CHARACTERIZATION OF CASPASES IN THE APOPTOTIC PATHWAY OF AEDES AEGYPTI

by

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Abstract

Caspases are a conserved family of cysteine proteases that play important roles in apoptosis and innate immunity as well as other cellular processes. Eleven caspase genes have been annotated in the mosquito *Aedes aegypti.* Amongst these, previous studies have demonstrated functional roles for AeDronc, CASPS7 and CASPS8 in the *Ae. aegypti* apoptosis pathway, while CASPS18 and CASPS19 have also been functionally characterized. A previous study from our research group showed that AeIAP1 has preferential binding for CASPS7 compared to CASPS8. In this study, it was confirmed that AeIAP1 has a higher capacity to inhibit CASPS7 than CASPS8. Furthermore, five of the remaining *Ae. aegypti* caspases, namely CASPS15, CASPS16, CASPS17, CASPS20 and CASPS21, were characterized. An attempt was made to classify these caspases as initiator or effector caspases, based on factors such as the length of their prodomain, sequence similarity to known *Drosophila* initiator and effector caspases, and their substrate specificity. The functions of these caspases in apoptosis was examined in the *Ae. aegypti* cell line Aag2, by using RNA interference to reduce their expression and test the effect on apoptosis. Recombinant CASPS16, 17, 20 and 21 were produced in bacteria and the abilities of these recombinant proteins to cleave different caspase substrates were examined. From the resulting data, it was concluded that CASPS17 and CASPS21 are likely to be effector caspases since they preferred a effector caspase substrate. When considering the prodomain length, CASPS17 has a short prodomain, but CASPS21 has a long prodomain, which is normally associated with initiator caspases. CASPS20 did not show preference for any specific substrate and has a short prodomain. Since it did not have a specific preference of substrate, it is likely to be an effector caspase based on prodomain length. CASPS16 showed a slightly higher preference for the

initiator caspase substrate WEHD, and has a long prodomain. Based on these results, CASPS16 is likely an initiator caspase.

To examine the potential roles of CASPS15, 16, 17, 20 and 21 in apoptosis, their expression in Aag2 cells was knocked down using RNA interference. Successful knockdown was verified by qRT-PCR. After silencing specific caspases, the cells were exposed to two different apoptotic stimuli, ultraviolet radiation (UV) or the RNA synthesis inhibitor actinomycin D (ActD). Following apoptotic treatment, apoptosis was measured by two methods; caspase activity was measured using an effector caspase substrate, and phosphatidyl serine exposure on the outer leaflet of the plasma membrane, which occurs in apoptotic cells, was measured by Annexin V staining and flow cytometry. In cells where CASPS15, 16, 17, 20 or 21 had been knocked down and the cells were then treated with UV or ActD, it was observed that effector caspase activity and Annexin V staining were both significantly lower than in UV- or ActD-treated cells that had received control double-stranded RNA. Together these results suggest that all of these caspases are involved in apoptosis in Aag2 cells. This study serves as a starting point for further research on *Ae. aegypti* caspases and their roles in specific cellular processes.

Table of Contents

List of Figures

List of Tables

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Dedication

This thesis is dedicated to my parents, Binod and Bijaya Bhandary. Thank you for everything you have done for me. Thank you for always providing me the best education and opportunities. Thank you for letting me pursue my dreams even when you had to send me afar. Thank you for giving me the freedom and confidence to achieve my goals. Thank you for teaching me to become humble and kind always. Most importantly, thank you for making me the person I am. Everything I am today and will ever become, I owe it all to you both. I am truly blessed to be your daughter and will always strive you make you proud.

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Chapter 1 - Introduction

Apoptosis:

Apoptosis is a regulated programmed cell death mechanism by which cells that are deleterious to the organism or are no longer needed are removed (Kerr et al., 1972). Multicellular organisms produce cells in excess that do not play any role in the body. Some of the cells produced get damaged during production while others are not involved in any specific function in the adult organism. In humans, billions of cells need to die every day in order for tissues and organs to maintain normal functioning and size (Raff, 1998). Thus, apoptosis is an important mechanism to get rid of unwanted cells for the normal functioning of the body. The regulation of apoptosis is as important as other processes like cell division and cell migration (Hengartner, 2000). Apoptosis is also required during embryonic development. During embryogenesis, apoptosis is involved in processes such as development of lumina of tubular structures, formation of digits in the limbs and involution of vestigial organs. Apoptosis is also a natural phenomenon responsible for tumor regression. On the other hand, excess apoptosis can cause developmental defects like congenital malformations, whereas insufficient amount of apoptosis is seen in tumor formation during cancer (Kerr et al., 1972). There are certain diseases that alter the rate of apoptosis occurring in the organism. For example, when cells are infected with viruses, they will often try to kill themselves using apoptosis. In neurodegenerative diseases like Alzheimers, neurons are known to kill themselves by the mechanism of apoptosis. Apoptosis is also activated in the cases of organ transplant, insulin-dependent diabetes and AIDS (Ulukaya et al., 2011).

While undergoing apoptosis there are distinct morphological changes that occur in the cell. Firstly, the cells start to shrink and chromatin is cleaved into oligonucleosomal fragments following the condensation of chromatin. The plasma membrane undergoes blebbing and

spherical pockets of cellular contents called apoptotic bodies are formed. The apoptotic bodies thus formed are phagocytized by surrounding cells (Shi et al., 2004). Unlike in apoptosis, when a cell is undergoing necrosis it increases in size and the membranes break open, releasing the cellular contents to the surrounding cells which activates the inflammatory response (Raff et al., 1998).

Apoptosis can broadly be characterized into two separate stages. At the first stage the cell commits to undergo cell death and in the second stage distinct morphological changes occur in the cell to execute the cell death process (Takahashi et al., 1996). When a cell is undergoing apoptosis, homeostasis needs to be disrupted and repair mechanisms in the cells need to be turned off. The cell also needs to arrest itself in the same cell cycle state and apoptotic inhibitors need to be inactivated. After these changes occur, the cell loses its structural integrity and begins to undergo morphological changes. The apoptotic bodies thus formed will have specific signals for their disposal (Nicholson et al., 1999). Therefore, the process of apoptosis is a highly regulated biological process with very stringent requirements for its activation and execution.

There are many discriminating characteristics that differentiate apoptosis from other forms of cell death like necrosis. In the case of necrosis, the cell membrane is damaged whereas in apoptosis the cell membrane initially remains intact with blebbing, and the membrane eventually becomes damaged in later stages. In terms of the cell structure, in necrosis the cell swells and lyses whereas in the case of apoptosis the cell actually shrinks and apoptotic bodies are formed. When a cell undergoes necrosis, lysosomal enzymes are released thus triggering inflammatory reaction in the body whereas inflammation does not occur following apoptosis (Ulukaya et al., 2011).

There are certain proteins that are required for the execution of apoptosis. Some of these proteins are responsible to activate or increase while others are required to stop or decrease the rate of apoptosis in the cells. There needs to be a proper balance of the various regulators in the cell so that the normal rate of apoptosis is maintained. Some of the major regulators of apoptosis are explained in detail below.

Caspases

Caspases are cysteinyl aspartate-specific proteases that cleave substrates specifically at aspartic acid (Asp) residues. High specificity for Asp in the P_1 position of the substrate is the major distinguishing characteristic of caspases. Although most caspases cleave at the Asp in the P1 position, the *Drosophila* initiator caspase Dronc has the capacity to cleave substrates after a Glutamate (Glu) at P1 position, including when it autoactivates and when it cleaves *Drosophila* inhibitor of apoptosis protein 1 (DIAP1) (Hawkins et al., 2000). The catalytic site of most caspases includes a 5 amino acid motif QACXG (where X is R, Q or G). Contrary to most caspases, Dronc has PFCRG motif in its active site. Caspases are synthesized as inactive zymogens. The zymogens contain a prodomain followed by large (p20) and small (p10) subunits. During activation, a series of cleavages separate the large subunit from the small subunit and the prodomain is removed. Active caspases consist of a heterotetramer made up of two small and two large subunits. When a cell receives an apoptotic signal, caspases are among the first group of proteins to be activated and these in turn cleave cellular substrates like lamins and poly (ADP-ribose) polymerase, causing morphological changes in the cell that are hallmarks of apoptosis (Cohen, 1997).

The first evidence indicating that caspases are important in apoptosis came from studies done in the nematode *Caenorhabditis elegans,* where it was found that the protein product of the gene *ced-3*, which was required for apoptosis, was homologous to the mammalian cysteine protease interleukin-1β- converting enzyme (ICE) (Thorberry et al., 1992). It was further observed that over-expression of ICE (later renamed caspase-1) in mammalian cells caused apoptosis (Miura et al., 1993). Since the discovery of the importance of caspases in apoptosis there have been many other discoveries showing the role of apoptosis in neuronal cell death and other mammalian cells (Gagliardini et al., 1994; Nicholson et al., 1995). Along with the discovery of caspases in mammals and *C. elegans*, one of the other early discoveries of caspases was Sf caspase-1, which was cloned from an insect, the fall armyworm (*Spodoptera frugiperda*) in 1997 (Ahmad et al., 1997). Caspase homologs have now been identified in numerous organisms including *C. elegans*, *Drosophila* and mammals, indicating that the apoptotic pathway is evolutionary conserved among organisms.

Inhibitors of Apoptosis (IAPs)

Inhibitors of Apoptosis (IAPs) are one of the main regulators that are responsible for inhibiting apoptosis. IAPs were originally discovered in baculoviruses (Crook et al., 1993). Soon after their discovery in baculoviruses, a homolog of baculovirus IAPs was found in *Drosophila*, DIAP1. DIAP1 was discovered while conducting a genetic screen for mutations that improved the effect of endogenous IAP-antagonist Reaper in *Drosophila* developing eyes (Hay et al., 1995). Along with DIAP1, the other IAPs present in *Drosophila* include Drosophila IAP2 (DIAP2), DETERIN and Drosophila BIR repeat-containing ubiquitin-conjugating (dBRUCE) (Orme et al., 2009). Neuronal apoptosis inhibitory protein (NAIP) was the first characterized IAP in mammals and was discovered while searching for the gene responsible for muscular atrophy (Roy et al., 1995).

Along with NAIP, other IAPs in mammals include XIAP, cIAP1 and cIAP2, which are the IAPs known to inhibit caspases-3, -7, and -9 respectively (Deveraux & Reed, 1999; Deveraux et al., 1998; Deveraux et al., 1997; Roy et al., 1997). In *Drosophila*, the major IAP protein that is responsible for regulating apoptosis is DIAP1 (also known as Thread) (Hay et al., 1995).

IAP proteins contain one or more Baculovirus IAP Repeat (BIR) domains, a domain which is 70-80 amino acids long. The BIR domains are crucial in the anti-apoptotic activity of IAPs. The structure of BIR domains consists of a three-stranded antiparallel □-sheet and five □-helices with three conserved cysteines and one histidine chelating a zinc atom. The BIR domains of IAPs directly interact with other binding partners like IAP antagonists and caspases to regulate apoptosis (Franklin et al., 2003; Hinds et al., 1999; Sun et al., 1999, 2000). Most IAP proteins also contain a Really Interesting New Gene (RING) domain near their carboxy-terminal end (Vaux and Silke, 2005). The RING domain has ubiquitin E3 ligase activity and promotes autoubiquitylation as well as transubiquitylation of proteins that bind to the RING domains (Vaux and Silke, 2005; Vucic et al., 2011). Some IAPs like mammalian c-IAP1 and c-IAP2 also possess a caspase recruitment domain (CARD). The CARD domain is present in some caspases as well. Like the other domains in IAPs, the CARD domain is also involved in binding of IAP with its regulators (Hofmann et al., 1997).

In baculoviruses, it was shown that IAPs prevent apoptosis caused by viruses (Clem, 2001). The mammalian XIAP protein was shown to inhibit activation of the inflammasome and thus activation of caspase-1 (Vince et al., 2012). XIAP is also able to inhibit both initiator (caspase-9) and effector (caspase-3 and caspase-7) caspases activated during the intrinsic apoptotic pathway in mammals (Bratton et al., 2002; Bratton et al., 2001; Srinivasula et al., 2001). *Drosophila* DIAP1 is known to inhibit apoptosis by the process of ubiquitylation and proteasomal

degradation of caspases and the IAP-antagonistic proteins Reaper, Hid, and Grim (Holley et al., 2002; Wilson et al., 2002).

Along with their roles in apoptosis, IAPs are known to play roles in various human diseases especially cancer. There is increased expression of IAPs in various tumor types (Fulda and Vucic, 2012; Hunter, et al., 2007; LaCasse et al., 1998). IAPs are important regulators of survival signaling pathways (Silke and Brink, 2010). Recently, IAPs have been used as targets for various therapeutic processes for diseases like cancer (Silke and Vucic, 2014).

IAP Antagonists

IAP antagonists are proteins that act on IAPs to inhibit their activity. Thus, these proteins are pro-apoptotic and their elevated levels correspond to occurrence of apoptosis. IAP antagonists are unique proteins because unlike other regulators of apoptosis like IAPs and caspases, these do not share high levels of sequence similarity among IAP antagonists of the same organism or even between different organisms. The only similarity among IAP antagonists is a conserved N-terminal motif called the IAP binding motif (IBM). This motif allows the binding of IAP antagonists to the BIR domains that are present in IAPs. In *Drosophila*, it has been observed that IAP antagonists, when overexpressed, compete with caspases for binding sites in DIAP1 and thus cause apoptosis (Zachariou et al., 2003). Another mechanism of induction of apoptosis in *Drosophila* by IAP antagonists is by the stimulation of DIAP1 by auto-ubiquitylation. Some IAP antagonists including Reaper also globally inhibit protein translation to cause apoptosis (Wilson et al., 2002; Ryoo et al., 2002; Yoo et al., 2002; Colon-Ramos et al., 2006).

IAP antagonists present in *Drosophila* are Reaper, Hid, Grim and Sickle. These four proteins have different affinity towards binding to the BIR domains in DIAP1. Reaper and Grim show equal preference for binding to the BIR1 and BIR2 domains, whereas Hid and Sickle have higher

affinity for BIR2 than BIR1. In the mosquito *Aedes aegypti*, two IAP antagonists, Michelob_x (Mx) and IMP have been discovered. As in the case of other IAP antagonists, the most obvious homology between these two proteins is the IBM sequence (Zhou et al., 2005).

There are several IAP antagonists in humans and among these, SMAC (second mitochondria-derived activator of caspases) also known as DIABLO (direct IAP-binding protein with low pI) is the most characterized (Du et al., 2000; Verhagen et al., 2000). Smac forms dimers and interacts with the BIR domains of XIAP (Wu et al., 2000). Human High temperature requirement protein A2 (HtrA2/Omi) and ARTS are other IAP antagonists in humans (Hegde et al., 2002; Gottfried et al., 2004). XIAP-associated factor 1 (XAF1) is an endogenous inhibitor of XIAP (Varkey et al., 1999). An IAP antagonist named Ibm1 has also been discovered in the genome of the silkworm *Bombyx mori*. Ibm1 was first lepidopteran reaper/grim ortholog discovered, and contains an IBM motif. Ibm1 is also known to bind with the BmIAP1 protein in *Bombyx mori* and induce apoptosis in insect cells (Bryant et al., 2009).

Defense Repressor 1 (Dnr1)

Along with the other regulators of apoptosis, Dnr1 has also been shown to be involved in apoptosis. It has been identified as an inhibitor of Dredd activity in *Drosophila* S2 cells (Foley and O'Farrell, 2004). Along with its role in controlling apoptosis, Dnr1 has also been shown to be involved in the innate immunity in *Drosophila* (Foley et al., 2009).

Studies from our laboratory have shown that AeDnr1 plays a role in apoptosis in *Ae. aegypti*. It was observed that silencing of AeDnr1 in Aag2 cells caused spontaneous apoptosis. Using RNAi epistasis experiments, it was determined that AeDnr1 requires the initiator caspase Dronc and the effector caspase CASPS8 to induce apoptosis. Among the IAP antagonists it was determined that AeDnr1 requires Mx but not IMP (Devore, 2007).

Caspase functions

Along with the major role that caspases play in apoptosis, there are other several biological processes that involve specific roles of caspases. Caspases have been shown to play role in non-apoptotic cell death pathways like necroptosis, pyroptosis and autophagy.

Necroptosis is a programmed type of necrosis, the process of cell death in which the dying cell and its organelles swell, eventually rupturing the plasma membrane and releasing all the cellular contents (Vanlangenakker et al., 2011).

