# Identification of a *Drosophila melanogaster* ICE/CED-3-related protease, drICE

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Cysteine proteases of the ICE/CED-3 family (caspases) are required for the execution of programmed cell death (PCD) in a wide range of multicellular organisms. Caspases are implicated in the execution of apoptosis in Drosophila melanogaster by the observation that expression of baculovirus p35, a caspase inhibitor, blocks cell death in vivo in Drosophila. We report here the identification and characterization of drICE, a D.melanogaster caspase. We show that overexpression of drICE sensitizes Drosophila cells to apoptotic stimuli and that expression of an N-terminally truncated form of drICE rapidly induces apoptosis in *Drosophila* cells. Induction of apoptosis by rpr overexpression or by cycloheximide or etoposide treatment of Drosophila cells results in proteolytic processing of drICE. We further show that drICE is a cysteine protease that cleaves baculovirus p35 and Drosophila lamin DmO in vitro and that drICE is expressed at all the stages of Drosophila development at which PCD can be induced. Taken together, these results strongly argue that drICE is an apoptotic caspase that acts downstream of rpr. drICE is therefore the first unequivocal link between the molecular machinery of Drosophila cell death and the conserved machinery of Caenorhabditis elegans and vertebrates. Identification of drICE should facilitate the elucidation of upstream regulators and downstream targets of caspases by genetic screening.

Keywords: apoptosis/caspase/Drosophila melanogaster/ ICE/protease

#### Introduction

Programmed cell death (PCD) is an evolutionarily conserved, active, regulated process of cell suicide which is used to delete unwanted or damaged cells (Raff, 1992). Genetic studies on the nematode worm *Caenorhabditis elegans* have identified three principal genes that are involved directly in the regulation and execution of cell death: *ced-9*, *ced-3* and *ced-4* (Ellis and Horvitz, 1991; Hengartner *et al.*, 1992). Although *ced-4* has no known homologues (Yuan and Horvitz, 1992), the *ced-9* and *ced-3* genes are highly structurally and functionally conserved. *ced-9* is a negative regulator of cell death in the nematode and shares sequence homology with the mammalian *bcl-2* family of genes (Hengartner and Horvitz, 1994). The *bcl-2* family also regulates cell death in

mammals and *bcl-2* itself can partially substitute for *ced-9* function in *C.elegans. ced-3*, whose function is absolutely required for all cell deaths in the worm, is a cysteine protease with homology to the mammalian ICE-related proteases (caspases) (Yuan *et al.*, 1993).

The first identified caspase was Interleukin-1β-Converting Enzyme (ICE) (Cerretti et al., 1992; Thornberry et al., 1992) but at least 10 mammalian caspases have now been identified (reviewed in Takahashi and Earnshaw, 1996). All caspases thus far characterized are synthesized as inactive proenzymes that are activated by proteolytic processing to generate cysteine proteases with specificity for an aspartic acid residue at the P1 position. The sites at which this proteolytic processing takes place during caspase activation themselves conform to caspase substrate sites, suggesting the possibility of both autoactivation and caspase proteolytic cascades. The mechanism by which such cascades are initiated remains unclear although recent work on the CD95- and TNF-signalling pathways (reviewed in Fraser and Evan, 1996) has suggested that at least part of the upstream signalling impinging on caspase regulation may operate via the prodomain of these proteases.

There is strong evidence for the involvement of caspases in the execution of cell death. Caspase inhibitors, including the viral proteins crmA (Ray et al., 1992) and p35 (Clem et al., 1991) and synthetic tetrapeptide inhibitors, block cell death induced by a wide range of stimuli in all organisms so far studied (reviewed in Fraser et al., 1996). Caspase activity is required for cytoplasmic extracts of apoptotic cells to have the ability to induce the nuclear events of apoptosis (Lazebnik et al., 1994; Nicholson et al., 1995) (breakdown of the nuclear lamina, PARP cleavage and DNA condensation and degradation). Finally, there is strong homology between caspases and CED-3, whose activity is absolutely required for programmed cell death in the nematode.

While the basal machinery of apoptosis appears to be conserved between C.elegans and vertebrates, there are no identified homologues of any of the components of this machinery in Drosophila. Widespread PCD does occur during Drosophila development and can additionally be induced by stimuli such as DNA damage (Abrams et al., 1993). The cell deaths are identical at the morphological level to those that occur in other organisms suggesting a common mechanism. A genetic screen to identify genes required for cell death during Drosophila development (White et al., 1994) isolated a genomic region that was subsequently shown to contain at least three genes whose products can induce cell death: rpr (White et al., 1996), hid (Grether et al., 1995) and grim (Chen et al., 1996). Overexpression of any single one of these genes induces extensive cell death even in the absence of the other two. In all three cases, the deaths seem to be caspase dependent

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since they are blocked by coexpression of baculovirus p35 (a broad spectrum caspase inhibitor).

