drICE is an essential caspase required for apoptotic activity in *Drosophila* cells

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Caspases are involved in the execution of cell death in all multicellular organisms so far studied, including the nematode worm, fruit fly and vertebrates. While *Caenorhabditis elegans* has only a single identified caspase, CED-3, whose activity is absolutely required for all developmental programmed cell deaths, most mammalian cell types express multiple caspases with varying specificities. The fruit fly *Drosophila melanogaster* is genetically tractable, less complex than vertebrates and possesses two known caspases, DCP-1 and drICE. The fly may therefore provide a good model system for examining the hierarchy and relative roles of individual caspases in the execution of apoptosis. We have examined the role of drICE in *in vitro* apoptosis of the *D.melanogaster* cell line S2. We show that cytoplasmic lysates made from S2 cells undergoing apoptosis induced by either *reaper* (*rpr*) expression or cycloheximide treatment contain a caspase activity with DEVD specificity which can cleave p35, lamin DmO, drICE and DCP-1 *in vitro*, and which can trigger chromatin condensation in isolated nuclei. Using antibodies specific to drICE, we show that immunodepletion of drICE from these lysates is sufficient to remove most measurable *in vitro* apoptotic activity, and that re-addition of exogenous drICE to such immunodepleted lysates restores apoptotic activity. We conclude that, at least in S2 cells, drICE can be the sole caspase effector of apoptosis.

Keywords: apoptosis/caspase/*D.melanogaster*/drICE/S2 cells

Introduction

In the nematode worm *Caenorhabditis elegans*, programmed cell death is regulated and executed by three genes: *ced-9*, *ced-4* and *ced-3* (Ellis *et al.*, 1991). *Ced-9* encodes a suppressor of cell death and is homologous to the mammalian *bcl-2* gene family (Hengartner and Horvitz, 1994). *Ced-4* has no known homologues (Yuan and Horvitz, 1992), but amongst other functions (James *et al.*, 1997) may play a role in activation of CED-3 (Liu *et al.*, 1997). CED-3, which is thought to be the final effector of programmed cell death in the worm (Shaham and Horvitz, 1996), is homologous to the mammalian caspases (Yuan *et al.*, 1993) which are also implicated in the execution of cell death in mammalian cells.

Caspases are cysteine proteases that are synthesized as inactive pro-enzyme precursors and activated by proteolytic processing (reviewed by Takahashi and Earnshaw, 1996). They have a near-unique substrate specificity for aspartic acid at the P1-position and have been implicated in the execution of mammalian cell death by a number of studies. First, inhibition of caspases, whether by peptide inhibitors [e.g. zVAD.fmk (Slee *et al.*, 1996)] or by expression of the polypeptide caspase inhibitors baculovirus *p35* (Clem *et al.*, 1991; Rabizadeh *et al.*, 1993; Beidler *et al.*, 1995; Martinou *et al.*, 1995) or poxvirus *crmA* (Ray *et al.*, 1992; Gagliardini *et al.*, 1994), prevents many of the hallmarks of apoptosis, including chromatin condensation and DNA fragmentation. Second, caspases become processed to their active, mature forms during apoptosis and many cellular proteins [e.g. PARP (Lazebnik *et al.*, 1994), lamins (Oberhammer *et al.*, 1994) and DFF (Liu *et al.*, 1997)] are cleaved during apoptosis at caspase consensus sites. Finally, cytoplasmic lysates of apoptotic cells (which we call Cytoplasmic Apoptotic Lysates or CALs throughout) contain caspase activity that is both necessary and sufficient to trigger chromatin condensation, and nuclear and DNA fragmentation *in vitro* (Lazebnik *et al.*, 1994). Thus, caspase activity appears to be required for the execution of cell death both in the nematode and in mammalian cells.

However, whereas CED-3 is the sole reported *C.elegans* caspase, mammals possess many caspases. Most of these have a broad tissue expression pattern and are frequently co-expressed in individual cells. Moreover, because the proteolytic processing that converts an inactive pro-caspase to its mature active form occurs at caspase consensus sites, there exists the probability that caspases exist in inter-dependent proteolytic cascades. Both the nature of the hierarchies of caspase cascades and the extent to which the multiple caspases are functionally redundant remain key questions in mammalian cell death, neither of which can be addressed by reference to the nematode worm with its identified single caspase. Unfortunately, the large number of mammalian caspases makes assigning specific roles to individual caspase members very difficult, and a less complex organism would be preferable.