Pyroptosis is a specific cell death pathway that is known to be involved in the removal of macrophages infected with microbes and intracellular pathogens. This process is activated when there is acute bacterial or viral infection or an abundance of bacterial toxins. Human caspase-1 and caspase-11 are known to be involved in pyroptosis (Miao et al., 2011). Autophagy is a cell death mechanism in which the cellular contents are degraded in the lysosomes. Human caspase-6 and 8 are known to play a role in autophagy (Norman et al., 2010). *Drosophila* effector caspase Dcp-1 has also been shown to be involved in autophagy (DeVorkin and Gorski, 2014). Along with the above mentioned cell death mechanisms, caspases have been known to be involved in mitotic catastrophe. Mitotic catastrophe occurs when the cells die during mitosis due to buildup of DNA damage due to irregularities during the process of cell cycle progression. Caspase activation, specifically caspase-2, is known to be involved in the regulation of this process along with other proteins like cyclin-dependent kinases (Castedo et al., 2004).

Along with their roles in the various apoptotic and non-apoptotic cell death mechanisms, caspases have been known to play roles in inflammation and immunity. Mammalian caspase-1 is known to play a role in processing and maturation of the interleukins IL-1β and IL-18 (Ghayur et al., 1997). The *Drosophila* caspase Dredd is involved in the immune deficiency (IMD) pathway

of the innate immune response, which is involved in the immune response against gram negative bacterial infection in *Drosophila*. The caspase Dredd specifically cleaves the NF-$_k$B-related protein Relish following exposure to gram negative bacteria, thus activating the IMD pathway (Stoven et al., 2003).

Mammalian caspases have been found to play a role in the differentiation of many cell types. Caspase-3 has been found to be involved in the differentiation of lens cells in the eye (Ishizaki et al., 1998). Caspases-2, 3 and 9 have been known to play role in differentiation of erythoid cells (Zermati et al., 2001). Similar to the roles in mammals, some of the *Drosophila* caspases have also shown to play a role in differentiation and fate determination of various cells. In mutants of *Drosophila* Apaf-1 ortholog (*dark*), the flies have defects in development and have extra cells. This effect is known to be mediated by Dronc (Kurunaga et al., 2006; Kumar S, 2007).

Caspases have also been shown to play a role in the process of tissue regeneration. In mammals, caspase-3 and caspase -7 deficient mice have decreased levels of liver regeneration and wound healing (Li et al., 2010). In addition, in invertebrates such as *Hydra* and in amphibians like *Xenopus*, the process of regeneration is associated with increased level of apoptosis (Chera et al., 2009; Tseng et al., 2007).

It has also been shown that caspases are involved in basal lamina remodeling in insects. Matrix metalloproteases (MMPs) were able to activate effector caspases which lead to the remodeling of the basal lamina in the midgut (Means and Passarelli, 2010). Due to this this remodeling, systemic viral infection was established in the insect. The insects used in this study were orally infected with baculoviruses and the presence of laminin in the midgut was examined using immunohistochemistry using anti-laminin antibody. These insets were infected with and without

pan caspase inhibitor zVAD-fmk. It was observed that when caspase activity was blocked using inhibitor zVAD-fmk, the disruption of laminin did not occur (Means and Pasarelli, 2010). Along with these specific roles, caspases have been known to be involved in tumor formation, aging and neural development (Shalini et al., 2014). All the different roles played by caspases show them to be important versatile players in various apoptotic and non-apoptotic processes. The study of caspases will thus be important in understanding other biological processes besides apoptosis.

Caspase Characterization

Caspases can be classified into two different groups according to their position in the apoptosis pathway. The caspases that are first activated by external or internal signals for apoptosis are called the "initiator" caspases. Initiator caspases have long prodomains that contain protein-protein interaction motifs such as the death effector domain (DED) or the caspase activation and recruitment domain (CARD) (Fig. 1). These motifs bind to adapter proteins and are involved in the autoactivation of initiator caspases, as described further below. The other group of caspases, the "effector" caspases, are activated by initiator caspases and cleave the cellular substrates that are involved in apoptosis. Effector caspases act downstream of the initiator caspases in the apoptosis pathway. Effector caspases have short prodomains lacking discernable motifs (Degterev et al., 2003).

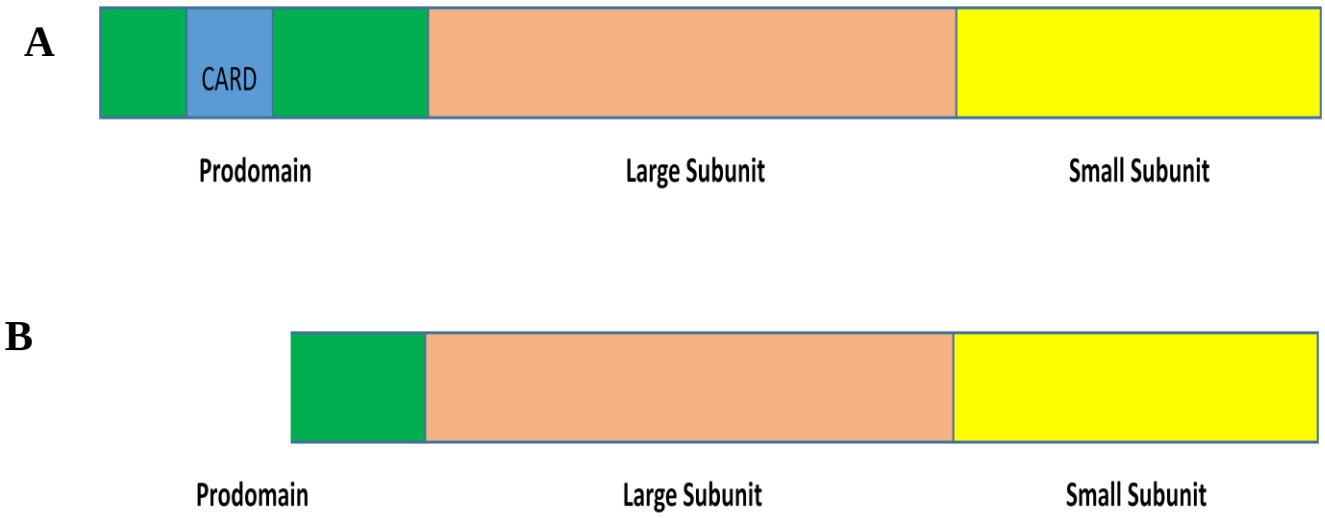

Figure 1.1. The primary structure of caspases.

The prodomain, large and small subunits in the proteins are denoted for A) initiator caspases and B) effector caspases. Initiator caspases have long prodomains with binding motifs like CARD or DED domains that are responsible for interaction of caspases with adapter proteins, allowing autoactivation. Effector caspases have short prodomains that lack binding motifs. Effector caspases require cleavage by other caspases in order to be activated.

Caspase activation

The activation of initiator caspases has been explained using two different models. The first, known as the induced proximity model, explains that initiator caspases auto-process themselves when brought in close proximity with each other. According to this model, CARD and DED domains in caspases interact with comparable motifs in adaptor proteins like Fas-associated death domain (FADD), which bring two or more caspase molecules close to each other. When the caspases come together, autoactivation occurs (Salvesen and Dixit, 1999). The second model, known as the proximity-induced dimerization model, postulates that initiator caspases like caspase-8 and -9 dimerize when they come close to each other. The dimerization of these two

initiator caspases is facilitated by the death-inducing signaling complex (DISC) and the apoptosome, respectively (Boatright et al., 2003; Donepudi et al., 2003; Renatus et al., 2001; Yan et al., 2005).

Caspase Structure

Much of the structural knowledge of caspase structure comes from studying human caspase-7. A functional caspase is a homodimer of heterodimers, consisting of 2 large (~20 kDa each) and 2 small (~10kDa each) subunits (Fig. 1.2). The active site is formed by five protruding loops, loops L1, L2, L3 and L4 from one monomer and loop L2' from adjacent monomer. This active site is highly conserved among all caspases. The substrate binding groove consists of the L1 and L4 loops. The catalytic Cys that is essential for enzymatic activity is positioned at the end of the L2 loop. The orientation of the four loops determines the substrate specificity of caspases (Chai et al., 2001b; Yan and Shi, 2005).

During the inactive zymogen state, the loops are in closed confirmation. Upon activation, all loops except L2 move away from their resting state and expose the substrate binding groove present in the L1 and L4. In the zymogen state, L2 is also twisted which causes a shift in the position of catalytic cysteine away from the active state (Yan and Shi, 2005).

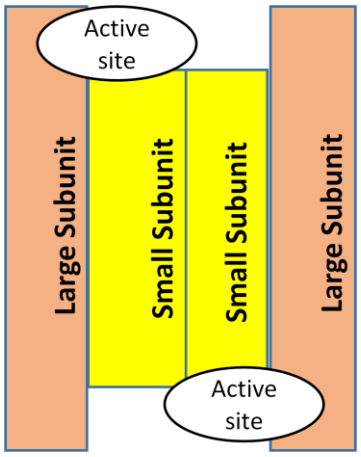

Figure 1.2: Active caspases form a heterotetramer.

Diagrammatic representation of an active caspase. An active caspase consists of a heterotetramer made up of 2 large and 2 small subunits. The prodomains are cleaved off during the activation of caspases, thus are not included in the active form of the protein. An active caspase contains 2 active sites.

Caspases recognize at least four adjacent amino acids in their substrates that are in positions named P4-P3-P2-P1. They cleave the substrate after the C-terminal residue in P1 position which is usually an Asp residue. Initially, caspases were known to be specific only for Asp at P1 position, but there is evidence that some caspases, specifically *Drosophila* Dronc, can also cleave after Glu residues (Hawkins et al., 2000; Srinivasula et al., 2001). The binding pockets in the caspases are called S4-S3-S2-S1 and these are complimentary to P4-P3-P2-P1 residues in the substrate. The conformation at the substrate-binding groove among caspases is highly similar. Specifically, the S2 and S4 positions are highly conserved whereas S1 and S3 positions are nearly identical among all caspases (Shi, 2002a; 2002b).

Caspases can also be differentiated according to their preference of substrates. Substrate specificity of human caspases was accessed *in vitro* using peptide substrates coupled to aminomethyl-coumarin (AMC). This assay was used to classify human caspases into three classes based on their residue preference in the P4 position of the substrate. The first group of caspases (Class I) preferred bulky, hydrophobic side chains in the P4 substrate position with the optimal sequence of WEHD. The caspases included in this group were caspase -1, -4 and -5. The second group of caspases (Class II) preferred intermediate-sized residues in the P4 substrate position with sequence similarity to (L/V)EXD. The caspases in this group were caspase -6, -8, -9 and -11. The third group of caspases (Class III), which includes Caspase -2, -3 and -7,

preferred small charged residues in the P4 position of their substrate and the sequence of substrate is DEXD, where X is V, T or H (Thornberry et al., 1997; Degterev et al., 2003).

Caspase regulation

There are various mechanisms of caspase regulation. Caspases are regulated at the transcriptional translational as well as post translational levels (Suzuki et al., 2001b). γ-interferon is known to enhance caspase activity in human U937 leukemia cells (Tamura et al., 1996). In mammalian apoptosis, the activity and processing of caspases is due to regulation by death receptors and cytochrome c. The activity of caspases has also been known to be altered by posttranslational modifications like phosphorylation and dephosphorylation of the active caspases (Earnshaw et.al., 1999). Along with the above-mentioned processes, phenomena such as nitrosylation in the active sites, oxidation, ubiquitination by IAPs and other proteins have also shown to positively or negatively affect caspase activation (Degterev et al., 2003).

Apoptosis pathways in different organisms

Apoptosis in mammals

The first caspase, interleukin-1β-converting enzyme (ICE), was identified in humans (Thornberry et al., 1992; Cerreti et al., 1992). There have been 14 caspases identified in mammals. Among the 14, at least 7 have been known to play a role in apoptosis. Caspase-2, caspase-8, caspase-9 and caspase-10 are examples of initiator caspases, while caspase-3, caspase-6 and caspase-7 are effector caspases.

Apoptosis pathways in mammals can be broadly divided into two major categories; extrinsic and intrinsic pathways. The extrinsic pathway involves death receptors that are present on the surface of cells. This pathway is activated in response to external signals that are received by cells convincing the cell to die for the overall well-being of the organism (Degterev et al., 2003). The

receptors contain intracellular death domains (DD) that recruit adaptor proteins like Fas-Associated protein with Death Domain (FADD) and Tumor necrosis factor receptor type 1-associated Death domain protein (TRADD). The adaptor proteins interact with domains of molecules involved in apoptosis, like certain initiator caspases, and trigger apoptosis (Yan et al., 2005). The intrinsic pathway is the pathway that is activated by intracellular stimuli such as DNA damage, cytotoxic drugs or other types of stress (Degterev et al., 2003). Upon receiving an intrinsic apoptotic signal, the mitochondria release several molecules like cytochrome c, Apoptosis inducing factor (AIF) and others into the cytoplasm of the cell. After reaching the cytoplasm, cytochrome c will activate Apoptotic protease activating factor (APAF1) which in turn will activate caspase-9 and thus turn on the cascade of caspase activation (Riedl and Shi, 2004).

A lot of knowledge on apoptosis and the role of specific caspases has been gained by using knockout mice that lack specific caspases. Mice deficient in caspase-8 have abnormal heart development and die early during embryonic development (Varfolomeev et al., 1998). Mutant mice deficient in caspase-3 and caspase-9 die before birth and exhibit brain malformations that occur due to decreased apoptosis in neuroepithelium (Kuida et al., 1996; Zheng et al., 1999; Woo et al., 1998; Hakem et al., 1998). Mutant mice with altered caspase-1, caspase -2 or caspase-11 do not suffer changes in apoptosis patterns *in vivo* (Kuida et al., 1995; Li et al., 1995; Bergeron et al., 1998; Wang et al., 1998).

Apoptosis in the nematode *Caenorhabditis elegans*

Cell-death abonormality-3 (CED-3) is the only apoptotic caspase in *C. elegans* and thus functions as both an initiator and effector caspase. There are four genes that act sequentially during apoptosis in *C. elegans* (Horvitz, 2003). Adaptor protein CED-4, which is homologous to

mammalian protein Apaf-1, activates CED-3 through oligomerization. When the cell is not undergoing apoptosis, the anti-apoptotic protein CED-9, which is homologous to Bcl-2 protein in mammals, suppresses CED-4 activity by direct physical interaction. When a cell receives an apoptotic signal, another protein, EGL-1, removes the negative regulation of CED-9 on CED-4, thus CED-4 activates CED-3 and the cell undergoes apoptosis. The process of apoptosis is highly regulated by these four proteins and it had been documented that 131 cells die due to apoptosis in *C. elegans* in specific locations during distinct developmental stages (Horvitz, 2003; Shi, 2004a, 2004b). Mutant *C. elegans* lacking CED-3 do not lose the cells that normally die due to apoptosis during development, but nevertheless they seem to develop normally.

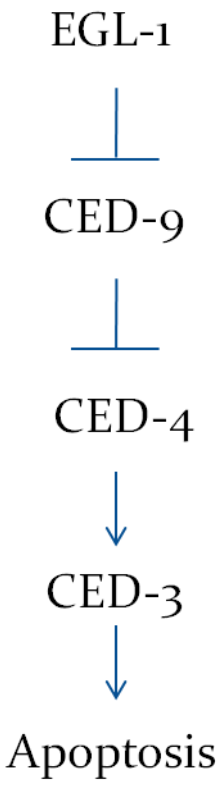

Figure 1.3: Summary of apoptosis in *C. elegans*.

The regulators of apoptosis act in a cascade, and in the above figure the arrow represents activation whereas blunt arrows represent inhibition. In *C. elegans*, CED-3 is a caspase which

acts as both an initiator and effector caspase. CED-3 is regulated by adaptor protein CED-4. CED-9 is an antiapoptotic protein in *C. elegans* which is negatively regulated by another protein, EGL-1.

Apoptosis in *Drosophila melanogaster*

There are 7 caspases in *Drosophila*. Dronc, Dredd and Strica are initiator caspases and Drice, Decay, Damm and Dcp1 are effector caspases. *Drosophila* Apaf-1 (Kanuka et al., 1999) or Dark (Rodriguez et al., 1999) is a protein with homology to mammalian Apaf-1 that is important in activating the initator caspase Dronc. When activated, Dronc in turn cleaves and activates initiator caspase Drice. During apoptosis, DIAP1 is inactivated by one or more of the four IAP antagonist proteins Reaper, Hid, Grim and Sickle through direct physical interaction. This activates Dronc, which activates Drice which in turn targets the cellular substrates for apoptosis (Shi, 2004).

