Debcl, a Proapoptotic Bcl-2 Homologue, Is a Component of the Drosophila melanogaster Cell Death Machinery

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Abstract. Bcl-2 family of proteins are key regulators of apoptosis. Both proapoptotic and antiapoptotic members of this family are found in mammalian cells, but no such proteins have been described in insects. Here, we report the identification and characterization of Debcl, the first Bcl-2 homologue in Drosophila melanogaster. Structurally, Debcl is similar to Bax-like proapoptotic Bcl-2 family members. Ectopic expression of Debcl in cultured cells and in transgenic flies causes apoptosis, which is inhibited by coexpression of the baculovirus caspase inhibitor P35, indicating that Debcl is a pro-apoptotic protein that functions in a caspase-dependent manner. debcl expression correlates with developmental cell death in specific Drosophila tissues. We also show that debcl genetically interacts with diap1 and dark, and that debcl-mediated apoptosis is not affected by gene dosage of rpr, hid, and grim. Biochemically, Debcl can interact with several mammalian and viral prosurvival Bcl-2 family members, but not with the proapoptotic members, suggesting that it may regulate apoptosis by antagonizing prosurvival Bcl-2 proteins. RNA interference studies indicate that Debcl is required for developmental apoptosis in Drosophila embryos. These results suggest that the main components of the mammalian apoptosis machinery are conserved in insects.

Key words: Bcl-2 family • BH domains • baculovirus P35 • Dark • caspase

Introduction

Programed cell death by apoptosis is essential to remove unwanted and superfluous cells during animal development and metamorphosis to maintain tissue homeostasis (reviewed in Jacobson et al., 1997; Vaux and Korsmeyer, 1999). Genetic studies in Caenorhabditis elegans have identified at least four genes, egl-1, ced-3, ced-4, and ced-9, that are essential for the regulation of all developmentally programmed death of somatic cells (reviewed in Metzstein et al., 1998). EGL-1, CED-3, and CED-4 are required for cell death to occur, whereas CED-9 is essential for cell survival (Yuan and Horvitz, 1992; Yuan et al., 1993; Hengartner and Horvitz, 1994; Conradt and Horvitz, 1998). In this developmental cell death pathway, EGL-1 functions upstream of CED-9, while CED-9 interacts with and regulates CED-4, which is required for CED-3 activation (Metzstein et al., 1998). The main apoptotic machinery has been conserved during evolution, and homologues of these C. elegans proteins are found in mammals. As expected, the pathways of cell death are considerably more complex in mammals, where EGL-1, CED-3, and CED-9 are represented by multiple family members. Although there is only one mammalian counterpart of CED-4, named A paf-1, currently known (Zou et al., 1997), adaptor molecules that act to recruit caspases to death complexes and mediate their activation can be seen as functional homologues of CED-4 (reviewed in Kumar, 1999; Kumar and Colussi, 1999).

CED-3, a cysteine protease of the caspase family, is the main downstream effector of apoptosis in C. elegans (Yuan et al., 1993). There are at least 14 mammalian homologues of CED-3, some of which play key roles in apoptosis (reviewed in Cryns and Yuan, 1998; Nicholson, 1999). The adaptor proteins, CED-4 in C. elegans, and A paf-1 in mammals, are essential for the activation of CED-3 and caspase-9, respectively (Li et al., 1997; Yang et al., 1998). CED-9 and its mammalian homologues, including Bcl-2,
Bcl-xL and Bcl-w, act as inhibitors of caspase activation and function upstream of CED-4/A paf-1 (reviewed in A dams and Cory, 1998; Gross et al., 1999). EGL-1 and its mammalian homologues share a small region of homology (BH3 domain) with CED-9/Bcl-2 proteins and act as proapoptotic proteins upstream of CED-9/Bcl-2 (Conradt and Horvitz, 1998; Gross et al., 1999). In addition to these proteins that are distantly related to Bcl-2, mammalian cells also express a number of proapoptotic members of the Bcl-2 family, such as Bax, Bak, and Bok. These proteins, termed the Bax subclass of proteins, contain three Bcl-2 homology (BH) domains, BH1, BH2, and BH3, but lack the NH2-terminal BH4 domain present in some (e.g., CED-9, Bcl-2, Bcl-xL, and Bcl-w) prosurvival members of the Bcl-2 family (A dams and Cory, 1998; Gross et al., 1999). Interestingly, no homologues of the Bax subclass of proteins have been found in C. elegans.