Drosophila melanogaster requires caspase activation for the execution of cell death (Hay *et al.*, 1994). The three pro-apoptotic genes so far cloned in the fly [*rpr* (White *et al.*, 1994), *hid* (Grether *et al.*, 1995) and *grim* (Vucic *et al.*, 1997)] all activate a caspase-containing machinery. This machinery contains at least two caspases, drICE (Fraser and Evan, 1997) and DCP-1 (Song *et al.*, 1997). DCP-1 displays a broad expression pattern and drICE is expressed throughout development. Both can induce apoptosis when over-expressed, and drICE is proteolytically processed during apoptosis in the S2 *Drosophila* cell line.

In this paper, we employ a biochemical approach to determine the contribution of drICE to the execution of apoptosis in *Drosophila*. We show that in lysates of S2 cells undergoing apoptosis (S2 cell CALs) following *rpr* induction or cycloheximide treatment, there is a DEVD-cleaving caspase activity that can process p35, lamin DmO, drICE and DCP-1. We show that specific immunodepletion of endogenous drICE removes all detectable caspase activity from these CALs, and that immunopurified drICE is a DEVD-cleaving caspase. We also show that drICE immunodepletion substantially impairs the ability of the apoptotic lysates to induce chromatin condensation of HeLa nuclei, and that this can be restored by re-addition of bacterially-expressed active drICE.

Results

Lysates of apoptotic S2 cells contain caspase activity

The *Drosophila* S2 cell-line requires caspase activity for the execution of apoptosis following expression of *rpr* or following treatment with cycloheximide or etoposide (Fraser and Evan, 1997; A.G.Fraser and G.I.Evan, unpublished data). drICE is proteolytically processed to its mature form in cells following *rpr* expression or treatment with cycloheximide (Fraser and Evan, 1997) and may therefore play a role in the execution of apoptosis in these cells. In order to dissect the contribution made by drICE to the execution of apoptosis, we used an *in vitro* biochemical approach by which caspase activity can be analysed more easily than within intact cells.

Apoptosis was induced in S2 cells either by *rpr* expression or by cycloheximide treatment and cytoplasmic apoptotic lysates (CALs) prepared from these cells as described in Materials and methods. The CALs were then analysed for caspase activity in three ways (see Materials and methods for details). First, synthetic colorimetric reporter peptide substrates were employed to assess caspase activity and determine whether it was DEVD- or YVAD-specific. Second, the competence of the CALs in cleaving known caspase substrates was determined by adding *in vitro* translated ³⁵S-labelled caspase substrates and analysing the cleavage products on SDS gels. The substrates chosen were the baculovirus protein p35, which is both substrate (Bump *et al.*, 1995; Xue and Horvitz, 1995) and inhibitor for all known caspases, lamin DmO (Gruenbaum *et al.*, 1988), which is known to be cleaved by drICE *in vitro* and to be processed during S2 cell apoptosis (Fraser and Evan, 1997) and drICE itself which is also processed during apoptosis in S2 cells. Finally, intact isolated HeLa cell nuclei were added to the CALs to determine the ability of the lysates to induce chromatin condensation, an *in vitro* activity known to be caspase-dependent (Lazebnik *et al.*, 1993).

Our analyses of lysates prepared from S2 cells undergoing apoptosis induced by either *rpr* expression or cycloheximide treatment gave essentially identical results. Such lysates contain detectable DEVD- but not YVAD-cleaving activity (Figure 1A), and cleave p35, lamin DmO and drICE (Figure 1B). The *Drosophila* caspase DCP-1 is also processed to its mature subunits by these lysates (data not shown). In addition, when added to these lysates, HeLa cell nuclei undergo dramatic chromatin condensation

(Figure 1C) which is completely prevented by the synthetic caspase inhibitor DEVD.cho at 5 nM. In the absence of CAL, isolated nuclei were completely stable over the duration of the experiments. The drICE, DmO and p35 cleaving activity in these lysates is also completely inhibited by 5 nM DEVD.cho, but a 50 µM YVAD.cho concentration must be used to achieve similar inhibition [Figure 2 shows data for drICE and DmO; analogous results are seen for p35 (data not shown)]. The complete inhibition of all substrate cleavage activity and chromatin condensation activity by DEVD.cho at 5 nM concentration suggests that a DEVD-containing chromogenic substrate will detect the activity of all critical caspases involved in these processes.

