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The *Drosophila* DIAP1 Protein Is Required to Prevent Accumulation of a Continuously Generated, Processed Form of the Apical Caspase DRONC*

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Although loss of the inhibitor of apoptosis (IAP) protein DIAP1 has been shown to result in caspase activation and spontaneous cell death in *Drosophila* cells and embryos, the point at which DIAP1 normally functions to inhibit caspase activation is unknown. Depletion of the DIAP1 protein in *Drosophila* S2 cells or the Sf-IAP protein in *Spodoptera frugiperda* Sf21 cells by RNA interference (RNAi) or cycloheximide treatment resulted in rapid and widespread caspase-dependent apoptosis. Co-silencing of *dronc* or *dark* largely suppressed this apoptosis, indicating that DIAP1 is normally required to inhibit an activity dependent on these proteins. Silencing of *dronc* also inhibited DRICE processing following stimulation of apoptosis, demonstrating that DRONC functions as an apical caspase in S2 cells. Silencing of *diap1* or treatment with UV light induced DRONC processing, which occurred in two steps. The first step appeared to occur continuously even in the absence of an apoptotic signal and to be dependent on DARK, because full-length DRONC accumulated when *dark* was silenced in non-apoptotic cells. In addition, treatment with the proteasome inhibitor MG132 resulted in accumulation of this initially processed form of DRONC, but not full-length DRONC, in non-apoptotic cells. The second step in DRONC processing was observed only in apoptotic cells. These results indicate that the initial step in DRONC processing occurs continuously via a DARK-dependent mechanism in *Drosophila* cells and that DIAP1 is required to prevent excess accumulation of this first form of processed DRONC, presumably through its ability to act as a ubiquitin-protein ligase.

The IAP[1] proteins are conserved from yeast to humans and are also found in certain viruses that infect invertebrates, including baculoviruses and entomopoxviruses (1). IAP proteins are identified by the presence of 1–3 copies of a motif called a baculovirus IAP repeat (BIR) at the amino terminus. Many IAP proteins also contain a RING finger motif at the carboxyl terminus, some of which have been shown to possess E3 activity (2). Most IAP proteins from insects and vertebrates are capable of inhibiting apoptosis when overexpressed, whereas IAP homologs in nematodes and yeast instead play a role in regulating cytokinesis (1).

IAP proteins inhibit apoptosis stimulated by a variety of signals in both insect and mammalian cells, presumably at least in part through their ability to inhibit caspases, a family of cysteine proteases that mediate many of the morphological and biochemical changes associated with apoptosis (3). Following a death signal, apical or signaling caspases become activated through proteolytic processing. These apical caspases in turn proteolytically activate other caspases, called effector caspases, that go on to cleave various target proteins, leading to apoptosis (4). In mammalian cells, two major pathways leading to apical caspase activation have been described, the extrinsic and intrinsic pathways. The extrinsic pathway primarily involves the activation of the apical caspases caspase-8 and -10 by death receptors such as fas and tumor necrosis factor receptor, whereas the intrinsic pathway involves the release of factors from mitochondria such as cytochrome *c* that results in the activation of apical caspase-9 via the Apaf-1 protein, an oligomerizing factor required for the activation of caspase-9 in mammals (5). Upon binding to cytochrome *c*, Apaf-1 forms large oligomeric complexes known as apoptosomes that recruit and activate caspase-9 (6, 7). In mammals, either caspase-8 or -9 is capable of activating effector caspases such as caspase-3 or -7, which then cleave apoptotic substrates, leading to apoptosis. A link between the extrinsic and intrinsic pathways is observed in certain cells that involves the cleavage of the Bcl-2 family member Bid by caspase-8, which leads to release of cytochrome *c* from mitochondria and activation of caspase-9 (8, 9).

Certain IAP proteins have been shown to inhibit both apical and effector caspases, such as mammalian XIAP, where the third BIR domain of XIAP directly binds and inhibits caspase-9, whereas a short linker region between the first and second BIR domains binds and inhibits caspases 3 and 7 (10). Inhibition of caspase-9 is relieved by Smac/DIABLO, another protein that is released from mitochondria following a death signal and binds to BIR3 of XIAP, releasing caspase-9 (11, 12). The *Drosophila* DIAP1 protein is also capable of inhibiting death induced by ectopic caspase expression in yeast and in the fly eye (13–15). This activity is important for the anti-apoptotic function of DIAP1 because loss of DIAP1 results in caspase activation and the death of most, if not all, cells in the embryo

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[1] The abbreviations used are: IAP, inhibitor of apoptosis; RNAi, RNA-mediated interference; dsRNA, double stranded RNA; Z-VAD-fmk, Z-Val-Ala-DL-Asp-fluoromethyl ketone; FBS, fetal bovine serum; RT, reverse transcriptase; E3, ubiquitin-protein ligase; BIR, baculovirus IAP repeat; CAT, chloramphenicol acetyltransferase.

(15–18). In contrast, the *xiap* knockout mouse has no discernible phenotype, although the levels of c-IAP1 and c-IAP2 were higher than normal in embryonic fibroblasts derived from XIAP-deficient mice, suggesting compensation because of the loss of XIAP (19).

