



# STRICA, a novel *Drosophila melanogaster* caspase with an unusual serine/threonine-rich prodomain, interacts with DIAP1 and DIAP2

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## Abstract

The recently published genome sequence of *Drosophila melanogaster* predicts seven caspases in the fly. Five of these caspases have been previously characterised. Here, we describe the *Drosophila* caspase, STRICA. STRICA is a caspase with a long amino-terminal prodomain that lacks any caspase recruitment domain or death effector domain. Instead, the prodomain of STRICA consists of unique serine/threonine stretches. Low levels of *strica* expression were detected in embryos, larvae, pupae and adult animals. STRICA is a cytoplasmic protein that, upon overexpression, caused apoptosis in cultured *Drosophila* SL2 cells that was partially suppressed by DIAP1. Interestingly, unlike other fly caspases, STRICA showed physical association with DIAP2, in cotransfection experiments. These results suggest that STRICA may have a unique cellular function. *Cell Death and Differentiation* (2001) 8, 387–394.

**Keywords:** apoptosis; caspase; prodomain; IAPs

**Abbreviations:** CARD, caspase recruitment domain; DED, death effector domain; IAP, inhibitor of apoptosis

## Introduction

Programmed cell death in metazoans is mediated by a family of cysteine proteases termed caspases, which cleave their substrates following an aspartate residue.<sup>1–5</sup> To date 14 caspases have been cloned in mammals.<sup>1–4</sup> Gene targeting studies in mouse suggest that some caspases play spatially restricted roles in apoptosis, whereas others seem mainly involved in the processing and activation of proinflammatory cytokines.<sup>6</sup> In *Drosophila melanogaster* there are seven caspases as predicted by the sequenced *Drosophila* genome, five of which, DCP-1, DREDD/DCP-2, DRICE,

DRONC and DECAY have been previously published,<sup>7–12</sup> whereas the other two, DAMM and STRICA, have not been characterised.<sup>5</sup>

Caspases are classified into two broad groups, based on the presence or absence of a long amino-terminal prodomain. The prodomains in some caspases contain specific protein–protein interaction motifs that appear to function in caspase activation either by mediating procaspase oligomerisation, or interaction of the caspase precursor with adaptor molecules leading to caspase autoprocessing.<sup>13,14</sup> Among the published *Drosophila* caspases, DREDD and DRONC contain long prodomains carrying death effector domains (DEDs) and a caspase recruitment domain (CARD), respectively.<sup>5</sup> In the case of mammalian caspase-9, interaction with the adaptor molecule Apaf-1 occurs through the CARD present in each protein.<sup>15,16</sup> Caspase-8 also associates with an adaptor molecule, FADD via DED interactions.<sup>17</sup> It is likely that DRONC and DREDD are functional homologues of caspase-9 and caspase-8 respectively, as evident by the ability of DRONC to associate with the *Drosophila* Apaf-1 homologue Dark,<sup>18–20</sup> and of DREDD with *Drosophila* FADD, dFADD.<sup>21</sup> DCP-1, DRICE, DECAY and DAMM lack long prodomains and are thus similar to downstream effector caspases in mammals.<sup>5</sup> *dcp-1* mutation results in larval lethality and melanotic tumors.<sup>7</sup> 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.<sup>22</sup> Heterozygosity at the *dredd* locus suppresses cell death induced by ectopic expression of *rpr*, *hid* and *grim* in transgenic models,<sup>9</sup> 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.<sup>23–25</sup>

Several lines of evidence suggest that DRONC is a key upstream caspase in the fly cell death machinery. *dronc* mRNA is upregulated several fold by ecdysone in larval salivary glands and midgut prior to histolysis of these tissues.<sup>11</sup> Furthermore, the 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.<sup>18,26,27</sup> Furthermore, loss of *dronc* function in early *Drosophila* embryos using the RNA interference technique results in a block of apoptosis, indicating that DRONC is required for programmed cell death during embryogenesis.<sup>18</sup> *In vitro* antibody depletion experiments suggest that DRICE is required for apoptotic activity in a *Drosophila* cell line.<sup>28</sup> Furthermore, DRICE physically associates with DRONC and can be processed by DRONC, suggesting that it may be downstream of DRONC in the apoptotic pathway.<sup>26</sup> The precise functions

of DECAY and DAMM in programmed cell death in *Drosophila* are currently not well established.

Two proteins, *Drosophila* inhibitor of apoptosis 1 and 2 (DIAP1 and DIAP2) suppress programmed cell death in the fly eye induced by REAPER, HID or GRIM overexpression.<sup>29</sup> Both DIAP1 and DIAP2 interact with REAPER, HID and GRIM.<sup>30,31</sup> Loss of function mutations of *diap1* suggest that this gene is required for the survival of cells.<sup>32,33</sup> DIAP1 inhibits the activity of a number of *Drosophila* caspases including DCP-1, DRICE and DRONC, and has been shown to interact directly with DRICE and DRONC.<sup>18,26,34,35</sup> Based on these results, it is proposed that REAPER, HID and GRIM mediate cell death by inhibiting DIAP1 function as a caspase inhibitor.<sup>36</sup> While the role of DIAP1 is clearly required for cell survival, DIAP2 function is more puzzling. Although *diap2* overexpression in the eye inhibits cell death by *rpr*, *hid* and *grim* overexpression, and DIAP2 can associate with REAPER, HID and GRIM proteins, the deficiencies removing the *diap2* locus have no effect on *rpr* or *hid*-dependent apoptosis in the eye.<sup>29,36</sup> Also, DIAP2 has not been shown to interact with any of the *Drosophila* caspases thus far.<sup>36</sup>

