# TURNING ON DEATH IN THE FLY: REGULATION OF APOPTOSIS IN *DROSOPHILA MELANOGASTER*

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*All accumulated wealth is eventually dispersed;*

*What goes up must come down.*

*All meetings end in partings;*

*Every life concludes in death.*

-The Dharmapada

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# ABSTRACT


## Turning on Death in the Fly: Regulation of Apoptosis in *Drosophila Melanogaster*

Apoptosis, an evolutionarily conserved form of cell suicide, is implemented by a family of cysteine proteases termed caspases. Caspases are constitutively expressed in most cells and function in a proteolytic cascade activated by diverse extracellular and intracellular death stimuli. What molecules govern caspase activity so that it is limited to doomed cells? What mechanisms do these regulators employ? Caspases and their regulators have been identified in a wide variety of species. This dissertation describes the analysis of caspase regulators in *Drosophila melanogaster*, which has led to the elucidation of some mechanisms that control caspase activity.

In *Drosophila*, three genes, *reaper* (*rpr*), *head involution defective* (*hid*), and *grim*, are essential for most normally occurring cell death, and are sufficient to initiate caspase-dependent death when expressed in cells that typically live. The *Drosophila* IAP homologue DIAP1 is a dosage-dependent suppressor of normally occurring, as well as *rpr*- and *hid*-dependent cell death. We show that DIAP1 physically interacts with RPR, HID, and GRIM, and binds to and inhibits the activity of *Drosophila* caspases. Using a yeast-based caspase activity reporter system and an *in vitro* reconstitution assay, we find that HID blocks DIAP1's ability to inhibit caspase activity and provide evidence that RPR and GRIM can behave similarly to activate caspases. These observations define a novel point at which caspase activity can be regulated and suggest that one mechanism by which RPR, HID, and GRIM can promote apoptosis is by disrupting productive IAP-caspase interactions.

Supporting this hypothesis we show that DIAP1 is required to block apoptosis-inducing caspase activity during *Drosophila* embryonic development. Elimination of DIAP1 function results in global early embryonic cell death and a large increase in DIAP1-inhibitable caspase activity. DIAP1 is still required for cell survival when expression of *rpr*, *hid,* and *grim* is eliminated. Since the death program is constitutively expressed in most cells, the mechanism of cell death activation defined by RPR, HID, and GRIM may be quite general.

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# CHAPTER 1

## Introduction: Mechanisms of Caspase Regulation

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Apoptosis is an evolutionarily conserved, intrinsic process by which the organism abolishes undesired cells. The most common form of cell death, apoptosis is indispensable during development as well as in the adult metazoan animal. Its roles include: structure remodeling, lymphocyte control, tissue size homeostasis, and removal of abnormal and potentially harmful cells (reviewed in Jacobson et al., 1997). Apoptosis is executed by specialized proteolytic machinery involving a family of proteases termed caspases (Alnemri et al., 1996). Caspases are the essential components of many, if not all apoptotic pathways. They exist in most living cells as zymogens which are activated by proteolytic processing in response to death-inducing stimuli. Caspase activation is mediated either autocatalytically or by another protease, triggered by cofactor binding or inhibitor removal. Regulated caspase activation permits accumulation of large amounts of precursor that can be rapidly activated on demand (Cryns and Yuan, 1998; Nicholson and Thornberry, 1997; Salvesen and Dixit, 1997; Thornberry and Lazebnik, 1998). Since procaspases are widely expressed at high levels and have low but significant protease activity, efficient mechanisms must exist to prevent inappropriate caspase activation.

## Caspases: effectors of apoptosis

Studies of developmental cell death in the nematode *Caenorhabditis elegans* have provided important clues about the molecular nature of the death program. A group of genes, termed *ced* for cell death-defective, regulates cell death in worms (Ellis et al., 1991). One of these genes, *ced-3*, is required for carrying out cell death and is a member of a family of cysteine proteases that form the core of the apoptotic machinery (Yuan et al., 1993). These proteases effect death by cleaving cellular substrates at specific *asp*artate residues and are thus called c*asp*ases (Alnemri et al., 1996). Since the discovery that CED-3 protein bears marked sequence similarity to interleukin-1β-converting enzyme (ICE,

caspase-1), a mammalian protease responsible for proteolytic maturation of pro-interleukin-1β, many additional caspase family members have been isolated in a variety of organisms (Thornberry et al., 1992).

Although caspases are critical for the execution of many apoptotic pathways, their exact physiological roles have not been well defined. Gene targeting studies have shed light on the distinctive role of individual caspases in cell death as well as in other biological processes (reviewed in Los et al., 1999). In particular there are caspases that, rather than regulate apoptosis, primarily mediate immune responses by controlling proinflammatory cytokine processing. Caspases may also be involved in the regulation of cell proliferation, a process which is likely to share some machinery involved in programmed cell death. For example, cell division during proliferation requires enzymes involved in cytoskeletal rearrangement and the disruption of cell adhesion, two events that function during apoptosis. A primary phenotype of flies lacking zygotic *Drosophila* caspase-1 (DCP-1) is the absence of imaginal discs and gonads, hinting at a possible role in regulating cell proliferation in these tissues (Song et al., 1997).

Several lines of evidence indicate that caspases are important for apoptosis. First, worms without functional CED-3 have a complete absence of developmental programmed cell death (Yuan et al., 1993). Second, targeted deletion of caspase and caspase regulator genes in mice have shown a definitive role for caspases in apoptosis. Caspase-3, caspase-8, and caspase-9 knockout animals all die at birth due to serious defects in developmental apoptosis (Los et al., 1999). Ablation of the caspase-8 regulator, FADD, is embryonic lethal with mutant mice exhibiting a phenotype similar to that of caspase-8 deficient animals (Yeh et al., 1998). Mice lacking apoptotic protease-activating factor 1 (Apaf-1), a caspase-9 regulator, have a phenotype even more severe than caspase-9 knockout mice. Both Apaf-1 and caspase-9 knockout mice exhibit brain hyperplasia, craniofacial alterations, and persistence of interdigital webs (Cecconi et al., 1998; Hakem et al., 1998;

Kuida et al., 1995; Yoshida, 1998). Third, loss of a functional caspase in flies also indicates caspase involvement in cell death regulation: flies lacking DCP-1 develop melanotic tumors and die as larvae (Song et al., 1997). Fourth, ectopic expression of the viral protein caspase inhibitor CrmA blocks apoptosis in a number of different systems (Ray et al., 1992). Addition of the cell-permeable peptide caspase inhibitor Z-VAD-fmk [benzyloxycarbonyl-Val-Ala-Asp(OMe) fluoromethyl ketone] to cultured cells, including thymocytes, hepatocytes, and neuronal cells, potently inhibits apoptosis induced by a wide range of stimuli (Armstrong et al., 1996; Chow et al., 1995). Similarly, the pan-caspase inhibitor baculovirus P35 blocks normally occurring developmental cell death in the *Drosophila* embryo and eye when ectopically expressed in these tissues (Hay and Rubin, 1994).

Caspase substrates

Identification of caspase substrates has provided insight into how caspases promote the cellular changes that occur during programmed cell death. Pro- and anti-apoptotic regulatory proteins are likely to be the first caspase targets following a death stimulus. Cleavage of these targets results in signal amplification and caspase inhibitor inactivation. For example, caspase-3 cleaves apoptotic regulators Bcl-2 (Bcl, *B cell lymphoma*) and Bcl-$X_L$, destroying their anti-apoptotic function and releasing proapoptotic fragments (reviewed in Gross et al., 1999). Bcl-2 and Bcl-$X_L$ act as death inhibitors upstream in pathways resulting in caspase activation. Proapoptotic Bcl-2 family member BID is cleaved by caspase-8, releasing a fragment that induces an efflux of cytochrome *c* , as well as other apoptotic effectors, from the mitochondria. Cytochrome *c* is required for the activation of Apaf-1, which promotes activation of procaspase-9 (Li et al., 1997).

Another category of caspase substrates includes housekeeping or structural proteins whose cleavage is required for the ordered disassembly of the cell. Apoptotic cells die a morphologically distinguished death characterized by condensation and fragmentation of nuclear chromatin, compaction of cytoplasmic organelles, a decrease in cell volume, and alterations to the plasma membrane resulting in recognition and phagocytosis of the dying cell (Kerr et al., 1972). Caspases cleave several proteins involved in cytoskeleton regulation, including gelsolin and focal adhesion kinase (Kothakota et al., 1997; Wen et al., 1997). Cleavage of these proteins results in deregulation of their activity. A caspase-3-activated fragment of gelsolin severs actin filaments, promoting both cytoplasmic and nuclear apoptosis, including DNA fragmentation. Caspases directly cleave lamins, causing lamina to collapse and contributing to chromatin condensation (Orth et al., 1996; Takahashi et al., 1996). Proteolysis of adhesion molecules such as β-catenin by caspases may lead to disruption of cell-cell contacts (Brancolini et al., 1997). Altogether, cleavage of these structurally related substrates is believed to promote cellular packaging and subsequent engulfment of dead cell debris by phagocytes.

The nuclear changes during apoptosis are associated with internucleosomal cleavage of DNA, recognized as a "DNA ladder" by agarose gel electrophoresis. Although DNA fragmentation now appears to be a relatively late event in the apoptotic process and in some models may be separated from early critical steps, its measurement is simple and it is often used as a major criterion to determine if a cell is apoptotic. Many proteins involved in DNA synthesis and repair, such as the nuclear replication factor MCM3, the large subunit of DNA replication factor C and human Rad51, have been identified as caspase substrates in apoptotic cells (Flygare et al., 1998; Rheaume et al., 1997; Schwab et al., 1998; Ubeda and Habener, 1997). Moreover, caspase-3 cleaves the

caspase-activated DNase (CAD) inhibitor ICAD, allowing the released nuclease to cleave DNA and promote nuclear condensation (Liu et al., 1997; Sakahira et al., 1998).

## Caspase structure

Caspases are formed as latent precursors consisting of a prodomain and a region containing the two subunits that comprises the catalytic domain. These zymogens are activated *in vivo* following proteolytic cleavages after specific aspartate residues. In general, there is an initial cleavage or two that separates the small C-terminal subunit from the rest of the molecule and allows formation of an active protease that cleaves off its own prodomain. Based on the crystal structure of caspase-1 and caspase-3, mature caspases are heterotetramers composed of two large subunits and two small subunits (Mittl et al., 1997; Rotonda et al., 1996; Walker et al., 1994; Wilson et al., 1994). The caspase active site is composed of residues from both subunits and contains a positively charged $S_1$ subsite that binds the $P_1$ aspartate of the substrate. Caspases contain an active site cysteine within a conserved QAC*X*G motif (where *X* is R, Q or G). Substrate specificity varies with individual caspases depending on the composition of their residues present in the substrate binding site. The tertiary structure and residues within the active site can vary significantly among different caspases, consistent with the diversity of their biological functions.

The prodomain and large and small subunits are derived from the proenzyme by cleavage following aspartates in regions that often resemble consensus caspase target sites. This observation suggests that caspases can be activated either autocatalytically or in a cascade by enzymes with similar specificity. Based on their structure and order in cell death pathways, caspases can be classified as either initiator or effector caspases. Initiator caspases, which often contain large prodomains, can promote activation of other family

members by cleaving them, generating a proteolytic cascade of caspase activation. The prodomains of several apical caspases contain protein modules that are conserved in many apoptosis regulatory molecules. Death effector domains (DEDs) and caspase recruitment domains (CARDs) are two types of motifs found in prodomains of initiator caspases. Both domains consist of six anti-parallel α-helices contained in a similar three-dimensional fold. These motifs associate with homologous regions in other proteins, thus prompting binding of proteins to one another (Eberstadt et al., 1998; Huang et al., 1996). The DED and CARD have been proposed to play a regulatory role in apoptosis by coupling caspase regulators to procaspases.

## Mammalian caspases

Mammalian caspases comprise a group of at least fourteen members that can promote apoptosis. Based on substrate specificity studies and phylogenetic analysis, caspases can be classified into three subfamilies (reviewed in Cohen, 1997; Cryns and Yuan, 1998; Nicholson and Thornberry, 1997). The ICE-like family consists of caspase-1, -4, -5, -13, and -14 as well as murine caspase-11 and -12. The CED-3 subfamily includes caspase-3, -6, -7, -8, -9, and -10. A third subfamily has only one member, caspase-2. Members of a caspase subfamily recognize similar peptide sequence target sites within their substrates, and some can cleave the same molecules, indicating functional redundancy within the caspase family.

## *Drosophila* caspases

The apoptosis death program is highly conserved among vertebrates and invertebrates. Several *Drosophila* caspases have been identified recently. They include DCP-1,

DREDD/DCP-2, DRICE, DRONC, DECAY (Chen et al., 1998; Dorstyn et al., 1999; Fraser and Evan, 1997; Song et al., 1997).

## Caspase activation

There is strong evidence that the death program is constitutively expressed in most cells (Jacobson et al., 1997; Shaham and Horvitz, 1996; Steller, 1995). Various receptor-mediated apoptosis occur in the presence of inhibitors of either RNA or protein synthesis (Itoh et al., 1991). Even enucleated cells undergo apoptosis upon Fas activation suggesting that all of the components necessary for apoptotic signal transduction are present and that Fas activation simply triggers this machinery (Schulze-Ostoff et al., 1994).

Initiation of the apoptotic program involves the activation of latent caspase zymogens. In the cascade model for effector caspase activation, a proapoptotic signal culminates in the activation of an initiator caspase which, in turn, activates effector caspases. Effector caspases then carry out proteolytic cleavages of cellular proteins ultimately resulting in cell death. Events likely to be involved in caspase activation include: the removal of inhibitors, association with proapoptotic cofactors, conformational changes, and proteolytic processing. Current evidence suggests that caspase processing may occur by autoactivation, transactivation, or proteolysis by other proteases such as granzyme B (Greenberg, 1996).

Proapoptotic signals can originate from outside the cell through means such as receptor signaling, granzyme B uptake, and death peptide ingestion. Alternatively, the cell can sense internal stress, such as damaged DNA, and choose to commit suicide. Different upstream death signals are mediated by distinct initiator caspases. Three major caspase activation pathways have been elucidated to some extent. One pathway, which involves a

complex called the "apoptosome," functions similarly in organisms ranging from nematodes to mammals. Another route, featured in mammalian cell death, is triggered by and directly linked to "death receptors." A third apoptotic pathway operates through the inhibition of caspase inhibitors.

Models for caspase autoactivation

1. The "facilitated autocatalysis" model

The "facilitated autocatalysis" model is based on the hypothesis that procaspases are present in cells in an inactive conformation or complex that precludes autocatalysis. Cofactors promote activation by changing the conformation of the precursors either directly or by removing an inhibitor. Induction of apoptosis by arginine-glycine-aspartate (RGD) peptides may utilize such a mechanism. In a cell culture model, soluble RGD peptides cause apoptosis by activating procaspase-3 in cells (Buckley et al., 1999). In this scenario, the RGD peptide may out-compete an RGD motif in procaspase-3 and disrupt an intramolecular interaction with a DDM sequence. When this bond is blocked, the procaspase changes conformation, allowing it to become activated. The structure of the unprocessed pro-form of caspase-3 is needed to verify the existence of an intramolecular interaction between RGD and DDM. Experiments using purified components and site-directed mutagenesis may aid in understanding the exact mechanism of caspase-3 activation by RGD peptides.

2. The "induced proximity" model

A caspase cascade may initiate by autoproteolysis when two or more zymogen molecules aggregate. The activation of molecules by their clustering is a mechanism utilized in other biological processes, such as the activation of tyrosine kinases and complement cascade

proteins (Davie et al., 1991; Ullrich and Schlessinger, 1990). Overexpression of wildtype caspases, but not catalytically inactive mutants, results in caspase processing and activation (Orth et al., 1996). This suggests that autoactivation may be facilitated by high proenzyme concentration. Affinity-labeling experiments demonstrate that procaspase molecules have weak proteolytic activity (Muzio et al., 1998; Yamin et al., 1996). When zymogens are brought together in close contact, their low activity may be sufficient for trans-proteolytic generation of active enzyme. Once formed, active enzyme may cleave and activate downstream procaspases, thereby initiating a proteolytic cascade leading to apoptosis. *In vivo*, adapter molecules mediate oligomerization of long prodomain containing initiator procaspases. Using specific domains, adapter molecules connect procaspases to apoptotic sensors such as mitochondria and death receptors. CARDs, DEDs, and death domains (DDs) are three types of domains found in adapter molecules. Comparable to DEDs and CARDs, DDs associate via like-like interactions. A CARD domain can be found in many apoptotic molecules, including Apaf-1 and the prodomain of caspase-9. DDs are present in several death-inducing receptors of the tumor necrosis factor (TNF)-receptor family, including Fas, TNF-R1, and others. They are also present in many cytoplasmic adapter proteins that interact with the DD-containing receptors. The adapter molecule FADD (*F*as-*a*ssociated *d*eath *d*omain protein) contains both a DD and DED. Two DEDs are present in tandem in both caspase-8 and caspase-10 (reviewed in Wolf and Green, 1999).

Pathways of caspase activation

1. The apoptosome

Genetic studies of developmental cell death in *C. elegans* have been very successful in identifying the basic components of the apoptotic machinery. Through the use of genetic

screens, a pathway for caspase activation involving several worm proteins has been delineated. All of these proteins have homologues in mammals and fly. They include: CED-9, a Bcl-2-like protein; EGL-1, a BH3 (*B*cl-2 *H*omology region *3*)-only protein; CED-4, an Apaf-1-like protein which contains both a CARD and an ATPase domain; and CED-3 (Conradt and Horvitz, 1998; Hengartner and Horvitz, 1994; Yuan and Horvitz, 1992; Yuan et al., 1993; Zou et al., 1997). In a process requiring the activity of the CED-4 ATPase domain, the CED-4 CARD domain binds to a homologous region in the prodomain of CED-3. Although CED-4 and CED-3 associate, in cells that live, the CED-9 protein blocks CED-4 from aggregating and activating the CED-3 procaspase. It is thought that CED-9 localizes the complex (dubbed the "apoptosome") to intracellular membranes. In cells that die, apoptotic cofactor EGL-1 binds to CED-9, thereby releasing the complex of CED-4 and CED-3. The CED-4 can now aggregate, which brings the bound CED-3 procaspase molecules close enough together so that they can activate each other (reviewed in Hengartner, 1997). The exact mechanism of CED-3 activation is not clear. The induced proximity resulting from CED-4 clustering may be sufficient for CED-3 autoactivation. Conceivably, in a reaction inhibited by CED-9 binding, CED-4 catalyzes the hydrolysis of ATP, which could provide energy required for a conformational change and autocatalytic activation of CED-3.

In mammals, CED-3 and CED-4 homologous molecules caspase-9 and Apaf-1 are important for developmental cell death and oncogene- and p53-dependent apoptosis (Cecconi et al., 1998; Fearnhead et al., 1998; Soengas et al., 1999; Yoshida, 1998). The mammalian apoptosome is more complicated than the worm machinery in that it appears to have an additional regulatory component involving the mitochondria as a death sensor. Apaf-1, like CED-4, consists of a CARD and a CED-4-like (Walker A and B loops composing the ATPase) domain (Zou et al., 1997). However, unlike CED-4, Apaf-1 also contains a WD40-repeat region required for cytochrome *c* binding. An apoptotic stimulus

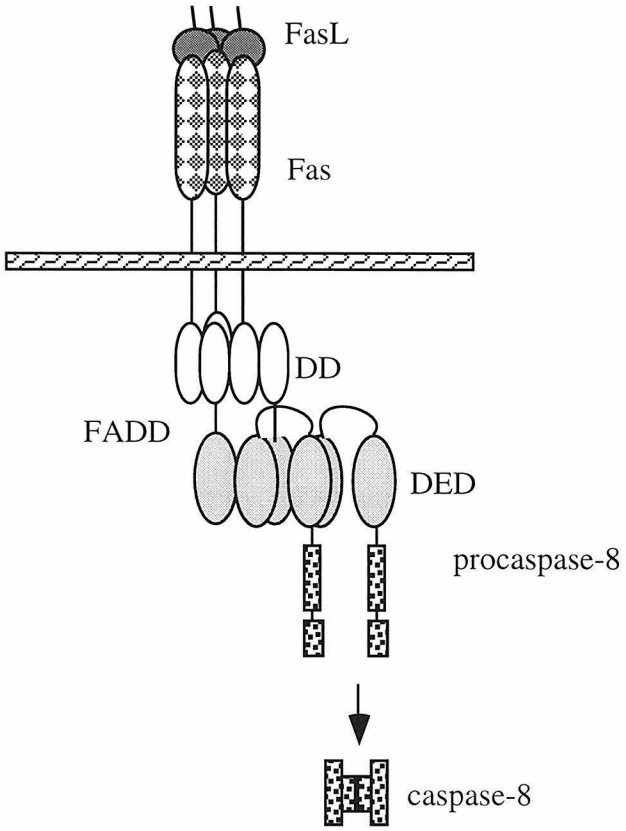
releases cytochrome *c* from the mitochondrial intermembrane space into the cytoplasm. Proapoptotic BH3-containing molecules, such as Bax and Bak, are believed to be involved in this process (Shimizu et al., 1999). They stimulate opening of the voltage-dependent anion channel (VDAC), a mitochondrial channel through which the cytochrome *c* permeates. Conversely, anti-apoptotic protein Bcl-$X_L$ closes VDAC by binding to it directly and blocking cytochrome *c* release. Apaf-1 is activated through a conformational change induced by the binding of ATP and cytochrome *c*. Activated Apaf-1 molecules oligomerize, providing a platform for caspase-9 activation. The oligomerized Apaf-1 complex recruits procaspase-9 to the apoptosome via its CARD domain. Consequently, the "induced proximity" of procaspase-9 molecules by cytochrome *c*-activated Apaf-1 stimulates their proteolytic processing to mature caspases. Once activated, caspase-9 can cleave and activate procaspase-3 and other downstream caspases.

2. "Death receptors" and DISC operation

The TNFR family systems regulate immune-related cell death and survival . They allow the cell to adjust to extracellular insult through brief, intense responses which are characterized by a remarkable duality. On one hand, they can induce the cell to commit suicide. On the other hand, in the appropriate environment, they promote repair and growth. Two of the best characterized TNFR family members are Fas and TNF-receptor-1 (TNF-R1).

The Fas ligand, FasL, rapidly signals apoptosis by triggering the formation of a death-inducing signaling complex (DISC) (Chinnaiyan et al., 1995). When Fas is ligated, its clustered DDs bind a homologous domain in FADD. Subsequently, FADD recruits a complex consisting of procaspase-8 and a DED-like (DRD)-domain containing molecule, FLASH ["*FL*ICE (caspase-8)-*as*sociated *h*uge" protein] via homophilic interactions requiring DEDs (Imai et al., 1999). FLASH's DRD binds to the DEDs in procaspase-8.

*Figure 1.* Simplified diagram of the DISC.


FasL
Fas
DD
FADD
DED
procaspase-8
caspase-8

Interestingly, FLASH contains a CED-4-like domain through which it can self-associate and bind ATP. In the DISC, FLASH may function in a similar manner to CED-4/Apaf-1 in an apoptosome-like complex. Following recruitment to the DISC, procaspase-8 is cleaved and processed, generating active caspase-8. As mentioned above, one caspase-8 substrate is the Bcl-2 family member BID. Cleavage of BID results in cytochrome *c* release, leading to apoptosome formation and caspase-9 activation (Li et al., 1998). Caspase-8 also directly activates caspase-3, -6, and -7. Caspase-3 can cleave and convert Bcl-2 to a BH3-only death effector, amplifying the caspase activation cascade by feeding into the vicious "circle of death" (Cheng et al., 1997).

The apoptotic signaling pathway triggered by ligation of TNF shares some aspects with that of FasL (Ashkenazi and Dixit, 1998). TNF-R1 signal transduction can lead to apoptosis in some cell types; however, in most cells TNF-induced apoptosis is blocked by suppressive factors believed to be controlled by pathway involving NFκB. When TNF-R1 is ligated, it trimerizes. Subsequently, DD-containing adapter TRADD (*T*NF*R*-*a*ssociated *d*eath *d*omain) binds to the clustered receptors' DDs. Oligomerized TRADD recruits various signaling molecules to the receptor complex, depending on the repertoire of proteins available in the cell. For example, the DD-containing Ser/Thr kinase receptor-interacting protein (RIP) is required in the complex for the activation of NFκB (Kelliher et al., 1998; Ting, 1996). In a death-conducive environment FADD couples the TNFR1-TRADD complex to activation of caspase-8.

Many questions regarding DISC operation remain. For example, it is unclear whether active caspase-8 must remain complexed with, or if it is released from, the DISC in order to be fully active. Other questions include: how many procaspase molecules are required for activation? Does activation require dimerization? Does processing occur in

*cis* or *trans*? Are there other molecules involved in DISC operation? What is FLASH's role in caspase-8 processing? Answers to these questions will help to clarify the molecular mechanisms involved in caspase activation downstream of death receptor signaling.

The "induced proximity" hypothesis argues that caspases remain dormant in the cell until activated because they are present at low concentration as monomers. Whether caspase zymogens are indeed monomers in living cells remains to be tested. Analysis of nonprocessable mutant procaspases, generated by replacement of the two Asp cleavage sites separating the large and small subunits with Ala, revealed that procaspases vary in their level of intrinsic enzymatic activity (Muzio et al., 1998; Stennicke et al., 1999). For example, procaspase-8 possesses approximately 1% of the proteolytic activity of mature caspase-8. Procaspase-9 is nearly as active before as it is after processing; however, there is evidence that purified, recombinant, processed caspase-9 is approximately two thousand-fold more active when associated with cytosolic factors supplemented with cytochrome *c* and dATP than in isolation (Stennicke et al., 1999). This and other data suggest that caspase-9 requires complex formation with cytosolic factors, possibly the apoptosome, to be fully active (Lazebnik, in press).

3. REAPER, HID, and GRIM

In *Drosophila melanogaster*, caspases are important for cell death, although the role of particular caspases in apoptotic pathways is currently unknown (Hay and Rubin, 1994). The fly death activators REAPER (RPR), head involution defective (HID), and GRIM act upstream in pathways resulting in caspase-dependent cell death (Bump et al., 1995; Xue and Horvitz, 1995). Chapter 3 of this thesis describes a "facilitated autocatalysis"-like model in which RPR, HID, and GRIM promote apoptosis by directly inhibiting a caspase inhibitor (Wang et al., 1999). In this paradigm, the caspase inhibitor blocks death at the

level of either pro- or mature caspase. Thus, in certain situations, RPR, HID, and GRIM act to directly activate already processed mature caspases. In other cases, they liberate inhibitor-bound procaspases which subsequently undergo processing via other caspase activation pathways, such as the DISC or apoptosome.

The *Drosophila* homologue of Apaf-1 is Dark (*Drosophila A*paf-1-*r*elated *k*iller) (Rodriguez et al., 1999). RPR-, HID-, and GRIM-induced death is partly suppressed by a loss of Dark in embryos, suggesting that the three activators act as upstream regulators of apoptosome-mediated caspase activation.

## Caspase inhibition

Cells employ a variety of methods to ensure that caspase activation is restricted to doomed cells. Inappropriate triggering of caspase activation is controlled at many stages; there are inhibitory mechanisms that function: 1) upstream of caspase activation, 2) at the procaspase level, and 3) directly on mature caspases. Decoy receptors and DED/CARD-containing inhibitory molecules block signal transduction of death receptors. Compartmentalization of death-inducing factors is another way to control caspase activation: the apoptotic cofactor cytochrome *c* is kept sequestered in the mitochondrial inner membrane space until appropriate death stimuli promote its release into the cytosol, where it activates Apaf-1. Procaspases and mature caspases can be directly regulated by protein phosphorylation. There are several known caspase inhibitor proteins. Work on viruses, which attenuate apoptosis to circumvent the normal host response to infection, has led to the identification of several caspase inhibitors. These include CrmA, P35 and SLP49, and a family of IAP (*i*nhibitor of *ap*optosis) proteins. Of the viral caspase inhibitors, only the IAPs are known to have mammalian members thus far.

Decoy receptors

A subgroup of TNF receptor homologues consists of decoy receptors, which function as inhibitors, rather than transducers of signaling. This subgroup includes two members that are cell-surface molecules, decoy receptor (DcR)1 and DcR2 (Ashkenazi and Dixit, 1998). Two additional members, osteoprotegerin (OPG) and DcR3 are secreted, soluble proteins. A death ligand closely related to FasL called Apo2L was discovered through DNA database screening (Pitti et al., 1996). Like FasL, Apo2L potently induces apoptosis in tumor cells. It can bind several cell-associated receptors, including DR4, DR5, DcR1, DcR2, as well as the secreted OPG. DR4 and DR5 cytoplasmic regions both contain DDs, and signal apoptosis upon ligation. DcR1 is a glycosyl phosphatidylinositol-anchored cell surface protein that resembles DR4 and DR5 but lacks a cytoplasmic tail. DcR2 has a short cytoplasmic domain that does not signal apoptosis. The extracellular domains of DcR1 or DcR2 compete with those of DR4 and DR5 for ligand binding; thus the DcRs appear to function as decoys that block Apo2L from triggering apoptosis through DR4 and DR5. DcR3 competes with Fas for FasL binding and inhibits apoptosis induction by Fas (Pitti et al., 1998). Therefore, DcR3 is a decoy receptor for FasL. By modulating the ratio of decoy receptors to death receptors, a cell can regulate its response to death ligands. Expressing decoy receptors at high levels may be a mechanism to protect against Apo2L toxicity.

FLIPs and ARC

The multiplicity of interacting proteins in DISC formation provides points of ramification, allowing induction of different effects stemming from one receptor. Adapter-like molecules can block death receptor signaling in a manner similar to decoy receptors. These include the FLIPs (*FL*ICE-*i*nhibitory *p*roteins), and ARC (*a*poptosis *r*epressor with *C*ARD). FLIPs exist in a short and long form, referred to as $FLIP_S$ and $FLIP_L$,

respectively. $FLIP_S$ contains two DEDs, much like its viral counterparts, vFLIPs. vFLIPs block death induction by interfering with the binding of DED-containing caspases to FADD. Similarly, $FLIP_S$ can effectively inhibit apoptosis triggered by all known death receptors. $FLIP_L$ contains the same two DEDs found in $FLIP_S$ plus a caspase-like domain in which a tyrosine has been substituted for the cysteine normally found in the active site. The exact role $FLIP_L$ plays in caspase regulation is controversial; some studies show that it inhibits while others indicate a proapoptotic function for $FLIP_L$ (Goltsev et al., 1997; Hu et al., 1997; Irmler et al., 1997; Shu et al., 1997). Possible explanations for the discrepancy in activity are that $FLIP_L$'s function may vary depending on its expression level or the differentiation state of the cell. As an apoptotic inhibitor, $FLIP_L$ may act similarly to $FLIP_S$ and vFLIPs. In addition, $FLIP_L$ may bind to prodomains of caspase-8 and caspase-10 via its caspase-like domain and prevent proteolytic self-processing of the caspases (Irmler et al., 1997).

Another decoy adapter molecule, ARC, is a human protein expressed in skeletal muscle and heart (Koseki et al., 1998). It contains a CARD that interacts with the prodomains of caspase-2 and caspase-8, and blocks caspase-8 processing. The factors that determine how procaspases select between their cofactors and their decoys, or how cofactors select between procaspases and their decoys, are not known.

