Human Bcl-2 cannot directly inhibit the Caenorhabditis elegans Apaf-1 homologue CED-4, but can interact with EGL-1

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Summary

Although the anti-apoptotic activity of Bcl-2 has been extensively studied, its mode of action is still incompletely understood. In the nematode Caenorhabditis elegans, 131 of 1090 somatic cells undergo programmed cell death during development. Transgenic expression of human Bcl-2 reduced cell death during nematode development, and partially complemented mutation of ced-9, indicating that Bcl-2 can functionally interact with the nematode cell death machinery. Identification of the nematode target(s) of Bcl-2 inhibition would help clarify the mechanism by which Bcl-2 suppresses apoptosis in mammalian cells. Exploiting yeast-based systems and biochemical assays, we analysed the ability of Bcl-2 to interact with and regulate the activity of nematode apoptosis proteins. Unlike CED-9, Bcl-2 could not directly associate with the caspase-activating adaptor protein CED-4, nor could it inhibit CED-4-dependent yeast death. By contrast, Bcl-2 could bind the C. elegans pro-apoptotic BH3-only Bcl-2 family member EGL-1. These data prompt us to hypothesise that Bcl-2 might suppress nematode cell death by preventing EGL-1 from antagonising CED-9, rather than by inhibiting CED-4.

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Introduction

Programmed cell death (PCD) removes unwanted cells in metazoans. It is implemented by a morphologically and biochemically defined process – apoptosis – in which latent proteases termed caspases become activated and then digest the cell from within. Although much progress has been made in recent decades in identifying and characterising the components of apoptosis pathways, key aspects of apoptotic signalling remain poorly understood. In particular, the mechanism(s) of action of the first identified cell death regulator, Bcl-2, is still not resolved.

Analyses of cell death in the nematode worm Caenorhabditis elegans have highlighted the evolutionary conservation of cell death pathway components and have provided valuable clues into the more complex mammalian apoptosis machinery. In addition to their comparative genetic simplicity (Aravind et al., 2001), nematodes offer considerable advantages in the study of apoptosis because developmental cell death is programmed. Indeed, of the 1090 somatic cells generated during C. elegans development, 131 cells are fated to die (Sulston et al., 1983).

An essential component of the nematode cell death machinery is a cysteine aspartyl protease (caspase) designated CED-3 (Yuan et al., 1993). Oligomerisation of an adaptor molecule, CED-4, promotes the proteolytic activation of CED-3 (Chinnaiyan et al., 1997a; Chinnaiyan et al., 1997b; Irmler et al., 1997; Seshagiri and Miller, 1997; Wu et al., 1997a; Yang et al., 1998). The tail-anchored membrane protein CED-9 inhibits cell death by directly interacting with CED-4 (Chinnaiyan et al., 1997b; Spector et al., 1997; Wu et al., 1997a; Wu et al., 1997b; Yan et al., 2005) and is required for the survival of the majority of nematode cells during development (Hengartner et al., 1992). Transcriptionally controlled expression of EGL-1 is necessary for death of the 131 cells developmentally destined to die (Conradt and Horvitz, 1999). EGL-1 exerts its pro-apoptotic activity by binding to CED-9, thus releasing CED-4 to activate CED-3 (Conradt and Horvitz, 1998; del Peso et al., 1998; Fairlie et al., 2006; Kim et al., 2004; Yan et al., 2005; Yan et al., 2004).

Counterparts of C. elegans proteins exist in mammalian cells. Relatives of CED-3 comprise the human caspase family (reviewed by Salvesen, 2002). Apaf-1 is the only known mammalian equivalent of CED-4. In conjunction with cytosolic cytochrome c and dATP, Apaf-1 can activate caspase-9. This in turn triggers caspase-3 activation and cell death (reviewed by Ferraro et al., 2003). CED-9 has a similar...
structure to Bcl-2, bearing all four so-called Bcl-2 homology (BH) domains (BH1-BH4) and a C-terminal membrane anchor (Hengartner and Horvitz, 1994). EGL-1 only has a BH3 domain, like the mammalian ‘BH3-only’ pro-apoptotic family members Bim, Bad, Noxa and Puma (reviewed by Marsden and Strasser, 2003). EGL-1 antagonises CED-9 in the same manner as the mammalian BH3-only proteins antagonise pro-survival Bcl-2 family members (Chittenden et al., 1995; Letai et al., 2002; Wang et al., 1996).

The conservation of cell death pathways was highlighted by the ability of the mammalian CED-9 homologue Bcl-2 to inhibit PCD during nematode development (Hengartner and Horvitz, 1994; Vaux et al., 1992). This indicated that Bcl-2 could functionally interact with at least one C. elegans apoptosis pathway component. Given the similarity between Bcl-2 and CED-9, CED-4 seemed to be the most likely target for inhibition by Bcl-2. Consistent with this theory, Bcl-2 could also suppress the excessive cell death in worms homozygous for a ced-9 loss-of-function mutation (Hengartner and Horvitz, 1994). This supported the notion that Bcl-2 interacted with the nematode apoptosis machinery downstream of CED-9, and probably with CED-4. However, as those experiments were performed at an early developmental stage, maternal CED-9 may have persisted (Hengartner et al., 1992), so the possibility of Bcl-2 acting at or upstream of CED-9 could not be excluded.

