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Alternative splicing generates multiple transcripts of the Inhibitor of Apoptosis Protein 1 in *Aedes* and *Culex* spp. mosquitoes

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Abstract

We determined the sequences of cDNA encoding Inhibitor of Apoptosis Protein 1 (IAP1) homologues from *Aedes triseriatus*, *Ae. albopictus*, *Ae. aegypti*, *Culex pipiens* and *Cx. tarsalis*. The cDNAs encode translation products that share ≥84% sequence similarity. The IAP1 mRNA of each mosquito species exists as 3 to 5 distinct variants due to the presence of heterogeneous sequences at the distal end of their 5'UTRs. Partial genomic sequencing upstream of the 5' end of the *Ae. triseriatus* IAP1 gene, and analysis of the *Ae. aegypti* genomic sequence, suggest that these mRNA variants are generated by alternative splicing. Each IAP1 mRNA variant from *Ae. triseriatus* and *Cx. pipiens* was detected by RT-PCR in all mosquito life-stages and adult tissues examined, and the relative concentration of each *Ae. triseriatus* IAP mRNA variant in various tissues was determined.

Keywords

Inhibitor of apoptosis; Alternative splicing; *Aedes* mosquitoes; *Culex* mosquitoes

INTRODUCTION

Apoptosis, or programmed cell death, is an evolutionarily conserved process that regulates the normal development and homeostasis of multicellular organisms by removing unwanted, damaged, mutated, or infected cells (Reed, 2000; Zimmerman et al., 2001; Twomey and McCarthy, 2005). Inhibitor of Apoptosis Proteins (IAPs) play a key role in this process (Deveraux and Reed, 1999; Salvesen and Duckett, 2002). IAPs were first identified in baculoviruses and have since been identified in other viruses, invertebrates and vertebrates (Crook et al., 1993; Deveraux and Reed, 1999; Salvesen and Duckett, 2002). IAPs bind to and inhibit the activity of caspases, a family of cysteine proteases that orchestrate rapid cellular destruction by cleaving various target proteins (Shi, 2002; Shi, 2004). The anti-apoptotic activities of IAPs are antagonized by IAP binding motif (IBM) proteins, a family of proapoptotic proteins that share an IAP-binding tetrapeptide motif (Bergmann et al., 2003; Vaux and Silke, 2003; Verhagen et al., 2006). Members of this family include Reaper, Hid, Grim, Sickle, and Jafrac2 in *Drosophila*, michelob_x in *Anopheles gambiae*, the michelob_x homolog in *Aedes aegypti*, and Smac/DIABLO, Omi/HtrA2 and GSPT1/eRF3 in mammals.

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The defining characteristic of an IAP is the presence of at least one baculovirus IAP repeat (BIR) domain, with many IAPs also containing a C-terminal RING finger motif (Miller, 1999; Salvesen and Duckett, 2002). At least one BIR is required for anti-apoptotic activity, whereas the requirement for the RING motif depends upon the IAP and/or death-inducing stimuli. BIRs contain a highly conserved arrangement of cysteine and histidine residues that belong to a putative zinc-binding fold. BIRs mediate the interactions between IAPs and various other proteins, including caspases and IBM proteins (Roy et al., 1997; Vucic et al., 1997; Salvesen and Duckett, 2002). BIRs also mediate the formation of IAP homodimers, the apparent functional form of these proteins (Hozak et al., 2000). While BIRs are present in all IAPs, they are not exclusive to IAPs. In the nematode *Caenorhabditis elegans*, the BIR-1 protein contains a BIR domain, but functions in regulating cytokinesis rather than the inhibition of apoptosis (Fraser et al., 1999). BIR containing proteins that function in cell division have also been described in yeast and mammals (Verhagen et al., 2001). RING motifs can function as E3 ligases by binding to E2 ubiquitin-conjugating enzymes and recruiting E2s to target proteins (Huang et al., 2000; Yang et al., 2000; Olson et al., 2003). IAPs have been shown to catalyze the ubiquitination of caspases and various IBM proteins, targeting them for proteasomal degradation. The ring motifs of IAPs can also promote auto-ubiquitination and subsequent degradation, which presumably promotes apoptosis.

Only a few apoptosis-related genes from mosquitoes have been characterized, despite the central role that these vectors play in disease transmission (Blitvich et al., 2002; Zhou et al., 2005; Li et al., 2007). Studies on apoptosis regulation in insects have mostly focused on *Drosophila* (Kornbluth and White, 2005). However, the recent completion of the genome sequence of *Anopheles gambiae*, the principal malaria vector, has provided considerable insight into the genetic basis of apoptotic regulation in mosquitoes (Christophides et al., 2002). Interestingly, a larger number of apoptosis-related genes are encoded in the genome of *An. gambiae* than *Drosophila*, possibly due to the role that mosquitoes play in pathogen transmission. For example, 7 IAPs were identified in *An. gambiae* whereas only 4 IAPs (designated DIAP1, DIAP2, Deterin and Bruce) exist in *Drosophila* (Hay et al., 1995; Jones et al., 2000; Christophides et al., 2002; Vernooy et al., 2002). The recent availability of the *Ae. aegypti* genome sequence, the principal vector of both yellow fever and dengue viruses, should further increase our knowledge of mosquito apoptotic regulation (<http://msc.tigr.org/aedes/aedes.shtml>).

We recently identified and characterized a DIAP1 homologue from the La Crosse virus vector, *Ae. triseriatus* (Say) (designated AtIAP1) (Blitvich et al., 2002). The AtIAP1 translation product is a 403 amino acid protein that contains 2 BIRs and a RING finger motif. AtIAP1 mRNA was detectable by RT-PCR using primers that amplified a 1461 nt region (nt -252 to +1209) in all mosquito life-stages (embryos, first–fourth instar larvae, early and late pupae, adults) and adult tissues (midguts, ovaries, salivary glands) examined. In contrast, the AtIAP1 protein was detectable by western blot only in certain developmental stages (first instar larvae, early pupae, adults) and tissues (ovaries). Taken together, these data suggest that expression of AtIAP1 is post-transcriptionally regulated. More recently, an IAP1 from *Ae. albopictus* (designated AaIAP1) was characterized; this is the only other mosquito gene with significant homology to a known apoptotic suppressor to be studied in detail (Li et al., 2007). Expression of AaIAP1 protected lepidopteron cells from Hid-induced apoptosis, and vertebrate cells from bluetongue virus-induced apoptosis.

The 5' untranslated region (5'UTR) in the AtIAP1 mRNA is unusually long, and possesses significant secondary structure when analyzed using the MFOLD RNA folding server (<http://bioweb.pasteur.fr/seqanal/interfaces/mfold-simple.html>), suggesting that it has an important role in post-transcriptional regulation (Blitvich et al., 2002). Unusually long 5'UTRs are also present in the mRNAs of other IAPs, such as X-linked inhibitor of apoptosis (XIAP) and human

inhibitor of apoptosis 2 (HIAP2) (1.6 kb and 1.2 kb, respectively), suggesting that this feature is common among IAPs. The 5'UTR of the XIAP1 mRNA contains an internal ribosomal entry site (IRES) that regulates translation in response to serum starvation and low dose gamma irradiation, but not etoposide-induced apoptosis, in human cell lines (Holcik et al., 1999; Holcik and Korneluk, 2000; Nevins et al., 2003). HIAP2 translation is regulated by an inducible IRES in response to endoplasmic reticulum stress (Warnakulasuriyarachchi et al., 2004). Translation of HIAP2 mRNA has also been shown to be regulated by a short 5'UTR open reading frame (μ ORF) located just 8 nt upstream of the HIAP2 initiation codon, suggesting that multiple post-transcriptional mechanisms can regulate IAP translation (Warnakulasuriyarachchi et al., 2003).

In our previous study, we identified heterogeneous sequences at the distal end of the 5'UTR in the *AtIAP1* mRNA (Blitvich et al., 2002). Four cDNA clones were analyzed by 5' rapid amplification of cDNA ends (5' RACE) and automated sequencing. The 5'UTRs encoded by these cDNAs varied in length. The 401 nt immediately upstream of the open reading frame (ORF) were identical for each clone, but the distal nucleotide sequences possessed no significant similarity. At the time, it was not known whether this represented an artifact of our experimental approach, a splicing event, or unusual genomic organization. To investigate this phenomenon further, the *AtIAP1* gene and additional *AtIAP1* cDNA clones were sequenced, as were cDNAs encoding IAP1s from *Ae. albopictus* (Skuse), *Ae. aegypti* (Linnaeus), *Culex pipiens* Linnaeus and *Cx. tarsalis* Coquillett.

