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Induction of apoptosis by *Drosophila reaper, hid* and *grim* through inhibition of IAP function

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Induction of apoptosis in *Drosophila* requires the activity of three closely linked genes, *reaper*, *hid* and *grim*. Here we show that the proteins encoded by *reaper*, *hid* and *grim* activate cell death by inhibiting the anti-apoptotic activity of the *Drosophila* IAP1 (*diap1*) protein. In a genetic modifier screen, both loss-of-function and gain-of-function alleles in the endogenous *diap1* gene were obtained, and the mutant proteins were functionally and biochemically characterized. Gain-of-function mutations in *diap1* strongly suppressed *reaper*-, *hid*- and *grim*-induced apoptosis. Sequence analysis of these alleles revealed that they were caused by single amino acid changes in the baculovirus IAP repeat domains of *diap1*, a domain implicated in binding REAPER, HID and GRIM. Significantly, the corresponding mutant DIAP1 proteins displayed greatly reduced binding of REAPER, HID and GRIM, indicating that REAPER, HID and GRIM kill by forming a complex with DIAP1. These data provide strong *in vivo* evidence for a previously published model of cell death regulation in *Drosophila*.

Keywords: caspase/cell death/development/DIAP1

Introduction

Programmed cell death or apoptosis is crucial to normal embryonic development and metamorphosis of multicellular organisms (Thompson, 1995; Jacobson *et al*., 1997; McCall and Steller, 1997). In *Drosophila*, the induction of apoptosis requires the products of *reaper*, *hid* and *grim*, genes encoded by the 75C1,2 region of the *Drosophila* third chromosome (White *et al*., 1994, 1996; Grether *et al*., 1995; Chen *et al*., 1996). *reaper*, *hid* and *grim* are transcriptionally regulated in response to diverse death-inducing signals and appear to link distinct signaling pathways with the cell death program (Nordstrom *et al*., 1996; Kurada and White, 1998). In addition, HID is post-transcriptionally regulated by the RAS-MAP kinase pathway in response to cell survival signals (Bergmann *et al*., 1998a,b). Phosphorylation of HID by activated MAPK inhibits the pro-apoptotic activity of HID (Bergmann *et al*., 1998a). Ectopic expression of *reaper*, *hid* and *grim* induces cell death in different tissues of transgenic animals and in cultured insect and mammalian cells (Grether *et al*., 1995; Hay *et al*., 1995; Chen *et al*., 1996; Pronk *et al*., 1996; White *et al*., 1996; Vucic *et al*., 1997, 1998a; McCarthy and Dixit, 1998; Haining *et al*., 1999). This ectopic cell death is blocked by baculovirus p35, a caspase inhibitor protein (Bump *et al*., 1995) and by chemical inhibitors of caspases (Hay *et al*., 1994; Grether *et al*., 1995; Chen *et al*., 1996; White *et al*., 1996; McCarthy and Dixit, 1998; Haining *et al*., 1999), indicating that *reaper*, *hid* and *grim* induce apoptosis by activating a caspase pathway. Several caspase-like molecules have been identified in *Drosophila* (Fraser and Evan, 1997; Song *et al*., 1997; Chen *et al*., 1998; Dorstyn *et al*., 1999), but the precise mechanism of their activation is not clear.

Apoptosis is negatively regulated by another conserved and important class of molecules, the inhibitor of apoptosis proteins (IAPs) (Deveraux and Reed, 1999). IAPs were first described in baculoviruses based on their ability to rescue the p35 mutant phenotype and were shown to exert anti-apoptotic activity (Crook *et al*., 1993; Birnbaum *et al*., 1994; Clem and Miller, 1994; Miller, 1997). Since then, several cellular homologs from both vertebrates and invertebrates have been described (Clem and Duckett, 1997; Deveraux and Reed, 1999). Typically, IAP family genes have at least one and often two or three tandem baculovirus IAP repeat (BIR) motifs and may have an additional C-terminal RING finger domain (Clem and Duckett, 1997; Deveraux and Reed, 1999). Some mammalian IAPs have been implicated in human diseases, including spinal muscular atrophy (SMA) (Roy *et al*., 1995) and cancer (Ambrosini *et al*., 1997). In *Drosophila*, two IAP homologs have been reported: *diap1* and *diap2* (Hay *et al*., 1995; Duckett *et al*., 1996; Uren *et al*., 1996). *diap1* is encoded by the *thread* (*th*) locus (Lindsley and Zimm, 1992), and loss-of-function mutations in *diap1* are lethal and enhance *reaper*-, *hid*- and *grim*-induced cell death (Hay *et al*., 1995). Ectopic expression of either *diap1* or *diap2* suppresses apoptosis (Hay *et al*., 1995; Vucic *et al*., 1997, 1998a). Baculovirus, *Drosophila* and mammalian IAPs can physically interact with REAPER, HID and GRIM and antagonize their pro-apoptotic properties (Vucic *et al*., 1997, 1998a; McCarthy and Dixit, 1998). Human XIAP, cIAP1 and cIAP2 can bind to and inhibit caspase 3, 7 and 9 *in vitro* (Deveraux *et al*., 1997, 1998; Roy *et al*., 1997). These observations have led to the proposal that IAPs are apoptosis antagonists exerting their inhibitory effect at several levels in the apoptotic cascade and, in *Drosophila*, inhibit REAPER, HID and GRIM from activating effectors downstream in the death program (McCarthy and Dixit, 1998; Vucic *et al*., 1998b). More recently, however, results obtained primarily from a yeast expression system suggest that REAPER, HID and GRIM block DIAP1 from inhibit-

Table I. Phenotypes and molecular changes associated with the *diap1* alleles

Screen obtained from	*diap1* mutant	Modifier phenotype		Recessive phenotype	Mutation
		reaper	*hid*		
Gain-of-function					
hid	*45-2s*	strong suppressor	suppressor	semi-lethal[a]	G88D
reaper	◆*6-3s*	strong suppressor	strong suppressor	semi-lethal[a]	G88S
hid	*21-2s*	weak suppressor	weak suppressor	semi-lethal[a]	P105S
hid	◆*23-4s*	strong suppressor	strong suppressor	lethal	G269S
hid	*23-8s*	strong suppressor	strong suppressor	lethal	G269S
Loss-of-function					
–	◆th^4	enhancer	enhancer	lethal	H283Y
–	◆th^5	enhancer	enhancer	lethal	W273stop
reaper	*11-3e*	strong enhancer	strong enhancer	lethal	N117K