S2 cell CALs therefore contain all the predicted proteolytic activities attributable to the presence of active caspases. This caspase activity is a DEVD-cleaving and DEVD-inhibitable activity and appears to be similar, irrespective of whether apoptosis was triggered by cycloheximide treatment or by ectopic expression of *rpr*.

drICE p11 antibody specifically immunodepletes drICE

Previous experiments in which specific *in vitro* activities of CALs (e.g. PARP cleavage or lamin A cleavage) have been attributed to caspases have used peptide-based reagents in order to inhibit (Lazebnik *et al.*, 1994) or deplete (Nicholson *et al.*, 1995) caspases from the CALs. These reagents (e.g. biotin-DEVD.cho), while having preferences for certain subgroups of caspases, are not specific for individual caspases. Furthermore, they are used at concentrations sufficient to inhibit (and also deplete) almost all known caspases. It has therefore never been possible to assign specific roles for individual caspases in the execution of apoptosis using these *in vitro* assay systems.

Antibodies raised against highly variable regions of caspases could have specificity for individual caspase members, and we decided to employ this approach to determine the contribution of drICE to the activity of the S2 CALs. Polyclonal rabbit antibodies were raised against KLH-conjugated peptides (see Materials and methods) derived from either the very C-terminus of drICE (Abp11) or the large subunit (Abp19). The antibodies were assayed both for their ability to immunodeplete drICE from lysates and for their immunodepletion specificity for drICE and not DCP-1.

Abp11 immunodepletes ³⁵S-labelled drICE efficiently, whereas Abp19 fails to do so (Figure 3B) and serves as a control antibody throughout the subsequent experiments. Abp11 also only immunodepletes ³⁵S-labelled drICE and not ³⁵S-labelled DCP-1 when both are present. DCP-1 is 55% identical to drICE over their full lengths, and is 42% identical even over the region against which the Abp11 was raised (Figure 3A). The C-terminus of caspases is the most variable region between family members, and the 42% identity between DCP-1 and drICE over this region is higher than for other family members (Figure 3A). However, since Abp11 does not deplete even the highly similar DCP-1, it is reasonable to assume that immunodepletions of drICE carried out with Abp11 are specific for drICE and do not co-deplete other caspases.

A(i) **A(ii)** **B** **C**

Fig. 1. DEVD- and YVAD-cleaving activities of cycloheximide- and *rpr*-CALs. (A) CALs made from S2 cells undergoing apoptosis following 25 μ g/ml cycloheximide treatment (i) or induction of *rpr* expression from a metallothionein promoter (ii) were diluted to 100 μ g/ml final protein concentration and incubated with either a DEVD- or a YVAD-containing colorimetric substrate as described in Materials and methods. The substrate cleavage was monitored spectrophotometrically. (B) 35 S-labelled drICE, lamin DmO and baculovirus p35 are cleaved by *rpr*-CALs. 35 S-labelled drICE, lamin DmO and baculovirus p35 were synthesized as described in Materials and methods and incubated at 37°C for 2 h either in Enzyme Dilution Buffer (–) or in the presence of *rpr* CAL (+). The protein products were subjected to SDS-PAGE on a 12.5% polyacrylamide gel and exposed overnight to Kodak XAR-5 film. Size marker sizes are given in kDa. (a) drICE is processed to give rise to intermediates 1 (equivalent to predicted size of drICE without its 28 amino acid prodomain) and 2 (equivalent to predicted size of drICE with its 28 amino acid prodomain but without its small subunit), and to the mature p19 large subunit (3) and p11 small subunit (4). (b) DmO is processed to bands 1 and 2. (c) p35 is processed to bands 1 and 2. The 29 kDa band in both lanes marked by an asterisk is an internal initiation translation product of p35. (C) *rpr*-CALs contain a DEVD-inhibitable activity that activates chromatin condensation in HeLa nuclei. HeLa nuclei purified as described in Materials and methods were incubated at 37°C for 3 h in *rpr*-CALs in the absence or presence of 5 nM DEVD.cho. Nuclei were stained with acridine orange and observed by fluorescence microscopy.

