Caspases are main effectors of apoptosis in metazoans. Genome analysis indicates that there are seven caspases in *Drosophila*, six of which have been previously characterized. Here we describe the cloning and characterization of the last *Drosophila* caspase, DAMM. Similar to mammalian effector caspases, DAMM lacks a long prodomain. We show that the DAMM precursor, along with the caspases DRONC and DECAY, is partially processed in cells undergoing apoptosis. Recombinant DAMM produced in *Escherichia coli* shows significant catalytic activity on a pentapeptide caspase substrate. Low levels of *damm* mRNA are ubiquitously expressed in *Drosophila* embryos during early stages of development. Relatively high levels of *damm* mRNA are detected in larval salivary glands and midgut, and in adult egg chambers. Ectopic expression of DAMM in cultured cells induces apoptosis, and similarly, transgenic overexpression of DAMM, but not of a catalytically inactive DAMM mutant, in *Drosophila* results in a rough eye phenotype. We demonstrate that expression of the catalytically inactive DAMM mutant protein significantly suppresses the rough eye phenotype due to the overexpression of HID, suggesting that DAMM may be required in a *hid*-mediated cell death pathway.

Programmed cell death in metazoans is mediated by caspases, a family of cysteine proteases, which cleave their substrates following an Asp residue (1–4). A number of caspases have been described in both vertebrates and invertebrates. To date 14 caspases have been cloned in mammals (1–4). Gene targeting studies in the mouse suggest that some caspases play a signal specific and spatially restricted role in apoptosis, whereas others seem mainly involved in the processing and activation of proinflammatory cytokines (reviewed in Ref. 5). Four caspases exist in the nematode *Caenorhabditis elegans*, but only one, CED-3, is essential for all developmentally programmed cell death, and the functions of the remaining three are not known (6). In *Drosophila melanogaster* six caspases, named DCP-1, DREDD/DCP-2, DRICE, DRONC, DECAY, and STRICA have been cloned so far (7–13). In addition to the already described caspases, analysis of the *Drosophila* genomic database predicts one more caspase, which we have termed DAMM (GenBank™ accession number AF240763; death-associated molecule related to Mch2) (14). Among the *Drosophila* caspases, DREDD and DRONC contain long prodomains carrying death effector domains (DEDs) and a caspase recruitment domain (CARD), respectively, suggesting that these two caspases may act as upstream initiator caspases (reviewed in Ref. 14). STRICA also has a long prodomain, but it lacks any CARD or DED structures (13). On the other hand, DCP-1, DRICE, and DECAY lack long prodomains and are thus similar to downstream effector caspases in mammals. *dcp-1* mutants are larval lethal and exhibit melanotic tumors (7). Additionally, DCP-1 is required for *Drosophila* oogenesis, as *dcp-1* mutants show a defect in transfer of nurse cell cytoplasmic contents to developing oocytes (15). 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 (8). Heterozygosity at the *dredd* locus suppresses cell death induced by the ectopic expression by *rpr*, *hid*, and *grim* in transgenic models, indicating that DREDD concentration may be a rate-limiting step in this pathway. In addition to its function in apoptosis, DREDD also plays a key role in the innate immune response (16, 17).

*dronc* mRNA is widely expressed during development and is up-regulated several-fold by edcsyne in larval salivary glands and midgut prior to histolysis of these tissues (11). Heterozygosity at the *dronc* locus or the expression of a catalytically inactive DRONC mutant suppress the eye phenotype caused by *rpr*, *grim*, and *hid*, consistent with the idea that DRONC functions in the RPR, GRIM, and HID pathway (18–20). DRONC also interacts, both biochemically and genetically, with the CED-4/ApaF-1 fly homolog, Dark (20). Furthermore, loss of DRONC function in early *Drosophila* embryos because of *dronc* RNA ablation results in a decrease in apoptosis, indicating that DRONC is required for programmed cell death during embryogenesis (20). These results suggest that DRONC is a key upstream caspase in mediating developmentally programmed cell death in *Drosophila*.