In Drosophila melanogaster, six caspases have been discovered so far (Fraser and van E, 1997; Inohara et al., 1997; Song et al., 1997; Chen et al., 1998; Dorstyn et al., 1999a,b). In addition, a CED-9/Bcl-2-like homologue, termed Dark/Dapaf-1/HAC-1, has recently been described (Kanuka et al., 1999; Rodriguez et al., 1999; Zou et al., 1999). Although the caspase(s) regulated by Dark is currently unknown, Dark can interact with two known Drosophila caspases, Dredd and Dronc (Kanuka et al., 1999; Rodriguez et al., 1999). So far, no CED-9/Bcl-2-like protein has been reported in the fly. Given the conservation of the cell death machinery, it is anticipated that Drosophila also has Bcl-2-like proteins. In this paper, we describe the identification of two Bcl-2 homologues in Drosophila, one of which, named Debcl, was characterized in detail. Debcl is a proapoptotic member of the Bcl-2 family that contains BH1, BH2, and BH3 domains. We show that Debcl is structurally related to the mammalian proapoptotic Bcl-2 family of proteins and functions in the execution of physiological cell death in Drosophila.

Materials and Methods

Cloning of Debcl and 48A-E cDNAs

Debcl and the 48A-E Bcl-2-like proteins were identified as genomic regions encoding putative Bcl-2 family members by TBLASTN searches using Bcl-2 protein sequence (accession numbers of the genomic sequence entries are indicated below). Full-length debcl cDNA sequence of 1,535 bp was obtained from BDGP clones GH 01265 and LD 12719, purchased from Research Genetics. A 950-bp partial cDNA clone for the 48A-E homologue was isolated from a mixed stage Drosophila embryo cDNA library using a 450-bp probe derived from Drosophila genomic DNA by PCR. Sequencing of this clone confirmed that it also encoded a Bcl-2 family member (Fig. 1 C). However, since the predicted reading frame in the sequence is open at the 5′ end, it is likely that the cDNA clone is not full length.

Plasmid Vectors for Expression in Cultured Cells

The 900-bp coding region of debcl was PCR amplified by Pfu polymerase (Stratagene) with an in-frame NH2-terminal HA tag and cloned into mammalian expression vector pcDNA3 (Invitrogen) and inducible Drosophila expression vector, pRMHa.3 (Bunch et al., 1988). pRMHa.3-Debcl was characterized in detail. Debcl is a Bcl-2–like protein. A, Genomic structure of the debcl gene at 42E–43A. The noncoding regions of the exons are shown as hatched boxes. B, Debcl protein structure. The relative positions of the three BH domains (BH1, BH2, and BH3) and a membrane anchor (MA) are shown as hatched boxes. C, An alignment of the Debcl sequence with Bok and 48A-E homologues. The sequence of the 48A-E homologue was obtained from a partial cDNA sequence isolated by us and the genomic sequence in the data base. The protein sequence of this clone is likely to be incomplete at the NH2 terminus. Residues identical in all three proteins are shown in black boxes and those similar shown in gray boxes. The positions of the two residues in the BH3 domain of Debcl, which were mutated in functional studies in Fig. 5 A, are indicated by an asterisk. D, A Kyte-Doolittle plot of the Debcl protein showing the putative MA region.

mRNA Expression Analysis

Total RNA from various developmental stages of Drosophila or adult flies was prepared using RNAzol B according to the manufacturer’s (Tel-Test Inc.) protocol. Poly A–enriched RNA was prepared using oligo dT magnetic beads (Dynal). Northern blots were prepared and hybridized with a 900-bp debcl open reading frame (ORF) probe as described (Dorstyn et al., 1999a,b). For reverse transcriptase (RT)-PCR, 1 μg total RNA was reverse transcribed using a first strand cDNA synthesis kit (A marsham Pharmacia Biotech). A aliquots of cDNA were subjected to 30 cycle PCR using primers from debcl ORF that generate a PCR product of ~450 bp.
labeled riboprobes were prepared using T7 and SP6 RNA polymerases from linearized pcDNA3-debcl as a template. Digoxigenin labeling was performed according to the manufacturer's instructions (Roche Biochemicals). In situ hybridization to Drosophila embryos and larval tissues was essentially as described (Dorstyn et al., 1999a,b), except that hybridization signals were further amplified using Tyramide Signal Amplification (TSA™) indirect system according to the protocol supplied by the manufacturer (New England Nuclear Life Science Products).

