

## Characterization of *Aedes Dredd*: A novel initiator caspase from the yellow fever mosquito, *Aedes aegypti*

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### Abstract

Caspases play an essential role during programmed cell death in all metazoans. These enzymes are cysteine proteases and comprise a multi-gene family with more than a dozen mammalian family members. Although caspases have been characterized in many animals, including *Drosophila melanogaster*, little is known about the caspases that exist in mosquitoes. Here we describe the identification and characterization of *Aedes Dredd* (AeDredd), a novel caspase in the yellow fever mosquito, *Aedes aegypti*. AeDredd contains two N-terminal death effector domains and the well conserved caspase catalytic domain. Multiple sequence alignments and functional substrate assays of recombinant protein suggest that AeDredd is an orthologue of *Drosophila Dredd* and human caspase-8, both central effectors of the death receptor-mediated apoptotic pathway. AeDredd exhibits substrate specificity most similar to human caspase-8. AeDredd transcripts were found in all developmental stages with highest expression in early pupae. Within adults, AeDredd was found in all the tissues examined, with the highest transcript levels detected in fat body tissues. This is the first functional characterization of a death domain-containing caspase in an insect vector of human disease, and will initiate studies on the role of apoptosis in the innate immune response of vectors towards intracellular parasites such as viruses.

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### 1. Introduction

Apoptosis is a physiological process of programmed cell death that occurs in all multi-cellular organisms. Programmed cell death is essential for the removal of unwanted cells and is critical for restricting cell numbers and for tissue patterning during development (Raff, 1998; Kaufmann and Hengartner, 2001; Hengartner, 2000). Dysregulation of the cell death process is postulated to play a role in the pathogenesis of a variety of human diseases (Twomey and McCarthy, 2005). Apoptosis also

plays a key role in the development, regulation and function of the immune system and can be a response of cells to general stress, including the stress incurred as a result of infection with intracellular pathogens, particularly viruses (Benedict et al., 2002; Teodoro and Branton, 1997).

In vertebrates, apoptosis is a rapid first response to infection that stimulates the adaptive immune system. Insects, such as mosquitoes that transmit parasitic diseases to humans, lack this adaptive immune response and rely solely on innate immune mechanisms to combat pathogens (Khush et al., 2002; Lowenberger, 2001; Lowenberger et al., 1999). This immune response may include protease cascades leading to coagulation and melanization, cellular responses such as phagocytosis and encapsulation, and the production of antimicrobial peptides (Loker et al., 2004; Beutler, 2004). These classic invertebrate immune responses can act directly upon accessible extracellular pathogens but are likely less effective against intracellular

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pathogens such as viruses. Although apoptosis has been described as an anti-viral defense in most metazoans, it has not been described in this role in mosquitoes. The extent to which the apoptotic cascade exists in mosquitoes is unknown but is essential in determining its potential role in mosquito immunity.

Apoptotic signals converge upon the same evolutionarily conserved mechanism that results in the activation of caspases, a family of cysteine proteases. Caspases induce the morphological and biochemical changes characteristic of apoptosis; chromatin condensation, DNA fragmentation and blebbing of the plasma membrane (Thornberry and Lazebnik, 1998; Kornbluth and White, 2005; Aravind et al., 2001). In this process caspases may function directly in cell disassembly or in initiating this disassembly through a biochemical cascade involving the sequential activation of different caspases that amplify their proteolytic effects (Raff, 1998).

All caspases exist in cells as inactive precursors or zymogens that contain three domains: an amino terminal domain, a large subunit (~20 kD) and a small subunit (~10 kD), which together form the C14 catalytic subunit (Raff, 1998). Activation requires proteolytic cleavage between these domains and the formation of a heterodimer by the small and large subunits (Thornberry and Lazebnik, 1998). Each heterodimer comprises six  $\beta$ -strands that form a twisted  $\beta$ -sheet with five loops, which together form the catalytic site (Blanchard et al., 2000). This catalytic site forms a cleft comprising four pockets, S1–S4, spanning both the small and the large subunits. The four pockets determine substrate binding and specificity.

Caspases can be broadly classified as initiators, effectors, and inflammatory caspases. Initiator caspases activate downstream effector caspases, either directly through proteolysis, or indirectly through an intermediate signaling mechanism (Raff, 1998; Shi, 2004). The effector caspases then cleave specific proteins such as CAD (the caspase activated DNA deoxyribonuclease) that ultimately leads to cell death (Enari et al., 1998). Initiator caspases have long prodomains that contain specific protein–protein interaction motifs, the caspase recruitment domains (CARD) and death effector domains (DED) that serve as docking sites for adaptor proteins that activate initiator caspases (Weber and Vincenz, 2001). Initiator caspases coordinate the signals initiated by both internal sensors (intrinsic pathway, mitochondria dependent) and external stimuli (extrinsic pathway, death-receptor-mediated) (Hengartner, 2000; Ho and Hawkins, 2005). In the intrinsic pathway CARD domain interactions between Apaf-1 and procaspase-9 enhance caspase-9 activation of caspase-3 (Boatright et al., 2003). In the extrinsic pathway, death receptors such as Fas bind to the adaptor FADD (Fas-associated protein with death domain) which in turn recruits multiple copies of procaspase-8 which initiate autocatalytic processing and the activation of caspase-3. In *Drosophila*, there are three putative initiators (Dronc, Dredd and Strica) and four putative effector caspases (Drice, Dcp-1, Decay, and

Damm) (Kumar and Dumanis, 2000; Richardson and Kumar, 2002). Dronc and Dredd have long prodomains containing CARD and death inducing domains (DID), respectively, suggesting that these two caspases act as initiator caspases (Dorstyn et al., 1999; Chen et al., 1998).

We describe here the characterization of *Aedes Dredd* (AeDredd), the first initiator caspase characterized in a non-drosophilid insect. AeDredd contains the well conserved caspase C14 subunit and exhibits strong caspase activity. AeDredd also contains two putative N-terminal death domains suggesting that a death receptor mediated pathway exists in mosquitoes. The characterization of apoptotic pathways in mosquitoes may elucidate how vectors eliminate or regulate the number of intracellular parasites they harbor and transmit.

## 2. Materials and methods

### 2.1. Insect maintenance cDNA generation

*Aedes aegypti* (Liverpool strain) mosquitoes were reared as described previously (Lowenberger et al., 1999). Total RNA was extracted from different developmental stages (larvae, callow pupae, black pupae, and whole adult mosquitoes) or from individual tissues (salivary glands, ovaries, midguts, and fat body tissues) dissected from adult females three days after emergence. Tissues were dissected, rinsed in ice cold sterile *Aedes* saline solution (Hayes, 1953) and placed directly into Tri-reagent (Sigma, Oakville, Ont.). RNA extraction, purification, and cDNA synthesis using 3  $\mu$ g of total RNA was done as described previously (Lowenberger et al., 1999).

### 2.2. Identification and sequencing of AeDredd

Partial sequences of AeDredd were identified in a series of TBLASTN searches of the TIGR *Aedes* Gene Indices (<http://www.tigr.org/tdb/tgi/>) as expressed sequence tags (EST) displaying significant homology to the C14 subunit of *Drosophila* and mammalian caspases. An additional EST was identified showing homology to the prodomain of *Drosophila* caspase Dredd. Primers designed against the EST sequences and 5'-3' RACE PCR were used to obtain a single transcript encoding the two partial ESTs. Amplified PCR products were cloned and sequenced using Big Dye v3.1 chemistry as described (Lowenberger et al., 1999).

### 2.3. Tissue comparison

For an estimation of tissue specific expression and mRNA concentration we used real time quantitative (qPCR). All qPCR reactions were performed with a Rotor-Gene 3000 (Corbett Research) using the Platinum SYBR Green Supermix-UDG (Invitrogen, Carlsbad, CA). We used 2.5  $\mu$ l cDNA with 12.5  $\mu$ l of Platinum SYBR Green Supermix, 1  $\mu$ l (10  $\mu$ M) sense primer (5'-GAACATTGAAAG-CAACTTCAACCG-3'), 1  $\mu$ l (10  $\mu$ M) antisense primer

(5'-GCCATTTTGTCCACCTCGG-3') in 25  $\mu$ l reactions under the following conditions: 50 °C for 2 min, 95 °C for 2 min, followed by 35 cycles of 95 °C for 10 s, 61.5 °C for 15 s, 72 °C for 30 s. Quantity values were generated using the 2(- $\Delta\Delta$ CT) method as described previously (Livak and Schmittgen, 2001). The expression of AeDredd was compared per unit of  $\beta$ -actin, a normalizing gene that had stable expression levels in all conditions tested. All cDNAs were screened with primers known to span a 5' intron to verify that samples contained no genomic DNA contamination. All data represent triplicate runs of independently generated cDNAs.

#### 2.4. Modeling of AeDredd

We used the metaserver ([www.bioinfo.pl](http://www.bioinfo.pl)) that applies the 3D jury method (Ginalski et al., 2003) to annotate the structure of AeDredd. The 3D jury score gave us values >150 for the best models of AeDredd based on the structure of different caspase structural templates (1flj, li4e, lqx3, lm72, lqtn). A value >50 corresponds to an accuracy of 90%. The Carbon- $\alpha$  model of AeDredd generated by the metaserver from the caspase-8 template (pdb:1QTN) was selected. Side chains were added using the maxsprout software (<http://www.ebi.ac.uk/maxsprout>) and refined using scwrl3 software (<http://dunbrack.fccc.edu/SCWRL3.php>) (Canutescu et al., 2003). The quality of the model was confirmed by performing a structural superimposition of the template (1QTN) to the model of AeDredd (RMSD = 0.68 Å over 872 atoms). Manual inspection of the sequence and spatial conservation of key amino acids involved in the hydrophobic core and catalytic sites between 1QTN and AeDredd were used to validate our model. The heterotetramer of AeDredd was obtained by crystallography symmetry based on 1QTN. The transformation of these atomic coordinates was performed using SPDBV (<http://ca.expasy.org/spdbv/>). Models were verified for favorable geometrical and stereochemical properties using Verify3D (Luthy et al., 1992) and PROCHECK. For molecular visualizations we used the graphics software PyMol ([pymol.sourceforge.net/](http://pymol.sourceforge.net/)). The secondary structure associated with the AeDredd prodomain was predicted using the BioInfoBank Meta server (<http://bioinfo.pl/Meta/>).

#### 2.5. Expression of recombinant AeDredd, caspase assays, and inhibition assays

Recombinant AeDredd was generated by transforming *E. coli* BL21 (DE3) cells with a truncated form of AeDredd lacking the putative prodomain (residues 1–276) (Fig. 1a). The C14 subunit was amplified by PCR and cloned into pET-46 EK/LIC (Novagen, Madison, WI). pET-AeDredd LB-Amp<sup>50</sup> starter cultures were grown for 3 h, subcultured into fresh medium, and grown at 37 °C to an OD of 0.5, induced with 2 mM IPTG and grown for 16 h at 22 °C. Cells were pelleted and lysed using Easy-Lyse Bacterial

Protein Extraction Solution (Epicentre, Madison, WI) following manufacturer's instructions. Cleared *E. coli* lysates from cells expressing AeDredd were assayed for protein concentration using the Bradford Assay (BioRad Hercules, CA), and 100  $\mu$ g of total protein from each lysate was incubated with 200  $\mu$ M chromophore *p*-nitroaniline labeled substrates (BioVision Inc. Mountain View, CA) according to manufacturer's recommendations for 3 h at 37 °C. The release of -pNA was monitored by a spectrophotometer at 405 nm. Activity was considered biologically significant when absorbance was greater than two-fold higher than the uninduced controls (Biovision, Inc Mountain View, CA). We assessed the ability of a general caspase inhibitor, z-VAD-fmk (Biovision Inc. Mountain View, CA), to inhibit caspase activity. For each assay, a given amount of z-VAD-fmk was mixed with 100  $\mu$ g of AeDredd lysate (total protein) and allowed to incubate for 1 min. The 200  $\mu$ M IETD-pna was added and each reaction was allowed to incubate for 3 h at 37 °C. The data represent three replicates, measured in duplicate.

#### 2.6. Ecdysone and UV treatments

Because some apoptotic processes are regulated by developmental changes in hormones, or due to other factors such as UV damage we assessed the effects of ecdysone and UV damage on AeDredd expression. Early third instar larvae were rinsed with fresh ddH<sub>2</sub>O and maintained individually for 3 h in tissue culture wells containing 100  $\mu$ l of water and 30  $\mu$ M 20-Hydroxyecdysone (Sigma, Oakville, Ont.) for 24 and 48 h at 22–25 °C. Water volumes were increased to 1 ml/well and finely ground Tetramin<sup>®</sup> fish flakes was added as a food source 3 h after initial ecdysone exposure. At specific time points, whole larvae were placed directly in Tri-Reagent and RNA extraction, cDNA synthesis and qPCR were done as described above. Larvae treated with water alone served as the control. To determine the effects of UV treatment on AeDredd expression, we exposed freshly dissected adult gut tissues to UV-irradiation. Gut tissues from adult *A. aegypti* were dissected, rinsed in *Aedes* saline solution (Hayes, 1953) and placed individually in 50  $\mu$ l of 1X PBS. Gut tissues were then exposed to 90 mJ/cm<sup>2</sup> UV-irradiation through the PBS for 30 min. Following irradiation, guts were incubated at room temperature for an additional 60 min, then transferred directly into Tri-Reagent for RNA extraction, cDNA synthesis and qPCR as described above. Controls comprised gut tissues incubated at room temperature with no UV exposure. Data represent duplicate experiments.

### 3. Results

#### 3.1. Identification of AeDredd

We performed a TBLASTN search of the TIGR *Aedes* genomic database using protein sequence queries of both

the C14 subunits and the prodomains of *Drosophila* and mammalian caspases. We identified one EST draft sequence that shared 22% sequence identity with the C14 subunit of both *Drosophila* and mammalian caspases and one small EST draft sequence that shared 12% identity with the prodomain of *Drosophila* Dredd. Through 5'-3'RACE PCR we obtained a cDNA with an open reading frame that encodes a novel 493 residue caspase named AeDredd (GenBank Accession: DQ860099). This putative protein is similar to the product of the gene annotated as *Aedes* caspase-8 (EAT33580) from the *Aedes aegypti* genome project that subsequently has become available. Comparisons with the conserved domain database indicate the AeDredd putative protein contains a C-terminal C14 caspase subunit (246aa) and an N-terminal prodomain 238aa in length. Multiple sequence alignments with mammalian and invertebrate caspases indicate that AeDredd contains all the essential residues required for catalysis (His-237, Gly-238, and Cys-285) and the residues involved in coordinating the P1 aspartate (Arg-179, Gln-283, Arg-341, Ser-343), using caspase-1 nomenclature

(Cerretti et al., 1992). The pentapeptide sequence encompassing the catalytic Cys is QACQG (Fig. 1a).

Phylogenetic analysis clusters AeDredd with *Drosophila* Dredd and with the human initiator caspases-8, -9, and -10 (data not shown). The C-terminus of AeDredd shares significant sequence identity with the C14 subunits of all mammalian and insect caspases, with the highest similarity with *Drosophila* Dredd (36% identity, 54% similarity). AeDredd shares 26% identity (43% similarity) with the C14 subunit of human caspase-8, 23% identity (41% similarity) with human caspase-10, and 23% identity (38% similarity) with the C14 subunit of human caspase-9 (Fig. 1a).

Although caspase prodomains tend to be much more divergent than the catalytic segments, we have identified limited sequence similarities between the prodomain of AeDredd and the prodomains of *Drosophila* Dredd, human caspase-8, and human caspase-10. Human caspases -8 and -10, carry two N-terminal DED. *Drosophila* Dredd carries two non-orthologous but functionally similar death inducing domains (Tibbetts et al., 2003). The prodomain of

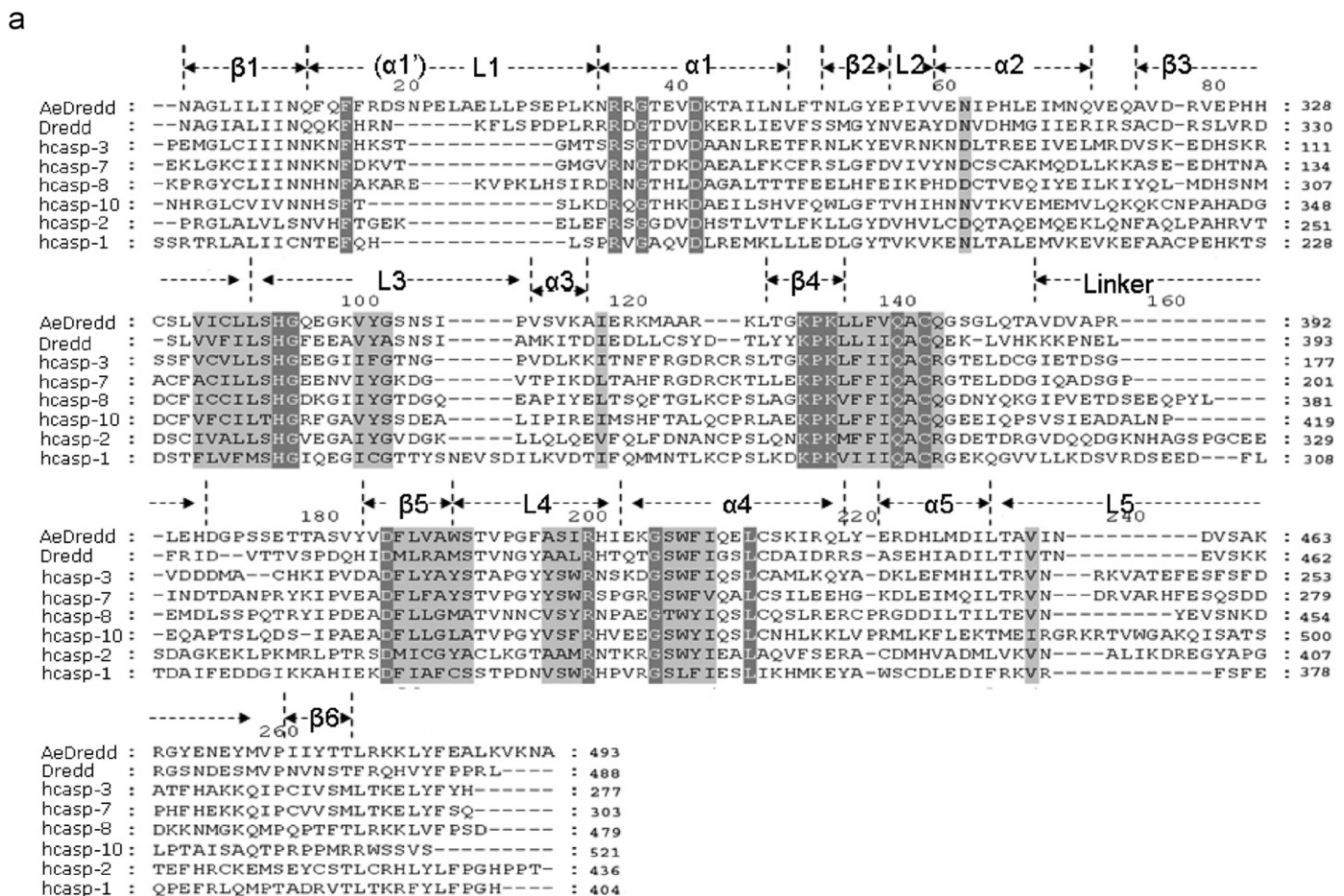


Fig. 1. (a) Clustal X alignment of the partial enzymatic domains of *Aedes* Dredd (AeDredd) with human and *Drosophila* caspases, highlighting the conservation of residues critical for substrate binding and catalysis. The residues highlighted in gray contribute to the active site of the enzyme and the stabilization of the P1 aspartate residue in the substrate. Spatially aligned  $\alpha$ -helices,  $\beta$ -sheets and loops are indicated. (b) Alignment of AeDredd with the prodomains of *Drosophila* Dredd, human caspase-8, human caspase-10. Black and gray boxes outline the predicted  $\alpha$ -helical bundles of AeDredd and *Drosophila* Dredd, respectively. The predicted  $\alpha$ -helices within each bundle are numbered H1–H6. Both AeDredd and *Drosophila* Dredd contain two bundles each with six  $\alpha$ -helices. Secondary structure was predicted using the BioInfo Meta Server (<http://bioinfo.pl/Meta/>).

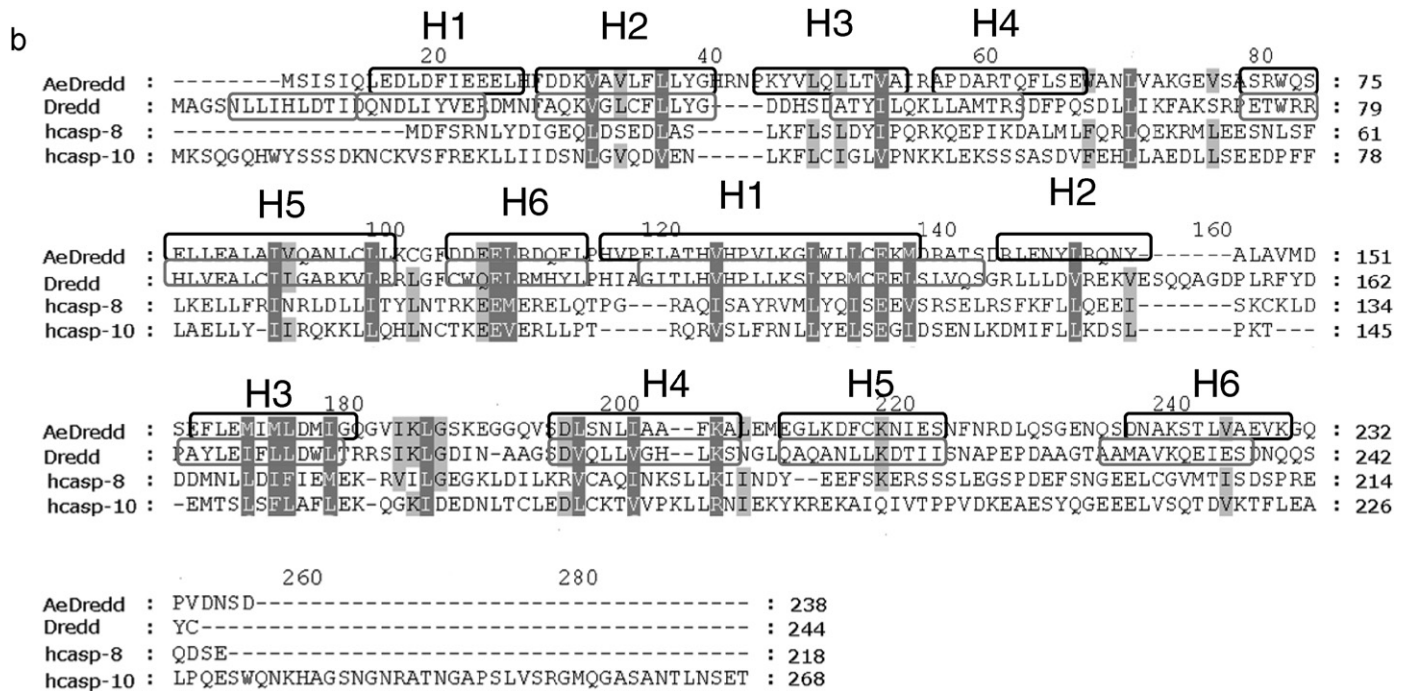


Fig. 1. (Continued)

AeDredd shares significant identity with the prodomains of *Drosophila* Dredd (22% identity; 43% similarity), human caspase-8 (11% identity; 31% similarity) and human caspase-10 (11% identity; 38% similarity) (Fig. 1b). The prodomain of human caspase-9 carries one N-terminal CARD and is shorter than the prodomains of AeDredd, *Drosophila* Dredd and human caspases-8 and -10. AeDredd shares 11% identity (25% similarity) with the prodomain of human caspase-9.

Since sequence identity below 25%, as measured by pairwise amino acid sequence alignments only, is not statistically significant and makes structural predictions unreliable, we used a fold recognition program (BioInfo-Bank Metaserver) to predict secondary structures present in the prodomain of AeDredd. The program predicts two six- $\alpha$ -helical bundles in the prodomain of AeDredd, nearly identical to those found in *Drosophila* Dredd, and similar to those found in human caspases-8 and -10, whereas the prodomain of human caspase-9 contains only one six  $\alpha$ -helical bundle (Fig. 1b). These data suggest that AeDredd is an orthologue of *Drosophila* Dredd and human caspases-8 and -10.

### 3.2. Expression during development

Quantitative real time PCR (qPCR) analysis revealed low levels of AeDredd transcripts present in the whole bodies of all developmental stages screened. AeDredd transcripts were present at similar levels in early and late instar larvae, late pupae, and adults (Fig. 2a). Transcript levels in callow pupae were five- to six-fold higher than levels found in other developmental stages.

### 3.3. Tissue specific expression

AeDredd transcripts were found in differing levels in all individual tissues screened in this study. Transcript levels were highest in the fat body tissues; up to four-fold higher than the levels found in salivary glands and ovaries. AeDredd transcripts were two-fold higher in midgut tissues than levels found in salivary glands and ovaries (Fig. 2b).

### 3.4. Enzymatic activity and substrate specificity

We expressed the C14 subunit of AeDredd in *E. coli*, and incubated the cell lysates with a variety of colorimetric tetrapeptide substrates to analyze substrate preference. AeDredd displayed significant cleavage on IETD-pna (optimal for caspase-8), VDVAD-pna (optimal for caspase-2) and YVAD-pna (optimal for caspase-1) compared with the control lysate from uninduced cells (Fig. 3a). Under identical conditions, recombinant AeDredd and recombinant caspase-8 showed similar activities on the IETD-pna substrate (data not shown). AeDredd showed low, but significant, activity on LEHD-pna and VEID-pna, optimal substrates for caspases-9 and 6, respectively. The enzymatic activity of AeDredd on IETD-pna was suppressed by all concentrations of the general caspase family inhibitor z-VAD-fmk (Fig. 3b) confirming the role of AeDredd as a caspase.

### 3.5. Homology modeling

The metaserver predicted the C14 caspase domain as a putative fold for AeDredd. The superimposition of

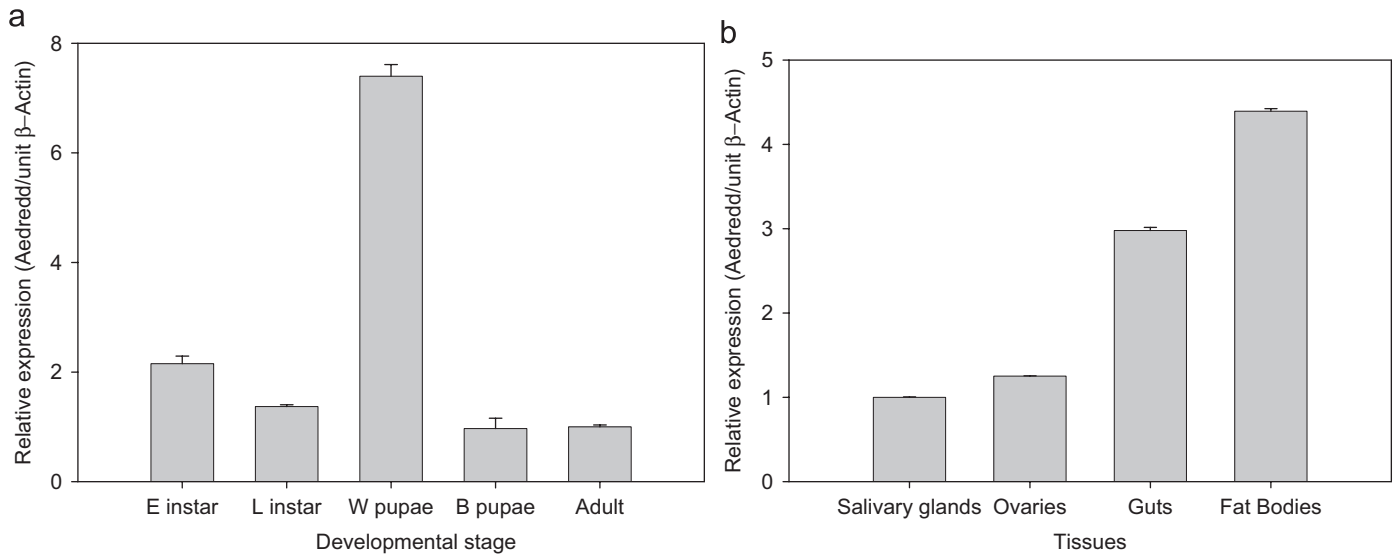


Fig. 2. Relative expression of *Aedes Dredd* (AeDredd) in different developmental stages (a) and individual adult tissues (b) using real-time quantitative PCR. Adult samples and salivary gland tissues were designated as the standard and arbitrarily set to 1. The vertical axis represents the fold increase in transcript number in different developmental stages or different adult tissues relative to their given control. AeDredd values were normalized using the housekeeping gene  $\beta$ -actin.

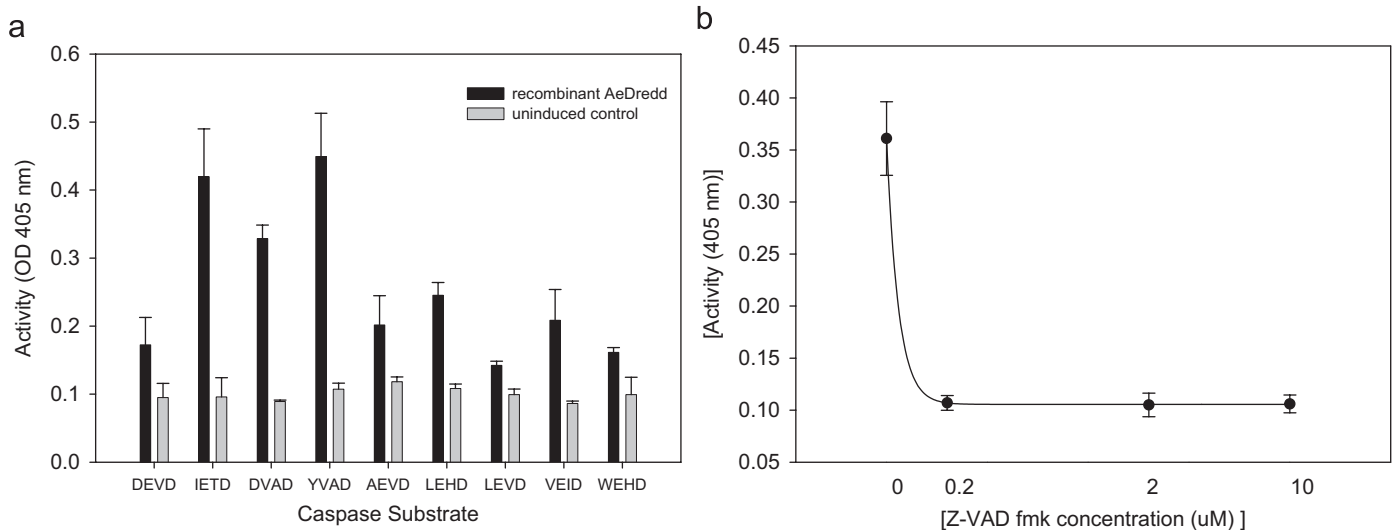


Fig. 3. Functional analysis of *Aedes Dredd* (AeDredd). (a) Activity of recombinant AeDredd on caspase-specific colorimetric peptide substrates. Activities greater than two-fold above the uninduced control were considered significant. (b) *E. coli* lysates containing the recombinant C14 subunit of AeDredd were treated with varying amounts of the general caspase inhibitor, z-VAD fmk. z-VAD fmk inhibited caspase activity, as measured by the ability of recombinant AeDredd to use IETD-pna, at concentrations as low as 0.2  $\mu$ M.

AeDredd with human caspase-8 predicts the AeDredd C14 caspase domain is composed of a central core of  $\beta$ -sheets surrounded by five  $\alpha$ -helices typical of caspases (Fig. 4a). The five  $\alpha$ -helices form five loops (Loops 1–5) near the active site that are important for substrate specificity (Blanchard et al., 2000). A prominent feature of caspase-8 is the presence of a significantly larger Loop 1 (7–10 residues between  $\beta$ 1 and helix  $\alpha$ 1), when compared to caspases-1 and -3. The insertion contains a helical segment,  $\alpha$ 1, not observed in caspase-1 or -3 (Fig. 1a) (Watt et al., 1999). AeDredd has an insertion of 11 residues, four residues larger than Loop 1 in caspases-8 and -11 and -14

residues larger than caspases-3 and -1, respectively (Figs. 4a and 1a). Most of the residues forming Loop 1 confer the same properties as those forming Loop 1 in caspase-8. The additional four residues are largely hydrophobic in nature. Loops 2 and 4 in AeDredd are four and 13 residues, respectively, identical in size to caspases-1, -3, and -8. Loop 3 of AeDredd consists of 17 residues, similar in size and structure to caspases-8 and -3 but smaller than caspase-1 (Watt et al., 1999). Loop 3 forms one side of the substrate binding cleft (the S4 pocket) and therefore directly affects the specificity at the P4 position of substrates. The Loop 5 region in AeDredd is 20 residues

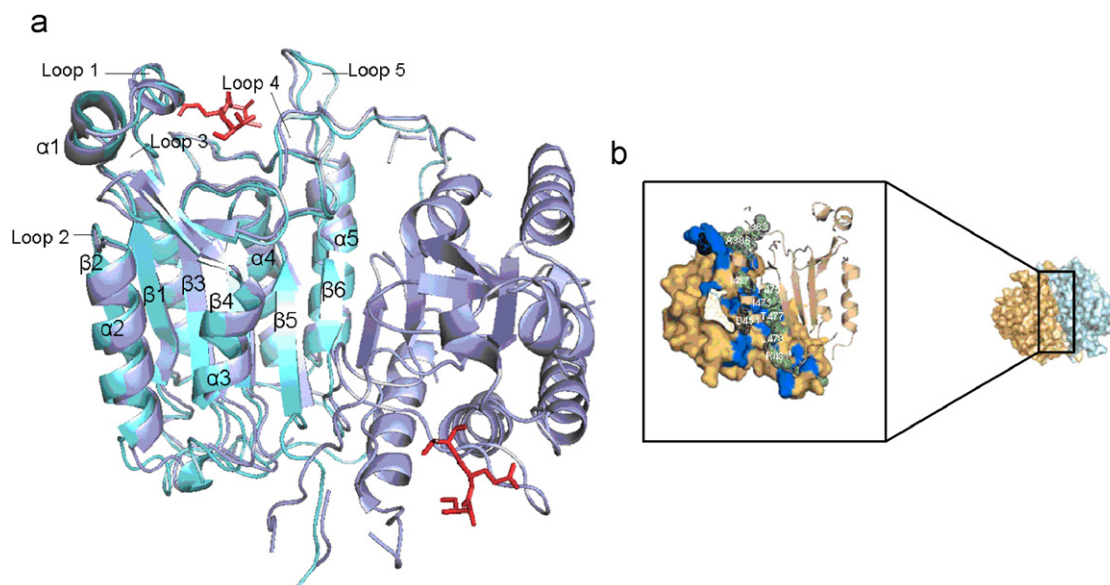


Fig. 4. (a) Ribbon diagram of the superimposition of *Aedes Dredd* (AeDredd) (purple) onto caspase-8 (1QTN) (blue). AeDredd shares the same basic tertiary structure common to all caspases; a central  $\beta$ -sheet surrounded by five  $\alpha$ -helices. The loops around the active site important for caspase substrate specificity are labeled Loops 1–5. The inhibitor (z-IETD) of caspase-8 is shown as a red stick model. (b) Most residues that form contact at the dimer interface by hydrogen bonding in caspase-8 (1QTN) are conserved in the AeDredd model. The homodimer of caspase-8 shown is represented here as the gold surface diagram with the residues important for dimer–dimer interactions shown in blue. The AeDredd homodimer is shown as a ribbon diagram. The conserved residues involved in dimer–dimer interactions are shown as green spheres. Graphics were produced using PyMol.

in length, similar in size and structure to caspase-8, and intermediate in size between caspases-1 and -3 (Fig. 1a). AeDredd is more similar to caspase-8 in size and structure in all five loop regions suggesting that AeDredd is more similar to caspase-8 than to other human caspases. To predict if AeDredd functions as a heterodimer, we examined our model for the conservation of residues known to be important for dimer/dimer interactions in caspase-8. The model indicates that most of the pairs of amino acids that form contact at the dimer interface by hydrogen bonding in caspase-8 are conserved in the AeDredd model (Fig. 4b).

The active site within the heterodimer is a cleft composed of four pockets, S1–S4 that span the large and the small subunits (Fig. 5) (Thornberry and Lazebnik, 1998; Fuentes-Prior and Salvesen, 2004). All caspases hydrolyze peptide bonds on the carboxyl side of an aspartate residue, termed the P1 residue. The P1 residues of substrates fit into the S1 pocket and are tethered by four residues that are conserved in known caspases; Arg-179, Gln-283, Arg-341 and Ser-347 (standard caspase-1 numbering). The first two residues are contributed by the large subunit and the remaining two by the small subunit. Each of these residues are spatially conserved in the S1 pocket of AeDredd except for Arg-179 (Arg-281 in AeDredd), which is found deeper in the pocket relative to its position in caspase-8 (Fig. 5) (Watt et al., 1999), which may be due to improper side chain packing in the model. The S2 and S3 pockets play key roles in anchoring the substrates and substrate specificities (Fuentes-Prior and Salvesen, 2004; Blanchard et al., 2000, 1999). The two residues in the S2 and S3 pockets, Val-410 and Tyr-412 in caspase-8, are replaced by

Ala-421 and Ile-423, which, although different, confer similar hydrophobic properties (Fig. 1a). The P4 residue, three upstream of the P1 aspartate in substrates and the size of the S4 pocket (to which the P4 residue binds) also contributes to substrate specificity. The residues contributing to the S3 and S4 pockets of caspase-8 are Tyr-412, Arg-413, Pro-415, Trp-419 and Asn-414 (Blanchard et al., 1999). The equivalent residues in AeDredd are Ile-423, Arg-424, Ile-426, Trp-431 and His-425 (Fig. 1a). The substitution of His-425 for Asn-414 maintains the hydrophobic nature of the pocket. Once in the active site, the peptide backbone of the bound substrate forms bonds with Ser-339 (Ser-411 in caspase-8 and Ser-422 in AeDredd), which is spatially conserved in nearly all caspases, including AeDredd (Fig. 5). Following substrate binding, catalysis utilizes a cysteine protease mechanism involving a catalytic dyad composed of Cys-285 and His-237, plus an oxyanion hole involving Gly-238 and Cys-285 (standard caspase-1 numbering). The residues important for catalysis, Gln-376, Cys-378, Gly-379, His-338, and Gly-339 are all spatially conserved in AeDredd with respect to their positions in caspase-8 (Fig. 5).

### 3.6. The effects of ecdysone and UV-irradiation on AeDredd expression

We examined transcript levels of AeDredd in third instar larvae after a 24 or 48 h exposure to ecdysone. We detected no significant changes in AeDredd transcript levels at any time points examined in this study (Fig. 6a). This is in complete contrast to another caspase characterized from *A. aegypti* whose expression closely follows pulses of

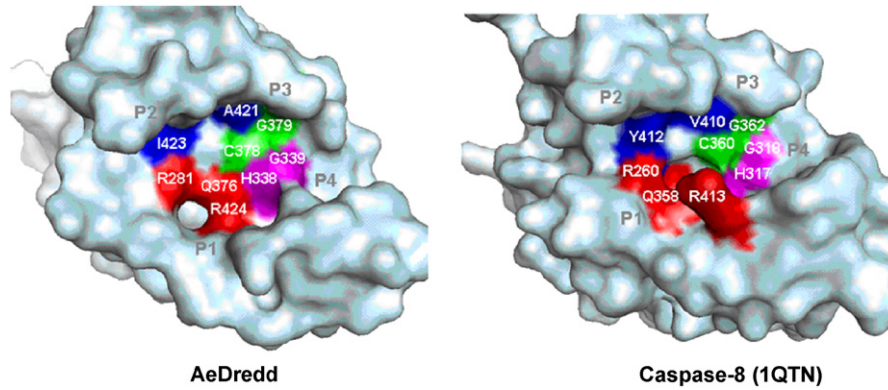


Fig. 5. Molecular surface representation of the active site regions of (a) *Aedes Dredd* (AeDredd) and (b) human caspase-8 (1QTN) generated using Pymol. This model shows spatial conservation of the pockets (P1–P4) forming the substrate binding cleft between AeDredd and caspase-8 (P1 (red), P2 (blue), P3 (blue), P4 (green and pink)). The residues that form each of the pockets are conserved except for V-410 and Y-412 in caspase-8 which have been substituted by A-421 and I-423 in AeDredd. The residues important for catalysis, Q-376, C-378, G-379, H-338, and G-339 are all spatially conserved with respect to their positions in caspase-8.

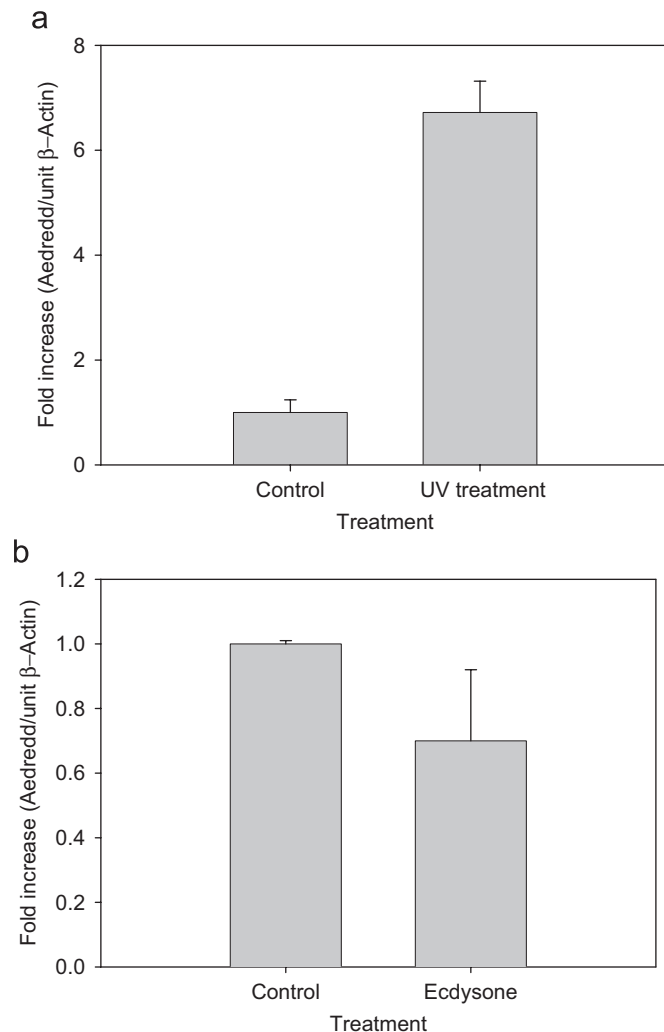


Fig. 6. Relative expression of *Aedes Dredd* (AeDredd) in response to UV-irradiation (a) and ecdysone treatment (b) using real-time quantitative PCR. Control samples were designated as the standard and arbitrarily set to 1. The vertical axis represents the fold increase in transcript number relative to their given control. AeDredd values were normalized using the housekeeping gene  $\beta$ -actin.

ecdysone (Cooper et al., unpublished). To examine the effects of UV exposure on AeDredd expression, we exposed adult guts to UV-irradiation. Following a 30 min exposure, AeDredd transcripts were six-fold higher in the UV-treated tissues than the controls (Fig. 6b).

#### 4. Discussion

Programmed cell death depends on a set of conserved structural molecules that transmit and regulate the death signal. Signals initiating cell death converge on the highly conserved caspase family of proteins. Because they are responsible for most of the morphological changes associated with apoptosis, caspases can be thought of as central executioners in the apoptotic pathway (Cohen, 1997). All caspases are expressed as proenzymes that contain an N-terminal prodomain and a C14 catalytic subunit and can be classified according to their structure, function, or substrate specificity. Classification based on function distinguishes between caspases involved in the cytokine maturation and inflammatory response and those primarily involved in apoptotic signaling (Ho and Hawkins, 2005). AeDredd contains a long N-terminal prodomain and the well conserved C14 catalytic domain. Through multiple sequence alignments, homology modeling and functional characterization, we propose that AeDredd is an orthologue of the initiator caspases *Drosophila Dredd* and human caspase-8.

The long prodomains of initiator caspases contain protein–protein interaction domains, known as death domains, which play important roles in apoptotic signaling. The sequence similarity across the death domain superfamily is low (4.7–25.3%), making it difficult to predict relationships based on primary sequence alone (Weber and Vincenz, 2001). DED and CARD domains, although dissimilar in sequence, possess similar structures comprising a conserved backbone of six  $\alpha$ -helices, with key



conserved hydrophobic residues in each  $\alpha$ -helix. Despite the lack of obvious sequence similarity in death domains, we have identified significant sequence identity (22%) between the N-terminal prodomains of AeDredd and *Drosophila* Dredd and limited sequence identity (11%) between the prodomains of AeDredd and human caspase-8. The Bioinfobank metasever predicts the prodomain of AeDredd to contain two sets of six  $\alpha$ -helices, nearly identical to those seen in *Drosophila* Dredd, making it the first death domain-containing caspase found in a non-drosophilid insect (Fig. 1b).

