

Destabilizing Influences in Apoptosis: Sowing the Seeds of IAP Destruction

Minireview

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Inhibitor of apoptosis proteins (IAPs) can block apoptosis through interactions with members of the caspase family of cysteine proteases. Recent developments suggest that ubiquitin-proteasome mediated destruction of the *Drosophila* IAP, DIAP1, is a key event during the initiation of programmed cell death in the fly.

During the past decade, we have been faced with a seemingly endless stream of papers concerned with the molecular control of apoptosis. Thankfully, the dust is beginning to settle somewhat, and a basic blueprint for cell death has now emerged. However, because the devil is in the details, our understanding of the regulation of apoptosis is far from complete. Here, the regulation of the central elements of the cell death machinery will be discussed in the context of recent developments in this area.

Maybe Not a Thousand Cuts, but Getting There

During apoptosis, cells undergo a form of controlled demolition that results in their removal with the minimum of collateral damage to surrounding cells. The key participants in this dismantling process are a family of cysteine proteases (the caspases) that exist as dormant proenzymes in most, if not all, cells. During apoptosis, a battery of caspases becomes activated through proteolytic processing at internal aspartic acid residues. Active caspases then proceed to dismantle the cell by targeting numerous proteins for limited proteolysis (see Slee et al., 2001, and references therein). To date, approximately 220 mammalian caspase substrates have been identified, but it is very likely that this number will increase significantly upon completion of global proteomic analyses of apoptotic cells. Undoubtedly, many of the proteins targeted by caspases during the terminal phase of apoptosis are “innocent bystanders” that happen to contain the appropriate cleavage motifs. However, given the remarkable conservation of the apoptotic phenotype, from nematodes to mammals, it is highly likely that a conserved group of caspase substrates exist. Proteolysis of the latter substrates is what probably leads to the stereotypical alterations to the cellular architecture that occur during apoptosis.

Because of the destructive potential of the caspases, stringent controls are necessary to regulate the activation and/or activity of these enzymes. In mammals, this is achieved in two main ways. First, caspases are synthesized as largely inactive proenzymes (zymogens) that require recruitment to scaffold proteins or membrane receptors that facilitate close proximity with other cas-

pase molecules—thereby enabling autoprocessing and activation. Only certain caspases can be activated in this manner (i.e., those that possess particular motifs called CARDs or DEDs within their N termini), and it is these executive caspases that set the wrecking ball rolling. Upon activation, the latter caspases can rapidly seal the fate of the cell by recruiting additional effector caspases through direct proteolytic processing of their latent forms. Much of the nasty business of cellular demolition is perpetrated by the effector caspases, although the details of this phase remain patchy at present.

The second major restraint on caspase activity is provided by a diverse family of proteins called the IAPs (inhibitor of apoptosis proteins). IAPs contain at least one, but usually three, BIR motifs that are capable of interacting with caspases and occluding their catalytic pockets. Certain IAPs also possess C-terminal RING domains. Many proteins that contain the latter motif act as E3 ubiquitin ligases and can participate in reactions that culminate in the transfer of polyubiquitin chains to their target proteins. Polyubiquitinated proteins are typically rapidly bound and destroyed by the proteasome. IAPs preferentially interact with active caspases, although evidence that they can promote polyubiquitination of these proteases *in vivo* has been lacking. However, numerous studies have shown that IAPs can impede caspase activity and delay the onset of apoptosis.

Paths to Destruction

Two major pathways to apoptosis-associated caspase activation have been defined in mammals: the extrinsic death receptor pathway and the intrinsic or mitochondrial pathway.

The intrinsic pathway is initiated through release of mitochondrial cytochrome *c* into the cytosol in response to divergent cellular stresses. Cytochrome *c* release is a major checkpoint on the road to apoptosis in mammals, as this protein can provoke the assembly of a caspase-9 activating complex in the cytosol, termed the apoptosome. In mammalian cells, it has been established that cytochrome *c* is critical for the assembly of the apoptosome, most likely through binding to the Apaf-1 C terminus and displacing this region away from the oligomerization surface. The latter event also facilitates binding of dATP (or ATP) by Apaf-1, which may lock the molecule into a more open configuration and permit oligomerization and caspase-9 recruitment. Upon activation within the apoptosome, caspase-9 can propagate a cascade of further caspase activation events by direct processing of effector caspases downstream.