The precise roles of DRICE, DECAY, and STRICA 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 (21). Similar to *dronc*, *decay* expression is high in larval midgut and salivary glands, but *decay* expression is not

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF240763.

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1 The abbreviations used are: DAMM, death-associated molecule related to *Mch2*; CARD, caspase recruitment domain; DED, death effector domain; *Drosophila* inhibitor of apoptosis; HA, hemagglutinin; CHAPS, 3-(3-cholamidopropyl)dimethylammoniomethyl-1-propanesulfonic acid; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; afe, amino-trifluoromethylcoumarin; ame, amino-methylcoumarin.
regulated by edcysone (12). Accumulation of dronc and decay mRNA in salivary glands and midgut may be required to sensitize these tissues for deletion by apoptosis during metamorphosis.

The presence of multiple caspases in Drosophila indicates that apoptotic pathways in insects are likely to be of similar complexity to those in mammals. To fully understand the role of apoptotic pathways in insects, it is important to analyze cell physiology, it is important to analyze various caspases in a given model organism. In this paper, we describe the initial characterization of DAMM, the last caspase in Drosophila.

**EXPERIMENTAL PROCEDURES**

**Cloning of damm cDNA—**

Cloning of damm cDNA—damm was identified using a TBLASTN search of the Berkeley Drosophila Genome Project database, as a model organism. From this region of homology, damm specific primers were designed and used in combination with library-specific vector primers to amplify the full-length open-reading frame of damm from a Drosophila larval cDNA library. The 5’-end of the damm open-reading frame was confirmed using 5’-rapid amplification of cDNA ends (RACE) (Life Technologies). The cDNA sequence of damm has been deposited in the GenBankTM database under accession number AF240763. Multiple sequence alignments and construction of phylogenetic trees were carried out using Bionavigator software packages ClustalW and Protpars.

**Plasmid Constructs—**

All caspases in a given model organism. In this paper, we describe the initial characterization of DAMM, the last caspase in Drosophila. In Vivo Caspase Cleavage Assays—Full-length damm in pcDNA3 was used as a template for the production of [35S]methionine-labeled protein using a coupled transcription/translation kit (Promega). 5 µl of translated product was incubated with recombinant enzyme lysates for 30 min at 37 °C. Electrophoresis was performed on a 4% SDS-polyacrylamide gel transferred to polyvinylidene membrane (Dupont) and exposed to x-ray film.

**Transient Transformation—** NIH3T3 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. For cell death assays, 2 × 10^6 cells were plated per 35-mm dish the day before transfection. 2 µg of plasmid DNA comprising either 2 µg of empty vector, 2 µg of pRM-damm (C156G) together with 1 µg of empty vector, pcDNA3-diap1Myc, pcDNA3-p35Hα, pRSP-be12, pcDNA3-ermA, or pcDNA3-mihA, was cotransfected with 0.5 µg of a β-galactosidase expression plasmid (pEF-βgal) (27). All transfections in mammalian cells were carried out using Fugene6 transfection reagent (Roche Molecular Biochemicals) according to manufacturer’s instructions. Cells were fixed and stained with X-gal at 24 or 48 h post-transfection, and β-galactosidase-positive cells were scored for apoptotic morphology as previously described (27).

Cell death assays in insect cells were carried out using Schneider L2 (SL2) cells as previously described (28, 29). SL2 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. To fully understand the role of apoptotic pathways in insects, it is important to analyze cell physiology, it is important to analyze various caspases in a given model organism. In this paper, we describe the initial characterization of DAMM, the last caspase in Drosophila.

**Processing in SL2 Transfectants—**

SL2 cells were transfected with pRM-dronc, pRM-damnFLAG, or pRM-decayFLAG as described above. 24 h following transfection, cells were induced to express respective constructs by the addition of CuSO4 at a final concentration of 0.7 mM. Cells were fixed and stained with X-gal 48 h post-CuSO4 treatment, and β-galactosidase-positive cells were scored for apoptotic morphology as previously described (29). Cell survival was calculated as the percent of transfected (β-galactosidase-positive) cells in the CuSO4-treated population relative to the percent of transfected cells in the untreated population. All calculations were normalized against the 100% survival value of transfected cells. The results, shown as average percent survival ± S.E.M., were derived from three independent experiments.