**Cell Death Assays**

Schneider L2 SL2 cells were maintained and transfected using Cellfectin (Life Technology) as described (Chen et al., 1996). For death assays, 1.5 x 10^5 SL2 cells were cotransfected with 1.6 µg vector or pMT-debcl (wild-type or mutants) and 0.4 µg µg PA-C-GFP reporter. 24 h later, cells were split into two halves, one of which was treated with 0.7 mM CuSO_4 for 8 h (for immunoblotting) or 16 h (for fluorescence microscopy). Where indicated, 50 µM ω-A-D-fmk (Enzyme Systems Inc.) was added to cultures at the time of addition of CuSO_4. A fter fixation with 4% paraformaldehyde, GFP positive cells were counted by fluorescence microscopy. Cell survival was calculated as the percentage of GFP positive cells in CuSO_4-treated cells, relative to the percent of GFP positive cells in untreated cells. The results, shown as average percentages ± SEM, were derived from three independent experiments. To check copper-induced Debcl expression, an 8-h CuSO_4 treatment, cells were lysed in SDS-PAGE buffer and lysates subjected to immunoblotting using an αHA antibody (Roche Biochemicals). SL2 cells were transfected using Fugene6 (Roche Biochemicals) with 1.0 µg of pcDNA3-3-debcl, and 0.2 µg of a β-galactosidase expression plasmid (pEF-β-gal; Kumar et al., 1994). Where indicated, cells were cotransfected with P35, Bcl-2, Bcl-xL, M IHA, and Bcl-x expression constructs (described in Uren et al., 1996; Dorstyn and Kumar, 1997; Huang et al., 1997). In these experiments, we used 3 µg of the specific inhibitor expression construct mixed with 1.0 µg of pcDNA3-3-debcl and 0.2 µg of pEF-β-gal. Cells were fixed and stained with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) 48 h after transfection, and β-galactosidase positive cells were scored for apoptotic morphology (Kumar et al., 1994; Dorstyn and Kumar, 1997). Data, presented as percent apoptotic cells as a fraction of total β-galactosidase positive cells ± SEM, were derived from three independent experiments.

**Analysis of Apoptotic Cells in Embryos**

For TUNEL, embryos were devitellinized and prepared as described (Chen et al., 1996). TUNEL was performed using a kit (Roche Biochemicals) and embryos were mounted in 70% glycerol. A acridine orange staining was according to a published protocol (A Brams et al., 1993).

**Transgenic Flies and Genetic Interaction Studies**

The PCR-amplified 900-bp debcl coding region tagged with HA (see above) was cloned into the pUA ST plasmid (Brand and Perrimon, 1993). Transgenic flies were generated and maintained essentially as described (Richardson et al., 1995). A homozygous line on the 2nd chromosome was used for the analysis of cell death in larval tissues. For genetic interaction studies, a line on the 3rd chromosome, UAS-debcl#6, was used to generate the strain GMR-GAL4/CyO; UAS-debcl#6/TM6B, which gave rise to adults with severely ablated eyes. This strain was crossed to strains containing a deficiency of a baculoviral P35 expression construct by lipofection as described previously (Richardson et al., 1995), and the eye phenotypes were scored for apoptotic morphology (Kumar et al., 1994; Dorstyn and Kumar, 1997). A total of 128 adult flies was obtained from the Bloomington stock center.

**Debcl/Bcl-2 Interaction Studies**

292T cells were cotransfected with HA-tagged Debcl in pcDNA3 and FLAG-tagged control vector or various Bcl-2 family proteins in the presence of a baculoviral P35 expression construct by lipofection as described (Huang et al., 1997). 36 h after transfection, cells were labeled with 100-200 µCi/ml ^35S-methionine (New England Nuclear) and cell lysates were prepared in lysis buffer (20 mM Tris-HCl, pH 7.4, 135 mM NaCl, 1% Triton X-100, 10% glycerol, supplemented with 0.5 µg/ml Pefabloc, 100 µg/ml soybean trypsin inhibitor, and 1 µg/ml each of leupeptin, apro tinin, and pepstatin). In some experiments, unlabeled cell extracts from transfected cells were used for immunoprecipitation/immunoblotting analyses. Equivalent trichloro-acetic acid precipitable amounts (5 x 10^6 cpm) or 1 mg of cell lysates were used for each immunoprecipitation with 5 µg of a control iso type-matched αE (Berkeley Antibody Co., Inc). αFLAG M2 (Sigma Chemical Co.) or αHA 11 (Berkeley Antibody Co. or Roche Biochemicals) antibodies. Immunoprecipitations were performed according to a previous protocol (Moriishi et al., 1996) and the immunoprecipitated material fractionated by SDS-PAGE. For in vitro labeling of αHA antibody, the signals were detected by fluorography after signal enhancement using Amplifi (A mersham Pharmacia Biotech). For unlabeled proteins, signals were detected by immunoblotting using the ECL detection system (Amersh am Pharmacia Biotech).

**RNA Interference (RNAi) Methods**

RNAi methods were essentially as described (Bhat et al., 1999; M isquitta and Paterson, 1999). debcl sense and antisense RNA transcripts were synthesized using the Ambion Megascript kit using linearized pcDNA3-debcl as a template. After purification and annealing, double-stranded RNA was dissolved in injection buffer (5 mM KCl in 0.1 M phosphate buffer, pH 7.8) at 0.75 mg/ml. Precellularized embryos were injected at 50% egg length as described (Bhat et al., 1999; Misquitta and Paterson, 1999). Embryos were aged at 18°C to stage 16 before TUNEL staining.