The amino acid changes deduced from the nucleotide sequence of the *diap1* gene of homozygous mutant embryos and their genetic properties are listed. All mutants except th^4 and th^5 were obtained from the modifier screens for GMR-*reaper*, and GMR-*hid*, th^4 and th^5 have been reported previously (Hay *et al*., 1995) and were obtained from J.A.Kennison (NIH). Mutants representative of amino acid changes in both BIR domains were used in subsequent cell culture and biochemical studies and are indicated by a ◆.
[a]These gain-of-function mutants were semi-lethal with lethality ranging from 25 to 40% and observed mostly in the larval and pupal stages of development.

ing caspase activity (Wang *et al*., 1999). Consistent with this observation, loss-of-function *diap1* mutants are embryonic lethal, with the widespread induction of apoptosis (Wang *et al*., 1999; this study). We now report the isolation and characterization of several gain-of-function and loss-of-function mutants in the endogenous *diap1* gene. The gain-of-function mutant DIAP1 proteins are impaired for binding to REAPER and HID. This suggests that the association of REAPER and HID with DIAP1 is critical for the induction of apoptosis by these pro-apoptotic genes *in vivo*. Collectively, these data provide strong support for the idea that REAPER, HID and GRIM kill by inhibiting DIAP1's ability to antagonize caspase function.

Results

Mutations in diap1 modify reaper- and hid-induced cell death phenotypes

Ectopic expression of *reaper*, *hid* and *grim* under the control of an eye-specific promoter (GMR) results in ectopic cell death in the developing retina and causes rough and reduced eyes (Grether *et al*., 1995; Hay *et al*., 1995; Chen *et al*., 1996; White *et al*., 1996). The phenotypes caused by GMR-*reaper* and GMR-*hid* transgenes are dosage dependent and very sensitive to the dosage of other cell death genes acting downstream or in parallel pathways. This was the basis of a mutagenesis screen to isolate modifiers of *reaper*- and *hid*-induced cell death (J.Agapite, K.McCall and H.Steller, unpublished) (Bergmann *et al*., 1998a). Among a large number of dominant modifiers of apoptosis obtained in this screen, at least 10 were mutations in *diap1*. As expected, loss-of-function *diap1* alleles were strong enhancers of both *reaper*- and *hid*-induced eye phenotypes (Hay *et al*., 1995) (Figure 1; Table I). However, we also obtained several gain-of-function mutants of *diap1* that potently inhibited cell death and restored the eye morphology to near wild-type levels (Figure 1; Table I). Another class of mutants that enhance *reaper*-induced death but suppress the *hid* phenotype was also identified.

The gain-of-function mutants were also tested for suppression of *reaper*- and *hid*-induced cell death in the wing (J.Agapite and H.Steller, unpublished) and the suppression of larval lethality induced by the expression of *hid* under a heat shock promoter (Bergmann *et al*., 1998a). In all assays tested, the *diap1* gain-of-function mutants suppressed *reaper*- and *hid*-induced cell death very efficiently (data not shown) and the *diap1* mutant phenotype was recapitulated outside the context of the developing eye.

Gain-of-function mutations in diap1 map to the BIR domains

Molecular analysis revealed that all five gain-of-function alleles were associated with single amino acid changes in the BIR domain of DIAP1 (Table I; Figure 2). Significantly, this domain of DIAP1 has been implicated in the binding of REAPER, HID and GRIM (Vucic *et al*., 1998a,b). The proline residue mutated to serine in $th^{21\text{-}2s}$ is highly conserved among different BIR domains (Uren *et al*., 1998). In $th^{6\text{-}3s}$ and $th^{45\text{-}2s}$ as well as in $th^{23\text{-}4s}$ and $th^{23\text{-}8s}$, amino acid substitution of a specific glycine residue in corresponding locations in BIR1 and BIR2, respectively, was found to cause the gain-of-function phenotype. The flanking amino acid of the mutated glycine is a conserved non-polar residue (valine, leucine or isoleucine) in 100% of all BIR domains (Uren *et al*., 1998). Two previously reported loss-of-function *thread* alleles, th^4 and th^5 (Hay *et al*., 1995), were also included in the molecular analysis. th^4 is a change in a highly conserved histidine residue (Uren *et al*., 1998), and the th^5 mutation introduces a premature truncation resulting in the deletion of several amino acids at the C-terminus of DIAP1 (Figure 2).

To confirm that the missense mutation in the primary protein sequence of *diap1* was responsible for the observed phenotype, we employed a cell death assay in transfected culture cells. Expression of *reaper* and *hid* can induce apoptosis in several cultured cell types (Pronk *et al*., 1996; Bergmann *et al*., 1998a; McCarthy and Dixit, 1998; Vucic *et al*., 1998a; Haining *et al*., 1999), which is inhibited efficiently by co-transfecting IAPs (McCarthy and Dixit, 1998; Vucic *et al*., 1998a; Haining *et al*., 1999). Representative gain-of-function and loss-of-function *diap1* alleles were tested in *Drosophila* Schneider (S2) cells for their ability to overcome *reaper*- and *hid*-induced cell death.