In this study, we explored potential nematode targets of Bcl-2 inhibition, using a yeast-based functional system, two-hybrid assays, and by direct protein binding in vitro. Our findings indicate that, unlike CED-9, Bcl-2 cannot interact with CED-4, inhibit its activity, or affect its localisation in yeast. However, Bcl-2 was able to bind to the BH3-only protein EGL-1 and alter its localisation in yeast, raising the possibility that this interaction might account for the ability of Bcl-2 to inhibit PCD in C. elegans.

Results
Reconstituting the core worm apoptotic pathway in yeast
Heterologous yeast-based systems, most notably the yeast two-hybrid system, have been extensively harnessed to probe the molecular pathways of higher eukaryotes. Here, we capitalised on the previously noted ability of active caspases to kill the yeast Saccharomyces cerevisiae (Hawkins et al., 2001; Hawkins et al., 1999; Hawkins et al., 2000a; Hawkins et al., 2000b; Jabbour et al., 2002; Jabbour et al., 2004; Kang et al., 1999), to reconstitute the core nematode apoptotic pathway and to explore the ability of Bcl-2 to interact with it. Caspase toxicity in yeast depends both on the enzyme adopting an active conformation, as well as its substrate specificity. Neither of two proteins implicated in endogenous yeast cell death pathways – YCA1 (Madeo et al., 2002) and Aif1p (Wissing et al., 2004) – is required for caspase-dependent yeast lethality (Puryer and Hawkins, 2006), implying that the toxicity reflects fortuitous proteolysis of essential proteins rather than triggering of an endogenous yeast ‘apoptotic’ pathway.

As we reported previously (Jabbour et al., 2004), yeast survived expression of either CED-3 or CED-4 alone (Fig. 1A, lanes 2 and 3). However, CED-4 triggered CED-3 processing when the two proteins were co-expressed (Jabbour et al.,...
2004), killing the yeast (lane 4). Mutation of a cysteine residue in the active site of CED-3 to serine rendered the protein inactive; co-expression of this mutant protein together with CED-4 was tolerated by yeast (lane 5). Likewise, mutation of a crucial lysine residue in an ATP-binding loop of CED-4 (Chinnaiyan et al., 1997b; Saraste et al., 1990) to isoleucine inactivated it, as demonstrated by the inability of the mutant to cooperate with CED-3 to kill yeast (lane 6). Together, these findings confirm that yeast death in this system requires the activity of both CED-3 and CED-4.

Expression of CED-9 inhibited yeast death caused by CED-3 and CED-4 co-expression (lane 7). With the aim of reconstituting the entire nematode core apoptotic pathway, we next tested the effect of EGL-1 expression in yeast expressing CED-3, CED-4 and CED-9. EGL-1 was able to antagonise the protective activity of CED-9, leading to yeast death (lane 8). This effect was specific, as EGL-1 expression did not affect yeast survival when expressed alone or in combination with CED-3, CED-4, CED-9, CED-3 plus CED-9, or CED-4 plus CED-9 (lanes 9–14). To allow visualisation of CED-4 within the yeast, it was C-terminally tagged with GFP. CED-9 and EGL-1 were N-terminally FLAG-tagged and myc-tagged, respectively. Neither CED-4, CED-9 nor EGL-1 activity was altered by the addition of the tags (Fig. 1B).

**Bcl-2 inhibits Bax-dependent but not CED-4-dependent yeast toxicity**

We used this reconstituted core nematode apoptosis pathway to test directly whether Bcl-2 could functionally substitute for CED-9. CED-9 was able to inhibit CED-4-dependent yeast killing when its expression was directed by the constitutively active ADH promoter (Fig. 2A, compare lanes 2 and 3). A C-terminal truncation mutant (CED-9\(^{-1-251}\)) was also protective (lane 4). Conversely, Bcl-2 was unable to inhibit this CED-4-dependent lethality when expressed under the control of either the ADH promoter (lane 5) or the stronger GAL1/10 (GAL) promoter (lane 6). Fusion of the N-terminus of CED-9 to Bcl-2 (CED-9\(^{-1-80}\)-Bcl-2) had been previously demonstrated to confer increased protection against nematode developmental cell death, compared with wild-type Bcl-2 (Xue and Horvitz, 1997). However, the addition of this N-terminal portion of CED-9 to Bcl-2 did not enable it to inhibit CED-4-dependent yeast killing (lane 7).

