THE ROLE OF APOPTOSIS DURING INFECTION OF *AEDES AEGYPTI* BY SINDBIS VIRUS

by

HUA WANG

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AN ABSTRACT OF A DISSERTATION

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Abstract

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In Chapter 2, the effects of inducing or inhibiting apoptosis on SINV replication were tested in mosquito cells. It was observed that recombinant SINVs expressing pro-apoptotic genes caused extensive apoptosis in mosquito cells, with decreased virus production after the cells underwent apoptosis. Infection of mosquito cells with SINV expressing the caspase inhibitor P35 inhibited actinomycin D-induced apoptosis, but had no observable effects on virus replication. This study was the first to test directly whether inducing or inhibiting apoptosis affects arbovirus replication in mosquito cells.

Chapter 3 examined the effects of silencing apoptosis regulatory genes on SINV replication and dissemination in *A. aegypti*. Genes which either positively or negatively regulate apoptosis were silenced by RNA interference in mosquitoes, which were then infected with a recombinant SINV expressing green fluorescent protein (GFP). Reciprocal effects were observed on both the occurrence and intensity of expression of GFP in various tissues. These results suggest that

systemic apoptosis positively influences SINV replication in *A. aegypti*. This was the first direct study to explore the role of apoptosis in determining mosquito vector competence for arboviruses.

Finally, in Chapter 4, the mechanisms of apoptosis were explored in *A. aegypti*. Overexpression of IAP antagonists caused extensive cell death in mosquito cells, while silencing the expression of IAP antagonists attenuated apoptosis. The results showed that the IAP binding motif (IBM) of IAP antagonists was critical for their binding to AeIAP1. The IAP antagonists released initiator and effector caspases from AeIAP1 by competing for the binding sites and caused caspase-dependent apoptosis. These findings imply that the mechanisms of IAP antagonists regulating apoptosis are conserved between mosquitoes and the model insect where apoptosis has been mainly studied, *Drosophila melanogaster*.

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Approved by:

Major Professor
Dr. Rollie J. Clem

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Dedication

This dissertation is dedicated to my parents Yushan Wang and Peifang Cong.

CHAPTER 1 - Introduction

Mosquito-borne diseases are among the top global health issues we are facing today. Each year, over 500 million people are infected with mosquito-borne diseases, including malaria, yellow fever and dengue fever, which not only cause several million deaths, but also cause much more long-term disability and suffering. The mosquito *Aedes aegypti*, which vectors yellow fever and dengue viruses, is widely distributed in subtropical and tropical countries, and over 2 billion people are at the risk for infection with dengue viruses (Halstead, 2008, Kyle & Harris, 2008). These issues highlight the necessity of vector control for the prevention of mosquito-borne diseases. One critical aspect for vector control is to interrupt vector competency for pathogens, which addresses the importance for basic science to explore the interaction between the vectors and pathogens. This dissertation is focused on one of most important vectors, *A. aegypti*, the vector for dengue virus and yellow fever virus. Since sindbis virus (SINV) is an arthropod-borne virus (arbovirus) which is capable of being vectored by *A. aegypti*, and since SINV has been well characterized at the molecular level, we are using the SINV-*A. aegypti* model to predict virus transmission in mosquitoes and determine whether apoptosis plays a role in the control of vector competency.

Sindbis Virus

SINV is the type species of the genus *Alphavirus* within the family *Togaviridae*, and has a positive-sense, non-segmented, single-stranded RNA (ssRNA) genome, which is characterized by a 5' cap and a 3'-terminal poly(A) and is around 11.7 kb in length

(Strauss *et al.*, 1984). SINV is transmitted by mosquitoes and the virus circulates between mosquito vectors and vertebrate hosts. Birds are the primary vertebrate hosts for SINV, and migratory birds are able to transport SINV widely. Not surprisingly, SINV have been isolated from Africa, Asia, Europe and Australia. SINV is mainly vectored by *Culex species*, but *Aedes* species are also able to transmit SINV.

Pathogenesis

SINV is among Old World alphaviruses along with Semliki Forest virus, chikungunya virus, O'nyong-nyong virus, and Ross River virus. The classification of Old World and New World alphaviruses is defined by the geographic distribution and the diseases caused. Old World viruses are distributed in Europe, Asia, Africa, New Zealand and South America, while New World viruses are found in the Americas (Strauss & Strauss, 1994). Old World alphaviruses are usually not life-threatening to humans, but cause illnesses with symptoms such as fever (sometimes hemorrhagic fever), chills, headache, eye pain, arthralgia/arthritis, diarrhea, vomiting, and rash (Ryman & Klimstra, 2008). New World alphaviruses, such as Venezuelan, western, and eastern equine encephalitis viruses, are more virulent, and cause acute encephalitis which can result in death in humans (Ryman & Klimstra, 2008).

Alphaviruses are transmitted by mosquitoes to vertebrate hosts, such as birds, cattle, and humans. This often results in acute infection with a short-term and high-titer viremia in vertebrate hosts, which in turn pass the viruses back to other mosquitoes through blood meals. However, alphaviruses cause persistent and life-long infection in mosquito vectors, which facilitates virus circulation between mosquitoes and hosts.

Virion structure

The SINV virion is spherical, 70 nm in diameter, with an icosahedral structure, and contains a nucleocapsid surrounded by a virion envelope. The capsid is composed of 240 copies of the capsid protein, and is around 41 nm in diameter (Pletnev *et al.*, 2001). The capsid proteins are arranged in a regular T=4 icosahedral lattice. Each capsid protein consists of 264 amino acids with a mass of about 30KD. The capsid protein has multiple functions and is involved in each stage of the SINV life cycle. According to structure and function analysis, the capsid protein can be divided into three regions, region I (1-80 amino acids), region II (81-113 amino acids), and region III (114-264 amino acids). Region I is involved in nonspecific binding with viral genomic RNA, based on the property of basic amino acids with a high degree of positive charge for neutralizing the viral genomic RNA. In the center of region I there is a coiled-coil, helix I (38-55 amino acids), which is important for core assembly, stabilizing the core protein dimer through the coiled-coil interaction (Hong *et al.*, 2006, Perera *et al.*, 2003, Perera *et al.*, 2001). Region II interacts with genomic RNA and is involved in oligomerization of the core proteins to encapsidate genomic RNA (Hong *et al.*, 2006, Owen & Kuhn, 1996). Region III is a chymotrypsin-like serine proteinase which forms the core protein dimer by auto-cleavage (Choi *et al.*, 1991), and interacts with E2 glycoprotein on the outer shell of the core (Hong *et al.*, 2006).

The virion envelope consists of a lipid bilayer derived from the host, in which two virus-encoded glycoproteins, E1 and E2 are embedded. During assembly, E1 and precursor E2 (PE2) form a heterodimer, which is further cleaved to form a mature E1-E2 dimer during transport to the cell surface. Three copies of E1-E2 heterodimers form a

spike on the surface of SINV. There are 80 spikes from 240 heterodimers protruding on the envelope characterized as $T = 4$ icosahedral symmetry. According to protein structural analysis, E1 lies flat on the surface and E2 protrudes from it; E2 interacts with the nucleocapsid through the domain underlying the membrane (Pletnev *et al.*, 2001, Strauss & Strauss, 2001). The analysis of E1 from alphaviruses and glycoprotein E from flaviviruses supported that these two viral families are related to each other based on envelope structure and evolved from a common ancestor (Lescar *et al.*, 2001, Pletnev *et al.*, 2001, Strauss & Strauss, 2001).

Genome structure

The positive-ssRNA genome of SINV has two large open reading frames. The 5' two thirds of the genome encodes the nonstructural proteins (nsP1-nsP4), forming the replicase-transcriptase complex for viral RNA synthesis or replication. The structural proteins (capsid protein, envelope proteins E2 and E1, and two small proteins E3 and 6K) are encoded in the 3' one third of the genome (Strauss & Strauss, 1994).

There are several important features in the SINV genome, such as *cis*-RNA elements, which are also highly conserved among alphaviruses. First, the 5' portion of the SINV genome RNA has multi-functions and is divided into two *cis*-acting elements, the 5' UTR (1-154 nt) and the 51-nt conserved sequence element (CSE) (155-205 nt). The 5' UTR contains a conserved predicted secondary structure, a short stem-loop, while the 51-nt CSE forms two conserved smaller stem loops among alphaviruses (Frolov *et al.*, 2001, Garmashova *et al.*, 2006).

- The 5' UTR of the SINV genome initiates the translation of the nonstructural polyprotein, upon the transduction of SINV genome into cells (Frolov *et al.*, 2001, Garmashova *et al.*, 2006).
- The 5' UTR of the SINV genome and its complement in the 3' end of the negative-strand RNA, function as core promoter elements, being recognized by the RNA-dependent-RNA polymerase (RdRP) complex, for the synthesis of negative- and positive-strand RNAs (Frolov *et al.*, 2001, Garmashova *et al.*, 2006).
- The 51-nt CSE acts as a replication enhancer, which is located in the nsP1 coding sequence. Mutation of the 51-nt CSE has less SINV replication effects in mammalian cells than in mosquito cells, where it is critical for SINV replication (Frolova *et al.*, 2002, Garmashova *et al.*, 2006, Ou *et al.*, 1982).

Second, the 3' terminus of SINV carries a poly(A) tail and a 19-nt conserved AU-rich CSE. It has been proposed to serve as a core promoter and cooperating 5' UTR of positive-strand RNA for the initiation of negative-strand RNA synthesis (Frolov *et al.*, 2001, Frolova *et al.*, 2002, Raju & Huang, 1991). Third, the 24-nt CSE is upstream of and includes the start of the subgenomic RNA. The complement of this sequence in the negative-strand RNA functions as a core promoter for the transcription of structural genes (Frolov *et al.*, 2001).

Transcription, translation, and genome replication

The SINV genome replicates in infected cells following two steps. First, the viral genomic RNA serves as a template for the synthesis of the complementary negative-strand RNA. Second, the complementary negative-strand RNA provides a template for

the synthesis of both new genomic RNA and subgenomic RNA. The synthesis of negative-stand RNA is only detected in the first 3-4 h post infection, not at the late stages, while positive-strand RNA is present at all stages (Frolov *et al.*, 2001, Strauss & Strauss, 1994).

The genomic RNA, with a 5' cap and 3' poly A, is able to function as a cellular messenger RNA for translation of viral nonstructural proteins (nsPs) by host cell machinery immediately after introduction of the genomic RNA into the cytosol. The 5' two-thirds of the genome is translated into two polyproteins, nsP123 and nsP1234, which are processed into four nsPs. Each nsP has different functions in virus replication. nsP1 participates in capping viral RNAs through its guanine-7-metheyltransferase and guanyltransferase activities; nsP2 plays multiple roles during replication, acting as a protease, an RNA helicase, and a 5' triphosphatase; nsP3 macro domain is critical for SINV replication; nsP4 is an RNA-dependent RNA polymerase (Garmashova *et al.*, 2006, Strauss & Strauss, 1994).

Genome replication and subgenomic RNA transcription are regulated by the *cis*-acting RNA elements (5' UTR, 19-, 24-, and 51-nt CSEs), and the viral nonstructural polyproteins. Viral nonstructural polyproteins have different template specificity for RNA synthesis by the sequential production of RNA replicase/transcriptase through proteolytic cleavage. Nonstructural polyprotein nsP1234 is processed in *cis* into nsP123 and nsP4 by nsP2, a papain-like protease. The nsP123/nsP4 complex is capable of full-length negative-strand RNA synthesis, but does not efficiently synthesize positive-strand RNA. Further cleavage of P123 into nsP1, nsP2, and nsP3, by nsP2 in *trans*, produces a replicase complex (nsP1/nsP2/nsP2/nsP4), which is capable of synthesis of the positive-

strand RNAs, 49S genome RNA and 26S subgenomic RNAs, and shuts off synthesis of negative-strand RNA (Garmashova *et al.*, 2006, Huang *et al.*, 2001, Thal *et al.*, 2007).

SINV structural proteins are translated from the 26S subgenomic mRNA, as soon as the subgenomic RNA is transcribed from full-length negative sense RNA. The viral capsid polypeptide is first to be translated. The capsid polypeptide auto-cleaves the C-terminal tryptophan-serine bond by its serine-protease activity. Later, the capsid protein assembles to encapsidate the 49S genomic RNA. Following the capsid protein, a signal sequence is translated from the subgenomic mRNA, which facilitates translocation of the downstream polypeptide into the endoplasmic reticulum. The final translation products are PE2, 6K, and E1. PE2 and E1 are transmembrane proteins, and 6K is a small membrane-embedded protein. The PE2, 6K and E1 are transported together through secretory vesicles from the endoplasmic reticulum to the Golgi complex, eventually localizing to the plasma membrane. During transportation, the proteins undergo modifications including oligosaccharide addition and trimming, fatty acylation, and proteolytic processing. Eventually, the heterodimer of PE2 and E1 is transformed into E2 and E1 (Xiong *et al.*, 1989).

Engineering SINV expression systems

Recombinant SINV expression systems have been developed by inserting an additional copy of the viral subgenomic promoter into the genome to facilitate expression of foreign genes (Foy *et al.*, 2004, Hahn *et al.*, 1992, Olson *et al.*, 2000, Raju & Huang, 1991). An early generation infectious SINV clone is TE/3'2J, which contains a second copy of the viral subgenomic mRNA promoter downstream of the primary subgenomic promoter at the 3' end of the structural protein coding region in the viral genome (Hahn

et al., 1992). Foreign genes can be inserted downstream of the duplicated subgenomic promoter, allowing their expression. However, TE/3'2J suffers from two drawbacks: 1) instability of the insert due to deletions that occur after several passages and remove the foreign gene without affecting any of the viral genes and 2) low oral infectivity, since TE strains of SINV are adapted to replication in tissue culture. To overcome the instability problem, later infectious clones contained the duplicated subgenomic promoter inserted upstream of the structural genes, so that deletions removing the foreign gene also would remove structural genes, rendering the virus replication incompetent (Pierro *et al.*, 2003). To overcome the infectivity problem, infectious clones were generated from MRE16, a strain of SINV with high oral infectivity (Foy *et al.*, 2004, Myles *et al.*, 2004). Using these improvements the SINV infectious clones 5'dsMRE16ic and TE5'2J were constructed from the MRE16 SINV strain and the mouse neurovirulent TE12 SINV strain, respectively (Foy *et al.*, 2004, Pierro *et al.*, 2003, Pierro *et al.*, 2007).

Insect Innate Immunity

Most of the knowledge about insect innate immunity has come from studies of the fruit fly *Drosophila melanogaster* (Brennan & Anderson, 2004, Lemaitre & Hoffmann, 2007). Upon being infected with pathogens, insects are able to combat the infection through a serial of physical barriers along with immune responses. Epithelia (such as gut and trachea) act as physical barriers against pathogens; phagocytosis and encapsulation of pathogens are mediated by hemocytes; the fat body produces antimicrobial peptides (AMPs) (Lemaitre & Hoffmann, 2007). The regulation of AMP genes is by two distinct pathways, Toll receptor and Imd pathways. The recognition of fungal and Gram-positive bacterial infection is mediated by the activation of Toll pathway to express Drosomycin

and other AMPs, while Gram-negative bacteria activate Imd-mediated signaling cascade to express Diptericin and other AMPs (Brennan & Anderson, 2004, Lemaitre & Hoffmann, 2007).

For viral infection, three cell signaling pathways (Toll, Jak-STAT and JNK) have been identified to play important roles in immune defense against viruses. The Toll pathway is up-regulated in *A. aegypti* after a SINV blood meal based on data from a microarray study (Sanders *et al.*, 2005), and inhibiting or activating the Toll signaling pathway leads to a negative or positive effect, respectively, on dengue virus replication in *A. aegypti* (Xi *et al.*, 2008). Infection of *Drosophila* with *Drosophila* C virus (DCV) induced activation of STAT (signal transducer and activator of transcription) and Jak kinase Hopscotch is involved in immune defense against DCV infection (Agaisse & Perrimon, 2004, Galiana-Arnoux *et al.*, 2006). After SINV infection, the transcript levels of the JNK pathway and several protease inhibitors (serpin) genes were shown to be altered by microarray analysis (Sanders *et al.*, 2005). Also, after O'nyong-nyong virus infection of *Anopheles gambiae*, heat-shock protein cognate 70B is up-regulated, and silencing this gene results in higher levels of virus replication (Sim *et al.*, 2007).

Apoptosis, autophagy and RNA interference are considered to be intracellular pathways which can lead to inhibition of viral infection or intrinsic innate immune mechanisms. Recent studies have shown that autophagy plays a direct antiviral role against vesicular stomatitis virus in *Drosophila* (Deretic, 2009, Shelly *et al.*, 2009). More detail about apoptosis and RNA interference as anti-viral defense mechanisms will be discussed below.

Apoptosis

Apoptosis has been considered an antiviral defense in mammalian and insect systems (Chiou *et al.*, 1994, Clarke & Clem, 2003, Crook *et al.*, 1993, Cuconati *et al.*, 2002). The baculovirus *Autographa californic*a M nucleopolyhedrovirus (AcMNPV) was one of the first viruses found to regulate host apoptosis. The *p*35 gene from AcMNPV was identified as an early gene during virus infection of *Spodoptera frugiperda* (SF-21) cells (Friesen & Miller, 1987) and the mutation of *p*35 in AcMNPV caused apoptosis in SF-21 cells during infection (Clem *et al.*, 1991, Friesen & Miller, 1987). Later, studies showed that a *p*35 mutant of AcMNPV caused apoptosis accompanied with reduced production of viral progeny in SF-21 cells, a delay in the transcription and translation of early and late viral genes, a lack of expression of very late genes, and a total cessation of protein synthesis late in the apoptotic process (Clem *et al.*, 1991, Hershberger *et al.*, 1992). In addition, a 1,000-fold higher dose of *p*35 mutant AcMNPV was required for 50% lethality in *S. frugiperda* larvae than wild-type AcMNPV (Clem & Miller, 1993, Clem *et al.*, 1994), indicating that apoptosis could serve as an anti-viral defense in *S. frugiperda*. This was later verified by studying the pathology of infection of *S. frugiperda* larvae by *p*35 mutant AcMNPV, which resulted in extensive apoptosis in the fat body and epithelium with reduced viral infectivity, replication and spread, compared with wild-type AcMNPV infection (Clarke & Clem, 2003). Adenovirus E1B 19K was also indentified as a viral anti-apoptotic gene. E1B 19K is a Bcl-2 homolog and inhibits apoptosis by interacting with Bak and Bax, and infection with E1B 19K mutant viruses induces apoptosis with impaired progeny virus production in human HeLa cells (Chiou *et al.*, 1994, Cuconati *et al.*, 2002, Cuconati & White, 2002).

There are several intriguing observations that indicated apoptosis might be involved during arbovirus infection in mosquitoes, in which cell death was observed in midguts and/or salivary glands in mosquitoes after infection with arboviruses (Bowers *et al.*, 2003, Girard *et al.*, 2005, Girard *et al.*, 2007, Mims *et al.*, 1966, Vaidyanathan & Scott, 2006, Weaver *et al.*, 1992, Weaver *et al.*, 1988). Since midgut and salivary glands are considered to be infection barriers, and arboviruses must escape from the initial infection site to salivary gland lumen (Campbell *et al.*, 2008a), any physical changes in these barriers could have anti- or pro-infection effects. However, no causative data exist that directly link apoptosis to effects on viral vector competence in mosquitoes. Some studies reported that apoptosis is an anti-viral defense in hosts at the early stage of virus infection; while apoptosis might also promote virus release at the late stage of infection (White, 2001).

RNA interference

The mechanism of RNA interference (RNAi) was discovered in *C. elegans* in 1998 (Fire *et al.*, 1998). Since then, this mechanism has been ubiquitously found in many other organisms, *E. coli*, plants, *Drosophila*, yeast, zebrafish, and mammalian cells (Elbashir *et al.*, 2001, Gottesman, 2004, Tuschl *et al.*, 1999).

RNAi pathway

RNAi is triggered by dsRNA at a posttranscriptional level and is specific for the mRNA, which is complementary to the antisense strand of the dsRNA (Fire *et al.*, 1998). The process of RNAi is carried out by the molecular machinery in cells. The major players in RNAi are small RNAs, which are around 21-30 nucleotides and classified into

three types, small interfering RNAs (siRNAs), microRNAs (miRNAs) and Piwi-associated interfering RNAs (piRNA). These small RNAs are generated from different cellular machineries. siRNAs are produced from long dsRNA in the cytosol, after being cleaved into small pieces by the endonuclease Dicer (RNase III). The sense strand is further degraded by the endonuclease Ago2, and the antisense strand binds to RNA-induced silencing complex (RISC). The RISC complex is orientated by the antisense strand based on complementarity to the target mRNA and subsequently degrades the mRNA (Bernstein *et al.*, 2001, Hammond *et al.*, 2000, Hammond *et al.*, 2001).

miRNAs are first generated as pre-miRNA in the nucleus from endogenous hairpin-shaped transcripts by the endonuclease Drosha (RNase III), and then are exported into the cytosol and processed by Dicer to form mature miRNA. miRNAs mediate post-transcriptional gene regulation by base pairing to mRNA within 3' untranslated region. Through the recognition of partially complementary mRNA sequences, miRNAs degrade or repress target messenger RNAs.

piRNAs are around 30 nucleotides in length, and found in the germline cells of flies and vertebrates. piRNAs are Dicer-independent and processed to form effector complexes as RNA-induced transcriptional silencing complex (RITS) (Zamore, 2007). piRNAs control the expression of transposons in the genome by directing silencing processes.

RNA silencing directs methylation of chromatin and keeps chromatin condensed and suppresses transcription to help maintain genome integrity (Hall *et al.*, 2002, Mette *et al.*, 2000, Sijen *et al.*, 2001, Volpe *et al.*, 2002), and also stabilizes the genome by keeping endogenous transposons silent (Ketting *et al.*, 1999, Tabara *et al.*, 1999). RNA

silencing guides the cleavage of mRNA to arrest translation and regulate the development of organisms by miRNAs (Lagos-Quintana *et al.*, 2001, Lau *et al.*, 2001, Lee & Ambros, 2001).

RNAi-based antiviral response

The discovery of the RNAi mechanism suggested that RNAi could be a defense weapon against virus infection, because during virus infection, a lot of intermediate viral dsRNAs are produced (viral genome, dsRNA and secondary structure of RNA during virus synthesis), and these can initiate RNAi machinery recognition. Evidence that the RNAi pathway protects organisms against viral infection was first found in plants (Noad *et al.*, 1997, Ratcliff *et al.*, 1997), and later the anti-viral mechanism was found in worms (Lu *et al.*, 2005, Wilkins *et al.*, 2005), in *Drosophila* (Galiana-Arnoux *et al.*, 2006, Li *et al.*, 2002, Yu *et al.*, 2006, Zambon *et al.*, 2006) and in mosquitoes (Keene *et al.*, 2004).

First, RNAi-core machinery mutant animals are highly permissive to virus infection and exhibit high mortality after virus infection (Galiana-Arnoux *et al.*, 2006, Keene *et al.*, 2004, Lu *et al.*, 2005, Wilkins *et al.*, 2005, Yu *et al.*, 2006, Zambon *et al.*, 2006). In *Drosophila*, mutants (*dcr-2, ago-2, r2d2*) allow high level of virus load (FHV, *Drosophila* C and X viruses and SINV) (Galiana-Arnoux *et al.*, 2006, Yu *et al.*, 2006, Zambon *et al.*, 2006). Silencing Argonaute-2 results in a significant increase of DCV and Cricket Paralysis virus replication in *Drosophila*, while silencing Argonaute-2 expression in adult *Anopheles gambiae* mosquitoes makes them more susceptible to O'nyong-nyong virus (ONNV) (*Alphavirus*; *Togaviridae*) (Keene *et al.*, 2004). Also, silencing expression of *dcr2*, *r2d2*, or *ago2* in *A. aegypti* increases dengue virus or SINV

replication in the vector and promotes virus transmission in mosquitoes (Sanchez-Vargas *et al.*, 2009).

Second, viral dsRNAs induce specific antiviral defense. SINV-dsRNA-immunized *Drosophila* are resistant to SINV infection (van Rij *et al.*, 2006), while ONNV-derived nsP3-dsRNA specifically inhibited ONNV spread in *A. aegypti* mosquitoes when they were treated with co-injection of nsP3 dsRNA and ONNV (Keene *et al.*, 2004). In addition, inverted-repeat RNA derived from DENV-2 inhibits DENV-2 replication in mosquito cells and adult mosquitoes (Adelman *et al.*, 2002).

Third, viruses can interfere with or suppress the RNA silencing machinery. The most well studied example is the dsRNA-binding protein FHV-B2, which directly suppresses the RNAi pathway and is necessary for accumulation of viral RNAs (Fenner *et al.*, 2007, Li *et al.*, 2002, van Rij *et al.*, 2006, Yu *et al.*, 2006). FHV-B2 also inhibits the RNAi-based antiviral response in *A. aegypti* to SINV (Cirimotich *et al.*, 2009, Myles *et al.*, 2008). Expression of B2 by SINV causes accumulation of viral siRNA up to 10% of the total cellular small RNAs (~21 bp) in mosquitoes, a higher load of SINV, and lower survival rate (Cirimotich *et al.*, 2009, Myles *et al.*, 2008). Interestingly, the mammalian virus proteins vaccinia virus E3L and influenza virus NS1 are dsRNA-binding proteins that can bind both long and short dsRNA and serve as interferon antagonist proteins in mammalian cells; these viral proteins have been shown to be able to suppress of RNA silencing in *Drosophila* S2 cells and in *A. gambiae* cells (Li *et al.*, 2004). These data indicate that viruses have evolved mechanisms to evade the anti-viral defense under RNAi pressure.

Apoptotic Pathways

Apoptosis is a type of programmed cell death and the mechanism of apoptosis is evolutionarily conserved among species. Apoptosis is an important process to delete unwanted, damaged, and infected cells. Thus apoptosis is critical for animals to properly maintain homeostasis, development, and immune responses (Hay & Guo, 2006b, Hay *et al.*, 2004).

The evolution of cell death

Cell death has garnered attention by scientists for over a century (Conti *et al.*, 2005, Majno & Joris, 1995). In early times, cell death was defined using morphological criteria by pathologists. In 1858, cell death was named necrosis, a passive process and degeneration, in Lecture XV of Virchow's Cellular Pathology (Majno & Joris, 1995). Before apoptosis was named, spontaneous cell death was studied with available nuclear stains. In 1885, Walther Flemming found the nuclei were breaking up with the half-moons of pyknotic chromatin in cells when he studied normal rabbit ovarian follicles, and he named such a process chromatolysis. Later, the same observation was also being seen in lactating mammary glands by Franz Nissen in 1886, in breast cancer tissue by Ströbe in 1914, and in embryonic tissue by Glücksmann in 1951 (Majno & Joris, 1995).

In the late 1950s, research on lysosomes led to introduction of the concept of cell suicide by De Duve (Conti *et al.*, 2005, Kerr, 2002, Majno & Joris, 1995). Subsequent studies of ischemic liver injury in the 1960s revealed two types of cell death, classical necrosis with ruptured lysosomes, and a newly defined cell death with formation of scattered small cells containing condensed nuclear chromatin and intact lysosomes (Kerr, 2002, Majno & Joris, 1995). In 1971, Kerr described the ultrastructural changes of the

new death in ischemic rat liver by electron microscopy, and he named the new death as shrinkage necrosis (Kerr, 2002).

A year later, Kerr, Wyllie, and Currie published a review paper (Kerr *et al.*, 1972) where they defined this shrinkage necrosis as apoptosis. *"The term apoptosis is proposed for a hitherto little recognized mechanism of controlled cell deletion, which appears to play a complementary but opposite role to mitosis in the regulation of animal cell populations."* Kerr et al. realized that apoptosis was universally involved in many normal tissues, ischemic organs, and tumors, participating in embryonic development, pathological atrophy, and regression of tumors. They described two stages of apoptosis by ultrastructural observation. The first stage is nuclear and cytoplasmic condensation and apoptotic body formation, while the second stage is phagocytosis of these apoptotic bodies by other cells (Kerr *et al.*, 1972). Interestingly, through the early observations of cell death/apoptosis by morphology, scientists predicted that cell death may be a pre-ordained, genetically programmed phenomenon (Kerr, 2002, Kerr *et al.*, 1972). In 1980, Wyllie found that the activation of endonuclease caused excision of nucleosome chains, which was associated with the morphological changes of apoptosis (Wyllie, 1980). The nucleosomal fragments from apoptotic cells can be observed in a ladder pattern following electrophoresis, while the chromatin from necrotic cells results in a smear pattern (Wyllie, 1980).

C. elegans apoptosis

We have to thank the great gift of nature to science, *C. elegans*, which provides an excellent animal model for us to discover the fundamental knowledge of apoptosis in genetic and molecular aspects, as was studied by Horvitz and his colleagues in the early

1990s (Brenner, 2003, Horvitz, 2003). Otherwise, our knowledge of apoptosis would have been remained at the level of describing morphological changes for a while.

In 1977, Sulston and Horvitz found that 131 invariant cells out of a total of 1090 cells underwent apoptosis during worm development, which brought to Horvitz's attention the further study of genes that control programmed cell death (Horvitz, 2003). Through genetic screening for mutants defective in cell death, Horvitz's research group found four genes involved in the core molecular genetic pathway for apoptosis in *C. elegans* in the 1990s. Three proapoptotic genes are *egl-1* (egg-laying defective), *ced-3* (cell-death abnormal), and *ced-4*, because loss-of-function mutation in these genes leads to survival of all of the cells which are supposed to die (Ellis & Horvitz, 1986, Trent *et al.*, 1983). One apoptosis inhibitor gene is *ced-9*, in which loss of function leads to extensive apoptosis (Hengartner *et al.*, 1992). In this genetic pathway, *egl-1* is upstream of *ced-9* to promote apoptosis, *ced-9* is upstream of *ced-4* to inhibit apoptosis, and *ced-4* is upstream of *ced-3* to induce apoptosis (Horvitz, 2003). Each of these gene products has one or more corresponding homologs in mammals. EGL-1 is a proapoptotic BH3 only protein, CED-9 is a Bcl-2 like protein, CED-4 is a caspase adaptor similar to Apaf-1, and CED-3 is a caspase. These resemblances highlight the evolutionary conservation of the apoptotic pathway between nematodes and mammals.

