

A GH3-like Domain in Reaper Is Required for Mitochondrial Localization and Induction of IAP Degradation*

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Reaper is a potent pro-apoptotic protein originally identified in a screen for *Drosophila* mutants defective in apoptotic induction. Multiple functions have been ascribed to this protein, including inhibition of IAPs (inhibitors of apoptosis); induction of IAP degradation; inhibition of protein translation; and when expressed in vertebrate cells, induction of mitochondrial cytochrome *c* release. Structure/function analysis of Reaper has identified an extreme N-terminal motif that appears to be sufficient for inhibition of IAP function. We report here that this domain, although required for IAP destabilization, is not sufficient. Moreover, we have identified a small region of Reaper, similar to the GH3 domain of Grim, that is required for localization of Reaper to mitochondria, induction of IAP degradation, and potent cell killing. Although a mutant Reaper protein lacking the GH3 domain was deficient in these properties, these defects could be fully rectified by appending either the C-terminal mitochondrial targeting sequence from Bcl-x_L or a homologous region from the pro-apoptotic protein HID. Together, these data strongly suggest that IAP destabilization by Reaper in intact cells requires Reaper localization to mitochondria and that induction of IAP instability by Reaper is important for the potent induction of apoptosis in *Drosophila* cells.

Programmed cell death in the fly *Drosophila melanogaster* is regulated by a group of genes situated adjacent to one another on chromosome 3. A chromosomal deletion that removes three of these genes, *reaper*, *hid*, and *grim* (the H99 deletion), leads to the loss of developmental cell deaths as well as a loss of the majority of cell deaths resulting from cell-damaging stimuli such as X-irradiation (1). Moreover, ectopic expression of any of these individual genes leads to autonomous cell death in both cultured fly cells and cells of the intact fly (2–4). It has also

been demonstrated that Reaper, Grim, and HID¹ can induce apoptosis in vertebrate cells, suggesting that these central regulators of fly cell death can engage evolutionarily conserved apoptotic pathways (5–7).

Under most circumstances, apoptotic cell death is executed by a group of aspartate-directed cysteine proteases known as caspases (reviewed in Ref. 8). In a healthy cell, caspases are inactive, allowing cell survival. However, in response to diverse apoptotic stimuli, cells can initiate signaling pathways leading to the activation of caspases and consequent proteolytic cleavage of key intracellular substrates. Acting in opposition to the caspases is a family of proteins known as the IAPs, which can bind to caspases and inhibit their enzymatic activity. In *Drosophila*, it appears that loss of IAP function is sufficient to induce apoptosis, suggesting that the *Drosophila* caspases are poised for activation, but are normally held in check by the IAPs (9).

Although *reaper*, *grim*, and *hid* (as well as a nearby pro-apoptotic gene, *sickle*) do not share overall sequence homology, careful examination of their sequences revealed an area of limited homology at their extreme N termini. Both biochemical and genetic experiments revealed that this domain, termed the IAP-binding motif (IBM), can bind to and functionally inhibit the IAPs. Specifically, these proteins can displace IAPs from caspases, thereby alleviating IAP-mediated caspase inhibition (10–12).

Aside from inhibiting IAP function, Reaper, Grim, and HID proteins have all been reported to promote proteosomal degradation of the IAP proteins (13–18). The IAP proteins examined in these experiments, including DIAP1 from *Drosophila* and XIAP, c-IAP1 (cellular inhibitor of apoptosis-1), and c-IAP2 from human cells, all have RING domains that can act as ubiquitin ligases (reviewed in Ref. 19). Because caspase-independent IAP degradation induced by Reaper, HID, and Grim depends upon an intact IAP RING domain, it has been generally concluded that these proteins can stimulate IAP auto-ubiquitination. Interestingly, the IAPs try to “retaliate” by promoting ubiquitination of Reaper, Grim, and HID, so the relative abundances of the IBM and IAP proteins reflect a balance between IAP auto-ubiquitination and ubiquitination of the IBM motif-containing IAP antagonists (20). We (15) and others (18) have also reported that Reaper and Grim can inhibit general protein translation, which may contribute to apo-

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¹ The abbreviations used are: HID, head involution-defective; IAPs, inhibitors of apoptosis; IBM, IAP-binding motif; DIAP1, *Drosophila* inhibitor of apoptosis-1; XIAP, X-linked inhibitor of apoptosis; GST, glutathione S-transferase; GH3, Grim helix 3; EGFP, enhanced green fluorescent protein; Z-VAD-fmk, benzylloxycarbonyl-Val-Ala-methoxy-Asp-fluoromethyl ketone; GFP, green fluorescent protein; FACS, fluorescence-activated cell sorting; RFP, red fluorescent protein.

ptosis by preventing resynthesis of short-lived apoptotic inhibitors such as the IAPs.

Although IAP inhibition may be sufficient for apoptosis in fly cells, this may not be the case in vertebrates. Indeed, XIAP knockout mice do not exhibit any overt phenotypes (21). Although this could well indicate some functional redundancy among the IAPs, it may also reflect a requirement for additional events in the activation of caspases and cell death in vertebrate cells. In particular, many apoptotic stimuli in vertebrate systems promote release of cytochrome *c* from the intermembrane space of the mitochondria to the cytoplasm (reviewed in Ref. 22). Once cytoplasmic, the released cytochrome *c* binds to Apaf-1, which recruits and activates the initiator caspase, caspase-9. Also released from the mitochondria is SMAC, an IAP inhibitory protein that, in its cytoplasmically released form, carries an N-terminal IBM motif (23, 24). Like Reaper, HID, and Grim, SMAC can interfere with IAP-mediated caspase inhibition. However, unlike the fly IBM proteins, SMAC does not appear to be a potent cell killer, consistent with the hypothesis that vertebrate caspases require positive activation as well as a relief of IAP inhibition for full activation. It is currently unclear whether this reflects differences intrinsic to the IBM proteins themselves or differences in apoptotic regulation in the parent systems.

We have reported that recombinant Reaper protein can induce caspase activation in cell-free *Xenopus* egg extracts (25). In this system, mitochondria are absolutely required for caspase activation by Reaper. Indeed, Reaper can induce the release of cytochrome *c* from mitochondria in a pathway requiring a Reaper-binding protein known as Scythe (26). Although we have found that Scythe is a member of the BAG family of proteins that can modulate the chaperone activity of Hsp70/Hsc70, the precise means by which Reaper and Scythe cooperate to promote cytochrome *c* release is not yet clear (27). Although Reaper with a free N terminus can both inhibit and induce degradation of *Xenopus* IAPs, GST-Reaper protein lacking a free N terminus (and therefore unable to bind or modulate the IAPs) or Reaper bearing a deletion of the IBM motif (Reaper-(16–65)) can still induce caspase activation by triggering cytochrome *c* release. Similarly, Reaper-(16–65) has been shown to induce apoptosis when expressed in human cells (6). This same protein has also been reported to induce apoptosis in fly cells (albeit weakly), raising the issue of whether regions of Reaper lying outside of the IBM domain are important for its function (28).

Because Reaper lacking the IBM motif has some apoptotic activity in fly cells and can induce mitochondrial cytochrome *c* release in vertebrate cells, it has been speculated that Reaper might also have some mitochondrial effects in fly cells. Indeed, it has been reported that cytochrome *c* from fly cells, although not fully released in response to apoptotic induction, undergoes a conformational change, exposing previously masked epitopes (29). Moreover, there is a fly homolog of Apaf-1, known variously as DARK, HAC-1, or D-Apaf-1, that can, like Apaf-1, recruit and activate a fly caspase (DRONC) (9, 30–32). That being said, it has also been reported that RNA interference ablation of cytochrome *c* in cultured fly cells does not impair Reaper-induced apoptosis (33). In addition, the question of whether DARK is an obligatory participant in Reaper/Grim/HID-induced apoptosis is controversial.

In analyzing mutants of Grim, Claveria *et al.* (34) identified a pro-apoptotic region of Grim lying outside of the IBM motif, which they termed the GH3 domain. Grim lacking this domain was grossly defective in apoptotic induction; and intriguingly, wild-type (but not GH3 domain-deleted) Grim protein, localized to mitochondria. However, it was not clear why mitochondrial

localization of Grim in the fly system might be important. These observations, coupled with the residual apoptotic induction by Reaper-(16–65), prompted us to explore the possible functions of Reaper lying within the C-terminal 50 amino acids of the protein. We report here that a GH3-like domain of Reaper is responsible for localizing Reaper to mitochondria. Surprisingly, we have discovered that a mutant Reaper protein lacking the GH3 domain is unable to induce IAP ubiquitination and destruction, whereas it maintains the ability to inhibit IAP-caspase interactions. This mutant protein is partially defective in apoptotic induction. We have found that restoration of mitochondrial localization achieved by appending the tail of either Bcl-x_L or HID to the GH3 mutant Reaper protein is sufficient to restore both cell killing and IAP destruction. These data demonstrate the importance of mitochondrial localization in cell killing by Reaper, highlight the importance of IAP destruction (as opposed to simple inhibition) for Reaper-induced apoptosis, and provide an unexpected link between mitochondrial localization and the ability of Reaper to promote IAP destruction.

