DECAY, a Novel Drosophila Caspase Related to Mammalian Caspase-3 and Caspase-7*

Loretta Dorstyn‡§¶,**, Stuart H. Read‡, Leonie M. Quinn¶,**, Helena Richardson¶,**, and Sharad Kumar‡§¶‡‡

From the ‡Hanson Centre for Cancer Research, Institute of Medical and Veterinary Science, Frome Road, Adelaide, SA 5000, Australia and the Departments of ¶Genetics and **Medicine, the University of Adelaide, Adelaide SA 5001, Australia

Caspases are key effectors of programmed cell death in metazoans. In Drosophila, four caspases have been described so far. Here we describe the identification and characterization of the fifth Drosophila caspase, DECAY. DECAY shares a high degree of homology with the members of the mammalian caspase-3 subfamily, particularly caspase-3 and caspase-7. DECAY lacks a long prodomain and thus appears to be a class II effector caspase. Ectopic expression of DECAY in cultured cells induces apoptosis. Recombinant DECAY exhibited substrate specificity similar to the mammalian caspase-3 subfamily. Low levels of decay mRNA are ubiquitously expressed in Drosophila embryos during early stages of development but its expression becomes somewhat spatially restricted in some tissues. During oogenesis decay mRNA was detected in egg chambers of all stages consistent with a role for DECAY in apoptosis of nurse cells. Relatively high levels of decay mRNA are expressed in larval salivary glands and midgut, two tissues which undergo histolysis during larval/pupal metamorphosis, suggesting that DECAY may play a role in developmentally programmed cell death in Drosophila.

Programmed cell death in metazoans is mediated by caspases, a family of cysteine proteases, which cleave their substrates following an Asp residue (1–5). A number of caspases have been described in both vertebrates and invertebrates. To date fourteen caspases have been cloned in mammals, some of which play a critical role in apoptosis, whereas others seem mainly involved in the processing and activation of proinflammatory cytokines (1–5). Although four caspases exist in the nematode Caenorhabditis elegans, only one, CED-3, is essential for all developmentally programmed cell death (6, 7). In Drosophila melanogaster four caspases, named DCP-1, DRED/DCP-2, drICE and DRONC, have been reported so far (8–12). Among these caspases, DRED and DRONC contain long prodomains carrying death effector domains and a caspase recruitment domain (CARD), respectively, suggesting that these two caspases may act as upstream (class I) caspases. On the other hand, DCP-1 and drICE lack long prodomains and are thus similar to downstream effector (class II) caspases in mammals. Currently, loss of function mutants are only available for dcp-1. dcp-1 mutation results in larval lethality and melanotic tumors (8). Additionally, DCP-1 is required for Drosophila melanogaster development, as dcp-1 is one of two essential genes involved in transmission of nurse cell cytoplasmic contents to developing oocytes (13). The transcript for dredd accumulates in embryonic cells undergoing programmed cell death and in nurse cells in the ovary at a time that coincides with nurse cell death (9). drone mRNA is widely expressed during development and appears to be up-regulated by ecdysone in larval salivary glands and midgut before histolysis of these tissues (12). The precise roles of drICE and DRONC in programmed cell death in Drosophila have not been established. However, in vitro antibody depletion experiments suggest that drICE is required for apoptotic activity in the S2 Drosophila cell line (14). Accumulation of drone mRNA in salivary glands and midgut may be required to sensitize these tissues for deletion by apoptosis during metamorphosis. These recent studies suggest that specific caspases may mediate tissue and stage specific programmed cell death during Drosophila development.

To fully understand the role of various caspases in cell physiology, it is important to identify all caspases in a given model organism. In this study, we describe the characterization of DECAY, the fifth Drosophila caspase. DECAY is highly similar to class II executioner caspases such as mammalian caspase-3 and caspase-7. We show that decay gene expression is widespread in developing fly embryos, and DECAY has substrate specificity similar to caspase-3 subfamily of caspases.

EXPERIMENTAL PROCEDURES

Identification and Sequencing of Decay cDNA—Decay was identified through a homology search with mammalian caspases as a GenBank™ expressed sequence tag (accession number AI259958). The expressed sequence tag clone was obtained from Berkley Drosophila Genome Project in pOT2 vector and was sequenced in full. This clone (clone ID LP9492) contained a 1.79-kb base pair insert, which was much longer than the 1.1-kilobase predicted size of the transcript (see below). A careful examination of the sequence suggested that ~700 base pair of the 3′ sequence in this clone may be derived from fusion of a heterologous cDNA. The 5′ 1,101 base pair of the sequence, including a 20 residue poly(A) tail, is likely to represent the authentic full-length decay cDNA containing the entire coding region. This sequence has been deposited in the GenBank™ data base under accession number AF130469.

