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Drosophila Homologs of Baculovirus Inhibitor of Apoptosis Proteins Function to Block Cell Death

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Summary

Apoptotic cell death is a mechanism by which organisms eliminate superfluous or harmful cells. Expression of the cell death regulatory protein REAPER (RPR) in the developing Drosophila eye results in a small eye owing to excess cell death. We show that mutations in *thread* (*th*) are dominant enhancers of RPR-induced cell death and that *th* encodes a protein homologous to baculovirus inhibitors of apoptosis (IAPs), which we call Drosophila IAP1 (DIAP1). Overexpression of DIAP1 or a related protein, DIAP2, in the eye suppresses normally occurring cell death as well as death due to overexpression of *rpr* or *head involution defective*. IAP death-preventing activity localizes to the N-terminal baculovirus IAP repeats, a motif found in both viral and cellular proteins associated with death prevention.

Introduction

Apoptosis is a form of regulated cell death in which superfluous or harmful cells are removed from an organism (Ellis et al., 1991). Cells undergoing apoptosis go through a series of morphological changes that include cell shrinkage, membrane blebbing, chromatin condensation, and cell fragmentation (Kerr et al., 1972). Signals that trigger apoptosis occur as a part of normal development and adult homeostasis, as well as in response to stimuli that indicate a cell is potentially harmful or abnormal (reviewed by Ellis et al., 1991; Steller, 1995; Thompson, 1995). Once initiated, apoptosis is thought to proceed through a common pathway. Homologous proteins that function in cell death regulation have been identified in Caenorhabditis elegans and mammals (reviewed by Steller, 1995), implying that this pathway is highly conserved throughout evolution.

Viruses have provided a rich source of proteins that perturb host cell death signaling (reviewed by Shen and Shenk, 1995). The induction of cell death is an important response to viral infection, since host-induced cell death may limit the spread of a viral infection (Shen and Shenk, 1995). Some viruses can block the host apoptotic response by expressing proteins that interfere with the cells ability to transduce a particular cell death signal or that interfere with the function of components of a common cell death effector pathway (reviewed by Gooding, 1994; Shen and Shenk, 1995). Thus, viral proteins that mediate suppression of host cell death are valuable probes for cell death signal transduction pathways.

Cell death regulation has been shown to be important for the baculovirus life cycle (reviewed by Clem and Miller, 1994b). Baculoviruses are large double-stranded DNA viruses that encode proteins that function to block cell death following viral infection: p35 in the case of nuclear polyhedrosis viruses of Autographa californica (AcNPV) (Clem et al., 1991) and Bombyx mori (BmNPV) (Kamita et al., 1993), and inhibitors of apoptosis (IAPs) in the case of Orgyia pseudotsugata (Birnbaum et al., 1994) and Cydia pomonella granulosis viruses (Crook et al., 1993). Blocking death of the infected cell allows the virus to replicate to a high titer (Clem and Miller, 1993). Expression of either p35 or viral IAPs (v-IAPs) is also sufficient to block cell death induced by stimuli other than viral infection (Clem and Miller, 1994a, 1994b), suggesting that they act at points common to multiple cell death signaling pathways. p35 functions in many organisms to block cell death (Rabizadeh et al., 1993; Hay et al., 1994; Sugimoto et al., 1994), through its ability to inactivate interleukin-1β-converting enzyme (ICE)-like cysteine proteases (Bump et al., 1995; Xue and Horvitz, 1995). ICE-like proteins are thought to play an important evolutionarily conserved role in bringing about cell death (reviewed by Martin and Green, 1995). v-IAPs block death in Drosophila (this work) and lepidopteran cells (Birnbaum et al., 1994; Crook et al., 1993; Clem and Miller, 1994a), but little is known about the mechanism.

Baculoviruses contain many genes that are homologous to cellular proteins (Ayres et al., 1994), and there is evidence that they can acquire genes from other viruses or the host genome (reviewed by Blissard and Rohrmann, 1990). The genomic region containing p35 in AcNPV and BmNPV appears to be derived from an insertion of foreign DNA (Gombart et al., 1989), but it is not known whether this DNA is derived from another virus or host DNA. These observations suggest that homologs of viral death inhibiting proteins might be present in the insect genome.

In Drosophila, the 75C1-2 region is important for normally occurring cell death during embryogenesis (White et al., 1994). Two genes in this region, *reaper* (*rpr*) and *head involution defective* (*hid*), are likely to be key regulators of cell death. *rpr* is expressed in cells in the embryo that are selected to die, and ectopic expression of *rpr* in other cells is sufficient to cause cell death (White et al., 1994). Mutations that remove *rpr* but not *hid* have not been identified, so it has not been possible to demonstrate that *rpr* is necessary to bring about cell death. *hid* function is required for some, but not all, normally occurring cell death in the embryo. As with *rpr*, overexpression of *hid* results in ectopic death, when expressed in the embryo or the eye (Grether et al., 1995). In contrast with *rpr*, *hid* is expressed broadly in the embryo in cells that live as well as in cells that die (Grether et al., 1995). Thus, *hid* or *hid*-dependent cell death activity must be regulated posttranscriptionally. Genes required for death in the eye have not been identified; however, given their role in embryogenesis, it is likely that *rpr* and *hid* are involved. The observation that p35

expression can block *rpr*-dependent (this work; H. Steller, personal communication) and *hid*-dependent cell death (Grether et al., 1995) in Drosophila as well as in evolutionarily distant organisms (Rabizadeh et al., 1993; Hay et al., 1994; Sugimoto et al., 1994) strongly suggests that at least some molecules functioning downstream of *rpr* and *hid* are evolutionarily conserved.

We used the developing Drosophila eye to screen for genes important in cell death regulation. The eye was chosen because it is nonessential for viability or fertility of the fly, because it has developmentally important normally occurring cell death (Cagan and Ready, 1989; Wolff and Ready, 1991), and because proteins can be specifically expressed in cells of the eye, including cells that normally die, using the vector pGMR (for *glass* [*gl*] multimer reporter) (Hay et al., 1994). GL is a transcription factor that is expressed in all cells in and posterior to the morphogenetic furrow in the eye (Ellis et al., 1993). pGMR contains a multimer of GL-binding sites from the Drosophila *Rh1* gene that are sufficient to drive expression of coding regions placed downstream of these binding sites (Ellis et al., 1993; Hay et al., 1994). Expression of cell death regulators under regulatory control of pGMR produces externally visible phenotypes; a large, somewhat rough eye in the case in which normally occurring death is prevented (Wolff and Ready, 1991; Hay et al., 1994) or a small eye in the case in which ectopic death is induced (Grether et al., 1995; this work).