CED-3 was found to have homology to mammalian interleukin-1 beta-converting enzyme (ICE), a cysteine protease involved in cytokine maturation. Therefore the study of CED-3 paved the first insight into molecular mechanisms of apoptosis (Miura *et al.*, 1993, Yi *et al.*, 2007). CED-3 is the only caspase playing a central role in apoptosis in *C. elegans*. CED-3 is a unique caspase, because it functions as both an initiator and effector

caspase. CED-3 has a caspase-recruitment domain (CARD), which is also present in mammalian (Caspases-8 and -10) and *Drosophila* initiator caspases (Dronc and Dredd). The presence of a CARD classifies CED-3 as an initiator caspase, and indeed CED-3 cleaves the initiator caspase substrate IETD-AFC (Taylor *et al.*, 2007). However, CED-3 also processes effector caspase substrates such as DEVD-AFC, and a wide range of worm proteins (e.g. actin, tubulin and chaperone) have been identified as its natural substrates (Taylor *et al.*, 2007).

In most cells, CED-3 is present as an inactive zymogen, as are other caspases. But when cells are exposed to apoptotic stimuli, the CED-3 zymogen is recruited by CARD/CARD interaction between CED-3 and CED-4, which also contains a CARD domain. Therefore, CED-4 is responsible for the oligomerization of CED-3 and allows CED-3 auto-activation (Yang *et al.*, 1998). However, in living cells, CED-4 physically interacts with CED-9 and is constitutively inhibited by CED-9. When cells undergo apoptosis, EGL-1 is transcriptionally activated and binds to CED-9, resulting in the dissociation of CED-4 and CED-9. Thus, free CED-4 tetramerizes as an apoptosome and recruits CED-3 to auto-activate (Yan & Shi, 2005).

Interestingly, two BIR (baculovirus IAP repeat) proteins were found in *C. elegans*, which do not suppress apoptosis as inhibitor of apoptosis (IAP) proteins normally do but are required for embryonic cytokinesis (Fraser *et al.*, 1999). Thus, it poses the question how CED-3 remains as an inactive zymogen without auto-activation. A recent study found a caspase homolog-3 (CSP-3) that is similar in sequence to the small subunit of CED-3, but does not contain a caspase large subunit with a cysteine in its active site and is predicted to lack proteolytic activity (Geng *et al.*, 2008, Shaham *et*

al., 1999). CSP-3 was found to associate with the large subunit of the CED-3 zymogen and inhibit CED-3 auto-cleavage to generate active forms. CSP-3 is a negative regulator of CED-3, similar to mammalian c-FLIP as a caspase decoy of Caspase-8, because it does not block CED-4 induced CED-3 activation or inhibit CED-3 protease activity (Bump *et al.*, 1995, Geng *et al.*, 2008).

Mammalian apoptosis

In mammalian cells, apoptosis is mediated through either the intrinsic or extrinsic pathway. The intrinsic pathway is also known as the mitochondria apoptotic pathway, while the extrinsic pathway is initiated by the binding of an extracellular death ligand to a death receptor (Riedl & Shi, 2004, Yan & Shi, 2005).

Extrinsic pathway

The death receptors are a family of transmembrane proteins with multiple cysteine-rich repeats in their extracellular domains and cytoplasmic tails containing death domains (DD) extended in the cytosol. These death receptors include tumor necrosis factor receptor 1(TNFR1), Fas (APO-1), APO-3 (DR3), TNF-related apoptosis inducing ligand recepor1 (TRAILR1), TRAILR2, DR6, ectodysplasin A receptor (EDAR) and nerve growth factor receptor (Li & Yuan, 2008).

For Fas or TRAIL receptor, upon interacting with its extracellular ligand, the death receptor is activated and facilitates the assembly of an intracellular death-inducing signaling complex (DISC) through interaction with the C-terminal DD of FADD. The N-terminal death effector domain (DED) of FADD recruits caspase-8 or -10 through a homotypic interaction with the DED in its prodomain. Thus, three copies of caspase-8 or

-10 interact with FADD through the same DED, forming a DISC to initiate caspase autoprocessing and activation at the plasma membrane. If the activated caspase-8 is sufficiently released into the cytosol, and further cleaves and activates effector caspases-3 and -7, this is able to directly induce apoptosis (Scaffidi *et al.*, 1998). If an insufficient amount of DISC formation leads to reduced levels of activated caspase-8 and -3, the death signaling can be amplified by the mitochondrial apoptotic pathway through truncated Bid (tBid), which results from the cleavage of the BH3-only proapoptotic Bcl-2 family member Bid by activated caspase-8. tBID translocates to mitochondria, triggers the release of cytochrome c, and induces activation of caspase-9, which then can activate caspases-3 and -7 (Li *et al.*, 1998, Luo *et al.*, 1998, Scaffidi *et al.*, 1998).

The activation of TNFR1 by TNFα can induce two different pathways resulting in two distinct cell fates. One pathway is via the formation of complex I through DD recruitment of TNFR1, TRADD (TNFR1-associated death domain protein), RIP (receptor-interacting protein), and TRAFs (TNF receptor-associated factors), which further activates NF-κB by regulating inflammatory cytokines and inducing the expression of caspase-8 inhibitor FLIP for cell survival. Later, TRADD and RIP1 dissociate from TNFR1, and then associate with FADD via DD interaction, recruiting caspase-8 to form complex II and inducing apoptosis (Micheau & Tschopp, 2003).

Intrinsic pathway

The intrinsic or mitochondria pathway is induced by signals from inside the cells, such as DNA damage, cytotoxic drugs, Ca^{2+} fluxes or viral infection (Green *et al.*, 2004, Li & Yuan, 2008). Mitochondrial membrane integrity is under control of Bcl-2 family proteins, which contain at least one Bcl-2 homology (BH) region. Anti-apoptotic Bcl-2

proteins (Bcl-2, Bcl-XL, Bcl-w, and Mcl-1) mainly locate in the outer mitochondrial membrane, regulating mitochondria integrity by limiting membrane permeabilization. Upon intracellular stimuli, BH3-only proteins (Bad, Bid, Bim, Hrk, Puma, Noxa and others) are activated, and interact with anti-apoptotic Bcl-2 proteins via their BH3 domains to remove the anti-apoptotic inhibition (Huang *et al.*, 2000, Wang, 2001). BH3-only proteins translocate to mitochondria from other cellular compartments, and then cooperate with mitochondrial membrane proteins, Bax and Bak, which are belong to proapoptotic Bcl-2 family. Later, Bax and Bak oligomerize and cause release of proapoptotic proteins from the intermembrane space by an unknown mechanism, which may involve pore formation within the mitochondrial outer membrane. The proapoptotic proteins from the intermembrane space, such as cytochrome c, Samc/DIABLO, HtrA2/Omi, AIF, and endonuclease G, are released into the cytosol and promote apoptosis (Korsmeyer *et al.*, 2000, Lau *et al.*, 2001, Lindsten *et al.*, 2000, Marzo *et al.*, 1998a, Marzo *et al.*, 1998b).

The release of cytochrome c promotes assembly of the apoptosome, consisting of Apaf-1, cytochrome c and dATP/ATP (Liu *et al.*, 1996). Apaf-1 is the main component of the apoptosome, containing an N-terminal CARD, an expanded nucleotide-binding oligomerization domain (NOD), and a C-terminal (tryptophan-aspartic acid) WD40 repeat. The apoptosome is a wheel-shaped signaling platform containing seven Apaf-1 molecules; each Apaf-1 bound one molecule of cytochrome c via a WD40 repeat and caspase-9 through CARD interaction (Nava *et al.*, 1997, Zou *et al.*, 1999). Recruitment of caspase-9 by the apoptosome leads to its autocatalytic activation, and caspase-9 then activates effector caspases (caspase-3 and -7) (Zou *et al.*, 1999). IAP antagonists (Smac)

are released from mitochondria to counteract the ability of IAPs to inhibit caspases (Chai *et al.*, 2000, Du *et al.*, 2000, Wu *et al.*, 2000). Therefore, the induction of apoptosis is concerted by multiple proapoptotic proteins.

Drosophila apoptosis

Drosophila melanogaster provides a unique model to study the regulation of apoptosis in response to different apoptotic stimuli, by using powerful genetic tools and molecular biology techniques (Hay & Guo, 2006b, Hay *et al.*, 2004, Steller, 2008). The core of the *Drosophila* apoptosis machinery consists of the adaptor protein Dark (the main component of apoptosome), the initiator caspase Dronc, the effector caspase Drice, and the IAP protein DIAP1 (Hay & Guo, 2006b).

The *Drosophila* genome encodes three initiator caspases (Dronc, Dredd, and Dream/Strica) with long N-terminal prodomains, and four effector caspases (Drice, Dcp-1, Decay, and Damm) containing short prodomains (Hay & Guo, 2006b, Riedl & Shi, 2004). Dronc is the best studied *Drosophila* caspase and plays a critical role as an initiator caspase (Chew *et al.*, 2004). Dronc has also been shown to be an ecdysone-inducible caspase, mediating steroid hormone ecdysone-induced apoptosis during metamorphosis (Dorstyn *et al.*, 1999, Dorstyn *et al.*, 2002, Waldhuber *et al.*, 2005). Dronc is involved in most apoptosis processes during embryogenesis, development, spermatid individualization, and stress (Chew *et al.*, 2004, Daish *et al.*, 2004, Huh *et al.*, 2004, Waldhuber *et al.*, 2005, Xu *et al.*, 2005). Dronc is the only CARD-carrying caspase in the *Drosophila* genome and is recruited by DARK through the CARD interaction, resulting in Dronc autoprocessing and activation (Muro *et al.*, 2004, Yan *et al.*, 2006). Dronc has specific cellular substrates, including itself, Drice, Dcp-1 and

DIAP1, and Dronc has been shown to be capable of cleaving after either glutamate or aspartate residues. Dronc autoprocesses itself at Glu352 for its activation, resulting in a catalytically active dimer (Hawkins *et al.*, 2000, Muro *et al.*, 2004), and following the second cleavage after Glu143 to remove the CARD domain from the catalytic core domain, Dronc is released from apoptosome (Yan *et al.*, 2006). Recent studies demonstrated that Dronc activation relies on dimerization through the apoptosome, and that the initial autoprocessing at Glu352 is not necessary for activation of Dronc but stabilizing the active dimer (Dorstyn & Kumar, 2008, Snipas *et al.*, 2008). Dronc cleaves DIAP1 between BIR1 and BIR2 domains of DIAP1 at Glu205, which occurs in both normal and apoptotic *Drosophila* S2 cells to maintain efficient inhibition of apoptosis (Muro *et al.*, 2005, Yan *et al.*, 2004a). Dronc also cleaves and activates Drice, and in turn activated Drice induces the cleavage of Dronc after Asp135 for further activation (Dorstyn & Kumar, 2008, Muro *et al.*, 2005).

The effector caspases Drice and Dcp-1 are highly homologous to each other, similar to mammalian effector caspases, caspase-3 and -7. However, Drice plays a more essential role as an effector caspase than Dcp-1. In *vivo* studies have been shown that *dcp-1* mutants are healthy and possess few defects in normally occurring cell death, while *drice* mutants have severe defects in apoptosis during embryogenesis and development and are resistant to radiation and IAP antagonist-induced cell death (Muro *et al.*, 2006, Xu *et al.*, 2006). In *Drosophila* S2 cells, silencing of Drice by RNAi also inhibits a variety of apoptotic stimuli (Leulier *et al.*, 2006, Means *et al.*, 2006, Muro *et al.*, 2002, Muro *et al.*, 2004).

DIAP1 is a pivotal caspase inhibitor in *Drosophila*. Loss of *diap1* results in cell death in many contexts, such as embryos, larvae, the adult eye, and S2 cells (Hay & Guo, 2006b). DIAP1 negatively regulates the initiator and effector caspases by physical binding and polyubiquitination. DIAP1 contains two baculovirus IAP repeats (BIRs) and a C-terminal RING domain, which acts as an E3 ubiquitin ligase. The BIR2 domain of DIAP1 physically interacts with Dronc through a 12-residue peptide, which is located in the linker sequence between the CARD domain and the catalytic subunit of Dronc (Chai *et al.*, 2003). The RING domain of DIAP1 functions as E3 ubiquitin ligase and promotes the ubiquitination and degradation of Dronc (Chai *et al.*, 2003, Wilson *et al.*, 2002). The BIR1 domain of DIAP1 first binds to effector caspases (Drice and DCP-1) through an IAP binding motif (IBM) which is generated by cleavage of the p20 subunit, and the binding further blocks the enzymatic activity through steric occlusion (Tenev *et al.*, 2005, Yan *et al.*, 2004b). After the physical binding, the N-terminal 20 amino acids of DIAP1 are removed by active Drice and the lack of auto-inhibition of DIAP1 by the N-terminal sequence is essential for the inhibition of Drice (Ditzel *et al.*, 2003, Yan *et al.*, 2004b). However, the physical interaction between DIAP1 and Drice, or DIAP1 and Dronc cannot completely inhibit these caspases, and the RING domain of DIAP1 promotes the ubiquitination and degradation of these caspases (Chai *et al.*, 2003, Wilson *et al.*, 2002). Interestingly, the non-degradative polyubiquitination of Drice and Dcp-1 by DIAP1 reduces the caspase activity by steric interference with binding of substrate (Ditzel *et al.*, 2008). Steric blocking of caspase active sites and polyubiquitination by DIAP1 are important for the inactivation of effector caspase.

IAP antagonists (*reaper*, *hid* and *grim*) were first discovered through molecular genetic studies of apoptosis in *Drosophila* (Grether *et al.*, 1995). A large chromosomal deletion removing all three genes protected embryos from apoptosis caused by X-ray (Grether *et al.*, 1995), while ectopic expression of these genes induced extensive apoptosis in both transgenic flies and cell culture and cell death was blocked by expression of the baculovirus caspase inhibitor P35 (Chen *et al.*, 1996, Grether *et al.*, 1995). These genes, especially *rpr*, are transcriptionally activated by a variety of apoptotic stimuli, such as radiation, stress, developmental signals and steroid hormones (Steller, 2008). IAP antagonists are a group of proteins with a highly conserved N-terminal motif, the IBM, which allows binding to the BIR1 and BIR2 of DIAP1. IAP antagonists mainly replace the interaction between caspases and IAP to induce apoptosis by competing with caspases for the binding sites of IAP (Yan *et al.*, 2004a). The interaction of IBM and DIAP1 requires the exposure of Ala at N terminus of IAP antagonists. The endoplasmic reticulum protein Jafrac-2 exposes an IBM after the N-terminal signal peptide is cleaved off, resulting in the release of mature Jafrac-2 from the ER (Tenev *et al.*, 2002). These IAP antagonists display different preferences for binding of DIAP1 BIR domains. Rpr and Grim show equal preference to BIR1 and BIR2 domains, but Hid, Sickle and Jafrac2 have higher affinity for BIR2 than for BIR1 (Zachariou *et al.*, 2003). Another proapoptotic feature of IAP antagonists is that they stimulate DIAP1 auto-ubiquitination by the RING domain, or ubiquitination by other E3 ligases for further degradation by the proteosome. Rpr and Grim can also inhibit global protein translation (Colon-Ramos *et al.*, 2006). Rpr, Grim and Sickle share the GH3 domain, which is required for mitochondria localization. Even without the IBM domain,

Rpr, Grim and Sickle can still stimulate cell death through their GH3 domains (Claveria *et al.*, 2002, Zhou *et al.*, 2005).

Mosquito apoptosis

Since the availability of genome sequences for *A. aegypti* and *A. gambiae* (Holt *et al.*, 2002, Nene *et al.*, 2007), the study of apoptosis in mosquitoes has been expanded (Bryant *et al.*, 2008, Waterhouse *et al.*, 2007). The most important caspase inhibitor in mosquitoes, IAP1, has been identified in *A. aegypti*, *A. albopictus* and *A. triseriatus* (Beck *et al.*, 2007, Blitvich *et al.*, 2002, Li *et al.*, 2007). *A. albopictus* IAP1 protected mammalian BSR cells from bluetongue virus-induced apoptosis, and rescued insect Sf9 cells from Hid-induced apoptosis (Li *et al.*, 2007). Also the topical application of *A. aegypti* IAP1 dsRNA to the dorsal thorax of female adult *A. aegypti* caused significant death in mosquitoes (Pridgeon *et al.*, 2008b). Because the midgut is the first barrier against many pathogens, the relative higher expression level of *A. aegypti* IAP1 in adult midgut indicates IAP1 might play an important role in immunity (Bryant *et al.*, 2008). The expression level of *A. aegypti* IAP1 was upregulated responding to UV exposure and heat-shock treatment, suggesting that IAP1 might function as an important stress regulator (Pridgeon *et al.*, 2008a). Phylogenetic analysis of caspases revealed that the effector caspases AeCASPS7 and AeCASPS8 are closest to *Drosophila* Drice and Dcp-1, while the initiator caspases AeDronc and AeDredd are orthologs of *Drosophila* Dronc and Dredd (Bryant *et al.*, 2008, Cooper *et al.*, 2007a, Cooper *et al.*, 2007b). However, there have been expansions of effector Decay and initiator Damm caspase homologs in *A. aegypti* (Bryant *et al.*, 2008).

Aedes and *Anopheles michelob_x* (Mx) have been studied in the *Drosophila* context (Zhou *et al.*, 2005), and expression of Mx or IMP in *A. albopictus* C6/36 cells induced cell death (Bryant *et al.*, 2008, Wang *et al.*, 2008, Zhou *et al.*, 2005). However, it is still not clear what the functions of these antagonists are in *A. aegypti* and how AeIAP1, antagonists and caspases, interact at the molecular level in *A. aegypti*.

In this dissertation, several aspects of apoptosis in mosquitoes and its effect on virus infection were examined: the effects of inducing or inhibiting apoptosis on SINV replication in mosquito cells, the role of IAP antagonist proteins in the core apoptosis pathway of *A. aegypti*, and the effects of silencing apoptosis regulatory genes on SINV replication and dissemination in *A. aegypti*. The results show that recombinant SINVs expressing pro-apoptotic genes caused extensive apoptosis in mosquito cells with decreased virus production after the cells underwent apoptosis, systemic apoptosis positively influenced SINV replication and dissemination in *A. aegypti*, and the mechanisms of IAP antagonists regulating apoptosis were conserved between *A. aegypti* and the model insect where apoptosis has been mainly studied, *Drosophila melanogaster*. The model of the core apoptosis pathway in mosquitoes proposed in this dissertation is illustrated in Figure 1.1.

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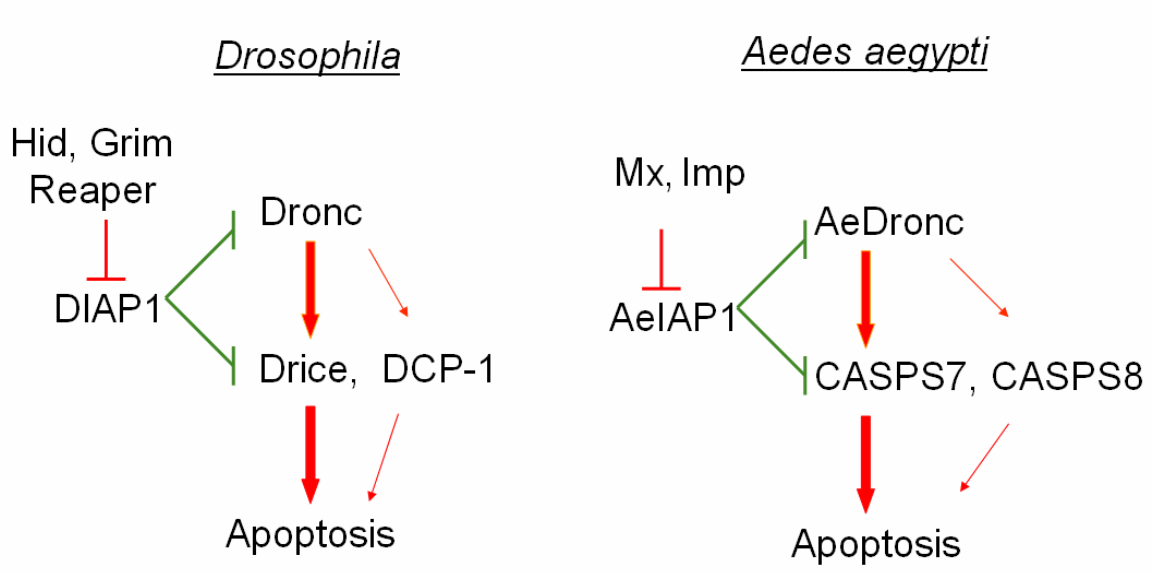

Figure 1.1 Comparison of the core apoptotic pathways between *Drosophila* and *A. aegypti*

CHAPTER 2 - Effects of inducing or inhibiting apoptosis on Sindbis virus replication in mosquito cells

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Abstract

Sindbis virus (SINV) is a mosquito-borne virus in the genus *Alphavirus*, family *Togaviridae*. Like most alphaviruses, SINVs exhibit lytic infection (apoptosis) in many mammalian cell types, but are generally thought to cause persistent infection with only moderate cytopathic effects in mosquito cells. However, there have been several reports of apoptotic-like cell death in mosquitoes infected with alphaviruses or flaviviruses. Given that apoptosis has been shown to be an antiviral response in other systems, we have constructed recombinant SINVs that express either pro-apoptotic or anti-apoptotic genes in order to test the effects of inducing or inhibiting apoptosis on SINV replication in mosquito cells. Recombinant SINVs expressing the pro-apoptotic genes *reaper* (*rpr*) from *Drosophila* or *michelob_x* (*mx*) from *Aedes aegypti* caused extensive apoptosis in cells from the mosquito cell line C6/36, thus changing the normal persistent infection observed with SINV to a lytic infection. Although the infected cells underwent apoptosis, high levels of virus replication were still observed during initial infection. However, virus production subsequently decreased compared to persistently infected cells, which continued to produce high levels of virus over the next several days. Infection of C6/36 cells with SINV expressing the baculovirus caspase inhibitor P35 inhibited actinomycin D-induced caspase activity and protected infected cells from actinomycin D-induced apoptosis, but had no observable effect on virus replication. This study is the first to directly test whether inducing or inhibiting apoptosis affects arbovirus replication in mosquito cells.

Introduction

Each year several million people die of arthropod-borne diseases including malaria, yellow fever, and dengue fever (Hill *et al.*, 2005). *Sindbis virus* (SINV) (genus *Alphavirus*; family *Togaviridae*) is an arthropod-borne virus (arbovirus) having a positive-sense, single-stranded RNA genome of 11.7 kb, with a 5'cap and a 3' poly(A) tail (Strauss & Strauss, 1994). SINV is an important tool to study the interaction between viruses and mosquitoes because full-length infectious cDNA clones are available which have been engineered to allow expression of foreign genes, and because SINV can infect *Aedes aegypti*, a mosquito vector which is important in the transmission of dengue and yellow fever viruses.

SINVs generally cause acute cell death in most types of mammalian cells, and infected cells display typical characteristics of apoptosis (Levine *et al.*, 1993, Nava *et al.*, 1998). However, SINVs are generally thought to cause only moderate cytopathic effects in mosquito cells with a persistent infection (Karpf & Brown, 1998). Expression of the apoptotic inhibitory gene *bcl-2* can convert the pattern of SINV infection in mammalian cells from lytic to persistent (Levine *et al.*, 1993). In addition, the ability of SINV to cause apoptosis in neurons correlates with pathogenesis in mice (Lewis *et al.*, 1996). The reasons why SINV infection does not cause apoptosis in mosquito cells are still unknown. Cell and species specificity of SINV-induced cell death implies that cellular and viral regulators of apoptosis play important roles in determining the outcome of SINV infection. However, it is important to keep in mind that most of the information in this area comes from studies performed using mosquito cell lines. Less is known about SINV infection *in vivo*, and the possibility remains that SINV could cause apoptosis in certain

cell types in mosquitoes, or in certain mosquito species. Indeed, there are a number of reports of cell death in mosquitoes infected with arboviruses (including the alphaviruses SINV, Semiliki Forest virus, and Eastern and Western equine encephalitis viruses, as well as the flavivirus West Nile virus), some of which are consistent with apoptosis (Bowers *et al.*, 2003, Girard *et al.*, 2005, Mims *et al.*, 1966, Weaver *et al.*, 1992, Weaver *et al.*, 1988). In addition, correlation between apoptosis and resistance to West Nile virus infection has been observed in midgut cells of a refractory lab strain of *Culex pipiens pipiens* (Vaidyanathan & Scott, 2006), and apoptosis that occurs in the salivary glands of *Culex pipiens quinquefasciatus* late in infection also correlates with reduced transmission potential for West Nile virus (Girard *et al.*, 2005, Girard *et al.*, 2007). However, despite these intriguing observations, no causative data exist that directly link apoptosis to effects on viral vector competence in mosquitoes.

Apoptosis is executed by initiator and effector caspases (cysteinyl aspartate-specific proteases), which become activated following an apoptotic stimulus and cleave a number of cellular substrates. Caspases are negatively regulated by cellular IAP (inhibitor of apoptosis) proteins, and IAPs are themselves negatively regulated by IAP antagonists. IAP antagonists are characterized by sharing a highly conserved N-terminal motif, an IAP-binding motif (IBM). *Drosophila* Reaper (Rpr) and *Ae. aegypti* Michelob_x (Mx) are examples of IAP antagonists which contain an IBM and function as pro-apoptotic proteins (Zhou *et al.*, 2005). On the other hand, the baculovirus caspase inhibitor P35 is a potent inhibitor of effector caspases from a wide variety of organisms (Clem, 2007). Following cleavage of P35 by an active caspase, a covalent bond is

formed between P35 and the active site cysteine of the caspase (Fisher *et al.*, 1999, Xu *et al.*, 2001).

Recombinant SINV expression systems have been developed by inserting an additional copy of the viral subgenomic promoter in the genome to facilitate expression of foreign genes (Foy *et al.*, 2004, Hahn *et al.*, 1992, Olson *et al.*, 2000, Pierro *et al.*, 2003, Raju & Huang, 1991). The SINV infectious clones 5'dsMRE16ic and TE5'2J each contain a duplicated subgenomic promoter upstream of the normal subgenomic promoter in the viral genome. TE5'2J was generated from the mouse neurovirulent TE12 SINV strain, while 5'dsMRE16ic was engineered from the MRE16 SINV strain (Foy *et al.*, 2004, Pierro *et al.*, 2003, Pierro *et al.*, 2007). TE5'2J viruses replicate well in cell lines, but poorly infect mosquito midguts after oral infection. In contrast, 5'dsMRE16ic viruses are able to efficiently infect and disseminate from midgut epithelial cells after oral infection (Foy *et al.*, 2004, Myles *et al.*, 2004). In this study, we have used these SINV constructs to express pro-apoptotic and anti-apoptotic proteins in order to begin testing whether apoptosis can play a role in governing interactions between alphaviruses and mosquitoes.

Materials and Methods

Cell culture

BHK-21 cells were propagated in Dulbecco's Modification of Eagle's Medium (DMEM) (Mediatech) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen). *Aedes albopictus* C6/36 cells were propagated in Leibovitz's medium (Gibco) with 10% FBS. BHK-21 cells were cultured at 37^{o}C with 8% CO_2, and C6/36 cells were maintained at 27^{o}C.

Recombinant virus construction

The coding regions of the *mx*, *rpr* and *p35* cDNAs were amplified by PCR and cloned into the SINV DNA infectious clones p5'dsMRE16ic (MRE) (Foy *et al.*, 2004, Myles *et al.*, 2004) or pTE5'2J (TE) (Pierro *et al.*, 2003) in the sense and antisense orientation. Additional clones were constructed containing in-frame fusions with the HA epitope tag at the C- (Mx and Rpr) or N terminus (P35), sites which have been shown previously to not affect protein function. The insert sequences of all of the plasmids were verified by nucleotide sequencing. The GFP-expressing viruses MRE/GFP and TE/GFP have been previously described (Foy *et al.*, 2004, Pierro *et al.*, 2003).

Virus production

Capped transcripts of SINV RNA were produced using AmpliScribe™ SP6 High Yield Transcription Kit (EPICENTRE Biotechnologies) and m^{7}G(5')ppp(5')G Cap Analog (Ambion). Ten µl of each transcript reaction were transfected into BHK-21 cells using Lipofectamine™ 2000 (Invitrogen) and 100 µl Opti-MEM® I Reduced Serum

Medium (Opti-MEM) (Invitrogen). After 3 days, medium containing virus was harvested, aliquoted and stored at -80^{o}C. Virus titers were determined by tissue culture infectious dose ($TCID_{50}$) assay in BHK-21 cells. The $TCID_{50}$ of each sample was converted to p.f.u. ml^{-1} by multiplying by 0.69 (O'Reilly *et al.*, 1994). All of the virus stocks used in this study came directly from transfected BHK-21 cells without any further passage, and were only frozen and thawed once before use.

Virus growth curves and $TCID_{50}$ assay

One million C6/36 cells were infected at an m.o.i. of 0.1 or 10 in a 6-well plate. After a one hour absorption period with Leibovitz's medium, the cells were washed three times with PBS, and 2 ml of Leibovitz's medium containing 10% FBS was added into each well. At 0, 1, 2, 3, 4, and 5 days p.i., 100 µl of cell medium containing virus was collected and frozen at -80^{o}C until being subjected to $TCID_{50}$ assay as described above. In the non-cumulative assay, after each time point the cells were washed three times with PBS and the medium was replaced.