To check copper-induced protein expression after 48 h of CuSO4 treatment, cells were lysed in SDS-PAGE buffer, and lysates were subjected to immunoblotting using an rat monoclonal αHα antibody (Roche Molecular Biochemicals) or a mouse monoclonal αFLAG antibody (Sigma Chemical Co.).

**In Vitro Caspase Cleavage Assays—** Full-length damm in pcDNA3 was used as a template for the production of [35S]methionine-labeled protein using a coupled transcription/translation kit (Promega). 5 µl of translated product was incubated with recombinant enzyme lysates for 30 min at 37 °C. Electrophoresis was performed on a 4% SDS-polyacrylamide gel transferred to polyvinylidene membrane (Dupont) and exposed to x-ray film.

**Northern and In Situ mRNA Analysis—**

Northern and In Situ mRNA Analyses were carried out using Fugene6 as described previously (29). Cell survival was calculated as the percent of transfected (β-galactosidase-positive) cells in the CuSO4-treated population relative to the percent of transfected cells in the untreated population. All calculations were normalized against the 100% survival value of transfected cells. The results, shown as average percent survival ± S.E.M., were derived from three independent experiments.

To check copper-induced protein expression after 48 h of CuSO4 treatment, cells were lysed in SDS-PAGE buffer, and lysates were subjected to immunoblotting using an rat monoclonal αHα antibody (Roche Molecular Biochemicals) or a mouse monoclonal αFLAG antibody (Sigma Chemical Co.).

**Decay Interaction Studies—**

In Vitro Caspase Cleavage Assays—Full-length damm in pcDNA3 was used as a template for the production of [35S]methionine-labeled protein using a coupled transcription/translation kit (Promega). 5 µl of translated product was incubated with recombinant enzyme lysates for 30 min at 37 °C. Electrophoresis was performed on a 4% SDS-polyacrylamide gel transferred to polyvinylidene membrane (Dupont) and exposed to x-ray film.

**Transient Transformation—**

NIH3T3 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. For cell death assays, 2 × 10^6 cells were plated per 35-mm dish the day before transfection. 2 µg of plasmid DNA comprising either 2 µg of empty vector, 2 µg of pRM-damm (C156G) together with 1 µg of empty vector, pcDNA3-diap1Myc, pcDNA3-p35Hα, pRSP-be12, pcDNA3-ermA, or pcDNA3-mihA, was cotransfected with 0.5 µg of a β-galactosidase expression plasmid (pEF-βgal) (27). All transfections in mammalian cells were carried out using Fugene6 transfection reagent (Roche Molecular Biochemicals) according to manufacturer’s instructions. Cells were fixed and stained with X-gal at 24 or 48 h post-transfection, and β-galactosidase-positive cells were scored for apoptotic morphology as previously described (27).

Cell death assays in insect cells were carried out using Schneider L2 (SL2) cells as previously described (28, 29). SL2 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. To fully understand the role of apoptotic pathways in insects, it is important to analyze cell physiology, it is important to analyze various caspases in a given model organism. In this paper, we describe the initial characterization of DAMM, the last caspase in Drosophila.
FIG. 1. DAMM sequence and its relationship to Drosophila caspases. A, genomic organization of the damm gene. The non-coding regions of exons 1 and 5 are shown as open boxes. The 5' boundary of exon 1 and 3' boundary of exon 5 have not been characterized. B, an amino acid sequence alignment of the seven Drosophila caspases. The deduced amino acid sequence of DAMM consists of 255 amino acid residues. Alignments were obtained using ClustalW program. Residues conserved in at least six 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 and mammalian caspases.
DAMM, A New Drosophila Caspase