Plasmid Constructs—The 0.85-kilobase coding region of decay was amplified from the original pOT2-decay vector by polymerase chain reaction using Pco polymerase (Roche Biochemicals) and the following oligonucleotides: Primer A, 5′-GGCCGATCCGGCGCCATGCGAGCAAAC-3′; and Primer B, 5′-CGGGAGATCTCAGCATCGG-3′. This sequence is underlined. Primer A contained a consensus Kozak sequence that required alteration of the initiation site from the original sequence and a BamHI cloning site. Primer B contained a consensus Kozak sequence that required alteration of the initiation site from the original sequence and a BamHI cloning site. Primer B contained a consensus Kozak sequence that required alteration of the initiation site from the original sequence and a BamHI cloning site.
FIG. 1. DECAY sequence and its relationship to other known Drosophila caspases. A, deduced amino acid sequence of DECAY consists of 287 amino acid residues. The pentapeptide sequence QACRG, encompassing the catalytic Cys150, is underlined. B, an amino acid sequence alignment of the known Drosophila caspases. AC005466 is the GenBank accession number of contig of Drosophila genomic sequence that contains the coding region for a putative caspase. The partial sequence for this caspase shown here, which lacks the amino-terminal region, is derived from a single exon. Alignments were obtained using CLUSTAL W program at European Bioinformatics Institute. Residues conserved in at least five caspases are shown in black boxes. Similar residues in at least five caspases or those identical in four caspases are shown in gray boxes. C, phylogenetic relationship between various Drosophila caspases.
tained an EcoRI site and sequence encoding a FLAG tag. Amplified product was purified and cloned directionally into pcDNA3 (Invitrogen). The catalytic Cys<sup>150</sup> residue of DECAY was mutated to a Gly residue by Quikchange mutagenesis (Stratagene) using pT2-decay as template. Mutant decay cDNA encoding DECAY(C150G) protein was cloned directly into pcDNA3 as described above. Primer C, 5′-GGCAATTC-CAATGCGACCAAGATCCCAT containing a NdeI site and primer D, 5′-GGCGGATCCCGGGTTCTGGCTTAAACGCA-G containing a BamHI site (sequence corresponding to decay is underlined in both primers) were used to amplify wild type and catalytic cysteine mutant DECAY for directional cloning into pET32b vector (Novagen).

Recombinant Caspases and Caspase Assays—Recombinant DECAY was generated by transformation of Escherichia coli BL21 cells with DECAY-6xHis or DECAY(C150G)-6xHis constructs in pET32b. Overnight cultures were subcultured in 10 and grown at 37 °C for 2 h. Cultures were induced with 1 mM isopropyl-1-thio-

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\text{Galactopyranoside (Roche Molecular Biochemicals).}
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while searching for new molecules with homology to various mammalian caspases, using TBLASTN program, we identified an expressed sequence tag in the GenBank<sup>TM</sup> data base which encoded a partial caspase-like molecule. The sequencing of the entire clone revealed that the cDNA has an open reading frame of 287 amino acid residues with a high degree of homology to mammalian caspases, particularly those related to the caspase-3 subfamily (Fig. 1). We named this new molecule DECAY, for Drosophila executioner caspase related to Apopain/Yama. DECAY shares approximately 39% identity (54% similarity) with Spodoptera frugiperda caspase-1, 37% identity (56% similarity) with mammalian caspase-3 and caspase-7, 35% identity (53–55% similarity) with Drosophila caspases DCP-1 and drICE, and 32–33% identity (48–52% similarity) with caspase-8 and caspase-10.

An alignment of all known Drosophila caspases showed that DECAY is most homologous to an unpublished putative caspase encoded by genomic sequence contained in a data base entry (accession number AC005466), followed by DCP-1 and...
DECAY, a New Drosophila Caspase

The text discusses the identification and characterization of a new Drosophila caspase called DECAY. DECAY is characterized by its lack of a long amino-terminal prodomain and its relationship to DRONC and DREDD, the two class I Drosophila caspases. It is more similar to S. frugiperda caspase-1 and mammalian caspase-3 and caspase-7 than all known Drosophila caspases. DECAY is the only Drosophila caspase that carries a QACRG sequence encompassing the putative catalytic Cys150 residue.