Caspase assay

To detect caspase activity, 1 x 10^5 cells were infected at an m.o.i. of 0.01 or 1. At 6, 12, and 24 hpi, cells were harvested and centrifuged at 500 x g for 5 min. Cell pellets were washed with PBS and resuspended in 100 µl lysis buffer (20 mM HEPES KOH, pH 7.5, 50 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 250 mM sucrose). One complete mini EDTA-free protease inhibitor tablet (Roche Molecular Biochemicals) was added per 50 ml lysis buffer. Cells were lysed by four cycles of freezing-thawing and 50 µg of protein was mixed in 100 µl reaction buffer (100 mM

HEPES buffer, pH 7.4 containing 2 mM DTT, 0.1% CHAPS, 1% sucrose) with 200 μM Ac-DEVD-AFC (MP Biomedicals), an effector-type caspase substrate, and incubated for 15 min at 37°C. The fluorescence (excitation 405 nm, emission 535 nm) in the reactions was monitored over 1 hr at 25°C using a Victor^3 1420 Multilabel counter (Perkin-Elmer), and the values of the final measurements are shown.

TUNEL staining and flow cytometry analysis

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) was performed using the *In Situ* Cell Death Detection Kit, TMR red (Roche Applied Science). Two million C6/36 cells were infected at an m.o.i. of 0.1. Cells were harvested and washed 3 times with PBS, then pelleted by centrifugation at 500 x g for 5 min, and resuspended in 2% paraformaldehyde freshly prepared in PBS for 1 hr at room temperature. After washing once with PBS, cell pellets were resuspended in fresh permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice. Cells were washed twice with PBS and resuspended in 50 μl TUNEL reaction mixture (5 μl enzyme solution with 45 μl label solution) for 1 hour at 37°C. Cells were washed twice with PBS and resuspended in 250 μl PBS with 1 μM TO-PRO-3 (Invitrogen) for nuclear counterstaining. Cells were detected using FL2 and FL4 in a FACSCalibur (Becton Dickinson), and data were analyzed with WinList5.0 (Verity Software House).

DNA fragmentation assay

C6/36 cells (2×10^6) were infected at an m.o.i. of 1. At 24 hpi, cells were harvested and pelleted as described above. The cell pellet was resuspended in 100 μl

lysis buffer (10 mM Tris-HCl, pH 8.0; 100 mM NaCl; 25 mM EDTA; 0.5% SDS; 0.1 mg/ml Proteinase K). The lysate was extracted twice with phenol/chloroform and ethanol precipitated. The precipitate was washed with 75% ethanol and resuspended in 100 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) containing 100 µg ml^{-1} RNase. Twenty µl of each sample were analyzed by agarose gel electrophoresis and the bands were visualized by ethidium bromide staining. To visualize nuclei, cells (48 hpi) were stained with 5 µg ml^{-1} Hoechst 33258 for 20 min before observation by UV microscopy.

Cell viability assay (MTT assay)

To determine cell viability, C6/36 cells (1 x 10^5) were infected at an m.o.i. of 0.01 in a 96-well-plate. Every 24 h, the cell medium was replaced with fresh medium. At each time point, cells were centrifuged at 500 x g for 5 min and washed once with PBS. Cells were incubated with 100 µl 1% [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) (Sigma) for 4 h at 27^{o}C. Cells were washed with PBS again, and 150 µl acidic isopropanol (0.04 M HCl in absolute isopropanol) was added, followed by rocking on a shaking platform for 15 min at room temperature. Absorbance was determined at 550 nm.

For the actinomycin D (ActD)-induced cell death experiment, C6/36 cells (4 x 10^5) were infected at an m.o.i. of 1 in a 24-well-plate. At 24 hpi, 1 µg ml^{-1} ActD (Clontech Laboratories) and/or 100 µM z-VAD-FMK (MP Biomedicals) were added. After 24 h of ActD treatment, cell viability was determined by MTT assay as described above.

Immunoblotting

C6/36 cells (2×10^6) were infected at an m.o.i. of 1. At 6 hpi, 100 μM z-VAD-FMK was added to the medium. At 24 hpi, cells were collected in 100 μl of SDS-PAGE loading buffer, heated at 100°C for 5 min and resolved by 15% SDS-PAGE, and then transferred to PVDF. Proteins were detected with a 1:1000 dilution of anti-HA antibody (Covance) or anti-β actin antibody, and a 1:10,000 dilution of goat anti-mouse IgG–horseradish peroxidase (Bio-Rad) and SuperSignal West Pico Chemiluminescent substrate (Pierce).

Results

Construction of recombinant SINVs and expression in C6/36 cells

The IAP antagonist genes *michelob_x* (*mx)* from *Ae. aegypti* and *reaper* (*rpr*) from *Drosophila melanogaster* have been shown to induce apoptosis when expressed in insect cells (Pronk *et al.*, 1996, Zhou *et al.*, 2005), while expression of the baculovirus *p35* gene blocks apoptosis by inhibiting caspases (Clem & Miller, 1994). In order to test whether inducing or inhibiting apoptosis would have an effect on SINV replication, a series of recombinant SINVs were constructed by inserting the coding regions of *mx*, *rpr*, or *p35* into the TE5'2J (TE) and 5'dsMRE16ic (MRE) SINV infectious clones in sense or antisense orientation, and in sense orientation with an HA epitope (Fig. 2.1A and B). To examine protein expression, C6/36 cells infected with viruses expressing HA-tagged proteins were harvested at 24 hpi and analyzed by western blotting. All of the foreign genes were expressed in infected C6/36 cells, with the level of expression being generally higher from the TE viruses than from the MRE viruses (Fig. 2.1C; note the longer exposure of the MRE blot).

SINVs expressing IAP antagonists induce apoptosis

In initial experiments, C6/36 cells infected with viruses expressing Mx or Rpr in the sense orientation underwent lysis within the first 24-48 hpi, while cells infected with all of the other viruses did not lyse, but instead exhibited typical signs of persistent infection. To quantify the death of C6/36 cells infected with SINVs expressing Mx or Rpr, cell viability was quantified by MTT assay, which measures metabolic activity. C6/36 cells that were infected with viruses containing antisense inserts or viruses

expressing GFP or P35 continued to proliferate similar to mock-infected cells, although by 4-5 days p.i., infected cells were slightly fewer in number than mock-infected cells, consistent with moderate cytopathic effect induced by SINV infection in these cells (Fig. 2.2A and B). Consistent with the higher level of foreign protein expression from TE viruses than MRE viruses (Fig. 2.1C), TE/Mx and TE/Rpr viruses induced cell death faster than MRE/Mx and MRE/Rpr. Cell blebbing and apoptotic bodies were first observed in TE/Mx- and TE/Rpr-infected cells at 12 hpi, while MRE/Mx- and MRE/Rpr-infected cells began blebbing at 18 hpi (data not shown). By 48 hpi, nearly all the cells infected by SINV expressing Mx or Rpr had died, while their counterpart antisense-virus-infected cells continued to proliferate (Fig. 2.2A-C). We also tested the viability of cells infected with the viruses expressing epitope-tagged IAP antagonists. TE/Mx-HA induced cell death in C6/36 cells, but TE/Rpr-HA, MRE/Mx-HA, and MRE/Rpr-HA did not, possibly due to the epitope tag interfering with protein function (data not shown).

To determine whether the death caused by recombinant SINVs expressing Mx or Rpr in C6/36 cells was due to apoptosis, we examined several parameters, including caspase activation (Fig. 2.3). At 6 hpi, all of the infected cells exhibited caspase activity similar to that of mock-infected cells. However, at 12 hpi, TE/Mx and TE/Rpr infection caused increased levels of caspase activity, in contrast to MRE/Mx and MRE/Rpr infection, which remained fairly low (Fig. 2.3). By 24 hpi, MRE/Mx- and MRE/Rpr-infected C6/36 cells exhibited extensive cell blebbing and dramatically increased caspase activity. TE/Mx- and TE/Rpr- infected C6/36 cells appeared to exhibit less caspase activity at 24 hpi compared with MRE/Mx- and MRE/Rpr-infected cells, but presumably this was because many of the TE/Mx- and TE/Rpr-infected cells had already completed

apoptosis and undergone secondary necrosis by this time, resulting in leakage of intracellular proteins into the culture supernatant.

Chromatin degradation, nuclear condensation and nuclear fragmentation are also hallmarks of apoptosis. At 24 hpi, we examined the DNA from infected C6/36 cells by agarose gel electrophoresis . MRE/Mx-, MRE/Rpr-, TE/Mx- and TE/Rpr-infected cells exhibited genomic DNA fragmentation into oligonucleosomal ladders characteristic of apoptotic cells (Fig. 2.4A and B). In contrast, the genomic DNA from cells infected by anti-sense viruses or viruses expressing P35 was intact, similar to that of mock-infected cells (Fig. 2.4A and B). We also observed genomic DNA condensation and nuclear fragmentation in C6/36 cells infected with MRE/Mx, MRE/Rpr, TE/Mx and TE/Rpr at 24 hpi, in contrast to the uniform nuclear staining observed in C6/36 cells infected with anti-sense viruses and mock-infected cells (Fig. 2.4C).

We quantified apoptotic cells using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) and flow cytometry (Fig. 2.5). As expected, TE/Mx- and TE/Rpr-infected cells became TUNEL-positive more quickly than MRE/Mx- and MRE/Rpr-infected cells. At 12 hpi, 47% of TE/Mx- and 51% of TE/Rpr-infected cells were TUNEL-positive, while the proportions of MRE/Mx- and MRE/Rpr-infected cells that were TUNEL-positive were 24.5% and 25.9% respectively. At 24 and 36 hpi, the proportion of TUNEL-positive MRE/Mx- and MRE/Rpr-infected cells increased to a range of 40-50%, while 55-65% of TE/Mx- and TE/Rpr-infected cells were TUNEL-positive. In contrast, approximately 10% of MRE/P35-, MRE-, TE/P35-, and TE-infected cells were TUNEL-positive at each time point, which was similar to the background staining seen in mock-infected cells.

P35 expression by SINV protects C6/36 cells from apoptotic stress

To test whether SINV-mediated expression of the caspase inhibitor P35 can protect C6/36 cells from apoptotic stress, we analyzed the viability of infected cells after treatment with actinomycin D (ActD), which induces apoptosis in many insect cell lines. After 24 h treatment with ActD we observed that over 90% of C6/36 cells were apoptotic as judged by their morphology (Fig. 2.6C). By MTT assay, we found that mock-infected, ActD-treated cells were only around 20% viable compared to untreated cells (Fig. 2.6A), while the relative viability of MRE- and TE-infected cells decreased from 60% to 20% after ActD treatment (Fig. 2.6A). However, the viability of MRE/P35- and TE/P35-infected cells was only slightly reduced after ActD treatment (Fig. 2.6A). The death induced by ActD was inhibited by the caspase inhibitor z-VAD-FMK (85% viability compared to 20% in cells treated with ActD alone) (Fig. 2.6A).

Without ActD treatment, MRE-, MRE/P35-, TE-, and TE/P35-infected cells had similar levels of caspase activity as mock-treated cells (Fig. 2.6B). After ActD treatment, MRE- or TE-infected cells showed an increase in caspase activity, but MRE/P35- or TE/P35-infected cells had no change in caspase activity compared to non-ActD-treated cells, indicating caspase inhibition by P35 (Fig. 2.6B).

Replication of recombinant SINVs

To assess the effect of apoptosis on SINV replication, virus growth curves were performed. To measure the production of virus during each 24 h period following infection (non-cumulative assay), the cells were washed three times with PBS at each time point after removal of virus-containing culture supernatant. Mx- and Rpr-expressing recombinant viruses caused lytic replication in C6/36 cells, and as expected, the amount

of virus production significantly decreased after the death of C6/36 cells. The viral titers of both MRE/Rpr and MRE/Mx viruses peaked at 2 dpi (Fig. 2.7A), while TE/Rpr and TE/Mx -infected C6/36 cells exhibited the highest level of virus at 1 dpi (Fig. 2.7C), consistent with the viability results (Fig. 2.2A and B). Viruses containing any inserts, including antisense inserts or GFP, tended to produce lower levels of virus than the empty vectors MRE or TE, presumably due to their increased genome size (Pierro *et al.*, 2003). In addition, the recombinant TE viruses produced around a 10-fold higher amount of virus than the corresponding recombinant MRE viruses, although the TE and MRE empty vectors produced roughly equivalent titers. This was not unexpected, given that the TE strain is adapted to replication in cell culture (Olson *et al.*, 2000, Pierro *et al.*, 2003). Infection with high versus low m.o.i. did not significantly affect the final viral titers (Fig. 2.7B and D).

We also examined cumulative virus replication by removing a small amount of culture medium at each time point without replacing the medium or washing the cells. Generally, the production of each virus reached a plateau at 2 dpi in C6/36 cells (Fig. 2.8A and B) and 1 dpi in BHK-21 cells (Fig. 2.8C and D), and a high level of virus remained in the culture supernatant for the rest of the experiment. Similar to the above results, the MRE and TE recombinant viruses containing inserts produced approximately 10-fold less progeny virus than viruses without inserts (Fig. 2.8). All of the viruses caused cell death in BHK-21 cells within 1 day, including the viruses expressing P35. In C6/36 cells, the viruses expressing Mx or Rpr caused extensive apoptosis within 1-2 days, while the rest of the viruses caused only moderate cytopathic effect. Despite this difference, the recombinant viruses in each type of parental clone (TE or MRE) had

similar growth patterns in both cell lines. While MRE/Mx, MRE/Rpr, TE/Mx, and TE/Rpr-infected C6/36 cells were almost all dead after 2 days, cells infected with the other viruses remained alive; however, the level of virus in the medium did not increase significantly over the next 3 days for any of the viruses (Fig. 2.8A and B).

Discussion

We are using SINV as a model to study the effect of inducing or inhibiting apoptosis on the ability of mosquito cells to permit arbovirus replication. Arboviruses usually do not induce apoptosis in mosquito cell lines; however, there are reports of cytopathic effects resembling apoptosis in arbovirus-infected mosquitoes, leading to the question of whether apoptosis could be an anti-viral response in certain tissues or in some arbovirus-mosquito combinations. The effects of apoptosis on arbovirus replication have not been previously investigated. In this study we have characterized the effects of expressing apoptotic regulatory genes on cell viability and virus replication in the mosquito cell line C6/36.

The genetic factors that govern susceptibility to arbovirus infection in mosquitoes are poorly understood. One pathway that increasingly appears to be important in regulating the level of virus replication in mosquitoes is RNA interference (RNAi) (Campbell *et al.*, 2008b, Keene *et al.*, 2004, Sanchez-Vargas *et al.*, 2004). Besides RNAi, there are other pathways that are also likely to be involved in mosquito anti-viral immunity, but at this time little evidence exists in this area. Transcript levels of members of the Toll and JNK pathways, as well as several serpin genes, were shown to be altered following SINV infection of *Ae. aegypti* (Sanders *et al.*, 2005), suggesting that known innate immune pathways may be stimulated by virus infection in mosquitoes. In addition, reducing or activating Toll pathway signaling has effects on dengue virus replication in *Ae. Aegypti* (Xi *et al.*, 2008). Finally, heat shock protein cognate 70B of *Anopheles gambiae* is upregulated by O'nyong-nyong virus infection, and that silencing

of this gene results in higher levels of O'nyong-nyong replication in *An. gambiae* mosquitoes (Sim *et al.*, 2007).

Apoptosis is another attractive candidate anti-viral response in mosquitoes, given its importance in other virus-host systems (Clem, 2007, Hay & Kannourakis, 2002). It has been postulated that there are at least three barriers to successful infection and dissemination of arboviruses in mosquitoes: the midgut infection barrier (the ability to establish infection and replicate in midgut epithelium), the midgut escape barrier (the ability to penetrate the midgut and establish replication in other tissues), the salivary gland infection barrier (the ability to infect salivary glands), and the salivary gland escape barrier (the ability to enter the salivary gland lumen) (Black *et al.*, 2002). A successful apoptotic response in the midgut or salivary gland could thus limit the ability of a virus to replicate and be disseminated.

In this study, we expressed the IAP antagonists Mx and Rpr and the caspase inhibitor P35 to either purposely induce or inhibit apoptosis during SINV infection. While SINV normally causes non-lytic, persistent infection in mosquito cell lines, expression of Mx or Rpr from SINV caused apoptosis in C6/36 cells, as determined by cell morphology, caspase activity, and DNA fragmentation. Expression of P35, on the other hand, inhibited apoptosis induced by ActD treatment. This result, together with the fact that P35 is a broad-spectrum caspase inhibitor which inhibits apoptosis in a wide variety of situations (Clem, 2007), suggests that this virus could be used to test the effect of inhibiting apoptosis on vector competence in mosquitoes. The viruses expressing P35 still induced apoptosis in BHK cells, despite expressing P35. The reason for this is unclear, but it may be because SINV induces apoptosis rapidly in BHK cells, perhaps

before sufficient amounts of P35 can be expressed from the subgenomic promoter. In a previous report, SINV-mediated expression of another caspase inhibitor, CrmA, inhibited apoptosis in BHK cells (Nava *et al.*, 1998), but different strains of SINV and BHK cells were used.

The two SINV expression systems used in this study, MRE and TE, differ from each other in their ability to replicate in cultured cells, and in their ability to infect mosquitoes following a blood meal. TE is derived from a laboratory strain of SINV that is well adapted to replication in cultured cells. As a consequence, we observed higher levels of foreign gene expression in C6/36 cells with TE-based viruses, and we also saw that TE viruses expressing Mx or Rpr caused apoptosis faster than their MRE-based counterparts. Higher levels of virus replication were also observed for the TE-based viruses than for the MRE-based viruses when a foreign gene insert was present in the genome, although MRE without any additional insert replicated at equivalent levels to TE in either BHK or C6/36 cells. MRE, on the other hand, is derived from a field isolate of SINV, and has higher oral infectivity in mosquitoes than TE (Foy *et al.*, 2004). It will thus be interesting to determine how purposely inducing or inhibiting apoptosis affects the infectivity and dissemination of these viruses in mosquitoes following infection via a blood meal.

Neither induction nor inhibition of apoptosis had significant effects on the initial burst of replication of SINV in C6/36 cells. This may be in part due to the expression of these foreign genes from the viral subgenomic promoter, which is not expressed until after the viral genome has been replicated. In mammalian cells, SINV also replicates to high titers in spite of the apoptosis that is typically associated with infection, and

blocking apoptosis does not have a significant effect on the levels of replication (Nava *et al.*, 1998). However, cells that were infected by viruses expressing Mx or Rpr died after the initial burst of replication, and thus were not able to maintain high levels of virus replication over time. In an infected mosquito, this could be an important factor in determining vector competence. Sustained virus replication is presumably required for virus escape from the midgut and dissemination to other tissues, including the salivary glands. Therefore, if infected cells die after producing a burst of initial virus replication, virus dissemination may be adversely affected. In addition, other mechanisms may operate *in vivo* to limit virus replication. For example, early and rapid recognition of apoptotic cells by phagocytic cells (hemocytes) could result in enhanced clearance of infected cells and destruction of newly formed virus before it is able to bud from the infected cell. Thus, apoptosis could have a negative effect on the ability of SINV to productively infect and be transmitted by mosquitoes.

It is generally thought that arbovirus infection has little or no negative consequences for mosquito vectors in terms of cytopathology or decreased fecundity or life span. However, there have been reports of cytopathic effects in mosquitoes infected with arboviruses (including West Nile virus and several alphaviruses) including observations of apoptosis occurring in midgut or salivary gland (Bowers *et al.*, 2003, Girard *et al.*, 2005, Mims *et al.*, 1966, Weaver *et al.*, 1992, Weaver *et al.*, 1988), as well as negative effects on mosquito life span (Cooper *et al.*, 2000). It is likely that, if apoptosis has a negative effect on vector competence, there would be little apoptosis observed in successful virus-vector combinations. In these situations, the virus may either actively inhibit apoptosis or avoid inducing apoptosis altogether. Thus, apoptosis

may be more likely to occur in mosquitoes which do not have the ability to vector a particular virus, and which can mount a successful anti-viral response. To date the role of apoptosis in determining viral vector competence in mosquitoes has not been studied experimentally. The recombinant viruses characterized in this study will be useful tools to study the effects of apoptosis on determining the outcome of arbovirus infection *in vivo* in mosquito vectors.

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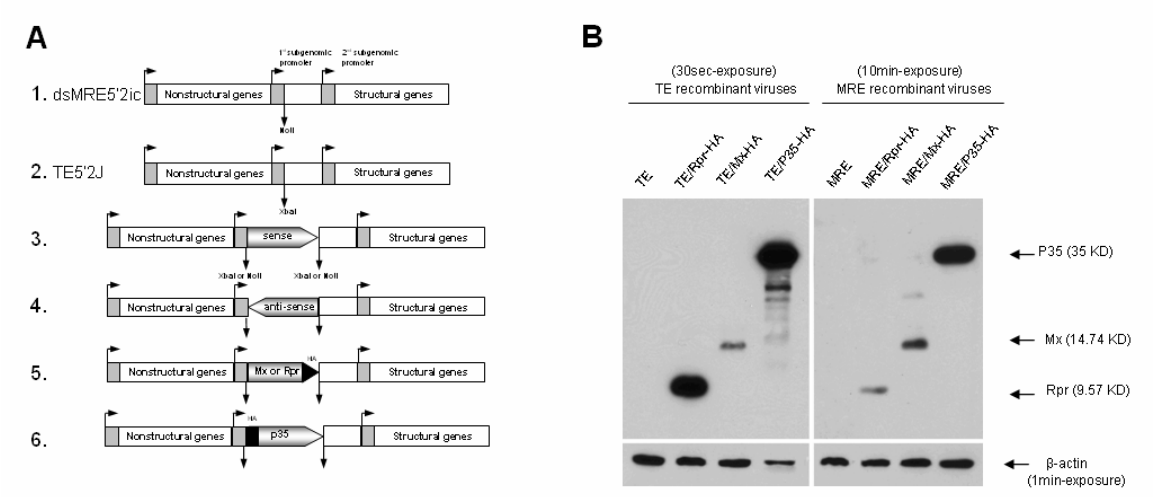

Figure 2.1 Recombinant SINVs and foreign gene expression in C6/36 cells by SINV

(A) Schematic of recombinant SINVs constructed in this study. Constructs expressing the sense, antisense, or epitope tagged forms of each gene under the 5' subgenomic promoter were prepared in both the MRE and TE infectious clones. In the tagged versions, the HA-tag was inserted at the N terminus of P35, and at the C terminus of Mx and Rpr. (B) Detection of foreign gene expression by immunoblotting. C6/36 cells were infected with the indicated HA-tagged recombinant and wild-type viruses (m.o.i. = 1). Lysates were prepared at 24 hpi, and analyzed by immunoblotting using anti-HA antibody. Antibody against β-actin was used as a loading control.

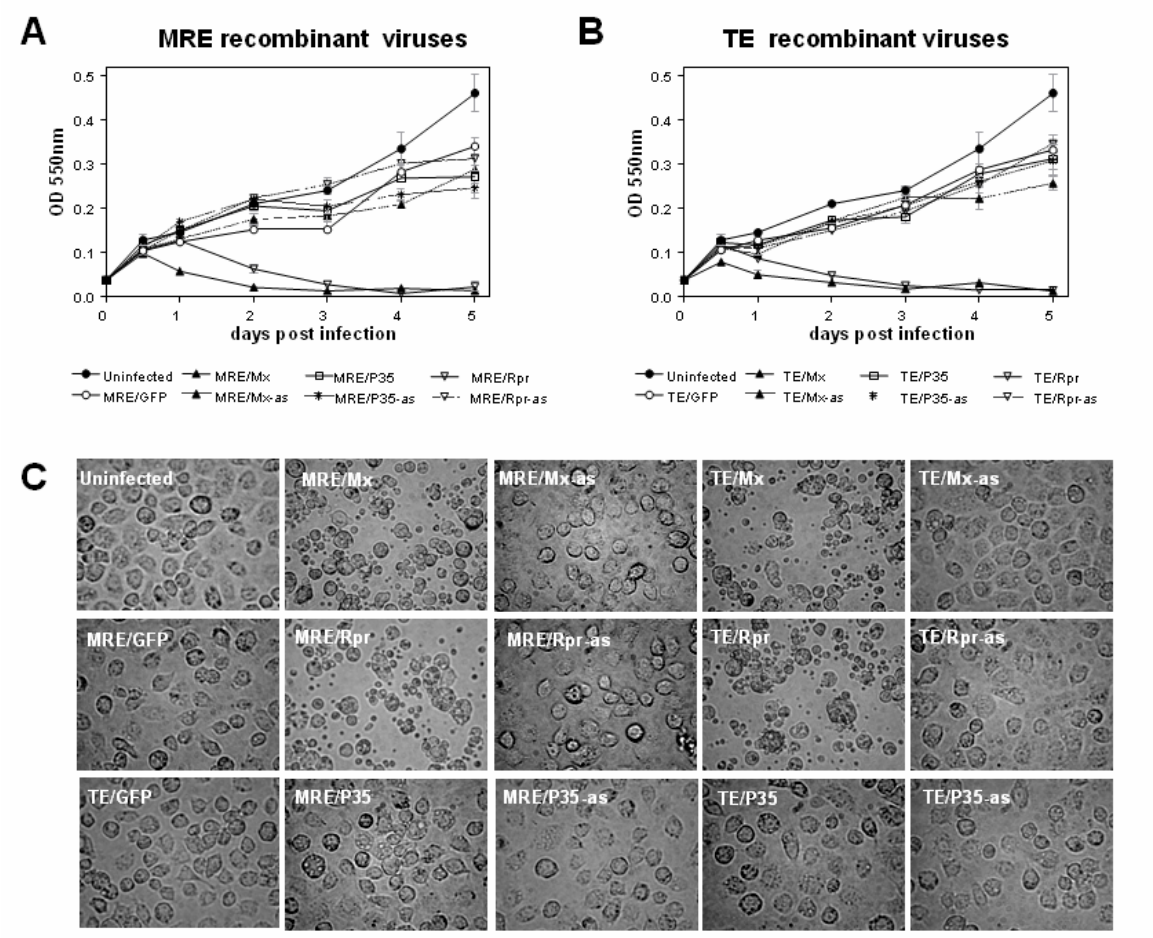

Figure 2.2 Recombinant SINVs expressing Mx or Rpr cause lytic infection in C6/36 cells

(A and B) C6/36 cells were mock-infected or infected with the indicated viruses (m.o.i. = 0.01) and cell viability was determined by MTT assay. Data are shown as mean + SEM of four to six independent experiments. The treatments differed significantly by two way ANOVA ($P < 0.0001$). (C) Morphology of infected cells. C6/36 cells were infected with the indicated SINVs (m.o.i. = 0.01) and photographed (magnification, 400X) at 48 hpi.

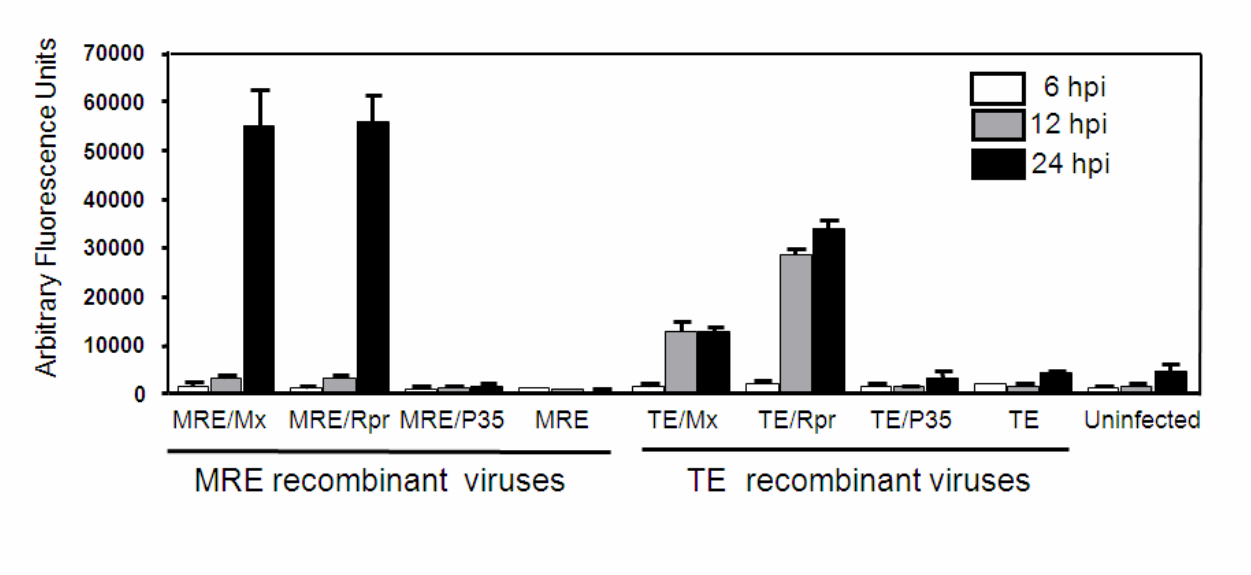

Figure 2.3 Activation of caspases by recombinant SINVs expressing Mx or Rpr

C6/36 cells were either mock-infected or infected with the indicated SINVs (m.o.i. = 0.01). At 6, 12, and 24 hpi, cell lysates were prepared and caspase activity was determined using Ac-DEVD-AFC as a substrate. Data are shown as mean + SEM of three experiments. The treatments differed significantly as judged by one way ANOVA ($P < 0.0001$).