RESULTS

Identification of DAMM—While searching for new molecules with homology to various mammalian caspases using the TBLASTN program, we identified a genomic sequence contained in an entry (accession no. AC005466) in the Berkeley Drosophila Genome Project database that encoded a partial caspase-like molecule. We cloned the corresponding cDNA for this gene by a combination of PCR and 5'-RACE. The cDNA contained a predicted open-reading frame of 255 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 DAMM, for death-associated molecule related to Mch2. A comparison of the damm cDNA sequence and the annotated Drosophila genome sequence reveals that the coding region for DAMM is contained in 5 exons (Fig. 1A). This differs from the predicted gene structure for damm in the flybase. DAMM shares ~29% amino acid sequence identity and 43% sequence similarity with caspase-6 (Mch-2) and 27% sequence identity and 46% sequence similarity with caspase-3. Of the Drosophila caspases, DAMM is most homologous to the newly identified caspase STRICA (Fig. 1C) (13), sharing 44% sequence identity and 60% sequence similarity. The degree of homology between these two caspases is particularly striking given that DAMM does not possess the long prodomain of STRICA. Among other Drosophila caspases, DAMM shares 28% sequence identity with DCP-1 and 26% identity with DECAY, DRICE, and DREDD. DAMM shares the least homology with DRONC among the Drosophila caspases with 23% sequence identity and 39% similarity.

damm mRNA Expression during Drosophila Development—In RNA blots, damm was present as an ~0.9-kilobase transcript in most developmental stages, larvae, pupae, and in the adult fly (Fig. 2). Relatively high levels of damm transcript were detected in early 3rd instar larvae and in the adult fly (Fig. 2). We further analyzed the expression pattern of damm during Drosophila development by in situ hybridization to embryos and larval tissues using a digoxigenin-labeled antisense mRNA probe (Fig. 3). damm is expressed at low levels through-out embryogenesis and shows no up-regulation at stage 11 (Fig. 3), when programmed cell death first becomes evident in Drosophila (32). In addition to data shown for stage 5 and later

Fig. 2. Expression of damm mRNA during Drosophila development. Approximately 20 μg of total RNA isolated from various developmental stages and from adult flies was analyzed by Northern blotting using a probe encompassing nucleotides 1–690 of the damm open-reading frame. damm transcript is present as a single, 0.9-kilobase band. The lower panel depicts the corresponding ribosomal RNA bands in each sample as visualized by ethidium bromide staining of the gel prior to transfer to membrane.

DAMM Transgenic Flies and Genetic Interactions—Wild-type damm or damm156G mutant cDNA, tagged with FLAG was cloned into the pGMR vector and transgenic flies were generated and maintained as previously described (12, 29, 30). For testing the interaction of GMR-damm156G or GMR-hid, crosses were carried out embryonically (31). Progeny were scored by examining eye phenotypes using a light microscope, as previously described (31).

Fig. 3. In situ mRNA analysis of damm expression during Drosophila development. damm mRNA was detected by in situ hybridization with a digoxigenin-labeled antisense mRNA probe. A, a stage 5 embryo showing a uniform level of damm expression. B, a stage 10/11 embryo. C, a stage 14 embryo showing damm expression in specific tissues. CNS, central nervous system; SG, salivary gland; HG, hindgut; D and E, the higher magnification of the panels shown in C. Arrowheads indicate examples of specific cells showing damm expression. F, a stage 17 embryo. SG, salivary gland; HG, hindgut; MG, midgut. G, a stage 14 embryo hybridized with a control damm sense probe showing no staining. All embryos are oriented with anterior to the left. H, the brain lobes (BL) from third instar larvae showing low damm expression. VG, ventral ganglion. I, a third instar midgut (MG) displaying slightly higher damm expression than seen in brain lobes. GC, gastric caeca; PV, proventriculus. J, a third instar salivary gland showing a high level of damm expression. K, a third instar salivary gland hybridized with a control damm sense probe showing no staining. Panels J and K are oriented with the salivary gland duct toward the top left hand corner. L, a stage 10B adult egg chamber showing high level damm expression in both the nurse cells (NC) and the oocyte (OC). M, a stage 10B adult egg chamber hybridized to control damm sense probe exhibiting no staining. Panels L and M are oriented with the oocyte to the right.

