Diverse Domains of THREAD/DIAP1 Are Required to Inhibit Apoptosis Induced by REAPER and HID in Drosophila

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ABSTRACT

Significant amounts of apoptosis take place during Drosophila development. The proapoptotic genes *reaper* (*rpr*), *grim*, and *head involution defective* (*hid*) are required for virtually all embryonic apoptosis. The proteins encoded by these genes share a short region of homology at their amino termini. The Drosophila IAP homolog THREAD/DIAP1 (TH/DIAP1), encoded by the *thread* (*th*) gene, negatively regulates apoptosis during development. It has been proposed that RPR, GRIM, and HID induce apoptosis by binding and inactivating TH/DIAP1. The region of homology between the three proapoptotic proteins has been proposed to bind to the conserved BIR2 domain of TH/DIAP1. Here, we present an analysis of loss-of-function and gain-of-function alleles of *th*, which indicates that additional domains of TH/DIAP1 are necessary for its ability to inhibit death induced by RPR, GRIM, and HID. In addition, that analysis of loss-of-function mutations demonstrates that *th* is necessary to block apoptosis very early in embryonic development. This may reflect a requirement to block maternally provided RPR and HID, or it may indicate another function of the TH/DIAP1 protein.

THE active elimination of cells by apoptosis is a fundamental aspect of normal development and homeostasis in multicellular organisms. In the embryo of Drosophila, apoptosis is controlled by three genes that are clustered in a small region of the third chromosome (White *et al.* 1994). These genes, *head involution defective* (*hid*), *grim*, and *reaper* (*rpr*), act to regulate all embryonic apoptosis (White *et al.* 1994; Grether *et al.* 1995; Chen *et al.* 1996). In the absence of all three genes, embryonic apoptosis is virtually eliminated. Overexpression of any one of these genes is sufficient to induce apoptosis in a wide variety of cell types (Grether *et al.* 1995; Chen *et al.* 1996; White *et al.* 1996).

Apoptosis is negatively regulated in Drosophila by the gene *thread* (*th*; Hay *et al.* 1995). This gene encodes a protein, TH/DIAP1, that is homologous to the inhibitor of apoptosis or IAP proteins, first identified in baculovirus (Crook *et al.* 1993). In the virus, these proteins act to block the death of the infected host cell (Clem and Miller 1994). The viral proteins have been shown to block apoptosis in a wide variety of systems (Hay *et al.* 1995; Rothe *et al.* 1995; Uren *et al.* 1996; Vucic *et al.* 1997b). IAP homologs have been found in both Drosophila and mammals (Hay *et al.* 1995; Rothe *et al.* 1995; Roy *et al.* 1995; Duckett *et al.* 1996; Uren *et al.* 1996; Ambrosini *et al.* 1997). The mammalian homologs have been proposed to provide survival signals in the tumor necrosis factor signaling pathway and in oncogenic transformation (Rothe *et al.* 1995; Uren *et al.* 1996; Ambrosini *et al.* 1997).

The highly conserved baculovirus IAP repeats (BIRs) play a major role in the antiapoptotic function of the IAPs. IAPs may have one, two, or three BIRs. There is also commonly a carboxyl-terminal "ring" domain consisting of four pairs of cysteines and histidines (Deveraux and Reed 1999). Both of these domains are predicted to bind zinc. In the viral proteins, both the BIR and ring domains are required to inhibit apoptosis under some, but not all, conditions (Clem and Miller 1994; Harvey *et al.* 1997; Vucic *et al.* 1998b), while in the Drosophila and mammalian proteins, the BIR domains alone are sufficient to inhibit apoptosis (Hay *et al.* 1995; Deveraux *et al.* 1997; Roy *et al.* 1997).

In transgenic Drosophila, ectopic expression of TH/DIAP1 has been shown to block apoptosis induced by overexpression of RPR and HID (Hay *et al.* 1995). RPR and TH/DIAP1 interact physically when overexpressed in lepidopteran SF-21 cells (Vucic *et al.* 1997a). Similarly, both HID and GRIM each can physically interact with TH/DIAP1 in this overexpression assay (Vucic *et al.* 1998a). In these studies, the binding of RPR, GRIM, and HID to TH/DIAP1 appears to require the BIR domains of TH/DIAP1 and a short stretch of conserved residues at the amino-terminus of the death inducers (Vucic *et al.* 1998a).

IAPs have also been shown to bind to and inhibit the activity of caspases, a class of proteases that effect cell death (reviewed in Devereax and Reed 1999). In particular, TH/DIAP1 binds to a Drosophila caspase, drICE, and inhibits apoptosis induced by activated drICE and

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by mammalian caspase 3 in SF-21 cells (Kaiser *et al.* 1998). TH/DIAP1 also binds to the caspase DCP-1 and inhibits its activity (Hawkins *et al.* 1999; Wang *et al.* 1999). Thus, one model for the proapoptotic action of RPR, GRIM, and HID is that they bind to TH/DIAP1, inhibiting the ability of TH/DIAP1 to block caspase activity (Wang *et al.* 1999). The activated caspases would then cause apoptosis. In this model, TH/DIAP1 acts downstream of RPR, GRIM, and HID.

In this article, we report the identification of a potent gain-of-function mutation in *th*. This mutation was unique in that it protected against RPR- and GRIM-induced cell death, but not against HID-induced cell death. The specificity of this protection prompted us to investigate the ability of other *th* alleles to inhibit apoptosis. We found that several other alleles showed specificity in their interactions with RPR, GRIM, and HID. We have identified the molecular lesions in these mutations. Our data support the model that TH/DIAP1 inhibits HID-induced death differently than it inhibits RPR- and GRIM-induced death.

In addition, we examined the lethal phenotype of *th* alleles. As described below, the analysis of embryos homozygous for loss-of-function alleles of *th* indicates that this gene is required to block apoptosis very early in embryonic development.

MATERIALS AND METHODS

Fly stocks: Fly matings were conducted using standard procedures, and the cultures were maintained at 25°. The following alleles of *thread* were used: *Df(3L)brm11*, th^4, th^5, th^{6B}, th^7, th^8 (Brizuela *et al.* 1994), $th^{81.03}$, and $th^{109.07}$ (Moore *et al.* 1998). th^{SL} and th^9 were generated by chemical mutagenesis (see below).

Mutagenesis for modifiers of *GMRrpr*: To identify suppressors or enhancers of the *GMRrpr* eye phenotype, *GMRrpr 81* (White *et al.* 1996) homozygous male flies were starved for 2 hr and exposed overnight to 25 mm EMS in 1% sucrose. Males were then mated to *GMRrpr81/GMRrpr81; GMRrpr97/GMRrpr97* females.The progeny of this cross were screened for changes in eye size and roughness. All flies with eyes that were larger or smaller than the eyes of siblings (carrying three copies of GMRrpr) were retested by crossing to *CyO2xGMRrpr; TM2/MKRS* (Kurada and White 1998). Those that showed consistent suppression were mapped by segregation and by recombination with the *rucuca* mapping chromosome (Ashburner 1989b).

