Published in final edited form as:

Insect Biochem Mol Biol. 2013 May ; 43(5): 444–454. doi:10.1016/j.ibmb.2013.02.005.

SfDronc, an initiator caspase involved in apoptosis in the fall armyworm *Spodoptera frugiperda*

Ning Huang^a, Srgjan Civciristov^b, Christine J. Hawkins^b, and Rollie J. Clem^{a,*}

^aMolecular, Cellular and Developmental Biology Program, Division of Biology, Kansas State University, Manhattan, KS 66503

^bDepartment of Biochemistry, La Trobe University, Bundoora 3086, Victoria, Australia

Abstract

Initiator caspases are the first caspases that are activated following an apoptotic stimulus, and are responsible for cleaving and activating downstream effector caspases, which directly cause apoptosis. We have cloned a cDNA encoding an ortholog of the initiator caspase Dronc in the lepidopteran insect *Spodoptera frugiperda*. The *SfDronc* cDNA encodes a predicted protein of 447 amino acids with a molecular weight of 51 kDa. Overexpression of SfDronc induced apoptosis in Sf9 cells, while partial silencing of *SfDronc* expression in Sf9 cells reduced apoptosis induced by baculovirus infection or by treatment with UV or actinomycin D. Recombinant SfDronc exhibited several expected biochemical characteristics of an apoptotic initiator caspase: 1) SfDronc efficiently cleaved synthetic initiator caspase substrates, but had very little activity against effector caspase substrates; 2) mutation of a predicted cleavage site at position D340 blocked autoprocessing of recombinant SfDronc and reduced enzyme activity by approximately 10-fold; 3) SfDronc cleaved the effector caspase Sf-caspase-1 at the expected cleavage site, resulting in Sf-caspase-1 activation; and 4) SfDronc was strongly inhibited by the baculovirus caspase inhibitor SpliP49, but not by the related protein AcP35. These results indicate that SfDronc is an initiator caspase involved in caspase-dependent apoptosis in *S. frugiperda*, and as such is likely to be responsible for the initiator caspase activity in *S. frugiperda* cells known as Sf-caspase-X.

Keywords

apoptosis; caspase; dronc; RNAi; *Spodoptera frugiperda*

1. INTRODUCTION

Apoptosis is a normal physiological process responsible for removing unwanted or damaged cells including virus-infected cells. Caspases are a family of cysteine proteases that play important roles in the execution of apoptosis, and can be divided into two groups, initiator and effector caspases (Li and Yuan, 2008). These enzymes are initially synthesized as inactive zymogens and must undergo an activation process to acquire full catalytic activity. All caspases share a similar overall organization of domains: an N-terminal prodomain,

© 2013 Elsevier Ltd. All rights reserved.

*Corresponding author: Rollie J. Clem, Ackert Hall, Division of Biology, Kansas State University, Manhattan, KS 66506, Tel: 785-532-3172, Fax: 785-532-6653, rclem@ksu.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

followed by large and small catalytic subunits. Following an apoptotic stimulus, initiator caspases are activated by dimerization, which is promoted by adaptor proteins such as *Drosophila* Ark (Apaf-1-Related Killer), the ortholog of Apaf-1 (Apoptotic Protease Activating Factor-1) in mammals, that bind to specific domains in the prodomain of initiator caspases, such as *Drosophila* Dronc (*Drosophila* Nedd2-like Caspase) or mammalian caspase-9. In the case of Ark/Apaf-1 and Dronc/caspase-9, the binding of the adaptor stimulates the formation of a large complex called the apoptosome. Activated initiator caspases cleave themselves in the apoptosome, resulting in higher catalytic activity, and also cleave and activate effector caspases. In both cases, activation involves an initial cleavage event that releases the C-terminal small catalytic subunit, followed by a second cleavage releasing the prodomain from the large subunit (Li and Yuan, 2008). Activated effector caspases selectively cleave various cellular substrates, leading to the characteristic morphological and biochemical manifestations of apoptosis, including plasma membrane blebbing, chromatin condensation and DNA fragmentation (Lord and Gunawardena, 2012).

In the model insect *Drosophila melanogaster*, the initiator caspase Dronc is required for most if not all apoptosis (Chew et al., 2004; Daish et al., 2004; Xu et al., 2005). Similarly, silencing Dronc expression by RNAi in cultured cells inhibits apoptosis (Igaki et al., 2002; Muro et al., 2002). DmDronc is a somewhat unusual caspase in being able to recognize and cleave substrates that contain either a glutamate or an aspartate residue at the cleavage site, while other known caspases only cleave after aspartate (Hawkins et al., 2000; Snipas et al., 2008). Under physiological conditions, Dronc activation involves dimerization, which requires binding to Ark and formation of the apoptosome (Kanuka et al., 1999; Rodriguez et al., 1999; Zhou et al., 1999). Following dimerization, autocatalytic cleavage occurs between the large and small subunits, which stabilizes the dimer and enhances enzyme activity (Snipas et al., 2008). Like many caspases, Dronc can autoactivate in the absence of Ark when the protein is present at high concentration, such as during purification of recombinant protein from bacteria (Hawkins et al., 2000). A low level of chronic apoptosome formation occurs naturally in *D. melanogaster* cells and embryos, meaning that cell survival depends on the continuous presence of the DIAP1 protein, an E3 ubiquitin ligase responsible for inhibiting Dronc (Wang et al., 1999; Dorstyn et al., 2002; Muro et al., 2002; Zimmermann et al., 2002). Activated Dronc cleaves and activates the effector caspases DrICE and DCP-1, leading to apoptosis, mainly through the activity of DrICE (Xu et al., 2009).

A recent analysis of Lepidopteran caspase sequences classified these proteins into 6 clades, with Dronc orthologs being placed in a clade called Lep-Caspase-5 (Courtiade et al., 2011). Although orthologs of Dronc can be identified by sequence homology in the currently available insect genomes, only two other Dronc orthologs have been characterized in any detail, those of the mosquito *Aedes aegypti* (Cooper et al., 2007; Liu and Clem, 2011) and the lepidopteran *Bombyx mori* (Suganuma et al., 2011). As is the case with DmDronc, both AeDronc and BmDronc are required for apoptosis, since silencing their expression renders cells resistant to apoptotic stimuli (Liu and Clem, 2011; Suganuma et al., 2011).

Spodoptera frugiperda, the fall armyworm, is a pest insect of crops and ornamental plants (Sparks, 1979). The complete genome sequence of *S. frugiperda* has not been determined, but some EST sequences are available. Cell lines derived from *S. frugiperda*, namely IPLB-SF21-AE and its clonal isolate Sf9, have been heavily exploited for expression of foreign proteins using the baculovirus expression system. In addition, *S. frugiperda* larvae and cell lines have been extensively utilized as a model system for understanding baculovirus replication and apoptosis (Clem, 2007). Despite the use of *S. frugiperda* cell lines as a model for studying apoptosis, only three apoptosis regulatory genes (two effector caspases, Sf-caspase-1 and Sf-caspase-2, and an IAP, Sf-IAP) have been identified in this insect (Ahmad et al., 1997; LaCount, 1998; Huang et al., 2000). Nonetheless, using *S. frugiperda* cell lines,

the functions of several anti-apoptotic baculovirus proteins have been studied, including the caspase inhibitors AcP35 from *Autographa californica* M nucleopolyhedrovirus (AcMNPV) and SpliP49 from *Spodoptera littoralis* nucleopolyhedrovirus (SpliNPV), and baculovirus IAP proteins. By studying the activation of Sf-caspase-1 in cell lines after AcMNPV infection, it was determined that AcP35 inhibits the activity, but not the cleavage and activation, of Sf-caspase-1 (LaCount et al., 2000; Manji and Friesen, 2001). Meanwhile, SpliP49 is able to inhibit both Sf-caspase-1 cleavage/activation and activity (Zoog et al., 2002). Based on this observation, it was postulated that SpliP49 is capable of inhibiting an unknown initiator caspase activity in *S. frugiperda*, which was named Sf-caspase-X (LaCount et al., 2000), and which is responsible for cleaving and activating Sf-caspase-1. However the identity of Sf-caspase-X has remained elusive for over a decade.