In wild-type flies, damm transcript is expressed in almost all tissues analyzed. Additionally, we did not detect any significant difference in damm mRNA expression between transgenic and control flies (data not shown). The interaction of damm with the proapoptotic genes hid (12, 29) and rpr (30) was examined to determine whether damm shares similar properties with these genes. damm156G flies were crossed to GMR-hid or GMR-rpr, crosses were carried out at 18 °C. Progeny were scored by examining eye phenotypes using a light microscope.
FIG. 4. Enzymatic activity of recombinant DAMM on fluorogenic peptide substrates. A, recombinant DAMM was expressed in *E. coli* as His₆-tagged protein, and protein expression detected by immunoblotting of bacterial lysates using an αHis₆ tag antibody. Note that following induction with isopropyl-1-thio-β-D-galactopyranoside, both DAMM and DAMM C156G mutant proteins were expressed at similar levels. All lanes contain ~10 μg of *E. coli* lysate. B, equivalent amount of *E. coli* lysates containing recombinant DAMM or DAMM C156G were incubated with various fluorogenic caspase substrates at 37 °C for 30 min and release of -amc and -afc was monitored by a fluorimeter. Data (±S.E.M.) are derived from three separate experiments performed in triplicate.

FIG. 5. Effects of DAMM protein expression in cultured cells. A, effect of DAMM expression in transfected mammalian cells. Various expression constructs were cotransfected with pEF-gal into NIH3T3 cells by lipofection. At 48 h post-transfection, cells were fixed, stained with X-gal, and blue cells were observed for apoptotic morphology. *Bars* represent apoptotic cells as percent of total β-galactosidase-positive cells ± S.E.M. At least 300 blue cells were scored for each dish. The data shown were derived from three independent experiments. B, effect of DAMM expression in *Drosophila* SL2 cells. SL2 cells were cotransfected with various caspase and inhibitor constructs in conjunction with pCASPERhs-lacZ. At 24 h post-transfection, cells were treated with heat shock, followed by CuSO₄, to induce expression of the β-galactosidase and caspase/inhibitor constructs, respectively. 48 h later, cell loss because of apoptosis was calculated by counting the number of residual β-galactosidase-positive cells. *Bars* represent the percent of β-galactosidase-positive cells in CuSO₄-treated samples as compared with untreated samples. Values were normalized against vector alone-transfected samples (100%). The data shown were derived from three independent experiments. C, expression of DAMM and inhibitor proteins 48 h following CuSO₄ treatment. Protein expression of respective constructs was analyzed by Western blotting using antibodies to DAMM FLAG or Diap1HA, Diap2HA, P35HA. The data shown in C are derived from a single experiment. Data from multiple similar experiments indicated that all proteins were expressed at roughly equal levels, and all proteins had similar stability in transfected cells. Therefore, the variations in the intensity of bands in C are likely to be caused by the loading variations, rather than protein stability.

embryos (Fig. 3, A–F) *damm* mRNA was also detected in stage 1–4 syncytial embryos (not shown), suggesting that it is maternally deposited into the embryo, because zygotic expression does not begin before stage 5 (33). In stage 5 cellularized embryos, *damm* mRNA is ubiquitously expressed (Fig. 3A), but in later stages higher levels of *damm* transcript were evident in specific cells and tissues (Fig. 3, C–F). For example, specific cells in developing salivary gland (Fig. 3D) and midgut (Fig. 3E) showed staining for *damm* transcript. No staining was seen when a *damm* sense probe was hybridized to embryos at various stages of development (Fig. 3G and data not shown).