To examine the possibility that Bcl-2 expressed in this system was inactive, and that this inactivity accounted for its inability to inhibit CED-4, we investigated whether Bcl-2 could inhibit Bax-dependent yeast death. Confirming previously published observations (Hananada et al., 1995), Bcl-2 was able to inhibit yeast lethality triggered by expression of Bax (Fig. 2A, compare lane 8 with lanes 11 and 12), confirming that Bcl-2 was active, at least by this criterion. As reported earlier (Tao et al., 1997), CED-9 had no effect on Bax-dependent yeast death (lane 9). The CED-9\(^{-1-80}\)-Bcl-2 chimaeric protein inhibited Bax killing slightly more efficiently than wild-type Bcl-2 (compare lanes 11 and 13).

To test whether Bcl-2 was capable of inhibiting lower levels of CED-4, CED-4 expression was reduced using a methionine-repressible promoter (Fig. 2B). The addition of methionine decreased the degree of CED-4-dependent lethality; however, even in this context, high levels of Bcl-2 (expressed using the GAL1/10 promoter) failed to relieve CED-4-dependent yeast toxicity (Fig. 2B, lane 5).

**Bcl-2 fails to interact with CED-4 directly**

The data presented above suggested that Bcl-2 does not directly inhibit CED-4, contrary to a model in which Bcl-2 functions analogously to CED-9. To determine whether this lack of inhibitory capability reflected an inability of Bcl-2 to interact with CED-4, a yeast two-hybrid method was employed. yeast co-expressing CED-4 and CED-9 (as fusions to Gal4 domains) were able to grow on plates lacking histidine, indicating interaction between these two proteins (Fig. 2C, lane 5). By contrast, growth of yeast co-expressing CED-4 and Bcl-2 (fused to Gal4 domains) was histidine dependent, demonstrating that these proteins failed to interact (lane 6).

**CED-9 relocates CED-4 in yeast cells, but Bcl-2 does not**

Previous work has shown that CED-9 alters the subcellular localisation of CED-4 in nematode cells (Chen et al., 2000). We investigated whether CED-9 could affect CED-4 localisation in our yeast system and, if so, whether Bcl-2 could act similarly. We expressed these proteins in yeast as GFP fusion proteins. Confocal microscopy was then used to determine their localisation relative to the endoplasmic reticulum protein Kar2p (Rose et al., 1989), MitoTracker (which stains mitochondria) and 4′,6-diamidino-2-phenylindole (DAPI; which stains DNA). Fusion to GFP did not affect the abilities of CED-9 and Bcl-2 to inhibit CED-4-dependent or Bax-dependent yeast death, respectively (data not shown).

When expressed without other nematode proteins, CED-4-GFP was detected in the perinuclear region of S. cerevisiae cells (Fig. 3), similar to its localisation in nematode and insect cells (Chen et al., 2000; Seiffert et al., 2002), but contrasting with its cytosolic localisation in mammalian cells (Wu et al., 1997b). GFP-CED-9 fluorescence was detected in yeast transformants both in the perinuclear area (co-localising with Kar2p) and around the cortex of the cell (co-localising with MitoTracker and Kar2p). In approximately half of the cells, some perivacuolar localisation was also observed (Fig. 3). A similar distribution was seen in yeast co-expressing CED-4 (Fig. S1, supplementary material). A previous analysis of CED-9 distribution within nematode cells revealed co-localisation with MitoTracker (the endoplasmic reticulum was not visualised in the study) (Chen et al., 2000). FLAG-CED-9 co-expression changed the distribution of CED-4-GFP in most yeast cells, targeting it to the perinuclear region and cell periphery (Fig. 3). GFP-Bcl-2 was detected throughout the endoplasmic reticulum of yeast transformants (Fig. 3), whether or not CED-4 was co-expressed (Fig. S1, supplementary material). Consistent with our data indicating that Bcl-2 could not bind to CED-4, co-expression of Bcl-2 did not alter CED-4-GFP localisation (Fig. 3).

**Bcl-2 does not cooperate with CED-9 to inhibit CED-4**

To determine whether Bcl-2 could cooperate with low levels of CED-9 to inhibit CED-4 indirectly, yeast bearing the CED-3 and CED-4 expression vectors were co-transformed with a vector in which CED-9 was expressed under the control of an inducible copper promoter (CUP1). Copper levels were titrated to vary CED-9 expression. In the absence of added copper, only
low levels of CED-9 were induced, conferring only weak protection against CED-4-dependent yeast death (Fig. 4, compare lanes 2 and 4). Bcl-2, expressed under the control of either the ADH promoter or stronger GAL1/10 (GALL) promoter, was unable to enhance CED-9 protection against CED-4-dependent yeast death (lanes 5 and 6).

Bcl-2 interacts with the BH3-only Bcl-2 family member EGL-1

We next considered the possibility that Bcl-2 might interact with the BH3-only protein EGL-1. Using a two-hybrid assay, yeast co-expressing EGL-1 and either CED-9 or Bcl-2 (fused to Gal4 domains) showed histidine-independent growth (Fig. 5A, lanes 5 and 6). This indicated that EGL-1 could not only interact with CED-9, but could also bind to Bcl-2.