In *Drosophila*, a pathway similar to the intrinsic pathway in mammals is beginning to be characterized (for a recent review see Ref. 20). A protein with homology to Apaf-1, known as DARK, Hac-1, or Dapaf-1 (21–23), has been shown to be important for apoptosis stimulated by a variety of signals (24, 25). In addition, the *Drosophila* caspases DRONC and DRICE have been shown to accumulate in large complexes reminiscent of apoptosomes (26). However, cytochrome *c* release does not appear to occur in *Drosophila* cells (26, 27), and the role of cytochrome *c* in *Drosophila* apoptosome formation is not clear.

Based on these and other results, it has been hypothesized that a low level of constitutive caspase activity is present in cells, and IAP proteins promote survival by suppressing amplification of the caspase cascade. Disruption of IAP-caspase interactions thus provides an attractive approach to sensitizing cells to death signals. However, an important unanswered question is which IAP-caspase interactions are rate-limiting for the survival of cells that normally live? We have used the RNAi technique to dissect this pathway in insect cells. Our results demonstrate that in unstimulated *Drosophila* S2 cells, DIAP1 is required to inhibit an activity dependent on DRONC and DARK. Furthermore, DRONC is continuously processed in normal cells through a mechanism that requires DARK. However, this initially processed form of DRONC does not normally accumulate to significant levels because it is subject to degradation by the proteasome. Removal of DIAP1 results in the accumulation of processed DRONC, activation of the downstream caspase DRICE, and apoptosis.

EXPERIMENTAL PROCEDURES

Cell Lines—Spodoptera frugiperda Sf21 cells were maintained in TC-100 medium (Invitrogen) supplemented with 10% tryptose broth and 10% fetal bovine serum (FBS) (Invitrogen). *Drosophila* S2 cells were maintained in Schneider's medium (Invitrogen) supplemented with 10% FBS.

RNAi Procedure—The RNAi technique was performed essentially as described (28). Complementary RNA strands were transcribed *in vitro* using the AmpliScribe kit (EpiCentre Technologies), mixed, and annealed by heating to 65 °C for 15 min and then allowing to cool to room temperature. For each gene, the following sequences were used to generate dsRNA (based on the start codon of the coding sequence): Sf-*iap* and CAT, all of the coding sequence; *dark*, nucleotides 1561–2821; *dronc*, nucleotides 16–1160; *diap1*, nucleotides 406–1319; Sf-actin, an unpublished 523 nucleotide partial cDNA sequence (obtained from Dr. Lois Miller, University of Georgia). Sf21 or S2 cells were plated overnight in 6-well plates at 5×10^5 or 1×10^6 cells per well, respectively, in TC-100 medium supplemented with 10% FBS. The following day, the medium was removed, and 80 μg of dsRNA suspended in 1 ml of TC-100 medium without FBS was added, followed by vigorous shaking. After 4 (Sf21) or 5.5 h (S2), 1 ml of TC-100 medium with 20% FBS was added to bring the final FBS concentration to 10%. Mock-treated cells were subjected to the same procedure except that no RNA was added.

To perform co-silencing, the RNAi procedure was performed as above for the first gene. After 24 h cells were placed in TC-100 media without FBS, and *diap1* dsRNA was added as well as another dose of the first dsRNA. Control cells were treated the same except cells received either *diap1* dsRNA alone or no dsRNA. In the cycloheximide experiment, RNAi was performed on the indicated genes, and after 24 h 100 μg/ml of cycloheximide (Calbiochem) was added.

Apoptosis Assays—At various times after adding dsRNA, cell viability was determined either by counting intact, non-blebbing cells in three high power fields (Sf21 cells) or by resuspending the cells and counting intact cells using a hemocytometer (S2 cells) and comparing to the number of intact cells in the mock-treated control at 3 h. The experiments were performed in duplicate three independent times.

DNA Laddering—To detect DNA laddering, cells were treated with 0.2 μg/ml actinomycin D (Invitrogen) or dsRNA, and apoptotic bodies and cells were collected 7 h later by centrifugation and lysed in 0.4 M Tris, pH 7.5, 0.1% SDS, and 0.1 M EDTA. Following phenol/chloroform extraction and ethanol precipitation, DNA was analyzed by 1% agarose gel electrophoresis and visualized by ethidium bromide staining.

Transfection Assay—Plasmids (3 μg) expressing green fluorescent protein, baculovirus p35, or Op-IAP from a *Drosophila* heat shock promoter were introduced into 5×10^5 Sf21 cells using lipid-mediated transfection in TC-100 media without FBS. After 4 h, the lipid/DNA mixture was replaced with TC-100 media containing 10% FBS. After an additional 16 h, the cells were heat-shocked at 42 °C for 30 min, and 4 h later dsRNA was added. For the caspase inhibitor experiment, S2 cells were mock-treated or treated with *diap1* dsRNA as above, except that 100 μM Z-Val-Ala-DL-Asp-fluoromethyl ketone (Z-VAD-FMK, Alexis Biochemicals) or 0.5% Me_2SO (carrier control) was added with the dsRNA. Inhibitor or carrier concentration was maintained when FBS was added at 4 h. At 24 h after addition of dsRNA, cell viability was determined as described above. The experiment was performed in triplicate.

RT-PCR—Sf21 cells were treated with dsRNA as described above, and at various times after treatment total RNA was isolated using Trizol reagent (Invitrogen). Total RNA (3 μg) was used in a reverse transcriptase reaction with a gene-specific primer. From the resulting 50-μl reaction, 2 μl of cDNA was then used as a template in a PCR with nested primers specific for the sequence of interest. In each case, at least one of the primers used for PCR bound to nontranslated sequences or to sequences outside of the region of the open reading frame used to produce dsRNA. Dilutions (1:2 and 1:4) of cDNA were also used for PCR to assess better the relative amounts of PCR product. The PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining.