EXPERIMENTAL PROCEDURES

S2 Cell Culture—Details of S2 cell culture and transfection were described previously (20), but S2 cells were maintained in *Drosophila* serum-free medium supplemented with L-glutamate (Invitrogen) and transfected with Cellfectin (Invitrogen). Generally, 12×10^6 S2 cells grown in T-25 flasks were transfected by mixing 10 μ g of Reaper, Reaper mutant, or SMAC with 1 μ g of EGFP/pCasper (a construct in which EGFP is driven by the constitutive ubiquitin promoter) and 48 μ l Cellfectin. To monitor transfection efficiency, 20% of the transfection mixture was plated with 50 μ M Z-VAD-fmk. The remaining cells were returned to the T-25 flasks. After 24 h, DNA mixtures were removed, and cells were resuspended in fresh serum-free medium (new Z-VAD-fmk was added to the control transfection following the media change). After 8 h, cells were treated with copper sulfate and analyzed after an additional 16 h by flow cytometry; at least 100,000 live cells were counted to determine percent GFP-positive cells. A minimum of four transfections per construct were analyzed per experiment; error bars represent S.D. values across all analyses, normalized for transfection efficiency. Steady-state DIAP1 levels in the presence of Reaper, Reaper mutants, and SMAC were determined by transfection as described above, but in the presence of constant 50 μ M Z-VAD-fmk. 24 h after the media change, 1.5×10^6 GFP-positive S2 cells were collected by FACS, subjected to an additional 12 h of 700 μ M copper sulfate treatment (in the presence of Z-VAD-fmk), and lysed in 1% Nonidet P-40. Protein concentrations were determined by the Bradford assay (Bio-Rad) and normalized. Lysates were briefly sonicated with SDS-PAGE buffer and analyzed by immunoblotting with anti-DIAP1 antibody (a gift from Dr. Bruce A. Hay). Equal protein loading was confirmed by immunoblotting with anti-tubulin antibody.

Live S2 Cell Confocal Fluorescence Microscopy—10 μ g of Reaper-GFP or 15 μ g of RFP-DIAP1 was transfected into *Drosophila* cells in the presence of 50 μ M Z-VAD-fmk. 36 h after transfection, 700 μ M CuSO₄ was added. Where indicated, cells were incubated with MitoTracker Red (Molecular Probes, Inc.) for 10 min and then returned to fresh medium (with copper and Z-VAD-fmk). Live cells in chambered coverglass trays (Lab-Tek) were examined by confocal fluorescence microscopy.

Xenopus Extract Preparation, DEVD Assay, DIAP1 Stability Assay, and Scythe Binding—Preparation of cleared (ultracentrifuged S extract) interphase egg extracts was carried out as described (15). These extracts were supplemented with 2 mM ATP, 5 mg/ml creatine kinase, and 20 mM phosphocreatine. DIAP1 stability assays (15) were performed as described previously.

Reaper Peptide—Reaper and Reaper deleted for the GH3 domain (Δ GH3 Reaper) generated as full-length untagged synthetic peptides were prepared as described previously (20).

Mutant Construction—Reaper mutants were constructed by overlap PCR and were subcloned into the EcoRI/BamHI sites of pRmHa-3 for analysis in insect cells, into the NcoI/HindIII sites of pGEX-KG for bacterial expression, into the BamHI/NotI sites of pEBB for expression in human cells, and into the BamHI/XbaI sites of pGALL-HIS3 (12) for expression in *Saccharomyces cerevisiae*. RFP-DIAP1 was constructed by subcloning RFP upstream of DIAP1 in pRmHa-3. For fluorescence-

based localizations, overlap PCR was used to subclone EGFP in-frame with and downstream of the *reaper* sequence. In some instances (*i.e.* with GFP-XLE and HIDC-GFP), an additional overlap PCR step was required to append the hydrophobic tail of Bcl-x_L (XLE for x_L tail at the end) or HID (HIDC for HID C-terminal tail) to the 3'-end of Δ GH3 Reaper-GFP.

Reaper-IAP Interaction Yeast Screen—This screen was performed as described previously (12), except that wild-type and mutant Reaper proteins were compared in their relative abilities to restore lethality to yeast grown on galactose.

Human Cell Culture—Details of human 293T cell culture, transfection, constructs, immunoblotting, affinity precipitation, and pulse-chase analyses were as described (20). Apoptotic induction by *reaper* mutants was assayed visually by cotransfection with GFP and scoring the number of apoptotic figures per field and by quantitative colorimetric assay for cleavage of DEVD-*p*-nitroanilide (BIOMOL Research Labs Inc.). For immunostaining, cells were transfected with *reaper*/pEBB. After 24 h, cells were incubated with MitoTracker Red and fixed on ice with 4% paraformaldehyde, followed by permeabilization with Triton X-100. Fixed and permeabilized cells were blocked in 2% bovine serum albumin and incubated with anti-Reaper antibody (diluted 1:500) labeled with fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody for visualization by immunofluorescence. Hoechst dye was used to visualize DNA.

RESULTS

Previous analyses of the Reaper protein have suggested that regions lying outside of the N-terminal IBM motif contribute to its full apoptotic activity (6, 28). Moreover, sequence alignment of the Reaper and Grim proteins suggested that Reaper might contain a domain similar to the GH3 domain found in Grim (34). We therefore examined more carefully the predicted structure of Reaper using Predict Protein from the Swiss Model server² and 3D-PSSM from the 3D-PSSM web server.³ These analyses predicted a globular protein that would adopt a helical structure over much of its length (from Gln¹⁰ to Thr⁴⁷). When the amino acid sequences contained within this region of Reaper were arrayed on a helical wheel projection, it was evident that residues within the core of Reaper (amino acids 24–41) form an amphipathic helix with hydrophobic residues (Ile²⁴, Leu²⁷, Phe³⁴, Leu³⁵, Val³⁸, and Val³⁹) lying on one face of the helix and hydrophilic residues (Arg²⁶, Glu²⁹, Ser³⁰, Arg³³, Thr³⁷, and Glu⁴¹) lying on the other (Fig. 1A), as had been proposed for the GH3/Trp block region of Grim (34). Using the KINEMAGE program,⁴ these analyses were extended to produce a three-dimensional representation, with C- α atoms plotted to scale, assuming a perfect α -helical arrangement and spacing (Fig. 1B). Note the extended hydrophobic stretch of residues in Reaper between residues 32 and 42; this region is analogous to the GH3 domain of Grim and will hereafter be referred to in this study as the GH3 domain of Reaper.

The GH3 Domain Is Essential for Mitochondrial Localization of Reaper—As the GH3 domain of Grim had been previously implicated in localizing Grim to the mitochondria (34), we speculated that the GH3 domain of Reaper might also serve as a mitochondrial targeting sequence. To first determine whether Reaper localizes to mitochondria, we transfected *Drosophila* S2 cells with a construct encoding wild-type Reaper fused at its C terminus to GFP. Initial examination of the wild-type protein revealed a punctate cytoplasmic pattern that overlapped quite well with MitoTracker Red (Fig. 2A). Note that inclusion of Z-VAD-fmk was necessary to preserve MitoTracker Red staining in the face of Reaper expression, consistent with previous reports of the disruption of *Drosophila* mitochondria by caspases (29). MitoTracker Red staining could also be maintained in Reaper-transfected cells by prior RNA inter-

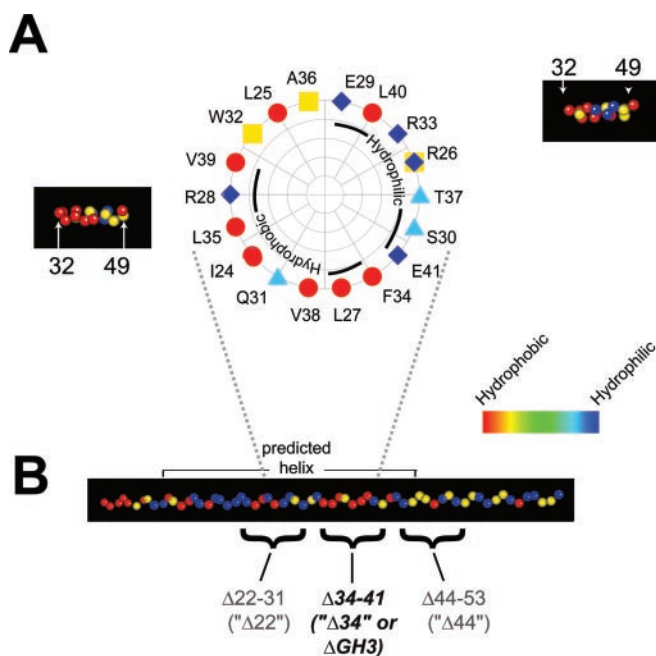


FIG. 1. Identification of a GH3-like amphipathic helix in Reaper. A, helical wheel projection of Reaper residues 24–41. Hydrophobic amino acids are represented by red circles, neutral amino acids by yellow squares, weak hydrophilic amino acids by triangles, and strong hydrophilic amino acids by blue diamonds. Residues are labeled with the single letter amino acid code and position. Note that this region forms an amphipathic helix. Left inset, schematic of C- α atoms of amino acids 32–49 arranged in an ideal α -helix (to scale). C- α atoms are colored as described for the helical wheel projection. Note the hydrophobic surface of this region. Right inset, schematic showing the opposite, hydrophilic face of amino acids 32–49. B, three-dimensional representation of Reaper with the extended hydrophobic stretch shown. The deletion mutants used in this study are also noted.

ference ablation of DARK (data not shown).

