

## Cleavage of the Apoptosis Inhibitor DIAP1 by the Apical Caspase DRONC in Both Normal and Apoptotic *Drosophila* Cells\*

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**In *Drosophila* S2 cells, the apical caspase DRONC undergoes a low level of spontaneous autoprocessing. Unintended apoptosis is prevented by the inhibitor of apoptosis DIAP1, which targets the processed form of DRONC for degradation through its E3 ubiquitin protein ligase activity. Recent reports have demonstrated that shortly after the initiation of apoptosis in S2 cells, DIAP1 is cleaved following aspartate residue Asp-20 by the effector caspase DrICE. Here we report a novel caspase-mediated cleavage of DIAP1 in S2 cells. In both living and dying S2 cells, DIAP1 is cleaved by DRONC after glutamate residue Glu-205, located between the first and second BIR domains. The mutation of Glu-205 prevented the interaction of DIAP1 and processed DRONC but had no effect on the interaction with full-length DRONC. The mutation of Glu-205 also had a negative effect on the ability of overexpressed DIAP1 to prevent apoptosis stimulated by the proapoptotic protein Reaper or by UV light. These results expand our knowledge of the events that occur in the *Drosophila* apoptosome prior to and after receiving an apoptotic signal.**

IAP<sup>1</sup> proteins were first discovered in baculoviruses and have now been found in a wide range of organisms (1). IAPs are identified by the presence of at least one baculovirus IAP repeat (BIR), a roughly 70-amino acid domain shown to be important for protein-protein interactions. Many IAPs also contain a C-terminal RING finger domain, and several of these have been shown to possess E3 ubiquitin ligase activity (2–8).

Although all IAP proteins contain a BIR domain, not all BIR-containing proteins can inhibit apoptosis, and some BIR-containing proteins have been implicated in cell signaling and cell cycle regulation (9). Several IAPs have been shown to directly bind and inhibit the activity of a family of proapoptotic proteins known as caspases (10, 11). Caspases are cysteine

proteases and are synthesized as zymogens, which must usually be proteolytically cleaved to become activated (12). They are divided into two types depending on the size of an N-terminal prodomain found on all caspases. Initiator caspases are identified by a long prodomain, whereas a short prodomain is indicative of an effector caspase. Upon reception of a death signal, upstream initiator caspases are first activated and in turn cleave and activate downstream effector caspases leading to apoptosis.

In mammalian cells, caspase-9 functions as the initiator caspase for the intrinsic death pathway and upon induction of apoptosis associates with APAF-1, the mammalian CED-4 homolog. Caspase-9 and APAF-1 binding occur through their mutual CARD domains and along with the cofactors cytochrome *c* and dATP form the apoptosome. The apoptosome is a large complex that induces dimerization and activation of caspase-9 (13). Activated caspase-9 then activates effector caspases including caspase-3 and caspase-7, which bring about the morphological and biochemical events associated with apoptosis. In mammalian cells, apoptosome formation is thought to occur only after the cell has received an apoptotic signal.

In *Drosophila*, DREDD and DRONC have been identified as initiator caspases and have been shown to interact with DARK, the *Drosophila* APAF-1/CED-4 ortholog (14, 15). Studies have shown that in unstimulated *Drosophila* BG2 cells DRONC is found in a large molecular weight complex (16), and in normal living *Drosophila* S2 cells, DRONC undergoes continuous autoprocessing via a DARK-dependent mechanism (17, 18). Other cofactors such as cytochrome *c* do not seem to be necessary for apoptosome formation in *Drosophila* (16, 19–21), although the details of this process are not well understood. The *Drosophila* DIAP1 protein is required for cell viability in the developing embryo, as demonstrated by extensive apoptosis early in development and embryonic lethality in a DIAP1 mutant fly (22). In addition silencing of DIAP1 by RNAi in S2 cells causes spontaneous apoptosis, as does silencing of an *iap* gene in Sf21 cells, a lepidopteran insect cell line (17, 20, 23). This result differs from mammalian cells in which a knock-out mouse of a mammalian IAP, XIAP, was shown to be essentially normal, although the levels of cIAP1 and cIAP2 were elevated suggesting that they may have compensated for the absence of XIAP (24). Thus, insect cells appear to differ from mammalian cells in that apoptosome formation and apical caspase activation are always occurring, and continuous IAP expression is required for cell viability.

Certain IAPs have been determined to themselves be caspase substrates, and during apoptosis XIAP and cIAP1 are cleaved in a caspase-dependent manner. XIAP cleavage was observed in Jurkat cells during Fas-induced apoptosis and resulted in reduced levels of XIAP protein and progression of apoptosis (25). Cleavage of cIAP1 also reduced protein levels and in addition resulted in the production of a proapoptotic C-termi-

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<sup>1</sup> The abbreviations used are: IAP, inhibitor of apoptosis; E3, ubiquitin-protein isopeptide ligase; DIAP1, *Drosophila* IAP1; BIR, baculovirus IAP repeat; RNAi, RNA interference; GST, glutathione *S*-transferase; eGFP, enhanced green fluorescent protein; z, benzoyloxycarbonyl; FMK, fluoromethyl ketone; dsRNA, double-stranded RNA.

nal fragment suggesting that in mammals caspase cleavage reduces IAP levels and may also have other effects on IAP function (26).