Reversion of th^{SL}: Homozygous th^{SL} males were mutagenized with EMS and crossed to *GMRrpr81/GMRrpr81; GMRrpr97/GMRrpr97* females. The progeny were screened for loss of the suppressing capability of th^{SL} by identifying flies that showed eyes at least as small as those of *GMRrpr81/+*; *GMRrpr97/+* flies.

Sequencing of the *th* alleles: DNA was isolated from adult flies homozygous for viable alleles (th^{SL}) or from dead embryos homozygous for lethal alleles (th^4, th^5, th^{6B}, th^7, th^9, $th^{81.03}$, and $th^{109.07}$). Homozygous dead embryos were obtained from matings of *th/+* flies. Collections of embryos 0–16 hr after egg laying (AEL) were aged for at least 24 hr, and the dead embryos were picked under a microscope and processed for DNA isolation. DNA extraction from 200 adults or 400 embryos was performed using QIAmp blood kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. The *th* open reading frame was then amplified by polymerase chain reaction (PCR) under standard conditions using the Expand Long Template PCR system (Boehringer Mannheim, Indianapolis). The following oligonucleotide primers were used: 5′-CGACT TCAGAGGAAAGGAGCCAGA-3′ and 5′-CGCTTATAATTAA CACATTCCGCCCAC-3′. DNA sequences were obtained using the Thermo Sequenase radiolabeled terminator cycle sequencing system (Amersham, Arlington Heights, IL). The sequencing of the entire coding region of *th* was performed on both strands for all alleles.

Embryonic protection experiments: *hs-rpr* (White *et al.* 1996) flies were crossed to $th^{SL}/TM2$ flies, and embryos were collected on molasses plates, aged for 3–8 hr, and heat shocked for 1 hr at 37°. The embryos were transferred to standard fly food and scored for adult viability. *TM3hs-hid* (FlyBase 1994) flies were crossed to $th^{SL}/D\ gl^3$ or $th^{6B}/D\ gl^3$. At 8–10 hr postcollection, embryos were heat shocked for 20 min at 37°. The embryos were transferred to standard fly food and scored for adult viability.

Anti-TH/DIAP1 antibody production and immunostaining: The TH/DIAP1 polyclonal antibody was raised in rabbits (Poconos Rabbit Farm) against full-length TH/DIAP1 expressed and purified using the Xpress System protein purification kit (Invitrogen, San Diego, CA). The antibody was subsequently affinity purified with TH/DIAP1 His-tagged fusion protein covalently bound to cyanogen-bromide-activated Sepharose (Pharmacia, Piscataway, NJ). This antibody recognized a single band of the correct size on Western blots of eye discs. For immunostaining, eye discs were fixed in 2% paraformaldehyde, washed in BSS (Ashburner 1989b), preincubated in BSS with 5% goat serum and 0.3% Triton X-100 (BSN), and incubated overnight at 4° in preabsorbed anti-TH/DIAP1 antibody diluted 1:1000 in BSN. Preabsorbed Texas red-conjugated anti-rabbit secondary antibody (Cappel, Aurora, OH) was used at a concentration of 1:1000. Tissues were mounted in Fluoromount-G (Southern Biotechnology, Birmingham, AL) and viewed on a TCS NT4D confocal microscope (Leica, Deerfield, IL). The specificity of the antibody was confirmed by preincubating the antibody with TH/DIAP1 fusion protein, which eliminated all staining.

pUAS constructs and transformation: Full-length pUASthV85M and pUASthwt were generated in a two-part cloning: 3′ fragments of the genes were obtained by *Sal*I digestion of pHSP70PLVI$^+$EpiD-iap1V85M and pHSP70PLVI$^+$EpiD-iap1, which were kindly provided by Dr. Lois Miller (University of Georgia, Athens, GA). The *Sal*I fragments were purified and redigested with *Mfe*I, and the *Sal*I-*Mfe*I fragments were purified. These fragments contain the 3′ end of the cDNA, starting at base 617 (Hay *et al.* 1995). The G-to-A mutation at base 684 found in the SL mutation was introduced by site-directed mutagenesis by the Miller lab, using the Clontech (Palo Alto, CA) Transformer system. The 5′fragment was generated by PCR amplification from genomic DNA extracted from *yw* flies, as described above. The following oligonucleotide primers were used: 5′-CGACTTCAGAGGAAAGGAGC CAGA-3′ and 5′TAATGCTTCTTCGGCATTGATCGGCACA-3′. The PCR products were gel purified and cut with *Eco*RI and *Mfe*I, followed by further gel purification. This fragment contains bases 357–616 of the cDNA. The vector GAL UAS (pUAST) was cut with *Eco*RI and *Xho*I and gel purified. The three DNA pieces were ligated together using T4 DNA ligase (Boehringer Mannheim), and constructs were confirmed by sequencing. Flies bearing transgenes were generated by *P*-element-mediated germline transformation (Ashburner 1989a).

TUNEL staining: Embryos were dechorionated and fixed for 30 min in 4% paraformaldehyde in 0.1 m phosphate buffer,

pH 7.4. The vitelline membrane was removed with methanol. The embryos were rehydrated through 75, 50, and 25% methanol/PBT (PBS with 0.1% Tween-20) and treated with proteinase K (10 μg/ml in PBS) for 5 min, washed twice in PBT, postfixed for 20 min in 4% paraformaldehyde in PBS, washed five times for 5 min in PBT, and incubated for 1 hr at 37° in the equilibration buffer provided in the Apoptag kit from Intergen. The embryos were incubated overnight at 37° with TdT (reaction buffer with TdT 2:1 from the Apoptag kit, with 0.3% Triton X-100). The reaction was stopped by incubating the embryos for 4 hr at 37° with stop solution (from the Apoptag kit). The embryos were washed three times for 5 min in PBT and blocked for 1 hr with 2 mg/ml BSA and 5% goat serum in PBT. They were incubated overnight at 4° with 1:2000 preabsorbed antidigoxygenin antibody (alkaline phosphate conjugated, Boehringer Mannheim). The embryos were washed four times for 20 min in PBT, rinsed twice for 20 min in NTMT buffer (0.1 m Tris-HCl, pH 9.5, 50 mm $MgCl_2$, 0.1 m NaCl, 0.1% Tween-20), and incubated for 10 min with 3.5 μl of NBT and 4.5 μl of X-phosphate provided in the DIG Nucleic Acid detection kit (Boehringer Mannheim) in 1 ml of NTMT. The reaction was stopped by washing in PBT. The embryos were mounted in Vectashield (Vector Laboratories, Burlingame, CA).