To confirm this observation, in vitro translated [35S]methionine-labelled FLAG-Tab-1, [35S]methionine-labelled CED-9 or [35S]methionine-labelled Bcl-2 were incubated with bacterial lysates containing recombinant HIS-tagged p35, HIS-tagged EGL-1 or HIS-tagged CED-4. The

Fig. 2. Unlike CED-9, Bcl-2 cannot inhibit CED-4-dependent yeast death. (A) Yeast were transformed and spotted as described in the legend to Fig. 1. The ability of Bcl-2 to inhibit Bax-dependent and CED-4-dependent yeast death was tested, using Bcl-2 expressed from either the same promoter as CED-9 (ADH) or a very strong galactose-inducible promoter (GALL). The resulting expression levels were visualised by anti-Bcl-2 immunoblotting. Immunoblotting was also performed to monitor expression of GFP-tagged CED-4 and FLAG-tagged CED-9. An asterisk indicates a crossreacting yeast protein recognised by the anti-GFP antibody. Coomassie staining indicates protein loading. (B) Lowering expression of CED-4 using a methionine-repressible promoter (MET) yielded only weak CED-4-dependent yeast death upon co-expression with CED-3. Bcl-2 expression, directed by the intermediate-strength (ADH) or very strong (GALL) promoters, could not suppress even this weak death stimulus. (C) The two-hybrid yeast strain HF7c was transformed with the indicated plasmids or empty vector controls. Transformants were spotted onto minimal medium either containing histidine (growth confirms the presence of the plasmids) or lacking histidine (growth indicates interaction between the prey and bait proteins).
protein complexes that formed were then isolated by binding to nickel-agarose beads. CED-9 bound to CED-4 (Fig. 5B, lane 8), but the FLAG-Tab-1 negative control did not (lane 5). Validating our results from the yeast systems, radiolabelled Bcl-2 did not interact with CED-4 (lane 11). Both CED-9 and Bcl-2 could bind EGL-1, but FLAG-Tab-1 could not (lanes 9, 12 and 6 respectively). Neither CED-9, Bcl-2 nor FLAG-Tab-1 interacted with the p35 negative control (lanes 4, 7 and 10).

We took advantage of the ability of Bcl-2 to suppress Bax-mediated yeast death, to monitor the capacity of Bcl-2 to interact functionally with EGL-1. Yeast co-expressing Bax and Bcl-2 were protected from Bax-mediated yeast death (Fig. 5C, lane 3). This protection could be antagonised by either the mammalian BH3-only protein Bim (lane 4) or the nematode protein EGL-1 (lane 5).

CED-9 and Bcl-2 relocalise EGL-1 in yeast cells

We expressed GFP-tagged EGL-1 in yeast and used confocal microscopy to investigate its subcellular localisation, either in isolation or when co-expressed with CED-9 or Bcl-2. The tag did not

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Fig. 3. Unlike CED-9, Bcl-2 does not alter CED-4 localisation in yeast. Yeast were transformed with the indicated plasmids. Transformants were either stained for nucleic acid using DAPI (blue; upper three rows) or mitochondria using MitoTracker (red; middle three rows). The endoplasmic reticulum was visualised by anti-Kar2p immunofluorescence (red; lower three rows). GFP-tagged CED-4, CED-9 and Bcl-2 were detected (green fluorescence). GFP-CED-4 localisation was also observed in the presence of either FLAG-CED-9 or Bcl-2. Bars, 5 μm.

Fig. 4. Bcl-2 does not cooperate with CED-9 to inhibit CED-4-dependent yeast death. Yeast were transformed with the specified plasmids and spotted on inducing or repressing medium, as described in the legend to Fig. 1. FLAG-tagged CED-9 was expressed either by the constitutive ADH promoter or an inducible copper promoter (CUP1). The copper concentration in the inducing medium was varied to control FLAG-CED-9 expression levels in yeast transformed with the pCUP1-(TRP1)-FLAG-CED-9 plasmid. In the absence of added copper, only weak protection was afforded. The impact of Bcl-2 on this weak protection was tested by co-expressing Bcl-2, either using the intermediate-strength promoter (ADH) or the very strong promoter (GALL). Transformants were also grown in liquid galactose-containing media with no added copper. Lysates generated from those cultures were immunoblotted to visualise FLAG-CED-9 and Bcl-2 levels. Coomassie staining allowed visualisation of protein loading.
Bcl-2 binds EGL but cannot inhibit CED-4

alter the ability of EGL-1 to antagonise CED-9 in yeast (data not shown). GFP-EGL-1 was detected at low levels in the cytoplasm and the nucleus when expressed alone in yeast (Fig. 6). Co-expression of either CED-9 or Bcl-2 yielded a stronger GFP-EGL-1 signal, which was concentrated in the perinuclear region and cell periphery (Fig. 6). These distributions resembled those of GFP-CED-9 and GFP-Bcl-2 (Fig. 3), suggesting that each protein can bind to, and relocalise, EGL-1.