Here, we report cloning and initial characterisation of the *Drosophila* caspase, STRICA. STRICA contains an unusual and novel prodomain rich in Ser and Thr residues. We show that *strica* mRNA is widely expressed during development and overexpression of *strica* induces apoptosis in *Drosophila* SL2 cells. We also demonstrate that unlike other fly caspases, STRICA interacts with DIAP2.

## Results

### Identification of STRICA

While searching for new molecules with homology to various mammalian caspases using the TBLASTN program, we identified a genomic sequence contained in a database entry (accession no. AC014299) in the GenBank database which encoded a partial caspase-like molecule. Using a genomic PCR product, and with the help of an EST, we cloned the full-length cDNA for this molecule. A comparison with the *Drosophila* genomic sequence in GenBank revealed that *strica* cDNA sequence is encoded by 3 exons (Figure 1A), which differs from the gene structure predicted in the flybase. *strica* cDNA has an open-reading frame of 527 amino acid residues and the predicted protein shares significant homology with the mammalian caspases (Figure 1B,C). Analysis of the primary structure of this new caspase molecule revealed the presence of a long amino-terminal prodomain, rich in Ser and Thr residues. The prodomain sequence shares no homology with any of the other known caspase prodomains and lacks any CARD or DED-like motifs. This new molecule was named STRICA, for Ser/Thr/Rich Caspase.

Based on amino acid sequence comparison with other known mammalian and insect caspases, STRICA is most homologous to the long prodomain-containing caspases DREDD, and caspase-8 (Figure 1C). Interestingly, the caspase domain of STRICA shares the highest degree of identity with the caspase DAMM (GenBank accession no.

AF240763). The QACKG sequence surrounding the catalytic Cys residue is identical in DAMM and STRICA. None of the other five fly caspases share this sequence.

### *strica* expression during *Drosophila* development

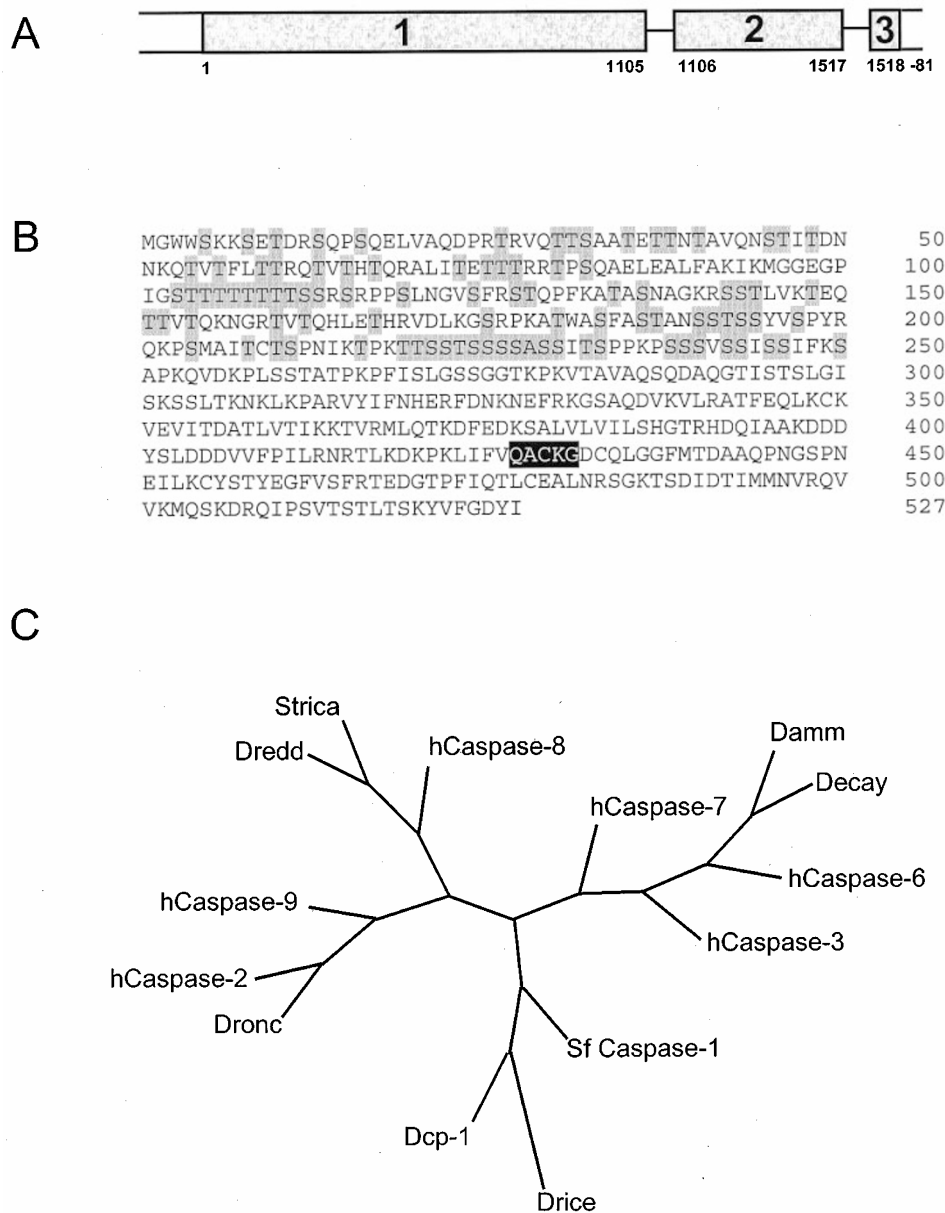
Northern blot analysis of *strica* expression showed very weak bands of around 2.2 kb in several developmental stages (data not shown) suggesting that *strica* is expressed at low levels during development. RT-PCR analysis of *strica* revealed that *strica* is expressed at all stages of development, however, expression was somewhat lower in late larval stages and higher during pupation and in adult flies (Figure 2). The expression pattern of *strica* during fly development was further analyzed by *in situ* hybridisation to *Drosophila* embryos and larval tissues using a digoxigenin-labeled antisense mRNA probe (Figure 3). In early embryos, *strica* mRNA was present uniformly, but became more concentrated in some tissues later in embryogenesis (Figure 3A–C). *strica* expression was clearly evident in the midgut and salivary glands from third instar larvae (Figure 3E). These tissues are known to undergo massive cell death during larva to pupa metamorphosis and are completely removed during pupation.<sup>37</sup> mRNA for other fly caspases, including *dronc* and *decay*, are known to accumulate in larval midgut and salivary gland before the histolysis of these tissues.<sup>11,12</sup> Relatively high levels of *strica* transcript was evident in the nurse cell compartment of stage 10a and 10b ovaries (Figure 3I). At later stages, when nurse cells dump their cytoplasmic contents into the oocyte<sup>38</sup> *strica* mRNA was clearly seen in the oocyte. These results indicate that the relatively high levels of *strica* RNA in early embryos (Figure 3A; data not shown for pre-cellularised embryos) are likely to be derived maternally.

