An essential role for the caspase Dronc in developmentally programmed cell death in *Drosophila*°

Leonie M. Quinn§, Loretta Dorstyn#, Kathryn Mills#, Paul A. Colussi#, Po Chen†, Michelle Coombe§, John Abrams†, Sharad Kumar#†‡¶ and Helena Richardson§†‡¶

From the §Department of Genetics, The University of Adelaide, North Terrace, Adelaide, SA 5001, Australia, the #Hanson Centre for Cancer Research, Institute of Medical and Veterinary Science, Frome Road, Adelaide, SA 5000, Australia; and the †Department of Cell Biology, The University of Texas, Southwestern Medical Center, Dallas, TX 75235-9039, USA

*Running Title:* Dronc is required for cell death in *Drosophila.*
SUMMARY

Dronc is a caspase recruitment domain-containing Drosophila caspase that is expressed in a temporally and spatially restricted fashion during development. Dronc is the only fly caspase known to be regulated by the hormone ecdysone. Here, we show that ectopic expression of dronc in the developing fly eye leads to increased cell death and an ablated eye phenotype that can be suppressed by halving the dosage of the genes in the H99 complex (reaper, hid and grim) and enhanced by mutations in diap1. In contrast to previous reports, we show that the dronc eye ablation phenotype can be suppressed by coexpression of the baculoviral caspase inhibitor p35. Dronc also interacts, both genetically and biochemically, with the CED-4/Apaf-1 fly homolog, Dark. Furthermore, extracts made from Dark homozygous mutant flies, have reduced ability to process Dronc, showing that Dark is required for Dronc processing. Finally, using RNA interference technique, we show that loss of Dronc function in early Drosophila embryos results in a dramatic decrease in cell death indicating that Dronc is important for programmed cell death during embryogenesis. These results suggest that Dronc is a key caspase mediating programmed cell death in Drosophila.

INTRODUCTION

Caspases are cysteine proteases that act as central effectors of programmed cell death (PCD) (1-3). These proteases are synthesized as precursor molecules that are processed in cells undergoing apoptosis to generate two subunits that fold into a tetrameric active enzyme conformation. In mammals, there are 14 caspases described so far that can be grouped into two classes based upon the length of their prodomain (1-3). Caspases containing a long prodomain, such as caspase-2, -8, -9 and -10 appear to be activated first by a proximity induced autoprocessing mechanism, involving clustering of procaspace molecules, often assisted by specific adaptor molecules (4, 5). These caspases contain specific protein-protein interaction domains in the prodomain region that mediate their interaction with their respective adaptors, or mediate dimerization of procaspace molecules (4, 5). For example, caspase-2 and caspase-9
contain caspase recruitment domains (CARD) in their prodomain, while caspase-8 and caspase-10 contain two copies of death effector domains (DED) (6-8). The CARD in caspase-2 is required for homodimerization (9, 10), whereas the CARD in caspase-9 interacts with the mammalian CED-4-like adaptor Apaf-1 (11). Cytochrome c- and dATP-dependent oligomerization of Apaf-1, followed by interaction of oligomerized Apaf-1 with procaspase-9 results in proximity induced activation of caspase-9 (11, 12). One of the DEDs in caspase-8 interacts with the DED in the adaptor FADD, a molecule that helps recruit procaspase-8 to activated death receptors of the TNFR family (6, 7). Again, this adaptor-mediated recruitment of the procaspase molecules is believed to be sufficient for caspase activation (8). Once the caspases containing long prodomains are activated, they are believed to activate downstream caspases that lack specific protein-protein interaction domains, thereby initiating a cascade of caspase activation (1-3).

In Drosophila melanogaster, three proteins, Reaper, Hid and Grim, play critical roles in apoptosis (13). These proteins act upstream of caspase activation and appear to be required to counteract the caspase inhibitors Diap1 and Diap2, Drosophila homologs of the baculovirus inhibitor of apoptosis, IAP, P35 (13, 14). In Drosophila, specific mutations in diap1 (thread), show increased cell death in the embryo (15-18) and Diap1 has been shown to bind to and inhibit the activity of Drosophila effector caspases (18, 19). A CED-4/Apaf-1 homolog called Dark/Hac-1/Dapaf-1 (20-22), and a pro-apoptotic CED-9/Bcl-2 homolog, Debcl/Drob-1/dBorg-1 (23-25) have recently been described in Drosophila. There are seven known caspases in Drosophila, including 5 published ones, Dcp-1, Dredd, Drice, Dronc, and Decay (26-33) and two unpublished ones, Damm and Strica (accession numbers AF240763 and AF242734, respectively). Dredd and Dronc contain long prodomains, carrying DEDs and a CARD, respectively, suggesting that these two caspases may act as upstream caspases. Strica also contains a long prodomain, but it lacks any CARD/DED sequences. Strica also contains a long prodomain, but it lacks any CARD/DED sequences. On the other hand, Dcp-1, Drice, Decay and Damm lack long prodomains and are thus similar to downstream effector caspases in mammals. A dcp-1 mutation results in larval lethality and melanotic tumors (26). Additionally, dcp-1 mutants show a defect in transfer of nurse cell cytoplasmic contents to developing oocytes, suggesting that it may also be required for Drosophila oogenesis (34). As no mutants for other Drosophila caspases are currently available, their precise functions remain
unknown. However, a number of indirect observations point to a role for other *Drosophila* caspases in apoptosis *in vivo*. For example, *dredd* mRNA accumulates in embryonic cells undergoing programmed cell death and in nurse cells in the ovary at a time that coincides with nurse cell death (27), *dronc* mRNA although widely expressed during development, is upregulated by ecdysone in larval salivary glands and midgut prior to histolysis of these tissues (30), and antibody depletion experiments suggest that Drice is required for apoptotic activity in the S2 *Drosophila* cell line (35). Furthermore, a deficiency uncovering *dredd* dominantly suppresses the ablated eye phenotype due to ectopic expression of *rpr, hid or grim*, and a mini-gene of *dredd* reverses this suppression, showing that *dredd* is important for PCD *in vivo* (27).