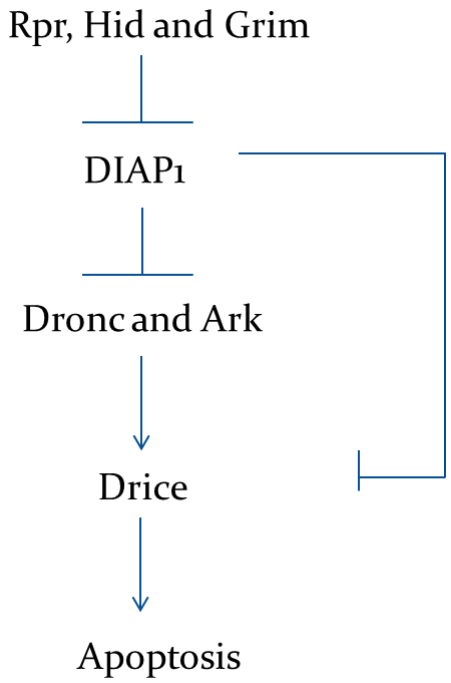

Figure 1.4: Summary of the apoptosis pathway in *Drosophila*.

Regulators of apoptosis in *Drosophila*. The caspases Dronc and Drice are normally inhibited by DIAP1. Rpr, Hid and Grim are IAP antagonists that regulate DIAP1. During apoptosis, inhibition by DIAP1 of Dronc is lost and Dronc activates Drice, causing apoptosis.

Apoptosis in *Aedes aegypti*

The apoptosis pathway in *Ae. aegypti* has not been studied to the same extent as in *Drosophila*. The proteins involved in apoptosis, including caspases and IAPs, were initially annotated during searches for immune-related genes using phylogenetic analysis in the malaria vector *Anopheles gambiae, Drosophila melanogaster* and in the dengue vector *Ae. aegypti* (Waterhouse et al., 2007). Apoptosis-related genes in *Ae. aegypti* were further annotated using their homology to known apoptosis-related genes in *Drosophila* (Bryant et al., 2008). This study led to the discovery of four additional apoptosis-related genes in *Ae. aegypti*. Currently, there are 11 caspases, three IAPs, one Dnr1, and two IAP antagonist homologs annotated in *Ae. aegypti*. Of the 11 *Ae. aegypti* caspases, only a minority have been characterized to date. AeDronc and AeDredd are characterized as initiator caspases whereas CASPS7 and CASPS8 are characterized as effector caspases. Further studies are required for the characterization of the other *Ae. aegypti* caspases as initiator or effector caspases.

Differential expression of certain caspases, namely CASPS16, Aedronc and Aedredd, was observed in a refractory strain of *Ae. aegypti* (Cali-MIB) when compared to the susceptible (Cali-S) strain in the presence of dengue virus (Ocampo et al., 2013). This suggests a potential role of these caspases in innate immune response against viral infections in *Ae. aegypti*.

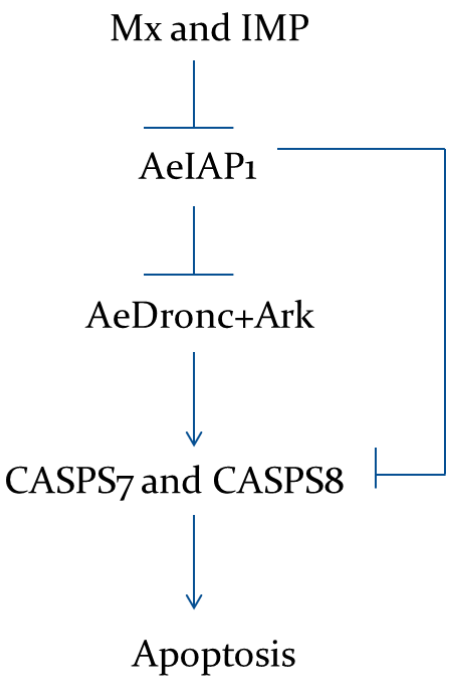

Figure 1.5: Summary of apoptosis pathway in *Ae. aegypti*.

In *Ae. aegypti*, AeDronc is the initiator caspase that activates effector caspases CASPS7 and CASPS8. AeIAP1 inhibits AeDronc and AeIAP1 is inhibited by IAP antagonists MX and IMP. During apoptosis, the inhibition of AeIAP1 in AeDronc is lost and AeDronc gets activated with the help of adaptor protein Ark. When activated, AeDronc in turn activates effector caspases CASPS7 and CASPS8, thus turning on apoptosis.

Table 1.1: Regulators of apoptosis in *Drosophila* and *Ae. aegypti*

Proteins	***Drosophila***	***Ae. aegypti***
Caspases	Dredd, Dronc, Drice, Dcp-1, Strica, Damm, Decay	AeDronc, AeDredd, CASPS7, CASPS8, CASPS15, CASPS16, CASPS17, CASPS18, CASPS19, CASPS20, CASPS21
IAPs	DIAP1, DIAP2, DETERIN, dBRUCE	AeIAP1, AeIAP2, AeIAP5
IAP antagonists	Rpr, Hid, Grim	MX, IMP
Other proteins	Ark	Ark, Dnr1

The various regulators of apoptosis in *Drosophila* and *Ae. aegypti* are listed above. *Drosophila* has 7 caspases whereas *Ae. aegypti* has 11 known caspases. *Drosophila* has 4 IAPs and *Ae. aegypti* has 3. The high conservation of the apoptosis pathway in these two organisms is depicted by the common regulators in these two species.

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Chapter 2 - Characterization of caspases in the apoptotic pathway of *Aedes aegypti*

ABSTRACT

Caspases are a conserved family of cysteine proteases that play important roles in apoptosis and innate immunity as well as other cellular processes. Eleven caspase genes have been annotated in the mosquito *Aedes aegypti*. A previous study involving two *Ae. aegypti* effector caspases, CASPS7 and CASPS8, found that AeIAP1 has preferential binding for CASPS7 compared to CASPS8. In this study, it was confirmed that AeIAP1 has a stronger ability to inhibit CASPS7 compared to CASPS8. Furthermore, five previously unstudied *Ae. aegypti* caspases, namely CASPS15, CASPS16, CASPS17, CASPS20 and CASPS21, were characterized. An attempt was made to classify these caspases as initiator or effector caspases, based on factors such as the length of their prodomain, sequence similarity to known *Drosophila* initiator and effector caspases, and their substrate specificity. The functions of these caspases in apoptosis were also examined in the *Ae. aegypti* cell line Aag2 by using RNA interference to reduce their expression and testing the effect on apoptosis. Substrate specificity assays using recombinant versions of the caspase proteins CASPS16, 17, 20 and 21 indicated that CASPS17 and 21 are likely to be effector caspases since they preferred DEVD, a known effector caspase substrate, even though CASPS 21 has long prodomain, which is normally associated with initiator caspases. CASPS20 did not show preference for any specific substrate but also has a short prodomain, and so was also predicted to be an effector caspase. CASPS16 showed a slightly higher preference for the initiator caspase substrate WEHD, and has a long prodomain, indicating it is likely an initiator caspase. After silencing the expression of each caspase in Aag2 cells, apoptosis was measured

after UV and actinomycin D (ActD) treatment by two different criteria; caspase activity was measured using an effector caspase substrate, and phosphatidyl serine exposure on the outer leaflet of the plasma membrane, which occurs in apoptotic cells, was measured by Annexin V staining and flow cytometry. In cells where CASPS15, 16, 17, 20 or 21 expression had been knocked down and the cells were then treated with UV or ActD, it was observed that effector caspase activity were significantly lower than in UV- or ActD-treated cells that had received a control double-stranded RNA. Similarly, using flow cytometry we also observed that the percentage of cells stained with Annexin V was significantly lower in cells where CASPS17, 20, and 21 have been knocked down compared to control double-stranded RNA. These results suggest that all five of these caspases are involved in apoptosis in Aag2 cells. This study serves as a starting point for further research on *Ae. aegypti* caspases and their roles in apoptosis and other cellular processes.

INTRODUCTION

Apoptosis is a natural phenomenon occurring in metazoans ranging from hydra to mammals. Although there is similarity in the regulators and pathway of apoptosis among closely related organisms, there are certain mechanisms and players that are specific to each organism. *Drosophila melanogaster* has been used as the basis for studying apoptosis pathway in other insects, including mosquitoes, due to the homology of proteins that play roles in the execution of apoptosis. As in *Drosophila*, apoptosis in the mosquito *Aedes aegypti* also involves caspases, IAP proteins, and IAP antagonists (Denton et al., 2013; Lui and Clem, 2011).

There are seven caspases in *Drosophila*, among which three are categorized as initiator caspases, while the remaining four are effector caspases. The initiator caspases include Dronc, Dredd, and Dream, whereas Drice, Dcp-1, Decay, and Damm are classified as effector caspases. Dronc and Drice are the most important caspases involved in apoptosis in *Drosophila*. Dredd plays a role in immune pathways, whereas Dcp-1 plays a supportive role in apoptosis (Hay and Guo, 2006). Little is known about the functions of the other *Drosophila* caspases. Decay is known to have weak apoptotic affect in mammalian cells (Dorstyn et al., 1999). Strica is known to induce cell death at low levels in specific tissues like eyes, salivary glands and egg chamber while the specific role of Damm is unknown (Harvey et al., 2001; Doumanic et al., 2001).

The eleven caspases annotated in *Ae. aegypti* are AeDronc, AeDredd, CASPS7, CASPS8, CASPS15, CASPS16, CASPS17, CASPS18, CASPS19, CASPS20, and CASPS21. These caspases were annotated using phylogenetic analysis and comparisons with *Drosophila* caspases. The expression patterns of the *Ae. aegypti* caspase genes were also examined at the transcript level in various developmental stages of *Ae. aegypti* (Bryant et al., 2008). In addition, six *Ae. aegypti* caspases have been characterized at the functional level. *Ae. aegypti* initiator caspases

AeDronc and AeDredd were studied by examining the conserved domains present in their sequences and comparison with domains in known *Drosophila* and human caspases. These caspases were also studied for their preferred substrates during apoptosis using known substrates for human caspases. The expression patterns of these two genes was also studied at various developmental stages and tissues in *Ae. aegypti* (Cooper et al., 2007a, 2007b).

Functional studies have also been performed on the regulators of apoptosis identified in *Ae. aegypti*. It was observed that silencing of the AeIAP1 gene using RNA interference (RNAi) in the *Ae. aegypti* cell line Aag2 caused spontaneous apoptosis (Liu and Clem, 2011). It was also observed that silencing *Ae. aegypti* Ark or AeDronc repressed apoptosis caused by introducing apoptotic stimuli. When the effector caspases CASPS7 or CASPS8, which are most closely related to *Drosophila* Drice and Dcp-1, were silenced it was observed that the degree of apoptosis repression was less than when AeDronc was silenced. When CASPS7 and CASPS8 were co-silenced, it did not cause significant change in apoptosis compared to them being individually silenced. These findings indicate that AeDronc, AeArk, AeIAP1, CASPS7 and CASPS8 play roles in the apoptosis pathway in *Ae. aegypti* (Liu and Clem, 2011). To study the physical interaction of AeIAP1 with caspases, the BIR domains of AeIAP1 were tested for their interactions with caspase proteins. It was observed that AeDronc bound more strongly to the BIR1 domain than the BIR2 domain. On the other hand, AeDredd only interacted with BIR1. In the same study, it was observed that *Drosophila* Dronc interacted only with BIR2 of DIAP1 and not BIR1 (Wang and Clem, 2011).

Sequence analysis of one of the caspases identified in *Ae. aegypti,* CASPS18, revealed substitutions in the catalytic site including a substitution in the catalytic cysteine. Due to this difference at this crucial position, this caspase did not have caspase activity. However, when

CASPS18 was co-expressed with the highly similar CASPS19 (or even when the two recombinant proteins were incubated together), the caspase activity of CASPS19 was significantly increased. It was therefore concluded that CASPS18 is a decoy caspase (Bryant et al., 2010). Interestingly, even though overexpression of CASPS19, with or without CASPS18, resulted in high levels of caspase activity in a lepidopteran cell line, it did not cause apoptosis suggesting that CASPS18 and CASPS19 do not play a role in apoptosis (Bryant et al., 2010). However, the possible roles of CASPS18 and 19 in apoptosis have not been tested in *Ae. aegypti* cells.

Phylogenetic analysis of the caspases in *Drosophila* and mosquitoes has shown that all of the *Drosophila* caspases have homologs in *Ae. aegypti*, but some mosquito caspases have been duplicated, and in some cases it is difficult to assign one-to-one orthology. In addition, one *Ae. aegypti* caspase, CASPS20, appears to be specific to Culicine mosquitoes (Bryant et al., 2010). AeDronc and AeDredd are orthologous to the *Drosophila* initiator caspases Dronc and Dredd, respectively. While looking for orthologs for the *Drosophila* effector caspases Drice and Dcp-1 in mosquitoes, it was difficult to assign orthology because Drice and Dcp-1 are more closely related to each other than to any mosquito caspases. However, CASPS7 and CASPS8 are the closest *Ae. aegypti* relatives to Drice and DCP-1. The caspases CASPS15, CASPS16, CASPS17 and CASPS21 from *Ae. aegypti* are most closely related to Dream and Damm, while CASPS18 and CASPS19 are most homologous to the effector caspase Decay (Bryant et al., 2010).

Some of the caspases in *Drosophila* seem to have originated by gene duplication. For example Dream and Damm appear to have arose from a gene duplication event, but they differ in the length of their prodomains, with Dream having a long prodomain, whereas Damm has a short prodomain. Similar duplication events seem to have occurred in the evolution of mosquitoes as

well, but to an even greater degree. During the evolution of *Ae. aegypti*, gene duplications have given rise to CASPS18 and CASPS19, CASPS15 and CASPS16, and CASPS 17 and CASPS21. These caspase pairs share high sequence similarity and interestingly, CASPS 15, 16, 17 and 21 all group phylogenetically with Dream and Damn in *Drosophila* which are themselves also products of gene duplication (Bryant et al., 2008).

Length of the caspase prodomain is often used as a predictor of whether a caspase functions as an initiator or an effector caspase (Degterev et al., 2003). Comparing the lengths of prodomains of the caspases in *Ae. aegypti*, AeDronc, AeDredd, CASPS15, CASPS16, CASPS21 have long prodomains (defined as greater than 100 amino acids upstream of a conserved arginine residue in the large subunit), whereas CASPS7, CASPS8, CASPS18, CASPS19, CASPS17 and CASPS20 have short prodomains. Although the length of prodomain and homology to known *Drosophila* caspases have been used to predict whether the *Ae. aegypti* caspases are initiator or effect caspases, these factors do not necessarily always provide enough information to allow this type of classification.

The *Ae. aegypti* caspases are compared in Table 2.1 in terms of the length of their prodomain, their closest *Drosophila* homolog, and whether they are known to play a functional role in apoptosis. CASPS18 and CASPS19 have only been studied by overexpression in lepidopteran cells, thus there some uncertainty about their roles in apoptosis.

Apart from AeDronc, CASPS7, CASPS8, CASPS18 and CASPS19, the functions of the other *Ae. aegypti* caspases that were annotated in 2008 have not been examined. The aim of this study was to functionally characterize the other *Ae. aegypti* caspases, namely CASPS15, CASPS16, CASPS17, CASPS20 and CASPS21. For this characterization, we used RNAi and substrate preference determination using recombinant proteins as our major approaches. The findings from

this study provide valuable insights into whether these specific caspases play a functional role in apoptosis in *Ae. aegypti* and also help to characterize them as initiator or effector caspases.

MATERIALS AND METHODS

Cells

Aag2 cells, which were originally derived from embryonic tissue of *Aedes aegypti*, were grown in Schneider's medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals). For all the RNAi, RNAi epistasis and flow cytometry experiments, the cells were plated in Falcon ^{TM} 12 well tissue culture plates.

Plasmids

Plasmids containing green florescent protein (GFP), AeDronc, AeCASP7, AeCASP8, AeCASP20, and AeCASP21 were produced by cloning the corresponding PCR products into the pCRII vector using Dual Promoter TA Cloning Kit (Invitrogen) according to manufacturer's protocol. Plasmids for AeCASP16 and AeCASP17 contain the full length caspase sequences cloned into the PET-23a vector, and were produced via gene synthesis by Genscript^{TM}.

Apoptosis treatment

Ultraviolet radiation (UV) and actinomycin D (Act D) were used as the apoptotic treatments in this study. For UV treatment, the plates were placed on a UV transilluminator for 4 minutes at maximum intensity. For Act D treatment, each well was treated with 1µg/ml of Act D.

RNAi

Double-stranded RNAs (dsRNAs) for specific opening reading frames for the genes of interest were synthesized using the T7 High Yield Transcription Kit (Epicentre) using the manufacturer's protocol. The protocol was multiplied to produce 200 µl of dsRNA per reaction and these were divided into aliquots of 20 µl and stored before use. The dsRNA were stored in aliquots of 20 µl in -20^{o}C.