RESULTS

Identification of a gain-of-function allele of *th* as a dominant suppressor of *GMRrpr*: To understand the mechanisms underlying apoptosis in Drosophila, we took advantage of the cell death phenotype caused by RPR overexpression. Directed overexpression of RPR in the developing eye using the GMR vector (*GMRrpr*) results in a dose-dependent elimination of the eye due to ectopic apoptosis (White *et al.* 1996). We and others have shown that this phenotype is sensitive to dominant modification by mutations in genes that act to inhibit apoptosis (Hay *et al.* 1995; Bergmann *et al.* 1998a; Kurada and White 1998).

A screen was carried out to look for modifiers of the *GMRrpr* phenotype. We mutagenized flies carrying two copies of the GMRrpr transgene and crossed them to flies carrying four copies of the transgene. Among the progeny, which showed a highly reproducible three-copy *GMRrpr* phenotype, we looked for flies that showed either a larger eye, indicating suppression or the *GMRrpr* phenotype, or a smaller eye, indicating enhancement. From the 64,000 haploid genomes scored, we identified 19 mutations that strongly suppressed *GMRrpr*. To investigate whether these mutations suppressed the killing activity of RPR or the expression of the GMRrpr transgene, we tested these mutations for the ability to suppress the rough-eye phenotype of *GMRp21* (de Nooij and Hariharan 1995). All but one of the strong suppressor mutations also suppressed the *GMRp21* phenotype, indicating that these mutations probably inhibited expression from the GMR promoter. The remaining mutation, preliminarily called *Su*(*GMRrpr*)*SL*, was quite striking, as it restored the *GMRrpr* eye to a wild-type appearance (Figure 1, A and B).

Preliminary recombination mapping placed *Su*(*GMRrpr*)*SL* very closely to the *th* gene (Hay *et al.* 1995). As loss-of-function (LOF) mutations in *th* have been previously shown to dominantly enhance RPR killing, we speculated that *Su*(*GMRrpr*)*SL* might be a gain-of-function (GOF) *th* mutation. To explore this possibility,

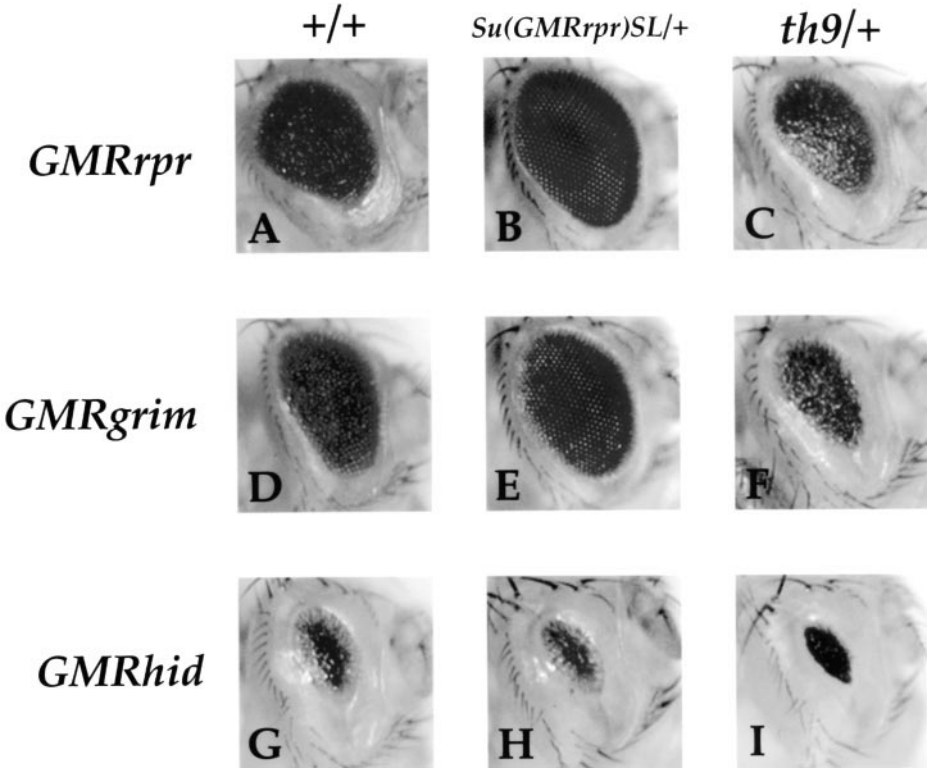


Figure 1.—A gain-of-function allele of *th* suppresses GMRrpr- and GMRgrim-induced cell death. The rough-eye phenotype of flies expressing two copies of a GMRrpr transgene (A) or one copy of a GMRgrim transgene (D) can be rescued to wild type by *Su(GMRrpr)SL* (B and E). The small- and rough-eye phenotype of flies carrying one copy of the GMRhid transgene (G) is slightly enhanced by the presence of one copy of *Su(GMRrpr)SL* (H). A second mutation in *Su(GMRrpr)SL*, th^9, completely reverts its ability to suppress GMRrpr- and GMRgrim-induced cell death. This allele dominantly enhances the rough-eye phenotypes of *GMRrpr*, *GMRgrim*, and *GMRhid* (C, F, and I).