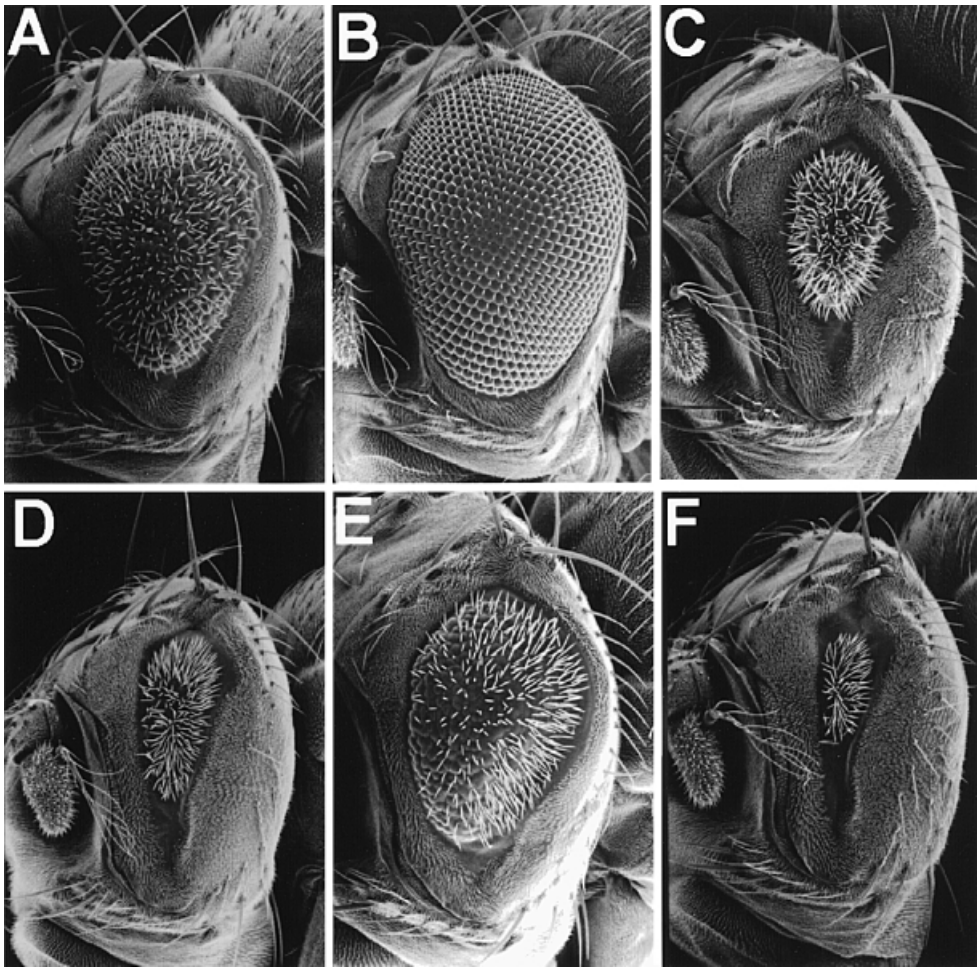

Fig. 1. Mutations in *diap1* modify *reaper*- and *hid*-induced cell death. A gain-of-function mutation in *diap1*, $th^{6\text{-}3s}$, suppresses the GMR-*reaper* (**B**) (GMR-*rpr*/$th^{6\text{-}3s}$) and GMR-*hid* (**E**) (GMR-*hid*/$th^{6\text{-}3s}$) induced eye phenotypes. Reduction of *diap1* activity as in $th^{11\text{-}3e}$ enhances the eye phenotypes associated with GMR-*rpr* (**C**) (GMR-*rpr*/$th^{11\text{-}3e}$) and GMR-*hid* (**F**) (GMR-*hid*/$th^{11\text{-}3e}$). The unmodified phenotypes are shown in (**A**) (GMR-*rpr*/+) and (**D**) (GMR-*hid*/+). The wild-type eye (not shown) is similar to that in (B).

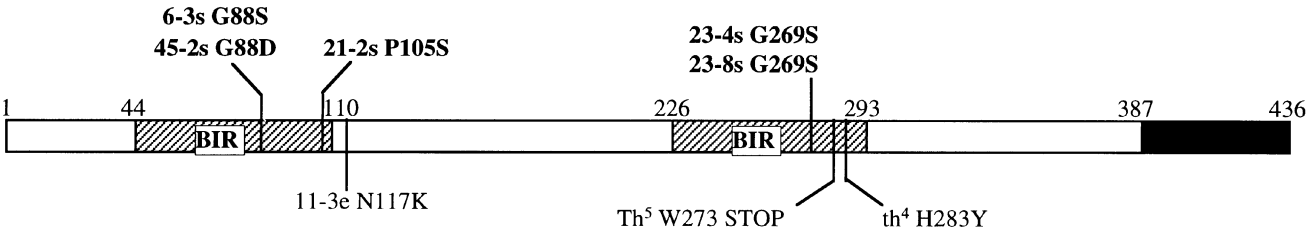

Fig. 2. Schematic representation of the *diap1* mutants. The DIAP1 protein is boxed, with the BIR domains indicated as cross-hatched regions and the RING domain in black. The gain-of-function mutants are indicated in bold and the loss-of-function mutants are in normal face. The figure is drawn to scale.

All *diap1* mutants tested expressed protein at levels comparable to wild-type, indicating that these mutations did not significantly affect the expression and stability of the protein (Figure 3). Gain-of-function mutants showed an ability to inhibit *reaper*- and *hid*-induced cell death by 40–50% above wild-type levels. All the mutants exhibited the same phenotypes as observed *in vivo* (Table I), except for the th^4 allele, which showed a near wild-type level of protection against *hid*-induced death (Figure 3, see below).

Gain-of-function mutants of DIAP1 show impaired binding to REAPER and HID

REAPER, HID and GRIM can bind to DIAP1 via the BIR domains (Vucic *et al*., 1998b), and all the strong *diap1* gain-of-function suppressor mutations that we obtained in our modifier screen map to a specific region in the BIR domains (Figure 2). Therefore, we hypothesized that these mutations may have altered the binding of DIAP1 to REAPER, HID and GRIM. Based on previous findings that REAPER, HID and GRIM can interact functionally and physically with IAPs (Vucic *et al*., 1997, 1998a), two simple models can be proposed to explain the role of *diap1* in regulating apoptosis. In the first model, *diap1* acts upstream of *reaper*, *hid* and *grim* to prevent them from activating the cell death program (McCarthy and Dixit, 1998; Vucic *et al*., 1998b). Alternatively, *reaper*, *hid* and *grim* may induce apoptosis by inhibiting *diap1* from blocking cell death (Wang *et al*., 1999). According