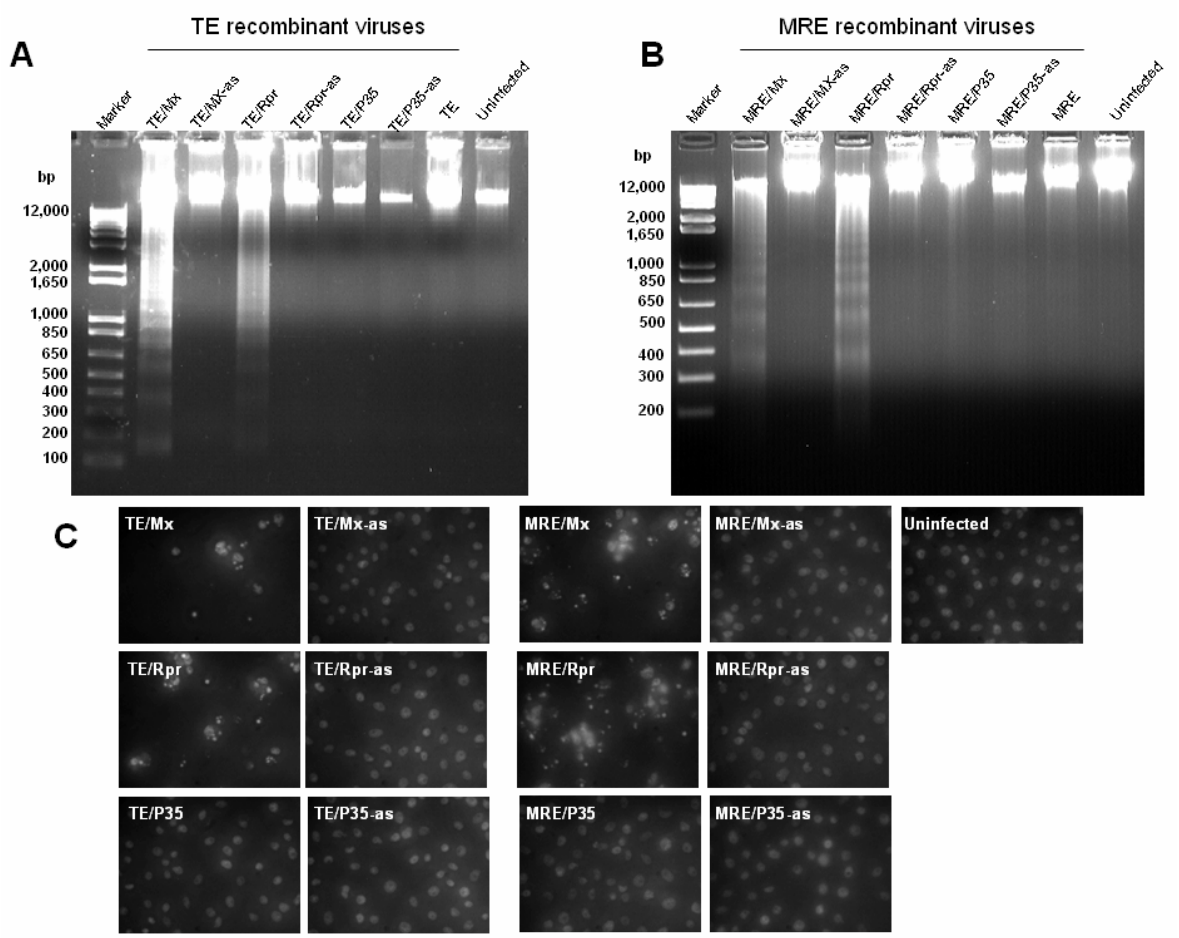

Figure 2.4 SINVs expressing Mx or Rpr cause DNA fragmentation indicative of apoptosis

(A and B) C6/36 cells were mock-infected or infected with the indicated SINVs (m.o.i. = 1). At 24 hpi, cells were lysed and DNA was analyzed by agarose gel electrophoresis and ethidium bromide staining. (C) C6/36 cells were infected with the indicated SINVs (m.o.i. = 0.01) for 48 h, stained with Hoechst 33258, and photographed (magnification, 400X).

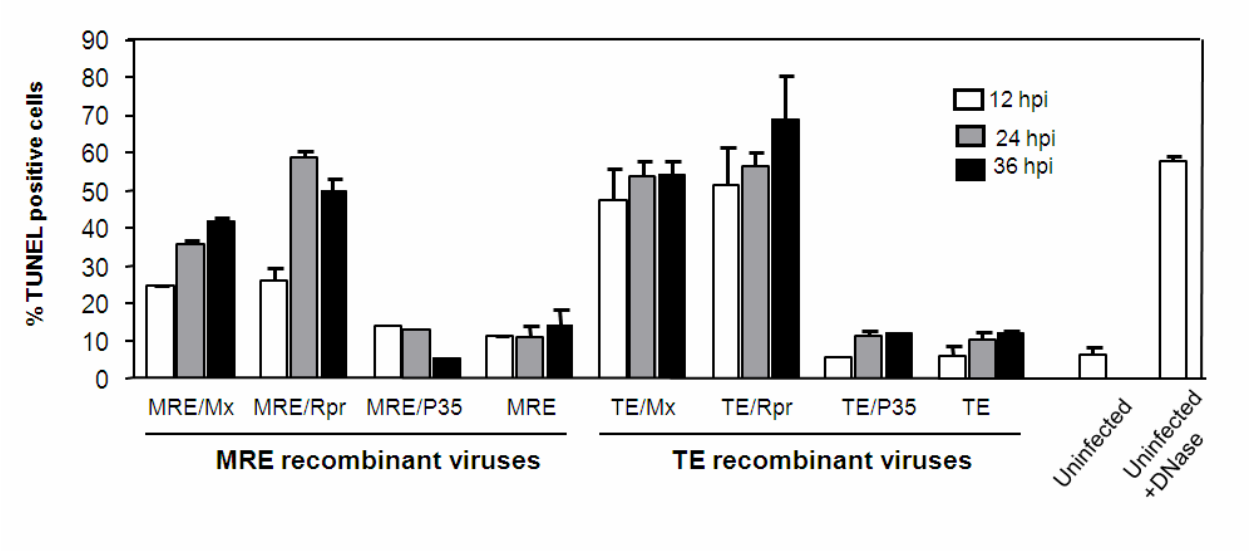

Figure 2.5 Apoptosis caused by SINVs expressing Mx or Rpr as assayed by TUNEL-staining

C6/36 cells were infected with the indicated SINVs (m.o.i. = 0.1) and harvested at 12, 24, and 36 hpi. The cells were subjected to TUNEL assay and analyzed by flow cytometry. A sample of mock-infected cells was treated with DNase as a positive control for DNA fragmentation. Data are shown as mean $\pm$ SEM of four experiments. The treatments differed significantly as judged by one way ANOVA ($P < 0.0001$).

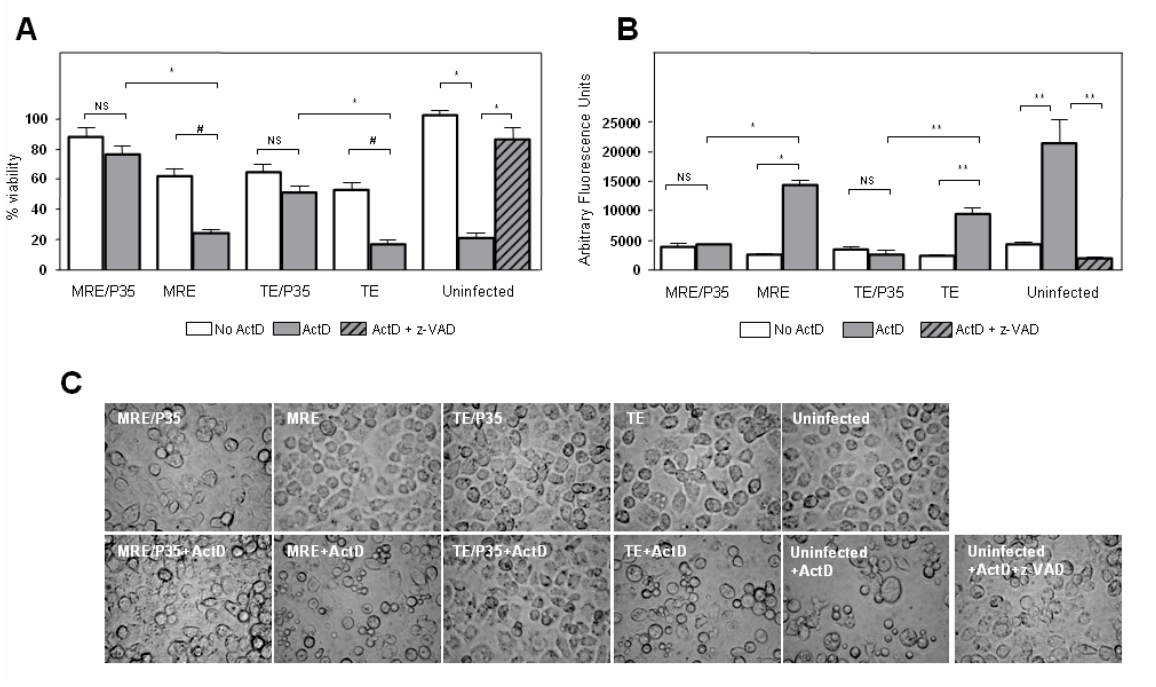

Figure 2.6 P35 expression by SINV protects C6/36 cells from ActD-induced cell death

(A) C6/36 cells were mock-infected or infected with the indicated SINVs (m.o.i. = 1), and at 24 hpi treated with ActD or ActD + z-VAD-FMK. MTT assay was performed 24h after ActD treatment. Data are shown as mean + SEM of three experiments (NS, non-significant, **P* < 0.0001, #*P* = 0.0002 by Student's t test). (B) C6/36 cells were mock-treated or infected with SINVs (m.o.i. = 1), and at 24 hpi treated with ActD or ActD + z-VAD-FMK. Cell lysates were prepared 18h after ActD treatment, and caspase activity was determined using Ac-DEVD-AFC as a substrate. Data are shown as mean + SEM of four experiments (NS, non-significant, **P* < 0.0001, ***P* < 0.005 by Student's t test). (C) C6/36 cells were infected with the indicated SINVs (m.o.i. = 1), and at 24 hpi treated with ActD. Cells were photographed (magnification, 400X) 24 h after ActD treatment.

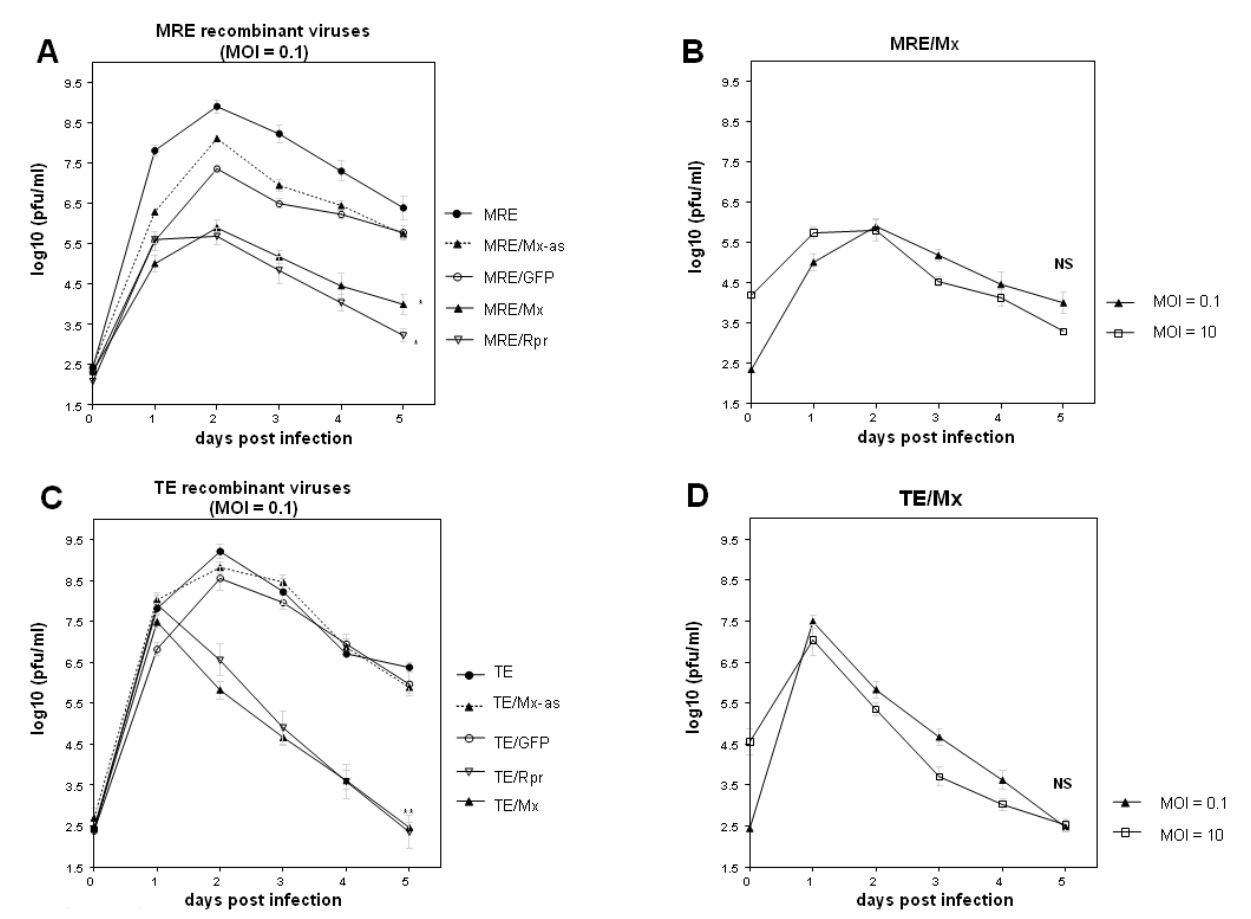

Figure 2.7 Non-cumulative virus growth curves in C6/36 cells

Cells were infected with the indicated viruses at m.o.i. of 0.1 (A and C), and 0.1 or 10 (B and D). The cells were washed three times with PBS after harvesting each time point. Data are shown as mean + SEM of four experiments. The treatments in panels A and C differed significantly by two way ANOVA ($P < 0.0001$) while differences between treatments in panels B and D were not significant.

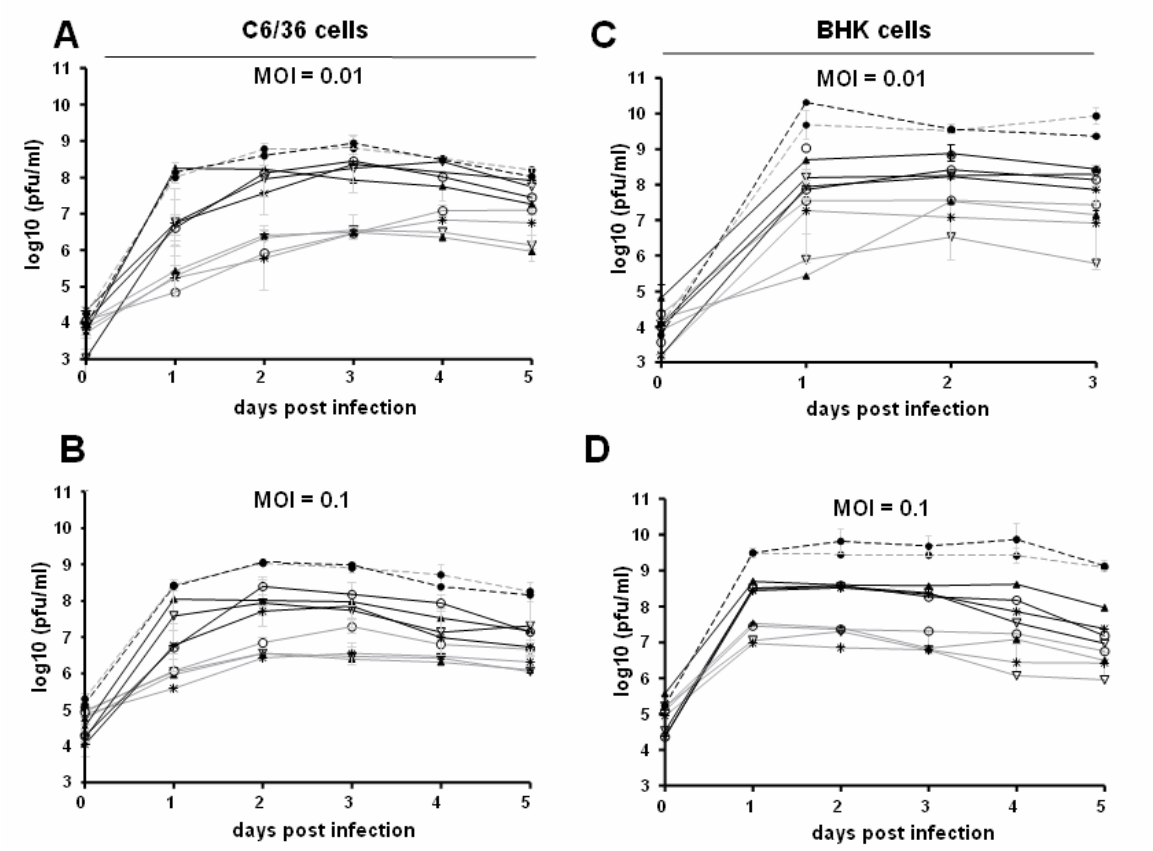

Figure 2.8 Cumulative virus growth curves in C6/36 and BHK-21 cells

C6/36 cells (A and B) or BHK-21 cells (C and D) were infected with the indicated viruses at an M.O.I. of 0.01 (A and C) or 0.1 (B and D). Supernatants were collected at the indicated times pi without replacing the medium or washing the cells. Data are shown as mean + SEM of three to five experiments. The treatments in panels A-D were significantly different by two way ANOVA ($P < 0.0001$).

CHAPTER 3 - Effects of silencing apoptosis regulatory genes on Sindbis virus replication and dissemination in *Aedes aegypti*

Abstract

Sindbis virus (SINV) is the type species of the genus *Alphavirus* in the *Togaviridae* family, and is associated with occasional outbreaks of disease in Africa, Europe and Asia. SINV is an arthropod-borne virus (arbovirus) which is most often vectored by mosquito species in the genus *Culex*, but can be vectored by the yellow fever mosquito, *Aedes aegypti*. Since *A. aegypti* is the major vector of yellow fever and dengue fever, and SINV has been well characterized at the molecular level (including the development of gene expression systems based on the SINV genome), SINV infection of *A. aegypti* is often used as a model to study arbovirus-mosquito interactions. We are interested in determining whether apoptosis plays a role in the ability of arboviruses to replicate in mosquitoes. Genes which either positively or negatively regulate apoptosis in *A. aegypti* were silenced by RNA interference in adult female mosquitoes, and the mosquitoes were then fed a blood meal containing the SINV infectious clone 5'dsMRE16ic expressing green fluorescent protein (5'dsMRE16ic-EGFP). When expression of the anti-apoptotic protein AeIAP1 was silenced, positive effects were observed in the occurrence and intensity of expression of GFP in mosquito tissues, while silencing of the initiator caspase *Aedronc* had negative effects on virus spread. We also observed increased caspase activity, changes in midgut morphology, and higher mosquito mortality following silencing of *Aeiap1*, even in the absence of virus infection. These results suggest that systemic apoptosis caused by *Aeiap1* silencing can positively influence SINV replication in *A. aegypti*, possibly due to loss of integrity of infection barriers. This is the first observation that silencing *Aeiap1* induces systemic apoptosis in

mosquitoes, and the first direct study to explore the role of apoptosis in determining mosquito vector competence for arboviruses.

Introduction

Mosquito-borne diseases are an urgent global health issue. Each year, over 500 million people are infected with mosquito-borne diseases, including malaria, yellow fever and dengue fever, which cause significant morbidity and mortality world-wide. Moreover, traditional mosquito control measures have become increasingly less effective over the past several decades. These issues highlight the necessity for more sophisticated strategies for mosquito vector control in the prevention of mosquito-borne diseases. One of the critical aspects for vector control is to interrupt vector competency for pathogens, which requires more detailed knowledge of the molecular interactions between the vectors and pathogens. Sindbis virus (SINV) is an important tool to study the interaction between viruses and mosquitoes because SINV can be vectored by *Aedes aegypti*, which is an important vector in the transmission of dengue and yellow fever viruses.

Currently, we have limited knowledge about anti-viral defense mechanisms in mosquitoes at the cellular level. The most studied mechanism is RNA interference (RNAi). Studies have found that the replication of several arboviruses (O'nyong-nyong (Keene *et al.*, 2004), dengue virus (Sanchez-Vargas *et al.*, 2009, Sanchez-Vargas *et al.*, 2004), and SINV (Campbell *et al.*, 2008b) are repressed by mosquitoes through activation of the RNAi machinery. During replication of the viral RNA genome, double-stranded replication intermediates are formed and recognized by the RNAi machinery, stimulating degration of viral RNA. In addition to RNAi, the Toll pathway is involved in the anti-viral defense. The Toll pathway is up-regulated in *A. aegypti* after a SINV blood meal (Sanders *et al.*, 2005), and reducing or activating the Toll signaling pathway leads to positive or negative effects on dengue virus replication in *A. aegypti* (Xi *et al.*, 2008).

After SINV infection, transcript levels of JNK pathway genes and several serpin genes were shown to be altered (Sanders *et al.*, 2005). Also, after O'nyong-nyong virus infection of *Anopheles gambiae*, heat-shock protein cognate 70B was found up-regulated, and silencing this gene resulted in higher level of virus replication (Sim *et al.*, 2007).

Apoptosis has been considered an antiviral defense in mammalian and insect systems (Chiou *et al.*, 1994, Clarke & Clem, 2003, Crook *et al.*, 1993, Cuconati *et al.*, 2002). There are several intriguing observations indicating that apoptosis might be involved during arbovirus infection in mosquitoes, in which cell death in midgut and/or salivary gland was observed after infection with arbovirus (Bowers *et al.*, 2003, Girard *et al.*, 2005, Girard *et al.*, 2007, Mims *et al.*, 1966, Vaidyanathan & Scott, 2006, Weaver *et al.*, 1992, Weaver *et al.*, 1988). Since midgut and salivary gland are considered to be infection barriers for arboviruses, which must escape from the initial site of infection in the midgut epithelium into the hemocoel and then into the salivary gland lumen (Campbell *et al.*, 2008a), any physical changes in these barriers could potentially have either positive or negative effects on virus replication and dissemination. However, no causative data exist that directly link apoptosis to effects on virus vector competence in mosquitoes.

Silencing of *Aeiap1* by dsRNA induces dramatic apoptosis in mosquito Aag2 cells (Q. Liu and R. J. Clem, unpublished data). In a recent report, topical application of *Aeiap1* dsRNA caused high mortality in *A. aegypti* (Pridgeon *et al.*, 2008b). It has also been shown that environmental stress leads to increased *Aeiap1* transcript levels in *A. aegypti* (Pridgeon *et al.*, 2008a). These data suggest that AeIAP1 may play an important role in apoptosis *in vivo*. In this study, we directly tested the effects of inducing or

inhibiting apoptosis on virus replication and dissemination in midgut and salivary gland barriers. We found that silencing of *Aeiap1* in adult female mosquitoes directly induced high mortality, similar to the previous study (Pridgeon *et al.*, 2008b). Furthermore, we showed that silencing *Aeiap1* leads to widespread apoptosis *in vivo*. Interestingly, we found that silencing of *Aeiap1* in mosquitoes also caused increased SINV infection and dissemination, perhaps due to the weakening of infection barriers.

Materials and Methods

Insect rearing

A. aegypti mosquitoes RexD (Rexville D, Puerto Rico) strain, obtained from Dr. Carol Blair at Colorado State University, and Orlando strain obtained from Dr. James Becnel at USDA laboratory were reared at 27^{o}C, 80% humidity, under a 12 h dark/12 h light regime. Adults were maintained on 10% sucrose solution, sugar, raisins and fresh water. Naive adult females were collected at 1 day post-eclosion.

Gene silencing by RNAi

Genes of interest (the full length coding sequence of *Aeiap1*, *Aedronc* or *cat*) were cloned into pCRII-TOPO vector (Invitrogen). The full length genes were PCR amplified from the pCRII-vector and T7 polymerase promoter sites were incorporated onto both ends by using the following vector primers: forward 5'-CTAATACGACTCACTATAGG GCAGGAAACAGCTATGAC -3' and reverse 5'- CAGTCACGACGTTGTAAAACGAC GGCC -3'. The PCR products were used as templates to synthesize double-stranded RNA (dsRNA) by using the AmpliScribe T7 High Yield Transcription kit (EPICENTRE Biotechnologies). The dsRNA was concentrated to 5 µg/µl in DEPC-treated water, and 69 nl of the dsRNA was injected into 1-day post-eclosion females using a Nanoject II injector (Drummond Scientific). Three days after dsRNA injection, live mosquitoes were collected for gene expression analysis or for viral blood feeding.

Expression analysis

RNA was isolated from homogenized tissues of pooled individuals using Trizol (Invitrogen) and treated with Turbo DNA-free DNase (Ambion) to degrade contaminated genomic DNA. Equal amounts of RNA (1-3 µg) was used to synthesize cDNA using reverse transcriptase (Promega) and oligo dT primer. The resulting cDNA was analyzed for expression of *Aeiap1*, *Aedronc* and *actin6* with primers as previously described (Bryant *et al.*, 2008). Expression was initially analyzed by agarose gel electrophoresis to verify the correct size for the amplicons. Real time PCR was performed using the BioRad iCycler Optical module. Cycle thresholds (Ct) were determined using iQ SYBR Green Supermix according to the instructions of the manufacturer (BioRad). The relative expression level of genes was normalized to *actin* and calculated using the equation $2^{-\Delta Ct}$, where ΔCt = Ct (*AeIAP1* or *AeDronc*) – Ct (*actin*) (Bryant *et al.*, 2008).

Caspase assay

To detect caspase activity, mosquito tissues of pooled individuals were homogenized in lysis buffer (20 mM HEPES KOH, pH 7.5, 50 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 250 mM sucrose) with complete mini EDTA-free protease inhibitor (Roche Applied Science). Protein concentration was determined by BCA protein assay (Thermo scientific), and 50 µg of protein were mixed in 100 µl reaction buffer (100 mM HEPES buffer, pH 7.4 containing 2 mM DTT, 0.1% CHAPS, 1% sucrose) with Ac-DEVD-AFC (MP Biomedical), an effector caspase substrate, and incubated for 15 min at 37^{o}C. Fluorescence (excitation 405 nm, emission 535 nm) was monitored as previously described (Wang *et al.*, 2008).

Transmission electron microscopy

Dissected mosquito midguts were fixed in 2% paraformaldehyde and 2% glutaraldehyde in 0.1 sodium cacodylate buffer (pH 7.2-7.4) for 16 h at room temperature with constant rotation. Samples were washed (3 x 5 min) in 0.1 M sodium cacodylate buffer at room temperature with constant rotation. Samples were treated with post-fix in 2% Osmium tetroxide and 0.1M sodium cacodylate buffer at room temperature with constant rotation. The samples were dehydrated in an ascending acetone series (50 to 100%) and infiltrated with EMBED 812/Araldite resin at room temperature with constant rotation. Samples were embedded in a flat mold with 100% resin for further polymerization in a drying oven at 60^{o}C for 24 h. Images were collected with a CM 100 (FEI Company) transmission electron microscope.

Midgut staining

The embedded tissue was sectioned (0.5 - 1.5 μm thick) under a microscope using an ultratome. The sections were placed on a glass microscope slide and dried on a hot plate. A drop of epoxy-tissue stain (Toluidine Blue and Basic Fuchsin) (Electron microscopy sciences) was added to cover the sections on the warmed slide, and the slide was put back on the hot plate until a silver rim was formed. The sections were rinsed with distilled water to remove any excess stain. After the slide dried, the sections were covered with a cover-slip. The images were collected at 1000X magnification with ZEISS Axioplan2 upright microscope.

SINV and viral infection in RexD mosquitoes

SINV clone 5'dsMRE16ic-EGFP was a gift from Ken E. Olson (Colorado State University) (Foy *et al.*, 2004). Virus was generated and titrated in BHK-21 cells, and the propagation of virus was accomplished in C6/36 cells that were infected with P-0 virus for 2 days. More details are provide in previous studies (Wang *et al.*, 2008). Blood feeding was conducted in a closed hood within a certified Arthropod Containment Level 2 facility. Tissue culture fluid containing SINV clone 5'dsMRE16ic-EGFP ($10^{6.5}$ pfu/ml) was mixed 1:1 with defibrinated sheep blood (Colorado Serum Company), warmed to 37°C, and placed in a Hemotek 5W1 Membrane Feeding System (Discovery Workshops), where mosquitoes were allowed to probe and feed through a stretched sheet of Parafilm for 45 min. Fully engorged mosquitoes were collected and maintained in the insectary with food and water until assayed at 7 d p.i. (Pierro *et al.*, 2008). To visualize EGFP expression, pre-fixed tissues by paraformaldehyde were analyzed using UV microscopy. The infection in midgut tissue was scored as the percentage of infection area multiplied by intensity of GFP expression (0/none, 1/low, 2/moderate and 3/high) (Olson *et al.*, 2000).

Results

Silencing Aeiap1 causes death in Aedes aegypti mosquitoes

Several reports have shown that silencing *diap1* in cell culture (S2 cells) induces extensive apoptosis (Muro *et al.*, 2002) and mutations in *diap1* are embryonic lethal in *Drosophila* (Zou *et al.*, 1999). Silencing *Aeiap1* in mosquito Aag2 cells also causes dramatic apoptosis, and the silencing of *Aedronc* protects Aag2 cells from apoptosis induced by UV or cytotoxic drugs (Q. Liu and R. J. Clem, unpublished data). A recent study reported that topically applied *Aeiap1* dsRNA causes death of female adults of the Orlando strain of *A. aegypti* (Pridgeon *et al.*, 2008b), but the mechanism of death was not investigated. We hypothesized that the death observed in mosquitoes following *Aeiap1* RNAi is due to widespread apoptosis in mosquito tissues.

To begin to test this hypothesis, we injected adult female *A. aegypti* RexD and Orlando strains with 350 ng dsRNA to silence the apoptotic regulatory genes *Aeiap1* or *Aedronc* and analyzed mortality in these two strains after dsRNA injection. *Aeiap1* dsRNA caused significant mortality in both strains of *A. aegypti*, with mosquitoes of both strains exhibiting around 30-40% survival at 14 days after injection with *Aeiap1* dsRNA (Fig. 3.1). When we increased the amount of *Aeiap1* dsRNA from 350 to 2100 ng, there was no significant increase in mortality of the RexD strain (data not shown). However, RexD mosquitoes injected with control *cat* dsRNA or PBS, and even mock-injected mosquitoes (which were subjected to the same handling procedures but not injected), exhibited less survival than Orlando (Fig. 3.1). These data indicate that the RexD strain was more sensitive to injection trauma and handling procedures, which included being

held at cold temperatures for short periods of time, than the Orlando strain. Interestingly, in the RexD strain, there was a statistically significant difference (Logrank test, *P<0.0001*) in mortality between mosquitoes injected with *Aedronc* and control *cat* dsRNA, but not between *Aedronc* dsRNA and PBS injection (Fig. 3.1A). These data suggest that introduction of dsRNA, even non-specific dsRNA, into the hemocoel induces a stress response in *A. aegypti*, and silencing *AeDronc* helps protect against this stress in the RexD strain. This was not evident in the Orlando strain, which is more stress-resistant. For this reason, and because the RexD strain has been used previously in studies of SINV (Bryant *et al.*, 2008, Pierro *et al.*, 2008), the RexD strain was used in the remainder of this study.