Overexpression of *rpr* in the eye using pGMR (GMR–*rpr*) gives rise to dose-dependent cell death (this work; H. Steller, personal communication), resulting in flies that have small eyes. We have screened for mutations that enhance or suppress this eye phenotype. The premise of this screen is that, in the sensitized background of GMR–*rpr*, a 2-fold reduction in the dose of a downstream gene (making the fly heterozygous for a gene in diploid cells) will alter *rpr*-dependent signaling efficiency and result in a change in the adult eye phenotype. Dominant suppressors may identify genes required to carry out *rpr*-dependent functions, while enhancers may identify genes that act as cell death inhibitors.

We screened a collection of chromosomal deletions that covers about half of the Drosophila genome and identified both enhancers and suppressors of *rpr*-dependent death. One enhancer, *E(rpr)3-1*, was chosen for more extensive characterization. Here we report that this enhancer corresponds to lethal mutations in *thread* (*th*) and that *th* encodes a cellular homolog of baculovirus IAPs, DIAP1. A second DIAP, DIAP2, was identified through a database search. These proteins can function to block cell death in response to multiple stimuli, and loss-of-function phenotypes in the eye and ovary suggest DIAP1 is required for cell survival. IAP death-preventing activity is contained in an N-terminal baculovirus IAP repeat (BIR)-containing domain.

Results and Discussion

E(rpr)3-1 Encodes DIAP1, Which Is Homologous to Baculovirus IAPs

To identify genes required for regulating cell death in Drosophila, we took advantage of the fact that it is possible to overexpress proteins specifically in cells of the developing eye posterior to the morphogenetic furrow using the vector pGMR (Hay et al., 1994). The morphogenetic furrow is a moving front, posterior to which pattern formation occurs. pGMR drives expression both in cells that would normally live and cells that would normally die. We generated a

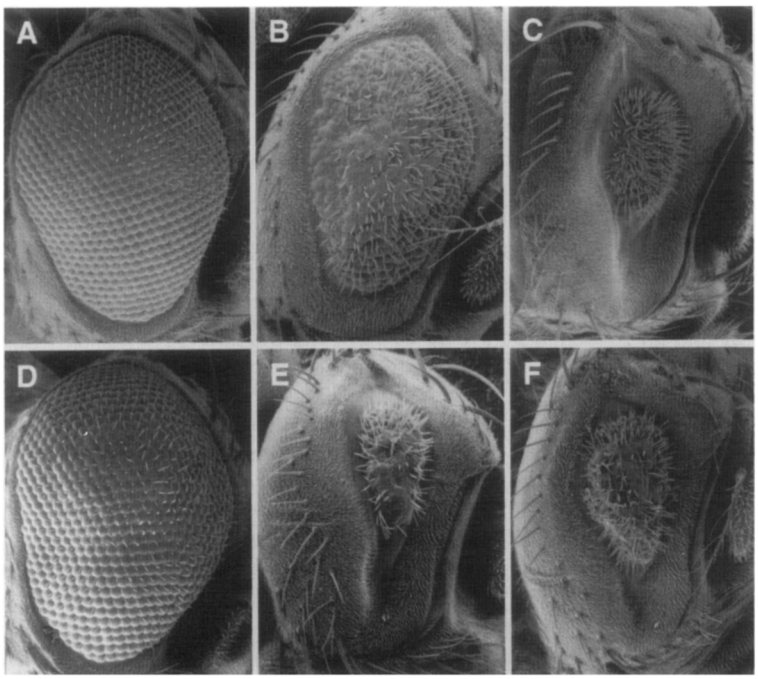

Figure 1. Scanning Electron Micrographs of Compound Eyes

The following genotypes are shown: wild type (A); GMR–*rprM*/+ (B); GMR–*rprS*/+ (C); GMR–p35/+;GMR–*rprS*/+ (D); GMR–*rprM*/+; *Df-th117*/+ (E); GMR–*rprM*/+; th^5/+ (F). Expression of RPR in the developing eye results in a decrease in adult eye size that can be moderate (B) or severe (C) depending on the site of insertion of the transgene. This death-induced decrease in eye size is prevented by coexpression of baculovirus p35 (D) and enhanced by a 2-fold decrease in *th* dose (E and F).

number of independent lines carrying a GMR-*rpr* transgene (pGMR-*rpr* flies). While phenotypes displayed by individuals within a line are similar, different lines display eyes of various reduced sizes, presumably owing to genomic position effects on the expression level of the transgene (see Spradling and Rubin, 1983). Two of these lines, GMR-*rprM* and GMR-*rprS*, which have a moderate or severe reduction in eye size, respectively, were characterized and utilized further (Figures 1B and 1C).

Third instar eye discs from these lines have excess death posterior to the morphogenetic furrow, as visualized by acridine orange staining (data not shown). The presence of acridine orange staining cells and cell fragments has been shown in many systems, including Drosophila, to reflect the presence of apoptotic cell death accurately (Spreij, 1971; Cagen and Ready, 1989; Wolff and Ready, 1991; Abrams et al., 1993). Acridine orange staining was eliminated (data not shown) and eye size restored (Figure 1D) by coexpression of baculovirus p35. Since p35 prevents cell death in multiple contexts in Drosophila (Hay et al., 1994), C. elegans (Sugimoto et al., 1994), and mammals (Rabizadeh et al., 1993) without other obvious effects on cell function, these results indicate that *rpr* overexpression is primarily inducing cell death and that induced cell death is the cause of the *rpr*-dependent small eye phenotype.

To identify endogenous genes that function in the *rpr*-dependent cell death pathway, GMR-*rprM* and GMR-*rprS* flies were crossed to a collection of chromosomal deletions that cover about half of the Drosophila genome (see Experimental Procedures). A small number of regions were identified as dominant modifiers of the GMR-*rpr* eye phenotype (data not shown). In this report we focus on one such modifier, *E(rpr)3-1*, that was identified by deletions covering the 72D1-2 region. These deletions dramatically enhance GMR-*rpr*-dependent death (Figure 1E; compare with Figure 1B). A search of translated nucleotide databases, using the TBLASTN program (Altschul et al., 1990), identified a sequence-tagged site (GenBank accession number G01117) that was derived from a P1 genomic clone that maps to this region (Berkeley Drosophila Genome Project, personal communication), with significant similarity to the RING finger domain of baculovirus IAPs encoded by O. pseudotsugata nuclear polyhedrosis virus (OpIAP) and C. pomonella granulosis virus (CpIAP). This fragment was amplified by polymerase chain reaction (PCR) and used to screen both genomic and cDNA libraries (see Experimental Procedures). The longest cDNA isolated was sequenced and found to contain an open reading frame with significant overall homology to baculovirus IAPs (Figure 2B; see Figure 3). We therefore have named this gene *DIAP1*.

