Down-regulation of DIAP1 Triggers a Novel Drosophila Cell Death Pathway Mediated by Dark and DRONC*

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Members of the inhibitor of apoptosis protein (IAP) family can inhibit caspases and cell death in a variety of insect and vertebrate systems. Drosophila IAP1 (DIAP1) inhibits cell death to facilitate normal embryonic development. Here, using RNA interference, we showed that down-regulation of DIAP1 is sufficient to induce cell death in Drosophila S2 cells. Although this cell death process was accompanied by elevated caspase activity, this activation was not essential for cell death. We found that DIAP1 depletion-induced cell death was strongly suppressed by a reduction in the Drosophila caspase DRONC or the Drosophila apoptotic protease-activating factor-1 (ApaF-1) homolog, Dark. RNA interference studies in Drosophila embryos also demonstrated that the action of Dark is epistatic to that of DIAP1 in this cell death pathway. The cell death caused by down-regulation of DIAP1 was accelerated by overexpression of DRONC and Dark, and a caspase-inactive mutant form of DRONC could functionally substitute the wild-type DRONC in accelerating cell death. These results suggest the existence of a novel mechanism for cell death signaling in Drosophila that is mediated by DRONC and Dark.

Multicellular organisms eliminate unwanted or damaged cells by a genetically regulated cell death process called programmed cell death or apoptosis (1, 2). Programmed cell death is mediated by a family of cysteine proteases, termed caspases (3). An initiator caspase, caspase-9, is activated by apoptotic protease-activating factor-1 (ApaF-1)1 in the presence of cytochrome c, which is released from mitochondria to the cytosol in response to a variety of apoptotic stimuli, thereby triggering a proteolytic caspase cascade (3). The activation of caspases is also negatively controlled by endogenous inhibitory proteins. Members of the inhibitor of apoptosis protein (IAP) family can bind to and directly inhibit caspases through their baculovirus IAP repeat domains (4). The function of IAPs can be inhibited by the mitochondrial factor Smac/DIABLO (5, 6) or HtrA2/Omi (7–11), which is also released to the cytosol in response to cell death stimuli, resulting in caspase activation. IAPs are RING finger domain proteins and can also function as ubiquitin ligases. A previous study demonstrated that the levels of IAPs are regulated by their own ubiquitination-dependent degradation such that IAPs are selectively lost upon exposure to cell death stimuli in a proteasome-dependent manner (12).

The molecular mechanisms of programmed cell death are highly conserved throughout evolution (13, 14). In Drosophila, there are seven caspases (DCP-1, drICE, Dredd, DECAY, STRICA, and DAMM, including a functional counterpart of caspase-9, DRONC), an Apaf-1 homolog (Dark/Dapaf-1/HAC-1), and two Bcl-2 family proteins (Drobl/Deblk/dBorg-1/dBok and Buffy/dBorg-2). The Drosophila genome also encodes four IAPs, DIAP1, DIAP2, Deterin, and dBruce. Caspase-dependent cell death induced by a Drosophila killer protein, Reaper or Hid, is strongly inhibited by the overexpression of DIAP1 or DIAP2 (15). In addition, a diap1 loss-of-function mutant embryo displays global cell death with an elevated caspase activity (16), indicating that DIAP1 is required to inhibit cell death and facilitate normal embryonic development. Although a diap1 mutant embryo exhibits massive apoptotic cell death, accompanied by cell rounding, membrane blebbing, and DNA fragmentation, no acridine orange staining is seen (16), suggesting that the mechanism of the cell death caused by the loss of DIAP1 function is somehow different from the cell death induced by the overexpression of Reaper or Hid. Here, using the RNA interference (RNAi) technique, we performed loss-of-function analyses of the cell death induced by the down-regulation of DIAP1. Our findings demonstrate a novel cell death pathway triggered by DIAP1 depletion that is executed independently of caspase activity but depends on Dark and DRONC.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfection, and Western Blotting—S2 cells were cultured at 26 °C and transfected using Cellfectin (Invitrogen) as described previously (17). For Western blotting, an anti-FLAG M2 monoclonal antibody (1:500, Sigma), an anti-HA polyclonal antibody (1:500, MBL), and an anti-β-tubulin E7 monoclonal antibody (1:500, Hybridoma Bank) were used.