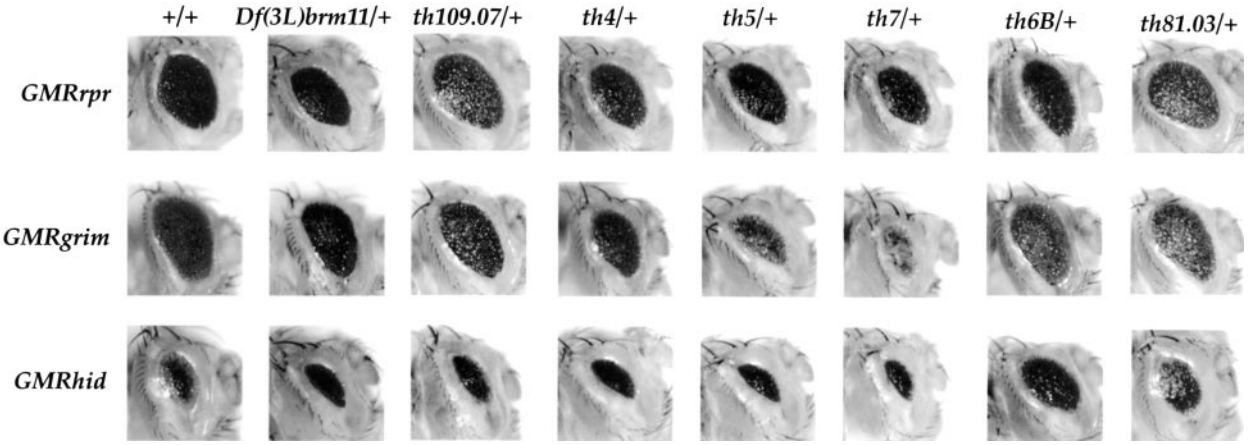


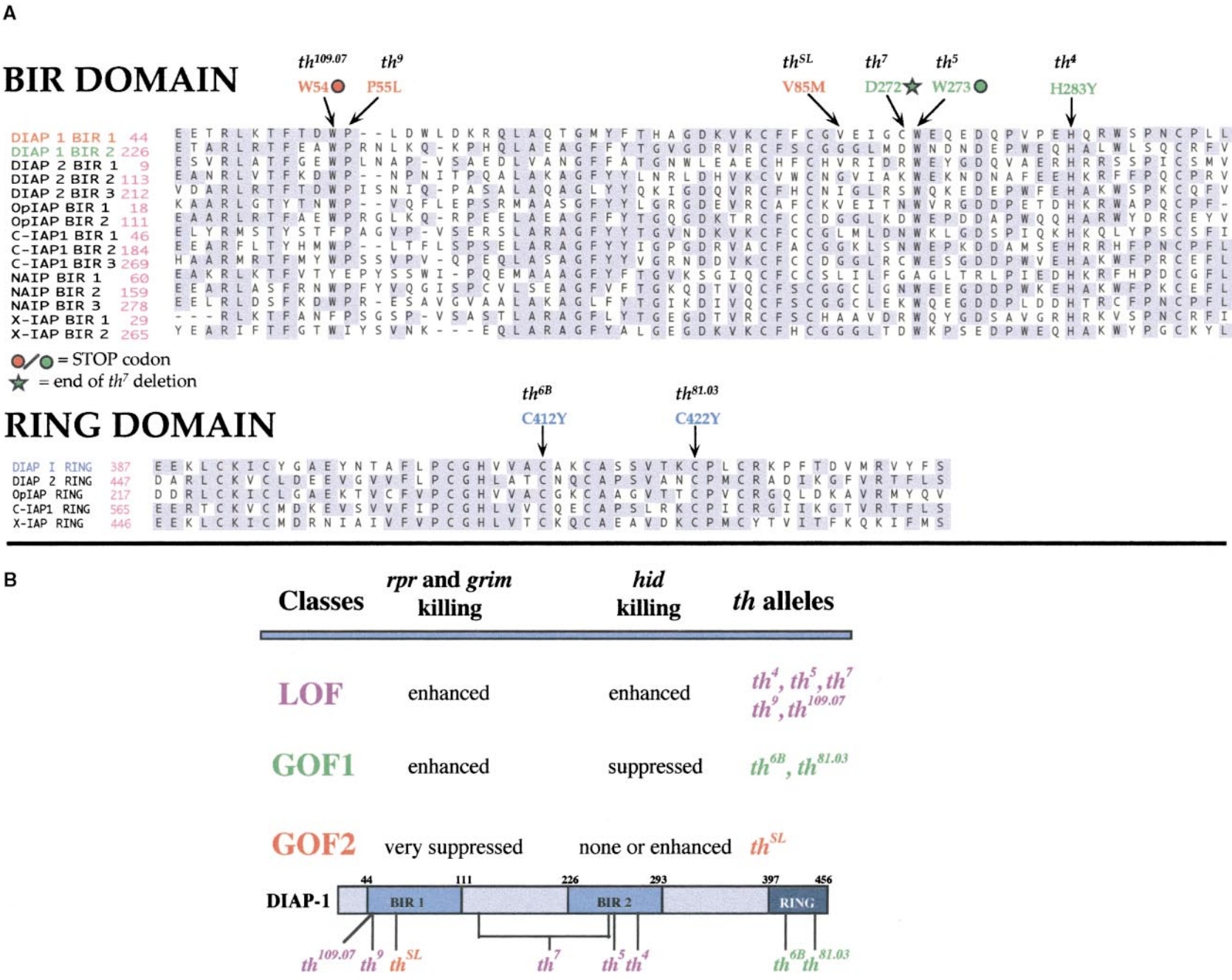
Figure 2.—Different *th* alleles enhance or suppress RPR-, GRIM-, or HID-induced cell death in the eye. Overexpression of RPR (top row), GRIM (middle row), and HID (bottom row) in the eye produces a rough and small eye (leftmost column). Heterozygosity for a deficiency that removes the *th* gene [*Df(3L)brm11*] enhances all three of these eye phenotypes (second column). Similar enhancement is achieved in the heterozygous loss-of-function alleles $th^{109.07}$ (third column), th^{4} (fourth column), th^{5} (fifth column), and th^{7} (sixth column). Enhancement of the *GMRrpr* and *GMRgrim* phenotype is also observed in th^{6B} (seventh column) and $th^{81.03}$ (last column) heterozygotes. Surprisingly, these two alleles suppress the *GMRhid* eye phenotype and, therefore, are defined as gain-of-function alleles (GOF1).

we tested the proximity of *Su*(*GMRrpr*)*SL* to *th* by further recombination mapping. No recombinants were obtained between *Su*(*GMRrpr*)*SL* and th^{1} in >1000 chromosomes scored.

If *Su(GMRrpr)SL* was a GOF *th* mutation, then reversion of the dominant phenotype should generate LOF *th* alleles. We mutagenized the *Su(GMRrpr)SL* stock and tested for reversion of the *Su(GMRrpr)SL* phenotype. Out of 42,000 mutagenized chromosomes scored, we obtained 3 revertants of the *Su(GMRrpr)SL* phenotype. Two of these mutations reverted *Su(GMRrpr)SL* to the baseline GMRrpr phenotype, while one line (now called th^{9}) enhanced the *GMRrpr* phenotype (Figure 1C). The mutations that did not enhance the *GMRrpr* phenotype complemented *th* LOF alleles, and they may represent second site suppressors of the mutation, or weak *th* LOF alleles. The th^{9} allele did not complement other *th* alleles when tested for lethality and, thus, was likely to be a *th* LOF allele. Sequencing has confirmed that th^{9} is a mutation in the *th* gene (see below). To eliminate the concern that th^{9} was not a revertant of *Su(GMRrpr)SL*, but a *th* allele acting as a closely linked second site suppressor, we tested whether another *th* LOF allele was able to suppress the phenotype of *Su(GMRrpr)SL*. We found that the phenotype of *CyO2xGMRrpr; Su(GMRrpr) SL* / th^{5} was the same as that of *CyO2xGMRrpr; Su(GMRrpr) SL* / + (data not shown). Thus, it is highly likely that *Su(GMRrpr)SL*, now called th^{SL}, is a GOF mutation in *th*, which is reverted by a second mutation in the same gene, the th^{9} mutation.