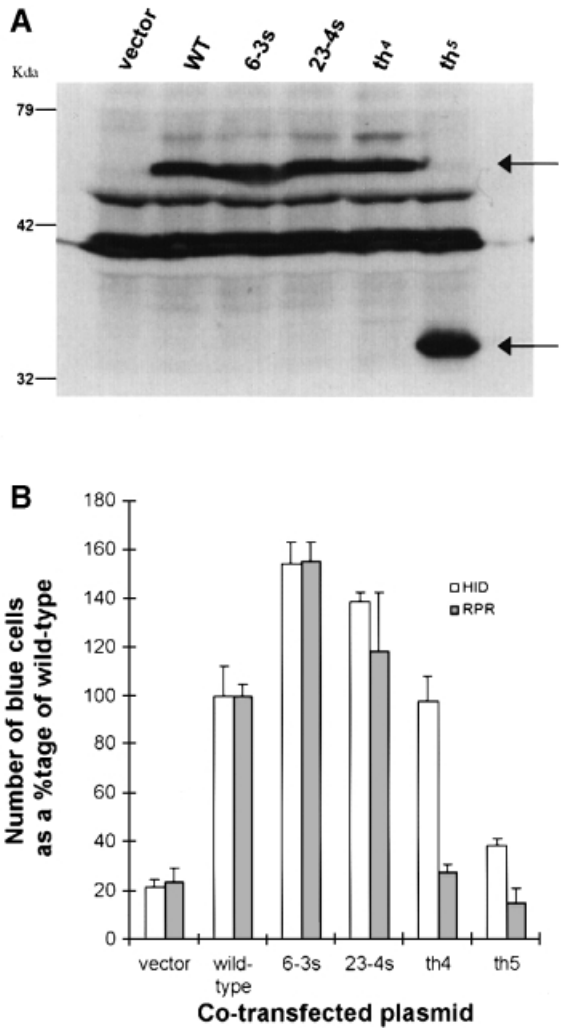

Fig. 3. *diap1* mutant alleles maintain their phenotype when expressed in S2 cells. (**A**) The wild-type and mutants of *diap1* were expressed in S2 cells under the control of a viral promoter with a FLAG epitope tag at the N-terminus of the protein. Protein expression levels were analyzed by immunoblotting equal amounts of protein extracts using an epitope-specific antibody. Epitope-tagged versions of wild-type and mutant proteins are expressed at comparable levels and are indicated with arrows. The th^5 mutant is a truncated protein due to the introduction of a premature stop codon. (**B**) The wild-type and mutant *diap1* constructs were co-transfected with plasmids for the expression of REAPER and HID in a ratio of 3:4. A β-galactosidase plasmid was also co-transfected as a marker. The numbers of viable cells with β-galactosidase activity were counted and expressed as a percentage of the number of viable blue cells in the wild-type *diap1*-transfected wells, to assess the ability of the mutants of *diap1* to inhibit *reaper*- and *hid*-mediated apoptosis. Except for th^4, all the mutants maintained the phenotypes observed *in vivo* with respect to *reaper*- and *hid*-induced eye ablation (see Table I and text).

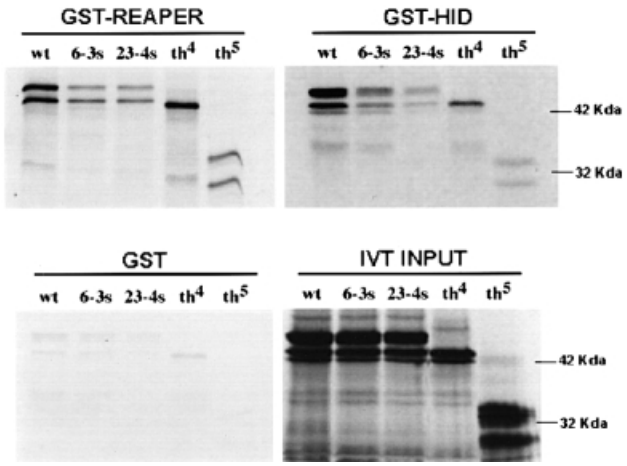

Fig. 4. Gain-of-function mutations in *diap1* diminish binding to REAPER and HID. Equal amounts of ^{35}S-labeled wild-type and mutant DIAP1 proteins (IVT input) were tested for association with GST, GST–REAPER and GST–HID in an *in vitro* binding assay. Wild-type DIAP1 protein shows specific binding to GST–REAPER and GST–HID. Mutant DIAP1 proteins encoded by $th^{6\text{-}3s}$ and $th^{23\text{-}4s}$ show significantly reduced binding of GST–REAPER and GST–HID as compared with wild-type DIAP1. In contrast, a protein corresponding to th^4, a loss-of-function allele of *diap1*, retains essentially normal affinity for REAPER and HID. The lower band visible in most lanes is due to initiation of translation at the second methionine in DIAP1 during *in vitro* synthesis. The th^5 mutant translates into a truncated protein due to the introduction of a premature stop codon. The IVT input represents 10% of the *in vitro* translated protein used in each pull-down reaction.

to the later model, mutations in *diap1* that inhibit binding of REAPER, HID and GRIM would be less susceptible to inactivation by the pro-apoptotic proteins and hence show increased anti-apoptotic activity. To test this hypothesis directly, we performed *in vitro* binding assays of wild-type and mutant DIAP1 protein to GST–REAPER and GST–HID. As predicted, gain-of-function mutants of *diap1* ($th^{6\text{-}3s}$ and $th^{23\text{-}4s}$) showed greatly diminished binding to both REAPER and HID as compared with wild-type DIAP1 (Figure 4). The effect of these single amino acid changes appears to be quite specific, since the mutant DIAP1 proteins retain potent anti-apoptotic activity (Figures 1 and 3; Table I), presumably by direct interaction with caspases (Deveraux *et al.*, 1997, 1998; Roy *et al.*, 1997; Hawkins *et al.*, 1999). Since the anti-apoptotic activity of IAPs requires a functional BIR domain, it is highly unlikely that these *diap1* gain-of-function mutations have a general effect on the overall structure of the BIR domains.