MATERIALS AND METHODS

Mosquitoes

Ae. triseriatus (Say) mosquitoes (originally collected as eggs in La Crosse, Wisconsin) were reared at 24°C and 75% relative humidity with a photocycle of 16:8 (L:D). *Ae. albopictus* (Skuse) mosquitoes (originally collected as eggs in San Juan, Puerto Rico) were reared at 27°C and 80% relative humidity with a photocycle of 12:12 (L:D). *Ae. aegypti* (Linnaeus) mosquitoes (also collected as eggs in Puerto Rico) were reared at 27°C and 80% relative humidity with a photocycle of 12:12 (L:D). *Cx. pipiens* Linnaeus mosquitoes (obtained from Iowa State University in 2002) were reared at 27°C and 80% relative humidity with a photocycle of 12:12 (L:D). *Cx. tarsalis* Coquillett mosquitoes (obtained from Bakersfield, California in 2004) were reared at 27°C and 80% relative humidity with a photocycle of 12:12 (L:D). Larvae and pupae were reared in 15 × 25 × 15 cm polypropylene plastic trays containing approximately 500 ml of tap water supplemented with 30 mg of pulverized tetramin fish food/mouse chow (1:1).

RNA isolation

Total RNA was extracted from whole adult mosquitoes (*Ae. triseriatus*, *Ae. albopictus*, *Ae. aegypti*, *Cx. pipiens*, *Cx. tarsalis*) and from various developmental stages (embryos, larvae, pupae) and organs (ovaries, midguts, salivary glands) of *Ae. triseriatus* and *Cx. pipiens* using Trizol Reagent (Invitrogen, Carlsbad, CA). Briefly, whole mosquitoes were homogenized in 2 ml of Trizol Reagent using a mortar and pestle on ice. Organs were dissected from adult female mosquitoes (n = 20) and homogenized in 200 μ l of Trizol Reagent by vigorous pipetting and vortexing. Numbers of whole mosquitoes used in each reaction are as follows: embryos (5000), first instar larvae (500), second instar larvae (50), third instar larvae (40), fourth instar larvae (30), early pupae (20), late pupae (20) and adults (20). Following homogenization, total RNA was extracted following the manufacturer's instructions.

Reverse transcription

An aliquot of total RNA (1–2 µg) was mixed with 100 ng of poly(A) tail-specific primer (5'-GGC CAC GCG TCG ACT AGT ACT TTT TTT TTT TTT TTT T-3') and heated at 70°C for 10 min. After briefly chilling on ice, the RNA was reverse transcribed at 42°C for 1 hour using 200 units of Superscript II reverse transcriptase (Invitrogen, Gaithersburg, MD) with each dNTP at 500 µM in 1x reaction buffer [20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂, 10 mM DTT]. Samples were heated at 70°C for 15 min and treated with 2 units of RNase H (Invitrogen, Gaithersburg, MD) at 37°C for 20 min.

Polymerase chain reaction

PCR amplifications were performed using 1 µl of cDNA template, 0.1 unit of *Taq* polymerase, 30 ng of each primer and each dNTP at 200 µM in 1x PCR buffer [20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂]. Reactions were performed as follows: 94°C for 3 min, thirty cycles of 94°C for 45 sec, 50°C for 45 sec and 72°C for 2 min, followed by a final extension at 72°C for 7 min. PCR amplifications were first performed using degenerate primers designed from conserved regions in the *AtIAP1* and *DIAP1* cDNA sequences (forward: 5'-TGC TTC AGY TGC GGY GGY GGY CTC AWG GAT TGG-3'; reverse: 5'-GGT RAA SGG YTT CCG RCA SAG CGG ACA CTT-3'). The resulting PCR products were sequenced and used to design additional primers. The nucleotide sequence of the reverse primer used to amplify the extreme 3' end of each *IAP1* cDNA matched the 5' half of the poly(A)-specific reverse transcription primer (5'-GGC CAC GCG TCG ACT AGT AC-3'). Control RTPCRs were performed using the *Cx. pipiens* actin-1 cDNA sequence (Genbank Accession DQ023309; forward primer: 5'-ATG TGG GAC GAA GAA GTT GCT GCT C-3', reverse primer: 5'-GCT CAA TGG GAT ACT TCA GCG TCA G-3').

5' RACE

The nucleotide sequences at the 5' end of each *IAP1* mRNA were determined by a series of 5' RACE (5' rapid amplification of cDNA ends) reactions. Briefly, total RNA was reverse transcribed using an *IAP1*-specific primer. Complementary DNAs were purified using a QIAquick spin column (QIAGEN, Chatsworth, CA) and oligo(dC) tails were added to the 3' ends using 15 units of terminal deoxynucleotidyl transferase (Invitrogen, Gaithersburg, MD) in 1x tailing buffer [10 mM Tris-HCl (pH 8.4), 25 mM KCl, 1.5 mM MgCl₂ and 0.02 mM dCTP]. Tailing reactions were performed at 37°C for 30 min and then terminated by heat-inactivation (65°C for 10 min). Oligo dC-tailed cDNAs were purified by ethanol precipitation, then PCR amplified using a consensus forward primer specific to the C-tailed termini (5'-GAC ATC GAA AGG GGG GGG GGG-3') and one of several reverse primers specific to the mosquito *IAP1* cDNA sequence.

cDNA cloning and sequencing

PCR products were inserted into the pCR4-TOPO cloning vector (Invitrogen, Carlsbad, CA) and ligated plasmids were transformed into competent TOPO10 *E. coli* cells (Invitrogen, Carlsbad, CA). Cells were grown on LB agar containing ampicillin (50 µg/ml) and kanamycin (50 µg/ml), and colonies were screened for inserts by PCR amplification. An aliquot of each PCR product was examined by 1% agarose gel electrophoresis and several PCR products were purified using a QIAquick spin column (QIAGEN, Chatsworth, CA) and sequenced using an ABI 377 DNA sequencer at Colorado State University.

Quantitative Reverse Transcriptase PCR of *AtIAP1* Variants

RNA was extracted from *Ae. triseriatus* life stages and tissue types according to the previously described methods; however, the number of individuals per sample was reduced to the following: embryos (100), first instar larvae (50), second instar larvae (25), third instar larvae

(10), fourth instar larvae (5), pupae (5), adult males (5), non-blood-fed female adults (5), and 24 hours post blood-fed female adults (5). Larvae and pupae were collected less than 24 hours after molting. Ovarian, midgut, and salivary gland tissue extractions were performed on tissue dissected from five adult female mosquitoes. All groups were sampled in triplicate. After RNA extraction each sample was analyzed using the Qiagen Quantitect SYBR Green RT-PCR kit (Qiagen, Chatsworth, CA) to determine the quantities of each mRNA splice variant and actin (primer sequences can be found in Table 1) on an Opticon 2 Real-Time Thermocycler (Bio-Rad, Hercules, CA). Reactions were run in duplicate and amplified according to the manufacturer's protocols (using 20 μ L reactions instead of 50 μ L reactions). The fluorescence threshold was set at 0.014 using the logarithmic scale and the CT values for each sample were recorded and averaged among duplicates. The average CT value of each sample was then averaged among triplicate samples of a particular life stage or tissue type. For a given life stage or tissue type, the Δ CT for each AtIAP1 mRNA variant was calculated by subtracting the lowest AtIAP1 mRNA variant CT value for that stage or tissue from the remaining CT values for that stage or tissue. The Δ CT value was then used as an exponent for the number two to determine the fold difference between the most abundant mRNA variant (the lowest CT value) and the other AtIAP1 mRNA variants (in a perfect PCR reaction each amplification cycle theoretically represents a doubling of the amplicon or conversely a two-fold decrease in the amount of the original target RNA). The reciprocal of this value was calculated (this does not change the relative concentrations of each mRNA variant, but serves to make the graph more intuitive because larger bars represent a greater amount of transcript). The most abundant AtIAP1 mRNA variant was set at a relative frequency of 1. An example calculation for the quantification of mRNA variant 1 of the pupal life stage follows:

Average Sample AtIAP variant CT = (CT for pupae sample 1 replicate 1 AtIAP variant 1 + CT for pupae sample 1 replicate 2 AtIAP variant 1)/2

Life stage average AtIAP variant CT = (pupae sample 1 average sample AtIAP variant CT + pupae sample 2 average sample AtIAP variant CT + pupae sample 3 average sample AtIAP variant CT)/3

Δ CT for sample = Life stage average AtIAP variant CT – Lowest life stage Average AtIAP variant CT at the particular life stage

Variant Fold Difference compared to most abundant mRNA variant = 2^{Δ CT for life stage

Genomic sequencing of *AtIAP1*

Because of the lack of an *Ae. triseriatus* genome sequence database, the genomic sequence of *AtIAP1* was determined using the Universal GenomeWalker Kit (Clontech, Palo Alto, CA). *Ae. triseriatus* DNA was extracted from colony mosquitoes and digested according to the manufacturer's specifications. Adaptors were then ligated to the digested DNA and PCR amplified using a gene specific primer in conjunction with an adaptor specific primer. Several walks were done to obtain as much sequence upstream of the *AtIAP1* gene as possible.

Genomic sequence analysis of *AeIAP1*

The locations of the heterogeneous sequences of the *Ae. aegypti* inhibitor of apoptosis 5'UTR were determined by a BLAST search of the *Ae. aegypti* genome on the TIGR website (<http://msc.tigr.org/aedes/aedes.shtml>). This search revealed that the *AeIAP1* gene is located on supercontig 1.368 of the genome (GenBank accession no. [CH477553](#)). Using this sequence, we were able to determine the location of the 5' heterogeneous UTR sequences in reference to the coding region of the *AeIAP1* gene.

RESULTS

Comparison of the ORFs

Using a combination of RT-PCR and 5'-RACE, we identified homologues of the *Drosophila* IAP1 in *Ae. triseriatus*, *Ae. albopictus*, *Ae. aegypti*, *Cx. pipiens* and *Cx. tarsalis* mosquitoes (designated AtIAP1, AaIAP1, AeIAP1, CpIAP1 and CtIAP1, respectively). The IAP1 cDNAs encode predicted translation products of 401 (*Ae. aegypti*), 402 (*Ae. albopictus*), 403 (*Ae. triseriatus*), 409 (*Cx. pipiens*) and 410 (*Cx. tarsalis*) amino acids (Fig. 1). Alignment of the deduced amino acid sequences revealed that the IAP1s from *Cx. pipiens* and *Cx. tarsalis* shared the greatest similarity (96% similarity, 93% identity), and the IAP1s from *Ae. albopictus* and *Cx. tarsalis* were the most divergent (84% similarity, 80% identity). Each amino acid sequence contains two BIR domains (BIR1 and BIR2) and a C-terminal RING finger. Fifty-three of the 65 (82%) amino acids in the BIR1 are strictly conserved between all mosquito species examined. Species with the most similar BIR1s were *Cx. pipiens* and *Cx. tarsalis* (97% similar, 97% identical). Sixty-one of the 66 (92%) amino acids in the BIR2 are also strictly conserved between all species examined. Species with the most similar BIR2s were *Cx. pipiens* and *Cx. tarsalis* (98% similarity, 100% identity). The 36-amino acid RING finger of each mosquito IAP1 is identical.

The predicted AaIAP1 translation product consists of 402 amino acids, assuming that the first ATG of the cDNA sequence serves as the initiation codon. The context of this codon (gatATGG) conforms to the consensus sequence (a/gccATGG) required for optimal translation initiation at the critical -3 and +4 positions (Kozak, 1986). Three other in-frame ATG codons are located shortly downstream, including one at position +13 to +15 which aligns with the predicted initiation codons for AtIAP1, CpIAP1 and CtIAP1 (Fig. 1). However, this ATG was not selected as the AaIAP1 initiation, codon because it is not in the context required for optimal translation initiation. The first in-frame ATG of the AeIAP1 cDNA sequence was also selected as the initiation codon. This context of this codon (ataATGG) conforms to the consensus sequence required for optimal translation initiation at the critical -3 and +4 positions. Two other in-frame ATG codons are located shortly downstream, including one that aligns with the predicted initiation codons for the AtIAP1, CpIAP1 and CtIAP1 but does not conform to the context required for optimal translation initiation. While the ATG surrounded by the optimal translation initiation sequence is the most likely translation initiation codon in both AaIAP1 and AeIAP1, the possibility that one of suboptimal ATG codons is the actual translation start site or an additional translation initiation site involved in increased translation efficiency cannot be ruled out (Kochetov, 2005).

Identification of multiple IAP1 mRNA variants

Multiple IAP1 mRNA variants were identified in each mosquito spp. (Table 2). Three IAP1 mRNA variants were detected in *Ae. aegypti* and *Cx. pipiens*, four were detected in *Ae. albopictus* and *Cx. tarsalis*, and five were detected in *Ae. triseriatus*. These variants contain different sequences at the distal ends of their 5'UTRs, as well as a long homogeneous 5'UTR region ranging from 305 nt (*Cx. tarsalis*) to 401 nt (*Ae. triseriatus*). Alignments of the heterogeneous 5' UTR sequences can be found in the supplemental information (Supplemental Figs. 1A-1E). RNA folding predictions of each 5' UTR sequence can also be found in the supplemental information (Supplemental Figs 2A-2E). Secondary structure predictions differ between mRNA variants within each species, with the most noticeable differences occurring in the variable portion of the 5' UTR.

The 5 IAP1 mRNA variants from *Ae. triseriatus* were identified by analyzing 30 cDNA clones by 5'RACE and automated sequencing (Table 2). The 5'UTRs of these clones are 450 nt (n = 1), 550 nt (n = 1), 604 nt (n = 9), 642 nt (n = 7) and 725 (n = 12) in length. The 401 nt immediately

upstream of the ORF are identical for each clone. The 5 transcripts are denoted as AtIAP1 mRNA variants 1 to 5; variant 1 is the shortest transcript, and variant 5 is the longest. A similar nomenclature has been used for the IAP1s of the other mosquito species.

Forty IAP1 cDNA clones from *Ae. albopictus* were examined (Table 2). The 5'UTRs of these clones are 592 nt (n = 4), 606 nt (n = 3), 739 (n = 25) and 915 nt (n = 1) in length. The 389 nt immediately upstream of the ORF are identical for each clone. The 7 other AaIAP1 cDNA clones contain a truncated 5'UTR consisting of only the 193 nt immediately upstream of the ORF. Truncated 5'UTRs were also observed for the IAP1 mRNA of *Ae. aegypti*. Briefly, 29 AeIAP1 cDNA clones were examined. The 5'UTRs of these clones are 619 nt (n = 5), 659 nt (n = 1) and 749 nt (n = 6) in length. The 393 nt immediately upstream of the ORF are identical for each clone. The 17 other cDNA clones contained only the 179 nt immediately upstream of the ORF. Two other *Ae. aegypti* IAP1 sequences are available in the public domain. One sequence was submitted to Genbank in February 2004 (GenBank accession no. [AAS66751](#)), and the second was determined by the *Ae. aegypti* Genomic Sequencing Consortium; both IAP1 sequences contain relatively short 5'UTRs (182 and 185 nt, respectively) similar to the length of the truncated 5'UTRs reported here.

Twelve and nineteen IAP1 cDNA clones were sequenced from *Cx. pipiens* and *Cx. tarsalis*, respectively (Table 2). The 5'UTRs of the CpIAP1 cDNA clones are 431 nt (n = 6), 537 nt (n = 2) and 677 (n = 4) in length. The 352 nt immediately upstream of the ORF are identical for each clone. The 5'UTRs of the CtIAP1 cDNA clones are 348 nt (n = 1), 349 nt (n = 1), 382 (n = 4) and 484 (n = 5) in length. The 305 nt immediately upstream of the ORF are identical for each clone. The 8 other CtIAP1 cDNA clones contain only the 124 nt (n = 6) or 128 nt (n = 2) immediately upstream of the 5'UTR.