In *Drosophila* S2 cells, DIAP1 is also a caspase substrate and is cleaved by the *Drosophila* effector caspase DrICE. Using purified proteins Meier and co-workers (27) determined that DIAP1 was cleaved by DrICE at position Asp-20. DrICE cleavage at amino acid Asp-20 of DIAP1 produces a processed form that possesses an asparagine at the N terminus and is recognized by the N-end rule degradation pathway. In agreement with this hypothesis it was determined that mutations at amino acid Asp-20 inhibited the degradation of DIAP1 when expressed in cells. The mutation of Asp-20 also reduced the ability of DIAP1 to inhibit apoptosis stimulated by overexpression of Reaper; however, effects on other death stimuli were not reported (27). In addition, it has been shown that DrICE binds BIR1 of DIAP1 and that cleavage of DIAP1 by DrICE at Asp-20 is required for DrICE binding and inhibition (28). However, a more recent report concluded that DrICE cleavage of DIAP1 at position Asp-20 was not important for the ability of DIAP1 to inhibit apoptosis stimulated by overexpression of Reaper or Hid (29).

In this study we observed a cleavage product of *Drosophila* DIAP1 in *Drosophila* S2 cells that differed in size from the DrICE-mediated cleavage product reported previously. This cleavage product was unstable, because it was only seen in the presence of a proteasome inhibitor. However, it was evident in both normal and dying cells. Accumulation of the DIAP1 cleavage product was inhibited by the caspase inhibitor z-VAD-FMK or by silencing of DRONC or DARK by RNAi. Using recombinant proteins, we found that DRONC readily cleaved DIAP1 *in vitro*, resulting in a digestion product similar in size to that seen in cells. The DRONC cleavage site in DIAP1 was mapped to amino acid Glu-205 in the spacer region separating BIR1 from BIR2 and the C-terminal RING domain. *In vitro* co-immunoprecipitations revealed that mutations at amino acid Glu-205 inhibited binding of DIAP1 to processed DRONC but not to full-length DRONC. In addition, when the Glu-205 mutant was overexpressed in S2 cells, its ability to protect cells against apoptosis induced by Reaper overexpression was reduced, suggesting that cleavage of DIAP1 by DRONC is involved in apoptosis regulation.

#### MATERIALS AND METHODS

**Protein Purification**—The vector expressing GST-DIAP1 was provided by Bruce Hay (Wang *et al.*, Ref. 22). The mutant versions of DIAP1 were generated using polymerase chain reaction and the QuikChange site-directed mutagenesis kit (Stratagene). The DRONC coding region was introduced into pET23(a) (Novagen) to produce pDronc-His6 (C-terminally tagged). All DIAP1 and DRONC constructs were expressed in *Escherichia coli* BL21 (DE3) pLysS (Stratagene). For protein purification bacteria were grown at room temperature to OD<sub>600</sub> of 0.4 and were induced with 0.1 mM IPTG for 1 h at room temperature. After induction bacteria were frozen overnight at -80 °C and then thawed in lysis buffer (200 mM Tris-HCl, pH 8.0, 0.4 M ammonia sulfate, 10 mM MgCl<sub>2</sub>, and 10% glycerol + proteasome inhibitor mixture (Roche)). Bacteria were then sonicated on ice at 50% pulse for 3 min in 15-s intervals. The soluble and insoluble fractions were separated by centrifugation at 12,000 × *g* for 10 min. The DIAP1 soluble fractions were purified using glutathione-Sepharose beads (Sigma), and DRONC soluble fractions were purified using cobalt Talon resin (Clontech) per manufacturer's instructions. Protein concentrations were then determined by comparing purified proteins to known concentrations of bovine serum albumin on a Coomassie Blue-stained SDS-polyacrylamide gel.

**DIAP1 Cleavage**—For DRONC cleavage of DIAP1, 200 ng of wild type GST-DIAP1 or the single and double mutants were mixed with increasing concentrations (10–500 ng) of wild type DRONC-His in 30 μl of buffer A (25 mM Tris-HCl, pH 8.0, 50 mM NaCl, and 10 mM dithiothreitol). The reaction mixtures were then incubated at 30 °C for 1 h. After incubation the DIAP1 reactions were stopped by adding SDS-

PAGE sample buffer and were then run on a 15% SDS-polyacrylamide gel. DIAP1 protein was then detected by Western blot using an anti-DIAP1 monoclonal antibody (obtained from Bruce Hay) at 1:3,500, anti-mouse IgG-horseradish peroxidase antibody at 1:6,500, and Super-signal West Pico Chemiluminescent Substrate (Pierce).