**Accession Numbers**

debcl sequence has been deposited in GenBank/EMBL/DBJ under accession number AF178430. The EST clones (BDGP clones GH01265 and LD12719) from which debcl sequence was derived have the accession numbers AA151093 and A1062455, respectively. debcl genomic sequence is contained in A C007624. Coding region for 48A-E Bcl-2-like protein is contained in A C007473.

**Results**

**Two Bcl-2 Homologues in Drosophila**

To identify Bcl-2-like proteins, we searched the Dro sophila sequence database using the TBLASTN program and identified two putative Bcl-2 homologues in the genomic regions 42E-43A and 48A-E. The 42E-43A region was represented in two EST clones, which were sequenced in full. The 1,535-bp full-length cDNA for the putative Bcl-2 homologue on 42E-43A contains a 410-bp 5′ untranslated region, a 900-bp coding sequence, and a 225-bp 3′ untranslated region. A comparison of the cDNA and genomic se quences identified three exons in this gene (Fig. 1 A). The cDNA encoded a Bcl-2-like protein consisting of 300 amino acids with an estimated molecular mass of ~33 kD. This was confirmed by in vitro translation of transcribed RNA (data not shown). We have named this protein Dbcl (pronounced debacle) for death executioner Bcl-2 homologue. Dbcl contains three of the BH domains, BH1, BH2, and BH3 (Fig. 1, B and C), but lacks the NH2-terminal BH4 domain found in some antiapoptotic members of the Bcl-2 family. The COOH terminus of Dbcl contains a putative hydrophobic membrane anchor, similar to that found in many Bcl-2-like proteins (Fig. 1, C and D).

Dbcl is most similar to the other putative Drosophila Bcl-2-like protein on region 48A-E, sharing 42% identity and 62% similarity in a 169 amino acid stretch (Fig. 1 C). A mong the published mammalian Bcl-2 family members, the homology is mostly limited to the regions that comprise the three BH domains. In this region, Dbcl shares the highest degree of homology (35% identity, 52% similarity) with Bok, a proapoptotic Bax subfamily member.
debcl expression was determined by RNA blotting, RT-PCR, and in situ hybridization to Drosophila embryos and tissues. In most cases low levels of debcl mRNA expression was detected. In RNA blots, a 1.5-kb transcript was evident in most developmental stages, but expression was relatively high in 0–4-h embryos and adult female flies (Fig. 2 A). In late embryos, larvae, and pupae debcl expression was somewhat reduced and barely detectable by Northern blotting. However, RT-PCR analysis indicated that debcl mRNA was present in all developmental stages examined (Fig. 2 B).

Because of low expression of debcl, we used tyramide amplification after hybridization to detect debcl mRNA expression in situ (Fig. 3). In early embryos, debcl mRNA was present uniformly, but became more concentrated in the tissues of the gut later in embryogenesis (Fig. 3, A–D). The relatively high levels of debcl RNA in early embryos (Fig. 3 A; data not shown for precellularized embryos) are likely to be derived maternally, as zygotic transcription does not begin until stage 5. From stage 14 embryos, debcl mRNA was detected in regions in the head that correspond to the pharynx and clypeolabrum, where many TUNEL positive cells are detected (Fig. 3 G). Expression could also be detected in a segmentally reiterated pattern in stage 14 embryos (Fig. 3 C) that may correlate with the TUNEL positive cells that are detected in the nervous system at this stage (Figs. 3 G and 4 C). During 3rd instar larval development, debcl expression was detected in the brain lobes in the outer proliferative center (Fig. 3 J), in the posterior part of the eye imaginal disc (Fig. 3 K), and in the gut (Fig. 3 N) where TUNEL positive cells were clearly seen (Figs. 3 O and 4, E and G). debcl expression was also clearly evident in the salivary glands, particularly in the ducts (Fig. 3 L). Because of background staining problems in salivary glands, acridine orange staining instead of TUNEL was used to detect apoptotic cells in this tissue. Using this technique, no apoptotic cells were detected in 3rd instar salivary glands (Fig. 4 K), suggesting that debcl expression may precede cell death in this tissue. High levels of debcl expression was detected in the nurse cell compartment of stage 10a ovaries (Fig. 3 P), which undergo apoptosis at stage 10b (Foley and Cooley, 1998). Thus, debcl expression late in embryogenesis, during larval development, and during oogenesis significantly correlates with tissues undergoing apoptosis.