### STRICA localises to the cytoplasm in transfected cells

To assess the cellular localisation of STRICA, cells were transiently transfected with expression constructs containing either the wild-type STRICA or STRICA with its catalytic Cys residue replaced with a Gly residue (C<sub>429</sub>G), both tagged at their carboxyl termini with the Flag epitope. Expression of Flag-tagged STRICA was then assessed by immunofluorescence using an anti-Flag monoclonal antibody. In both mammalian 293T cells and the *Drosophila* SL2 cells, STRICA protein was localised throughout the cytoplasm, completely exclusive of the nucleus (Figure 4). In 293T cells, STRICA appeared to be localised to the cytoskeleton, however, costaining with actin suggested that STRICA does not colocalise with actin fibres (data not shown).

### STRICA does not dimerise in yeast two-hybrid assays

Caspases with long prodomains are believed to be activated by a proximity-induced autocatalytic process.<sup>13,14</sup> In this model, CARDS or DEDs in the prodomains mediate oligomerisation of the procaspase molecules directly, or via specific adaptor molecules. In the case of mammalian caspase-2, the CARD region in the prodomain is sufficient



**Figure 1** (A) The genomic organisation of the *strica* gene is shown. The gene consists of three exons separated by two small intronic sequences. Numbers denote the coding region of the gene. The 5'- and 3'-most exon boundaries are not known. (B) The primary sequence of the STRICA protein is shown, with the catalytic Cys and surrounding residues highlighted in black. The putative prodomain region of STRICA is rich in Ser and Thr residues as highlighted in grey. (C) A phylogenetic tree of relationships between the *Drosophila* caspase family members and various mammalian caspases. The sequences for various caspases were derived from the GenBank database

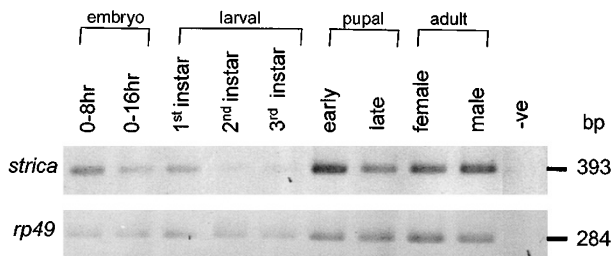
for homodimerisation in yeast two-hybrid assays.<sup>39,40</sup> Although the long prodomain of STRICA bears no similarity to any other known sequence in the database, we were interested to test whether the prodomain has the ability to homodimerise in yeast two-hybrid assays. Various STRICA constructs were generated in the yeast two-hybrid vectors pAS2.1 and pACT2. Pairs of constructs were simultaneously transformed into yeast strain Hf7C and plated onto medium lacking Trp and Leu to select for yeast colonies expressing both plasmids. To select for colonies expressing proteins

capable of interaction, the same transformation was plated onto medium lacking Trp/Leu/His. All STRICA transformants were able to grow on -Leu/-Trp medium demonstrating that these cells were expressing both constructs, however no growth was observed on -Leu/-Trp/-His plates (Table 1). Under similar conditions, two controls including murine p53/SV40 Large T antigen, and the caspase-2 prodomain were able to grow on both plates, as expected. These results suggest that, unlike the caspase-2 prodomain, the STRICA prodomain does not have the ability to homodimerise.