Apoptosis in Drosophila embryos is regulated by three genes, *rpr*, *grim*, and *hid*. All of these genes are able to induce apoptosis when overexpressed, and LOF *th* alleles enhance killing by all three genes (Hay *et al.* 1995; Bergmann *et al.* 1998b). This supports a central role for TH / DIAP1 in all three apoptotic pathways. It therefore seemed likely that th^{SL} would suppress apoptosis induced by GRIM and HID in the eye. As expected, apoptosis induced by GRIM was strongly suppressed by th^{SL} (Figure 1E). To our surprise, th^{SL} did not suppress HID-induced apoptosis, but rather, slightly enhanced this apoptosis (Figure 1H). This suggested that different alleles of *th* might affect killing by RPR, GRIM, and HID differently, and that different parts of the TH / DIAP1 protein might be important for interaction with these different apoptosis inducers.

Figure 3.—Molecular characterization of *th* alleles. (A) The homology shared by the BIR and ring domains of TH / DIAP1 with other members of the IAP protein family. Both BIR domains of TH / DIAP1 are shown, as well as another Drosophila IAP (DIAP2; Hay *et al.* 1995), the baculoviral protein OpIAP (Birnbaum *et al.* 1994), and the mammalian family members C-IAP1 (Rothe *et al.* 1995), NAIP (Roy *et al.* 1995), and X-IAP (Liston *et al.* 1996). Single-amino-acid changes in the first BIR for $th^{109.07}$, th^{9}, and th^{SL} alleles are marked in red, while in green are the point mutations in the second BIR for th^{4} and th^{5} and the end of the in-frame deletion of th^{7}. The th^{7} deletion starts in the spacer between the first and second BIR domain, at proline 130. The single cysteine-to-tyrosine mutations present in both th^{6B} and $th^{81.03}$, located in highly conserved residues of the ring, are marked in blue. (B) A graphic summary of all *th* alleles characterized with regard to their effect on the *GMRrpr*, *GMRgrim*, and *GMRhid* eye phenotypes. The relative location of the mutations in the DIAP1 protein are also shown, using the following color code: purple for LOF alleles, green for GOF1 alleles, and red for the GOF2 allele.


A
BIR DOMAIN
th109.07 W54
th9 P55L
thSL V85M
th7 D272
th5 W273
th4 H283Y
DIAP 1 BIR 1 44
DIAP 1 BIR 2 226
DIAP 2 BIR 1 9
DIAP 2 BIR 2 113
DIAP 2 BIR 3 212
OpIAP BIR 1 18
OpIAP BIR 2 111
C-IAP1 BIR 1 46
C-IAP1 BIR 2 184
C-IAP1 BIR 3 269
NAIP BIR 1 60
NAIP BIR 2 159
NAIP BIR 3 278
X-IAP BIR 1 29
X-IAP BIR 2 265
= STOP codon
= end of th7 deletion
RING DOMAIN
th6B C412Y
th81.03 C422Y
DIAP I RING 387
DIAP 2 RING 447
OpIAP RING 217
C-IAP1 RING 565
X-IAP RING 446
B
Classes
rpr and grim killing
hid killing
th alleles
LOF
enhanced
enhanced
th4, th5, th7 th9, th109.07
GOF1
enhanced
suppressed
th6B, th81.03
GOF2
very suppressed
none or enhanced
thSL
DIAP-1
44
111
226
293
397
456
BIR 1
BIR 2
RING
th109.07 th9 thSL th7 th5 th4 th6B th81.03

This theory provoked our interest in looking at the effects of other mutations in the *th* gene to determine if these showed a preferential interaction with either RPR-, GRIM-, or HID-induced cell death. We have found that certain alleles do show interactions with only one of these inducers of apoptosis (Figure 2). On the basis of these results, we can classify these mutations as LOF or GOF. The GOF alleles fall into two categories: those that suppress RPR- and GRIM-, but not HID-induced death, and those that suppress HID-, but not RPR- and GRIM-induced death.

We have gone on to identify the molecular lesions for many of these alleles of *th* (Figure 3A). We looked at both the dominant phenotypes of these alleles in the eye and the recessive phenotypes in the embryo. Below is a summary of our molecular and phenotypic characterization of each of these classes of alleles. These data are summarized in graphic format in Figure 3B.

Gain-of-function alleles: These are the most interesting mutations that we tested. We have classified them as GOF mutations on the basis of their ability to dominantly suppress apoptosis, in contrast to the enhancement seen with LOF alleles, described below. We have divided these mutations into two classes. GOF1 is represented by two alleles, th^{6B} and $th^{81.03}$. In contrast to what we saw with th^{SL}, both of these mutations suppress killing by HID, and they have no effect or slightly enhance killing by RPR and GRIM (Figure 2). The second class of GOF mutation (GOF2) consists of the th^{SL} allele. As described above, this mutation suppresses killing by RPR and GRIM, but slightly enhances killing by HID (Figure 1).

th^{6B} and $th^{81.03}$: Both of these alleles result from mutations that change canonical cysteines in the ring domain to tyrosines. These mutations are likely to disrupt the secondary structure of the ring domain, as these residues are thought to coordinate zinc. Previous data have suggested that the BIR domains of TH/DIAP1 alone are more potent inhibitors of apoptosis induced by both RPR and HID than by the full-length molecule (Hay *et al.* 1995; Vucic *et al.* 1998a). However, our data suggest that these mutations disrupt the ring structure in a way that only activates the protein in the context of HID killing. The differences in these conclusions may reflect differences in the level of protein expression, as the previous results were based on overexpression, while our data reflects changes in the endogenous gene.

th^{SL}: We considered whether the ability of th^{SL} to suppress RPR- and GRIM-induced death might result from generally increased levels of TH/DIAP1. To examine this question further, we looked at TH/DIAP1 protein levels by immunostaining and by Western blot. Confocal analysis of wild-type and th^{SL} eye discs stained with a polyclonal anti-TH/DIAP1 antibody showed that TH/DIAP1 levels and distribution are not detectably affected by the th^{SL} mutation (Figure 4). In addition, previous experiments indicated that overexpression of wild-type TH/DIAP1 results in the suppression of apoptosis induced by HID, as well as by RPR and GRIM (Hay *et al.* 1995). Therefore, we believe that increased expression cannot explain the specific protection th^{SL} offers against RPR- and GRIM-induced death.