Debcl Is a Proapoptotic Protein

To investigate whether Debcl is a pro- or antiapoptotic protein in vivo, we generated transgenic flies with the debcl cDNA under control of the yeast UAS-GAL4 promoter. Ectopic expression was then achieved by crossing these flies to various GAL4 drivers. To express debcl in all tissues at various developmental stages, UAS-debcl flies were crossed to hsp70-GAL4 flies and embryos or larvae were heat shocked. Heat shock-induced expression of debcl resulted in enhanced levels of TUNEL positive cells in the embryo (Fig. 4, B and D) and in larval tissues (Fig. 4, F and H; data not shown).

Tissue specific drivers were then used to express debcl during larval development. Ectopic expression of debcl in the posterior region of the eye imaginal disc using the GMR-GAL4 driver (Ellis et al., 1993) resulted in increased acridine orange staining cells in the posterior region of the eye (Fig. 4 I). Similarly, expression throughout the eye imaginal disc of 2nd instar larvae using the eyeless-GAL4 driver (Hauck et al., 1999) resulted in increased TUNEL positive cells in the anterior and posterior regions of the eye (Fig. 4 J). We predicted that expression of debcl from eye specific drivers would result in adults with ablated eyes, as does expression of rpr, hid, and grim from the GMR enhancer (White et al., 1994; Grether et al., 1995; Chen et al., 1996). Surprisingly, despite the increase in apoptotic cells seen in the imaginal discs, the adult flies from these crosses exhibited only a mild rough eye phenotype (data not shown), possibly because of the excess number of cells that are normally generated during eye development. However, other UAS-debcl lines, which presumably have a much higher level of expression, resulted in adults with severely ablated eyes when crossed to GMR-GAL4 (see below). We also expressed debcl in the larval salivary gland using a salivary gland specific driver, 109-88-GAL4, which resulted in a massive increase in acridine orange staining cells (Fig. 4 L) and a reduction in the size of the salivary glands (not shown). Thus, debcl induces cell death when ectopically expressed in a number of different tissue types during Drosophila development, indicating that Debcl is a proapoptotic protein of the Bcl-2 family.

Debcl Function Requires the BH3 Domain and Is Caspase-dependent

To characterize further the biological activity of Debcl, we...
expressed debcl in Drosophila SL2 cells under the control of an inducible insect promoter. Within 16 h of transfection, Debcl induced apoptosis in a majority of the transfected SL2 cells (Fig. 5 A). By 48 h, all debcl transfected cells had been lost (not shown). This cell death was partially inhibited by the cell permeable peptide caspase inhibitor zVAD-fmk and much more effectively by baculovirus caspase inhibitor P35, indicating that Debcl-induced apoptosis is, at least in part, mediated by caspases. While zVAD-fmk is an efficient inhibitor of many mammalian caspases, it is not known whether it can inhibit Drosophila caspases as effectively. Therefore, the partial inhibition of
Debcl-induced cell death by zVAD-fmk may reflect its inability to efficiently inhibit all Drosophila caspases. To confirm that Debcl’s cell killing function is dependent on caspase activity, we crossed debcl transgenic flies with GMR-p35 flies. As discussed below and shown in Fig. 6, in the resulting flies the effect of Debcl in eye ablation was significantly reduced.

In several proapoptotic Bcl-2 members, the BH3 domain is essential for their killing function (Adams and Cory, 1998; Gross et al., 1999). To determine whether the BH3 domain in Debcl was required for its proapoptotic function, we generated two substitution mutants (L146G and E151G) of the Debcl BH3 domain and analyzed their killing activity in SL2 cells. The L146 residue is conserved in the BH3 domains of most proapoptotic Bcl-2 members, whereas 151E corresponds to an acidic residue in most BH3 domains. Whereas the L146G mutation partially inhibited apoptosis induction by Debcl, E151G mutation completely abrogated Debcl-mediated cell killing (Fig. 5 A).

