grim, a novel cell death gene in *Drosophila*

Po Chen, William Nordstrom, Bridget Gish,¹ and John M. Abrams²

Department of Cell Biology and Neuroscience, University of Texas Southwestern Medical Center, Dallas, Texas 75235-9039 USA; ¹Department of Molecular Biology and Oncology, University of Texas Southwestern Medical Center, Dallas, Texas 75235-9148 USA

A genomic interval at 75C1,2 is required for programmed cell death in *Drosophila*. We identified a new activator of apoptosis, *grim*, which maps between two previously identified cell death genes in this region *reaper* (*rpr*) and *head involution defective* (*hid*). Expression of *grim* RNA coincided with the onset of programmed cell death at all stages of embryonic development, whereas ectopic induction of *grim* triggered extensive apoptosis in both transgenic animals and in cell culture. Cell killing by *grim* was blocked by coexpression of p35, a viral product that inactivates ICE-like proteases, and did not require the functions of *rpr* or *hid*. The predicted *grim* protein shares an amino-terminal motif in common with *rpr*. However, *grim* was sufficient to elicit apoptosis in at least one context, where *rpr* was not. The *grim* gene product might thus function in a parallel circuit of cell death signaling that ultimately activates a common set of downstream apoptotic effectors.

[Key Words: Apoptosis; programmed cell death; *Drosophila*]

Received May 3, 1996; revised version accepted June 6, 1996.

Programmed cell death (PCD) or apoptosis is an essential regulator of tissue homeostasis as animals develop and age (Saunders and Fallon 1967; Truman 1984; Hurler 1988; Ellis et al. 1991; Oppenheim 1991; Raff 1992). The importance of elucidating the molecular basis underlying this process is underscored by a number of recent studies that implicate the misregulation of PCD in the pathophysiology of several human diseases, including AIDS (Meynard et al. 1992; Gougen and Montagnier 1993), neurodegenerative disease (Roy et al. 1995; Liston et al. 1996; Vito et al. 1996), and cancer (for review, see Williams 1991).

Several lines of evidence suggest that the physiology of apoptosis is quite highly conserved. First, the morphological changes associated with programmed cell deaths are strikingly similar in both vertebrates and invertebrates (Kerr et al. 1972; Wyllie et al. 1980; Kerr and Harmon 1991; Abrams et al. 1993). Second, at least two essential cell death genes in *Caenorhabditis elegans*, *ced-3* and *ced-9*, are members of gene families that encode apoptotic functions in vertebrates (for review, see Steller 1995; White 1996). Third, viral proteins that suppress apoptosis in their hosts (p35 and crmA) can exhibit potent anti-apoptotic activity in a wide range of heterologous species (Rabizadeh et al. 1993; Hay et al. 1994; Sugimoto et al. 1994; Grether et al. 1995; Pronk et al. 1996; White et al. 1996; W. Nordstrom, P. Chen, H. Steller, and J.M. Abrams, in prep.).

In *Drosophila melanogaster*, a genomic interval defined by the H99 deletion mutation is required for embryonic PCD (Abrams et al. 1993; White et al. 1994; Abrams 1996). This region spans ~300 kb of DNA that includes at least two cell death genes, *reaper* (*rpr*) (White et al. 1994) and *head involution defective* (*hid*) (Grether et al. 1995). The former gene product is thought to share similarities with the "death domain" of the FAS/TNFR1 protein family (Cleveland and Ihle 1995; Golstein et al. 1995a,b), whereas the latter shares no extensive sequence similarity to known proteins. Although the distribution of RNA from both genes generally corresponds to embryonic patterns of apoptosis, only *rpr* appears to be selectively expressed in all cells that will later undergo programmed cell death.

Although no apoptosis occurs in embryos bearing homozygous deletions of the entire H99 interval (Abrams et al. 1993; White et al. 1994), null mutations at *hid* display only mild cell death defects (Grether et al. 1995) and, to date, no single-gene mutants of *rpr* have been identified. Therefore, the precise number of cell death genes uncovered by H99 is not known. Phenotypes associated with two informative deletions in the region, X14 and X25 (White et al. 1994; Grether et al. 1995), raised the possibility that perhaps one or more additional cell death genes might reside between *hid* and *rpr*. Both strains partially uncover the H99 interval from the distal boundary, thereby eliminating *hid* yet preserving *rpr*. However, although both deletions exhibit mild and indistinguishable PCD phenotypes as homozygotes

²Corresponding author.

(Grether et al. 1995), X25 uncovers a far more severe phenotype when placed in *trans* to H99 than does X14. Whereas X14/H99 embryos show subtle cell death defects similar to those observed for *hid* null alleles, X25/H99 transheterozygotes exhibit a severe reduction in apoptosis frequency that can be easily visualized by staining with acridine orange (Grether 1994; J.M. Abrams, unpubl.). Because the relevant breakpoint of X25 is ~60 kb more proximal than that of X14 (Grether 1994), it was possible that one or more additional cell death functions mapped to the interval bounded by these breakpoints.