We have previously shown that overexpression of Dronc induces cell death in mammalian cells (30). In two recent studies (36, 37) it was shown that ectopic expression of *dronc* promotes apoptosis in the developing *Drosophila* eye that can be suppressed by co-expression of *diap1*. Furthermore, in yeast, Diap1 inhibited Dronc activity and this inhibition was abrogated by co-expression of Hid or Grim (37). Diap1 binds to the prodomain of Dronc, and consistent with this, expression of a truncated version of Dronc lacking the prodomain resulted in a more severe ablated eye phenotype that could not be rescued by co-expression of Diap1 (36). These studies also showed that a mutated version of *dronc* (containing a mutation in the caspase active site) acted in a dominant negative manner to suppress *rpr* and *hid*-induced cell death (36). Likewise a deficiency removing the *dronc* gene was able to dominantly suppress *rpr* and *hid*-induced cell death in the eye (36), showing that Dronc mediates cell death by Rpr and Hid. In addition, the studies showed that Dronc associates with the effector caspase Drice and is able to process Drice to the active form (36, 37). Surprisingly, it was found that Dronc activity was not inhibited by the baculoviral caspase inhibitor, P35, nor could Dronc cleave P35 (36, 37).

Here we demonstrate that ectopic expression of *dronc* at various developmental stages results in apoptosis. As previously demonstrated (36, 37), we show that *dronc* overexpression results in an ablated eye phenotype that can be modified by halving or increasing the dosage of *diap1*. In addition, we show that the *dronc* eye phenotype can be suppressed by reducing the dosage of the *H99* complex genes or of the *APAF-1/ced-4* homolog, *dark*. In contrast to previous studies, we show that the *dronc* eye ablation phenotype can be blocked by
coexpression of the baculoviral caspase inhibitor p35 (13). Confirming these genetic interactions, Dronc can form complexes with Diap1, Grim, Dark and P35, however the interaction between Dronc and Grim or P35 are indirect. We also show that Dark is required for Dronc activation, since Dronc is poorly processed in extracts from Dark homozygous mutant flies. Since specific dronc mutants are currently unavailable, we used the technique of RNA interference (RNAi) to ablate dronc mRNA in embryos. Our results show that Dronc is essential for cell death during embryogenesis and suggest a central function for Dronc in the cell death effector machinery in Drosophila.

MATERIALS AND METHODS

DroncTransgenic Flies and Genetic Interaction Studies - Wild type dronc or the droncC318G (droncCG) mutant constructs, tagged with GFP were cloned into the pUAST plasmid (38), by ligating a NotI-EcoRI fragment containing dronc-GFP from pEGFPN1-dronc or pEGFPN1-droncCG (30) into the corresponding sites of pUAST. Transgenic flies were generated as described (39). Several lines of UAS-dronc or UAS-droncCG were crossed to the eye specific enhancer GMR-GAL4 to analyze the eye phenotype. Many of the UAS-dronc lines were lethal at 25 °C, while other lines gave only a few adult survivors with severe eye defects. None of the UAS-droncCG lines when crossed to GMR-GAL4 resulted in lethality or eye defects. Two less severe lines UAS-dronc#80 and UAS-dronc#23 (which both contained a double insert of the transgene on the 2nd and the 3rd), were used for genetic analysis. For both of these lines, recombinants were generated containing UAS-dronc and GMR-GAL4 (2nd chromosome) and were made homozygous for the UAS-dronc insert on the 3rd.

For genetic interaction tests, both UAS-dronc GMR-GAL4 lines were crossed at 25 °C or 29 °C to wild type (w1118) flies or strains containing GMR-p35, GMR-diap1, GMR-diap2 (15), a deficiency of rpr, hid and grim, Df(3L)H99, deficiencies of diap1, Df(3L)brm11 and Df(3L)stf-13, a specific loss-of-function allele of diap1, thread5 (15), a deficiency of diap2, Df(2R)Jp1, or P allele mutations of dark, darkCD4 (hypomorphic allele), darkCD8 (hypomorphic allele) and l(2)k11502 (hypomorphic allele) (20). For testing the interaction of a dronc deficiency (Df(3L)AC1 at 67A2-67D13) with GMR-hid (40) or GMR-rpr (41), crosses were
carried out at 25 °C and 18 °C, respectively. Progeny were scored by examining the eye phenotypes using light or scanning electron microscopes, as previously described (42). Deficiency, GMR-p35 (3rd chromosome) and l(2)k11502 stocks were obtained from the Bloomington stock center.

αDronc Antibody - A fragment of dronc cDNA encoding the 126 carboxyl-terminal amino acids was amplified by PCR and cloned into the EcoRI site of pGEX-4T3 (Pharmacia) to generate a GST-dronc fusion construct. The GST-Dronc fusion protein was purified and used to inoculate rabbits. GST-interacting antibodies were removed by passing serum through a GST-Sepharose column. αDronc polyclonal antibodies were further purified by affinity chromatography using a Dronc-antigen column.