We confirmed that Reaper-GFP staining in the mitochondria in live S2 cells reflected the true localization of Reaper by observing that immunostained Reaper expressed in fixed human cells also co-localized with MitoTracker Red (Fig. 2B); GST-tagged recombinant Reaper added to *Xenopus* egg extracts could also be copurified with mitochondria (data not shown). Moreover, Reaper-GFP was as potent as untagged Reaper in apoptotic induction in S2 cells (data not shown).

To determine whether the GH3 domain is required for the mitochondrial localization of Reaper, we produced Reaper-GFP fusions bearing deletions of the GH3 domain (deleted for amino acids 34–41) or bearing similarly sized deletions flanking the GH3 domain (deletions spanning amino acids 22–31 or 44–53). As predicted by the GH3 domain homology, we found the Reaper GH3 domain (but not the flanking regions) to be essential for Reaper mitochondrial localization. However, unlike Grim, which was reported to adopt a punctate non-mitochondrial pattern when the GH3 domain was deleted (34), deletion of the Reaper GH3 domain converted Reaper to a diffusely cytoplasmic protein (Fig. 3A). Moreover, although wild-type Reaper appeared to be excluded from nuclei, Δ GH3 Reaper could be found throughout the cytoplasm and nucleus.

The Reaper GH3 Domain Is an Autonomous Mitochondrial Localization Sequence—Although the GH3 domain is essential for mitochondrial localization, it was not clear whether this region alone serves to target Reaper to the mitochondria. Therefore, to determine whether the Reaper GH3 domain is sufficient to confer mitochondrial localization, we appended Reaper amino acids 29–45 to GFP and examined the subcellular localization of the fusion protein in live S2 cells. As shown

² Available at dodo.cpmc.columbia.edu/predictprotein.

³ Available at www.bmm.icnet.uk/.

⁴ Available at kinemage.biochem.duke.edu.

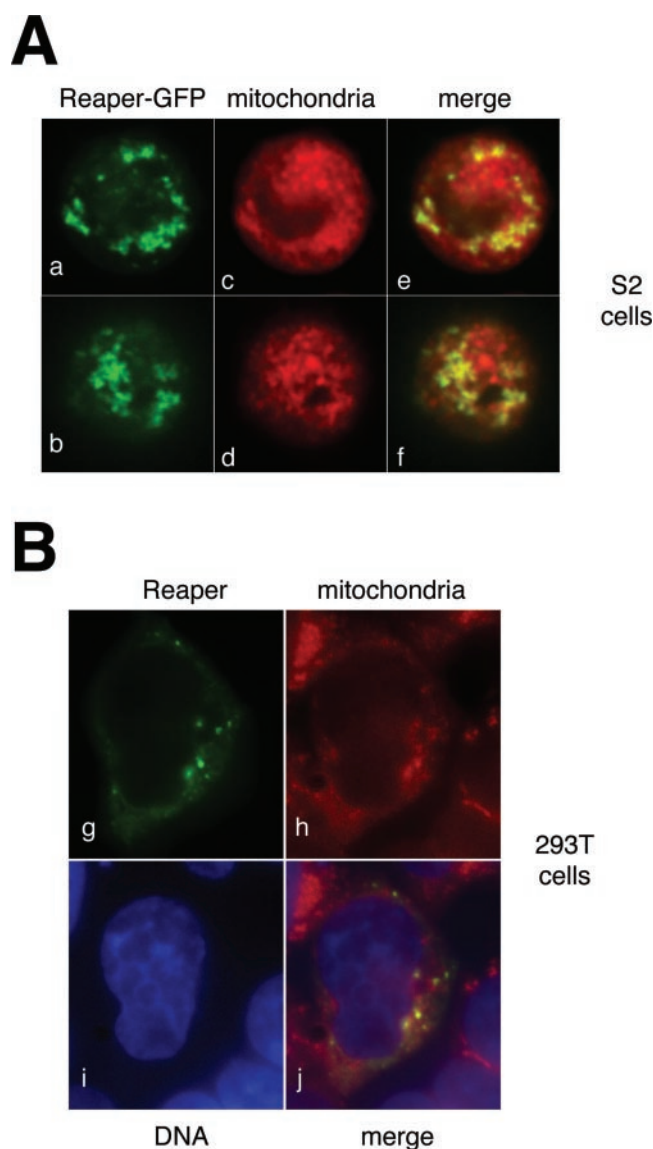


FIG. 2. Reaper localizes to mitochondria in *Drosophila* S2 cells. *A*, the localization of a C-terminal GFP fusion of Reaper was examined in *Drosophila* S2 cells. Two representative cells are shown with GFP fluorescence in *green* and MitoTracker Red in *red*, followed by the overlay. *B*, untagged Reaper was transfected into human 293T cells, which were fixed; permeabilized; and subjected to immunofluorescence using polyclonal anti-Reaper antibody (*panel g*), MitoTracker Red (*panel h*), and Hoechst dye (*panel i*). *Panel j* shows the human cell three-color merge.

in Fig. 3*B*, the GH3 domain fused to GFP displayed the same mitochondrial localization as wild-type Reaper. Thus, it appears that the GH3 domain is both necessary and sufficient to promote the mitochondrial association of Reaper.

Reaper GH3 Mutants Are Not Defective in Either IAP Inhibition or IAP Co-localization—Although Reaper has been shown to induce mitochondrial cytochrome *c* release in heterologous systems, it is not clear what role, if any, mitochondria might play in Reaper-induced fly cell apoptosis, particularly since the involvement of cytochrome *c* in activation of the fly apoptosome is controversial. Since ablation of DIAP1 is sufficient to cause fly cell apoptosis, and Reaper can both inhibit IAP function and induce IAP destruction, it was attractive to speculate that the defective mitochondrial localization of the GH3 mutant could in some way impact Reaper-IAP dynamics. Because short peptides encoding IBM-like motifs similar to the Reaper N terminus are sufficient to disrupt IAP-caspase inter-

actions *in vitro*, we did not think it likely that GH3 mutants would be deficient in the direct inhibition of IAP activity. However, to address this issue, we took advantage of a genetic screen developed by Hay and co-workers (12) in which the *Drosophila* executioner caspases drICE and DCP-1 were overexpressed in *S. cerevisiae*. Caspase overexpression via the potent *GAL* promoter is lethal to yeast, and viability is rescued by *CUP1*-driven expression of DIAP1. Expression of Reaper (also driven by the *GAL* promoter) de-suppresses caspase activity, thereby killing the yeast. Using this assay, we tested wild-type reaper, reaper deleted for the GH3 domain (deletion of amino acids 34–41), and reaper bearing point mutations within the GH3 domain for their abilities to release drICE or DCP-1 from DIAP1. As shown in Fig. 4 (*A* and *B*), all Reaper proteins examined in this assay were able to relieve IAP-mediated caspase inhibition. Moreover, the Reaper proteins were not themselves toxic to yeast since *GAL*-driven Reaper did not impair growth of yeast on galactose *versus* dextrose when yeast were transformed with *GAL*-driven Reaper alone (Fig. 4*C*). These observations demonstrate that the GH3 mutations do not interfere with the ability of Reaper to inhibit IAP-mediated caspase inhibition. Consistent with these data, when GH3 mutants were cotransfected with DIAP1 into tissue culture cells, they co-immunoprecipitated with DIAP1 as well as wild-type Reaper, suggesting that they have the potential to bind and inhibit DIAP1 function (see Fig. 6*A*).

Although the Reaper GH3 mutants retained their intrinsic ability to inhibit IAP function, it was possible that these mutants would not co-localize with DIAP1 in an intact fly cell (as opposed to a tissue culture cell lysate or yeast cell) due to perturbation of Reaper mitochondrial targeting. We found that wild-type Reaper-GFP and RFP-DIAP1 coexpressed in S2 cells co-localized in a punctate perinuclear pattern (Fig. 5*A*) very similar to that described by Miller and co-workers (35). Intriguingly, although GH3 domain-deleted Reaper left the mitochondria, DIAP1 traveled with it, adopting a similarly diffuse cytoplasmic pattern, albeit with nuclear exclusion (Fig. 5*B*). (This is probably because unbound Reaper-GFP is small enough to diffuse through the nuclear pores, whereas RFP-DIAP1 or the DIAP1-Reaper complex is not.) Therefore, although the correct subcellular localization of the Reaper GH3 mutant is disrupted, the IAP likely remains co-localized with it in intact cells.