In this study, we characterized a homolog of Dronc from *S. frugiperda*, which we have named SfDronc. We demonstrate that SfDronc has the expected characteristics of an initiator caspase, including the ability to cleave and activate Sf-caspase-1 at the expected cleavage site, and that SfDronc is involved in apoptosis in Sf9 cells. In addition, SfDronc activity is inhibited by SpliP49 but not by AcP35. These results indicate that SfDronc is likely to be Sf-caspase-X, the long-sought initiator caspase involved in apoptosis in *S. frugiperda*.

2. MATERIALS AND METHODS

2.1. Cells and virus

Sf9 cells (Invitrogen) were maintained at 27°C in TC-100 medium (Invitrogen) supplemented with 10% fetal bovine serum (Atlanta Biologicals), penicillin G (60 g/ml), streptomycin sulfate (200 g/ml), and amphotericin B (0.5 g/ml). The construction and amplification of the *p35* mutant virus vAcP35KO-PG was described previously (Huang et al., 2011b).

2.2. Identification and sequencing of SfDronc cDNA

SfDronc was initially identified as a partial sequence in a TBLASTN search of the SPODOBASE database of expressed sequence tag (EST) sequences using *Drosophila* Dronc as a query. Accession no. Sf1P04353-5-1 contained an incomplete ORF with significant homology to the C-terminus of *Drosophila* Dronc, as well as 3' UTR sequence. Repeated attempts to clone longer products containing the SfDronc sequence by 5' RACE were initially unsuccessful, presumably due to the GC-rich property of the sequence. A BLAST search of an RNASeq database using *Drosophila* Dronc as the query (courtesy of Dr. Philippe Fournier) revealed a short sequence with homology to the N-terminal portion of Dronc, (F67SK7T01BH193_x10), which allowed for the design of primers to amplify the full length cDNA. To amplify the full length SfDronc open reading frame, total RNA was isolated from Sf9 cells using Trizol (Invitrogen) and cDNA was synthesized using ImProm-II reverse transcriptase (Promega) (Huang et al., 2011b). The *SfDronc* coding region was then amplified using the forward and reverse primers SfDronc-ORF-F and SfDronc-ORF-R (Table S1). PCR was performed using Advantage®-GC 2 PCR kit (Clontech) under the following conditions: 3 min at 98°C, then 1 cycle for 30 s at 98°C, 30 s at 70°C and 90 s at 68°C, 1 cycle for 30 s at 98°C, 30 s at 63°C and 90 s at 68°C, 1 cycle for 30 s at 98°C, 30 s at 56°C and 90 s at 68°C, 25 cycles for 30 s at 98°C, 30 s at 60°C, and 90 s at 68 °C. The resulting PCR product was subcloned into pCR®II vector (Invitrogen) and sequenced using methods optimized for GC-rich templates (GENEWIZ). 5' and 3' RACE was used to amplify the 5' and 3' untranslated regions and confirm the N-terminus of the SfDronc ORF. Sequencing of several independently derived cDNAs was performed to verify the accurate sequence. A total of 8 single nucleotide polymorphisms were identified between the

SfDronc ORF sequence derived from Sf9 cells and the composite sequence derived from Spodbase and the RNAseq database (Table S2). These SNPs, which were present in multiple independently amplified cDNA sequences, were located at nucleotide positions 49, 135, 165, 174, 184, 468, 1125, and 1255 (with the first nucleotide in the initiation codon being at position 1). Three of these SNPs resulted in non-synonymous changes: at nucleotide 49/amino acid 17 (I to V), nucleotide 184/amino acid 62 (T to P), and nucleotide 1255/amino acid 419 (V to M). In the SfDronc clones used in these experiments, nucleotide 184 was changed to match that in the RNAseq sequence (C to A), and thus the clones used differ from accession number JX912275 by encoding a threonine residue at amino acid 62. A cDNA encoding proline at position 62 was found to also be enzymatically active (data not shown).

2.3 GenBank accession number

The sequence obtained from Sf9 cell cDNA was deposited in GenBank under accession number JX912275.

2.4. Construction of plasmids

pET23a-SfDronc, pET23a-AcP35, and pET23a-Sf-caspase-1 containing a His-tag at their C-termini were constructed by cloning the appropriate open reading frames into the restriction sites NdeI/HindIII (SfDronc) or NdeI/XhoI (AcP35 and Sf-caspase-1) of pET-23a(+) (Novagen). SpliP49-pET23a-noT7 was constructed by cloning a version of the SpliP49 ORF containing a C-terminal GSGSGS linker (amplified using oligos P49-1 and P49-2, Table S1) into pET23a bearing a modified polylinker to encode a C-terminally His-tagged protein. Ectopic expression plasmid pIE-1-SfDroncHA-polyA containing SfDronc with a hemagglutinin (HA) tag at the C-terminus was constructed for overexpression of SfDronc in Sf9 cells. The SfDronc open reading frame was amplified by PCR with primers SfDronc-NcoI-F and SfDronc-HA-tag-NcoI-R (Table S1) and subcloned into pCR[®]II vector to TA-SfDroncHA. After the correct sequence was confirmed, IE-1 promoter and polyA sequences were amplified by PCR using pGR09-10 (Huang et al., 2011a) as a template and inserted into restriction sites EcoRV/XbaI and HindIII/SpeI of TA-SfDroncHA.

Point mutations were made by a previously described method (Chiu et al., 2008) using the primers shown in Table S3. pET23a-SfDronc, pET23a-Sf-caspase-1, or pIE-1-SfDroncHA-polyA were used as PCR templates, using Vent Polymerase (New England Biolabs). The PCR conditions for Sf-caspase-1 were: 94°C for 2min, followed by 25 cycles of 94°C for 15s, 60°C for 20s and 68°C for 5min, and 68°C for 7min, and the PCR for SfDronc using Advantage[®]-GC 2 PCR kit (Clontech). PCR conditions for SfDronc were: 98°C for 3 min, followed by 1 cycle of 98°C for 30 s, 70°C for 30 s and 68°C for 5 min, 1 cycle of 98°C for 30 s, 63°C for 30 s and 68°C for 5 min, 1 cycle of 98°C for 30 s, 56°C for 30 s and 68°C for 5 min, and 25 cycles of 98°C for 30 s, 60°C for 30 s, and 68°C for 5 min, with a final extension at 68°C for 7 min. The PCR products were digested with DpnI (New England Biolabs) for 2 h and denatured for 3 min at 99°C, and then hybridized using two cycles of 5 min at 65°C and 15 min at 30°C. An aliquot of 10 µl from each hybridization mixture was transformed into *E. coli* strain DH5α. The presence of the desired mutations and the absence of any undesired mutations were confirmed by DNA sequencing.