To identify a mutation in *DIAP1*, the cDNA was used to probe a genomic blot of DNA from lethal P element insertion lines in this region (Spradling et al., 1995) and one line, *l(3)j5C8*, showed a polymorphism (data not shown). Sequencing of genomic DNA flanking the site of insertion showed that it was inserted in the 5′ untranslated region of the *DIAP1* transcription unit (Figure 2A). The lethality

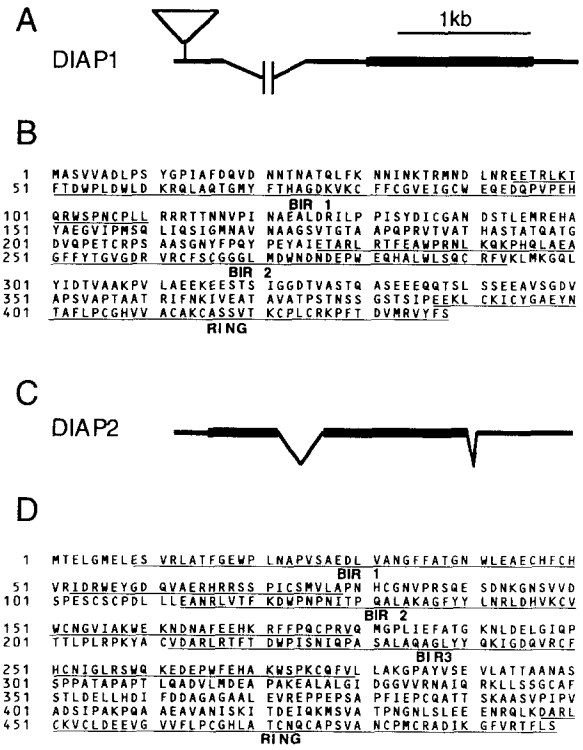

Figure 2. Genomic Structures and Amino Acid Sequences of DIAP1 and DIAP2

The genomic structure of the *DIAP1* (A) and *DIAP2* (C) transcription units were determined by sequencing genomic DNA corresponding to the longest cDNAs isolated: *DIAP1-NB1* (2017 bp) and *DIAP2-NB5* (2085 bp). The insertion site of the P element *l(3)j5C8* is indicated by the triangle and corresponds to base 34 of the *DIAP1-NB1* cDNA. The position of the open reading frame encoding DIAP1 or DIAP2 proteins are indicated by the heavier line. Genomic maps are drawn to scale except for the approximately 6 kb intron present in the 5′ noncoding region of *DIAP1* (A). The positions of the BIRs and the RING finger are noted in the amino acid sequences of DIAP1 (B) and DIAP2 (D).

is due to the P element insertion since excision of the P element generated viable revertants. Complementation tests with *l(3)j5C8* showed that it is allelic to *th*. Five ethyl methanesulfonate–induced lethal *th* alleles, th^4 to th^8, were obtained from J. A. Kennison (National Institutes of Health). These *th* alleles dominantly enhance GMR-*rprM* (see Figure 1F; compare with Figure 1B) as well as a death-induced small eye phenotype caused by overexpression of GMR-*hid* (Grether et al., 1995) (data not shown). th^1 mutants are viable, but the aristae lack normally occurring side branches (Lindsley and Zimm, 1992). th^1 is probably a weak cr tissue-specific allele since it does not enhance GMR-*rpr*. The missing aristae branch phenotype of th^1 is consistent with a defect in cell death suppression, but this has not been directly demonstrated.

IAPs Are an Evolutionarily Conserved Family of Proteins

We used the *DIAP1* coding sequence to search available databases and identified another Drosophila sequence that displays similarity to *DIAP1* and v-IAPs (GenBank ac-

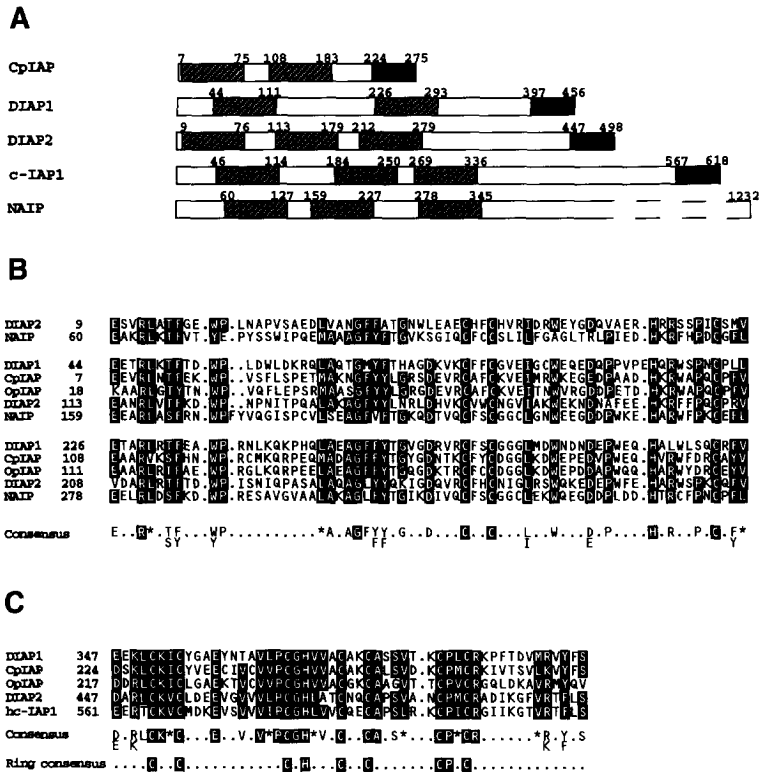

Figure 3. DIAPs Share Domains of Homology with Viral and Mammalian IAPs and NAIP

(A) The locations of the BIRs and the RING finger are noted in the block diagram of each protein. BIRs are indicated by cross-hatched boxes and the RING finger by a closed box. All proteins are drawn to scale except for the C-terminal region of NAIP. The v-IAPs that prevent cell death, OpIAP and CpIAP, are 58% identical to each other, but are less closely related to AcIAP (30% identity; diagram not shown), which does not prevent death. DIAP1 is most closely related to OpIAP and CpIAP (46% identity). DIAP1 is less closely related to DIAP2 (36% identity), the mammalian c-IAPs described by Rothe et al. (1995) (30% identity), and AcIAP (28% identity). DIAP2 is less similar to OpIAP and CpIAP (39% and 40% identity, respectively) and more closely related to mammalian c-IAPs (36% identity) than is DIAP1.