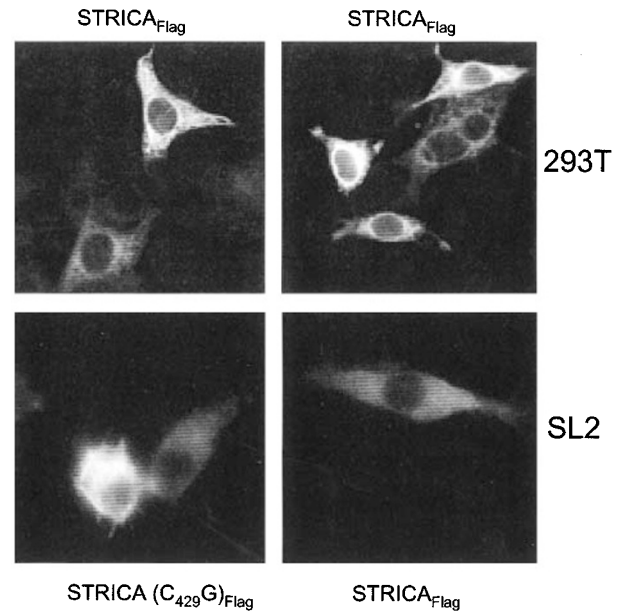
### Ectopic expression of STRICA induces apoptosis in SL2 cells

Many caspases, when overexpressed in cultured cells, induce apoptosis to some degree.<sup>1–4</sup> We therefore analyzed whether STRICA is able to induce apoptosis in transfected cells. We expressed *strica* in *Drosophila* SL2 cells from a copper-inducible insect promoter. At 48 h following induction of protein expression, around 50% of cells transfected with the wild-type *strica* construct were lost when compared to cells transfected with vector alone (Figure 5). An expression construct carrying a substitution mutant of the putative catalytic Cys (C<sub>429</sub>G) residue of STRICA was unable to induce cell death suggesting that the caspase activity of STRICA was responsible for its apoptosis-inducing ability (Figure 5). STRICA-induced apoptosis in SL2 cells was

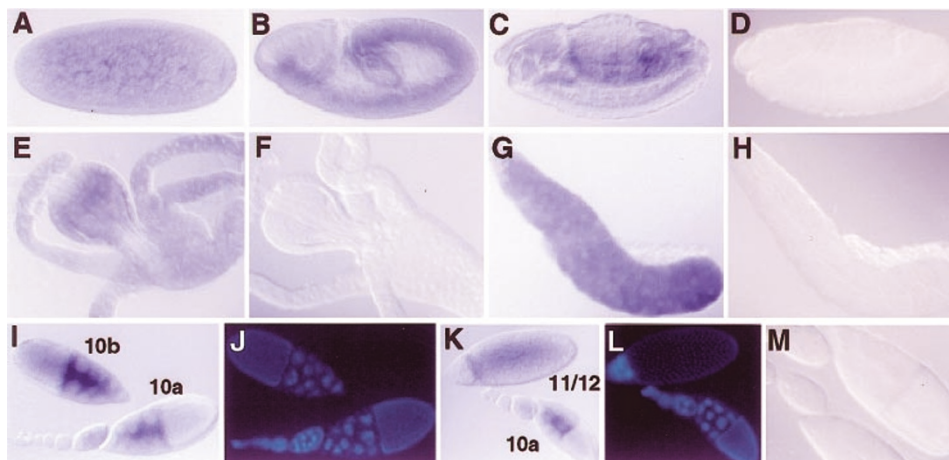
significantly inhibited by DIAP1. However, DIAP2 and the baculovirus apoptosis inhibitor P35 did not appear to significantly affect STRICA-induced apoptosis in this assay system (Figure 5).



**Figure 2** *strica* mRNA is expressed throughout *Drosophila* development. cDNA synthesised from total RNA was used as a template for PCR. The expected 393 bp *strica* product was detected in all stages of development. Expression is somewhat lower in late larval stages, before being upregulated in pupal stages and maintained in the adult. *Drosophila* ribosomal protein 49 (*rp49*) expressed ubiquitously throughout *Drosophila* development was used as an internal control. *strica* and *rp49* cDNA amplification was carried out simultaneously using appropriate primers in each individual reaction