2.5. Gene silencing by RNA interference (RNAi)

Sf9 cell cDNA or full-length open reading frame sequences were used as templates for PCR, with the T7 promoter sequence TAATACGACTCACTATAGGG added at each 5' terminus. The primers used to produce templates for double-stranded RNAs (dsRNAs) are shown in Table S4. Synthesis of dsRNAs was done using the resulting PCR products as templates by *in vitro* transcription using the T7-Scribe[™] Standard RNA In Vitro Transcription Kit

(Cellsript). The transfection of dsRNA into Sf9 cells was done as previously described (Huang et al., 2011b) using 150 µg of each dsRNA.

2.6. Plasmid transfection

The transfection of ectopic expression plasmid pIE-1-SfDroncHA-polyA, catalytic site mutant pIE-1-SfDroncHAC310A-polyA or cleavage site mutant pIE-1-SfDroncHAD340A-polyA into Sf9 cells was done as previously described (Huang et al., 2011a) using lipofectin and 3 µg of each plasmid.

2.7. Chemical and UV treatments

Treatment with actinomycin D (Fisher Scientific) or UV was done as described previously (Huang et al., 2011a; Huang et al., 2011b). At 24 h post transfection of dsRNA, Sf9 cells were infected with vAcP35-KOPG (MOI=5), treated with actinomycin D at a final concentration of 250 ng/ml, or treated with UV by placing on a transilluminator for 8 min. Cells were harvested at 4 h after ActD or UV treatment or at 48 h post virus infection for caspase activity and cell viability assay and immunoblotting. Where indicated, the pan caspase inhibitor Z-Val-Ala-Asp-(OMe)-Fluoromethylketone (Z-VAD-FMK) (MP Biomedicals) was added to Sf9 cells at a final concentration of 20 µM in TC-100 containing 10% FBS.

2.8. Recombinant protein purification

Plasmids pET23a-SfDronc, pET23a-SfDroncD340A, pET23a-SfDroncE344A, pET23a-AcP35, pET23a-Sf-caspase-1 and SpliP49-pET23a-noT7 were transformed into *E. coli* strain BL21(DE3)/pLysS. Recombinant, C-terminally His-tagged proteins were isolated using Talon resin (Clontech) as previously described (Wu and Passarelli, 2010). For expression, BL21(DE3)/pLysS cells containing expression plasmids were grown to a concentration of $A_{600} = 0.6$ in 1 L LB broth containing 100 µg/ml ampicillin, induced by addition of IPTG (isopropyl-β-D-thiogalactopyranoside) at a final concentration of 1 mM, and incubated for 20 h at 18°C. *E. coli* cells were harvested and lysed in 50 ml cold extraction buffer (300 mM NaCl, 50 mM NaH₂PO₄, 20 mM imidazole, pH 7.5) containing a Complete Mini EDTA-free protease inhibitor cocktail tablet (Roche), followed by 12 cycles of sonication for 10 s each. After centrifugation at 12,000 rpm for 15 min at 4°C, the clarified cell lysate was transferred to new tubes, mixed with 500 µl Talon resin, and incubated at 4°C for 3 h with constant rotation. The resin was washed with extraction buffer and transferred to a 2 ml gravity column, washed three times with extraction buffer, and eluted with extraction buffer containing 100 mM imidazole. Active site titration was performed as published (Stennicke and Salvesen, 1999) using Z-VAD-FMK (Merck).

2.9. SDS-PAGE and Immunoblotting

Purified recombinant proteins, reticulocyte lysates, or cultured Sf9 cells that had been washed and resuspended with PBS were mixed with an equal volume of 2× Laemmli buffer, incubated at 100°C for 5 min, and subjected to 15% SDS-PAGE. Proteins were visualized by Coomassie Blue staining or transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). The membranes were incubated with the primary antisera or antibodies (all diluted 1:3000) anti-Sfscap1 (LaCount et al., 2000) (provided by Paul Friesen), anti-GAPDH (Abcam), or monoclonal anti-HA (Covance) for 2 h, washed and incubated with horseradish peroxidase-conjugated secondary antibodies (diluted 1:3000) (Abcam) for 2 h. Where actin was detected as a loading control, monoclonal anti-β-actin-peroxidase (Sigma) was used (diluted 1:2000). Antibody binding was detected using SuperSignal West Pico chemiluminescent substrate (Pierce).

2.10. Caspase activity and cell viability assays

Cell viability assays were performed by manual cell counting as previously described (Huang et al., 2011a) and normalized by setting the highest value to 100%.

The substrate preference of purified recombinant SfDronc-His₆ was assayed using fluorogenic substrates Ac-LETD-AFC, Ac-DEVD-AFC, Ac-VEID-AFC, Ac-LETD-AFC, Ac-VDVAD-AFC, Ac-IETD-AFC and Ac-WEHD-AFC purchased from Enzo Life Sciences (Farmingdale, NY, USA), and Ac-TQTD-AFC and Ac-TQTE-AFC, which were custom-made by Life Research (Burwood East, Victoria, Australia). SfDronc-His₆ was pre-activated for 10 min at 37 °C in reaction buffer (50 mM Tris pH 7.4, 100 mM NaCl, 0.65 M Na-Citrate, 10 mM DTT). Active site-titrated SfDronc (350 nM) was mixed with 100 μM of each fluorogenic substrate in activity buffer to assess specificity, and the maximal rate of cleavage was determined in relative fluorescence units per minute. To determine cleavage efficiency, 1–1000 μM of selected fluorogenic substrates were incubated with 308 nM of active site-titrated, pre-activated SfDronc, in activity buffer. Fluorescence (excitation 410 nm, emission 500 nm) was measured every minute for 1 h. The maximal slope of each curve was calculated. A standard curve, using free AFC (Sigma), was used to convert fluorescence emissions into concentration of AFC produced during the cleavage reactions. Prism 5.0 software was used to determine k_{cat} and K_M using the equation $Y = Et * k_{cat} * X / (K_M + X)$, where Et is the concentration of enzyme catalytic sites, K_M is the Michaelis-Menten constant, k_{cat} is the turnover number, X is the concentration of substrate, and Y is change in fluorescence.

To measure caspase activity in Sf9 cells, cells were harvested at 4 h after ActD or UV treatment or at 48 h post-infection, washed with PBS and resuspended in 100 μl lysis buffer (20 mM HEPES KOH, pH 7.5, 50 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 250 mM sucrose) containing a Complete Mini EDTA-free protease inhibitor cocktail tablet. Fifty μl cell lysate was incubated with 50 μl reaction buffer (100 mM HEPES, pH 7.4, 2mM DTT, 0.1% CHAPS, 1% sucrose) at 37°C for 15 min. Caspase substrate Ac-DEVD-AFC (MP Biomedicals) was then added at a final concentration of 40 μM and the fluorescence (excitation wavelength of 405 nm and emission wavelength of 505 nm) was monitored every 15 min for 2 h at 25°C using a Victor³ 1420 Multilabel counter (Perkin-Elmer). The maximal values obtained were normalized by setting the sample with the highest value at 100. For caspase inhibition assays, 1 μM SfDronc-His₆ or 50 nM Sf-caspase-1-His₆ were incubated with increasing concentrations of AcP35-His₆ or SpliP49-His₆ (0–10μM) in reaction buffer (100 mM HEPES buffer, pH 7.4 containing 2 mM DTT, 0.1 % CHAPS, 1 % sucrose). Following incubation at 37°C for 30 min, caspase substrate was added to the reactions at a concentration of 40 μM. Ac-IETD-AFC (MP Biomedicals) was used for SfDronc and Ac-DEVD-AFC was used for Sf-caspase-1. Fluorescence was measured once per minute using a Victor3 1420 Multilabel counter (Perkin-Elmer) for 1 h. Maximal activity was plotted as the change at the steepest part of the curve in relative fluorescence units (RFU) per minute, relative to caspase alone which was set at 100.