We also examined the expression of *damm* in third instar larval tissues and during oogenesis. Low levels of *damm* expression were observed in brain lobes (Fig. 3H), which contain apoptotic cells at this stage (34). A relatively high level of *damm* expression was observed in midgut (Fig. 3I) and salivary glands (Fig. 3J) from late third instar larvae, preceding the onset of apoptosis in these tissues, which occurs after pupariation (35). During oogenesis *damm* mRNA is detected in egg chambers of all stages and in the nurse cells (Fig. 3L and data not shown). No staining was seen when a *damm* sense probe was hybridized to salivary glands (Fig. 3K), midgut (data not shown), or egg chambers at various stages of development (Fig. 3M and data not shown). Several other *Drosophila* caspases, including DCP-1 (15), DRONC (11), DECAY (12), and STRICA (13) are also expressed in egg chambers; however, the function of these caspases, except for DCP-1, in oocyte and nurse cell death remains unclear.

Caspase Activity of DAMM—To investigate the caspase activity of DAMM, we expressed full-length wild-type and C156G mutant DAMM fused to His₆ in *E. coli*. Lysates prepared from cultures induced with IPTG were tested for DAMM expression using an antibody against the 6xHis tag. Full-length DAMM
The induction of DAMM under the control of a copper inducible promoter. 48 h following transfection with pRM-DRONC, pRM-dammFLAG, or pRM-daccyFLAG and were induced to express respective constructs 24 h post-transfection by the addition of CuSO₄, as described under “Experimental Procedures.” At the indicated times post-treatment with 25 μg/ml cycloheximide, cells were harvested and analyzed by SDS-PAGE and Western blotting using aDRONC or aFLAG antibodies.

was clearly detectable in E. coli lysates, however, processed fragments were not visible (Fig. 4A; data not shown). The E. coli lysates containing DAMM were incubated with a variety of fluorogenic tetrapeptide caspase substrates to analyze substrate preference. DAMM did not show any activity on DEVD-amc, but displayed substantial cleavage activity on VDAD-amc compared with a control lyase expressing catalytically inactive DAMM C156G mutant (Fig. 4B). Because DAMM is more similar to caspase-6 than to any other known mammalian caspase, we tested whether DAMM can cleave the optimal caspase-6 tetrapeptide substrate, VEID-amc, and in vitro translated Nedd4 protein, which we have previously shown to be cleaved by caspase-6 (36). DAMM displayed no activity on either substrate (Fig. 4B and data not shown). Interestingly, DAMM showed small but significant cleavage activity on LEHD-amc and TVAD-afc, optimal substrates for caspase-4/caspase-5, and caspase-1, respectively, and to a much lesser extent on IETD-amc, a caspase-9 substrate (Fig. 4).

Ectopic Expression of DAMM Induces Apoptosis in Cultured Cells—Many caspases, when overexpressed in cultured cells, induce apoptosis to some degree. We therefore analyzed whether DAMM is able to induce apoptosis in transfected cells. In NIH3T3 cells, at 48 h following transfection, around 10% of cells transfected with the wild-type damm construct showed apoptotic morphology when compared with cells transfected with the empty vector or an expression construct carrying the C156G mutant DAMM (Fig. 5A). A similar level of apoptosis has been observed when DECAY is ectopically expressed in NIH3T3 cells (12). We and others have previously demonstrated that caspases lacking a long prodomain are not as efficient at inducing apoptosis because of the fact that they may rely on the activity of upstream caspases for their initial activation (37, 38). DAMM-induced apoptosis in NIH3T3 cells was partially inhibited by the Drosophila inhibitor of apoptosis protein Diap1 as well as the baculo virus apoptosis inhibitor P35 and other inhibitors Bcl-2, CrmA, and MIHA. damm overexpression also induced a small degree of apoptosis in Drosophila SL2 cells (Fig. 5B). In these cells, damm was transfected under the control of a copper inducible promoter. 48 h following the induction of damm expression, ~10% of cells had undergone apoptosis, as noted by their loss. Dammm-induced apoptosis in these cells was inhibited by the Drosophila inhibitors Diap1 and Diap2 as well as by P35 (Fig. 5B). The expression of respective proteins was confirmed by Western blotting of lysates from transfected cells (Fig. 5C).