(B) Alignments of the BIRs. Identical and similar residues are boxed. Amino acids designated as similar are: S, T; W, Y, F; I, L; D, E; and R, K. Conservation of a hydrophobic residue (I, L, M, V) is indicated by an asterisk. Residues are boxed if eight or more of the 12 repeats shown share an identical or similar residue. Consensus amino acids are indicated below the alignments. Invariant amino acids are boxed.

(C) Alignments of the RING finger domains. Residues are boxed if they are found to be identical or similar in at least four of the five RING domains. Conservative substitutions are as in (B). A consensus for the IAP RING finger motif is shown below the sequence alignments, and invariant residues are boxed. The consensus RING motif (Freemont, 1994) is noted below the IAP RING consensus.

cession number M96581). The predicted coding region was amplified by PCR and used to screen both cDNA and genomic libraries. The longest cDNA was sequenced and found to contain an open reading frame with extensive similarity to other IAPs (Figures 2C and 2D). We named this gene *DIAP2*. Also present in the GenBank database are multiple mammalian expressed sequence tags that display significant similarity to portions of DIAPs and v-IAPs. The sequence of one of these (GenBank accession number R07927) was used to clone a partial cDNA that also contained an open reading frame predicting an IAP-homologous protein (data not shown). This protein was also identified by Rothe et al. (1995 [this issue of *Cell*]).

DIAPs Share Common Domains with v-IAPs and Mammalian IAPs and Human Neural Apoptosis Inhibitory Protein

Members of the IAP family previously described in baculoviruses (v-IAPs) have a common structure in which the N-terminal region contains two tandem repeats of about 70 amino acids, termed the BIR motifs (Birnbaum et al., 1994; Figures 3A and 3B), which are separated from each other by a variable length linker with little homology. At the C-terminus of the v-IAPs is a RING finger motif (reviewed by Clem and Miller, 1994b), which is separated from the second BIR by a variable length linker with little homology. DIAP1 has a similar structure, while DIAP2 and mammalian cellular IAPs (c-IAPs) described by Rothe et al. (1995) have three N-terminal BIRs (Figure 3A). The product of the human neural apoptosis inhibitory protein gene (NAIP) (Roy et al., 1995) has three N-terminal BIRs, but it lacks the C-terminal RING finger (Figure 3A).

The most homologous regions of the IAPs are the BIRs and the RING finger. An alignment of the BIRs of IAPs and NAIP is shown in Figure 3B. The conserved spacing of cysteines and histidines within this motif suggests the possibility of metal ion coordination (Birnbaum et al., 1994).

The RING finger motif present at the C-terminus of IAPs is also present in a number of nuclear and cytoplasmic proteins involved in diverse cellular processes (reviewed by Freemont, 1993). This motif is thought to form a single domain with two independent zinc-binding sites (Barlow et al., 1994). IAP RING domains contain many other conserved residues in addition to those found in the canonical RING consensus sequence (Figure 3C). A comparison of DIAP1 and DIAP2 RING domains with those of other IAPs suggests that there may be two families of IAP RING fingers (Figure 3C). The DIAP1 RING finger is more similar to the RING domains of OpIAP and CpIAP than to those of DIAP2 or c-IAPs, while the DIAP2 RING finger has a much greater similarity to the c-IAP RING finger than to those of OpIAP and CpIAP or DIAP1. Since DIAP1 and OpIAP and CpIAP have two BIRs, whereas DIAP2 and mammalian c-IAPs have three, it may be that DIAP1 and DIAP2 identify members of distinct IAP subfamilies.

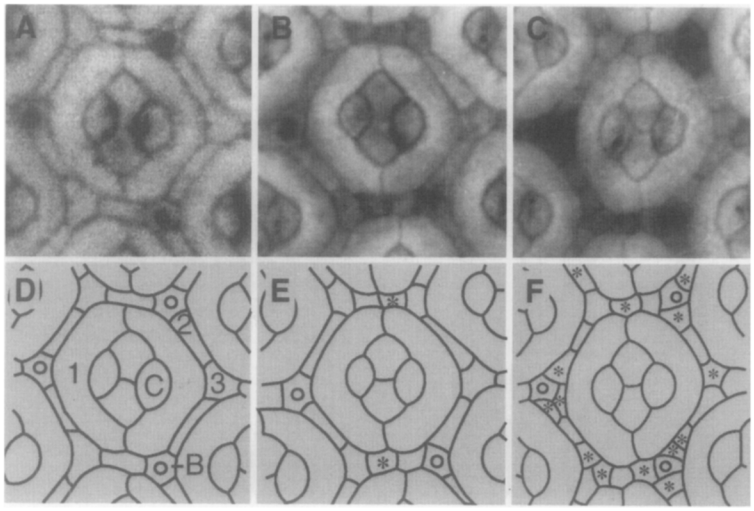

Figure 4. Pupal Eye Phenotypes of Flies Expressing GMR-*DIAP1* or GMR-*DIAP2*

Genotypes are as follows: wild type (A); GMR-*DIAP1*/+ (B); GMR-*DIAP2*/+ (C); wild type (D); GMR-*DIAP1*/+ (E); GMR-*DIAP2*/+ (F). (A)–(C) show 55-hr-old pupal eyes stained with cobalt sulfide to reveal the cell outlines at the apical surface of the developing retina. (D)–(F) show schematic drawings of the cobalt sulfide stains shown in (A)–(C), respectively. In the wild-type 55-hr eye (A and D), the apical surface of each ommatidium displays the outline of four cone cells (designated C) and two primary pigment cells (1), surrounded by a characteristic array of six secondary pigment cells (2), three tertiary pigment cells (3), and three bristles (designated B). Only a single example of each cell type is labeled. Primary pigment cells surround the cone cells, each secondary pigment cell is shared by two ommatidia, and tertiary pigment cells are located at the vertex of three ommatidia. Extra potential secondary and tertiary pigment cells present earlier in development have been removed by cell death. To count the number of extra pigment cells surrounding each ommatidium, we defined a sample area as the hexagonal area bounded by straight lines linking the cone cells of the six surrounding ommatidia. Secondary and tertiary pigment cells were counted as surrounding a central ommatidium if more than 50% of their apical surface area was within the sample area. In all panels, anterior is to the right. Expression of GMR-*DIAP1* (B and E) or GMR-*DIAP2* (C and F) results in the presence of extra secondary and tertiary pigment cells. Extra pigment cells in (E) and (F) are indicated by an asterisk within or next to a cell. These cells have been chosen arbitrarily since it is not possible to differentiate the extra pigment cells from those pigment cells that would have developed in the wild-type situation.