Immunoblotting—S2 cells were treated with UV light by placing on a transilluminator for 10 min, with 50 μg/ml MG132 (Sigma), or with dsRNA as above, except that each well contained 6×10^6 cells. At the indicated times, cells were harvested and lysed in SDS-PAGE loading buffer. Cell lysates were analyzed by immunoblotting using a monoclonal antibody against DIAP1 (18), polyclonal antisera raised against a His_6-tagged version of the DRONC p20 subunit (rabbit 51) (18), or full-length DRICE, and SuperSignal chemiluminescent reagent (Pierce).

RESULTS AND DISCUSSION

Silencing of iap Genes Induces Caspase-dependent Apoptosis in Insect Cells—A homozygous loss of function mutation in DIAP1 has been shown to result in widespread apoptosis during *Drosophila* embryogenesis (15–17). To determine the effect of depleting IAP proteins from cultured insect cells, two *iap* genes from different insect species, Sf-*iap* from the lepidopteran insect *S. frugiperda* (29) and *diap1* from *Drosophila melanogaster*, were silenced using RNAi. Within 4 h after addition of Sf-*iap* dsRNA to Sf21 cells or *diap1* dsRNA to S2 cells, membrane blebbing was observed in both cell lines consistent with apoptosis (Fig. 1, *C* and *G*). This morphology was indistinguishable from that observed after treatment with actinomycin D, ultraviolet light, or cycloheximide, known inducers of apoptosis in Sf21 and S2 cells. The blebbing intensified over time, and by 24 h more than 99% of the cells had undergone apoptosis (Fig. 1, *D* and *H*). Only very low background levels of apoptosis were observed in mock-treated cells or cells treated with control dsRNAs including dsRNA corresponding to the bacterial chloramphenicol acetyltransferase (CAT) gene or Sf-actin (Fig. 1, *A*, *B*, *E*, and *F* and data not shown). Apoptosis was also not observed when either the plus or minus strands of Sf-*iap* RNA were added separately to Sf21 cells (data not shown). These results are similar to those recently reported by others (24–26, 30) using the RNAi technique to silence *diap1* in S2 cells.

DNA laddering was observed in Sf21 cells treated with Sf-*iap* dsRNA similar to that seen following treatment with actinomycin D (31) (Fig. 2*A*), confirming that the cells died by apoptosis. Positive terminal deoxynucleotidyltransferase dUTP nick end labeling staining indicated that S2 cells treated with *diap1* dsRNA also underwent DNA fragmentation and apoptosis (data not shown).

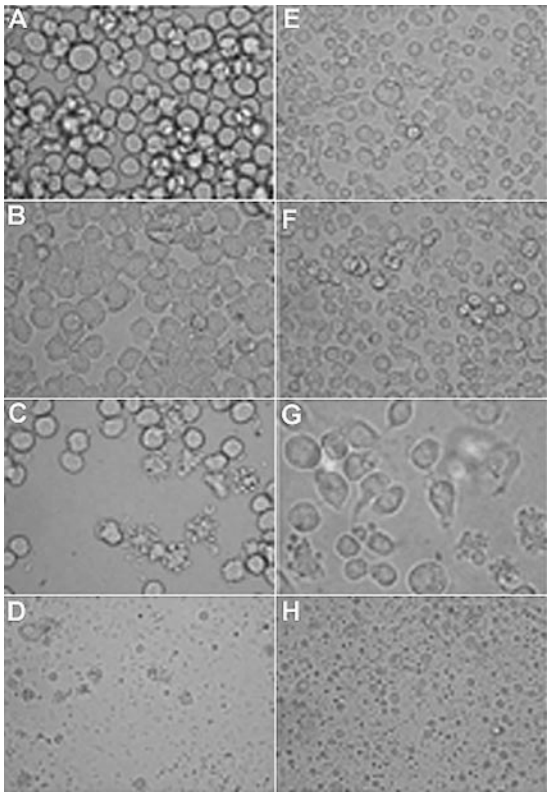

FIG. 1. **Silencing of endogenous *iap* genes induces apoptosis in cultured insect cells.** Sf21 (*A–D*) or S2 (*E–H*) cells were either untreated (*A* and *E*) or treated with either control chloramphenicol acetyltransferase (CAT) dsRNA (*B* and *F*) or dsRNA corresponding to Sf-*iap* (*C* and *D*) or *diap1* (*G* and *H*). Photographs were taken at 4 (*C* and *G*) or 24 h (*A*, *B*, *D–F*, and *H*) after addition of dsRNA. Cell blebbing was first detected at 3–4 h following addition of Sf-*iap* or *diap1* dsRNA (*C* and *G*), and nearly all cells were dead by 24 h (*D* and *H*). The magnification for each photograph was ×400 except (*G*), which was ×1000.