Dronc Interaction Studies - The analysis of the interaction of Dronc with Diap1, Diap2, Rpr, Grim or Hid was carried out in mammalian 293T cells after transfection of the tagged constructs, pcDNA3-dronc-GFP and pcDNA3-diap1-HA, or pcDNA3-diap2-myc, or pcDNA3-rpr-flag, or pcDNA3-hid-flag, or pcDNA3-grim-flag, or with only dronc-GFP. For the Dronc and P35 interaction, SL2 cells were transfected with HA- and 6xHis-tagged Dronc and HA-tagged P35, both in pRMHa.3 vector. Following induction of expression by CuSO4 (23), 6xHis-tagged Dronc and associated proteins were precipitated by Talon resin (Clontech) and Dronc and P35 detected by immunoblotting using an anti-HA antibody. For analyzing Dronc/Dark interaction, SL2 cells were transfected with Myc-tagged Dark(1-411) alone or with Flag-tagged Dronc. Myc-tagged Dark(1-411) was immunoprecipitated with an αMyc antibody and pelleted protein complexes and the original lysates were analyzed after SDS-PAGE by immunoblotting with an αFlag antibody. pMT-dark(1-411) was described previously (20). pMT-droncC/A-flag was constructed by ligating a BamHI/ApaI fragment from pCDNA3-droncC/A-flag (a gift from N. Inohara) into pMT vector. Cell culture and immunoprecipitations were carried out as previously described (23).
**Protein Binding Assays**- Full length P35\_\text{HA}, Grim\_\text{FLAG}, and Diap1\_\text{MYC} were in vitro translated from pCDNA3 templates using TNT T7 coupled Reticulolysate Lysate System (Promega). P35\_\text{HA} and Grim\_\text{FLAG} were purified by immunoprecipitating overnight at 4 °C in a total volume of 400 µl of caspase assay buffer (0.1 M Hepes pH 7, 0.1% CHAPS, 10% polyethelene glycol, 10 mM dithiothreitol., supplemented with complete™ protease inhibitors (Boehringer)). Proteins were pulled down with protein G-sepharose (Pharmacia) and unbound protein washed off with wash buffer (50 mM Tris-HCl pH 7, 150 mM KCl, 2 mM dithiothreitol, 0.025% Triton-X 100). 5 µl of P35\_\text{HA} and Grim\_\text{FLAG}, immobilised on protein G-sepharose, was added to either purified Dronc-GST or and/or 35S-Diap1 in a total volume of 400 µl caspase assay buffer. After incubation at 4 °C for 3 h, beads were washed twice with 100 volumes of wash buffer and twice in 100 volumes of phosphate buffered saline before SDS-PAGE and immunoblotting to a PVDF membrane.

**Western Blotting**- PVDF membranes were blocked overnight at 4 °C, in 5% skim milk in phosphate buffered saline containing 0.05% Tween20 (PBS-T). Blots were probed with purified anti-Dronc polyclonal antibody at a dilution of 1:200 for 2 h at room temperature. This was followed by incubation with anti-rabbit IgG conjugated with horseradish peroxidase (Amersham) for 1 h. Signals were detected by ECL (Amersham).

**Recombinant Dronc and Caspase Assays**- Full length Dronc was amplified by PCR and cloned into the EcoRI site of pGEX4T1 (Pharmacia). Plasmid pGEX4T1-dronc was then transformed into Escherichia coli strain BL21 (DE3) cells. After induction with 1mM IPTG at 37 °C for 3 h, the Dronc-GST fusion protein was prepared from the soluble fraction and affinity purified on glutathione sepharose as per manufacturer’s protocol (Pharmacia). Protein lysates were prepared as follows: Wild type (w\textsuperscript{1118}) or dark\textsuperscript{CD8} adult flies were frozen in liquid nitrogen and homogenised using a mortar and pestle. Approximately 10-20 lysed flies were resuspended in 300-500 µl caspase assay buffer. Lysates were centrifuged at 13000 rpm for 10 min at 4 °C, and supernatants were then removed and centrifuged again for 10 min. Protein concentration of
lysates was determined using the BCA kit as per manufacturers protocol (PIERCE). A total of 100 µg of protein from cleared lysates was assayed for caspase activity by incubating with 100 µM DEVD-7- amino-4-methylcoumaride (-amc) (Enzyme System Products), VDVAD-amc (California Peptide Research) or VEID-amc (Bachem), in a volume of 40 µl in caspase assay buffer at 37 °C for 30 min. Fluorescence was quantified on a Luminescence Spectrometer (Perkin-Elmer) (excitation 385 nm; emission 460 nm).

In vitro Cleavage of Dronc- The cDNA construct pOT2-dronc was used as a template for the production of 35S-methionine (ICN) labelled Dronc protein using a TNT T7 coupled Reticulolysate Lysate System (Promega). 5 µl of labelled protein was incubated in proteolysis assays at 37 °C for 3 h with protein extracts prepared from wild type or darkCD8 flies (100 µg total protein) in caspase assay buffer in a total volume of 20 µl. 20 µl of 2 x protein loading buffer (100 mM Tris-HCl pH 6.8, 200 mM dithiothreitol, 20% glycerol, 4% SDS, 0.2% Bromophenol Blue) was then added and proteins denatured by boiling for 5 min, centrifuged at 13000 rpm for 5 min and cleavage products resolved by 15% SDS-polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidine difluoride membrane (NEN Life Science Products) using semi-dry apparatus (Biometra) and visualised by autoradiography.

Cell Culture and Transient Transfections- Schneider L2 (SL2) cells were maintained in Schneider Cell Medium (GIBCO) supplemented with 10% fetal calf serum at 27 °C. For cell death assays 2x10^6 cells were seeded into 60 mm dishes in 2 ml medium the day before transfection. Cells were co-transfected with 1.5 µg vector or pMT-dronc (wild type, C318G mutant or minus pro-domain [MPD] containing amino acid residues 114-450 from Dronc) and 0.5 µg of the heat shock inducible pCasper.hs-lacZ reporter, using Cellfectin reagent as per manufacturer protocol (GIBCO). Where indicated pMT-MPD was co-transfected with pMT-p35, diap1, diap2 or droncC318G expression constructs at a ratio of 1:2. 16 h after transfection, cells were heat shocked at 37 °C for 30 min and then allowed 30 min recovery time at 27 °C.
This was repeated another two times. Following heat shock, cells were split into halves in two 35 mm dishes, one dish treated with 0.7 mM CuSO₄. Where indicated, 50 µM z-VAD-fluoromethylketone (Enzyme Systems Inc) was added to cells at the time of CuSO₄ addition. 48 h after CuSO₄ induction, cells were stained for β-galactosidase and survival percentage calculated as the % β-gal positive cells in CuSO₄ treated cells relative to % β-gal positive cells in untreated dishes.

**Immunohistochemistry and Staining of Apoptotic Cells** - Antibody staining of Drosophila tissues was as previously described (42). The 22C10 antibody developed by S. Benzer was obtained from the Developmental Studies Hybridoma Bank, University of Iowa. Acridine orange staining (43) and TUNEL assays (44) were used to detect apoptotic cells. The α-GFP antibody (rabbit) was obtained from P. Silver.