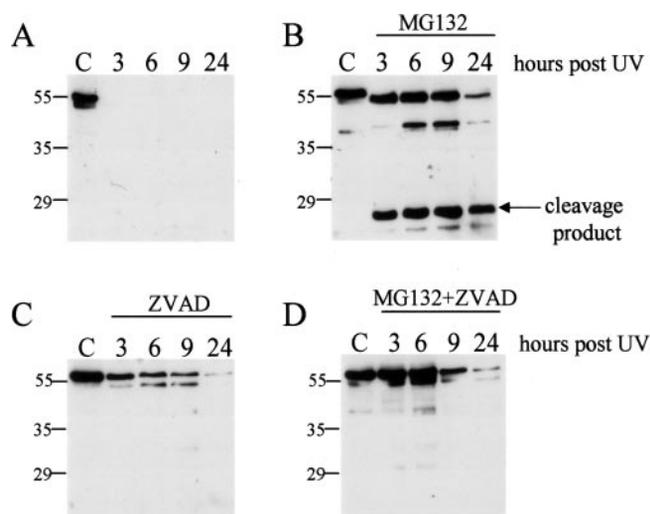
**S2 Cell Viability**—S2 cells were co-transfected with a plasmid expressing enhanced green fluorescent protein (eGFP) under the *Drosophila* heat shock promoter and either wild type DIAP1, N-DIAP1 (Δ205–438 DIAP1), C-DIAP1 (Δ1–205 DIAP1), or the single and double point mutants under the control of the constitutively active baculovirus IE1 promoter. The complete sequences of all of the mutant constructs were verified to ensure there were not any inadvertent mutations introduced during their construction. For Reaper viability, cells were also transfected with a plasmid expressing Reaper, and 48 h after transfection the number of viable green fluorescent cells was determined by counting three fields of view under 400× magnification. Control cells not receiving Reaper were set at 100%. For UV light viability, 20 h after transfection cells were UV light-irradiated by placing the plates on a transilluminator for 10 min. At 24 h after UV light treatment the number of viable green fluorescent cells was counted, and the percentage of viable cells remaining was determined by setting the number of green fluorescent cells at 100% 1 h after UV light treatment. To detect the expressed proteins, transfected cells were subjected to Western blotting using anti-DIAP1 antibody as described above, except that less lysate was used to better illustrate the difference in levels between endogenous DIAP1 and the exogenously expressed DIAP1 proteins.

**Co-immunoprecipitations**—Using recombinant GST-DIAP1 and recombinant DRONC-His, 200 ng of each DIAP1 construct was added to 500 ng of wild type or catalytically inactive DRONC in 30 μl of buffer A. The reactions were added to protein G beads (Sigma), bound to anti-DRONC antibody (obtained from Bruce Hay), and rocked overnight at 4 °C. The beads were then washed, and bound protein was eluted in SDS-PAGE sample buffer. The eluted protein was run on a 12% SDS-polyacrylamide gel, and DIAP1 protein was detected by Western blot using anti-DIAP1 antibody at 1:1,000 and anti-mouse IgG-horseradish peroxidase antibody at 1:10,000. For the co-immunoprecipitations of endogenous protein, 6 million S2 cells were plated overnight and then UV light-treated for 10 min. Cells were harvested at various times post UV light and lysed in Nonidet P-40 buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). The incubation with anti-DRONC bound protein G beads (Sigma) was performed as for recombinant protein. DIAP1 protein was then detected by Western blot using anti-DIAP1 antibody at 1:4,000 and anti-DRONC antibody at 1:7,000.

**Conditions for RNAi in *Drosophila* Cell Culture**—RNAi methods were essentially as described (17, 30). In brief, S2 cells were plated overnight at 6 million cells/well in 6-well plates in TC100 medium (Invitrogen) containing 10% fetal bovine serum (Invitrogen). The cells were then placed in TC100 medium without serum and *dronc* or *dark* dsRNA was added directly to the medium to a final concentration of 40 μg/ml followed by vigorous shaking. The cells were incubated for 5.5 h at 25 °C at which time serum was added to a final concentration of 10% serum. To allow time for protein turnover the cells were incubated for 24 h before proceeding with the rest of the experiment. We have previously shown this amount of time to be sufficient to significantly reduce DRONC and DARK protein levels (17).