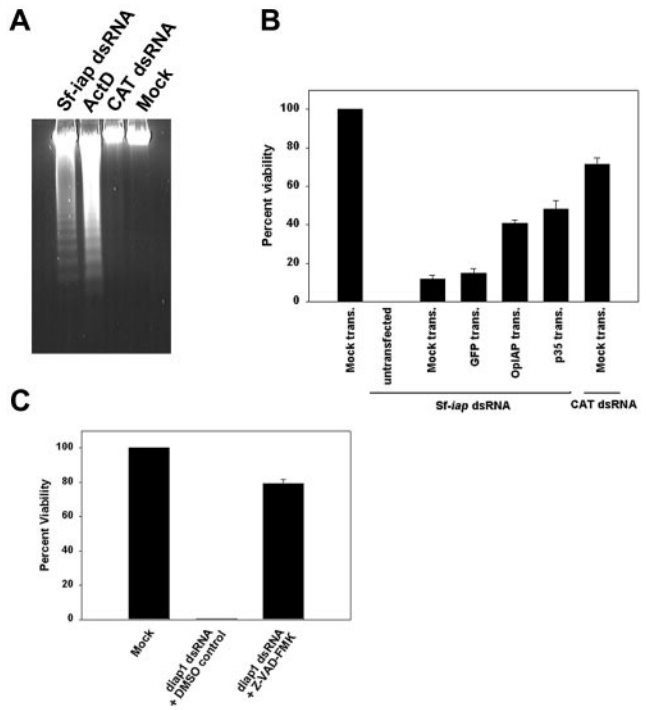

FIG. 2. **Cell death induced by *iap* silencing is due to apoptosis and is caspase-dependent.** *A*, DNA fragmentation was observed in Sf21 cells treated 7 h with Sf-*iap* dsRNA or actinomycin D (*ActD*) but not in cells treated with CAT dsRNA or mock-treated cells. *B*, Sf21 cells were mock-transfected or transfected (*trans.*) with plasmids encoding either control green fluorescent protein (*GFP*) or baculovirus P35 or Op-IAP, and 24 h later Sf-*iap* dsRNA or control CAT dsRNA was added as indicated *below* the graph. Cell viability was determined by apoptotic morphology at 24 h after dsRNA addition, and the means ± S.E. are shown. The transfection process inhibited apoptosis stimulated by Sf-*iap* RNAi to a slight degree for unknown reasons. *C*, S2 cells were either mock-treated or treated with *diap1* dsRNA, and at the same time either 0.5% Me_2SO (*DMSO*, carrier control) or Z-VAD-FMK was added, and cell viability was determined after 24 h. The means ± S.E. are shown.

To determine whether caspases were involved in the death induced by *iap* silencing, we examined the effect of caspase inhibitors on death induced by IAP depletion. Sf21 cells were transfected with a plasmid vector expressing the baculovirus caspase inhibitor P35, and then the cells were treated with Sf-*iap* dsRNA. Transient expression of P35 inhibited the apoptosis induced by the addition of Sf-*iap* dsRNA (Fig. 2*B*). In addition, expression of the baculovirus *iap* gene Op-*iap* also inhibited this apoptotic signal (Fig. 2*B*). In each case, the amount of inhibition was similar to the average transfection efficiency (54%), indicating that these anti-apoptotic genes were highly effective at inhibiting this apoptotic signal. In S2 cells, apoptosis induced by loss of DIAP1 was inhibited by the chemical caspase inhibitor Z-VAD-FMK (Fig. 2*C*), indicating that loss of DIAP1 also resulted in caspase activation and caspase-dependent apoptosis.

To confirm that the addition of dsRNA was in fact silencing endogenous gene expression, the levels of mRNA for Sf-*iap* or actin were examined by RT-PCR in Sf21 cells (Fig. 3*A*). Within 2 h after adding dsRNA, corresponding transcripts from either gene were undetectable. There was no effect on the levels of actin message when Sf-*iap* dsRNA was added or vice versa, confirming the specificity of RNAi. In these experiments, the dsRNA was added to cells in the absence of serum, and serum was then added 4 h later. For unknown reasons, the RNAi effect in Sf21 cells appeared to be reversed by the later addition of serum. For both Sf-*iap* and actin, silenced message returned to near normal levels after the addition of serum, and delaying the addition of serum also delayed the reappearance of the transcripts (Fig. 3 and data not shown). This reversal effect of serum was not observed in S2 cells.

The levels of DIAP1 protein also decreased rapidly after addition of *diap1* dsRNA to S2 cells, with the protein becoming undetectable by immunoblotting within 7.5 h (Fig. 3*B*). These results are consistent with a short half-life for DIAP1 protein, which has been shown to be ~30–45 min in S2 cells following cycloheximide treatment (18, 32).

DRONC and DARK Are Required for Apoptosis Stimulated by Depletion of DIAP1—The fact that depletion of DIAP1 stimulates caspase-dependent apoptosis suggests that DIAP1 normally promotes cell viability at least in part by inhibiting the activity of one or more caspases. However, the caspase(s) inhibited by DIAP1 *in vivo* have not been identified. An obvious candidate for a caspase targeted by DIAP1 was the caspase DRONC, which is widely expressed in the developing *Drosophila* embryo and is required for developmentally programmed embryonic cell death (33). In addition, DIAP1 binds to both the pro-domain and core subunits of DRONC (14), and death induced by ectopic DRONC expression in the fly and in yeast has been shown to be inhibited by DIAP1 (13, 14). S2 cells were treated with *dronc* dsRNA for 24 h, which reduced the amount of full-length DRONC protein to a non-detectable level (see Fig. 5*D*). This treatment was followed by addition of *diap1* dsRNA to induce apoptosis. Remarkably, in cells that had been treated with *dronc* dsRNA, death induced by silencing of *diap1* was largely suppressed, whereas cells that had been pre-treated with control dsRNA underwent almost complete apoptosis (Fig. 4*A*). After 12 h, the surviving cells that were pre-treated with

