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HAC-1, a *Drosophila* Homolog of APAF-1 and CED-4, Functions in Developmental and Radiation-Induced Apoptosis

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Summary

We have identified a *Drosophila* homolog of *Apaf-1* and *ced-4*, termed *hac-1*. Like mammalian APAF-1, HAC-1 can activate caspases in a dATP-dependent manner in vitro. During embryonic development, *hac-1* is prominently expressed in regions where cells undergo natural death. Significantly, *hac-1* transcription is also rapidly induced upon ionizing irradiation, similar to the proapoptotic gene *reaper*. Loss of *hac-1* function causes reduced cell death, and reducing the dosage of *hac-1* suppresses ectopic cell killing upon expression of the *dcp-1* procaspase in the retina but has little effect on *reaper*, *hid*, and *grim*-mediated killing. Our data indicate that caspase activation and apoptosis in *Drosophila* are independently controlled by at least two distinct regulatory pathways that converge at the level of caspase activation.

Introduction

Apoptosis, a morphologically distinct form of programmed cell death, is essential for normal development and tissue homeostasis in both invertebrates and vertebrates (Thompson, 1995; Jacobson et al., 1997; Raff, 1998; Vaux and Korsmeyer, 1999). During the last few years, rapid progress has been made in identifying some of the molecules that are responsible for the regulation and execution of apoptosis. Initially, genetic studies in the nematode *Caenorhabditis elegans* defined a core program for programmed cell death (Ellis and Horvitz, 1986; Ellis et al., 1991; Yuan et al., 1993; Hengartner and Horvitz, 1994b; Hengartner, 1996; Horvitz, 1999). In particular, this work revealed the importance of an unusual class of cysteine proteases, termed caspases (for cysteine aspartic acid–specific protease) for cell death (Yuan et al., 1993). Subsequently, a large number of mammalian caspases have been isolated and shown to play an important role in apoptosis (Takahashi and Earnshaw, 1996; Nicholson and Thornberry, 1997; Salvesen and Dixit, 1997; Thornberry et al., 1997). Caspases are synthesized as inactive zymogens, which are widely expressed in both dying and live cells. The activation of caspases during apoptosis involves several internal cleavages in the proenzyme, which lead to the removal of an inhibitory N-terminal prodomain and the generation of a large (p20) and small (p10) subunit. Caspases can be activated through cleavage by active "initiator" caspases in a caspase cascade (Li et al., 1997), and by autoproteolysis following aggregation of two or more zymogen molecules (MacCorkle et al., 1998; Muzio et al., 1998; Yang et al., 1998). Upon activation of "executioner" caspases, a wide variety of specific intracellular proteins are cleaved in different cellular compartments, and it is thought that their breakdown ultimately leads to the characteristic morphological changes of apoptosis (Nicholson and Thornberry, 1997). The activation of caspases appears to be tightly controlled by both positive and negative regulators. On the one hand, members of the IAP (inhibitor of apoptosis protein) family can inhibit caspases and apoptosis in a variety of insect and vertebrate systems (Uren et al., 1998; Deveraux and Reed, 1999). On the other hand, caspase activation is stimulated by a family of proteins that include *C. elegans* CED-4 and mammalian apoptosis protease-activating factor-1, APAF-1 (Yuan and Horvitz, 1992; Zou et al., 1997). The *ced-4* gene is required for programmed cell death in *C. elegans*, and mice deficient for APAF-1 have reduced programmed cell death (Ellis and Horvitz, 1986; Cecconi et al., 1998; Yoshida et al., 1998). Furthermore, *ced-4* acts genetically upstream of *ced-3*, and CED-4 can physically interact with pro-CED-3 and certain mammalian procaspases (Shaham and Horvitz, 1996; Chinnaiyan et al., 1997; Seshagiri and Miller, 1997; Wu et al., 1997). APAF-1, which shares significant amino acid homology with CED-4, can bind to the prodomain of procaspase-9 and activate it in the presence of cytochrome c and dATP in a cell-free system (Li et al., 1997; Zou et al., 1997). These observations suggest that CED-4/APAF-1-like proteins have an important and direct function in the activation of caspases that has been conserved from nematodes to mammals.

The cell-killing activity of *ced-4* is negatively regulated by *ced-9*, which encodes a protein homologous to the BCL-2 family of mammalian cell death regulators (Hengartner et al., 1992; Hengartner and Horvitz, 1994a; White, 1996; Reed, 1997b). Likewise, there is evidence that BCL-2-like proteins can control APAF-1 activity, but the precise mechanism has remained somewhat controversial. Because APAF-1 requires cytochrome c as a cofactor for caspase activation in vitro, it has been proposed that the release of cytochrome c from mitochondria into the cytosol is a critical regulatory step and that this release is blocked by BCL-2-like proteins (Kim et al., 1997; Kluck et al., 1997; Li et al., 1997; Reed, 1997a, 1997b; Yang et al., 1997; Green and Reed, 1998). However, the conditions under which the release of cytochrome c is used to activate apoptosis in situ remain to be determined.

A major gap in our current understanding of apoptosis is how specific death-inducing signals turn on the death program. Not surprisingly, the control of programmed cell death is remarkably complex. Apoptosis can be induced by a wide variety of stimuli that may originate

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either from within cells or from their extracellular environment (Raff, 1992; Raff et al., 1993; Steller, 1995; Nagata, 1997; Pettmann and Henderson, 1998). Molecular genetic studies of programmed cell death in *Drosophila* offer unique opportunities for expanding our knowledge in this area. In *Drosophila*, as in vertebrates, the regulation of apoptosis is highly plastic and involves a wide variety of intracellular and extracellular signals (Steller and Grether, 1994; McCall and Steller, 1997; Bergmann et al., 1998b). The induction of apoptosis in *Drosophila* requires the activities of three closely linked genes, *reaper*, *grim*, and *head involution defective (hid)*, which kill by activating a caspase pathway (White et al., 1994, 1996; Grether et al., 1995; Chen et al., 1996). Several *Drosophila* caspases have been identified. Two of them, DCP-1 and drICE, are very similar in structure and biochemical specificities (Fraser and Evan, 1997; Song et al., 1997; Chen et al., 1998; Dorstyn et al., 1999), and *reaper* and *grim* can genetically interact with *dcp-1* and *drice* in vivo (Song et al., submitted). Significantly, *reaper*, *hid*, and *grim* are transcriptionally regulated by a variety of death-inducing stimuli. In particular, the expression of *reaper* is controlled by a range of different signals, including the steroid hormone ecdysone, segmentation and patterning genes, and DNA-damaging agents, and *hid* expression is repressed by Ras signaling (White et al., 1994; Nordstrom et al., 1996; Jiang et al., 1997; Robinow et al., 1997; Kurada and White, 1998; Draizen et al., 1999; Lamblin and Steller, submitted). Therefore, it appears that these genes act as integrators for relaying different death-inducing signals to the core death program.