Partial sequencing of the *Ae. triseriatus* IAP1 gene

Using genome walking, approximately 6 kb of new sequence data were generated and three of the five heterogeneous 5'UTR sequences in the *Ae. triseriatus* genome were identified (GenBank accession no. [EF059537](#), Fig. 2). The heterogeneous sequence from AtIAP1 mRNA variant 1 was located immediately upstream of the homogeneous 5'UTR sequence, while the heterogeneous sequence from AtIAP1 mRNA variant 4 was 2,110 base pairs upstream of the homogeneous 5'UTR sequence. The heterogeneous 5'UTR sequence from AtIAP1 mRNA variant 3 was also found, but the reverse primer bound incorrectly, prohibiting determination of the exact genomic position of the sequence. The heterogeneous sequences at the distal ends of AtIAP1 mRNA variants 2 and 5 were not amplified from the *Ae. triseriatus* genome. Because two of the five heterogeneous 5' UTR sequences, variants 1 and 4, were found upstream of the *AtIAP1* ORF, alternative splicing is a more likely explanation for mRNA variants than multiple gene copies.

Genomic location of *Ae. aegypti* 5' heterogeneous UTR sequences

Two of the three heterogeneous 5'UTR sequences of *AeIAP1* were located in the known genome sequence of *Ae. aegypti* (Fig. 2). The heterogeneous sequence of AeIAP1 mRNA variant 2 begins 470 base pairs upstream of the genomic sequence of the 5' homogeneous UTR sequence. The heterogeneous sequence of AeIAP1 mRNA variant 1 begins 119.78 kb upstream of the genomic sequence for the 5' homogeneous UTR sequence. Both of these sequences are nearly identical between the cDNA sequence from our lab strain (Rexville D) and the genomic sequence of *Ae. aegypti* Liverpool strain found in Genbank. The heterogeneous sequence of AeIAP1 mRNA variant 3 was not found in the *Ae. aegypti* genome when a Blast search was performed. The *Ae. aegypti* IAP1 gene (and the *Ae. triseriatus* IAP1 gene) contains sequence motifs that are similar to the splice site motifs found in *D. melanogaster* introns (Fig. 2) (Lim and Burge, 2001). These sequences are found directly following the 5' UTR heterogeneous

sequences (the 5' intron splice site motif), preceding the 5' UTR homogeneous sequences (the 3' intron splice site motif), and 70 – 135 base pairs before the beginning of the 5' UTR homogeneous sequences (the 3' intron branch site motif). A similar pattern is seen in the genomic arrangement of *DIAP1* (Fig. 2), which is predicted to generate multiple mRNA variants through alternative splicing (Adams et al., 2000).

Life-stage specific expression of the AtIAP1 mRNA variants

We examined the life-stage and tissue-specific expression of each IAP1 mRNA variant from *Ae. triseriatus* using quantitative reverse-transcriptase PCR (Figs. 3A and 3B). Total RNA was extracted from 9 *Ae. triseriatus* life-stages, namely embryos (8 days old), larvae (first, second, third, and fourth instars within 24 hours after molting), pupae (within 24 hours of pupation) and adults (pre-bloodfed females, post-bloodfed females, and males), as well as three tissue types: salivary glands, ovaries, and midguts from pre-bloodfed (4 days post-eclosion) and 24 hours post blood-fed (fed at 4 days post-eclosion) females. All five mRNA variants were detected in all life-stages and tissue types albeit in varying concentrations (Figs. 3A and 3B). mRNA variant 2 had the lowest expression level in all tissues and was generally followed by variant 1 (with exceptions being seen in ovarian tissue and pupae). Variant 3 was the most abundant transcript in all *Ae. triseriatus* life stages except L2 and L3 larvae, where variant 4 was the most abundant. There were obvious differences in adult female tissue expression patterns of AtIAP1 mRNA variants: in salivary glands and midguts variant 4 predominated, whereas in ovarian tissue variant 3 was the most abundant.

Life-stage specific expression of the CplAP1 mRNA variants

We also examined the life-stage and tissue-specific expression of each IAP1 mRNA variant of *Cx. pipiens* (Fig. 4). Total RNA was extracted from 7 *Cx. pipiens* life-stages, namely embryos (24 hr), larvae (first/second, third, and fourth instars), pupae (early and late) and adults. Total RNA was also extracted from midguts, ovaries and salivary glands from adult female mosquitoes. RT-PCRs were performed using variant-specific primers that amplified regions of ~200 nt. In this species, the mRNA variants were ubiquitously expressed in all life-stages (Fig. 4A) and tissues examined (Fig. 4B). Control RT-PCR reactions for *Cx. pipiens* actin mRNA were used to assure equal loading in each lane.

DISCUSSION

A recent study provided evidence that AaIAP1 functions as an inhibitor of apoptosis (Li et al., 2007). Although a similar function has not been reported for AtIAP1, AeIAP1, CpIAP1 or CtIAP1, it seems plausible that these BIR-containing proteins do indeed function as apoptosis inhibitors based on their sequence homology to AaIAP1. We have shown that these putative IAP1s from *Aedes* and *Culex* spp. mosquitoes exist as distinct mRNA variants due to the presence of heterogeneous sequences at the distal ends of their 5'UTRs. Analysis of the *AtIAP1* and *AeIAP1* genome sequences indicated that these mRNA variants are created through alternative splicing from a single gene rather than the transcription of separate genes. This represents an interesting finding, as alternative splicing has been reported infrequently in mosquitoes (Kokoza and Raikhel, 1997; Ranson et al., 1998; Seo et al., 2003). However, alternative splicing has been reported frequently in other organisms. An estimated 35-60% of all human genes are alternatively spliced (Mironov et al., 1999; Stamm et al., 2005).

Many other apoptosis-regulatory genes are expressed as distinct mRNA variants or protein isoforms as a result of alternative splicing, which demonstrates that this mode of gene regulation plays a major role in the control of apoptosis (Wu et al., 2003; Schwerk and Schulze-Osthoff, 2005). Alternative splicing of transcripts from a single gene appears to generate multiple DIAP1 mRNA variants (Adams et al., 2000). Three DIAP1 mRNA variants

were predicted during the computational analysis of the *Drosophila* genome. Each transcript shares the same ORF and 78 nt region of the 5'UTR, but the distal ends of their 5'UTRs contain heterogeneous sequences of 352, 197 or 158 nt (GenBank accession nos. [NM_079377](#), [NM_168644](#), [NM_168645](#); isoforms A, B and C, respectively). Alternative splicing from a single gene also was predicted to generate two DIAP2 mRNA variants with heterogeneous sequences at the distal ends of their 5'UTRs (GenBank accession nos. [NM_057779](#) and [NM_176182](#); isoforms A and B, respectively). The biological significance of these DIAP1 and DIAP2 mRNA variants is not known. Most members of the Bcl-2 family are alternatively spliced (Akgul et al., 2004). For example, transcripts of the *Bcl-2* gene, the prototype member of this family, generate 2 discrete mRNA isoforms with different subcellular locations and biological functions (Tsujimoto and Croce, 1986; Tanaka et al., 1993).

While there is no information available on the developmental or tissue-specific distribution of the mRNA variants of DIAP1 or DIAP2, the expression profiles of several mammalian IAPs that exist as alternative splice variants have been determined. The tissue-specific expression of 3 survivin mRNA variants was assessed by RT-PCR in adult mice, with survivin₁₂₁ detectable in all 10 tissues examined, survivin₁₄₀ detectable only in the thymus and testis, and survivin₄₀ not detectable in any of the tissues examined (Conway et al., 2000). Differential expression patterns were also reported for the two Livin splice variants (Ashhab et al., 2001). Livin α mRNA was not detectable by RT-PCR in any of the 8 human fetal tissues examined, whereas Livin β mRNA was detectable only in fetal kidney, heart and spleen.