***drICE* is the only DEVD-specific caspase in S2 cell CALs**

drICE is expressed in S2 cells and is processed during apoptosis. Endogenous drICE in S2 cell CALs, derived from *rpr*- or cycloheximide-induced apoptotic S2 cells, was immunopurified using Abp11, or using Abp19 as a control (see Materials and methods for immunodepletion conditions). Both the immunopurified drICE and the immunodepleted lysates were assayed for DEVD- and YVAD-cleaving activity, and the results are shown for *rpr* CALs in Figure 4A and B (analogous results were seen for cycloheximide-induced CALs). Immunopurified endogenous drICE has only DEVD-cleaving activity and no detectable YVAD-cleaving activity, whereas an equivalent procedure carried out using non-immunodepleting control antiserum Abp19 has no detectable activity. As expected, no YVAD activity was observed in any fraction (data not shown). Three rounds of repeated immunodepletion using Abp11 (but not Abp19) removes all detectable DEVD-cleaving activity from the CALs (Figure 4B). This suggests that drICE is the sole DEVD-cleaving caspase in these CALs.

***drICE* is required for substrate cleavage by S2 cell CALs**

CALs from which drICE had been immunodepleted using Abp11 (Abp11 CALs) or in which a non-depleting control antiserum had been used (Abp20 CALs) were assayed for their ability to cleave the substrates p35, lamin DmO and drICE. Abp11 CALs did not cleave either of the three substrates *in vitro*, whereas the Abp19 CALs still cleaved the substrates (Figure 5 shows data for p35 and drICE; analogous results were seen for DmO cleavage). The data shown in Figure 5 are from *rpr*-induced CALs, but analogous results were seen for cycloheximide-induced CALs. drICE is therefore the sole caspase responsible for cleavage of these substrates in the CALs examined.

***drICE* is required for activation of chromatin condensation by S2 cell CALs**

Immunodepleted CALs and non-depleted control CALs (Abp11 and Abp19 CALs respectively), derived from both *rpr*- and cycloheximide-induced apoptotic S2 cells, were assayed for their ability to induce chromatin condensation in purified HeLa nuclei. While Abp19 CALs induced

Fig. 2. Inhibition of the drICE and lamin DmO cleavage activity in *rpr*-CALs by DEVD.cho and YVAD.cho. ³⁵S-labelled drICE and lamin DmO synthesized as described in Materials and methods were incubated in *rpr*-CALs at 37°C for 2 h in the presence of varying concentrations of either DEVD.cho or YVAD.cho. The protein products were subjected to SDS-PAGE on a 12.5% polyacrylamide gel and exposed overnight to Kodak XAR-5 film.

chromatin condensation at similar rates to the initial lysates, Abp11 CALs had greatly reduced activity as judged by a greater proportion of non-apoptotic nuclei. Data are represented as percentages of total nuclei exhibiting condensation of chromatin following acridine orange staining (Figure 6A and B). Morphology of non-apoptotic nuclei in these experiments was identical to that of control nuclei from non apoptotic cells. This loss in activity is directly attributable to loss of drICE activity, since re-addition of bacterially-synthesized active drICE restored the activity of the Abp11 CALs (Figure 7).