Discussion
The PCD pathway of the nematode worm *C. elegans* has been extensively studied, and the main components of the pathway have been identified (Fig. 7A). Components of this core cell death machinery are conserved between metazoans. The first mammalian apoptotic protein to be identified was Bcl-2 (Vaux et al., 1988). Although Bcl-2 has been intensively studied, its precise mechanism of action is still contentious. Three general mechanisms have been proposed to account for the anti-apoptotic activity of Bcl-2: direct binding to a CED-4 homologue such as Apaf-1; direct regulation of mitochondrial permeability; and/or influencing the impact of other Bcl-2 family members on mitochondrial permeability.

As a proportion of cellular Bcl-2 localises to the outer mitochondrial membrane, and Bcl-2 family members resemble bacterial pore-forming proteins, it has been hypothesised that Bcl-2 regulates mitochondrial permeability (reviewed by Hengartner, 2000) and the resultant release of pro-apoptotic mitochondrial proteins such as cytochrome c and DIABLO/Smac (Du et al., 2000; Kluck et al., 1997; Verhagen...
The ability of Bcl-2 and its close relatives to form pores in synthetic membranes (reviewed by Desagher and Martinou, 2000) provides evidence in favour of this model. An alternative hypothesis is that Bcl-2 might block apoptosis of mammalian cells by inhibiting a CED-4-like protein (such as Apaf-1). This idea emerged from investigations of the effect of transgenic Bcl-2 expression on nematode cell death. Transgenic overexpression of Bcl-2 reduced cell death during development both in wild-type and ced-9 mutant worms (Hengartner and Horvitz, 1994; Vaux et al., 1992). The simplest explanation for these results is that Bcl-2 can functionally replace CED-9 to suppress cell death in worms, presumably by inhibiting CED-4 (Fig. 7B). Supporting this notion, subsequent studies indicated that a close relative of Bcl-2, Bcl-xL, was able to bind CED-4 (Chinnaiyan et al., 1997b; Huang et al., 1998), and that Bcl-2 and Bcl-xL could partially inhibit mammalian cell death induced by overexpression of CED-3 and CED-4 (Chinnaiyan et al., 1997b; Huang et al., 1998). Some experiments suggested that Bcl-xL might directly inhibit the mammalian counterpart of CED-4, Apaf-1 (Hu et al., 1998; Inohara et al., 1998; Pan et al., 1998; Song et al., 1999), however subsequent studies refuted this (Conus et al., 2000; Hausmann et al., 2000; Moriishi et al., 1999).

Using yeast two-hybrid and pull-down experiments, we found that Bcl-2 was unable to bind CED-4. We also observed that, unlike CED-9, Bcl-2 could not inhibit CED-4-dependent yeast death nor influence the subcellular localisation of CED-4. Together, these results demonstrate that suppression of C. elegans developmental cell death by Bcl-2 could not be a result of its direct inhibition of CED-4. These data imply that the protection conferred by Bcl-2 against mammalian cell death stimulated by enforced expression of CED-3 and CED-4 (Chinnaiyan et al., 1997b; Huang et al., 1998) was most probably indirect. The mechanism by which co-expression of CED-3 and CED-4 induces apoptosis in mammalian tissue culture cells is not known, but this death could result from indirect triggering of the intrinsic (mitochondrial) pathway in response to the expression of the nematode proteins. As Bcl-2 effectively inhibits the intrinsic pathway, this might account for its ability to protect mammalian cells from death triggered by co-expression of CED-3 and CED-4.

A third model for the mechanism of action of Bcl-2 relates to its ability to regulate the activity of other family members through heterodimeric interactions. Bcl-2 can bind to a subset of BH3-only proteins with varying affinities (Chen et al.,...
Although the yeast-based system described here permits existence in C. elegans by reversing the antagonism by EGL-1 of CED-9. Consistent with this notion, data have recently been published indicating that some mammalian anti-apoptotic Bcl-2 family members might promote survival by opposing the pro-apoptotic activity of mammalian BH3-only proteins (Kuwana et al., 2005). Bcl-2 binds strongly to the BH3-only protein Bim (Chen et al., 2005; Hsu et al., 1998; O’Connor et al., 1998), and this interaction was confirmed in our yeast system because Bim was able to antagonise Bcl-2-mediated Bax inhibition. Our data demonstrate that EGL-1 can bind Bcl-2, and Bcl-2 can alter the subcellular localisation of EGL-1 in yeast. These data lead us to speculate that Bcl-2 might suppress PCD in C. elegans by reversing the antagonism by EGL-1 of CED-9. Consistent with this notion, data have recently been published indicating that some mammalian anti-apoptotic Bcl-2 family members might promote survival by opposing the pro-apoptotic activity of mammalian BH3-only proteins (Kuwana et al., 2005).