#### RESULTS

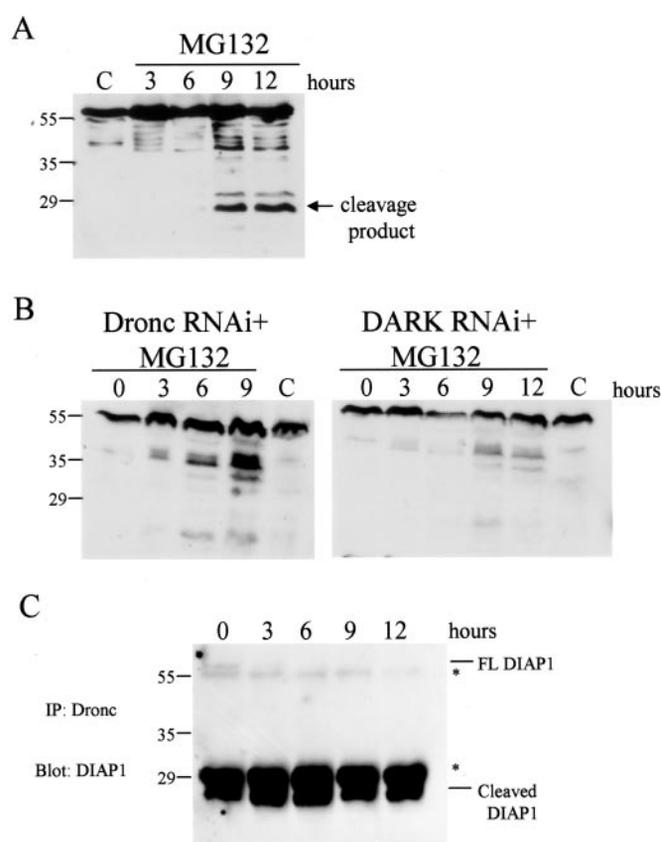
**DIAP1 Protein Levels Are Regulated by Both the Proteasome and Caspase Cleavage**—Recent studies have found that the *Drosophila* inhibitor of apoptosis protein, DIAP1, is short lived and upon induction of apoptosis is rapidly removed (7, 31). Using *Drosophila* S2 cells we confirmed that endogenous DIAP1 protein levels were undetectable 3 h after UV light treatment (Fig. 1A). Several IAPs, including DIAP1, have recently been shown to possess E3 ubiquitin ligase activity and induce the degradation of themselves and other apoptotic proteins (5, 31). We therefore were interested in investigating the effects of the proteasome inhibitor MG132 on the rate of DIAP1 degradation. MG132 treatment of UV light-irradiated cells delayed, but did not completely prevent, the degradation of DIAP1 and also resulted in the appearance and accumulation of a 27-kDa immunoreactive band (Fig. 1B). In addition, MG132 treatment somewhat delayed the progression of apo-



**FIG. 1. Caspase-mediated DIAP1 cleavage occurs in apoptotic S2 cells following UV light irradiation.** *A*, *Drosophila* S2 cells were UV light-irradiated, and lysates were collected at the times shown after irradiation and analyzed by Western blotting with anti-DIAP1 monoclonal antibody. The lanes labeled *C* contain lysates from control, untreated cells. *B–D*, cells were treated at the time of irradiation with the proteasome inhibitor MG132 (*B*), the caspase inhibitor z-VAD-FMK (*C*), or both MG132 and z-VAD-FMK (*D*). The positions of molecular mass markers are indicated on the left.

ptosis, which may have been because of the slower degradation of full-length DIAP1 (data not shown). Because some mammalian IAP proteins are cleaved by caspases (25, 26), we suspected that the 27-kDa protein was the result of caspase cleavage. To examine this, S2 cells were treated with the pan-caspase inhibitor z-VAD-FMK with or without MG132 prior to inducing apoptosis. Interestingly, the 27-kDa band was not present when cells were treated with z-VAD-FMK alone (Fig. 1*C*) or with both z-VAD-FMK and MG132 (Fig. 1*D*). Moreover, z-VAD-FMK treatment also caused a delay in the decrease in full-length DIAP1 (Fig. 1*C*) indicating that the amount of full-length DIAP1 is regulated by both proteasomal degradation and caspase cleavage in apoptotic S2 cells.

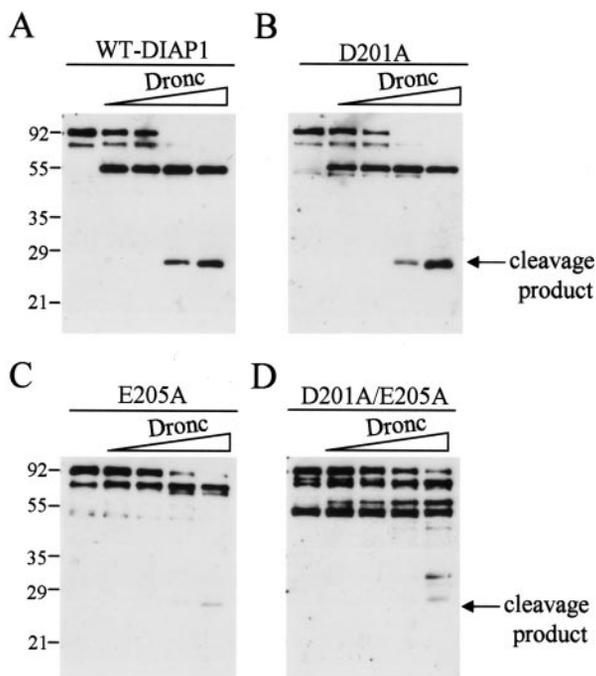
**DRONC Continuously Cleaves DIAP1**—In normal, living *Drosophila* S2 cells, DIAP1 is required to inhibit spontaneous apoptosis (17, 20, 23). We therefore monitored DIAP1 protein levels in non-apoptotic cells treated with MG132. DIAP1 Western blots showed no over-accumulation of full-length DIAP1 following proteasome inhibition but rather showed an accumulation of the 27-kDa fragment of DIAP1, which at times also appeared to be modified in a manner consistent with ubiquitination (Fig. 2*A*). MG132 treatment itself had no effect on cell viability (data not shown). These results suggested that DIAP1 is continuously cleaved in non-apoptotic S2 cells, possibly by one or more caspases. The initiator caspase DRONC was a good candidate for the caspase that cleaves DIAP1, because DIAP1 is known to bind and ubiquitinate DRONC (7), DRONC has previously been shown to also be continuously processed and degraded in normal S2 cells, and DIAP1 RNAi results in the accumulation of processed DRONC (17, 18). To investigate whether DRONC is required for cleavage of DIAP1 we used RNAi to silence either DRONC or DARK, the APAF-1/CED-4 homolog that is required for DRONC activation. Twenty-four hours after the addition of either *dronc* or *dark* dsRNA, S2 cells were treated with MG132. Immunoblot analysis indicated that silencing of either DRONC or DARK greatly reduced the accumulation of the DIAP1 27-kDa fragment (Fig. 2*B*). To determine whether endogenous DRONC and DIAP1 interact in S2 cells, cells were UV light-irradiated, and at various times lysates were immunoprecipitated using anti-DRONC antiserum.