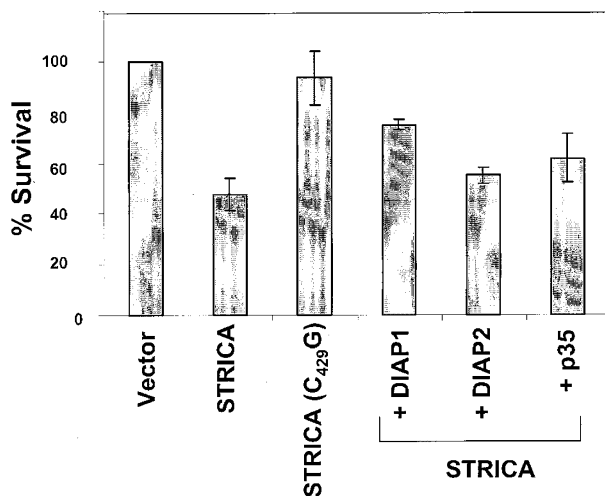
**Figure 4** Localisation of STRICA protein in transfected cells. Flag-tagged STRICA or STRICA(C<sub>429</sub>G) expression constructs were transfected into 293T cells (upper panels) and *Drosophila* SL2 cells (lower panels). For 293T cells, 24 h post-transfection, cells were fixed and stained with Flag antibody and analyzed by fluorescence microscopy. For SL2 cells, STRICA expression was induced by addition of CuSO<sub>4</sub> for 24 h prior to fixation, immunostaining and visualisation. In both cell types, STRICA expression is predominantly cytoplasmic



**Figure 3** *In situ* analysis of *strica* expression during development. A *strica* antisense RNA probe labelled with digoxigenin was used to detect expression *in situ*. (A) Expression of *strica* in a stage 4 syncytial blastoderm shows remnants of maternally deposited mRNA. (B, C) Germ band extended (stage 9–10) and germ band retracted (stage 13–14) with antisense *strica* probe. (D) Germ band retracted (stage 13–14) with sense control *strica* probe. Late 3rd instar larval midgut with antisense *strica* probe, (E) and negative control sense probe (F). Late 3rd instar larval salivary glands with antisense *strica* (G) and sense control probe (H). Adult ovaries (stage 10a and 10b) with sense *strica* probe (I) and stained with Hoechst DNA stain (J). Adult ovaries before (stage 10a) and after (stage 11/12) nurse cell cytoplasmic dumping (K). (L) Hoechst staining of the ovaries shown in (K). (M) Adult ovaries stained with sense *strica* probe

**Table 1** The prodomain of STRICA is not a dimerisation domain. Various STRICA constructs were expressed in the yeast two-hybrid vectors pAS2.1, which carries a TRP1 nutritional marker gene, and pACT2, which carries a LEU2 marker gene. HF7C competent yeast cells were transformed with pairs of constructs and plated onto selection medium. Positive interactors were able to grow on medium lacking His

Gal4BD construct in vector pAS2.1	Gal4AD construct in vector pACT2	Growth on – LEU-TRP	Growth on – LEU-TRP-HIS
Empty vector	Empty vector	+	–
Caspase-2 prodomain	Caspase-2 prodomain	+	+
p53	SV40 Large T antigen	+	+
p53	Empty vector	+	–
STRICA (C <sub>429</sub> G)	STRICA (C <sub>429</sub> G)	+	–
STRICA (C <sub>429</sub> G)	STRICA MPD	+	–
STRICA (C <sub>429</sub> G)	STRICA prodomain	+	–
STRICA prodomain	STRICA prodomain	+	–



**Figure 5** Overexpression of STRICA in cultured SL2 cells induces cell death. *Drosophila* SL2 cells were cotransfected with either 2  $\mu$ g empty pRmHa3 vector, 1  $\mu$ g pRmHa3-*strica*+1  $\mu$ g empty pRmHa3, or 1  $\mu$ g pRmHa3-*strica* (C<sub>429</sub>G)+1  $\mu$ g empty pRmHa3, and 0.5  $\mu$ g pCaSpeR- $\beta$ gal expression constructs. Expression was induced 24 h post-transfection by the addition of CuSO<sub>4</sub> to a final concentration of 0.7 mM. Loss of cells was determined 48 h post-induction by the ratio of blue cells in copper-induced transfectants to the number of blue cells in uninduced cells. A minimum of 200 cells were counted in each of three duplicate experiments. Where indicated, STRICA expression constructs were cotransfected in equal amounts with DIAP1, DIAP2 and p35 expression constructs in pRmHa3. Wild-type STRICA expression resulted in approximately 50% loss of cells, whereas the Cys mutant did not induce significant apoptosis

### STRICA interacts with with DIAP1 and DIAP2

Recent data suggest that DIAP1, but not DIAP2, is able to interact with a number of *Drosophila* caspases including DRONC, DCP-1 and DRICE.<sup>18,26,34–36</sup> To determine whether STRICA is capable of such interaction, cells were cotransfected with STRICA, or STRICA lacking the prodomain (STRICA-MPD), and DIAP1 or DIAP2. Cell lysates were immunoprecipitated and immuno-complexes analyzed by immunoblotting. DRONC, that has been shown to interact with DIAP1, but not DIAP2, in a prodomain-dependent manner,<sup>26</sup> was used as a positive control. As expected, DRONC was coprecipitated with DIAP1. Full-length STRICA coprecipitated with both DIAP1 and DIAP2 (Figure 6). Interestingly, STRICA-MPD was still able to interact with both DIAP1 and DIAP2, suggesting that the prodomain of

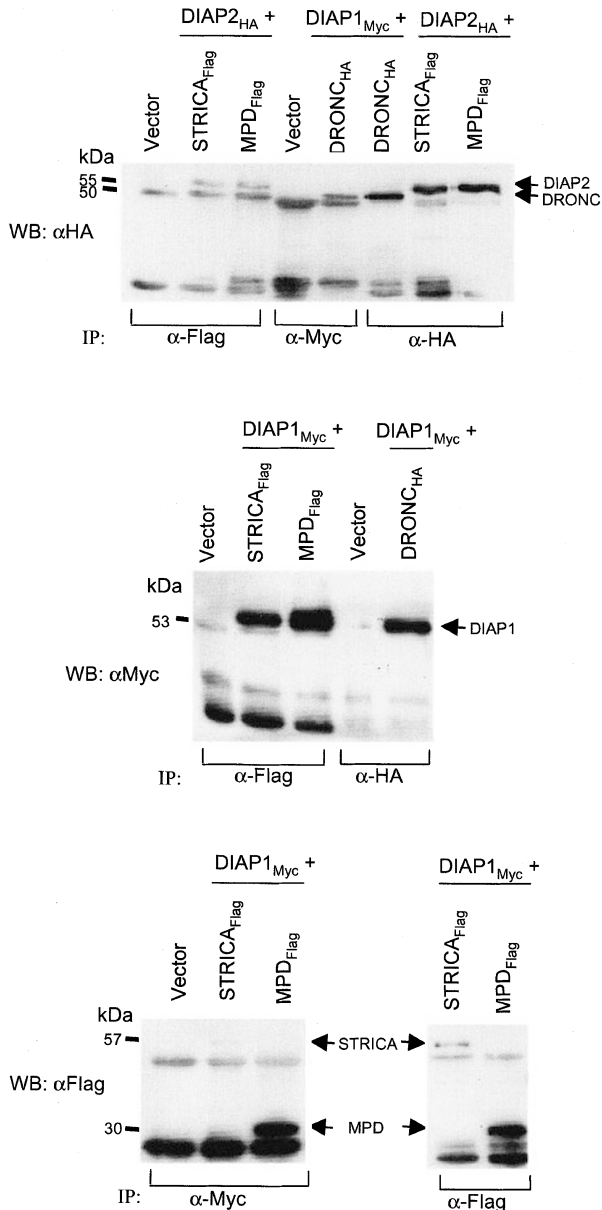
STRICA is not required for interaction with these inhibitors. We also tested whether STRICA and the baculovirus caspase inhibitor P35 can interact. However, in cotransfection experiments, no interaction between P35 and STRICA could be seen (data not shown).

### Discussion

Here, we have reported the identification and preliminary characterisation of the newest member of the *Drosophila* family of caspases, STRICA. STRICA was identified by a database homology search, and cloned from a *Drosophila* larval cDNA library. The resulting 527 amino acid polypeptide contains all the residues conserved throughout the caspase superfamily as well as a long amino-terminal prodomain rich in serine and threonine residues. Although the role of this novel prodomain is not well elucidated, it is clearly not required for oligomerisation of STRICA procaspase molecules, and does not mediate interaction with the *Drosophila* inhibitors of apoptosis, DIAP1 and DIAP2. Given the lack of homology of this domain to other known prodomains which contain various protein–protein interaction motifs, the role of the prodomain of STRICA is an interesting point for further investigation, which may reveal novel mechanisms of caspase activation and regulation. STRICA is expressed throughout *Drosophila* development at relatively low levels, and is seen in tissues which are known to undergo developmental apoptosis. STRICA localises to the cytoplasm of transfected cells. Although STRICA induces cell death when overexpressed in SL2 cells, the *in vivo* role of STRICA in apoptosis requires further investigation.

STRICA interacts with DIAP1, the role of which has been well defined in the apoptotic pathway. Interestingly, although DIAP2 does not significantly inhibit STRICA-induced apoptosis, interaction between these two proteins was clearly evident in transfected cells. The function of DIAP2 is less clear than that of DIAP1. Unlike DIAP1, DIAP2 has not been shown to interact with any other known caspases thus far.<sup>36</sup> The physiological relevance of the interaction between DIAP2 and STRICA is not known, however the lack of inhibition of STRICA-induced cell death by DIAP2 may indicate an involvement of STRICA and DIAP2 in a non-apoptotic cellular pathway.

So far we have not been able to test the biochemical activity of STRICA. Recombinant STRICA produced in *E. coli* does not show any activity on commercially available



**Figure 6** STRICA interacts with DIAP1 and DIAP2. 293T cells were cotransfected with various tagged cDNAs in pcDNA3 expression vector. Two  $\mu\text{g}$  of Flag-tagged STRICA or STRICA lacking the prodomain (MPD) were cotransfected with 2  $\mu\text{g}$  of either Myc-tagged DIAP1 or HA-tagged DIAP2. Forty-eight hours after transfection, cells were harvested and lysed. Both DIAP1 and DIAP2 were detected in lysates immunoprecipitated with an anti-Flag antibody. STRICA and STRICA-MPD were detected in lysates immunoprecipitated with anti-Myc, but not with anti-HA (data not shown). In a control experiment, HA-tagged DRONC coimmunoprecipitated with DIAP1, as expected. Expression of each construct was confirmed by immunoprecipitating with the appropriate antibody, followed by immunoblotting with the same antibody. All STRICA constructs used in these experiments contained the C429G mutation

caspase substrates, including YVAD, DEVD, VDVA, VEID and LHTD (data not shown). This suggests that either recombinant STRICA is not catalytically active, or that STRICA has a substrate specificity that is substantially

different from other caspases and not represented by the caspase substrates we have tested so far. We have also not detected processing of STRICA by other caspases (data not shown). Thus, the requirement for procaspase processing for STRICA activation is yet to be fully understood.

We have been unable to detect processing of STRICA in transiently transfected insect cells exposed to apoptotic stimuli (data not shown). This apparent lack of STRICA processing in apoptotic cells may suggest a role for this caspase in pathways additional to and/or outside of apoptosis. DIAP1 and DIAP2 have been shown to interact with Thick Veins, a Ser/Thr kinase receptor for Decapentaplegic (DPP), a *Drosophila* TGF- family member.<sup>41</sup> It may be that both STRICA and DIAP2 function in the DPP signalling pathway. To explore this possibility further, it will be necessary to identify other binding partners for STRICA and to generate and analyze the STRICA mutant phenotype. There are several potential Ser/Thr phosphorylation sites in the STRICA prodomain, which may mediate the binding of STRICA to other signalling molecules.

## Materials and Methods

### Cloning of *strica* cDNA and homology analysis

*strica* was identified using a TBLASTN search of the Berkeley *Drosophila* Genome Project database, as a region of genomic DNA sequence displaying significant homology to mammalian caspases. From this region of homology, *strica*-specific primers were designed and were used in conjunction with library-specific vector primers to amplify the partial open reading frame of *strica* from a *Drosophila* larval cDNA library. The 5' end of the *strica* open reading frame was obtained from EST clone LP09213 (Research Genetics). The cDNA sequence of *strica* has been deposited in the GenBank database under accession number AF242735. Multiple sequence alignments and construction of phylogenetic trees were carried out using Bionavigator software packages ClustalW and Protpars at the Australian National Genome Information Services server.

### Plasmid constructs

A 393 bp sequence, from residue 1108 to residue 1500, spanning the active site-encoding sequence of *strica* was amplified by PCR using *Taq* DNA Polymerase (Perkin Elmer), and cloned by overhanging T bases into pGEM<sup>®</sup>-T Easy (Promega) and sequenced. The 965 bp of the *strica* open reading frame starting from position 612 to the 3' end was amplified from a *Drosophila* larval cDNA library and cloned directionally into pcDNA3 vector (Invitrogen) using the following nucleotides: Primer A 5'-GCCGGTACCATGGCCATAACTTGCACATCTCCC and primer B 5'-CGCTCTAGATCACCTTGTTCATCGTCCTTGAATATAATCTCCAAAAGG. Primer B contained an *Xba*I restriction site (italicised) and a Flag tag-encoding sequence (underlined). Eight hundred and thirty-four bp of the 5'-most region of *strica* was amplified from an EST clone (LP09213) encoding an incomplete *strica/transferrin precursor* fusion and was directionally cloned into pcDNA3 using the following primers: Primer C 5'-GCCGGTACCATGGGTTGGTGGAGCAAGAAAAGCG and Primer D 5'-CTTGGTGCTCCTGACGATCC. Full-length *strica* was obtained by a 3-way cloning step using an internal *Ban*I restriction site in the region

overlapping between each incomplete pcDNA3 clone. The resulting pcDNA3-*strica*<sub>Flag</sub> clone was sequenced. Primers A, C and G (below) contained *KpnI* restriction sites (italicised). The putative catalytic Cys429 residue of STRICA was mutated to a Gly residue by Quikchange mutagenesis (Stratagene) using pcDNA3-*strica*<sub>Flag</sub> as a template. *strica*<sub>Flag</sub> and *strica*(C429G)<sub>Flag</sub> were amplified from pcDNA3-*strica*<sub>Flag</sub> and pcDNA3-*strica*(C429G)<sub>Flag</sub> using *Pwo* DNA polymerase (Roche Biochemicals) using primers B and C and cloned into insect vector pRmHa.3.<sup>42</sup> Minus prodomain (MPD) constructs were made by amplifying *strica* using Primer B and Primer G: 5'-GGCGGTACCATGGCGAAGCCGCTCAGTTCTACCGC, and cloning into pcDNA3.

Apoptosis inhibitors *diap1*, *diap2* and *p35* were amplified using *Pwo* polymerase (Roche Biochemicals) and the following primers: DIAP1A 5'-CGGAATTCATGGCATCTGTTGTAGCTGATC, DIAP1B 5'-CGCGGATCCGCGTCATGCGTAGTCTGGCACGTCGTATGGGTAAGAAAAATATACGCGCATC, DIAP2A 5'-CGGAATTCATGACG-GAGCTGGGCATG, DIAP2B 5'-CGCGGATCCGCGTCATGCG-TGGCACGTCGTATGGGTAATCGATTG-CTTAAGTGC, P35A 5'-CGGAATTCATGTGTGTAATTTTCCG, P35B 5'-CGCG-GATCCGCGTCATGCGTAGTCTGGCACGTCGTATGGGTAATTAAT-TTAC. In each case the forward primers contained an *EcoRI* site (in italics), while the reverse primers contained a *BamHI* site (in italics) and sequence encoding an HA tag (underlined). *diap1*<sub>HA</sub>, *diap2*<sub>HA</sub> and *p35*<sub>HA</sub> were cloned into pRmHa.3<sup>42</sup> for expression in insect cells and into pcDNA3 for expression in mammalian cells.