Our model would readily account for the ability of Bcl-2 to inhibit developmental cell death in wild-type worms (Hengartner and Horvitz, 1994; Vaux et al., 1992), as exogenous Bcl-2 would antagonise the EGL-1 protein expressed in the 131 cells normally destined to die, thus permitting their survival. Bcl-2 was also observed to suppress cell death in homozygous ced-9 loss-of-function worms (Hengartner and Horvitz, 1994). If CED-9 were completely absent, CED-4 would be expected to be able to activate CED-3 whether EGL-1 was bound to Bcl-2 or not. However, it is important to note that, whereas the genotype of the worms was homozygous ced-9 loss-of-function, substantial maternally derived CED-9 might persist in neurons at the late L3 larval stage when the experiments were performed (Hengartner et al., 1992). We therefore propose the following model to reconcile these apparently discordant data (Fig. 7C). At the late L3 stage, the ventral nerve cord cells might express low levels of EGL-1 that are insufficient to displace enough CED-9 from CED-4 to activate CED-3, therefore permitting the survival of the ventral nerve cord cells. However, in ced-9 loss-of-function worms bearing only maternally derived CED-9, total CED-9 levels would be lower. In this context, the putative small amounts of EGL-1 protein would be sufficient to release the maternally derived CED-9 from CED-4, which could then activate CED-3. In this model, overexpression of Bcl-2 would sequester EGL-1, thus allowing the maternal CED-9 to prevent CED-4 from activating CED-3, and protecting the cells from apoptosis.

Although EGL-1 is the best-studied nematode BH3-only protein, two others have also been reported: ceBNIP3 and CED-13. Unlike EGL-1, CED-13 deficiency had no impact on developmental cell death in C. elegans (Schumacher et al., 2004). It therefore seems unlikely that CED-13 would account for the ability of Bcl-2 to suppress nematode developmental apoptosis, even if CED-13 could bind Bcl-2. To date, ceBNIP3 has only been studied in mammalian over-expression systems (Cizeau et al., 2000; Yasuda et al., 1998). Further studies will be needed to determine whether the predicted ceBNIP3 protein exists in C. elegans and, if so, whether it plays a role in PCD and might therefore represent another target of Bcl-2 inhibition.

In summary, our data demonstrate that Bcl-2 cannot bind or inhibit CED-4. This indicates that the pro-survival activity of Bcl-2 must occur through a distinct mechanism from that of CED-9. However, Bcl-2 can interact with the BH3-only protein EGL-1, suggesting a model in which sequestration of EGL-1 accounts for the ability of Bcl-2 to inhibit PCD in C. elegans. Although the yeast-based system described here permits extremely robust analyses of pathways comprising up to four exogenous components (CED-3, CED-4, CED-9, EGL-1), extension of this system to express five transgenes (CED-3, CED-4, CED-9, EGL-1, Bcl-2), yielded a dramatic increase in intra-class variability when independent transformants were assayed. Unfortunately, this irreproducibility associated with the five-component system prevented us from directly testing the hypothesis that Bcl-2 can prevent EGL-1-mediated antagonism of CED-9. Experiments with transgenic nematodes might be one approach to evaluate the validity of this model. We would predict that, if Bcl-2 acts in worms by preventing EGL-1 from antagonising CED-9, transgenic expression of Bcl-2 would offer no protection from PCD in nematodes bearing loss-of-function mutations in both CED-9 and EGL-1. If this model of Bcl-2 activity is verified and extends to the mammalian system, the ability of Bcl-2 to dimerise with pro-apoptotic BH3-only family members might promote survival by opposing the pro-apoptotic activity of mammalian BH3-only proteins (Kuwana et al., 2005).