Drosophila proteins Grim, Reaper, and Hid are able to induce apoptosis in mammalian cells (McCarthy and Dixit, 1998; Claveria et al., 1998; Haining et al., 1999), despite the fact that mammalian homologues of these proteins have not been found. To determine whether Debcl can also induce apoptosis in mammalian cells, we cloned debcl cDNA in a mammalian expression vector and transfected it into NIH 3T3 cells. Most of the debcl-transfected cells underwent apoptosis (Fig. 5 B). When Debcl was cotransfected with expression vectors carrying caspase inhibitors P35, MIHA, or IAP (reviewed in Ekert et al., 1999), a substantial decrease in apoptosis was evident. These results indicate that to C. Strong TUNEL positive cells are observed in the gut (out of the plane of focus). E, A wild-type 3rd instar larval brain lobe (dorsal view) after heat shock showing low levels of TUNEL staining cells in the brain hemispheres (arrowheads) and in the ventral ganglion. F, A hsp70-GAL4 × UAS-debcl 3rd instar larval brain lobe (dorsal view) after heat shock-induced expression showing an increase in TUNEL positivity relative to E. Note that most of the TUNEL positive cells in the ventral ganglion are out of the plane of focus but extend all the way to the posterior end. G, A wild-type 3rd instar larval eye-antennal disc after heat shock showing only a few TUNEL positive cells. The arrowhead indicates the morphogenetic furrow (also in H, I, and J) after which there are a cluster of TUNEL positive cells. H, A hsp70-GAL4 × UAS-debcl 3rd instar larval eye-antennal disc after heat shock expression showing a large increase in TUNEL positive cells anterior to the morphogenetic furrow (arrow). K, A cridine orange staining of an eye disc from eyegless-GAL4 × UAS-debcl flies, which results in expression throughout the eye disc during 2nd instar larval development, and is strong in the anterior region in 3rd instar larvae showing an increase in TUNEL positive cells anterior to the morphogenetic furrow (arrow). L, A cridine orange staining of a 3rd instar larval salivary gland showing essentially no staining, even after long exposure. L, A cridine orange staining of a 3rd instar larval salivary gland from a 109-88-GAL4 × UAS-debcl, which results in strong expression in the embryonic and larval salivary glands (not shown), showing that there is strong staining of the large polyploid nuclei.
Dedebcl-induced killing is dependent upon its BH3 domain and requires caspase function. In addition to caspase inhibitors, coexpression of prosurvival Bcl-2 and Bcl-xL proteins also significantly inhibited Debcl-induced apoptosis (Fig. 5 B).

Genetic Interactions of debcl with p35, H99, diap1, and dark

After screening a number of UAS-debcl lines, two lines (UAS-debcl#26 on chromosome III and UAS-debcl#18 on chromosome II) were found that, when crossed to GMR-GAL4, gave rise to adults with severely ablated eyes (Fig. 6, B and D). To use this phenotype to examine genetic interactions, a stock was generated containing GMR-GAL4 (2nd chromosome) and UAS-debcl#26. To examine whether the rough eye phenotype was due to the activity of caspases, we crossed GMR-p35 to these flies and examined the eye phenotype of the progeny. As shown in Fig. 6 E, GMR-p35 significantly improved the severe rough eye phenotype of GMR-GAL4; UAS-debcl#26 eyes (Fig. 6 D). These results confirm that Debcl functions in a caspase-dependent fashion upstream of caspase activation.

To determine the involvement of rpr, hid, or grim in the GMR-GAL4; UAS-debcl#26 eye phenotype, we crossed these flies to a deficiency that removes all three genes (Df(3L)H99). If rpr, hid, or grim are rate limiting for Debcl function, then suppression of the GMR-GAL4; UAS-debcl#26 eye phenotype would be expected. However, no significant suppression of this phenotype was observed (Fig. 6 F), suggesting that the GMR-GAL4; UAS-debcl#26 eye phenotype is not dependent on the gene dosage of rpr, hid, or grim.

Next, we tested whether the inhibitor of apoptosis (IAP) homologue, diap1, genetically interacted with debcl, by examining the GMR-GAL4; UAS-debcl#26 eye phenotype when the dosage of diap1 was halved. Halving the dosage of diap1, using two different deficiencies, resulted in a strong enhancement of the GMR-GAL4; UAS-debcl#26 eye phenotype (Fig. 6 G). Furthermore, there was a significant reduction in the number of flies expected containing either of the diap1 deficiencies and GMR-GAL4; UAS-debcl#26. This was possibly due to leaky expression of the GMR-GAL4; UAS-debcl#26 construct in other tissues during development and the enhancement of this effect by reducing the dose of diap1. Thus, diap1 genetically interacts with debcl. We did not observe any genetic interaction between debcl and diap2 when a diap2 deficiency was crossed with GMR-GAL4; UAS-debcl#26 (data not shown).

Recently, a mutation in the Drosophila Apaf1/ced4 homologue, dark, has been described (Rodriguez et al., 1999). To assess the effect of reducing the dosage of dark on the GMR-GAL4; UAS-debcl#26 eye phenotype, a hypomorphic allele of dark (darkCD) was crossed to GMR-GAL4/CyO; UAS-debcl#26/T M6 flies. As shown in Fig. 6 H, reducing the dosage of dark suppressed the rough eye phenotype of GMR-GAL4; UAS-debcl#26 flies. Therefore, dark genetically interacts with debcl.