In vitro translation was carried out in 50 μl reactions using the template plasmids pET23a-Sf-caspase-1 or pET23a-Sf-caspase-1-D95A and the TNT Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's instructions. Seven μl of each *in vitro* translation reaction was mixed with 0.5 μM SfDronc-His₆ or SfDronc-C310A-His₆, 50 μl reaction buffer (100 mM HEPES, pH 7.4, 2mM DTT, 0.1% CHAPS, 1% sucrose), and 40 μM Ac-DEVD-AFC, and extraction buffer (300 mM NaCl, 50 mM NaH₂PO₄, 20 mM imidazole, pH 7.5) was added to bring the total volume to 100 μl. The reactions were incubated at 37°C for 30 min, after which fluorescence was measured as described above.

3. RESULTS AND DISCUSSION

3.1. SfDronc sequence analysis and phylogeny

BLAST searches of available *S. frugiperda* sequences at Spodobase (<http://bioweb.ensam.inra.fr/spodobase/>) identified a partial cDNA sequence with significant homology to *D. melanogaster* Dronc. After cloning the full length cDNA as described in Materials and Methods, an ORF was identified and named *SfDronc*. The *SfDronc* cDNA sequence was deposited in GenBank and assigned accession number JX912275. The full length cDNA contains 5' and 3' untranslated regions of 167 and 547 bp in length, respectively, while the predicted SfDronc ORF is 1344 nt long and encodes a predicted protein of 447 amino acids with a molecular mass of 51 kDa. Alignment of the predicted amino acid sequence of SfDronc with Dronc orthologs of *B. mori* (BmDronc), *A. aegypti* (AeDronc) and *D. melanogaster* (DmDronc) (Fig. 1A) revealed that SfDronc shares 53.6% and 24.8% amino acid identity with BmDronc and DmDronc respectively. Analysis of the SfDronc sequence revealed several features that are typical of caspases, and in particular initiator caspases. SfDronc contains a well-conserved putative catalytic site (QMCRG) centered at position 310, which is similar to the catalytic site found in BmDronc (QACRG) and most other known caspases, which have the general consensus sequence QAC(R/Q/G)(G/E). Notably, the catalytic sites found in Dronc orthologs from dipterans including 12 different *Drosophila* species (PFCRG) and the mosquitoes *A. aegypti*, *Anopheles gambiae*, and *Culex quinquefasciatus* (SICRG) stand out as unusual due to substitution of the glutamine in the first position of the catalytic site. Homology searches indicated the presence of a caspase recruitment domain (CARD) in a long N-terminal prodomain (Fig. 1A), which is also present in other Dronc homologs, and in *Drosophila* Dronc is responsible for binding to Ark in the apoptosome (Yuan et al., 2011). The predicted secondary structure of the remainder of SfDronc consisted of a series of five alpha helices and six beta sheets that are highly conserved in other caspases (Watt et al., 1999). BLAST analysis against available sequences in GenBank indicated that the closest relatives of SfDronc are sequences found in other lepidopteran insects, including *Spodoptera litura*, *Helicoverpa armigera*, *Lymantria dispar*, *B. mori*, and *Pieris rapae*. However, with the exception of BmDronc, these other lepidopteran caspases remain uncharacterized as hypothetical ORFs. Phylogenetic analysis of a number of caspases from insects indicated that the insect Dronc orthologs form an independent clade that is separate from other initiator and effector caspases (Fig. 1B).

3.2. Silencing the expression of SfDronc reduces apoptosis in Sf9 cells

Infection of Sf9 cells with mutants of the baculovirus AcMNPV lacking the caspase inhibitor P35 induces caspase-dependent apoptosis (reviewed in (Clem, 2007)). Apoptosis interrupts viral replication in these cells prior to formation of viral occlusion bodies, which are large, virion-containing protein crystals that are easily visible in the nucleus at late times after infection. To determine whether SfDronc is involved in the induction of apoptosis by AcMNPV, we silenced the expression of *SfDronc* in Sf9 cells by RNAi, and then infected the cells with vAcP35-KOPG, a version of AcMNPV that lacks P35 and expresses GFP (Huang et al., 2011b). Silencing of *SfDronc* was effective at the mRNA level, as observed by RT-PCR, but only partially effective at the protein level, as determined by silencing the expression of a transiently expressed, tagged version of SfDronc (Figs. S1 and S2). Cells which had been transfected with dsRNA corresponding to either *SfDronc* or the negative control bacterial sequence *chloramphenicol acetyl transferase* (*cat*) became equally infected, as indicated by GFP expression (Fig. 2A). However, while infected cells that had been transfected with control dsRNA underwent apoptosis, as demonstrated by apoptotic morphology, a decrease in the number of viable cells, and an increase in caspase activity, the infected cells which had received *SfDronc* dsRNA remained largely alive at 48 h post-

infection (Fig. 2A–C). Silencing *SfDronc* also allowed vAcP35-KOPG to complete its replication cycle in Sf9 cells, as shown by the presence of viral occlusion bodies in the nuclei of some of the infected cells (Fig. 2D).

To test whether SfDronc is involved in apoptosis triggered by other stimuli, we also tested the effect of silencing *SfDronc* on apoptosis triggered by treatment with actinomycin D or UV. As seen in Fig. 3, apoptosis triggered by either stimulus was significantly inhibited by silencing *SfDronc*, indicating that SfDronc is important for apoptosis in Sf9 cells that is stimulated by diverse stimuli. The observation that apoptosis was not completely blocked by silencing *SfDronc* is likely due to incomplete silencing; although we cannot rule out the possibility that additional initiator caspase(s) are also involved in apoptosis in *S. frugiperda*, we view this as unlikely since Dronc is responsible for essentially all apoptosis in *Drosophila*.

As a comparison, we also tested the effect of silencing the effector caspase *Sf-caspase-1*. Silencing of *Sf-caspase-1* was not complete at the mRNA level, as demonstrated by RT-PCR (Fig. S1), but nonetheless a decrease in Sf-caspase-1 protein was observed following treatment with *Sf-caspase-1* dsRNA (Fig. S2). RNAi of *Sf-caspase-1* also reduced the level of apoptosis stimulated by virus infection, actinomycin D or UV, but not to as great an extent as silencing *SfDronc* (Figs. 2 and 3). Again, this could be due to the fact that *Sf-caspase-1* was not completely silenced. However, there also may be other effector caspases, such as Sf-caspase-2 (LaCount, 1998), expressed in Sf9 cells that can partially function even when levels of Sf-caspase-1 are reduced. This latter situation exists in *A. aegypti*, where CASPS7 and CASPS8 are both effector caspases that are involved in apoptosis (Liu and Clem, 2011).