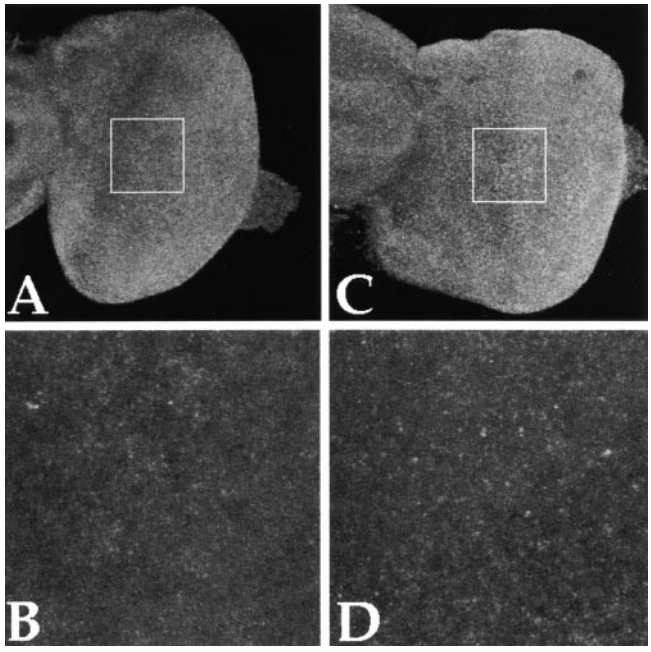


Figure 4.—The th^{SL} allele does not increase TH/DIAP1 protein levels. Eye discs from (A and B) wild-type and (C and D) homozygous th^{SL} larvae were stained with a polyclonal antibody to the TH/DIAP1 protein and then visualized by confocal microscopy. All panels show projections of staining throughout the disc. The protein is expressed ubiquitously. There is no evidence that the th^{SL} mutation modifies either the levels (A and C) or the subcellular localization (B and D) of the protein.

Sequencing of the open reading frame of th^{SL} has revealed a valine-to-methionine change in the first BIR domain. This residue is not highly conserved, although Op-IAP also has a valine in this position (Figure 3A). However, the recently published solution structure of BIR3 from c-IAP-1 places this residue very close to the zinc-binding site of the BIR (Hinds *et al.* 1999).

To confirm that this was in fact the mutation that was responsible for the th^{SL} phenotype, we generated transgenic flies that expressed wild-type TH/DIAP1 or the V85M th^{SL} allele, using the gal4/upstream activating sequence (UAS) binary expression system. As expected, the overexpression of the wild-type protein in the eye was able to suppress GMRrpr-induced apoptosis. However, we found that in two independent insertion lines, the V85M mutant form of the protein resulted in stronger suppression of GMRrpr than the wild-type form (Figure 5). This indicated that the V85M mutation was responsible for the th^{SL} phenotype, and suggests that this mutant form of the protein specifically inhibits the proapoptotic effects of RPR.

We also found that the V85M transgene was also able

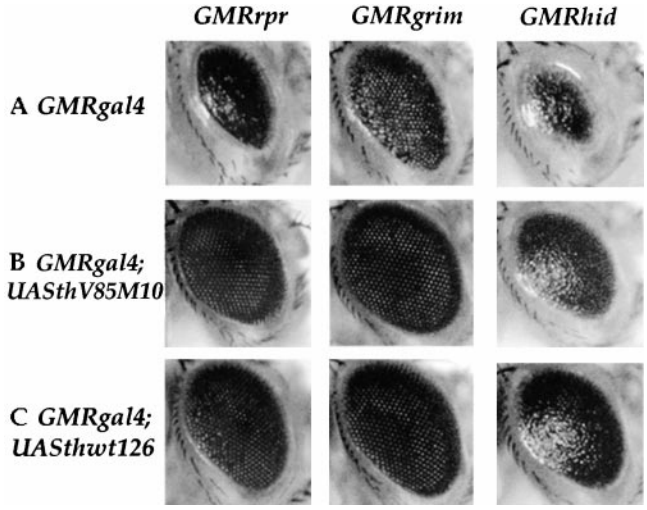


Figure 5.—Expression of th^{SL} from a transgene can suppress GMRrpr-, GMRgrim-, and GMRhid-induced cell death in the eye. Transgenic flies for either the wild-type or the V85M mutant version of TH/DIAP1 were generated using the UAS expression system. These flies were tested for their ability to suppress GMRrpr-, GMRgrim-, and GMRhid-induced cell death in the eye. Eye-specific expression was activated using a GMRgal4 driver. Two independent lines for the wild type and two for the GOF (V85M) constructs were examined. Control phenotypes for *GMRrpr*, *GMRgrim*, and *GMRhid* (A). The V85M transgene completely protects against RPR- and GRIM-induced death (B), while the wild-type transgene cannot completely rescue the *GMRrpr* eye phenotype (C). In contrast, the wild-type transgene can better protect against GMRhid-induced cell death, compared to the V85M transgene.

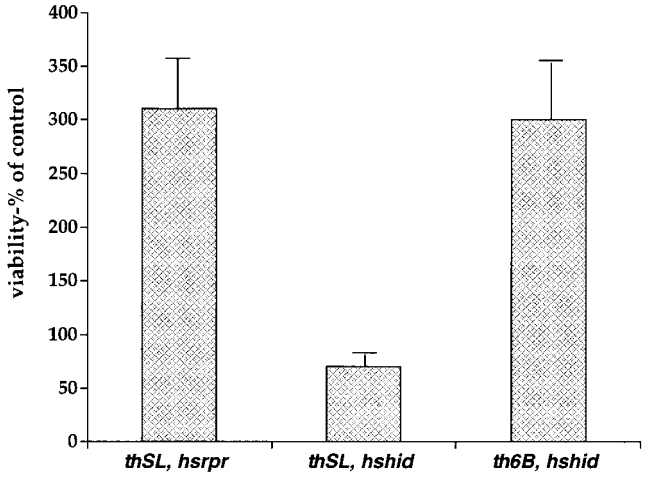


Figure 6.—The GOF alleles also protect against apoptosis in the embryo. Flies carrying a transgene that expressed RPR under the heat shock promoter (*hsrpr*) were crossed to th^{SL}/TM2 flies. Embryos were collected, heat shocked at 5–10 hr AEL and allowed to develop to adults (see materials and methods). The results are expressed as the ratio of *hsrpr* with the *th*GOF allele (*hsrpr*/+; *thGOF*/+) to those without it (*hsrpr*/+; TM2/+). *TM3-hshid* flies were crossed to $th^{SL}/D\ gl^3$ or $th^{6B}/D\ gl^3$ flies. Embryos were collected and heat shocked as described in materials and methods and were allowed to develop to adults. The results are expressed as the ratio of *hshid* flies with the *th*GOF allele (*TM3-hshid*/*thGOF*) to those without it (*TM3-hshid*/*D gl*^{3}). The results shown are the mean and standard error of two or more experiments. th^{SL} increases the viability of *hsrpr* flies more than threefold, but slightly enhances hshid-induced death, while th^{6B} protects against hshid-induced death, also by about threefold.

to suppress GMRhid-induced death, although not as strongly as the wild-type transgene (Figure 5). This corresponds to our finding that th^{SL} behaves as a weak LOF mutation in hid killing. In the context of overexpression, this slightly weakened protein can still protect against hid activity, but not as well as the wild-type protein.