In this report we describe our characterization of a newly identified cell death gene, *grim*, which maps between the relevant breakpoints of X14 and X25. A genomic cosmid spanning the *grim* gene restored apoptosis to H99 embryos, and *grim* RNA was specifically found in regions associated with PCD. Moreover, ectopic expression of a *grim* cDNA triggered extensive cell death in embryos, eye discs, and cultured cells. These cell deaths did not require Rpr or Hid and could be prevented by coexpression of the viral anti-apoptotic protein p35. We therefore propose that the apoptotic function encoded by *grim* is upstream of putative cysteine proteases (targets of p35) and that Grim activity parallels that of RPR and HID. Finally because *grim* triggered extensive apoptosis in at least one developmental context where Rpr was not sufficient, it is possible that these proteins enter a common apoptotic pathway at different sites.

Results

Identification and molecular characterization of *grim*

To test for possible cell death functions in the interval between the proximal breakpoints of DfX14 and DfX25, we produced four independent germ-line transformants with a cosmid clone that spans the appropriate region. Three of these were tested in a homozygous H99 background for restoration of embryonic cell death by acridine orange (AO) staining (Abrams et al. 1993). We found that substantial numbers of apoptotic cell deaths are restored to virtually all mutant embryos bearing four doses of the *grim* cosmid (Fig. 1). All three transformant lines

Figure 1. A cosmid spanning *grim* restores apoptosis to H99 embryos. AO staining of an embryo from the parental yw strain (A), a H99 homozygote (B), and H99 embryos carrying four doses of the *grim* cosmid transgene (C,D). The embryo in D is typical of a majority of rescued mutants, whereas the phenotype shown in C is observed only occasionally.

examined showed similar levels of rescue, and this activity was dose dependent, as four doses of the cosmid showed more rescue than two doses. Note that these cell deaths occur in the absence of two other cell death genes in the H99 region, *rpr* and *hid*. The partial nature of this phenotypic rescue was reminiscent of the behavior of H99 transformants bearing a *rpr* cosmid. We therefore counted apoptotic cells in H99 embryos bearing similar doses of the *rpr* or the *grim* transgene. Because they are only partially rescued for cell death and still defective for head involution, embryos with these genotypes are easily identified. In these studies we found that the extent of rescue conferred by four doses of *grim* was indistinguishable from that conferred by four doses of *rpr*. It is worth noting here that artifactual explanations for the restoration of PCD can be ruled out because other transgenes, including at least one cosmid, did not rescue apoptosis in H99 embryos (White et al. 1994; Grether et al. 1995).

We identified one transcription unit that mapped to a single ~3-kb *HpaI*–*NotI* fragment within this cosmid clone at a position that lies ~20 kb distal to the proximal breakpoint of X25. This fragment was used to isolate eight hybridizing clones from an embryonic cDNA library (see Materials and methods). These all contained inserts of ~1.6-kb, and the clone selected for sequence analysis (clone A) included an extensive poly(A) tail. By virtue of its proximity to *rpr* and its potent cell killing activity (see below) we assigned the name *grim* to this gene.

grim cDNA detected a single 1.6-kb transcript by Northern blot analysis of embryonic RNA and, as expected, also recognized a ~5-kb *EcoRI* genomic band that is present in wild-type flies but absent in homozygous H99 embryos (not shown). These data confirmed that our cDNA clones correspond to expressed sequences from within the H99 interval. Using PCR analyses of genomic DNA and partial sequencing of the *grim* cosmid, no apparent intronic sequences were found. By mapping the cDNA clone onto the cosmid, we determined that *grim* is transcribed toward the telomere in an orientation that parallels the direction of *rpr* and *hid* expression.

The complete sequence of *grim* cDNA clone A was determined (Fig. 2) and confirmed by sequencing genomic-derived PCR products and parts of the *grim* cosmid. The longest deduced open reading frame (ORF) in the 5' → 3' direction is a protein of 138 amino acids that was validated in tests for apoptosis functions (see Table 1; Fig. 5, below). The predicted Grim protein has an estimated pI of 5.8, no helical *trans*-membrane domains (Rost and Sander 1994), and shares no significant homology to any sequence in the data base as analyzed using BLAST (Altschul et al. 1990). However, direct inspection shows that the amino-terminal end of this predicted protein shares very notable similarity to Rpr and less similarity to Hid (Fig. 2). Ten of the first 14 residues of Grim are identical to the corresponding position in Rpr, whereas three of the remainder are conserved substitutions. This region of Rpr also shares notable but less similarity to the Hid protein (Grether et al. 1995). A

[illegible]

three-way comparison within this region suggests that Rpr and Grim share the most similarity to each other while Grim and Hid share the least.