3.3. Silencing expression of SfDronc decreases proteolytic processing of Sf-caspase-1 triggered by UV

Treatment of Sf9 cells with apoptotic stimuli triggers the activation of Sf-caspase-1, which is caused by cleavage of pro-Sf-caspase-1 at amino acid position D195, located between the large and small subunits. Cleavage at D195 results in the release of two Sf-caspase-1 peptides, the free small subunit and the prodomain-large subunit, and is sufficient to activate Sf-caspase-1. This initial cleavage event has been postulated to be carried out by the unidentified caspase, Sf-caspase-X (LaCount et al., 2000). A second cleavage event, probably performed by Sf-caspase-1 itself, then releases the free large subunit from the prodomain. These cleavage events can be monitored by immunoblotting using a polyclonal antiserum raised against the large subunit of Sf-caspase-1, which was kindly provided to us by Paul Friesen (LaCount et al., 2000). UV treatment of Sf9 cells that had been transfected with control *cat* dsRNA caused Sf-caspase-1 activation, which could be observed by appearance of the prodomain-large subunit and free large subunit at 3 and 6 h post UV treatment (Fig. 4). RNAi of *SfDronc* resulted in decreased processing of pro-Sf-caspase-1 following treatment with UV, as seen by reduced levels of the cleaved forms compared to *cat* RNAi (Fig. 4). This decrease suggests that SfDronc is responsible for cleavage and activation of Sf-caspase-1. Again, although RNAi of SfDronc did not completely abolish Sf-caspase-1 activation, this is likely due to incomplete silencing of SfDronc, but we cannot completely rule out the possibility that another initiator caspase can cleave Sf-caspase-1 after UV treatment.

3.4. Recombinant SfDronc undergoes autocatalytic cleavage at position D340, prefers initiator caspase substrates, and requires an aspartate in the P₁ position

To study the enzymatic activity of SfDronc, full length C-terminally His₆-tagged SfDronc (SfDronc-His₆) was expressed and purified from *E. coli*. As has been observed with many

other recombinant caspases, recombinant SfDronc-His₆ underwent autoactivation during purification, cleaving itself into free large and small subunits (Fig. 5A). In the case of DmDronc, dimerization induces autocatalytic cleavage at a glutamate residue (E352) located between the large and small subunits, and this cleavage event stabilizes the DmDronc dimer and greatly enhances enzyme activity (Hawkins et al., 2000; Snipas et al., 2008). To determine the cleavage site between the large and small subunits in SfDronc, we compared the sequences of SfDronc and DmDronc in the region surrounding E352 in DmDronc. Based on the sequence of SfDronc in this region, we hypothesized that one of two sites in SfDronc, either D340 or E344, was the cleavage site. We mutated each of these amino acids to alanine and tested the effect on SfDronc activity. Analysis by SDS-PAGE showed that the mutant protein SfDronc-E344A-His₆ still underwent autocatalytic cleavage similar to wild type SfDronc-His₆, but the mutant SfDronc-D340A-His₆ did not undergo autocatalytic cleavage (Fig. 5A). Furthermore, purified SfDronc-D340A-His₆ had 9.9-fold less enzymatic activity against the fluorogenic substrate Ac-IETD-AFC than wild type SfDronc-His₆ (Fig. 5B). This is compatible with previous observations made with DmDronc which have shown that cleavage at E252 is not required for activation, but only enhances DmDronc activity (Snipas et al., 2008). SfDronc-E344A-His₆ also had reduced activity against Ac-IETD-AFC, but the reduction was only 3.7-fold (Fig. 5B), and could be due to charge differences affecting the protein structure in this region. Thus SfDronc undergoes autocatalytic cleavage at position D340, which results in enhanced enzyme activity.

To examine the substrate specificity of recombinant SfDronc, purified SfDronc-His₆ was incubated with several different fluorogenic caspase substrates and enzyme activity was measured by release of fluorescence. Recombinant SfDronc-His₆ had the highest activity against the initiator caspase substrates Ac-VEID-AFC, Ac-IETD-AFC, and Ac-LETD-AFC, but had little activity against the substrates Ac-TQTD-AFC, Ac-LEHD-AFC, Ac-DEVD-AFC, Ac-WEHD-AFC, and Ac-VDVAD-AFC (Fig. 5C and Table 1). It is interesting to note that Ac-IETD-AFC and Ac-LETD-AFC both contain the sequence ETD, which is the same sequence found at the cleavage site in Sf-caspase-1 that is utilized by Sf-caspase-X (TETD¹⁹⁵). Additionally, even though SfDronc exhibited a low amount of activity against Ac-TQTD-AFC, no cleavage activity was observed against Ac-TQTE-AFC (Fig. 5C), suggesting that SfDronc requires aspartate in the P₁ position of its substrates, unlike DmDronc which can cleave substrates containing either aspartate or glutamate at the P₁ position (Hawkins et al., 2000).

3.5. SfDronc directly cleaves and activates Sf-caspase-1

The initiator caspase activity known as Sf-caspase-X cleaves Sf-caspase-1 at the site TETD¹⁹⁵↓G, resulting in activation of Sf-caspase-1 (LaCount et al., 2000). To test whether SfDronc was able to carry out this cleavage event, we mixed recombinant SfDronc and Sf-caspase-1 and monitored cleavage of Sf-caspase-1 by immunoblotting with anti-Sf-caspase-1 antiserum (Fig. 6A). To prevent autoactivation of Sf-caspase-1, we used either an active site mutant (Sf-caspase-1-C178A-His₆) or a cleavage site mutant (Sf-caspase-1-D195A-His₆), both of which are unable to undergo autoactivation. SfDronc-His₆ was able to cleave Sf-caspase-1-C178A-His₆, resulting in accumulation of the large subunit + prodomain (Fig. 6A, lane 1). This cleavage event was dependent on the presence of the D195 residue at the cleavage site, indicating that cleavage occurred at the expected site, D195 (lane 2). Furthermore, cleavage of Sf-caspase-1-His₆ at D195 was abolished by a mutation in the active site cysteine residue of SfDronc (C310A), confirming that SfDronc catalytic activity was required for Sf-caspase-1 cleavage (Fig. 6A, lanes 3 and 4).

In addition to showing that SfDronc was able to cleave Sf-caspase-1 at the expected site, we also wanted to confirm that cleavage by SfDronc was able to activate the enzymatic activity of Sf-caspase-1. To do so, we produced Sf-caspase-1 and Sf-caspase-1-D195A by *in vitro*

translation in reticulocyte lysate, and added recombinant SfDronc-His₆. Similar amounts of wild type Sf-caspase-1 and Sf-caspase-1-D195A were produced by *in vitro* translation, as determined by immunoblotting (data not shown). Wild type Sf-caspase-1 produced by *in vitro* translation did not autoactivate due to its relatively low concentration in the reticulocyte lysate (Fig. 6B). However, addition of recombinant SfDronc-His₆ caused robust activation of Sf-caspase-1, as measured by cleavage of the effector caspase substrate DEVD-AFC (Fig. 6B). Activation of Sf-caspase-1 by SfDronc was dependent on both the wild type cleavage site in Sf-caspase-1, as shown by the inability of SfDronc to activate Sf-caspase-1-D195A, and on the enzymatic activity of SfDronc, as shown by the lack of activation by SfDronc-C310A-His₆. Together, these results demonstrate that SfDronc is able to directly cleave Sf-caspase-1 at the cleavage site D195, resulting in the activation of Sf-caspase-1.

3.6. Transient expression of SfDronc induces apoptosis in Sf9 cells

To test whether overexpressing SfDronc would induce apoptosis in Sf9 cells, we constructed three plasmids expressing different versions of SfDronc that were HA-tagged at the C-terminus, with expression driven by the baculovirus *ie-1* promoter: wild type SfDronc (pIE-1-SfDroncHA-polyA), a catalytic site mutant (pIE-1-SfDroncHA-C310A-polyA), and a cleavage site mutant (pIE-1-SfDroncHA-D340A-polyA). Following transfection of these plasmids individually into Sf9 cells, apoptosis was observed at 24 h post transfection with pIE-1-SfDroncHA-polyA but not with either of the mutant plasmids (Fig. 7A–B). This apoptosis was inhibited by the general caspase inhibitor Z-VAD-FMK (Fig. 7B). Immunoblotting with an antibody against the HA tag showed that the three proteins were expressed at similar levels (Fig. 7C). We were not able to consistently detect cleaved SfDronc in the transfected cells; presumably the fraction of transiently expressed protein that becomes autoactivated, while enough to stimulate apoptosis in some cells, is low compared to the total amount of expressed protein. Nonetheless, these results indicate that the ability of SfDronc to induce apoptosis when overexpressed in Sf9 cells requires SfDronc enzymatic activity, and that reducing the activity of the enzyme by blocking its cleavage at D340 abolishes its ability to kill cells.

3.7. Enzymatic activity of recombinant SfDronc is inhibited by SpliP49 but not by AcP35

The previously observed initiator caspase activity in *S. frugiperda*, attributed to Sf-caspase-X, is inhibited by SpliP49 but is resistant to AcP35 (Manji and Friesen, 2001; Zoog et al., 2002). To determine whether SfDronc is compatible with this inhibitor profile, recombinant SfDronc-His₆, SpliP49-His₆ and AcP35-His₆ were used for caspase inhibition assays. Recombinant Sf-caspase-1-His₆, which is inhibited by both SpliP49 and AcP35 (LaCount et al., 2000; Zoog et al., 2002), was used as a control. SpliP49-His₆ inhibited both Sf-Dronc-His₆ and Sf-caspase-1-His₆ with similar kinetics (Fig. 8A). At a 1:1 ratio of SpliP49:caspase, Sf-Dronc-His₆ activity was reduced by 68%, while Sf-caspase-1-His₆ activity was reduced by 42%. However, while AcP35-His₆ had the expected ability to inhibit Sf-caspase-1-His₆, it was not able to inhibit SfDronc-His₆, even when up to a 100-fold molar excess of AcP35-His₆ was used (Fig. 8B and data not shown). Preliminary results indicated that SfDronc was also not inhibited by MaviP35, a P35 homolog from *Maruca vitrata* NPV (Brand et al., 2011) (data not shown). Thus SfDronc is inhibited by SpliP49 but is resistant to AcP35, which is consistent with the inhibitor profile of Sf-caspase-X.

3.8. Conclusions

Our data indicate that SfDronc is likely to be directly responsible for the initiator caspase enzymatic activity that was previously attributed to Sf-caspase-X (LaCount et al., 2000). First, Dronc is the sole apoptotic initiator caspase in *D. melanogaster*, and all insect genomes sequenced to date contain only a single Dronc ortholog, making it likely that SfDronc is the major apoptotic initiator caspase in *S. frugiperda*. Second, silencing of *SfDronc* expression

reduced the cleavage of Sf-caspase-1 and inhibited apoptosis in Sf9 cells. Although the inhibition of apoptosis was not complete, silencing of SfDronc was also not complete at the protein level. Third, SfDronc was able to directly cleave Sf-caspase-1 at the same cleavage site as Sf-caspase-X, position D195, resulting in biochemical activation of Sf-caspase-1. Finally, the activity of SfDronc was inhibited by SpliP49, but not by AcP35. All of these characteristics strongly indicate that SfDronc is the major initiator caspase in *S. frugiperda*, and that SfDronc is activated upon apoptotic stimulation and cleaves effector caspases, including Sf-caspase-1, leading to apoptosis. Discovery of SfDronc will facilitate further investigation of apoptosis mechanisms in *S. frugiperda*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Dr. Philippe Fournier (Institut National de la Recherche Agronomique) for providing unpublished *S. frugiperda* sequence data, which was critical for cloning the full-length *SfDronc* cDNA. We also thank Dr. Paul Friesen (University of Wisconsin-Madison) for providing anti-Sf-caspase-1 antiserum, and Katelyn O'Neill for assistance with recombinant protein purification. We gratefully acknowledge Dr. George Rohrmann (Oregon State University) for providing financial support of NH during a portion of this project. This work was supported by NIH grants R01AI091972 and P20RR016475/GM103418, by the Kansas Agricultural Experiment Station and by the Australian National Health and Medical Research Council (#602525) and an Australian Research Council Future Fellowship to CJH (#FT0991464). Contribution no. 13-090-J from the Kansas Agricultural Experiment Station.

REFERENCES

- Ahmad M, Srinivasula S, Wang L, Litwack G, Fernandes-Alnemri T, Alnemri E. *Spodoptera frugiperda* caspase-1, a novel insect death protease that cleaves the nuclear immunophilin FKBP46, is the target of the baculovirus antiapoptotic protein P35. *J. Biol. Chem.* 1997; 272:1421–1424. [PubMed: 8999805]
- Brand IL, Green MM, Civciristov S, Pantaki-Eimany D, George C, Gort TR, Huang N, Clem RJ, Hawkins CJ. Functional and biochemical characterization of the baculovirus caspase inhibitor MaviP35. *Cell Death Dis.* 2011; 2:e242. [PubMed: 22170098]
- Chew SK, Akdemir F, Chen P, Lu WJ, Mills K, Daish T, Kumar S, Rodriguez A, Abrams JM. The apical caspase *dronc* governs programmed and unprogrammed cell death in *Drosophila*. *Dev. Cell.* 2004; 7:897–907. [PubMed: 15572131]
- Chiu J, Tillett D, Dawes IW, March PE. Site-directed, Ligase-Independent Mutagenesis (SLIM) for highly efficient mutagenesis of plasmids greater than 8kb. *J. Microbiol. Methods.* 2008; 73:195–198. [PubMed: 18387684]
- Clem RJ. Baculoviruses and apoptosis: A diversity of genes and responses. *Curr. Drug Targets.* 2007; 8:1069–1074. [PubMed: 17979666]
- Cooper DM, Thi EP, Chamberlain CM, Pio F, Lowenberger C. *Aedes Dronc*: a novel ecdysone-inducible caspase in the yellow fever mosquito, *Aedes aegypti*. *Insect Mol. Biol.* 2007; 16:563–572. [PubMed: 17725799]
- Courtiade J, Pauchet Y, Vogel H, Heckel DG. A comprehensive characterization of the caspase gene family in insects from the order Lepidoptera. *BMC Genomics.* 2011; 12:357. [PubMed: 21740565]
- Daish TJ, Mills K, Kumar S. *Drosophila* caspase DRONC is required for specific developmental cell death pathways and stress-induced apoptosis. *Develop. Cell.* 2004; 7:909–915.
- Dorstyn L, Read S, Cakouros D, Huh JR, Hay BA, Kumar S. The role of cytochrome c in caspase activation in *Drosophila melanogaster* cells. *J. Cell Biol.* 2002; 156:1089–1098. [PubMed: 11901173]
- Hawkins CJ, Yoo SJ, Peterson EP, Wang SL, Vernooy SY, Hay BA. The *Drosophila* caspase DRONC cleaves following glutamate or aspartate and is regulated by DIAP1, HID, and GRIM. *J. Biol. Chem.* 2000; 275:27084–27093. [PubMed: 10825159]

- Huang N, Clem RJ, Rohrmann GF. Characterization of cDNAs encoding p53 of *Bombyx mori* and *Spodoptera frugiperda*. *Insect Biochem. Mol. Biol.* 2011a; 41:613–619. [PubMed: 21497653]
- Huang N, Wu W, Yang K, Passarelli AL, Rohrmann GF, Clem RJ. Baculovirus infection induces a DNA damage response that is required for efficient viral replication. *J. Virol.* 2011b; 85:12547–12556. [PubMed: 21917957]
- Huang Q, Deveraux QL, Maeda S, Salvesen GS, Stennicke HR, Hammock BD, Reed JC. Evolutionary conservation of apoptosis mechanisms: Lepidopteran and baculoviral inhibitor of apoptosis proteins are inhibitors of mammalian caspase-9. *Proc. Natl. Acad. Sci. U.S.A.* 2000; 97:1427–1432. [PubMed: 10677478]
- Igaki T, Yamamoto-Goto Y, Tokushige N, Kanda H, Miura M. Downregulation of DIAP1 triggers a novel *Drosophila* cell death pathway mediated by Dark and Dronc. *J. Biol. Chem.* 2002; 277:23103–23106. [PubMed: 12011068]
- Kanuka H, Sawamoto K, Inohara N, Matsuno K, Okano H, Miura M. Control of the cell death pathway by Dapaf-1, a *Drosophila* Apaf-1/CED-4-related caspase activator. *Molec. Cell.* 1999; 4:757–769. [PubMed: 10619023]
- LaCount, DJ. Ph.D. thesis. University of Wisconsin-Madison; 1998. Virus replication events and host factors involved in baculovirus-induced apoptosis.
- LaCount DJ, Hanson SF, Schneider CL, Friesen PD. Caspase inhibitor P35 and inhibitor of apoptosis Op-IAP block in vivo proteolytic activation of an effector caspase at different steps. *J. Biol. Chem.* 2000; 275:15657–15664. [PubMed: 10747956]
- Li J, Yuan J. Caspases in apoptosis and beyond. *Oncogene.* 2008; 27:6194–6206. [PubMed: 18931687]
- Liu Q, Clem RJ. Defining the core apoptosis pathway in the mosquito disease vector *Aedes aegypti*: the roles of *iap1*, *ark*, *dronc*, and effector caspases. *Apoptosis.* 2011; 16:105–113. [PubMed: 21107703]
- Lord CE, Gunawardena AH. Programmed cell death in *C. elegans*, mammals and plants. *Eur. J. Cell Biol.* 2012; 91:603–613. [PubMed: 22512890]
- Manji GA, Friesen PD. Apoptosis in motion: An apical, P35-insensitive caspase mediates programmed cell death in insect cells. *J. Biol. Chem.* 2001; 276:16704–16710. [PubMed: 11278634]
- Muro I, Hay BA, Clem RJ. The *Drosophila* DIAP1 protein is required to prevent accumulation of a continuously generated, processed form of the apical caspase DRONC. *J. Biol. Chem.* 2002; 277:49644–49650. [PubMed: 12397080]
- Rodriguez A, Oliver H, Zou H, Chen P, Wang X, Abrams JM. Dark is a *Drosophila* homologue of Apaf-1/CED-4 and functions in an evolutionarily conserved death pathway. *Nat. Cell Biol.* 1999; 1:272–279. [PubMed: 10559939]
- Snipas SJ, Drag M, Stennicke HR, Salvesen GS. Activation mechanism and substrate specificity of the *Drosophila* initiator caspase DRONC. *Cell Death Differ.* 2008; 15:938–945. [PubMed: 18309328]
- Sparks AN. A review of the biology of the fall armyworm. *Florida Entomologist.* 1979; 62:82–87.
- Stennicke HR, Salvesen GS. Caspases: preparation and characterization. *Methods.* 1999; 17:313–319. [PubMed: 10196102]
- Suganuma I, Ushiyama T, Yamada H, Iwamoto A, Kobayashi M, Ikeda M. Cloning and characterization of a *dronc* homologue in the silkworm, *Bombyx mori*. *Insect Biochem. Mol. Biol.* 2011; 41:909–921. [PubMed: 21911060]
- Wang SL, Hawkins CJ, Yoo SJ, Muller H-AJ, Hay BA. The *Drosophila* caspase inhibitor DIAP1 is essential for cell survival and is negatively regulated by HID. *Cell.* 1999; 98:453–463. [PubMed: 10481910]
- Watt W, Koeplinger KA, Mildner AM, Heinrikson RL, Tomasselli AG, Watenpaugh KD. The atomic-resolution structure of human caspase-8, a key activator of apoptosis. *Structure.* 1999; 7:1135–1143. [PubMed: 10508785]
- Wu W, Passarelli AL. Autographa californica multiple nucleopolyhedrovirus Ac92 (ORF92, P33) is required for budded virus production and multiply enveloped occlusion-derived virus formation. *J. Virol.* 2010; 84:12351–12361. [PubMed: 20861245]

- Xu D, Li Y, Arcaro M, Lackey M, Bergmann A. The CARD-carrying caspase Dronc is essential for most, but not all, developmental cell death in *Drosophila*. *Development*. 2005; 132:2125–2134. [PubMed: 15800001]
- Xu D, Woodfield SE, Lee TV, Fan Y, Antonio C, Bergmann A. Genetic control of programmed cell death (apoptosis) in *Drosophila*. *Fly*. 2009; 3:78–90. [PubMed: 19182545]
- Yuan S, Yu X, Topf M, Dorstyn L, Kumar S, Ludtke SJ, Akey CW. Structure of the *Drosophila* apoptosome at 6.9 angstrom resolution. *Structure*. 2011; 19:128–140. [PubMed: 21220123]
- Zhou L, Song Z, Tittel J, Steller H. HAC-1, a *Drosophila* homolog of APAF-1 and CED-4 functions in developmental and radiation-induced apoptosis. *Molec. Cell*. 1999; 4:745–755. [PubMed: 10619022]
- Zimmermann KC, Ricci J-E, Droin NM, Green DR. The role of ARK in stress-induced apoptosis in *Drosophila* cells. *J. Cell Biol*. 2002; 156:1077–1087. [PubMed: 11901172]
- Zoog SJ, Schiller JJ, Wetter JA, Chejanovsky N, Friesen PD. Baculovirus apoptotic suppressor P49 is a substrate inhibitor of initiator caspases resistant to P35 in vitro. *EMBO J*. 2002; 21:5130–5140. [PubMed: 12356729]

Highlights

- An initiator caspase, SfDronc, was identified in *Spodoptera frugiperda*
- SfDronc is orthologous to the apoptotic initiator caspase Dronc found in *Drosophila melanogaster* and other insects
- Silencing of SfDronc expression inhibits apoptosis in *S. frugiperda* cells, and SfDronc cleaves and activates the effector caspase Sf-caspase-1
- Protease activity of SfDronc is inhibited by the baculovirus caspase inhibitor SpliP49, but not by the related inhibitor AcP35
- SfDronc is likely to be Sf-caspase-X, the long-sought apoptotic initiator caspase in *S. frugiperda*

A

B **Fig. 1.**

The sequence of SfDronc. A) The predicted amino acid sequence of SfDronc is shown aligned to Dronc homologs from *Bombyx mori* (BmDronc), *Aedes aegypti* (AeDronc), and *Drosophila melanogaster* (DmDronc). Identical or similar residues conserved in all four proteins are shown in white on a black background. The CARD domain and predicted alpha helices and beta sheets are indicated above the sequence. The predicted catalytic site, centered at position 310, is outlined by a box, while the cleavage site at position D340 is marked by an asterisk. The alignment was performed using ClustalX 2.1 and modified by GeneDoc 3.2. InterProScan Version 4.8 was used to predict the conserved motifs in SfDronc, and JPred3 was used to predict secondary structure. B) Phylogenetic analysis of selected insect caspases. Amino acid sequences from *D. melanogaster* (DrICE, Dcp1, Decay, Dredd, and DmDronc), *S. frugiperda* (Sf-caspase-1 and SfDronc), *B. mori* (BmICE, BmDredd, and BmDronc), *A. aegypti* (AeDronc), *T. castaneum* (TcDronc), *P. rapae* (Pr-

caspase-5), *L. dispar* (Ld-caspase-5), *H. armigera* (Ha-caspase-5), and *S. litura* (Spli-caspase-5) were aligned by ClustalX 2.1 and the phylogenetic tree was constructed by MEGA 5.05 using the neighbor-joining method. GenBank accession numbers of the sequences used are listed in Table S5.