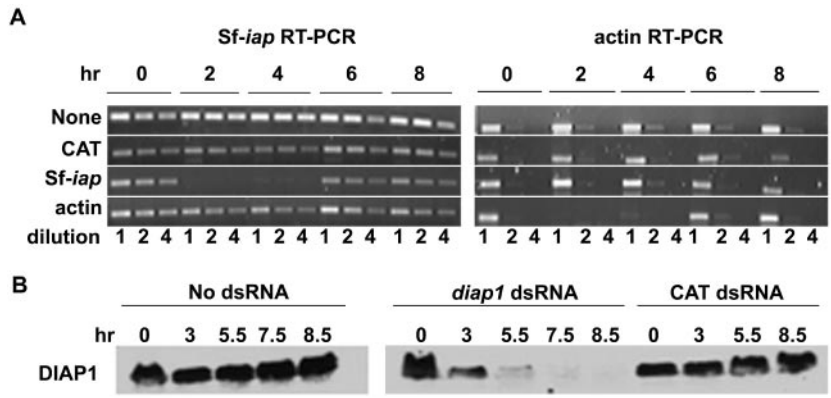

FIG. 3. **Sf-*iap* and *diap1* are specifically silenced by addition of the corresponding dsRNA.** *A*, levels of Sf-*iap* (*left panel*) or Sf-actin (*right panel*) mRNA were determined by RT-PCR at 2-h intervals following treatment with the dsRNAs indicated on the *left*. The cDNA samples were also diluted 2- or 4-fold prior to performing RT-PCR to indicate relative transcript levels, and the dilution is shown *below* each lane. *B*, the levels of DIAP1 protein were determined by immunoblotting at the times shown following mock treatment or treatment with *diap1* or CAT dsRNA. Coomassie Blue staining of the same lysates verified equal protein loading (data not shown).

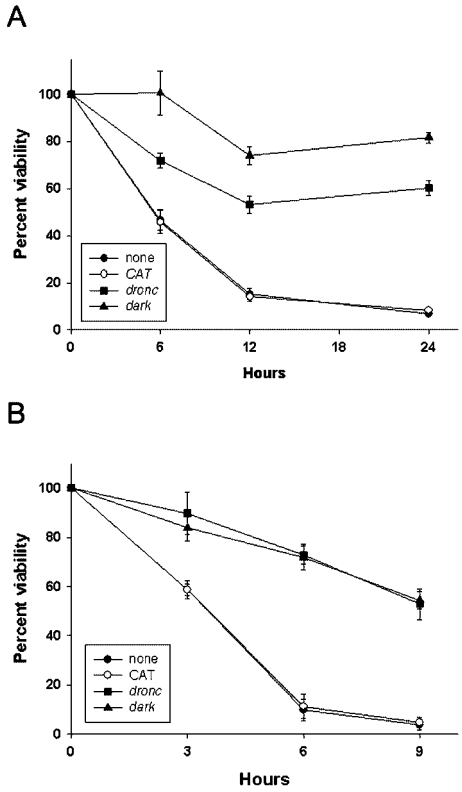

FIG. 4. **Co-silencing of DRONC or DARK suppresses apoptosis induced by silencing of DIAP1 or by cycloheximide in S2 cells.** *A*, cells were treated with the indicated dsRNAs, and 24 h later *diap1* dsRNA was added. Cell viability was assessed by apoptotic morphology at various times after addition of *diap1* dsRNA. *B*, cells were treated with the indicated dsRNAs, and 24 h later the cells were treated with cycloheximide, and cell viability was measured as above. In both *A* and *B*, means ± S.E. are shown.

dronc dsRNA began to divide, resulting in an apparent increase in viability. Thus, death in these surviving cells appeared to be completely inhibited, not just delayed, by depletion of DRONC.

In mammals, activation of caspase-9 requires dATP, cytochrome *c*, and Apaf-1 (34). The homolog of Apaf-1 in *Drosophila* is DARK (also known as Dapaf-1 or Hac-1) (21–23). *In vitro* activation of DRONC is decreased in extracts made from mutant fly embryos lacking DARK, indicating that DARK may play a role in DRONC activation similar to that of Apaf-1 in caspase-9 activation (33). In order to determine whether DARK is also required for apoptosis stimulated by depletion of DIAP1, S2 cells were treated with *dark* dsRNA, and 24 h later *diap1* dsRNA was added to induce apoptosis. Co-silencing of *dark* and *diap1* also suppressed apoptosis induced by loss of DIAP1 and resulted in even higher cell viability than cells co-silenced for *dronc* and *diap1* (Fig. 4*A*). Also, similar to *dronc* dsRNA-treated cells, *dark* dsRNA-treated cells surviving after 12 h of *diap1* dsRNA treatment began to divide. Together, these data indicate that an important function of DIAP1 in S2 cells is to inhibit the activity of DRONC, and that DRONC activity is in turn dependent on DARK.

S2 cells treated with cycloheximide undergo caspase-dependent apoptosis within 3–4 h (18). Similar to *diap1* RNAi, silencing *dronc* or *dark* prior to cycloheximide treatment dramatically delayed apoptosis (Fig. 4*B*). Silencing *dronc* also strongly inhibited apoptosis stimulated by UV light (data not shown). These data are in agreement with the recent report (25) that depletion of DARK by RNAi protected S2 cells from stress-related apoptotic stimuli, including ultraviolet light and cycloheximide.