Materials and Methods
Plasmid construction
Yeast expression constructs
The constructs pADH-(TRP1), pADH-(HIS3), pGALS-(LEU2), pGALS-(LEU2)-CED-3, pADH-(HIS3)-CED-4, pADH-(HIS3)-CED-4 GFP, pADH-(TRP1)-CED-3, pADH-(TRP1)-CED-3 FLAG, pADH-(TRP1)-CED-3 FLAG-CED-9, pADH-(TRP1)-CED-3 FLAG-CED-9 were all thawed. pADH-(TRP1)-CED-3 FLAG-CED-9 was cut out of pEF Bcl-2 (Strasser et al., 1995) and cloned into pBluescript II SK+ (Stratagene) to give pBS-Bcl-2 UTR+N. The direction was determined by digesting pADH-(TRP1)-CED-3 FLAG-CED-9 with NotI and ligated into either pADH-(HIS3)-CED-4 or pADH-(HIS3)-CED-4 GFP cut with BamHI and EcoRI to give pADH-(HIS3)-CED-4 and pADH-(HIS3)-CED-4 GFP cut with BamHI and EcoRI to give pADH-(HIS3)-CED-4 and pADH-(HIS3)-CED-4 GFP respectively. PCR was used to determine insert orientation. pMET-(HIS3)-CED-4 was generated by amplifying the C-terminal portion of CED-4, using oligonucleotides 5 and 4, the PCR product was cut with BamHI and EcoRI to give pADH-(HIS3)-CED-4. As the N-terminal section of CED-4 was lost during this process, the plasmid was cut with BamHI and treated with alkaline phosphatase, and ligated to the BamHI-BamHI fragment of pGALL-(HIS3)-CED-4 to give pGALL-(HIS3)-CED-4. The N-terminal portion of CED-4 was ligated into pGAL1-(TRP1)-FLAG-CED-9. As the N-terminal section of CED-4 was lost during this process, the plasmid was cut with BamHI and treated with alkaline phosphatase, and ligated to the BamHI-BamHI fragment of pGALL-(HIS3)-CED-4 to give pGALL-(HIS3)-CED-4. The N-terminal portion of CED-4 was ligated into pCUP1-(TRP1) by digesting pADH-(TRP1)-FLAG-CED-9 with EcoRI and NotI and ligating the insert into pGALL-(HIS3) to give pGALL-(HIS3)-FLAG-CED-9. The C-terminal portion of CED-9 and the actin promoter was then excised with BamHI and SpI and cloned into pCUP1-(TRP1) to give pCUP1-(TRP1)-CED-9. The tagged N-terminal end of CED-9 was then excised from pGALL-(HIS3)-FLAG-CED-9 with EcoRI and NotI and cloned into pCUP1-(TRP1)-FLAG-CED-9. To N-terminally GFP-tag CED-9 and CED-9 1-251, GFPS65T was inserted fragment. FLAG-tagged CED-9 was cloned into pCUP1-(TRP1)-CED-9. PCR was used to verify the presence and direction of the insert fragment. FLAG-tagged CED-9 was cloned into pCUP1-(TRP1)-CED-9. As the N-terminal section of CED-4 was lost during this process, the plasmid was cut with BamHI and treated with alkaline phosphatase, and ligated to the BamHI-BamHI fragment of pGALL-(HIS3)-CED-4 to give pGALL-(HIS3)-CED-4. As the N-terminal section of CED-4 was lost during this process, the plasmid was cut with BamHI and treated with alkaline phosphatase, and ligated to the BamHI-BamHI fragment of pGALL-(HIS3)-CED-4 to give pGALL-(HIS3)-CED-4. As the N-terminal section of CED-4 was lost during this process, the plasmid was cut with BamHI and treated with alkaline phosphatase, and ligated to the BamHI-BamHI fragment of pGALL-(HIS3)-CED-4 to give pGALL-(HIS3)-CED-4. As the N-terminal section of CED-4 was lost during this process, the plasmid was cut with BamHI and treated with alkaline phosphatase, and ligated to the BamHI-BamHI fragment of pGALL-(HIS3)-CED-4 to give pGALL-(HIS3)-CED-4. As the N-terminal section of CED-4 was lost during this process, the plasmid was cut with BamHI and treated with alkaline phosphatase, and ligated to the BamHI-BamHI fragment of pGALL-(HIS3)-CED-4 to give pGALL-(HIS3)-CED-4.
PstI digestion. The N-terminal part of the coding region was amplified with oligonucleotides 8 and 9, cut with EcoRI and PstI and ligated into pBS-Bcl-2. The fragment was excised with PstI-NruI and ligated into pBS-Bcl-2 N cut with BamHI and XhoI to give pBS-Bcl-2. The full-length coding region was then isolated by cutting with EcoRI and NotI and cloned into pGALs-(URA3) as a XhoI-EcoRI fragment.

**Yeast two-hybrid assay**

Yeast (HF7c) were transformed with desired plasmids as described previously (Hawkins et al., 2000a). In this strain, HIS3 expression is induced by a reconstituted Gal4 transcription factor. Genes cloned into pGBT9 are translated as fusion proteins to the Gal4 DNA binding domain. Genes cloned into pACT3-1 are translated as fusion proteins to the Gal4 activation domain. Protein interaction was detected on medium lacking histidine (and containing 2 mM 3-amino-triazole in Fig. 5A).

**Yeast transformations and death assays**

The Saccharomyces cerevisiae strain W303a was used to perform yeast death assays, as previously described (Hawkins et al., 2000a).

**Immunoblotting and SDS-PAGE staining**

Yeast were prepared for immunoblotting as previously described (Hawkins et al., 2000a). Lysates were also subjected to SDS-PAGE and stained with Coomassie Brilliant Blue (BioRad) to visualise protein loading. Antibodies used for immunoblotting in this study were anti-FLAG (clone M2; Sigma), anti-myc (clone 9B11; Cell Signaling), anti-GFP (Jabbour et al., 2004), anti-Bcl-2a (clone 8C8; Oncogene Research Products), anti-Bax (Upstate), anti-His (Invitrogen), anti-rabbit-HRP (Amersham) and anti-mouse-HRP (Sigma).