By hybridizing to a filter containing Drosophila genomic P1 clones, DECAY was localized to the Fas1 contig located within chromosome region 89C6-D4. DECAY has a substrate specificity similar to the Caspase-3 Subfamily. To confirm that DECAY is indeed a caspase, expression constructs were co-transfected with pEF-bgal into 293T and NIH3T3 cells by lipofection. At 24 h post-transfection, cells were fixed, stained with X-gal, and blue cells observed for apoptosis. DECAY was found to be a cytoplasmic component in transfected cells and was more active on the pentapeptide substrate VDVAD-amc. As expected, the C150G mutant DECAY did not exhibit any appreciable caspase activity.

Poly(ADP-ribose) polymerase (PARP) is one of the key cellular substrates of caspase-3. To check whether PARP can serve as a substrate for DECAY in vitro, we incubated a

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**Fig. 3.** A, effect of DECAY expression in transfected mammalian cells. Various expression constructs were co-transfected with pEF-bgal into 293T and NIH3T3 cells by lipofection. At 24 h post-transfection, cells were fixed, stained with X-gal, and blue cells observed for apoptosis. B, ectopically expressed DECAY localizes mainly to a cytoplasmic compartment in transfected cells. NIH3T3 or 293T cells were transfected with the empty vector (left side panels) or a DECAY-FLAG (right side panels) expression construct. At 18 h post-transfection, cells were fixed, and expression of DECAY-FLAG protein detected using an anti-FLAG primary antibody followed by a fluorescein isothiocyanate-coupled secondary antibody. Cells were observed and photographed using a fluorescence microscope. Although DECAY expression induces significant cell death in both cell lines (as shown in Fig. 3A), selected fields with mostly nonapoptotic cells are shown in this figure.

**Fig. 4.** Expression of decay mRNA. Approximately 20 µg of total RNA (A) or 1–2 µg of poly(A)+-enriched RNA (B) isolated from various developmental stages and adult flies were analyzed by Northern blotting using decay open reading frame as a probe. decay transcript is detected as a single approximately 1.1-kilobase band in all samples examined. The lower panels in both A and B depict portions of the ethidium bromide-stained gels corresponding to ribosomal RNA bands before transfer to membrane.
**FIG. 5. In situ mRNA analysis of decay expression during Drosophila development.** decay mRNA was detected by in situ hybridization with a digoxigenin-labeled antisense mRNA probe. A, a stage 5 syncitial embryo showing uniformly low levels of decay expression; B, a stage 7 embryo showing decay expression throughout the embryo. The regions of higher staining are due to tissue folding. C, a stage 13 embryo showing decay expression occurs at higher levels in the middle of the embryo corresponding to the gut tissue but is absent from the dorsal cells of the amnioserosa. D, a stage 8 embryo hybridized with the decay sense control probe showing no staining. E, a third instar larval salivary gland showing high levels of decay mRNA. F, a third instar midgut showing high levels of decay expression. G, a late third instar eye imaginal disc showing very low levels of decay expression. H, brain lobes from third instar larvae showing ubiquitous low level of decay expression. decay sense control on late third instar larval tissues showed no staining (data not shown). I, a stage 10a adult egg chamber showing high expression of decay mRNA in the nurse cells but not in the oocyte (on the right). J, adult egg chambers showing that decay mRNA is increased at stage 9 compared with earlier stages. K, Hoechst 33258 staining of DNA in adult egg chambers, showing the morphology of nuclei. At stage 12 the nurse cells (see large nuclei on the left in K) are undergoing apoptosis, and decay mRNA has been dumped into the oocyte (shown in J). The oocyte is surrounded by follicle cells (see small nuclei in K), whereas the germinal vesicle is out of the plane of focus. L, decay sense control on adult egg chambers showing no staining.

**FIG. 6. decay transcript is not up-regulated by ecdysone.** Salivary glands and midgut were dissected out of larvae at the indicated stages and either treated with 1 mM ecdysone for 1 h, or left untreated. Total RNA prepared from untreated and ecdysone-treated tissues was subjected to Northern blot analysis using decay and dronc cDNA probes. The lower panel depicts a portion of the ethidium bromide-stained gel before transfer to membrane. The last lane in the gel contains total RNA from early pupae, which express relatively high levels of dronc transcript.

$^{35}$S-labeled truncated PARP protein that carries the caspase-3 cleavage site (18) with recombinant DECAY and measured its cleavage by electrophoresis and autoradiography. As shown in Fig. 2B, this protein was efficiently cleaved by DECAY. The cleavage products generated by DECAY were identical in size to those generated by caspase-3 suggesting that both caspases cleave following the same DEVD sequence in PARP.