## Compartmentalization

Compartmentalization of caspases and their cofactors is another way of regulating caspase activation. For example, cytochrome *c* is kept sequestered in the intermembrane space of the mitochondria, where it is an essential component of the respiratory electron-transport chain. As mentioned above, it also functions as a potent activator of apoptosis when released into the cytosol. In addition to cytochrome *c*, there are some procaspases that reside within the intermembrane space in certain tissues (Susin et al., 1999). For

example, procaspase-9 can be released from the mitochondria and activated in the cytosol of certain neuronal cell lines and canine ischaemic brain tissue (Krajewski et al., 1999). Once activated, it facilitates apoptosis by activating downstream caspases. Neurons and myocardiocytes have a high requirement for ATP and may therefore be more susceptible to apoptosis, as ATP is a driving force behind apoptosome formation. Localization of caspase-9 zymogen to the mitochondria provides extra protection against accidental activation of the caspase cascade.

Phosphorylation

The serine/threonine kinase known as Akt can exert its anti-apoptotic effects in a variety of ways. PI3K, an effector of Ras, generates second messengers that activate Akt (Downward, 1998). Akt phosphorylation of $Ser^{196}$ of mature caspase-9 inhibits protease activity directly (Cardone et al., 1998). Furthermore, it blocks procaspase-9 autocatalytic self-processing. It is not known how phosphorylation inhibits caspase activity. Phosphorylation may allosterically affect subunit dimerization or alter the catalytic machinery of the substrate cleft through conformational changes. Akt also phosphorylates the proapoptotic BH3-only protein Bad, preventing Bad from binding and inhibiting Bcl-$X_L$ (Datta et al., 1997). Consequently, Akt indirectly blocks caspase-9 processing by precluding apoptosome formation. Even more upstream in another caspase-activation pathway, Akt phosphorylates mammalian members of the Forkhead family, causing them to be retained in the cytoplasm where they cannot impel transcription of the gene encoding FasL (Brunet et al., 1999; Kops et al., 1999).

CrmA, P35, SLP49

Viruses can block caspase activity to inhibit host cell death. They do this with genes whose products directly bind and block caspases. The cowpox virus-encoded cytokine

response inhibitor A (CrmA) forms a stable complex with caspases and can effectively block both TNF- and Fas-mediated apoptosis (Gagliardini et al., 1994; Ray et al., 1992; Tewari et al., 1995). The product of the *p35* gene of *Autographa californica* multiply embedded nucleopolyhedrovirus (Ac*M*NPV) inhibits a broad range of caspases, acting as a "suicide" inhibitor of ICE-like and CED-3 family caspases (Bump et al., 1995; Xue and Horvitz, 1995). P35 is cleaved by caspases, generating two polypeptides that form a stable inhibitory complex with the caspase. There are two homologues of Ac*M*NPV P35, *Bombyx mori* NPV (BmNPV) P35, and SLP49 from *Spodoptera littoralis* NPV (SINPV) (Du et al., 1999). Thus far, *p35*-like genes have only been identified in viruses, but it would not be surprising if they are found to exist in metazoan genomes as well.

Inhibitor of apoptosis protein (IAP)

The IAPs constitute a subfamily of the BIR (*b*aculovirus *I*AP *r*epeat)-containing proteins (BIRPs) (reviewed in Miller, 1999). BIRPs are found in viruses, yeast, and metazoans. Evidence suggests that some BIRPs regulate cytokinesis and/or mitotic spindle function during cell division while others regulate apoptosis. IAPs were originally identified in baculovirus, where they were found to block host cell death induced by viral infection. Subsequently, the *Drosophila* IAP homologue DIAP1 was identified in a genetic screen as a modifier of RPR-induced cell death (Hay et al., 1995). DIAP1 and another fly IAP homologue, DIAP2, act as dosage-dependent suppressors of RPR- and HID-induced cell death in transgenic flies. Overexpression of DIAP1 can block death upon coexpression with the apoptotic inducer RPR in insect cell cultures (Vucic et al., 1997). Ectopic expression of certain mammalian IAPs in cultured cells can suppress apoptosis induced by a variety of stimuli including TNF and Fas (Ambrosini et al., 1997; Duckett et al., 1996).

IAPs block cell death at distinct steps in apoptotic pathways induced by Fas or another pro-apoptotic molecule, Bax (Deveraux et al., 1998). The mammalian IAPs

XIAP, cIAP1, and cIAP2 can directly bind to and inhibit procaspase-9 as well as mature caspase-9 (Deveraux et al., 1998). Since these IAPs can inhibit caspase-9, Bax-mediated caspase-3 processing through cytochrome *c* release is also blocked. XIAP and other IAPs can also directly inhibit mature caspase-3 and caspase-7 activity (Deveraux et al., 1997; Roy et al., 1997). Studies using purified, recombinant proteins *in vitro* have shown that DIAP1 and several mammalian IAPs, including XIAP, cIAP, and cIAP2, can directly inhibit procaspase processing and/or mature caspase activity by direct interaction (Deveraux et al., 1997; Hawkins et al., 1999; Roy et al., 1997). Although some studies indicate that certain BIR domains of IAPs are sufficient for this binding, the exact nature of the interaction between IAPs and pro- and mature caspases has not been characterized.

Many IAPs contain a RING finger C-terminal to one or more BIRs. However, the requirement of the RING finger for anti-apoptotic activity differs depending on the IAP and/or the nature of the apoptotic stimulus (Clem and Miller, 1994; Takahashi et al., 1998; Vucic et al., 1997). There is evidence that the different BIR domains within an IAP can perform distinct functions. During Fas-mediated apoptosis in cultured cells, ectopically expressed XIAP is cleaved by caspase (Deveraux et al., 1999). After cleavage, XIAP is separated into two molecules: one that contains two BIR domains, BIR1-2, and another part consisting of the third BIR domain and the RING finger (BIR3-RING). BIR1-2 inhibits Fas-mediated apoptosis, although with slightly less potency than full-length XIAP. In contrast, ectopic expression of BIR3-RING enhances Fas-induced cell death while potently inhibiting Bax-induced cytochrome *c*-mediated apoptosis. Studies using purified proteins revealed that BIR1-2 can directly inhibit caspase-3 and caspase-7 while BIR3-RING only inhibits caspase-9. Therefore, IAPs have potential for inhibiting different caspases depending on the repertoire of their domains. In addition to the BIRs and RING, cIAP1 and cIAP2 also contain an internal CARD domain. Although its function has not been determined, the IAP CARD can bind to ARC through a homophilic

interaction (Koseki et al., 1998). Some RING finger-containing proteins have the ability to facilitate ubiquitin-conjugating enzyme (UBC or E2)-dependent ubiquitination (Lorick et al., 1999). Since many IAPs contain RING fingers, it is conceivable that they play a role in modulating protein levels via ubiquitination. An attractive possibility is that IAPs target proapoptotic molecules, such as caspases, for degradation. Conversely, IAPs may target themselves for degradation upon receipt of appropriate death stimuli.

IAPs can bind other proteins, including several molecules implicated in cell death and survival. Among these are TNFR-associated factors (TRAFs), RPR, HID, GRIM, and DOOM (Harvey et al., 1997; Rothe et al., 1995; Vucic et al., 1997). In some cases, IAP may act to suppress cell death via their association. Other interactions may serve to disrupt IAP inhibition of caspases, resulting in caspase activation and, subsequently, cell death.

How are caspase inhibitors involved in the regulation of apoptosis?

Caspase inhibitors may function to establish thresholds that determine the concentration of active effector caspases required to initiate cell disassembly. Consequently, they avert the dire consequences of accidental proenzyme activation.

## Overview of thesis

The core components of the death machinery and their regulators have been identified in wide variety of species, including *C. elegans*, *Drosophila*, and mammals. Despite these major findings, our knowledge of the central mechanisms that underlie apoptosis is still rudimentary. Given the complexity of regulatory circuits that modulate caspase activity, there are likely to be many more molecules involved in the processes described above. Because the yeast *Saccharomyces cerevisiae* lack many of the specialized proteolytic

systems found in cells of higher eukaryotes, it provides an ideal environment to carry out screens for proteases, their regulators and their targets. Chapter 2 of this thesis describes a yeast-based system to identify caspases and their regulators. Chapter 2 also describes the isolation and *in vitro* characterization of a *Drosophila* caspase inhibitor.

How is the cell death program activated in unwanted cells and kept quiescent in others? Given that the basic mechanism of apoptosis is widely conserved, *Drosophila* provides an attractive system to address this question because it is highly tractable for experimental analysis. The strength of the *Drosophila* model system lies in its usefulness in assessing the requirement for a gene in normal apoptosis, and in placing the gene in pathways relative to previously characterized functions. How do the *Drosophila* death activators RPR, HID, and GRIM promote apoptosis? Chapter 3 describes a proposed mechanism by which RPR, HID, and GRIM trigger death: by disrupting IAP-mediated inhibition of caspase activity. Furthermore, chapter 3 also describes the role of DIAP1 as a caspase inhibitor that is required for cell survival even in the absence of RPR, HID, and GRIM.

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# CHAPTER 2

A Cloning Method to Identify Caspases and Their Regulators in Yeast: Identification of *Drosophila* IAP1 as an Inhibitor of the *Drosophila* Caspase DCP-1

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## Abstract

Site-specific proteases play critical roles in regulating many cellular processes. To identify novel site-specific proteases, their regulators and substrates, we have designed a general reporter system in *Saccharomyces cerevisiae*, in which a transcription factor is linked to the intracellular domain of a transmembrane protein by protease cleavage sites. Here we explore the efficacy of this approach using caspases, a family of aspartate-specific cysteine proteases, as a model. Introduction of an active caspase into cells that express a caspase-cleavable reporter results in the release of the transcription factor from the membrane, and subsequent activation of a nuclear reporter. We show that known caspases activate the reporter, that an activator of caspase activity stimulates reporter activation in the presence of an otherwise inactive caspase, and that caspase inhibitors suppress caspase-dependent reporter activity. We also find that whereas low or moderate levels of active caspase expression do not compromise yeast cell growth, higher level expression leads to lethality. We have exploited this observation to isolate clones from a *Drosophila* embryo cDNA library that block DCP-1 caspase-dependent yeast cell death. Among these clones we identified the known cell death inhibitor DIAP1. We showed, using bacterially synthesized proteins, that GST-DIAP1 directly inhibits DCP-1 caspase activity, but that it had minimal effect on the activity of a second *Drosophila* caspase, drICE.

## Introduction

Site-specific proteolysis plays a critical role in regulating a number of cellular processes. An important class of site-specific proteases are a group of cysteine proteases known as caspases (Alnemri et al., 1996). Extensive genetic and biochemical evidence indicates that caspases play roles as cell death signaling and effector molecules in a number of different contexts, thus making them attractive potential therapeutic targets. Caspases identified to date have been found primarily based on homology to the *C. elegans* caspase CED-3 and mammalian caspase 1, and through biochemical purification (reviewed in Nicholson and Thornberry, 1997; Salvesen and Dixit, 1997; Thornberry and Lazebnik, 1998). Viral and cellular activators and inhibitors of caspase function have also been identified in genetic and biochemical screens for regulators of apoptosis (Teodoro and Branton, 1997; Villa et al., 1997). These approaches to isolating caspases and their regulators are limited by the fact that some proteases that cleave a caspase target site and their regulators may not share primary sequence homology with the proteins identified to date, or they may be expressed only in specific tissues with limited availability for biochemical purification. Furthermore, it is clear that caspases regulate processes other than cell death, including cytokine secretion in mammals (Cerretti et al., 1992; Thornberry et al., 1992; Ghayur et al., 1997; Zhang et al., 1998; Wang et al., 1998) and cell proliferation and oogenesis in *Drosophila* (Song et al., 1997; McCall and Steller, 1998). It seems likely, given the early stage of the field, that more roles exist. Caspases and caspase regulators involved in these processes may be missed in screens that focus strictly on cell death related phenotypes. Thus, molecules that possess caspase or caspase regulatory activity may not have been identified yet. As an alternative approach to identifying novel caspases or caspase regulators it would be useful to have assays for caspase function that are based strictly on protease activity.

Because of the importance of site-specific proteolysis, we sought to develop a versatile system that would allow the identification of novel site-specific proteases, regulators of the activity of known site-specific proteases, or their substrates. Because caspase cleavage sites have been well defined, and activators and inhibitors of caspases have been identified, we set out to establish a prototype system that would allow positive selection for caspase-like proteases, their activators, and their inhibitors. Our approach to identifying these molecules employs reporters for caspase activity that function in living cells. Yeast, though eukaryotic, lacks many of the specialized proteolytic systems found in cells of higher eukaryotes. Thus it constitutes an ideal background in which to carry out function-based screens for these proteases, their regulators and their targets. Reporters for the activity of specific proteases in bacteria and eukaryotes have been developed using several strategies that involve cleavage-dependent alterations in the activity of specific proteins (McCall et al., 1994; Xu et al., 1998; Sices and Kristie, 1998; Struhl and Adachi, 1998; Lecourtois and Schweisguth, 1998; Stagljar et al., 1998). To visualize caspase activity, we created a fusion protein in which a transcription factor is linked to the intracellular domain of a transmembrane protein by caspase cleavage sites. Expression of this protein in yeast, in the presence of an active caspase, should result in release of the transcription factor from the membrane, followed by transcriptional activation of a reporter. As described below, using such a reporter system, we can visualize caspase activity in yeast, and identify proteins that act as caspase activators and inhibitors. Caspase inhibitors can also be identified by virtue of their ability to suppress caspase overexpression-dependent yeast cell death.

## Materials and Methods

## Constructs

**Yeast strains**. The W303α strain (*MATα, can1-100, leu2-3,-112, his3-11,-15, trp1-1, ura3-1, ade2-1*) was used to monitor caspase activity using the *lacZ* reporter system. EGY48 (MATa, *ura3, trp1, his3, LexAop$_6$-LEU2*) (Invitrogen) was used to monitor caspase-dependent cell killing.

**Construction of caspase target site fusion proteins.** The reporter, CLBDG6, was generated using PCR and standard techniques (details provided on request). This protein consists of, from N- to C-terminus, amino acids 1-401 of a type 1 transmembrane protein, human CD4 (Maddon et al., 1985), a linker consisting of 6 tetrapeptide caspase target sites that bracket the specificity of known caspases and granzyme B (Thornberry et al., 1997) - DEVDG-WEHDG-IEHDG-IETDG-DEHDG-DQMDG -, each of which is followed by a glycine residue, which acts as a stabilizing residue in the N-end rule degradation pathway in yeast (reviewed in Varshavsky, 1996), and finally, a transcription factor containing the LexA DNA binding domain (Horii et al., 1981). A second construct, designated CLBGG6, was generated that encodes a protein identical to CLBDG6 except that the essential P1 aspartates of the six caspase cleavage sites are replaced with glycines, rendering them nonfunctional.

**Construction of yeast expression plasmids.** Plasmids for expression of genes in yeast were derived from the pRS series (Sikorski and Hieter, 1989). To express genes in yeast under galactose-inducible control we utilized two GAL1 promoter fragments: a long version, extending from base 1-815, called GALL, and a shorter and somewhat weaker version, extending from base 406-815, called GALS (Johnston and Davis, 1984). The yeast actin terminator, bases 2107-2490 (Genbank accession L00026), was used for all constructs. GALL promoter and actin terminator fragments were inserted into pRS313 (*HIS3*), pRS314 (*TRP1*) and pRS315 (*LEU2*), generating pGALL-(*HIS3*), pGALL-

(*TRP1*) and pGALL-(*LEU2*). CLBDG6 and CLBGG6 coding regions were cloned into pGALL-(*TRP1*). GALS promoter and actin terminator fragments were inserted into pRS315, generating pGALS (*LEU2*). To express genes under the control of the copper inducible CUP1 promoter, we utilized a promoter fragment extending from base 1079-1533 of the CUP1 locus (Genbank accession K02204). Site-directed mutagenesis was used to mutate the *CUP1* promoter to prevent activation in response to glucose starvation . The mutated CUP1 promoter and actin terminator fragments were inserted into pRS315 (*LEU2*), generating pCUP1-(*LEU2*).

The coding region for the *C. elegans* caspase CED-3 (Miura et al., 1993) was introduced into pCUP1-(*LEU2*). Site-directed mutagenesis was used to generate an inactive version of CED-3 in which the active site cysteine was changed to serine (CED-3CS). Full length human caspase 7 (caspase 7FL) (Duan et al., 1996a), caspase 7 lacking the N-terminal 53 amino acid prodomain (caspase $7^{53}$), and the caspase 8 isoform corresponding to MACH$\alpha$2 (Boldin et al., 1996) or Mch5 (Fernandes-Alnemri et al., 1996) (caspase 8FL), were introduced into pCUP1-(*LEU2*). Full length DCP1 (Song et al., 1997) was introduced into pGALS-(*LEU2*) and pGALL-(*LEU2*). Site-directed mutagenesis was used to change the active site cysteine to serine (DCP-1CS). The resulting full length coding region was introduced into pGALL-(*LEU2*). Full length drICE (Fraser and Evan, 1997) was inserted into pGALL-(*LEU2*). Full length human caspase 9 (Duan et al., 1996b; Srinivasula et al., 1996) was introduced into pGALL-(*LEU2*). The region encoding amino acids 1-530 of Apaf-1 (Zou et al., 1997) (Apaf-$1^{530}$) was introduced into pGALL-(*HIS3*).

Full length DIAP1 (FLDIAP1) and a version of DIAP1 C-terminally truncated following residue 381 (DIAP1BIR) (Hay et al., 1995) were introduced into pGALL-

(*HIS3*). The mouse IAP MIHA (Uren et al., 1996) and baculovirus p35 (Clem et al., 1991) were introduced into pGALL-(*HIS3*).

## Yeast transformation and characterization

Plasmids were introduced into yeast by lithium acetate transformation. For caspase activity assays in which *lacZ* expression was monitored, yeast were transformed with pSH18-34 (*URA3*) (Invitrogen), which carries a LexA-responsive *lacZ* gene. These cells were then transformed with either pGALL-CLBDG6-(*TRP1*) or pGALL-CLBGG6-(*TRP1*). Caspase expression plasmids or an empty expression vector were introduced into these backgrounds and characterized as described below. For caspase activation and inhibition assays, a fourth plasmid was also introduced, either expressing Apaf-$1^{530}$, p35, MIHA, or with no insert. To carry out X-gal filter assays for β-galactosidase (β-gal) activity, transformants were plated on selective plates with glucose (2%) as the sugar source. After three days, duplicate colonies were picked and resuspended in 1ml of sterile Tris/EDTA, pH 8.0. One µl of each sample was streaked on a minimal medium glucose plate. After two days, a nylon membrane was used to lift the streaked yeast (yeast side upwards) onto a complete medium plate containing 2% galactose and 1% raffinose (gal/raf media) and 3µM copper sulfate. After various periods of induction the filters were processed for X-gal staining (Breeden and Nasmyth, 1985). To quantitate β-gal activity, three tubes of liquid selective gal/raf medium were inoculated with single colonies from each transformation plate, grown for 24 hours, then diluted 1:10 into fresh gal/raf selective medium containing the indicated concentration of copper sulfate, and grown for a further 10 hours. ONPG assays were performed as described by Miller (Miller, 1972). To assay

caspase-dependent cell death and protection by inhibitors, colonies carrying the relevant plasmids were streaked from 2% glucose selective media plates onto gal/raf selective media plates. The plates were photographed after three days.

## Expression and purification of recombinant *Drosophila* IAPs and caspases

The DIAP1 coding region was amplified by PCR using primers that generated an N-terminal myc epitope (EQKLISEEDL) and introduced into the GST expression vector pGEX4T-1 (Pharmacia). The GST-myc-DIAP1 fusion protein was expressed in *E. coli* strain BL21(DE3)pLysS (Novagen) and affinity purified on glutathione-Sepharose by standard methods. The eluted protein was dialyzed against buffer A [25 mM Tris (pH 8.0), 50 mM NaCl, 10 mM DTT]. Following dialysis, the protein was frozen in aliquots after addition of glycerol to 10%.

DCP-1, initiating at codon 31 (DCP-$1^{31}$), was introduced into pET23a(+) (Novagen), generating a DCP-$1^{31}$-$His_6$ fusion. A similar procedure was used to generate a drICE-$His_6$ fusion protein, in which DrICE initiates at codon 81 (drICE$^{81}$-His6). DCP-$1^{31}$-$His_6$ and drICE$^{81}$-$His_6$ were expressed in the *E. coli* strain BL21(DE3)pLysS. Protein expression and affinity purification from the soluble fraction were carried out using ProBond resin (Invitrogen), by standard methods. Eluted protein was dialyzed against buffer A and subsequently snap frozen in buffer A containing 10% glycerol.

## *Drosophila* cDNA library construction, and DCP-1 inhibitor screening

*Drosophila* embryonic polyA+ mRNA (Clontech) was converted into cDNA using a Superscript cDNA synthesis kit (GIBCO). cDNAs larger than 600 bp were ligated into a

modified version of pGALL-(*HIS3*) in which the polylinker was expanded to contain XhoI and NotI sites. ElectroMax DH10B cells (GIBCO) were transformed with the ligation mix and used to amplify the library, which contained 5 X $10^6$ primary transformants.

W303α yeast carrying pGALL-DCP-1-(*LEU2*) were transformed with 26ug of library plasmid DNA, grown in YPD for 3 hrs, washed twice to remove glucose, and plated on gal/raf selective plates. A total of 140,000 transformants were screened. Colonies were picked after 4 days of growth at 30°C. PCR was carried out on DNA isolated from individual colonies using DIAP1-specific primers.

**In vitro protease assays**

DCP-$1^{31}$-His6 and DrICE$^{81}$-$His_6$ caspase activity were measured fluorometrically by following the release of 7-amino-4-trifluoromethyl-coumarin (AFC) from Ac-DEVD-AFC (Enzyme Systems Products) using the fmax fluorescence microplate reader (Molecular Devices) with an excitation wavelength of 405 nm and an emission wavelength of 510 nm. The ability of GST-DIAP1 to inhibit caspase activity was determined from caspase activity assay progress curves, in which substrate hydrolysis (100 μM) by DCP-$1^{31}$-$His_6$ (0.2 nM) or drICE$^{81}$-$His_6$ (0.62 nM) was measured in the presence of GST (0.48 μM) or GST-mycDIAP1 (0.16 μM), in caspase activity buffer (50 mM HEPES pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.1% CHAPS, 10% sucrose and 5 mM dithiothreitol).

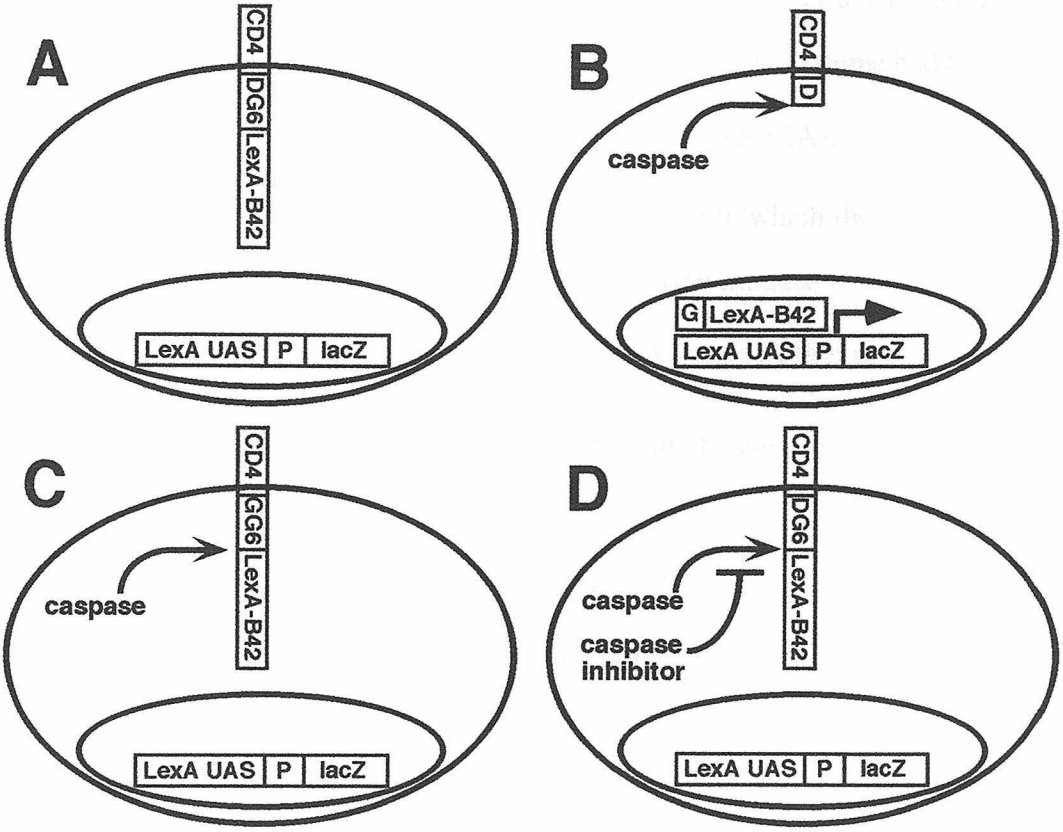
## Results and Discussion

Our approach to monitoring caspase activity in vivo was to create cells in which caspase activity stimulates transcriptional activation of a reporter. We created a fusion protein substrate for caspase cleavage in which the transcription factor LexA-B42 (LB) is linked to the truncated cytoplasmic domain of a membrane protein, CD4 (C), by a short linker (DG6) consisting of six different caspase cleavage sites that bracket the specificities of known caspases and the serine protease granzyme B, which cleaves caspases and other targets at sites of similar sequence (Material and Methods for details). When this molecule, referred to as CLBDG6, is expressed in a reporter strain in which a LexA-dependent promoter drives *lacZ* expression (LexA/β-gal reporter), levels of β-gal activity should depend on the presence of an active caspase able to cleave one or more of the introduced target sites, thereby releasing LexA-B42 from membrane association (Figure 1A, B).

### A reporter for caspase activity in yeast

We introduced CLBDG6 into the LexA/β-gal reporter strain in a plasmid, pGALL-CLBDG6, in which expression is induced in response to galactose. We introduced into this background a copper-inducible expression plasmid, pCUP1, containing either no insert or different versions of the caspase CED-3. Transformants were initially streaked on glucose medium. Colonies from these streaks were then replica plated onto gal/raf medium containing 3μM copper to induce expression of CLBDG6, and from the pCUP1 plasmid. After 12 hours of induction, levels of β-gal activity were determined using an X-gal assay in which cells that do not express β-gal remain white, while those that do turn shades of

*Figure 1.* A genetic system for monitoring caspase activity in yeast using a transcriptional reporter. Yeast were created that express a chimeric type-1 transmembrane protein (CLBDG6) in which the N-terminal signal sequence and transmembrane domain (CD4) is followed by a linker consisting of 6 tetrapeptide caspase target sites (indicated in bold) that bracket the specificity of known caspases and granzyme B (Thornberry et al., 1997) - **DEVD**G-**WEHD**G-**IEHD**G-**IETD**G-**DEHD**G-**DQMD**G -, each of which is followed by a glycine residue, which acts as a stabilizing residue in the N-end rule degradation pathway in yeast (Varshavsky, 1996). C-terminal to the caspase target site linker is a transcription factor domain, LexA-B42. The LexA-dependent transcriptional reporter consists of LexA binding sites (LexA UAS) and a promoter (P) upstream of the bacterial *lacZ* gene (*lacZ*) (A). The cells in (A) act as caspase activity reporters since expression of an active caspase results in CLBDG6 cleavage at the caspase target sites, releasing LexA-B42, which enters the nucleus and activates *lacZ* transcription (B). A version of CLBDG6 in which the P1 aspartates are changed to glycines (CLBGG6) cannot be cleaved by caspases. Cells expressing CLBGG6 act as false positive reporters for molecules that activate *lacZ* expression independent of cleavage at caspase target site (C). If cells in (B) express a caspase inhibitor as well as an active caspase, caspase activity, and thus caspase-dependent release of LexA-B42 is inhibited, and β-gal levels are decreased compared to cells that express the caspase alone (D).

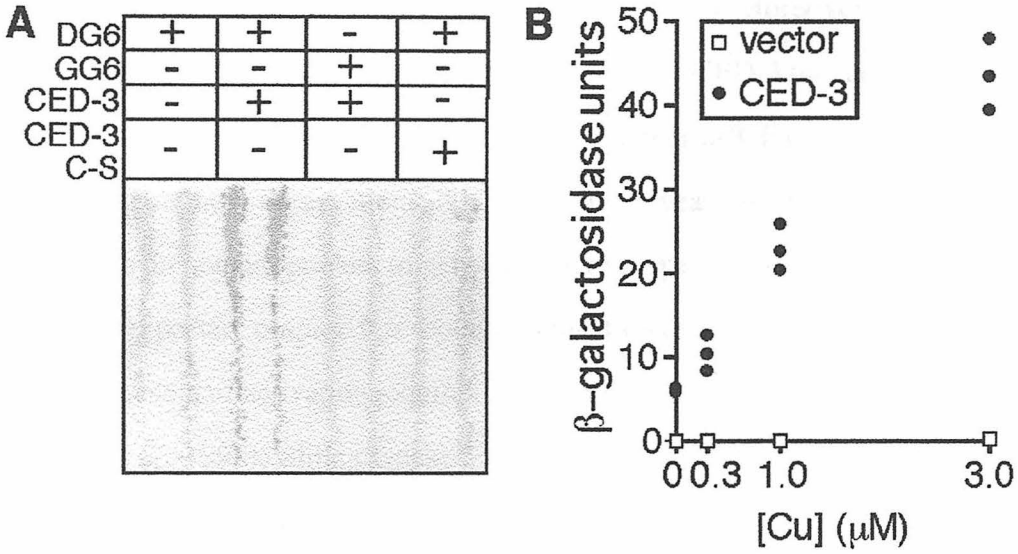

A
CD4
DG6
LexA-B42
LexA UAS
P
lacZ
B
CD4
D
caspase
G
LexA-B42
LexA UAS
P
lacZ
C
CD4
GG6
LexA-B42
caspase
LexA UAS
P
lacZ
D
CD4
DG6
LexA-B42
caspase
caspase
inhibitor
LexA UAS
P
lacZ

blue. Reporter cells that expressed CLBDG6 alone remained white in this assay (Figure 2A), indicating that yeast contains negligible amounts of proteases capable of cleaving caspase target sites under standard growth conditions. However, when expression of the *C. elegans* caspase CED-3 (pCUP1-CED-3) was induced, a high level of β-gal activity was observed (Figure 2A), which increased in a copper concentration-dependent manner (Figure 2B). Importantly, caspase activity was required for reporter activation, because expression of an inactive CED-3 mutant in which the active site cysteine had been changed to serine (CED-3CS) did not result in β-gal expression (Figure 2A).

Finally, expression of wildtype CED-3 in a reporter strain in which the essential P1 aspartates of the caspase target sites in CLBDG6 had been mutated to glycines (CLBGG6) (Figure 1C), did not result in β-gal activity (Figure 2A), arguing that the CED-3-dependent induction of β-gal activity was a direct result of cleavage of CLBDG6 at the caspase target sites.