For specific RNAi experiments, Aag2 cells were plated in 12 well tissue culture plates and allowed to attach. When the cells were attached, media (Schneider's with FBS) was removed and washed using Schneider's media without FBS. 1 ml of media (without FBS) was added to each well and 40 µg of the specific dsRNA of interest was added to the specific well. The plate with cells was left rocking at room temperature for 4-6 hrs and the cells were then supplemented with 1 ml of Schneider's media with 20% FBS. Two sets of cells were plated for each dsRNA in each experiment. The first set of cells was harvested 24 hours after dsRNA addition. These cells were used for qRT-PCR analysis to confirm the knockdown of the specific genes. After 24 hours, UV or Act D were applied to the cells. A second set of cells were harvested 12 hours after exposure to apoptotic induction and these cells were used for caspase activity assay.

qRT-PCR

Real time PCR was done on cells harvested 24 hours after dsRNA application. After harvesting, total RNA was extracted from the cells using standard Trizol ™ (Invitrogen) protocol. 0.5 µg of RNA was used in a reverse transcription reaction to produce the complementary DNA (cDNA) for the samples. The reverse transcription reaction was performed using cDNA Synthesis with SuperScript® III RT kit (ThermoFisher Scientific) following the standard manufacturer's protocol using an oligo-dT primer. 20 µl of cDNA was produced from each reaction. All the cDNA samples produced were diluted 1:20 and 2 µl of each cDNA was used for each qRT-PCR reaction. 2 µl of 10 µM primer (produced by Integrated DNA technologies ™) and 7 µl of 2X SYBR Green Mastermix (ThermoFisher Scientific) were added into the final PCR mixture of 15 µl.

The plates were run in a PCR cycle consisting of step 1: 95°C for 3 min, step 2: 30 cycles of 95°C for 15 sec followed by 55-60°C for 30 sec, step 3: 95°C for 1 min with the melt curve

cycle at the end. For each sample the threshold cycles (Ct) value was recorded. Standard curves for the genes were produced by plotting the Ct value obtained for each dilution against the log of its concentration. The Ct values for the experimental samples were then plotted onto this dilution series standard curve. The relative expression value (REVs) for each gene was produced using ribosomal protein S7 as housekeeping gene. t-test was used to determine the significance for each data set.

Caspase activity assay

Caspase activity of the cells was measured using substrate N-acetyl-Asp-Glu-Val-Asp - 7-amino-4-trifluoromethyl-coumarin (Ac-DEVD-AFC). The cells were harvested after the apoptotic treatment (UV or ActD) and collected by centrifuging at 5000g for 2 min. The media was removed and the cells were washed using PBS. After washing, the cell pellets were resuspended in 100 µl lysis buffer (20mM HEPES*KOH, pH 7.5, 50 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1mM DTT). The cells were lysed by 3 freeze (-80 °C) and thaw (room temperature) cycles. For each of the caspase assay, the protein concentration of sample was determined using BCA assay. 50 µM of each sample was added to white opaque 96 well plates and reaction buffer (100 mM HEPES, pH 7.4, 2 mM DTT, 0.1% CHAPS, and 1% sucrose) added to the each reaction making the total volume 100 µl. The samples were incubated at 37^{o}C for 30 minutes. Caspase substrate Ac-DEVD-AFC (MP Biomedicals) was added at concentration of 20 µM after incubation and fluorometric measurements (excitation 405 nm, emission 535 nm) of the samples were performed using a Victor3 1420 Multilabel Counter (Perkin Elmer).

The samples were read every minute for 90 minutes. The change in activity over time (ΔRFU/time) was calculated by calculating the slope of the activity/time graph over the time

period that the slope changed the most. The first 30 minutes of the assay was the time considered for this calculation in most cases, as that time frame had the most change in activity. The percent change in activity was calculated by setting the caspase activity of the control sample (dsGFP) as 100 % and converting all the other samples using this term.

Active site titration assay

To determine the amount of active caspase present in a purified preparation, active site titration was performed. For this assay purified recombinant caspases were incubated with increasing concentrations of the irreversible caspase inhibitor ZVAD-FMK. Reaction buffer (100 mM HEPES, pH 7.4, 2 mM DTT, 0.1% CHAPS, and 1% sucrose) was added to each sample bringing the total volume of reaction to 100 µl and the reactions were incubated at 37^0C for 30 min. After incubation, 20 µm caspase substrate (Ac-DEVD-AFC) was added to each reaction. The fluorescence (excitation 405 nm, emission 535 nm) was measured over time. For each sample, the rate or slope (y-axis) was plotted against inhibitor concentration (x axis). The concentration of active caspase in each preparation was determined as the x-intercept of the above mentioned graph.

Caspase inhibition assay using AeIAP1

Equivalent concentrations of active AeCASPS7 and AeCASP8 (8µM) were incubated with the increasing (6 µM – 64 µM) concentrations of AeIAP1 at 37 ^{o}C for 30 min. Caspase reaction buffer (100 µl) was added to this reaction. After 30 min, 20 µM caspase substrate (Ac-DEVD-AFC) was added to each sample. Fluorometric analysis (excitation 405nm, emission 535 nm) of the samples were performed using Victor3 1420 Multilabel Counter (Perkin Elmer). The change in activity (ΔRFU/time) for each sample was calculated using above mentioned procedure for caspase activity.

Recombinant protein production

His-tagged AeCASP7, AeCASP15, AeCASP16 AeCASPS20 and AeCASPS21 as well as GST-tagged AeIAP1 were produced by expression in BL21 strain of *E. coli.* The bacteria cultures were grown as 50 ml cultures overnight at 37 ^{o}C. These overnight cultures were used to start a 1 liter culture and these were allowed to reach Optical Density (OD) of 0.6 at 37 ^{o}C. After the OD reached 0.6, the cells were induced for protein production using 0.05 M IPTG and incubated at 18^{o}C overnight. The cell pellets were collected after induction by centrifuging the samples at 12000g for 20 min each. The cell pellets were collected and resuspended in 40 ml lysis buffer (200 mM Tris-Cl pH 8.0, 0.4 M ammonium sulfate, 10 mM MgCl2, 10% glycerol, and protease inhibitor cocktail (Roche)) and sonicated for 3 min (15 sec bursts with 10 sec break in between). The supernatant was collected after centrifugation (12000g for 5 minutes) of the lysed samples. The supernatants were further purified using TALON^{TM} resin for His-tagged proteins and glutathione sepharose-conjugated beads for GST tagged protein following the manufacturer's protocol. The protein concentration of the supernatant and purified proteins was measured using BCA assay. Equal concentrations of proteins were used for each caspase activity assay. You need to describe how you ended up having to use the unpurified supernatant.

RNAi epistasis

To perform the double knockdown experiments, 20 µg dsRNA corresponding to each of two caspases were added, making the total dsRNA in each reaction 40 µg. When single caspases were knocked down in each of these experiments, 40 µg of one dsRNA was added per reaction. Therefore, the total amount of dsRNA added to each treatment cells remained the same. The apoptotic signal UV or Act D was applied 24 hrs after the dsRNA treatment as mentioned in all experiments.

Flow cytometry

Flow cytometry was performed using the PE Annexin V Apoptosis Detection Kit I™ (BD Pharmingen) on Aag2 cells using a BD Fortessa X-20 flow cytometer. The kit provided two dyes, Annexin V conjugated with PE, which stained the apoptotic cells in a population and 7-Amino-Actinomycin (7-AAD) which is a nucleic acid-binding dye and only stains cells with damaged (permeable) membranes, ie necrotic or late stage apoptotic cells.

To prepare the samples, Aag2 cells were plated in 12 well plates and allowed to attach. After attachment, UV or Act D was applied to the cells. 12 hours after application of apoptotic signal, the cells were harvested and washed twice using cold PBS. The cells were then resuspended in 250 µl of 1X Binding Buffer (provided in the kit). After resuspending in Binding Buffer, 5 µl of the specific dye PE Annexin V or 7-AAD were added to the respective samples. For the PE Annexin V with 7-AAD sample, 2.5 µl (total 5 µl) of each dye was also added to treatment cells. The samples were then gently vortexed and incubated for 15 min at room temperature (25°C) in the dark. After 15 minutes, 250 µl of 1X Binding Buffer again was added to each sample. The samples were then transported on ice to be analyzed by flow cytometry.

Substrate specificity assay

The substrates used in this assay were purchased from MP Biomedicals and are represented in the Table 2.2.

Supernatants from lysed bacteria expressing the recombinant proteins CASPS15, 16, 17, 20 or 21 were incubated with the 50 µM concentration of above-mentioned substrates and caspase activity was measured using a Victor plate reader after incubation at 37 ^{0}C for 30 min. The change in activity was measured as described above.

RESULTS

Ability of AeIAP1 to inhibit CASPS7 versus CASPS8

Previous experiments indicated that the caspase inhibitor AeIAP1 is able to bind more strongly to CASPS7 compared to CASPS8 (Devore, 2009). To determine if the stronger binding correlated with the inhibitory capacity of AeIAP1, we tested the ability of recombinant AeIAP1 to inhibit the ability of an equivalent concentration of active CASPS7 and CASPS8 to cleave a synthetic effector caspase substrate, N-acetyl-Asp-Glu-Val-Asp -7-amino-4-trifluoromethyl-coumarin (Ac-DEVD-AFC). In order to compare the ability of AeIAP1 to inhibit these effector caspases, recombinant CASPS7 and CASPS8 were expressed and purified from bacteria. We observed that equal concentrations of recombinant CASPS7 and CASPS8 (as determined by BCA assay) had varying levels of caspase activity, with CASPS7 having higher activity than CASPS8 (data not shown). When caspases are expressed in bacteria, autoactivation occurs due to concentration effects. However, the extent of activation can vary between protein preparations. In addition, contaminating proteins can be present at different concentrations, affecting the total amount of protein as determined by BCA assay. For our study, it was important to use equal concentrations of active CASPS7 and CASPS8 to determine the extent of inhibition of the caspases by AeIAP1. The amount of active caspase in each reaction was thus determined by performing active site titration using the irreversible caspase inhibitor N-Benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone **(**ZVAD-FMK). Since the concentration of ZVAD-FMK added is accurately known, this concentration can be used to determine the amount of active caspase in a specific batch of caspase protein. In active site titration, the amount of active

caspase in the reaction can be determined by the amount of inhibitor required to bring the caspase activity to zero.

When increasing amounts of ZVAD-FMK were mixed with CASPS7 or CASPS8 protein, the same concentration of CASPS7 protein as determined by BCA assay had approximately twice the amount of active caspase compared to CASPS8 (Fig. 2.1A and Fig. 2.1B). The concentration of active CASPS7 was calculated as 4.49 µM, while that of CASPS8 was 9.34 µM.

When equal concentrations of active CASPS7 or 8 were incubated with recombinant AeIAP1 protein, the results indicated that AeIAP1 was able to inhibit both caspases, but that it inhibited CASPS7 more efficiently than CASPS8 (Fig. 2.2). At an 8:1 ratio of AeIAP1:caspase, CASPS7 was inhibited by 6.4-fold, while CASPS8 was inhibited by only 1.6-fold. This correlates with the results from previous studies from our laboratory suggesting that AeIAP1 is likely to inhibit apoptosis by inhibiting CASPS7 rather than CASPS8 (Devore, 2009).

The roles of several uncharacterized *Ae. aegypti* caspases in apoptosis

The roles of CASPS7, CASPS8, and Dronc in apoptosis have been previously examined (Liu and Clem, 2011). To determine whether any other caspases play roles in the apoptosis pathway in *Ae. aegypti*, we used RNA interference (RNAi) to knock down the expression of several previously uncharacterized *Ae. aegypti* caspases in Aag2 cells, and studied the effects on apoptosis, including caspase activity and cell viability. dsRNAs specific for each caspase were used to individually knock down CASPS15, CASPS16, CASPS17, CASPS20 and CASPS21 in Aag2 cells. The expression of each of the caspases was significantly decreased after treatment by the specific dsRNA, as measured by qRT-PCR (Fig. 2.3). dsRNA for green florescent protein (dsGFP) was used as a non-specific dsRNA negative control. As an additional control, some of the cells were not treated with any dsRNA and represented the normal level of caspase

expression in Aag2 cells. The level of significance was determined by using t-test to compare dsGFP and dsRNA for the specific caspases. The silencing of a caspase, for example CASPS17 did not affect the expression of other caspases like CASPS15, CASPS16 and others. However, the silencing of CASPS15 decreased the expression of CASPS16 because of their high degree of similarity. But the silencing of other caspases like CASPS17, CASPS20, CASPS21 did not affect the expression of other caspases even the ones that were closely related. Therefore, the silencing of caspases appeared specific except for CASPS15 and 16 (data not shown).

After demonstrating that the caspases were effectively and specifically knocked down by RNAi, dsRNA-treated Aag2 cells were introduced to two different kinds of apoptotic stimuli. Ultraviolet radiation (UV) is known to cause apoptosis by damaging cellular DNA, whereas actinomycin D (Act D) is an inhibitor of RNA synthesis and is known to cause apoptosis in a variety of insect cell lines, due at least in part to inhibition of IAP expression (Miller et al., 1993). In separate experiments, UV and ActD were used as two different stimuli for apoptosis activation in Aag2 cells.

Aag2 cells were treated with dsRNA for specific caspases and 24 hrs later, the cells were exposed to apoptotic stimuli. Caspase activity of the cells, as measured using DEVD-AFC was significantly decreased when each of the caspases was silenced, compared to cells treated with dsGFP (Fig. 2.4A and B). The level of significance was determined using t-test. The lower caspase activity in the cells treated with caspase dsRNAs indicates that the rate of apoptosis occurring in these cells was lower than in the control cells treated with dsGFP. The results indicated that the caspase activity was decreased to a similar degree by knocking down each of the caspases. This result suggests that these caspases all contribute to apoptosis in *Ae. aegypti*.

After knocking down individual caspases in Aag2cells, we sought to determine whether knocking down two caspases at a time had an additive effect. For double knockdowns, we picked dsRNA pairs that shared sequence similarity. CASPS15 and CASPS16, as well as CASPS21 and CASPS17 were chosen as pairs since these share high sequence similarity. After treatment with the combinations of dsRNA, the cells were exposed to UV and caspase activity was measured. Surprisingly, we did not observe an additional effect on the decrease in caspase activity when two genes were knocked down compared to single genes being knocked down. The caspase activity of individually silenced cells were not significantly different than cells with double dsRNA treatment. The level of significance was determined using t-test. When CASPS15 and CASPS16 were individually knocked down, the change in activity was similar to double knockdowns with CASPS15 and CASPS16 together, but this may have been due to the fact that silencing of these two genes was not specific. However, similar results were obtained for CASPS17 and 21 (Fig. 2.5A), which were able to be specifically silenced. There was a trend towards a slight decrease in caspase activity in the double knockdowns compared to single, but the change was not significant. Similar results were observed when Act D was used as the apoptotic stimulus for the single and double knockdown treatments (Fig. 2.5B). In summary, there was no significant difference when knocking down two caspases compared to single knockdowns as determined by t-test.

For the next set of RNAi epistasis experiments, we wanted to determine whether knocking down a known apoptotic initiator caspase, AeDronc, with the other caspases would cause an additional impact on caspase activity following an apoptotic stimulus. For this experiment, Aag2 cells were treated with dsRNA for each caspase alone, or together with AeDronc. UV and Act D were used as apoptotic stimuli again and caspase activity was measured 12 hours post apoptotic treatment.

We again observed a slight decrease in caspase activity when knocking down various caspases with AeDronc, compared to knocking down the caspases by themselves, but the change was not significant as determined by t-test. This was the case when either UV (Fig. 2.6A) or Act D (Fig. 2.6B) was used as the apoptotic stimulus.

Substrate specificity of uncharacterized *Ae. aegypti* caspases

The substrate specificity of caspases is one of the factors that determine their characterization as initiator or effector caspases. Since several *Ae. aegypti* caspases have not been characterized, we wanted to get more information about these caspases and their potential roles in apoptosis. We thus produced recombinant proteins for CASPS16, CASPS 17, CASPS20 and CASPS21 using the *E. coli* expression system. After induction of caspase expression, the caspases were purified using TALON^{TM} resin, and the caspase activity of the cell supernatant, washes and elutions collected was measured. Unfortunately, the caspase activity in the unpurified supernatant was significantly higher than the washes and elution fractions, suggesting that the purification method was not working well for these caspases (Fig. 2.7A-D).