DIAPs Function to Block Normally Occurring Cell Death

To ask whether DIAP1 and DIAP2 proteins are sufficient to function as cell death inhibitors, we generated transgenic lines expressing full-length *DIAP1* or *DIAP2* from the pGMR vector, pGMR-*DIAP1* and pGMR-*DIAP2*, respectively.

Between 35 and 50 hr postpupation, 1500–2000 surplus cells in the developing Drosophila eye are eliminated by cell death (Cagan and Ready, 1989; Wolff and Ready, 1991). This death is necessary for the formation of the ordered cellular lattice that makes up the adult eye (Cagan and Ready, 1989). Failure of this death to occur results in the presence of extra secondary and tertiary pigment cells (Wolff and Ready, 1991; Hay et al., 1994). We quantitated the amount of normally occurring cell death prevented by expression of the DIAP constructs by counting the number of cells present in a sample area, defined as described in the legend to Figure 4. In the wild-type eye, the number of secondary and tertiary pigment cells is essentially invariant, at 12 cells per sample area, extra cells being present in less than 1% of ommatidia (Wolff and Ready, 1991). Expression of baculovirus p35 under GMR control causes a complete loss of normally occurring cell death in the eye, resulting in the presence of 16 ± 2.9 (n = 50) extra secondary and tertiary pigment cells per sample area (Hay et al., 1994). *DIAP1* or *DIAP2* expression in the eye is sufficient to partially block cell death, giving rise to an average of 5 ± 1.4 (n = 50) or 10 ± 2.0 (n = 50) extra cells per sample area, respectively (Figures 4B and 4E and Figures 4C and 4F, respectively; compare with Figures 4A and 4D).

GMR-driven *DIAP1* and *DIAP2* expression is also sufficient partially to block a second wave of normally occurring eye cell death that takes place between 60 and 70 hr postpupation, in which the perimeter clusters, a population of stunted ommatidia that end each ommatidial row, are removed (data not shown).

Tissue in situ hybridization using the *DIAP1* cDNA reveals that it is expressed throughout the eye disc and in the embryo in most, if not all, cells, although there are expression level differences in specific cell types (data not shown). Embryos homozygous for *th* loss-of-function mutations do not show a massive increase in cell death, as visualized by acridine orange staining. However, we cannot rule out that excess death is present in specific tissues in these mutant embryos. Our failure to see an obvious effect on cell death may not be surprising in view of similar studies performed in C. elegans and mammals. *ced-9* encodes the C. elegans homolog of the mammalian apoptosis inhibitor, BCL2 (Hengartner and Horvitz, 1994). Homozygous *ced-9* loss-of-function mutants derived from heterozygous mutant mothers hatch and grow to a normal size. Only moderate amounts of extra cell death are seen in these animals. Large amounts of embryonic cell lethality are seen only with embryos derived from homozygous *ced-9* loss-of-function mutants, suggesting the presence of a maternal component (Hengartner et al., 1992). In mice, although *Bcl2* is clearly an important cell death regulator, homozygous loss-of-function mutants survive to adulthood (Veis et al., 1993). The lack of significant ectopic cell death in most tissues in these animals may reflect the action of other BCL2-related proteins that also function to prevent cell death (reviewed by Boise et al., 1995). Thus, the lack of a dramatic increase in embryonic cell death in *th* mutants may be due to a significant maternal contribu-

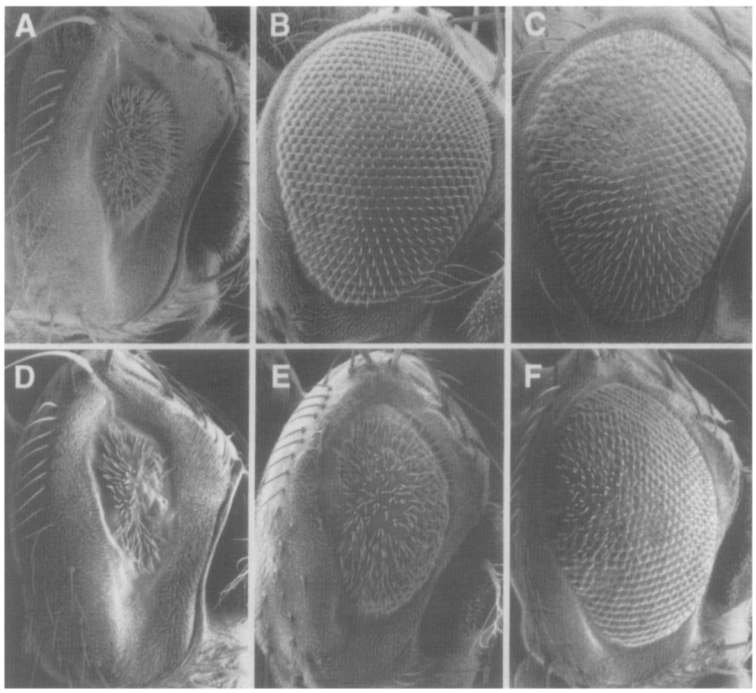

Figure 5. Expression of GMR–*DIAP1* or GMR–*DIAP2* Suppresses GMR–*rpr*- or GMR–*hid*-Dependent Death

Scanning electron micrographs of adult eyes. Genotypes are as follows: GMR–*rprS*/+ (A); GMR–*rprS*/+;GMR–*DIAP1*/+ (B); GMR–*rprS*/+; GMR–*DIAP2*/+ (C); GMR–*hidS*/+ (D); GMR–*hidS*/+;GMR–*DIAP1*/+ (E); GMR–*hidS*/+;GMR–*DIAP2*/+ (F). The GMR–*rprS*-dependent small eye phenotype (A) is nearly completely suppressed by coexpression of GMR–*DIAP1* (B) or GMR–*DIAP2* (C), while the GMR–*hidS*-dependent small eye phenotype (D) is partially suppressed by expression of GMR–*DIAP1* (E) or GMR–*DIAP2* (F).

tion of DIAP1 protein or to a redundancy in function with DIAP2. Furthermore, translational regulation may restrict DIAP1 function to specific subpopulations of cells expressing the transcript. Understanding the role of DIAP1 in embryonic development will best be addressed using antibodies to DIAP1 in conjunction with other tissue-specific markers.

As a second approach to asking whether *DIAP1* is required for cell survival, we made mitotic clones of loss-of-function *th* alleles in the eye and germline clones in the ovary. When clones in the eye were made using a mini-*white* (*w*) marker that allows us to distinguish the homozygous wild-type twin spot from the heterozygous background, twin spots were present, indicating that recombination had occurred. However, no w^- homozygous mutant clones were seen (data not shown). For germline clones in the ovary, experiments with a wild-type chromosome were conducted in parallel to those using th^- chromosomes. Eggs were obtained from crosses utilizing the wild-type chromosome, but not from crosses utilizing the th^- chromosome. These results are consistent with the possibility that *DIAP1* is required for cell survival, but do not exclude the possibility that *DIAP1* is required for cell proliferation or some other aspect of cell function. Mutations in *DIAP2* have not been identified.

DIAPs Function to Block Cell Death Induced by RPR or HID

Overexpression of either DIAP1 or DIAP2 under GMR control suppresses GMR–*rprS*- and GMR–*hid*-dependent death in the eye (Figure 5). DIAP1 or DIAP2 overexpression suppresses *rpr*-dependent death nearly completely (Figures 5A–5C) and GMR–*hid*-dependent death moderately (Figures 5D–5F). Overexpression of CpIAP also blocks some *rpr*-dependent death, although not as well as the Drosophila proteins (data not shown). Unlike DIAP1, deficiencies that remove DIAP2 (*52D1-2*) do not enhance GMR–*rpr* phenotypes (data not shown); however, DIAP2 overexpression does block *rpr*- and *hid*-dependent death (Figures 5C and 5F), and DIAP2 is expressed in the third instar eye disc (data not shown). These results can be reconciled if DIAP2 is not rate limiting for DIAP-mediated death preventing activity in the eye.

The contexts in which DIAP1 functions to prevent death in vivo are unknown. Normally occurring cell death is likely to employ multiple signals functioning in parallel, which may ultimately converge on common effectors. The induction of *rpr* transcription in cells that are going to die is likely to be one of these signals. However, signals that activate *rpr* transcription may also regulate other pathways that normally act in concert with *rpr* to bring about death. *hid* may identify one such pathway.

N-Terminal Fragments of IAPs Containing the BIRs Are Sufficient to Prevent Normally Occurring and Ectopically Induced Cell Death

The most significant similarity among the IAPs and NAIP are the BIRs, suggesting these repeats may be important for cell death preventing activity. Clem and Miller (1994a) made hybrids between OpIAP, which can prevent cell death, and AcIAP, which does not affect cell death. They found that chimeric IAPs that contained AcIAP BIR domain or RING finger sequences were unable to block cell death, suggesting that both the BIR and RING domains were required for cell death inhibition. We asked which domains of DIAP1 are sufficient for death preventing activity by making two pGMR expression constructs. The first (pGMR–*DIAP1*–BIR) contains the N-terminal portion of

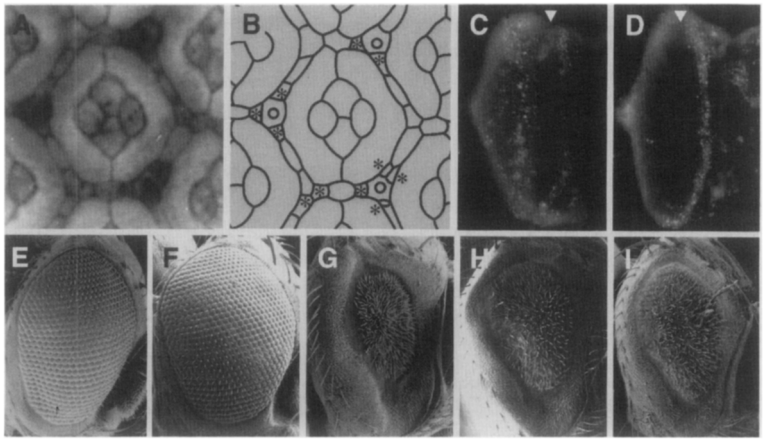

Figure 6. Eye Phenotypes of Flies Expressing Fragments of *DIAP1* and c-*IAP1*

Genotypes are as follows: GMR-*DIAP1*-BIR/+ (A); GMR-*DIAP1*-BIR/+ (B); wild type (C); GMR-*DIAP1*-BIR/+ (D); GMR-*rprS*/+;GMR-*DIAP1*-BIR/+ (E); GMR-*hidS*/+;GMR-*DIAP1*-BIR/+ (F); GMR-*rprS*/+;GMR-c-*IAP1*/+ (G); GMR-*rprS*/+;GMR-c-*IAP1*-BIR/+ (H); GMR-*DIAP1*-RING/+ (I).

(A) A cobalt sulfide-stained 55-hr-old pupal eye from a fly expressing GMR-*DIAP1*-BIR. GMR-*DIAP1*-BIR expression results in the presence of many additional secondary and tertiary pigment cells; indicated by asterisks in (B). Expression of GMR-*DIAP1*-BIR prevents normally occurring cell death in the eye, resulting in the presence of extra secondary and tertiary pigment cells.

(B) Schematic drawings of the central ommatidia present in the cobalt sulfide stain shown in (A).

(C and D) Acridine orange-stained third instar eye imaginal discs. Anterior is to the right. The morphogenetic furrow is indicated by an arrow.

(C) Wild-type third instar eye disc from larvae X-ray-irradiated with 8000 rads and stained with acridine orange. Note the presence of acridine orange-stained dying cells and fragments both anterior and posterior to the morphogenetic furrow.

(D) pGMR-*DIAP1*-BIR expression blocks essentially all irradiation-induced death posterior to the morphogenetic furrow, where the GMR promoter is active.

(E-I) Scanning electron micrographs of adult eyes. GMR-*DIAP1*-BIR expression completely suppresses the pGMR-*rprS*-dependent (E) and pGMR-*hidS*-dependent (F) small eye phenotypes; compare (E) with Figure 5A and (F) with Figure 5D. GMR-c-*IAP1* expression has little, if any, effect on the GMR-*rprS*-dependent small eye phenotype (G), while expression of GMR-c-*IAP1*-BIR partially suppresses the pGMR-*rpr*-dependent small eye phenotype (H); compare [G] and [H] with Figure 5A. (I) shows that expression of GMR-*DIAP1*-RING gives rise to a small eye phenotype.