Debcl Interacts with Bcl-2 and its Prosurvival Homologues

Since Debcl induces cell death, which is partly inhibited by the overexpression of Bcl-2 and Bcl-xL, an attractive hypothesis is that Debcl binds to and neutralizes prosurvival Bcl-2 homologues. Because no prosurvival Bcl-2–like proteins have been identified so far in Drosophila, we tested if Debcl can bind to any of the known mammalian or viral prosurvival homologues of Bcl-2. In transient overexpres-
sion experiments in mammalian cells, Debcl associated with Bcl-2 and most of its functional homologues, although the binding to Bcl-xL, Mcl-1, and adenovirus E1B19K protein was relatively weaker (Fig. 7). For these interaction studies, we used a method involving radiolabeled cell extracts that allows the simultaneous detection of two interacting proteins in the same sample (Fig. 7 A). We also used conventional immunoblotting of the immunoprecipitated proteins and obtained similar results (Fig. 7 B). These data clearly show that Debcl can interact with most of the known prosurvival Bcl-2 proteins and is likely to induce cell death by the same molecular mechanisms as other proapoptotic Bcl-2-related proteins. These results further provide evidence for the functional conservation of Bax-like proteins in mammals and flies. We also tested whether Debcl interacts with the proapoptotic members of Bcl-2 family. In coimmunoprecipitation experiments, Debcl did not associate with any of the BH3-only proteins (Bik, Bid, Bad, and Bim) or the BH1-, BH2-, and BH3-containing proteins (Bax and Bak; data not shown).

Debcl Is Required for Embryonic Cell Death

Currently, no specific debcl mutants are available. Therefore, to examine the in vivo function of Debcl, we carried out RNAi studies to inhibit debcl gene function. RNAi is a
powerful technique to disrupt the function of specific genes (Hunter, 1999). Additionally, RNAi has the advantage of ablating maternally contributed mRNA that is difficult to achieve genetically. It was originally used in C.

Figure 8. debcl is required for developmental cell death in embryos. RNAi was used to ablate debcl function in embryos. Pre-cellularized embryos were injected with double-stranded debcl RNA and aged to stage 16 before fixation and TUNEL labeling. A, A uninjected control embryo showing the normal pattern of TUNEL labeling. B–E, Typical examples of injected embryos from the debcl RNAi experiment showing that the number of TUNEL positive cells is dramatically reduced compared with the control (A; see Fig. 3H). F, A buffer-injected control shows that the injection procedure does not inhibit apoptosis, but instead an increase in TUNEL positive cells is observed (compare A and F).

**Figure 7.** Debcl interacts with multiple prosurvival Bcl-2 family members. HA-tagged Debcl protein was coexpressed with the control vector or FLAG-tagged Bcl-2 family protein and a P35 expression vector. A, 35S-labeled cell lysates were immunoprecipitated (IP) with an isotype-matched control antibody (top), an αEE (control) antibody (middle), or an αHA antibody (3rd panel). In the lower panel, extracts from untransfected 293T cells were immunoprecipitated with the control, αHA, and αFLAG antibodies to determine nonspecific interactions. B, Further immunoblot analyses of immunoprecipitates were carried out to confirm the identity of various tagged proteins in transfected cells. In these experiments, unlabeled lysates prepared from transfected cells were used for immunoblotting (WB) and IPs. The top two panels depict the same blot probed sequentially with the rat αHA and the mouse αFLAG antibodies, respectively. In the αFLAG panel, the faint band around 35 kD is residual Debcl signal in the stripped blot. In the bottom two panels, mouse αFLAG immunoprecipitated proteins were immunoblotted with the rat αHA and the mouse αFLAG antibodies, respectively. The Ig light (L) and heavy (H) chain bands are indicated. Note that while αFLAG antibody can pull down both FLAG-tagged proteins (indicated by *) and the associated Debcl protein, αHA antibody mostly immunoprecipitates HA-tagged Debcl, not the associated proteins (not shown). This result suggests that the binding of αHA antibody to HA-Debcl precludes interaction between Debcl and Bcl-2 family members.
Debcl binds to many of the mammalian prosurvival Bcl-2 homologues. Thus, it is likely that Debcl functions in a manner analogous to the mammalian or worm proapoptotic Bcl-2-like proteins (A dams and Cory, 1998; Gross et al., 1999). These proteins probably act by binding to, and neutralizing the activity of, Bcl-2 or its closest relative, Bok, which has a single Bcl-2 homologue, CED-9, with the exception of diap1, but not diap2, genetically interacts with Debcl. Since diap1’s function as a prosurvival gene is antagonized by the genes of H99 complex (Wang et al., 1999), the enhancement of Debcl transgenic phenotype when diap1 dosage is halved simply may be due to reduced caspase inhibition in these flies. Future studies involving generation of Debcl mutant flies and crossing these flies with rpr, hid, and grim transgenic flies should address whether the H99 genes and Debcl indeed lie in the same death pathway.