The observation that depletion of *dronc* or *dark* did not completely suppress apoptosis induced by a reduction in DIAP1 (Fig. 4*A*) may have been due to small amounts of DRONC or DARK protein remaining after 24 h of dsRNA treatment. Alternatively, there may be other apical caspases, such as DREDD (35) or STRICA (36) that can become activated following loss of DIAP1. This latter possibility is supported by the greater protection seen with co-silencing of *dark* than with *dronc* (Fig. 4*A*). Nevertheless, our results indicate that DRONC appears to be the major apical caspase that is activated following depletion of DIAP1, because the majority of cells are protected and continue dividing following *diap1* and *dronc* RNAi.

DIAP1 Inhibits Excess Accumulation of Processed DRONC—In addition to DRONC, another caspase, DRICE, is also known to be activated in *Drosophila* embryos lacking DIAP1 (18). Furthermore, immunodepletion of DRICE from apoptotic S2 cell lysates removed all of the detectable chromatin condensing activity from the lysates, suggesting that DRICE plays a vital role in apoptosis (37). These findings and our data showing the requirement for DRONC and DARK in apoptosis stimulated by depletion of DIAP1 or UV light led us to examine the activation of DRONC and DRICE following treatment with these stimuli. Lysates from S2 cells treated with *diap1* dsRNA or UV light were immunoblotted for DRONC and DRICE. Within 3 h after treatment with either stimulus a processed form of DRONC, hereafter referred to as Pr1, was detected (Fig. 5*A*). By 6 h, a second, smaller processed form of DRONC, hereafter referred to as Pr2, was also seen. Pr1 disappeared as Pr2 accumulated over time, suggesting but not proving that Pr1 was being further processed into Pr2. Full-length DRONC also disappeared over time, although the decrease in full-length DRONC was sometimes difficult to detect because it runs as a tight doublet with a nonspecific background band (best seen in the *right-hand panel* of Fig. 5*A*). On the other hand, DRICE processing was not detected until 6 h after treatment with either stimulus (Fig. 5*B*). The appearance of the Pr2 form of DRONC coincided

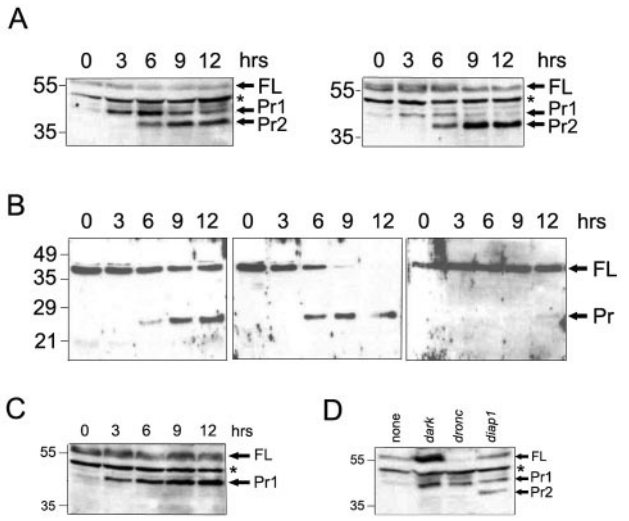

FIG. 5. **DRONC undergoes continuous processing in S2 cells, and DIAP1 is required to prevent accumulation of processed DRONC.** *A*, DRONC is processed into two forms, Pr1 and Pr2, following treatment of S2 cells with UV light (*left*) or *diap1* dsRNA (*right*). Cell lysates were harvested at the times shown and immunoblotted for DRONC protein. *FL*, full-length DRONC. The *asterisk* indicates a nonspecific background band. *B*, DRICE also undergoes processing following apoptotic stimuli, but it is processed later than DRONC, and its processing is dependent on DRONC. Cells were treated with UV light (*left*) or *diap1* dsRNA (*middle*), and lysates were harvested at the times shown and immunoblotted for DRICE protein. In the *right-hand panel*, S2 cells were treated with *dronc* dsRNA for 24 h to deplete DRONC protein, and then *diap1* dsRNA was added, and cells were harvested at the times shown after *diap1* dsRNA addition, and lysates were immunoblotted for DRICE. *FL*, full-length DRICE; *Pr*, processed DRICE. *C*, proteasome inhibition results in accumulation of the Pr1 processed form of DRONC. S2 cells were treated with MG132 and harvested at the times shown. Lysates were immunoblotted for DRONC protein. *D*, silencing of *dark* results in over-accumulation of full-length DRONC. S2 cells were treated with the indicated dsRNAs for 24 h, except *diap1*, which was treated for 9 h. Cell lysates were harvested and immunoblotted for DRONC protein. *A–D*, the migration of molecular mass markers is indicated to the *left* in kDa. In each case, similar results were obtained in several independent experiments.