GH3 Mutant Reaper Protein Cannot Destabilize IAPs—Although the Δ GH3 mutant retained the ability to displace caspases from DIAP1, it remained possible that the mutant was defective in some other IAP-mediated function. We have reported previously that IAPs can ubiquitinate Reaper and that Reaper can stimulate IAP degradation (15, 20). We would predict that a Reaper mutant either particularly susceptible to IAP-mediated degradation or unable to promote IAP self-destruction would be a poor apoptotic inducer. In examining our Reaper mutants, we performed a previously described fluorescence stability assay (20) in *Drosophila* S2 cells and found that all proteins (when assayed 12 h post-transfection to ensure that endogenous DIAP1 was still present) had half-lives similar to that of wild-type Reaper (data not shown). Moreover, as we had shown previously that Reaper undergoes DIAP1-mediated ubiquitination (20), we examined the Reaper deletion mutants to see if removal of the GH3 domain prevents ubiquitin conjugation to Reaper. As expected, the GH3 domain-deleted protein was similar to wild-type Reaper in its susceptibility to DIAP1-mediated ubiquitination (Fig. 6*A*).

Acting in opposition to IAP-mediated degradation of Reaper is the Reaper-stimulated destruction of the IAPs. We sought to determine whether GH3 mutant proteins might be defective in this activity. Using an *in vitro* reconstitution assay we reported

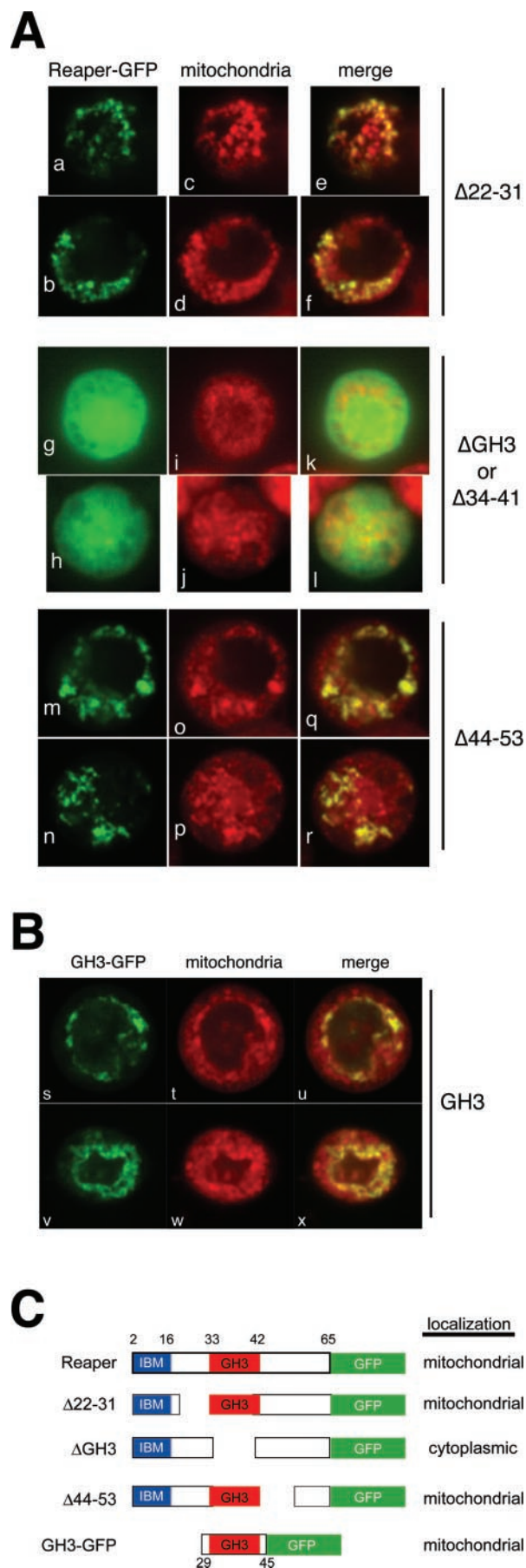


FIG. 3. The Reaper GH3 domain is necessary and sufficient for mitochondrial localization. A, Reaper-GFP mutants were transfected into *Drosophila* S2 cells that were treated as described in the

previously (15), a synthetic Reaper peptide missing its GH3 domain, but containing the full remainder of the Reaper coding sequence, was unable to effect DIAP1 destruction like its wild-type counterpart in *Xenopus* egg cytosol (Fig. 6B). Furthermore, an additional centrifugation of the cytosol used in this *in vitro* assay at $200,000 \times g$ to remove contaminating membranes (Fig. 6B, *Double Spun*) (see “Discussion”) rendered even the wild-type peptide unable to effect DIAP1 destruction.

We next examined the effect of the Reaper GH3 domain on the steady-state level of DIAP1 in *Drosophila* S2 cells. For this purpose, wild-type or Δ GH3 reaper driven by the metallothionein promoter was transfected into S2 cells along with GFP in the presence of Z-VAD-fmk and sorted to collect 1.5×10^6 GFP-positive cells. Wild-type and mutant Reaper proteins were then induced with copper sulfate and harvested after 24 h. Note that Z-VAD-fmk was clearly effective at inhibiting apoptosis, as all populations (including the vector control) contained equivalent percentages of GFP-positive cells, and no loss of GFP-positive cells was observed. Equivalent amounts of cell lysates were then immunoblotted with anti-DIAP1 antibody. As shown in Fig. 6C, DIAP1 levels were markedly decreased in cells transfected with wild-type Reaper compared with cells transfected with vector alone. In contrast, the levels of DIAP1 were comparable in Δ GH3 Reaper- and vector-transfected cells, suggesting that Δ GH3 Reaper is unable to promote IAP destruction.

To verify the steady-state DIAP1 results, we examined the ability of wild-type and Δ GH3 Reaper to induce IAP instability in human 293T cells (where it is less technically challenging to do pulse-chase analysis compared with S2 cells). As we reported previously (15), and as shown for DIAP1, Reaper was quite effective in destabilizing XIAP. Moreover, Δ GH3 Reaper was also defective in killing human cells in culture (data not shown). Accordingly, we performed pulse-chase analysis on human XIAP in the presence of Reaper mutants. These results confirmed that the Δ GH3 mutant is defective in promoting IAP destabilization (Fig. 6D).

GH3 Domain-deleted Reaper Is a SMAC-like Molecule— Given the shared ability of Reaper and the mammalian SMAC protein to inhibit IAP function, we wished to determine whether SMAC, which lacks any obvious GH3 domain-like sequence, would be able to destabilize IAPs. We found that expression of processed SMAC (cytoplasmically expressed with an exposed N terminus) did not induce IAP destabilization in S2 cells (Fig. 7A). Moreover, processed SMAC or a peptide of only its IBM domain was also unable to destabilize DIAP1 in *Xenopus* egg extracts (Fig. 7B). Thus, Δ GH3 Reaper is, in effect, an SMAC-like molecule: it can still bind to IAPs and compete for BIR domain binding to promote caspase release, but it has lost the ability to bring about RING domain-mediated IAP destruction.

It has been hypothesized that both IAP inhibition and destabilization contribute to full apoptotic induction by Reaper in fly cells (13–18, 36). Consistent with this suggestion, we found that the GH3 mutant Reaper protein was partially defective in apoptotic induction. Specifically, we transfected *Drosophila* S2 cells with wild-type Reaper, Δ GH3 Reaper, or control deletion mutants, along with a plasmid encoding GFP (at one-tenth the level of Reaper DNA). The percentage of GFP-positive cells was

legend to Fig. 2. Shown are two cells, each transfected with Δ 22–31 Reaper-GFP (panels a–f), Δ GH3 Reaper-GFP (deletion of amino acids 34–41; panels g–l), and Δ 44–53 Reaper-GFP (panels m–r). B, amino acids 29–45 of Reaper were fused to GFP and transfected into *Drosophila* S2 cells that were treated as described in the legend to Fig. 2. C, shown is a schematic of the Reaper deletions made and their respective localizations.

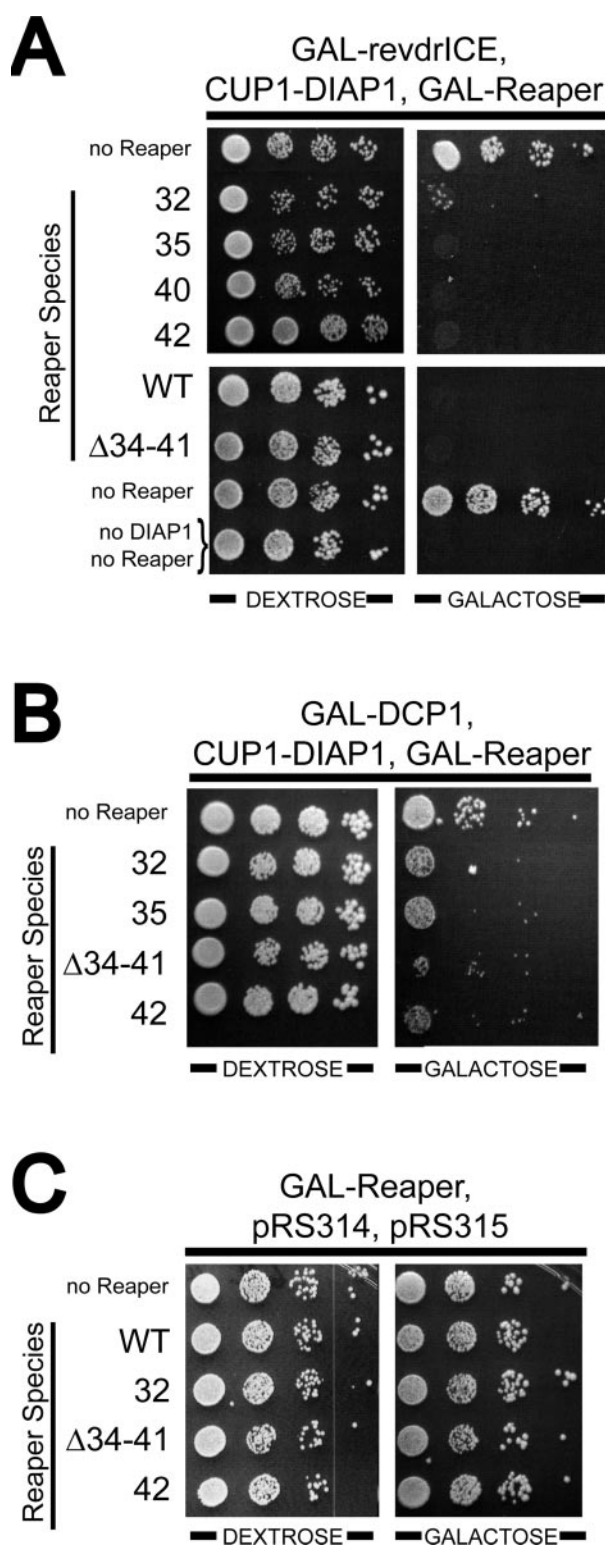


FIG. 4. Reaper GH3 mutants retain IAP inhibitory functions. *A*, GAL-driven wild-type (WT) or mutant reaper proteins were transformed into *S. cerevisiae* strain W303 α together with drICE, also under GAL control, and DIAP1, under the control of the CUP1 promoter. Dilution series were made of each mutant, and replicas were plated onto either dextrose-selective (left panels) or galactose-selective (right panels) medium. Note that revdrICE refers to the drICE in which the p10 subunit of drICE was cloned N terminal to the prodomain and the p20 subunit to obtain constitutive activity (12). For point mutants, 32 is W32I/R33L, 35 is L35Q/A36R, 40 is L40Q, and 42 is T42N/L43R. *B*, the same assay as described for *A* was carried out, but with GAL-DCP1 in place of GAL-drICE. *C*, *S. cerevisiae* strain W303 α was transformed with wild-type or mutant reaper genes (with vector controls in place of caspase and DIAP1).

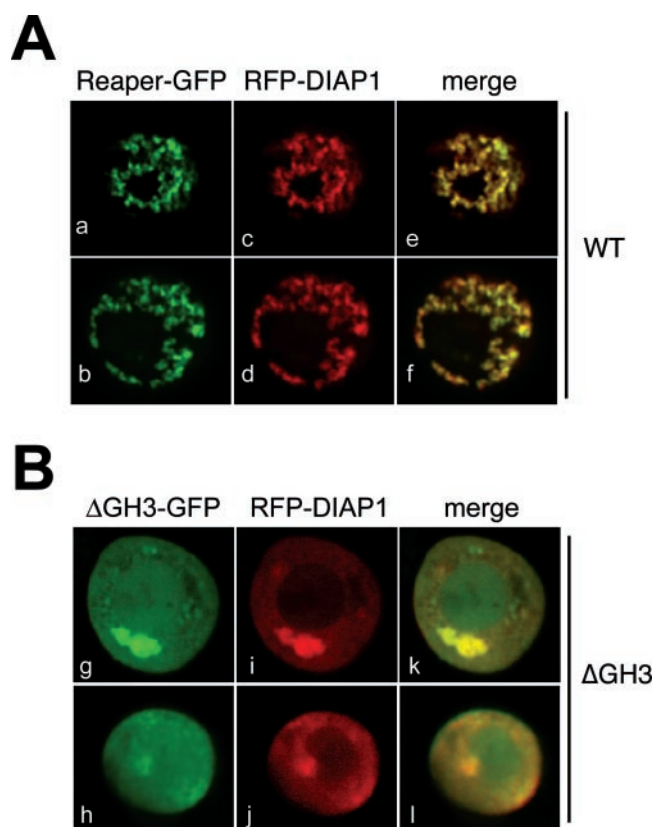


FIG. 5. Reaper GH3 mutants co-localize with DIAP1. Wild-type Reaper-GFP (WT; *A*) or Δ GH3 Reaper-GFP (*B*) was cotransfected into S2 cells with RFP-DIAP1 in the presence of Z-VAD-fmk. Two-color confocal fluorescence microscopy of live S2 cells was performed as described under "Experimental Procedures." Reaper-GFP fluorescence is shown in green, and RFP-DIAP1 is shown in red. Merges are shown in panels e, f, k, and l. Note the exclusion of DIAP1 from the nucleus (panels i and j), whereas Δ GH3 Reaper is found throughout the cell (panels g and h). Wild-type Reaper and DIAP1 formed a punctate perinuclear pattern.

determined by FACS analysis and normalized for transfection efficiency. As shown in Fig. 7C, the GH3 domain deletion mutant was impaired in inducing S2 cell death, whereas mutants bearing similarly sized deletions on either side of the GH3 domain were as lethal as wild-type Reaper. Interestingly, SMAC was also a less potent inducer of S2 cell death than full-length Reaper, consistent with the notion that IAP inhibition alone is not as effective as the combination of inhibition and induced degradation in promoting caspase activation (data not shown). It should also be noted that changing all of the lysines in the GH3 mutant to arginine (which we have shown previously both stabilizes and enhances the biological activity of wild-type Reaper) did not rectify the apoptotic deficiencies of this mutant, consistent with the idea that increased susceptibility to IAP-mediated degradation does not underlie the impaired activity of the GH3 mutant (Fig. 7D). Rather, we hypothesize that the inability to promote IAP destabilization renders the GH3 mutant less active than the wild-type protein.

Restoring Mitochondrial Localization Restores Δ GH3 Reaper-mediated IAP Destabilization and Apoptosis—The experiments described above raised the issue of whether mislocalization of the Δ GH3 mutant is causally related to its inability to promote IAP degradation. Moreover, we wished to determine whether restoring mitochondrial localization would restore full apoptotic activity to the Δ GH3 mutant. To bring the Δ GH3 mutant back to the mitochondria, we took advantage of a recent study identifying the residues in mammalian Bcl-x_L that

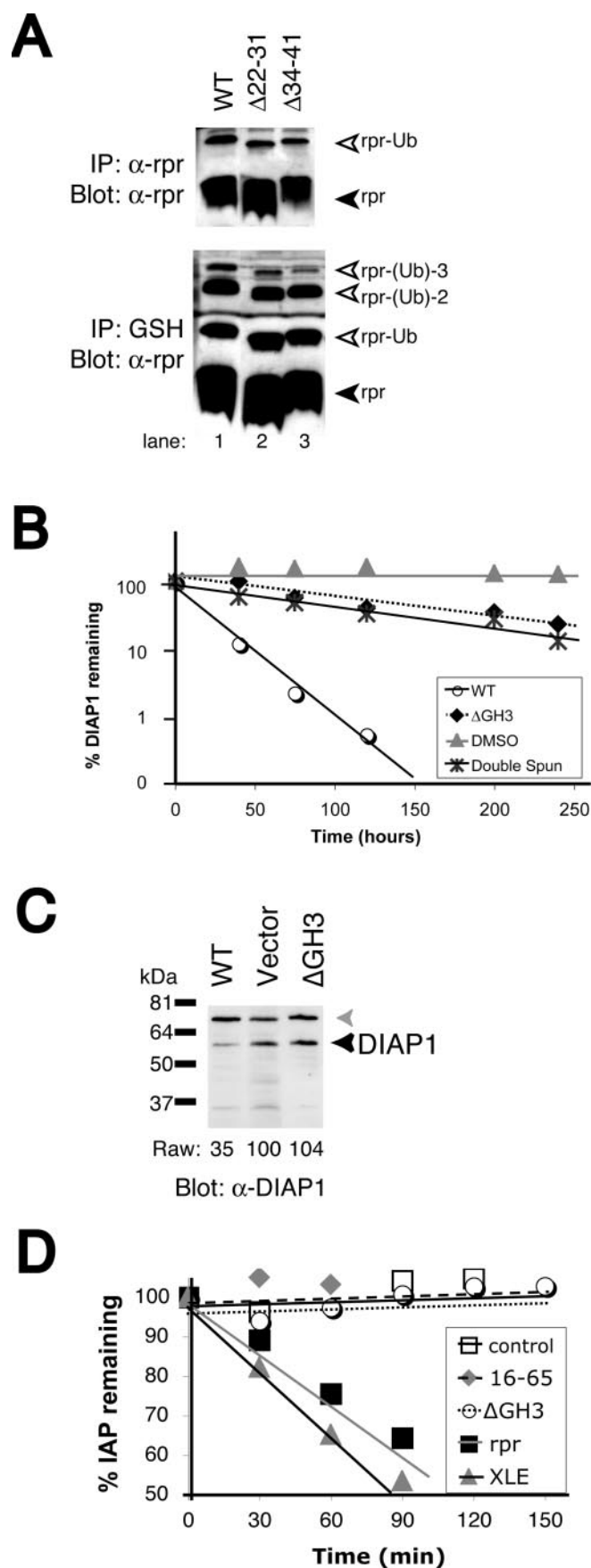


FIG. 6. Reaper GH3 mutants cannot destabilize DIAP1. A, 293T cells were cotransfected with Reaper (*rpr*) constructs and GST-DIAP1. Lysates were immunoprecipitated (IP) using either polyclonal anti-Reaper antiserum or glutathione beads. All Reaper proteins quantitatively precipitated with and were also ubiquitinated by GST-DIAP1

confer its mitochondrial localization (37). Amino acids 213–233 of Bcl-x_L form the hydrophobic tail believed to anchor Bcl-x_L in the outer mitochondrial membrane. These residues were either inserted into the *reaper* sequence in place of the GH3 domain (referred to as XLM for x_L tail in the middle) or fused to the end of the Δ GH3 mutant (referred to as XLE for x_L tail at the end). Additional variants were constructed fusing GFP to these proteins, and their localizations were ascertained by confocal microscopy of live cells. As shown in Fig. 8A, although the XLM chimera failed to localize to the mitochondria, displaying, instead, a largely diffuse staining pattern (with some punctate non-mitochondrial concentrations), the C-terminal fusion of the Bcl-x_L residues in the XLE chimera did indeed restore mitochondrial localization of Reaper.

The killing activities of the XLE and XLM mutant Reaper proteins were determined using the FACS-based assay described above. As shown in Fig. 8C, the apoptotic activity of the XLE mutant was very similar to that of wild-type Reaper, whereas the XLM mutant was as defective as the parent GH3 mutant in cell killing. Indeed, we assayed this panel of mutants at a range of copper concentrations to produce varying levels of protein within the cells. At all concentrations tested, even as high as 700 μ M CuSO₄, the Δ GH3 and XLM *reaper*-transfected cells exhibited a survival rate 3.5-fold that of the XLE or wild-type *reaper*-transfected S2 cells (Fig. 8C), whereas XLE and wild-type Reaper were identical. These data demonstrate that mitochondrial localization is sufficient to complement the GH3 mutant defect.

Although the Bcl-x_L tail restored apoptotic function and mitochondrial localization to the Δ GH3 mutant, it remained formally possible that the mitochondrial localization conferred upon the Δ GH3 protein a pro-apoptotic function distinct from the original IAP destabilization defect. Note, however, that all XLE-associated deaths are IAP-dependent, as deletion of the Reaper IBM motif from the XLE mutant abrogated its killing activity (Fig. 8D). To measure the effect of the XLE protein on IAP steady-state levels and half-lives, we transfected Δ GH3, wild-type, or XLE *reaper* into S2 cells with GFP and isolated GFP-positive cells by FACS (in the presence of Z-VAD-fmk, as described above) (Fig. 7A). As shown in Fig. 8E, the Bcl-x_L mitochondrion-anchoring tail appended to the GH3 domain-deleted protein (XLE) completely restored the ability of Reaper to promote IAP degradation. Therefore, restoring the mitochondrial targeting function is sufficient to restore IAP loss. To confirm that this loss is due to increased IAP degradation, we performed pulse-chase analysis on XIAP in Reaper-transfected 293T cells (Fig. 6D). Consistent with the loss of DIAP1 in S2 cells, the XLE protein markedly shortened the XIAP half-life, whereas the Δ GH3 mutant did not (Fig. 6D). These data imply

(open arrowheads). Ub, ubiquitin. B, the synthetic wild-type (WT) or Δ GH3 Reaper peptide was incubated in *Xenopus* egg cytosol, and DIAP1 stability was assayed. In addition, in one example, the ability of the wild-type peptide to effect DIAP1 destruction was determined in cytosol that was spun for an additional 15 min at 200,000 \times g to pellet contaminating membranes (Double Spun). DMSO, dimethyl sulfoxide. C, wild-type Reaper, Δ GH3 Reaper, or vector alone was transfected into S2 cells in the presence of Z-VAD-fmk. After 26 h, GFP-positive cells were isolated by FACS and induced with copper sulfate for an additional 24 h. Cell lysates were analyzed by Western blotting for endogenous DIAP1 levels. Equal loading was confirmed by immunoblotting with anti-tubulin antibody (data not shown). The parent DIAP1 species (black arrowhead) was quantified by densitometry and normalized to a nonspecific background band (gray arrowhead). D, Reaper or Reaper mutants (16–65 refers to Reaper lacking amino acids 1–15; for XLE, see Fig. 8) were transfected with GST-XIAP into human 293T cells, which were then subjected to pulse-chase analysis for XIAP. At the indicated times, cells were harvested and processed by SDS-PAGE and autoradiography and then quantified.

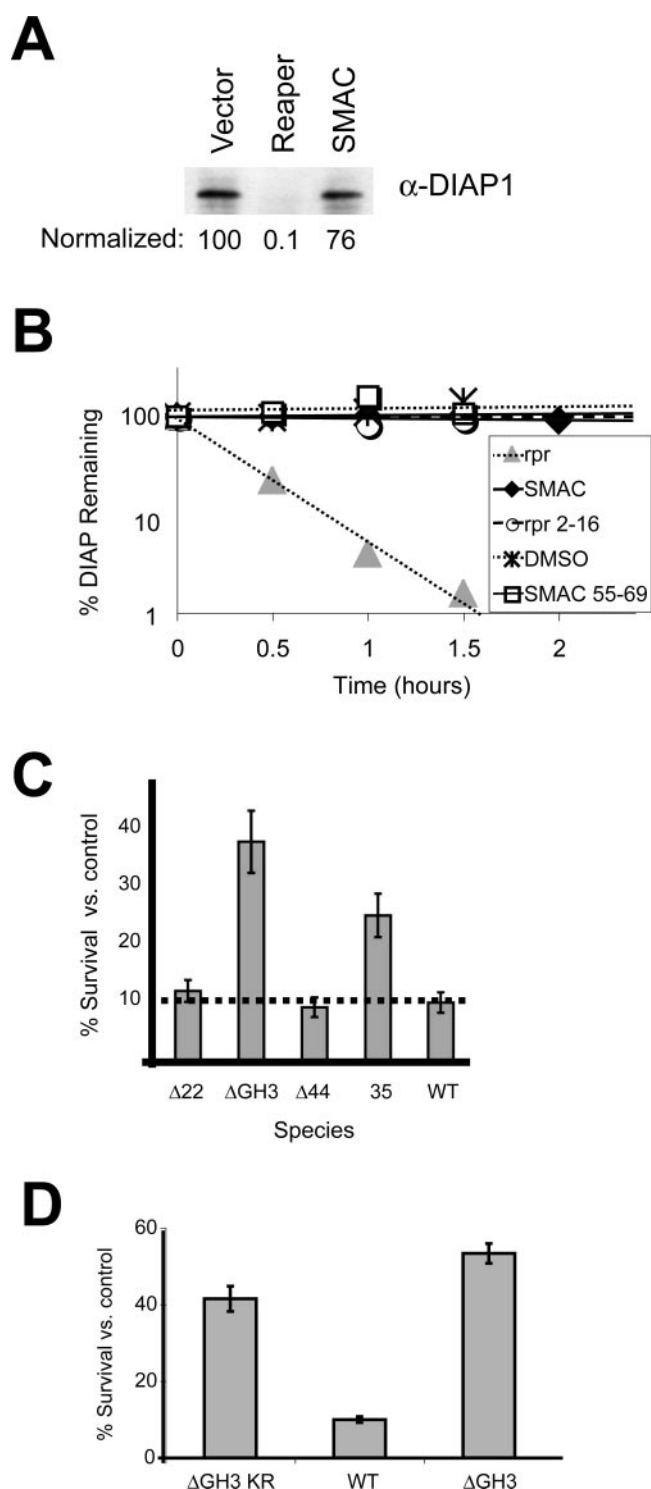


FIG. 7. SMAC does not destabilize IAPs and GH3 mutant Reaper is a weak apoptotic inducer. A, Reaper, SMAC, or the vector control was transfected into S2 cells in the presence of Z-VAD-fmk. After 36 h, GFP-positive cells were isolated by FACS and then induced with copper sulfate for an additional 24 h. Cell lysates were analyzed by Western blotting for endogenous DIAP1 levels. Equal loading was confirmed by blotting with anti-tubulin antibody (data not shown). The results from quantification by densitometry are also shown. B, full-length Reaper (*rpr*) peptide, the synthetic Reaper IBM domain (*rpr* 2–16), the synthetic SMAC IBM domain (*smac* 55–69), the dimethyl sulfoxide (*DMSO*) vehicle, or recombinant active $\Delta 2$ –55 SMAC (*smac*) was mixed with radiolabeled DIAP1 and incubated in *Xenopus* egg cytosol (not double spun). The resulting radiolabeled protein levels were analyzed by autoradiography and quantified by densitometry. C, *Drosophila* S2 cells were transfected with wild-type (*WT*) or mutant Reaper constructs along with GFP as a transfection marker. Cells surviving after a 12-h induction with 7 μ M copper sulfate were quantified by flow

cytometry. Equal transfection efficiency was verified for all constructs by transfecting a sample of each in the presence of Z-VAD-fmk. Error bars represent S.D. values of four independent trials. $\Delta 22$, $\Delta 22$ –31 Reaper-GFP; $\Delta 34$, $\Delta GH3$ Reaper-GFP (deletion of amino acids 34–41); $\Delta 44$, $\Delta 44$ –53 Reaper-GFP; 35, L35Q/A36R Reaper. D, wild-type Reaper, $\Delta GH3$ Reaper, or a GH3 domain deletion mutant in which all lysines were changed to arginine ($\Delta GH3$ KR) was analyzed by the S2 survival assay described for C.

that mitochondrial targeting is important for Reaper-induced IAP degradation in intact cells.

The C-terminal Tail of HID Can Restore the Ability of $\Delta GH3$ Reaper to Induce Apoptosis—The pro-apoptotic protein HID has also been described as a mitochondrially localized protein (5). Although HID does not contain an obvious GH3 domain, we were interested to find that the HID C-terminal tail is notably similar to the Bcl-x_L C-terminal tail (Fig. 8F). In fact, this observation may account for the reported ability of Bcl-x_L to displace HID from mitochondria (5), perhaps by competing for similar mitochondrial docking sites. Interestingly, loss-of-function mutants in *hid* in which this portion of HID alone has been truncated have been identified genetically (38). Unfortunately, analysis of *hid* function via overexpression of the wild-type protein is complicated by its post-translational regulation by *Drosophila* Ras (39, 40). To address the potential role of this mitochondrial targeting sequence in another manner, we wondered if we might convert the $\Delta GH3$ Reaper protein into an effective apoptotic inducer by appending the HID tail to the end of the Reaper sequence (as the Reaper-HID chimera lacks the requisite phosphorylation sites for MAPK regulation) (Fig. 8B). Indeed, appending the HID tail to Reaper lacking the GH3 domain promoted its re-localization to mitochondria (Fig. 8G) and the induction of both apoptosis (Fig. 8H) and DIAP1 destruction (Fig. 8I). These data strongly support our conclusion that Reaper-induced DIAP1 destruction requires mitochondrial localization.

DISCUSSION

Reaper has been shown to have dual effects on IAPs, both displacing them from caspases and inducing their degradation. In previous efforts to analyze the contributions of various regions of Reaper to its biological function, it was discovered that the ability to inhibit IAP function resides in the extreme N terminus of the protein. However, this region did not appear to be sufficient for IAP destabilization, implicating additional regions of Reaper in the process. In this report, we have identified a region of Reaper, lying between amino acids 34 and 41, termed the GH3 domain of Reaper, that proved to be critical for both localizing Reaper to the mitochondria and promoting IAP destabilization. Surprisingly, restoration of mitochondrial localization by appending the tail of either Bcl-x_L or HID to a GH3 domain-deleted Reaper mutant also restored IAP instability, suggesting that mitochondrial localization may be important for this biological function of Reaper. In addition, the impaired apoptotic activity of the $\Delta GH3$ mutant suggests that IAP destabilization, and not just IAP inhibition, is important for potent cell killing by Reaper.

IBM Motif-containing Proteins Do Not Necessarily Destabilize IAPs—As we reported previously, the mutant Reaper-(16–65) protein, which cannot bind IAPs, is also unable to induce IAP ubiquitination (15). Thus, the IBM domain is required for Reaper to stimulate IAP destruction. However, an IBM domain alone does not appear to be sufficient to induce IAP degradation, as the mammalian protein SMAC, which is unrelated to Reaper outside of the IBM domain, was unable to induce DIAP1 degradation in either fly cells or *Xenopus* egg extracts, even when expressed in its truncated, “activated” form. In

cytometry. Equal transfection efficiency was verified for all constructs by transfecting a sample of each in the presence of Z-VAD-fmk. Error bars represent S.D. values of four independent trials. $\Delta 22$, $\Delta 22$ –31 Reaper-GFP; $\Delta 34$, $\Delta GH3$ Reaper-GFP (deletion of amino acids 34–41); $\Delta 44$, $\Delta 44$ –53 Reaper-GFP; 35, L35Q/A36R Reaper. D, wild-type Reaper, $\Delta GH3$ Reaper, or a GH3 domain deletion mutant in which all lysines were changed to arginine ($\Delta GH3$ KR) was analyzed by the S2 survival assay described for C.