**FIG. 2. DIAP1 undergoes continuous caspase cleavage in normal S2 cells.** *A*, S2 cells were treated with either Me<sub>2</sub>SO as a carrier control (lane labeled *C*) or MG132 dissolved in Me<sub>2</sub>SO and at various times after addition of the drug, lysates were harvested and subjected to Western blotting with anti-DIAP1 antibody. *B*, S2 cells were treated with dsRNA corresponding to DRONC or DARK for 24 h, and then MG132 was added to the cells, and lysates were prepared at the times shown after addition of MG132 and analyzed by Western blotting with anti-DIAP1 antibody. *C*, control untreated cells. *C*, S2 cells were treated with dsRNA corresponding to DIAP1 and at the times shown cell lysates were harvested and immunoprecipitated with anti-DRONC antiserum and immunoprecipitated DIAP1 protein was detected by Western analysis. Asterisks mark the position of the heavy and light immunoglobulin chains used for the immunoprecipitation (*IP*). The migration of full-length (*FL*) DIAP1 and cleaved DIAP1 are indicated at the right.

Anti-DIAP1 Western blots of the immunoprecipitated samples showed that in non-apoptotic cells, DRONC interacted with both full-length DIAP1 and the 27-kDa cleavage fragment of DIAP1, although the interaction with cleaved DIAP1 appeared much stronger (Fig. 2*C*, lane 1). These results indicate that in non-apoptotic cells, activated DRONC is necessary for DIAP1 cleavage and support our previous finding that a portion of the DRONC protein in non-apoptotic cells is activated but is inhibited prior to activating effector caspases (17).

**DRONC Cleaves DIAP1 in the Spacer Region between BIR1 and BIR2**—To further verify that DRONC was directly responsible for DIAP1 cleavage, we examined the ability of DRONC to cleave DIAP1 in reactions using recombinant, purified proteins. When recombinant DIAP1 was incubated with increasing concentrations of recombinant DRONC, DIAP1 was readily cleaved, resulting in cleavage products of ~50 and 27 kDa (Fig. 3*A*). The recombinant DIAP1 protein used in this experiment was fused with glutathione *S*-transferase (*GST*) at the N terminus, and an anti-*GST* immunoblot showed that the 50-kDa cleavage product included the N terminus of DIAP1 (data not shown). We therefore concluded that the 27-kDa fragment, which was similar to the cleavage product seen in S2 cells, represented the C-terminal portion of DIAP1.



**FIG. 3. DIAP1 is cleaved *in vitro* by DRONC at position Glu-205.** GST-tagged wild type (WT) DIAP1 or DIAP1 with point mutations at positions 201 (*D201A*) or 205 (*E205A*) or both positions was incubated with increasing concentrations of recombinant His-tagged DRONC, and cleavage of DIAP1 was monitored by Western blotting. Molecular mass markers are indicated at the left.

To determine the DRONC cleavage site, we examined the amino acid sequence of DIAP1 for potential cleavage sites that would result in a C-terminal cleavage product of ~27 kDa. DRONC is unusual among caspases in that it has been shown to cleave after both glutamate and aspartate residues (32). Residues Asp-201 and Glu-205 are both in the spacer region of DIAP1 between the first and second BIRs and, if cleaved, would result in a C-terminal fragment of approximately the correct size. We therefore mutated DIAP1 at these residues by changing these residues to alanines (*E205A* and *D201A*) and expressed them in bacteria to generate recombinant proteins. After incubation with increasing concentrations of active DRONC, samples were immunoblotted using anti-DIAP1 antibody, and it was observed that both wild type DIAP1 and the *D201A* mutant were cleaved by DRONC into two fragments of 50 and 27 kDa (Fig. 3, A and B). In contrast DRONC cleavage of the *E205A* mutant was greatly inhibited (Fig. 3C) and was only observed when incubated with high concentrations of DRONC. In addition, the *E205A/D201A* double mutant showed little to no cleavage even when incubated with high concentrations of DRONC (Fig. 3D), suggesting that the cleavage products observed with the *E205A* mutant may have been because of DRONC cleavage at amino acid Asp-201 and/or cleavage at other less efficient sites. These results indicate that DRONC cleaves DIAP1 after amino acid Glu-205 and support a recent publication that reported the same DRONC cleavage site *in vitro* (28).