DAMM, DRONC, and DECAY Are Processed during Apoptosis of SL2 Cells—To analyze whether DAMM can be processed by various Drosophila caspases, in vitro translated DAMM was incubated with lysates expressing DAMM, DRONC, DECAY, DCPR-1, or DRICE. In a similar manner to DECAY (12), no significant DAMM processing was evident by any of the caspases tested (data not shown). These results suggest that DAMM may require a caspase/protease, other than those tested here, for processing and activation.

We further analyzed the processing of DAMM in Drosophila SL2 cells undergoing apoptosis in response to cycloheximide treatment. In this study, we also analyzed the processing of DRONC, a key initiator caspase in the Drosophila cell death pathway (20), and DECAY, which, like DAMM, does not show any processing in vitro (12). We show that all three caspases are processed in SL2 transfecants in response to cycloheximide treatment (Fig. 6). As expected of an upstream initiator caspase, DRONC processing occurred early and by 8 h after cycloheximide treatment, DRONC precursor was completely cleaved to smaller fragments. The processing of DAMM and DECAY became apparent following that of DRONC, with processed products visible 16 h after the addition of cycloheximide.
DAMM, A New Drosophila Caspase

Photographs of wild-type and transgenic GMR-hid eye phenotype is suppressed by catalytically inactive 3rd instar larval eye disc from wild type (and analysis of DAMM-FLAG transgene expression in larval eye discs. Interestingly, the GMR-damm/phenotype was significantly enhanced as compared with controls, by γ-irradiation (data not shown), suggesting that ectopic expression of damm sensitizes cells to apoptosis. All transgenic lines expressing the catalytically inactive C156G mutant DAMM protein showed normal eye phenotype (Fig. SF), suggesting that the rough eye phenotype in GMR-damm F was dependent upon the caspase activity of DAMM.

Expression of Mutant damm Inhibits hid-induced Cell Death in the Fly Eye—Catalytically inactive mutants of some caspases have been shown to act as dominant negative molecules. For example, catalytically inactive mutant of DRONC immunostained with an anti-FLAG antibody. Eye discs are orientated with posterior upwards. Arrowheads in A and B indicate morphogenetic furrow. The GMR enhancer drives expression of DAMM-FLAG in cells posterior to the morphogenetic furrow, which show staining with the anti-FLAG antibody. Examples of positive-staining cells are indicated by arrows. C–J represent light microscopic pictures of the wild-type and various transgenic fly eyes. Eyes are oriented with posterior to the left. C, wild type (Canton-S); D, GMR-damm H; E, GMR-damm F. Note that GMR-damm line F (E) shows a rough eye phenotype compared with wild-type (C), whereas GMR-damm line H (D) does not. F, GMR-damm C156G; G, GMR-hid/+; H, GMR-hid/+ GMR-damm C156G/+; I, GMR-rpr/+; J, GMR-rpr/+ GMR-damm C156G/+.”
has been shown to suppress cell death mediated by the Drosophila death activators REAPER and HID in the transgenic fly eye model (18, 19). To test whether DAMM plays a role in HID and REAPER-mediated cell death pathways, we crossed GMR-dammC156G flies with GMR-hid and GMR-rpr flies. As expected, flies containing one copy of GMR-hid or GMR-rpr showed a severely ablated eye phenotype (Fig. 8, G and I). Flies containing one copy each of GMR-hid and GMR-dammC156G showed a significant improvement of this ablated eye phenotype, and the eyes appeared larger and more structured than in GMR-hid/+ flies (Fig. 8H). Conversely, no significant suppression of the GMR-rpr ablated eye phenotype was seen in flies carrying single copies of GMR-rpr and GMR-dammC156G (Fig. 8J). These results suggest that DAMM may be required for HID, but not REAPER-mediated cell death.