**Confocal analyses of yeast cells**

Yeast were transformed with the appropriate plasmids and transformants were grown to stationary phase in liquid repressing minimal media. For galactose-inducible expression, the yeast were then incubated in 0.2% peptone, 0.1% yeast extract, 2% galactose. Yeast were then either stained to label mitochondrial or nucleic acid as described below. Microscopy was undertaken with a Leica TCS4 SP2 spectral confocal scanner or an Olympus Fluoview 300 confocal laser scanning microscope.

**Yeast mitochondrial staining**

Yeast mitochondria were stained using MitoTracker CMH2-X-Ros (Molecular Probes) as per the manufacturer’s instructions. Briefly, 1 μM MitoTracker CMH2-X-Ros was added to the yeast, which were then incubated in the dark at 30°C for 30 minutes, with shaking. They were then washed and resuspended in distilled water, then mounted on coverslips 1:1 with 1% low-melt agarose.

**Yeast nucleic acid staining**

Yeast were fixed by adding a final concentration of 3.7% (vol/vol) formaldehyde to yeast in complete medium, then incubated for 30 minutes with occasional mixing before being washed three times in PBS. Yeast were placed on a slide treated with poly-L-lysine (Sigma) and adhered for 20 minutes before being washed twice with PBS. A drop of 300 nM DAPI (Sigma) was added to the yeast and incubated for 15 minutes. Excess DAPI was removed by washing the slide three times with PBS and ensuring that the final wash was completely aspirated, before two drops of 1% low-melt agarose were added.

**Immunofluorescence**

Yeast were washed with phos/sorb (0.1 M KH2PO4, 1.2 M sorbitol pH 6.5) then incubated with zymolase 20T (from ICN; 600 μg/ml in phos/sorb) for 45 minutes at 30°C, flicking every 10 minutes. The yeast were pelleted at 550 g for 5 minutes, then washed twice with phos/sorb and resuspended in phos/sorb. Formaldehyde (3.7% final concentration) was added to the cells, which were incubated for 30 minutes, gently flicking every 10 minutes to mix. The yeast were pelleted (350 g, 5 minutes), washed three times, then permeabilised by incubation for 1-2 hours at room temperature (protected from light), then washed three times with phos/sorb. The cells were then immobilised by addition of a drop of 1% low-melt agarose.

**Puriﬁcation of recombinant proteins**

pET23a-p35 has been previously reported (Jabbour et al., 2004). EGL-1 was amplified using oligonucleotides 15 and 16 and ligated into the pET23a-XbaI fragment. To clone GFP-tagged EGL-1, GFP was amplified using oligonucleotides 17 and 18, the PCR product was digested with NdeI and cloned into pGALs-(URS3) to give pGALS-(URS3)-myc-EGL-1. To clone GFP-tagged EGL-1, the N-terminal part of the coding region was amplified with primers 26 and 27. The product was cut with EcoRI and ligated into pGBT9. CED-9 was subcloned into pACT3-1 first by excising the CED-9 terminal portion of CED-4 was cloned into pGBT9 cut with BamHI and ligated into pGBT9-CED-4-C to give pGBT9-CED-4. Yeast mitochondrial staining is described below. Microscopy was undertaken with a Leica TCS4 SP2 spectral confocal scanner or an Olympus Fluoview 300 confocal laser scanning microscope.

**Yeast mitochondrial staining**

Yeast mitochondria were stained using MitoTracker CMH2-X-Ros (Molecular Probes) as per the manufacturer’s instructions. Briefly, 1 μM MitoTracker CMH2-X-Ros was added to the yeast, which were then incubated in the dark at 30°C for 30 minutes, with shaking. They were then washed and resuspended in distilled water, then mounted on coverslips 1:1 with 1% low-melt agarose.

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**Puriﬁcation of recombinant proteins**

pET23a plasmids were transformed into BL21(DE3)pLysS bacteria (Novagen). Transforms were grown overnight at 37°C and then expanded 1:5 (p35) or 1:8 (CED-4 and EGF-1) in fresh media containing 1 mM IPTG. Protein expression was induced at 30°C for 4 hours (p35) or 8 hours (CED-4 and EGF-1). The bacteria were harvested, lysed in an immunoprecipitation buffer (50 mM HEPES, 150 mM NaCl, 1% NP40, 5 mM imidazole, protease inhibitor cocktail (Set 1, Calbiochem)) and incubated with in vitro translated proteins that were generated using an in vitro transcription/translation kit (Promega). After incubation at 4°C for
1 hour (rotating), protein complexes were isolated by binding to NiNTA beads at 4°C for 30 minutes, with rotation. After binding, beads were washed three times in immunoprecipitation buffer, then proteins were eluted by boiling in sample buffer for SDS-PAGE, prior to autoradiography and immunoblotting.

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