We also detected signal for *grim* RNA that was subcellularly localized inside some, but not all, macrophages (Fig. 3E,F) [Abrams et al. 1992, 1993]. This distribution, also found with probes for *rpr* [White et al. 1994; Zhou et al. 1995] and *hid* RNAs [Grether et al. 1995], probably reflects hybridization to mRNAs inside corpses that have recently been engulfed by phagocytic cells and

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may be characteristic of cell death activators mapping to 75C1,2 in *Drosophila*.

grim is sufficient to induce cell death that can be blocked by coexpression of p35

To determine whether *grim* encodes apoptotic activity, we examined the consequences of ectopic *grim* expression in a variety of experimental contexts. A *grim* cDNA was cloned into the pGMR vector (pGMR-*grim*) that directs specific expression to the developing eye (Hay et al. 1994), and this construct was used to isolate a total of eight independent germ-line transformants. As homozygotes, seven of these exhibit the severe eye-ablation phenotype illustrated in Figure 4C, whereas one strain showed no obvious effects. Heterozygotes for the GMR-*grim* transgene exhibit varying degrees of intermediate phenotypes (Fig. 4B), with eyes that are substantially smaller than wild type and display a characteristic rough appearance.

We also tested whether this eye-ablation phenotype could be affected by the anti-apoptotic protein p35, a baculovirus protein that functions to block cell death by inactivating proteases of the ICE/CED-3 family (Clem et al. 1991; Miura et al. 1993; Clem and Miller 1994; Bump et al. 1995; Xue and Horvitz 1995). To examine this issue, we produced strains that simultaneously express both *grim* and p35 transgenes (Hay et al. 1994) in the developing eye. Figure 4D shows that eyes of these co-expressing flies are restored to a wild-type size and normal organization. Thus, p35 can suppress the retinal cell death phenotype associated with ectopic *grim* expression.

We also examined the consequence of *grim* expression in cultured cells. *Drosophila* SL2 cells (Schneider 1972) were transfected with pMT-*grim*, a plasmid designed to conditionally express the *grim* cDNA from an upstream metal-inducible promoter (Bunch et al. 1988; Kovach et al. 1992). In both transient assays and in stably transfected populations, potent cell killing associated with *grim* induction was observed. Within 4 hr of metal treatment, considerable numbers of apoptotic figures can be found in cultures transfected with pMT-*grim* and by 9 hr apoptotic death is very extensive (Fig. 5B). This effect is indistinguishable from cultures in which *rpr* has been similarly induced (W. Nordstrom, P. Chen, H. Steller, and J.M. Abrams, in prep.) and is not observed in controls.

We used two independent methods to measure apoptosis in our studies. Figure 5C shows the results of flow cytometry assays that rely on the appearance of "hypodiploid nuclei" in samples stained with propidium iodide. In cultured cells, the appearance of nuclei with a "sub-G1" DNA content is a characteristic determinant of apoptosis and can be used to assess the extent of apoptotic death (Tounekti et al. 1995; W. Nordstrom, P. Chen, H. Steller, and J.M. Abrams, in prep.). In transiently transfected samples, a substantial fraction of pMT-*grim* cells underwent apoptosis after 16 hr of metal treatment, whereas only background levels of cell death

occurred in the absence of copper or in control cells that were transfected either with empty vector or an irrelevant protein expressed from the same vector (pMT-HA-hook) (Kramer and Phistry 1996). Note that the percent apoptotic nuclei in Figure 5C is not normalized for transient transfection efficiencies, which typically range from ~40%–60% in our assays. Therefore, the extent of apoptosis after *grim* induction is likely to be substantially higher than is indicated by the absolute percentage of hypodiploid nuclei.

We corroborated these findings with an alternative assay for cell killing that also detects *rpr*-induced apoptosis (W. Nordstrom, P. Chen, H. Steller, and J.M. Abrams, in prep.) and relies on the loss of cotransfected reporter activity after induction of the test plasmid (Hsu et al. 1995). In this case, the number of cells positive for a cotransfected actin promoter-driven LacZ reporter, pAct-LacZ, was determined 16 hr after induction (64 hr after transfection; see Materials and methods). Treatment with metals alone had no effect on the number of

Figure 4. A dramatic phenotype caused by ectopic expression of *grim* in the developing eye can be prevented by coexpression of the baculovirus p35 protein. Scanning electron micrographs of the compound eye of the parental strain yw (A), pGMR-*grim1*/+ (B), pGMR-*grim1*/pGMR-*grim1* (C), and pGMR-*grim1*/pGMR-*grim1*; pGMR-p35/pGMR-p35 (D). Transgenic flies containing a single copy of pGMR-*grim1* (B) exhibit a reduced number of disordered ommatidia and mechanosensory bristles. Two copies of the pGMR-*grim1* transgene (C) causes a complete elimination of ommatidia with only a few bristles remaining. Seven of eight independent transformants had this phenotype. The compound eyes of flies homozygous for both pGMR-*grim1* and pGMR-p35 transgene insertions (D) are restored to the wild-type size. Note: Apparent differences in magnification between samples reflects different positioning on electron microscope stage.

Figure 5. Induction of *grim* expression triggers apoptosis in culture, which is prevented by p35. (A) Photomicrographs of SL2 cells; (B) cells stably transfected by pMT-*grim* treated for 9 hr with CuSO_4 . Arrows in B indicate cells with a characteristic apoptotic appearance. The pictures were taken under a 40 $\times$ lens using a CCD camera. (C) Flow cytometric assays for apoptosis in transiently transfected cultures. *Drosophila* SL2 cells were transiently transfected alone with pActin-LacZ (0.2 μg) or cotransfected with pActin-LacZ (0.2 μg) plus empty vector pRmHa.3 (2 μg), pMT-HA-hook (2 μg), pMT-*grim* (2 μg), pMT-*grimORF* (2 μg), or pMT-*grim*/pMT-p35 (2/3 μg), and processed for FACSscan as described in Materials and methods. Shown are percent of apoptotic (hypodiploid) nuclei obtained from triplicate samples of control (open) or copper (hatched)-treated samples.

LacZ-positive cells nor did controls with empty vector or an irrelevant protein (Table 1). In contrast, a drastic reduction of β -gal-expressing cells occurred in metal-treated cells that had been transfected with pMT-*grim*, and many of the remaining lacZ-positive cells in the *grim*-induced cultures exhibited classic apoptotic morphology. We conclude that the *grim* gene product is a potent activator of apoptosis. To confirm that the ORF predicted from our cDNA sequence analysis is responsible for cell killing, we tested pMT-*grimORF*, a plasmid that directs metal-inducible expression of the predicted 138 amino acid protein (see Fig. 2). As illustrated in Table 1 and Figure 5C, this construct encodes levels of apoptotic activity that are indistinguishable from the full-length cDNA.

We also took advantage of this culture system to test

more directly the effects of p35 on *grim*-induced cell killing. For these studies we used pMT-p35 a metal-inducible version of p35 (W. Nordstrom, P. Chen, H. Steller, and J.M. Abrams, in prep.). Consistent with our observations in the developing eye (Fig. 4), coexpression of p35 completely abrogated *grim*-mediated apoptosis in this context as well (Table 1; Fig. 5C). This result indicates that the function of *grim* may reside upstream of one or more ICE-like proteases that are targeted by p35.

We also produced germ-line transformants with a heat-inducible transgene, heat shock-*grim* and found that ectopic expression of *grim* in this context also caused extensive cell death throughout the embryo (two independent strains tested). Within 1 hr of heat treatment, excessive apoptosis was scored by AO and TUNEL staining (Fig. 6) in >70% of HS-*grim* embryos, whereas no appreciable elevation of apoptosis was observed in control embryos that were heat-shocked but lacked the transgene (Fig. 6A) (see also White et al. 1994; Grether et al. 1995). Gross morphological abnormalities as a consequence of ectopic cell death and severe organismal lethality were two additional phenotypes associated with heat-induced *grim* expression. After heat treatment (see Materials and methods), no HS-*grim* embryos hatched,

Figure 6. Apoptosis induced by *grim* in wild-type and H99 embryos. *yw* embryos (A,D) or embryos bearing a heat-inducible *grim* transgene, HS-*grim* (C,F) or a heat-inducible *rpr* transgene, *hsrpr* (B,E) were heat-treated for 30 min and analyzed 1 hr later (see Materials and methods). (A–C) Stage 13/14 embryos stained with AO. (D–F) Stage 9 embryos labeled for apoptotic cells by TUNEL. Note that although *grim* and *rpr* both induce cell death during later stages of embryonic development (cf. B and C with A), only *grim* triggers apoptosis at the earlier stage (cf. F and E). *grim*-induced apoptosis also occurs in embryos deleted for the H99 region (G,H). AO staining is shown in a heat-shocked H99 embryo (G) or in a heat-shocked HS-*grim2*, H99 embryo (H). Embryos homozygous for H99 were identified by their *hid* phenotype.

Table 1. Cell death induced by *Grim*

Copper treatment	Blue cells per field					
	Actin-LacZ	pRmHa.3	HA-hook	<i>grim</i>	<i>grimORF</i>	<i>grim/p35</i>
No	150 ± 9	150 ± 13	138 ± 9	147 ± 5	160 ± 16	141 ± 6
Yes	141 ± 7	162 ± 15	148 ± 12	39 ± 6	31 ± 5	135 ± 5

Drosophila SL2 cells were transiently transfected with pActin-LacZ (0.2 µg) alone, or cotransfected with pActin-LacZ (0.2 µg) plus empty vector pRmHa.3 (2 µg), pMT-HA-hook (2 µg), pMT-*grim* (2 µg), pMT-*grimORF* (2 µg), or pMT-*grim* and pMT-p35 (2 and 3 µg, respectively). Cells from each well were split to two wells 48 hr after transfection, and copper was added to one of the wells. Cells were fixed and stained with X-gal 16 hr later as described in Materials and methods. β -Galactosidase activity data are presented as average number of β -gal-positive (blue) cells ($\pm$ S.D.) per field (10 $\times$ lens) for at least three fields from each of three independent transfections.

whereas >85% of control embryos exposed in parallel did.

The conditional phenotypes associated with HS-*grim* are similar to those documented for HS-*rpr* transformants (White et al. 1996). We therefore conducted a detailed comparison of the apoptotic responses of these strains at various embryonic stages. Interestingly, we found that induction of HS-*grim* could trigger substantial cell deaths during early embryogenesis at a time when HS-*rpr* failed to provoke any apoptotic response (Fig. 6E). Because these are stages prior to the initiation of PCD in wild-type embryonic development (Abrams et al. 1993) we used TUNEL to score cell death and also confirm their apoptotic character. Although HS-*rpr* was unable to induce cell death at stage 9 or earlier, many TUNEL-positive cells were frequently observed in HS-*grim* embryos at this stage (Fig. 6F). These findings were corroborated in two independent HS-*rpr* strains and two independent HS-*grim* strains and are therefore not dependent on insertion site effects. The ability of *grim* to induce cell death in a developmental context where *rpr* does not suggests that the activity or regulation of these proteins may be distinct.

The cell death function of grim is independent of rpr and hid

To determine whether *grim*-induced apoptosis requires other functions in the H99 region, we tested the ability of HS-*grim* transgenes to trigger cell death in homozygous embryos that are deleted for this interval. In a collection of embryos from parents that are heterozygous for H99 and homozygous for the HS-*grim* transgene, 25% will be cell death defective and easily scored by an absence of AO staining. After heat treatment, however, prominent AO staining occurred in 100% of embryos from parents of this genotype (as in Fig. 6C) suggesting that *grim*-induced apoptosis occurred even in embryos homozygous for H99. At later embryonic stages we were able to still distinguish the *hid* defect even though cell death was restored to H99 homozygotes. Figure 6, G and H, illustrates this observation showing that within 1 hr after heat treatment, induced *grim* expression is sufficient to trigger extensive cell death among embryos that bear the characteristic *hid* phenotype. Appreciable induction of apoptosis does not occur in the absence of

heat shock nor does it occur in heat-shocked embryos from the parental strains. Moreover, several irrelevant control transgenes (including *engrailed*, *hedgehog*, and *disco*) also expressed from the *hsp70* promoter have been tested similarly and do not trigger apoptosis in H99 embryos (Grether et al. 1995). Our results clearly show that *grim*-induced apoptosis does not require the function of other cell death genes in the H99 interval.

Because expression of *rpr* RNA anticipates both normal PCD (White et al. 1994) and ectopically induced apoptosis (W. Nordstrom, P. Chen, H. Steller, and J.M. Abrams, in prep.) we investigated the possibility that *grim*-induced cell death might be associated with cross-regulation at the *rpr* locus. However, when HS-*grim* embryos were examined after heat shock by in situ hybridization for altered expression of *rpr*, no significant effects on the distribution of *rpr* RNA were observed (not shown). Conversely, it was also possible that the functions of *rpr* and *hid* might influence *grim* expression. We therefore examined *grim* RNA expression in heat-shocked *rpr* (*hsrpr*) embryos (White et al. 1996) and in embryos homozygous for *hid*^{WR+X1}, a null mutation in *hid* (Abbot and Lengyel 1991; Grether et al. 1995). Again, no evidence for cross-regulatory interactions was obtained, as the distribution of *grim* RNA in both instances was indistinguishable from wild type (not shown). Taken together, these results establish that *grim* functions independently of *rpr* or *hid* as an activator of PCD.

Discussion

Deletion mutations that uncover a ~300-kb genomic interval at 75C1,2 prevent all apoptotic deaths in the *Drosophila* embryo. So far, two activators of PCD, *rpr* (White et al. 1994) and *hid* (Grether et al. 1995), have been mapped to this interval. However, several lines of evidence were consistent with, or implicated, at least one additional cell death function uncovered by the H99 deletion. Strains X14 and X25 have proximal breakpoints within the H99 interval, and although both deletions retain *rpr* and remove *hid*, they behave differently when placed in *trans* to H99. This observation prompted us to test for additional cell death functions in the region with specific emphasis on DNA proximal to *hid* and distal to *rpr*. In this report we describe a gene in this location, *grim*, which is itself a potent activator of cell death and

defines the third apoptotic function in the H99 genomic interval.

grim encodes a potent apoptotic function

Several lines of evidence established that *grim* encodes an activator of PCD. First, germ-line transformation of genomic DNA spanning the *grim* locus resulted in restoration of cell death to H99 mutant embryos. Although only partial rescue of the cell death defect by the *grim* cosmid was observed, the extent of rescue was dose dependent and similar to levels of rescue obtained by corresponding doses of genomic *rpr* DNA. Second, the amino-terminal portion of Grim shares conspicuous similarity to Rpr, a protein already well established as an activator of cell death in *Drosophila* (White et al. 1994, 1996; Pronk et al. 1996; W. Nordstrom, P. Chen, H. Steller, and J.M. Abrams, in prep.). Third, the induction of *grim* either in cultured cells or in ectopic tissues triggered extensive cell death that was apoptotic in appearance, prevented by a viral inhibitor of apoptosis, and detected by methods that are selective for this form of cell death [AO (Abrams et al. 1993) and TUNEL (Gavrieli et al. 1992)]. These results argue strongly for an apoptotic mode of cell killing induced by *grim*. Finally, the distribution of *grim* transcript during embryogenesis is coincident with patterns of embryonic cell death, and we occasionally detect compartmentalized signals for *grim* RNA inside phagocytes. This feature is an unusual staining characteristic that has also been observed with probes for *rpr* (White et al. 1994; Zhou et al. 1995) and *hid* (Grether et al. 1995) and probably reflects hybridization to RNAs that persist within engulfed cell corpses. These observations suggest that like RNA expressed from *rpr* and *hid*, that from the *grim* gene anticipates the onset of cell death.

Although not yet proven formally, the consequences of ectopic *grim*ORF expression strongly suggest that *grim* activity is mediated by a protein product that provides an associated cell autonomous function. The mechanism by which *grim* elicits the apoptosis program remains to be determined as does the functional significance of amino-terminal motif shared among *grim*, *rpr*, and *hid*. In contrast to reported alignments between Rpr and some death domain proteins (Cleveland and Ihle 1995; Golstein et al. 1995a,b) no similar alignments were detected for Grim.

Genetics of the H99 cell death interval

grim is the third gene mapped to the H99 deletion mutation thus far. In addition to *rpr* and *hid*, evidence from synthetic deletions (see introductory section) had implicated at least one more cell death function in the region. Although *grim* clearly meets all requisite criteria for this predicted function, we cannot rule out the possibility that additional PCD genes may reside in the H99 interval. Genetic approaches to this problem are hampered by the fact that only alleles of *hid* were recovered from very extensive screens for chemically induced lethal muta-

tions in the H99 interval (Abbot and Lengyel 1991; White et al. 1994; Grether et al. 1995). These lesions have been examined carefully and, although they do exhibit partial cell death phenotypes, *hid* mutants are distinctly less severe than the complete apoptotic failure exhibited by H99 (Grether et al. 1995). It is plausible that *hid*, *rpr*, and *grim* might encode partially redundant functions, which could account for the absence of single-gene lesions that phenocopy H99 and our inability to recover single gene lesions in *grim* or *rpr*. According to this scenario, more fully penetrant cell death phenotypes could require the disruption of at least two of the three known elements in the region; therefore, single-gene mutations other than *hid* might be inaccessible by screens for lethal or visible phenotypes. Conversely, simultaneous rescue of both *rpr* and *grim* functions might restore individuals to a phenocopy of the *hid* mutation. We investigated this possibility by examining H99 embryos that were homozygous for both *grim* and *rpr* cosmid transgenes. This genotype did not result in a phenocopy of *hid*, as these individuals were indistinguishable from those bearing four doses of either *grim* or *rpr*. Because expression from cosmid transgenes may be compromised in our transformants, solid conclusions regarding the number of distinct cell death functions uncovered by H99 cannot be drawn from these results.

In *Drosophila* and in other species, tight linkage can occur among groups of genes that share closely related functions (for review, see Lawrence 1992; Krumlauf 1994). Typically, the individual members within conserved gene clusters share a common orientation of transcription and a considerable degree of sequence similarity. Therefore, the H99 cell death interval apparently shares some, but not all, of the classic features of a complex: *grim*, *hid*, and *rpr* share commonality of orientation and function, yet outside a very limited stretch these proteins share little or no sequence similarity. Because individual members within a gene cluster are typically thought to have arisen by evolutionary duplication and divergence (for review, see Kenyon 1994) it is possible that organization of the H99 cell death cluster may have evolved by different mechanisms.

Models of apoptosis signaling by *grim*

The induction of *grim* was sufficient to trigger apoptosis in embryos that were homozygous for the H99 deletion. This observation rules out an absolute requirement for either *rpr* or *hid* during *grim*-mediated cell death. Among a number of inducible transgenes tested thus far (Grether et al. 1995), only *rpr* and *hid* also triggered significant apoptosis in the H99 background. Because coexpression of p35 blocks apoptosis triggered by *grim*, *rpr*, and *hid* (Grether et al. 1995; White et al. 1996; W. Nordstrom, P. Chen, H. Steller, and J.M. Abrams, in prep.; this paper), all three might ultimately activate a common pathway involving conserved ICE/CED-3-like proteases that are targets for inactivation by p35. Moreover, *grim* induction was able to trigger apoptosis at a time in early development when no such effects were observed with *rpr*. This

distinction could reflect differences in cell death signaling that are uncovered during early development. For instance, a negative regulator of *rpr* that does not block *grim* might be present at this stage, or alternatively, an effector required for *rpr* but not for *grim* might be absent at this stage. These gene products might thus be viewed as parallel switches that can ultimately activate a common circuit of apoptotic downstream effectors. Alternatively, two or more entirely parallel pathways, each of which is blocked separately by p35, could define the effector circuits downstream of these genes. In either case, *grim*, *rpr*, and *hid* might represent alternate switches or nodes that could be activated by similar yet distinct sets of converging apoptotic signals. Preliminary evidence (P. Chen, W. Nordstrom, and J.M. Abrams, unpubl.) to support this notion comes from assays that monitor differential regulation of these gene products in response to stimuli that provoke ectopic cell death.

Materials and methods

Drosophila stocks

Df(3L)H99, *In(3L)hid^{WR+X1}*, were described previously (Abbot and Lengyel 1991). Isolation of X14 and X25 was described in Grether (1994) and White et al. (1994) and that of *hsrpr46A* and *hsrpr53* were described in White et al. (1996). GMR-p35 (Hay et al. 1994) was obtained from B. Hay and G. Rubin. The wild-type strain used was Canton S, and the parental strain for germ-line transformations was *yw^{67c23}*. All genetic symbols are according to Lindsley and Zimm (1992).

Cosmid transformation and phenotypic analysis

A cosmid from a genomic walk (Grether 1994; White et al. 1994) was determined to span the *grim* gene. A mixture of 437 µg/ml of cosmid DNA and 100 µg/ml of Δ2-3 helper plasmid DNA was injected into several thousand *yw* embryos (Rubin and Spradling 1982). Four independent transformed lines were isolated. Two of these map to the third chromosome (*grim*-CosB and *grim*-CosD), one maps to the second (*grim*-CosA), and one is on the X Chromosome (*grim*-CosC). Two of these (lines A and C) were introduced into the H99 background, and a recombinant of *grim*-CosD was constructed to obtain *w*; *grim*-CosD, *H99 ri,p/TM3, Sb* flies. Levels of rescue by *grim* cosmid, were determined by staining with AO (Abrams et al. 1993) and found to be similar among the three lines, A, C, and D. To construct a line with four *grim* transgenes in the H99 background, homozygous *grim*-CosA flies were mated with *w*; *CyO/+*; *grim*-CosD, *H99 ri,p/TM3 Sb ri,p* individuals. *CyO*, *Sb* virgins and male *CyO*; *grim*-CosD, *H99 ri,p* progeny were mated, and in the following generation, individuals of the *grim*-CosA; *grim*-CosD, *H99 ri,p/TM3, Sb ri,p* genotype were selected by eye color and *ri*.

Isolation, characterization, and sequencing of *grim* cDNA

A 3-kb *HpaI*-*NotI* fragment from the *grim* cosmid was used to screen an embryonic cDNA library in λgt10 (Poole et al. 1985). Eight clones with similar sized inserts were isolated from ~500,000 clones screened. One of these, clone A, was confirmed to map to the H99 interval by Southern hybridization to both wild-type DNA and DNA isolated from H99 embryos. The insert was subcloned into the *EcoRI* site of pBluescript SK+ and sequenced by dideoxy-DNA sequencing using Sequenase

version 2.0 DNA Sequencing Kit (U.S. Biochemical) and ³⁵S-labeled dATP (Amersham).

Southern and Northern hybridization

Genomic DNA from adult flies was isolated according to Ashburner (1989). To isolate DNA from homozygous H99 embryos, samples were aged so that all embryos were older than ~18 hr and dechorionated before sorting. At this stage and beyond, mutants can be readily identified by virtue of their head defect and inability to hatch. DNA was isolated from ~300–600 embryos as in Ashburner (1989) except that the phenol/chloroform extraction was skipped and the DNA was precipitated with 0.5 volume of isopropanol. DNAs from ~100 sorted H99 embryos per lane were typically used for Southern hybridizations (Sambrook et al. 1989). As expected, probes mapping inside the H99 interval fail to produce hybridizing signals on H99 DNA, whereas probes outside the interval do. RNA was isolated from 7- to 12-hr embryos in a procedure similar to Chirgwin et al. (1979) and was processed for Northern hybridizations according to Sambrook et al. (1989).

Histological methods

AO staining was performed as in Abrams et al. (1993). Embryos were staged according to Campos-Ortega and Hartenstein (1985). TUNEL (Gavrieli et al. 1992) was as described performed (White et al. 1994). A *grim* RNA probe for use in whole-mount in situ hybridizations (Tautz and Pfeifle 1989) was transcribed from the linearized cDNA using the DIG RNA labeling kit (Boehringer Mannheim). Hybridizations were performed on 0- to 16-hr embryos from *yw* and H99/TM3 stocks. No signal was detected in H99 homozygotes. *In(3L)hid^{WR+X1}* embryos were also examined with the *grim* probe and identified by their head involution defect (Abbot and Lengyel 1991). To look for possible cross-regulation between *grim* and *rpr*, heat-shocked HS-*grim2.1* (see below) embryos were hybridized with a *rpr* probe (White et al. 1994), and heated *hsrpr46A* embryos were hybridized with the *grim* probe.

Construction of HS-*grim* transgenic strains and phenotypic analysis

An *EcoRI* fragment containing *grim* cDNA was inserted in the sense orientation into the pCaSpeR/*hsp70* vector (Grether et al. 1995) to generate the HS-*grim* plasmid. A mixture of 500 µg/ml of HS-*grim* DNA and 100 µg/ml of Δ2-3 helper plasmid DNA was injected into *yw* embryos. Two independent transformants (from a total of seven isolated) were mapped and examined in detail. For heat shock treatment, embryos from homozygous strains were collected at 25°C, aged at 18°C, heat-treated in a 39°C water bath for 30 min, and allowed to recover at 25°C for 1 hr. Subsequently, the samples were processed for either AO staining, TUNEL labeling, or in situ hybridization. Embryos from flies of homozygous HS-*grim2.1*, HS-*grim3*, and HS-*grim2.1*; *H99/TM3 Sb* were checked for *grim*-induced cell killing. Embryos from untransformed *yw* and *H99/TM3 Sb* flies were also subjected to the same heat shock treatment as a control. For comparisons to *rpr*-induced cell death, homozygous *hsrpr46A* and *hsrpr53A* (White et al. 1996) embryos were treated in parallel. To determine lethality, known numbers of 0- to 8-hr homozygous embryos were heat-treated and empty egg cases were counted after 24 hr at 25°C. The results cited are the combined results of two separate trials in which a total of 197 HS-*grim* embryos and 187 *yw* embryos were treated in parallel.

Construction of pGMR-grim transgenic strains and phenotypic analysis

An *EcoRI* fragment containing *grim* cDNA was cloned into the pGMR vector (Hay et al. 1994) in the sense orientation to generate the pGMR-*grim* transgene. This plasmid was injected into *yw* embryos at 500 µg/ml, together with the $\Delta 2-3$ transposase helper plasmid at 100 µg/ml to produce transgenic flies as described above. Seven of eight independent lines isolated had rough eyes that were substantially reduced in size, a phenotype consistent with increased cell death in the developing eye. One strain, pGMR-*grim1*, maps to the second chromosome and shows a representative phenotype that is more severe when homozygous. This strain was mated to pGMR-p35 (Hay et al. 1994) to generate flies homozygous for pGMR-*grim1* and pGMR-p35. For scanning electron microscopy, samples were coated with gold-palladium and examined in a JEOL 120-kV electron microscope.

Construction of pMT-grim, pMT-grimORF, cell culture, transfection, and cell death assays

pMT-*grim* was made by inserting a 1.1-kb *EcoRI*-*RsaI* fragment of *grim* cDNA into *EcoRI*-*BamHI*/blunt-digested pRmHa.3 vector (Bunch et al. 1988). The large *EcoRI*-*NcoI* fragment of pMT-*grim* was blunted using Klenow fragment and circularized to generate pMT-*grimORF*, which includes the hypothetical start codon through 429 bases downstream of the stop codon. Stably transfected cell lines were produced by cotransfection of 2 µg of test plasmid with 0.2 µg pCohygro (Van der Straten et al. 1989) into 1×10^6 Schneider L2 cells (Schneider 1972) cultured in Sf900 II SFM (GIBCO BRL) with 50 µg/ml of gentamycin (Sigma). After transfections with pMT-*grim* or pMT-*grimORF* DNA alone, or in combination with a similar amount of pMT-p35 plasmid (W. Nordstrom, P. Chen, H. Steller, and J.M. Abrams, in prep.), cells were selected with a supplement of 300 µg/ml of hygromycin (Sigma). For induction of the metallothionein-responsive promoter, cells were exposed to 700 mM CuSO₄.

For transient transfections, SL2 cells were plated at a density of 1×10^6 cells/well in six-well plates. Transfections were done with CellFECTIN (GIBCO BRL) according to the manufacturer's instruction. Forty-eight hours after transfection, cells from each well were split into two wells, and copper was added to one of the two wells. Two procedures were used to measure apoptosis frequency after gene induction in transiently transfected cells. One measure relies on the appearance of hypodiploid nuclei in flow cytometric assays (Tounekti et al. 1995) of propidium iodide-stained cells. After various treatments, cells were washed off the plates, fixed with 2% formaldehyde in PBS for 15 min, stained with 50 µg/ml of propidium iodide in the dark at 4°C, and analyzed within 24 hr on a Becton Dickinson FACScan flow cytometer using LYSIS II software. The alternative method relies on loss of cotransfected reporter gene activity to measure apoptosis (Hsu et al. 1995). An amount of 0.2 µg of the reporter plasmid (pActin-LacZ) was cotransfected with 2 µg of test plasmids. Forty-eight hours later, samples were induced, and after another 16 hr, β -gal activity was visualized by fixing cells for 30 min with 2% formaldehyde and staining with X-gal as described (Hsu et al. 1995).

Acknowledgments

We thank L. Cherbas for pActin-LacZ plasmid, B. Hay and G. Rubin for pGMR-p35 flies and the pGMR vector, H. Kramer for pMT-HA-*hook*, P. Friesen and L. Miller for p35 DNA, and H. Steller and K. White for the *hsrpr* strains. We also thank P. Lee, J. Varkey, and K. Plunk for excellent technical assistance. We

are grateful to Carol Lewis and Wilshirl Rowan for general help. This work was supported by grants to J.M.A. from the National Institutes of Health (AG12466) and from the Texas Advanced Technology Program (003660061).

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Note added in proof

The sequence data reported in this paper will appear in GenBank under accession number U61976.

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Genes Dev. 1996, **10**:

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