DIAP1, which includes the BIRs and the C-terminal linker, but not the RING finger; the second (pGMR-*DIAP1*-RING) begins just downstream of the most C-terminal BIR and includes the C-terminal linker and the RING finger.

We examined pupal eyes of GMR-*DIAP1*-BIR flies using cobalt sulfide staining to determine whether expression of DIAP1 lacking the RING finger was sufficient to block normally occurring cell death. We also asked whether GMR-*DIAP1*-BIR expression was able to block death due to X-ray irradiation or overexpression of *rpr* or *hid*. Eyes of GMR-*DIAP1*-BIR flies have 16 ± 3.1 (n = 50) extra cells per sample area, in contrast with those of GMR-*DIAP1*, which have only 5 ± 1.4 extra cells (n = 50) (Figures 6A and 6B; compare with Figures 4B and 4E). The number of extra cells in GMR-*DIAP1*-BIR eyes is similar to that of flies expressing GMR-p35 (16 ± 2.9; n = 50). Since p35 expression completely blocks cell death in the developing eye posterior to the morphogenetic furrow (Hay et al., 1994), GMR-*DIAP1*-BIR blocks most, if not all, normally occurring cell death in the eye.

Many cells, including those of Drosophila, undergo apoptosis following X-ray irradiation (Ashburner, 1989; Abrams et al., 1993). X-ray irradiation causes large amounts of death in the third instar eye disc anterior and posterior to the morphogentic furrow (Figure 6C) (Hay et al., 1994). X-ray irradiation-induced death posterior to the furrow is completely prevented by expression of GMR-*DIAP1*-BIR (Figure 6D), as it is by expression of p35 (Hay et al., 1994). GMR-*DIAP1*-BIR also blocks death due to expression of pGMR-*rpr* and GMR-*hid* (Figures 6E and 6F). Moreover, GMR-*DIAP1*-BIR expression blocks GMR-*hid*-dependent death in the eye much more efficiently than does expression of GMR-*DIAP1* (compare Figure 6F with Figure 5E).

We also tested the ability of pGMR-c-*IAP1* and pGMR-c-*IAP1*-BIR to prevent *rpr*-dependent death. Expression of full-length mammalian c-*IAP1* is unable to prevent GMR-*rprS*-dependent death (Figure 6G). However, expression of c-*IAP1*-BIR results in an increase in eye size (Figure 6H), indicating some death preventing ability.

Since Drosophila and human IAPs show very low homology outside the BIR domains N-terminal to the RING finger, it is likely that the repeats define an important motif that is able to mediate cell death suppression. Since expression of BIR-containing fragments blocks death in response to multiple stimuli, they may function at a point common to multiple death signaling pathways. Based on our observations, the results of Clem and Miller (1994a) can be explained if placing an inappropriate RING finger C-terminal to the BIRs creates a protein in which the BIRs cannot function to prevent cell death.

The human NAIP gene also contains three N-terminal BIRs and has been implicated in death preventing activity based on its association with spinal muscular atrophy, a disease in which there is inappropriate death of spinal motor neurons (Roy et al., 1995). Our observations that expression of BIR-containing fragments blocks cell death supports a role for NAIP as a cell death inhibitor.

We also generated a number of lines transgenic for the pGMR-*DIAP1*-RING construct. The eye size differed among lines, ranging from wild type to very small (Figure 6I). These phenotypes are dose dependent, with multiple copies of the transgene resulting in an increase in the severity of the small eye phenotype (data not shown). The *DIAP1*-RING-dependent small eye phenotype is associated with extra cell death in the third instar eye disc, as visualized by acridine orange (data not shown). The cell death associated with *DIAP1*-RING finger overexpression

may reflect a possible role of the RING finger as a negative regulator of the BIR death-preventing activity (see below). Alternatively, the cell death phenotype caused by expression of the RING finger may be a secondary consequence of its effects on cell function or development.

Concluding Remarks

Here we report that Drosophila encodes proteins homologous to baculovirus IAPs and that these proteins function to prevent normally occurring cell death and death induced by diverse stimuli. Our observations on overexpression of full-length and N-terminal fragments of DIAP1 and loss-of-function *th* phenotypes suggest that the function of full-length DIAP1 is to prevent death and that this death preventing activity resides in the BIRs. The observation that removal of the RING finger results in a protein with increased death-preventing activity suggests a model in which the RING acts as a negative regulator of BIR-dependent death preventing activity. Our results suggest that the DIAPs act to prevent death by regulating components common to multiple death pathways, but they do not distinguish where, with respect to known death regulators, these proteins act.

Rothe et al. (1995) have found that mammalian c-IAPs interact with tumor necrosis factor receptor–associated factor 1 (TRAF1) and 2 (TRAF2) and that the N-terminal BIR motif–containing domain is sufficient for these interactions. Our observations that a c-IAP1 protein lacking the RING domain is able partially to suppress a RPR-dependent small eye phenotype also suggests that death-preventing activity localizes to the BIRs. The BIRs may associate with TRAFs or TRAF-like proteins to prevent death, or they may have other unidentified interaction partners that mediate cell death-preventing activity. The contexts in which the DIAPs function in vivo are not known, but the loss-of-function phenotypes of *DIAP1* we observed during eye development and oogenesis suggest a requirement for cell survival.

In at least some cells, death activators and death inhibitors are constitutively present (Hengartner et al., 1992; Martin, 1993), consistent with the proposal that many, if not all, cells are poised to die and must constantly receive signals to repress the cell death pathway to survive (Raff, 1992). Whether a cell lives or dies depends on the balance of these activities. Death induction may occur through activation of a death-inducing stimulus or removal of a death-preventing stimulus. By overexpressing DIAP1 in cells that normally die, we are able to tip this cellular balance toward death prevention, resulting in cell survival.

We have shown that the *DIAP1*–BIR constructs are better able to prevent death due to multiple stimuli (HID overexpression, normally occurring death, or death induced by X-ray irradiation) than full-length *DIAP1*. These results raise the possibility that DIAP1 death-preventing activity is down-regulated by a RING finger–dependent process in cells that are targeted for death. This hypothesis is not inconsistent with the observation that overexpression of full-length DIAP can prevent cell death; it simply means that not all DIAP1 activity is down-regulated. Such down-regulation provides a potential mechanism for death induction independent of changes in other death activators. If it is generally the case that IAPs are negatively regulated by the C-terminal RING finger, this may have important clinical implications. Truncation of a coding region, through introduction of a stop codon or a frameshift, is likely to be a relatively common sort of mutation. Such events may give rise to IAPs that act to block death inappropriately. It will be interesting to see whether truncated forms of IAPs are associated with conditions in which there is excess cell survival.