Discussion

In this paper, we have described the identification of two Bcl-2 homologues in Drosophila. We have shown that one of these, Debcl, is a proapoptotic protein. Given that the existence of a proapoptotic Bcl-2 protein in Drosophila is now established, it can be envisaged that antiapoptotic Bcl-2 proteins are also present in insects. Unlike in C. elegans, which has a single Bcl-2 homologue, CED-9, Drosophila contains at least two such proteins. Interestingly, Debcl-like proteins, which are structurally similar to the mammalian Bax subclass, are not found in C. elegans. Also, considering that Drosophila contains at least six caspases, most of which have been implicated in apoptosis execution, it is likely that the degree of complexity of apoptotic pathways in the fly is much closer to that in mammals than in the worm.

Although debcl expression is low during embryonic and larval development, the expression appears to correlate with cell death in various tissues. With the exception of early embryos, our TUNEL data suggest that debcl may be expressed mainly in the cells that are destined to die. The function of higher debcl mRNA levels in early embryos is currently not known. Transgenic experiments show that ectopic Debcl expression is accompanied by potent apoptosis induction. This may explain why Debcl expression is mostly limited to cells that are committed to undergo programmed cell death during development. The closest mammalian relative of Debcl, Bok, is mainly expressed in adult reproductive tissues (Hsu et al., 1997). In adult female flies, Debcl expression is relatively high and may be mainly contributed by the ovaries. Thus, Debcl may function throughout embryonic and larval development, and also in the apoptosis of nurse cells in the adult ovaries. Our in vitro and in vivo data with P35 clearly shows that debcl-induced cell death is caspase-dependent and that Debcl lies upstream of caspases in the death pathway. Additionally, an inhibition of Debcl eye phenotype in dark mutant flies further substantiates the finding that Debcl lies upstream of the dark-mediated caspase activation pathway (Fig. 9). Given that Debcl RNAi suppresses most of the programed cell death in embryos, Debcl is likely to be a critical regulator of cell death during embryogenesis. Since embryos injected with the debcl double-stranded RNA do not progress into larval development, it was not possible to study the function of Debcl later in development using RNAi technique.

Our genetic data show that gene dosage of rpr, hid, and grim have no effect on Debcl-induced apoptosis. This suggests that Debcl either lies downstream of the H99 genes or on a separate pathway (summarized in Fig. 9). Our data also show that diap1, but not diap2, genetically interacts with Debcl. Since diap1’s function as a prosurvival gene is antagonized by the genes of H99 complex (Wang et al., 1999), it is possible that Debcl is a component of this pathway (Fig. 9). On the other hand, as diap1 is known to inhibit caspases (Wang et al., 1999), the enhancement of Debcl transgenic phenotype when diap1 dosage is halved simply may be due to reduced caspase inhibition in these flies. Future studies involving generation of Debcl mutant flies and crossing these flies with rpr, hid, and grim transgenic flies should address whether the H99 genes and Debcl indeed lie in the same death pathway.

Figure 9. Possible position of Debcl in the Drosophila cell death pathway leading to caspase activation. Several genetic and biochemical studies have established the possible hierarchy between various components of the Drosophila cell death machinery (reviewed in Abrams, 1999). The studies described in this paper place Debcl upstream of the P35-inhibitable, Dark-mediated caspase activation pathway. Debcl may lie downstream of the proteins of the H99 complex (Reaper, Grim, and Hid). However, further experiments are required to firmly place Debcl and H99 in the same genetic pathway.
tive protein with BH1, BH2, and BH3 domains (Fig. 1 D) available cDNA and genomic sequence can encode a puta-
vicious, such as Bax and Bak that contain the BH1, BH2, 
vided by observations that some proapoptotic Bcl-2 rel-
provided by altering cell fates and generating dominant phenotypes. Development. 118: 401–415.
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An additional level of complexity in mammals is pro-
vided by observations that some proapoptotic Bcl-2 rel-
tives, such as Bax and Bak that contain the BH1, BH2, and BH3 regions, may induce caspase-independent death (Xiang et al., 1996; Aptonsson et al., 1997), possibly by forming pores in membranes (Aptonsson et al., 1997). Al-
though Decbl belongs to this subclass of proteins, as dis-
cussed above, our studies indicate that Decbl-induced apop-
tosis is caspase-dependent. Mutation data indicate that
those containing voltage-dependent anion channel (VDAC; 
regulating the mitochondrial membrane pores, such as 
function by maintaining organelle integrity, possibly by 
regulating the release of apoptogenic factors, such as cytochrome c from the mitochondria (Green and Reed, 1998). Bcl-2 or its homologues may perform this 
does not require genes within the H99 deficiency. Development. 125:1075– 
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