These results establish that yeast can be used as a cell-based reporter system for caspase activity. In order for a caspase to be identified in this assay, the caspase must be active in yeast. Physiological activation of caspases occurs through multiple mechanisms, including recruitment and oligomerization at the plasma membrane, cleavage by caspases or other proteases able to recognize a caspase target site, interactions with members of the CED-4/Apaf-1 family of proteins, and autoactivation. In some cases overexpression alone is sufficient to induce autoactivation, while in other cases significant activation requires interactions with other proteins (reviewed in Cryns and Yuan, 1998; Nicholson and Thornberry, 1997; Salvesen and Dixit, 1997). Thus it is likely that only proteases in which the primary translation product is active, or in which the protease is able to autoactivate, will be identified in the simplest reporter-based caspase screen. However,

*Figure 2*. Yeast expressing CLBDG6 act as reporters for CED-3 caspase activity. W303α yeast were transformed with pSH18-34, which carries a LexA-responsive *lacZ* transcriptional cassette (the LexA/β-gal reporter strain). These cells were transformed with pGALL expression plasmids carrying CLBDG6 (DG6) or CLBGG6 (GG6). These cells also carry a copper-inducible pCUP1 plasmid, which contains either wildtype CED-3 (CED-3), an inactive C to S mutant version of CED-3 (CED-3 C-S), or nothing. Duplicate colonies from each transformation were streaked onto gal/raf medium to induce GAL1-dependent expression of the caspase substrates, and then lifted onto complete media plates with 3μM copper sulfate to induce caspase expression. After a 12 hour induction an X-gal assay was performed on the filter. Only cells expressing CLBDG6 and wild-type CED-3 have significant β-gal activity (A). Cultures from three transformants carrying pSH18-34, pGAL-CLBDG6 and either the empty pCUP1 vector or pCUP1-CED-3 were grown to stationary phase, then diluted into medium containing the indicated levels of copper sulfate and grown for a further 10 hours. ONPG assays were performed and β-gal activity was determined. β-gal activity in the CED-3-expressing cells increased as a function of copper concentration (filled circles, CED-3). No β-gal activity was found in the cultures carrying only the empty pCUP1 vector (open boxes, vector) (B).


A
DG6
GG6
CED-3
CED-3 C-S
+ + - +
- - + -
- + + -
- - - +
B
β-galactosidase units
50
40
30
20
10
0
vector
CED-3
0 0.3 1.0 3.0
[Cu] (μM)

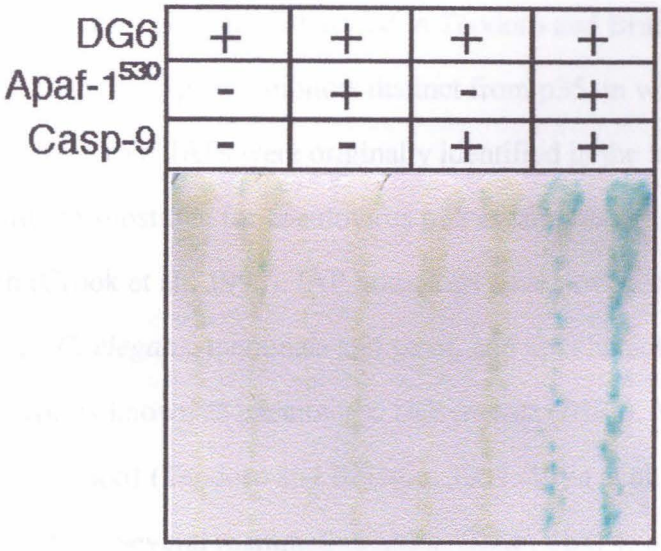
more complex screens for caspases that can activate following forced oligomerization or association with potential caspase activators (Li et al., 1997; Srinivasula et al., 1998; Yang et al., 1998b; Yang et al., 1998a) can be envisioned. We have tested several other caspases in this reporter system. Expression of mammalian caspase 7[53] (below) and full length caspase 8 (data not shown) resulted in reporter-dependent *lacZ* expression. Expression of human caspase 3, caspase 9 or *Drosophila* drICE failed to activate reporter expression (data not shown), even though active forms of these caspases are known to efficiently cleave peptides with the same sequence as the target sites introduced into the CLBDG6 (Thornberry, et al., 1997; Fraser and Evan, 1997). Moreover, although overexpression of wild-type, but not an inactive mutant of CED-3 induced yeast cell death (below), similar overexpression of caspase 3, caspase 9 or drICE had no effect on cell growth. Based on these observations, it is likely that in yeast the procaspase forms of these caspases do not autoprocess to generate active caspase heterodimers.
This result is expected: caspase 9 is thought to function as an upstream caspase, in which a major mechanism of activation requires association with Apaf-1 (Li et al., 1997; Srinivasula et al., 1998), while caspase-3 is thought to act as a downstream caspase, in which a principal mechanism of activation is cleavage by other caspases (reviewed in Cryns and Yuan, 1998; Nicholson and Thornberry, 1997; Salvesen and Dixit, 1997). drICE activation may be regulated by either of these mechanisms.

## Activators of caspase-dependent reporter activation

The fact that certain caspases do not activate in yeast suggests that it should be possible to screen for their activators as molecules that induce reporter expression in the presence of an otherwise inactive caspase. To demonstrate this we carried out an experiment in which caspase activity was monitored in yeast that expressed full length caspase 9, alone or in

combination with a fragment of Apaf-1 that is constitutively active with respect to caspase 9 processing activity in vitro (Srinivasula et al., 1998). Transformants of the pGALL-CLBDG6 LexA/β-gal reporter strain were generated that carried either two empty vectors, an empty vector and pGALL-Apaf-$1^{530}$, an empty vector and pGALL-caspase 9, or pGALL-caspase 9 and pGALL-Apaf-$1^{530}$. Transformants were initially streaked on glucose medium. Colonies from these streaks were then replica plated onto gal/raf medium to induce GALL-dependent expression. After 16 hrs of induction, levels of β-gal activity were determined using an X-gal assay. Reporter cells that expressed either nothing, caspase 9, or Apaf-$1^{530}$ alone remained white in this assay, indicating that caspase activity was not induced (Figure 3). However, colonies that expressed both caspase 9 and Apaf-$1^{530}$ showed robust β-gal activity, suggesting the occurrence of Apaf-$1^{530}$-mediated activation of the otherwise inactive procaspase 9 (Figure 3). We used β-gal activity, as assayed following replica plating of colonies, as the basis for our caspase reporter assay because caspases are conditionally expressed following replica plating. Thus, their identification is feasible even if their expression is toxic to cells. However, caspase activity screens can also be adapted to positive, survival-based screening assays by requiring LexA-dependent expression of a yeast auxotrophic marker such as HIS3 or URA3. False positives in these caspase reporter assays could arise because introduced proteins bind to the LexA binding sites and activate transcription directly, or because they encode proteases that cleave CLBDG6, but not at the caspase target sites. Both classes of false positives can be identified by the fact that they should still activate *lacZ* expression when introduced into a LexA/β-gal reporter strain that expresses CLBGG6, the false positive reporter strain (Figure 1C).

*Figure 3*. Expression of Apaf-1 induces caspase 9-dependent reporter activation. W303α containing pSH18-34 and pGALL-CLBDG6 were transformed with pGALL plasmids so as to carry either two empty pGALL vectors, Apaf-$1^{530}$ and an empty pGALL vector, caspase 9 and an empty pGALL vector, or Apaf-1 and caspase 9. Two colonies from each transformation were streaked onto selective glucose medium plates, grown for several days, and then replica plated onto complete gal/raf medium. After 16 hours an X-gal assay was performed. Only cells expressing both Apaf-1 and caspase 9 show significant β-gal activity.


DG6
Apaf-1530
Casp-9
+ + + +
- + - +
- - + +

**Suppression of caspase-dependent reporter activation by caspase inhibitors**

Once a reporter for the activity of a specific caspase has been established in yeast, it should be possible to screen for inhibitors of that activity by identifying cells that express the protease, but in which reporter activity is repressed (Figure 1D). Here we demonstrate the feasibility of this approach by showing that two different families of caspase inhibitors, exemplified by baculovirus p35, and the murine IAP MIHA, suppress caspase-dependent reporter activation. p35 is a broad specificity caspase inhibitor in which inhibition is associated with cleavage (reviewed in Teodoro and Branton, 1997). The IAPs comprise a second class of caspase inhibitors distinct from p35, in which cleavage of the inhibitor does not play a role. IAPs were originally identified in the baculovirus system by virtue of their ability to substitute for baculovirus p35 as suppressers of viral infection-induced cell death (Crook et al., 1993). IAP homologs have now been found in other viruses, *Drosophila*, *C. elegans*, mammals and yeast, and are characterized by one or more N-terminal repeats known as baculovirus IAP repeats (BIRs). Many also have a C-terminal RING finger motif (Teodoro and Branton, 1997; Villa et al., 1997; Clem et al., 1991; Uren et al., 1998). Several mammalian IAPs: XIAP, cIAP1, and cIAP2, bind to and directly inhibit caspases -3 and -7 in vitro (Deveraux et al., 1997; Roy et al., 1997). We introduced pGALL-CLBDG6 and either an empty pCUP1 plasmid or a pCUP plasmid containing CED-3 or caspase $7^{53}$ into the LexA/β-gal reporter strain along with either an empty pGALL expression vector, a pGALL expression vector carrying p35, or a pGALL expression vector carrying MIHA (murine XIAP). Transformants were grown in galactose-containing medium to induce expression of CLBDG6 and the caspase inhibitor, and then transferred to medium containing galactose and 3μM copper, to induce expression of the caspase. β-gal activity was determined following a 10 hr copper

induction. As shown in Figure 4 (Figure 4A, B), expression of p35 inhibited both CED-3 and caspase $7^{53}$ activity roughly fivefold. A similar, though somewhat weaker (approximately twofold) inhibition of caspase $7^{53}$ activity was seen in the presence of MIHA (Figure 4B), indicating that caspase-IAP interactions can be detected in this assay.

**Caspase overexpression-dependent yeast cell death**

In the above assay, the presence of a caspase inhibitor is indicated by a decrease in β-gal activity. However, in many situations it would be useful if caspase inhibition was coupled to a positive reporter output. A direct approach to identifying caspase inhibitors rests on the observation that high level expression of active caspase causes yeast cell death. Low level expression of the *Drosophila* caspase DCP-1 from the induced CUP-1 promoter did not significantly compromise yeast cell growth. Higher level expression from the GAL promoter, however, did result in cell lethality. Cells were able to grow on galactose-containing media if they carried an empty pGALL expression vector, but not if they carried a pGALL-DCP-1 expression construct (Figure 5A). This effect of DCP-1 expression was due to cell death since a greater than 250-fold decrease in the number of colony forming units was seen when cells carrying the pGALL-DCP-1 expression plasmid were grown for 12 hours in liquid gal/raf medium, thus inducing high level DCP-1 expression, and then plated on glucose-containing medium (data not shown). Importantly, GALL-DCP-1-expression-dependent cell killing depended on caspase activity because expression of an inactive mutant form of DCP-1 (DCP-1 C285S) did not cause cell death (Figure 5A). Caspase-mediated cell death in yeast may be a general phenomenon because other caspases that are active in yeast, including full length CED-3, full length caspase 8, and caspase $7^{53}$,

*Figure 4*. Expression of caspase inhibitors suppresses caspase-dependent reporter activation in yeast. The LexA/β-gal reporter strain carrying pGALL-CLBDG6 was transformed with either an empty pCUP1 plasmid or pCUP1-CED-3, and either an empty pGALL vector or pGALL-p35. Three colonies from each transformation were grown for 24 hours in selective gal/raf medium. Cultures were diluted 1:10 into fresh gal/raf medium containing 3μM copper sulfate, grown for a further 10 hours, after which ONPG assays for β-gal activity were performed. Cultures from caspase transformants showed significant β-gal activity, which was suppressed by GALL-dependent expression of baculovirus p35 (A). In an experiment similar to that described in (A), expression of caspase $7^{53}$ was induced in cells that express baculovirus p35 or the mouse IAP MIHA. Expression of caspase $7^{53}$ resulted in a significant increase in cellular β-gal activity, which was suppressed by GALL-dependent expression of p35 or MIHA (B).

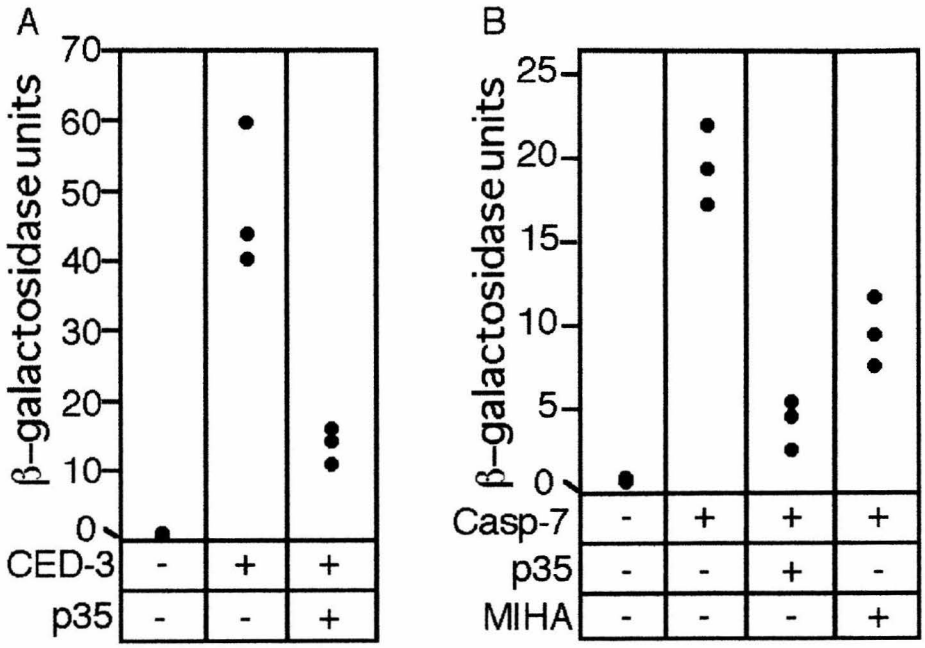
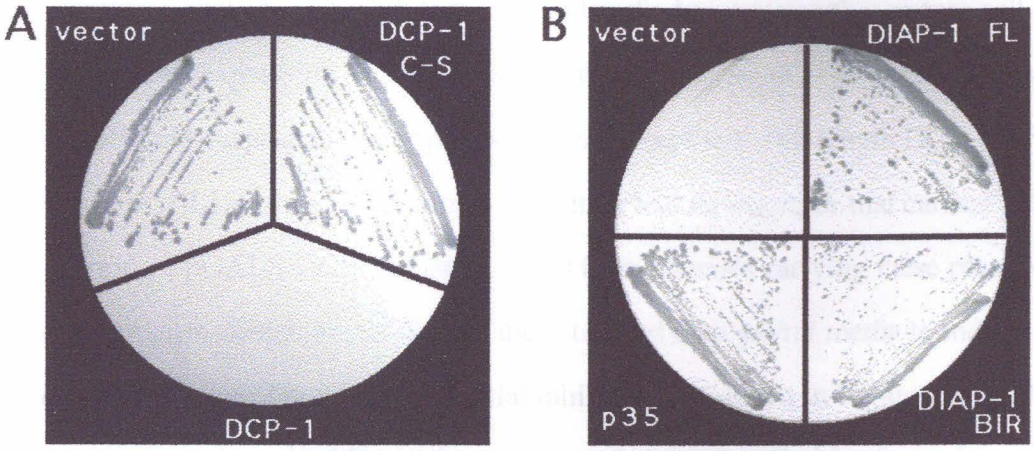

A
β-galactosidase units
70
60
50
40
30
20
10
0
CED-3 - + +
p35 - - +
B
β-galactosidase units
25
20
15
10
5
0
Casp-7 - + + +
p35 - - + -
MIHA - - - +

*Figure 5*. High level expression of the *Drosophila* caspase DCP-1 kills yeast, and is prevented by coexpression of baculovirus p35 or DIAP1. EGY48 yeast were transformed with pGALL plasmids containing either full length DCP-1 (FLDCP-1), an active site C-S mutant of DCP-1 (DCP-1 C-S), or no insert (vector). Transformants were streaked from selective glucose-containing medium onto selective gal/raf inducing medium. Cells expressing either an empty pGALS vector or the DCP-1 C-S active site mutant grow on galactose-containing medium, while cells expressing full length DCP-1 do not (A). EGY48 yeast carrying pGALS-FLDCP-1 were transformed with pGALL vectors carrying full length DIAP1 (DIAP1), the DIAP1 BIR repeats ( DIAP1 BIR), baculovirus p35 (p35), or nothing (vector). GALL-dependent expression of p35, full length DIAP1, and to a somewhat lesser extent, the DIAP1 BIR repeats, block DCP-1-dependent cell death (B).


A
vector
DCP-1
C-S
DCP-1
B
vector
DIAP-1 FL
p35
DIAP-1
BIR

block colony formation when expressed under control of the strong GALL promoter (data not shown).

## Caspase inhibitors suppress caspase overexpression-dependent cell death

To demonstrate that caspase inhibitors can be identified as proteins that restore cell viability to yeast expressing an active caspase, we carried out an experiment in which the broad specificity caspase inhibitor p35 was coexpressed with full length DCP-1. We introduced pGALL expression plasmids that either had no insert, or that carried p35, into cells carrying a pGALS-DCP-1 plasmid. Cells from colonies carrying these plasmids were grown on glucose-containing media and then streaked onto gal/raf media to induce expression of the caspase and the potential inhibitor. As shown in Figure 5B, pGALS-dependent expression of DCP-1 in the presence of an empty pGALL expression vector resulted in no cell growth. In contrast, coexpression of baculovirus p35 with DCP-1 resulted in a dramatic rescue of cell growth.

To determine if a yeast survival-based screen can be used to identify novel caspase inhibitors, we transformed yeast carrying pGALS-DCP-1 with a *Drosophila* pGALL embryonic cDNA expression library and plated these cells on gal/raf medium. In a screen of about $1.4 \times 10^5$ transformants, approximately 50 positives were obtained. These were tested by PCR and all found to correspond to DIAP1, which was originally identified as an inhibitor of *reaper* or *hid* overexpression-induced, caspase-dependent cell death in the fly eye (Hay et al., 1995). In the fly eye, and in cell culture (Vucic et al., 1998; Vucic et al., 1997; Harvey et al., 1997; Hawkins et al., 1998), it was also found that cell death could be suppressed by expression of an N-terminal DIAP1 fragment containing the two BIR repeats, but lacking the C-terminal RING finger domain. To determine if the same fragment of DIAP1 was sufficient to block DCP-1-dependent cell killing in yeast, we

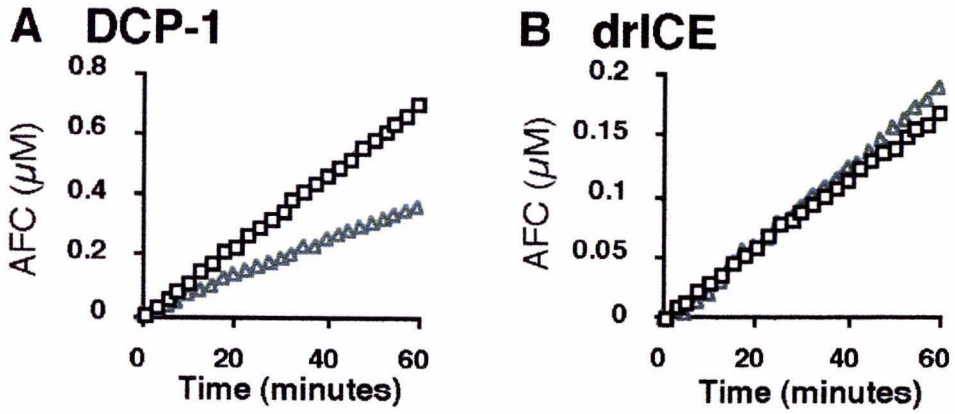
carried out an experiment in which full length DIAP1, an N-terminal fragment of DIAP1, or an empty vector, was expressed under GALL control, in the presence of GALS-driven DCP-1. As shown in Figure 5B, inhibition of DCP-1-dependent cell death was seen when DIAP1 or the N-terminal fragment of DIAP1 containing only the DIAP1 BIR repeats was coexpressed with DCP-1. To determine if the observed interaction between DIAP1 and DCP-1 was direct, we generated bacterially synthesized GST-DIAP1, as well as $His_6$ tagged versions of prodomainless DCP-1 (DCP-$1^{31}$-$His_6$) and drICE (drICE$^{81}$-$His_6$). As shown in Figure 6, GST-DIAP1 inhibited DCP-$1^{31}$-$His_6$ caspase activity, but had little, if any, effect on that of drICE$^{81}$-$His_6$. Thus, these results demonstrate that caspase inhibitors can be identified as molecules that block GAL-driven, caspase-dependent cell death.

## Concluding Remarks

We have developed a yeast cell-based assay for the activity of one group of proteases, the caspases, in which caspase activity is monitored either by the cleavage-dependent release of a transcription factor from its transmembrane anchor and subsequent activation of a reporter, or by induction of cell killing. Both reporter activation and cell killing are suppressed by known caspase inhibitors. We have exploited this fact to directly isolate caspase inhibitors from a *Drosophila* embryo cDNA library.

Yeast carrying the transcription-based caspase reporter should be useful as a background in which to carry out screens for proteins that cleave a caspase target site and for their regulators. Because yeast can be transformed with high efficiency, it also constitutes an ideal system in which to carry out large scale mutagenesis studies of particular proteases or their regulators. It may also be possible to screen for cellular targets of specific caspases by using artificial substrate libraries in which cDNA fragments substitute for the caspase target site linker in the caspase substrate fusion protein

*Figure 6.* GST-DIAP1 inhibits the caspase activity of bacterially synthesized DCP-1, but not drICE. Purified GST-DIAP1 (0.16μM) (open triangles) or GST (.48μM) (open squares) was incubated with a fixed amount of DCP-$1^{31}His_6$ (0.2nM) in caspase activity assay buffer containing 100μM of the Ac-DEVD-AFC substrate. Release of AFC was monitored fluorometrically over time. GST-DIAP1 inhibits DCP-1-dependent caspase activity (A). In similar experiments in which 0.62nM drICE$^{81}His_6$ was incubated in caspase activity buffer with GST (0.48μM), or GST-DIAP1, (0.16μM), no inhibition of drICE activity by GST-DIAP1was seen (B).


A DCP-1
AFC (μM)
0.8
0.6
0.4
0.2
0
0
20
40
60
Time (minutes)
B drICE
AFC (μM)
0.2
0.15
0.1
0.05
0
0
20
40
60
Time (minutes)

CLBDG6. A transcription-based reporter strategy similar to that described here may also provide a way to monitor caspase activity in cells of higher eukaryotes. Finally, substituting caspase cleavage sites for those of other site-specific proteases in the CLBDG6 reporter should enable the identification and study of these proteins and their regulators.

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# CHAPTER 3

## The *Drosophila* Caspase Inhibitor DIAP1 Is Essential for Cell Survival and Is Negatively Regulated by HID

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## Summary

*Drosophila* REAPER (RPR), HID and GRIM induce caspase-dependent cell death and physically interact with the cell death inhibitor DIAP1. Here we show that HID blocks DIAP1's ability to inhibit caspase activity, and provide evidence suggesting that RPR and GRIM can act similarly. Based on these results we propose that RPR, HID and GRIM promote apoptosis by disrupting productive IAP-caspase interactions, and that DIAP1 is required to block apoptosis-inducing caspase activity. Supporting this hypothesis we show that elimination of DIAP1 function results in global early embryonic cell death and a large increase in DIAP1-inhibitable caspase activity, and that DIAP1 is still required for cell survival when expression of *rpr*, *head involution defective* and *grim* is eliminated.

## Introduction

Programmed cell death, or apoptosis, is an evolutionarily conserved process by which organisms remove damaged or unwanted cells (reviewed in Wyllie et al., 1980; Raff, 1992). Central components of the machinery that carries out this process are caspases, a family of aspartate-specific, cysteine-dependent proteases (Alnemri et al., 1996). Caspases are made as zymogens comprising three functional modules: a prodomain and two catalytic subunits known as the large, or p20, and the small, or p10, subunits. In general caspases are activated in vivo following cleavage at aspartate residues that separate the prodomain from the catalytic region, and that separate the large and small subunits of the catalytic domain. The aspartates cleaved in caspase zymogens often resemble consensus target sites of known caspases. This has suggested that caspases may function in a cascade in which initiator caspases, activated by upstream death signals, cleave and activate a set of executioner caspases that carry out proteolytic cleavages of cellular proteins, leading ultimately to cell death and phagocytosis (reviewed in Nicholson and Thornberry, 1997; Salveson and Dixit, 1997; Cryns and Yuan, 1998; Thornberry and Lazebnik, 1998).

Because caspases have the potential to initiate cascades of proteolysis it is important that their activity be tightly regulated. The only cellular caspase inhibitors identified to date all share homology with a family of cell death inhibitors identified in baculoviruses, known as inhibitors of apoptosis, or IAPs (Crook et al., 1993; Birnbaum et al., 1994; reviewed in Deveraux and Reed, 1999; LaCasse et al., 1998). IAP homologous proteins have been identified in other viruses, *Drosophila*, *C. elegans*, mammals and yeast (reviewed in Uren et al., 1998). These proteins are characterized by one or more N-terminal repeats of a motif known as the baculovirus IAP repeat (BIR). Many also have a C-terminal RING finger motif. Some IAP homologous proteins have

been shown to act as cell death inhibitors. In several cases this activity is localized to the BIR repeat-containing region of the protein (reviewed in LaCasse et al., 1998; Uren et al., 1998; Deveraux and Reed, 1999), which has also been shown to mediate caspase binding and inhibition (Deveraux et al., 1997; Roy et al., 1997; Deveraux et al., 1998; Takahashi et al., 1998; Hawkins et al., 1999).

Proteins that initiate cell death by disrupting IAP-caspase interactions have not been described. However, the products of the *Drosophila reaper* (*rpr*), *head involution defective* (*hid*) and *grim* genes are interesting candidates. All three loci are located in the 75C region of the *Drosophila* genome, and deletion of this region eliminates death induced by many different stimuli, indicating that important cell death activators reside within (White et al., 1994). Furthermore, individual overexpression of any one of these genes is sufficient to initiate caspase-dependent cell death in many cells that normally live (Grether et al., 1995; Hay et al., 1995; Chen et al., 1996; White et al., 1996; Vuvic et al., 1997, 1998; Zhou et al., 1997; Wing et al., 1998). Two *Drosophila* IAPs, DIAP1, the product of the *th* locus (Hay et al., 1995), and DIAP2 (Hay et al., 1995; Ducket et al., 1996; Uren et al., 1996), suppress *rpr*-, *hid*- and *grim*-dependent cell death when overexpressed (Hay et al., 1995; Vucic et al., 1997, 1998; Wing et al., 1998), and DIAP1 and DIAP2 can immunoprecipitate RPR, HID and GRIM from insect cells (Vucic et al., 1997, 1998). Furthermore, DIAP1 binds a processed form of the caspase drICE in insect cells (Kaiser et al., 1998), and inhibits the activity of a second caspase, DCP-1 (Hawkins et al., 1999). These observations suggest several models of how *Drosophila* IAPs, RPR, HID, and GRIM, and *Drosophila* caspases might interact to regulate cell death (Kaiser et al., 1998). In one model, RPR, HID or GRIM activate caspases through an IAP-independent pathway. In this model *Drosophila* IAPs suppress apoptosis by acting as a sink for these proteins and by inhibiting caspase activation or activity initiated by their action. In a second model DIAP1 functions primarily as a caspase inhibitor, and RPR, HID and/or

GRIM initiate caspase-dependent cell death by preventing IAPs from productively interacting with caspases.

The yeast *Saccharomyces cerevisiae* provides an ideal system to study interactions between caspases and molecules that regulate their activity (Hawkins et al., 1999; Kang et al., 1999). While yeast cells do not exhibit apoptosis, and database searches have failed to uncover yeast homologs of the core apoptosis regulators identified in worms, flies and mammals, overexpression of DCP-1 or drICE results in yeast cell death that is blocked by DIAP1. Here we used yeast expressing these proteins as a system to assay the ability of RPR, HID and GRIM to disrupt IAP-caspase interactions. We found that all three proteins, while nontoxic on their own, killed yeast coexpressing DCP-1 or drICE, and DIAP1, suggesting that they were blocking DIAP1's ability to function as a caspase inhibitor. We pursued the basis for this activity further with HID and found, both in yeast and in vitro, that proteins containing the N-terminal 37 residues of HID, which are sufficient to induce apoptosis in insect cells (Vucic et al., 1998), suppressed DIAP1's ability to inhibit DCP-1 activity. We also found that the DIAP1 loss-of-function phenotype consists of an embryo-wide set of cellular changes reminiscent of apoptotic cell death, and that these were associated with the activation of DIAP1-inhibitable caspase activity. Furthermore, double mutants that remove zygotic *rpr*, *hid*, *grim*, and DIAP1 function showed phenotypes similar to those of the DIAP1 loss-of-function mutant alone. These observations suggest that a principal function of DIAP1 is to promote cell survival by blocking caspase activity, and that at least one mechanism by which RPR, HID and GRIM promote apoptosis is by disrupting IAP-caspase interactions.

## Results and Discussion

**RPR, HID and GRIM block DIAP1's ability to suppress caspase-dependent cell death in yeast**

Yeast were transformed with two plasmids: one in which DCP-1 expression was driven by the inducible GAL1 promoter, and a second in which DIAP1 expression was driven by the constitutive Adh promoter (hereafter referred to as DCP-1-DIAP1 yeast). These cells were then transformed with a second GAL1 expression plasmid that was either an empty vector, or that carried GAL1-driven RPR, HID or GRIM. Transformants were spotted as a series of tenfold serial dilutions onto glucose plates to indicate the number of cells, and onto galactose plates to induce expression of DCP-1 and RPR, HID or GRIM. DCP-1-DIAP1 yeast carrying an empty vector survived on galactose, but DCP-1-DIAP1 yeast expressing HID or GRIM did not (Figure 1A). Expression of RPR under GAL1 control did not kill DCP-1-DIAP1 yeast in which DIAP1 expression was driven by the Adh promoter, but it was able to kill DCP-1-DIAP1 yeast in which DIAP1 expression was driven by a weaker promoter, the copper-inducible CUP-1 promoter in the presence of 30μM Cu++ (Figure 1A). Importantly, expression in yeast of RPR, HID or GRIM alone under GAL1 control had no effect on cell growth as compared with yeast expressing only the empty vector (Figure 1A). These results suggest that RPR, HID and GRIM are able to block DIAP1's ability to inhibit DCP-1 caspase activity.

Three other *Drosophila* caspases, drICE and DCP-2/Dredd, and DRONC have been implicated as effectors of apoptosis (Fraser et al., 1997, 1998; Chen et al., 1998; Dorstyn et al., 1999). We wanted to determine if RPR, HID and GRIM played a similar role in regulating their activity. We focused our attention on drICE. A version of drICE lacking the prodomain is not inhibited by DIAP1 (Hawkins et al., 1999), but a version of drICE that contains prodomain sequences is (Hawkins and Hay, unpublished observations). Full length drICE expressed in yeast under GAL1 control neither kills nor

*Figure 1*. RPR, HID and GRIM block DIAP1's ability to suppress caspase-dependent yeast cell death.

(A) RPR, HID and GRIM kill yeast kill yeast expressing DCP-1 and DIAP1.