To determine whether the genes targeted by dsRNA were being silenced, we harvested surviving RexD mosquitoes at 3 days after dsRNA injection and examined *Aeiap1* and *Aedronc* transcript levels. Semi-quantitative RT-PCR revealed that the mRNA levels of *Aeiap1* in both midgut and carcass were significantly decreased compared to *cat* dsRNA and PBS treatments (Fig. 3.2B). Quantitative RT-PCR verified that the transcript levels of *Aeiap1* and *Aedronc* were decreased in midgut after treatment with the corresponding dsRNA compared to mock-injected mosquitoes (Fig. 3.2A and C). Interestingly, mosquitoes injected with PBS or *cat* dsRNA had relatively higher *Aeiap1* transcript levels and lower *Aedronc* transcript levels than mock individuals (Fig 3.2A and C), which may have been due to injection trauma.

To test whether silencing *Aeiap1* induced systemic apoptosis in mosquitoes, we first investigated whether caspase activity was induced, since silencing *diap1* in *Drosophila* or *Aeiap1* in *A. aegypti* induces caspase activation and apoptosis. We found

that the midgut tissue from *Aeiap1* dsRNA-injected mosquitoes had a higher level of caspase activity than the other treatments (mock-injected, PBS, *cat* and *Aedronc* dsRNA) at 1, 2, and 3 days after injection (Fig. 3.3A). In the rest of the carcass, caspase activity was only marginally increased by *Aeiap1* RNAi (Fig. 3.3B).

Gross examination of midguts from *A. aegypti* injected with *Aeiap1* dsRNA revealed that the midguts were often misshapen and fragile compared to midguts from control mosquitoes, including ones injected with *cat* or *Aedronc* dsRNA (data not shown). To study this more closely, we examined the morphological features of the midguts by microscopy of stained tissue sections. In mock-injected and *cat* dsRNA-treated samples, microvilli were intact and the midgut epithelium was highly organized (Fig. 3.4). However, in *Aeiap1* dsRNA-injected mosquitoes, the midgut microvilli were highly disrupted and the epithelium was disorganized (Fig. 3.4). Examination by transmission electron microscopy revealed the presence of condensed chromatin, a hallmark of apoptosis, in the nuclei of epithelial cells from midguts treated with *Aeiap1* dsRNA at 2 days after injection (Fig. 3.5). These data further confirmed that apoptotic morphological features were present in midguts after mosquitoes were treated with *Aeiap1* dsRNA.

Apoptosis regulates SINV infection and dissemination in mosquitoes

During infection in mosquitoes, viruses have to overcome several barriers, including the midgut infection barrier, midgut escape barrier, salivary gland transmission barrier and salivary gland escape barrier (Campbell *et al.*, 2008a). We hypothesized that systemic apoptosis, such as that induced by injection of *Aeiap1* dsRNA, might negatively impact the integrity of these barriers, and such a condition might facilitate virus infection

and dissemination. On the other hand, however, apoptosis might be expected to have a negative effect on virus replication and dissemination, as it does in the case of other viruses.

To test the effects of widespread apoptosis on virus infection and dissemination in mosquitoes, we orally infected adult female *A. aegypti* with 5'dsMRE16ic-EGFP, a strain of SINV that is efficient at midgut infection and dissemination in *A. aegypti* and expresses GFP (Foy *et al.*, 2004). Mosquitoes were injected with *cat*, *Aeiap1*, or *Aedronc* dsRNAs, and 3 days later given a blood meal containing 5'dsMRE16ic-EGFP. At 7 days post infection, the occurrence of infection and the infection pattern was examined by observing GFP expression. No significant difference was observed between dsRNA treatments in the ability of 5'dsMRE16ic-EGFP to establish infection in the midgut (Fig. 3.6A). However, by scoring the infected midguts individually, we found that *Aeiap1* dsRNA-treated midguts had higher infection scores (calculated by multiplying the percentage of GFP-positive midguts by the intensity of GFP expression) than *cat* dsRNA treatment, while *Aedronc* dsRNA-treated midguts had lower infection scores than *cat* dsRNA-treated (Fig. 3.6B). Similar infection patterns were also observed in foregut and hindgut (Fig. 3.6C-F).

To investigate SINV dissemination, we examined the occurrence of infection in salivary glands and eyes. We found *Aeiap1* dsRNA-treated mosquitoes had the highest percentage of infection in both salivary glands and eyes, while the mosquitoes injected with *Aedronc* dsRNA had lower dissemination rates than either *Aeiap1* or *cat* dsRNA-injected mosquitoes in these two organs (Fig. 3.7A and B).

Discussion

Successful transmission of arboviruses between vertebrate hosts is dependent on the survival and competency of the arthropod vector. Arboviruses such as SINVs usually do not induce apoptosis in mosquito cell lines, but are generally thought to cause only moderate cytopathic effects in mosquito cells with a persistent infection (Wang *et al.*, 2008). However, there are several intriguing reports of cytopathic effects, including apoptosis-like cell death, being observed in midgut and salivary glands infection barriers in arbovirus-infected mosquitoes (Bowers *et al.*, 2003, Girard *et al.*, 2005, Girard *et al.*, 2007, Mims *et al.*, 1966, Vaidyanathan & Scott, 2006, Weaver *et al.*, 1992, Weaver *et al.*, 1988). These reports raise the possibility that the vector competency of mosquitoes might be determined in part by apoptosis. However, there are no causative data that directly link apoptosis to effects on vector competency for viruses.

In this study, we modulated the apoptotic pathway *in vivo* by silencing the initiator caspase *Aedronc* or the central apoptosis inhibitor *Aeiap1*. Although we did not attempt to determine in this study whether RNAi of *Aedronc* inhibited apoptosis *in vivo*, doing so is highly effective at inhibiting apoptosis in the *A. aegypti* cell line Aag2 (Q. Liu and R. J. Clem, unpublished data). Therefore, it is likely to inhibit apoptosis *in vivo*. Following inhibition or activation of apoptosis, we demonstrated a reciprocal effect on viral replication and dissemination: inhibition of apoptosis limited infection and dissemination, while activation of apoptosis exacerbated infection. However, these observations were based on systemic induction or inhibition of apoptosis before infection. Systemic apoptosis may compromise infection barriers and cause the mosquitoes to become more susceptible to viruses. In a natural infection, only a small number of cells

are initially infected in the midgut or salivary gland. Thus, a successful apoptotic response in one of these infection barriers would not necessarily need to involve a large number of cells, and could still limit the infection and dissemination of a virus. Overall, the extent of apoptosis could play a role in determining vector competency.

Injection of *Aeiap1* dsRNA also caused high levels of mosquito mortality, although not as high as previously reported (Pridgeon *et al.*, 2008b). This correlated with pathology in the injected mosquitoes, including caspase activation in midgut and carcass, disrupted midgut epithelium, and fragmented and condensed chromatin in the nucleus of midgut epithelial cells. This correlation suggests that systemic apoptosis is the cause of high mortality in mosquitoes injected with *Aeiap1* dsRNA, and is the first observation of systemic pathological effects in mosquitoes after inhibiting an anti-apoptotic gene.

The major pathology we observed following injection of *Aeiap1* dsRNA was in the midgut. This may be due to either the midgut being more amenable to RNAi following intrahemocoelic injection, or to midgut cells being more sensitive to apoptosis following silencing of *Aeiap1*. It is also likely that apoptosis occurs in other organs that we did not examine in detail, such as salivary gland, but the main effects were in the midgut. In addition to effects on the midgut epithelium, other physiological changes could also be occurring in midguts after *Aeiap1* RNAi, such as loss of integrity of peritrophic membrane, changes in expression of innate immunity genes, or alteration of midgut pH. All these factors could possibly cause mosquitoes to be more susceptible to infection after treatment with *Aeiap1* dsRNA. If using *Aeiap1* dsRNA as an insecticide for mosquitoes, we should consider possible effects on innate immunity, which eventually could make them more susceptible to virus infection.

In both RexD and Orlando strains, we observed 60-70% mortality following injection of *Aeiap1* dsRNA. However, compared to the Orlando strain, RexD mosquitoes were more sensitive to injection and cold from a 4^{o}C chill table, which was used to keep them unconscious during manipulation.

Some reports have indicated cross talk between the apoptosis pathway and innate immune pathways (Pridgeon *et al.*, 2008a). Mosquitoes injected with either PBS or *cat* dsRNA exhibited increased *Aeiap1* transcript levels compared to mock-injected insects, suggesting that the stress of injection trauma may induce *Aeiap1* transcription. This is consistent with a report showing that various stress stimuli caused induction of *Aeiap1* transcription (Pridgeon *et al.*, 2008a). Injection trauma (PBS or *cat* dsRNA) also caused decreased *Aedronc* expression levels compared to mock-injected. It is possible that increased *Aeiap1* and decreased *Aedronc* expression could both be caused by stress response signaling pathways. The observation that RexD mosquitoes injected with *Aedronc* dsRNA had a higher percentage of survival than those injected with *cat* dsRNA also suggests that inhibiting apoptosis by knocking down *Aedronc* can counteract the stress from cold and injection. Thus, possible scenario could be that stress-responsive signaling pathways such as JNK or NF-κb are activated by stress signals, and act in part through anti-apoptotic effects.

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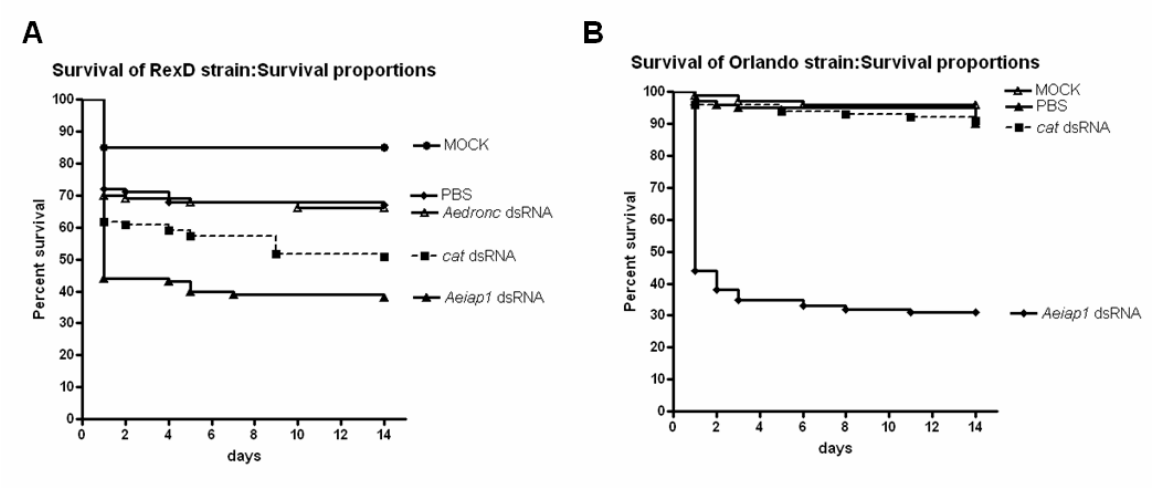

Figure 3.1 Mortality of *A. aegypti* mosquitoes after injection of dsRNA

RexD strain (A) and Orlando strain (B) adult female mosquitoes were intrathoracically injected with 69 nl (350 ng) of the indicated dsRNA or PBS, or mock-injected (MOCK). Each treatment included 100 mosquitoes. The survival of the two strains was analyzed for 14 days. The survival curves were analyzed by Logrank test, $P < 0.0001$.

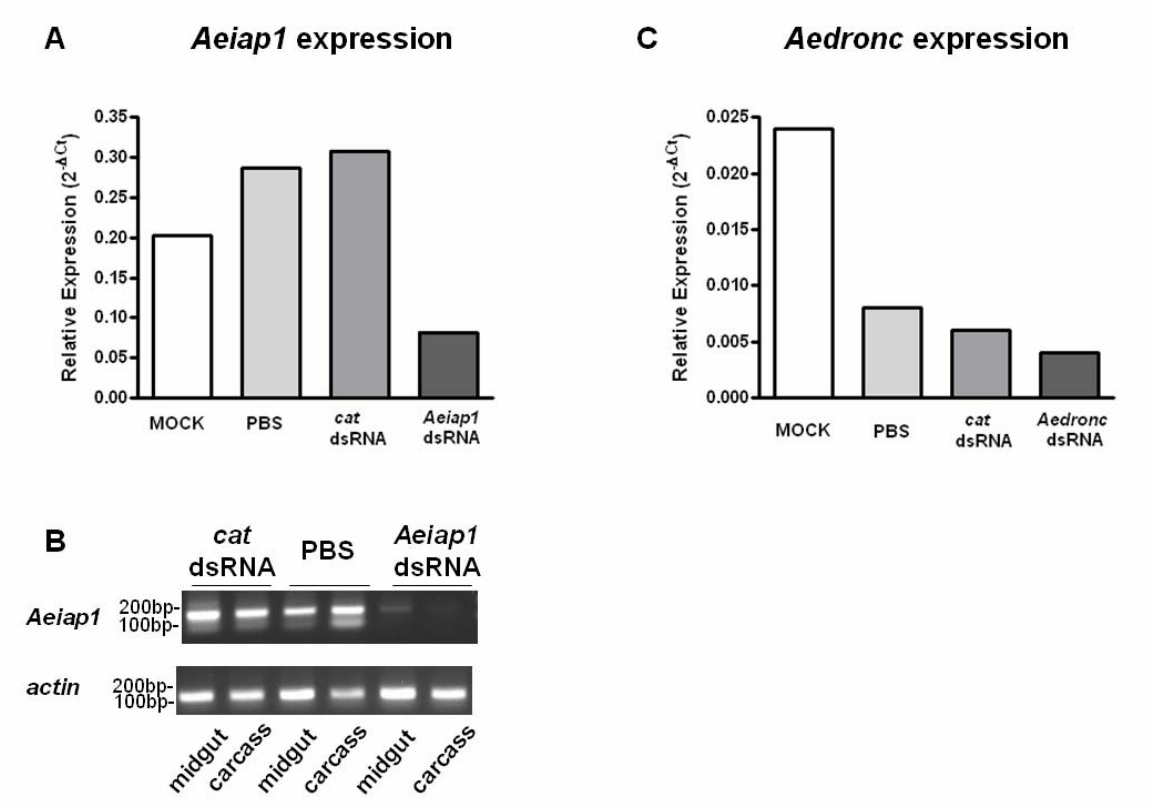

Figure 3.2 Analysis of gene expression in mosquitoes silenced for *Aeiap1* or *Aedronc*

Adult female RexD mosquitoes were injected with the indicated dsRNAs, PBS, or mock-injected, and gene expression was analyzed at 3 days after injection. (A) *Aeiap1* expression in midgut (relative to *actin6*) as assayed by real time RT-PCR. (B) Semi-quantitative RT-PCR analysis of *Aeiap1* and *actin6* in midgut and carcass with treatments (*cat* dsRNA, PBS and *Aeiap1* dsRNA). (C) *Aedronc* expression in midgut (relative to *actin6*) by real time RT-PCR. All the experiments were repeated at least three times, and the results shown here were the one of the representatives.

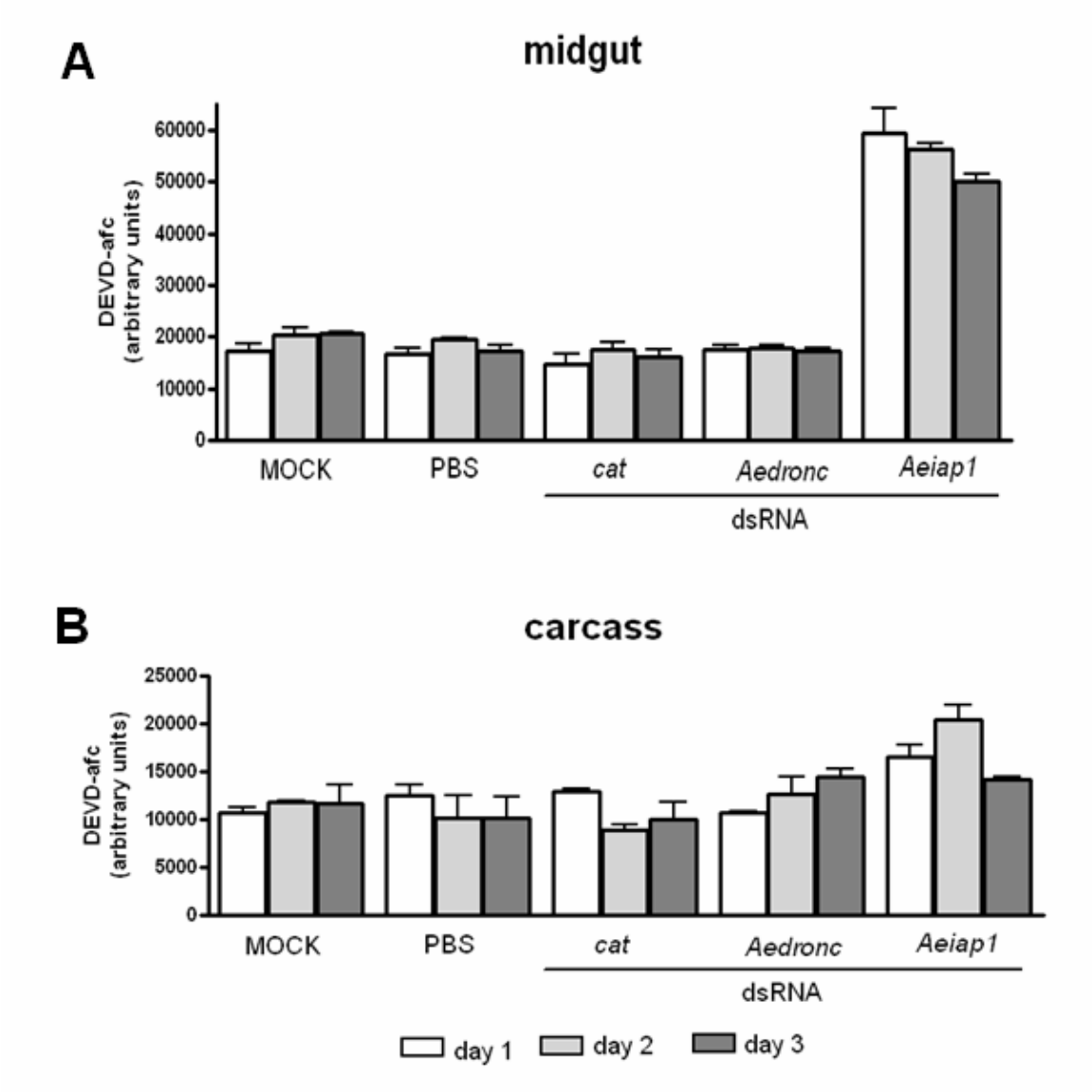

Figure 3.3 Caspase activity in midgut (A) and carcass (B) following silencing of *Aeiap1* or *Aedronc*

Adult female RexD mosquitoes were intrathoracically injected with 69 nl of the indicated dsRNA or PBS, or non-injected (MOCK). Mosquitoes were dissected at 1, 2, or 3 days post injection. Pooled tissues of 10 individuals were homogenized in lysis buffer and caspase activity was determined using Ac-DEVD-AFC as a substrate. Data are shown as mean $\pm$ SEM of three independent experiments ($P < 0.0001$ by one way ANOVA).

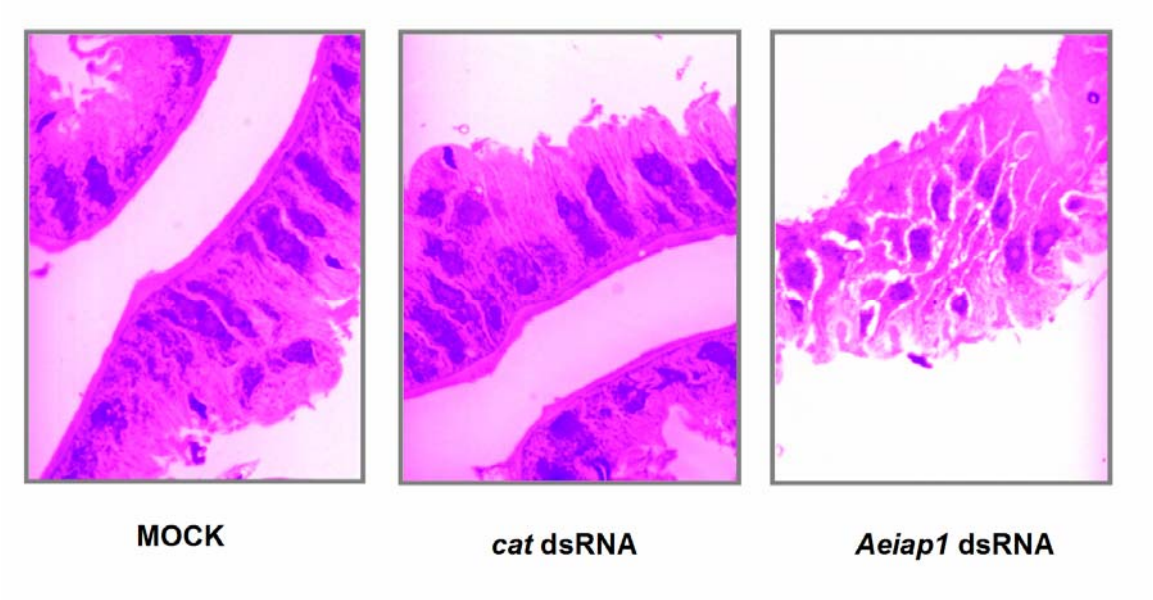

Figure 3.4 Midgut morphology after injection with *Aeiap1* dsRNA

RexD mosquitoes were intrathoracically injected with dsRNA (*cat* or *Aeiap1*) or non-injected (MOCK). Mosquitoes were harvested and dissected at 3 days post injection. The tissue was fixed, embedded, sectioned and stained with epoxy-tissue stain. The tissue was photographed at 1000X magnification.

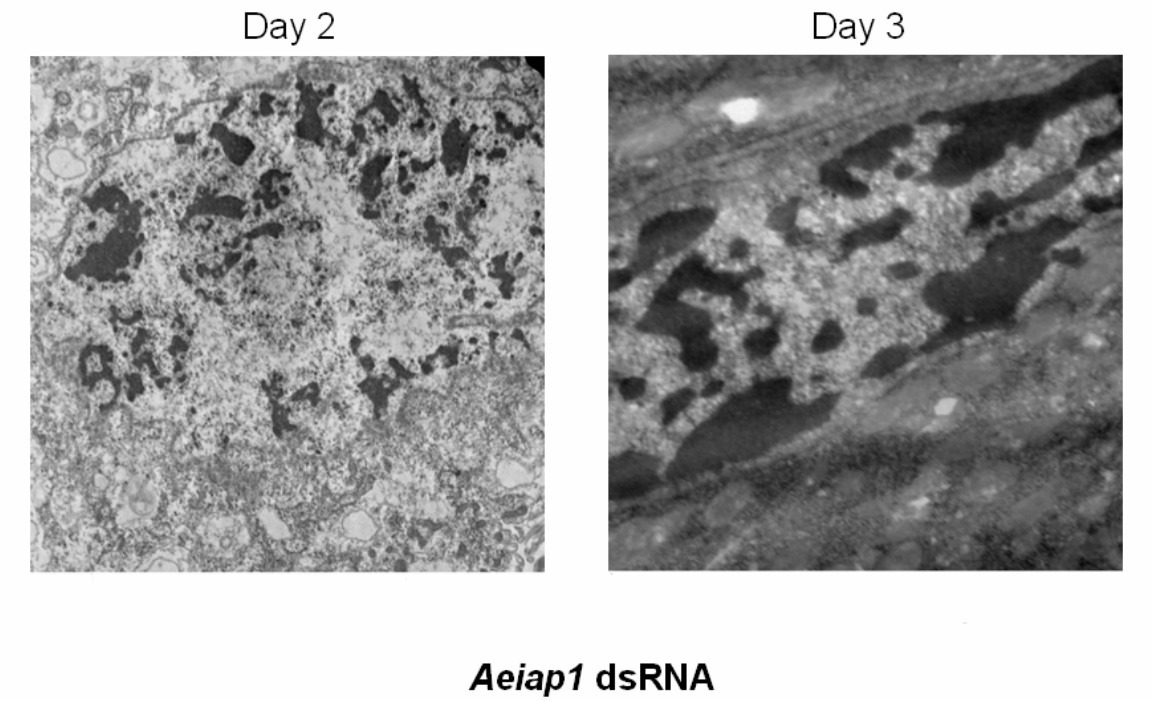

Figure 3.5 Transmission electron microscopy of midgut from mosquito injected with *Aeiap1* dsRNA at 2 & 3 days post injection

Condensed chromatin, a hallmark of apoptosis, in the nucleus of an epithelial cell.

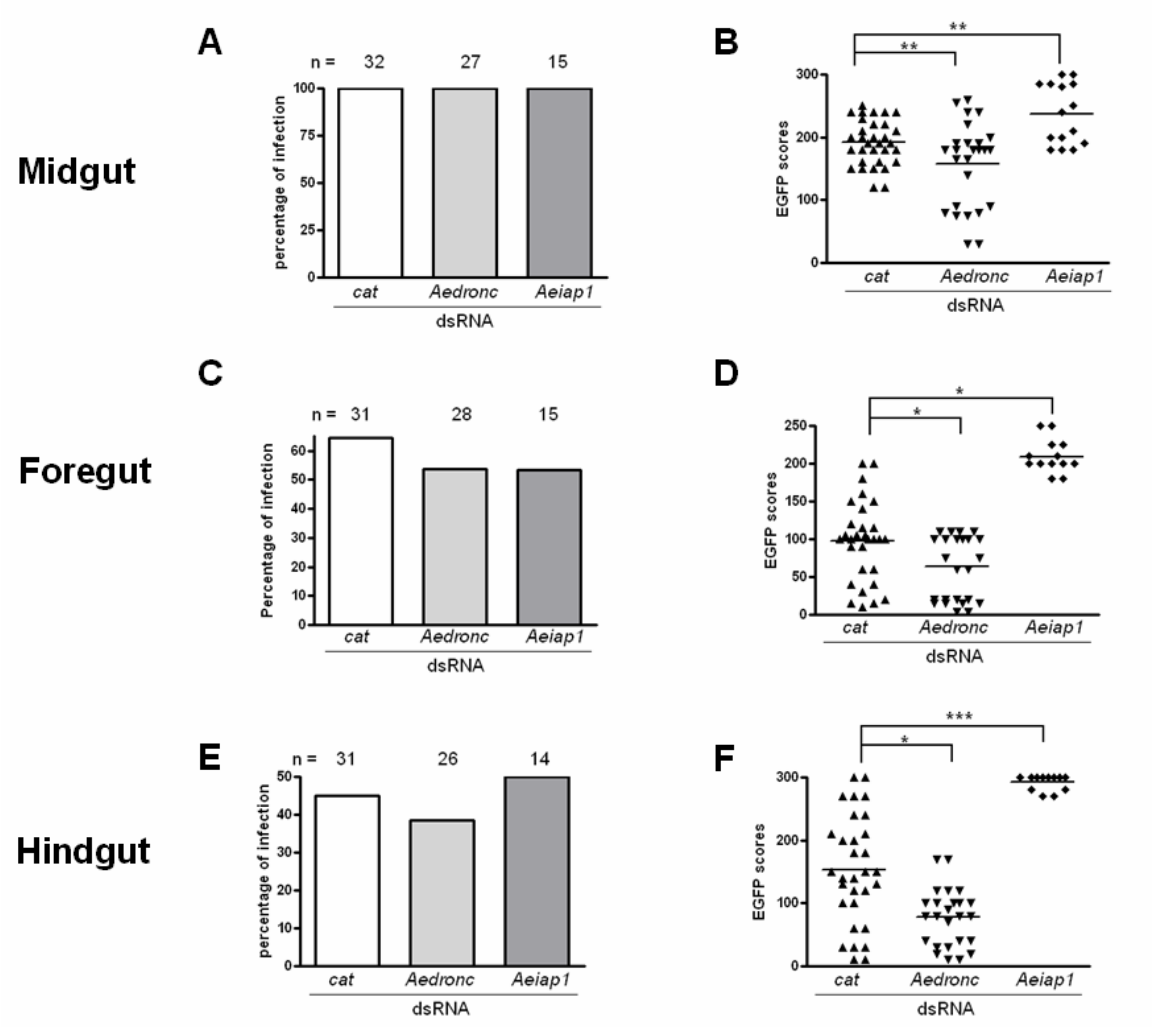

Figure 3.6 Virus infection patterns in mosquitoes with silenced *Aeiap1* or *Aedronc*

Adult female RexD mosquitoes were injected with dsRNA (*cat*, *Aedronc* or *Aeiap1*) and 3 days later mosquitoes were orally infected with 5'dsMRE16ic-EGFP. At 7 days post infection, virus infection patterns were analyzed in midgut (A and B), foregut (C and D), and hindgut (E and F) by EGFP occurrence (A, C and E) or EGFP scores (B, D and F). EGFP scores were calculated as the percentage of infection area multiplied by intensity of EGFP (none/0, low/1, moderate/2 or high/3). (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$ by Student t-test).