**Glu-205 Mutations Inhibit DRONC Binding *in Vitro* and Have Reduced Ability to Inhibit Apoptosis**—Recent reports have shown that DIAP1 is also cleaved by the effector caspase DrICE at amino acid Asp-20 (27, 28). These reports found that DrICE cleavage of DIAP1 was required for DrICE binding to DIAP1 and was important for DIAP1 activity. We therefore hypothesized that DRONC cleavage of DIAP1 may also play a role in DRONC binding to DIAP1 and be important for DIAP1 activity. To examine the effects of DIAP1 cleavage on DRONC

binding, various purified DIAP1 mutant proteins were incubated with bacterially expressed DRONC that was either fully processed (active) or a catalytically inactive DRONC mutant (C318S) that was full-length. Immunoprecipitation of these samples indicated that wild type DIAP1, and the *D201A* mutant bound both processed and full-length DRONC (Fig. 4). DIAP1 was cleaved by active DRONC and both the N- and C-terminal fragments of DIAP1 were immunoprecipitated, indicating that the both fragments remained bound to DRONC following cleavage, at least stably enough to be immunoprecipitated. However the *E205A* mutant bound only full-length DRONC and was unable to bind active DRONC (Fig. 4), indicating that presence of an intact cleavage site in DIAP1 is important for this processed form of DRONC to bind DIAP1.

To investigate the role of DRONC cleavage on the anti-apoptotic activity of DIAP1, untagged wild type DIAP1, N-DIAP1 (amino acids 1–205 of DIAP1), C-DIAP1 (amino acids 205–438 of DIAP1 with an initiating methionine), or the single, double, and triple combinations of the point mutants *D20A*, *D201A*, and *E205A* were transiently expressed in S2 cells, and the cells were co-transfected with a construct expressing Reaper to induce apoptosis (Fig. 5A). As previously reported (27), mutation of Asp-20 reduced the ability of DIAP1 to protect against Reaper-induced death by ~50%. In addition, mutation of residue Glu-205 also reduced the anti-apoptotic function of DIAP1 against Reaper-induced death (Fig. 5A), although not to the same degree as mutation of Asp-20. Mutation of Asp-201 had no effect on Reaper-induced apoptosis. Interestingly, although the *D20A/D201A* mutant behaved similarly to the *D20A* mutant, the ability of the *D20A/E205A* double mutant to inhibit Reaper-induced death was similar to the *E205A* mutant. This would fit with an interaction between DIAP1 and DRONC being epistatic to the interaction between DIAP1 and DrICE. Expression of C-DIAP1 also had a reduced ability to inhibit Reaper-induced death, whereas expression of N-DIAP1 had no anti-apoptotic activity, and expression of both C- and N-DIAP1 together was similar to expression of C-DIAP1 alone.