To circumvent this problem, bacterial supernatants containing unpurified recombinant capsases were used in caspase assays. As a negative control for background protease activity, a CASPS7 construct with a mutation in the active site (Cys2Ala) was expressed and used in caspase assays. The substrates used were a commercially available set of caspase substrates consisting of several conjugated peptides that have been used to classify human caspases (MP Biomedicals). Lysates containing the individual recombinant caspases were mixed with the various substrates and activity was measured. As expected, lysate containing mutant CASPS7 protein had very low caspase activity, indicating that there is negligible background activity in the bacterial lysate (Fig. 2.8A). CASPS16 did not have a strong preference for any of the substrates tested, however

activity with WEHD-AFC was significantly higher compared to others (Fig. 2.8B). CASPS17 showed preference for the substrates DEVD-AFC, WEHD-AFC, VEID-AFC and LEHD-AFC compared to the other substrates (Fig. 2.8C). CASPS20 showed similar caspase activity with all the tested substrates, although it had the highest activity with VEID-AFC (Fig. 2.8D). CASPS21 exhibited preference for DEVD-AFC, WEHD-AFC, VEID-AFC and LEHD-AFC compared to the other substrates (Fig. 2.8E). The significance of caspase preference was determined using one way Analysis of Variance (ANOVA). Overall, the caspases demonstrated varying preferences for the known human caspase substrates.

Flow cytometry was used as another method to determine the levels of apoptosis when individual caspases or pairs of caspases were silenced. Annexin V and 7-Amino-Actinomycin (7-AAD) were used to stain to the cells. Annexin V is a 35-36 kDa Ca2+ dependent phospholipid-binding protein that has a high affinity for the phospholipid phosphatidylserine (PS), and binds to cells with exposed PS. Since PS is translocated from the inner leaflet to the outer leaflet of the plasma membrane during the early stages of apoptosis, Annexin V staining can be used to measure cells undergoing apoptosis (Koopman G, et al 1994). For the flow cytometry analysis, Annexin V conjugated with the fluorochrome Phycoerythrin (PE) was used to stain apoptotic cells. 7-AAD is a DNA stain that is only taken up by cells that have damaged membranes, thus it stains necrotic and late apoptotic cells.

It was first important to determine whether flow cytometry could be successfully used to measure apoptosis in Aag2 cells, as use of this technique in this cell line has not been reported previously to our knowledge. Fig. 2.9 depicts the raw data from a representative flow cytometry analysis that was performed to optimize the staining procedure. The x-axis represents Annexin V-PE staining, whereas the y-axis represents the 7-AAD staining. A higher number of Annexin

V-positive cells were observed when the cells were exposed to UV compared to untreated cells (Fig. 2.9A and B). Similarly, in cells stained with 7-AAD there were higher number of 7-AAD positive cells in the UV-treated sample compared to the untreated (Fig. 2.9C and D). In cells treated with both Annexin V and 7-AAD, there was higher number of Annexin V, 7-AAD as well as dual positive cells compared to the untreated group (Fig. 2.9E and F). These results suggest that flow cytometry with Annexin V and 7-AAD staining can be used to measure apoptotic Aag2 cells.

The accumulated data from three biological replicates of Aag2 cells stained with Annexin V, 7-AAD or Annexin V + 7-AAD and either UV-treated or untreated cells are shown in Fig. 2.10. For each treatment, the percentage of cells stained with Annexin V, 7-AAD or both stains are represented (Fig. 2.10). In the case of both Annexin V- and 7-AAD-stained cells, significantly higher percentage of positive cells was observed in UV-treated cells compared to untreated cells as determined by t-test. When the cells were stained with both dyes, there was also a higher number of dual-positive cells in the UV-treated group compared to untreated cells (Fig. 2.10). These results correlate with the results from the caspase activity assay, indicating higher apoptosis after UV treatment.

Flow cytometry was then used to determine the effects of silencing individual caspases cells on UV-stimulated apoptosis. Aag2 cells were treated with the indicated dsRNAs, and 24 hrs later the cells were treated with UV. At 12 hrs after UV treatment, the cells were harvested and stained with Annexin V and 7-AAD. It was observed that there was significantly higher (determined by t-test) proportion of Annexin V and 7-AAD-stained cells in all the dsRNA treatments after UV exposure (Fig. 2.11). Thus, this method can thus be used to compare the number of apoptotic cells in cells knocked down with one caspase versus another.

While comparing the percentage of Annexin V positive cells between the dsGFP and caspase dsRNA treatment groups using t-test, it was observed CASPS15, CASPS20 and CASPS21 had significantly lower number of Annexin positive cells after UV treatment compared to dsGFP treated cells. This indicates less number of apoptotic cells are present after UV treatment in the caspase dsRNA treated cells compared to dsGFP treated cells. This results confirms with the results obtained from measuring caspase activity after silencing of CASPS15, CASPS20 and CASPS21 (Fig 2.4A), providing further evidence for the role of these caspases in apoptosis in Aag2 cells.

DISCUSSION:

In this study, we have characterized the substrate specificity of several previously uncharacterized *Ae. aegypti* caspases, and also investigated the roles of these caspases in apoptosis using RNAi. The genomes of mosquitoes that have been examined thus far, including *Ae. aegypti*, have an expanded number of caspase homologs compared to other insects whose genomes have been sequenced (Bryant et al., 2008). Our results provide an initial assessment of the functional relevance of some of these additional caspase homologs.

We also examined the ability of two previously characterized *Ae. aeygpti* caspases, CASPS7 and CASPS8, to be inhibited by the caspase inhibitor AeIAP1. Our results indicated that CASPS7 and CASPS8 were both inhibited by AeIAP1, but the inhibition of CASPS7 was stronger than that of CASPS8. This suggests that AeIAP1 has a preference for inhibiting CASPS7. Previous study of the interaction of AeIAP1 with CASPS7 and CASPS8 was performed using *in vitro* translated AeIAP1 protein and recombinant caspase proteins. Pulldown assay using these proteins indicated that AeIAP1 bound more strongly to CASPS7 than CASPS8 (Devore, 2009). Therefore, our results agree with the previous protein interaction data, and support the conclusion that AeIAP1 acts preferentially on CASPS7 versus CASPS8 in the apoptosis pathway of *Ae. aegypti*.

To study the substrate preference of the effector caspases CASPS15, CASPS16, CASPS17, CASPS20 and CASPS21, we expressed the caspases as recombinant, His-tagged proteins, using an *E coli* expression system. We observed high levels of expression of the caspases in the bacterial cell supernatants, indicating that the recombinant proteins were soluble. However, we were not able to isolate useable levels of the caspases using the His tag for purification, despite

multiple attempts. Potential reasons for the difficulty in purifying the recombinant proteins may be either the protein did not bind properly with the TALON resin or it could not successfully be eluted from the beads by the elution buffer used. Due to this it was difficult to obtain useable concentrations of recombinant caspase protein. Therefore, we used the supernatant fraction of the bacterial cell lysates, which contained the expressed *Ae. aegypti* caspase proteins, to perform the substrate preference assay. The expression of caspases in the supernatants was confirmed because the supernatant had significantly higher caspase activity assay compared to supernatant obtained after identical expression and treatment of the active site mutant of CASPS7. Since the caspases were expressed and were active in the supernatant, we decided to use it for our substrate specificity assay. Although using purified recombinant proteins would be preferred, since we observed specific caspase activity in the supernatant our approach could be used as well. A similar approach of using supernatants obtained after lysis of *E coli* cells was used previously to determine the substrate preference for AeDredd (Cooper et al., 2007).

The substrates used in our assay are known substrates for human caspases that have been used to classify caspases by their substrate specificity (MP Biomedicals,Inc.). Although substrates that are customized for insect caspases are not available, due to the high structural similarity among all caspases, the human caspase substrates are very helpful for basic understanding of the insect caspases, and have been widely used to characterize other insect caspases (Lui et al., 2005; Degterev et al., 2003).

Caspases are commonly classified as initiator or effector caspases, mainly based on two criteria: 1) substrate specificity and 2) the length of their prodomain, as well as the presence of known structural domains in the prodomain of initiator caspases that bind to adapter proteins and allow their activation. The substrate specificity assay was one of the methods used to determine

whether the caspases are initiator or effector caspases. In the case of *Drosophila* and human caspases, it has been observed that certain substrates are preferred by initiator versus effector caspases. The preferred substrate for the *Drosophila* initiator caspase Dronc is VDVAD (Hawkins et al., 2000), while the *Drosophila* effector caspases Drice, Dcp-1 and Decay prefer DEVD (Kumar and Doumanis 2000, Dorstyn et al 1999). We attempted to use this knowledge obtained by using known human caspase substrates, combined with prodomain length, to differentiate among *Ae. aegypti* caspases.

From our recombinant protein data, we can tentatively conclude that CASPS17 and CASPS21 are likely to be effector caspases since they preferred the substrate DEVD compared to VDVAD. When comparing the prodomains, CASPS17 has a short prodomain, consistent with its substrate preference. CASPS21 has a long prodomain, which seems inconsistent with its preference for DEVD. However, when the prodomain of CASPS21 was scanned using PROSITE© there were no recognizable interaction motifs found that are normally present in initiator caspase prodomains. Thus, we conclude that CASPS21 is likely to be an effector caspase, despite its long prodomain. CASPS20 did not show preference for any specific substrate and also has a short prodomain, indicating it is also likely to be an effector caspase. CASPS16 showed slightly higher preference for WEHD, and has a long prodomain. Based on this results, it is likely to be an initiator caspase.

Our results indicate that CASPS15, CASPS16, CASPS17, CASPS20 and CASPS21 are expressed in Aag2 cells and the expression of these genes can be successfully reduced using RNAi. The expression of the transcripts of these caspases in Aag2 cells was previously confirmed by RT-PCR (Lui and Clem, 2011). In the same paper it was shown that Aag2 cells can uptake dsRNA and be used for RNAi experiments. The expression pattern of all of the above

mentioned caspases was also analyzed in different developmental stages of *Ae. aegypti* including larvae, pupae and adults as well as in midgut tissue in adult females (Bryant, 2008).

Silencing of the effector caspases CASPS7 or CASPS8, which are the closest relatives to DrICE, caused a decrease in apoptosis (Lui and Clem, 2011). When comparing CASPS7 and CASPS8, silencing CASPS7 produced a more dramatic effect compared to silencing CASPS8. Co-silencing both of them decreased caspase activity similar to when only CASPS7 was silenced and co-silencing did not completely deplete caspase activity (Lui and Clem, 2010). In our study, we observed that silencing of CASPS7, CASPS15, CASPS16, CASPS17, CASPS20 or CASPS21 each resulted in a roughly equivalent reduction in apoptosis. This suggests these caspases are all potentially involved in the apoptosis pathway in *Ae. aegypti*. It is difficult to accurately determine the extent of involvement of specific caspases in this study since silencing of each of the caspases showed a similar effect on apoptosis. Previously, in a study of caspases AeDronc, CASPS7 and CASPS8, it was observed that silencing of AeDronc had the most change in caspase activity compared to other two effector caspases (Liu and Clem, 2011). However, in our study we could not differentiate the effect of silencing of one caspase versus another. One possible reason for this may be incomplete silencing of the caspases at the protein level, which makes it difficult to assess the effects of gene silencing. It may also be helpful in the future to co-silence individual caspases with AeIAP1, and to study the biochemical interactions of AeIAP1 with these different caspases. Based on the interaction of each caspase with AeIAP1, it may be possible to better predict the importance of specific caspases in the apoptosis pathway in *Ae. aegypti*.

In our study, we observed that co-silencing of caspases that share high sequence similarity (CASPS15 and 16, and CASPS17 and 21) did not result in a significant change in the effect on

apoptosis compared to silencing the caspases individually (Fig. 2.5). This suggests that although the proteins have high sequence similarity they do not share functional redundancy. The lack of difference between single and double knockdown can also result from the activation of other caspases during apoptosis. This result can also be explained by the fact that while knocking down these caspases using RNAi, there might still be some level of expression of the caspases. We also did not observe any significant difference when the caspases were knocked down with the known initiator caspase AeDronc compared to the caspases being knocked down alone (Fig. 2.6). This might have resulted from the activation of other effector caspases by the apoptotic stimuli as described above. This scenario is similar to when the co-silencing of two known effector caspases CASPS7 and CASPS8 did not show any additional effect on the rate of apoptosis (Lui and Clem, 2011).

In addition to using caspase activity as a measurement of apoptosis, we also attempted to use Annexin V staining and flow cytometry. One of the hallmarks of apoptosis is the translocation of phospholipid phosphatidylserine (PS) from the inner to the outer surface of the plasma membrane. Annexin V is a 35-36 kDa Ca2+ dependent phospholipid-binding protein which possesses a high binding affinity for PS. This unique feature of Annexin V can be used to identify apoptotic cells when conjugated to a fluorochrome such as Phycoerythrin (PE) (Vermes et al., 1995). Annexin V staining has the ability to determine apoptosis at a much earlier state compared to other methods that are based on nuclear changes (Koopman et al., 2000; Martin et al.,1995).

We were able to successfully stain Aag2 cells using this method and we saw a significant difference in the number of apoptotic cells before and after UV treatment (Fig. 2.9). Although we observed a significant number of apoptotic cells, the difference was smaller than expected. This

may be because Annexin V is very sensitive in detection of apoptosis and is able to determine apoptosis at a much earlier stage than other methods that we have being using like caspase activity assay. Thus, if the treated cells had been collected earlier, the difference might have been greater. And moreover, while performing the steps that lead to staining, we had to leave the cells in the 12- well plates for at least 48 hours from the time dsRNA added to staining (steps include dsRNA addition, UV treatment and collection of cells). This much time might be too long for optimal results using Annexin V with these particular cells. This might be a reason why we were not able to observe a dramatic effect using flow cytometry on UV or ActD-treated cells compared to caspase activity assay data. Nevertheless, this study is the first to use the flow cytometry method for measuring the rate of apoptosis in Aag2 cells and we did observe a significant difference between number of apoptotic cells before and after UV treatment. Overall, this study serves as a good starting point to begin to understand the functions of these previously uncharacterized caspases and study their specific roles in the apoptosis pathway or in other pathways in *Ae. aegypti*. Like other mosquitoes studied so far, *Ae. aegypti* has a large number of caspases compared to most other insects, and we know from this and previous studies that all of the predicted caspases are expressed in Aag2 cells and in the organism, indicating that they are probably all authentic, expressed genes (i.e., not pseudogenes). This study represents the first attempt to study the functions of several of the *Ae. aegypti* caspases, namely CASPS15, CASPS16, CASPS17, CASPS20 and CASPS21. Apart from being involved in apoptosis these caspases might also be involved in other functions such as autophagy or immune response. They might also function in processes like cell migration or differentiation (Accorsi et al., 2015). We have shown that expression of several of the caspases can be decreased in Aag2 cells using RNAi, and we found that many of the caspases appear to play roles in apoptosis. Since all of the

caspases studied here except CASPS20 can be phylogenetically grouped with known *Drosophila* caspases, it is surprising to see that they play a role in apoptosis pathway in *Ae. aegypti* since not all of the Drosophila caspases play a role in apoptosis. Among the caspases characterized though, it is likely that some may play more important roles compared to others. For example, we observed that AeIAP1 preferentially inhibits CASPS7 compared to CASP8. To determine the extent to which these individual caspases function in apoptosis, further functional studies using known caspase regulators like IAPs and IAP antagonists need to be performed.

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TABLES

Table 2.1: Summary of caspases in *Ae. aegypti*:

Name	**Closest *Drosophila* relative**	**Prodomain length**	**Known functional role in apoptosis?**
AeDronc	Dronc	Long	Yes
AeDredd	Dredd	Long	Unknown
CASPS7	Drice and Dcp-1	Short	Yes
CASPS8	Drice and Dcp-1	Short	Yes
CASPS15	Dream and Damm	Long	Unknown
CASPS16	Dream and Damm	Long	Unknown
CASPS17	Dream and Damm	Short	Unknown
CASPS18	Decay	Short	No?
CASPS19	Decay	Short	No?
CASPS20	Unknown	Short	Unknown
CASPS21	Dream and Damm	Long	Unknown

Table 2.2: List of substrates used for assay

Substrate	Substrate for mammalian caspase	Characterization
Ac-YVAD-AFC	1	Initiator
Ac-VDVAD-AFC	2	Initiator
Ac-DEVD-AFC	3, 6, 7, 8 and 10	Effector
Ac-WEHD-AFC	1, 4 and 5	Initiator
Ac-VEID-AFC	6	Effector
Ac-LEHD-AFC	4, 5 and 9	Initiator
Ac-IETD-AFC	8	Initiator

FIGURES

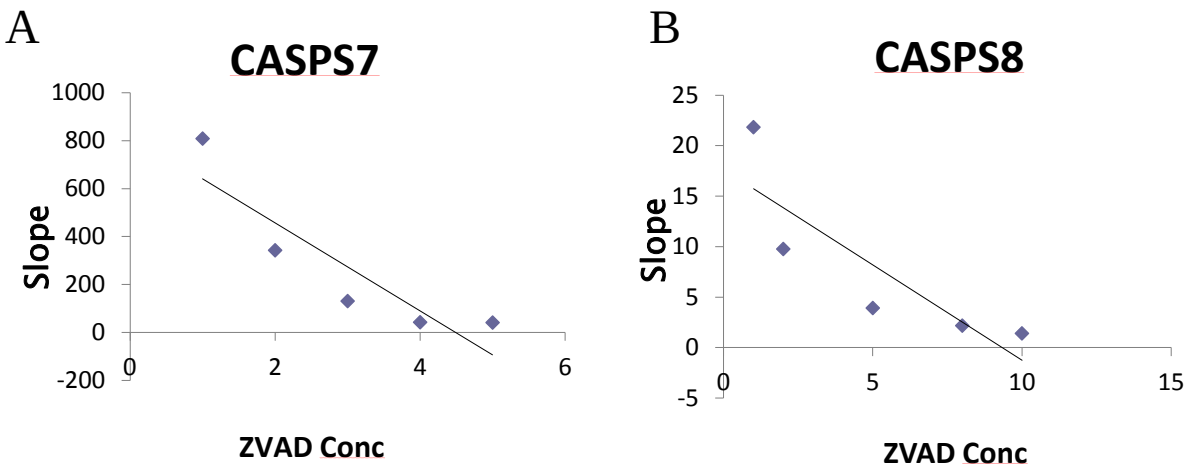


Figure 2.1: Active site titration of CASPS7 and CASPS8.