Worm and Fly Apoptosomes: Seeking a Role for Cytochrome c

Caspase activation is also central to the programmed cell deaths (PCD) that have been extensively studied in *C. elegans* and *Drosophila*. The extrinsic death receptor pathway appears to be absent in nematodes (and probably also in the fly), but these animals do utilize an apoptosome pathway to apoptosis. In *C. elegans*, the apoptosome is composed of an Apaf-1 homolog, CED-4,

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which can recruit and activate the nematode caspase, CED-3. However, in sharp contrast to what is seen in mammals, the worm apoptosome does not appear to have a requirement for cytochrome *c* for its assembly. Instead, CED-3 activation is regulated by CED-9, which sequesters CED-4 on the outer mitochondrial membrane until a signal to die is received.

In *Drosophila*, an Apaf-1/CED-4-related molecule has also been found (called DARK/HAC-1/Dapaf-1), and Kumar and colleagues have recently reported that DARK may form apoptosomes by recruitment of the fly caspase-9 homolog, DRONC (Dorstyn et al., 2002). However, a burning question in the field has been whether the fly regulates assembly of its apoptosomes through interactions between DARK and cytochrome *c*, as in mammals. Although initial experiments indicated that DARK interacted with cytochrome *c* (Rodriguez et al., 1999), two recent studies suggest that fly apoptosomes do not require this protein as a cofactor for their assembly. Both groups examined the release of mitochondrial cytochrome *c* in *Drosophila* cell lines during apoptosis initiated through growth factor deprivation, UV irradiation, or exposure to cytotoxic drugs—stimuli that provoke mitochondrial cytochrome *c* release in mammalian cells. Cytochrome *c* release was not observed under any of the conditions examined, despite the fact that these stimuli engaged a DARK-dependent pathway to apoptosis (Dorstyn et al., 2002; Zimmermann et al., 2002). Furthermore, using double-stranded RNA interference (RNAi) to knock down cytochrome *c* expression in *Drosophila* Schneider cells, Green and colleagues found no impairment of apoptosis under conditions where cytochrome *c* expression was almost entirely (>95%) ablated (Zimmermann et al., 2002).

Proponents of a cytochrome *c*-regulated fly apoptosome could point out that the residual cytochrome *c* (post RNAi-mediated ablation) could still be sufficient for apoptosome assembly. Indeed, while Abrams and colleagues have also previously reported that mitochondrial cytochrome *c* release does not occur during *Drosophila* PCD, they did detect enhanced cytochrome *c* immunoreactivity (suggestive of a conformational change) in cells destined to die (Varkey et al., 1999). Interestingly, enhanced immunoreactivity of cytochrome *c* was found to be caspase-dependent, suggesting that it might be a consequence rather than an instigator of apoptosis, or that it might represent a feedback amplification loop. In addition, *Drosophila* Reaper protein has been found to promote mitochondrial cytochrome *c* release upon addition to a *Xenopus* cell-free system (Evans et al., 1997). Thus, although the recent data suggest that the *Drosophila* apoptosome may be regulated in a cytochrome *c*-independent manner, it remains formally possible that cytochrome *c* may participate in *Drosophila* PCD in some guise.

A puzzle not explained by the observations that cytochrome *c* fails to be released during *Drosophila* PCD is why the C terminus of DARK contains multiple WD-40 repeats, a feature that it shares with Apaf-1 but not CED-4. The WD-40 repeat region of Apaf-1 has been established as the cytochrome *c* binding domain, and this region represses the assembly of the apoptosome, probably through interactions with the oligomerization and caspase-9 recruitment regions of Apaf-1 at the N

terminus. The simple explanation for this paradox is that the WD-40 repeat region in DARK, while capable of binding cytochrome *c*, may not act as a regulatory domain as it does in Apaf-1. The WD-40 repeat region may have acquired its regulatory function later in evolution through exploiting its ability to act as a cytochrome *c* “sensor” within the cytosol. An alternative explanation, not ruled out by the recent studies, is that DARK may be regulated by an entirely different mitochondrial component. However, other recent observations (see below) also suggest that fly and mammalian apoptosomes are regulated in fundamentally different ways.

DARK Tales of Reaper, Hid, and Grim: An Apoptosome on a Hair Trigger?