The gain-of-function mutations in *diap1* reduce binding to REAPER and HID up to 14-fold (Figure 4). The residual association with REAPER and HID may be due to the presence of one intact BIR domain in both the mutants (Figure 2). However, it is notable that mutations in either the first or the second BIR domain can reduce binding of HID and REAPER by >2-fold, suggesting that both BIR domains may cooperate for the efficient binding of REAPER and HID. On the other hand, we cannot exclude the possibility that other regions of DIAP1 are also involved in binding of REAPER, HID and GRIM, perhaps involving domains distinct from the N-terminal region of these proteins. In any event, our results show that gain-of-function alleles of *diap1* with increased anti-apoptotic activity are associated with reduced *in vitro* binding activity to REAPER and HID. These findings are not reconciled easily with the model that DIAP1 protects against cell death by inhibiting the pro-apoptotic activity of REAPER and HID. Rather, they suggest that REAPER and HID activate apoptosis by binding to and inactivating DIAP1.

Not all BIR domain mutations have impaired binding to REAPER, HID and GRIM. The th^4 mutant, which harbors a single amino acid change in BIR2 (Figure 2), binds to REAPER and HID *in vitro* (Figure 4). Although

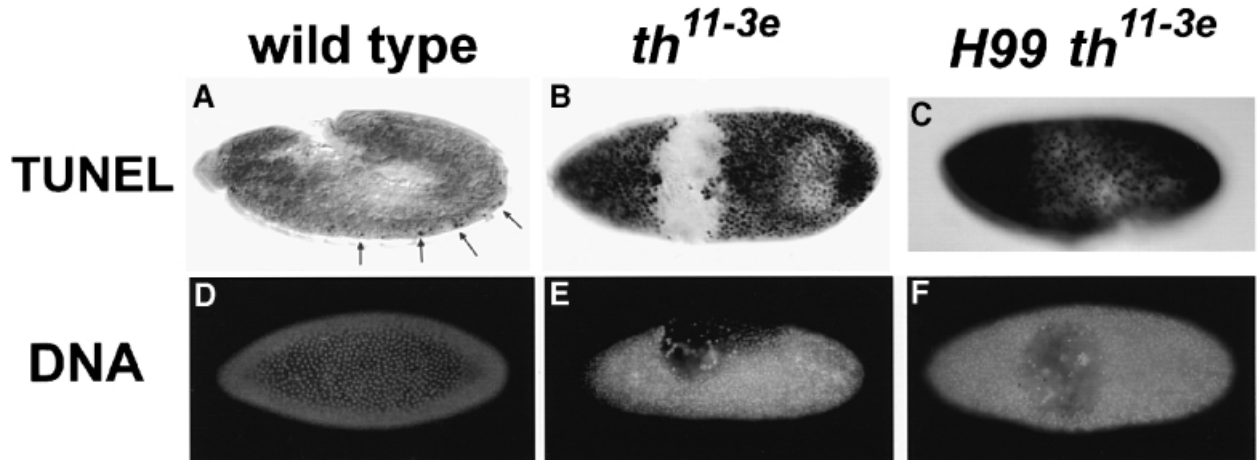

Fig. 5. *diap1* function is essential to prevent apoptosis. Embryos were labeled using the TUNEL procedure (A, B and C) to detect apoptotic nuclei (White *et al*., 1994) or with Oligreen (D, E and F) to stain DNA. (**A**) In wild-type embryos, the first evidence of apoptosis is seen at stage 11; the arrows point to clusters of apoptotic cells. (**B**) A $th^{11\text{-}3e}$ embryo aged to a physiological age of 7 h (7 h AEL). In *diap1* loss-of-function mutants, such as $th^{11\text{-}3e}$ and th^{5}, essentially all nuclei of embryos are TUNEL positive 7–8 h AEL. (**C**) $th^{11\text{-}3e}$ Df(3L)H99 double mutant embryos. As with $th^{11\text{-}3e}$ embryos, essentially all nuclei of the early H99 $th^{11\text{-}3e}$ double mutant embryos are TUNEL positive. Therefore, induction of apoptosis in $th^{11\text{-}3e}$ embryos does not require the activity of *reaper*, *hid* and *grim*, indicating that *diap1* functions downstream from these genes. (**D**) Stage 5 (cellular blastoderm) wild-type embryo. (**E**) $th^{11\text{-}3e}$ embryo aged to 7 h AEL. At this stage, mutant nuclei have condensed chromatin characteristic of apoptosis, and the embryo is organized abnormally. (**F**) $th^{11\text{-}3e}$ Df(3L)H99 double mutant embryos. The absence of *reaper*, *hid* and *grim* does not attenuate the $th^{11\text{-}3e}$ phenotype, since the phenotype of $th^{11\text{-}3e}$ and the double mutant embryo is virtually indistinguishable (compare E and F).

this is a loss-of-function allele in *Drosophila*, presumably by lacking the ability to inhibit caspases, overexpression of th^{4} in cultured cells can block *hid*-induced cell death (Figure 3). In order to explain this apparent paradox, we propose that overexpression of the mutant protein at unphysiologically high levels in cultured cells protects against *hid*-induced apoptosis by binding to and sequestering HID. While this proposed mechanism is not reflective of the normal function of DIAP1, it explains results from previous cell transfection studies suggesting that IAPs may function upstream of REAPER, HID and GRIM to inhibit apoptosis (McCarthy and Dixit, 1998; Vucic *et al*., 1998b).

Loss of diap1 results in the induction of apoptosis

Loss-of-function *diap1* mutants are embryonic lethal (Hay *et al*., 1995) (Table I). Since *diap1* has anti-apoptotic activity, we investigated whether zygotic loss of *diap1* function may lead to excessive apoptosis during embryogenesis. Using the TUNEL technique to label apoptotic nuclei, essentially all nuclei of homozygous $th^{11\text{-}3e}$ embryos stained positive by 7–8 h after egg laying (AEL) (Figure 5). In contrast, only a few TUNEL-positive cells were observed in correspondingly aged wild-type embryos (stage 11), the first time point at which cell deaths are normally seen in *Drosophila* embryos (Figure 5A) (Abrams *et al*., 1993). Due to the extraordinary amount of apoptosis, *thread* loss-of-function embryos appeared highly disorganized at this time (Figures 5B and E, and 6B–D). However, time course analyses revealed that these embryos develop normally until the cellular blastoderm stage and begin to gastrulate, but become abnormal shortly after the formation of the ventral furrow (J.Tittel and H.Steller, unpublished observations). Analysis of $th^{11\text{-}3e}$ embryos by electron microscopy revealed many nuclei with features characteristic of apoptosis, including condensed chromatin and electron-dense nuclei (Figure 6B–D). These experiments reveal a striking dependence of cell survival on zygotic *diap1* function. We conclude that *diap1* is required to prevent apoptosis in most, if not all cells of the *Drosophila* embryo.

diap1 functions downstream of reaper, hid and grim

If *diap1* functions upstream of *reaper*, *hid* and *grim*, the excessive apoptosis that we observed in *diap1* loss-of-function embryos should be blocked in the absence of *reaper*, *hid* and *grim.* In contrast, if *reaper*, *hid* and *grim* function upstream of *diap1*, apoptosis in *diap1* loss-of-function embryos should be independent of these pro-apoptotic genes. To distinguish between these two models, we constructed double mutant embryos that are homozygous for both H99 and $th^{11\text{-}3e}$, a deletion that removes *reaper*, *hid* and *grim*. Wild-type, $th^{11\text{-}3e}$ and H99$th^{11\text{-}3e}$ embryos were analyzed for their phenotypic morphology and by TUNEL labeling as before. We found that the H99$th^{11\text{-}3e}$ phenotype was indistinguishable from the $th^{11\text{-}3e}$ phenotype observed earlier (Figure 5). Similar results were reported recently by Wang *et al*. (1999). The failure of the H99 deletion to inhibit cell death in *diap1* loss-of-function embryos is not consistent with the idea that DIAP1 blocks apoptosis by inhibiting REAPER, HID and GRIM.

Discussion

In this study, we report the isolation of novel gain-of-function and loss-of-function mutants in one of the *Drosophila* IAP genes, *diap1*. Gain-of-function mutants of *diap1* strongly suppressed *reaper*- and *hid*-induced cell death and correspond to single amino acid changes in the BIR domain of the DIAP1 protein. The gain-of-function mutant DIAP1 proteins displayed impaired binding to REAPER and HID, indicating that the ability of REAPER and HID to bind DIAP1 is critical for the regulation of DIAP1 *in vivo*. Loss-of-function mutations in *diap1* caused

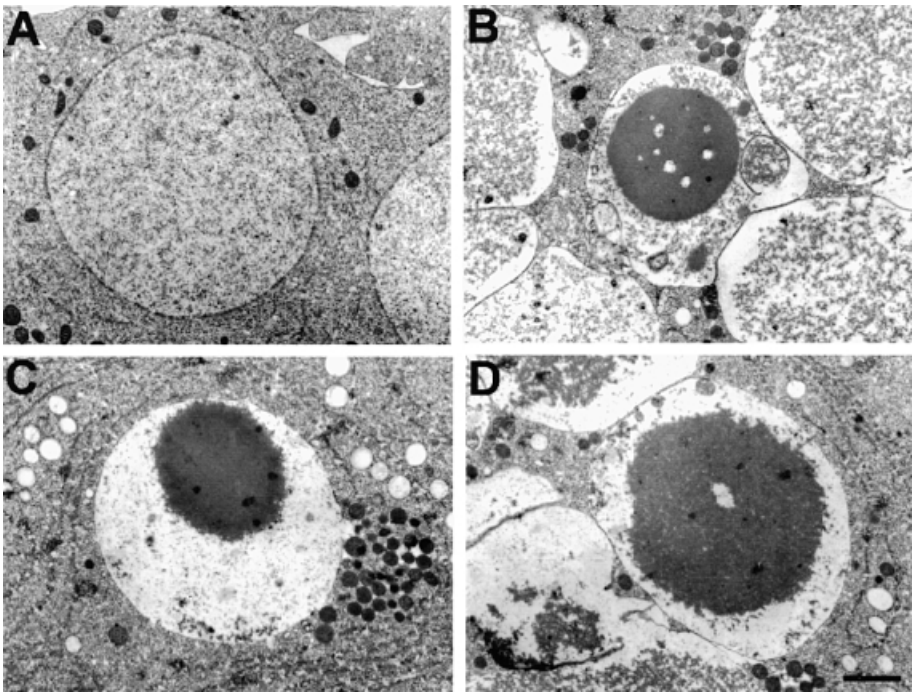

Fig. 6. Cell deaths in the *diap1* mutant embryo display the ultrastructural features of apoptosis. (**A**) Electron micrograph of a stage 6 wild-type embryo. All nuclei have normal 'healthy' morphology, and no signs of condensed chromatin can be detected until ~7 h AEL (stage 11) (Abrams *et al*., 1993). (**B**–**D**) Homozygous $th^{11\text{-}3e}$ mutant embryos were selected by morphology 4 h after egg laying and processed for electron microscopy. Representative micrographs of electron-dense nuclei from $th^{11\text{-}3e}$ homozygous mutant embryos showing condensed chromatin characteristic of apoptosis. The scale bar as represented in (D) is 1 μm.

widespread induction of cell death resulting in embryonic lethality, and a deletion in *reaper*, *hid* and *grim* was unable to rescue the *diap1* loss-of-function phenotype (Wang *et al*., 1999; this study). These results indicate that *diap1* is required for cell survival and is genetically downstream of *reaper*, *hid* and *grim*.

Based on overexpression studies in cultured cells, it was initially proposed that IAPs function upstream of *reaper*, *hid* and *grim* to prevent them from activating caspases (Vucic *et al*., 1997, 1998a). Subsequently, IAPs were also reported to bind to and inhibit caspases (Deveraux *et al*., 1997, 1998; Roy *et al*., 1997; Kaiser *et al*., 1998). In order to reconcile this apparent paradox, it was proposed that IAPs act on multiple targets and at different steps in the apoptotic pathway (Kaiser *et al*., 1998; McCarthy and Dixit, 1998; Vucic *et al*., 1998b). Based on data obtained primarily from a yeast expression system, Wang *et al*. (1999) have suggested a more simple linear model for the induction of apoptosis by *reaper*, *hid* and *grim*. According to this 'double inhibition model', REAPER, HID and GRIM induce apoptosis by binding directly to and preventing DIAP1 from inhibiting caspase activation (Figure 7). The *diap1* mutants analyzed in this study provide evidence for the function of *reaper* and *hid* *in vivo* and concur with the conclusions obtained by Wang *et al*. (1999) from their study in yeast. The decreased binding of REAPER and HID to DIAP1 correlates with a gain-of-function *diap1* phenotype, suggesting that the association with DIAP1 is important for the pro-apoptotic activity of REAPER, HID and GRIM. These data strongly support the idea that in *Drosophila*, REAPER and HID induce apoptosis by binding to and inhibiting DIAP1 function (Figure 7; Wang *et al*., 1999). We believe that the previously proposed function of IAPs upstream of *reaper*, *hid* and *grim* is simply an artifact of unphysiologically high levels of protein expression in heterologous systems. When IAP expression constructs are introduced into cultured cells under the control of strong promoters and at high copy numbers, the levels of proteins expressed far exceed those of the endogenous cellular IAP proteins. Under these unphysiological conditions, cellular IAPs can display properties that do not reflect their normal mechanism of action. In particular, our results with th^{4} and other *diap1* alleles (data not shown) demonstrate that mutant proteins that completely lack anti-apoptotic activity *in vivo* can still inhibit cell death *in vitro* as long as they can bind to REAPER, HID and GRIM (Figures 3 and 4). Conversely, gain-of-function *diap1* alleles that display reduced binding to REAPER, HID and GRIM have strongly increased anti-apoptotic function *in vivo* (Figures 1 and 4), but show reduced protection in heterologous cell transfection assays (data not shown). These results clearly reveal the limitations of overexpression studies in cultured cells for determining the normal mechanism of action of these proteins in the cell death pathway.

A mutant of HID that fails to bind IAPs has no pro-apoptotic activity (Vucic *et al*., 1998a; Haining *et al*., 1999; Wang *et al*., 1999), implying that HID functions primarily, if not exclusively, by inhibiting IAPs. Although the precise mechanism by which IAPs inhibit apoptosis is not known, there are strong reasons to believe that the primary mode of action is a direct inhibition of caspases and/or their activation (Hawkins *et al*., 1999; Wang *et al*., 1999; Meier *et al*., 2000). Mammalian IAPs can inhibit caspases *in vitro* (Deveraux *et al*., 1997, 1998; Roy *et al*., 1997), and DIAP1 has been shown to interact with

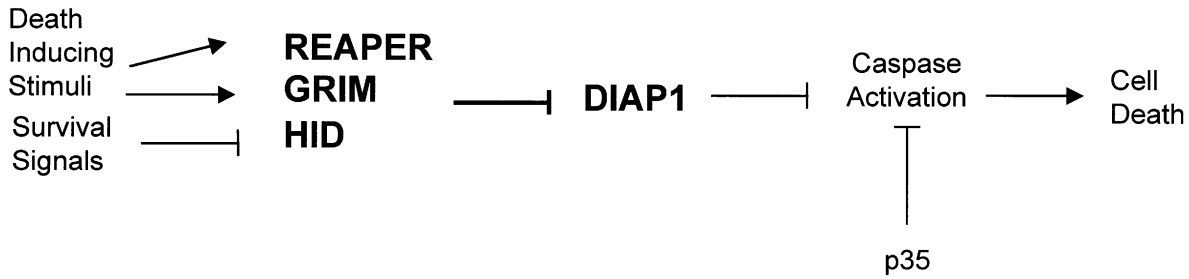

Fig. 7. *diap1* functions downstream of *reaper*, *hid* and *grim* to inhibit caspase activation. The 'double inhibition' model presented here and by Wang *et al.* (1999) indicates that *diap1* is required to keep the caspases in check. The binding of *reaper*, *hid* and *grim* to *diap1* results in the release of inhibition and therefore the activation of caspases and apoptosis.

and inhibit *Drosophila* caspases DrICE and DCP1 in heterologous systems (Kaiser *et al*., 1998; Hawkins *et al*., 1999). Although these inhibitory activities have been detected with active caspases, we prefer the model that DIAP1 interacts with pro-caspases to prevent caspase activation (Meier *et al*., 2000; Song *et al*., 2000). In order to explain the *diap1* loss-of-function phenotype, we propose that in the absence of inhibition by DIAP1, caspases are activated either by a weak autocatalytic activity or by the presence of DARK/HAC-1/dAPAF-1, the *Drosophila* APAF1/CED4-like activator (Kanuka *et al*., 1999; Rodriguez *et al*., 1999; Zhou *et al*., 1999).

How general is the proposed model of IAP regulation and function? In mammals, as in *Caenorhabditis elegans* and *Drosophila*, caspases are widely expressed as weakly active zymogens in virtually all cells. This includes cells that survive for the lifetime of the animal, such as neurons. Therefore, powerful control mechanisms must be in place to prevent the inappropriate activation of caspases in cells that should live, and it appears that some IAPs play a critical role in this regard. On the other hand, these 'brakes on death' must somehow be removed in cells that are selected to die. In *Drosophila*, this appears to be mediated by REAPER, HID and GRIM. Although no mammalian homologs of *reaper*, *hid* and *grim* have been reported yet, we expect that a similar regulation must occur in mammalian cells. Consistent with this idea, *reaper*, *hid* and *grim* can activate apoptosis in mammalian cells, and REAPER has been shown to activate caspases and apoptosis-like events in a vertebrate cell-free system (Evans *et al*., 1997; McCarthy and Dixit, 1998; Haining *et al*., 1999). Many of the residues involved in the *diap1* gain-of-function mutants have been highly conserved during evolution, indicating that the domain that is necessary for binding to REAPER/HID/GRIM is under considerable selective pressure. Therefore, we consider it likely that the inhibition of IAP function is a general mechanism of cell death induction. If so, gain-of-function mutations similar to those described here may contribute to aberrant regulation of apoptosis in human diseases, such as cancer.

Materials and methods

Genetic screen and fly stocks

The genetic screen performed to isolate modifiers of GMR-*hid* has been described previously (Bergmann *et al*., 1998a). In a similar screen for dominant modifiers of GMR-*reaper*-induced eye ablation, 180 000 mutagenized F_1 progeny were screened and 46 dominant modifiers were identified (J.Agapite, K.McCall and H.Steller, unpublished). The following mutant and transgenic fly strains were used for the genetic analysis: GMR-*hid* and GMR-*reaper* (Bergmann *et al*., 1998a), $th^{11\text{-}3e}$ and $th^{6\text{-}3s}$ (this study). H99$th^{11\text{-}3e}$ recombinants were generated by standard *Drosophila* genetic techniques. All crosses were performed at 25°C.

Molecular analysis and plasmid construction

Single embryos of the *diap1* mutants over a balancer with a LacZ marker were selected 4 h AEL and squashed in 10 µl of Gloor and Engel's buffer (10 mM Tris pH 8.2, 1 mM EDTA, 25 mM NaCl, 200 µg/ml proteinase K). The squashed single embryo was incubated at 37°C for 30 min and at 95°C for 2 min to inactivate proteinase K. A 4 µl aliquot of the single embryo DNA was used in each PCR with primers specific for the open reading frame (ORF) of *diap1*. Primers specific for LacZ were used in an independent PCR to identify homozygous *diap1* mutant embryos. The PCR product from homozygous mutant embryos was either sequenced directly or cloned into pIE1-3 vector (Novagen) with a FLAG epitope tag at the N-terminus using restriction sites engineered into the primers. Cleaned PCR product or plasmid DNA carrying the mutant *diap1* coding sequence was subjected to automated sequencing to determine the molecular nature of the mutation. Mutations were confirmed by sequencing the *diap1* ORF from multiple independent homozygous mutant single embryos. Similar procedures were used to clone and sequence the ORF of wild-type *diap1* from the background strain as control.

Cell culture assays

SL2 cell transient transfection experiments were performed as described (Bergmann *et al*., 1998a). Briefly, 0.15 µg of the FLAG epitope-tagged wild-type or mutant *diap1* cDNA in the pIE1-3 vector (Novagen) were co-transfected with 0.2 µg of *reaper* or *hid* cDNA in the pIE1-3 vector into each well of a 24-well plate of SL2 cells. pIE1-3 LacZ (18 ng) was included in all transfections as a reporter. Transfections were performed using Cellfectin (Gibco-BRL) according to the manufacturer's instructions. SL2 cells adhere poorly to culture dishes and apoptotic cells float up into the culture medium. At 24 h post-transfection, cells still adhering to the dish are viable. Therefore, 24 h post-transfection, the cells still adhering to the dish were fixed and stained for LacZ activity and the number of blue cells was counted as an indicator of cell survival. All plasmid combinations were transfected in triplicate and the mean and standard deviation calculated. For Western blots, extracts were made from SL2 cells transfected with FLAG-tagged wild-type or mutant pIE1-3 *diap1* plasmids and resolved by SDS–PAGE. After transfer of the proteins to nitrocellulose membranes, the blots were probed with a monoclonal antibody directed to the FLAG epitope tag (Kodak). An anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody was used and the immune complexes were detected by enhanced chemiluminescence (Amersham).

TUNEL labeling of embryos

For TUNEL and Oligreen staining, Canton S, $th^{11\text{-}3e}$ or H99$th^{11\text{-}3e}$ embryos were aged for 16 h at 18°C before processing. TUNEL was carried out as previously described (White *et al*., 1994) with some modifications. Briefly, embryos were fixed in 4% paraformaldehyde, in phosphate-buffered saline (PBS) with heptane, and devitellinized with methanol. After several washes with PBS plus 0.1% Tween-20 (PBT), the embryos were treated with proteinase K (10 µg/ml), washed in PBT and treated with reagents from the Apotag Plus kit (Oncorr). For detection, an alkaline phosphatase-conjugated antibody to digoxigenin and reagents from the genius kit (both from Boehringer Mannheim)

were used. For Oligreen (Molecular Probes) staining to visualize the nuclei, fixed and washed embryos were incubated with a 1:5000 dilution of Oligreen in PBS. Embryos were examined and photographed on a Zeiss Axiophot microscope.

Electron microscopy

$th^{11\text{-}3e}$ homozygous mutant embryos were aged for 4–5 h AEL and selected under a dissection microscope. Embryos were dechorionated and fixed with 1.5% glutaraldehyde, 1% paraformaldehyde and 0.75% acrolein in 0.1 M cacodylate buffer (pH 7.4). Fixed embryos were devitellinized by hand peeling and post-fixed in 1% osmium tetroxide. Thin sections were cut after embedding in an eponate mixture and photographed on a Jeol 1200EX II microscope.

In vitro binding assays

Full-length coding sequences of *reaper* and *hid* in the pGEX vector (Amersham Pharmacia Biotech) (kindly provided by Vivian Hua and Andreas Bergmann) or the pGEX vector alone were used to generate recombinant GST, GST–REAPER and GST–HID proteins in *Escherichia coli*. [^{35}S]Methionine-labeled wild-type and mutant DIAP1 proteins were generated in an *in vitro* transcription–translation system (Promega) using T7 expression plasmids. Equivalent amounts of the labeled proteins, as determined by SDS–PAGE analysis of one-tenth of the *in vitro* translation mix, were incubated with 2 µg of the GST or GST fusion proteins immobilized on glutathione–Sepharose beads. The binding was performed for 2 h at 4°C in the buffer described previously (Vucic *et al*., 1997) containing 1% NP-40 and washed four times with the same buffer. Protein-bound Sepharose beads were boiled in SDS-containing sample buffer and resolved on 10% SDS–PAGE gels. Gels were fixed, dried and autoradiographed.

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