Recombinant caspases were incubated with increasing concentrations of the irreversible caspase inhibitor ZVAD-FMK, and caspase activity was measured over time. Because ZVAD-FMK binds 1:1 with active caspase and the binding is irreversible, the concentration of ZVAD-FMK required to bring the caspase activity to zero equals the concentration of active caspase in the preparation. The concentration of ZVAD-FMK required to bring caspase activity zero for CASPS7 was 4.49 µM and for CASPS8 was 9.34 µM.

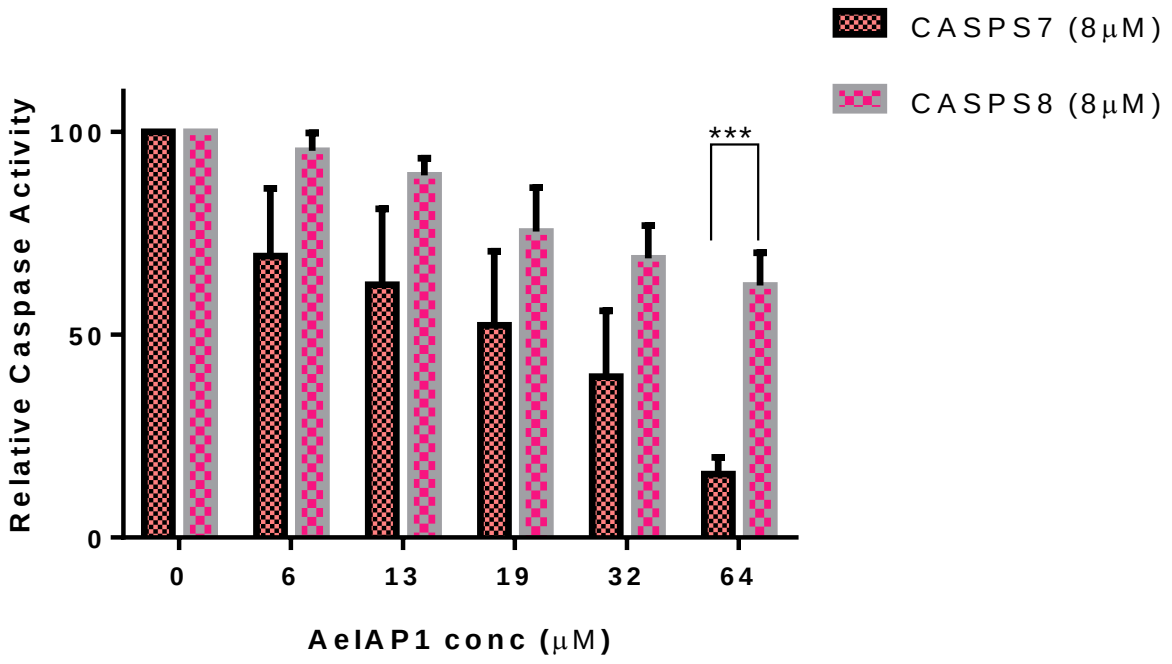
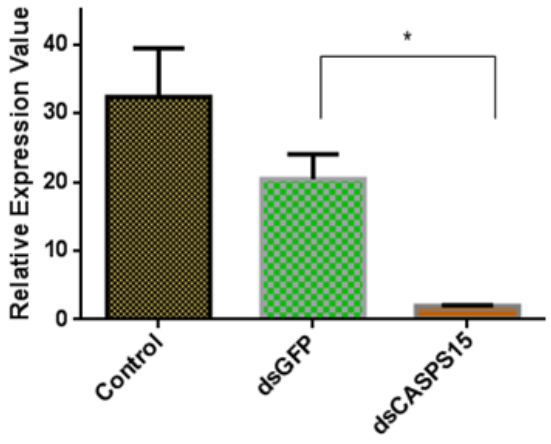


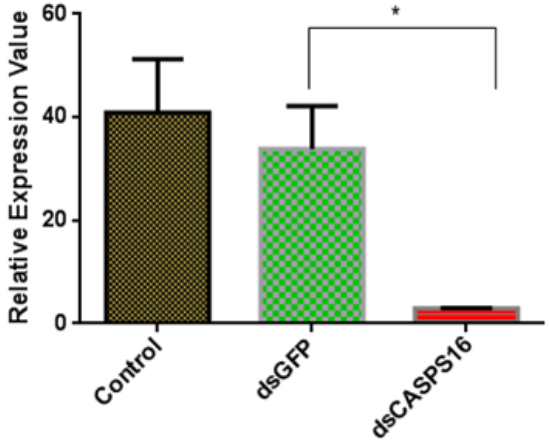
Figure 2.2: Caspase inhibition assay by AeIAP1.

Increasing concentrations of recombinant AeIAP1 were incubated with equal concentrations of active,recombinant CASPS7 and CASPS8, and then the ability to cleave DEVD-AFC was determined. At 64μM of AeIAP1, the activity of Caspase 7 was significantly lower than Caspase 8 (calculated using t-test). The level of significance in each graph is demonstrated by the '*' where * is p-value less than 0.05, ** is p-value less than 0.005, *** is p value less than 0.0005 and **** is p value less than 0.00005. "NS" in graphs represents at p-value greater than 0.05 thus the difference is not significant. Each bar represents the average of 3 biological replicates (+/- SE).

A


CASPS15
Relative Expression Value
40
30
20
10
0
*
Control
dsGFP
dsCASPS15

B


CASPS16
Relative Expression Value
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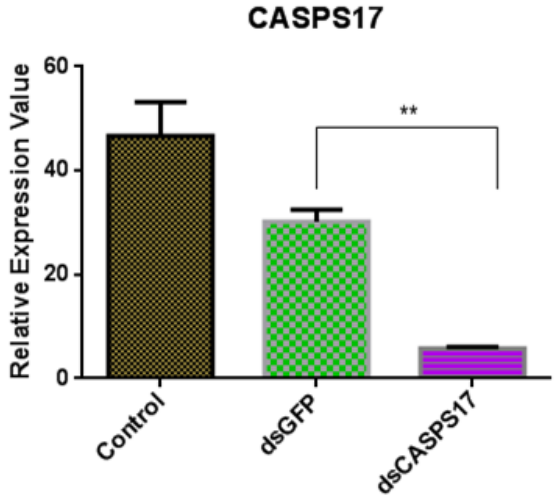
40
20
0
*
Control
dsGFP
dsCASPS16

C


CASPS17
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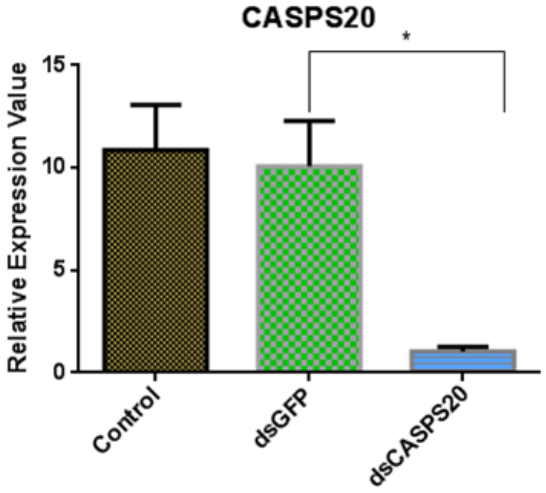
20
0
**
Control
dsGFP
dsCASPS17

D


CASPS20
Relative Expression Value
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10
5
0
*
Control
dsGFP
dsCASPS20

E

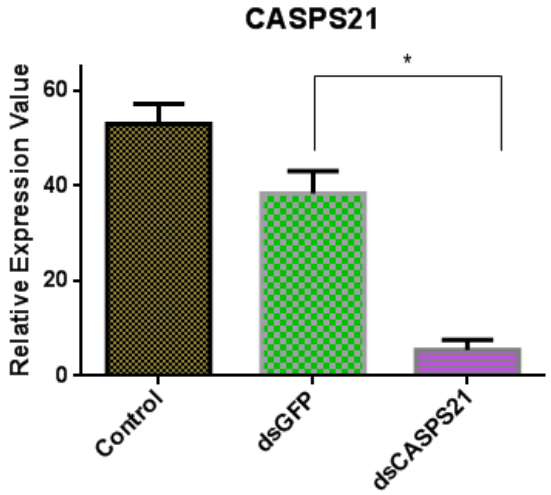
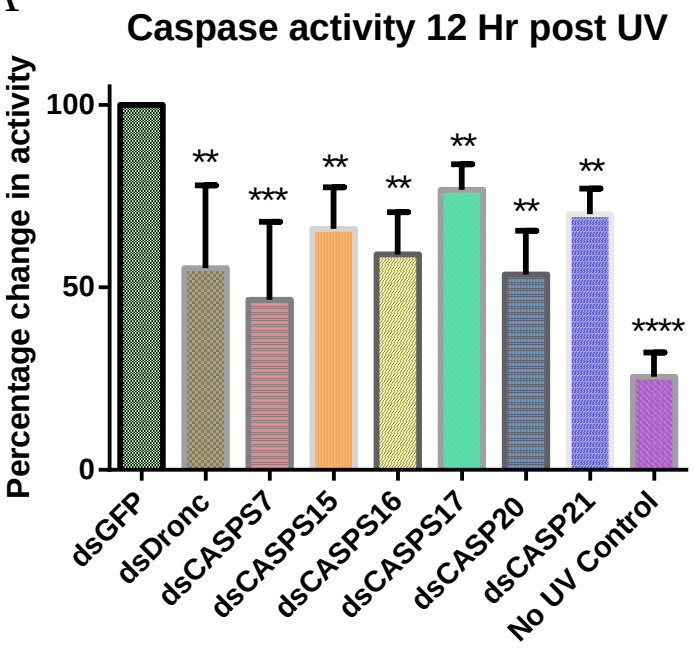

CASPS21
Relative Expression Value
60
40
20
0
*
Control
dsGFP
dsCASPS21

Figure 2.3: Knockdown confirmation of individual caspases.

Aag2 cells were left untreated (Control) or treated with dsRNAs corresponding to control GFP, CASPS15, CASPS16, CASPS17, CASPS20 or CASPS21. 24 hrs later, the cells were harvested and the levels of mRNA for each gene were determined by qRT-PCR. Relative expression value for each treatment was calculated using the ct values. For each of the dsRNA treatment, the relative expression was significantly lower than dsGFP treatment (calculated using t-test) confirming a successful knockdown of the genes. Each bar represents the average of 3 biological replicates (+/- SE).

A


Caspase activity 12 Hr post UV
Percentage change in activity
100
50
0
**
***
**
**
**
**
**
****
dsGFP
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dsCASPS16
dsCASPS17
dsCASP20
dsCASP21
No UV Control

B

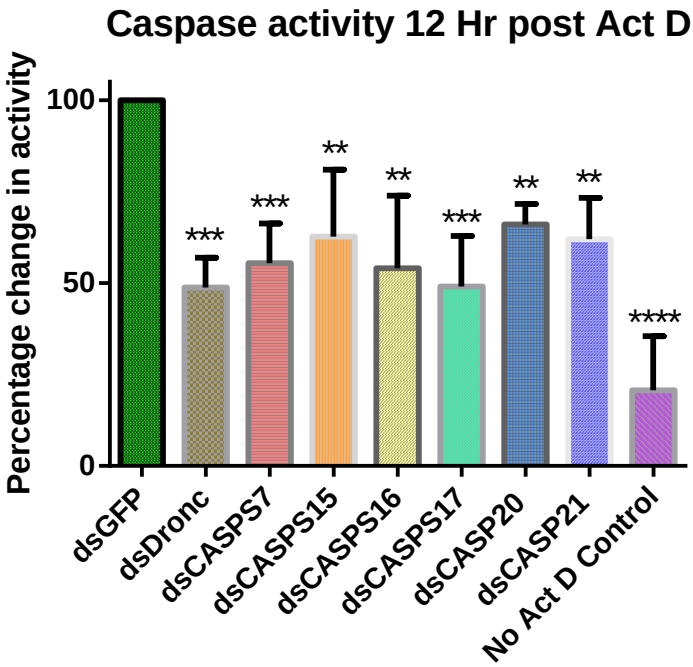
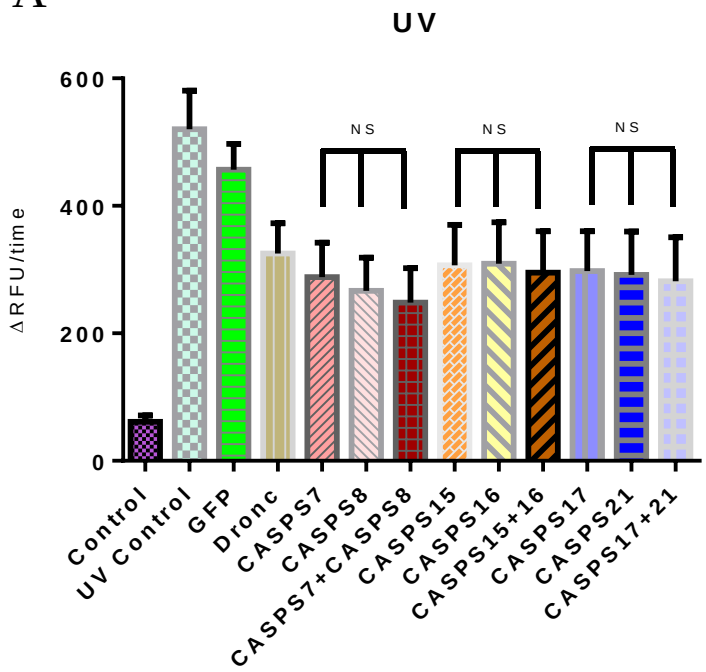

Caspase activity 12 Hr post Act D
Percentage change in activity
100
50
0
***
***
**
**
***
**
**
****
dsGFP
dsDronc
dsCASPS7
dsCASPS15
dsCASPS16
dsCASPS17
dsCASP20
dsCASP21
No Act D Control

Figure 2.4: Caspase activity of dsRNA-treated cells collected 12 hours post-UV or -ActD treatment.

Aag2 cells were treated with the indicated dsRNAs, and 24 hrs later the cells were exposed to A) UV or B) ActD. The caspase activity of the cells was measured 12 hrs later. The caspase activity of caspase dsRNA treated cells were significantly lower than dsGFP treated cells (calculated using t- test). Each bar on the graph represents the average of 3 biological replicates (+/- SE).