Cell Death Assay and Caspase Assay—Cell death assays were performed as described previously (17). In brief, S2 cells were transfected with pUAST-derived expression plasmids and a driver plasmid, pWAGAL4, together with a reporter plasmid, pCaspese-hs-lacZ, which encodes β-galactosidase under the control of the hsp70 promoter. Twenty-four hours after transfection, the cells were heat shocked at 37 °C and cultured at 26 °C for another 24 h. At 48 h, the cells were lysed and assayed for β-galactosidase activity in a reaction mixture containing o-nitrophenyl-β-D-galactopyranoside. Caspase activity was determined

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† The abbreviations used are: Apaf-1, apoptotic protease-activating factor-1; IAP, inhibitor of apoptosis protein; DIAP, Drosophila IAP; RNAi, RNA interference; HA, hemagglutinin; MCA, 4-methylcoumaryl-7-amide; ds, double-stranded; GFP, green fluorescent protein; z-Dcb, benzoyloxycarbonyl-Asp-CH2-OC(O)-dichlorobenzene; mDRONC, mutant DRONC.
dsRNA was used as a control dsRNA that shared no homology with any sequences in the Drosophila genome. dsRNA was dissolved in injection buffer (5 mM KCl in 0.1 mM phosphate buffer, pH 8.0) at 0.75 mg/ml. Precellularized embryos from wild-type or above.

For the cell death assay, S2 cells were transfected with either pUAS-GFP or pUAS-reaper together with driver plasmid pWAGAL4 with or without pUAS-p35. Cell viability was determined by cell death assay as described under “Experimental Procedures.” B, S2 cells were transfected with either cod-9 dsRNA or diap1 dsRNA together with pWAGAL4 plus pUAS-GFP or pUAS-p35. Note that p35 had a slightly suppressive effect on the diap1 dsRNA-induced cell death. C and D, S2 cells were cultured with or without 10 μg/ml cycloheximide (CHX) for 12 h and then lysed, and the cell lysate was subjected to caspase assay using 10 μM DEVD-MCA (C) or 10 μM VEID-MCA (D) with or without adding the pan-caspase inhibitor zD-dcb (100 μM) (Peptide Institute). The inhibition of the DEVDDase activity was also observed by adding 10 or 50 μM zD-dcb (61 or 94% inhibition, respectively), and a complete inhibition of the VEIDase activity was also observed by 10 or 50 μM zD-dcb (data not shown). E, S2 cells were transfected with either pUAS-GFP or pUAS-reaper together with pWAGAL4 in the absence or presence of various concentrations of zD-dcb. F, S2 cells were transfected with either cod-9 dsRNA or diap1 dsRNA in the absence or presence of zD-dcb as in E. Note that zD-dcb did not suppress the DIAP1 depletion-induced cell death.

epression of the DIAP1 protein compared with the control cod-9 dsRNA (Fig. 1C). In addition to killing cells, the down-regulation of DIAP1 strongly stimulated caspase-3-like DEVD cleavage activity, but not caspase-1-like YYAD cleavage activity, similar to the overexpression of Reaper (Fig. 1D). However, cleavage of the tetrapeptide VEID, which is preferentially cleaved by DRONC, was not significantly increased (Fig. 1D).