### In situ mRNA expression analysis

For *in situ* RNA analysis, antisense and sense digoxigenin labelled riboprobes were prepared using T7 and SP6 RNA polymerases from a linearized pGEM<sup>®</sup>-T Easy-*strica* clone containing a 393 bp fragment of *strica* cDNA. Digoxigenin labelling was performed according to the manufacturer's instructions (Roche Biochemicals). *In situ* hybridisation to *Drosophila* embryos and larval tissues were essentially as described.<sup>11,43</sup>

### Reverse transcriptase PCR

Total RNA was prepared from each *Drosophila* developmental stage from early embryo to adult fly using TRIzol reagent (Life Technologies) as per the manufacturer's instructions. Random-primed cDNA was synthesised from 5 µg total RNA using 1st strand cDNA synthesis kit (Amersham Pharmacia). One µl from each 33-µl reaction was used as a template in a PCR reaction using the following primers: Primer E 5'-CAAACCAAGGACTTTGAGGAC and Primer F 5'-CACTTGACGCA-CATTCAT. As a positive control, a region spanning from bp 196 to bp 480 of the *Drosophila* ribosomal protein 49 (*rp49*) gene was amplified. Products were separated by agarose gel electrophoresis, stained with ethidium bromide, and scanned using a fluorimager.

### Immunofluorescence

SL2 cells and 293T cells were seeded into 35-mm dishes and transfected as described.<sup>12,43</sup> Twenty-four hours post-transfection, cells were washed once in PBS and fixed in 4% paraformaldehyde/0.18% Triton X-100 in PBS. Cells were washed and incubated with anti-Flag monoclonal antibody (SIGMA Chemical Co). Cells were then incubated in anti-mouse-FITC and mounted beneath coverslips in 80% glycerol. STRICA expression was visualised by fluorescence microscopy.

### Apoptosis assays

Cell death assays in insect cells were carried out using Schneider L2 (SL2) cells. SL2 cells were maintained and transfected using Cellfectin (Life Technology) as described.<sup>43</sup> For death assays,  $1.5 \times 10^6$  SL2 cells were cotransfected with either 2 µg vector or 1 µg pRM-*strica*<sub>Flag</sub> together with 1 µg of respective pRmHa.3 inhibitor constructs, pRM*diap1*<sub>HA</sub>, pRM*diap2*<sub>HA</sub> or pRM*p35*<sub>HA</sub> and 0.5 µg pCaSpeR-βgal reporter.<sup>44</sup> All death assays were performed in duplicate. Twenty-four hours post transfection, cells were induced to express pCaSpeR-βgal by three cycles of heat shock at 37°C for 30 min followed by 27°C for 30 min. Following heat shock, one sample of each duplicate transfection was induced to express the respective pRmHa.3 constructs by the addition of CuSO<sub>4</sub> at a final concentration of 0.7 mM. Cells were fixed and stained with X-gal 48 h post-CuSO<sub>4</sub> induction as previously described.<sup>45</sup> Cell survival was calculated as the percentage of transfected (β-galactosidase positive) cells in the CuSO<sub>4</sub> treated population relative to the percentage of transfected cells in the untreated population. All calculations were normalised against the 100% survival value of vector-transfected cells. The results, shown as average percentages ± S.E.M., were derived from three independent experiments. To check copper-induced protein expression after 48 h of CuSO<sub>4</sub> treatment, cells were lysed in SDS-PAGE buffer and lysates were subjected to immunoblotting using an αHA antibody (Roche Molecular Biologicals) or αFlag antibody (Sigma Chemical Co.).

### STRICA interaction studies

293T cells were transfected using FuGene (Boehringer Mannheim). Two µg of pcDNA3-*strica*(C429G)<sub>Flag</sub> or pcDNA3-*strica*(C429G)<sub>MPD</sub><sub>Flag</sub> were cotransfected with either 2 µg pcDNA3 vector or 2 µg of pcDNA3-*diap1*<sub>myc</sub>, pcDNA3-*diap2*<sub>HA</sub> or pcDNA3-*p35*<sub>HA</sub>. As a positive control, pcDNA3-*dronc*<sub>HA</sub> was cotransfected with pcDNA3-*diap1*<sub>myc</sub>. Twenty-four hours post transfection, cell lysates were prepared in lysis buffer (20 mM NaCl, 0.2% Triton X-100, 10% glycerol, 20 mM Tris-HCl pH 7.5, supplemented with a 1 × protease inhibitor cocktail (Complete<sup>™</sup>, Roche) Lysates were immunoprecipitated with 2 µg of either αFlag M2 (Sigma Chemical Co.), αHA 11 (Roche) or αMyc (Roche). Immunoprecipitated protein was separated by SDS-PAGE and analyzed by immunoblotting using the appropriate antibodies.

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