The number of IAP1 mRNA variants identified in each mosquito species varied from three to five. However, these findings may be a consequence of our experimental approach, and not represent a true difference in the number of IAP1 mRNA variants between species. Longer 5'UTR sequences may not have been identified because they are less likely to be RT-PCR amplified or cloned into the TOPO vectors than shorter sequences. Furthermore, some 5'UTRs may not have been RT-PCR amplified due to the presence of unusually stable secondary structures that were not heat-denatured during the reverse transcription. Interestingly, several AaIAP1, AeIAP1 and CtIAP1 cDNA clones encoded truncated 5'UTRs. It is unclear whether this represents a splicing event or an artifact of our RT-PCR or 5' RACE procedures. However, it is important to note that the *Ae. aegypti* Genomic Sequencing Consortium (<http://msc.tigr.org/aedes/aedes.shtml>) employed a different sequencing strategy from that used here and reported that the 5'UTR of the *Ae. aegypti* IAP1 consists of 185 nt. This corresponds closely to the size (179 nt) of the truncated 5'UTR region identified in several AeIAP1 cDNA clones.

We searched the available *Ae. aegypti* genome sequence for the 3 heterogeneous sequences that we identified at the distal end of the AeIAP1 mRNA. Two of these heterogeneous sequences were found. The heterogeneous sequence from AeIAP1 mRNA variant 2 was located 470 bp upstream of the homogeneous 5'UTR sequence whereas the heterogeneous sequence from AeIAP1 mRNA variant 1 was approximately 120 kb upstream of the homogeneous 5'UTR sequence. The *AeIAP1* genomic sequence is located on supercontig 1.368, which is made up of many smaller contig sequences. There are still gaps between some of the sequences that make up this supercontig, which may be why we did not find the heterogeneous sequence from AeIAP1 mRNA variant 3.

Using genomic walking, we sequenced approximately 6 kb of the *Ae. triseriatus* genome. This region contains 3 of the 5 heterogeneous sequences at the distal end of the AtIAP1 mRNA. The two other heterogeneous sequences were not identified. The heterogeneous sequences may be located too far upstream to be detected using our PCR-based approach. Indeed, as already noted, one of the heterogeneous sequences in the AeIAP1 mRNA was located 120 kb upstream of the homogeneous 5'UTR sequence.

Approximately 95% of intron boundaries in *Drosophila melanogaster* can be predicted exactly using computer programs. Lim and Burge have studied the sequence of several genomes, including *D. melanogaster*, and have determined the most frequently occurring sequences found at the 5' splice site, the 3' splice site, and the branch site of introns (Lim and Burge, 2001). These predictions were made using computer programs and revealed that the most common splice signals are quite similar to sequences found in both *Ae. triseriatus* and *Ae. aegypti* genomes. Computer analysis shows that the sequence “AAGGTAAGTTT” is the most common splice site for the 5' end of an intron in *D. melanogaster*. With the exception of the underlined base pairs in this sequence, there is some variability found at each site, particularly the first and last two bases of the sequence (Lim and Burge, 2001). Similar sequences were found directly following all of the variable 5'UTR sequences in the *D. melanogaster*, *Ae. aegypti* and *Ae. triseriatus* genomes (Fig. 2). Similarly, “TACTAAT” make up the most common nucleotides in the branch site generally located near the 3' end of the intron. Again, there is some variability, most of which is seen in the first two and the last base of the sequence (Lim and Burge, 2001). Sequences similar to this one were found 70 - 135 base pairs upstream from the homogeneous region of the 5'UTR in the *D. melanogaster*, *Ae. aegypti*, and *Ae. triseriatus* genomes (Fig. 2). Finally, the 3' splice site sequence of *D. melanogaster* is generally “CAGAT” preceded by approximately 17 thymidines (Lim and Burge, 2001). This “CAGAT” sequence is found at the beginning of the homogeneous 5'UTR sequence (i.e. the 3' end of the intron separating the heterogeneous and homogeneous 5'UTR sequences) in the genomes of *D. melanogaster*, *Ae. aegypti*, and *Ae. triseriatus* (Fig. 2). The *D. melanogaster* genome also has several thymidines preceding the 3' splice sequence. While the *Ae. aegypti* and *Ae. triseriatus* sequences are not directly preceded by a string of thymidines, they are in a fairly thymidine-rich area of the genome. These sequence motifs indicate that *AeIAP1* and *AtIAP1* genes undergo alternative splicing to create mRNA variants.

We previously examined the life-stage and tissue-specific expression of *AtIAP1* mRNA by RT-PCR using primers specific to a homogeneous region of the cDNA sequence (Blitvich et al., 2002). *AtIAP1* mRNA was detected in all developmental stages (embryos, first-fourth instar larvae, early and late pupae, adults) and adult tissues (midguts, ovaries, salivary glands) examined. Here, we have extended our earlier observations and shown that each mRNA variant of *AtIAP1* is also detectable, although at different concentrations, by quantitative RT-PCR in all of these life-stages and tissues. All three mRNA variants of *CpIAP1* also were detectable by variant-specific RT-PCR in all developmental stages and adult tissues examined. Taken together, these data suggest that constitutive expression of each *IAP1* mRNA variant is common to *Culicine* mosquitoes.

It is likely that translational control of the mosquito *IAP1* mRNA is dependent upon the heterogeneous region of the 5'UTR. Each mRNA variant may be translated in response to a particular apoptosis-inducing stimulus or in a certain life-stage or tissue. For example, our previous studies showed that *AtIAP1* protein is found only in the ovaries (Blitvich et al., 2002), while current studies show that the ovaries have a very different *AtIAP1* mRNA expression profile from the midguts and salivary glands (Fig. 3B). It is possible that in adult female ovaries only *AtIAP1* mRNA variant 3 is translated to make protein. It is also interesting to note that mRNA variants 3 and 5 are the two most prominent variants in the life stages where the mosquitoes are transitioning to the next stage of development (i.e. embryos, L4 larvae, and early pupae) (Fig. 3A). These variants could play a role in creating *AtIAP1* protein that is used for structural metamorphosis.

Differential translation of each mosquito *IAP1* mRNA variant could be achieved if the heterogeneous region of each 5'UTR forms distinct secondary structures recognized by different translational activators and repressors. Analysis of the 5'UTR sequence of all culicine *IAP1* mRNA variants by the method of Markham and Zuker (2005), using the DINAMelt web

server, revealed that each 5'UTR differed considerably in respect to predicted secondary structure, particularly in the heterogeneous region of the UTR (Supplemental Figs. 2A-2E). The different structures observed could condition translation efficiency in different tissues or life stages. Differential translational regulation has been reported for other mRNA variants that contain distinct 5'UTRs; examples include human surfactant protein A, estrogen receptor- α and insulin (Kos et al., 2002;Shalev et al., 2002;Wang et al., 2005).

Our current study shows that mosquito IAP RNAs contain variations in the 5'UTR that are produced through alternative splicing, but this does not affect protein sequence. Constitutive expression of all mRNA variants can be seen in all life stages and tissue types of *Cx. pipiens* and *Ae. triseriatus*. However, it is likely that different IAP mRNA variants are predominantly translated following different apoptosis-inducing stimuli or in different mosquito life-stages or tissue types.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES CITED