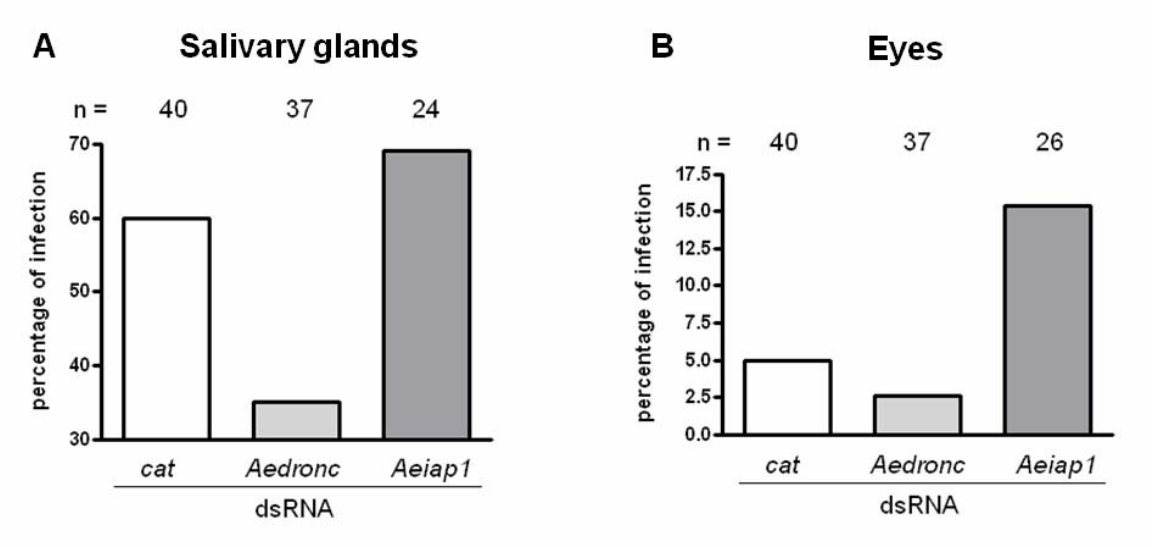

Figure 3.7 Virus dissemination in mosquitoes with silenced *Aeiap1* or *Aedronc*

Mosquitoes were injected with the indicated dsRNAs and 3 days later mosquitoes were orally infected with 5'dsMRE16ic-EGFP. At 7 days post infection, virus infection patterns were analyzed in salivary glands (A) and eyes (B) by the percentage of individuals exhibiting EGFP expression.

CHAPTER 4 - The role of IAP antagonist proteins in the core apoptosis pathway of the mosquito disease vector *Aedes aegypti*

Abstract

While apoptosis regulation has been studied in great detail in *Drosophila melanogaster*, similar studies in other insects are lacking. In *Drosophila*, the inhibitor of apoptosis (IAP) protein DIAP1 is the major negative regulator of caspases, while IAP antagonists induce apoptosis, in part, by binding to DIAP1 and inhibiting its ability to regulate caspases. In this study, we characterized the roles of two *Aedes aegypt*i IAP antagonists, Michelob_x (Mx) and IMP, in apoptosis. Overexpression of IMP or Mx caused apoptosis in *A. aegypti* Aag2 cells, while silencing of *imp* or *mx* attenuated apoptosis in Aag2 cells. IMP and Mx directly bound to the IAP protein AeIAP1, and the IAP binding motif (IBM) of IMP and Mx was critical for this binding. AeIAP1 also bound and inhibited both initiator and effector caspases from *A. aegypti*, and IMP and Mx competed with caspases for binding to AeIAP1. Addition of recombinant IMP or Mx, but not cytochrome c, to Aag2 cytosolic extract caused caspase activation. These findings demonstrate for the first time that the mechanisms by which IAP antagonists regulate apoptosis are largely conserved between mosquitoes and *Drosophila*.

Introduction

Apoptosis is a genetically controlled mechanism of cell death which is important in development, tissue homeostasis, and innate immunity. Apoptotic cells are characterized by nuclear chromosomal condensation and fragmentation, cell membrane blebbing, and formation of apoptotic bodies. These features of apoptosis are directly caused by caspases, a family of cysteine proteases which are activated following an apoptotic stimulus. A core apoptosis pathway exists in nematodes, insects, and vertebrates (referred to as the intrinsic pathway in vertebrates) which regulates apoptosis by controlling the activation of caspases. In broad terms, the core apoptotic pathway controls the activation of a class of caspases called initiator caspases, which occurs by formation of the apoptosome, a complex that promotes initiator caspase dimerization. Activated initiator caspases cleave and activate effector caspases, which cleave key cellular substrates, leading to the stereotypical morphological changes associated with apoptosis.

The major components of the core apoptotic pathway are largely conserved among metazoans, but there are significant differences in how caspase activation is regulated between phyla (Hay & Guo, 2006a, Yan & Shi, 2005). At this point, our knowledge of apoptosis regulation in insects comes almost entirely from studies done in a single insect species, *Drosophila melanogaster*. Given the immense diversity which exists among insects, and the drastically different evolutionary pressures between insect groups, it is important to examine the process of apoptosis regulation in other insects. For example, even fruitflies and mosquitoes, which are both members of the order *Diptera*, are separated by 300 million years of evolution, and these two groups of insects have been exposed to very different evolutionary pressures, with

mosquitoes having an aquatic larval stage and relying on vertebrate blood feeding for reproduction.

In *D. melanogaster*, there are three initiator caspases (Dronc, Dredd, and Strica), and four effector caspases (DrIce, Dcp-1, Damm and Decay). Among these, Dronc and DrIce are the most important in carrying out apoptosis, with Dcp-1 having an auxiliary role. Unlike the situation in vertebrates, where an apoptotic signal is required for cytochrome c release from mitochondria and subsequent apoptosome formation, Dronc activation occurs constitutively, apparently due to the lack of a requirement for cytochrome c in apoptosome formation (Dorstyn *et al.*, 2004, Dorstyn *et al.*, 2002, Means *et al.*, 2006). Dronc is recruited by the oligomerizing factor Ark into an apoptosome complex, resulting in Dronc activation, probably through dimerization followed by auto-cleavage (Dorstyn & Kumar, 2008, Muro *et al.*, 2004, Shi, 2008, Snipas *et al.*, 2008). Activated Dronc cleaves and activates Drice, leading to apoptosis (Dorstyn & Kumar, 2008, Muro *et al.*, 2005). Dcp-1 is also activated, but does not appear to play a significant role in apoptosis (Fraser *et al.*, 1997, Muro *et al.*, 2006).

In unstimulated *Drosophila* cells, apoptosis is avoided only because the IAP protein DIAP1 is able to bind and ubiquitylate Dronc and DrIce. Thus, interruption of the expression of DIAP1 protein or the ability of DIAP1 to bind to Dronc or DrIce leads to rapid apoptosis, even in the absence of an exogenous apoptotic signal (Bump *et al.*, 1995, Challa *et al.*, 2007, Igaki *et al.*, 2007, Wang *et al.*, 1999). DIAP1 contains two baculovirus IAP repeat (BIR) domains and a RING domain; the BIR domains are responsible for the physical interaction with caspases, while the RING domain confers E3 ubiquitin ligase activity.

The BIR2 domain of DIAP1 interacts with Dronc by binding to a twelve residue motif located between the prodomain and the large catalytic subunit of Dronc (Chai *et al.*, 2003).

Effector caspase (DrICE and DCP-1) binding is accomplished by the BIR1 domain of DIAP1, which binds to a motif that is revealed following cleavage of the caspases, and the resulting binding blocks enzymatic activity through steric occlusion (Tenev *et al.*, 2005, Yan *et al.*, 2004b). After binding, the N-terminal 20 amino acids of DIAP1 are removed by active DrICE, which relieves auto inhibition of DIAP1 by the N-terminal sequence (Ditzel *et al.*, 2003, Yan *et al.*, 2004b). However, the physical interaction between DIAP1 and DrICE or Dronc cannot completely inhibit these caspases. The RING domain of DIAP1 is also required because of its ability to promote caspase ubiquitylation, which can result in caspase degradation via the proteosome (Chai *et al.*, 2003, Wilson *et al.*, 2002). In addition, non-degradative polyubiquitylation of DrIce and Dcp-1 by DIAP1 reduces the activation of these caspases by steric interference with binding of substrate (Ditzel *et al.*, 2008).

IAP antagonists are a group of proteins which share little sequence similarity other than a highly conserved N-terminal motif called the IAP binding motif (IBM), which allows binding to BIR1 and BIR2 of DIAP1. Elevated levels of IAP antagonists induce apoptosis, in part by competing with caspases for the binding sites of DIAP1. IAP antagonists in *D. melanogaster* include the cytoplasmic proteins Rpr, Hid, Grim and Sickle. These IAP antagonists have different binding affinities for DIAP1 BIR domains, with Rpr and Grim showing equal preference for binding to the BIR1 and BIR2 domains, while Hid and Sickle has higher affinity for BIR2 than BIR1 (Zachariou *et al.*, 2003). In addition to competing for IAP binding with caspases, IAP antagonists also induce apoptosis through other mechanisms, including stimulation of DIAP1 ubiquitylation (Yoo *et al.*, 2002) and inhibition of global protein translation, a property shared by Rpr and Grim (Colon-Ramos *et al.*, 2006). Rpr, Grim and Sickle also contain a GH3

domain, which can stimulate cell death in the absence of an IBM (Claveria *et al.*, 2002, Zhou *et al.*, 2005).

The availability of mosquito genome sequences has allowed the initiation of the study of apoptosis in mosquitoes. A number of mosquito genes have been identified that share sequence homology with apoptosis regulatory genes in *Drosophila* (Bryant *et al.*, 2008, Cooper *et al.*, 2007a, Cooper *et al.*, 2007b, Li *et al.*, 2007). Expression of *Aedes albopictus* IAP1 protects mammalian cells from bluetongue virus-induced apoptosis and rescues insect Sf9 cells from apoptosis induced by overexpression of Hid (Li *et al.*, 2007). In addition, silencing of *Aedes aegypti* IAP1 (AeIAP1) in adult females caused significant death in mosquitoes, but the mechanism involved was not explored (Pridgeon *et al.*, 2008b). The core apoptosis pathway appears to be largely conserved in *A. aegypti*, as silencing of *Aeiap1* causes spontaneous apoptosis in *A. aegypti* Aag2 cells, and apoptosis is dependent on AeDronc and AeArk (unpublished data by Q. Liu and R. J. Clem). Eleven caspases are encoded in the genome of *A. aegypti*, and these include clear orthologs of Dronc and Dredd. The remaining caspases have undergone expansion following the divergence of flies and mosquitoes, making it difficult to assign orthologs for DrICE or Dcp-1. Phylogenetic analysis of caspases suggests that the effector caspases AeCASPS7 and AeCASPS8 are the closest relatives to *Drosophila* DrICE and DCP-1. Silencing AeCASPS7 or AeCASPS8 inhibits apoptosis in Aag2 cells (unpublished data, Q. Liu and R. J. Clem).

IAP antagonists that have been identified in *A. aegypti* include Michelob_x (Mx) and IMP. Expression of these genes in *A. albopictus* C6/36 cells induces apoptosis, which is dependent on the N-terminal IBM (Bryant *et al.*, 2008, Wang *et al.*, 2008, Zhou *et al.*, 2005). However, it is not clear whether or how the *A. aegypti* IAP antagonists normally function in

apoptosis, and if they do, whether the mechanisms involved are similar to those used by IAP antagonists found in *D. melanogaster*.

In this study, we have examined the proapoptotic functions of Mx and IMP in *A. aegypti* cells. By silencing expression of *mx* and *imp*, we found that both genes are involved in apoptosis. We further characterized the ability of IAP antagonists and *A. aegypti* caspases to bind AeIAP1, and examined the competition between IAP antagonists and caspases for binding to AeIAP1. This work is the first to systematically characterize the interactions between IAP, IAP antagonists, and caspases in mosquitoes.

Materials and Methods

Cell culture

A. aegypti Aag2 cells and *D. melanogaster* S2 cells were maintained in Schneider's medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Atlanta Biologicals). *A. albopictus* C6/36 cells were cultured in Leibovitz's medium (Invitrogen) containing 10% FBS. *Spodoptera frugiperda* SF-21 cells were propagated in TC-100 medium (Invitrogen) with 10% FBS. BHK-21 cells were propagated in Dulbecco's modified Eagle's medium (DMEM) (Mediatech) supplemented with 10% FBS. Aag2, C6/36, S2 and SF-21 cells were maintained at 27^{o}C, and BHK21 cells were cultured at 37^{o}C in 8% CO_2.

Recombinant viruses

The coding regions of *A. aegypti imp* and *A. albopictus mx* cDNAs were amplified by PCR and cloned into the SINV DNA infectious clone pTE5'2J (TE) in the sense and antisense orientation. Construction of TE/Rpr, TE/Mx and TE viruses have been described previously (Wang *et al.*, 2008). Viruses were generated using BHK-21 cells and virus titres were determined by tissue culture infectious dose ($TCID_{50}$) assay in BHK-21 cells as previously described (Wang *et al.*, 2008).

Caspase activity assay

Caspase activity was measured using Ac-DEVD-AFC or Ac-IETD-AFC (Enzyme Systems Products) as a substrate. Cell lysate was prepared and assays were performed as previously described (Wang *et al.*, 2008).

RNAi procedure

Full-length ORFs were PCR-amplified such that T7 polymerase promoter sites were incorporated onto both ends. The PCR products were used as template to synthesize dsRNA using AmpliScribe T7 High Yield Transcription kit (EPICENTRE Biotechnologies). One million Aag2 cells were plated in Schneider's medium without serum and the dsRNA was added directly to the medium at a concentration of 10 μg ml^{-1}. After the cells were incubated with dsRNA for 1-2 h, FBS was added to a final concentration of 10%. The cells were incubated for 12 h before proceeding with the rest of the experiments.

RT-PCR

Aag2 cells were treated with dsRNA and at the indicated time points after treatment, total RNA was isolated using Trizol reagent (Invitrogen). One μg total RNA was used as a template to generate cDNA in a 10 μl reverse transcriptase reaction (Promega) with poly(dT) primer. Two microliters of cDNA was then used as template for PCR with gene-specific primers.

Transfection Assay

The coding regions of *Aeiap1, CASPS7*, *CASPS8*, *Aedronc*, *mx*, *imp*, and *Drosophila rpr* and *diap1* were inserted under control of a hsp70 promoter in the pHSP70PLVI+ vector (Clem & Miller, 1994). One million SF-21 or C6/36 cells were transfected with 5 μg of each plasmid and 5 μl lipofectamine in medium without serum. After 6 hours, cells were washed 3 times and replaced with medium plus 10% FBS. Twenty hours later, cells were incubated at 42°C for 30 min and harvested three hours after the heat shock.

Recombinant protein and peptide preparation

N-terminally tagged GST-AeIAP1, GST-AeIAP1 BIR1 (containing amino acid residues 24-118 of AeIAP1), GST-AeIAP1 BIR2 (residues 188-278) and GST-AeIAP1 BIR1+2 (residues 17-349) were cloned into the pGEX-3x vector. GST-DIAP1 was inserted in the pGEX-4T-1 vector. C-terminally tagged CASPS7-His6, CASPS8-His6, Rpr-His6, Rpr-IBM-His6, IMP-His6, IMP-IBM-His6, Mx-His6 and Mx-IBM-His6 were cloned into pET23b. His6-AeDronc (N-terminally tagged) was inserted into pET32. GB-His6/pET30a(+) was a gift from Katsura Asano (Kansas State University). These vectors were transformed into BL21pLysS(DE)3 *E. coli* (Stratagene). Bacterial cultures were grown at room temperature to OD_{600} = 0.6, at which time they were induced with 0.1M IPTG for 3 h. Proteins were purified using either glutathione-agarose beads (Sigma) for GST-tagged proteins or with Talon Metal Affinity Resin (Clontech) for the His6-tagged proteins, according to the instructions of the manufacturer. The IBM peptide (AIAFYK-biotin) was synthesized by New England Peptide and the Met-Hid peptide (MAVPFYLPEGGK-biotin) was previously described (Means *et al.*, 2006, Wright & Clem, 2002).

Pull down assay

To examine the interaction of IAP antagonists (Mx, IMP and Rpr) with full length AeIAP1, 3.5 µM of recombinant His-tagged IAP antagonists or corresponding mutants were incubated with 10 µl of in vitro translation reaction containing AeIAP1 in a100 µl final reaction with NP-40 lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 1 mM dithiothreitol, 1 mM phenylmethyl sulfonyl fluoride) at 30^{o}C for 1 h. Full length AeIAP1 was made using the TNT T7/SP6 Coupled Reticulocyte Lysate System (Promega). After incubation, the reaction was mixed with 40 µl of Talon Metal Affinity Resin (Clontech) and rocked at 4^{o}C

for 2 h. The beads were washed three times with NP-40 lysis buffer. The bound proteins were pulled down with beads and dissolved in Laemmli buffer by heating the samples at 100^{o}C for 5 min. Proteins were separated by 15% SDS-PAGE. After electrophoresis, gels were treated with fixing solution (30% methanol and 10% acetic acid) for 1 h and soaked in 16% salicylic acid for 5 min. After drying, gels were exposed to film at -80^{o}C.

To examine IAP binding motif (IBM) interaction with BIR domains of AeIAP1, 5 µg of IBM peptide (AIAFYK-bio) was added to 40 µl of streptavidin-conjugated beads (Sigma) with NP-40 lysis buffer up to 500 µl as a final volume and rocked for 1 h at 4^{o}C to allow the peptide to bind to the beads. The beads were washed three times with NP-40 lysis buffer and incubated with 0.5 µM of GST-AeIAP1 BIR1 or BIR2 recombinant protein in 500 µl of NP-40 lysis buffer for 1 h at 4^{o}C. After incubation, the beads were washed three times with NP-40 lysis buffer and bound protein was dissolved in Laemmli buffer by heating beads at 100^{o}C for 5 min. Proteins were analyzed by immunoblotting using anti-GST monoclonal antibody conjugated to horse radish peroxidase (Santa Cruz Biotechnology) at 1:1000.

To test caspase interaction with AeIAP1, 10 µl of ^{35}S-labeled *in vitro* translation reaction for each caspase were incubated with 2.5 µM of a GST-tagged protein in a 100 µl reaction with NP-40 lysis buffer at 30^{o}C for 1 h. After incubation, the reaction was added to 40 µl of glutathione-agarose beads (Sigma) and rocked overnight at 4^{o}C. The beads were washed three times with NP-40 lysis buffer and the bound proteins were separated by SDS-PAGE. The gels were dried and exposed to film at -80^{o}C.

To study the interaction of active caspases and AeIAP1, cleaved ^{35}S labeled AeDronc or active CASPS7 or CASPS8 recombinant proteins were used. To obtain cleaved ^{35}S labeled AeDronc, 10 µl of full length *in vitro* translated AeDronc was incubated with 0.5 µM active

recombinant AeDronc at 30°C for 1 hr. The active ^{35}S labeled AeDronc and the active CASPS7 or CASPS8 recombinant proteins were incubated with 0.5 μM GST-tagged protein (GST-BIR1, GST-BIR2, GST-AeIAP1 or GST) at 30°C for 1 hr, and further incubated with 40 μl of glutathione-agarose beads for 1 hr at 4°C, followed by washing and visualization as described above.

Viability assays

The viability of Aag2 cells was measured by MTT assay as previously described (Wang *et al.*, 2008).

Cell fractionation

Cell fractionation was performed as previously described (Means *et al.*, 2006). Cells were harvested by centrifugation at 500 *g* for 5 min. Cell pellets were re-suspended in 1 ml of Caspase Buffer A (Means *et al.*, 2006) (20 mM Hepes•KOH, pH 7.5, 50 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT in 250 mM sucrose supplemented with protease inhibitor cocktail). Cells were lysed using 50 strokes of a Dounce homogenizer and cells were further centrifuged at 500 *g* for 10 min at 4°C. After the supernatant was centrifuged at 10,000 *g* for 15 min at 4°C, the pellet (P10) was re-suspended in 1ml of Caspase Buffer A with 10% glycerol and stored at -80°C. After the supernatant was further centrifuged at 100,000 *g* for 30 min at 4°C, 10% glycerol was added to the resulting supernatant (S100) and stored at -80°C.

Immunoblotting

Protein or cell lysate were mixed with SDS-PAGE loading buffer, heated at 100°C for 5 min and resolved by 15% SDS-PAGE, and then transferred to PVDF membrane. The GST-tagged or His-tagged recombinant proteins were detected with 1:1000 anti-GST-HRP or anti-

His-HRP antibody (Santa Cruz Biotechnology). The P10 and S100 fractions from cells were immunoblotted for cytochrome c using 1:1000 anti-cytochrome c antibody (Santa Cruz Biotechnology) and 1:5000 anti-mouse IgG-HRP.

Results

Over expression of IAP antagonists induces apoptosis in mosquito cells

Four IAP antagonist genes, *A. aegypti michelob_x* (*m*x), *A. albopictus michelob_x* (*almx*), *A. aegypti imp*, and *D. melanogaster reaper* (*rpr*) have been shown to induce apoptosis when expressed in *A. albopictus* C6/36 cells (Bryant *et al.*, 2008, Wang *et al.*, 2008, Zhou *et al.*, 2005). To determine whether expression of these IAP antagonists also induces apoptosis in Aag2 cells, we expressed these genes using a recombinant sindbis virus (SINV) expression system. The SINV system was used to express foreing proteins, because of low transfection efficiency of expression plasmids in existing *A. aegypti* cell lines. We previously constructed a series of recombinant SINVs which express Mx and Rpr by inserting the coding regions of these genes into the TE5'2J(TE) SINV infectious clone in sense and antisense orientations (Wang *et al.*, 2008). For this study, additional clones were constructed expressing *Aamx* and *imp* (sense and antisense). Aag2 cells which were infected with TE/Mx, TE/alMx, TE/IMP or TE/Rpr underwent apoptosis within the first 24 h p.i., while cells infected with the SINVs containing antisense inserts or the parental TE virus did not die, and instead exhibited typical signs of persistent infection. At 24 h p.i., Aag2 cells expressing the IAP antagonists exhibited extensive plasma membrane blebbing and formation of apoptotic bodies (Fig. 4.1A). Similar results were obtained when C6/36 cells were infected with the SINVs expressing IAP antagonists (data not shown) (Wang *et al.*, 2008). Thus, overexpression of IAP antagonists induced apoptosis in Aag2 cells.

To quantify the death of Aag2 cells infected with SINVs expressing IAP antagonists, cell viability was quantified by MTT assay, which measures metabolic activity (Wang *et al.*, 2008).

Although MTT assay does not specifically measure apoptotic death, apoptotic cells lose metabolic activity within a short time, and the MTT assay is a convenient assay to determine cell viability. Aag2 cells infected with TE/Mx, TE/alMx, TE/IMP or TE/Rpr had significantly lower viability than mock-infected cells or cells infected with the control viruses containing antisense inserts (Fig. 4.1B).

We further examined initiator (Ac-IETD-AFC) and effector (Ac-DEVD-AFC) caspase activity in Aag2 cells. Consistent with the extensive morphological signs of apoptosis, Aag2 cells expressing IAP antagonists exhibited dramatically increased caspase activity compared to uninfected cells and cells infected with control viruses (Fig 4.1C and D). These data, along with the morphological characteristics of the dying cells (Fig. 4.1A), confirmed that cell death induced by overexpression of IAP antagonists was due to apoptosis.

Silencing of IAP antagonists attenuates apoptosis in Aag2 cells

Although overexpression of IAP antagonists induced apoptosis in Aag2 cells, this does not confirm that these proteins are normally involved in carrying out apoptosis. To study the role of *A. aegypti* IAP antagonists in apoptosis, we used RNA interference (RNAi) to silence expression of *Aemx* and *imp* in Aag2 cells. Transcript levels of *mx* and *imp* were dramatically decreased by 12 h after dsRNA treatment and remained low through at least 48 h, as compared with a control dsRNA treatment and mock-treated cells (Fig 4.2).

Next, we tested whether silencing *mx* or *imp* could protect cells from apoptosis stimulated by actinomycin D (ActD) or *Aeiap1* dsRNA. Both of these stimuli have been shown to induce apoptosis in many insect cell lines, including Aag2 (Means *et al.*, 2006, Wang *et al.*, 2008) (unpublished data, Q. Liu and R. J. Clem). After cells were treated with dsRNA of *mx* or *imp* for 24 h, cells were treated with 50 ng ml^{-1} of ActD or 5 μg ml^{-1} of *Aeiap1* dsRNA for 12 h. Cell

viability was then measured using MTT assay (Fig. 4.3A and C), and RT-PCR was used to confirm that the genes were being silenced (Fig. 4.3B and D). A pan-caspase inhibitor, Z-VAD-FMK, inhibited the majority of ActD- or *Aeiap1* RNAi-stimulated cell death, indicating that cell deaths was caspase-dependent. Silencing of *mx* or *imp* increased the viability of ActD- or *Aeiap1* dsRNA-treated cells, while silencing *mx* and *imp* together had an additive effect (Fig. 4.3A and C). These data indicate that Mx and IMP function in the apoptotic pathway(s) stimulated by ActD and *Aeiap1* dsRNA.

IAP antagonists physically interact with AeIAP1

To study the biochemical interactions between IAP antagonists and AeIAP1, several recombinant proteins were expressed in and purified from *E. coli* (Fig. 4.4B and D). Recombinant Mx, IMP, and Rpr proteins were C-terminally His-tagged, and either included or lacked the N-terminal IBM (Fig. 4.4B-C). In addition, a series of N-terminally GST-tagged AeIAP1 proteins were expressed and purified, including full length AeIAP1, BIR1 alone, BIR2 alone, or BIR1 and 2 together (Fig. 4.4D-E).

To test whether Mx or IMP interacts with AeIAP1, we used pull down assays. Initially, equal amounts of His-tagged recombinant IAP antagonist proteins were incubated with *in vitro* translated, ^{35}S-labeled AeIAP1, and the resulting protein complexes were purified using the His-tag. Mx, IMP and Rpr were each able to pull down AeAIP1, and this interaction was dependent on the presence of the IBM in the IAP antagonists (Fig. 4.5A). Mx and IMP appeared to have a higher affinity for AeIAP1 than Rpr, which is consistent with the species of origin of the proteins, and a single amino acid difference in the IBM sequence of Rpr versus Mx and IMP (Fig. 4.5A).

To further define the sites of interaction between AeIAP1 and the IAP antagonists, GST-tagged BIR1 and BIR2 of AeIAP1 were pulled down with a biotin-labeled IBM peptide representing the first six amino acids of both Mx and IMP, following removal of the initiating Met residue (AIAFYK-biotin) (Fig. 4.4A). We found that the IBM peptide bound equally well to both BIR1 and BIR2, indicating that Mx and IMP can bind to either BIR domain. Consistent with previous results using *D. melanogaster* and mammalian proteins (Liu *et al.*, 2000, Wu *et al.*, 2000, Zachariou *et al.*, 2003), the interaction required the first Ala residue of the IBM to be exposed at the N terminus, since a control peptide with an N-terminal Met (Met-Hid) was not able to bind BIR1 and had greatly reduced binding to BIR2 (Fig. 4.5B).

AeIAP1 inhibits IAP antagonist-induced caspase activation

To test whether AeIAP1 can counteract the proapoptotic activity of IAP antagonists, we examined caspase activity in C6/36 cells and lepidopteran SF-21 cells expressing IAP antagonists with or without AeIAP1 or DIAP1. Expression of Mx, IMP or Rpr caused caspase activation in these two cell lines, while co-expression of AeIAP1 or DIAP1 decreased the level of caspase activity induced by the IAP antagonists (Fig. 4.6A-B). We also observed that expression of AeIAP1 or DIAP1 attenuated IAP antagonist-induced apoptosis in C6/36 and SF-21 cells (data not shown).

AeIAP1 physically interacts with caspases

To test whether AeIAP1 physically interacts with caspases, we first examined the interaction between GST-tagged AeIAP1 or its BIR domains and *in vitro* translated caspase proteins by pull down assays using glutathione beads (Fig. 4.7A). The effector caspases CASPS7 and CASPS8 mainly interacted with BIR1; however, CASPS7 also interacted to a

lesser degree with BIR2 and appeared to have a higher affinity for AeIAP1 than CASPS8, based on the amount of specific binding versus the input for each protein. Initiator caspase AeDronc also bound to both BIR1 and 2, although more binding was observed to BIR1, while AeDredd and *Drosophila* Dronc only interacted with BIR1 (Fig 4.7A).

The assay in Fig. 4.7A used full length (unprocessed) caspase protein. *In vitro* translated caspase proteins are normally full length, while recombinant caspases expressed in bacteria often undergo auto-activating cleavage due to the effect of concentration during the purification process (Muro *et al.*, 2004). To test whether there was a difference in the interaction between AeIAP1 and activated (cleaved) caspases versus full length caspases, we activated *in vitro* translated AeDronc by incubation with a small amount of recombinant Dronc, and then tested the interaction between AeIAP1 and activated Dronc by pull down assay. *In vitro* translated, full length AeDronc was partially processed by active recombinant AeDronc into 4 fragments, plus some remaining full-length protein (Fig. 4.7C). The 40 KD fragment consisted of the prodomain and large (p20) subunit, the 23 KD fragment was the large subunit, the 17 KD fragment was the prodomain, and the 11.8 KD fragment was the small (p10) subunit. Activated AeDronc still interacted with both BIR1 and BIR2, but appeared to bind more to BIR1 (Fig. 4.7C), similar to the results observed with full-length AeDronc (Fig. 4.7A).

To test the interaction between AeIAP1 and processed CASPS7 and CASPS8, purified CASPS7 and CASPS8 from *E. coli* was incubated with AeIAP1 and interacting caspase proteins were visualized by western blotting with anti-His antibody. The interaction between active CASPS7 and BIR1 was stronger than between CASPS8 and BIR1, and CASPS7 interacted more strongly with BIR1 than BIR2 (Fig. 4.7D), consistent with the results obtained using unprocessed CASPS7 and CASPS8 (Fig. 4.7A). Active CASPS7 was not pulled down by full length GST-

AeIAP1. A possible explanation for this is that the active CASPS7 may cleave AeIAP1 near its N terminus, removing the GST domain and not allowing purification of AeIAP1-associated proteins, since *Drosophila* DrICE cleaves DIAP1 after residue 18 (Yan *et al.*, 2004b).