Ectopic Expression of DECAY in Cultured Cells—Many caspases, when overexpressed in cultured cells, induce apoptosis to some degree. We therefore analyzed whether DECAY is able to induce apoptosis in transfected cells. In 293T cells, at 24 h following transfections, around 35% of cells transfected with the wild-type decay construct showed apoptotic morphology when compared with cells transfected with the empty vector or an expression construct carrying the C150G mutant DECAY (Fig. 3A). In NIH3T3 cells, by 24 h post-transfection, a small number (~10%) of cells transfected with the wild-type decay were apoptotic (Fig. 3A). By 48 h, decay transfected cells showing apoptotic morphology increased slightly to around 15% (data not shown). This level of cell death induced by DECAY overexpression is similar to that induced by caspase-3 under similar conditions (22, 27). We also assessed the effect of DECAY overexpression in MCF-7 cells and Drosophila S2 cells. In both cases levels of apoptosis similar to those seen in NIH3T3 cells were observed (data not shown).

Using FLAG-tagged DECAY, we further investigated the subcellular localization of DECAY protein in transfected cells by immunofluorescence analysis employing an anti-FLAG antibody and a fluorescein isothiocyanate-coupled secondary antibody. In both NIH3T3 and 293T cells, most of the DECAY-FLAG protein was present in the cytoplasmic compartment (Fig. 3B).

decay mRNA Expression During Drosophila Development—In RNA blots, decay was present as an approximately 1.1-kilobase transcript in most developmental stages, larvae, pupae, and in the adult fly (Fig. 4). Relatively high levels of decay transcript were detected in the adult fly (Fig. 4B). We further analyzed the expression pattern of decay during fly development by in situ hybridization to Drosophila embryos and larval tissues using a digoxigenin-labeled antisense mRNA probe (Fig. 5). decay is expressed at low levels throughout embryogenesis and shows no specific up-regulation at stage 11 (Fig. 5, A–C, and data not shown) when programmed cell death
first becomes evident in *Drosophila* (28). decay mRNA was present in stage 1–4 syncitial embryos (not shown), suggesting that it is maternally deposited into the embryo, because zygotic expression does not begin before stage 5 (29). In stage 6–7 cellularized embryos, decay mRNA is ubiquitously expressed (Fig. 5B), but in later stages decay mRNA is present at higher levels within the gut (Fig. 5C). We also examined the expression of decay in third instar larval tissues and during oogenesis (Fig. 5, E–L). High levels of decay expression was observed in salivary glands and midgut tissue from third instar larvae (Fig. 5, G and H), preceding the onset of apoptosis in these tissues, which occurs after pupariation (30). Only very low levels of decay expression were observed throughout third instar larval eye imaginal discs and brain lobes (Fig. 5, G and H), which contain apoptotic cells at this stage (31). However, up-regulation of decay expression was not observed in eye disc or brain lobe cells undergoing apoptosis.

During oogenesis decay mRNA is detected in egg chambers of all stages but was present at higher levels in the nurse cells after stage 10a (Fig. 5F and data not shown). In stage 12 egg chambers, decay mRNA was absent from nurse cells that have initiated apoptosis and present at high levels in the developing oocyte (Fig. 5, J and K), consistent with dumping of the nurse cell cytoplasm into the oocyte that occurs at this stage (32). The expression of decay mRNA in egg chambers is consistent with a role for decay in apoptosis of the nurse cells.

We have previously shown that *drone* mRNA is up-regulated when isolated salivary glands and midgut from second instar larvae are exposed to edecysone (12). Because larval salivary glands and midgut show relatively high expression of decay transcript, it was of interest to check whether decay mRNA is also regulated by edecysone. As shown in Fig. 6, no up-regulation of decay transcript was evident in ecyson treated salivary glands and midgut. Under similar conditions, *drone* transcript was up-regulated at least 5-fold in response to edecysone (Fig. 6).

**Conclusions**—We have described here preliminary characterization of a new *Drosophila* caspase DECAY. Presence of multiple caspases in *Drosophila* suggest that cell death pathways in the fly are likely to be complex. DECAY is most similar to caspase-3-like effector caspases and shares a similar substrate specificity. Low levels of decay transcript are widely expressed during *Drosophila* embryogenesis. Higher expression of decay mRNA in larval salivary glands and midgut suggests a possible role for DECAY in programmed deletion of these obsolete tissues during metamorphosis and tissue remodeling. Additionally, moderate expression of decay mRNA in nurse cells suggests a possible role for DECAY in nurse cell death. Because high levels of decay transcript are also found in the adult animals, DECAY may also be involved in regulating the normal cell turnover in the adult. Generation of loss-of-function decay mutant, or RNA ablation studies would shed further light on the role of DECAY in programmed cell death in *Drosophila*.

Mammalian caspases have been proposed to belong to two groups. The upstream, initiator, or class I caspases carrying specific protein-protein interaction domains are autoactivated when several molecules are clustered in close proximity follow-