A unique feature of *Drosophila* is that the products of three closely linked genes, *reaper* (*rpr*), *head involution defective* (*hid*), and *grim*, control essentially all developmental-related PCD in this organism (White et al., 1994). *Rpr*, *Hid*, and *Grim* share a short region of homology at their N termini, known as the RHG motif, and this region is both necessary and sufficient for binding to the *Drosophila* IAPs, DIAP1, and DIAP2. While the RHG proteins were initially thought to contain proapoptotic activities that could be repressed by the fly IAPs, recent studies suggest the opposite—that the RHG proteins act by repressing the caspase-inhibitory function of DIAP1 and DIAP2 (Wang et al., 1999; Goyal et al., 2000). *Drosophila* embryos that lack *rpr*, *hid*, and *grim* expression due to deletion of the H99 interval are essentially devoid of all PCD (White et al., 1994). However, these cell death-defective mutants display extensive apoptosis when *DIAP1* expression is also eliminated, suggesting that loss of DIAP1 is sufficient to promote apoptosis independent of the RHG-proteins (Wang et al., 1999). This interpretation is further supported by observations that gain-of-function mutations in *DIAP1* that reduced binding of the RHG proteins strongly suppressed apoptosis induced by the latter (Goyal et al., 2000).

DIAP1 is an essential regulator of PCD in *Drosophila*, as loss of this IAP is sufficient to trigger widespread apoptosis, resulting in embryonic lethality shortly after gastrulation (Wang et al., 1999). Studies from several laboratories have recently shown that apoptosis due to DIAP1 loss is both DARK- and DRONC-dependent, suggesting that loss of DIAP1 is sufficient to activate the apoptosome (Rodriguez et al., 2002; Zimmermann et al., 2002). This result is puzzling, as it suggests that the fly apoptosome may be on a hair trigger, with DIAP1 playing a critical role in repressing its activation/activity. This contrasts sharply with the situation in mammals and nematodes, where the IAPs appear to play a less important role in making life or death decisions. For example, disruption of *XIAP* appears to have no deleterious consequences in the mouse, and overexpression of the human IAPs merely delays apoptosis but does not result in clonogenic survival. Furthermore, no role has been found for IAPs in the regulation of PCD in *C. elegans*, despite extensive genetic analysis of cell death controls in this organism. Taken together with the observation that cytochrome *c* may not be a required cofactor for apoptosome assembly in the fly, these data suggest that caspase activation in *Drosophila* may be regulated primarily through neutralization of DIAP1-mediated repression of DARK-dependent caspase activation.

If the latter view is correct, then it suggests that DARK/DRONC apoptosomes may have a propensity to assemble spontaneously, but that this is normally prevented from feeding forward through DIAP1-mediated inhibition of DRONC activity. An alternative possibility is that in addition to neutralization of DIAP-1 function, a second signal—provided by other cells within the fly—is required to promote DARK-dependent activation of DRONC. However, this would appear to be ruled out by the observation that RNAi-mediated ablation of DIAP1 expression is sufficient to drive DARK-dependent apoptosis in cultured *Drosophila* cells, in the absence of any apparent death stimulus (Zimmermann et al., 2002)

Support for the hair trigger model is also provided by recent gel filtration experiments reported by Kumar's laboratory. They observed that incubation of dATP-supplemented *Drosophila* BG2 cell extracts at 27°C was sufficient to recruit DRONC into large complexes (>700 kDa) that probably represent assembled apoptosomes (Dorstyn et al., 2002). However, lack of appropriate antibodies precluded confirmation that DARK is a component of these complexes, so further analysis is clearly required here.

Reaper Sows the Seed of DIAP1 Destruction

So, if Rpr, Hid, and Grim activate DARK-dependent apoptosis by neutralization of DIAP1, do they all achieve this through a similar mechanism? A series of recent papers would seem to suggest not.

Previous studies on *DIAP1/thread* mutant alleles provided evidence that Rpr, Hid, and Grim may interfere with DIAP1 function in distinct ways (Lisi et al., 2000). Recent observations from several laboratories have provided further evidence of this and have revealed a molecular basis for the differential effects of the RHG proteins on DIAP1 function.

To ask whether RHG proteins could regulate DIAP1 protein levels, Steller and colleagues used a model where they coexpressed *rpr* and the caspase-inhibitor *p35* in the posterior compartment of the *Drosophila* wing imaginal disc (*p35* was used to block death of the cells overexpressing Reaper). Using this approach, they noted that endogenous DIAP1 protein levels were significantly reduced (by a posttranscriptional mechanism) in the *rpr*-overexpressing cells, as compared to the wild-type cells of the anterior wing compartment (Ryoo et al., 2002). However, expression of *hid* within the same model failed to downregulate DIAP1 levels, once again suggesting that Rpr and Hid interfere with DIAP1 function through different mechanisms (Ryoo et al., 2002). Because RING domain mutants of DIAP1 resisted elimination by Rpr, they then explored whether DIAP1 auto-ubiquitination was the mechanism underlying the disappearance of this protein. Using an *in vitro* ubiquitination assay, Ryoo et al. provide convincing evidence that the binding of Rpr to DIAP1 activates the intrinsic E3 ubiquitin-ligase activities of this protein toward itself. Thus, Rpr-induced DIAP1 downregulation *in vivo* is almost certainly due to proteasome-mediated destruction of this protein as a consequence of polyubiquitination (Figure 1). In the same study, Steller and colleagues also identified a *Drosophila* mutant, *ubcd1/effete* (which encodes an E2 ubiquitin-conjugating enzyme), that dominantly suppressed Rpr-induced cell death. UBCD1 was found to interact physically with DIAP1 and to promote

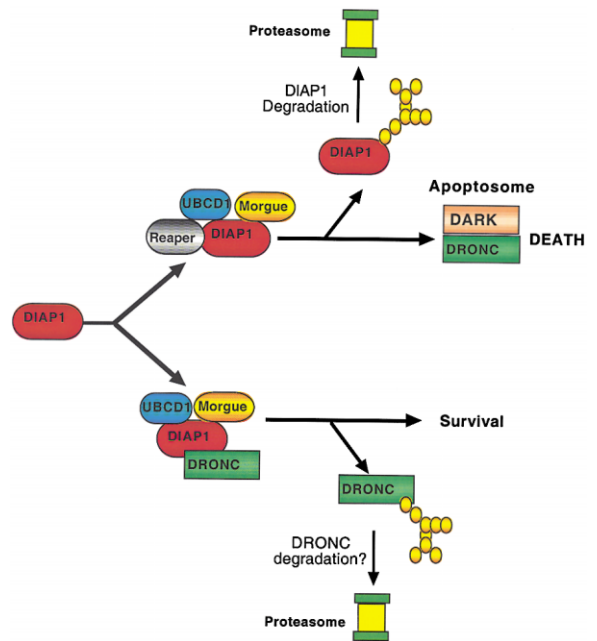


Figure 1. Regulation of DIAP1 Stability by Reaper

Autoubiquitination of DIAP1 is stimulated by the binding of Reaper (and possibly by Hid) in a reaction catalyzed by an E1 ubiquitin activating enzyme (not depicted) and an E2 conjugase (UBCD1), assisted by Morgue (E2-like). DIAP1 degradation results in the assembly of the DARK/DRONC apoptosome, which is likely to contain several DARK/DRONC heterodimers (only a single dimer is depicted). In the absence of Reaper (or other RHG proteins), DIAP1 may inactivate DRONC through a similar complex of enzymes, although this remains speculative.

Rpr-induced DIAP1 polyubiquitination *in vitro*. These data strongly suggest that the binding of Rpr to DIAP1 targets the latter for destruction through a mechanism in which UBCD1 acts as the E2 ubiquitin conjugase and DIAP1 as the E3 ligase component (Figure 1).

However, the plot thickens further due to the discovery of a fourth component of this ubiquitination reaction, called Morgue, by two independent groups (Wing et al., 2002; Hays et al., 2002). Using different approaches, the Cagan and Nambu laboratories discovered that *morgue* mutants could suppress cell death associated with targeted expression of *rpr*, *grim*, or *hid* in the *Drosophila* eye. In the cells of the interommatidial lattice, DIAP1 protein levels were found to be considerably higher in *morgue* mutants, suggesting that Morgue may regulate DIAP1 expression or stability. Morgue belongs to a class of enzymes known as ubiquitin conjugating enzyme variants (UEVs) that are similar to E2 conjugases (such as UBCD1) but lack a critical cysteine that is present in all E2s. Morgue also interacts with DIAP1 and probably acts in concert with UBCD1 to polyubiquitinate DIAP1. In support of this idea, coexpression of Rpr and Morgue was found to promote DIAP1 polyubiquitination and degradation in a *Drosophila* cell line, while expression of Morgue alone stabilized DIAP1 levels (Wing et al., 2002; Hays et al., 2002).

Using an entirely different approach based upon experiments in mammalian cell lines and *Xenopus* egg extracts, Kornbluth and colleagues come to a similar

conclusion that Rpr can promote DIAP1 degradation (Holley et al., 2002). Remarkably, they also report that overexpression of *rpr* in a human embryonic kidney cell line (HEK293T) triggered degradation of the human IAPs, XIAP, and cIAP1. This suggests that the mammalian IAP binding proteins, Smac/Diablo and Omi/Htra2, may also regulate IAP function through activating their autoubiquitination capabilities.

However, a fly in the ointment is provided by the Hay laboratory, who report that all three RHG proteins can downregulate DIAP1 expression but that Hid, rather than Rpr, stimulated DIAP1 autoubiquitination (Yoo et al., 2002). Intriguingly, the Hay and Kornbluth laboratories also report that Grim and Rpr can exert generalized suppressive effects on protein translation, which may be partly responsible for DIAP1 downregulation in cells expressing these proteins (Yoo et al., 2002; Holley et al., 2002). However, the significance of these observations and the mechanism underlying the effects of Rpr and Grim on translation remain to be explored in detail.

The observed differences between the groups may reflect differences in the tissues studied or expression efficiencies of the *rpr*, *grim*, and *hid* constructs used. However, the broad message from all of the studies is that destabilization of DIAP1, by targeting this protein for proteasome-mediated destruction, is at least one of the ways in which DARK-dependent PCD is triggered in the fly.

Quenching Caspase Activation through IAP-Mediated Polyubiquitination?

Previous studies by Ashwell and colleagues have shown that mammalian IAPs can autoubiquitinate, and that this is accelerated by cell death triggers, at least in thymocytes (Yang et al., 2000). Because IAPs can interact with active caspases, it follows that IAPs may also act as E3 ligases for caspases to put a brake on caspase activity. Evidence in support of this idea is provided by Meier and colleagues, who report that DIAP1 can promote the ubiquitination of DRONC; however, it is not clear whether this targets DRONC for proteasome-mediated destruction (Wilson et al., 2002; Figure 1). In the mammalian context, it has been reported that XIAP and cIAP2 can act as E3 ligases for caspase-3 and caspase-7. However, the latter result is based largely upon *in vitro* ubiquitination reactions and experiments conducted with an artificial, constitutively active, caspase-3 construct. Thus, there remains a lack of conclusive evidence that caspases are targeted for destruction as a result of interactions with IAPs.

Notwithstanding this, it is tempting to speculate that IAPs may serve to damp down spontaneous or inadvertent caspase activation through their E3 ubiquitin ligase properties. This would not necessarily result in degradation of the caspase, merely inactivation. Molecules that divert the attention of the IAPs away from this activity (such as the RHG proteins) and toward autoubiquitination would tip the balance in favor of death. However, although things may work this way in the fly, they may not do so in mammals. For instance, Smac/Diablo overexpression is not sufficient to trigger apoptosis, suggesting that IAP neutralization alone is not enough to drive apoptosis in higher organisms. This may reflect some redundancy between the mammalian IAPs (assuming that they cannot all be neutralized by one pro-

tein) or that the requirement for cytochrome *c* as a cofactor for mammalian apoptosome assembly acts as an efficient safety catch.

Finally, we still have a major gap to fill in our knowledge concerning how CED-9/Bcl-2 relatives integrate into the *Drosophila* paradigm. Perhaps the fly apoptosome is also subject to direct regulation by a Bcl-2 relative, as in the worm? Or maybe *Drosophila* places all of its faith in the IAPs? Could DIAP1 also target a Bcl-2-like molecule for degradation upon RHG protein binding? Clearly, there is more work to be done to resolve these issues. To paraphrase Arthur Conan Doyle: when we have spun the web, they may take the flies, but not before!

Selected Reading

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