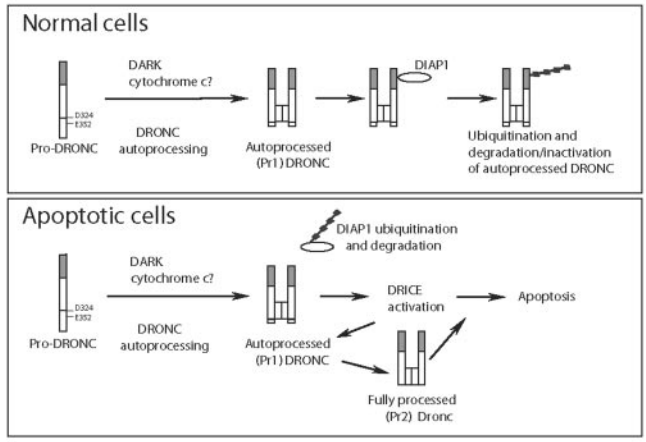

FIG. 6. **A model for how DIAP1 promotes *Drosophila* cell viability.** In normal living cells (*upper panel*), DRONC undergoes continuous autoprocessing via a DARK-dependent mechanism. It is not clear whether cytochrome *c* is involved in this process. DIAP1 promotes degradation of autoprocessed DRONC via its E3 activity and may also directly inhibit the enzymatic activity of autoprocessed DRONC. Upon receiving a death stimulus (*lower panel*), DIAP1 levels decrease, and autoprocessed DRONC levels increase. This leads to activation of effector caspases such as DRICE, which in turn further cleave DRONC and other apoptotic substrates, resulting in apoptosis. The prodomain and the large and small subunits of DRONC are shown, with the prodomain *shaded*.

with the onset of DRICE processing, and thus may be a result of cleavage of Pr1 by DRICE or another effector caspase. Both Pr1 and Pr2, as well as full-length DRONC, were affinity-labeled with biotinylated Z-VAD-fmk indicating that they are enzymatically active processed forms of DRONC and not merely inactive degradation products (data not shown).

Because of the length of their prodomains, it is widely assumed that DRONC and DRICE are apical and effector caspases, respectively. Our observation that DRONC is processed earlier than DRICE following stimulation of apoptosis supports this hypothesis. In order to determine whether processing of DRICE is dependent on DRONC, we examined DRICE processing following depletion of DRONC. S2 cells treated with *dronc* dsRNA for 24 h and then treated with *diap1* dsRNA were immunoblotted for DRICE and showed almost no DRICE processing (Fig. 5*B*). DRICE processing was also almost completely inhibited when S2 cells were treated with UV light after *dronc* RNAi (data not shown). In both cases, the cells remained viable throughout the experiment. These results are consistent with DRONC being an apical caspase that is required for processing of the effector caspase DRICE following an apoptotic stimulus. In addition, these results also indicate that in normal, living cells, DIAP1 promotes cell viability by inhibiting DRONC activity and not that of DRICE, because DRICE processing does not occur in the absence of active DRONC.

Fly embryos lacking DIAP1 spontaneously undergo massive apoptosis, as do S2 cells depleted of DIAP1 by RNAi. This suggests that not only does DIAP1 inhibit DRONC activity, but also that this activity is constitutively present in cells because DIAP1 is required to prevent spontaneous apoptosis. Because DIAP1 can bind to DRONC, this inhibition may be direct and/or it may be due to DIAP1 acting as an E3 and causing the degradation of DRONC by the proteasome. To determine whether DRONC is targeted for proteasome degradation, S2 cells were treated with the proteasome inhibitor MG132 and immunoblotted for DRONC. Interestingly, MG132-treated cells consistently exhibited an accumulation of the Pr1 form of processed DRONC (Fig. 5*C*), even though MG132 treatment itself had no effect on cell viability. However, MG132 treatment did not result in an increase in the levels of full-length DRONC (Fig 5*C*). In addition, there was no further processing of DRONC to the Pr2 form seen in cells undergoing apoptosis induced by either UV treatment or *diap1* dsRNA (compare Fig. 5, *C* and *A*). Thus, even in the absence of an apoptotic signal, DRONC is continuously processed to the Pr1 form, and the Pr1 form is subject to proteasome-mediated degradation. Given the fact that *diap1* RNAi or UV light cause a rapid decrease in DIAP1 levels, and the recent report (32) that DRONC is a target for ubiquitination by DIAP1, it appears that DIAP1 is required to prevent accumulation of the Pr1 processed form of DRONC, probably by directing its ubiquitination.

These results also indicate that there are least two steps involved in DRONC processing *in vivo*. We suggest that the first cleavage, resulting in the Pr1 form, may be due to autocatalytic processing, whereas the second cleavage resulting in Pr2 is specifically seen in dying cells and may be due to cleavage by an effector caspase such as DRICE. Although we do not have direct evidence to support this conclusion, we believe it is the simplest explanation for the observed results based the following evidence. 1) The apparent molecular weight of Pr1 is consistent with an *in vitro* autocatalytic cleavage event for DRONC as demonstrated previously (13). DRONC was shown previously to autoprocess itself *in vitro* after a glutamate residue, Glu-352, and the apparent molecular weight of Pr1 is similar to that expected if cleavage occurred at Glu-352 (40.3

kDa). Furthermore, the size of Pr2 is consistent with Pr1 being further cleaved at the canonical caspase cleavage site (DEYD) located at the boundary between the large and small subunits at position 324 (expected size of 37.0 kDa). 2) The timing of the appearance and disappearance of Pr1 and Pr2 suggests (but does not prove) a precursor-product relationship between these two forms of processed DRONC (Fig. 5*A*). 3) Because DRONC is believed to be an apical caspase, it would be expected that the initial cleavage event is autocatalytic. Pr1 is the first cleavage product of DRONC observed following an apoptotic stimulus and occurs before DRICE activation, whereas the appearance of Pr2 correlates with DRICE activation.