The effect of th^{SL} on RPR-induced death was not confined to the eye; th^{SL} was able to protect against organismal death resulting from RPR overexpression in the embryo. Like HID-induced death in the eye, HID-induced embryonic death was not inhibited by the th^{SL} mutation, but rather, was slightly enhanced. In contrast, th^{6B} of the GOF1 class protected against HID-induced death in the embryo (Figure 6).

Loss-of-function alleles: We have found that five of the alleles we tested, th^4, th^5, th^9, $th^{109.07}$, and th^7, can be classified genetically as LOF. All of these alleles dominantly enhance the ability of RPR, GRIM, and HID to kill in the eye, approximately to the same extent as a deletion that takes out the gene (Figure 2). Sequencing has revealed that all of these alleles are due to changes in conserved residues in the BIR domains (Figure 3A). The molecular lesions found in these mutations are summarized below.

th^4: One of the canonical histidine residues in the second BIR is mutated to a tyrosine in th^4. A histidine-to-alanine mutation at this site has been shown to inhibit the ability of Op-IAP to inhibit HID-induced apoptosis in SF-21 cells (Vucic *et al.* 1998b).

th^5: There is a stop mutation near the middle of the second BIR of th^5. This mutation acts as a strong LOF mutation. GMRgrim-induced apoptosis is enhanced by th^5 mutations to a slightly greater extent than by the deletion, suggesting that a single complete BIR may have slight dominant-negative properties (Figure 2). On the basis of work done with Op-IAP in Lois Miller's lab, this mutation would be predicted to prevent binding of DIAP1 to HID (Vucic *et al.* 1998b).

th^9: This allele was isolated as a revertant of the th^{SL} allele and, thus, contains two mutations: the original V85M mutation in BIR1 seen in th^{SL} (see above), as well as a second mutation in BIR1 at Pro 55. This residue is conserved in almost all BIRs, except BIR1 of NAIP and BIR3 of X-IAP (Figure 3A).

$th^{109.07}$: This is likely to be a complete null, as there is a stop at the beginning of BIR1 at amino acid 54. It shows a slightly weaker ability to enhance RPR killing than a deletion of the gene (Figure 2). This may reflect additional enhancing activities on the deletion chromosome.

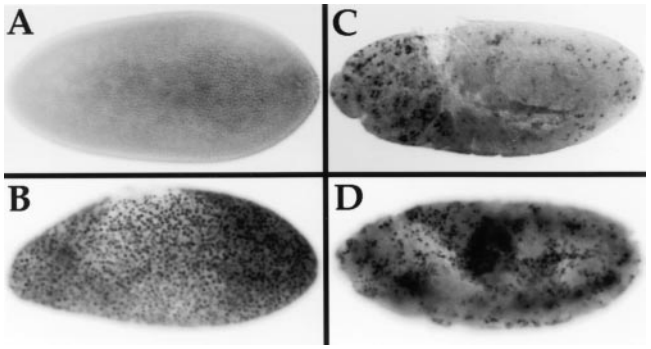


Figure 7.—Early embryonic cell death phenotype of *th* mutants, as assayed by TUNEL staining. Normally occurring embryonic cell death is not present in wild-type blastoderm stage embryos. None of the nuclei of wild-type embryos 2–4 hr old AEL stains with TUNEL (A). In contrast, in a collection of th^{7}/TM2 embryos at 5–11.5 hr AEL, 20% of the embryos show a blastoderm morphology and massive TUNEL labeling. All the labeled nuclei appear to be at the surface of the embryo (B). Embryos homozygous for a GOF1 allele ($th^{81.03}$) are also heavily labeled with TUNEL at 5–12 hr AEL (D). These embryos, however, appear to progress further in development than the LOF embryos. Wild-type embryos at the same stage have significantly lower amounts of apoptosis (C).

th^{7}: This allele appears to show dominant-negative properties, as it enhances killing by all three inducers to a greater extent than the deletion of the region (Figure 2). This allele is an in-frame deletion that removes the first half of BIR2, but leaves BIR1 and the ring intact. This suggests that the presence of BIR1 alone may have dominant-negative properties, or that the close proximity of the ring to BIR1 interferes with IAP function.

Embryonic phenotypes: All the LOF alleles described above are lethal when crossed to *Df(3L)brm11*, a deletion of the region, and when crossed to each other (data not shown). TUNEL staining was used to assess the role of apoptosis in the lethality. Embryos were collected from th^{5}, th^{7}, th^{9}, and $th^{109.07}$ stocks. In collections of embryos from 0 to 5 hr AEL, no ectopic apoptosis could be seen. At this stage, normal, developmentally regulated apoptosis had not yet begun.

In collections of embryos where the youngest embryos were ≥5 hr old, we found a striking phenotype. In a subset of embryos, the vast majority of nuclei stain with TUNEL (Figure 7B). A total of 20% of the embryos showed this phenotype in a collection from a th^{7}/*TM2* stock. These embryos appear to have been arrested prior to gastrulation, as the majority of labeled nuclei appear at the surface of the embryo. Gastrulation occurs at 3 hr AEL. We did not see any TUNEL labeling in mutant embryos until 5 hr AEL. As the onset of apoptosis is several hours after these embryos have arrested, it is possible that the ectopic apoptosis is secondary to the developmental defects. However, it is also possible that zygotic *th* is required to suppress maternally supplied *rpr*, *grim*, and *hid* activity (see discussion). Early activation of apoptosis in *th* mutant embryos has also been reported recently by Hay (Wang *et al.* 1999).

Both GOF1 allele classes are also lethal in combination with LOF *th* alleles. This indicates that an intact ring structure is essential for full activity of the TH/DIAP1 protein. However, we have found that embryos homozygous for these alleles progress further in development than the LOF mutants before widespread apoptosis begins (Figure 7D). This may reflect the distinct activity of these alleles with regard to RPR, GRIM, and HID killing. It is also possible that these alleles maintain residual activity in a developmentally required pathway independent of *rpr*, *grim*, and *hid* function.

DISCUSSION

Several mechanisms of action have been suggested for the antiapoptotic properties of the IAP family of proteins. Among these are the binding of the Drosophila IAPs to the proapoptotic proteins RPR, GRIM, and HID (Vucic *et al.* 1997a, 1998a). This interaction has been demonstrated in overexpression systems, and has been proposed to involve the homologous amino-terminal 14 amino acid sequences of the apoptosis initiators with the second BIR domain of the IAPs. The data presented here suggest that this is an oversimplification.

Another mechanism that has been proposed for IAP antiapoptotic activity is the direct binding and inhibition of caspases (reviewed in Deveraux and Reed 1999). TH/DIAP1 has been shown to bind to the Drosophila caspases drICE and DCP-1 and to inhibit their ability to induce apoptosis (Kaiser *et al.* 1998; Hawkins *et al.* 1999; Wang *et al.* 1999). Here again, this binding activity appears to rest within BIR2.

These physical interactions support a simple model of IAP action. In this model, IAPs act within viable cells to inhibit caspase function. The action of RPR, HID, and GRIM is to interfere with the ability of IAPs to inhibit caspases, thus inducing apoptosis.

On the basis of the model, the LOF mutations we have identified would be predicted to interfere with the ability of the TH/DIAP1 protein to inhibit caspase function. This is likely to be true for $th^{109.07}$, which lacks most of the protein, as well as for th^{5} and th^{4}, which affect conserved residues in BIR2. BIR2 has been shown to be sufficient to inhibit apoptosis induced by the active form of the Drosophila caspase drICE. The th^{9} mutation in BIR1 suggests that this BIR is also important for the full function in caspase inhibition. Alternatively, this change in BIR1 might have long-range effects on BIR2 structure or on protein stability.

It is interesting to note that th^{7}, which acts as a very strong LOF mutation and seems to show some dominant-negative properties, has only the BIR1 attached to the spacer and ring domains. Thus, despite the extensive homologies between the two BIR domains of the protein, a single BIR is not sufficient for TH/DIAP1 function, at least in the presence of an attached ring domain. Others have shown that BIR2 of TH/DIAP1 and Op-IAP, as well as the single BIR of survivin, are able to

inhibit apoptosis (Ambrosini *et al.* 1997; Vucic *et al.* 1998b).

Again, on the basis of the model above, the GOF mutations we have identified would be predicted to bind to caspases, but not to the inducers. The th^{SL} mutation maps to a weakly conserved residue in BIR1 and does not result in increased *th* protein levels. This suggests that BIR1 is important for RPR and GRIM binding, but not for HID binding, as HID activity is unaffected in this mutation. Even in the context of overexpression in the eyes of transgenic flies, this mutant IAP retains some specificity for RPR and GRIM killing. This implies that the simple model of BIR2 binding to the conserved NH_2-terminal sequences of RPR, GRIM, and HID is not accurate, and that other residues in the protein are differentially important for RPR and GRIM *vs.* HID binding.

The importance of regions outside of BIR2 for DIAP1 activity is supported by the analysis of the GOF1 class of mutations, th^{6B} and $th^{81.03}$. Both of these mutations suppress HID killing and would be predicted to inhibit HID binding. These mutations change conserved cysteines in the ring domain to tyrosines. This suggests that the ring is important for HID/DIAP1 interaction. However, Vucic *et al.* (1998b) have mapped the region of HID binding to DIAP1 and Op-IAP to BIR2, while the ring does not show any ability to bind to HID. In addition, these authors report that mutations in the ring, including those in conserved cysteines, had little effect on the ability of Op-IAP to protect against HID killing (Vucic *et al.* 1998b). These data, together with the finding that both GOF1 mutations are cysteine-to-tyrosine changes, suggest that these mutations might have a novel ability to interfere with binding of HID to BIR2. In addition, the observation that the GOF1 mutations slightly enhance RPR and GRIM killing suggests that these mutants are less potent inhibitors of caspases. This might result from weaker binding to caspases or from proteins that are slightly less stable. This second attribute would be predicted to enhance killing by any inducer that binds the IAP, but not to have an effect on HID, which is unable to bind.

In conclusion, our data support a model where RPR, GRIM, and HID interact with TH/DIAP1 to induce apoptosis. We have found that mutations that affect killing by RPR and GRIM or by HID can be isolated, indicating that these inducers interact with TH/DIAP1 in different ways. The GOF mutations that we have identified also provide us with useful tools to examine the roles of IAPs, *rpr*, *grim*, and *hid* during Drosophila development. The other Drosophila IAP homolog, DIAP2, has been shown to selectively inhibit RPR- and HID-induced but not GRIM-induced death (Wing *et al.* 1998).

Finally, we have shown that the TH/DIAP1 protein is required very early in Drosophila development. This has also been reported recently by others (Wang *et al.* 1999). In LOF *th* alleles, we see a developmental arrest at the blastoderm stage and, subsequently, a synchronous apoptosis of all the nuclei. Earlier reports that homozygous *th* embryos showed no ectopic apoptosis probably reflects the very early stage at which this apoptosis occurs.

At this time, we cannot distinguish a direct requirement for *th* to block apoptosis or a requirement for *th* in another developmental process. This developmental defect could then result in secondary apoptosis. The latter possibility is reasonable, as many failures in development result in ectopic apoptosis (Abrams *et al.* 1993). A BIR containing protein from *Caenorhabditis elegans* is required for cytokinesis in embryos (Fraser *et al.* 1999). However, it is also possible that developmental arrest occurs as a result of the initiation of apoptosis, which is manifest only as DNA damage several hours later.

Does this early requirement for *th* reflect a need to inhibit apoptosis induced by *rpr*, *grim*, and *hid*? Double mutants of *th* and *Df(3L)H99*, the deletion that removes *rpr*, *grim*, and *hid*, show a phenotype similar to *th* alone (Wang *et al.* 1999). This indicates that TH/DIAP1 is not required to suppress zygotic RPR, GRIM, and HID activity. However, *hid* and *rpr* mRNA can be seen in a subset of cells in the blastoderm embryo, as judged by *in situ* analysis (Nordstrom *et al.* 1996; Nassif *et al.* 1998; and K. White, unpublished observations). This may indicate that these gene products are supplied maternally. TH/DIAP1 may be required to suppress maternally supplied RPR, GRIM, or HID. We found that allelic differences in the stage at which apoptosis begins in the *th* mutants parallel the general ability of the alleles to inhibit apoptosis induced by RPR, HID, and GRIM. The strong LOF alleles arrest at the blastoderm stage, the GOF1 alleles arrest much later and the GOF2 allele is completely viable.

Many questions remain about how RPR, GRIM, and HID initiate apoptosis and how TH/DIAP1 protects cells. Our analysis has suggested that current models may be oversimplified. While BIR2 is certainly important for TH/DIAP1 antiapoptotic activity, BIR1 and the ring domain play a role in specific interactions with RPR and HID. In addition, our work indicates that overexpression of TH/DIAP1 does not completely reflect the activity of the gene *in vivo*. We expect that further investigation of these mutants should provide interesting insight into the functional mechanisms and regulation of *th*.

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