Discussion

Caspases are required for the execution of cell death in all metazoans (Takahashi and Earnshaw, 1996). However, while the situation in the worm is simple in that only one caspase has been identified (CED-3), in mammalian cells, multiple caspases are expressed. A key problem in understanding the regulation of mammalian cell death has therefore concerned the precise hierarchy of these caspases and the extent of functional redundancy of caspases. It may be that caspases are organised in proteolytic cascades in which certain caspases (e.g. caspase-8 and caspase-2) are 'apical' caspases responsible for initiating the cascade, whereas others like caspase-3 represent solely downstream 'effector' caspases required for the final substrate cleavages that result in cell death (reviewed in Fraser and Evan, 1996). It may also be that 'apical' caspases have downstream 'effector' roles in addition (e.g. caspase-8 is a DEVD-specific caspase and could therefore cleave PARP) or that in certain contexts an 'effector' caspase can be 'apical' (e.g. the activation of caspase-3 by granzyme B). Finally, and perhaps most importantly, while we are beginning to make progress in understanding some of the biochemical events that are downstream of caspase activation, exactly which caspases are required for which events and how much functional overlap exists between caspases is also unclear. We therefore turned to *D.melanogaster*, in which apoptosis is known to depend on caspase activation (Hay *et al.*, 1994). *Drosophila* contains at least two characterised

Fig. 3. (A) Alignment of C-termini of caspases. Caspases shown were aligned using PileUp. The percentage scores shown represent percentage identity between each full-length caspase with full-length drICE. The peptide sequence against which Abp11 was raised is shaded and a comparison of this region with DCP-1 is shown ('|' denotes identical, ':' denotes similar). (B) Comparison of abilities of antibodies raised to drICE C-terminus (Abp11) and drICE large subunit (Abp19) to immunodeplete drICE or DCP-1 from lysates. ³⁵S-labelled drICE and DCP-1 were synthesised *in vitro* and three rounds of immunodepletion carried out using 1:50 antibody dilutions as described in Materials and methods. The final immunodepleted lysates (S) and the first immunopurified protein sample (B) are shown above for antibodies Abp11 and Abp19.

caspases: drICE (Fraser and Evan, 1997) and DCP-1 (Song *et al.*, 1997), and cell death in *Drosophila* is regulated in response to many of the same stimuli as mammalian cell death (e.g. DNA damage, loss of survival signals and over-expression of genes that induce S-phase progression) (Steller *et al.*, 1994). We reasoned that while not as genetically simple as the worm, the situation in *Drosophila* may be less complex than for mammals in which 12 caspases are already known, most of which have a broad expression pattern. The existence of multiple (but not a multitude of) caspases therefore made *Drosophila* an ideal system to analyse the relative contributions of individual caspases to the biochemical events downstream

Fig. 4. DEVD-cleaving activities of (A) immunopurified drICE and (B) rpr-CALs following drICE immunodepletion. (A) Abp11 was used to immunopurify drICE from rpr-CALs as described in Materials and methods and the protein A-Sepharose-bound immunocomplex assayed for DEVD-cleaving activity (Abp11 beads). An identical procedure was carried out with Abp19 which is not able to immunoprecipitate drICE (Abp19 beads). (B) Abp11 was used to immunodeplete drICE from rpr-CALs as described in Materials and methods. Following three rounds of depletion, the CALs were assayed for DEVD-cleaving activity (Abp11snt). An identical procedure was carried out with Abp19 which is not able to immunoprecipitate drICE (Abp19snt).

Fig. 5. drICE and p35 cleaving activities of rpr-CALs are lost following drICE immunodepletion. 35 S-labelled drICE or p35 were incubated at 37°C for 2 h in CALs that had been immunodepleted as described in Materials and methods with Abp11 or with Abp19 (which is not able to immunoprecipitate drICE). Following the reaction, protein products were run on a 12.5% polyacrylamide gel which was subsequently dried and exposed overnight to Kodak XAR-5 film. (A) p35 is cleaved to p25 and p10 (not resolved on this gel). The intermediate band (marked by an asterisk) present in all lanes is an internal translational initiation product which is not cleaved. (B) drICE cleavage products.

of caspase activation, and hence perhaps draw conclusions as to the possible regulation and hierarchies of caspases in the more complex mammalian cell.

Rather than employ an *in vivo* approach (i.e. generation of a drICE homozygous null fly) to analyse the role of drICE in execution of cell death in *Drosophila*, we used a biochemical strategy in which it is easier to dissect individual pathways and downstream events. Apoptosis of the *Drosophila* S2 cell line induced by over-expression of *rpr* or by treatment with etoposide or cycloheximide

Fig. 6. Immunodepletion of drICE reduces the ability of cycloheximide- and rpr-CALs to induce chromatin condensation in HeLa nuclei. HeLa nuclei purified as described in Materials and methods were incubated at 37°C for the times shown with (A) cycloheximide CALs or (B) rpr CALs that had been immunodepleted as described in Materials and methods with Abp11 or with Abp19 (which is not able to immunoprecipitate drICE). Chromatin condensation was monitored by staining with acridine orange and inspection by fluorescence microscopy. The percentage of nuclei exhibiting condensed chromatin is shown as a percentage of the total number of nuclei. The results shown are the mean of triplicate counts.

Fig. 7. Addition of active drICE restores chromatin condensing activity to drICE-immunodepleted rpr-CALs. rpr-CALs that had been immunodepleted as described in Materials and methods with Abp11 were supplemented either with buffer alone (Abp11 depletion) or with sufficient bacterially-expressed drICE (as described in Materials and methods) to restore the initial DEVD-cleaving activity of rpr-CALs (Abp11 + drICE). HeLa nuclei were incubated at 37°C for the times shown, and chromatin condensation was monitored by staining with acridine orange and inspection by fluorescence microscopy. The chromatin condensing activity of the resulting CALs is expressed as a percentage of the activity of the initial rpr-CAL activity. The results shown are the mean of triplicate counts.

requires caspase activity and results in the proteolytic processing of drICE. We therefore used a biochemical approach to analyse the contribution of drICE to charac-

terised downstream apoptotic events in these cells (i.e. lamin DmO cleavage, drICE processing and chromatin condensation).

Cytoplasmic lysates of apoptotic cells contain caspase activity and can induce chromatin condensation in purified nuclei (Lazebnik *et al.*, 1993, 1994). In this paper, we show that CALs made from the *Drosophila* S2 cell line contain a DEVD- but not a YVAD-cleaving caspase activity. This activity appears to be highly similar whether apoptosis is triggered by *rpr* over-expression or by cycloheximide treatment.

This caspase activity can process p35, lamin DmO and drICE *in vitro*. drICE and lamin DmO are processed in S2 cells during apoptosis (Fraser and Evan, 1997) and p35 inhibits *rpr*-induced apoptosis in S2 cells, presumably by acting as a caspase substrate (Bump *et al.*, 1995; Xue and Horvitz, 1995). The substrates cleaved by S2 CALs *in vitro* are therefore identical to those cleaved in the intact cells and the same as those cleaved by drICE *in vitro* (Fraser and Evan, 1997). S2 cell CALs can also activate chromatin condensation in added HeLa nuclei. HeLa nuclei were used as they are large nuclei and the chromatin condensation changes are easily visible. In addition, they derive from human cells and hence cannot contain any endogenous drICE. This chromatin condensation activity of S2 cell CALs is inhibitable by DEVD.cho and therefore also requires caspase activity.

It might be argued that analysing caspase activity in the S2 CALs solely on the basis of DEVD- versus YVAD-cleaving activity might exclude caspases that, while poor cleavers of either DEVD- or YVAD-based peptide substrates, play an important role in the execution of apoptosis in these cells. However, the fact that DEVD.cho completely inhibits both substrate cleavage and activation of chromatin condensation in these CALs at 5 nM final concentration (compared with 50 µM for YVAD) suggests that at least all the 'effector' caspases would be detected by a DEVD-derived colorimetric reagent of the kind used. Activity against a DEVD-derived colorimetric reagent therefore represents a good measure of total 'effector' caspase activity in these lysates. That drICE cleavage is also blocked by 5 nM DEVD.cho shows that the caspase required for proteolytic activation of drICE itself is also detectable by a DEVD-derived colorimetric reagent. Such a caspase may either represent an 'apical' caspase (analogous to caspase-8) or an 'amplifier' caspase. A DEVD-specific caspase activity therefore appears to be required not only for all the 'effector' functions of caspases in S2 cells but also for the activation of drICE.

So far, we have shown that all the activities attributable to caspases in S2 cell CALs, i.e. cleavage of p35, lamin DmO and drICE, and activation of chromatin condensation, require a DEVD-cleaving caspase activity. We next attempted to ask how many of these properties of S2 CALs are directly attributable to drICE alone. To do this, we raised antisera against drICE that allow immunodepletion of drICE. This antiserum (Abp11) specifically depletes drICE from lysates while not appreciably depleting DCP-1. The peptide against which Abp11 was raised is the very C-terminus of drICE, which is the most variable region between the active subunits of caspases and therefore the best candidate for a 'drICE-specific region'. DCP-1 is not recognised by this antibody despite having unusually high

identity with drICE over this region (42%), and despite the fact that DCP-1 and drICE are amongst the most similar caspases cloned so far (55% identity overall). While this does not formally exclude the possibility that a more similar caspase to drICE than DCP-1 is co-depleted from these lysates, this appears improbable. Immunodepletion with Abp11 is therefore likely to deplete only drICE from the S2 CALs. This allows a biochemical approach to determine the contribution of drICE to the caspase activity of CALs.

We show that when drICE is immunopurified from S2 CALs, it has DEVD- and not YVAD-cleaving activity. We further show that immunodepletion of drICE using Abp11 from S2 CALs removes all DEVD-cleaving activity and all substrate cleaving activity. The substrates analysed (p35, lamin DmO, drICE and DCP-1) are all substrates for drICE *in vitro* (Fraser and Evan, 1997; A.G.Fraser and G.I.Evan, unpublished data). Immunodepletion of drICE also greatly reduces the ability of CALs to activate chromatin condensation. This activity is restored by re-addition of sufficient bacterially-expressed drICE to return DEVD-cleaving activity to pre-depletion levels.

Immunodepletion of drICE causes a greater loss of chromatin condensation activity in cycloheximide CALs as compared with *rpr*-CALs. This may be due either to the presence of additional active caspases in *rpr*-CALs that are not present in cycloheximide CALs and which we failed to detect by any other method, or else the *rpr* protein in the CALs can activate human caspases associated with the HeLa nuclei added. If the latter possibility is correct, the chromatin condensation detected would therefore be caused by two components: first, the direct effects of active drICE (which can be depleted), and second, a *rpr*-induced component (which cannot be depleted). While cycloheximide is most likely to kill at least in part via *rpr* induction, the levels of *rpr* expressed following cycloheximide treatment are very unlikely to be comparable with those arising following induction of expression of *rpr* mRNA from the metallothionein promoter, and hence the contribution of *rpr* to the chromatin condensation in the two CALs will be different. This could explain the differences seen. Of course, another possibility for differences between *rpr* and cycloheximide dependence upon drICE could be some effect of cycloheximide via its inhibition of protein synthesis.

drICE therefore appears to be required for all the detectable caspase-dependent activities of S2 cell CALs. This implies that it is the sole 'effector' caspase in these cells and that drICE alone can be sufficient for all the downstream effects of apoptosis, i.e. substrate cleavage and activation of chromatin condensation. More interestingly, however, drICE also appears to be required for the proteolytic activation of caspases in these lysates, since the drICE (and DCP-1) processing activity of S2 cell CALs is lost when drICE is immunodepleted. drICE could either have a role as both an apical caspase (activating drICE and DCP-1) and an effector caspase, or else there is an as yet unidentified apical caspase present at very low (and hence undetectable) levels which initiates a cascade which requires drICE activity both to amplify the signal and to play an effector role.

The model that emerges from these experiments is that even in an organism that contains multiple caspases, a

single caspase (in this case drICE) can be sufficient for all the downstream effects of caspases (substrate cleavage and activation of chromatin condensation) in an endogenous apoptotic pathway. The fact that this caspase is also required for activation of caspases (both drICE and DCP-1) suggests that it may also play a role upstream in a caspase cascade, whether as an 'apical' or as an 'amplifier' caspase, as well as an effector role. The questions of how *rpr*, *hid* or *grim* expression activates this drICE-dependent caspase activity, and of how relevant this 'single caspase is sufficient' model is for mammalian caspase organisation remain to be answered.

Materials and methods

Cell culture and transfections

HeLa cells were cultured in DMEM supplemented with L-glutamine and 10% FCS. S2 cells were grown in Gibco Schneider medium supplemented with L-glutamine and 10% FCS and transfected according to standard CaPO₄ protocols and selected with hygromycin (200 µg/ml). A *rpr* ORF was cloned into pMK33, which contains a hygromycin resistance marker, placing it under control of a metallothionein promoter. Expression of *rpr* was induced in pools of cells by addition of CuSO₄ to 0.7 mM final concentration.

Cytoplasmic lysates from apoptotic cells, preparation of nuclei and chromatin condensation assays

Apoptosis was induced either by activation of *rpr* expression by addition of CuSO₄ to 0.7 mM, or by addition of cycloheximide to a final concentration of 25 µg/ml. Cells were harvested when >50% showed initial apoptotic morphology (2.5 h for *rpr*, 8 h for cycloheximide). Cells were washed twice with cold PBS and lysates were prepared essentially as described in Cotter and Martin (1996), with the exception that ATP, creatine kinase and phosphocreatine (all Sigma) were added along with DTT (Sigma) immediately prior to use and not before freezing.

HeLa nuclei were prepared according to the method in Cotter and Martin (1996). Nuclei were added to CALs and incubated at 37°C for the times shown in each figure. Aliquots were taken at the times shown and chromatin condensation was assayed by acridine orange staining (10 µg/ml final concentration) and fluorescence microscopy and inspection.

Colorimetric protease assays

CALs were diluted in Enzyme Dilution Buffer (100 mM HEPES pH 6.8, 50 µM NaCl, 10 µM EGTA and 20 µM MgCl₂) to a final protein concentration of 0.1 mg/ml. Colorimetric reagents (Ac-DEVD.pna and Ac-YVAD.pna, both from Affiniti) were added to 100 µM final concentration and incubated at 37°C. Cleavage was monitored using a plate reader and monitoring OD₄₀₅. A blank reading of CAL alone was subtracted from all samples.

Antibodies, immunopurification and immunodepletion

Polyclonal anti-peptide antisera were raised in rabbits against KLH-conjugated peptides corresponding to amino acids 81–94 (Abp19) and 326–339 (Abp11) of drICE. Antisera were added to a 1:50 dilution and incubated at 4°C for 1 h. Protein A-Sepharose (Pharmacia) preswollen in PBS was added (50 µl of a 50% slurry) before a further 30 min incubation at 4°C. The beads were spun out at 2500 g in a microfuge, washed four times in Enzyme Dilution Buffer and retained for assaying. The supernatant was re-depleted twice more with a 1:50 antiserum dilution at each stage before final assays were carried out.

Synthesis of bacterially-expressed active drICE and analysis

An ORF corresponding to full-length drICE was cloned into pTrcHisB (Invitrogen) to allow expression of His₆-tagged drICE in *E.coli* under control of the *trc* promoter. The construct was transformed into the XL1blue *E.coli* strain and expression induced in a 400 ml liquid culture with 0.1 mM IPTG at 34°C. Bacteria were harvested 3 h after induction, washed twice in ice-cold PBS and finally resuspended in 100 mM HEPES pH 6.8 and sonicated on ice. The cell debris was spun out and the lysates diluted to 1 mg/ml final concentration with 100 mM HEPES pH 6.8. The bacterial lysate was assayed for DEVD-cleavage activity

and added to the immunodepleted CALs to restore the DEVD-cleaving activity to the initial level monitored in the CALs.

Synthesis of ³⁵S-labelled substrates and substrate cleavage assays

cDNAs encoding p35 and lamin DmO (Yin *et al.*, 1997) were cloned into pBluescript (Stratagene). drICE and DCP-1 cDNAs were cloned in pBN40. All were *in vitro* transcribed/translated for 90 min at 30°C using the Promega reticulocyte lysate TnT system in the presence of [³⁵S]methionine (Amersham). CALs were assayed for their ability to cleave synthesized substrates by mixing the TnT reaction to a 30% final concentration with CAL in a final volume of 50 µl at 37°C for times shown. The resulting products were analysed by SDS-PAGE and autoradiography.

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