**RNAi Methods** - dronc RNAi was preformed using previously described methods (45, 46). RNA transcripts were generated using the Ambion Megascript kit with linearised pOT2-dronc templates. Sense and antisense transcripts were purified, annealed and then dissolved in injection buffer (5 mM KCl in 0.1 mM phosphate buffer, pH 7.8) at 0.75 mg/ml. Precellularized embryos were injected at 50% egg length and aged until stage 11-13.

**RESULTS**

**Dronc Induces Apoptosis** - In order to examine the function of Dronc in a whole animal, transgenic flies were generated containing Dronc tagged with GFP or the inactive dronc mutant, dronc⁰⁰³₁₈₉ also tagged with GFP, under the control of the yeast UAS(GAL4) in pUAST (38). Expression of these constructs was then achieved by crossing flies to various GAL4 drivers. To show that the constructs were expressing, we crossed UAS-dronc and UAS-dronc⁰⁰³₁₈₉ flies to flies containing the GMR-GAL4 driver, allowing expression in the posterior region of 3rd instar larval eye imaginal discs (47). Eye imaginal discs from these 3rd instar larvae stained specifically in the posterior region with αGFP and αDronc antibodies (Fig. 1A-D), demonstrating that high
levels of specific expression were achieved and that the αDronc antibody was specifically
detecting Dronc protein. To determine whether ectopic overexpression of dronc could induce
cell death, we then carried out acridine orange staining of these eye imaginal discs to detect
dying cells. Expression of the droncC318G construct had little effect on the normal pattern of
dying cells in the eye imaginal disc (Fig. 1E; and data not shown), whereas wild type dronc
expression resulted in a massive induction of cell death in the posterior part of the eye disc (Fig.
1F). Expression of dronc during embryogenesis or in different tissues during larval
development using the heat shock inducible hsp70-GAL4 driver also resulted in ectopic cell
death (Fig. 1J).

To examine the phenotypic consequence of expression of dronc in the eye disc we
allowed progeny of the cross of GMR-GAL4 to the UAS-dronc construct to develop into
adults. Many died as pupae (data not shown), which has been previously observed and
attributed to the poor ability of the adults to break through the pupal case (36). The few adults
from this cross that survived, exhibited severely ablated eyes (Fig. 1H), as has been previously
observed (36, 37). By contrast, no death during the pupal stage was observed with flies from
the cross of GMR-GAL4 to UAS- droncC318G and adult flies showed normal eyes (Fig. 1G).
Thus the expression of dronc results in an almost complete ablation of the eye, similar to that
obtained with expression of the apoptosis inducers rpr, hid or grim from the GMR enhancer
(40, 41, 44, 48).

The GMR-Dronc Eye Phenotype is Suppressed by p35 - To examine genetic interactions
between Dronc and other apoptotic pathway genes, we chose two other UAS-dronc transgenic
lines (#23 and #80) that resulted in less lethality when crossed to GMR-GAL4, and generated a
recombinant 2nd chromosome for each of these transgenes with GMR-GAL4. When GMR-
GAL4 UAS-dronc#80 was crossed to wild type w1118 flies at 25 °C, adult flies that exhibited
slightly rough and mottled eyes were observed (Fig. 2G compared with 2A). A similar
phenotype has been observed in previous studies (36, 37) and has been shown to be due to
ablation of the pigment and photoreceptor cells (36). Similar results were observed for GMR-
GAL4, UAS-dronc#23 in this and in all other crosses described below (data not shown). This
phenotype became more severe when expression of dronc via GMR-GAL4 was increased by
raising the temperature to 29 °C (Fig. 2B). Since this eye phenotype can be modified by increasing the expression of dronc, it provided a dosage-sensitive system for examining genetic interactions between dronc and other genes of the apoptosis pathway. To test this further, we examined whether coexpression of the baculovirus caspase inhibitor P35, from the GMR enhancer was able to suppress the eye phenotype of GMR-dronc at 29 °C. As shown in Figure 2C, coexpression of GMR-p35 dramatically improved the eye ablation phenotype of GMR-dronc. Thus, in our system Dronc is sensitive to P35 in the Drosophila eye.

We also examined whether Dronc was able to induce cell death in the hemocyte-derived SL2 cells. Transfection of these cells with full-length Dronc surprisingly only resulted in 25% cell death (Fig. 3). Since previous studies have shown that Diap1 binds to the prodomain of Dronc and may inhibit Dronc function (36), we transfected a truncated version of Dronc lacking the prodomain (MPD-Dronc) into SL2 cells. This resulted in a significant increase in cell death (50%). Since previous studies had failed to observe an effect of the caspase inhibitor P35 on dronc-induced cell death (36, 37), we tested whether co-transfection of P35 could suppress MPD-Dronc induced cell death. In contrast to previous results, P35 was able to significantly suppress MPD-Dronc induced cell death in SL2 cells. This result is consistent with our previous observation showing that P35 inhibits Dronc-induced cell death in a mammalian overexpression system (30). However, it should be noted that co-expression of P35 did not rescue MPD-Dronc induced cell death as well as Diap1, z-VADfmk or the dominant negative Dronc mutant, DroncC318G, although rescue was significantly better than observed with Diap2 (Fig. 3).