The products of *reaper*, *grim*, and *hid* share a short stretch of conserved amino acids at their N terminus but otherwise encode novel proteins without any significant homology to other known genes. However, there are good reasons to believe that homologous molecules function in the control of apoptosis in vertebrates. First, the expression of Reaper, Grim, or Hid can induce apoptosis in mammalian cells, and recombinant Reaper can activate caspase activation and apoptosis-like events in a *Xenopus* cell-free system (Evans et al., 1997; Claveria et al., 1998; McCarthy and Dixit, 1998; Haining et al., 1999). Second, Reaper, Grim, and Hid interact physically and genetically with IAPs, a highly conserved family of antiapoptotic proteins (Hay et al., 1995; Vucic et al., 1997a, 1997b, 1998; Wang et al., 1999; Goyal et al., submitted). Finally, the proapoptotic activity of Hid is inactivated upon phosphorylation by MAP-kinase (Bergmann et al., 1998a), so Hid interacts directly with at least two highly conserved protein families. Collectively, these observations indicate that molecules homologous to Reaper, Grim, and Hid may be used to regulate apoptosis in vertebrates. However, the apparent absence of *Drosophila* homologs of CED-4/APAF-1 and BCL-2-like molecules has previously raised concerns that the regulation of apoptosis in *Drosophila* may be substantially different from that in *C. elegans* and mammals (see, for example, Meier and Evan, 1998; Rich et al., 1999).

We now report the identification and characterization of a *Drosophila* homolog of CED-4 and APAF-1, *hac-1* (for homolog of *Apaf-1* and *ced-4*). We show that *hac-1* is structurally and functionally very similar to *Apaf-1* and that it is required for normal cell death during embryonic development in *Drosophila*. Reducing *hac-1* activity in heterozygous animals suppresses cell killing by the *Drosophila* caspase *dcp-1* but has little effect on *reaper*, *hid*, and *grim*–induced ectopic apoptosis. Finally, we find that *hac-1* expression is regulated both in development and also by ionizing radiation. Our results indicate that death-inducing stimuli promote apoptosis in *Drosophila* by simultaneously activating two distinct regulatory pathways controlling caspase activation.

Results

Identification of a *Drosophila* Homolog of APAF-1/CED-4

We identified *hac-1* as a P1 sequence entry (GenBank accession number AC004335, located at cytological position 53F [Kimmerly et al., 1996]) of the Berkeley *Drosophila* Genome Project (BDGP) using a novel database search scheme. This entry encodes a putative P loop nucleotide-binding motif (corresponding to CED-4 $_{155}$LFLHGRAGSGKSVIA) as well as a motif that is shared by CED-4, APAF-1, and a family of plant disease-resistance proteins (corresponding to CED-4 $_{267}$RCLVTTR DVEISN, Figure 1B) (van der Biezen and Jones, 1998). Computational analysis using the GENESCAN (Berge and Karlin, 1997) program suggested that this part of genomic sequence (about 600 bp) is likely to be an exon of a larger gene. Two EST entries (from cDNA clone GH17715 and GH23583, Berkeley *Drosophila* Genome Project/HHMI EST Project, unpublished) are derived from the 3′ segment of this predicted gene. We also identified a P element insertion (Spradling et al., 1995), l(2)k11502, at the 5′ end of this gene, immediately upstream of the presumptive TATA box (Figure 1C). This P element insertion disrupts *hac-1* function and was subsequently used for phenotypic analyses (see below).

A combination of RT-PCR, genomic, and cDNA sequencing was used to determine the nucleotide sequence of the corresponding 5.5 kb mRNA. The results of this analysis predict an open reading frame, composed of ten exons, coding for a 1440–amino acid protein. The N terminus of the deduced protein shares significant homology with APAF-1 (20% identity and 41% similarity for amino acids 65–486) and the plant disease-resistant proteins (e.g., 19% identity, 34% similarity for amino acids 147–462 in comparison to RPR1, GenBank accession number AB019186). Like APAF-1, the *Drosophila* protein also has nine WD repeat elements following the CED-4 homology region (Figure 1A). We termed this protein Homolog of APAF-1/CED-4 (HAC-1). The sequence homology between HAC-1 and CED-4 is more limited and mainly restricted to the two nucleotide-binding motifs and the ARC domain (Figure 1B) (van der Biezen and Jones, 1998). Nevertheless, the biochemical and functional data presented below indicates that this homology is significant.

HAC-1 Can Induce Caspase Activation In Vitro

In order to investigate whether HAC-1 shares biochemical properties with APAF-1, we investigated the ability of the protein to stimulate *Drosophila* caspase activation in vitro. HAC-1 was expressed in 293 T cells by transient