AeIAP1 inhibits the activity of initiator and effector caspases

Having shown that AeIAP1 binds to AeDronc, CASPS7, and CASPS8, we next investigated whether AeIAP1 is able to inhibit the activity of these caspases, both in cells and *in vitro*. After C6/36 cells were transfected with CASPS7, CASPS8 or AeDronc, cells exhibited higher activity against the effector caspase substrate Ac-DEVD-AFC than mock-transfected cells, which received only transfection reagent (Fig. 4.8A). Cotransfection of AeIAP1 or DIAP1 with each of the caspases decreased the caspase activity (Fig 4.8A). However, since endogenous caspases were probably activated by overexpressed CASPS7, CASPS8, or AeDronc, it was not possible to conclude which caspase(s) were directly inhibited by AeIAP1.

To examine the ability of AeIAP1 to inhibit caspases directly, we purified recombinant IAP and caspase proteins from *E. coli* and performed *in vitro* caspase assays. AeIAP1 and DIAP1 inhibited AeDronc, while the baculovirus P35 protein did not (Fig. 4.8B), which is consistent with the known specificity of P35 for effector caspases (Bump *et al.*, 1995, Fisher *et al.*, 1999). AeIAP1 and DIAP1 also inhibited the activity of CASPS7 and CASPS8, which were also inhibited by P35 and Z-VAD-FMK (Fig. 4.8C and D). These data demonstrate that AeIAP1 functions as a caspase inhibitor through direct physical interaction with AeDronc, CASPS7, and CASPS8.

IAP antagonists release initiator and effector caspases from inhibition by AeIAP1

In *Drosophila*, it has been shown that IAP antagonists are able to compete with caspases for binding to DIAP1 (Chai *et al.*, 2003, Yan *et al.*, 2004b). To test whether this can also occur in *A. aegypti*, we performed an *in vitro* competition assay. The initiator caspase AeDronc exhibited high caspase activity alone, and its activity was inhibited when mixed with AeIAP1 (Fig. 4.9). However, the ability of AeIAP1 to inhibit AeDronc was reduced by the addition of IAP antagonists Mx, IMP or Rpr, as compared with the addition of IAP antagonist IBM mutants or control GB, B1 domain of streptococcal protein G (Reibarkh *et al.*, 2008) (Fig. 4.9A and B). Interestingly, Mx exhibited stronger ability to replace AeDronc from AeIAP1 than IMP, as demonstrated in the *in vitro* caspase assay and pull down assay (Fig. 4.9A and B). The ability of AeIAP1 to inhibit caspase CASPS7 was also reduced upon addition of recombinant Mx, IMP, or Rpr (Fig. 4.10). These results indicated that IAP antagonists can compete with caspases for binding to the BIR domains of AeIAP1, and thus release caspases from inhibition by AeIAP1.

The role of cytochrome c in caspase activation

In contrast to mammalian cells, where release of cytochrome c from mitochondria and its incorporation into the apoptosome is essential for caspase activation by the intrinsic apoptosis pathway, it has been shown that cytochrome c does not play a role in caspase activation in *Drosophila* (Dorstyn *et al.*, 2004, Dorstyn *et al.*, 2002, Means *et al.*, 2006). To test the role of cytochrome c in caspase activation in *A. aegypti*, we homogenized Aag2 cells in the presence of a high concentration of sucrose to prevent the rupture of mitochondria, and separated the cell lysate into P10 (heavy membrane) and S100 (cytosolic) fractions. Cytochrome c was only detected in the P10 fraction (Fig. 4.11A and D), consistent with its mitochondrial localization and with previous reports using *Drosophila* S2 cells (Means *et al.*, 2006). As expected, the S100

fraction from unstimulated Aag2, C6/36 and S2 cells did not exhibit significant caspase activity (Fig. 4.11B, C and E). However, incubation with recombinant Mx or IMP protein induced caspase activation, similar to previous results obtained using S2 cells (Means *et al.*, 2006). These results indicted that an excess of IAP antagonist protein was able to activate the caspases in S100 (Fig. 4.11B and C), presumably by relieving caspase inhibition by AeIAP1.

While the results above indicate that cytochrome c is not required for caspase activation in cytosolic extracts, we asked whether it was capable of activating caspases. Addition of cytochrome c and dATP to S100 from mammalian cells causes apoptosome formation and caspase activation, but not when S100 from S2 cells is used (Means *et al.*, 2006). Mixing S100 from Aag2 cells or S2 cells with purified cytochrome c protein and dATP did not show increased caspase activity as compared to mock treatment (Fig. 4.11E), which indicated that cytochrome c was not able to stimulate caspase activation in S100 from mosquito cells.

Discussion

The molecular pathways that regulate apoptosis are largely conserved among metazoans; however, there are significant differences in how apoptosis is regulated between nematodes, insects, and mammals. To date, apoptosis has only been studied in detail in a single insect, *D. melanogaster*. In this study we explored the mechanisms of apoptosis regulation in the mosquito *A. aegypti*. Although initial characterizations of a few genes involved in mosquito apoptosis have been reported, we are still in the early stages of understanding apoptosis in mosquitoes.

Due to low transfection efficiency in *A. aegypti* cell lines, we utilized recombinant SINVs to express the four IAP antagonists (Rpr, Mx, alMx and IMP) in both *A. aegypti* Aag2 and *A. albopictus* C6/36 cells. Overexpression of these IAP antagonists in both cell lines induced apoptosis with high levels of caspase activation, demonstrating these proteins are sufficient to induce apoptosis (Fig. 4.1A-D). Furthermore, they are likely to be necessary for apoptosis, since silencing expression of IAP antagonists Mx or IMP in Aag2 cells reduced apoptosis induced by ActD or *Aeiap1* RNAi (Fig. 4.3A and C). Silencing Mx and IMP together had an additive effect, but still did not provide complete protection; however, it is quite possible that additional IAP antagonists exist in the *A. aegypti* genome, which have not yet been discovered due to the low level of sequence similarity between most IAP antagonists. Also consistent with this is the observation that silencing of Mx or IMP provided only temporary (up to around 12 h) protection from ActD- and *Aeiap1* silencing- induced death, even when both genes were silenced. By 24 h, the protective effect was no longer evident, which is not the case when other genes such as *Aedronc* are silenced (data not shown).

IAP antagonists are thought to act upstream of IAPs, which raises the question why we observed partial protection against apoptosis induced by silencing *Aeiap1* when we co-silenced

Mx or IMP (Fig. 4.3A and C). One possibility, which we currently favor, is that the silencing of *Aeiap1* is incomplete, such that reducing the levels of Mx and/or IMP allows lower levels of AeIAP1 to still be able to function and prevent caspase activation. However, it is also possible that Mx and IMP function somehow downstream of AeIAP1, perhaps by perturbing mitochondrial function.

The IBM sequence is indispensable for the interaction between IAP antagonists and IAP, with the first 4 amino acids being especially critical (Yan & Shi, 2005). The IBM sequences of Mx and IMP are identical over the first 5 amino acids (Fig. 4.4A), raising the question of whether Mx and IMP can bind equally to AeIAP1. We found that *Drosophila* Rpr, whose IBM differs from that of Mx and IMP in the second residue, had less affinity for AeIAP1 than *Aedes* Mx and IMP (Fig. 4.5A). Even though IMP and Mx showed similar binding affinity to AeIAP1 and similar ability to displace CASPS7 from AeIAP1, Mx displaced AeDronc from AeIAP1 better than IMP (Fig 4.9A and B). However, further experiments are necessary to determine whether Mx and IMP are truly redundant, or whether they play different roles during apoptosis.

AeIAP1 interacts with caspases in different ways. Full length AeDronc and CASPS7 interacted with both the BIR1 and BIR2 domains, but binding was stronger to BIR1. These data indicate different binding preference from *Drosophila*, in which Dronc only binds to BIR2 of DIAP1 (Chai *et al.*, 2003) (data not shown). Interestingly, *Drosophila* Dronc only bound to BIR1 domain of AeIAP1. AeDredd and CASPS8 only interacted with BIR1 domain. However, AeIAP1 had higher affinity for AeDronc and CASPS7 than AeDredd and CASPS8. AeIAP1 also interacted with cleaved AeDronc, in a similar way as binding with full length AeDronc. Both activated CASPS7 and CASPS8 only interacted with BIR1 but not BIR2, however, activated CASPS7 still had stronger binding than activated CASPS8, and the active site mutant

CASPS7 and CASPS8 lost binding affinity to AeIAP1 (data not shown). Possibly because N terminus of CASPS7 p10 has Ala after the cleavage site, so the activated CASPS7 had higher affinity with AeIAP1 than CASPS8 (Fig. 4.7A and D).

We should take into consideration that mosquitoes are blood feeding animals. After they took a blood meal, they need to digest a large amount of blood, which is 2-10 times their body weight. During blood feeding, the midgut is exposed not only to blood for digestion, but also to pathogens. So the midgut will undergo dramatic stress during blood feeding. The mechanisms of caspase regulation in mosquitoes not only enhance our knowledge of apoptosis in species, but also help us to better understand the interaction between the pathogens and mosquitoes for further pest control with methods manipulating the apoptotic pathway.

Acknowledgements

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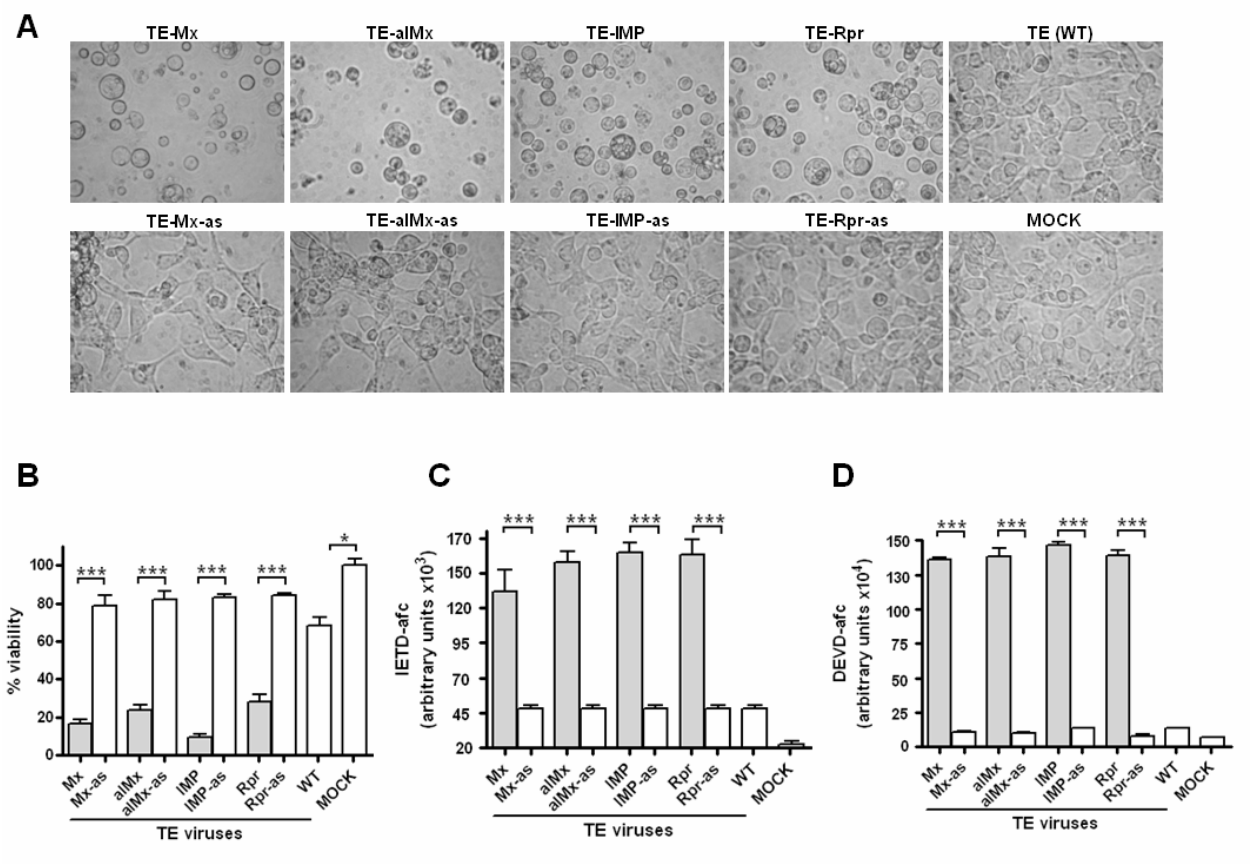

Figure 4.1 SINVs expressing Mx, IMP or Rpr cause apoptosis in Aag2 cells

(A to D) cells were mock-infected or infected with the indicated viruses (m.o.i. = 100). Analysis was conducted at 24 h p.i.. (A) Morphology of infected cells. Aag2 cells were photographed at 400X magnification. (B) Aag2 cell viability was determined by MTT assay. (C and D) At 24 h p.i., cell lysate was prepared and caspase activity was determined using Ac-IETD-AFC or Ac-DEVD-AFC as a substrate. Data are shown as mean $\pm$ SEM of three independent experiments (***$P < 0.0001$; *$P < 0.05$ by Student's t test).

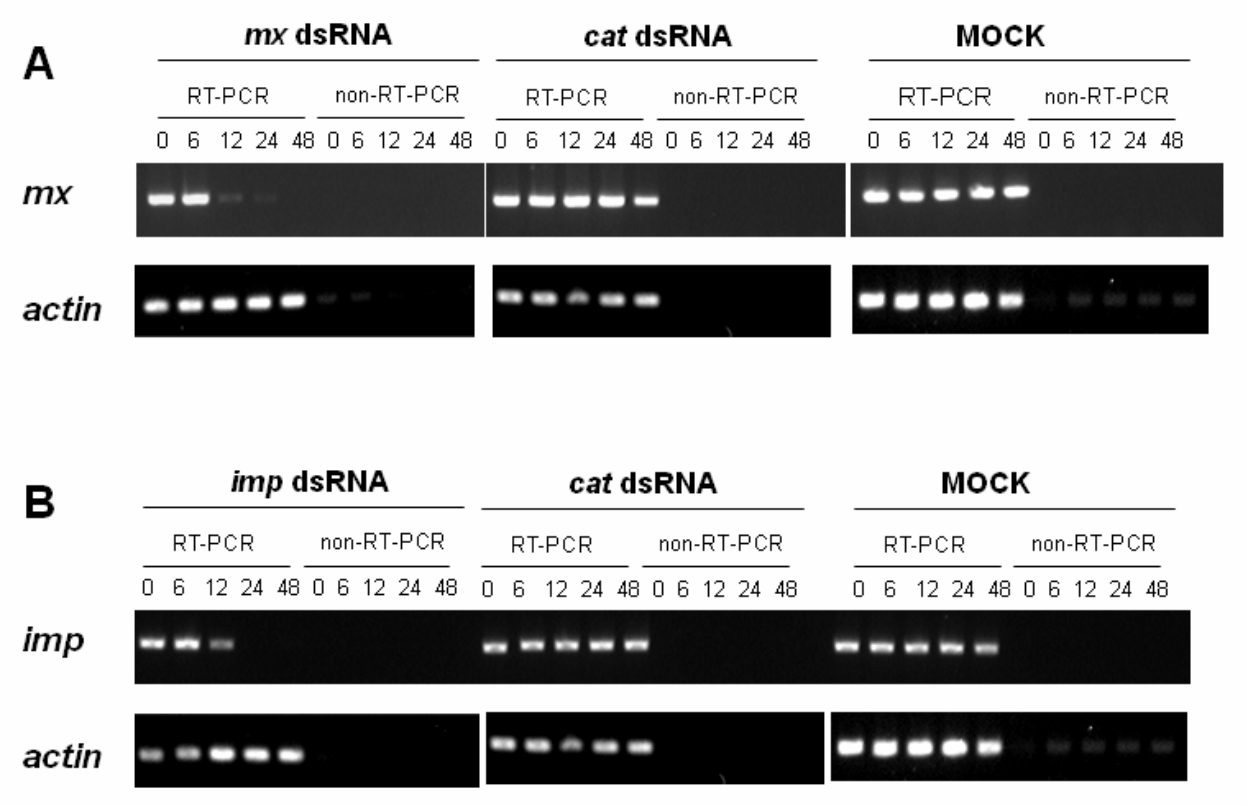

Figure 4.2 *mx* and *imp* are specifically silenced by addition of the corresponding dsRNA

(A) Levels of *mx* and *actin* mRNA were determined by RT-PCR at the indicated time points following treatment with *mx* dsRNA, *cat* dsRNA or mock treatment. As a control, PCR was performed without the reverse transcription step to confirm the lack of DNA contamination in the cells (non-RT-PCR). (B) Levels of *imp* and *actin* mRNA were determined by RT-PCR at the indicated time points following treatment with *imp* dsRNA, *cat* dsRNA or mock treatment.

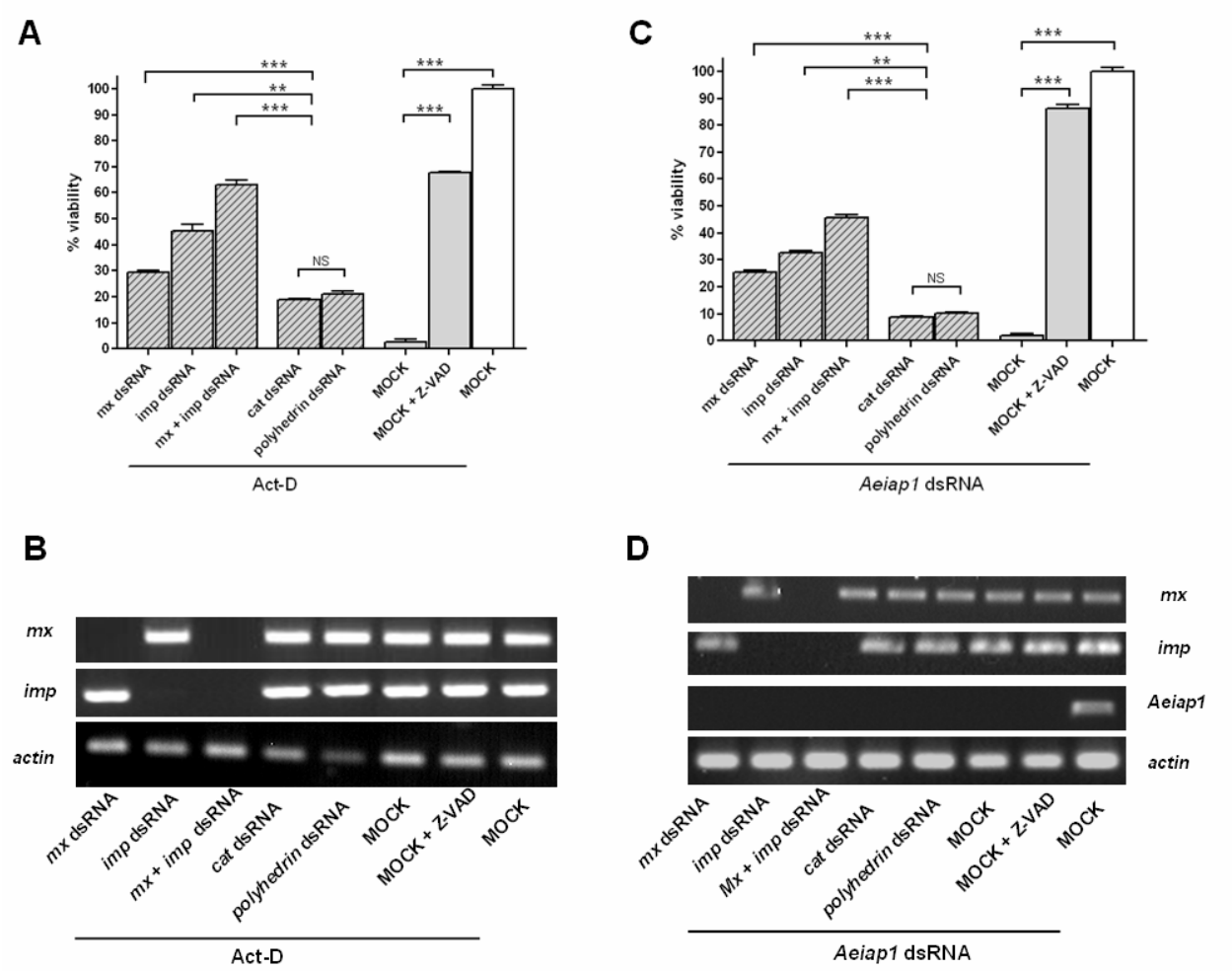

Figure 4.3 The silencing of *mx* and/or *imp* protects Aag2 cells from apoptotic stimuli

(A and B) After Aag2 cells were treated with indicated dsRNA for 24 h, cells were added with 50 ng ml^{-1} of ActD. Twelve hours later, Aag2 cell viability was determined by MTT assay (A) and the levels of *mx*, *imp* and *actin* mRNA were determined by RT-PCR (B). (C and D) After Aag2 cells were treated with indicated dsRNA for 24h, 5 μg ml^{-1} of *Aeiap1* dsRNA was added. Twelve hours later, Aag2 cell viability was determined by MTT assay (C) and the levels of *mx*, *imp* and *actin* mRNA were determined by RT-PCR (D). Data are shown as mean ± SEM of three independent experiments (***$P < 0.0001$; **$P < 0.001$ by Student's t test).

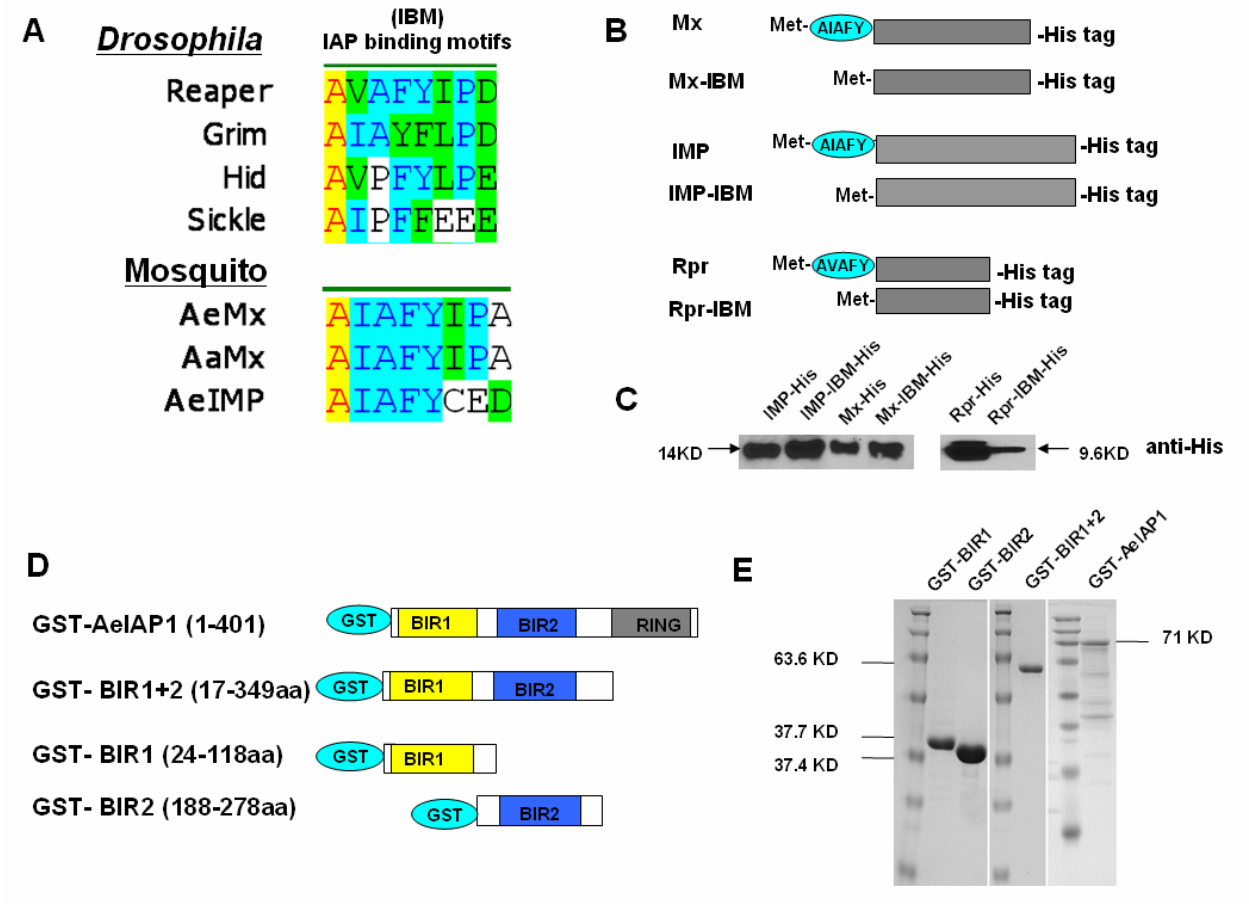

Figure 4.4 Expression of IAP antagonist, AeIAP1, and corresponding mutant recombinant proteins

(A) Conserved family of IAP-binding motifs (IBM). The tetrapeptide motif has the consensus sequence A-(V/I)-(A/P)-(F/Y). (B) Schematic of IAP antagonists and corresponding mutants constructed in this study. (C) Immunoblotting of IAP antagonist and mutant proteins with anti-His antibody. (D) Schematic of AeIAP1 and mutants constructed in this study. (E) Coommassie Blue staining of recombinant AeIAP1 and mutant proteins.

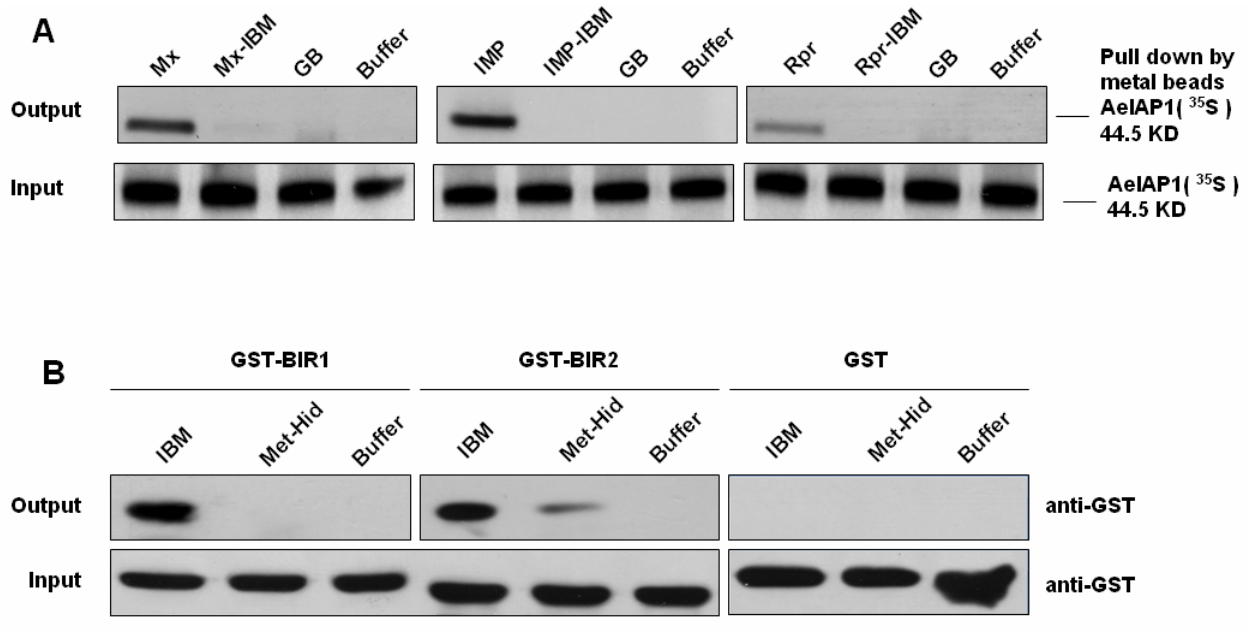

Figure 4.5 ***A. aegypti*** **IAP antagonists directly bind to AeIAP1, and binding is dependent on the IBM**

(A) The indicated His-tagged recombinant proteins were incubated with ^{35}S-labeled AeIAP1, after which protein complexes were purified using Talon resin and examined by autoradiography. GB represents B1 domain of streptococcal protein G (Reibarkh *et al.*, 2008) (B) Streptavidin-agarose beads were incubated with biotinylated peptides containing amino acids 2–6 of Mx and IMP, or 1–11(Met-Hid) of Hid (Wright & Clem, 2002), or buffer. Recombinant proteins (GST-BIR1, GST-BIR2, or GST) were added, and the protein that specifically bound was eluted and detected by immunoblotting with anti-GST antibody. Input represents 10% of the amount of the relevant protein added to the beads.

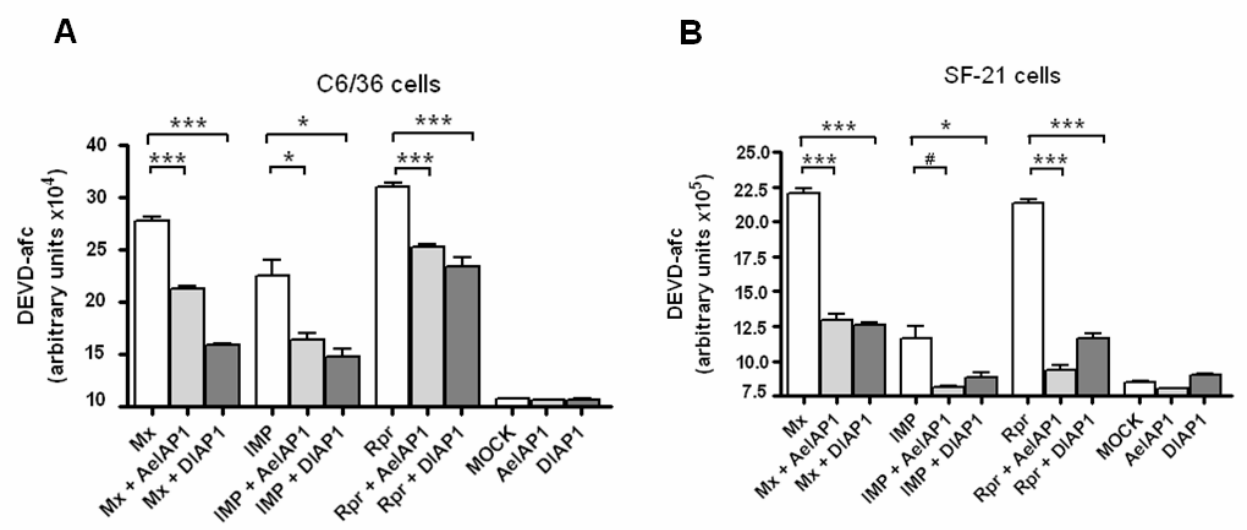

Figure 4.6 AeIAP1 and DIAP1 inhibit IAP antagonist-induced caspase activation

(A and B) C6/36 cells or SF-21 cells were transfected with IAP antagonist constructs with or without a plasmid expressing AeIAP1 or DIAP1. Cell lysates were prepared at 23 hrs post-transfection and caspase activity was determined using Ac-DEVD-AFC as a substrate. Data are shown as mean $\pm$ SEM of three independent experiments (***$P < 0.0001$; *$P < 0.01$; $^{\#}P < 0.05$ by Student's t test).