In this paper, we describe the cloning and characterization of drICE, a Drosophila melanogaster caspase. We show that drICE is expressed at all stages where death can occur during development, that it is a cysteine protease that cleaves p35 and lamin DmO, and that drICE is processed during apoptosis in *Drosophila* cells.

#### Results

#### Cloning of drICE

The suppression of cell death in Drosophila by p35 expression in vivo strongly indicates a role for caspases in the cell death machinery of *Drosophila*. To search for these caspases, we used a degenerate PCR-based strategy as described in Materials and methods. A unique band of ~200 bp was obtained after performing PCR on a 4-8 h embryonic D.melanogaster cDNA library (Brown and Kafatos, 1988) and this was used to probe the same cDNA library. The resulting full-length cDNA was sequenced and found to contain a single ORF, encoding a protein with 38.9% identity with human CPP32β (Fernandes-Alnemri et al., 1994; Nicholson et al., 1995; Tewari et al., 1995) and Mch2 (Fernandes-Alnemri et al., 1995) and 30.4% with *C.elegans* CED-3. The predicted protein, which we called drICE, contains all the residues required for catalysis by caspases. The catalytic cysteine (C211) sits in a QACQG pentapeptide, as is the case for certain mammalian caspases FLICE/MACH1 (Boldin et al., 1996; Muzio et al., 1996) and Mch4. The predicted small subunit contains a region similar to the P4 specificity loop of human CPP32β shown to be critical in determining its substrate specificity for an aspartic acid residue at the P4 position rather than a large hydrophobic residue (Rotonda et al., 1996) and drICE might therefore be predicted to share such a specificity. CED-3 also contains this P4specificity region and shares similar substrate specificity with CPP32 $\beta$ , from which we infer that all three proteases, from widely divergent organisms, probably share similar substrate specificity. drICE also contains an unusual N-terminal region which contains 30.8% Ser and 28.2% Gly (S32–Y69). The only other IRP known to contain a similar sequence is CED-3, whose N-terminal (S132-G206) highly Ser-rich (36.5%) region is of unknown function. Given the current emerging picture in which the N-terminal prodomains of caspases couple these enzymes to upstream regulators, the conservation of such a motif may have functional significance. Moreover, CED-3 is processed at D221 which is immediately C-terminal to the Ser-rich region and, since drICE also has an aspartic acid residue (D80) at the immediate C-terminal end of its Ser-rich region, this suggests that D80 might be the drICE N-terminal processing site.

#### Overexpression of drICE sensitizes S2 cells to apoptotic stimuli

To address whether drICE might be involved in the apoptotic pathway in *Drosophila*, we analysed the effects of its overexpression in the S2 Drosophila cell line. A full-length drICE ORF was cloned under the control of a metallothionein promoter and stably transfected into S2 cells. While overexpression of drICE had no direct effect

#### Α

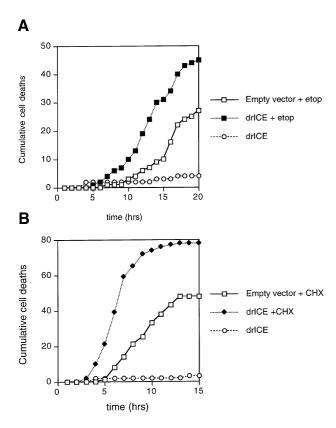
huice

| MDATNNGESADQVGIRVGNPEQPNDHTDALGSVGSGGAGSSGLVAGSSHP                                   | 50  |
|--|-----|
| YGSGAIGQLANGYSSPSSSYRKNVAKMVTDRHAAEYNMRHKNRGMALIFN                                   | 100 |
| HEHFEVPTLKSRAGTNVDCENLTRVLKQLDFEVTVYKDCRYKDILRTIEY                                   | 150 |
| SASQNHSDSDCILVAILSHGEMGYIYAKDTQYKLDNIWSFFTANHCPSLA                                   | 200 |
| ${\tt GKPKLFFI} \underline{{\tt OACOG}} {\tt DRLDGGVTMQRSQTETDGDSSMSYKIPVHADFLIAYS}$ | 250 |
| ${\tt TVPGFYSWRNTTRGTWFMQSLCAELAANGKRLDILTLLTFVCQRVAVDFE}$                           | 300 |
| SCTPDTPEMHOOKOIPCITTMLTRILRFSDKOLAPAGRV  | 339 |