To assess whether the cell death induced by diap1 dsRNA was dependent on caspase activation, the effects of caspase inhibitors were tested. Although baculovirus p35, a broad spectrum caspase inhibitory protein, completely inhibited the Reaper-induced cell death (Fig. 2A), diap1 dsRNA-induced cell death was only partially suppressed by p35 (Fig. 2B). Although p35 can strongly inhibit Drosophila DEVDase activity (17), it cannot block the DRONC activity (20). To further examine whether the caspase activity was required for DIAP1 depletion-induced cell death, we used a pan-caspase inhibitor zD-dcb. A treatment of the cells with zD-dcb blocked Reaper-induced cell death in a dose-dependent manner (Fig. 2E); however, it could not suppress the cell death caused by DIAP1 depletion (Fig. 2F). As shown in Fig. 2, C and D, a cell lysate from cycloheximide-treated S2 cells had elevated caspase activities including DRONC activity as assayed by cleavage of DEVDD and VEID. These activities were completely inhibited in vitro by adding
zD-dcb, suggesting that zD-dcb had a potential to block *Drosophila* caspase activity including DRONC. These results suggest that, although it strongly stimulates caspase activation, the cell death induced by the down-regulation of DIAP1 can be executed through a caspase-independent pathway.

To further analyze whether the caspases participated in DIAP1 depletion-induced cell death, we treated cells with dsRNAs synthesized from the cDNAs of *Drosophila* caspases, DRONC, dICE, and DCP-1, as well as the *Drosophila* Apaf-1, Dark. Each dsRNA worked as a specific inhibitor of the corresponding protein expression (Fig. 3A). As shown in Fig. 3B, Reaper-induced cell death was blocked almost completely by *drone* dsRNA and was also suppressed by dsRNA for *drice* or *dcdf*. In contrast to the observation that the pan-caspase inhibitor could not suppress the DIAP1 depletion-induced cell death, the cell death was completely inhibited by *drone* dsRNA (Fig. 3C). Furthermore, *dark* dsRNA strongly suppressed the *diap1* dsRNA-induced cell death. This was unexpected because in mammals IAPs function as inhibitors of caspases, and Apaf-1 functions upstream of the caspase cascade. Therefore, we further assessed *in vivo* whether Dark could function genetically downstream of DIAP1.

Precellularized embryos were injected with *diap1* dsRNA or buffer alone, and the effects on their hatch rates were analyzed. As shown in Table I, injection of *diap1* dsRNA resulted in a reduced hatch rate (10.6%) compared with buffer injection (34.9%). The *dpfK* allele is a loss-of-function hypomorphic *dark* mutant allele (19). We injected embryos from *dpfK* homozygous flies, and observed no reduction in the hatch rate of *diap1* dsRNA-injected embryos (22.6%) compared with buffer-injected embryos (21.0%). These data demonstrate that Dark functions genetically downstream of DIAP1 in the DIAP1 depletion-induced cell death pathway.

Our co-depletion analyses using S2 cells and *Drosophila* embryos indicated that the cell death induced by DIAP1 down-regulation was dependent on the presence of DRONC and Dark despite the fact that this cell death could not be inhibited by the pan-caspase inhibitor zD-dcb. To examine whether DRONC could mediate the cell death signaling independently of its caspase activity, we introduced a caspase-inactive mutant form of DRONC (mDRONC) (21) into the DIAP1-depleted background. The cell death caused by DIAP1 depletion was accelerated by overexpression of both of Dark and DRONC (Fig. 4),

![DIAP1 Down-regulation Triggers a Novel Cell Death Pathway](image)

**FIG. 3.** Cell death induced by DIAP1 depletion depends on **A**. S2 cells were transfected with the expression vectors for HA- or FLAG-tagged protein with the indicated dsRNA and subjected to Western analysis using an anti-HA, anti-FLAG, or anti-β-tubulin antibody. **B**. S2 cells were transfected with either pCaspeR-hs-GFP or pCaspeR-he-reaper together with dsRNA synthesized from CED-9, DRONC, dICE, DCP-1, or Dark cDNA. **C**. S2 cells were transfected with either *ced-9* dsRNA or *diap1* dsRNA together with *ced-9* dsRNA, *drone* dsRNA, *dric* dsRNA, *dcp-1* dsRNA, or *dark* dsRNA as in B. Note that *drone* dsRNA and *dark* dsRNA can strongly suppress the *diap1* dsRNA-induced cell death.

