Results

Caspase gene sequences were obtained from fifteen dipteran genomes and the most conserved sequences from each caspase, consisting of a region of ~195 amino acids surrounding the active site (Fig. 4.1) were aligned. The aligned sequences were then subjected to phylogenetic analysis, which yielded six different clades, three initiator and three effector (Fig. 4.2). In each clade we modeled after the *D. melanogaster* genes, since these are the only caspases with functional data. A neighbor joining tree is shown in Fig. 4.2 and the gene number per clade for each species is illustrated in Fig, 4.3.

Initiator caspases

Initiator Clade I, the Damm/Dream clade, had the most gene duplications/losses of the clades examined. Even within the genus *Drosophila*, not all species of *Drosophila* had a clear ortholog for either Damm or Dream. Species phylogenetically close to *D. melanogaster* had clear orthologs, but species outside of the *obscura* and *melanogaster* groups had multiple gene duplications/losses. For the mosquitoes, it was impossible to assign clear orthology. In *C. p. quinquefasciatus*, as previously shown for the other two mosquitoes (Bryant et al., 2008), these caspases have duplicated after the divergence of these species. For *An. gambiae*, s10 and s13 appeared to be haplotypes of the same gene, so s10 was omitted from the analysis.

Initiator Clades II and III, which include Dredd and Dronc, respectively, differed from Initiator Clade I in terms of pattern of evolution. The observation that losses or duplications of these two genes have not occurred in any of the species analyzed suggests that strong purifying selection exists for these two particular caspases across the order of *Diptera*. Two problems arose with the available gene models for Dredd caspases. These were FBpp0214869 in *D. simulans* and CpCASPL1 in *C. p. quinquefasciatus*. These models were reassessed by analyzing the trace archives at NCBI, and the improved gene models grouped appropriately in Init Clade II (Fig. 4.2).

Effector caspases

Effector Clade I includes *D. melanogaster* Decay. Unlike the other effector clades, Decay seems to have 1:1 orthology among all the *Drosophila* species examined. However, these genes have expanded in mosquitoes. CASPs19 seems to be shared among both *Culicinae* mosquitoes, while gene duplications have yielded the additional Decay paralogs CASPs18 in *Ae. aegypti* and CpCASPs30 and s31 in *C. p. quinquefasciatus*. In *An. gambiae*, gene duplications have resulted in seven copies for this clade. *An. gambiae* s5 and s11 appeared to be haplotypes of the same gene, so s11 was excluded from the analysis. Effector Clade I was the the only effector clade that had 1:1 orthology among the twelve *Drosophila* species, but contained gene expansions in mosquitoes.

Effector Clade II includes the main apoptotic effector caspases Dcp-1 and DrICE from *D. melanogaster*. Duplications for Dcp-1 have occurred in the *obscura* group (*D. pseudoobscura* and *D. persimilis*), while duplications for DrICE werefound in *D. grimshawi* and *D. willistoni*. Within Effector Clade II, there was also an ancestral cluster

of caspases among the *Drosophila* group (*D. virilis* FBpp0237835, *D. mojavnesis* FBpp0172086 and *D. grimshawi* FBpp0147044). This could be due to either duplications that have occurred just within these three species, or it could be due to gene loss in the other *Drosophila* species. While the first option is most parsimonious, this type of gene loss has been shown for vertebrate caspases (Eckhart et al., 2008). Amongst the mosquitoes, there was one copy for CASPs7 in all three mosquitoes analyzed, suggestive of this caspase being under more selective constraint than CASPs8, which in *C. p. quinquefasciatus* has duplicated, yielding caspases CpCASPs8 and s29. These duplications for these effector caspases suggest that these genes are not under as much constraining selective pressure as the initiator caspases Dronc and Dredd.

Effector Clade III, which contains CASPs20 from *Ae. aegypti*, has been lost from all of the *Drosophila* species examined and from *An. gambiae*, but six paralogs of CASPs20 are present in *C. p. quinquefasciatus*. Additional species will need to be examined to determine whether Effector Clade III is truly specific to the *Culicinae*.