The H99 Genes, dark and diap1 Genetically Interact with dronc - We then examined whether the GMR-dronc eye phenotype was sensitive to halving the dosage of the various Drosophila apoptosis regulatory genes. To assess whether the GMR-dronc eye phenotype was sensitive to the dosage of the H99 genes (reaper, hid and grim), we crossed GMR-dronc flies to a deficiency removing the H99 genes, Df(3L)H99 at 29 °C. As shown in Figure 2D, the H99 deficiency dominantly suppressed the GMR-dronc eye phenotype. Thus the cell death inducing activity of dronc is sensitive to the dosage of the H99 genes. Furthermore, as previously
observed (36), halving the dosage of dronc using a deficiency modified the ablated eye phenotype of GMR-hid and GMR-rpr, suggesting that dronc is downstream of hid and rpr (Fig. 2M-P). To determine whether there was a genetic interaction with dronc and dark, we examined whether decreasing the dosage of dark modified the eye phenotype of GMR-dronc at 29 °C. Three different P-element alleles of dark, darkCD4, darkCD8 and dark12,11502, showed suppression of the GMR-dronc eye phenotype (Fig. 2E, F and data not shown), indicating that Dark plays a role in promoting Drone-induced cell death in the eye. As observed previously (36, 37), halving the dosage of diap1 using deficiencies or the specific allele thread5, dominantly enhanced the GMR-dronc eye phenotype at 25 °C (Fig. 2G, I compared with 2G; and data not shown). In addition, these diap1 mutations dominantly enhanced the lethality associated with GMR-dronc, resulting in at least ten fold lower numbers of GMR-dronc/+; Df(diap1)/+ adult flies than expected. In contrast, a deficiency removing diap2 showed no effect on the GMR-dronc phenotype (Fig. 2J) and no lethal effects were observed. Thus diap1, but not a deficiency removing diap2, shows a dosage sensitive interaction with dronc. By contrast, ectopic expression of diap1 or diap2 from the GMR promoter showed suppression of the GMR-dronc ablated eye phenotype although GMR-diap2 resulted in much weaker suppression than GMR-diap1 (Fig. 2K, L compared with 2G). Thus both Diap1 and Diap2 are capable of directly or indirectly blocking Drone-mediated cell death.

Dronc Forms Complexes with Diap1, Grim, and P35 - We analysed the biochemical interaction of Dronc with various apoptosis regulators by coprecipitation methods. Previous studies have shown that Dronc can form a complex with Diap1 in mammalian 293T cells (36). When we transfected 293T cells with Dronc-GFP and Diap1-Myc or Diap2-Myc, Diap1, but not Diap2, was present in the Dronc immunoprecipitated complex (Fig. 4A and data not shown), showing that Dronc and Diap1 can interact. We have also observed that Dronc can form a complex with Diap1, but not with Diap2, in SL2 cells (not shown). Next we examined the H99 proteins, Rpr, Hid and Grim after transfecting 293T cells with Dronc-GFP and with Rpr-Flag, Hid-Flag or Grim-Flag constructs. Proteins associating with αGFP immunoprecipitated Dronc were immunoblotted with the αFlag antibody. As shown in Figure 4B, Dronc coimmunoprecipitated
with Grim, but not with Rpr or Hid, suggesting that Dronc forms a complex with Grim. We then examined whether Dronc could form a complex with P35 by transfecting SL2 cells with Dronc tagged with the HA and 6xHis epitopes and with HA-tagged P35. As shown in Figure 4C, P35 was present in the Dronc complex, indicating that these proteins can associate.

To determine whether the interactions of Dronc with Grim or P35 were direct we tested whether these proteins could interact in vitro. As shown in Fig. 5, while Grim could bind to Diap1 in vitro, Dronc was unable to bind to either Grim or P35. Thus under the conditions where Grim and Diap1 could interact, Dronc and P35 or Grim did not interact, suggesting that the interaction observed between Dronc and Grim or P35 in cells is indirect. Furthermore, the addition of Diap1 allowed Grim to be co-immunoprecipitated with Dronc (Fig. 5 lane 7), suggesting that the complex formed in vivo between Dronc and Grim may be mediated by Diap1.

The Amino-Terminal Region of Dark Associates with Dronc - The amino-terminal region of Dark containing the CARD and CED-4/Apaf-1 homology domains, but lacking the WD40 repeats, has been shown to bind to Dredd and Drice (14, 20). Furthermore in mammalian tissue culture cells, Dark has been shown to bind to Dronc (21). To investigate the region of Dark required for the interaction of Dark with Dronc, we transfected SL2 cells with a construct containing a Myc-tagged Dark amino-terminal region (Dark 1-411), containing the CARD and CED-4/Apaf-1 homology domains, alone or with a Flag-tagged-Dronc construct. Dark-Myc was immunoprecipitated with αMyc antibodies and pelleted complexes and cell lysates were analyzed by immunoblotting with αFlag antibodies (Fig. 4D). Dronc-Flag protein (50% of protein present in the lysate) was detected in the αMyc-Dark immunoprecipitate (Fig. 4D lane 4). Thus, the amino-terminal region of Dark containing the CARD domain and the CED-4/Apaf-1 homology region is sufficient for Dronc association in vivo.

Dark is Important for Dronc Processing - To test the requirement of Dark for Dronc activation (21, 21), we examined extracts prepared from dark<sup>CD8</sup> homozygous flies for caspase activity and for their ability to cleave Dronc in vitro. As shown in Fig. 6A, dark mutant flies had
reduced caspase activity compared with wild type for several caspase substrates. VEID is the preferred substrate for Dronc (37), although we have previously shown that VDVAD is also cleaved well by Dronc (30). DEVD is a caspase 3 substrate that is cleaved poorly by Dronc, but preferred by the downstream caspases, Dcp1, Decay and Drice (30, 33, 37). Thus dark mutant extracts contained lower cleavage activity towards both preferred Dronc substrates and preferred downstream caspase substrates. Lower caspase activity has also been observed previously in extracts from dark mutant embryos (21). Furthermore, dark mutant extracts showed considerably reduced ability to cleave Dronc to its active form (Fig 6B), showing that Dark is important for Dronc processing. Since darkCD8 (and darkCD4) are hypomorphic mutants, but it is not known whether they are completely null because a deficiency of the dark region is not available, the residual Dronc processing we observe may be due to residual Dark activity or to an alternative mechanism.