**TABLE I**

| Genotype | Injection | Hatch rate | n* |
|----------|-----------|------------|----|
| Wild type | Buffer    | 34.9%      | 106|
|          | *diap1* dsRNA | 10.6%    | 104|
| *dpfK*/*dpfK* | Buffer | 21.0% | 124|
|          | *diap1* dsRNA | 22.6% | 137|

*Total number of embryos is shown.*
consistent with the notion that DIAP1 functions genetically upstream of Dark. As with wild-type DRONC, mDRONC also accelerated cell death (Fig. 4), indicating that caspase activity of DRONC is not required for this phenomenon. These results suggest that the cell death signaling stimulated by DIAP1 down-regulation is mediated by DRONC and Dark through a mechanism independent of DRONC caspase activity.

DISCUSSION

In this study, we found that the down-regulation of DIAP1, but not DIAP2 (data not shown), is sufficient for inducing cell death in Drosophila S2 cells. This indicates that DIAP1 normally blocks a naturally stimulating cell death pathway. Unexpectedly, caspase inhibitors could not suppress the DIAP1 depletion-induced cell death, but it was blocked by the co-depletion of DRONC or Dark. Recently Zimmermann et al. (22) reported that down-regulation of DIAP1 in S2 cells induces a cell death pathway that can be blocked by the down-regulation of Dark. More recently Rodriguez et al. (23) showed that phenotypes caused by a loss of DIAP1 function were suppressed by a loss of Dark function. These are consistent with our observation, and we analyzed the mechanism of the cell death further. DIAP1 down-regulation-induced spontaneous cell death did not require caspase activity, but it depended on DRONC and Dark. It has been reported that DRONC can bind to both Dark and DRONC (19, 20). In mammals, the three-dimensional structure of the apoptosome (Apaf-1-cytochrome c-caspase-9 complex) suggests a model in which cytochrome c promotes the assembly of the apoptosome to activate caspase-9 (24). In Drosophila, on the other hand, it has been suggested that the apoptosome may form in the vicinity of the mitochondria in the absence of cytochrome c release (25). We also observed that DIAP1 depletion-induced cell death was not affected by cytochrome c dsRNA (data not shown). These observations and our data suggest that in Drosophila DIAP1 might normally inhibit the Dark-DRONC complex and that the down-regulation of DIAP1 might result in an “active” Dark-DRONC complex, triggering both caspase-dependent and -independent cell death pathways (Fig. 5).

The observation that the pan-caspase inhibitor z-dcb did not suppress the DIAP1 depletion-induced cell death suggests that DRONC may be able to induce cell death independent of its caspase activity. The observation that the caspase-inactive form of DRONC could functionally substitute the wild-type DRONC in accelerating DIAP1 depletion-induced cell death also supports the idea that the cell death can be mediated through non-caspase mechanisms. DRONC might have a pro-apoptosome-independent cell-killing activity that is activated by Dark. It is possible that DRONC is required simply as a bridging or scaffolding protein to bring other proteins together to transmit the cell death signaling. Although we cannot exclude the possibility that z-dcb could not completely inhibit the caspase activity of DRONC, it is apparent that the mode of cell death caused by the down-regulation of DIAP1 is distinct from Reaper-induced cell death. We also assessed the effects of dsRNAs synthesized from reaper, hid, grim, drob-1, and buffy/dborg-2 cDNAs on the diap1 dsRNA-induced cell death and found that none of them suppressed the cell death (data not shown). Further in vivo analysis should help elucidate the role of the caspase-independent cell death pathway regulated by DIAP1.

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