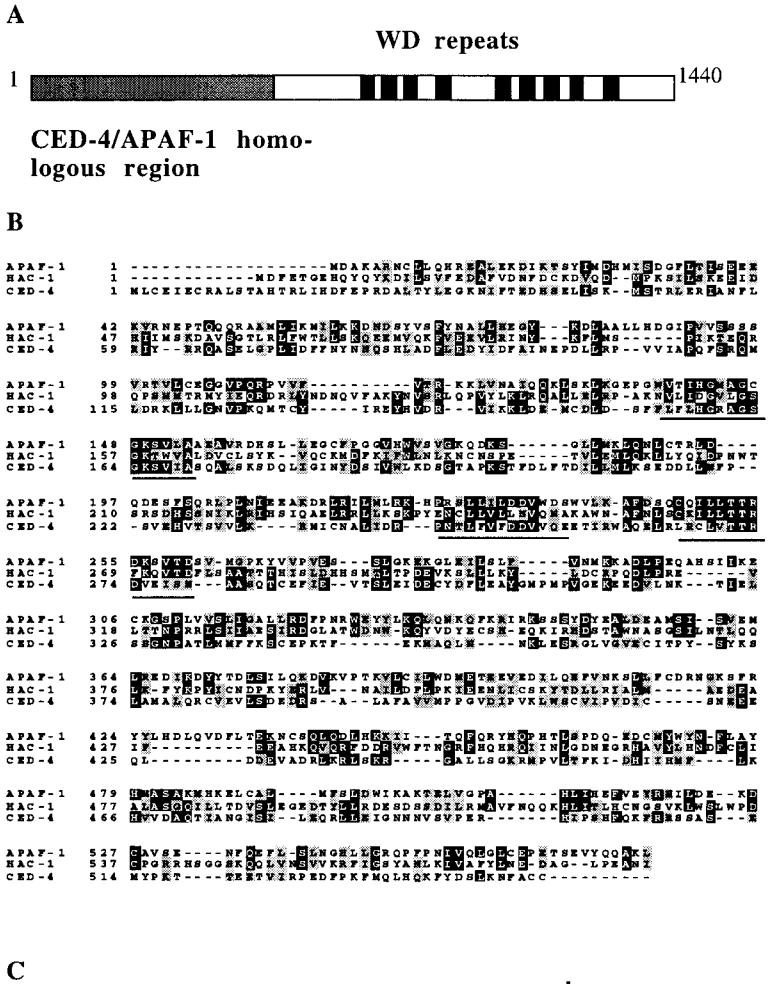

Figure 1. *hac-1* Encodes a Homolog of *ced-4/Apaf-1*

(A) is a schematic representation showing the domain structure of the HAC-1 protein. (B) shows the sequence alignment of the N termini of CED-4, APAF-1, and HAC-1. Identical and conserved residues are blocked as black or shadow, respectively. Of all the protein sequences in the database, HAC-1 is most similar to human APAF-1, and HAC-1 is less similar to CED-4, where the similarity is mainly limited to the three kinase motifs of the nucleotide-binding domain (B, underlined). In (C), the location of the P insertion l(2)k11502 relative to the genomic sequence is indicated by the arrow. Only one sequence motif (underlined) consistent with the TATA box consensus (TATAA/TA) is present between the P insertion site and the first ATG of the ORF. The sequence corresponding to the 5′ end of the longest RT-PCR product is shown in bold.

transfection, and the cytoplasmic fraction of these cells was tested for its ability to activate ^{35}S-labeled DCP-1 and drICE zymogens (Figure 2) (Fraser and Evan, 1997; Song et al., 1997). While extracts from control cells had no detectable caspase conversion activity (Figure 2, lane 2), extracts from cells expressing *hac-1* were able to induce processing of both drICE and DCP-1 proenzymes (Figure 2, lanes 3 and 4, and data not shown). Significantly, as previously observed for APAF-1 (Liu et al., 1996; Zou et al., 1997), this conversion was dependent on the presence of dATP (Figure 2, lanes 5 and 6). Therefore, HAC-1 appears to be similar to APAF-1 in its activity to stimulate caspase processing.

Distribution of *hac-1* during Embryogenesis

It is thought that the basic cell death program is constitutively expressed (Raff et al., 1993; Weil et al., 1996), and many cell death genes, including *apaf-1*, appear to be widely expressed (Zou et al., 1997). Figure 3 shows that *hac-1* mRNA is broadly distributed in preblastoderm embryos before the major onset of zygotic transcription, apparently due to maternal contribution (Figure 3A). However, by stage 7, the highest levels of *hac-1* mRNA were detected in the ventral neurogenic region, and around several invagination furrows (Figure 3B). Interestingly, these are regions in which prominent apoptosis occurs during subsequent development (Abrams et al., 1993; Nassif et al., 1998). Later, the highest levels of *hac-1* mRNA were found in the ectoderm and mesoderm of the procephalic region (Figure 3C). The expression of *hac-1* in the developing head overlaps significantly with that of the proapoptotic gene *hid* (Figures 3E and 3F; Grether et al., 1995). Again, abundant apoptosis is subsequently observed in this region. The mRNA distribution of *hac-1* is closely mirrored by the pattern of β-gal expression from the P element insertion l(2)k11502,

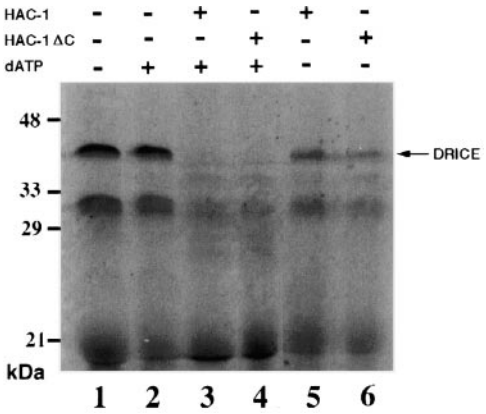

Figure 2. HAC-1 Induces *Drosophila* Caspase Activation In Vitro

The full-length HAC-1 protein and a C-terminal truncated version of HAC-1, derived from 293 T cell cytosol, activated DCP-1 and drICE in a dATP-dependent manner. 293 T cells were transiently transfected with expression vectors to overexpress the full-length or a C-terminal truncated HAC-1. The cytosolic fractions from these transfected cells activated DCP-1 and drICE in the presence of dATP. Lane 1, 1 μl of ^{35}S-labeled drICE in 10 μl of buffer A. Lane 2, 1 μl of ^{35}S-labeled drICE mixed with 10 μl of cytosol of untransfected 293 T cells, in the presence of 1 mM dATP, as control. Lane 3, 1 μl of ^{35}S-labeled drICE mixed with 10 μl of cytosol of 293 T cells transfected with pRK5-*hac-1* in the presence of 1 mM dATP. Lane 4, 1 μl of ^{35}S-labeled drICE mixed with 10 μl of cytosol of 293 T cells transfected with pRK5-*hac-1*Δ*C* in the presence of 1 mM dATP. Lane 5, the same as lane 3 except without dATP added. Lane 6, the same as lane 4 except without dATP added. DCP-1 results are essentially identical (data not shown).

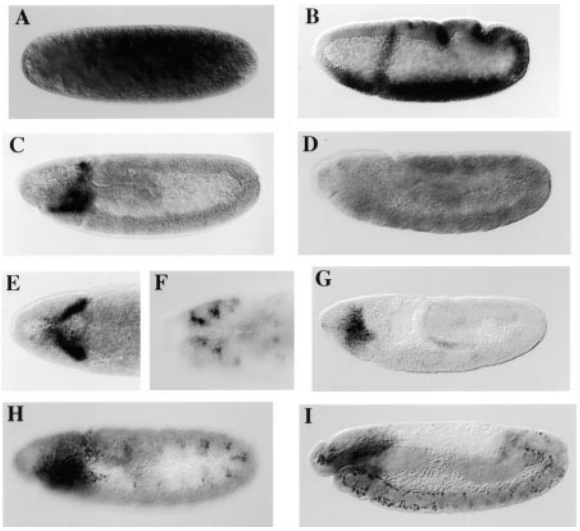

Figure 3. The Transcription of *hac-1* mRNA Is Regulated during Development

(A)–(C) show the distribution of *hac-1* mRNA in wild-type embryos ([A] stage 4; [B] stage 7; [C] stages 10–11). The mRNA signal in the procephalic region (C) is absent in embryos homozygous for l(2)k11502 (D). (E) and (F) compare the distribution of *hac-1* (E) and *hid* (F) mRNA in the procephalic region of stage 11 embryos. The expression patterns partially overlap. However, *hid* expression is restricted to clusters of cells in the ectoderm; *hac-1* is expressed in the ectoderm as well as the mesoderm, which is a source of macrophages. (G)–(I) show anti-β-gal staining of l(2)k11502/CyO embryos; the β-gal expression pattern largely mimics the mRNA distribution ([G] stage 10; [H] stage 11; [I] stage 12/1). Although a low level of β-gal immunoreactivity is present in most, if not all cells, cells in the procephalic region (G and H), and later in the head and macrophages (I) express much higher levels.