Top: GAL1-driven DCP-1 causes yeast cell death which is prevented by Adh-dependent expression of DIAP1 (DCP-1 + Adh-DIAP1 + vector). GAL1-driven HID (DCP-1 + Adh-DIAP1 + HID) or GRIM (DCP-1 + Adh-DIAP1 + GRIM) results in yeast cell death. Middle: GAL1-driven RPR kills yeast expressing DCP-1 and CUP1-driven DIAP1 DCP-1 + CUP-DIAP1 + RPR) in the presence of 30mM Cu++. Bottom: Expression of RPR, HID or GRIM in the presence of empty vectors does not affect yeast cell viability.

(B) RPR, HID and GRIM kill yeast expressing rev-drICE and DIAP1

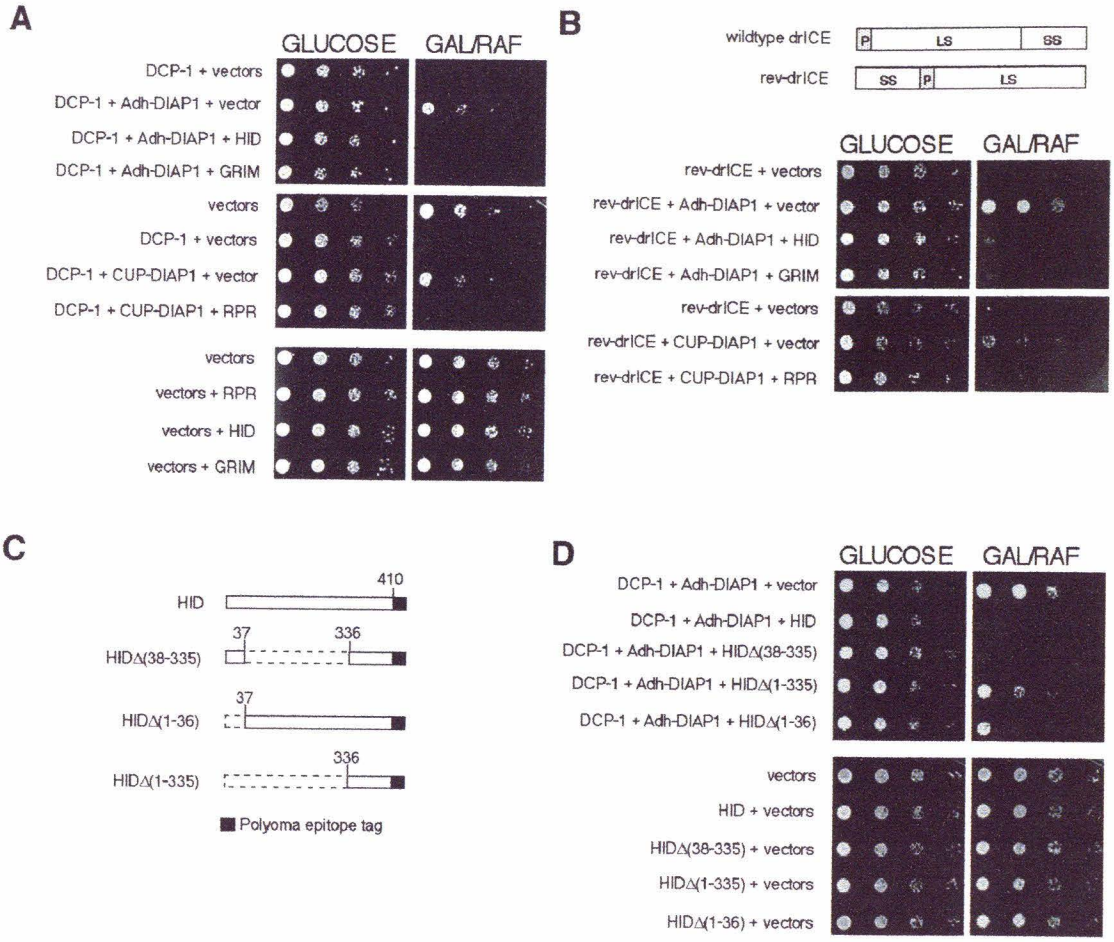
Top: drICE contains prodomain (P), large (LS) and small (SS) subunits. In reversed drICE (rev-drICE) the predicted drICE small subunit was placed N-terminal to drICE prodomain and large subunit sequences. Middle: Expression of HID or GRIM kills yeast expressing DCP-1 and Adh-DIAP1 (rev-drICE + Adh-DIAP1 + HID; rev-drICE + Adh-DIAP1 + GRIM).

Bottom: Expression of RPR kills yeast expressing rev-drICE and CUP-DIAP1 (rev-drICE + CUP-DIAP1 + RPR) in the presence of no added Cu++.

(C) Diagram of HID constructs. Numbers represent amino acid positions in full length HID. Solid lines indicate regions of HID present in the fusion protein, dashed lines sequences that are deleted. A polyoma epitope tag present at the C-terminus of each coding region is indicated by a black bar.

(D) Proteins containing the first 37 residues of HID block DIAP1's ability to inhibit DCP-1 activity in yeast.

Top: Effects of expression of HID fusion proteins on the viability of DCP-1 + Adh-DIAP1 yeast. Bottom: Effects of expression of HID fusion proteins and empty vectors on yeast viability.


A
GLUCOSE
GAL/RAF
DCP-1 + vectors
DCP-1 + Adh-DIAP1 + vector
DCP-1 + Adh-DIAP1 + HID
DCP-1 + Adh-DIAP1 + GRIM
vectors
DCP-1 + vectors
DCP-1 + CUP-DIAP1 + vector
DCP-1 + CUP-DIAP1 + RPR
vectors
vectors + RPR
vectors + HID
vectors + GRIM
B
wildtype drICE
rev-drICE
P
LS
SS
GLUCOSE
GAL/RAF
rev-drICE + vectors
rev-drICE + Adh-DIAP1 + vector
rev-drICE + Adh-DIAP1 + HID
rev-drICE + Adh-DIAP1 + GRIM
rev-drICE + vectors
rev-drICE + CUP-DIAP1 + vector
rev-drICE + CUP-DIAP1 + RPR
C
HID
410
HIDΔ(38-335)
37
336
HIDΔ(1-36)
37
HIDΔ(1-335)
336
Polyoma epitope tag
D
GLUCOSE
GAL/RAF
DCP-1 + Adh-DIAP1 + vector
DCP-1 + Adh-DIAP1 + HID
DCP-1 + Adh-DIAP1 + HIDΔ(38-335)
DCP-1 + Adh-DIAP1 + HIDΔ(1-335)
DCP-1 + Adh-DIAP1 + HIDΔ(1-36)
vectors
HID + vectors
HIDΔ(38-335) + vectors
HIDΔ(1-335) + vectors
HIDΔ(1-36) + vectors

activates a cleavage-dependent reporter, probably because the caspase does not significantly autoactivate (Hawkins et al., 1999). We created a form of drICE that is active in yeast and that contains prodomain sequences by generating a "reversed" form of drICE, rev-drICE, in which the drICE p10 domain was placed N-terminal to prodomain and p20 sequences (Figure 1B). Reversed forms of mammalian caspases that are not otherwise active are constitutively active (Srinivasula et al., 1998). As shown in Figure 1B, expression of rev-drICE under GAL1 control kills yeast and this death is prevented by coexpression of DIAP1. Importantly, as with DCP-1, expression of HID or GRIM or RPR killed yeast coexpressing rev-drICE and DIAP1.

**The N-terminus of HID mediates its ability to disrupt IAP-caspase interactions in yeast**

In insect cells the N-terminal 37 amino acids of HID, expressed in the context of a larger fusion protein, are necessary and sufficient to induce apoptosis and to mediate HID's binding to DIAP1 (Vucic et al., 1998). To test the activity of these sequences in yeast we generated GAL1 expression constructs similar to those of Vucic et al., (1998) in which the first 37 amino acids of HID were either present or absent (Figure 1C). HIDΔ(38-335) encodes a protein in which the first 37 amino acids of HID are fused to the C-terminal 74; HIDΔ(1-335) consists of only the last 74 residues of HID, and HIDΔ(1-36) encodes a protein that lacks the first 36 residues of HID, but contains the rest of the full length HID coding sequence. GAL1-dependent expression of HIDΔ(38-335) in DCP-1-DIAP1 yeast resulted in no growth. In contrast, DCP-1-DIAP1 yeast expressing HIDΔ(1-335) grew normally. HIDΔ(1-36) had weak killing activity in this assay (Figure 1D), but not in insect

cell death assays (Vucic et al., 1998), or in other in vitro assays described below. As with full length HID, expression of HIDΔ(38-335), HIDΔ(1-335) or HIDΔ(1-36) in isolation had no effect on yeast cell growth (Figure 1D). Thus, proteins carrying the N-terminal 37 residues of HID, which are sufficient to kill insect cells, are also able to kill DCP-1-DIAP1 yeast.

**HID suppresses DIAP1's ability to inhibit DCP-1 caspase activity in vitro**

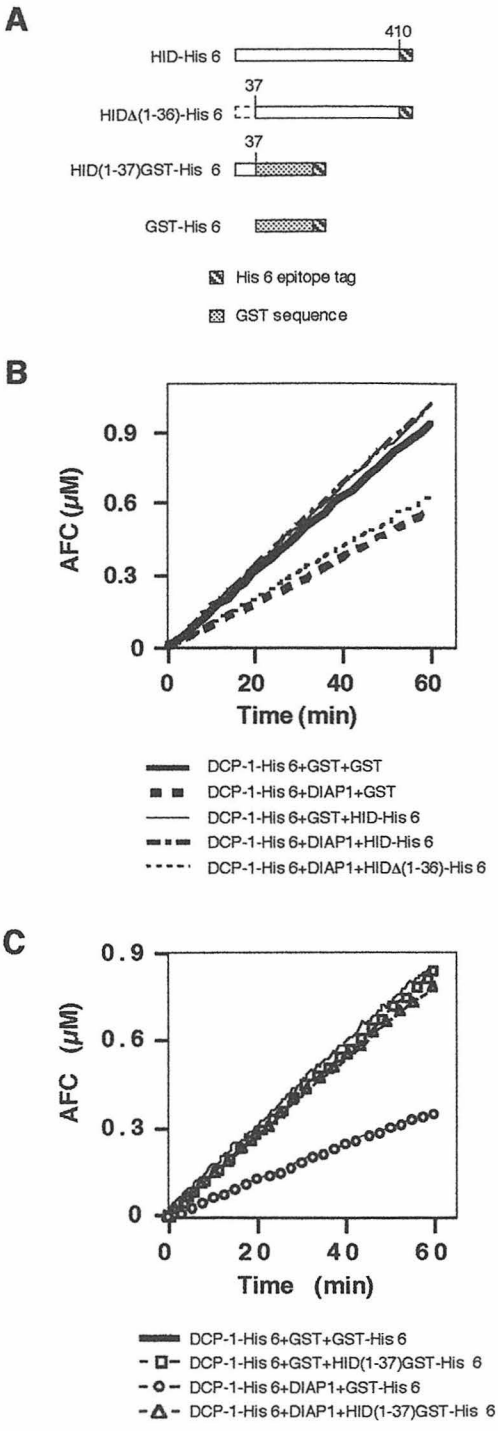
The results of our yeast experiments suggested that HID directly inhibits DIAP1. We tested this idea in vitro using purified proteins. Bacterially expressed and purified versions of DCP-1, DIAP1 and HID (Figure 2A) were mixed in various combinations, and DCP-1 caspase activity measured fluorometrically (Figure 2B, C). His6-tagged DCP-1 (DCP-1-His6) is active as a caspase, and this activity was inhibited by DIAP1 but not by GST (Hawkins et al., 1999; Figure 2B). Addition of HID alone to a reaction containing DCP-1-His6 did not alter DCP-1-His6 activity, indicating that HID has no direct effect on DCP-1-His6 (Figure 2B). However, when full length HID was incubated with DIAP1 and DCP-1-His6, DIAP1-dependent inhibition of DCP-1-His6 activity was lost (Figure 2B). Incubation of DIAP1 and DCP-1-His6 with HIDΔ(1-36)-His6 had little or no effect on DIAP1's ability to inhibit DCP-1-His6 activity, indicating that the first 37 residues of HID are necessary for this activity. To determine if the first 37 residues of HID were sufficient to mediate this effect, we carried out experiments using a version of HID that consists of the first 37 residues of HID fused to GST and a C-terminal His6 tag (HID(1-37)GST-His6). As shown in Figure 2C, addition of HID(1-37)GST-His6 to an assay containing DCP-1-His6 and DIAP1 blocked DIAP1's ability to inhibit DCP-1-His6 activity, while

*Figure 2*. Proteins containing the first 37 residues of HID block DIAP1's ability to inhibit DCP-1 activity in vitro.

(A) Schematic of HID constructs. HID coding regions are represented by open boxes, deleted regions by dashed lines.

(B) The N-terminal 36 residues of HID are required to inhibit DIAP1 function. DCP-1-His6 (0.2 nM) activity was measured by following the release of AFC fluorometrically from the Ac-DEVD-AFC substrate over time. The total protein concentration in each incubation was kept constant, with GST (0.2μM or 0.77μM) being used to replace DIAP1 (0.2μM), HID-His6 (0.44μM) or HIDΔ(1-36)-His6 (0.44μM), as the assay required.

(C) A protein containing the N-terminal 37 residues of HID, HID(1-37)GST-His6, is sufficient to block DIAP1's caspase inhibitory activity. Assays were performed as in (B).


A
410
HID-His 6
37
HIDΔ(1-36)-His 6
37
HID(1-37)GST-His 6
GST-His 6
His 6 epitope tag
GST sequence
B
AFC (μM)
0.9
0.6
0.3
0
0
20
40
60
Time (min)
DCP-1-His 6+GST+GST
DCP-1-His 6+DIAP1+GST
DCP-1-His 6+GST+HID-His 6
DCP-1-His 6+DIAP1+HID-His 6
DCP-1-His 6+DIAP1+HIDΔ(1-36)-His 6
C
AFC (μM)
0.9
0.6
0.3
0
0
20
40
60
Time (min)
DCP-1-His 6+GST+GST-His 6
DCP-1-His 6+GST+HID(1-37)GST-His 6
DCP-1-His 6+DIAP1+GST-His 6
DCP-1-His 6+DIAP1+HID(1-37)GST-His 6

addition of an equivalent amount of GST-His6 had no effect. Thus, consistent with our observations in yeast, purified HID directly blocks DIAP1's ability to inhibit DCP-1 caspase activity, and the first 37 amino acids of HID, at least in the context of a larger fusion protein, are necessary and sufficient for this activity.

To determine if HID directly interacts with DIAP1 or DCP-1, we carried out binding experiments. As expected from the results of our yeast and in vitro activity assays, GST-DIAP1 coupled to glutathione-Sepharose bound full length HID-His6 and DCP-1-His6, but did not appreciably bind HIDΔ(1-36)-His6. GST alone showed no interaction with any of these proteins (Figure 3A). Also, a maltose binding protein-DIAP1 fusion protein (MBP-DIAP1) bound to amylose resin was able to bind HID(1-37)GST-His6, while MBP bound to the same resin was not (Figure 3B). Furthermore, HID(1-37)GST-His6, bound to glutathione Sepharose beads was able to bind $^{35}$S-methionine labeled, in vitro translated DIAP1, but not in vitro translated DCP-1 (data not shown). These observations, in conjunction with the fact that HID-His6 or HID(1-37)GST-His6 alone had no effect on DCP-1-His6 caspase activity (Figure 2B,C), suggest that HID's effects on caspase activity are mediated through DIAP1.

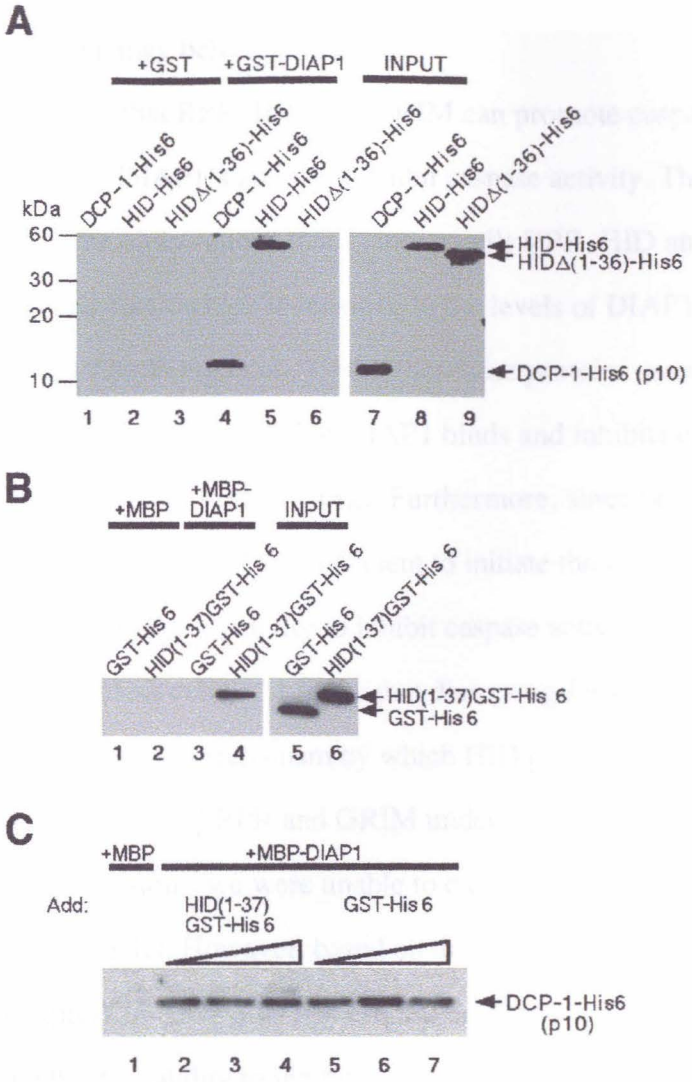
To explore the mechanism by which HID blocks DIAP1's ability to inhibit DCP-1, we asked if HID(1-37)GST-His6 was able to displace DCP-1-His6 from DIAP1. MBP-DIAP1 bound to amylose resin was preincubated with increasing amounts of GST or HID(1-37)GST-His6 followed by addition of DCP-1-His6. Binding reactions were followed by washes and analysis of the bead bound proteins by Western blotting. As shown in Figure 3C, DCP-1 binding to MBP-DIAP1 was not appreciably decreased by the presence of up to a 20-fold molar excess of GST-His6 or HID(1-37)GST-His6 over MBP-DIAP1. As expected, MBP beads incubated with HID(1-37)GST-His6 showed no binding to DCP-1-His6. Thus, we see no evidence that HID(1-37)GST-His6, at molar ratios with respect to DIAP1 greater than those used to demonstrate inhibition of DIAP1

*Figure 3*. Physical interactions between HID, DIAP1 and DCP-1

(A) DIAP1 binds DCP-1 and HID, and the first 37 residues of HID are required for this interaction. Glutathione Sepharose-bound GST or GST-DIAP1 was incubated in binding buffer with DCP-1-His6, HID-His6 or HIDΔ(1-36)-His6 (Figure 2A). Binding reactions were followed by washes and Western blotting. A fraction of the input of DCP-1-His6, HID-His6 and HIDΔ(1-36)-His6 is shown.

(B) DIAP1 binds proteins containing only the first 37 residues of HID. Amylose resin-bound MBP or MBP-DIAP1 was incubated with GST-His6 or HID(1-37)GST-His6 and processed as above.

(C) HID(1-37)GST-His6 does not prevent DCP-1-His6 from binding MBP-DIAP1. MBP-DIAP1 bound to amylose resin was incubated with increasing amounts of HID(1-37)GST-His6 or GST-His6. Following this preincubation a constant amount of DCP-1-His6 was introduced into the mixture. The beads were washed and processed for Western blotting using an anti-His antibody to detect DCP-1-His6. MBP beads incubated with the highest concentration of HID(1-37)GST-His6 and equivalent amounts of DCP-1-His6 were processed in a similar manner.


A
+GST
+GST-DIAP1
INPUT
kDa
DCP-1-His6
HID-His6
HIDΔ(1-36)-His6
60
30
20
10
HID-His6
HIDΔ(1-36)-His6
DCP-1-His6 (p10)
1 2 3 4 5 6 7 8 9
B
+MBP
+MBP-DIAP1
INPUT
GST-His 6
HID(1-37)GST-His 6
HID(1-37)GST-His 6
GST-His 6
1 2 3 4 5 6
C
+MBP
+MBP-DIAP1
Add:
HID(1-37) GST-His 6
GST-His 6
DCP-1-His6 (p10)
1 2 3 4 5 6 7

function in caspase activity assays (Figure 2C), prevents binding of DCP-1-His6 to MBP-DIAP1. These observations suggest that HID(1-37)GST-His6 does not prevent DIAP1 from inhibiting DCP-1 activity by blocking DCP-1 binding. It is important to note, however, that full length HID, which has a large C-terminal domain that can regulate HID activity (Bergmann et al., 1998), may behave differently.

Our observations suggest that RPR, HID and GRIM can promote caspase-dependent cell death by blocking DIAP1's ability to inhibit caspase activity. These conclusions are supported by the observations that in insect cells RPR, HID and GRIM promote caspase-dependent cell death which is sensitive to the levels of DIAP1 (Hay et al., 1995; Vucic et al., 1997, 1998; Wing et al., 1998), that these proteins associate with DIAP1 in vivo (Vucic et al., 1997, 1998), and that DIAP1 binds and inhibits caspases (Kaiser et al., 1998; Hawkins et al., 1999; this work). Furthermore, since proteins that contain only the first 37 amino acids of HID are sufficient to initiate the caspase-dependent death of insect cells and to block DIAP1's ability to inhibit caspase activity, but other regions of HID lack activity in insect cells, it is likely that disrupting DIAP1's ability to inhibit caspase activity is an important mechanism by which HID promotes cell death. Because of difficulties with solubility of RPR and GRIM under conditions that allowed productive DIAP1-caspase interactions, we were unable to carry out in vitro experiments similar to those carried out with HID. However, based on the fact that they behaved similarly to HID in yeast expressing DCP-1 or rev-drICE, and DIAP1, it seems likely that they also are able to block DIAP1's ability to inhibit caspase activity.

Our observations do not exclude the possibility that RPR, HID and GRIM promote apoptosis through other pathways as well. For example, RPR has been shown to promote apoptosis in a *Xenopus* system (Evans et al., 1997) through association with a novel protein, SCYTHE (Thress et al., 1998), and peptides corresponding to N-terminal sequences of RPR and GRIM block K+ channel function (Avdonin et al., 1998).

Furthermore, as described below, removal of DIAP1 in the embryo results in massive cell death, but the DIAP1 mutant cells do not show all the features of apoptosis seen in wildtype embryos, consistent with the idea that other pathways may be necessary to create a complete apoptotic response.

## DIAP1 mutant embryos show global early morphogenetic arrest and cell death associated with an increase in DIAP1-inhibitable caspase activity

If disruption of IAP-caspase interactions is an important mechanism by which cell death is initiated in *Drosophila*, then caspase activity must be continually held in check by DIAP1 and/or other IAPs in order to block an ever present death signal. To explore this possibility we characterized the embryonic phenotype of mutations (alleles of *th*) that disrupt DIAP1 function.

Homozygous *th* mutant embryos exhibit a severe terminal embryonic lethal phenotype and do not form a cuticle (Figure 5G, and data not shown). We monitored early development of loss of function *th* embryos from $th^5$, a strong loss-of-function allele, and a deficiency for the region, *Df(3R)brm11* (Figure 4). $th^5$ and *Df(3R)brm11* homozygous embryos undergo normal cellularization and initial gastrulation movements such as ventral furrow formation, cephalic furrow formation and posterior midgut invagination (Figure 4A-C). However, during germband extension, within 15 to 30 min after the onset of gastrulation movements, virtually all morphogenetic movements cease (Figure 4A-C). While the stage of initial arrest is somewhat variable in time (compare Figure 4B and C with respect to extent of ventral furrow formation and posterior midgut invagination), within the next hour all the cells adopt a rounded morphology and the yolk cell fragments and eventually moves to the surface of the embryo. The disruption of embryonic integrity is seen particularly clearly in Figures 4D-G, in which wildtype and $th^5$mutant embryos are

*Figure 4*. Removal of zygotic DIAP1 leads to morphogenetic arrest during germband extension and global cell shape changes.

(A) Wild type developmental series of living embryos derived from a$th^5$/TM3[ftz::lacZ] stock during gastrulation and germband extension (stages 6 through 9). Arrowheads mark the position of the cephalic furrow and arrows indicate the front of the extending germband.

(B), (C) $th^5$ and *Df(3L)brm11* homozygous embryos, respectively, at the same developmental stages as in (A).

(D), (F) Scanning electron micrographs of balancer bearing embryos from $th^5$/TM3[ftz::lacZ] stock. The right-hand panels show higher magnification of the general morphology of the embryonic surface. (D) shows an embryo at stage 8, (F) shows an embryo at stage 9.

(E), (G) Similar staged embryos homozygous for $th^5$.

Scale bars are 100 μm in (C) and (G) (left-hand panel), and 10μm in (G) (right-hand panel).

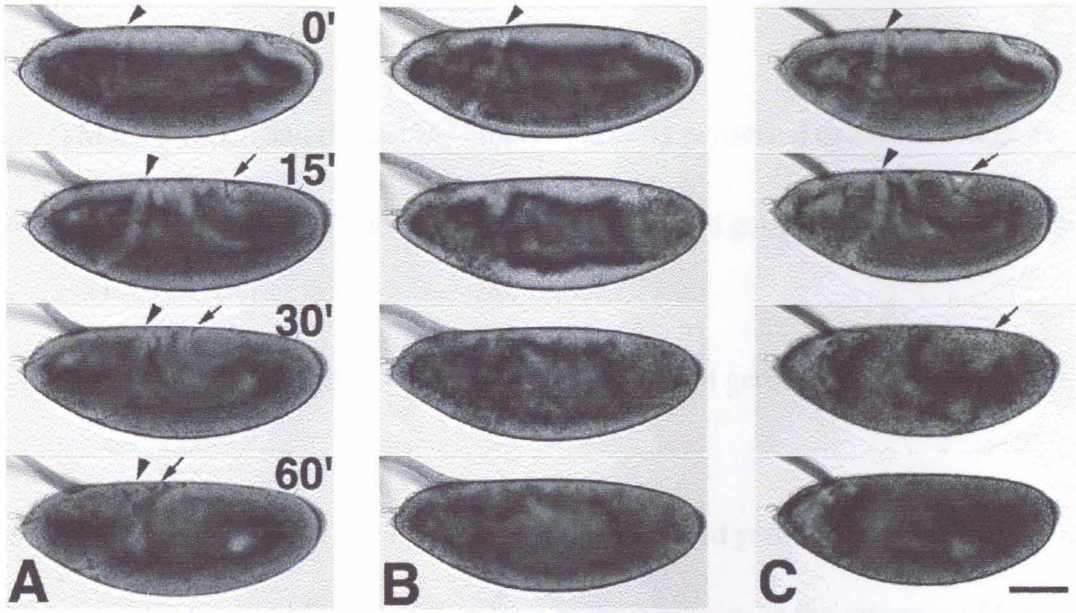

0'
15'
30'
60'
A
B
C
D
E
F
G

*Figure 5*. Cells in embryos homozygous mutant for DIAP1 show premature and increased DNA fragmentation and an increase in DIAP1-inhibitable caspase activity.

(A-F) Confocal images of staged embryos from stocks of $th^5$/TM3[ftz::lacZ], and (G, H)$th^6$/TM3[ftz::lacZ] double labeled for TUNEL (green) and β-galactosidase (red). Scalebar is 100μm.

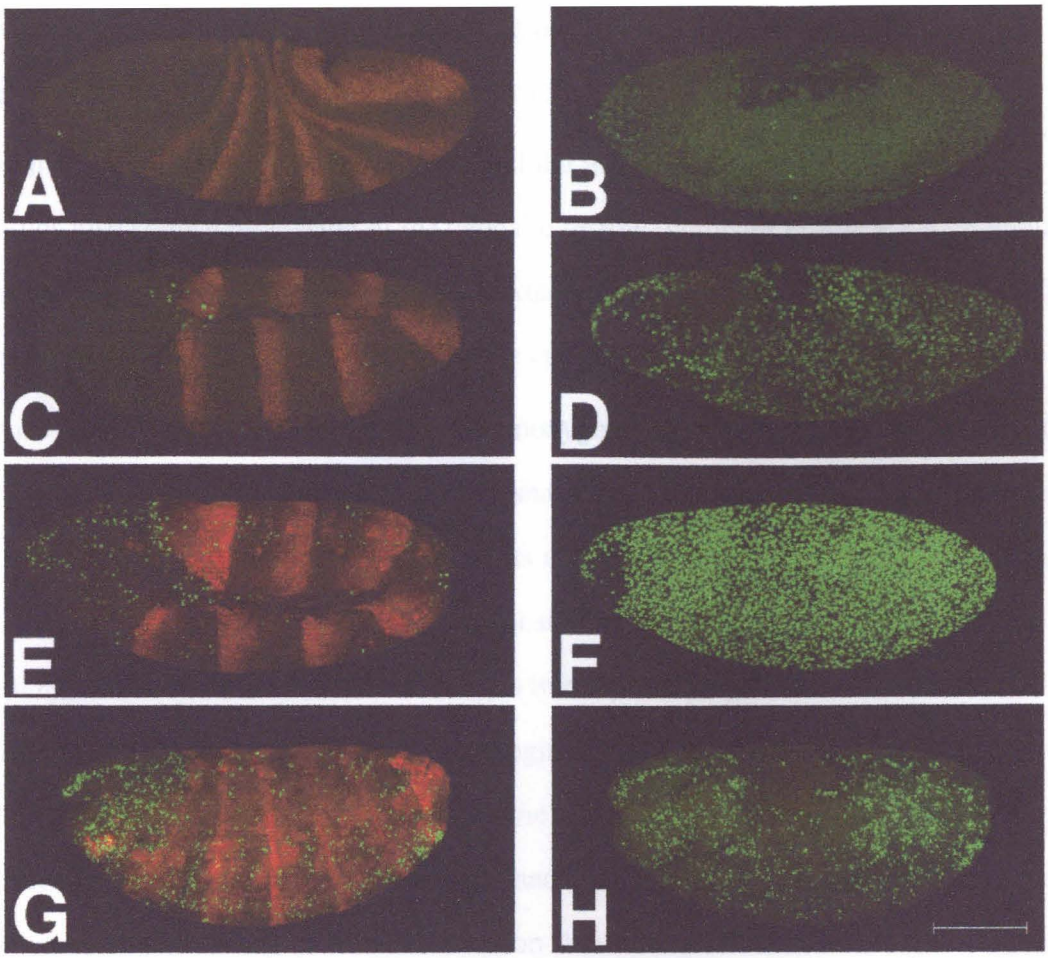
(A) $th^5$/TM3[ftz::lacZ], and (B) $th^5/th^5$ embryos at early germband extension (stage 8).

(C) $th^5$/TM3[ftz::lacZ], and (D) $th^5/th^5$ embryos at extended germband stage (early stage 9).

(E) $th^5$/TM3[ftz::lacZ], and (F) $th^5/th^5$ embryos at extended germband stage (later stage 9).

(G) $th^6$/TM3[ftz::lacZ], and (H) $th^6/th^6$ embryos at retracted germband stage (stage 14).

(I) Caspase activity of wildtype and *th* mutant embryo extracts in the presence of GST or DIAP1. 1μg of 2-4 hour embryo extract from stocks of *w*-, $th^5$/TM3 or $th^6$/TM3 w- was introduced into caspase activity buffer containing 100μM of the Ac-DEVD-AFC substrate and 0.5μg of either GST or DIAP1. Release of AFC was monitored fluorometrically over time.