#### B drICE MDATNNGESA DQVGIRVGNP EQPNDHTDAL GSVGSGGAGS SGLVAGSSHP mch2 срр32 ...MENTENS RARSRSRA LHSSDRHNYS SPPVNAFPSO PSSANSSFTG CSSLGYSSSR ced3 IDSVIPKGAQ ACQICITYIC EEDSYLAGTL GLSADQTSGN YLNMODSOGV huice 107 drICE YGSGAIGQLA NGYSSPSSSY RKNVAKMVTD RHAAE..YNM mch2 RRGHPAGGEE NMTETDAFYK REMFD......PAEK.YKM ..... 39 VDSKSIKNLE PKIIHGSESM DSGIS..... срр32 .LDNS..YKM ..... 39 ced3 NRSESKASGP TOYTEHEEDM NEVDAPTISE VEDEKTMYRN huice LSSFPAPQAV QDNPAMPTSS GSEGNVKLCS LEEAQRIWKQ KSAEIYPIMD 157 RHKNRGMALI DHRRRGIALI DYPEMGLCII FNHEHFE.VP TLKSRAGTNV FNHERFFWHL TLPERRTCA INNKNFHKST GMTSRSGTDV INNEHFE... QMPTRNGTKA drICE CENITRVIK QLDFEVTVYK RDN.TRRFS DIGFEVKCFN AANLRETFR NIKYEVRNKN mch2 89 срр32 ced3 FSSPRG KDNLTNLFR TCKD 284 ICNEEFD. SIPRRIGAEV huice KSSRTRI drICE DCRYKDLRT TEYSA.SONE SDSDCILVAL SHGEMGYTN 181 ADADCFVCVF SKRSSFVCVL SHGDSAILVI LSHGEGNHIY SHGEEGIIF SHGEENVII LLK AYDA.K... 133 cpp32 ced3 GTNG.P.... GVDDIP.... DLTREEIVEL MRDVS.KED 133 .KHE 328 huice NLTASDMTTE LEAFAHRPEH KTSDSTFLVF MSHGIREGIC GKKHSEQVPD FTANHCPSIA GKPKLFFIDA COCDRLDGGV FKGDKCHSIV GKPKIFFIDA CRCMOHDVPV FRGDRCRSIT GKPKLFIIDA CRCTELDCGI LNAANAPRIA NKPKIVFVOA CRCERRDNGF LNTKNCPSIK DKPKVIIIQA CRCDSPGVVW .YKLDNIWSF .IEIQTLTGL .VDLKKITNF drICE 221 mch2 IPLDVV<u>D</u>NQT срр32 172 .ISTHEIYDL ced3 PVLDSVDGVP huice FKDSVGVSGN 304 AYSTVPGFYS CYSVAEGYYS drICE MORSQTETDG DSSMSY.... KIPVHADELI AASVY.... mch2 EKI-DTNTTEV TIPAGADE 218 срр32 ..ETDSGVD KIPVDADFLY AYSTAPGYY 205 ced3 AFLRRGWDNR GPLFNFLGC VRPQVQQVWR KKPSQADILI RYATTAQYVS huice LSLPTTEEFE ...... K KAHIEKOFIA 339 drICE IQDLCEMLGK YGSSLEFTEL IQSLCAMLKQ YADKLEFMHI mch2 HRETVN SO RRVDFCKD.P 267 VAT EFESFSFD.A VAC GFQT...SQ срр32 ced3 ISAR 473 EMHQQKQIFC ITTM LTRIL SAIGKKYPC FASM LTKKL TFHAKKQIPC IVSM LTKEL GSNILKOMPE MTSR LLKKP QPDGRAOMPT TERVILTRCF drICE RESDKOLAPA GRV\* 339 mch2 HPFPKSN\*.. 293 срр32 YEYH\*.....YFWPEARNSA V\*. 503

Fig. 1. Amino acid sequence of drICE. (A) Predicted primary amino acid sequence of drICE. The QACQG pentapeptide surrounding the catalytic C211 is underlined. (B) Sequence alignment of drICE with nematode CED-3 and human CPP32 $\beta$ , Mch2 and ICE. The QACQG/ QACRG pentapeptide is in a shaded box, identical residues in all five proteins boxed and conserved residues are shaded. Known proteolytic processing sites have the P1 Asp underlined and the putative mature subunits of drICE are overlined. The locations of the PCR primers used for the initial degenerate PCR are shown.

on the cells, it significantly sensitized S2 cells to apoptosis (Figure 2A and B). A population of S2 cells overexpressing drICE died at a significantly increased rate relative to cells containing empty vector when induced to die by either cycloheximide or etoposide treatment. The observation that overexpression of drICE does not induce apoptosis in the absence of any apoptotic stimulus is consistent with a model in which caspases are principally post-transla-



**Fig. 2.** Overexpression of *drICE* sensitizes S2 cells to apoptosis. drICE was expressed in S2 cells under the control of a metallothionein promoter. 18 h after induction of expression of drICE with 0.7 mM CuSO<sub>4</sub>, cells were treated with either etoposide at 10  $\mu$ g/ml (**A**) or cycloheximide (CHX) at 5  $\mu$ g/ml (**B**) and cell deaths monitored by time-lapse videomicroscopy as described (Evan *et al.*, 1992).

tionally regulated and this led us to investigate the role of processing in drICE regulation and activation.

## Expression of a form of drlCE lacking its N-terminus induces rapid cell death in S2 cells

One of the critical processing events during caspase activation by proteolysis is the removal of an N-terminal prodomain. Given the tight regulation of the activity of full-length drICE in S2 cells even after overexpression, we decided to investigate the effects of expression of an N-terminally truncated form of drICE. An ORF corresponding to amino acids 81–339 of drICE was constructed, cloned under control of a metallothionein promoter and stably transfected into the S2 cells. Induction of expression of this ORF (drICE-N) rapidly resulted in apoptosis of S2 cells (Figure 3A) including characteristic blebbing of the cells, chromatin condensation and DNA degradation (Figure 3C and D). Overexpression of a catalytically inactive mutant drICE-N (C211A) had no effect on S2 cells (Figure 3A). The cell death induced by overexpression of drICE-N was completely blocked by the caspase inhibitors zVAD.fmk (Slee et al., 1996) and BocAsp.fmk (Figure 3B), consistent with the activity of drICE as a caspase.

### drICE is proteolytically processed during apoptosis in S2 cells

Apoptosis in S2 cells induced by overexpression of *rpr*, cycloheximide treatment or etoposide treatment is blocked by caspase inhibitors (Pronk *et al.*, 1996; data not shown).

S2 cells express drICE (Figure 4) and we therefore addressed the question of whether drICE is proteolytically processed during apoptosis in these cells as would be expected for a critical caspase in the apoptotic pathway. An *rpr* ORF was placed under the control of a metallothionein promoter and stably transfected into S2 cells. Following *rpr* induction, S2 cells underwent apoptosis (Figure 4A) and endogenous drICE was processed giving rise to both p21 (data not shown) and p12 subunits (Figure 4B). drICE processing was first detectable at time points where very little cell death could be seen in the cell pool. Analogous results were obtained for etoposide- and cycloheximide-induced apoptosis (data not shown).

### drICE is a cysteine protease that cleaves p35 and lamin DmO

Many caspases are known to autoprocess when over-expressed in *Escherichia coli* and we made use of this to purify mature processed drICE. We N-terminally Histagged drICE-N and produced this fusion protein under the control of the *trc* promoter in *E.coli*. The resulting protein was purified and found to contain three species when analysed by SDS-PAGE: a p30 form (His-drICE-N), a p21 (His-large subunit) and a small subunit of p12. The protein was >90% pure by SDS-PAGE (Figure 5A). Immunoblotting confirmed that the His tag was as expected on the p30 and p21 bands (Figure 5A).

We next assayed the ability of this purified protease to cleave p35 and lamin DmO. The baculovirus p35 protein inhibits all cell death in vivo in Drosophila and has been shown to act as a caspase inhibitor by acting as a substrate. If drICE is part of the basal machinery in *Drosophila*, it should therefore cleave p35. The Drosophila lamin B homologue, lamin DmO (Gruenbaum et al., 1988), is cleaved during S2 cell apoptosis (Figure 6) and therefore represents a potential effector substrate for drICE. 35Slabelled p35 or DmO proteins were incubated with the purified drICE enzyme which yielded the results shown in Figure 5B and 5C respectively. p35 is an excellent substrate for drICE, being cleaved to completion within 1 h at 37°C. While the cleavage of DmO is less efficient, the fragments obtained comigrate exactly with the DmO fragments in apoptotic S2 cells (data not shown). drICE activity in both reactions is completely inhibited by the addition of 5 mM iodoacetamide (Figure 5B and C) but not by a variety of other protease inhibitors (TLCK, TPCK, aprotinin and PMSF; data not shown), confirming that drICE is a cysteine protease.

#### drICE is expressed at all stages of development

Programmed cell death first becomes apparent during *Drosophila* development at ~7 h after egg laying (AEL) (Abrams *et al.*, 1993). However, it is possible to induce apoptosis at earlier times by DNA damaging agents (X-irradiation) or by overexpression of apoptotic activators such as *rpr* or *grim*. Since all these stimuli activate a caspase-dependent apoptotic programme, the caspases required for cell death must be present at all times at which death can be induced. A developmental Northern blot of drICE expression (Figure 7) shows clearly that drICE expression is highest at 2–6 h AEL. Intriguingly, this is not a period in which any detectable cell death is observed in the embryo: however, drICE is synthesized

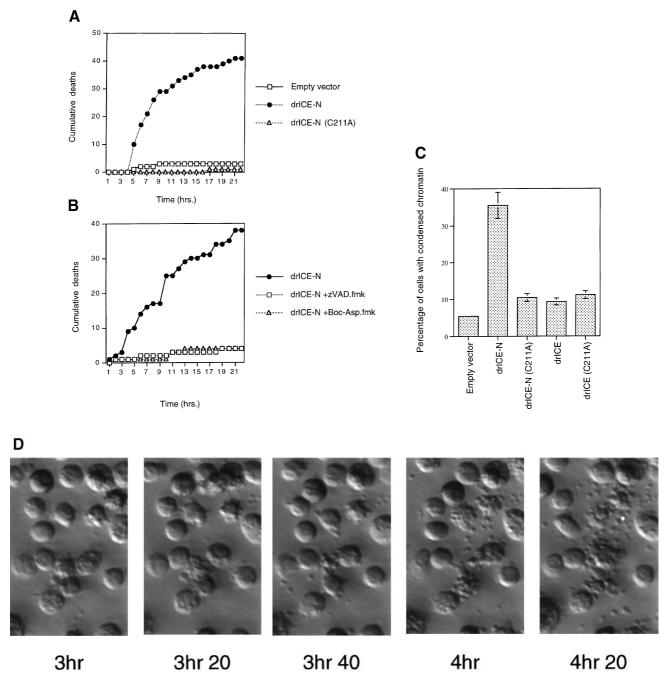


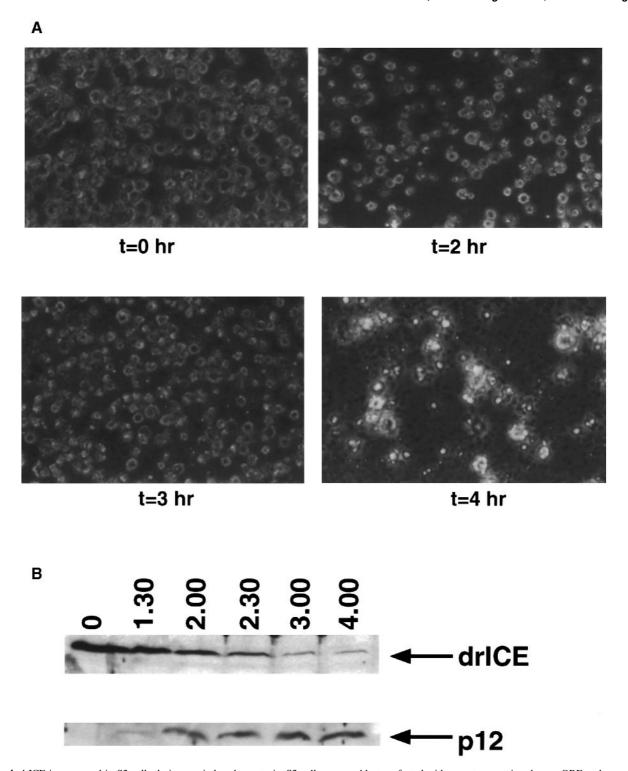
Fig. 3. Induction of apoptosis in S2 cells by an N-terminally truncated form of drICE. (A) Time-lapse videomicroscopic analysis of apoptosis of S2 cells following induction of expression of an N-terminally truncated form of drICE (drICE-N) or of expression of an inactive drICE-N mutant (C211A). (B) Suppression of drICE-N-induced apoptosis by zVAD.fmk and Boc-Asp.fmk as monitored by time-lapse videomicroscopy (Evan *et al.*, 1992). (C) Analysis of chromatin condensation following drICE-N induction by fluorescence microscopy and acridine orange staining. Percentages of cells with condensed chromatin (apoptotic cells) are expressed as percentages of total cell number. (D) Time course of drICE-N-induced cell death in S2 cells as visualized by time-lapse videomicroscopy and showing S2 apoptotic morphology. The times shown indicate time after induction of expression of drICE-N.

as an inactive pro enzyme, so mRNA expression gives no indication of enzyme activity. Thereafter, drICE expression remains detectable throughout *Drosophila* development, consistent with the notion that caspase machinery is present at all stages at which PCD can occur.

#### **Discussion**

The basal machinery controlling PCD appears to have been substantially conserved throughout metazoan evolution. The mammalian *bcl*-2 gene family encodes cell death regulator proteins that exhibit structural and functional homology with the product of the death-suppressing *ced*-9 gene of *C.elegans*. Likewise, the *ced*-3 'killer' gene of *C.elegans* encodes a member of the conserved caspase family of cysteine proteases, members of which play critical proapoptotic roles in mammalian apoptosis.

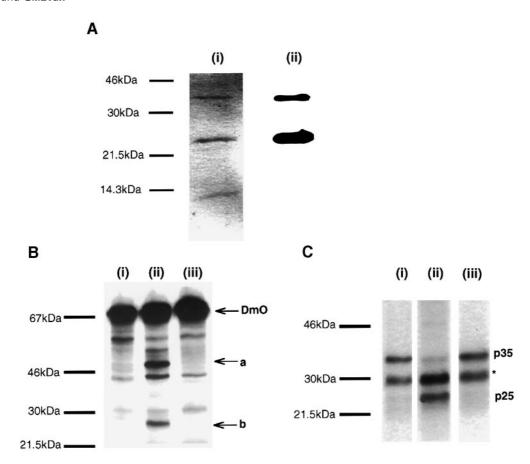
However, despite this conservation of the basal machinery between the nematode and mammals, neither *ced-9/bcl-2* nor caspase homologues has yet been identified



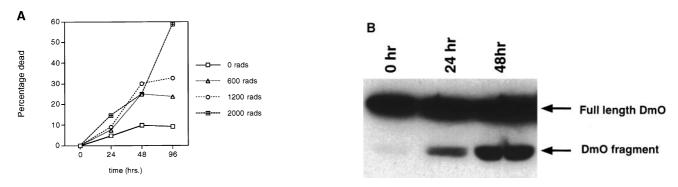
**Fig. 4.** drICE is processed in S2 cells during *rpr*-induced apoptosis. S2 cells were stably transfected with a vector containing the *rpr* ORF under control of a metallothionein promoter. Cell blebbing and cell death during apoptosis were determined by microscopic inspection. (**A**) S2 cells rapidly undergo apoptosis following *rpr* induction. (**B**) Cell lysates were prepared at the time points indicated (equivalent to time after *rpr* induction by 0.7 mM CuSO<sub>4</sub>), fractionated on an SDS gel, electroblotted and probed with a polyclonal antibody raised against a drICE C-terminal peptide, as described in Materials and methods.

in the fruit fly, *D.melanogaster*. Indeed, the proapoptotic machinery so far defined in *Drosophila* via genetic screening comprises three genes, *rpr*, *hid* and *grim* that share little if any homology with known components of the mammalian death machinery and none at all with the nematode. The only clear link thus far between the

apoptotic machinery in the fly and that in other organisms is that cell deaths in the fly, whether developmental or induced by DNA damage or overexpression of *rpr*, *hid* or *grim*, are all inhibited by expression of the baculovirus *p35* gene. Because p35 acts as a caspase inhibitor, this has been taken as evidence that cell deaths in the



**Fig. 5.** drICE is a cysteine protease that cleaves lamin DmO and p35 *in vitro*. (**A**) SDS–polyacrylamide gel characterization of purified bacterially expressed His<sub>6</sub>-tagged drICE. Lanes: (i) 100 ng purified His<sub>6</sub>-tagged drICE stained with Coomassie Blue; (ii) 10 ng purified His<sub>6</sub>-tagged drICE analysed by immunoblotting using the Invitrogen AntiXpress antibody directed against the N-terminal tag. (**B** and **C**) Purified bacterially expressed drICE cleaves *in vitro* translated <sup>35</sup>S-labelled lamin DmO to give fragments a and b; and <sup>35</sup>S-labelled p35 to generate p25 and p10 (not resolved). The asterix denotes an internal *in vitro* translation initiation product of p35. Both cleavages are inhibited by 5 mM iodoacetamide. Lanes: *in vitro* translated products incubated for 2 h at 37°C with (i) control (no protease added); (ii) plus 1 ng purified bacterially expressed His<sub>6</sub>-tagged drICE-N; (iii) as (ii) but with 5 mM iodoacetamide present.



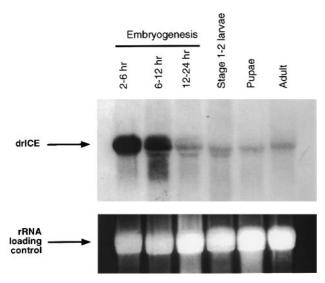
**Fig. 6.** Lamin DmO is cleaved during apoptosis in S2 cells. (**A**) S2 cells undergo apoptosis following X-irradiation, as assayed by exclusion of Trypan Blue. Percentages of dead cells were scored as percentage of total cells that no longer excluded Trypan Blue. (**B**) Lysates of S2 cells were prepared following X-irradiation with 2000 rads at the times shown, fractionated by SDS gel electrophoresis, electroblotted and probed with a monoclonal directed against lamin DmO.

fly, as in other organisms, utilize a caspase-containing machinery.

In this paper, we describe the cloning of a *D.melanogaster* caspase, drICE. drICE contains all the residues known to be involved in caspase catalysis and exhibits highest homology with the mammalian caspases Mch2 and CPP32 $\beta$  (38.9%). In addition to this overall homology with CPP32 $\beta$ , drICE also contains a region in its putative small subunit that corresponds to the P4-specificity loop

of CPP32β. Residues in this loop are critical in dictating the DXXD substrate specificity for CPP32β, suggesting that drICE also shares the same substrate specificity. The same P4-specificity loop is also present in the nematode CED-3 caspase which has a similar substrate specificity to CPP32β: thus, a critical apoptotic enzyme with DXXD substrate specificity appears to have been conserved through evolution.

Overexpression of full-length drICE sensitizes S2



**Fig. 7.** drICE mRNA is detectable at all developmental stages in *Drosophila*. 10 μg of total RNA per lane was fractionated on a 1.2% agarose gel, blotted and probed overnight with a  $^{32}$ P-labelled drICE ORF probe. The blot was washed at 65°C in 0.2× SSC, 0.1% SDS and exposed to Kodak XAR-5 film with an intensifying screen at -70°C for 48 h. Prior to blotting, the gel was stained with ethidium bromide in order to visualize the ribosomal RNAs as a loading control.

*Drosophila* cells to apoptosis but does not induce it alone. In contrast, overexpression of an N-terminally truncated form of drICE (drICE-N) is sufficient to trigger apoptosis rapidly in the same cell line. The difference between the effects of full-length and drICE-N in S2 cells is consistent with the notion that the N-terminal prodomain represses drICE activation. In this regard, it is interesting that the mammalian caspase proFLICE/MACH1 can be activated either via proteolytic processing by another protease (e.g. by granzyme B in vitro) or through interaction of its N-terminus with the death-domain adaptor FADD/ MORT-1 as part of the CD95- and TNF-killing pathways. In principle, therefore, drICE could also be activated via proteolytic abscission of its inhibitory N-terminus (resulting in a drICE-N-like molecule) by an upstream protease, or by interaction via the N-terminus with activators analogous to FADD/MORT1. The reported homology between rpr and the death domains of CD95 and TNFR1 (Golstein et al., 1995), which are required for the recruitment of FADD/MORT1 (Boldin et al., 1995; Chinnaiyan et al., 1995) and hence FLICE/MACH1 to the Death Inducing Signalling Complex (DISC) make the latter model particularly attractive. In this case, rpr could be involved in recruiting drICE (perhaps via other molecules) to an analogous 'DISC' resulting in drICE activation and apoptosis. Where mammalian FLICE/MACH1 activity is regulated by the action of external ligands (FasL or TNF), Drosophila drICE regulation would presumably be linked to transcriptional regulation of rpr.

However it is regulated, if drICE is a genuine and necessary part of the basal apoptotic machinery in the fly then processing to its mature, active form should accompany apoptosis. We have shown this to be the case: drICE is proteolytically processed to its mature subunits when

apoptosis is induced in *Drosophila* cells by *rpr* overexpression, or by the action of the topoisomerase II inhibitor etoposide or cycloheximide. Proteolytic processing of drICE is first detectable very early in such cultures, at a time when little overt apoptotic death is observed in the cell pool, suggesting that drICE processing is an early event in the execution of apoptosis.

drICE autoprocesses when expressed in E.coli. This autoprocessing is seen with most caspases expressed in bacteria—even when the processing sites of the caspase do not conform to the precise substrate specificity of the caspase being expressed. Probably the extremely high concentrations of enzyme in bacterial extracts allow more promiscuous cleavage specificities. The purified bacterially expressed drICE enzyme is an iodoacetamide-sensitive cysteine protease that cleaves baculovirus p35 and lamin DmO in vitro. Lamin DmO is cleaved during normal apoptosis in S2 cells and the resultant fragments are identical in size to those generated by bacterially expressed drICE in vitro, suggesting that drICE may function as the apoptotic laminase. Lastly, drICE mRNA is detectable throughout Drosophila development and thus drICE is present at all stages at which apoptosis can be induced.

Taken together, the above data firmly suggest that drICE is a genuine apoptotic caspase and as such is the first component of the conserved apoptotic machinery to be identified in *Drosophila*. The observations that drICE is processed during *rpr*-induced apoptosis and that drICE cleaves p35 *in vitro* are both consistent with the idea that drICE is a critical p35-inhibitable caspase that acts downstream from *rpr*. Elucidation of the precise nature of the molecular mechanism by which *rpr*, *hid* and *grim* trigger drICE activation is likely to prove highly revealing and the combination of *in vivo* genetics, cell culture and biochemical techniques that are possible in *Drosophila* should rapidly advance our understanding of caspases and the mechanisms by which their activity is regulated.