DISCUSSION

We have described here the characterization of a new Drosophila caspase, DAMM. Low levels of damm transcript are widely expressed during Drosophila embryogenesis. Higher expression of damm mRNA in larval salivary glands and midgut suggests a possible role for DAMM in the programmed deleton of these larval tissues during larval/pupal metamorphosis. Additionally, high expression of damm mRNA in egg chambers suggests a possible role for DAMM in nurse cell death. Because high levels of damm transcript are also found in adult animals, DAMM may additionally be involved in regulating normal cell turnover in the adult.

In mammals, initiator caspases carrying specific protein-protein interaction domains are believed to be autoactivated by a proximity-induced model (1–4). The downstream effector caspases on the other hand require processing by initiator caspases. Because DAMM lacks a long prodomain, we analyzed whether it could be processed by known Drosophila caspases in vitro. However, as these caspases were unable to process DAMM, and because DAMM expression in E. coli does not result in cleavage of the DAMM precursor, it is possible that DAMM either does not require proteolytic processing, or its full activation requires an as yet uncharacterized protease. In contrast to DAMM and DECAY, DRICE and DCP-1 are efficiently processed in vitro by the putative initiator fly caspase DRONC (18, 19). It is possible that the recently discovered prodomain containing caspase, STRICA (13), may be involved in processing DAMM and DECAY. Interestingly, we observed processing of DAMM, along with DRONC and DECAY, in cycloheximide treated SL2 cells. However, the processing of DAMM resulted in a slightly smaller product than the DAMM precursor, which may be generated by the removal of a small amino-terminal peptide. This observation suggests that DAMM precursor may not be cleaved into two subunits. Most mammalian caspase precursors have some intrinsic caspase activity, and recent data suggest that processing is not always a prerequisite for caspase activation (43). It is thus possible that mechanisms, other than full processing, may be involved in the activation of DAMM.

Among several commercially available caspase substrates examined, recombinant DAMM produced in E. coli showed highest activity on pentapeptide VDVAD-amc. As there was no cleavage of a similar tetrapeptide substrate DEVD-afc, it appears that DAMM, like caspase-2 and DRONC (11, 19, 44), prefers a residue in the P6 position of the substrate. DAMM also showed significant activity on LEHD-amc and YVAD-afc substrates. However, activity of DAMM on these substrates was 4–5-fold lower when compared with its activity on VDVAD-amc, suggesting that among the peptides tested, VDVAD is the optimal substrate for DAMM. Interestingly, despite its overall similarity to caspase-6, DAMM had no significant activity on caspase-6 substrate VEID-amc.

In our coprecipitation experiments, DAMM did not interact with Diap1 and Diap2. Diap1 is known to interact with a number of Drosophila caspases including DRONC, DRICE, STRICA, and DCP-1 (13, 18, 19, 41), whereas Diap2 has only been shown to interact with STRICA (13). The exact mechanisms by which insect IAPs interact with and inhibit caspases are not known, but recent data with mammalian XIAP show that it interacts with the processed forms of both effector caspases, such as caspase-3, -7, and the initiator caspase, caspase-9 (45–48). By analogy, Diap1 is also likely to associate with and inhibit processed forms of caspases, although it has also been shown to interact with the prodomain region of DRONC (18). We believe that the lack of interaction between Diap1 and DAMM may be because of the fact that DAMM is not significantly processed in our transient overexpression experiments.

The strongest evidence for a role for DAMM in fly cell death comes from our genetic studies showing that the expression of a catalytically inactive DAMM mutant protein significantly suppresses the eye phenotype because of ectopic expression of HID. Interestingly, cell death because of the overexpression of REAPER (Fig. 8) or DRONC (data not shown) was not inhibited by the catalytically inactive DAMM, suggesting that DAMM may function only in specific cell death pathway(s). Generation of a loss-of-function damm mutant and genetic-interaction analysis with the use of such a mutant would shed further light on the role of DAMM in programmed cell death in Drosophila.

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