Fig. 2.

SfDronc is involved in apoptosis stimulated by baculovirus infection. Sf9 cells were transfected with dsRNA corresponding to *SfDronc*, *Sf-caspase-1*, or the control sequence *cat*, and 24 h later infected with vAcP35KO-PG. Mock cells were treated with transfection agent, but were not infected. A) Cells were viewed at 48 h post infection using either bright field (upper) or fluorescence (lower). B) Caspase activity was measured at 48 h post infection using the substrate Ac-DEVD-AFC and is shown relative to *cat* dsRNA transfected cells. The results shown are mean $\pm$ SE of two experiments. C) Cell viability was determined at 48 h post infection and is shown relative to mock-transfected cells. The results shown are mean $\pm$ SE of four experiments. D) Micrograph showing the presence of viral

occlusion bodies in cells (indicated by white arrows) that were transfected with SfDronc dsRNA and infected with vAcP35KO-PG.

Fig. 3.

Effects of silencing *SfDronc* expression on apoptosis stimulated by UV or actinomycin D. A) Sf9 cells were transfected with dsRNA corresponding to *SfDronc*, *Sf-caspase-1*, or *cat* and 24 h later the cells treated with UV. Caspase activity and cell viability were determined at 4 h after UV treatment. B) Cells transfected with the indicated dsRNAs were treated 24 h later with actinomycin D (Act D) and caspase activity and cell viability were determined 4 h later. In both panels, mock-transfected cells were treated with transfection agent but not treated with UV or actinomycin D. Results are plotted as mean $\pm$ SE of 4–6 experiments, relative to *cat*-transfected or mock-treated cells.

Fig. 4.

Proteolytic processing of Sf-caspase-1 is decreased by silencing of *SfDronc*. Sf9 cells were transfected with dsRNA corresponding to *SfDronc* or control *cat*, and 24 h later treated with UV. As a control, some cells were treated with the pan-caspase inhibitor Z-VAD-FMK prior to UV treatment. At 3 and 6 h after UV treatment, cell lysates were isolated and subjected to immunoblotting using antiserum against Sf-caspase-1. Molecular mass markers are indicated on the left, while the migration of full length Sf-caspase-1 and cleaved subunits are indicated on the right. As a loading control, the same lysates were subjected to immunoblotting using anti-actin antibody.

Fig. 5. Enzymatic activity of bacterially-expressed recombinant SfDronc and SfDronc putative cleavage site mutants. A) His-tagged SfDronc or putative cleavage site mutants D340A and E344A were expressed in *E. coli*. Bacterial cells were lysed and separated into insoluble and soluble fractions by centrifugation. The soluble fractions were applied to TALON resin, and His-tagged proteins were eluted and analyzed by SDS-PAGE and Coomassie staining. Lanes 1, insoluble pellet; lanes 2, unpurified soluble fraction; lanes 3, proteins eluted from TALON resin. Molecular mass markers are indicated on the left, while the migration of full length SfDronc and cleaved subunits are indicated on the right. B) The enzymatic activity of wild type SfDronc and mutants D340A and E344A were compared by incubating the purified His-tagged proteins with Ac-IETD-AFC and monitoring substrate cleavage. The activity of the mutant proteins is plotted relative to wild type. C) The ability of His-tagged SfDronc to cleave various fluorogenic substrates was examined. SfDronc-His₆ (350 nM) was incubated with each substrate, and the rates of cleavage were monitored. Maximal activity is plotted as the change in relative fluorescence units (RFU) per minute. The means $\pm$ SE of three independent experiments are shown.

Fig. 6.

SfDronc directly cleaves and activates Sf-caspase-1. A) SfDronc-His₆ or the catalytic mutant SfDronc-C310A-His₆ were incubated with catalytic mutant Sf-caspase-1-C178A (lanes 1 and 3) or cleavage site mutant Sf-caspase-1-D195A (lanes 2 and 4). The reactions were analyzed by SDS-PAGE and immunoblotting with anti-Sf-caspase-1 antiserum. The migration of full length Sf-caspase-1 and the cleaved large subunit + prodomain are indicated on the right. Note that subsequent removal of the prodomain did not occur in lane 1 due to the lack of catalytically active Sf-caspase-1. B) Sf-caspase-1 or cleavage site mutant Sf-caspase-1-D195A were produced by *in vitro* translation using reticulocyte lysate. The *in vitro* translated proteins were incubated alone, with SfDronc-His₆, or with catalytic mutant

SfDronc-C310A-His₆. As an additional negative control, SfDronc-His₆ was incubated with unprogrammed reticulocyte lysate. The reactions were then subjected to caspase activity assay using Ac-DEVD-AFC as a substrate, and the maximal rates of substrate cleavage were plotted.

Fig. 7.

Overexpression of enzymatically active SfDronc induces apoptosis in Sf9 cells. Plasmids expressing HA-tagged wild type SfDronc or the mutants C310A or D340A were transfected into Sf9 cells. A) Photomicrographs were taken at 24 h after transfection. B) Cell viability was measured at 24 h after transfection. Z-VAD-FMK was added to some of the wells to inhibit apoptosis. C) Lysates were prepared at 24 h post transfection and immunoblotted using an antibody against the HA epitope tag to show expression of the tagged proteins.

A

B

Fig. 8.

Inhibition of SfDronc by SpliP49, but not AcP35. Recombinant SfDronc or Sf-caspase-1 were incubated with increasing concentrations of recombinant A) SpliP49 or B) AcP35, and then the ability of the caspases to cleave Ac-IETD-AFC (SfDronc) or Ac-DEVD-AFC (Sf-caspase-1) was determined. The levels of caspase activity are plotted relative to the caspases with no inhibitor added. The values shown are the mean $\pm$ SE from 3 independent experiments. The increased SfDronc activity seen when AcP35 is added is likely due to a stabilizing effect of higher total protein concentration on SfDronc.

Table 1

Activity of SfDronc against selected fluorogenic substrates.

	Ac-VEID-AFC	Ac-IETD-AFC	Ac-LETD-AFC	Ac-TQTD-AFC
average k_{cat} (per sec) $\pm$ SEM	0.166 $\pm$ 0.0410	0.189 $\pm$ 0.0373	0.0907 $\pm$ 0.0110	0.0237 $\pm$ 0.00608
average K_M (mM) $\pm$ SEM	0.572 $\pm$ 0.208	0.812 $\pm$ 0.239	0.752 $\pm$ 0.119	0.770 $\pm$ 0.269
average $[k_{\text{cat}}/K_M]$ ($M^{-1}s^{-1}$)	325 $\pm$ 47.0	246 $\pm$ 23.0	122 $\pm$ 6.88	33.5 $\pm$ 4.00