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A caspase-like decoy molecule enhances the activity of a paralogous caspase in the yellow fever mosquito, *Aedes aegypti*

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Abstract

Caspases are cysteine proteases that play critical roles in apoptosis and other key cellular processes. A mechanism of caspase regulation that has been described in mammals and nematodes involves caspase-like decoy molecules, enzymatically inactive caspase homologs that have arisen by gene duplication and acquired the ability to regulate other caspases. Caspase-like decoy molecules are not found in *Drosophila melanogaster*, raising the question of whether this type of caspase regulation exists in insects. Phylogenomic analysis of caspase genes from twelve *Drosophila* and three mosquito species revealed several examples of duplicated caspase homologs lacking critical catalytic residues, making them candidate caspase-like decoy molecules. One of these, CASPS18 from the mosquito *Aedes aegypti*, is a homolog of the *D. melanogaster* caspase Decay and contains substitutions in two critical amino acid positions, including the catalytic cysteine residue. As expected, CASPS18 lacked caspase activity, but co-expression of CASPS18 with a paralogous caspase, CASPS19, in mosquito cells or co-incubation of CASPS18 and CASPS19 recombinant proteins resulted in greatly enhanced CASPS19 activity. The discovery of potential caspase-like decoy molecules in several insect species opens new avenues for investigating caspase regulation in insects, particularly in disease vectors such as mosquitoes.

Keywords

apoptosis; *Drosophila*; insect; phylogenetic analysis

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INTRODUCTION

Caspases are a conserved family of cysteine proteases that play important roles in apoptosis and in other cellular processes (Li and Yuan, 2008). These enzymes are initially synthesized as inactive zymogens, which are cleaved upon activation into large (p20) and small (p10) subunits, releasing an N-terminal prodomain of varying length. The p20 and p10 subunits heterodimerize to form the active site, and two of these heterodimers further associate to form the active caspase holoenzyme. Following an appropriate signal, the first caspases activated are initiator caspases, which have the ability to auto-activate with the assistance of adaptor proteins. Activated initiator caspases cleave and activate effector caspases, which in turn target numerous substrates, leading to apoptosis and other signaling events.

The proteolytic activity of caspases is conferred by the catalytic dyad residues of the p20 subunit, which include a proton-donating histidine residue separated from the nucleophilic cysteine by ~40–50 amino acids, as well as additional neighboring residues in p20 and p10 that are highly conserved in caspases from invertebrates to mammals. Structural analysis has revealed that these conserved residues form a pocket surrounding the active site. Together, these key residues are responsible for substrate recognition and catalysis, and enzyme activity is compromised if they are altered (Fuentes-Prior and Salvesen, 2004).

Caspase zymogens are present in most cells, and their cleavage and activation must be carefully regulated. In mammals and in *Drosophila melanogaster*, an important mechanism of caspase regulation involves the inhibitor of apoptosis (IAP) proteins, which bind and inactivate caspases through a combination of active site inhibition and ubiquitylation (Wei et al., 2008). However, although the nematode *Caenorhabditis elegans* encodes proteins with homology to IAPs, they do not function in caspase regulation (Fraser et al., 1999). Instead, *C. elegans* encodes enzymatically inactive caspase homologs called CSP-2 and -3 which inhibit activation of the apoptotic caspase CED-3 (Geng et al., 2008; Geng et al., 2009). Similar inactive caspase homologs, termed caspase-like decoy molecules, are found in mammals and play important roles in regulation of caspases. Examples of these in mammals include COPI, INCA, ICEBERG, c-FLIP, and human caspase-12 (Lamkanfi et al., 2007). Some caspase-like decoy molecules are truncated caspase homologs, while others are full-length homologs that have substitutions in key amino acids involved in catalysis. While some caspase-like decoy molecules inhibit the activity of other caspases, there are also examples of caspase-like decoy molecules that have positive effects on other caspases. Despite their importance in caspase regulation in nematodes and mammals, caspase-like decoy molecules are not found in *D. melanogaster*, leading to the suggestion that this mode of regulation may not exist in insects (Brady and Duckett, 2009).

The genome of *D. melanogaster* encodes seven caspases, including the initiator caspases Dronc, Dredd, and Strica, and the effector caspases Damm, DrICE, Dcp-1, and Decay (reviewed in Yan et al., 2006). Of these, Dronc and DrICE are the key apoptotic caspases (Fraser et al., 1997; Quinn et al., 2000; Chew et al., 2004; Daish et al., 2004; Xu et al., 2005; Muro et al., 2006), with Dcp-1 playing a supportive role (Xu et al., 2006). *Drosophila* caspases also play important roles in diverse processes including innate immunity, ovarian cell death, and spermatogenesis (Leulier et al., 2000; Laundrie et al., 2003; Huh et al., 2004; Baum et al., 2007).

Recent phylogenomic analysis of caspases from *D. melanogaster* and two mosquitoes, *Aedes aegypti* and *Anopheles gambiae*, revealed interesting trends of gene duplication and/or loss in mosquitoes (Waterhouse et al., 2007; Bryant et al., 2008). Here we have taken advantage of the recent genome sequencing of eleven additional *Drosophila* species (Clark et al., 2007) and a third mosquito species, *Culex quinquefasciatus* (formerly known as *Culex pipiens*

quinquefasciatus), to examine the encoded caspases in fifteen Dipteran genomes. Only the initiator caspases Dronc and Dredd exhibit clear single-copy orthologous relationships across all fifteen genomes, while the other caspase clades exhibit extensive gene loss and/or duplication, even among the more closely related *Drosophilids*. Intriguingly, comparative sequence analysis revealed the presence, in both mosquitoes and *Drosophilids*, of several caspase homologs with substitutions in critical amino acids, suggesting that they are caspase-like decoy molecules. Functional analysis identified one of these caspase-like decoy homologs, CASPS18 from *A. aegypti*, as a positive regulator of a paralogous active caspase, CASPS19.

MATERIALS AND METHODS

Database mining and phylogenomic analysis

D. melanogaster caspases were used as queries to identify annotated caspase homologues in the additional eleven *Drosophila* genomes available at Flybase (<http://flybase.org/>). There are additional sequences in some of the *Drosophila* genomes with homology to caspases which may be unannotated caspase genes, but these were not included in this study. *A. gambiae* and *A. aegypti* caspase gene models were obtained from previously characterized annotations (Christophides et al., 2002; Waterhouse et al., 2007; Bryant et al., 2008). These mosquito caspases were employed to mine the *C. quinquefasciatus* genome available from the BROAD institute (http://www.broad.mit.edu/annotation/genome/culex_pipiens/Home.html). All curated mosquito gene models were submitted for public access to the ImmunoDB resource (<http://cegg.unige.ch/Insecta/immunodb/>) or to Flybase (<http://flybase.org/>). Available Expressed Sequence Tags (ESTs) served to confirm predicted gene model annotations and retrieval and assembly of sequence traces from the NCBI trace archives helped to improve possible whole genome assembly errors. The *A. gambiae* caspases S10 and S11 were excluded from analysis because they appear to be haplotypes of S13 and S5, respectively. Annotations of two of the available gene models for Dredd caspases, FBpp0214869/simulans and CqCASPL1 in *C. quinquefasciatus*, were improved by analyzing the trace archives at NCBI and the improved gene models grouped appropriately in Initiator Clade II.

A total of 132 amino acid sequences from fifteen species were used for phylogenomic analysis. The ~195 amino acid region comprising the active site and flanking sequences were used for alignment and tree building. Protein sequences were aligned using the Clustal W implementation of the MEGA3.1 suite (Kumar et al., 2004). Phylogenetic analysis was conducted using Neighbor Joining with software package PAUP* 4.0b10. Bootstrap values are based on 5,000 replicates.

Transfection and caspase assays

Transfections and caspase assays were performed as previously described (Bryant et al., 2008). Recombinant, C-terminally His₆-tagged CASPS18 and CASPS19 were expressed in *Escherichia coli* strain BL-21pLysS(DE)3 and purified using Talon resin (Clontech) as previously described (Means et al., 2006). cDNA from adult *A. aegypti* RexD mosquitoes was used as a template to isolate the full length cDNAs of *AeCASPS18*, *AeCASPS19* and *AeIAP1*. Caspases were cloned into the expression plasmid pHSP70PLVI+*Rpr*-epi (Vucic et al., 1997) by replacing *Rpr* with each caspase, yielding pHSP70PLVI+*AeCASPS19*-epi and pHSP70PLVI+*AeCASPS18*-epi, where epi indicates the presence of a C-terminal HA tag. *AeIAP1* was cloned into pHSP70PLVI+epi-*Opiap* (Vucic et al., 1997) by replacing *Opiap* with *AeIAP1*, yielding pHSP70PLVI+epi-*AeIAP1*. Transfections were done in C6/36 cells as previously described (Bryant et al., 2008). The total amount of DNA in each transfection was equalized using an irrelevant plasmid. At 24 hrs post-transfection, cells were analyzed for caspase activity as previously described (Bryant et al., 2008) by incubating lysate from

transfected cells with the human caspase-3 substrate DEVD-afc (MP Biomedicals) for 15 min, after which consecutive measurements were obtained at 15 min intervals.

To assess the ability of recombinant CASPS18 to enhance CASPS19 activity using DEVD-afc, 0.2 μ M CASPS19 and 0.1X (0.02 μ M), 0.5X (0.1 μ M), 1X (0.2 μ M), or 2X (0.4 μ M) CASPS18 were mixed together on ice, followed directly by addition of 100 μ M DEVD-afc. The reactions were then transferred to 37°C and fluorescence was measured every 15 min. For all substrates other than DEVD-afc, the conditions were the same except that 10-fold higher concentrations of CASPS19 and CASPS18 were used (2 μ M CASPS19 and 0.2, 1, 2, and 4 μ M CASPS18).

RESULTS

Phylogenomic analysis of Dipteran caspases

Phylogenomic analysis of 132 annotated caspase gene sequences from fifteen Dipteran genomes was performed using the conserved ~195 amino acid region surrounding the active site (Fig. 1 and Fig. S1). The analysis grouped the caspases into three initiator caspase clades and three effector caspase clades (Figs. 2 and 3). The caspases from *D. melanogaster* were used as the basis of comparison within the individual clades, since they are the best characterized in terms of both annotation and function. The gene number per clade for each species is summarized in Fig. S2.

Initiator Clades II and III, which include Dredd and Dronc, respectively, exhibited clear patterns of single-copy orthology among all fifteen species examined (Fig. 2). In contrast, Initiator Clade I, the Damm/Dream clade, exhibited complex patterns of gene duplications/losses, both within the genus *Drosophila*, as well as within the mosquitoes. *D. melanogaster* Damm and Dream arose by duplication after the divergence of flies and mosquitoes. In fact, within the *Drosophilids*, only the *melanogaster* and *obscura* groups contain orthologs of both Damm and Dream, while Dream but not Damm orthologs are present in the *willistoni*, *repleta*, *virilis*, and Hawaiian *Drosophila* groups, suggesting that Damm arose by duplication after the split of these groups. There have also been additional duplications and losses within the *Drosophilids*; *D. willistoni* contains four Dream homologs, while *D. mojavensis* contains three (Fig. 2). In *C. quinquefasciatus*, as previously shown for the other two mosquitoes (Bryant et al., 2008), these caspases experienced duplication events after the divergence of the three mosquito lineages. Although there were subsequent duplications within mosquitoes, these do not correspond to orthologs of Damm and Dream, but are instead orthologs of the ancestral Damm/Dream caspase.

Examination of Effector Clade I revealed that the *Drosophilids* all exhibit single-copy orthologs of *D. melanogaster* Decay (Fig. 3). Interestingly, however, multiple duplications in mosquitoes have resulted in a large group of Decay-like caspases. Within this group, CASPS19 appears as an orthologous pair in the *Culicinae* mosquitoes (*A. aegypti* and *C. quinquefasciatus*), and independent gene duplications/losses have yielded additional Decay paralogs CASPS18 in *A. aegypti* and CASPS30 and CASPS31 in *C. quinquefasciatus*, and at least seven paralogs in *A. gambiae* (Fig. 3).

Effector Clade II includes the main *D. melanogaster* apoptotic effector caspases DrICE and Dcp-1 (Fig. 3). Similar to Dream and Damm, DrICE and Dcp-1 arose by duplication after divergence of flies and mosquitoes. A clear duplication of Dcp-1 also occurred in the *obscura* group prior to the speciation of *D. pseudoobscura* and *D. persimilis*, and additional duplications have occurred in the DrICE subclade. Some sequence relationships within Effector Clade II are inconsistent with higher-level relationships between *Drosophilidae* and *Culicinae*. In the most extreme example, three *Drosophila* proteins (FBpp0237835/*virilis*,

FBpp0172086/mojavensis and FBpp0147044/grimshawi) do not cluster with the other main DrICE or Dcp-1 *Drosophila* proteins but are instead located basal to the group of mosquito CASPS7 sequences (Fig. 3). Interestingly, all three of the caspases in this group have substitutions in critical amino acid residues required for enzymatic activity (see below). Without strict catalytic functional constraints, these proteins may have experienced elevated divergence leading to the apparent inconsistency between the gene and species phylogenies (although they have remained grouped together, indicating they are not completely free of functional constraints). With regard to mosquito species in Effector Clade II, a single copy of CASPS7 is present in all three of the mosquito species analyzed, while CASPS8 has duplicated in *C. quinquefasciatus*, yielding caspases CASPS8 and CASPS29.

Effector Clade III appears as a *Culicinae*-specific group, containing CASPS20 from *A. aegypti* and six paralogs in *C. quinquefasciatus* (Fig. 3). Additional Dipteran species will need to be examined to determine whether Effector Clade III is truly specific to *Culicinae* mosquitoes.

Gene duplications leading to the evolution of putative decoy caspase molecules

In addition to the conserved GluAlaCys(Arg/Glu/Gly)(Gly/Asp) sequence found in the core active site of caspases, other key residues for catalysis include conserved Arg, His and Gly residues N-terminal to the core active site, and another conserved Arg located in p10 (Fuentes-Prior and Salvesen, 2004) (Fig. 1). As expected, we found strict conservation of these critical residues in most of the caspases examined. However, eight of the 132 caspases analyzed exhibited substitutions in one or more of these residues, and several of these genes appeared to be products of recent duplication events (Table I and Figs. 2 and 3).

For example, the protein sequences of FBpp0147044/grimshawi and FBpp0172086/mojavensis both lack critical residues and these genes appear to have been generated from duplication events in Effector Clade II, since both *D. mojavensis* and *D. grimshawi* have more copies of these genes in this clade than *D. melanogaster*. Likewise, FBpp0170482/mojavensis (Initiator Clade I), FBpp0247059/willistoni (Initiator Clade I), and FBpp0237835/virilis (Effector Clade II) each lack one or both of the conserved Arg residues, and each are located in the fruitfly genomes near paralogs that are predicted to be active caspases (Table 1). In *A. aegypti*, CASPS18 (Effector Clade I) encodes a Ser instead of the catalytic Cys and a Lys instead of a conserved Arg and appears to be a duplication of CASPS19. In *C. quinquefasciatus*, CASPS31 (Effector Clade I) exhibits substitutions of conserved His, Cys and Arg residues and is in close genomic proximity to CqCASPS30 and CqCASPS19, supporting the likely duplication events suggested by phylogenetic analyses. Similarly, in Effector Clade III, CqCASPS24 lacks the conserved Cys and Arg residues while the paralogous CqCASPS25 and CqCASPS26 genes encode all the critical residues, and all three genes are present on the same sequence contig, again suggesting recent duplication events.

The caspase-like decoy molecule CASPS18 enhances CASPS19 activity

We reported previously that *A. aegypti* CASPS18, which has substitutions in its active site Cys and in a conserved Arg, and its paralog CASPS19 are expressed in similar developmental expression patterns (Bryant et al., 2008). Thus, we considered the possibility that CASPS18 may regulate the function of CASPS19, similar to observations of caspase-like decoy molecules in other organisms. To examine this, CASPS18 and CASPS19 were expressed in *Aedes albopictus* C6/36 cells. Caspase activity was measured using DEVD-afc, an effector caspase substrate. Caspase activity was observed when CASPS19 was expressed, while expression of CASPS18 did not result in measurable activity, consistent with CASPS18 lacking a catalytic cysteine (Fig. 4). Interestingly, however, the cells expressing CASPS19 did not die, despite the presence of a large amount of effector caspase activity. Importantly, this lack of

apoptosis strongly indicates that the observed caspase activity was due to the overexpressed CASPS19, and not to the activation of endogenous effector caspases such as *A. albopictus* homologs of *A. aegypti* CASPS7 and CASPS8, which cause apoptosis when over-expressed in C6/36 cells. The caspase activity resulting from CASPS19 expression was inhibited by co-expression of AeIAP1, the ortholog of the caspase inhibitor DIAP1 (Fig. 4).

Intriguingly, co-expression of CASPS19 and CASPS18 resulted in significantly enhanced caspase activity compared to CASPS19 alone (Fig. 4), even though CASPS18 had no activity when expressed by itself. Again, cell death was not observed when the two caspases were co-expressed, suggesting that the increased activity was not due to the activation of other effector caspases, but rather to an increase in either the amount or activity of CASPS19, or to a reduction in the amount or activity of an inhibitor, such as AeIAP1.

In order to further investigate the mechanism of CASPS19 enhancement by CASPS18, recombinant CASPS18 and CASPS19 proteins were expressed in bacteria and purified. Similar to what was observed when the caspases were expressed in mosquito cells, recombinant CASPS18 did not have significant caspase activity, while recombinant CASPS19 exhibited significant activity against DEVD-afc (Fig. 5). Furthermore, addition of increasing ratios of CASPS18 to CASPS19 recombinant protein directly enhanced CASPS19 activity against DEVD-afc (Fig. 5). Addition of 0.1X CASPS18 had no observable effect, but 0.5X, 1X, and 2X CASPS18 significantly increased the ability of CASPS19 to cleave DEVD-afc. CASPS19 was more active in cleaving DEVD-afc than the initiator caspase substrates IETD-afc, LEHD-afc, or YVAD-afc, or the substrates VEID-afc or VDVAD-afc, but addition of 2X CASPS18 enhanced CASPS19 activity against all of the substrates tested (Fig. S4). These results indicate that CASPS18 enhances CASPS19 activity directly, not by influencing the expression of CASPS19 or the activity or expression of an inhibitor.

DISCUSSION

While the use of model organisms such as *D. melanogaster* has led to tremendous advancements in knowledge, model organisms may not be entirely representative of the large phylogenetic groups in which they reside. This study offers an example of the power of using comparative genome analysis of model and non-model organisms in combination with knowledge gained from studying model organisms. Our results illustrate that expansions and losses of caspase genes have occurred in the *Diptera*. Even within the genus *Drosophila*, many of the initiator and effector caspases have undergone gene expansions and losses when compared to *D. melanogaster*. Furthermore, gene expansion in the *Diptera* has been accompanied by the evolution of caspase genes which lack critical residues required for enzyme activity, and at least one of these caspase-like genes is able to positively regulate the activity of a paralogous caspase. The discovery of caspase-like decoy molecules in insects will complement the extensive knowledge gained from studying caspase regulation in *D. melanogaster*, and lead to a more in-depth and comprehensive understanding of caspase regulation in insects as a whole.

These results also add significantly to our understanding of caspase evolution in Dipteran insects, which include numerous important vectors of human disease. Several interesting points emerge from our analysis. The Dipteran initiator caspases Dronc and Dredd are present in each of the species we examined as single-copy orthologs. However, all of the other caspase clades have undergone frequent gene duplication/loss events. Extensive gene duplications/losses have been reported in the twelve *Drosophila* species for many other gene families, with over 40% of all gene families analyzed differing in size (Hahn et al., 2007). Gene losses have also been reported for caspase-18, 17 and 15 in vertebrates (Eckhart et al., 2008), which have been lost in humans but retained in other vertebrate species. Previous analyses comparing caspase

homologs in mosquitoes versus *D. melanogaster* have led to speculation that there is some specific requirement for additional caspases specifically in mosquitoes, perhaps due to their requirement for blood feeding. It is now clear that expansions in the number of caspase homologs have also occurred in other *Drosophila* species as well.

Enzymatically inactive caspases with the ability to regulate other caspases, known as caspase-like decoy molecules, have been reported in mammals and in nematodes. In humans, the caspase-1 gene has undergone a series of duplications resulting in the caspase-like decoy molecules COPI, INCA, and ICEBERG. These genes express truncated forms of caspase-1 that interfere with caspase-1 activation (reviewed in Lamkanfi et al., 2007). Similarly, *csp-2* and *csp-3* from *C. elegans* encode truncated caspase-like decoy molecules that bind to the zymogen form of the caspase CED-3 and inhibit CED-3 activation (Geng et al., 2008; Geng et al., 2009). In addition to these truncated molecules, there are examples of caspase-like decoy molecules that encode full-length caspase proteins, but which lack amino acids that are critical for enzymatic activity. In mammals, duplication of caspase-8 resulted in c-FLIP. While the splice variant c-FLIP_S encodes a truncated molecule which inhibits caspase-8, the variant c-FLIP_L is similar to full length caspase-8, but lacks several key amino acids, including the active site Cys. Interestingly, c-FLIP_L can regulate caspase-8 in both a negative and a positive manner (Micheau et al., 2002; Budd et al., 2006). Negative regulation by c-FLIP_L is due to inhibition of caspase-8 recruitment to its activation complex, but positive regulation is due to heterodimerization between c-FLIP_L and caspase-8, resulting in auto-activation of caspase-8. Another interesting example is human caspase-12, which has substitutions in key amino acids leading to a loss of enzymatic activity in the majority of the human population. Enzymatically active caspase-12 is associated with a higher risk of sepsis in humans (Saleh et al., 2004), suggesting that mutations that inactivate caspase-12 activity are beneficial. In mice, caspase-12 has been shown to bind to caspase-1 and inhibit its activity, and this inhibitory function is retained in active site mutants of caspase-12 (Saleh et al., 2006).

In this study, we have identified several insect caspase genes that encode full-length proteins with substitutions in amino acids that are known to be critical for enzymatic activity. One of these, *A. aegypti* CASPS18, is a paralog of the effector caspase Decay in *D. melanogaster* and CASPS19 in *A. aegypti*. Our results show that CASPS18 enhances the activity of its functional paralog CASPS19. This is the first report of an effector-type caspase-like decoy molecule in any organism, as the other caspase-like decoy molecules known are all paralogs of initiator caspases. CASPS18 and CASPS19 are expressed in similar developmental stages and tissues in *A. aegypti* (Bryant et al., 2008), consistent with a role for CASPS18 in regulating CASPS19 activity. Interestingly, high levels of CASPS19 activity did not cause apoptosis in C6/36 cells. Although this may seem surprising given that CASPS19 is able to cleave DEVD-afc with some degree of efficiency, this does not necessarily mean that the sequence DEVD is its optimal substrate cleavage site. Presumably, the in vivo substrates for CASPS19 differ from those of effector caspases involved in apoptosis. Consistent with this result, high levels of active Decay were recently shown to be present in dying midgut during *D. melanogaster* morphogenesis, but Decay was not required for midgut cell death (Denton et al., 2009). These results suggest that Decay orthologs may function in a non-apoptotic process, such as immunity. Although speculative, a possible role in immunity is also supported by the observation that CASPS18 is up-regulated in response to Dengue virus infection in *A. aegypti* (Xi et al., 2008).

The direct enhancement observed using recombinant proteins suggests that the mechanism of CASPS19 enhancement by CASPS18 is direct, rather than, for example, CASPS18 binding to a cellular inhibitor of CASPS19. This direct mechanism may involve heterodimerization between CASPS18 and CASPS19 subunits, resulting either in higher enzyme activity or in more efficient auto-activation of CASPS19, similar to what is seen in caspase-8 activation by c-FLIP_L. It should be noted that we cannot completely rule out the possibility that CASPS18

has catalytic activity, which somehow becomes activated when co-expressed with CASPS19, perhaps by heterodimerization. The presence of a serine rather than a cysteine in the active site could theoretically still allow nucleophile attack on the carbonyl carbon of the substrate scissile bond, and CASPS18 does still contain the necessary proton-accepting histidine residue in the active site. Although speculative, it is interesting to note that, compared to CASPS19 and Decay, CASPS18 has a four-amino acid insertion immediately downstream of the active site, perhaps to allow alternative contacts with substrate.