A
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C
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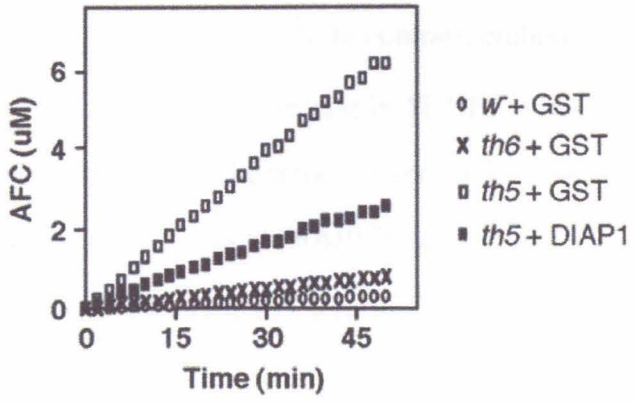
I


AFC (uM)
0
2
4
6
0
15
30
45
Time (min)
w+ GST
th6 + GST
th5 + GST
th5 + DIAP1

visualized using scanning electron microscopy. While some $th^5$ homozygous embryos showed normal morphology at the beginning of germband extension (data not shown), other embryos with a similar degree of extension movements showed extensive cell rounding, suggesting a disruption of intercellular adhesion (Figure 4D, E). All $th^5$ embryos also show a regression of the cephalic furrow (compare Figure 4A with Figure 4B and C, and Figure 4D with 4E). At the extended germband stage (stage 9), the surface of cells in control embryos is smooth and the cells are closely apposed to each other (Figure 4F). In contrast, by this time $th^5$ homozygotes have adopted a uniform cellular morphology in which all cells have a round shape, the surface of the cells contains many membrane blebs, and vesicular cell fragments are present similar to those seen in dying cells (Figure 4G). The yolk cell, normally located interiorly, is visible at the surface. Thus, zygotic loss of DIAP1 function results in early morphogenetic arrest followed by severe morphological abnormalities at the beginning of germband extension.

We used TUNEL labeling to determine whether the massive DNA fragmentation characteristic of cells undergoing apoptosis had occurred in cells of *th* mutants (Figure 5). Wildtype embryos during germband extension showed significant DNA fragmentation in only a few cells (Figure 5 A,C, E). In contrast, comparably staged $th^5$ embryos labeled extensively in most if not all cells. Initially, the TUNEL signal is relatively weak (Figure 5B, D), but the intensity increases within the following hour of development to a strong uniform labeling (Figure 5B,D,F). In contrast, embryos homozygous for the weaker allele $th^6$ do not show a significant increase in TUNEL positive cells as compared to comparably staged control embryos at the retracted germband stage (stage 14) (Figure 5G,H).

To determine if loss of DIAP1 in fact results in an increase in caspase activity, we measured caspase activity in extracts of 3-5 hour old embryos from wildtype, $th^5$ and $th^6$ stocks by following the release of Ac-DEVD-AFC fluorometrically (Figure 5I) . Extracts

from wildtype and $th^6$ embryos had very low levels of caspase activity. In contrast, extracts from $th^5$ embryos had very large amounts of caspase activity. Importantly, the increased caspase activity present in the $th^5$ embryonic extracts was inhibited by the addition of purified DIAP1 (Figure 5J), consistent with the idea that DIAP1 normally represses these caspases.

The caspase targets of DIAP1 in the *Drosophila* embryo or other tissues have not been identified. Five caspases have been identified in *Drosophila*: DCP-1 (Song et al., 1997), drICE (Fraser and Evan, 1997), DCP-2/Dredd (Inohara et al., 1997; Chen et al., 1998), DRONC (Dorstyn et al., 1999), and a fifth gene (C. J. H. and B. A. H., unpublished). DIAP1 is able to inhibit the activity of the three caspases we have tested: DCP-1 (Hawkins et al., 1999), drICE (this report) and DRONC (C. Hawkins, unpublished). The DIAP1 embryonic phenotype may result from the unrestrained activity of zygotically expressed versions of these caspases. Alternatively, DIAP1 may also function in the early embryo to block the activity of caspases introduced late during oogenesis into the developing oocyte from the degenerating nurse cells. (Cavaliere et al., 1998; Chen et al., 1998; Foley and Cooley, 1998; McCall and Steller, 1998; Dorstyn et al., 1999).

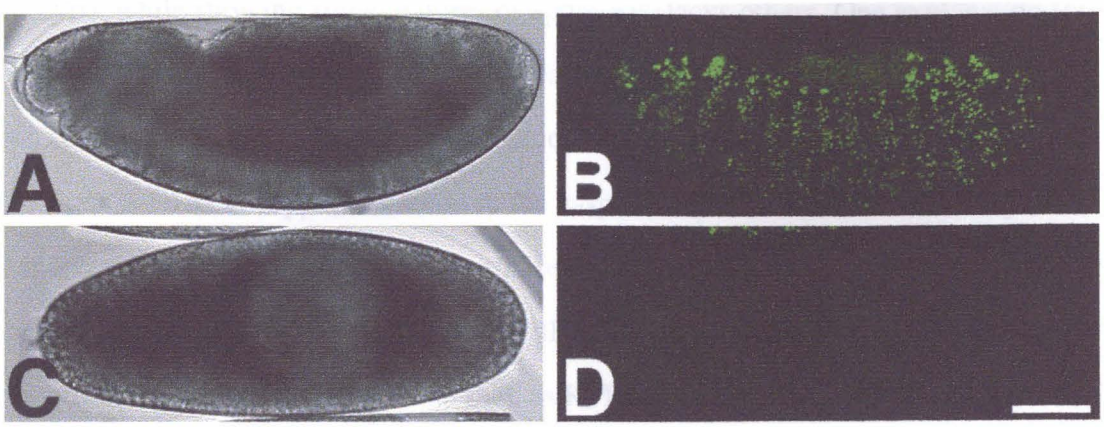
## DIAP1 loss-of-function mutant embryos do not stain with acridine orange, a marker of apoptotic cell death

Acridine orange (AO) is commonly used as a marker for apoptotic cell death. It appears to enter all cells, but is only retained (through unknown mechanisms) by cells undergoing apoptosis (c.f. Abrams et al., 1993). In light of the strong defects in *th* mutant embryos reported here, we repeated experiments described in Hay et al., (1995) reporting that *th* embryos did not show an increase in AO staining. Figure 6A shows a brightfield image of

*Figure 6*. Cells in DIAP1 loss-of-function embryos do not show an increase in acridine orange staining characteristic of cells undergoing apoptosis in wildtype embryos.

(A), (B) stage 14 $th^5$/TM3[ftz::lacZ] or TM3[ftz::lacZ]/ TM3[ftz::lacZ] embryo visualized using Nomarski optics (A) and confocal microscope following acridine orange staining (B).

(C), (D) $th^5$ homozygous embryo exhibiting the terminal phenotype. At this stage acridine orange staining is never observed in $th^5$ homozygotes (D). Scale bar is 50μm.

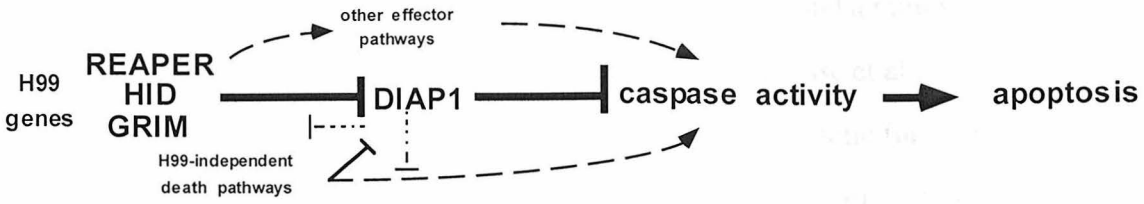

A
B
C
D

a stage 14 $th^5/TM3$ embryo. Figure 6B shows the same embryo stained with AO. Dying cells show bright green fluorescence and living cells do not stain. A $th^5$ homozygous embryo displaying the *th* terminal phenotype is shown in Figure 6C. All cells have rounded up and the yolk sac has moved to the surface. However, as shown in Figure 6D, no AO staining is seen, despite the fact that embryos at this stage show cell rounding, membrane blebbing, increased caspase activity and DNA fragmentation. Thus, the *th* phenotype, while showing some features of apoptosis, lacks others. One explanation for this is that the early embryo simply lacks the cellular machinery required to carry out apoptotic steps leading to AO retention. A second possibility is that the expression of other IAPs, such as DIAP2, suppresses some of the caspase-dependent events necessary for a full apoptotic cell death phenotype. A third possibility (discussed further below) is that loss of DIAP1 function, while sufficient to kill cells, is not sufficient to confer all aspects of apoptosis. In this scenario, proteins such as RPR, HID or GRIM, that are sufficient to trigger a full apoptotic response when overexpressed, may play dual roles: inhibiting IAP function and in addition activating other apoptotic pathways (Figure 7).

## DIAP1 is epistatic to *rpr*, *hid* and *grim*

To explore further the relationship between *rpr*, *hid*, *grim* and DIAP1, we made a double mutant with the H99 deficiency, which includes *rpr*, *hid* and *grim*, and $th^5$. Homozygous H99 embryos essentially lack apoptosis (White et al., 1994). However, double mutants show a terminal embryonic phenotype similar to that of $th^5$ alone (data not shown). This, in conjunction with the fact that DIAP1 is a caspase inhibitor, argues against models in which DIAP1's sole antiapoptotic role is to bind to and suppress RPR, HID and GRIM-dependent death pathways, since in this scenario the double mutant phenotype would be

*Figure 7.* Model for how DIAP1 regulates apoptosis. DIAP1 inhibits caspase activity (⊣) and is essential for cell survival. DIAP1's ability to inhibit caspase activity is suppressed by RPR, HID and GRIM (⊣), thus promoting caspase-dependent cell death. RPR and GRIM have other activities (Evans et al., 1997; Thress et al., 1998; Avdonin et al., 1999) suggesting that these proteins may also promote apoptosis through other pathways (dashed arrows). Free DIAP1 may inhibit apoptosis by binding these proteins, sequestering them from their targets (dashed-------| ). Thus DIAP1 may act both upstream and downstream of RPR, HID and GRIM to prevent cell death. Genes within the H99 interval are not required for some cell deaths (White et al., 1994; Foley and Cooley, 1998), indicating that other death pathways exist (dashed arrow). It is not known if these pathways act through DIAP1 or through other pathways


other effector
pathways
H99
genes
REAPER
HID
GRIM
DIAP1
caspase activity
apoptosis
H99-independent
death pathways

expected to be similar to that of the H99 mutant alone. Instead, our observations are consistent with models in which DIAP1 has an important antiapoptotic function as a caspase inhibitor and RPR, HID and GRIM function, at least in part, through DIAP1 to promote apoptosis (Figure 7).

DIAP1 may, however, have antiapoptotic functions in addition to caspase inhibition. IAPs in insects and mammals have been shown to bind a number of different proteins through the BIR repeats in addition to caspases (LaCasse et al., 1998). An attractive hypothesis is that some of these proteins have proapoptotic functions that do not involve disrupting IAP-caspase interactions, and that death preventing IAPs are able to suppress apoptosis induced by their expression by binding and sequestering them, preventing access to their targets. Candidates for such proteins in *Drosophila* are RPR, HID, GRIM and DOOM (Harvey et al., 1997), as well as uncharacterized molecules that mediate normally occurring apoptotic cell death that occurs independently of expression of genes in the H99 interval (White et al., 1994; Foley and Cooley, 1998) (Figure 7). DIAP1, and by extension other IAPs, may function to block death at multiple steps: at an upstream point free DIAP1 may titrate apoptosis inducers away from their targets, DIAP1-caspase complexes and components of other effector pathways, while at a downstream point it inhibits caspase activation or activity induced by these proteins (Figure 7).

## Concluding remarks

IAPs are the only cellular caspase inhibitors identified to date. We showed that RPR, GRIM, HID and HID fragments sufficient to cause the death of insect cells block DIAP1's ability to inhibit caspase activity. These observations provide a mechanism for RPR, HID and GRIM action and define a new point at which caspase activity can be regulated. The finding that the DIAP1 loss-of-function phenotype involves cell death associated with

increased caspase activity strongly suggests that the interactions we described between RPR, HID, GRIM, DIAP1 and caspases are physiologically important. Since most mammalian cells constitutively express caspases sufficient to carry out cell death (Weil et al., 1996), the mechanism of cell death activation defined by these proteins may be quite general. A prediction of our model of RPR, HID and GRIM function is that their ability to kill will depend on the status of a cell's caspases. In cells in which caspases are activated, or spontaneously undergoing autoactivation at some level, their expression may be sufficient to kill. However, in other cells in which caspase activity is either absent or more tightly regulated, they may function primarily to create a permissive condition for caspase-dependent cell death. Thus, just as IAPs in their role as caspase inhibitors may function as cellular buffers that create a requirement for a certain level of caspase activation in order to induce apoptosis, so RPR-, HID- and GRIM-like proteins may function to titrate this buffering activity so that cells are more or less sensitive to caspase-dependent death signals.

## Experimental Procedures

### Yeast Strain Constructions

All yeast strains used in this study are in the W303α background (*MATα, can1-100, leu2-3,-112, his3-11,-15, trp1-1 ,ura3-1, ade2-1*). Construction of pGALL-(*HIS3*), pGALL-(*LEU2*), and pGALL-DCP-1 was described previously (Hawkins et al., 1999). The p10 and prodomain-p20 fragments of drICE were amplified by PCR and inserted in tandem into pGALL-(*LEU2*) to make pGALL-rev-drICE. pAdh-(*TRP1*) was made by subcloning the promoter-terminator region from pHybZeo (Invitrogen) into pRS314. pCUP1-(*TRP1*) was constructed by replacing the GAL1 promoter of pGALL-(*TRP1*) (Hawkins et al., 1999) with the CUP1 promoter. The DIAP1 coding region was inserted into pAdh-(*TRP1*) and pCUP1-(*TRP1*) to generate pAdh-DIAP1 and pCUP-DIAP1. The HID coding region was amplified by PCR using primers that appended a C-terminal double polyoma epitope tag, and was inserted into pGALL-(*HIS3*) to generate pGALL-HID. pGALL-HID was digested with XhoI and religated to make pGALL-HIDΔ (38-335). C-terminally polyoma tagged versions of HID initiating at codon 37 (HIDΔ(1-36)) and HID lacking the first 335 amino acids (HIDΔ (1-335)) were amplified by PCR and inserted into pGALL-(*HIS3*). The coding regions of RPR and GRIM were amplified by PCR and inserted into pGALL-(*HIS3*) to generate pGALL-RPR and pGALL-GRIM.

### Cell Death Assays

Overexpression of active caspases in yeast results in cell death that is inhibited by coexpression of caspase inhibitors (Hawkins et al., 1999; Kang et al., 1999). Because expression of RPR, HID or GRIM alone has no effect on yeast growth (below) we infer that the yeast growth suppression seen in cells that express caspases, DIAP1 and RPR, HID or GRIM is due to cell death. To assay caspase-dependent cell death and protection by inhibitors, exponentially growing cultures of individual W303α transformants containing the relevant constructs were serially diluted tenfold and spotted onto 2% glucose selective medium plates or 2% galactose and 1% raffinose (gal/raf)-inducing selective medium plates. Plates were incubated at 25°C for 12 h, then 30°C and photographed after 2 (glucose) or 3-5 (gal/raf) days.

## Expression and Purification of Recombinant *Drosophila* IAPs, Caspases and HID

DCP-$1^{31}$-$His_6$ (DCP-1-His6) was prepared as described previously (Hawkins et al., 1999) and diluted into buffer A (50 mM Hepes [pH 7.5], 100 mM NaCl, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, 5 mM DTT). The DIAP1 coding region was amplified by PCR using primers that generated an N-terminal TEV Protease recognition site (ENLYFQG) and was introduced into the glutathione S-transferase (GST) expression vector pGEX4T-1 (Amersham Pharmacia). The GST-TEV-DIAP1 fusion protein was expressed in *Escherichia coli* strain BL21(DE3)pLysS and was affinity-purified on glutathione-Sepharose. The bound fusion protein was cleaved with TEV Protease in buffer A. The coding region of HID was introduced into pET23a(+) (Novagen), generating a HID-His6 fusion. A similar procedure was used to generate a version of HID initiating at codon 37 (HIDΔ (1-36)-His6). The first 37 amino acids of HID and the coding region of GST were

PCR amplified and introduced in tandem into pET23a(+) to generate HID(1-37)GST-His6. GST was introduced into pET23a(+) to generate GST-His6. These vectors were introduced into BL21(DE3)pLysS, protein expressed and affinity purified from the soluble fraction using ProBond resin (Invitrogen). Proteins were eluted and stored at -80°C in buffer D (50 mM Hepes [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, 20 mM 2-mercaptoethanol, 1% Triton X-100, 20% glycerol, 0.55 M imidazole).

A caspase cleavage site (DQVD) at residue 20 of DIAP1 (S.L. Wang, unpublished) was altered to DQVE in GST-mycDIAP1 (Hawkins et al., 1999), generating GST-DIAP1. GST and GST-DIAP1 were purified on glutathione sepharose beads. The mutated myc-DIAP1 coding sequence was inserted into pMAL-c2 (New England Biolabs), generating MBP-DIAP1. MBP and MBP-DIAP1 were purified on amylose resin.

### *In vitro* Protease Assays

HID's ability to inhibit DIAP1 was determined from caspase activity assay progress curves in which the release of 7-amino-4-trifluoromethyl-coumarin (AFC) from the synthetic substrate Ac-DEVD-AFC (100 μM) by DCP-1-His6 (0.2 nM) was measured in the presence of GST (0.2 μM) or DIAP1 (0.1 μM), and HID-His6 (0.44 μM), HIDΔ (1-36)-His6 (0.44 μM), or GST (0.77 μM). Caspase activity assays were performed in triplicate and prepared as follows: 2 μl DCP-1-His6 was incubated with 5 μl DIAP1 or GST for 5 minutes at 25°C. 71 μl buffer X (70.4 mM Hepes [pH 7.5], 140.8 mM NaCl, 1.4 mM EDTA, 0.14% CHAPS, 14.1% sucrose, 3.5 mM DTT, 5.6% glycerol, and 0.7% Triton X-100) and 20 μl HID-His6, HIDΔ (1-36)-His6, or GST-His6 was subsequently added and the samples were further incubated 23 minutes. After addition of 2 μl 5 mM

Ac-DEVD-AFC in DMSO to each sample, caspase activities were assayed at 27°C using a fluorometric plate reader (Molecular Devices, fmax) in the kinetic mode with excitation and emission wavelengths of 405 and 510 nm, respectively. HID(1-37)GST-His6 activity, used at a concentration of 1.6μM, was tested in a similar manner.

Caspase activity of embryos was monitored as follows: 0 to 2 hr old embryos were collected and aged for 3 hr at 25°C. Embryos were dechorionated with 50% bleach, rinsed, suspended in an equal volume of buffer E (buffer A containing 0.5% Triton X-100 and 4% glycerol), and homogenized. 1 μl of this extract (approximately 10mg/ml) was diluted into 100μl of buffer E (buffer A containing 0.5% Triton X-100 and 4% glycerol) in the presence of 100 μM Ac-DEVD-AFC. Assays were performed in triplicate with freshly prepared extracts.

**Protein Binding Assays**

Purified recombinant proteins (150 ng each) were incubated with either GST beads, MBP beads, GST-DIAP1 beads, or MBP-DIAP1 beads (200 ng) for 30 minutes on a rotator at 25°C in buffer C (100 mM Hepes [pH 7.5], 200 mM NaCl, 2 mM EDTA, 0.2% CHAPS, 20% sucrose, 40 mM 2-mercaptoethanol, 1% Triton X-100, 8% glycerol). Beads were then washed three times for five minutes with 1 ml buffer D lacking imidazole. Bead-protein complexes were subjected to 15% SDS-PAGE and immunoblotting. Proteins were detected with an anti-His antibody (Qiagen) and HRP-conjugated secondary antibodies followed by ECL (Amersham).

**HID-DCP-1 Competition Assay**

MBP beads or MBP-DIAP1 beads (0.5 μg) were mixed with 1, 5, or 20 μg GST-His6 or HID(1-37)GST-His6 for 1 h at 4°C in buffer A. 5 μg DCP-1-His6 was added and the samples further incubated ten minutes. After extensive washing the bead-protein complexes were subjected to 15% SDS-PAGE and immunoblotting. Proteins were detected with an anti-His antibody and HRP-conjugated secondary antibodies followed by ECL.

### *Drosophila* Strains

A *w*- Oregon R strain was used as wildtype. The deficiency *Df(3L)brm11* (72A3-4; 72D1-5) uncovers DIAP1. DIAP1 mutants, alleles of the *thread* (*th*) locus, and *Df(3L)brm11* were kept as balanced stocks over a version of the TM3 balancer that carries a ftz::lacZ transgene (TM3[ftz::lacZ]). $th^5$ is a strong loss of function mutation in DIAP1 since *Df(3L)brm11* shows a GMR-*rpr* enhancer phenotype similar to that of $th^5$ (Hay et al., 1995), and the embryonic lethal phenotype of $th^5$/ *Df(3L)brm11* (this work, data not shown) is similar to that of $th^5/th^5$. By these same criteria, $th^6$ is a much weaker allele.

### Cell Death Assays and Microscopy

Embryos were collected on yeasted apple juice plates. For observation of living embryos, syncytial blastoderm stages were selected under halocarbon oil 27 (Sigma) and mounted individually on a slide using 10 mm coverslips. Images were taken on a Zeiss Axiophot, from sets of 6 embryos per experiment every 15 min at 25°C.

For scanning electron microscopy staged collections of embryos were processed for staining with anti-β-galactosidase (β-gal) antibodies (Cappel) as described elsewhere

(Muller and Wieschaus, 1996). Homozygotes were isolated under the dissecting scope; the remaining embryos served as controls. The embryos were then processed for scanning electron microscopy as described in Muller and Weischaus (1996). Samples were viewed under a Hitachi S800 SEM.

TUNEL labeling was performed using an in situ Cell Death Detection kit (Roche). Embryos were fixed as described above, rinsed in PTX (PBS, 0.5% Triton X-100), and immunolabeled with antibodies against β-gal (Cappel) and Cy3-conjugated goat anti rabbit (Jackson) secondary antibodies. Embryos were then incubated in 100 mM sodium-citrate, 0.1% Triton X-100 at 65°C for 30 min, followed by rinsing twice in PTX and twice in TUNEL dilution buffer (Roche). Embryos incubated in TUNEL assay buffer (Roche) (30 min to 2 hrs) were supplemented with TUNEL enzyme (Roche) and the embryos were incubated a further 2 to 3 hrs. Embryos were rinsed in PTX and mounted in MOWIOL (Polysciences) containing DABCO (Sigma). Images were taken on a Leica TSC-NT confocal microscope. A range of 50 μm in depth was scanned in 16 optical sections and projected into one focal plane using the TCS-NT software.

Acridine orange staining was carried out as described in Hay et al., (1994). Confocal and Nomarksi images were taken on the confocal microscope.

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# CHAPTER 4

## Conclusion: Future Directions

Studies with *Drosophila* proteins in a yeast assay system and by *in vitro* reconstitution experiments have led to the isolation of a caspase inhibitor and the elucidation of a general mechanism by which it may modulate apoptosis. This work defines a novel point at which caspase activity can be regulated: caspases may be activated by the direct inhibition of a caspase inhibitor. In support of this proposed mechanism, we find that elimination of DIAP1 function results in global early embryonic cell death and a large increase in DIAP1-inhibitable caspase activity, and that DIAP1 is still required for cell survival when expression of *rpr*, *hid*, and *grim* is eliminated. Among many questions that arise from this work are the following: 1) What is the nature of the interaction between IAPs and pro- and mature caspases, and which domains or residues in DIAP1 facilitate interaction with HID? 2) Can these regions be altered such that DIAP1 still functions as a caspase inhibitor yet is rendered insensitive to regulation by RPR, HID, and GRIM? 3) What is the mechanism of full-length HID action? 4) At what level(s) of caspase activation are IAP/HID acting? 5) Although HID can activate caspases by inhibiting DIAP1 from blocking their activity in yeast and *in vitro*, does this in fact occur *in vivo*? 6) Are there other molecules involved in the "double inhibition" model of caspase activation? 7) On a more general level, what regulates RPR, HID, and GRIM? 8) Do these three activators have distinct cell-killing activities? 9) How do they activate the Apaf-1 homologue Dark? 10) Do the IAPs have a role in the Dark/Apaf-1 pathway? 11) Do sequence and/or functional RPR, HID, and GRIM homologues exist in mammals? A few key issues regarding questions 1-7 and 11 will be addressed in this essay.

A number of different approaches may be taken to obtain information on IAP-caspase and IAP-HID interactions, including mutational analysis, structural studies, and genetic screens performed in yeast or *Drosophila*. Solution structure and mutational analysis of IAP BIR domains indicate that particular linker sequences flanking the BIR domains are important for IAP-caspase interactions (Hinds et al., 1999; Sun et al., 1999).

Mutational analysis of IAPs, HID, and caspases should help to identify key residues mediating their interactions. Once minimal interaction domains are delineated by such studies, x-ray crystallography and solution structure analysis of these domains may provide information on the structural basis for their activity. As an alternative approach, one can utilize yeast-based screens using libraries of mutagenized molecules (i.e., DIAP1, HID, etc.) to identify residues required for IAP-HID interactions. *Drosophila* screens have uncovered a variety of DIAP mutants that exhibit varied phenotypes (K. White, unpublished data). Analysis of DIAP1 point mutants isolated as modifiers of HID-induced death but not RPR- or GRIM-mediated apoptosis is likely to provide information on the nature of HID-IAP interactions.

Although we have demonstrated that a fusion protein containing the first 37 amino acids of HID is sufficient to inhibit DIAP1 without displacing DCP-1, it is unknown whether the full-length protein behaves similarly. One could carry out binding/competition experiments with full-length HID to determine if it, too, fails to displace caspase from IAP. It is possible that full-length HID interferes with DIAP1 inhibition of caspase by both inducing a conformational change in the IAP, as well as catalyzing the release of caspase from IAP. BIAcore analysis measures, in real time, the association and dissociation of unlabeled ligand to an immobilized receptor, or vice versa, by changes in the adjacent refractive index. BIAcore technology can be used to examine the kinetics of caspase-IAP-HID complex formation. Generation of a version of HID which can be cleaved after its first 37 amino acids would allow one to test the functionality of a 37-mer peptide of HID. Is the 37-mer sufficient for IAP inhibition, or does activity require fusion to a larger molecule?

IAPs have the ability to inhibit both pro- and mature forms of certain caspases. In some cases, IAP can bind to and inhibit only the zymogen and not the active caspase. For example, while DIAP1 can inhibit active DCP-1, it appears to block DRICE-induced

apoptosis by inhibiting zymogen processing at a particular cleavage step (C. Hawkins & S. L. W., unpublished data; L. Miller, pers. comm.). The stage at which IAPs block caspase activity has implications for HID's mode of action. In situations where IAPs directly inhibit mature caspases, HID relieves IAP inhibition of already processed activated caspase. In other cases, HID may liberate inhibitor-bound procaspases which subsequently undergo processing via other caspase activation pathways.

In *Drosophila*, the role of particular caspases in apoptotic pathways is currently unknown. The *Drosophila* caspases DCP-1, DREDD/DCP-2, DRICE, DRONC, and DECAY all have caspase-like activation and/or proteolytic activity in experimental settings, but their order of activation in proteolytic cascades has not been determined (Chen et al., 1998; Dorstyn et al., 1999; Fraser and Evan, 1997; Song et al., 1997). DREDD and DRONC are candidate initiator caspases, since their prodomains contain a DED and CARD, respectively. DCP-1 and DRICE are structurally and biochemically similar to CED-3, indicating that they may be effector caspases.

Loss of zygotic DCP-1 function results in melanotic tumor development and larval lethality (Song et al., 1997). DCP-1 appears to play a role during nurse cell death (McCall, 1998). During oogenesis, nurse cells transfer their cytoplasmic contents to the developing oocytes and then die. Females carrying homozygous *dcp-1*$^{-}$ clones in their germline are sterile and their nurse cells show defects in cytoskeletal reorganization and nuclear breakdown that occurs during the transfer process. Normal nurse cells transfer the majority of their cytoplasm and presumably, active DCP-1, to the oocyte just prior to dying. However, the oocytes escape damage, suggesting the presence of a protective mechanism, feasibly IAP activity.

DREDD may also participate in the execution of nurse cell death, as the accumulation of *dredd* mRNA in nurse cells coincides with nurse cell death (Chen et al., 1998). *Dredd* mRNA also accumulates in embryonic cells undergoing apoptosis. DREDD

has a unique active site containing a glutamic acid in the position typically occupied by a glycine in the consensus sequence for caspases (QACXG). Interestingly, studies with DRONC indicate that it has a unique substrate cleavage ability in that it can cleave after glutamate residues (C. Hawkins, pers. comm.). DRONC's catalytic cysteine residue is contained within a unique sequence, PFCRG, which may contribute to DRONC's ability to recognize and cleave glutamate residues. DIAP1 can inhibit DRONC-induced yeast cell death, suggesting a role for the IAP as an upstream inhibitor of caspase cascade activation (C. Hawkins, pers. comm.).

The expression patterns and levels of RPR, HID, and GRIM, DIAP1, and caspases in the developing *Drosophila* embryo are currently unknown. Once the stoichiometric ratios at which HID inhibits DIAP1 from blocking caspase activity *in vitro* have been determined, immunohistochemical analysis could be carried out to verify whether or not these proteins are present at the appropriate amounts within cells at the time of apoptosis. Coimmunoprecipitation of IAP-caspase and HID-IAP-caspase complexes from *Drosophila* would support the hypothesis that IAP acts as a direct caspase inhibitor and HID is an IAP inhibitor *in vivo*. These types of experiments would help to clarify if HID inhibits DIAP1 activity *in vivo*, as it can in yeast and *in vitro*.

*In vivo* activation of the apoptotic program by HID inhibition of DIAP1 is likely to be more complicated than the simple interaction of these three components. What regulates HID? Does DIAP1 require any cofactors for maximal activity? *Hid* function is negatively coupled to Ras activation at both the RNA and protein levels (Bergmann et al., 1998; Kurada and White, 1998). Hyperactivation of the Ras-MAP kinase pathway downregulates *Hid* expression and blocks *Hid*-induced cell death. Site-directed mutation of MAPK consensus sites in *Hid* renders its killing properties insensitive to MAPK. Phosphorylation could inactivate HID in a number of ways which await evaluation. An attractive hypothesis is that phosphorylation induces a conformational change in HID

resulting in occlusion of DIAP1 binding sites. Alternatively, phosphorylation may mark HID for proteolytic degradation. Yet another hypothesis is that phosphorylation may allow HID inhibitors to recognize, bind, and inhibit it. Similarly, DIAP1 may be regulated by cofactors prior to association with DCP-1 or HID, or in the complex itself. Although the *in vitro* experiments demonstrate that no other cofactors are required for DIAP1 inhibition of caspase activity, these results do not exclude the possibility that IAPs may have additional properties that modify caspase activity. Since many IAPs contain RING fingers, it is conceivable that they play a role in modulating protein levels via ubiquitination. An attractive possibility is that IAPs target proapoptotic molecules, such as caspases, for degradation. Conversely, IAPs may target themselves for degradation upon receipt of appropriate death stimuli. In looking for additional regulatory molecules, the *Drosophila* model system provides a sophisticated range of tools for gene discovery and has already proven very useful in isolating cell death regulators. Yeast-based screens described in Chapter 2 provide a way of looking for caspase regulators strictly based on their protease activity.

To date, neither sequence nor functional mammalian homologues of RPR, HID, or GRIM have been identified. As these molecules are potent apoptosis inducers in vertebrate systems, it is conceivable that homologues exist (Evans et al., 1997; Haining et al., 1999; McCarthy and Dixit, 1998). Furthermore, the IAP-caspase interaction is conserved across species and is likely to have shared regulatory motifs. The yeast-based assay system described in this thesis could be modified and employed to search for mammalian RPR, HID, and GRIM-like molecules on functional grounds rather than by sequence homology. A very attractive candidate IAP to target in such a screen is the human IAP survivin.

Notably, *survivin* is expressed in a high proportion of the most common human cancers but not in normal, terminally differentiated adult tissues (Ambrosini et al., 1997).

Antisense-mediated reduction in *survivin* expression effected apoptosis in some tumor cell lines. Expression of *survivin* is enhanced at the G2/M phase of the cell cycle, and survivin protein associates with microtubules of the mitotic spindle during metaphase. It has been proposed that survivin may act to suppress a default death pathway that is activated during a G2/M checkpoint (Li et al., 1998). Survivin has been shown to suppress apoptosis induced by Fas, Bax, Taxol or overexpression of procaspases-3, 7, or 9 in cultured cells, and it can be coimmunoprecipitated with the processed forms of these caspases (Ambrosini et al., 1997; Tamm et al., 1998). These results imply that survivin is a caspase inhibitor.

With the sequencing of the *Drosophila* genome complete, fly homologues of mammalian genes are being identified at a breakneck pace. Not surprisingly, among these are several *Drosophila* homologues of mammalian and nematode genes encoding cell death regulators such as caspases, CED-4/Apaf-1 and Bcl-2-like proteins. The identification of these molecules in a number of organisms underscores the conserved nature of the death program and indicates the likelihood of the existence of RPR-, HID-, and GRIM-like death activators in other species in addition to *Drosophila.*

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# Appendix I

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Genetics



# A cloning method to identify caspases and their regulators in yeast: Identification of *Drosophila* IAP1 as an inhibitor of the *Drosophila* caspase DCP-1

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**ABSTRACT** **Site-specific proteases play critical roles in regulating many cellular processes. To identify novel site-specific proteases, their regulators, and substrates, we have designed a general reporter system in *Saccharomyces cerevisiae* in which a transcription factor is linked to the intracellular domain of a transmembrane protein by protease cleavage sites. Here, we explore the efficacy of this approach by using caspases, a family of aspartate-specific cysteine proteases, as a model. Introduction of an active caspase into cells that express a caspase-cleavable reporter results in the release of the transcription factor from the membrane and subsequent activation of a nuclear reporter. We show that known caspases activate the reporter, that an activator of caspase activity stimulates reporter activation in the presence of an otherwise inactive caspase, and that caspase inhibitors suppress caspase-dependent reporter activity. We also find that, although low or moderate levels of active caspase expression do not compromise yeast cell growth, higher level expression leads to lethality. We have exploited this observation to isolate clones from a *Drosophila* embryo cDNA library that block DCP-1 caspase-dependent yeast cell death. Among these clones, we identified the known cell death inhibitor DIAP1. We showed, by using bacterially synthesized proteins, that glutathione *S*-transferase–DIAP1 directly inhibits DCP-1 caspase activity but that it had minimal effect on the activity of a predomainless version of a second *Drosophila* caspase, drICE.**

Site-specific proteolysis plays a critical role in regulating a number of cellular processes. An important class of site-specific proteases are a group of cysteine proteases known as caspases (1). Extensive genetic and biochemical evidence indicates that caspases play roles as cell death signaling and effector molecules in a number of different contexts, thus making them attractive potential therapeutic targets. Caspases identified to date have been found primarily based on homology to the *Caenorhabditis elegans* caspase CED-3 and mammalian caspase 1 and through biochemical purification (reviewed in refs. 2–5). Viral and cellular activators and inhibitors of caspase function also have been identified in genetic and biochemical screens for regulators of apoptosis (reviewed in refs. 6–8). These approaches to isolating caspases and their regulators are limited by the fact that some proteases that cleave a caspase target site and their regulators may not share primary sequence homology with the proteins identified to date or they may be expressed only in specific tissues with limited availability for biochemical purification. Furthermore, it is clear that caspases regulate processes other than cell death, including cytokine secretion in mammals (9–13) and cell proliferation and oogenesis in *Drosophila* (14, 15). It seems likely, given the early stage of the field, that more roles exist. Caspases and caspase regulators involved in these processes may be missed in screens that focus strictly on cell death-related phenotypes. Thus, molecules that possess caspase or caspase regulatory activity may not have been identified yet. As an alternative approach to identifying novel caspases or caspase regulators, it would be useful to have assays for caspase function that are based strictly on protease activity.

Because of the importance of site-specific proteolysis, we sought to develop a versatile system that would allow the identification of novel site-specific proteases, regulators of the activity of known site-specific proteases, or their substrates. Because caspase cleavage sites have been well defined, and activators and inhibitors of caspases have been identified, we set out to establish a prototype system that would allow positive selection for caspase-like proteases, their activators, and their inhibitors. Our approach to identifying these molecules uses reporters for caspase activity that function in living cells. Yeast, though eukaryotic, lacks many of the specialized proteolytic systems found in cells of higher eukaryotes. Thus, it constitutes an ideal background in which to carry out function-based screens for these proteases, their regulators, and their targets. Reporters for the activity of specific proteases in bacteria and eukaryotes have been developed by using several strategies that involve cleavage-dependent alterations in the activity of specific proteins (16–21). To visualize caspase activity, we created a fusion protein in which a transcription factor is linked to the intracellular domain of a transmembrane protein by caspase cleavage sites. Expression of this protein in yeast, in the presence of an active caspase, should result in release of the transcription factor from the membrane, followed by transcriptional activation of a reporter. As described below, using such a reporter system, we can visualize caspase activity in yeast and can identify proteins that act as caspase activators and inhibitors. Caspase inhibitors also can be identified by virtue of their ability to suppress caspase overexpression-dependent yeast cell death.

## MATERIALS AND METHODS

### Constructs

**Yeast Strains.** The W303α strain (*MATα, can1–100, leu2–3,-112, his3–11,-15, trp1–1, ura3–1, ade2–1*) was used to monitor caspase activity by using the *lacZ* reporter system. EGY48 (MATa, *ura3, trp1, his3, LexAop$_6$-LEU2*) (Invitrogen) was used to monitor caspase-dependent cell killing.

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Abbreviations: β-gal, β-galactosidase; GST, glutathione *S*-transferase; AFC, 7-amino-4-trifluoromethyl-coumarin; BIR, baculovirus IAP repeats; gal/raf medium, medium containing 2% galactose and 1% raffinose.

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**Construction of Caspase Target Site Fusion Proteins.** The reporter, CLBDG6, was generated by using PCR and standard techniques (details provided on request). This protein consists of, from N to C termini, amino acids 1–401 of a type 1 transmembrane protein, human CD4 (22), a linker consisting of six tetrapeptide caspase target sites that bracket the specificity of known caspases and granzyme B (23)—DEVDG-WEHDG-IEHDG-IETDG-DEHDG-DQMDG—, each of which is followed by a glycine residue, which acts as a stabilizing residue in the N-end rule degradation pathway in yeast (reviewed in refs. 24), and finally, a transcription factor containing the LexA DNA binding domain (25). A second construct, designated CLBGG6, was generated that encodes a protein identical to CLBDG6 except that the essential P1 aspartates of the six caspase cleavage sites are replaced with glycines, rendering them nonfunctional.

**Construction of Yeast Expression Plasmids.** Plasmids for expression of genes in yeast were derived from the pRS series (26). To express genes in yeast under galactose-inducible control, we used two GAL1 promoter fragments: a long version, extending from base 1–815, called GALL, and a shorter and somewhat weaker version, extending from base 406–815, called GALS (27). The yeast actin terminator, bases 2,107–2,490 (GenBank accession no. L00026), was used for all constructs. GALL promoter and actin terminator fragments were inserted into pRS313 (*HIS3*), pRS314 (*TRP1*), and pRS315 (*LEU2*), generating pGALL-(*HIS3*), pGALL-(*TRP1*), and pGALL-(*LEU2*). CLBDG6 and CLBGG6 coding regions were cloned into pGALL-(*TRP1*). GALS promoter and actin terminator fragments were inserted into pRS315, generating pGALS-(*LEU2*). To express genes under the control of the copper-inducible CUP1 promoter, we used a promoter fragment extending from bases 1,079–1,533 of the CUP1 locus (GenBank accession no. K02204). Site-directed mutagenesis was used to mutate the *CUP1* promoter to prevent activation in response to glucose starvation (28). The mutated CUP1 promoter and actin terminator fragments were inserted into pRS315 (*LEU2*), generating pCUP1-(*LEU2*).

The coding region for the *C. elegans* caspase CED-3 (29) was introduced into pCUP1-(*LEU2*). Site-directed mutagenesis was used to generate an inactive version of CED-3 in which the active site cysteine was changed to serine (CED-3CS). Full length human caspase 7 (caspase 7FL) (30), caspase 7 lacking the N-terminal 53-aa prodomain (caspase $7^{53}$), and the caspase 8 isoform corresponding to MACH$\alpha$2 (31) or Mch5 (32) (caspase 8FL) were introduced into pCUP1-(*LEU2*). Full length DCP1 (14) was introduced into pGALS-(*LEU2*) and pGALL-(*LEU2*). Site-directed mutagenesis was used to change the active site cysteine to serine (DCP-1CS). The resulting full length coding region was introduced into pGALL-(*LEU2*). Full length drICE (33) was inserted into pGALL-(*LEU2*). Full length human caspase 9 (34, 35) was introduced into pGALL-(*LEU2*). The region encoding amino acids 1–530 of Apaf-1 (36) (Apaf-$1^{530}$) was introduced into pGALL-(*HIS3*). Full length DIAP1 (FLDIAP1) and a version of DIAP1 C-terminally truncated following residue 381 (DIAP1BIR) (37) were introduced into pGALL-(*HIS3*). The mouse IAP MIHA (38) and baculovirus p35 (39) were introduced into pGALL-(*HIS3*).

**Yeast Transformation and Characterization.** Plasmids were introduced into yeast by lithium acetate transformation. For caspase activity assays in which *lacZ* expression was monitored, yeast were transformed with pSH18–34 (*URA3*) (Invitrogen), which carries a LexA-responsive *lacZ* gene. These cells then were transformed with either pGALL-CLBDG6-(*TRP1*) or pGALL-CLBGG6-(*TRP1*). Caspase expression plasmids or an empty expression vector were introduced into these backgrounds and were characterized as described below. For caspase activation and inhibition assays, a fourth plasmid also was introduced, either expressing Apaf-$1^{530}$, p35, or MIHA or with no insert. To carry out X-gal filter assays for $\beta$-galactosidase ($\beta$-gal) activity, transformants were plated on selective plates with glucose (2%) as the sugar source. After 3 days, duplicate colonies were picked and resuspended in 1 ml of sterile Tris·EDTA (pH 8.0). One microliter of each sample was streaked on a minimal medium glucose plate. After 2 days, a nylon membrane was used to lift the streaked yeast (yeast side upwards) onto a complete medium plate containing 2% galactose and 1% raffinose (gal/raf media) and 3 $\mu$M copper sulfate. After various periods of induction, the filters were processed for X-gal staining (40). To quantitate $\beta$-gal activity, three tubes of liquid selective gal/raf medium were inoculated with single colonies from each transformation plate, were grown for 24 hr, then were diluted 1:10 into fresh gal/raf selective medium containing the indicated concentration of copper sulfate and were grown for a further 10 hr. *o*-nitrophenyl-$\beta$-D-galactoside assays were performed as described by Miller (41). To assay caspase-dependent cell death and protection by inhibitors, colonies carrying the relevant plasmids were streaked from 2% glucose selective media plates onto gal/raf selective media plates. The plates were photographed after 3 days.

**Expression and Purification of Recombinant *Drosophila* IAPs and Caspases.** The DIAP1 coding region was amplified by PCR using primers that generated an N-terminal myc epitope (EQKLISEEDL) and was introduced into the glutathione *S*-transferase (GST) expression vector pGEX4T-1 (Amersham Pharmacia). The GST-myc-DIAP1 fusion protein was expressed in *Escherichia coli* strain BL21(DE3)pLysS (Novagen) and was affinity-purified on glutathione-Sepharose. The eluted protein was dialyzed against buffer A (25 mM Tris, pH 8.0/50 mM NaCl/10 mM DTT). After dialysis, the protein was frozen in aliquots after addition of glycerol to 10%.

DCP-1, initiating at codon 31 (DCP-$1^{31}$), was introduced into pET23a(+) (Novagen), generating a DCP-$1^{31}$-$His_6$ fusion. A similar procedure was used to generate a drICE-$His_6$ fusion protein in which DrICE initiates at codon 81 (drICE$^{81}$-His6). DCP-$1^{31}$-$His_6$ and drICE$^{81}$-$His_6$ were expressed in the *E. coli* strain BL21(DE3)pLysS. Protein expression and affinity purification from the soluble fraction were carried out using ProBond resin (Invitrogen). Eluted protein was dialyzed against buffer A and subsequently was snap frozen in buffer A containing 10% glycerol.

***Drosophila* cDNA Library Construction and DCP-1 Inhibitor Screening.** *Drosophila* embryonic polyA+ mRNA (CLONTECH) was converted into cDNA by using a Superscript cDNA synthesis kit (GIBCO). cDNAs larger than 600 bp were ligated into a modified version of pGALL-(*HIS3*) in which the polylinker was expanded to contain *Xho*I and *Not*I sites. ElectroMax DH10B cells (GIBCO) were transformed with the ligation mix and were used to amplify the library, which contained $5 \times 10^6$ primary transformants.

W303$\alpha$ yeast carrying pGALL-DCP-1-(*LEU2*) were transformed with 26 $\mu$g of library plasmid DNA, were grown in yeast extract/peptone/dextrose for 3 hr, were washed twice to remove glucose, and were plated on gal/raf selective plates. A total of 140,000 transformants were screened. Colonies were picked after 4 days of growth at 30°C. PCR was carried out on DNA isolated from individual colonies by using DIAP1-specific primers.

***In Vitro* Protease Assays.** DCP-$1^{31}$-His6 and drICE$^{81}$-$His_6$ caspase activity were measured fluorometrically by following the release of 7-amino-4-trifluoromethyl-coumarin (AFC) from Ac-DEVD-AFC (Enzyme Systems Products, Livermore, CA) using the fmax fluorescence microplate reader (Molecular Devices) with an excitation wavelength of 405 nm and an emission wavelength of 510 nm. The ability of GST-DIAP1 to inhibit caspase activity was determined from caspase activity assay progress curves in which substrate hydrolysis (100 $\mu$M)

by DCP-$1^{31}$-$His_6$ (0.2 nM) or drICE$^{81}$-$His_6$ (0.62 nM) was measured in the presence of GST (0.48 $\mu$M) or GST-mycDIAP1 (0.16 $\mu$M), in caspase activity buffer (50 mM Hepes, pH 7.5/100 mM NaCl/1 mM EDTA/0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate/10% sucrose/5 mM DTT).

## RESULTS AND DISCUSSION

Our approach to monitoring caspase activity *in vivo* was to create cells in which caspase activity stimulates transcriptional activation of a reporter. We created a fusion protein substrate for caspase cleavage in which the transcription factor LexA-B42 (LB) is linked to the truncated cytoplasmic domain of a membrane protein, CD4 (C), by a short linker (DG6) consisting of six different caspase cleavage sites that bracket the specificities of known caspases and the serine protease granzyme B, which cleaves caspases and other targets at sites of similar sequence (*Material and Methods* for details). When this molecule, referred to as CLBDG6, is expressed in a reporter strain in which a LexA-dependent promoter drives *lacZ* expression (LexA/$\beta$-gal reporter), levels of $\beta$-gal activity should depend on the presence of an active caspase able to cleave one or more of the introduced target sites, thereby releasing LexA-B42 from membrane association. (Fig. 1 *A* and *B*).

**A Reporter for Caspase Activity in Yeast.** We introduced CLBDG6 into the LexA/$\beta$-gal reporter strain in a plasmid, pGALL-CLBDG6, in which expression is induced in response to galactose. We introduced into this background a copper-inducible expression plasmid, pCUP1, containing either no insert or different versions of the caspase CED-3. Transformants initially were streaked on glucose medium. Colonies from these streaks then were replica plated onto gal/raf medium containing 3 $\mu$M copper to induce expression of CLBDG6 and from the pCUP1 plasmid. After 12 hr of induction, levels of $\beta$-gal activity were determined by using an X-gal assay in which cells that do not express $\beta$-gal remain white whereas those that do turn shades of blue. Reporter cells that expressed CLBDG6 alone remained white in this assay (Fig. 2*A*), indicating that yeast contains negligible amounts of proteases capable of cleaving caspase target sites under standard growth conditions. However, when expression of the *C. elegans* caspase CED-3 (pCUP1-CED-3) was induced, a high level of $\beta$-gal activity was observed (Fig. 2*A*), which increased in a copper concentration-dependent manner (Fig. 2*B*). Of importance, caspase activity was required for reporter activation because expression of an inactive CED-3 mutant in which the active site cysteine had been changed to serine (CED-3CS) did not result in $\beta$-gal expression (Fig. 2*A*). Finally, expression of wild-type CED-3 in a reporter strain in which the essential P1 aspartates of the caspase target sites in CLBDG6 had been mutated to glycines (CLBGG6) (Fig. 1*C*) did not result in $\beta$-gal activity (Fig. 2*A*), arguing that the CED-3-dependent induction of $\beta$-gal activity was a direct result of cleavage of CLBDG6 at the caspase target sites.

These results establish that yeast can be used as a cell-based reporter system for caspase activity. In order for a caspase to be identified in this assay, the caspase must be active in yeast. Physiological activation of caspases occurs through multiple mechanisms, including recruitment and oligomerization at the plasma membrane, cleavage by caspases or other proteases able to recognize a caspase target site, interactions with members of the CED-4/Apaf-1 family of proteins, and auto-

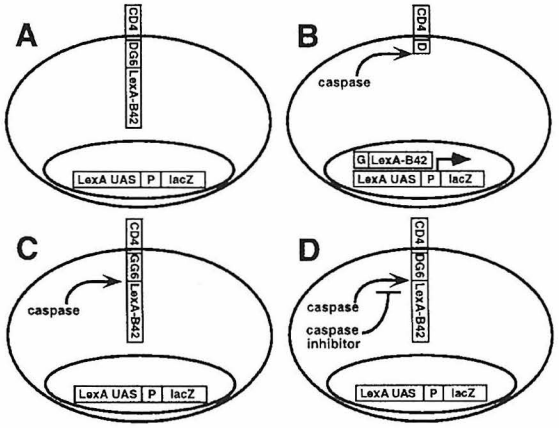


FIG. 1. A genetic system for monitoring caspase activity in yeast using a transcriptional reporter. Yeast were created that express a chimeric type-1 transmembrane protein (CLBDG6) in which the N-terminal signal sequence and transmembrane domain (CD4) is followed by a linker consisting of six tetrapeptide caspase target sites (indicated in bold) that bracket the specificity of known caspases and granzyme B (23)—DEVDG-WEHDG-IEHDG-IETDG-DEHDG-DQMDG—each of which is followed by a glycine residue, which acts as a stabilizing residue in the N-end rule degradation pathway in yeast (reviewed in ref. 24). C-terminal to the caspase target site linker is a transcription factor domain, LexA-B42. The LexA-dependent transcriptional reporter consists of LexA binding sites (LexA UAS) and a promoter (P) upstream of the bacterial *lacZ* gene (*lacZ*) (*A*). The cells in *A* act as caspase activity reporters because expression of an active caspase results in CLBDG6 cleavage at the caspase target sites, releasing LexA-B42, which enters the nucleus and activates *lacZ* transcription (*B*). A version of CLBDG6 in which the P1 aspartates are changed to glycines (CLBGG6) cannot be cleaved by caspases. Cells expressing CLBGG6 act as false positive reporters for molecules that activate *lacZ* expression independent of cleavage at caspase target site (*C*). If cells in *B* express a caspase inhibitor as well as an active caspase, caspase activity, and thus caspase-dependent release of LexA-B42, is inhibited, and $\beta$-gal levels are decreased compared with cells that express the caspase alone (*D*).

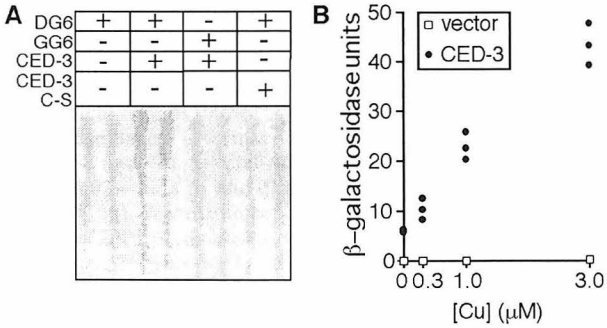


FIG. 2. Yeast expressing CLBDG6 act as reporters for CED-3 caspase activity. W303$\alpha$ yeast were transformed with pSH18–34, which carries a LexA-responsive *lacZ* transcriptional cassette (the LexA/$\beta$-gal reporter strain). These cells were transformed with pGALL expression plasmids carrying CLBDG6 (DG6) or CLBGG6 (GG6). These cells also carry a copper-inducible pCUP1 plasmid, which contains either wild-type CED-3 (CED-3), an inactive C to S mutant version of CED-3 (CED-3 C-S), or nothing. Duplicate colonies from each transformation were streaked onto gal/raf medium to induce GAL1-dependent expression of the caspase substrates and then were lifted onto complete media plates with 3 $\mu$M copper sulfate to induce caspase expression. After a 12-hr induction, an X-gal assay was performed on the filter. Only cells expressing CLBDG6 and wild-type CED-3 have significant $\beta$-gal activity (*A*). Cultures from three transformants carrying pSH18–34, pGAL-CLBDG6, and either the empty pCUP1 vector or pCUP1-CED-3 were grown to stationary phase, then were diluted into medium containing the indicated levels of copper sulfate and grown for a further 10 hr. *o*-nitrophenyl-$\beta$-D-galactoside assays were performed, and $\beta$-gal activity was determined. $\beta$-gal activity in the CED-3-expressing cells increased as a function of copper concentration (filled circles, CED-3). No $\beta$-gal activity was found in the cultures carrying only the empty pCUP1 vector (open boxes, vector) (*B*).

activation. In some cases, overexpression alone is sufficient to induce autoactivation whereas, in other cases, significant activation requires interactions with other proteins (reviewed in refs. 2–5). Thus, it is likely that only proteases in which the primary translation product is active, or in which the protease is able to autoactivate, will be identified in the simplest reporter-based caspase screen. However, more complex screens for caspases that can activate after forced oligomerization or association with potential caspase activators (42–46) can be envisioned. We have tested several other caspases in this reporter system. Expression of mammalian caspase $7^{53}$ (below) and full length caspase 8 (data not shown) resulted in reporter-dependent *lacZ* expression. Expression of human caspase 3, caspase 9, or *Drosophila* drICE failed to activate reporter expression (data not shown), even though active forms of these caspases are known to efficiently cleave peptides with the same sequence as the target sites introduced into CLBDG6 (23, 33). Moreover, although overexpression of wild-type but not an inactive mutant of CED-3 induced yeast cell death (below), similar overexpression of caspase 3, caspase 9, or drICE had no effect on cell growth. Based on these observations, it is likely that, in yeast, the procaspase forms of these caspases do not autoprocess to generate active caspase heterodimers. This result is expected: Caspase 9 is thought to function as an upstream caspase, in which a major mechanism of activation requires association with Apaf-1 (42, 43), whereas caspase-3 is thought to act as a downstream caspase, in which a principal mechanism of activation is cleavage by other caspases (reviewed in refs. 2–5). drICE activation may be regulated by either of these mechanisms.

**Activators of Caspase-Dependent Reporter Activation.** The fact that certain caspases do not activate in yeast suggests that it should be possible to screen for their activators as molecules that induce reporter expression in the presence of an otherwise inactive caspase. To demonstrate this, we carried out an experiment in which caspase activity was monitored in yeast that expressed full length caspase 9, alone or in combination with a fragment of Apaf-1 that is constitutively active with respect to caspase 9 processing activity *in vitro* (43). Transformants of the pGALL-CLBDG6 LexA/β-gal reporter strain were generated that carried either two empty vectors, an empty vector and pGALL-Apaf-$1^{530}$, an empty vector and pGALL-caspase 9, or pGALL-caspase 9 and pGALL-Apaf-$1^{530}$. Transformants initially were streaked on glucose medium. Colonies from these streaks then were replica plated onto gal/raf medium to induce GALL-dependent expression. After 16 hr of induction, levels of β-gal activity were determined by using an X-gal assay. Reporter cells that expressed either nothing, caspase 9, or Apaf-$1^{530}$ alone remained white in this assay, indicating that caspase activity was not induced (Fig. 3). However, colonies that expressed both caspase 9 and Apaf-$1^{530}$ showed robust β-gal activity, suggesting the occurrence of Apaf-$1^{530}$-mediated activation of the otherwise inactive procaspase 9 (Fig. 3).

We used β-gal activity, as assayed after replica plating of colonies, as the basis for our caspase reporter assay because caspases are expressed conditionally after replica plating. Thus, their identification is feasible even if their expression is toxic to cells. However, caspase activity screens also can be adapted to positive, survival-based screening assays by requiring LexA-dependent expression of a yeast auxotrophic marker such as HIS3 or URA3. False positives in these caspase reporter assays could arise because introduced proteins bind to the LexA binding sites and activate transcription directly or because they encode proteases that cleave CLBDG6, but not at the caspase target sites. Both classes of false positives can be identified by the fact that they should still activate *lacZ* expression when introduced into a LexA/β-gal reporter strain that expresses CLBGG6, the false positive reporter strain (Fig. 1*C*).

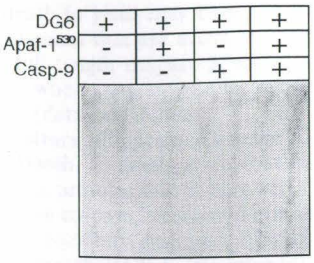


FIG. 3. Expression of Apaf-$1^{530}$ induces caspase 9-dependent reporter activation. W303α containing pSH18–34 and pGALL-CLBDG6 were transformed with pGALL plasmids to carry either two empty pGALL vectors, Apaf-$1^{530}$ and an empty pGALL vector, caspase 9 and an empty pGALL vector, or Apaf-$1^{530}$ and caspase 9. Two colonies from each transformation were streaked onto selective glucose medium plates, were grown for several days, and then were replica plated onto complete gal/raf medium. After 16 hr, an X-gal assay was performed. Only cells expressing both Apaf-$1^{530}$ and caspase 9 show significant β-gal activity.

**Suppression of Caspase-Dependent Reporter Activation by Caspase Inhibitors.** Once a reporter for the activity of a specific caspase has been established in yeast, it should be possible to screen for inhibitors of that activity by identifying cells that express the protease but in which reporter activity is repressed (Fig. 1*D*). Here, we demonstrate the feasibility of this approach by showing that two different families of caspase inhibitors, exemplified by baculovirus p35 and the murine IAP MIHA, suppress caspase-dependent reporter activation. p35 is a broad specificity caspase inhibitor in which inhibition is associated with cleavage (reviewed in ref. 6). The IAPs comprise a second class of caspase inhibitors distinct from p35 in which cleavage of the inhibitor does not play a role. IAPs originally were identified in the baculovirus system by virtue of their ability to substitute for baculovirus p35 as suppressers of viral infection-induced cell death (47). IAP homologs have now been found in other viruses, *Drosophila*, *C. elegans*, mammals and yeast and are characterized by one or more N-terminal repeats known as baculovirus IAP repeats (BIRs). Many also have a C-terminal RING finger motif (reviewed in refs. 6–8 and 48). Several mammalian IAPs—XIAP, cIAP1, and cIAP2—bind to and directly inhibit caspases-3 and -7 *in vitro* (49, 50). We introduced pGALL-CLBDG6 and either an empty pCUP1 plasmid or a pCUP plasmid containing CED-3 or caspase $7^{53}$ into the LexA/β-gal reporter strain along with either an empty pGALL expression vector, a pGALL expression vector carrying p35, or a pGALL expression vector carrying MIHA (murine XIAP). Transformants were grown in galactose-containing medium to induce expression of CLBDG6 and the caspase inhibitor and then were transferred to medium containing galactose and 3 μM copper to induce expression of the caspase. β-gal activity was determined after a 10-hr copper induction. As shown in Fig. 4 *A* and *B*, expression of p35 inhibited both CED-3 and caspase $7^{53}$ activity ≈5-fold. A similar though somewhat weaker (≈2-fold) inhibition of caspase $7^{53}$ activity was seen in the presence of MIHA (Fig. 4*B*), indicating that caspase-IAP interactions can be detected in this assay.

**Caspase Overexpression-Dependent Yeast Cell Death.** In the above assay, the presence of a caspase inhibitor is indicated by a decrease in β-gal activity. However, in many situations, it would be useful if caspase inhibition was coupled to a positive reporter output. A direct approach to identifying caspase inhibitors rests on the observation that high level expression of active caspase causes yeast cell death. Low level expression of the *Drosophila* caspase DCP-1 from the induced CUP-1 promoter did not significantly compromise yeast cell growth. Higher level expression from the GAL promoter, however, did

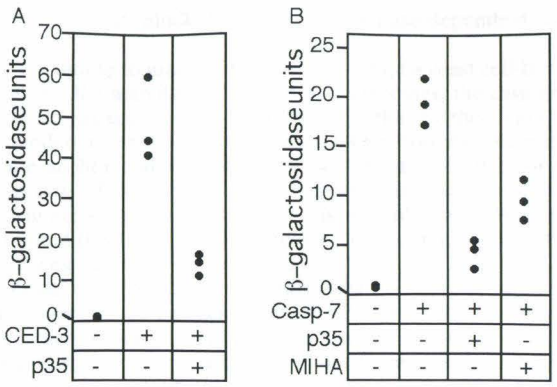


FIG. 4. Expression of caspase inhibitors suppresses caspase-dependent reporter activation in yeast. The LexA/β-gal reporter strain carrying pGALL-CLBDG6 was transformed with either an empty pCUP1 plasmid or pCUP1-CED-3 and either an empty pGALL vector or pGALL-p35. Three colonies from each transformation were grown for 24 hr in selective gal/raf medium. Cultures were diluted 1:10 into fresh gal/raf medium containing 3 μM copper sulfate and were grown for a further 10 hr, after which *o*-nitrophenyl-β-D-galactoside assays for β-gal activity were performed. Cultures from caspase transformants showed significant β-gal activity, which was suppressed by GALL-dependent expression of baculovirus p35 (*A*). In an experiment similar to that described in *A*, expression of caspase $7^{53}$ was induced in cells that express baculovirus p35 or the mouse IAP MIHA. Expression of caspase $7^{53}$ resulted in a significant increase in cellular β-gal activity, which was suppressed by GALL-dependent expression of p35 or MIHA (*B*).

result in cell lethality. Cells were able to grow on galactose-containing media if they carried an empty pGALL expression vector but not if they carried a pGALL-DCP-1 expression construct (Fig. 5*A*). This effect of DCP-1 expression was attributable to cell death because a >250-fold decrease in the number of colony forming units was seen when cells carrying the pGALL-DCP-1 expression plasmid were grown for 12 hr in liquid gal/raf medium, thus inducing high level DCP-1 expression, and then were plated on glucose-containing medium (data not shown). Of importance, GALL-DCP-1-expression-dependent cell killing depended on caspase activity because expression of an inactive mutant form of DCP-1 (DCP-1 C285S) did not cause cell death (Fig. 5*A*). Caspase-

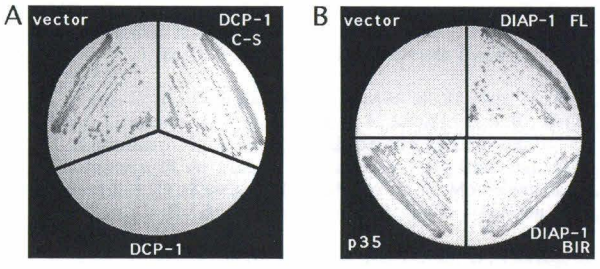


FIG. 5. High level expression of the *Drosophila* caspase DCP-1 kills yeast and is prevented by coexpression of baculovirus p35 or DIAP1. EGY48 yeast were transformed with pGALL plasmids containing either full length DCP-1 (FLDCP-1), an active site C-S mutant of DCP-1 (DCP-1 C-S), or no insert (vector). Transformants were streaked from selective glucose-containing medium onto selective gal/raf-inducing medium. Cells expressing either an empty pGALS vector or the DCP-1 C-S active site mutant grow on galactose-containing medium whereas cells expressing full length DCP-1 do not (*A*). EGY48 yeast carrying pGALS-FLDCP-1 were transformed with pGALL vectors carrying full length DIAP1 (DIAP1), the DIAP1 BIR repeats (DIAP1 BIR), baculovirus p35 (p35), or nothing (vector). GALL-dependent expression of p35, full length DIAP1, and, to a somewhat lesser extent, the DIAP1 BIR repeats block DCP-1-dependent cell death (*B*).

mediated cell death in yeast may be a general phenomenon because other caspases that are active in yeast, including full length CED-3, full length caspase 8, and caspase $7^{53}$, block colony formation when expressed under control of the strong GALL promoter (data not shown).

**Caspase Inhibitors Suppress Caspase Overexpression-Dependent Cell Death.** To demonstrate that caspase inhibitors can be identified as proteins that restore cell viability to yeast expressing an active caspase, we carried out an experiment in which the broad specificity caspase inhibitor p35 was coexpressed with full length DCP-1. We introduced pGALL expression plasmids that either had no insert, or that carried p35, into cells carrying a pGALS-DCP-1 plasmid. Cells from colonies carrying these plasmids were grown on glucose-containing media and then were streaked onto gal/raf media to induce expression of the caspase and the potential inhibitor. As shown in Fig. 5*B*, pGALS-dependent expression of DCP-1 in the presence of an empty pGALL expression vector resulted in no cell growth. In contrast, coexpression of baculovirus p35 with DCP-1 resulted in a dramatic rescue of cell growth.

To determine whether a yeast survival-based screen can be used to identify novel caspase inhibitors, we transformed yeast carrying pGALS-DCP-1 with a *Drosophila* pGALL embryonic cDNA expression library and plated these cells on gal/raf medium. In a screen of ≈$1.4 \times 10^5$ transformants, ≈50 positives were obtained. These were tested by PCR, and all were found to correspond to DIAP1, which was originally identified as an inhibitor of *reaper* or *hid* overexpression-induced, caspase-dependent cell death in the fly eye (37). In the fly eye, and in cell culture (51–54), it also was found that cell death could be suppressed by expression of an N-terminal DIAP1 fragment containing the two BIR repeats but lacking the C-terminal RING finger domain. To determine whether the same fragment of DIAP1 was sufficient to block DCP-1-dependent cell killing in yeast, we carried out an experiment in which full length DIAP1, an N-terminal fragment of DIAP1, or an empty vector was expressed under GALL control in the presence of GALS-driven DCP-1. As shown in Fig. 5*B*, inhibition of DCP-1-dependent cell death was seen when DIAP1 or the N-terminal fragment of DIAP1 containing only the DIAP1 BIR repeats was coexpressed with DCP-1. To determine whether the observed interaction between DIAP1 and DCP-1 was direct, we generated bacterially synthesized GST-DIAP1 as well as $His_6$-tagged versions of prodomainless DCP-1 (DCP-$1^{31}$-$His_6$) and drICE (drICE$^{81}$-$His_6$). As shown in Fig. 6, GST-DIAP1 inhibited DCP-$1^{31}$-$His_6$ caspase activity but had little if any effect on that of drICE$^{81}$-$His_6$. Thus, these results demonstrate that caspase inhibitors can be identified as

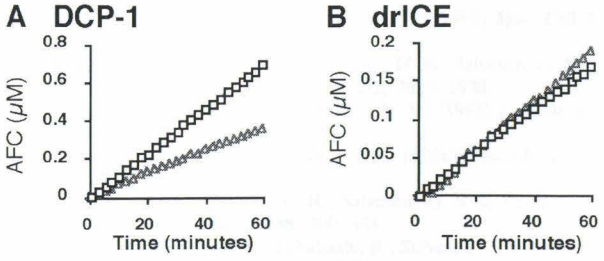


FIG. 6. GST-DIAP1 inhibits the caspase activity of bacterially synthesized DCP-$1^{31}$-$His_6$, but not drICE$^{81}$-$HIS_6$. Purified GST-DIAP1 (0.16 μM) (open triangles) or GST (0.48 μM) (open squares) was incubated with a fixed amount of DCP-$1^{31}His_6$ (0.2nM) in caspase activity assay buffer containing 100 μM of the Ac-DEVD-AFC substrate. Release of AFC was monitored fluorometrically over time. GST-DIAP1 inhibits DCP-1-dependent caspase activity (*A*). In similar experiments in which 0.62nM drICE$^{81}His_6$ was incubated in caspase activity buffer with GST (0.48 μM) or GST-DIAP1, (0.16 μM), no inhibition of drICE activity by GST-DIAP1was seen (*B*).

molecules that block GAL-driven, caspase-dependent cell death.

**Concluding Remarks.** We have developed a yeast cell-based assay for the activity of one group of proteases, the caspases, in which caspase activity is monitored either by the cleavage-dependent release of a transcription factor from its transmembrane anchor and subsequent activation of a reporter or by induction of cell killing. Both reporter activation and cell killing are suppressed by known caspase inhibitors. We have exploited this fact to directly isolate caspase inhibitors from a *Drosophila* embryo cDNA library.

Yeast carrying the transcription-based caspase reporter should be useful as a background in which to carry out screens for proteins that cleave a caspase target site and for their regulators. Because yeast can be transformed with high efficiency, it also constitutes an ideal system in which to carry out large scale mutagenesis studies of particular proteases or their regulators. It may also be possible to screen for cellular targets of specific caspases by using artificial substrate libraries in which cDNA fragments substitute for the caspase target site linker in the caspase substrate fusion protein CLBDG6. A transcription-based reporter strategy similar to that described here also may provide a way to monitor caspase activity in cells of higher eukaryotes. Finally, substituting caspase cleavage sites for those of other site-specific proteases in the CLBDG6 reporter should enable the identification and study of these proteins and their regulators.

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# Appendix II

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# The *Drosophila* Caspase Inhibitor DIAP1 Is Essential for Cell Survival and Is Negatively Regulated by HID

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## Summary

**_Drosophila_ Reaper (RPR), Head Involution Defective (HID), and GRIM induce caspase-dependent cell death and physically interact with the cell death inhibitor DIAP1. Here we show that HID blocks DIAP1's ability to inhibit caspase activity and provide evidence suggesting that RPR and GRIM can act similarly. Based on these results, we propose that RPR, HID, and GRIM promote apoptosis by disrupting productive IAP–caspase interactions and that DIAP1 is required to block apoptosis-inducing caspase activity. Supporting this hypothesis, we show that elimination of *DIAP1* function results in global early embryonic cell death and a large increase in DIAP1-inhibitable caspase activity and that *DIAP1* is still required for cell survival when expression of *rpr*, *hid*, and *grim* is eliminated.**

## Introduction

Programmed cell death, or apoptosis, is an evolutionarily conserved process by which organisms remove damaged or unwanted cells (reviewed in Wyllie et al., 1980; Raff, 1992). Central components of the machinery that carries out this process are caspases, a family of aspartate-specific, cysteine-dependent proteases (Alnemri et al., 1996). Caspases are made as zymogens, comprising three functional modules: a prodomain and two catalytic subunits known as the large, or p20, and the small, or p10, subunits. In general, caspases are activated in vivo following cleavage at aspartate residues that separate the prodomain from the catalytic region and separate the large and small subunits of the catalytic domain. The aspartates cleaved in caspase zymogens often resemble consensus target sites of known caspases. This has suggested that caspases may function in a cascade in which initiator caspases, activated by upstream death signals, cleave and activate a set of executioner caspases that carry out proteolytic cleavages of cellular proteins, leading ultimately to cell death (reviewed in Nicholson and Thornberry, 1997; Salvesen and Dixit, 1997; Cryns and Yuan, 1998; Thornberry and Lazebnik, 1998).

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Because caspases have the potential to initiate cascades of proteolysis, it is important that their activity be tightly regulated. The only cellular caspase inhibitors identified to date all share homology with a family of cell death inhibitors identified in baculoviruses, known as inhibitors of apoptosis, or IAPs (Crook et al., 1993; Birnbaum et al., 1994; reviewed in LaCasse et al., 1998 and Deveraux and Reed, 1999). IAP homologous proteins have been identified in other viruses, *Drosophila melanogaster*, *Caenorhabditis elegans*, mammals, and yeast (reviewed in Uren et al., 1998). These proteins are characterized by one or more N-terminal repeats of a motif known as the baculovirus IAP repeat (BIR). Many also have a C-terminal RING finger motif. Some IAP homologous proteins have been shown to act as cell death inhibitors. In several cases, this activity is localized to the BIR-containing region of the protein (reviewed in LaCasse et al., 1998; Uren et al., 1998; Deveraux and Reed, 1999), which has also been shown to mediate caspase binding and inhibition (Deveraux et al., 1997, 1998; Roy et al., 1997; Takahashi et al., 1998; Hawkins et al., 1999).

Proteins that initiate cell death by disrupting IAP–caspase interactions have not been described. However, the products of the *Drosophila reaper* (*rpr*), *head involution defective* (*hid*), and *grim* genes are interesting candidates. All three loci are located in the 75C region of the *Drosophila* genome, and deletion of this region eliminates death induced by many different stimuli, indicating that important cell death activators reside within (White et al., 1994). Furthermore, individual overexpression of any one of these genes is sufficient to initiate caspase-dependent cell death in many cells that normally live (Grether et al., 1995; Hay et al., 1995; Chen et al., 1996; White et al., 1996; Vucic et al., 1997, 1998; Zhou et al., 1997; Wing et al., 1998). Two *Drosophila* IAPs, DIAP1, the product of the *thread* (*th*) locus (Hay et al., 1995), and DIAP2 (Hay et al., 1995; Duckett et al., 1996; Uren et al., 1996), suppress *rpr*-, *hid*-, and *grim*-dependent cell death when overexpressed (Hay et al., 1995; Vucic et al., 1997, 1998; Bergmann et al., 1998; Wing et al., 1998), and DIAP1 and DIAP2 can immunoprecipitate RPR, HID, and GRIM from insect cells (Vucic et al., 1997, 1998). Furthermore, DIAP1 binds a processed form of the caspase drICE in insect cells (Kaiser et al., 1998) and inhibits the activity of a second caspase, DCP-1 (Hawkins et al., 1999). These observations suggest several models of how *Drosophila* IAPs, RPR, HID, GRIM, and *Drosophila* caspases might interact to regulate cell death (Kaiser et al., 1998). In one model, RPR, HID, or GRIM activates caspases through an IAP-independent pathway. In this model, *Drosophila* IAPs suppress apoptosis by acting as a sink for these proteins and by inhibiting caspase activation or activity initiated by their action. In a second model, DIAP1 functions primarily as a caspase inhibitor, and RPR, HID, and/or GRIM initiate caspase-dependent cell death by preventing IAPs from productively interacting with caspases.

The yeast *Saccharomyces cerevisiae* provides an

ideal system to study interactions between caspases and molecules that regulate their activity (Hawkins et al., 1999; Kang et al., 1999). While yeast cells do not exhibit apoptosis, and database searches have failed to uncover yeast homologs of the core apoptosis regulators identified in worms, flies, and mammals, overexpression of DCP-1 or drICE results in yeast cell death that is blocked by DIAP1. Here we used yeast expressing these proteins as a system to assay the ability of RPR, HID, and GRIM to disrupt IAP–caspase interactions. We found that all three proteins, while nontoxic on their own, killed yeast coexpressing DCP-1 or drICE and DIAP1, suggesting that they were blocking DIAP1's ability to function as a caspase inhibitor. We pursued the basis for this activity further with HID and found, both in yeast and in vitro, that proteins containing the N-terminal 37 residues of HID, which are sufficient to induce apoptosis in insect cells (Vucic et al., 1998), suppressed DIAP1's ability to inhibit DCP-1 activity. We also found that the *DIAP1* loss-of-function phenotype consists of an embryo-wide set of cellular changes reminiscent of apoptotic cell death and that these were associated with the activation of DIAP1-inhibitable caspase activity. Furthermore, double mutants that remove zygotic *rpr*, *hid*, *grim*, and *DIAP1* function showed phenotypes similar to those of the *DIAP1* loss-of-function mutant alone. These observations suggest that a principal function of DIAP1 is to promote cell survival by blocking caspase activity and that at least one mechanism by which RPR, HID, and GRIM promote apoptosis is by disrupting IAP–caspase interactions.

## Results and Discussion

### RPR, HID, and GRIM Block DIAP1's Ability to Suppress Caspase-Dependent Cell Death in Yeast

Yeast were transformed with two plasmids: one in which DCP-1 expression was driven by the inducible GAL1 promoter, and a second in which DIAP1 expression was driven by the constitutive Adh promoter (hereafter referred to as DCP-1-DIAP1 yeast). These cells were then transformed with a second GAL1 expression plasmid that was either an empty vector or carried GAL1-driven RPR, HID, or GRIM. Transformants were spotted as a series of 10-fold serial dilutions onto glucose plates to indicate the number of cells and onto galactose plates to induce expression of DCP-1 and RPR, HID, or GRIM. DCP-1-DIAP1 yeast carrying an empty vector survived on galactose, but DCP-1-DIAP1 yeast expressing HID or GRIM did not (Figure 1A). Expression of RPR under GAL1 control did not kill DCP-1-DIAP1 yeast in which DIAP1 expression was driven by the Adh promoter, but it was able to kill DCP-1-DIAP1 yeast in which DIAP1 expression was driven by a weaker promoter, the copper-inducible CUP1 promoter, in the presence of 30 μM $Cu^{2+}$ (Figure 1A). Importantly, expression in yeast of RPR, HID, or GRIM alone under GAL1 control had no effect on cell growth as compared with yeast expressing only the empty vector (Figure 1A). These results suggest that RPR, HID, and GRIM are able to block DIAP1's ability to inhibit DCP-1 caspase activity.

Three other *Drosophila* caspases, drICE, DCP-2/Dredd, and DRONC, have been implicated as effectors of apoptosis (Fraser and Evan, 1997; Fraser et al., 1997; Chen et al., 1998; Dorstyn et al., 1999). We wanted to determine if RPR, HID, and GRIM played a similar role in regulating their activity. We focused our attention on drICE. A version of drICE lacking the prodomain is not inhibited by DIAP1 (Hawkins et al., 1999), but a version of drICE that contains prodomain sequences is (C. J. H. and B. A. H., unpublished observations). Full-length drICE expressed in yeast under GAL1 control neither kills nor activates a cleavage-dependent reporter, probably because the caspase does not significantly autoactivate (Hawkins et al., 1999). We created a form of drICE that is active in yeast and contains prodomain sequences by generating a "reversed" form of drICE, rev-drICE, in which the drICE p10 domain was placed N-terminal to prodomain and p20 sequences (Figure 1B). Reversed forms of mammalian caspases that are not otherwise active are constitutively active (Srinivasula et al., 1998). As shown in Figure 1B, expression of rev-drICE under GAL1 control kills yeast, and this death is prevented by coexpression of DIAP1. Importantly, as with DCP-1, expression of HID, GRIM, or RPR killed yeast coexpressing rev-drICE and DIAP1.

### The N Terminus of HID Mediates Its Ability to Disrupt IAP–Caspase Interactions in Yeast

In insect cells, the N-terminal 37 amino acids of HID, expressed in the context of a larger fusion protein, are necessary and sufficient to induce apoptosis and to mediate HID's binding to DIAP1 (Vucic et al., 1998). To test the activity of these sequences in yeast, we generated GAL1 expression constructs similar to those of Vucic et al. (1998) in which the first 37 amino acids of HID were either present or absent (Figure 1C). HIDΔ(38–335) encodes a protein in which the first 37 amino acids of HID are fused to the C-terminal 74; HIDΔ(1–335) consists of only the last 74 residues of HID; and HIDΔ(1–36) encodes a protein that lacks the first 36 residues of HID but contains the rest of the full-length HID coding sequence. GAL1-dependent expression of HIDΔ(38–335) in DCP-1-DIAP1 yeast resulted in no growth. In contrast, DCP-1-DIAP1 yeast expressing HIDΔ(1–335) grew normally. HIDΔ(1–36) had weak killing activity in this assay (Figure 1D) but not in insect cell death assays (Vucic et al., 1998) or other in vitro assays described below. As with full-length HID, expression of HIDΔ(38–335), HIDΔ(1–335), or HIDΔ(1–36) in isolation had no effect on yeast cell growth (Figure 1D). Thus, proteins carrying the N-terminal 37 residues of HID, which are sufficient to kill insect cells, are also able to kill DCP-1-DIAP1 yeast.

### HID Suppresses DIAP1's Ability to Inhibit DCP-1 Caspase Activity In Vitro

The results of our yeast experiments suggested that HID directly inhibits DIAP1. We tested this idea in vitro using purified proteins. Bacterially expressed and purified versions of DCP-1, DIAP1, and HID (Figure 2A) were mixed in various combinations and DCP-1 caspase activity measured fluorometrically (Figures 2B and 2C). His6-tagged DCP-1 (DCP-1-His6) is active as a caspase, and this activity was inhibited by DIAP1 but not GST

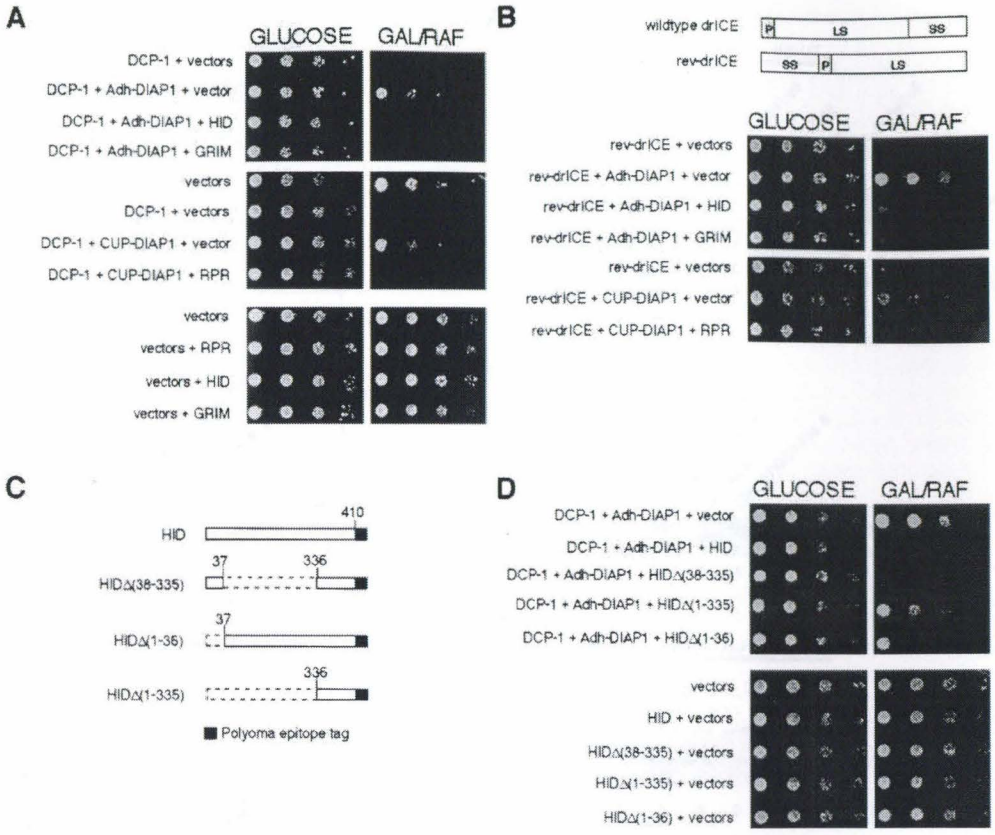



Figure 1. RPR, HID, and GRIM Block DIAP1's Ability to Suppress Caspase-Dependent Yeast Cell Death

(A) RPR, HID, and GRIM kill yeast expressing DCP-1 and DIAP1. Top: GAL1-driven DCP-1 causes yeast cell death that is prevented by Adh-dependent expression of DIAP1 (DCP-1 + Adh-DIAP1 + vector). GAL1-driven HID (DCP-1 + Adh-DIAP1 + HID) or GRIM (DCP-1 + Adh-DIAP1 + GRIM) results in yeast cell death. Middle: GAL1-driven RPR kills yeast expressing DCP-1 and CUP1-driven DIAP1 (DCP-1 + CUP-DIAP1 + RPR) in the presence of 30 μM $Cu^{2+}$. Bottom: Expression of RPR, HID, or GRIM in the presence of empty vectors does not affect yeast cell viability.

(B) RPR, HID, and GRIM kill yeast expressing rev-drICE and DIAP1. Top: drICE contains prodomain (P), large (LS), and small (SS) subunits. In reversed drICE (rev-drICE), the predicted drICE small subunit was placed N-terminal to drICE prodomain and large subunit sequences. Middle: Expression of HID or GRIM kills yeast expressing rev-drICE and Adh-DIAP1 (rev-drICE + Adh-DIAP1 + HID; rev-drICE + Adh-DIAP1 + GRIM). Bottom: Expression of RPR kills yeast expressing rev-drICE and CUP-DIAP1 (rev-drICE + CUP-DIAP1 + RPR) in the presence of no added $Cu^{2+}$.

(C) Diagram of HID constructs. Numbers represent amino acid positions in full-length HID. Solid lines indicate regions of HID present in the fusion protein; dashed lines indicate sequences that are deleted. A polyoma epitope tag present at the C terminus of each coding region is indicated by a black bar.

(D) Proteins containing the first 37 residues of HID block DIAP1's ability to inhibit DCP-1 activity in yeast. Top: Effects of expression of HID fusion proteins on the viability of DCP-1 + Adh-DIAP1 yeast. Bottom: Effects of expression of HID fusion proteins and empty vectors on yeast viability.

(Hawkins et al., 1999; Figure 2B). Addition of HID alone to a reaction containing DCP-1-His6 did not alter DCP-1-His6 activity, indicating that HID has no direct effect on DCP-1-His6 (Figure 2B). However, when full-length HID was incubated with DIAP1 and DCP-1-His6, DIAP1-dependent inhibition of DCP-1-His6 activity was lost (Figure 2B). Incubation of DIAP1 and DCP-1-His6 with HIDΔ(1–36)-His6 had little or no effect on DIAP1's ability to inhibit DCP-1-His6 activity, indicating that the first 37 residues of HID are necessary for this activity. To determine if the first 37 residues of HID were sufficient to mediate this effect, we carried out experiments using a version of HID that consists of the first 37 residues of HID fused to GST and a C-terminal His6 tag [HID(1–37)GST-His6]. As shown in Figure 2C, addition of HID(1–37)GST-His6 to an assay containing DCP-1-His6 and DIAP1 blocked DIAP1's ability to inhibit DCP-1-His6 activity, while addition of an equivalent amount of GST-His6 had no effect. Thus, consistent with our observations in yeast, purified HID directly blocks DIAP1's ability to inhibit DCP-1 caspase activity, and the first 37 amino acids of HID, at least in the context of a larger fusion protein, are necessary and sufficient for this activity.

To determine if HID directly interacts with DIAP1 or DCP-1, we carried out binding experiments. As expected from the results of our yeast and in vitro activity

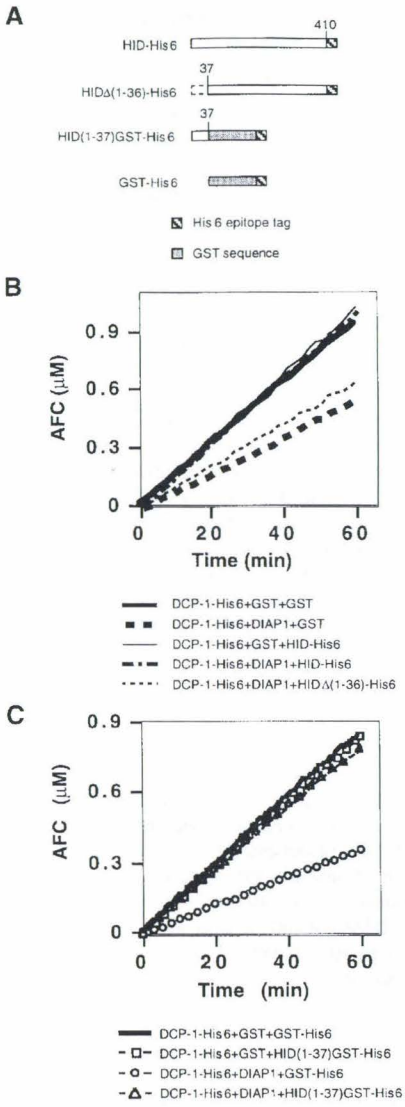



Figure 2. Proteins Containing the First 37 Residues of HID Block DIAP1's Ability to Inhibit DCP-1 Activity In Vitro

(A) Schematic of HID constructs. HID coding regions are represented by open boxes and deleted regions by dashed lines.

(B) The N-terminal 36 residues of HID are required to inhibit DIAP1 function. DCP-1-His6 (0.2 nM) activity was measured by following the release of AFC fluorometrically from the Ac-DEVD-AFC substrate over time. The total protein concentration in each incubation was kept constant, with GST (0.2 μM or 0.77 μM) being used to replace DIAP1 (0.2 μM), HID-His6 (0.44 μM), or HIDΔ(1–36)-His6 (0.44 μM) as the assay required.

(C) A protein containing the N-terminal 37 residues of HID, HID(1–37)GST-His6, is sufficient to block DIAP1's caspase inhibitory activity. Assays were performed as in (B).

assays, GST-DIAP1 coupled to glutathione–Sepharose bound full-length HID-His6 and DCP-1-His6 but did not appreciably bind HIDΔ(1–36)-His6. GST alone showed no interaction with any of these proteins (Figure 3A). Also, a maltose-binding protein–DIAP1 fusion protein

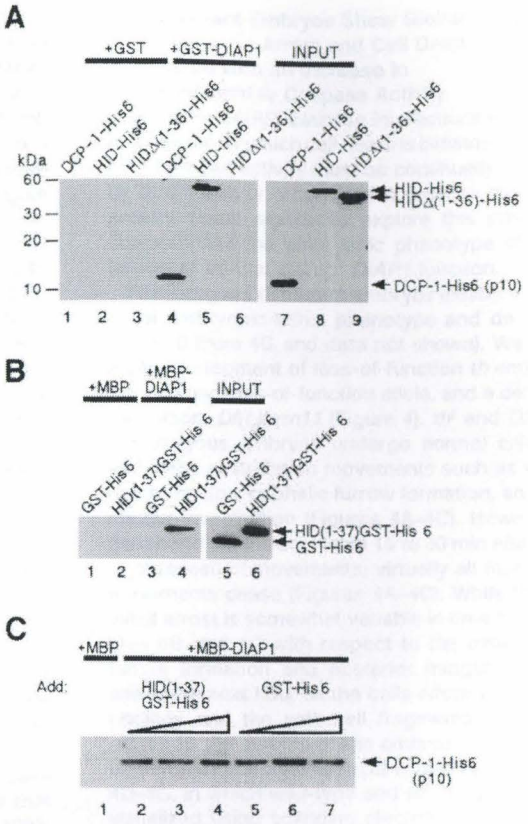



Figure 3. Physical Interactions between HID, DIAP1, and DCP-1

(A) DIAP1 binds DCP-1 and HID, and the first 37 residues of HID are required for this interaction. Glutathione-Sepharose-bound GST or GST-DIAP1 was incubated in binding buffer with DCP-1-His6, HID-His6, or HIDΔ(1–36)-His6 (Figure 2A). Binding reactions were followed by washes and Western blotting. A fraction of the input of DCP-1-His6, HID-His6, and HIDΔ(1–36)-His6 is shown.

(B) DIAP1 binds proteins containing only the first 37 residues of HID. Amylose resin–bound MBP or MBP-DIAP1 was incubated with GST-His6 or HID(1–37)GST-His6 and processed as above.

(C) HID(1–37)GST-His6 does not prevent DCP-1-His6 from binding MBP-DIAP1. MBP-DIAP1 bound to amylose resin was incubated with increasing amounts of HID(1–37)GST-His6 or GST-His6. Following this preincubation, a constant amount of DCP-1-His6 was introduced into the mixture. The beads were washed and processed for Western blotting using an anti-His antibody to detect DCP-1-His6. MBP beads incubated with the highest concentration of HID(1–37)GST-His6 and equivalent amounts of DCP-1-His6 were processed in a similar manner.

(MBP-DIAP1) bound to amylose resin was able to bind HID(1–37)GST-His6, while MBP bound to the same resin was not (Figure 3B). Furthermore, HID(1–37)GST-His6, bound to glutathione–Sepharose beads, was able to bind $^{35}$S-methionine-labeled, in vitro–translated DIAP1 but not in vitro–translated DCP-1 (data not shown). These observations, in conjunction with the fact that HID-His6 or HID(1–37)GST-His6 alone had no effect on DCP-1-His6 caspase activity (Figures 2B and 2C), suggest that HID's effects on caspase activity are mediated through DIAP1.

To explore the mechanism by which HID blocks DIAP1's ability to inhibit DCP-1, we asked if HID(1–37)GST-His6 was able to displace DCP-1-His6 from DIAP1. MBP-DIAP1 bound to amylose resin was preincubated with increasing amounts of GST or HID(1–37)GST-His6 followed by addition of DCP-1-His6. Binding reactions were followed by washes and analysis of the bead-bound proteins by Western blotting. As shown in Figure 3C, DCP-1 binding to MBP-DIAP1 was not appreciably decreased by the presence of up to a 20-fold molar excess of GST-His6 or HID(1–37)GST-His6 over MBP-DIAP1. As expected, MBP beads incubated with HID(1–37)GST-His6 showed no binding to DCP-1-His6. Thus, we see no evidence that HID(1–37)GST-His6, at molar ratios with respect to DIAP1 greater than those used to demonstrate inhibition of DIAP1 function in caspase activity assays (Figure 2C), prevents binding of DCP-1-His6 to MBP-DIAP1. These observations suggest that HID(1–37)GST-His6 does not prevent DIAP1 from inhibiting DCP-1 activity by blocking DCP-1 binding. It is important to note, however, that full-length HID, which has a large C-terminal domain that can regulate HID activity (Bergmann et al., 1998), may behave differently.

Our observations suggest that RPR, HID, and GRIM can promote caspase-dependent cell death by blocking DIAP1's ability to inhibit caspase activity. These conclusions are supported by the observations that in insect cells, RPR, HID, and GRIM promote caspase-dependent cell death that is sensitive to the levels of DIAP1 (Hay et al., 1995; Vucic et al., 1997, 1998; Bergmann et al., 1998; Wing et al., 1998), that these proteins associate with DIAP1 in vivo (Vucic et al., 1997, 1998), and that DIAP1 binds and inhibits caspases (Kaiser et al., 1998; Hawkins et al., 1999; this work). Furthermore, since proteins that contain only the first 37 amino acids of HID are sufficient to initiate the caspase-dependent death of insect cells and to block DIAP1's ability to inhibit caspase activity, but other regions of HID lack activity in insect cells, it is likely that disrupting DIAP1's ability to inhibit caspase activity is an important mechanism by which HID promotes cell death. Because of difficulties with solubility of RPR and GRIM under conditions that allowed productive DIAP1–caspase interactions, we were unable to carry out in vitro experiments similar to those carried out with HID. However, based on the fact that they behaved similarly to HID in yeast expressing DCP-1 or rev-drICE and DIAP1, it seems likely that they also are able to block DIAP1's ability to inhibit caspase activity.

Our observations do not exclude the possibility that RPR, HID, and GRIM promote apoptosis through other pathways as well. For example, RPR has been shown to promote apoptosis in a *Xenopus laevis* system (Evans et al., 1997) through association with a novel protein, SCYTHE (Thress et al., 1998), and peptides corresponding to N-terminal sequences of RPR and GRIM block $K^+$ channel function (Avdonin et al., 1998). Furthermore, as described below, removal of DIAP1 in the embryo results in massive cell death, but the *DIAP1* mutant cells do not show all the features of apoptosis seen in wild-type embryos, consistent with the idea that other pathways may be necessary to create a complete apoptotic response.

### *DIAP1* Mutant Embryos Show Global Early Morphogenetic Arrest and Cell Death Associated with an Increase in DIAP1-Inhibitable Caspase Activity

If disruption of IAP–caspase interactions is an important mechanism by which cell death is initiated in *Drosophila*, then caspase activity must be continually held in check by DIAP1 and/or other IAPs in order to block an ever-present death signal. To explore this possibility, we characterized the embryonic phenotype of mutations (alleles of *th*) that disrupt *DIAP1* function.

Homozygous *th* mutant embryos exhibit a severe terminal embryonic lethal phenotype and do not form a cuticle (Figure 4G and data not shown). We monitored early development of loss-of-function *th* embryos from $th^5$, a strong loss-of-function allele, and a deficiency for the region, *Df(L)brm11* (Figure 4). $th^5$ and *Df(3R)brm11* homozygous embryos undergo normal cellularization and initial gastrulation movements such as ventral furrow formation, cephalic furrow formation, and posterior midgut invagination (Figures 4A–4C). However, during germband extension, within 15 to 30 min after the onset of gastrulation movements, virtually all morphogenetic movements cease (Figures 4A–4C). While the stage of initial arrest is somewhat variable in time (compare Figures 4B and 4C with respect to the extent of ventral furrow formation and posterior midgut invagination), within the next hour all the cells adopt a rounded morphology and the yolk cell fragments and eventually moves to the surface of the embryo. The disruption of embryonic integrity is seen particularly clearly in Figures 4D–4G, in which wild-type and $th^5$ mutant embryos are visualized using scanning electron microscopy. While some $th^5$ homozygous embryos showed normal morphology at the beginning of germband extension (data not shown), other embryos with a similar degree of extension movements showed extensive cell rounding, suggesting a disruption of intercellular adhesion (Figures 4D and 4E). All $th^5$ embryos also show a regression of the cephalic furrow (compare Figure 4A with Figures 4B and 4C, and Figure 4D with 4E). At the extended germband stage (stage 9), the surface of cells in control embryos is smooth and the cells are closely apposed to each other (Figure 4F). In contrast, by this time $th^5$ homozygotes have adopted a uniform cellular morphology in which all cells have a round shape, the surface of the cells contains many membrane blebs, and vesicular cell fragments similar to those seen in dying cells are present (Figure 4G). The yolk cell, normally located interiorly, is visible at the surface. Thus, zygotic loss of *DIAP1* function results in early morphogenetic arrest followed by severe morphological abnormalities at the beginning of germband extension.

We used TUNEL labeling to determine whether the massive DNA fragmentation characteristic of cells undergoing apoptosis had occurred in cells of *th* mutants (Figure 5). Wild-type embryos during germband extension showed significant DNA fragmentation in only a few cells (Figures 5A, 5C, and 5E). In contrast, comparably staged $th^5$ embryos labeled extensively in most if not all cells. Initially, the TUNEL signal is relatively weak (Figures 5B and 5D), but the intensity increases within the following hour of development to a strong uniform labeling (Figures 5B, 5D, and 5F). In contrast,

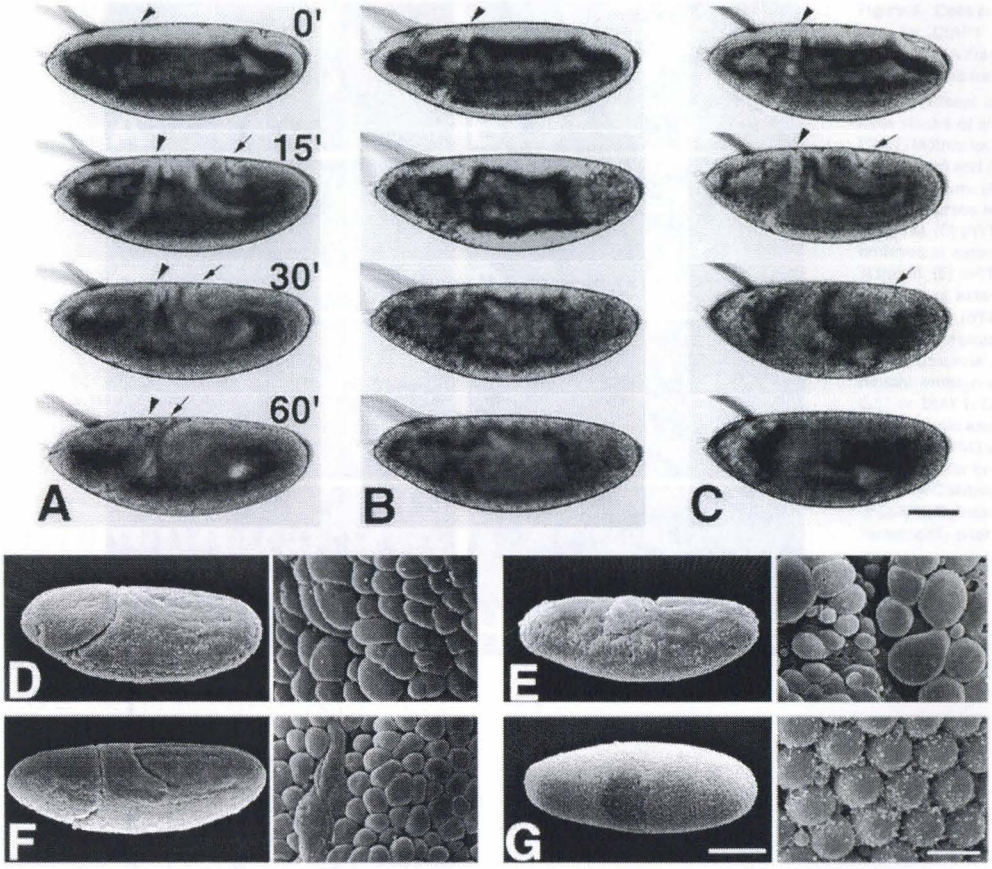



Figure 4. Removal of Zygotic DIAP1 Leads to Morphogenetic Arrest during Germband Extension and Global Cell Shape Changes

(A) Wild-type developmental series of living embryos derived from a $th^5$/TM3[ftz::lacZ] stock during gastrulation and germband extension (stages 6 through 9). Arrowheads mark the position of the cephalic furrow, and arrows indicate the front of the extending germband.

(B and C) $th^5$ and *Df(3L)brm11* homozygous embryos, respectively, at the same developmental stages as in (A).

(D and F) Scanning electron micrographs of balancer-bearing embryos from $th^5$/TM3[ftz::lacZ] stock. The right-hand panels show higher magnification of the general morphology of the embryonic surface. (D) shows an embryo at stage 8, and (F) shows an embryo at stage 9.

(E and G) Similarly staged embryos homozygous for $th^5$.

Scale bars are 100 μm in (C) and (G) (left-hand panel) and 10 μm in (G) (right-hand panel).

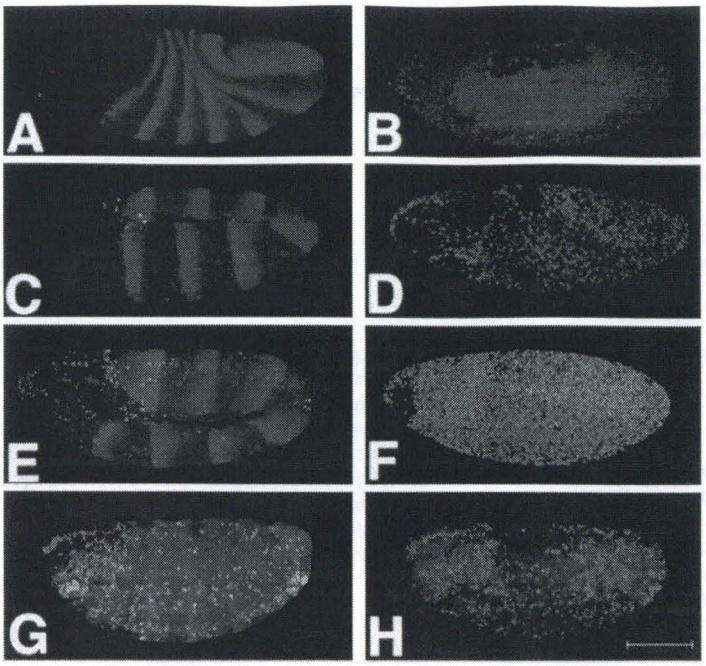
embryos homozygous for the weaker allele $th^6$ do not show a significant increase in TUNEL-positive cells as compared to comparably staged control embryos at the retracted germband stage (stage 14; Figures 5G and 5H).

To determine if loss of *DIAP1* in fact results in an increase in caspase activity, we measured caspase activity in extracts of 3- to 5-hr-old embryos from wild-type, $th^5$, and $th^6$ stocks by following the release of Ac-DEVD-AFC fluorometrically (Figure 5I). Extracts from wild-type and $th^6$ embryos had very low levels of caspase activity. In contrast, extracts from $th^5$ embryos had very large amounts of caspase activity. Importantly, the increased caspase activity present in the $th^5$ embryonic extracts was inhibited by the addition of purified DIAP1 (Figure 5I), consistent with the idea that DIAP1 normally represses these caspases.

The caspase targets of DIAP1 in the *Drosophila* embryo or other tissues have not been identified. Five caspases have been identified in *Drosophila*: DCP-1 (Song et al., 1997), drICE (Fraser and Evan, 1997), DCP-2/Dredd (Inohara et al., 1997; Chen et al., 1998), DRONC (Dorstyn et al., 1999), and a fifth gene (C. J. H. and B. A. H., unpublished). DIAP1 is able to inhibit the activity of the three caspases we have tested: DCP-1 (Hawkins et al., 1999), drICE (this report), and DRONC (C. J. H., unpublished). The *DIAP1* loss-of-function embryonic phenotype may result from the unrestrained activity of zygotically expressed versions of these caspases. Alternatively, DIAP1 may also function in the early embryo to block the activity of caspases introduced late during oogenesis into the developing oocyte from the degenerating nurse cells (Cavaliere et al., 1998; Chen et al., 1998; Foley and Cooley, 1998; McCall and Steller, 1998; Dorstyn et al., 1999).

### *DIAP1* Loss-of-Function Mutant Embryos Do Not Stain with Acridine Orange, a Marker of Apoptotic Cell Death

Acridine orange (AO) is commonly used as a marker for apoptotic cell death. It appears to enter all cells but is



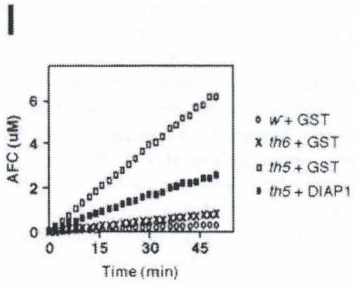




Figure 5. Cells in Embryos Homozygous Mutant for DIAP1 Show Premature and Increased DNA Fragmentation and an Increase in DIAP1-Inhibitable Caspase Activity

(A–F) Confocal images of staged embryos from stocks of $th^5$/TM3[ftz::lacZ] and (G and H)$th^6$/TM3[ftz::lacZ] double labeled for TUNEL (green) and β-galactosidase (red). Scale bar is 100 μm. (A) $th^5$/TM3[ftz::lacZ] and (B) $th^5/th^5$ embryos at early germband extension (stage 8). (C) $th^5$/TM3[ftz::lacZ] and (D) $th^5/th^5$ embryos at extended germband stage (early stage 9). (E) $th^5$/TM3[ftz::lacZ] and (F) $th^5/th^5$ embryos at extended germband stage (late stage 9). (G) $th^6$/TM3[ftz::lacZ] and (H) $th^6/th^6$ embryos at retracted germband stage (stage 14). (I) Caspase activity of wild-type and *th* mutant embryo extracts in the presence of GST or DIAP1. One microgram of extract of 3- to 5-hr-old embryos from stocks of $w^-$, $th^5$/TM3, or $th^6$/TM3 was introduced into caspase activity buffer containing 100 μM of the Ac-DEVD-AFC substrate and 0.5 μg of either GST or DIAP1. Release of AFC was monitored fluorometrically over time.

only retained (through unknown mechanisms) by cells undergoing apoptosis (see Abrams et al., 1993). In light of the strong defects in *th* mutant embryos reported here, we repeated experiments described in Hay et al. (1995) reporting that *th* embryos did not show an increase in AO staining. Figure 6A shows a bright-field image of a stage 14 $th^5$/*TM3* embryo. Figure 6B shows the same embryo stained with AO. Dying cells show bright green fluorescence, and living cells do not stain. A $th^5$ homozygous embryo displaying the *th* terminal phenotype is shown in Figure 6C. All cells have rounded up, and the yolk sac has moved to the surface. However, as shown in Figure 6D, no AO staining is seen despite the fact that embryos at this stage show cell rounding, membrane blebbing, increased caspase activity, and DNA fragmentation. Thus, the *th* phenotype, while showing some features of apoptosis, lacks others. One explanation for this is that the early embryo simply lacks the cellular machinery required to carry out apoptotic steps leading to AO retention. A second possibility is that the

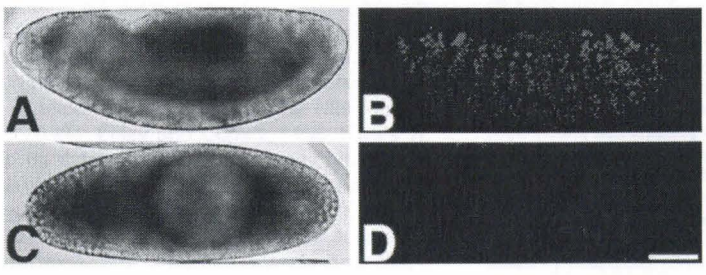



Figure 6. Cells in DIAP1 Loss-of-Function Embryos Do Not Show an Increase in Acridine Orange Staining Characteristic of Cells Undergoing Apoptosis in Wild-Type Embryos

(A and B) stage 14 $th^5$/TM3[ftz::lacZ] or TM3[ftz::lacZ]/TM3[ftz::lacZ] embryo stained with acridine orange and viewed under the confocal microscope using Nomarski (A) and fluorescence channels (B).

(C and D) $th^5$ homozygous embryo exhibiting the terminal phenotype. At this stage, acridine orange staining is never observed in $th^5$ homozygotes (D). Scale bar is 50 μm.

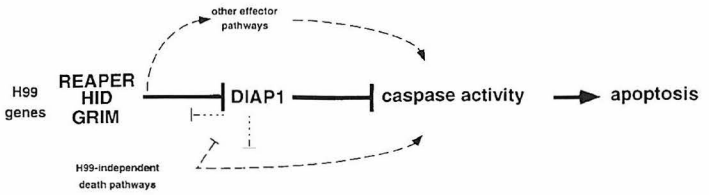



Figure 7. Model for How DIAP1 Regulates Apoptosis

DIAP1 inhibits caspase activity and is essential for cell survival. DIAP1's ability to inhibit caspase activity is suppressed by RPR, HID, and GRIM, thus promoting caspase-dependent cell death. RPR and GRIM have other activities (Evans et al., 1997; Avdonin et al., 1998; Thress et al., 1998), suggesting that these proteins may also promote apoptosis through other pathways. Free DIAP1 may inhibit apoptosis by binding these proteins, sequestering them from their targets. Thus, DIAP1 may act both upstream and downstream of RPR, HID, and GRIM to prevent cell death. Genes within the H99 interval are not required for some cell deaths (White et al., 1994; Foley and Cooley, 1998), indicating that other death pathways exist. It is not known if these pathways act through DIAP1.

expression of other IAPs, such as DIAP2, suppresses some of the caspase-dependent events necessary for a full apoptotic cell death phenotype. A third possibility (discussed further below) is that loss of *DIAP1* function, while sufficient to kill cells, is not sufficient to confer all aspects of apoptosis. In this scenario, proteins such as RPR, HID, or GRIM that are sufficient to trigger a full apoptotic response when overexpressed may play dual roles: inhibiting IAP function and, in addition, activating other apoptotic pathways (Figure 7).

### *DIAP1* Is Epistatic to *rpr*, *hid*, and *grim*

To explore further the relationship between *rpr*, *hid*, *grim*, and *DIAP1*, we made a double mutant with the H99 deficiency (which includes *rpr*, *hid*, and *grim*) and $th^5$. Homozygous H99 embryos essentially lack apoptosis (White et al., 1994). However, double mutants show a terminal embryonic phenotype similar to that of $th^5$ alone (data not shown). This, in conjunction with the fact that DIAP1 is a caspase inhibitor, argues against models in which DIAP1's sole antiapoptotic role is to bind to RPR, HID, and GRIM, thereby suppressing death pathways they activate, since in this scenario, the double mutant phenotype would be expected to be similar to that of the H99 mutant alone. Instead, our observations are consistent with models in which DIAP1 has an important antiapoptotic function as a caspase inhibitor and RPR, HID, and GRIM function, at least in part, through DIAP1 to promote apoptosis (Figure 7).

DIAP1 may, however, have antiapoptotic functions in addition to caspase inhibition. IAPs in insects and mammals have been shown to bind a number of different proteins through the BIRs in addition to caspases (reviewed in LaCasse et al., 1998). An attractive hypothesis is that some of these proteins have proapoptotic functions that do not involve disrupting IAP–caspase interactions and that death-preventing IAPs are able to suppress apoptosis induced by their expression by binding and sequestering them, preventing access to their targets. Candidates for such proteins in *Drosophila* are RPR, HID, GRIM, and DOOM (Harvey et al., 1997), as well as uncharacterized molecules that mediate normally occurring apoptotic cell death that occurs independently of expression of genes in the H99 interval (White et al., 1994; Foley and Cooley, 1998; Figure 7). DIAP1, and by extension other IAPs, may function to block death at multiple steps: at an upstream point, free DIAP1 may titrate apoptosis inducers away from their targets, DIAP1–caspase complexes and components of other effector pathways, while at a downstream point it inhibits caspase activation or activity induced by these proteins (Figure 7).

### Concluding Remarks

IAPs are the only cellular caspase inhibitors identified to date. We showed that RPR, GRIM, HID, and HID fragments sufficient to cause the death of insect cells block DIAP1's ability to inhibit caspase activity. These observations provide a mechanism for RPR, HID, and GRIM action and define a novel point at which caspase activity can be regulated. The finding that the *DIAP1* loss-of-function phenotype involves cell death associated with increased caspase activity strongly suggests that the interactions we described between RPR, HID, GRIM, DIAP1, and caspases are physiologically important. Since most mammalian cells constitutively express caspases sufficient to carry out cell death (Weil et al., 1996), the mechanism of cell death activation defined by these proteins may be quite general. A prediction of our model of RPR, HID, and GRIM function is that their ability to kill will depend on the status of a cell's caspases. In cells in which caspases are activated or spontaneously undergoing autoactivation at some level, their expression may be sufficient to kill. However, in other cells in which caspase activity is either absent or more tightly regulated, they may function primarily to create a permissive condition for caspase-dependent cell death. Thus, just as IAPs in their role as caspase inhibitors may function as cellular buffers that create a requirement for a certain level of caspase activation in order to induce apoptosis, so RPR-, HID-, and GRIM-like proteins may function to titrate this buffering activity so that cells are more or less sensitive to caspase-dependent death signals.

## Experimental Procedures

### Yeast Strain Constructions

All yeast strains used in this study are in the W303α background (*MATα*, *can1-100*, *leu2-3* and *-112*, *his3-11* and *-15*, *trp1-1*, *ura3-1*, *ade2-1*). Construction of pGALL-(*HIS3*), pGALL-(*LEU2*), and pGALL-DCP-1 was described previously (Hawkins et al., 1999). The p10 and prodomain-p20 fragments of drICE were amplified by PCR and inserted in tandem into pGALL-(*LEU2*) to make pGALL-rev-drICE. pAdh-(*TRP1*) was made by subcloning the promoter-terminator region from pHybZeo (Invitrogen) into pRS314. pCUP1-(*TRP1*) was constructed by replacing the GAL1 promoter of pGALL-(*TRP1*) (Hawkins et al., 1999) with the CUP1 promoter. The DIAP1 coding region was inserted into pAdh-(*TRP1*) and pCUP1-(*TRP1*) to generate pAdh-DIAP1 and pCUP-DIAP1. The HID coding region was amplified by PCR using primers that appended a C-terminal double

polyoma epitope tag and was inserted into pGALL-(*HIS3*) to generate pGALL-HID. pGALL-HID was digested with XhoI and religated to make pGALL-HIDΔ(38–335). C-terminally polyoma-tagged versions of HID initiating at codon 37 [HIDΔ(1–36)] and HID lacking the first 335 amino acids [HIDΔ(1–335)] were amplified by PCR and inserted into pGALL-(*HIS3*). The coding regions of RPR and GRIM were amplified by PCR and inserted into pGALL-(*HIS3*) to generate pGALL-RPR and pGALL-GRIM.

### Cell Death Assays

Overexpression of active caspases in yeast results in cell death that is inhibited by coexpression of caspase inhibitors (Hawkins et al., 1999; Kang et al., 1999). Because expression of RPR, HID, or GRIM alone has no effect on yeast growth (below), we infer that the yeast growth suppression seen in cells that express caspases, DIAP1 and RPR, HID, or GRIM is due to cell death. To assay caspase-dependent cell death and protection by inhibitors, exponentially growing cultures of individual W303α transformants containing the relevant constructs were serially diluted 10-fold and spotted onto 2% glucose selective medium plates or 2% galactose and 1% raffinose (gal/raf)–inducing selective medium plates. Plates were incubated at 25°C for 12 hr and then at 30°C and photographed after 2 (glucose) or 3–5 (gal/raf) days.

### Expression and Purification of Recombinant *Drosophila* IAPs, Caspases, and HID

DCP-$1^{31}$-$His_6$ (DCP-1-His6) was prepared as described previously (Hawkins et al., 1999) and diluted into buffer A (50 mM HEPES [pH 7.5], 100 mM NaCl, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, 5 mM DTT). The DIAP1 coding region was amplified by PCR using primers that generated an N-terminal TEV protease recognition site (ENLYFQG) and was introduced into the glutathione S-transferase (GST) expression vector pGEX4T-1 (Amersham Pharmacia). The GST-TEV-DIAP1 fusion protein was expressed in *Escherichia coli* strain BL21(DE3)pLysS and was affinity purified on glutathione–Sepharose. The bound fusion protein was cleaved with TEV protease in buffer A. The coding region of HID was introduced into pET23a(+) (Novagen), generating a HID-His6 fusion. A similar procedure was used to generate a version of HID initiating at codon 37 [HIDΔ(1–36)-His6]. The first 37 amino acids of HID and the coding region of GST were PCR amplified and introduced in tandem into pET23a(+) to generate HID(1–37)GST-His6. GST was introduced into pET23a(+) to generate GST-His6. These vectors were introduced into BL21(DE3)pLysS, protein expressed, and affinity purified from the soluble fraction using ProBond resin (Invitrogen). Proteins were eluted and stored at −80°C in buffer D (50 mM HEPES [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, 20 mM 2-mercaptoethanol, 1% Triton X-100, 20% glycerol, 0.55 M imidazole).

A caspase cleavage site (DQVD) at residue 20 of DIAP1 (S. L. W., unpublished) was altered to DQVE in GST-mycDIAP1 (Hawkins et al., 1999), generating GST-DIAP1. GST and GST-DIAP1 were purified on glutathione–Sepharose beads. The mutated myc-DIAP1 coding sequence was inserted into pMAL-c2 (New England Biolabs), generating MBP-DIAP1. MBP and MBP-DIAP1 were purified on amylose resin.

### In Vitro Protease Assays

HID's ability to inhibit DIAP1 was determined from caspase activity assay progress curves in which the release of 7-amino-4-trifluoromethyl-coumarin (AFC) from the synthetic substrate Ac-DEVD-AFC (100 μM) by DCP-1-His6 (0.2 nM) was measured in the presence of GST (0.2 μM) or DIAP1 (0.1 μM) and HID-His6 (0.44 μM), HIDΔ(1–36)-His6 (0.44 μM), or GST (0.77 μM). Caspase activity assays were performed in triplicate and prepared as follows. Two microliters DCP-1-His6 was incubated with 5 μl DIAP1 or GST for 5 min at 25°C. Seventy-one microliters buffer X (70.4 mM HEPES [pH 7.5], 140.8 mM NaCl, 1.4 mM EDTA, 0.14% CHAPS, 14.1% sucrose, 3.5 mM DTT, 5.6 % glycerol, and 0.7% Triton X-100) and 20 μl HID-His6, HIDΔ(1–36)-His6, or GST-His6 was subsequently added and the samples further incubated 23 min. After addition of 2 μl 5 mM Ac-DEVD-AFC in DMSO to each sample, caspase activities were assayed at 27°C using a fluorometric plate reader (Molecular Devices, fmax) in the kinetic mode with excitation and emission wavelengths of 405 and 510 nm, respectively. HID(1–37)GST-His6 activity, used at a concentration of 1.6 μM, was tested in a similar manner.

Caspase activity of embryos was monitored as follows. Zero to two-hr-old embryos were collected and aged for 3 hr at 25°C. Embryos were dechorionated with 50% bleach, rinsed, suspended in an equal volume of buffer E (buffer A containing 0.5% Triton X-100 and 4% glycerol), and homogenized. One microliter of this extract (approximately 10 mg/ml) was diluted into 100 μl of buffer E in the presence of 100 μM Ac-DEVD-AFC. Assays were performed in triplicate with freshly prepared extracts.

### Protein Binding Assays

Purified recombinant proteins (150 ng each) were incubated with either GST beads, MBP beads, GST-DIAP1 beads, or MBP-DIAP1 beads (200 ng) for 30 min on a rotator at 25°C in buffer C (100 mM HEPES [pH 7.5], 200 mM NaCl, 2 mM EDTA, 0.2% CHAPS, 20% sucrose, 40 mM 2-mercaptoethanol, 1% Triton X-100, 8% glycerol). Beads were then washed three times for 5 min with 1 ml buffer D lacking imidazole. Bead–protein complexes were subjected to 15% SDS-PAGE and immunoblotting. Proteins were detected with an anti-His antibody (Qiagen) and HRP-conjugated secondary antibodies followed by ECL (Amersham).

### HID-DCP-1 Competition Assay

MBP or MBP-DIAP1 beads (0.5 μg) were mixed with 1, 5, or 20 μg GST-His6 or HID(1–37)GST-His6 for 1 hr at 4°C in buffer A. Five micrograms of DCP-1-His6 was added and the samples further incubated for 10 min. After extensive washing, the bead–protein complexes were subjected to 15% SDS-PAGE and immunoblotting. Proteins were detected with an anti-His antibody and HRP-conjugated secondary antibodies followed by ECL.

### *Drosophila* Strains

A *w* Oregon R strain was used as wild type. The deficiency *Df(3L)brm11* (72A3–4; 72D1–5) uncovers *DIAP1*. *DIAP1* mutants, alleles of the *th* locus, and *Df(3L)brm11* were kept as balanced stocks over a version of the TM3 balancer that carries an ftz::lacZ transgene (TM3[ftz::lacZ]). $th^5$ is a loss-of-function mutation in *DIAP1*, since *Df(3L)brm11* shows a GMR-*rpr* enhancer phenotype similar to that of $th^5$ (Hay et al., 1995), and the embryonic lethal phenotype of $th^5$/*Df(3L)brm11* (this work, data not shown) is similar to that of $th^5/th^5$. By these same criteria, $th^6$ is a much weaker allele.

### Cell Death Assays and Microscopy

Embryos were collected on yeasted apple juice plates. For observation of living embryos, syncytial blastoderm stages were selected under halocarbon oil 27 (Sigma) and mounted individually on a slide using 10 mm coverslips. Images were taken on a Zeiss Axiophot from sets of six embryos per experiment every 15 min at 25°C.

For scanning electron microscopy, staged collections of embryos were processed for staining with anti-β-galactosidase (β-gal) antibodies (Cappel) as described elsewhere (Müller and Wieschaus, 1996). Homozygotes were isolated under the dissecting scope; the remaining embryos served as controls. The embryos were then processed for scanning electron microscopy as described in Müller and Wieschaus (1996). Samples were viewed under a Hitachi S800 SEM.

TUNEL labeling was performed using an in situ cell death detection kit (Roche). Embryos were fixed as described above, rinsed in PTX (PBS, 0.5% Triton X-100), and immunolabeled with antibodies against β-gal (Cappel) and Cy3-conjugated goat anti-rabbit (Jackson) secondary antibodies. Embryos were then incubated in 100 mM sodium-citrate, 0.1% Triton X-100 at 65°C for 30 min, followed by rinsing twice in PTX and twice in TUNEL dilution buffer (Roche). Embryos incubated in TUNEL assay buffer (Roche; 30 min to 2 hr) were supplemented with TUNEL enzyme (Roche) and incubated a further 2 to 3 hr at 37°C. Embryos were rinsed in PTX and mounted in MOWIOL (Polysciences) containing DABCO (Sigma). Images were taken on a Leica TSC-NT confocal microscope. A range of 50 μm in depth was scanned in 16 optical sections and projected into one focal plane using the TCS-NT software.

Acridine orange staining was carried out as described in Hay et al. (1994). Confocal and Nomarksi images were taken on the confocal microscope.

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