Dronc is Essential for Embryonic Cell Death - Since specific mutations in dronc are currently not available, we used the technique of RNA mediated interference (RNAi) to ablate dronc gene function during embryogenesis. RNAi, a technique developed in C. elegans, has recently been successfully used in Drosophila and mammalian cells to specifically ablate gene function (22, 23, 45, 46, 49-51). Dronc double-stranded mRNA was injected into precellularized embryos and samples were aged until stage 13. Embryos were analyzed by Dronc antibody staining to assess the efficiency of the Dronc protein ablation and TUNEL assays to reveal apoptotic cells. In addition, embryos were also stained with neural differentiation marker Mab 22C10 (52), to reveal whether ablation of dronc was affecting neural development. At stage 13, uninjected embryos show Dronc expression throughout the embryo and a large number of TUNEL positive cells (Fig. 7A, C). In contrast, in stage 13 dronc RNAi embryos Dronc protein was undetectable and very few TUNEL positive cells were observed (Fig. 7B, D, G, I). Buffer injected control embryos showed no decrease in cell death, but rather more cells were TUNEL positive (Fig. 7N). We analyzed at least 400 dronc RNAi injected embryos, and the results obtained were consistent for all embryos. Although dronc RNAi injected embryos failed to hatch, examination of embryonic structures using Nomaski optics showed no apparent gross structural defects (Fig. 7H, I). Furthermore, staining with neural marker 22C10 showed that
neural differentiation was normal (Fig. 7E, F, K, L, M). These results show that dronc is essential for induction of cell death during embryogenesis. Since Dronc shares very limited (<25%) nucleotide sequence homology with all Drosophila caspases, dronc RNAi is unlikely to affect the function of other caspases.

**DISCUSSION**

In this paper, we have shown that the Drosophila CARD-containing caspase, Dronc, mediates PCD in vivo. We show that overexpression of Dronc results in ectopic cell death in transgenic flies in different tissues and at various developmental stages. Conversely ablation of Dronc in early embryos using RNAi results in a dramatic decrease in the number of apoptotic cells during embryogenesis. Thus Dronc is an important mediator of PCD during Drosophila development. Although, ectopic overexpression of Dronc resulted in substantial cell death, not all cells where Dronc was expressed underwent PCD. This indicates that over expression of Dronc alone is not always sufficient to kill cells and that other factors are likely to be involved, perhaps in the activation of the zymogen form of Dronc to the active caspase. Furthermore, we and others have shown that the prodomain of Dronc has a negative regulatory role probably due to the binding of Diap1 to this region (36, 37). Our RNAi studies were only able to demonstrate that Dronc is essential for PCD during embryogenesis, since none of the injected embryos developed into larvae. Therefore it will be necessary to use other methods, to examine whether Dronc is essential for PCD in all tissues during Drosophila development.

By genetic and biochemical means we have explored the interaction of Dronc with other components of the apoptotic machinery. We demonstrated that the GMR-dronc eye phenotype can be suppressed by GMR-p35 and is sensitive to the dosage of the caspase inhibitor diap1, the H99 genes (reaper, grim and hid) and the apaf-1/CED-4 homolog, dark. In addition, in SL2 cells we show that a prodomain deleted version of Dronc, MPD-Dronc, is more potent at inducing cell death, consistent with the observations of Meier et al. (36). However in constrast to Meier et al. (36), we observed that this is sensitive to P35. Furthermore, Dronc can form a complex with P35, Grim, Dark and Diap1, but not with Hid, Rpr and Diap2. We also show that extracts from dark mutant flies have reduced caspase activity and lower levels of the cleaved active form of Dronc, suggesting that Dark is important for Dronc activation. Our genetic and
biochemical data, as well as recent work by others showing that dark and dronc are downstream of rpr, hid or grim-induced cell death and that Dronc forms a complex with and is activated by Dark (20, 21, 36, 37), is most consistent with the model shown in Fig. 8. However, since results reported here and by others have been obtained using overexpression systems, these genetic interactions need to be supported by analysis of specific dronc mutants.

Dronc, as a CARD containing caspase-9 homolog, is expected to function downstream of death signals and upstream of the effector caspases such as Drice, Dcp-1 and Decay (Fig. 8). The suppression of the GMR-dronc phenotype by coexpression of the caspase inhibitor P35, and the biochemical interaction we have observed between Dronc and P35, show that Dronc-induced cell death is caspase-dependent, as may be expected. Another recent study has also observed that P35 can inhibit Dronc-mediated cell death in S2 cells (53). However, this is in contrast to previous studies, where no genetic or biochemical interaction was observed between Dronc and P35 (36, 37). The difference in genetic interactions may simply be due to our GMR-dronc eye ablation phenotype being less severe than the phenotype assayed in previous studies (36, 37) and was therefore more sensitive to detecting more subtle genetic interactions. The difference between our biochemical data may be explained by differences in experimental design, since the studies of Meier et al. and Hawkins et al. (36, 37) were done in yeast where no other apoptosis components are present. Since we were unable to observe a direct binding between Dronc and P35 in vitro, it is possible that P35 interacts with, and inhibits a downstream caspase, rather than Dronc itself. One possible candidate is Drice, which has been shown to interact with both P35 (19) and Dronc (36), or Dcp-1, which can cleave P35 (37).

Based on homology and the ability of Dronc to form a complex with Dark, Dronc is expected to be a functional homolog of CED-3/caspase-9 (30; this study). Dronc, is therefore expected to be downstream of Dark and the proteins of the H99 complex, which are known to induce PCD by activating caspases (13, 27; Fig. 8). Consistent with this, we and others (36, 37) have shown that a dronc deficiency or expression of the dominant negative dronc mutant is able to suppress the ablated eye phenotype of GMR-hid and GMR-rpr. Since overexpression of upstream caspases generally results in autoactivation, ectopic expression of dronc was expected to be epistatic to the H99 genes and dark. However, we found that halving the dosage of the H99 genes or dark suppressed the GMR-dronc eye phenotype, suggesting that the H99 genes...
and dark are rate limiting for dronc function. This may be explained by the possibility that Dronc, when overexpressed as a zymogen, is not able to self-activate very efficiently and may therefore be dependent on the dosage of upstream activating genes. Another possibility is that the suppression of GMR-dronc by halving the dosage of the H99 genes may be a result of a feedback amplification loop between active caspases and Rpr, Hid and Grim.