1. Adams MD, Celniker SE, Holt RA, Evans CA, Gocayne JD, Amanatides PG, Scherer SE, Li PW, Hoskins RA, Galle RF, George RA, Lewis SE, Richards S, Ashburner M, Henderson SN, Sutton GG, Wortman JR, Yandell MD, Zhang Q, Chen LX, Brandon RC, Rogers YH, Blazej RG, Champe M, Pfeiffer BD, Wan KH, Doyle C, Baxter EG, Helt G, Nelson CR, Gabor GL, Abril JF, Agbayani A, An HJ, Andrews-Pfannkoch C, Baldwin D, Ballew RM, Basu A, Baxendale J, Bayraktaroglu L, Beasley EM, Beeson KY, Benos PV, Berman BP, Bhandari D, Bolshakov S, Borkova D, Botchan MR, Bouck J, Brokstein P, Brottier P, Burtis KC, Busam DA, Butler H, Cadieu E, Center A, Chandra I, Cherry JM, Cawley S, Dahlke C, Davenport LB, Davies P, de Pablos B, Delcher A, Deng Z, Mays AD, Dew I, Dietz SM, Dodson K, Doup LE, Downes M, Dugan-Rocha S, Dunkov BC, Dunn P, Durbin KJ, Evangelista CC, Ferraz C, Ferreira S, Fleischmann W, Fosler C, Gabrielian AE, Garg NS, Gelbart WM, Glasser K, Glodek A, Gong F, Gorrell JH, Gu Z, Guan P, Harris M, Harris NL, Harvey D, Heiman TJ, Hernandez JR, Houck J, Hostin D, Houston KA, Howland TJ, Wei MH, Ibegwam C, et al. The genome sequence of *Drosophila melanogaster*. Science 2000;287:2185–95. [PubMed: 10731132]
2. Akgul C, Moulding DA, Edwards SW. Alternative splicing of Bcl-2-related genes: functional consequences and potential therapeutic applications. Cell. Mol. Life. Sci 2004;61:2189–99. [PubMed: 15338051]
3. Bergmann A, Yang AY, Srivastava M. Regulators of IAP function: coming to grips with the grim reaper. Curr. Opin. Cell. Biol 2003;15:717–24. [PubMed: 14644196]
4. Blitvich BJ, Blair CD, Kempf BJ, Hughes MT, Black WC, Mackie RS, Meredith CT, Beaty BJ, Rayms-Keller A. Developmental- and tissue-specific expression of an inhibitor of apoptosis protein 1 homologue from *Aedes triseriatus* mosquitoes. Insect Mol, Biol, 2002;11:431–42. [PubMed: 12230542]
5. Christophides GK, Zdobnov E, Barillas-Mury C, Birney E, Blandin S, Blass C, Brey PT, Collins FH, Danielli A, Dimopoulos G, Hetru C, Hoa NT, Hoffmann JA, Kanzok SM, Letunic I, Levashina EA, Loukeris TG, Lycett G, Meister S, Michel K, Moita LF, Muller HM, Osta MA, Paskewitz SM, Reichhart JM, Rzhetsky A, Troxler L, Vernick KD, Vlachou D, Volz J, von Mering C, Xu J, Zheng L, Bork P, Kafatos FC. Immunity-related genes and gene families in *Anopheles gambiae*. Science 2002;298:159–65. [PubMed: 12364793]

6. Conway EM, Pollefeyt S, Cornelissen J, DeBaere I, Steiner-Mosonyi M, Ong K, Baens M, Collen D, Schuh AC. Three differentially expressed survivin cDNA variants encode proteins with distinct antiapoptotic functions. *Blood* 2000;95:1435–42. [PubMed: 10666222]
7. Crook NE, Clem RJ, Miller LK. An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif. *J. Virol* 1993;67:2168–74. [PubMed: 8445726]
8. Deveraux QL, Reed JC. IAP family proteins--suppressors of apoptosis. *Genes Dev* 1999;13:239–52. [PubMed: 9990849]
9. Fraser AG, James C, Evan GI, Hengartner MO. *Caenorhabditis elegans* inhibitor of apoptosis protein (IAP) homologue BIR-1 plays a conserved role in cytokinesis. *Curr. Biol* 1999;9(6):292–301. [PubMed: 10209096]
10. Hay BA, Wassarman DA, Rubin GM. *Drosophila* homologs of baculovirus inhibitor of apoptosis proteins function to block cell death. *Cell* 1995;83:1253–62. [PubMed: 8548811]
11. Holcik M, Korneluk RG. Functional characterization of the X-linked inhibitor of apoptosis (XIAP) internal ribosome entry site element: role of La autoantigen in XIAP translation. *Mol. Cell. Biol* 2000;20:4648–57. [PubMed: 10848591]
12. Holcik M, Lefebvre C, Yeh C, Chow T, Korneluk RG. A new internal-ribosome-entry-site motif potentiates XIAP-mediated cytoprotection. *Nat. Cell. Biol* 1999;1:190–2. [PubMed: 10559907]
13. Hozak RR, Manji GA, Friesen PD. The BIR motifs mediate dominant interference and oligomerization of inhibitor of apoptosis Op-IAP. *Mol. Cell. Biol* 2000;20:1877–85. [PubMed: 10669762]
14. Huang H, Joazeiro CA, Bonfoco E, Kamada S, Levenson JD, Hunter T. The inhibitor of apoptosis, cIAP2, functions as a ubiquitin-protein ligase and promotes in vitro monoubiquitination of caspases 3 and 7. *J. Biol. Chem* 2000;275:26661–4. [PubMed: 10862606]
15. Jones G, Jones D, Zhou L, Steller H, Chu Y. Deterin, a new inhibitor of apoptosis from *Drosophila melanogaster*. *J. Biol. Chem* 2000;275:22157–65. [PubMed: 10764741]
16. Kokoza VA, Raikhel AS. Ovarian- and somatic-specific transcripts of the mosquito clathrin heavy chain gene generated by alternative 5'-exon splicing and polyadenylation. *J. Biol. Chem* 1997;272:1164–70. [PubMed: 8995417]
17. Kornbluth S, White K. Apoptosis in *Drosophila*: neither fish nor fowl (nor man, nor worm). *J. Cell. Sci* 2005;118:1779–87. [PubMed: 15860727]
18. Kos M, Denger S, Reid G, Gannon F. Upstream open reading frames regulate the translation of the multiple mRNA variants of the estrogen receptor alpha. *J. Biol. Chem* 2002;277:37131–8. [PubMed: 12147702]
19. Kozak M. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell* 1986;44:283–92. [PubMed: 3943125]
20. Li Q, Li H, Blitvich BJ, Zhang J. The *Aedes albopictus* Inhibitor of Apoptosis 1 gene protects vertebrate cells from bluetongue virus-induced apoptosis. *Insect Mol. Biol* 2007;16(1):93–105. [PubMed: 17257212]
21. Lim LP, Burge CB. A computational analysis of sequence features involved in recognition of short introns. *Proc. Natl. Acad. Sci. USA* 2001;98:11193–8. [PubMed: 11572975]
22. Markham NR, Zuker M. DINAMelt web server for nucleic acid melting prediction. *Nucleic Acids Res* 2005;33:W577–W581. [PubMed: 15980540]
23. Miller LK. An exegesis of IAPs: salvation and surprises from BIR motifs. *Trends Cell. Biol* 1999;9:323–8. [PubMed: 10407412]
24. Mironov AA, Fickett JW, Gelfand MS. Frequent alternative splicing of human genes. *Genome Res* 1999;9:1288–93. [PubMed: 10613851]
25. Nevins TA, Harder ZM, Korneluk RG, Holcik M. Distinct regulation of internal ribosome entry site-mediated translation following cellular stress is mediated by apoptotic fragments of eIF4G translation initiation factor family members eIF4GI and p97/DAP5/NAT1. *J. Biol. Chem* 2003;278:3572–9. [PubMed: 12458215]
26. Olson MR, Holley CL, Yoo SJ, Huh JR, Hay BA, Kornbluth S. Reaper is regulated by IAP-mediated ubiquitination. *J. Biol. Chem* 2003;278:4028–34. [PubMed: 12446669]