The catalytic domain of AeDredd shares a high degree of sequence similarity with the catalytic domain of known caspases, with the highest similarity found with *Drosophila* Dredd, human caspase-8 and human caspase-10. Multiple sequence alignments indicate that all residues important for substrate binding and catalysis are conserved and the model predicts that the catalytic domain of AeDredd shares a similar tertiary structure common to all known caspases (Fig. 4a and b) with spatial conservation of residues forming the loops and pockets of the substrate binding cleft (Fig. 1a and 5) (Blanchard et al., 2000). An analysis of the residues known to be important for dimer–dimer interactions in caspase-8 and the position of the equivalent residues in AeDredd suggests there is spatial conservation of most residues important for interface interactions, suggesting the active form of AeDredd may also be a heterodimer (Fig. 4b). The information collected on both the prodomain and the C14 subunit indicates AeDredd is an initiator caspase and an orthologue of both *Drosophila* Dredd and human caspase-8.

Caspases show a high degree of specificity, with an absolute requirement for cleavage after an aspartic acid (Talanian et al., 1997). This specificity is important to the apoptotic process as it involves cleavage of a particular group of proteins in an ordered manner. A combinatorial approach used to determine substrate specificity classifies caspases into three specificity groups: Group I (caspases-1, -4, -5 and -13), with a preference for WEHD; Group II (caspases-2, -3, and -7) with a preference for DEXD; and Group III (caspases-6, -8, -9, -10), with a preference for (I/V/L)EXD (X indicates any amino acid). AeDredd showed significant activity on IETD-pna, VDAD-pna, and YVAD-pna and limited activity on LEHD-pna and VEID-pna. IETD-pna, VEID-pna and LEHD-pna are substrates preferred by the Group III caspases while YVAD and DVAD are optimal substrates from Groups I and II, respectively. The ability of AeDredd to use substrates specific to more than one group of caspases suggests it may act on a broad range of cellular proteins. There are small differences in size and hydrophobicity between caspase-8 and AeDredd which may affect substrate binding and specificity. In addition, the substrate binding cleft in AeDredd appears to be wider which may account for the broader range of substrates used. The recognition motif for Group III initiator caspases (IETD) resembles the activation site within several effector

procaspases, specifically caspases-3 and 7. The fact that AeDredd cleaves substrates containing IETD motifs, suggests it may indeed be an upstream initiator caspase for a downstream caspase-3 homologue in mosquitoes.

The identification of AeDredd as a potential initiator caspase suggests an adaptor protein is required for activation. The activation of both human caspase-8 and *Drosophila* Dredd are mediated by the adaptor protein FADD, that contains a C-terminal death domain and N-terminal DED domain (Chinnaiyan et al., 1995; Leulier et al., 2002; Naitza et al., 2002). The recruitment of FADD to the death complex occurs via homotypic interactions between death domains, while the association of FADD with caspase-8 or *Drosophila* Dredd occurs through their DEDs. Since AeDredd is an orthologue of *Drosophila* Dredd, a death domain-containing adaptor protein such as FADD may be required for AeDredd activation, which would suggest that a death receptor pathway mediated by FADD exists in mosquitoes. We know of no published data on the characterization of a FADD homologue in mosquitoes; however, it will now be possible to identify such proteins by using AeDredd as an interacting partner.

Low levels of AeDredd transcripts were detected in all the developmental stages and individual tissues examined. Transcript levels were highest in the fat body and midgut tissues. This result is not unexpected because the fat body and midgut tissues have some of the highest tissue turnover rates. Since apoptosis plays a central role in tissue remodeling, we would expect to detect higher basal levels of caspases in tissues with rapid renewal rates. Examination of AeDredd expression in different developmental stages indicated low constitutive levels in the early and late larvae, as well as black pupae and adults. Transcript levels were five- to six-fold higher in callow pupae than any other developmental stage. In mosquitoes, ecdysone pulses can be detected in the late larval instar and early pupal stages (Lan and Grier, 2004; Margam et al., 2006). Each pulse of ecdysone commits the mosquito to the next development stage and initiates tissue specific programmed cell death. In *Drosophila*, a number of apoptotic effector molecules such as *dronc*, *rpr* and *hid* are transcriptionally upregulated in response to the pulses of ecdysone (Cakouros et al., 2004; Daish et al., 2003). Although we detected the highest number of AeDredd transcripts in the early pupal stage, we were unable to induce AeDredd expression with ecdysone treatment, suggesting that its involvement in development is mediated by different developmental cues. This is in contrast to the initiator caspase *Drosophila* Dronc, which shows significant increases at both the transcript and protein levels following exposure to ecdysone. This again suggests that AeDredd is more similar to *Drosophila* Dredd than to *Drosophila* Dronc. AeDredd expression was upregulated in response to UV-irradiation suggesting that it functions as part of a signaling pathway involved in cellular damage control.

Although caspases were originally identified as effectors of apoptosis, there is increasing evidence that caspases

function in other physiological processes, namely immunity, NF- $\kappa$ B signaling and the production of antimicrobial peptides (Leulier et al., 2002; Naitza et al., 2002; Lamkanfi et al., 2006). In mammals, human caspases-8 and -10 mediate the NF- $\kappa$ B dependent inflammatory response in antiviral signaling and the production of antimicrobial peptides (Chaudhary et al., 2000; Hu et al., 2000; Su et al., 2005). In *Drosophila*, Dredd, functions as part of the immune deficiency pathway (IMD) (Leulier et al., 2000; Stoven et al., 2003). Loss of function mutations in the gene encoding *Drosophila* Dredd block the expression of all downstream genes in the pathways that produce immune peptides against Gram-negative bacteria. Another *Drosophila* caspase, Damm, is up-regulated in response to viral infection, although the significance of this up-regulation is unknown (Dostert et al., 2005). AeDredd also may function as an immune signaling molecule in mosquitoes, both as a central player in the IMD pathway, similar to the role played by *Drosophila* Dredd, but additionally as a potential antiviral signaling molecule, as is seen with human caspases-8 and -10 (Takahashi et al., 2006). It is interesting to note that AeDredd transcripts were highest in the fat body, the principle immune response tissue in *A. aegypti* (Lowenberger, 2001), and in the midgut which is the first tissues to come in contact with ingested pathogens. Because *A. aegypti* is the vector of a number of important human diseases, the role of AeDredd in immunity will be the subject of further studies.

Although the physiological role of programmed cell death in mammals has been determined by a large array of *in vivo* studies, many of these models indicate a high degree of genetic redundancy that hinders definitive conclusions. With this in mind, many studies have turned to lower animals as models to study programmed cell death. Insect models such as *Drosophila*, and now *A. aegypti*, provide a level of intermediate complexity between nematodes and mammals. The mosquito model provides us with the opportunity for a comparative study of apoptotic pathways in development and metamorphosis as well as the role apoptosis may play in regulating components of the innate immune response of insects that transmit parasites to humans. The complete dissection of the insect immune response will involve the elucidation of how novel pathways such as the apoptotic pathways interact with, and complement the better described mechanisms of innate immunity in insects. This information will enable us to understand additional factors that contribute to the exceptional vectorial capacity and vectorial competence of mosquitoes.

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