A


UV
ΔRFU/time
600
400
200
0
NS
NS
NS
Control
UV Control
GFP
Dronc
CASPS7
CASPS8
CASPS7+CASPS8
CASPS15
CASPS16
CASPS15+16
CASPS17
CASPS21
CASPS17+21

B

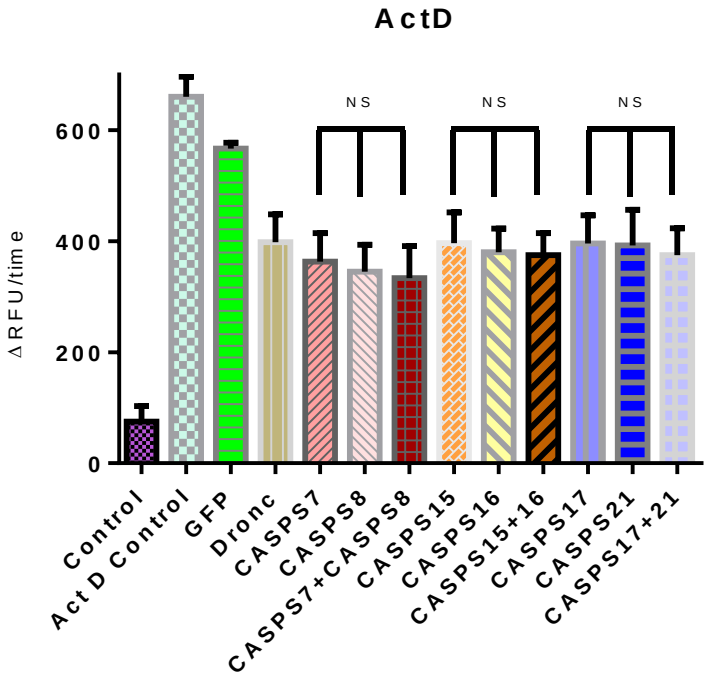
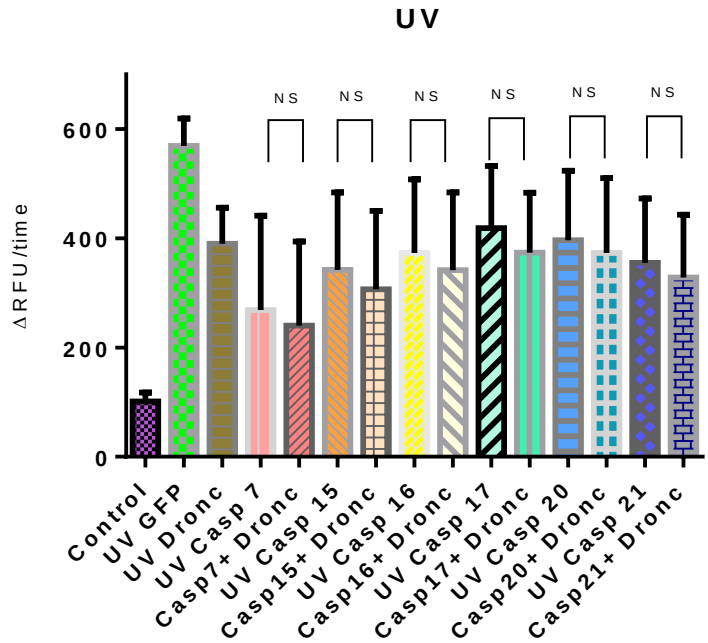

ActD
ΔRFU/time
600
400
200
0
NS
NS
NS
Control
Act D Control
GFP
Dronc
CASPS7
CASPS8
CASPS7+CASPS8
CASPS15
CASPS16
CASPS15+16
CASPS17
CASPS21
CASPS17+21

Figure 2.5: Caspase activity after double knockdown of caspases followed by UV treatment or Act D treatment.

The caspase activity of the cells was measured after silencing of individual caspases or two caspases at a time, and exposure of the cells to A) UV or B) Act D. Double knockdown of caspases did not have a significant effect (p-value greater than 0.05) denoted by "NS" in the graphs (calculated using t-tests) compared to caspases being knocked down individually. The activity of caspases knocked down individually or in pairs was however significantly lower dsGFP treated control (calculated using t-test) confirming the role of these genes in apoptosis. Each bar on the graph represents the average of 3 biological replicates (+/- SE).

A


UV
ΔRFU/time
600
400
200
0
NS
NS
NS
NS
NS
NS
Control
UV GFP
UV Dronc
UV Casp 7
Casp7+ Dronc
UV Casp 15
Casp15+ Dronc
UV Casp 16
Casp16+ Dronc
UV Casp 17
Casp17+ Dronc
UV Casp 20
Casp20+ Dronc
UV Casp 21
Casp21+ Dronc

B

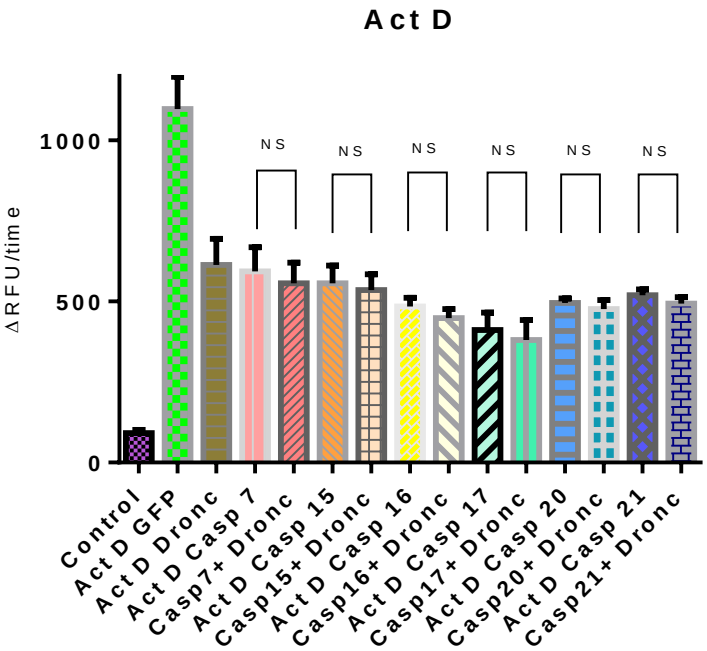

Act D
ΔRFU/time
1000
500
0
NS
NS
NS
NS
NS
NS
Control
Act D GFP
Act D Dronc
Act D Casp 7
Casp7+ Dronc
Act D Casp 15
Casp15+ Dronc
Act D Casp 16
Casp16+ Dronc
Act D Casp 17
Casp17+ Dronc
Act D Casp 20
Casp20+ Dronc
Act D Casp 21
Casp21+ Dronc

Figure 2.6: Caspase activity after double knockdown of caspases and AeDronc followed by UV or Act D treatment.

Caspase activity was measured after silencing of individual caspases or double knockdown of caspases with the initiator caspase AeDronc and exposure to A) UV or B) Act D). Double knockdown of caspases did not have a significant effect (p-value greater than 0.05) compared to caspases being knocked down individually, which is represented by "NS" in the graphs. The significance was determined using t-test where "NS" represents p-value greater than 0.05. Each bar on the graph represents the average of 3 biological replicates (+/- SE).

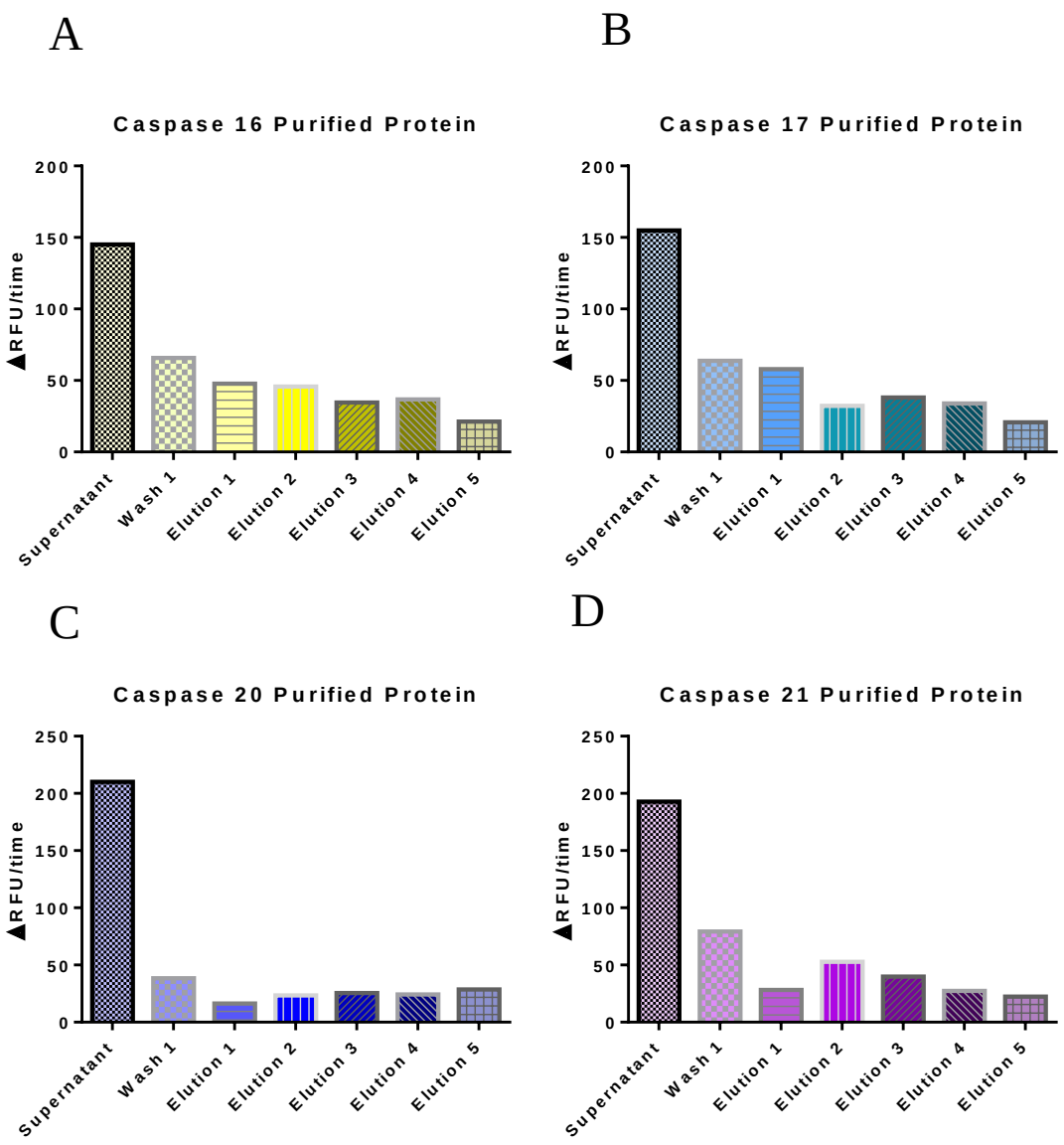


Figure 2.7: Caspase activity of supernatant and elutions after recombinant protein purification.

Recombinant caspases with His tags were expressed in *E. coli* and purified as described in Materials and Methods. The caspase activity was measured by using DEVD-AFC as a substrate. For all the caspases, the supernatant had significantly higher caspase activity compared to the elutions, indicating that purification was not successful. The above graphs are one replicate representative of multiple purification attempts.

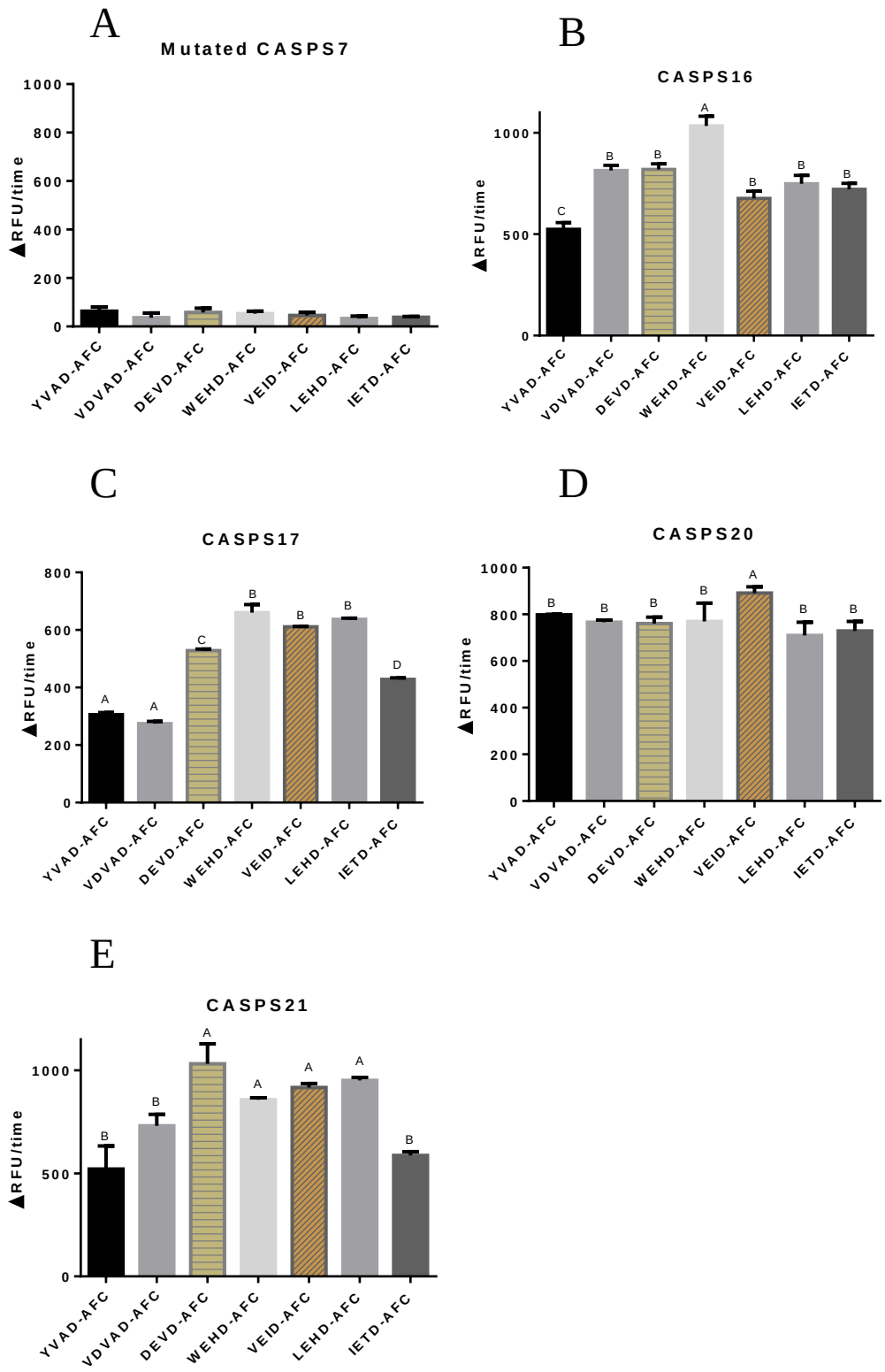
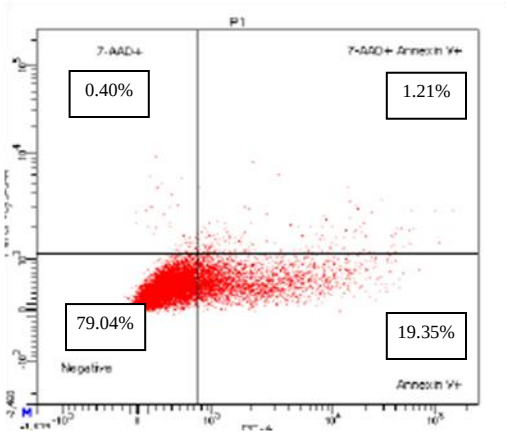

A
Mutated CASPS7
▲RFU/time
1000
800
600
400
200
0
YVAD-AFC
VDVAD-AFC
DEVD-AFC
WEHD-AFC
VEID-AFC
LEHD-AFC
IETD-AFC
B
CASPS16
▲RFU/time
1000
500
0
C
B
B
A
B
B
B
C
CASPS17
▲RFU/time
800
600
400
200
0
A
A
C
B
B
B
D
D
CASPS20
▲RFU/time
1000
800
600
400
200
0
B
B
B
B
A
B
B
E
CASPS21
▲RFU/time
1000
500
0
B
B
A
A
A
A
B

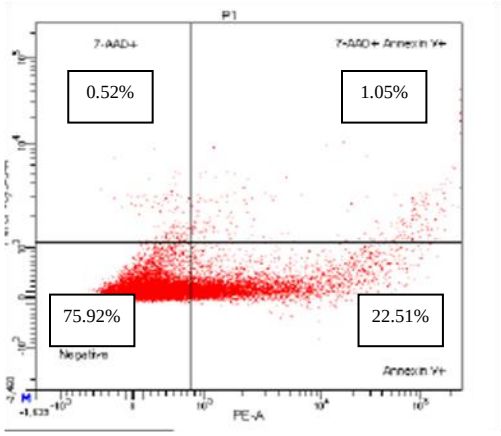
Figure 2.8: Substrate preference assays using clarified lysates containing recombinant active caspases.

Substrate preference for the active site mutant of CASPS7 (negative control) and other caspases were determined using recombinant caspases. Same letters on the graph represents groups that do not have significant difference (determined by comparing p-values where $p < 0.05$ is significant) whereas different letters represent significant difference in the activity. The level of significance for each substrate was calculated using one-way ANOVA. Each graph represents the average of 2 biological replicates (+/- SE).