Consistent with the genetic interaction, Dronc forms a complex with the H99 gene product Grim, when co-expressed in cells. However, we have shown that this interaction is not direct and may occur through Diap1, which can bind to Dronc and to Grim (this study, 36, 37). The significance of the in vivo interaction we have observed between Grim and Dronc is unclear and requires further investigation. As a CED-3/caspase-9 homolog, activation of Dronc is expected to require Dark, and consistent with this, Dronc and Dark form a complex in SL2 cells. A complex of Dark and Dronc has also been observed in 293T and to result in generation of the cleaved, active form of Dronc (21). In this study we have also observed that dark mutant extracts are defective in their ability to generate the cleaved active form of Dronc and have lower levels of active caspases. These results, together with our genetic data, suggest that Dronc is likely to be a functional homolog of CED-3/caspase-9, since it is activated by the Dark, the CED-4/Apaf-1 homolog. Furthermore, we have shown that the amino-terminal region of Dark containing the CARD and CED-4 homology domain is sufficient for Dronc binding.

The Drosophila apoptosis inhibitor Diap1 inhibits the activity of Drice and Dcp-1, and is antagonized by Rpr, Hid or Grim (18, 19). Our data showing a dose-dependent enhancement of GMR-Dronc by diap1 mutations and that Dronc and Diap1 form a complex is consistent with Diap1 also acting as an inhibitor of Dronc. A recent study (36) has also demonstrated that Diap1 and Dronc interact genetically and biochemically and that the pro-domain of Dronc is required for the Diap1 interaction. We also observed that expression of diap1 or diap2 (to a lesser extent) was able to suppress the GMR-dronc phenotype, indicating that Diap2 as well as Diap1 can prevent Dronc-mediated cell killing. However, since a diap2 deficiency did not show a dominant enhancement of GMR-dronc, and Diap2 did not form a complex with Dronc, it is likely that the suppression of GMR-Dronc by GMR-Diap2 is indirect, perhaps via the inhibition of downstream caspases. Our genetic and biochemical observations for a role for Diap1, but not Diap2, in suppressing Dronc function are consistent with previous studies showing that diap1
and diap2 function differently in inhibiting cell death (13, 15, 18). Halving the dosage of diap1, but not diap2, enhances rpr, hid or grim-induced cell death (15), while overexpression of diap1 or diap2 can inhibit rpr or hid-induced death, but only overexpression of diap1 can inhibit grim-induced cell death (13, 15, 18, 54). Additional studies are required to further explore the precise roles of Diap1 and Diap2 in the Drosophila cell death pathway.

In summary, we have shown that Dronc is essential for cell death in early embryos and that ectopic expression of Dronc can induce cell death in flies. Furthermore, we have provided evidence that Dronc is likely to be a functional homolog of CED-3/caspase-9 in flies. Our data showing genetic and physical interactions between Dronc and P35, Grim, Dark and Diap1, provide a framework for further investigation of the PCD pathway in flies.

ACKNOWLEDGMENTS

We are grateful to S. Read for technical assistance, N. Harvey, B. Hay, K. White, M. Miura, V. M. Dixit, G. Nunez and N. Inohara for reagents.
Footnotes:

* This work was supported by the Wellcome Trust and the National Health and Medical Research Council.

†† Wellcome Senior Research Fellows in Medical Science

‡ Equal senior authors, to whom correspondence should be addressed: Email: helena.richardson@adelaide.edu.au or sharad.kumar@imvs.sa.gov.au.
REFERENCES

FIGURE LEGENDS

Fig. 1. *dronc* induces cell death in transgenic flies. (A-H) *UAS-dronc-GFP* or *UAS-dronc*<sup>CG</sup>-GFP flies were crossed to *GMR-GAL4* and third instar larval imaginal discs or adult eyes analyzed. (A-D) Third instar larval eye imaginal discs co-stained with α*Dronc* antisera (red), αGFP (dark blue), and for DNA using Hoechst 33258 (light blue). (A, C) *GMR-GAL4/UAS-dronc*<sup>CG</sup>*.* (B, D) *GMR-GAL4/UAS-dronc*. (E, F) Eye imaginal discs from *GMR-GAL4/UAS-dronc*<sup>CG</sup> and *GMR-GAL4/UAS-dronc*, respectively, stained with acridine orange to detect dying cells. Scanning electronmicrographs of (G) *GMR-GAL4/UAS-dronc*<sup>CG</sup> and (H) of *GMR-GAL4/UAS-dronc* adult eyes. (I, J) *UAS-dronc-GFP* or *UAS-dronc*<sup>CG</sup>-GFP flies were crossed to flies containing the heat shock inducible transgene *hsp70-GAL4*. Expression of *dronc* was induced in stage 12 embryos by heat shock for 30 min at 37 °C followed by recovery for 1 h and apoptotic cells were detected by TUNEL. (I) TUNEL of a *hsp70-GAL4/UAS-dronc*<sup>CG</sup> stage 13 embryo showing wild type level of cell death (J) TUNEL of a *hsp70-GAL4/UAS-dronc* stage 13 embryo.

Fig. 2. *dronc* genetically interacts with *p35*, the *H99* genes, *dark* and *diap1*.

**Fig 3. Dronc-MPD induces death in SL2 cells.** Cell death assays in *Drosophila* SL2 cells. SL2 cells, cotransfected with *pMT-dronc, dronc*<sup>C318G</sup> or *dronc* minus the prodomain *MPD-dronc* and *pCasper.lacZ*, were treated with CuSO<sub>4</sub> for 48 hours to induce Dronc expression. Cell survival was quantified by comparing % β-gal positive cells in treated versus untreated dishes. Result shown as average percentages ± SEM were derived from three independent experiments. Cell death induced by MPD-Dronc was significantly inhibited by the caspase inhibitors, baculovirus P35, Diap1, z-VAD-fmk or by Dronc<sup>C318G</sup>, but not by Diap2.