#### Materials and methods

#### Cloning of drICE

Five nanogrammes of a 4–8 h embryonic *D.melanogaster* cDNA library (Brown and Kafatos, 1988) was used as template for PCR. An initial 30 cycle PCR of 1 min 94°C, 1 min 58°C, 1 min 72°C was carried out using primers F1 (5'-AAGCCSAAGITSDTSATCRTSCAGG) and a T7 anchor primer (5'-AATACGACTCACTATAG); 0.1% of this reaction was reamplified in a 30 cycle reaction of 1 min 94°C, 1 min 48°C, 30s 72°C using primers F2 (5'-CAGGCNTGYCGNGG) and R2 (5'-ACASCWSAWWATGWKTC) where S = C, G; D = A, G, T; R = A, G; W = A, T; K = G, T; I = deoxyinosine. All reactions were performed in 100 µl volume containing 1.5 mM MgCl<sub>2</sub>, 100 µM dNTP, 500 ng of each primer and 2.5 U of Taq polymerase in 1X BioTaq NH<sub>4</sub><sup>+</sup> buffer. The PCR product obtained was cloned into pCR 2.1 using the TA cloning kit according to manufacturer's instructions (Invitrogen) and then sequenced. The PCR product was subsequently used to probe the same library that had been used as a PCR template. The longest clone obtained was subcloned and sequenced both manually and using an automated sequencer. The amino acid alignment was carried out using the PILEUP program. The accession number of drICE is Y12261.

#### Culture and transfections of S2 cells

ORFs corresponding to full-length drICE, full-length FLAG-tagged drICE and drICE-N were generated by PCR. The C211A mutation was introduced using the Altered Sites II system (Promega) according to the manufacturer's instructions. All ORFs were cloned into pMK33, which contains a hygromycin resistance marker, placing them under the control of a metallothionein promoter. S2 cells were grown in Gibco Schneider

medium supplemented with L-glutamine and 10% fetal calf serum and transfected according to standard CaPO<sub>4</sub> protocols and selected with hygromycin (200  $\mu g/ml$ ). Expression was induced in pools of cells by addition of CuSO<sub>4</sub> to 0.7 mM final concentration. By time-lapse videomicroscopy, apoptotic deaths were recorded as described in Evan et al. (1992) based upon membrane blebbing. zVAD.fmk and BD.fmk were each added to the culture medium at a final concentration of 50  $\mu$ M. Chromatin condensation was assayed by acridine orange fluoresence (acridine orange used at a final concentration of 5  $\mu$ g/ml in PBS). Cycloheximide was added to a final concentration of 5  $\mu$ g/ml and etoposide to 10  $\mu$ g/ml.

### Antisera and Western blotting

Polyclonal anti-peptide antisera were raised against KLH-conjugated peptides corresponding to amino acids 81–94 (RHA) and 326–339 (p12). Anti-FLAG antibody purchased from Kodak. Anti-Xpress antibody purchased from Invitrogen. Anti-DmO monoclonal obtained as a personal gift from Professor David Glover. All antisera were used at 1:1000 dilution in TMT (1× TBS pH 8.1, 2% milk, 0.5% Tween). Horseradish peroxidase-conjugated secondary antibodies were purchased from Amersham and used at 1:2500 in TMT.

### Purification of processed drICE and protease assays

An ORF corresponding to drICE-N was cloned into pTrcHisB (Invitrogen) to allow expression of His<sub>6</sub>-tagged drICE-N in *E.coli* under control of the *trc* promoter. The construct was transformed into TOP10 *E.coli* and expression induced in a 50 ml liquid culture with 0.1 mM IPTG. Cells were harvested after 90 min and a native IMAC protein purification carried out according to manufacturer's instructions (Invitrogen). His<sub>6</sub>-tagged drICE-N was eluted with 150 mM imidizole and its purity assayed by gel electrophoresis. Immunoblotting of His<sub>6</sub>-tagged drICE-N was carried out according to standard procedures.

cDNAs encoding p35 and lamin DmO were cloned into pBluescript (Stratagene) and *in vitro* transcribed/translated for 1 h at 30°C using the Promega reticulocyte lysate TnT system in the presence of [35S]methionine (Amersham). Protease assays were then carried out using 1 ng purified bacterially expressed His<sub>6</sub>-tagged drICE-N in a 50 mM NaCl, 25 mM Tris–HCl pH 7.5 buffer using 50% volume of the TnT reaction in a final volume of 10 μl at 37°C for 2 h, either in the presence or absence of 5 mM iodoacetamide (Sigma). The resulting products were analysed by SDS–PAGE.

#### Northern blotting

10  $\mu g$  of total RNA prepared using Trizol according to manufacturer's instructions (GibcoBRL) were loaded per lane and blotted according to standard protocols. The blot was probed overnight with a  $^{32}P$ -labelled drICE ORF probe and washed at 65°C in 0.2× SSC, 0.1% SDS and exposed to Kodak XAR-5 film with an intensifying screen at -70°C for 48 h. The gel was stained with ethidium bromide prior to blotting to visualize the rRNAs as a loading control.

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