27. Ranson H, Collins F, Hemingway J. The role of alternative mRNA splicing in generating heterogeneity within the *Anopheles gambiae* class I glutathione S-transferase family. *Proc. Natl. Acad. Sci. USA* 1998;95:14284–9. [PubMed: 9826692]
28. Reed JC. Mechanisms of apoptosis. *Am. J. Pathol* 2000;157:1415–30. [PubMed: 11073801]
29. Roy N, Deveraux QL, Takahashi R, Salvesen GS, Reed JC. The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. *Embo J* 1997;16:6914–25. [PubMed: 9384571]
30. Salvesen GS, Duckett CS. IAP proteins: blocking the road to death's door. *Nat. Rev. Mol. Cell. Biol* 2002;3:401–10. [PubMed: 12042762]
31. Schwerk C, Schulze-Osthoff K. Regulation of apoptosis by alternative pre-mRNA splicing. *Mol. Cell* 2005;19:1–13. [PubMed: 15989960]
32. Seo SJ, Cheon HM, Sun J, Sappington TW, Raikhel AS. *J. Biol. Chem* 2003;278:41954–62. [PubMed: 12917414]
33. Shalev A, Blair PJ, Hoffmann SC, Hirshberg B, Peculis BA, Harlan DM. A proinsulin gene splice variant with increased translation efficiency is expressed in human pancreatic islets. *Endocrinology* 2002;143:2541–7. [PubMed: 12072386]
34. Shi Y. Caspase activation, inhibition, and reactivation: a mechanistic view. *Protein Sci* 2004;13:1979–87. [PubMed: 15273300]
35. Shi Y. Mechanisms of caspase activation and inhibition during apoptosis. *Mol. Cell* 2002;9:459–70. [PubMed: 11931755]
36. Stamm S, Ben-Ari S, Rafalska I, Tang Y, Zhang Z, Toiber D, Thanaraj TA, Soreq H. Function of alternative splicing. *Gene* 2005;344:1–20. [PubMed: 15656968]
37. Tanaka S, Saito K, Reed JC. Structure-function analysis of the Bcl-2 oncoprotein. Addition of a heterologous transmembrane domain to portions of the Bcl-2 beta protein restores function as a regulator of cell survival. *J. Biol. Chem* 1993;268:10920–6. [PubMed: 8496157]
38. Tsujimoto Y, Croce CM. Analysis of the structure, transcripts, and protein products of bcl-2, the gene involved in human follicular lymphoma. *Proc. Natl. Acad. Sci. USA* 1986;83:5214–8. [PubMed: 3523487]
39. Twomey C, McCarthy JV. Pathways of apoptosis and importance in development. *J. Cell. Mol. Med* 2005;9:345–59. [PubMed: 15963254]
40. Vaux DL, Silke J. Mammalian mitochondrial IAP binding proteins. *Biochem. Biophys. Res. Commun* 2003;304:499–504. [PubMed: 12729584]
41. Verhagen AM, Coulson EJ, Vaux DL. Inhibitor of apoptosis proteins and their relatives: IAPs and other BIRPs. *Genome Biol* 2001;2(7)e-publication
42. Verhagen AM, Kratina TK, Hawkins CJ, Silke J, Ekert PG, Vaux DL. Identification of mammalian mitochondrial proteins that interact with IAPs via N-terminal IAP binding motifs. *Cell. Death Differ.* 2006
43. Vernooij SY, Chow V, Su J, Verbrugghe K, Yang J, Cole S, Olson MR, Hay BA. *Drosophila* Bruce can potently suppress Rpr- and Grim-dependent but not Hid-dependent cell death. *Curr. Biol* 2002;12:1164–8. [PubMed: 12121627]
44. Vucic D, Kaiser WJ, Harvey AJ, Miller LK. Inhibition of reaper-induced apoptosis by interaction with inhibitor of apoptosis proteins (IAPs). *Proc. Natl. Acad. Sci. USA* 1997;94:10183–8. [PubMed: 9294184]
45. Wang G, Guo X, Floros J. Differences in the translation efficiency and mRNA stability mediated by 5'-UTR splice variants of human SP-A1 and SP-A2 genes. *Am. J. Physiol. Lung Cell. Mol. Physiol* 2005;289:L497–508. [PubMed: 15894557]
46. Warnakulasuriyarachchi D, Cerquozzi S, Cheung HH, Holcik M. Translational induction of the inhibitor of apoptosis protein HIAP2 during endoplasmic reticulum stress attenuates cell death and is mediated via an inducible internal ribosome entry site element. *J. Biol. Chem* 2004;279:17148–57. [PubMed: 14960583]
47. Warnakulasuriyarachchi D, Ungureanu NH, Holcik M. The translation of an antiapoptotic protein HIAP2 is regulated by an upstream open reading frame. *Cell. Death Differ* 2003;10:899–904. [PubMed: 12867997]
48. Wu JY, Tang H, Havlioglu N. Alternative pre-mRNA splicing and regulation of programmed cell death. *Prog. Mol. Subcell. Biol* 2003;31:153–85. [PubMed: 12494766]

49. Yang Y, Fang S, Jensen JP, Weissman AM, Ashwell JD. Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. *Science* 2000;288:874–7. [PubMed: 10797013]
50. Zhou L, Jiang G, Chan G, Santos CP, Severson DW, Xiao L. Michelob_x is the missing inhibitor of apoptosis protein antagonist in mosquito genomes. *EMBO Rep* 2005;6:769–74. [PubMed: 16041319]
51. Zimmermann KC, Bonzon C, Green DR. The machinery of programmed cell death. *Pharmacol. Ther* 2001;92:57–70. [PubMed: 11750036]

AtIAP1	----MTAQVLSIPRDFTDNKGKDESPFVGVTGASSVSVSYHTEVARLDSFRNWTVLFITK	56
AaIAP1	MAQMMMAQVLSIPRDFTDNKVKEENA-YG-QTGSSVPISYHTEVSRLESFRDWMVPFISK	58
AeIAP1	MAGVMMMAQVLSIPRDFTDNKVKEDNA-YG-HTATSVPISYHTEVARLESFRGWMVSFISK	58
CpIAP1	----MTAQVLSIPRDYTDNKVKDENTSYG---LTGTSVTYHIEVSRLESFLNWSIPFISK	53
CtIAP1	----MTAQVLSIPRDFTDNKVKDENT-YG---LTGPSVSVYHVEASRLLESFLNWCIPFISK	52
AtIAP1	AELARYGFYYVGPNDMVKCYFCRVEIGLWEPNDNVLSEHLRWSPYCPLLRKQTNNVPID	116
AaIAP1	TELARYGFYYVGPNDMVKCYFCRVEIGLWEQNDNVLSEHLRWSPYCPLLRKQTNNVPID	118
AeIAP1	TELARYGFYYVGPNDMVKCYFCRVEIGLWEPNDNVLSEHLRWSPYCPLLRKQTNNVPID	118
CpIAP1	FELARFGFYVGPNDMVKCFRCRVEIGLWEPNDNVLSEHLRWSPYCPLLRKPTNNQPLD	113
CtIAP1	<u>IELARFGFYVGPNDMVKCFRCRVEIGLWEPNDNVLSEHLRWSPYCPLLRKPTNNVPID</u>	112
AtIAP1	ASFLDQLPEPSYDTCGIRIRENSVAENAYSSSDRSSGSLSPSSSLTSESSMLSNGPIP	176
AaIAP1	ASFLDQLPEPSYDTCGIRIRENSVAENAYSSSDRSSGSLTSPSSSLTSESSMASNGPVV	178
AeIAP1	ASFLDQLPEPSYDTCGIRIRENSVAENAYSSSDRSSGSLTSPSSSLTSESSMASNGPVV	178
CpIAP1	ASFLDQLPEPSYDTCGIQIRQHSVAENAYSSSDRSSGSMSPSSSLTSESSMMSNASGG	173
CtIAP1	ASFLDQLPEPSYDTCGIRIRQHSVAENAYSSSDRSSGSMSPSSSLTSESSMMSASGG	172
AtIAP1	QQLPAPALP-QQ----KRPEYPNYAIEAKRLESYEDWPKFMKQKPKELSDAGFFYTGKGD	231
AaIAP1	QQMVAQASMPQQ----KRPEYPNYAIEAKRLECYEDWPKFMKQKPKELSDAGFFYPGKSD	234
AeIAP1	QQLSAPVLP-QQ----KLPEYPNYAIEAKRLESYEDWPKFMKQKPKELSDAGFFYTGKSD	233
CpIAP1	IGATELQPPQQQ---HKLPEYPNYAIEAKRLESYEDWPKFMKQKPKELSDAGFFYTGKSD	230
CtIAP1	VQVTEPHPPQQQQQHKLPEYPNYAIEAKRLESYEDWPKFMKQKPKELSDAGFFYTGKSD	232
AtIAP1	RVKCFSCGGGLKDWEAEDPEWEQHAWWYSNCEYLKLMKGEEYIAQCLAKKDNPSDQ--KG	289
AaIAP1	RVKCFSCGGGLKDWEAEDPEWEQHAWWYSNCEYLKLMKGEEYIAQCLAKKESSEQ--PG	292
AeIAP1	RVKCFSCGGGLKDWEAEDPEWEQHAWWYSNCEYLKLMKGEEYIAHCLAKKESSEE--NG	291
CpIAP1	RVKCFSCGGGLKDWEAGDEPEWEQHAWWYSNCEYLKLMKGEEYIAQCLAKKENSSEMSEQP	290
CtIAP1	<u>RVKCFSCGGGLKDWEAGDEPEWEQHAWWYSHCEYLKLMKGEEYIAQCLAKKESSEMAEQP</u>	292
AtIAP1	SDVGPSNSPQPSTSGVTSAAATSLPSSQSSSLASAAEEVNSPLGGSS-----SGMEEDED	344
AaIAP1	SDVGPSNSPQPSTSGVASAATSSLQSSPASSFTASAEESTAMVGCS-----S-----DED	343
AeIAP1	SDVGPSNSPQPSTSGVVSAAATSSLQSSQSSSLSVSAEEDNTAMVGCS-----S-----DED	342
CpIAP1	QDVGPSNSPQPSTSGVASAATSLPSSQSSSLSTSVEESNPMTSMAAPMSGSSGLEEDED	350
CtIAP1	QDVGPSGSPQPSTSGVASAATSLPSSQSSSLSTSVEEGNPMTSMAP-ASASSGLEEDED	351
AtIAP1	EPNRKLDTSRICKICYVNEYNTAFSPCGHVACAKCASSVTKCPLCRKPFTNMVRIYLM	403
AaIAP1	EPNRKQDTSRTCKICYVNEYNTAFSPCGHVACAKCASSVTKCPLCRKPFTNMVRIYLM	402
AeIAP1	EPNRKLDTSRTCKICYVNEYNTAFSPCGHVACAKCASSVTKCPLCRKPFTNMVRIYLM	401
CpIAP1	EPNRKQDTSRTCKICYVNEYNTAFSPCGHVACAKCASSVTKCPLCRKPFTNMVRIYLM	409
CtIAP1	<u>EPNRKQDTSRTCKICYVNEYNTAFSPCGHVACAKCASSVTKCPLCRKPFTNMVRIYLM</u>	410