**Fig. 4. Dronc forms a complex with Diap1, Grim, P35 and Dark.** (A) Lysates from 293T cells transfected with GFP-tagged Dronc alone or with Myc-tagged Diap1, were immunoprecipitated with αMyc antibodies (middle and bottom panel) and pelleted proteins were immunoblotted with αGFP to detect Dronc (middle panel) or with αMyc to show that Diap1-Myc was present in the cotransfected cells (bottom panel). As a control, cell lysates were immunoblotted with αGFP, to show that Dronc-GFP was expressed. (B) Lysates from 293T cells transfected with GFP-tagged Dronc protein alone or with Flag-tagged Grim, Rpr or Hid were immunoprecipitated (IP) with an αGFP antibody or an αFlag antibody (middle panel) and then immunoblotted with either αFlag (top two panels) or αGFP (bottom panel) antibodies. In the top panel, the upper part of the gel is not shown, but no bands corresponding to Hid were seen. Note that the smaller band in the Grim lane is likely to be a proteolytic fragment of Grim as previously noted by others (55). (C) Lysates from SL2 cells cotransfected with HA- and 6xHis-tagged Dronc and HA-tagged P35 were precipitated with Talon resin and then immunoblotted with αHA antibody (upper panel). In the control experiment, lysates were immunoprecipitated and immunoblotted with αHA antibody (lower panel). (D) Lysates prepared from SL2 cells transfected with Myc-tagged Dark(1-411) alone or with Flag tagged DroncC/A, were immunoprecipitated (IP) with αMyc antibody. Pelleted protein complexes (lanes labeled P) and the original lysates (lanes labeled L) were immunoblotted with αFlag.
antibody. The positions of Dronc and IgG heavy chain are indicated. Also note that an αFlag cross-reacting protein in the cell lysate does not co-immunoprecipitate with Dark.

**Fig 5. The interaction of Dronc with Diap1 or Grim is indirect.** Purified Dronc-GST was incubated with purified $^{35}$S-labelled P35$_{HA}$, Grim$_{FLAG}$ ± Diap1$_{MYC}$. $^{35}$S-labelled proteins were visualised by autoradiography after immunoprecipitation with α-HA, α-Myc or α-Flag (top panel) and binding to Dronc-GST visualised by blotting with α-Dronc polyclonal antibody. Small amounts of Dronc are observed binding to the sepharose beads alone (first lane). The lower molecular weight bands present in the Diap1 lanes are probably breakdown products. Dronc does not co-immunoprecipitate with Grim or P35, but does when Grim and Diap1 are incubated together with Dronc (last lane).

**Fig. 6. Dark is required for Dronc activation.** (A) The $dark^{CD8}$ mutant extract has lower caspase activity. Caspase activity of protein lysates from $dark^{CD8}$ mutant flies was compared with wild type ($w^{1118}$) flies by incubation with various fluorogenic caspase substrates, VDVAD-amc, DEVD-amc and VEID-amc at 37 °C for 30 min and release of amc was monitored on a fluorimeter. Equivalent amount of total protein was used in each sample. $dark^{CD8}$ flies consistently had lower activity on the substrates assessed. (B) $dark^{CD8}$ mutant extract has reduced ability to generate the processed forms of Dronc. The ability of protein lysates from $dark^{CD8}$ mutant flies and wild type ($w^{1118}$) flies to cleave in vitro translated Dronc was compared by incubation of Dronc protein for 3 h with the extracts followed by PAGE. Equivalent amount of total protein was used in each sample. $dark^{CD8}$ mutant fly extracts were less efficient in processing Dronc. The track indicated with the minus sign contains Dronc protein alone without lysate. The full-length Dronc protein is indicated by the arrowhead. Asterisks refer to intermediate and fully processed forms of Dronc.

**Fig. 7. Dronc is required for cell death in embryos.** RNAi was used to ablate $dronc$ function in embryos. Precellularized embryos injected with double-stranded $dronc$ RNA or uninjected controls were aged to stage 13 before fixation and staining for TUNEL (A, B, G, H,
I), with the αDronc antibody (C, D, J) and/or with the neural differentiaition marker, Mab 22C10 (E, F, K, L, M). (A) TUNEL on an uninjected embryo. (B, G) TUNEL on dronc double-stranded RNA injected embryos. (C) Uninjected embryo shown in A, stained with αDronc antiserum. (D, J) dronc double-stranded RNA injected embryo shown in B and G, respectively, stained with αDronc antiserum, showing no staining even after long exposure. (E) An uninjected embryo stained with Mab 22C10 (F, M) dronc double-stranded RNA injected embryos shown in B and G, respectively, stained with Mab 22C10. (H) High magnification view of the thoracic region of an uninjected embryo stained with TUNEL. (I) High magnification view of the thoracic region of a dronc double-stranded RNA injected embryo stained with TUNEL. (K) High magnification view of the thoracic region of an uninjected embryo stained with Mab 22C10. (L) High magnification view of the thoracic region of a dronc RNAi injected embryo stained with Mab 22C10. (M) dronc double-stranded RNA injected embryo shown in G, stained with Mab 22C10. (N) An example of a buffer injected control embryos at stage 11.

Fig. 8. Possible location of Dronc in Drosophila apoptotic pathway. Death signals induce Rpr, Grim and Hid, which lead to the activation of caspases. Dronc as a CARD-containing CED-3/caspase-9 homolog is activated by the CED-4/Apaf-1 homolog Dark, which is required for Rpr, Hid or Grim induced cell death (20). Diaps act by binding to pro-caspases and preventing their activation (13, 14). Rpr, Hid and Grim, by binding to Diap1 are thought to disrupt IAP-caspase complexes, leading to caspase activation (18). The baculovirus protein P35 acts to inhibit many caspases (14), but has not yet been shown to directly inhibit Dronc. In the Dronc pathway, P35 may function by inhibiting a downstream caspase, such as Drice.
An essential role for the caspase Dronc in developmentally programmed cell death in Drosophila
Leonie M. Quinn, Loretta Dorstyn, Kathryn Mills, Paul A. Colussi, Po Chen, Michelle Coombe, John Abrams, Sharad Kumar and Helena Richardson

J. Biol. Chem. published online September 12, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M002935200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts