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 (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 (Clém 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; Dukett 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 (Clém 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 Deveraux and Reed 1999). In particular, TH/DIAP1 binds to a Drosophila caspase, drICE, and inhibits apoptosis induced by activated drICE and...
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 pro-
cedures, and the cultures were maintained at 25°C. The follow-
ing alleles of thread were used: Df(3L)brm1, th1, th2, th5, th7,
th8 (Brizuela et al. 1994), th12, and th1287 (Moo re et al. 1998).
th4 and th9 were generated by chemical mutagenesis (see below).

Mutagenesis for modifiers of GMRrpr: To identify suppressors
or enhancers of the GMRrpr eye phenotype, GMRrpr81 (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 CO2XGMrpr;
TM2/KRS (Kurada and White 1998). Those that showed
consistent suppression were mapped by segregation and
recombination with the rucua mapping chromosome (Ash-
burner 1989b).

Reversion of th4c: Homozygous th5 males were mutagenized
with EMS and crossed to GMRrpr81/GMRrpr81; GMRrpr97/ GMRrpr97 females. The progeny were screened for loss of the
suppressing capability of th4c 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 (th4c) or from dead embryos
homozygous for lethal alleles (th1, th2, th5, th7, th9, th12, and
th1287). 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 em-
bryos were picked under a microscope and processed for DNA
isolation. DNA extraction from 200 adults or 400 embryos was
performed using QIAamp blood kit (Qiagen, Chatsworth, CA)
according to the manufacturer’s instructions. The th open
reading frame was then amplified by polymerase chain re-
once (PCR) under standard conditions using the Expand Long
Template PCR system (Boehringer Mannheim, Indianapolis).
The following oligonucleotide primers were used: 5’-CGACT
TCAGAGAGAAAGGCAGA-3’ and 5’-CGCTTATATTTA
CACATTCGCCACC-3’. DNA sequences were obtained using
the Thermo Sequenase radiolabeled terminator cycle se-
quencing system (Amersham, Arlington Heights, IL). The se-
queencing 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/+/TM 2 flies, and embryos were
collected on molasses plates, aged for 3–8 hr, and heat shocked
for 1 hr at 37°C. The embryos were transferred to standard fly
food and scored for adult viability. TH5shid (fly8ase 1994)
flies were crossed to th/+ / D [G] or th+/ D [G]. At 8–10 hr post-
collection, embryos were heat shocked for 20 min at 37°C. The
embryos were transferred to standard fly food and scored for
adult viability.

Anti-th/TH/DIAP1 antibody production and immunostaining:
The TH/DIAP1 polyclonal antibody was raised in rabbits (Po-
conos 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 Histagged 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% paraformalde-
hyde, washed in BSS (Ashburner 1989b), preincubated in
BSS with 5% goat serum and 0.3% Triton X-100 (BSN), and
incubated overnight at 4°C in preabsorbed anti-TH/DIAP1 anti-
body diluted 1:1000 in BSS. Preabsorbed Texas red-conju-
 gated 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 TCSNT4D confocal microscope (Leica, Deer-
field, 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 pU-
AsthV85M and pUASthw were generated in a two-part clone:
3’ fragments of the genes were obtained by Sall digestion of
mRNA of hsp70PLV1 ’EpD-IapIv85M and hsp70PLV1 ’EpD-
Iap1, which were kindly provided by Dr. Lois Miller (University
of Georgia, Athens, GA). The Sall fragments were purified
and redigested with MfeI, and the Sall-MfeI 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 oligonucleo-
tide primers were used: 5’-CGACTTTCAGAGAGAAAGGCAGA
CAGA-3’ and 5’-TAATGCTTCTTCGGCATTGATCGGACA-
TAATGCTTCTTCGGCATTGATCGGA-3’. The PCR products were gel purified and cut with EcoRI and
MfeI, followed by further gel purification. This fragment
contains bases 357–616 of the cDNA. The vector GAL UAS
(pUASt) was cut with EcoRI and Xhol 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 protease 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°C in the equilibration buffer provided in the ApopTag kit from Intergen. The embryos were incubated overnight at 37°C 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°C 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°C with 1:2000 preabsorbed antidigoxigenin 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 MgCl₂, 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 GM Rrpr. 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 Nooil 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](image-url)

**Figure 1.**—A gain-of-function allele of th suppresses GMRrpr- and GM Rgrim-induced cell death. The rough-eye phenotype of flies expressing two copies of a GMRrpr transgene (A) or one copy of a GM Rgrim 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, th⁹, completely reverts its ability to suppress GMRrpr- and GM Rgrim-induced cell death. This allele dominantly enhances the rough-eye phenotypes of GMRrpr, GM Rgrim, and GMRhid (C, F, and I).
we tested the proximity of Su(GMRrpr)SL to th by further recombination mapping. No recombinants were obtained between Su(GMRrpr)SL and th 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) 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 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 is a mutation in the th gene (see below). To eliminate the concern that th 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 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, is a GOF mutation in th, which is reverted by a second mutation in the same gene, the th 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 would suppress apoptosis induced by GRIM and HID in the eye. As expected, apoptosis induced by GRIM was strongly suppressed by th (Figure 1E). To our surprise, th 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 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 (third column), th (fourth column), th (fifth column), and th (sixth column). Enhancement of the GMRrpr and GMRgrim phenotype is also observed in th (seventh column) and th (last column) heterozygotes. Surprisingly, these two alleles suppress the GMRhid eye phenotype and, therefore, are defined as gain-of-function alleles (GOF1).

**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 XIAP (Liston et al. 1996). Single-amino-acid changes in the first BIR for th, th, and th alleles are marked in red, while in green are the point mutations in the second BIR for th and th and the end of the in-frame deletion of th. The th 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 and th, 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 GM Rpr, GM Grim, and GM R hid 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.
Alleles Distinguish RPR and HID Killing

th\textsuperscript{109.07} \quad th\textsuperscript{0} \quad th\textsuperscript{SL} \quad th\textsuperscript{5} \quad th\textsuperscript{4} \\
W54 \quad P52L \quad V8S \quad D272 \quad W273 \quad H283Y

- = STOP codon \\
\star = end of th\textsuperscript{7} deletion

RING DOMAIN

\textbf{ Classes } \quad \textbf{ rpr and grim killing } \quad \textbf{ hid killing } \quad \textbf{ th alleles } \\
\textbf{ LOF } \quad \text{enhanced} \quad \text{enhanced} \quad th\textsuperscript{4}, th\textsuperscript{5}, th\textsuperscript{7}, th\textsuperscript{109.07} \\
\textbf{ GOF1 } \quad \text{enhanced} \quad \text{suppressed} \quad th\textsuperscript{6B}, th\textsuperscript{81.03} \\
\textbf{ GOF2 } \quad \text{very suppressed} \quad \text{none or enhanced} \quad th\textsuperscript{SL}
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\(^{6B}\), 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\(^{6B}\) 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\(^{6B}\): We considered whether the ability of th\(^{6B}\) 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\(^{6B}\) eye discs stained with a polyclonal anti-TH/DIAP1 antibody showed that TH/DIAP1 levels and distribution are not detectably affected by the th\(^{6B}\) 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\(^{6B}\) offers against RPR- and GRIM-induced death.

Sequencing of the open reading frame of th\(^{6B}\) 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\(^{6B}\) phenotype, we generated transgenic flies that expressed wild-type TH/DIAP1 or the V85M th\(^{6B}\) 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\(^{6B}\) 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
Alleles Distinguish RPR and Hid Killing

**Figure 5.**—Expression of thSL 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. Cells expressing a transgene that expressed RPR under the heat shock promoter (hsrpr) were crossed to thSL/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 to hslrpr (hsrpr/++; thGOF/+-) to those without it (hsrpr/++; TM2/+-). TM3-hshid flies were crossed to thSL/Dgl3. TM3-hshid flies were crossed to thSL/Dgl3. The results shown are the mean ± standard error of two or more experiments. thSL increases the viability of hslrpr flies more than threefold, but slightly enhances hshid-induced death, while th88 protects against hshid-induced death, also by about threefold.

Loss-of-function alleles: We have found that five of the alleles tested, th\(^{4}\), th\(^{5}\), th\(^{6}\), 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\(^{6}\):** This allele was isolated as a revertant of the th\(^{5}\) allele and, thus, contains two mutations: the original V85M mutation in BIR1 seen in th\(^{5}\) (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.
th5: 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 1999). TH/DIAP1 has been shown to bind to the Drochapsophila caspases drICE and DCP-1 and to inhibit their 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 th109.07, which lacks most of the protein, as well as for th5 and th4, 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 th5 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 th5, 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 OPIAP, as well as the single BIR of survivin, are able to

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.
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 $\text{th}^{12}$ mutation maps to a weakly conserved residue in BIR1 and does not result in increased $\text{th}$ 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, $\text{th}^{12}$ and $\text{th}^{11,5}$. 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 $\text{th}$ alleles, we see a developmental arrest at the blastoderm stage and, subsequently, a synchronous apoptosis of all the nuclei. Earlier reports that homozygous $\text{th}$ 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 $\text{th}$ to block apoptosis or a requirement for $\text{th}$ in another developmental process. This developmental defect could then result in secondary apoptosis. The latter possibility is reasonable, as many failures in developmental 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 $\text{th}$ reflect a need to inhibit apoptosis induced by rpr, grim, and hid? Double mutants of $\text{th}$ and $\text{Df}(3L)H99$, the deletion that removes rpr, grim, and hid, show a phenotype similar to $\text{th}$ 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 $\text{th}$ 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 $\text{th}$.

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LITERATURE CITED


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