Figure 1.

Alignment of the deduced amino acid sequences of the mosquito IAP1s. The first, second and third underlined regions denote the BIR1, BIR2 and RING domains, respectively. The IAP1s from *Ae. triseriatus*, *Ae. albopictus*, *Ae. aegypti*, *Cx. pipiens* and *Cx. tarsalis* are denoted as AtIAP1, AaIAP1, AeIAP1, CpIAP1 and CtIAP1, respectively.

Figure 2.

Genomic schematics of *DIAP1*, *AelIP1*, and *AtIP1* genes. The schematics are drawn to scale (the full length *DIAP1* gene represents roughly 13 kb) with the exception of the dotted line in the *AelIP1* figure (this line represents roughly 115 kb of DNA) and dotted and dashed line in the *AtIP1* figure (this line represents an unknown genomic distance). Red boxes indicate untranslated regions, grey indicates the protein coding sequence, and blue indicates 5' UTR variable regions. Uppercase letters represent nucleotides located in the exon while lowercase letters represent nucleotides found in the intron. Bold letters indicate nucleotides matching the consensus sequences of *D. melanogaster* 5' splice site (sequences indicated by an *), 3' branch site (sequences indicated by a #), and 3' splice site (sequences indicated by a ~) intron motifs.

Figure 3.

Developmental- and tissue-specific expression of each IAP1 mRNA variant from *Ae. triseriatus*, showing the relative amount of each AtIAP1 mRNA variant found in each life stage or tissue type. The most abundant mRNA variant for each stage or tissue was recorded as a relative frequency of one and the remaining AtIAP1 mRNA variants were recorded as a percentage of the most abundant variant based on their expression levels. Variant 1 is on the left of each group and proceeds to variant 5 on the right. (A) Quantitative RT-PCR amplifications were performed using mRNA variant-specific primers and total RNA extracted from various life stages, namely: embryos, first instar larvae, second instar larvae, third instar larvae, fourth instar larvae, pupae (< 24 hrs after pupation), pre-bloodfed adult females, 24 hour post-bloodfed females, and adult males. (B) Quantitative RT-PCR amplifications were performed using mRNA variant-specific primers and total RNA extracted from various adult tissues, namely pre-bloodfed and 24 hour post-bloodfed female salivary glands, midguts, and ovaries.

Figure 4.

Developmental- and tissue-specific expression of each IAP1 mRNA variant from *Cx. pipiens*. (A) RT-PCR amplifications were performed using mRNA variant-specific primers and total RNA extracted from various life stages, namely: 24-h-old embryos (lane 1), first/second instar larvae (lane 2), third instar larvae (lane 3), fourth instar larvae (lane 4), early pupae (lane 5), late pupae (lane 6) and adults (lane 7). (B) RT-PCR amplifications were performed using mRNA variant-specific primers and total RNA extracted from various adult female tissues, namely midguts (lane 1) ovaries (lane 2) and salivary glands (lane 3). Control reactions were performed using primers specific to the *Cx. pipiens* actin cDNA sequence

Table 1Primers used for Q-RT-PCR of *Ae. triseriatus* AtIAP1 mRNA splice variants.

Primer Name	Primer Sequence (5' – 3')	mRNA Amplified
ActinF	GAA CAC CCA GTG CTG TTG AC	<i>Ae. triseriatus</i> Actin
ActinR	GTA CGA CCG GAA GCG TAC AG	<i>Ae. triseriatus</i> Actin
IAP1F	TCC ATT CGT CTT TGC TTG TC	<i>Ae. triseriatus</i> AtIAP1 variant #1
IAP1R	GCA ATT GCA CTG CTC TCT CTC	<i>Ae. triseriatus</i> AtIAP1 variant #1
IAP2F	CGA AAA AGG ATT TGC TGC TG	<i>Ae. triseriatus</i> AtIAP1 variant #2
IAP2R	CTC TTG GTC CAG ATG GGA AA	<i>Ae. triseriatus</i> AtIAP1 variant #2
IAP3F	CAT TGT GTC TCG CAT CGT CT	<i>Ae. triseriatus</i> AtIAP1 variant #3
IAP3R	GCG CTC TCT TCT TCA ATG GT	<i>Ae. triseriatus</i> AtIAP1 variant #3
IAP4F	CCA TTG TAA CTG GTC CTG GTC	<i>Ae. triseriatus</i> AtIAP1 variant #4
IAP4R	CTC TTG GTC CAG ATG GGA AA	<i>Ae. triseriatus</i> AtIAP1 variant #4
IAP5F	TCG ATT TGT GAT GTT TGG TG	<i>Ae. triseriatus</i> AtIAP1 variant #5
IAP5R	GCA ATT GCA CTG CTC TCT CTC	<i>Ae. triseriatus</i> AtIAP1 variant #5

Table 2

Overview of the IAP1 mRNA variants of each mosquito species.

Species	Number of IAP1 mRNA variants identified	Length (nt) of each 5'UTR	Length (nt) of homogeneous 5'UTR	Number of IAP1 cDNA clones sequenced
<i>Ae. triseriatus</i> ^a	5	450, 550, 604, 642, 725	401	30
<i>Ae. albopictus</i>	4	592, 606, 739, 915	389	40
<i>Ae. aegypti</i>	3	619, 659, 749	393	29
<i>Cx. pipiens</i>	3	431, 537, 677	352	12
<i>Cx. tarsalis</i>	4	348, 349, 382, 484	305	19

^aThe 5 different AtIAP1 transcripts are denoted as mRNA variant 1 to 5, with variant 1 being the shortest transcript, and variant 5 being the longest. A similar nomenclature has been used for the other species. GenBank accession nos. are as follows: AtIAP1 mRNA variants 1 to 5: [EF043244](#), [EF043245](#), [EF043246](#), [EF043247](#), and [EF043248](#), respectively; AaIAP1 mRNA variants 1 to 4: [EF043240](#), [EF043241](#), [EF043242](#), and [EF043243](#), respectively; AeIAP1 mRNA variants 1 to 3: [EF043237](#), [EF043238](#) and [EF043239](#), respectively; CplAP1 mRNA variants 1 to 3: [EF043249](#), [EF043250](#), and [EF043251](#), respectively and CtIAP1 mRNA variants 1 to 4: [EF043252](#), [EF043253](#), [EF043254](#), and [EF043255](#), respectively.