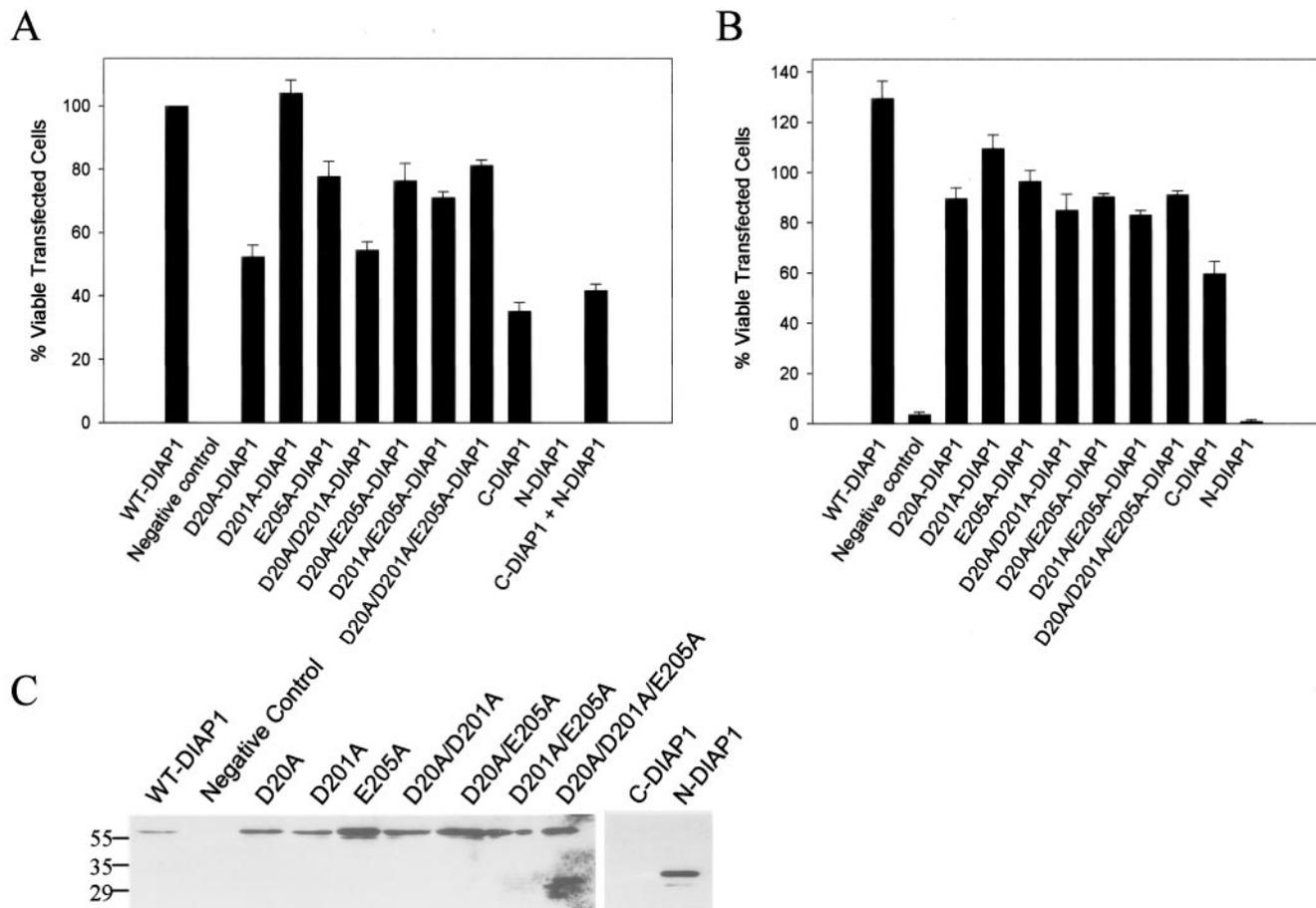
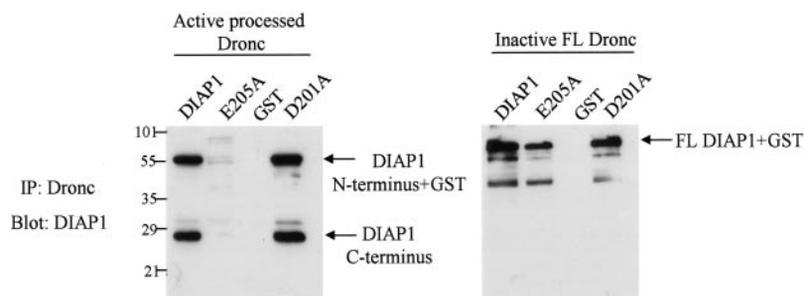
When a different death stimulus, UV light irradiation, was used, mutation of Asp-20 and/or Glu-205 had less of an inhibitory effect on anti-apoptotic function than against Reaper. However both mutants still had slightly reduced anti-apoptotic function compared with wild type or the Asp-201 mutant (Fig. 5B). As seen with Reaper overexpression, C-DIAP1 protected less well than any of the point mutants, whereas expression of N-DIAP1 did not protect at all.

All of the mutant proteins were expressed at levels equal to or higher than the wild type DIAP1 construct, with the exception of C-DIAP1 (Fig. 5C). Expression of C-DIAP1 did, however, provide some protection compared with the negative control, indicating that it was expressed, but the protein was probably highly unstable because of the presence of the RING domain. This is consistent with the observation that the 27-kDa DIAP1 cleavage product, which is essentially the same as C-DIAP1, was not observed unless cells were treated with proteasome inhibitor (Figs. 1 and 2).

## DISCUSSION

In previous studies we have shown that addition of MG132 to normal living S2 cells caused the over-accumulation of a processed form of DRONC, whereas full-length DRONC levels remained unaffected (17). This suggested that DRONC was being continuously processed and once processed, targeted for degradation by the proteasome. In this report we found that MG132 treatment of normal living S2 cells also caused the over-accumulation and modification of a C-terminal cleavage fragment of DIAP1. We also observed that similar to full-length DRONC, full-length DIAP1 protein levels were unaffected by

**FIG. 4. An intact cleavage site is required for interaction of DIAP1 and fully processed DRONC.** Recombinant active, processed DRONC or catalytically inactive, unprocessed DRONC was incubated with the indicated DIAP1 recombinant proteins and was then immunoprecipitated using anti-DRONC antibody. Immunoprecipitated (IP) proteins were then Western blotted using anti-DIAP1 antibody. *FL*, full-length.



**FIG. 5. Mutation of the DRONC cleavage site affects the ability of DIAP1 to inhibit apoptosis.** *A*, S2 cells were transfected with a plasmid expressing eGFP alone (*Negative control*) or co-transfected with plasmids expressing eGFP, the indicated DIAP1 mutant, and Reaper, and the viability of the transfected cells was determined 48 h later. *B*, S2 cells were transfected with a plasmid expressing eGFP alone (*negative control*) or co-transfected with plasmids expressing eGFP and the indicated DIAP1 mutant. Twenty hours after transfection cells were UV light-irradiated, and transfected cell viability was determined 24 h later. *C*, lysates from S2 cells transfected with eGFP alone (*negative control*), or the indicated DIAP1 constructs were analyzed by Western blotting using anti-DIAP1 antibody. *WT*, wild type.