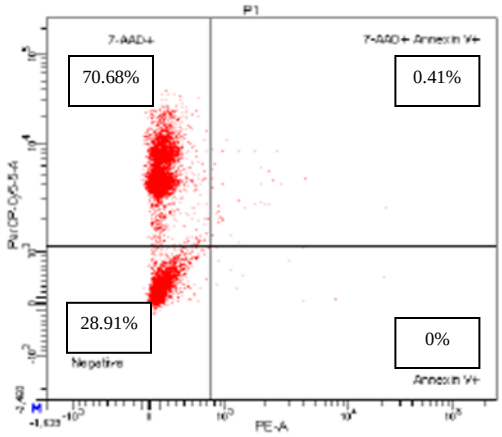
A


P1
7-AAD+
7-AAD+ Annexin V+
0.40%
1.21%
79.04%
19.35%
Negative
Annexin V+

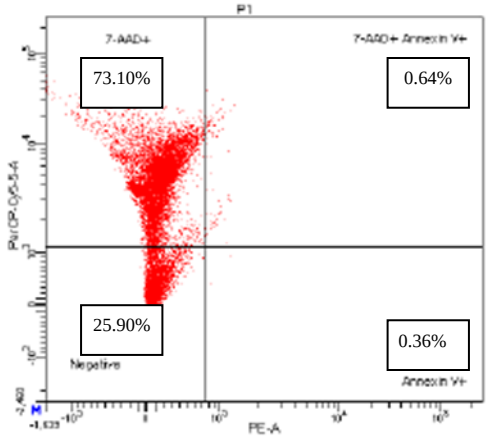
B


P1
7-AAD+
7-AAD+ Annexin V+
0.52%
1.05%
75.92%
22.51%
Negative
Annexin V+
PE-A

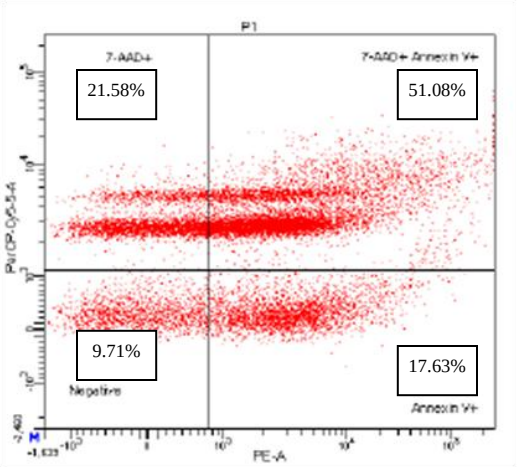
C


P1
7-AAD+
7-AAD+ Annexin V+
70.68%
0.41%
28.91%
0%
Negative
Annexin V+
PE-A

D


P1
7-AAD+
7-AAD+ Annexin V+
73.10%
0.64%
25.90%
0.36%
Negative
Annexin V+
PE-A

E


P1
7-AAD+
7-AAD+ Annexin V+
21.58%
51.08%
9.71%
17.63%
Negative
Annexin V+
PE-A

F

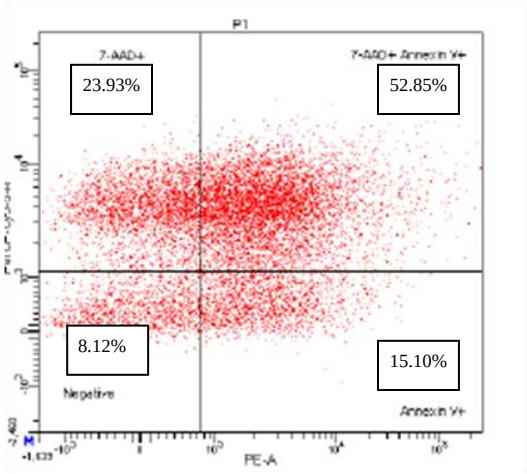
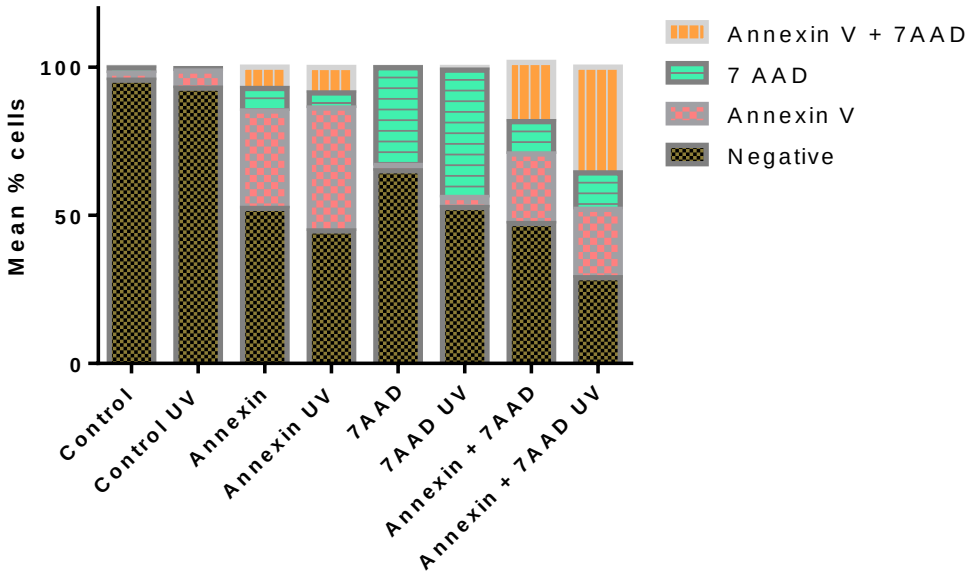

P1
7-AAD+
7-AAD+ Annexin V+
23.93%
52.85%
8.12%
15.10%
Negative
Annexin V+
PE-A

Figure 2.9: Raw data of flow cytomtery analysis on Aag2 cells stained with Annexin V and/or 7AAD.

Aag2 cells were stained with Annexin V and/or 7AAD with or without UV treatment. The X axis in each graph represents the staining with Annexin V, whereas the Y axis represents staining with 7AAD. A) Control cells and B) UV-treated cells stained with Annexin V; C) control cells and D) UV-treated cells stained with 7AAD; E) control cells and F) UV-treated cells stained with Annexin V and 7AAD. The percentage of cells is each quadrant is represented in the boxes.

A


Annexin V + 7AAD
7 AAD
Annexin V
Negative
Mean % cells
100
50
0
Control
Control UV
Annexin
Annexin UV
7AAD
7AAD UV
Annexin + 7AAD
Annexin + 7AAD UV

B

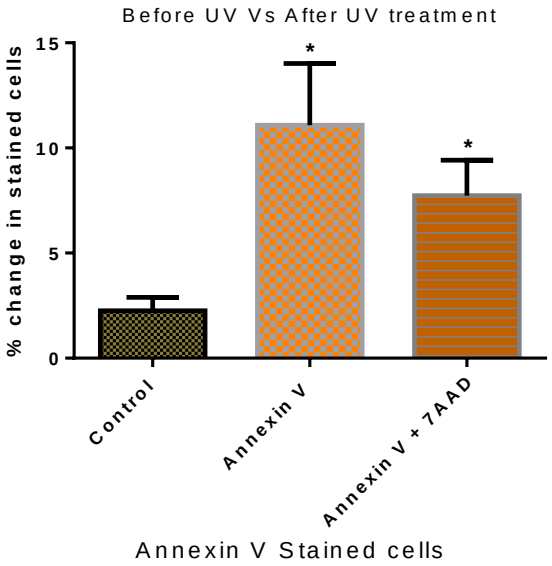
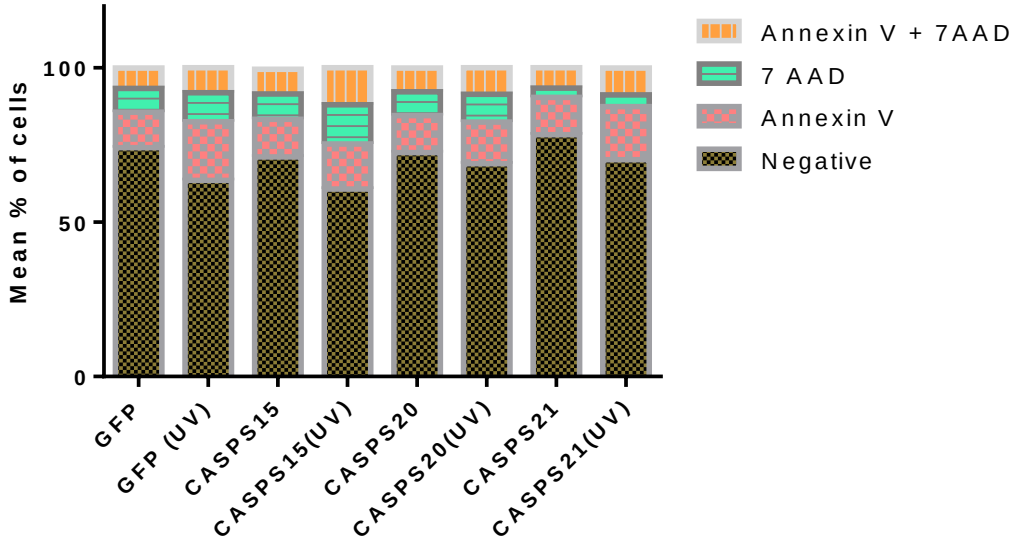

Before UV Vs After UV treatment
% change in stained cells
15
10
5
0
*
*
Control
Annexin V
Annexin V + 7AAD
Annexin V Stained cells

Figure 2.10: Mean percentages of cells stained by Annexin and 7AAD with or without UV treatment.

The mean percentages of untreated and UV-treated Aag2 cells that were stained with Annexin V and/or 7AAD were determined by flow cytometry. The samples labeled “Control” were not stained with either reagent. Higher percentages of cells were stained in Annexin V and Annexin+7AAD treatment after UV compared to control cells. The level of significance was determined using t-test. Each graph represents the average of 3 biological replicates (+/- SE).

A


Annexin V + 7AAD
7 AAD
Annexin V
Negative
Mean % of cells
100
50
0
GFP
GFP (UV)
CASPS15
CASPS15(UV)
CASPS20
CASPS20(UV)
CASPS21
CASPS21(UV)

B

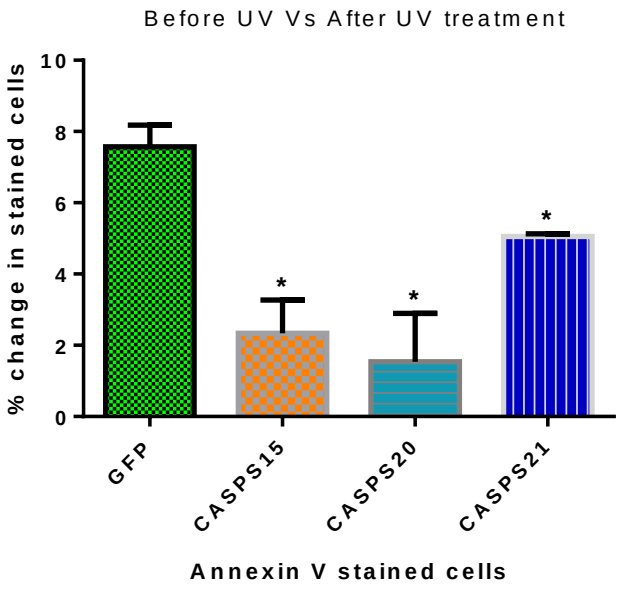

Before UV Vs After UV treatment
% change in stained cells
10
8
6
4
2
0
*
*
*
GFP
CASPS15
CASPS20
CASPS21
Annexin V stained cells

Figure 2.11: Mean percentage of cells stained by Annexin and/or 7AAD in caspase-knocked down cells with or without UV treatment.

The mean percentage of cells stained by Annexin V and/or 7AAD was calculated in each treatment after caspase knockdown. UV-treated cells had significantly higher percentage of stained compared to on UV treatment. Each bar represents 3 biological replicates. Panel B represents the comparison of percentage of Annexin stained cells with and without UV treatment. It was observed that knockdown of CASP21 had more Annexin stained cells after UV treatment compared to other caspase dsRNA treatment.

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Doumanis, J., Quinn, L., Richardson, H., & Kumar, S. (2001). STRICA, a novel *Drosophila melanogaster* caspase with an unusual serine/threonine-rich prodomain, interacts with DIAP1 and DIAP2. *Cell Death and Differentiation, 8*(4), 387-394. doi:10.1038/sj.cdd.4400864 [doi]

Harvey, N. L., Daish, T., Mills, K., Dorstyn, L., Quinn, L. M., Read, S. H., . . . Kumar, S. (2001). Characterization of the Drosophila caspase, DAMM. *The Journal of Biological Chemistry, 276*(27), 25342-25350. doi:10.1074/jbc.M009444200 [doi]

Hawkins, C. J., Yoo, S. J., Peterson, E. P., Wang, S. L., Vernooy, S. Y., & Hay, B. A. (2000). The Drosophila caspase DRONC cleaves following glutamate or aspartate and is regulated by DIAP1, HID, and GRIM. *The Journal of Biological Chemistry, 275*(35), 27084-27093. doi:10.1074/jbc.M000869200 [doi]

Hay, B. A., & Guo, M. (2006). Caspase-dependent cell death in drosophila. *Annual Review of Cell and Developmental Biology, 22*, 623-650. doi:10.1146/annurev.cellbio.21.012804.093845 [doi]

Koopman, G., Reutelingsperger, C. P., Kuijten, G. A., Keehnen, R. M., Pals, S. T., & van Oers, M. H. (1994). Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood, 84*(5), 1415-1420.

Kumar, S., & Doumanis, J. (2000). The fly caspases. *Cell Death and Differentiation, 7*(11), 1039-1044. doi:10.1038/sj.cdd.4400756 [doi]

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Liu, Q., Qi, Y., & Chejanovsky, N. (2005). *Spodoptera littoralis* caspase-1, a lepidopteran effector caspase inducible by apoptotic signaling. *Apoptosis : An International Journal on Programmed Cell Death, 10*(4), 787-795. doi:10.1007/s10495-005-0365-x [doi]

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Chapter 3 - Conclusions

Apoptosis is known to be an important antiviral mechanism in mammals as well as insects. When cells are infected with viruses, they often will try to kill themselves to inhibit the growth and propagation of the virus to other neighboring cells (Clarke and Clem, 2003, Clem and Miller, 1993, Scallan et al., 1997). Diseases that are caused by viruses carried by arthropod hosts (arboviruses) include dengue, yellow fever, and chikungunya fever. These diseases have huge public health concern and are known to cause significant mortality and morbidity in the affected regions (WHO 2007, 2008). *Aedes aegypti,* which is the subject of this study, is a vector for dengue, chikungunya, and yellow fever. Thus, the study of apoptosis in *Ae. aegypti* will help us better understand the mechanism of transmission of various arboviruses from insect vectors to the hosts. This knowledge can be used to develop future strategies to stop the transmission and replication of viruses in the mosquitoes. With improved knowledge of specific regulators of apoptosis in *Ae. aegypti* like caspases, we can better understand the apoptosis pathway in mosquitoes and use the knowledge to target specific areas of the pathway.

The caspases included in this study, CASPS15, CASPS16, CASPS17, CASPS20 and CASPS21, were shown to play a role in apoptosis in *Ae. aegypti*. Besides being involved in apoptosis, these caspases may be involved in other biological processes like immune response. For example, the *Drosophila* caspase AeDredd is known to be involved in NF-kB activation. Since there are more caspases identified in *Ae. aegypti* (11) compared to *Drosophila* (7), it is likely that these *Ae. aegypti* caspases serve other functions along with their apoptotic function.

The specific roles of the caspases included in this study can be further studied by studying their interactions with other known regulators of apoptosis in *Ae. aegypti*. The genetic interaction of these caspases with IAP1 can be studied using RNAi. Recombinant caspases, IAPs and IAP

antagonists can be produced and their direct interaction can be studied using binding assays. These assays will help us understand the regulators that interact with specific caspases during apoptosis in *Ae. aegypti*. Mutant forms of CASPS15, 16, 17, 20 and 21 with alterations to the specific domains of caspase structure can also be used to study in detail the contribution of specific regions of the caspases in apoptosis. Studying the predicted 3D structure of these caspases using X-ray crystallography will also help greatly in understanding their specific apoptotic and non-apoptotic functions. Apoptosis is a complex phenomenon that involves multiple regulators, and so further knowledge regarding the caspases discussed in this study will be beneficial in understanding apoptosis in mosquitoes and other insects.