DARK is required for efficient processing of DRONC *in vitro* (33) and in the absence of DARK, apoptosis induced by either *diap1* dsRNA addition or cycloheximide treatment is largely suppressed (Fig. 4). DARK may therefore play a role in the continuous autoprocessing of DRONC. S2 cells were treated with *dark* dsRNA and after 24 h immunoblotted for DRONC. Remarkably, these cells showed an accumulation of full-length DRONC (Fig. 5*D*), suggesting that DARK is indeed required for the continuous autoprocessing of DRONC. These cells also contained some of the Pr1 form of DRONC, which may have been due to low levels of DARK remaining after RNAi or to spontaneous DRONC dimerization and autoactivation that may occur without the need for DARK when DRONC accumulates to high levels, similar to the Apaf-1-independent activation observed when caspase-9 is present at higher than normal concentrations (38). The presence of Pr1 DRONC in cells treated with *dronc* dsRNA for 24 h (Fig. 5*D*) suggests that the half-life of Pr1 in nonapoptotic cells may be relatively long compared with the time required for autoprocessing of full-length DRONC. If our hypothesis is correct that DRONC first autoprocesses itself to Pr1 and only then is subject to proteasome degradation, then it would be expected that full-length DRONC would disappear faster than Pr1 following silencing of *dronc* by RNAi. In addition, cells treated with *dronc* dsRNA are not apoptotic, and thus Pr1 is not being further processed to Pr2, possibly further lengthening Pr1 half-life.

Together these results provide evidence for a model (Fig. 6) in which DRONC continuously undergoes processing to the Pr1 form in normal living cells, and this processed form, which we suggest is due to autoprocessing, is continuously degraded via the E3 activity of DIAP1. This initial processing step proceeds through a mechanism that requires DARK, perhaps involving apoptosome formation. In this model, DIAP1 is required to inhibit the over-accumulation of Pr1 DRONC through its ability to act as an E3. However, once a death signal is received and DIAP1 is removed, either through binding to apoptotic inducers such as Hid, Reaper or Grim, or by degradation (18, 39–42), this suppression is released and the Pr1 form of DRONC accumulates, activating effector caspases such as DRICE which can further cleave Pr1 DRONC to the Pr2 form as well as cleave other apoptotic substrates, leading to apoptosis. This model does not rule out the possibility that DIAP1 may also directly inhibit the enzymatic activity of full-length and/or partially processed DRONC. In fact, this possibility is suggested by the fact that MG132 treatment resulted in an over-accumulation of Pr1 DRONC, but these cells did not die (Fig. 5*C*).

Concluding Remarks—Prior to this work, the identity of the caspase(s) that are normally inhibited by IAP proteins in any living cells had not been determined. Our results indicate that continuous expression of the short lived IAP protein DIAP1 is required to inhibit the activity of the caspase DRONC and that DRONC acts as an apical caspase in *Drosophila* S2 cells. Importantly, our results showing induction of apoptosis in Sf21 cells following Sf-*iap* RNAi demonstrate that this pathway is probably conserved in other insects as well.

Although it is widely assumed that DIAP1 inhibits caspase activity in *Drosophila*, this is the first identification of a specific caspase that must be inhibited by DIAP1 to promote cell survival. The activation of mammalian caspase-9 requires Apaf-1, cytochrome *c*, and dATP (6). Our results suggest that the Apaf-1 homolog DARK is also required for activation of DRONC, as depletion of DARK caused an excess accumulation of full-length DRONC, and silencing of *dark* prior to reducing DIAP1 levels protected cells from apoptosis. However, unlike caspase-9, DRONC appears to undergo continuous autoprocessing, even in normal living S2 cells. It is possible that in insect cells, there is some level of constitutive apoptosome formation that may not require cytochrome *c* but may involve other factors. Recent data suggest that cytochrome *c* is not required for apoptosome formation in *Drosophila* cells (25, 26), although addition of cytochrome *c* further stimulated formation of an apoptosome-like complex (26).

We found that treatment of cells with the proteasome inhibitor MG132 resulted in over-accumulation of the larger Pr1 processed form of DRONC, even though these cells remained viable throughout the experiment. This result argues that this processed form, which we suggest may result from autoprocessing at Glu-352, is continuously produced in cells but is normally targeted for degradation by the proteasome. In contrast, our data do not indicate that full-length DRONC is a proteasome substrate *in vivo*, because there was no detectable excess accumulation of full-length DRONC following treatment with MG132. The involvement of DIAP1 in this degradation process is supported by the increased levels of processed DRONC following silencing of *diap1*, and by the recent report (32) that DIAP1 is capable of directing ubiquitination of DRONC.

In conclusion, the results of this study show that the apical caspase DRONC is continuously processed in living *Drosophila* cells, probably by an autocatalytic mechanism, and that DIAP1 is required to prevent accumulation of this processed form of DRONC. This initial processing step is dependent on the Apaf-1 homolog DARK and may occur by DARK promoting DRONC dimerization, similar to the mechanism by which Apaf-1 activates caspase-9. Removal of DIAP1 results in excess accumulation of processed DRONC, activation of the downstream effector caspase DRICE, and apoptosis. Our results thus have implications for therapeutic strategies aimed at disrupting IAP function in mammalian cells, because they suggest that targeting interactions between IAP proteins and apical caspases are likely to be more effective at inducing cell death than targeting interactions between IAP proteins and downstream effector caspases.

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