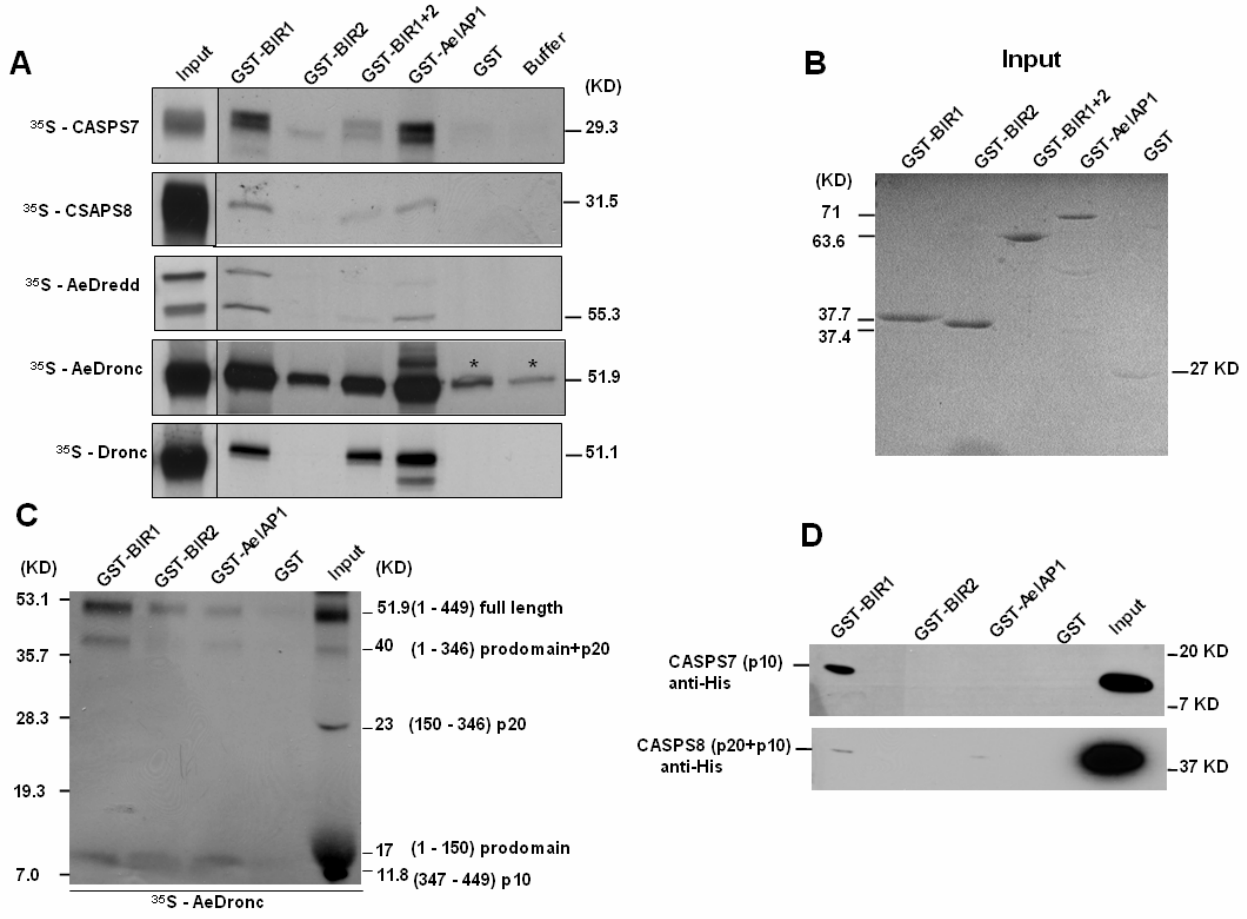

Figure 4.7 Initiator and effector caspases interact with AeIAP1 differentially

(A) *In vitro* translated caspases were incubated with recombinant protein (GST-BIR1, GST-BIR2, GST-BIR1+2, GST-AeIAP1, or GST) or buffer alone, and protein complexes were purified using glutathione-agarose beads and examined by autoradiography. (* indicates that the bands came from the overflow of previous GST-AeIAP1 loading) (B) Coommassie Blue staining of recombinant proteins used in the pull down assay. (C) Full-length *in vitro* translated AeDronc was incubated with active recombinant AeDronc to obtain processed AeDronc, which was then incubated with GST-BIR1, GST-BIR2, GST-AeIAP1 or GST and pulled down with glutathione-agarose beads and examined by autoradiography. (D) Active His-tagged CASPS7 or CASPS8 recombinant protein was incubated with a GST-tagged recombinant protein (GST-

BIR1, GST-BIR2, GST-AeIAP1 or GST) and pulled down with glutathione-agarose beads. Proteins that specifically bound were eluted and detected by immunoblotting with anti-His antibody.

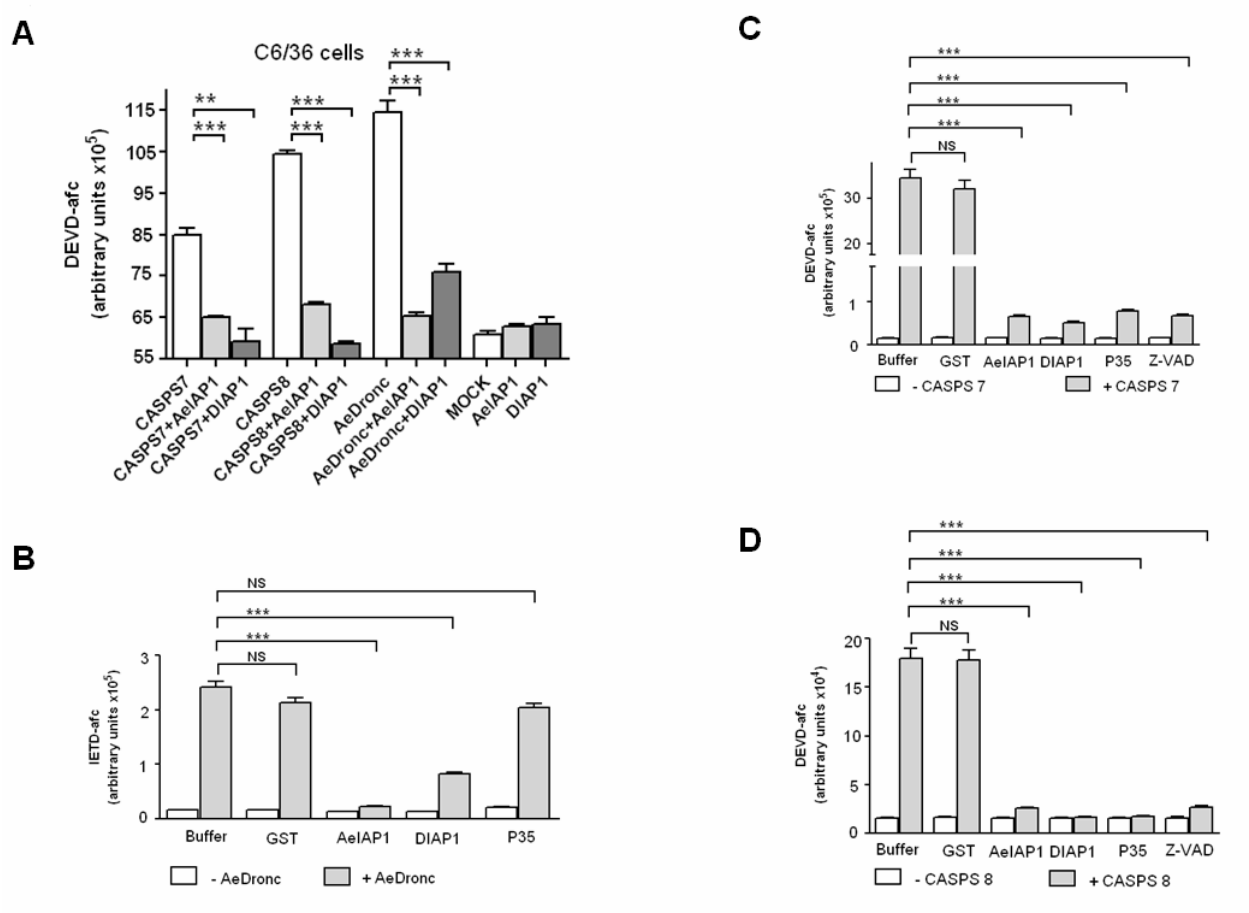

Figure 4.8 AeIAP1 and DIAP1 inhibit the activity of AeDronc and effector caspases CASPS7 and CASPS8

(A) C6/36 cells were transfected with CASPS7 or CASPS8 constructs, with or without a plasmid expressing AeIAP1 or DIAP1. Cell lysates were prepared at 23 hrs post transfection and caspase activity was determined using Ac-DEVD-AFC as a substrate. (B to D) Recombinant protein (AeIAP1, DIAP1, P35 or GST) (10 μM) or Z-VAD-FMK (100 μM) was incubated with 0.5 μM active caspases (AeDronc, CASPS7 or CASPS8) at 30^{o}C for 1 hour. Caspase activity was determined using Ac-IETD-AFC or Ac-DEVD-AFC as a substrate. Data are shown as mean $\pm$ SEM of three independent experiments (***$P < 0.0001$; **$P < 0.001$; NS, non-significant by Student's t test).

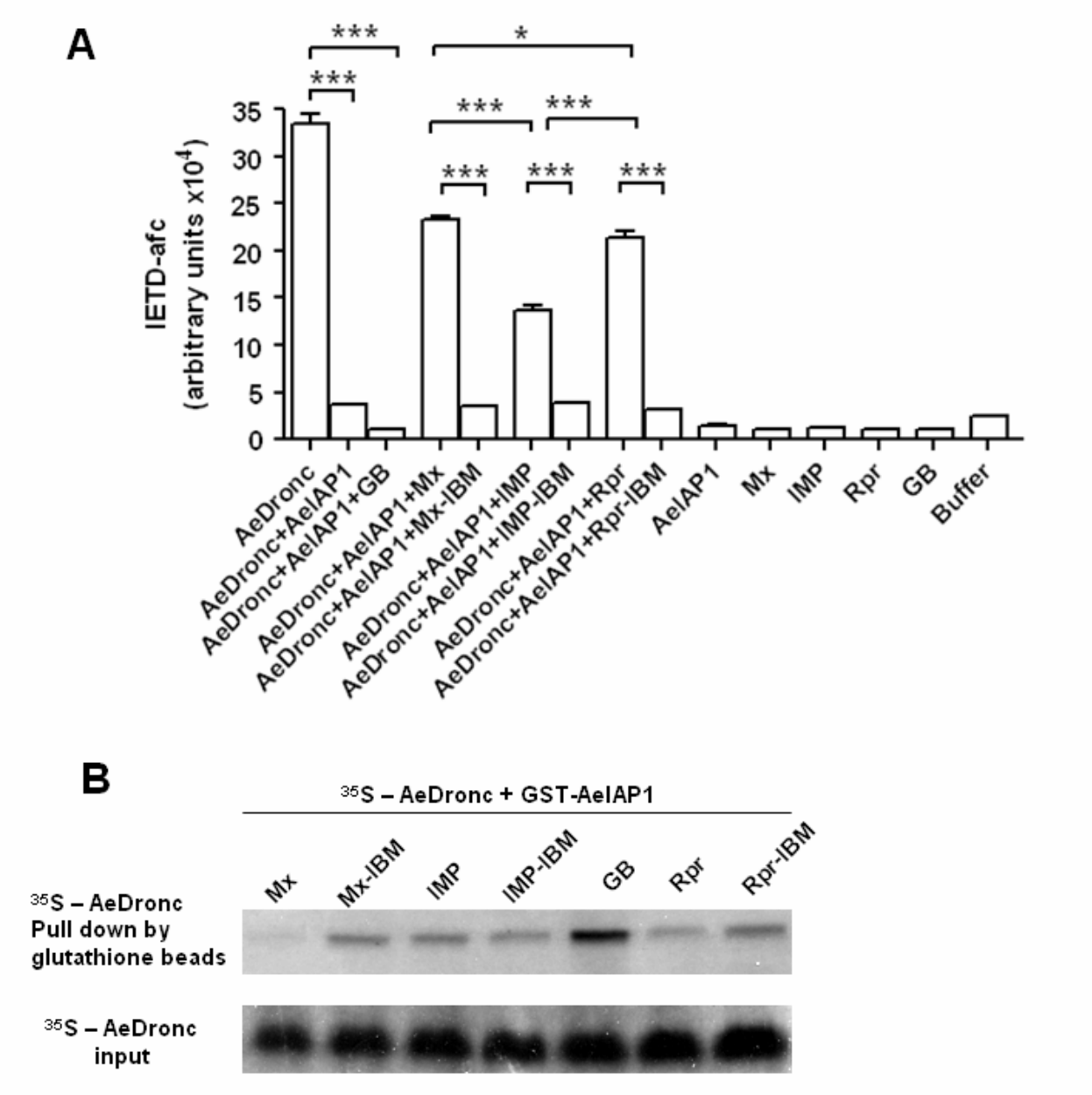

Figure 4.9 IAP antagonists release AeDronc from inhibition by AeIAP1

(A) Recombinant GST-AeIAP1 (10 μM) was incubated with 0.5 μM of active AeDronc, followed by addition of recombinant IAP antagonists (Mx, IMP or Rpr) (10 μM) or GB control protein (10 μM). After 1 hr incubation, caspase activity was determined using Ac-IETD-AFC as a substrate. Data are shown as mean ± SEM of three independent experiments (***$P < 0.0001$; *$P = 0.0255$ by Student's t test). (B) AeDronc was labeled with radioactive ^{35}S by *in vitro*

translation. Recombinant protein GST-AeIAP1 (2.5 μM) was incubated with 10 μl of the reaction at 30^{o}Cfor 1 hour. Later, the reaction was added with 2.5 μM IAP antagonists (Mx, Imp or Rpr) or corresponding mutants or GB control protein at 30^{o}C for 1 hour. The reaction was further incubated with 40 μl of glutathione agarose beads for 1 hour at 4^{o}C. Radioactive labeled AeDronc was pulled down with the beads and examined by autoradiography.

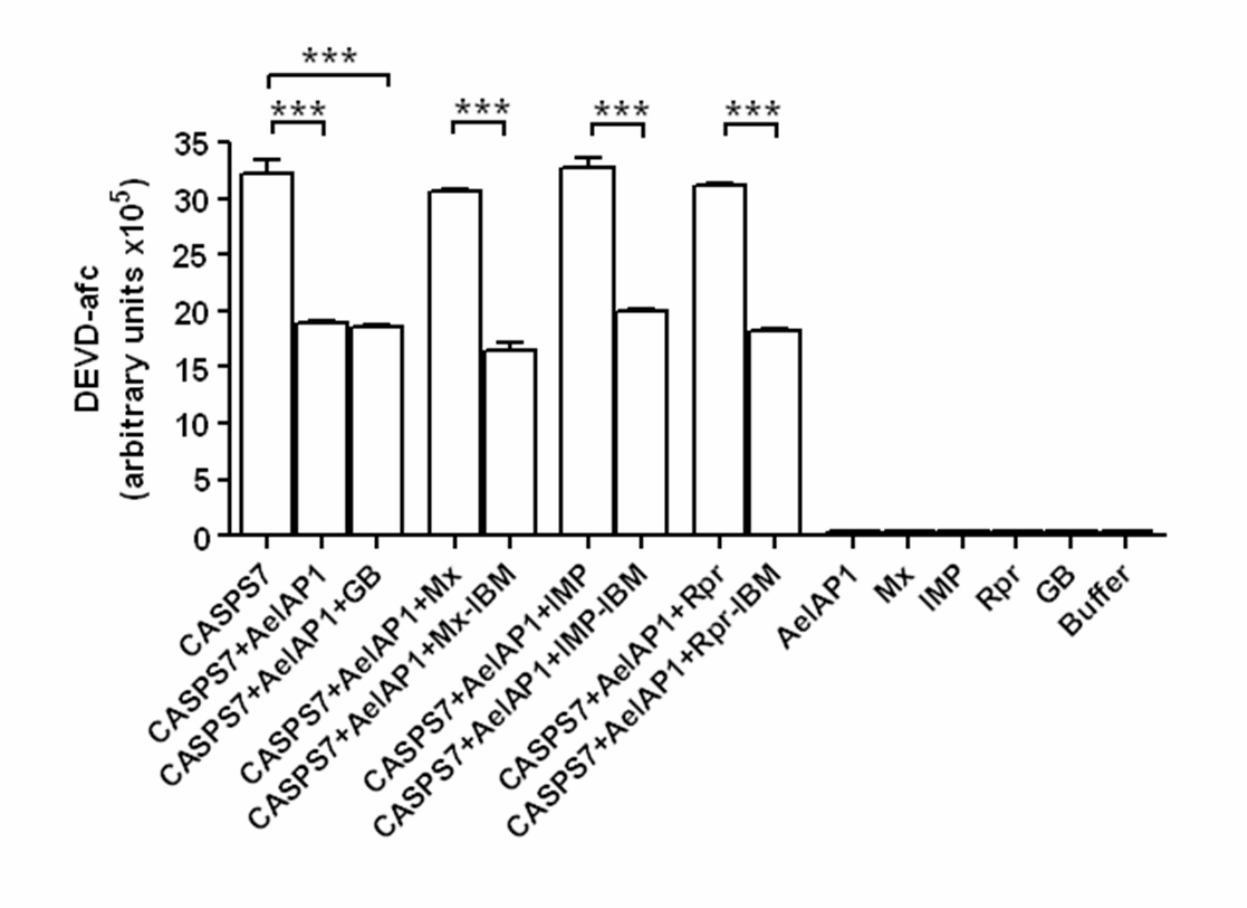

Figure 4.10 IAP antagonists release CASPS7 from inhibition by AeIAP1

Recombinant GST-AeIAP1 (10 μM) was incubated with 0.5 μM of active CASPS7, followed by addition of IAP antagonists (Mx, IMP or Rpr) or corresponding mutants or GB control protein. After incubation for 1 h, caspase activity was determined using Ac-DEVD-AFC as a substrate. Data are shown as mean ± SEM of three independent experiments (***$P < 0.0001$ by Student's t test).

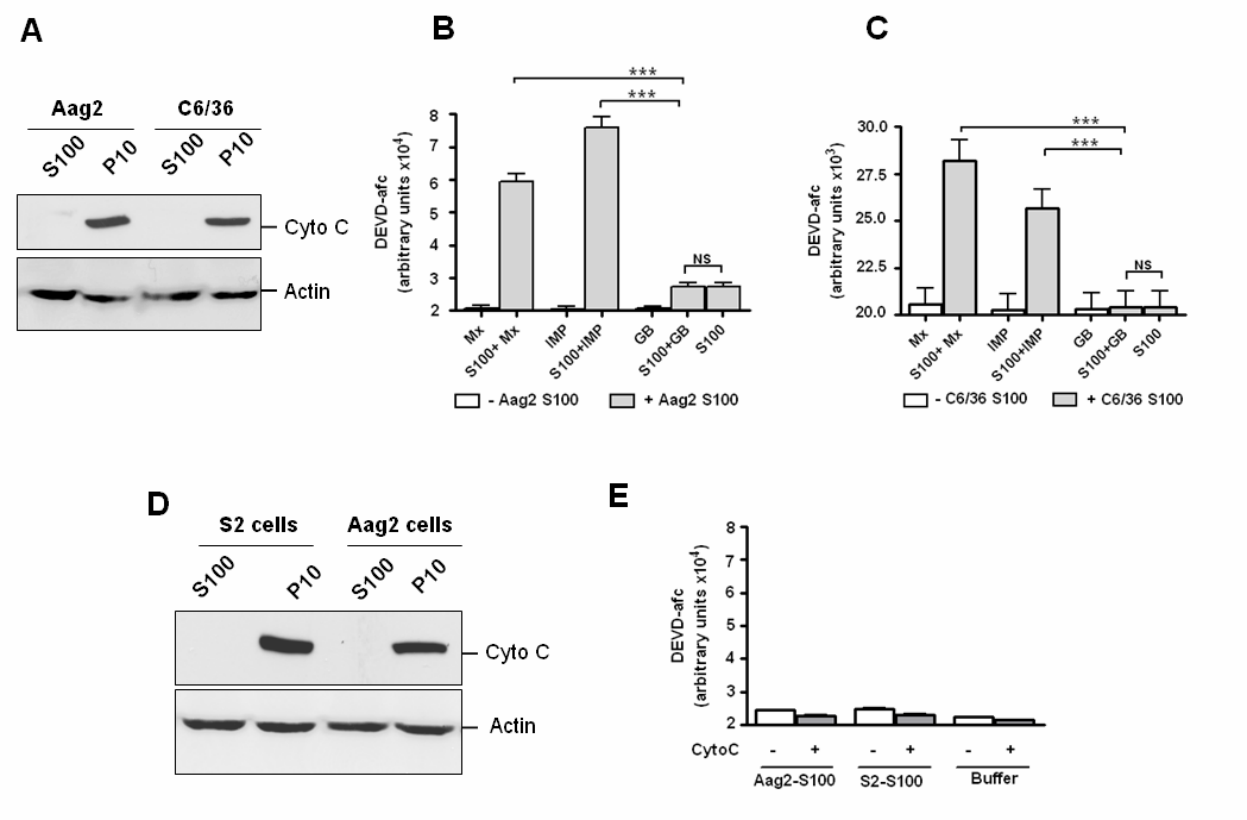

Figure 4.11 IAP antagonists induce caspase activation in mosquito cell lysate, and cytochrome C does not

(A) The P10 and S100 fractions isolated from mosquito Aag2 and C6/36 cells were analyzed by immunoblotting with anti-cytochrome C and anti-actin antibodies. (B and C) Fifty microgram of S100 lysate was incubated with recombinant protein (Mx or IMP or control GB) (10 μM) prior to determining caspase activity using Ac-IETD-AFC as a substrate. (D) The P10 and S100 fractions isolated from mosquito S2 and Aag2 cells were analyzed by immunoblotting as in (A). (E) Fifty microgram of S100 lysate was incubated with cytochrome C (2 μg), ATP (2 mM) and $MgCl_2$ (1 mM), and caspase activity was determined using Ac-DEVD-AFC as a substrate. Data are shown as mean ± SEM of three independent experiments (***$P < 0.0001$; NS, non-significant by Student's t test).

CHAPTER 5 - Conclusions

Mosquito-borne diseases are serious global health concerns and majority of the diseases are caused by arboviruses, such as yellow fever and dengue fever viruses. In this dissertation the interaction between *Aedes aegypti* and SINV was studied. Since apoptosis is considered to be an anti-viral defense in some mammalian and insect systems, and is not well defined in mosquitoes compared to *Drosophila melanogaster*, the role of apoptosis during infection of SINV in *A. aegypti* and the role of IAP antagonist proteins in the core apoptosis pathway of *A. aegypti* were specifically investigated.

In Chapter 2, effects of inducing or inhibiting apoptosis on Sindbis virus replication in mosquito cells were tested. After mosquito C6/36 cells were infected with recombinant SINVs expressing pro-apoptotic proteins or anti-apoptotic proteins, cells had different fates: apoptosis or apoptotic resistance. Since SINV normally infects mosquito cells persistently, apoptosis induced by recombinant SINV infection was carefully analyzed by several parameters, including cel viability, caspase activity, nuclear condensation and fragmentation, and DNA laddering. Recombinant SINV replication was also quantified using non-cumulative and cumulative methods. Even though pro-apoptotic SINVs replicated with an initial burst, the level of virus dramatically dropped after the cells died. The anti-apoptotic effects did not influence SINV replication in cells.

From this work, several issues remain to be investigated. First, the mechanism by which SINV inhibits apoptosis is still unresolved. Second, how apoptosis affects SINV transcription, translation and assembly remain to be studied. Third, during SINV infection of mosquito cells, a lot of vacuoles were observed, which is a major character of autophagy. That brings up the questions about the role of autophagy during SINV infection: how autophagy cross-talks to

apoptosis, or how autophagy and apoptosis inhibit or enhance each other. Fourth, SINV shows different tropisms in different mosquito cell lines. SINV replicates in *A. albopictus* C6/36 cells faster than *A. aegypti* Aag2 cells and recombinant SINV express higher levels of foreign proteins in C6/36 cells than in Aag2 cells (data not shown). These observations are consistent with the results obtained from adult *A. albopictus* and *A. aegypti* mosquitoes (Dohm *et al.*, 1995). Therefore, the understanding of *A. aegypti* suppresses SINV replication better than *A. albopictus* could help us to define a possible anti-viral defense mechanism in mosquitoes.

In Chapter 3, the effects of silencing apoptosis regulatory genes on SINV replication and dissemination were directly tested in *A. aegypti*. Pro-apoptotic (*Aedronc*) or anti-apoptotic (*Aeiap1*) genes were silenced in two strains of *A. aegypti*, RexD and Orlando strains. Silencing *Aeiap1* induced significant death in both strains, and midguts contained high caspase activity, highly disrupted microvilli, disorganized epithelia, and condensed chromatin in epithelial cells. Thus, the death observed in mosquitoes following *Aeiap1* RNAi may have been due to widespread apoptosis in mosquito tissues. At 3 days after injection with *Aedronc* or *Aeiap1* dsRNA, mosquitoes were fed a blood meal containing SINV infectious clone 5' dsMRE16ic-EGFP. Seven days later, EGFP expression was examined in midgut, foregut, hindgut salivary glands, and eyes. Silencing *Aedronc* promoted virus replication and dissemination in mosquitoes, while silencing *Aeiap1* had opposite effects.

Even though anti-viral effects of apoptosis were not observed in this study, future studies remain to explore these effects. The proapoptotic recombinant viruses from Chapter 2 could be used to study the natural infection of arboviruses in mosquito and to test the role of apoptosis in the model. Given that systemic RNAi has been shown to be an anti-viral defense in the

Drosophila system, how apoptosis facilities the RNAi response to spread the viral dsRNA being recognized by cells in insects is still unknown.

In Chapter 4, the roles of two *A. aegypt*i IAP antagonists, Michelob_x (Mx) and IMP, in apoptosis were characterized. Overexpression of IMP or Mx induced apoptosis in *A. aegypti* Aag2 cells, *A. albopictus* C6/36 cells, and *Spodoptera frugiperda* SF-21 cells, and apoptosis was attenuated by co-expression of AeIAP1. Recombinant IMP and Mx proteins directly bound to the BIR1 and BIR2 domains of AeIAP1 by IAP binding motif. As a central apoptotic regulator, AeIAP1 physically interacts with multiple caspases, including initiator caspases (AeDronc and AeDredd) and effector caspases (CASPS7 and 8), with different binding affinity and BIR preferences for these caspases. AeIAP1 inhibiting initiator and effector caspases was demonstrated *in vitro* and *in vivo*. The inhibition of caspases by AeIAP1 was removed by IMP and Mx; however, Mx displayed higher ability to replace AeDronc from AeIAP1. Addition of recombinant IMP or Mx to Aag2 cytosolic extract caused caspase activation, but not cytochrome c. Thus, mosquito IAP antagonists share a lot of functional features with *Drosophila* Rpr.

There are still several aspects we need to explore. First, the pathways that Mx and IMP are involved in remain to be examined in detail. In this study, IMP and Mx showed similar binding affinity to AeIAP1 and similar ability to displace CASPS7 from AeIAP1, but Mx displaced AeDronc from AeIAP1 better than IMP. What are the other caspases (such as CASPS 18, 19, and 16)Mx and IMP prefer to replace from AeIAP1? Do Mx and IMP have functions in inhibition of protein expression as Rpr does? Given that silencing Mx and IMP together had an additive effect, but still did not provide complete protection, there are probably additional IAP antagonists that need to be discovered. Second, the role of mitochondria still remains to be explored. Even though cytochrome C was not involved in activating caspases directly, a lot of

factors (such as AIF and EndoG) in mitochondria that are released during apoptosis have been found in *Drosophila*. Whether these mitochondrial factors promote apoptosis or help in apoptosome assembly in insects is still unresolved. Third, the redundancy of CASPS7 and CASPS8 *in vivo* is still unknown. In this study, the activity of recombinant CASPS7 was much higher than that of CASPS8, however the transfection of these two caspases induced similar cell death effects. Future studies could determine the pathways these two caspases are involved in, the roles they play in apoptosis, and whether CASPS8 can activate CASPS7 to cause similar phenotype as the activation of CASPS7.

Overall, the studies in this dissertation have been the first to test directly whether inducing or inhibiting apoptosis affects arbovirus replication in mosquito cells; the first observation that silencing *Aeiap1* induces systemic apoptosis in mosquitoes; the first direct study to explore the role of apoptosis in determining mosquito vector competency for arboviruses; and the first time demonstration that the mechanisms by which IAP antagonists regulate apoptosis are largely conserved between mosquitoes and *Drosophila*.

These studies help to expand the knowledge of apoptosis regulation in mosquitoes and insects, and the interaction between mosquitoes and arboviruses. These studies will contribute to the global efforts to control mosquito-borne diseases.

Dohm, D. J., Logan, T. M., Barth, J. F. & Turell, M. J. (1995). Laboratory transmission of Sindbis virus by Aedes albopictus, Ae. aegypti, and Culex pipiens (Diptera: Culicidae). *J Med Entomol* **32**, 818-21.