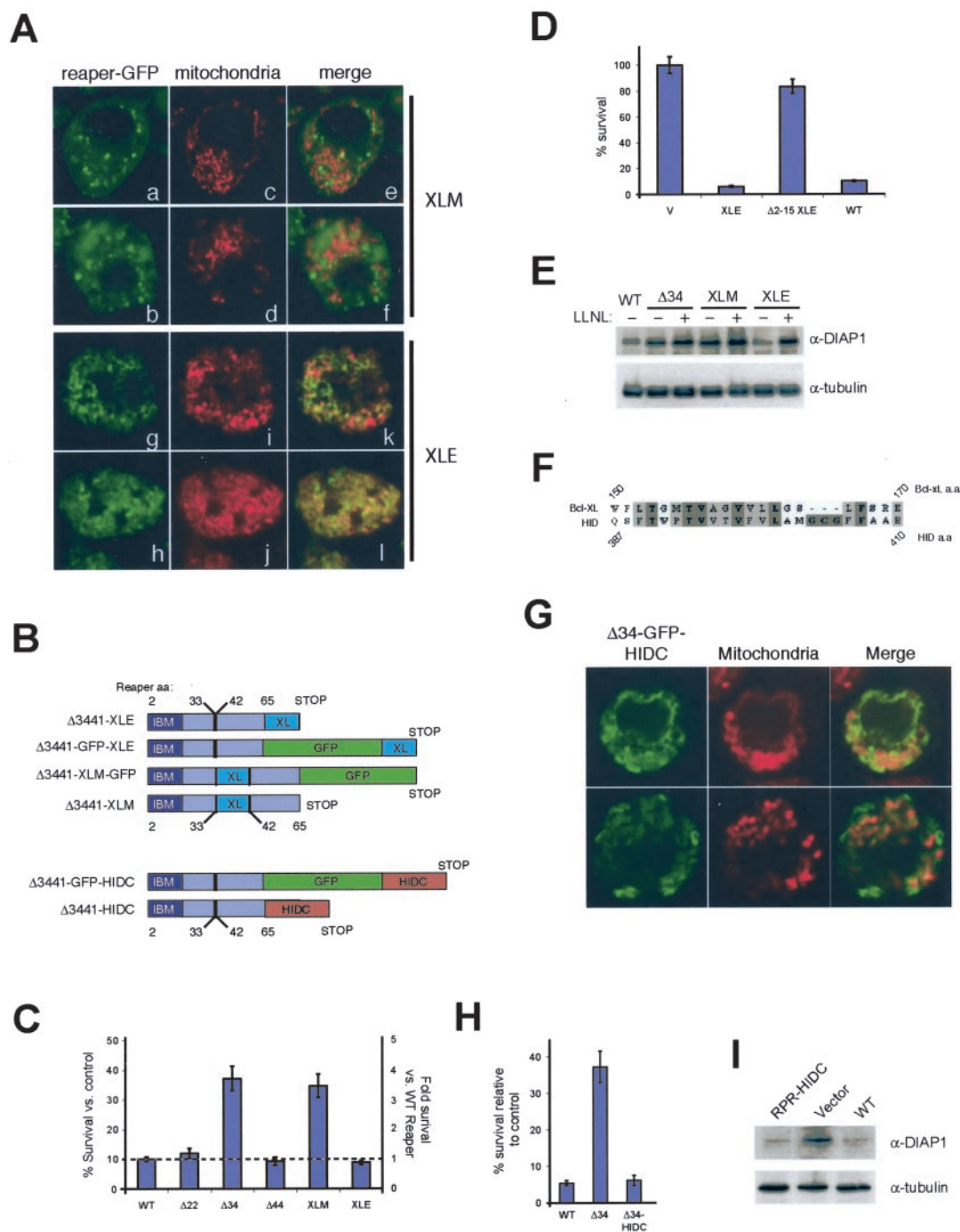


FIG. 8. Correcting mitochondrial localization of the Reaper GH3 mutant restores apoptotic activity. Reaper lacking the GH3 domain was fused to amino acids 213–233 of human Bcl-x_L (to make XLE), or those 21 amino acids from Bcl-x_L (minus the stop codon) were substituted for the GH3 residues of Reaper (to make XLM). In addition, the Bcl-x_L region was fused to the C terminus of ΔGH3 Reaper-GFP (to make GFP-XLE). GFP-tagged XLM was also constructed. **A**, GFP-XLE or XLM-GFP was transfected into *Drosophila* S2 cells and visualized as described in the legend to Fig. 3. *aa*, amino acids; Δ34, ΔGH3 Reaper (lacking amino acids 34–41); XL, Bcl-x_L tail. **B**, shown is a schematic of the constructs made. **C**, the *Drosophila* S2 cell survival assay was performed using all deletion mutants, expressed with 0.7 μM copper sulfate (percent survival versus control). In addition, a range of copper concentrations was used (from 0.7 to 700 μM) to induce varying levels of protein expression. For each mutant, at each concentration, survival (as measured by percent GFP-positive cells) was compared with survival in the presence of wild-type (WT) Reaper (*Fold survival vs. WT Reaper*). Note that survival plots are identical across a wide range of copper concentrations. *Error bars* represent S.D. values of results across six concentrations of copper sulfate (per construct). Δ22, Δ22–31 Reaper-GFP; Δ44, Δ44–53 Reaper-GFP; **D**, the *Drosophila* S2 survival assay was carried out using wild-type Reaper, XLE, XLE missing its IBM domain (Δ2–15 XLE), or vector in the presence of 7 μM copper sulfate. **E**, steady-state DIAP1 levels were determined in the presence of wild-type Reaper, ΔGH3 Reaper, XLE, or XLM (all with Z-VAD-fmk) in *Drosophila* S2 cells as described in the legend to Fig. 1B. In this experiment, *N*-acetyl-Leu-Leu-Nle-CHO (LLNL) was added (or not) to verify that destruction of DIAP1 was proteasome-dependent. **F**, shown is an alignment of amino acids 213–233 of Bcl-x_L and amino acids 387–410 of HID. Similarities are *lightly shaded*, whereas identities are *darkly shaded*. **G**, amino acids 387–410 of HID were appended to ΔGH3 Reaper-GFP (Δ34-GFP-HIDC), and its localization was determined in live S2 cells (with Z-VAD-fmk). **H**, the survival assay was carried out with S2 cells in the presence of wild-type Reaper, ΔGH3 Reaper, or ΔGH3 Reaper with amino acids 387–410 of HID fused to its C terminus (Δ34-HIDC) following induction with 7 μM copper sulfate. **I**, shown are the steady-state DIAP1 levels in the presence of wild-type Reaper, vector, or ΔGH3 Reaper-HIDC as described for **E**.

addition, IBM domain-exposed ubiquitin-SMAC (which undergoes cytosolic processing to expose its IBM domain (41)) was unable to induce XIAP instability in human 293T cells, and a peptide consisting of residues 2–16 of Reaper could not destabilize IAPs in *Xenopus* egg extracts.⁵ These data are consistent with a role for other regions of Reaper in promoting IAP degradation.

The GH3 Domain Is a Mitochondrial Localization Sequence—Since deletion of the GH3 domain prevented mitochondrial localization of Reaper, we suspected that it might serve as an autonomous mitochondrial targeting sequence. This was confirmed when we found that the GH3 domain alone could target GFP to mitochondria. It is not yet clear precisely how this domain associates with mitochondria. However, based on sequence alignment, as noted by Claveria *et al.* (34), we suspect that the GH3 domains of Reaper and Grim will be determined to function similarly. It is interesting that the Bcl-x_L tail sufficed to complement the GH3 domain deletion in Reaper, allowing a restoration of apoptosis, given the lack of any overt homology between these two domains. Indeed, a careful examination of the micrographs of native Reaper and Bcl-x_L tail-tagged ΔGH3 Reaper revealed that their “mitochondrial” localization was not precisely identical, in that wild-type Reaper appeared to be diffuse over mitochondrial “spots,” whereas Bcl-x_L tail-tagged ΔGH3 Reaper appeared to “ring” around the mitochondria, consistent with a Bcl-2-like outer membrane localization. Moreover, wild-type Reaper was concentrated largely at perinuclear mitochondria, whereas GFP-XLE was found across all mitochondria. Interestingly, the HID tail, which displays some homology to the Bcl-x_L tail, appeared to be more similar to the XLE protein than to wild-type Reaper, appearing to “ring” around the mitochondria as noted previously for wild-type HID by Steller and co-workers (5). These data might explain their observation that Bcl-x_L could compete HID from mitochondria (5) since HID and Bcl-x_L may share a similar, saturable, mitochondrial docking mechanism. Further biochemical experiments will be necessary to determine whether the Reaper GH3 domain confers binding to a specific mitochondrially localized protein (explaining its perinuclear concentration) or associates with the membrane bilayer via the hydrophobic face of its amphipathic helical region and is selective for perinuclear mitochondria for another, unknown reason.

Mitochondrial Localization and IAP Destabilization—Although it might be argued that the GH3 domain contributes independently to mitochondrial localization and induction of IAP degradation, the fact that both defects can be rectified by restoring mitochondrial localization speaks against this, particularly since mitochondrial re-localization by two different sequences (HID and Bcl-x_L tails) restored both apoptotic induction and IAP destruction. One plausible explanation for a mitochondrial requirement in Reaper-mediated IAP degradation is that Reaper (or Grim or HID) and the targeted IAP must co-localize at the mitochondrion with some cofactor (*e.g.* a ubiquitin carrier protein) required for degradation. This is an attractive explanation, but how then was Reaper-mediated IAP destruction successfully reconstituted in cytosolic extracts of *Xenopus* eggs (or in reticulocyte lysates)? Factors that are spatially separated in an intact cell can be randomly mixed in a cell-free lysate; and thus, it may be that mitochondrial localization is critical for IAP destruction only in the context of an intact cell. It is also plausible that mitochondrial components (or even intact mitochondria) were present as low level contaminants in the cell-free systems assayed. In this regard, it is

interesting to note that recentrifugation of the “purified” *Xenopus* egg cytosol at 200,000 × *g* produced a cytosolic extract unable to support Reaper-stimulated IAP degradation (Fig. 6B). Additionally, at least in our hands, although DIAP1 was slightly unstable in the face of Reaper peptide in reticulocyte lysates, its half-life was extended by at least an order of magnitude (to ~4 h) compared with that in fly cells or *Xenopus* extract (~20 min). It is also possible that mitochondrial localization *in vivo* allows Reaper to achieve a high local concentration, whereas *in vitro* experiments may have employed levels of Reaper sufficiently high to bypass this requirement.

An alternative hypothesis to explain the link between mitochondrial localization and IAP degradation stems from our previously reported data demonstrating that IAPs can ubiquitinate and destroy Reaper (20). According to this hypothesis, ΔGH3 Reaper would not induce IAP destruction because the IAP would “win” the battle, destroying the ΔGH3 protein before Reaper-induced IAP auto-ubiquitination could occur. According to this scenario, the mitochondria would be a “safe zone” wherein Reaper could induce IAP ubiquitination without itself being destroyed. Arguing against this idea is the observation that mutating all of the lysines in the ΔGH3 mutant (which we have shown previously prevents IAP-mediated Reaper ubiquitination) does not restore its cell killing ability. Therefore, it is very unlikely that the primary defect in the ΔGH3 mutant or the rectification of this defect by the Bcl-x_L tail results from stabilization of the Reaper protein.

Finally, perhaps the most provocative hypothesis to explain the link between mitochondrial localization and IAP destabilization postulates that proteins localized within the mitochondria and required for IAP destruction are released from mitochondria by Reaper in a GH3 domain-dependent manner. As recent reports have suggested that multiple mitochondrial constituents are co-released with cytochrome *c* in vertebrate cells, the ability of Reaper to induce release of such factor(s) from fly mitochondria might be reflected in the ability of Reaper to induce cytochrome *c* release in heterologous systems. In this regard, it is interesting to note that we have also found ΔGH3 Reaper to be defective in inducing caspase activation in *Xenopus* egg extracts, where we have shown that apoptosis depends absolutely on mitochondrial cytochrome *c* release.⁶ On the other hand, neither Reaper-(16–65) (Fig. 6D) nor the XLE protein in which the IBM domain was deleted (data not shown) could destabilize IAPs. These data strongly suggest that both the IBM and GH3 domains are required for Reaper to trigger IAP destabilization.

As mentioned above, it remains possible that the reconstitution of IAP degradation in cell-free systems reflects contamination of cytosolic extracts with mitochondrial constituents (which might be released by Reaper in intact cells and required for IAP degradation). In this respect, a recent report that DIAP1 can be degraded by a caspase-dependent N-end rule pathway raised the concern that such mitochondrial contaminants could allow Reaper-induced caspase activation, thereby leading to the observed IAP degradation (36). However, aside from the fact that caspase activation was never detected in these cytosolic extracts, the degradation we observed requires IAP intrinsic ubiquitin ligase activity, whereas the N-end rule pathway does not. Similarly, caspase inhibition (by Z-VAD-fmk or baculovirus p35) was utilized in all IAP destabilization experiments presented here (and previously (15)), and this did not prevent IAP destabilization in either intact human or fly cells. Finally, although SMAC in fly cells could promote some degree of caspase activation and cell death (in the absence of

⁵ C. L. Holley and M. R. Olson, unpublished data.

⁶ M. R. Olson and S. Kornbluth, unpublished data.

Z-VAD-fmk) (data not shown), SMAC failed to effect DIAP1 destruction, at least when caspases were inhibited.

In any case, the data presented in this study lead to the conclusion that simple inhibition of the IAP-caspase interaction is unlikely to be sufficient for inducing either IAP destabilization or apoptosis, even in fly cells. Rather, the C-terminal two-thirds of the Reaper protein must provide an additional function. The data presented here strongly suggest that this additional function depends upon the localization of Reaper to the mitochondria and is exerted, at least in part, through IAP destabilization.

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