Gene duplications leading to the evolution of putative caspase-like decoy molecules

Caspases cleave cellular substrates and this enzymatic activity is required in both apoptotic and non-apoptotic pathways. Certain residues are highly conserved in the active site of caspases, as shown in Fig. 4.1. The core active site includes the residues QAC(R/Q/G)(G/E). Amino-terminal to the active site are the highly conserved residues Arg, His and Gly and carboxy-terminal to the active site is another conserved Arg. In combination, these key residues are responsible for substrate recognition and catalysis. Numerous studies have shown that when these residues are mutated, catalytic activity is altered (reviewed in Fuentes-Prior et al., 2004).

We found extreme conservation of these critical residues in most of the caspases examined. However, eight of the 132 caspases we analyzed exhibited substitutions in one or more of these residues, and several of these appeared to be products of gene duplication events. These caspases and their amino acid substitutions are summarized in Table 4.1, and the phylogenetic relationships with their paralogs are shown in Fig. 4.2.

Genes FBpp0147044_grimshawi and FBpp0172086_mojavensis both lack critical residues. By phylogenetic analysis, these appear to be duplications from caspases in Effector Clade II, but neither gene resides in the genome near another paralogous gene found in this clade. However, *D. mojavensis* has three copies in this clade and *D. grimshawi* has four copies (Fig. 4.3). Even though for each species these paralogs are not on similar contigs, both species possess more copies than *D. melanogaster* (two copies) suggestive of possible duplications within these species.

FBpp0170482_mojavensis, FBpp0247059_willistoni, and FBpp0237835_virilis are lacking one or both of the conserved Arg residues. These genes appear to be recent duplications, since they each reside near paralogs, which are predicted to be functional caspases (Table 4.1). This phenomenon was also observed in the mosquitoes *Ae. aegypti* and *C. p. quinquefasciatus*. In *Ae. aegypti*, CASPs18 contains a Ser instead of a Cys and a Lys instead of an Arg and appears to be a duplication of CASPs19. In *C. p. quinquefasciatus*, CpCASPs31 lacks the conserved His, Cys and Arg residues and resides near CpCASPs30 and s19. Phylogenetic analysis suggested that CpCASPs30 and s31 are also duplications of CpCASPs19. Similarly, CpCASPs24 lacks conserved Cys and Arg residues while the paralogs CpCASPs25 and s26 contain these critical residues, and all three genes are relatively close together in the genome, suggestive of recent duplication.

The caspase-like decoy, AeCASPs18, enhances AeCASPs19 activity

We reported previously that both CASPs18 and CASPs19 in *Ae. aegypti* are expressed and have similar developmental expression patterns (Bryant et al., 2008). To examine the function of these caspases, CASPs18 and CASPs19, along with *D. melanogaster* Decay, were expressed in *Aedes albopictus* C6/36 cells. Caspase activity was measured using DEVD-afc, an effector caspase substrate. High levels of activity were observed when CASPs19 was expressed, while expression of the other caspases did not result in measurable activity (Fig. 4.4A). Interestingly, however, the cells expressing CASPs19 did not die, despite the presence of a large amount of effector caspase activity. This activity was inhibited when CASPs19 was co-expressed with AeIAP1 (Fig. 4.4A).

When CASPs19 and CASPs18 were co-expressed, we observed an enhancement of caspase activity (Fig. 4.4B), even though CASPs18 had no activity when expressed alone. Again, cell death was not observed, suggesting that the increased activity was not due to the activation of other effector caspases, but to an increase in the amount or activity of CASPs19. Similar results were observed when the caspases were expressed in the lepidopteran cell line SF-21 from *Spodoptera frugiperda* (data not shown), suggestive of a conserved mechanism.

Discussion

This study illustrates that, unlike previously postulated, expansion of caspase genes in insects is not specific to mosquitoes; even within the genus *Drosophila*, initiator and effector caspases have undergone gene expansions when compared to *D. melanogaster*. Logically, the caspase genes in the species further away from *D.*

melanogaster have undergone more gene duplications/losses. Interestingly enough, all fifteen dipteran genomes analyzed contained single copies of the initiator caspases Dronc and Dredd, suggestive of strong purifying selection for these caspases. The *Ae. aegypti* ancestral effector caspase CASPs20 was found to have undergone expansion in *C. p. quinquefasciatus,* while CASPs20 has apparently been lost in the *Drosophila* and *An. gambiae* genomes. This type of gene duplication/loss has also been reported in the twelve *Drosophila* species for other gene families, with over 40% of all gene families analyzed differing in size (Hahn et al., 2007) and with Caspase-18, 17 and 15 in vertebrates (Eckhart et al., 2008). Humans have lost these caspases while other vertebrate species have retained these genes in their genomes. Initiator Clade I proved to be the most active in terms of gene duplications/losses for initiator caspases. This type of evolutionary pattern, where there is species specific expansions, is similar to what is found with genes involved in innate immunity in insects (Sackton et al., 2007; Waterhouse et al., 2007). We also found that the main effector caspase DrICE from *D. melanogaster* was not as constrained as Decay. This suggests that Decay may play a more important role than currently appreciated, possibly in a non-apoptotic process.

Duplications that result in inactive enzyme homologs have been observed for many other enzyme families including protein tyrosine phosphatases, superoxide dismutase, phospholipase A2, amongst others. These duplicated enzymes have often been shown to regulate their functional counterpart (Pils et al., 2004). This is the first report of insect caspase-like decoy molecules, which are not present in the model organism *D. melanogaster*. In the model organism *C. elegans,* however, this type of molecule has been recently reported and shown to regulate the caspase CED-3 (Geng et

al., 2008). We found these caspase-like decoy molecules to be present in the *Drosophila* species *mojavensis, willistoni, grimshawi* and *virilis* and in the mosquitoes *Ae. aegypti* and *Culex p. quinquefasciatus,* but not in *An. gambiae*. The human caspase-1 and caspase-8 genes have undergone duplications, resulting in caspase-like decoy molecules. Duplication of caspase-8 resulted in c-FLIP (also called CFLAR) and this paralog is very important in regulating caspase-8 in both an inhibitory and activating manner (Micheau et al., 2002; Budd et al., 2006). Caspase-1 duplications resulted in COP1, INCA and ICEBERG, which regulate caspase-1 in a dominant negative manner (Humke et al., 2000; Druilhe et al., 2001; Lee et al., 2001; Lamkanfi et al., 2004; Lamkanfi et al., 2007). Unlike the paralogs for caspase-1, caspase-8 and CED-3, which are all initiator caspases, we report an effector caspase (CASPs19) with a paralog (CASPs18) that is predicted to be inactive, but enhances the activity of its functional counterpart. This is the first report of an effector caspase in any system possessing this type of regulation, and the first report of caspase-like decoy molecules in insects.

This discovery of caspase-like decoy molecules may potentially lead to an identification of a new regulatory process of caspase regulation in insects, however these molecules were not found in the model organism *D. melanogaster.* The fact that these caspase-like decoy molecules are only present in only six out of the fifteen dipteran genomes, raises the question of whether this is this a random evolutionary event or whether it is driven specifically for particular genes in particular species. For example, in *Culicinae* mosquitoes there are caspase-like decoy paralogs for decay. Perhaps for these mosquitoes there is a need for this novel regulation for decay that is not needed in *Drosophila* or *Anopheles*. Alternatively, it may be that these genes are under strong

purifying selection in *Drosophila*, preventing duplications in fruit flies, while in mosquitoes these genes are under relaxed pressure, which leads to duplications, eventually producing these caspase-like decoy molecules. This latter hypothesis is supported by the fact that there is just one copy of decay in all *Drosophila* species analyzed, while all the mosquitoes have undergone gene expansions. The discovery of these molecules in *Culicinae* raises the question of whether these caspase-like decoys are expressed and whether they regulate their parental gene.

Overexpression of CASPs19 from *Ae. aegypti* results in high levels of effector caspase activity, but does not result in apoptosis. This is despite the fact that overexpression of the effector caspases CASPs7 or s8 causes apoptosis (data not shown). We also found that CASPs19 activity could be inhibited by the IAP1 protein from *Ae. aegypti*, which is similar to the ability of *D. melanogaster* DIAP1 to inhibit caspases, suggestive of conservation of caspase regulation. Interestingly, CASPs18, which has a Ser instead of a Cys in the active site, actually enhanced caspase activity when co-expressed with CASPs19 as compared to CASPs19 alone. The mechanism of this enhancement is currently unknown, but presumably it is due to enhancement of CASPs19 activity, as opposed to activation of other effector caspases in the transfected cells, because activation of other effector caspases would likely result in death. Several possible mechanisms can be envisioned for how CASPs18 could enhance CASPs19 activity in either a *cis* or *trans* manner. In a *cis* manner, CASPs18 could heterodimerize with CASPs19, resulting in a more catalytically active caspase than a homodimer of CASPs19. Also we cannot rule out the possibility that CASPs18 has catalytic activity itself, which somehow becomes activated when co-expressed with CASPs19. In *trans*,

there may be a cellular inhibitor of CASPs19, and CASPs18 acts as a decoy for this inhibitor, leading to more active CASPs19. It has been shown that DIAP1 in *Drosophila* regulates caspases by ubiquitination (Wilson et al., 2002; Ditzel et al., 2008). Thus an attractive mechanism would be that CASPs18 acts as a decoy molecule for AeIAP1, leading to more active CASPs19 molecules. Further work is needed to decipher the mechanism of enhancement of CASPs19 by CASPs18. The discovery of these caspase-like decoy molecules in several dipteran insects provides new avenues for uncovering novel mechanisms of caspase regulation in insects.

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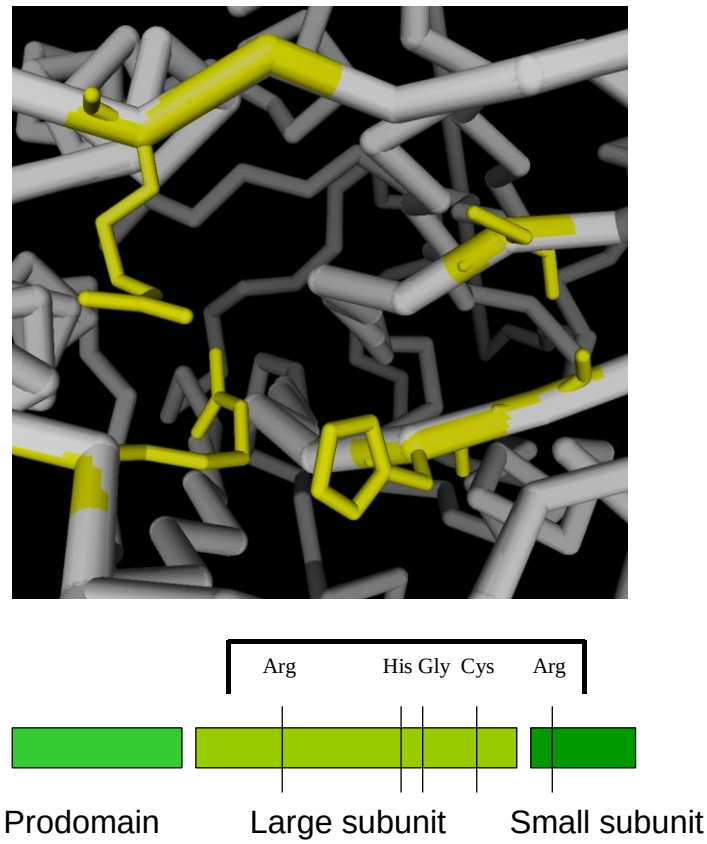
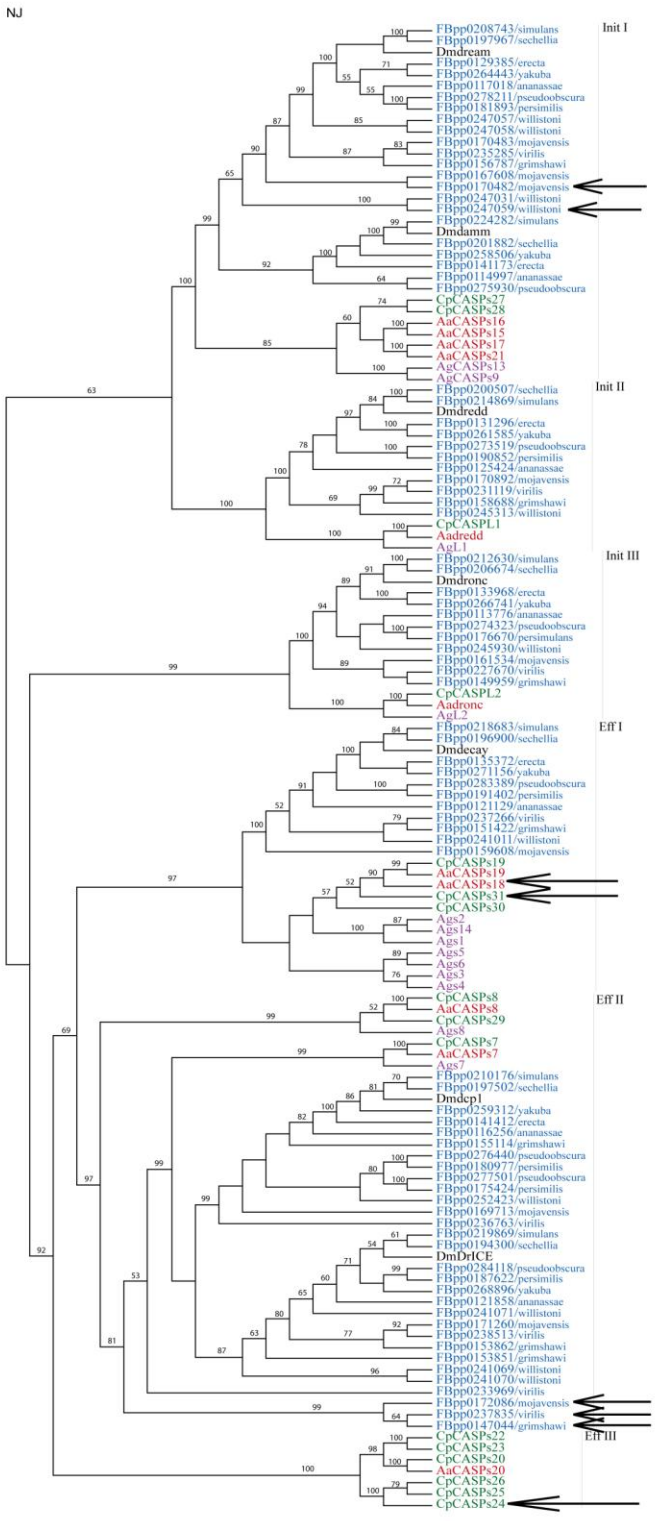


Figure 4.1 Caspase active site
Critical residues involved in binding substrates are shown. The top figure was made using the 3D Molecule Viewer component of Vector NTI Advance 10.3.0 (Invitrogen). The structure of the active site of human caspase-3 (NCBI entry 2CNN) is shown. The placement of critical residues in the active site are indicated in yellow. The bottom figure illustrates the domains of a typical caspase, the cleavage sites (shown as gaps), and the placement of the critical residues in the active site. The region used for the alignment is illustrated with a bracket.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Dmdream
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FBpp0117018/ananassae
FBpp0278211/pseudoobscura
FBpp0181893/persimilis
FBpp0247057/willistoni
FBpp0247058/willistoni
FBpp0170483/mojavensis
FBpp0235285/virilis
FBpp0156787/grimshawi
FBpp0167608/mojavensis
FBpp0170482/mojavensis
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Dmdamm
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CpCASPs28
AaCASPs16
AaCASPs15
AaCASPs17
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AgCASPs9
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FBpp0214869/simulans
Dmdredd
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FBpp0261585/yakuba
FBpp0273519/pseudoobscura
FBpp0190852/persimilis
FBpp0125424/ananassae
FBpp0170892/mojavensis
FBpp0231119/virilis
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Dmdronc
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FBpp0159608/mojavensis
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CpCASPs30
Ags2
Ags14
Ags1
Ags5
Ags6
Ags3
Ags4
CpCASPs8
AaCASPs8
CpCASPs29
Ags8
CpCASPs7
AaCASPs7
Ags7
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Dmdcp1
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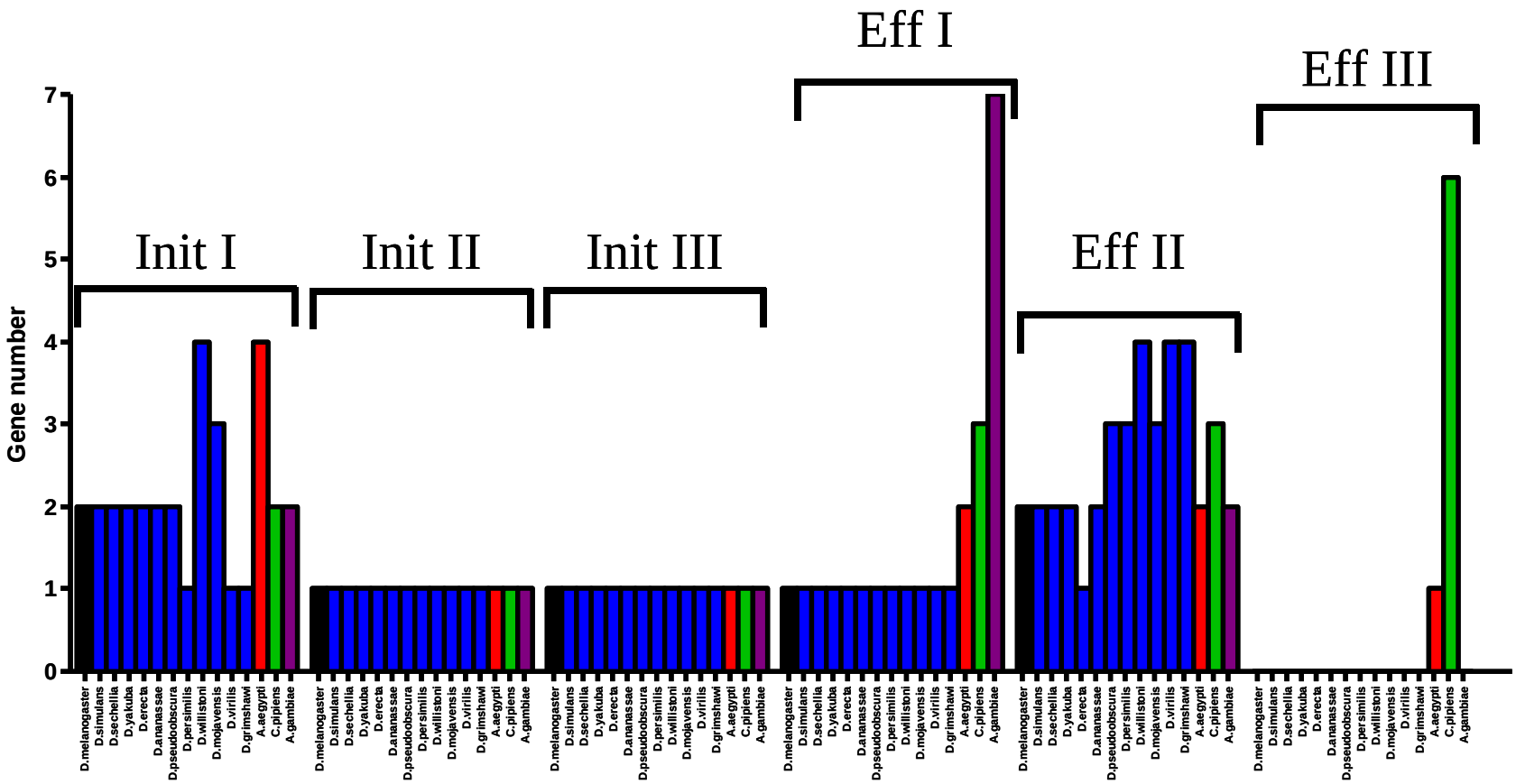
CpCASPs22
CpCASPs23
CpCASPs20
AaCASPs20
CpCASPs26
CpCASPs25
CpCASPs24
Init I
Init II
Init III
Eff I
Eff II
Eff III

Figure 4.2 Phylogenetic relationships among Dipteran caspases
Caspases from fifteen dipteran species were obtained, as outlined in methods, with the region surrounding the conserved residues of the active site aligned using Clustal W. This alignment was then used to make NJ trees using PAUP. *D. melanogaster* is shown in black, other *Drosophila* species are shown in light blue, *Ae. aegypti* is shown in red, *Cu. quinquefasciatus* is shown in green, and *An. gambiae* is shown in purple. The tree is divided into six clades with three initiator and three effector. Arrows designates caspase-like decoy molecules within the clades.


Init I
Init II
Init III
Eff I
Eff II
Eff III
Gene number
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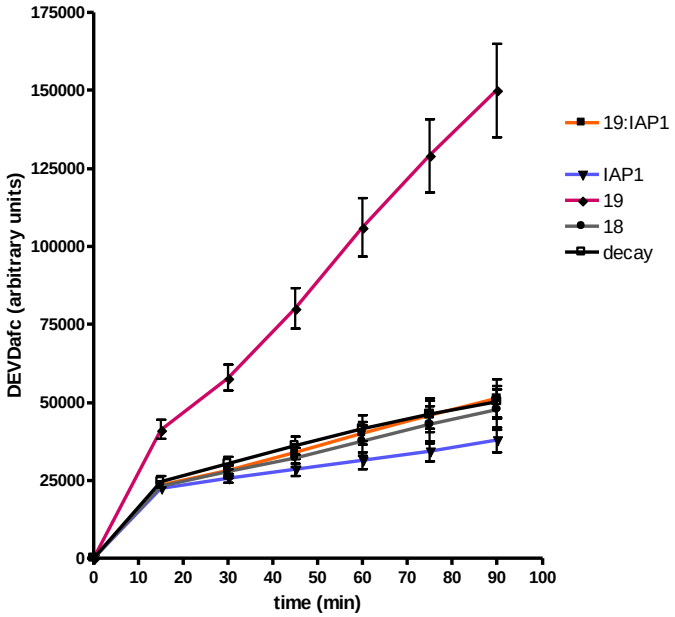
1
2
3
4
5
6
7
D.melanogaster
D.simulans
D.sechellia
D.yakuba
D.erecta
D.ananassae
D.pseudoobscura
D.persimilis
D.willistoni
D.mojavensis
D.virilis
D.grimshawi
A.aegypti
C.pipiens
A.gambiae

Figure 4.3 Number of caspase genes found per clade in each species
The number of genes in each species are shown for each clade. Each species in this graph is indicated using the same color scheme as in Fig. 4.2.

	Conserved residues for substrate recognition catalysis						
Gene	R	H	G	C	R	clade	neighboring caspases
FBpp0170482_mojavensis	no/H	no/L	no/C	no/T	yes	Init I	FBpp0170483, FBpp0167608
FBpp0247059_willistoni	yes	no/Y	yes	no/S	no/I	Init I	FBpp0247057, FBpp0247058, FBpp0247031
FBpp0147044_grimshawi	no/L	no/N	yes	yes	no/G	Eff II	ancestral to Eff II
FBpp0172086_mojavensis	yes	yes	yes	yes	no/S	Eff II	ancestral to Eff II
FBpp0237835_virilis	no/K	yes	yes	yes	no/S	Eff II	FBpp0233969/ancestral to Eff II
CpCASPs31	yes	no/Y	yes	no/W	no/E	Eff I	CpCASPs30, CpCASPs19
CpCASPs24	yes	yes	yes	no/P	no/L	Eff III	CpCASPs25, CpCASPs26
CASPs18	yes	yes	yes	no/S	no/K	Eff I	CASPs19

Table 4.1 Caspases lacking critical residues
This table illustrates caspases found that lack residues needed for catalytic activity. Resides that are absent are indicated and the substituted residue is indicated. Clade and neighboring caspases in the genome are also indicated.

A.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100000
75000
50000
25000
0
DEVDafc (arbitrary units)
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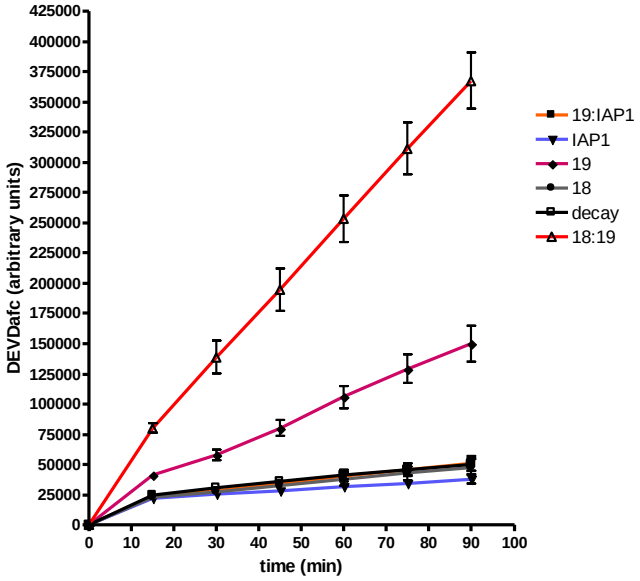
time (min)
19:IAP1
IAP1
19
18
decay