The discovery of these putative caspase-like decoy molecules in several Dipteran insects provides new avenues for research on the mechanisms of caspase regulation in insects. In particular, the potential positive or negative roles of caspases in transmission of disease agents by insect vectors can now be more completely investigated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

IAP inhibitor of apoptosis

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Fig. 1. Conserved residues in the caspase active site. A, The domains of a typical caspase and the positions of the critical residues which surround the core active site are shown. The region used for sequence alignment is indicated by a bracket. B, Structure of the active site of human caspase-3. Critical residues surrounding the active site are indicated in yellow. The model was produced using NCBI PDB ID: 2CNN and 3D Molecule Viewer from Vector NTI Advance 10.3.0 (Invitrogen).

Fig. 2.

Neighbor-joining tree illustrating phylogenetic relationships among Dipteran initiator caspases. The tree was constructed using both initiator and effector sequences, but for purposes of presentation, only the initiator caspase clades are shown here, while the effector caspase clades are shown in Fig. 3. The intact tree is presented in Fig. S3. *D. melanogaster* genes are shown in black, while other *Drosophila* species are shown in blue, *A. aegypti* is shown in red, *C. quinquefasciatus* is shown in green, and *A. gambiae* is shown in purple. Arrows indicate potential caspase-like decoy molecules. Relatively recent species-specific gene duplication events are indicated by circles. Numbers above branches indicate bootstrap support where

greater than 50%. In the case of sequences that are labeled by numbers followed by a species name, the numbers represent Flybase ID numbers.

Fig. 3. Neighbor-joining tree illustrating phylogenetic relationships among Dipteran effector caspases. Relatively recent gene duplication events are indicated as follows: circles indicate species-specific duplications, stars indicate *Culicinae*-specific duplications, and a square indicates a duplication in a *D. pseudoobscura*-*D. persimilis* common ancestor. For additional details, see the legend for Fig. 2.

Fig. 4.

Expression of CASPS18 in cells enhances caspase activity stimulated by expression of CASPS19. The indicated combinations of CASPS18, CASPS19, and AelAP1 were expressed in C6/36 cells, and caspase activity was measured in the resulting cell lysates by analyzing DEVD-afc cleavage at the times shown following addition of substrate. Values shown represent the mean $\pm$ s.e.m. of three independent experiments.

Fig. 5. Recombinant CASPS18 protein enhances the activity of CASPS19 *in vitro*. The activity of purified recombinant CASPS18, CASPS19, or mixtures of both caspases (0.1:1, 0.5:1, 1:1, or 2:1 ratios of CASPS18:CASPS19) was measured using DEVD-afc as a substrate. Values shown represent the mean $\pm$ s.e.m. of three independent experiments.

Table 1

Putative Dipteran caspase-like decoy molecules

Gene	Conserved residues					R	Clade	Neighboring caspases
	R	H	G	C	T			
FBpp0170482_mojavensis	no/H ¹	no/L	no/C	no/T	yes	yes	Init I	FBpp0170483, FBpp0167608
FBpp0247059_willistoni	yes	no/Y	yes	no/S	no/I	no/I	Init I	FBpp0247057, FBpp0247058, FBpp0247031
FBpp0147044_grimshawi	no/L	no/N	yes	yes	no/G	no/G	Eff II	none
FBpp0172086_mojavensis	yes	yes	yes	yes	no/S	no/S	Eff II	none
FBpp0237835_virilis	no/K	yes	yes	yes	no/S	no/S	Eff II	FBpp0233969
CqCASP31	yes	no/Y	yes	no/W	no/E	no/E	Eff I	CqCASP30, CqCASP19
CqCASP24	yes	yes	yes	no/P	no/L	no/L	Eff III	CqCASP25, CqCASP26
AeCASP18	yes	yes	yes	no/S	no/K	no/K	Eff I	AeCASP19

¹ Presence of the conserved residue is indicated by “yes”, while absence is indicated by “no” followed by the residue that is present instead