Experimental Procedures

Mosaic Analysis

Clones of cells homozygous for th^4, th^5, and th^6 in the eye were produced as described by Tomlinson et al. (1988). The dominant marker used was the P[*w*]G106 element at cytogenetic position 73A1 (T. Laverty, personal communication). th^4 and th^5 germline clones were generated as described by Hou et al. (1995).

Histology

Cobalt sulfide staining (Melamed and Trujillo-Cenoz, 1975) of staged pupal retinae was performed as described by Wolff and Ready (1991). Staging was carried out by aging white prepupae at 20°C. Flies were prepared for scanning electron microscopy as described by Kimmel et al. (1990). Fixation and sectioning of adult eyes were performed as described by Wolff and Ready (1991). Acridine orange staining was carried out as described by Spreij (1971). Tissue in situ hybridization was performed according to the method of Dougan and DiNardo (1992).

X-Ray Irradiation

X-ray irradiation was carried out as described in Hay et al. (1994).

Genetics

Fly cultures and crosses were carried out according to standard procedures. The screen for dominant modifiers of GMR–*rpr* will be described elsewhere. In brief, GMR–*rpr* virgin females were crossed to males from a collection of deficiency stocks for the second and third chromosomes provided by the Bloomington Drosophila Stock Center, and the eyes of the progeny were compared with those of GMR–*rpr*/+. Similar crosses were carried out with ethyl methanesulfonate–induced mutants for the 72D region provided by J. A. Kennison (National Institutes of Health).

The transposon present in the *l(3)j5C8* line was mobilized by matings to flies carrying a stable source of transposase activity (Robertson et al., 1988). A number of lines were established that had lost the w^+ marker gene contained in the P-*lacZ* element. In about 70% of these lines, the lethality was reverted.

pGMR Constructs and Transformation

The *rpr* coding sequence was isolated using the PCR and the primers 5′-GCGGAATTCACAACAATGGCAGTGGCATTC and 5′-CGCAGATCTGGGTTTTGGGTTGGCTCA. Products were gel purified, cut with EcoRI and BglII, and cloned into similarly cut pGMR to generate pGMR–*rpr*. Full-length *DIAP1* and *DIAP2* coding regions were ligated in pGMR as EcoRI fragments derived from their respective cDNAs by PCR. The *DIAP1*–RING construct was made by carrying out PCR on the full-length coding fragment with primers 5′-GCGGAATTCAAACCAGAATGGAGGAGAAGGAGGAGAGCAC and 5′-CGCGGATTCTGGGGTTATATTGAAAAATAT. This created a Kozak consensus sequence and an initial methionine in place of amino acid 312 and also placed EcoRI and BamHI sites at the 5′ and 3′ ends of the new coding region, respectively. The fragment generated was ligated into pGMR as above. To make the *DIAP1*–BIR construct, we carried out the same procedures using primers 5′-GCGGAATTCAGCTAACAACCAGAACACAAA and 5′-GCGGGATCCCTAGCCGGTGCTGTTTGTCGAGGGAGT to generate a fragment containing a stop codon following amino acid

381. To make pGMR–c-*IAP* and pGMR–c-*IAP*–BIR (amino acids 1–569), we blunted EcoRI–HindIII fragments containing the respective coding regions (provided by M. Rothe, Tularik, Incorporated) with Klenow and cloned them into pGMR cut with HpaI.

Flies bearing transgenes were generated by P element–mediated germline transformation (Spradling and Rubin, 1982). Flies overexpressing *hid* with very small eyes (GMR–*hidS*) were provided by H. Steller (Massachusetts Institute of Technology) (Grether et al., 1995). GMR–*hid* flies with a moderately reduced eye size for use in crosses to *th* and *th* deletions were generated by crossing a loss-of-function *gl* allele, gl^{60j}, into the GMR–*hidS* background, generating flies heterozygous for GMR–*hidS* and *gl*. Since the GMR promoter is driven by *gl*, reducing *gl* expression 2-fold results in less GMR-driven gene expression and a somewhat larger eye phenotype.

Isolation of the *DIAP1* and *DIAP2* Gene and cDNA

A Drosophila sequence-tagged site present in GenBank (accession number G01117) was found to have significant homology to the RING fingers of baculovirus IAPs. PCR primers 5′-GGCGAATTCGCTGGTCGAGTGCTGATCGCCTGTTGA and 5′-GGCGGATCCCCATGCGGTCATGTGGTGGCCTGCGCC were used to amplify this sequence, which was used to screen a Drosophila embryonic cDNA library (Brown and Kafatos, 1988). Genomic clones corresponding to the *DIAP1* cDNA sequence were isolated from a D. melanogaster cosmid library (Tamkun et al., 1992). Plasmid rescue from the *l(3)j5C8* line was carried out according to the method of Bier et al. (1989); sequencing from the P element ends was carried out using primers 5′-CCTCTCAACAAGCAAACGTG and 5′-GAATACTATTCCTTTCACTCG, which are complementary to the P element ends.

A similar strategy was used to isolate cDNA and genomic clones for *DIAP2*. GenBank entry M96581 shows homology to v-IAPs in multiple reading frames. Primers 5′-GGCGAATTCTGGAGAGCGTTCGCCTGGCCACAT and 5′-GCGGGATCCTGGCCGCAGGGCAGGAACACTACG flank a portion of this sequence that has homology to IAPs and were used to amplify a genomic fragment that was subcloned and used to screen and purify clones from cDNA and genomic libraries as above.

DNA Sequencing

DNA sequences were performed by the dideoxy chain termination procedure (Sanger et al., 1977) using the automated laser fluorescence system from Pharmacia. Templates were prepared by sonicating plasmid DNA and inserting the sonicated DNA into the M13mp10 vector. The entire coding regions of *DIAP1* and *DIAP2* were sequenced on both strands as well as genomic DNA that corresponds to the *DIAP1* and *DIAP2* coding regions. Sequences were analyzed using the Staden (R. Staden, Medical Research Council of Molecular Biology) and the Genetics Computer Group (University of Wisconsin) software packages.

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GenBank Accession Numbers

The accession numbers for the sequences reported in this paper are L49440 (*DIAP1*) and L49441 (*DIAP2*).