B.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300000
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250000
225000
200000
175000
150000
125000
100000
75000
50000
25000
0
DEVDafc (arbitrary units)
0 10 20 30 40 50 60 70 80 90 100
time (min)
19:IAP1
IAP1
19
18
decay
18:19

Figure 4.4 CASPs18 enhances CASPs19 activity, while IAP1 inhibits CASPs19
Caspases CASPs18, CASPs19 and Decay were overexpressed in C6/36 cells. Caspase activity was then determined by analyzing the amount of DEVD-afc cleavage. In panel A, IAP1 is shown to inhibit CASPs19 caspase activity, while in panel B, CASPs18 enhances CASPs19 even though CASPs18 itself has no caspase activity.

CHAPTER 5 - Summary

Work done in this dissertation is outlined below. Before I started, the knowledge of the genetic pathways regulating apoptosis in insects outside of *D. melanogaster* was limited with only a few known players in other insects. With the sequencing of more insect genomes many new genes have surfaced and their annotations have led to numerous studies. With an annotated genome, one can easily find a gene of interest in an afternoon as compared to "fishing" for genes by old-fashioned ways, which could easily take up to months to a year to find a gene.

In Chapter 2, we report a difference in phenotype between *Ac*MNPVΔP35 and wild-type virus in *Trichopusia ni* cells, which was viral entry defects and slower G2/M cell cycle arrest for the mutant virus. This difference in phenotype was abolished however, when the *Ac*MNPVΔP35 virus was grown in the presence of the chemical caspase inhibitor zVAD-fmk. This analysis (along with unstable virus titer data) suggests that caspases may be used against the virus either in *cis* or *trans* and this slower infection might explain the lack of liquefaction in the host *T. ni* when infected with *Ac*MNPVΔP35. This led us to suggest that infection with the mutant virus maybe enough to kill the larvae but the lack of efficient spread is not enough to end in liquefaction. We have data (not included in Chapter 2) that illustrated that the main viral genes responsible for liquefaction (v-chitinase and v-cath) were expressed by the mutant virus in TN-368 cells, with just slightly less expression of these late genes as compared to wild type virus,

which correlates with the entry defect phenotype (data not shown). This and other data in past studies also suggest a weaker infection with the mutant virus in *T. ni*.

In Chapter 3, we annotated the yellow fever mosquito genome for genes involved in apoptosis. We did systematic analysis for caspases and properly annotated genes involved in regulating caspases. In addition to annotating the caspases we found ARK, DNR1, BG4 and a new IAP antagonist called IMP (IAP-antagonist Michelob_x-like Protein). We analyzed expression analysis for these gene models as well and functionally characterized IMP. This body of work lays a strong foundation for future work on apoptosis in the yellow fever vector, which the Clem lab is already using.

In Chapter 4, we undertook a more systematic analysis of caspases in fifteen different dipteran genomes. By taking advantage of the availability of twelve *Drosophila* species and three different mosquito genomes from different genus, we found interesting trends of gene loss/duplications for caspases even in the *Drosophila* lineage. More importantly we found that recent duplications for particular caspases led to enzyme homologs which are predicted to be enzymatically inactive, called caspase-like decoy molecules. We found these molecules in six different dipterans, but these molecules were not found in the model organism *D. melanogaster*. We also illustrated that one of these caspase-like molecules in *Ae. aegypti* possesses the ability to enhance activity of the parental caspase. The mechanism behind this phenomenon deserves more attention.

With this body of work I was very fortunate to be involved in annotating mosquito genomes and being exposed to interesting insects like mosquitoes. I plan to continue my work with mosquitoes and with the wealth of knowledge that comes with an

annotated genome, this will be an easier task than without a sequenced and annotated genome.