which carries a *lacZ* reporter gene. The l(2)k11502 P element transposon is inserted near the transcriptional start site of *hac-1*, 81 bp upstream of the presumptive TATA box (Figure 1C). Although a basal level of *lacZ* expression could be detected in essentially all cells, high levels of expression were again seen in the procephalic region (Figure 3G). At later stages, both *lacZ* reporter expression and *hac-1* mRNA were detected in a segmentally repeated pattern. Finally, β-gal immunoreactivity was seen in macrophages starting from late stage 11 to the end of embryonic development. These observations indicate that *hac-1* is abundantly expressed in many but not all dying cells and that its expression is transcriptionally regulated during development.

hac-1 Is Required for Normal Cell Death during Embryonic Development

The insertion of l(2)k11502 appears to disrupt the expression of *hac-1* since embryos homozygous for the insertion displayed no head-specific in situ signals above the basal levels derived from maternally contributed mRNA (Figure 3D). In order to examine whether zygotic *hac-1* function is required for normal cell death, embryos heterozygous or homozygous for the l(2)k11502 insertion were labeled with TUNEL to visualize apoptotic cells. In wild-type and heterozygous embryos (Figure 4A), a large number of TUNEL-positive cells were consistently observed in the head region of stage 12 embryos. In contrast, significantly fewer TUNEL-positive cells were present in the head region of embryos homozygous for l(2)k11502 (Figures 4B and 4C). This phenotype was highly penetrant, but some variation among homozygous mutant embryos was seen (Figures 4B and 4C), possibly due to differences in the amount or perdurance of maternal *hac-1*. Therefore, although many cells can still die in homozygous mutant embryos, zygotic *hac-1* product is required for the normal pattern of apoptosis.

It is unlikely that elimination of zygotic *hac-1* function in l(2)k11502 homozygous embryos represents the true null phenotype, since *hac-1* mRNA is maternally contributed. To inactivate both zygotic and maternal *hac-1* mRNA, we used the RNA interference assay (RNAi). Previous work in *C. elegans* and, more recently, in *Drosophila* has demonstrated that RNAi can be an effective tool for studying gene function (Fire et al., 1998; Kennerdell and Carthew, 1998; Misquitta and Paterson, 1999). Double-stranded *hac-1* RNA corresponding to either the first 723 amino acids, or amino acids 134–332, encompassing the most closely conserved region between APAF-1 and CED-4, was made and injected into syncytial wild-type embryos. The patterns of cell death in these embryos were compared to uninjected and *lacZ* RNAi control embryos by TUNEL and acridine orange staining (Abrams et al., 1993; White et al., 1994). When injected into the posterior of the embryo, both *hac-1* dsRNAs significantly reduced the number of apoptotic cells in the injected part of the embryo (Figures 4D and

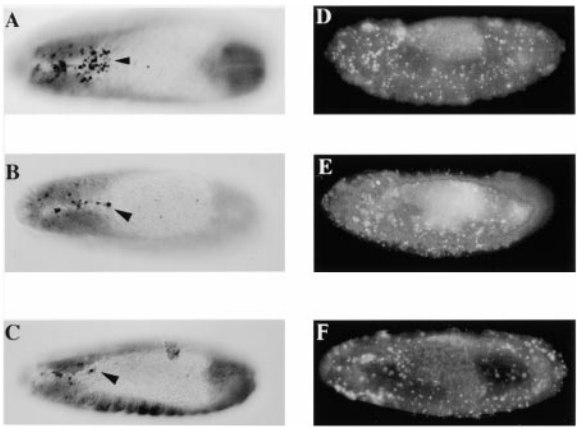

Figure 4. Apoptosis Is Reduced in *hac-1* Mutant Embryos

(A)–(C) depict TUNEL labeling of apoptotic cells in wild-type (A, stage 12/1) and l(2)k11502 mutant (B and C, stage 12/1) embryos. Apoptotic cells first appear in the head as well as the germband at late stage 10 to early stage 11 (White et al., 1994; Franc et al., 1999). The apoptotic cells in the procephalic region appear to be quickly phagocytosed by hemocytes/macrophages migrating away from the head mesoderm, as most of the TUNEL labeling is inside phagosomes within the macrophages (arrowheads) of both WT (A) and l(2)k11502 (B and C) embryos. However, fewer TUNEL-positive cells are seen in the head region of l(2)k11502 mutant embryos (two representative mutant embryos are shown in [B] and [C]). (D)–(F) depict acridine orange staining of stage 12 wild-type embryos. Injection of ds *hac-1* RNA, corresponding to Arg-143 to Arg-332, posteriorly, significantly reduces the number of acridine orange–positive cells in the posterior third of the embryo (E) when compared to uninjected embryos (D). No reduction in acridine orange staining is seen in ds *lacZ* RNA posteriorly injected control embryos (F). Ten to twenty percent of ds *hac-1* RNA injected embryos showed a reduction of acridine orange staining to the extent seen in (E).

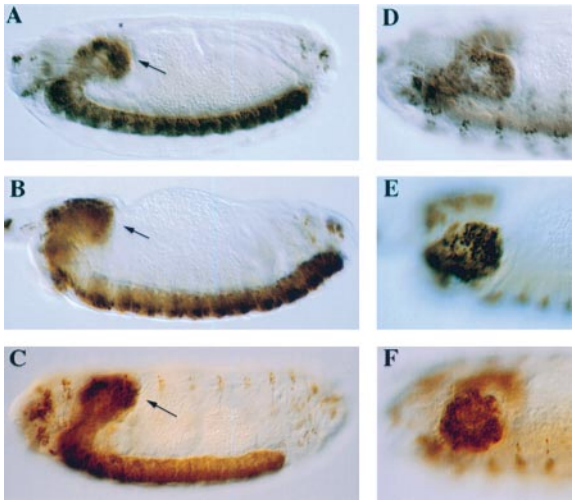

Figure 5. Extra Neurons Are Present in *hac-1* Mutant Embryos

Elimination of zygotic *hac-1* function leads to increased size and cell number in the embryonic nervous system. An anti-ELAV antibody was used to stain neurons of wild-type (A and D), *hid* mutant (B and E), and l(2)k11502 (C and F) embryos. (A)–(C) are sagittal views to show the involution of the head; note the shape of the head segments (arrows) as well as the space between the head and the epidermis (A, asterisk), which is missing in *hid* and l(2)k11502 mutants. (D)–(F) are focused on the brain to demonstrate enlarged size and extra ELAV-positive cells in the *hid* and l(2)k11502 mutant embryos. The focal plane that has the greatest number of neurons was chosen in wild-type as well as mutant embryos.

4E). Injecting the anterior of the embryo yielded similar results (data not shown), with the reduction of cell death restricted to the injected half of the embryo. Similar localized effects in RNAi experiments have been previously reported for other genes, such as *frizzled* and *frizzled-2* (Kennerdell and Carthew, 1998). In contrast, injecting a double-stranded *lacZ* RNA did not at all reduce the amount of apoptosis (Figure 4F) but abolished the expression of β-galactosidase (data not shown). Since RNAi produced a more severe reduction of apoptosis than elimination of zygotic *hac-1* function, it appears that maternal *hac-1* product contributes to the induction of cell death during normal embryonic development.

At least some of the "undead" cells in the l(2)k11502 mutant embryos appear to develop into extra neurons. When stained with the anti-Elav antibody, which recognizes all differentiated neurons, brains of l(2)k11502 homozygous mutant embryos contained more Elav-positive cells than wild type (Figure 5). This phenotype is very similar to that seen in null mutants of the proapoptotic gene *head involution defective (hid)* (Grether et al., 1995). In addition, like loss of *hid* function, many *hac-1* mutant embryos showed defects in head involution (Figure 5). These findings suggest that *hac-1*, like *hid*, is required to eliminate cells for proper morphogenesis of the *Drosophila* embryo.

hac-1 and *reaper*, *hid*, and *grim* Can Independently Promote Caspase Activation

The ectopic expression of *reaper*, *hid*, *grim*, and several *Drosophila* caspases can lead to ectopic apoptosis. For example, when these genes are expressed in the developing retina under the control of an eye-specific promoter, GMR (Hay et al., 1994), cell death is induced in a dosage-dependent manner, and this produces various degrees of eye ablation (reviewed in Bergmann et al., 1998b). This provides a highly sensitized background to investigate genetic interactions with other components of the cell death pathway. For example, eliminating one copy of the antiapoptotic gene *diap1* suppresses the eye ablation phenotypes of GMR-*reaper* and GMR-*hid*, and mutations in various components of the Ras-signaling pathway dominantly modify the GMR-*hid* phenotype (Hay et al., 1995; Bergmann et al., 1998a; Kurada and White, 1998). We investigated potential interactions of *hac-1* with several other proapoptotic genes by crossing the l(2)k11502 P insertion strain with transgenic fly strains carrying GMR-*reaper*, GMR-*hid*, GMR-*grim*, and GMR-*dcp-1* (Figure 6) (Grether et al., 1995; Chen et al., 1996; White et al., 1996; Song et al., submitted). Heterozygosity for *hac-1* resulted in only a very mild suppression of the eye phenotypes of GMR-*reaper/hid/grim* (Figures 6A–6F). In contrast, l(2)k11502 robustly suppresses the eye phenotype of GMR-*dcp-1* (Figures 6G and 6H). This indicates that endogenous *hac-1* is rate limiting for cell killing by DCP-1, but not for apoptosis induced by *reaper*, *hid*, and *grim* under these circumstances. Similar effects were seen upon expression of

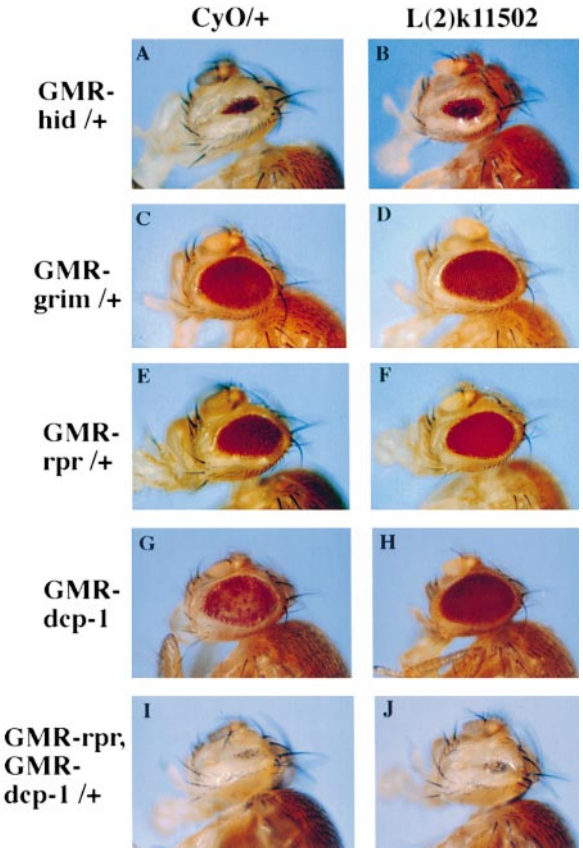

Figure 6. *hac-1* and *reaper*, *hid*, and *grim* Can Independently Promote Caspase Activation

Heterozygous l(2)k11502 has little effect on the eye phenotype of transgenic flies carrying one copy of GMR-*hid* (A and B), GMR-*grim* (C and D), and GMR-*rpr* (E and F). In contrast, it dominantly modifies the eye phenotype of transgenic flies carrying two copies of GMR-*dcp-1* (G and H). A strong synergism in cell ablation is observed when both *rpr* and *dcp-1* are expressed in the eye (compare E, G, and I) (Song et al., submitted). This activation of DCP-1 by RPR is independent of HAC-1 dosage since it is not modified by l(2)k11502 (J).

a truncated form of *dcp-1* lacking the prodomain (GMR-*dcp-1-N*, data not shown). This suggests that *hac-1* functions at a step either downstream of, or in parallel to, the removal of the *dcp-1* prodomain.

Genetic evidence suggests that *reaper* and *grim* induce apoptosis by activating *dcp-1* (Song et al., submitted). For example, when both *reaper* and *dcp-1* are expressed in the eye, they synergize to induce cell death (compare Figures 6E, 6G, and 6I). Expression of full-length *dcp-1* results in a weak phenotype, presumably due to poor activation of the zymogen in these conditions. In contrast, coexpression of *reaper* and *grim* with *dcp-1* leads to an eye ablation phenotype far more severe than the expression of any of these genes alone (Song et al., submitted). We investigated whether this synergy depends on *hac-1*. Since the eye phenotype of GMR-*dcp-1* is suppressed by heterozygosity for *hac-1*, we expected to see a significant modification of the GMR-*reaper* GMR-*dcp-1* eye phenotype. However, this was not observed. Upon introduction of l(2)k11502 into a GMR-*reaper* GMR-*dcp-1* background, only a very mild change in eye morphology could be detected (Figures 6I and 6J). We conclude that the activation of *dcp-1* by *reaper* is independent of *hac-1* function (see Figure 8).

hac-1 Is Induced by Irradiation

The mRNA distribution of *hac-1* during embryonic development suggests that the expression of this gene is developmentally regulated. This prompted us to examine whether *hac-1* expression can be induced by other death-inducing stimuli, such as DNA-damaging agents. Ionizing radiation induces apoptosis and expression of *reaper* in *Drosophila* embryos (Abrams et al., 1993; White et al., 1994; Nordstrom et al., 1996). Significantly, X-ray and UV irradiation also induces *hac-1* expression (Figure 7). Embryos from wild-type or l(2)k11502/CyO flies were irradiated with X-ray (4000 rads) or UV (50 mJ/cm^2 or 500 mJ/cm^2). In control embryos, expression above basal levels was only detected in the head region (Figures 3A and 3C). In contrast, ectopic *hac-1* expression was detected within 45 min after radiation exposure in the ectoderm (Figure 7B), mesoderm in the trunk (Figure 7H), and endoderm (Figure 7J). In embryos carrying the l(2)k11502 P element insertion, the *lacZ* reporter gene of the P element was also induced by X-ray irradiation in an essentially identical pattern (Figure 7D). Interestingly, *hac-1* expression is normally not detected in mesodermal and endodermal cells (Figures 7G and 7I). This strongly suggests that the increased levels of *hac-1* mRNA are the result of de novo transcription, and not reduced mRNA turnover. Furthermore, the pattern of ectopic *hac-1* expression corresponded very well with the pattern of TUNEL labeling of embryos subjected to the same radiation treatment (compare Figures 7B, 7D, and 7F), suggesting that the observed induction is functionally relevant. It appears that embryos between stages 8 and 11 are most sensitive to X-ray irradiation, since there was no detectable induction before stage 8, and induction in embryos after stage 12 was much weaker.

The expression of *hac-1* is also induced in response to UV irradiation (Figure 7L). Because UV rays do not penetrate far into the embryo, *hac-1* expression was only induced on the exposed side (Figure 7L). Again the ectopic *hac-1* expression correlated very well with TUNEL labeling of identically treated embryos (Figure 7N). However, there was one significant difference between UV and X-ray induction of *hac-1* expression. While X-ray irradiation failed to induce *hac-1* transcription prior to germ band elongation (stage 8), UV irradiation led to increased *hac-1* RNA as early as the blastoderm stage (data not shown). This suggests the possibility that distinct pathways are used for the induction of *hac-1* expression upon UV and X-ray irradiation.

Discussion

Genetic analyses of programmed cell death in *C. elegans* and *Drosophila* have identified different pathways for controlling the activation of caspases (reviewed in Bergmann et al., 1998b). In the absence of known *Drosophila* homologs of CED-4/APAF-1 and CED-9/

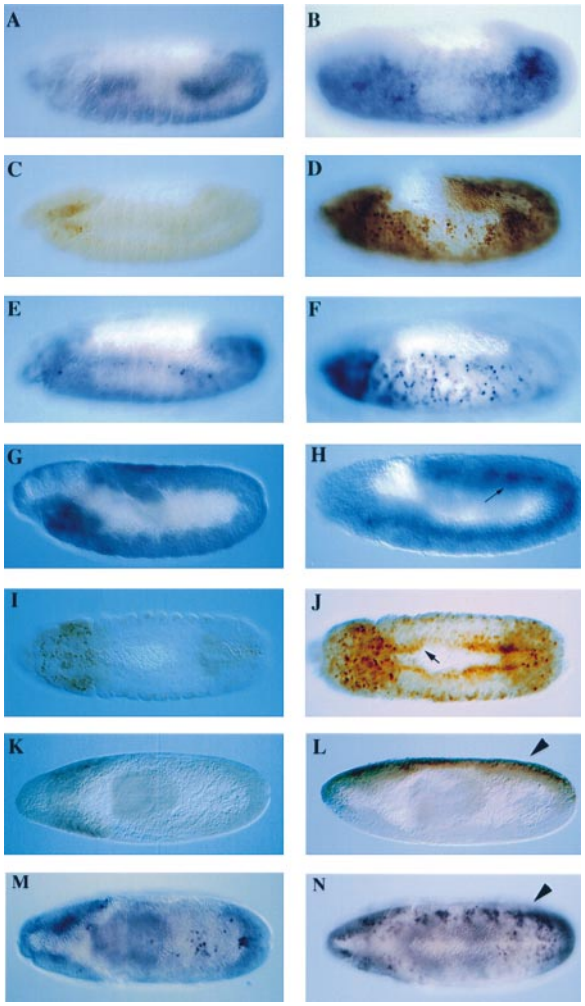

Figure 7. *hac-1* Is Induced by X-Rays and UV Irradiation

Embryos on the left side are nonirradiated control embryos that underwent the same histological analysis as their irradiated counterparts in the right panels. (A)–(F) depict stage 12 embryos. Many epidermal cells and neuroblasts express high levels of *hac-1* mRNA, 45 min after X-ray treatment (A and B; in situ hybridization). This is mimicked by the β-gal expression pattern in l(2)k11502/CyO embryos treated with X-Ray (C and D; anti-β-gal staining). (F) shows TUNEL labeling of embryos treated with X-rays. X-ray treatment also induces ectopic *hac-1* expression in mesoderm of the trunk (arrow in [H], in situ hybridization of stage 10 embryo) and endoderm cells (arrow in [J], β-gal staining of l(2)k11502/CyO stage 13 embryo), which normally have no detectable *hac-1* expression (G and I). UV irradiation induced *hac-1* expression on the exposed side (arrowheads) of the embryo, which is paralleled by the *lacZ* expression from the P insertion (L, stage 9 embryo). The UV-induced *hac-1* expression pattern corresponds well with the pattern of TUNEL labeling in embryos treated identically (N, stage 9).

BCL-2-like proteins, it may have appeared that insects adopted a distinct mechanism for the activation of a caspase-based death program (Meier and Evan, 1998; Rich et al., 1999). However, the present study indicates that *Drosophila*, like *C. elegans* and mammals, also utilizes CED-4/APAF-1-like molecules to induce caspases and cell death. The same gene has been independently isolated by Kanuka et al. (1999) (this issue of *Molecular*

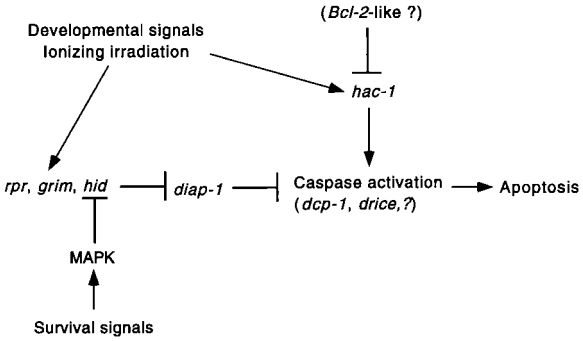

Figure 8. A Model for the Control of Caspase Activation in *Drosophila*

Genetic studies of cell death in *Drosophila* have initially defined *reaper*, *hid*, and *grim* as key activators of caspase-mediated death program. The products of these genes appear to kill by binding to and inhibiting that antiapoptotic activity of the DIAP1 protein (Wang et al., 1999; Goyal et al., submitted). The *hac-1* gene, identified based on its homology with APAF-1 and CED-4, appears to act in a separate pathway to stimulate caspase activation and cell death. At least under some circumstances, both pathways are simultaneously used to induce cell death in *Drosophila*. The expression of *reaper*, *grim*, *hid*, and *hac-1* is regulated at the transcriptional level during normal development, and transcription of both *reaper* and *hac-1* is rapidly induced by ionizing radiation. However, since *Drosophila* contains multiple caspases that may act in a proteolytic cascade, it is possible that *reaper*, *grim*, *hid*, and *hac-1* activate initially distinct caspases upstream of presumptive effector caspases, such as *dcp-1*. This would explain why genetic interactions among *reaper*, *grim*, *hid*, and *hac-1* are very weak.

Cell) and Rodriguez et al. (1999). Our initial characterization of *hac-1* also suggests that the activation of caspases can be simultaneously controlled by at least two separate pathways (Figure 8).

hac-1 Is a *Drosophila* Homolog of *Apaf-1* and *ced-4*

There are several reasons to conclude that *hac-1* is a *Drosophila* homolog of *Apaf-1* and *ced-4.* First, the predicted amino acid sequence of *hac-1* shares significant sequence homology with APAF-1, plant disease-resistant proteins, and CED-4. In particular, functionally important domains, including the nucleotide-binding motifs, are conserved in HAC-1. Interestingly, HAC-1 is most similar to APAF-1, and it also contains nine WD repeat elements in its C-terminal region that are not present in CED-4. Since it is thought that the WD repeat region of APAF-1 is the target for its activation by cytochrome c (Li et al., 1997; Hu et al., 1998, 1999), the conservation of this motif in HAC-1 suggests that similar interaction exists between HAC-1 and cytochrome c. However, at this point, there are no reports documenting the release of cytochrome c from mitochondria during apoptosis in *Drosophila*, and the functional significance of this domain remains to be investigated (Varkey et al., 1999).

HAC-1 is also functionally similar to APAF-1 and CED-4. As previously described for APAF-1, extracts from cells expressing HAC-1 stimulate caspase conversion in a dATP-dependent manner in vitro. Most importantly, we provide several lines of evidence to show that *hac-1* is required for the normal pattern of apoptosis

during *Drosophila* embryogenesis. The elimination of zygotic *hac-1* function leads to a prominent reduction of apoptosis, most noticeably in the head and brain region of the embryo. Furthermore, mutant embryos contain extra neurons, and a significant fraction of them display head involution defects that are strikingly similar to those previously described for mutations in the pro-apoptotic gene *hid* (Grether et al., 1995). However, unlike *ced-4* mutants, many cells die in the absence of zygotic *hac-1* function. Likewise, mice deficient for APAF-1 retain significant levels of cell death (Cecconi et al., 1998; Yoshida et al., 1998). There are several reasons for why cells may die in the absence of zygotic *hac-1* activity. First, *Drosophila* may have additional homologs of CED-4/APAF-1 that have a partially redundant function. Since the zygotic expression of *hac-1* is restricted to specific regions of the embryo, in particular the head, it is possible that the induction of apoptosis in other regions involves other family members. Second, maternally contributed mRNA and protein may provide an adequate supply for the death of many cells. Consistent with this notion, we find that the phenotypes generated by RNAi injection experiments are stronger than those of the P element insertion. Because any maternally contributed protein would not have been removed in these experiments, even stronger phenotypes may result upon the complete elimination of all *hac-1* products from the *Drosophila* embryo. Finally, not all cells selected to undergo apoptosis during normal development may utilize APAF-1/CED-4-like proteins. As discussed below, it appears that *reaper*, *hid*, and *grim* activate caspases by a pathway that is distinct from *hac-1*, and it is possible that certain cells rely primarily on one versus the other pathway for the induction of apoptosis (see Figure 8).

Two Distinct Pathways Converge for the Activation of Caspases in *Drosophila*

We observed strong genetic interactions between *hac-1* and the *Drosophila* caspase *dcp-1*, but only weak interactions with *reaper*, *grim*, and *hid*. In particular, the dosage of endogenous *hac-1* was rate limiting for ectopic cell killing in the fly retina induced by *dcp-1* but had little effect on killing by *reaper*, *hid*, and *grim*. On the other hand, *reaper* and *grim* interact genetically with *dcp-1* (Figure 6; Song et al., submitted). Furthermore, the synergy between *reaper* and *dcp-1* for the induction of cell death in the retina did not depend on the dosage of *hac-1*. The lack of significant genetic interactions between *hac-1* and *reaper*, *hid*, and *grim* indicates that these molecules act at some distance from each other in the cell death pathway, since genetic interactions are typically strongest among proximal components of a pathway. Therefore, we propose that *hac-1* promotes caspase activation through a pathway that is distinct from the one used by *reaper*, *hid*, and *grim*. According to our model, the activation of caspases during apoptosis in *Drosophila*, and presumably also in mammals, is controlled by the simultaneous action of two separate pathways (Figure 8). On one hand, Reaper, Hid, and Grim can activate caspases by inhibiting the antiapoptotic activity of *diap1*, one of the *Drosophila* IAPs (Wang et al., 1999; Goyal et al., submitted). On the other hand, *hac-1* appears to promote caspase activation by a mechanism that is similar to APAF-1. If one activating pathway is strongly induced, for example by overexpression of Reaper, the activity of components in the other pathway may become less important. This would explain why a reduction of *hac-1* dosage has little effect on *reaper*, *grim*, and *hid*-induced eye ablation. In addition, we consider it likely that the activation of *dcp-1* by *hac-1* involves one or more upstream caspases. A number of caspase-like sequences have been identified in *Drosophila* (Fraser and Evan, 1997; Song et al., 1997; Chen et al., 1998; Dorstyn et al., 1999), but the precise order in which they may act within a caspase cascade remains to be determined. Therefore, it is possible that *reaper*, *grim*, *hid*, and *hac-1* initially activate distinct upstream caspases, but we have not incorporated this potential complexity in our model at this time (Figure 8).

We expect that the activity of HAC-1 may also be controlled in a manner similar to that of APAF-1 and CED-4. Specifically, HAC-1 may be regulated by CED-9/BCL-2-like proteins. The recent identification of BCL-2-like sequences in *Drosophila* (L. Z. and H. S., unpublished results) should allow a critical test of this hypothesis. Significantly, *hac-1* is also regulated at the transcriptional level. Our results demonstrate that zygotic *hac-1* expression is not at all uniform and becomes restricted to specific regions of the embryo. Expression of *hac-1* in the cephalic region is similar to the expression of *hid* and correlates overall well with regions in which major morphogenetic cell death occurs subsequently. Furthermore, *hac-1* transcription is induced by both X-ray and UV irradiation in a manner similar to what has been previously reported for *reaper*. Therefore, it appears that at least some proapoptotic stimuli induce cell death by simultaneously turning on two different pathways for caspase activation (Figure 8).

Experimental Procedures

Fly Stocks

Canton-S or yw67C23 strains were used as wild type. The P element insertion line l(2)k11502/CyO was obtained from the Bloomington stock center. To facilitate the identification of a homozygous mutant, l(2)k11502 was also balanced with CyO{ftz-*lacZ*}. Transformant fly strains used in this study incude: GMR-*reaper* (White et al., 1994), GMR-*grim* (Chen et al., 1996), GMR-*hid* (Grether et al., 1995), and GMR-*dcp-1* and GMR-*dcp-1*,GMR-*rpr*/TM3 (Song et al., submitted).

Database Searching

Hidden Markov models of protein motifs were built using the alignment of identified families of cell death–related proteins (Grundy et al., 1997). The specificity of these domains for searching databases was tested, and search parameters were trained empirically by searching these domains against the finished *C. elegans* genome sequence (The *C. elegans* sequencing consortium, 1998). For the CED-4/APAF-1 family, three separate models were built corresponding to the three kinase motifs of the nuleotide-binding domain (van der Biezen and Jones, 1998). FASTA files containing *Drosophila* genomic or EST sequence were downloaded from the Berkeley *Drosophila* Genome Project. A simple searching algorithm with O(n) complexity was chosen and implemented in standard C/C++. The program was compiled using Borland C++ Builder and executed on an IBM compatible microcomputer.

hac cDNA Isolation

About 3 kb of *hac-1* sequence was obtained through RT-PCR. RT-PCR products were cloned into Bluescript and sequenced. After comparison with the genomic sequence (AC004335), one clone of

the RT-PCR products was chosen for further study because it only has two base pair differences with the genomic sequence, neither of which results in a coding change. The RT-PCR product was fused with cDNA clone GH17715 (Research Genetics) using a native PvuI restriction site located in the overlapping region. The fused product was termed clone A2177 and used as the full-length cDNA for functional assays. The cDNA sequence corresponding to aa 1–565, removing the WD repeats, was amplified using A2177 as template and used as *hac-1*Δ for functional assays described below.

Double-Stranded RNA Construction

For *hac-1*, either the sequence from Arg-143 to Arg-332 or the sequence from the start Met to Gly-723 plus 148 bases of 5′ UTR was inserted into Bluescript SK^+. The *lacZ* gene from pCaSpeR-AUG-β-gal (Thummel et al., 1988) was inserted into the BamH1 and Xba1 sites of pCS2+ (Rupp et al., 1994). dsRNA was synthesized essentially as described before (Kennerdell and Carthew, 1998). dsRNA was injected at concentrations between 0.6 and 1.0 mg/ml.

Injection Protocol

Canton S embryos were collected for 60 min at 25°C, dechorionated in 50% bleach, attached to a microscope slide with double-sided tape (3M Scotch), desiccated, and covered with Voltalef 10S oil. Embryos were injected in the syncytial stage, either in the posterior or anterior. The injected volume was approximately 2%–3% egg volume or 200–300 pL. Uninjected control embryos were also put on tape under oil, but not desiccated.

Cell Culture and Transfection

293 T cells were seeded in a 6-well plate, with 10^6 cells per well. After overnight culture in a 37°C incubator with 5% CO_2, the cells were transfected with 2 μg of pRK5-*hac-1* or pRK5-*hac-1*DC plasmid DNA per well with lipofectamine (GIBCO/BRL). Forty-eight hours after transfection, the cells were collected and washed with ice-cold PBS twice by centrifugation in an Eppendorf tube. Packed cells (100 μl) were resuspended in 200 μl buffer A (Li et al., 1997) and homogenized in a Dounce glass homogenizer on ice. The cytoplasmic fraction was collected by centrifugation of the homogenate at 14,000 rpm for 15 min in an Eppendorf centrifuge. Ten microliters of this supernatant was mixed with 1 μl of ^{35}S-labeled DCP-1 or drICE (in vitro transcription was performed as described in Song et al. [submitted]) in the presence or absence of 1 mM dATP and incubated at 37°C for 1 hr. The DCP-1 and drICE cleavage was analyzed by a 12% SDS-PAGE.

X-Ray and UV Irradiation

Embryos were collected from wild-type or l(2)k11502/CyO flies and aged at 25°C for various times. X-ray irradiation was performed as described (White et al., 1994). A Stratalinker 2400 (Stratagene, La Jolla, CA) with 264 nm UV source was used for UV irradiation. Embryos on egg plates were put inside the Stratalinker and exposed to 50 mJ/cm^2 or 500 mJ/cm^2 irradiation. After UV treatment, embryos were allowed to recover for either 45 min (for in situ hybridization) or for 75 min (for β-gal expression and TUNEL labeling) at 25°C, followed by 4% paraformaldehyde fixation.

Histology

In situ hybridization and immunocytochemistry were performed essentially as described before (Zhou et al., 1995, 1997). The 9F8A9 anti-ELAV MAb (O'Neill et al., 1994) was obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa, Department of Biological Science. In order to visualize the β-gal expression pattern in l(2)k11502 embryos, monoclonal anti-β-gal Ab was diluted 1:20,000. When the antibody was used at 1:1,000 (Zhou et al., 1995), the embryo carrying the P insertion l(2)k11502 turned brown instantly, and a specific pattern could not be observed, suggesting a high basal level of β-gal activity in most, if not all, cells. Acridine orange and TUNEL labeling was performed as previously described (Abrams et al., 1993; White et al., 1994).

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