MG132 treatment. We were able to conclude that DRONC directly cleaves DIAP1 in S2 cells based on several pieces of evidence. 1) Reducing the levels of either DRONC or DARK by RNAi inhibited the production of the 27-kDa fragment of DIAP1; 2) endogenous DRONC interacted with both full-length DIAP1 and the C-terminal DIAP1 cleavage fragment; and 3) experiments using recombinant, purified proteins revealed that DRONC cleaved DIAP1 and produced a cleavage product similar in size to that seen in S2 cells. It is unclear why this 27-kDa cleavage product has not been reported by other groups who have examined DIAP1 cleavage in S2 cells (27, 29), but it may be because of the antibody we used to detect DIAP1, which was different from that used by the other groups.

Our data, including that presented here and in previous publications (17, 18), point to a model wherein DRONC, DARK, DIAP1, and DrICE exist in an apoptosome-like complex and

autoactivation of DRONC occurs spontaneously in a DARK-dependent fashion, resulting in cleavage at residue Glu-352 of DRONC, separating the large and small subunits (18). In this model, activated DRONC then cleaves DIAP1 at Glu-205 and is subsequently ubiquitinated by DIAP1 and degraded. The C-terminal cleavage product of DIAP1, containing the RING domain, is also ubiquitinated and degraded. These events appear to occur continuously in unstimulated S2 cells. Interruption of the DIAP1-DRONC interaction, whether by Reaper, Hid, or Grim binding or by a decrease in DIAP1 levels, results in the accumulation of activated DRONC and subsequent cleavage and activation of DrICE. Cleavage of DIAP1 by DrICE at position Asp-20 then occurs (27), and this cleavage event has been reported to be required for inhibition of DrICE by DIAP1 (28). The observation that the D20A/E205A double mutant protected at a level similar to the E205A mutant (Fig. 5A) supports the

conclusion that cleavage at Glu-205 occurs prior to that at Asp-20, as does the observation that DRONC activation occurs prior to DrICE activation following a death signal (17).

The interaction between DIAP1 and either full-length DRONC or Pr1 DRONC, the predominant active form of DRONC in apoptotic cells (DRONC cleaved only at position Glu-352 between the large and small subunits) (18), appears to be different from the interaction of DIAP1 with fully active DRONC that is expressed in bacteria, which is cleaved at Glu-352 and also at an unidentified residue lying between the prodomain and the large subunit (18). The interaction between DIAP1 and full-length DRONC has been shown to involve BIR2 of DIAP1 and amino acids 114–125 of DRONC (33), but this sequence is probably not present in bacterially expressed active DRONC due to cleavage during processing (18). The interaction between bacterially expressed active DRONC and DIAP1 appears instead to involve the active site of DRONC and Glu-205 of DIAP1, because fully processed DRONC requires an intact cleavage site at position Glu-205 for interaction with DIAP1, whereas full-length DRONC does not (Fig. 4).

Mutation of either of the caspase cleavage sites in DIAP1 (Asp-20 or Glu-205) had a negative effect on the ability of DIAP1 to protect cells against Reaper-induced apoptosis; however, in both cases the mutant proteins still retained significant anti-apoptotic function. Similar results were previously reported for the D20A mutant against Reaper (27). These observations suggest that although caspase cleavage of DIAP1 may normally be important for its anti-apoptotic function, it is not absolutely essential. It is likely that the effect of mutating the cleavage site is being masked by overexpression of the mutant proteins in these experiments. The levels of overexpressed DIAP1 were much higher than the levels of endogenous DIAP1 in this system, which was not even detected with the amount of lysate used for analysis (Fig. 5C, compare the first two lanes). The effect of mutating Asp-20 or Glu-205 was even less on UV light-induced apoptosis. This could be because Reaper-induced apoptosis and UV light-induced apoptosis involve different pathways, or because Reaper-induced apoptosis may be a stronger death stimulus than the dose of UV light used in these experiments.

It was recently reported that BIR1 of DIAP1 is necessary and sufficient for inhibition of DrICE enzyme activity *in vitro* (28). However in our experiments expression of N-DIAP1, containing amino acids 1–205 and including all of BIR1, had no ability to protect S2 cells against apoptosis stimulated by either Reaper or UV light, even though the truncated protein was expressed at high levels (Fig. 5). Because RNAi of DrICE is highly effective at inhibiting apoptosis in S2 cells (18), this result indicates that merely inhibiting the catalytic activity of DrICE is not sufficient to prevent apoptosis and points to the importance of the RING domain in the anti-death function of DIAP1, as reported previously (27).

DIAP1 has a pivotal role in regulating apoptosis in *Drosophila*, and it is a critical determinant for whether cells die or live. Although mutating the DRONC cleavage site in DIAP1 did not completely eliminate DIAP1 function when DIAP1 was overex-

pressed, the interactions between DIAP1 and DRONC are clearly important for regulating apoptosis, and this study has reported and characterized a novel feature of that interaction. Future studies may reveal a more significant role for this cleavage event when the proteins are expressed at physiologically relevant levels.

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