Mutational analysis of the interacting cell death regulators CED-9 and CED-4

Sabine Ottilie[1,2], Yan Wang[1], Sean Banks[1], Julia Chang[1], Nicole J. Vigna[1], Suzanne Weeks[1], Robert C. Armstrong[1], Lawrence C. Fritz[1] and Tilman Oltersdorf[1]

[1] IDUN Pharmaceuticals, Inc. La Jolla, California, USA
[2] corresponding author: Dr Sabine Ottilie, IDUN Pharmaceuticals, Inc. 11085 N. Torrey Pines Road, Suite 300, La Jolla, California 92037, USA.
   Tel: 619-623-1330; Fax: 619-625-2677; E-mail address: sottilie@idun.com

Abstract

The genes *ced-3*, *ced-4* and *ced-9* are central components in the cell death pathway of the nematode *C. elegans*. *Ced-9*, which functions to inhibit cell death, is homologous to the Bcl-2 family of mammalian anti-apoptotic genes. The *ced-3* gene encodes a protein homologous to the caspases, a family of cysteine proteases involved in the execution of programmed cell death. It has recently been demonstrated that CED-4, an inducer of apoptosis for which no mammalian equivalent has been reported, can interact with CED-9 and Bcl-x$_L$. Here we confirm that CED-9 and CED-4 interact and using a series of deletion mutants, demonstrate that only short N-terminal deletions are tolerated in each molecule without loss-of-interaction. Two loss-of-function point mutations in different regions of CED-4 also lead to a significant loss of interaction suggesting further that the relevant interaction domains are not short linear sequences, but rather, are formed by more complex structural determinants in each molecule. Furthermore, we demonstrate that CED-4 not only interacts with Bcl-x$_L$ but also with its homologue, Bcl-2, and that the unstructured loop region present in Bcl-x$_L$ and Bcl-2 can regulate the CED-4 interaction. Lastly, we show that a BH3 peptide that can inhibit Bcl-2 family interactions also inhibits the interaction between Bcl-x$_L$ and CED-4.

Keywords: apoptosis; cell death; ced-9; ced-4; Bcl-2; Bcl-x$_L$; BH-3 peptide

Abbreviations: ZVAD, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; aa, amino acid; GST, glutathionine-S-transferase; SDS – PAGE, sodium dodecyl sulfate polyacrylamide gelelectrophoresis; EDTA, ethylenediaminetetraaceticacid

Introduction

Programmed cell death (PCD) is an intrinsic biochemical pathway that plays an essential role in development and tissue homeostasis. Genetic studies in the nematode *Caenorhabditis elegans* have identified a number of genes that participate in the death pathway. The *ced-3* gene product promotes cell death and is required to effect all developmentally regulated programmed cell deaths in *C. elegans*. *Ced-3* encodes a nematode homologue of the growing family of cysteine proteases termed caspases (Yuan *et al*, 1993). The *ced-9* gene product inhibits programmed cell death and encodes a nematode homologue of the mammalian Bcl-2 family (Hengartner *et al*, 1992; Hengartner and Horvitz, 1994). The *ced-4* gene, for which no mammalian homologue has been identified, exists as two alternatively spliced forms. The more abundant *ced-4S* promotes cell death, while *ced-4L* antagonizes cell death (Yuan and Horvitz, 1992; Shaham and Horvitz, 1996). Genetic studies in the worm have shown that *ced-9* acts upstream to prevent the action of *ced-3* and *ced-4*. In mammalian cells, biochemical analysis indicates that Bcl-2 acts upstream of at least some members of the caspase family of proteases (Armstrong *et al*, 1996; Chinnaiyan *et al*, 1996).

Recent studies using both cell-free systems and intact cells have demonstrated that the *ced-4* gene product can associate with the anti-apoptotic factor CED-9 and its mammalian homologue Bcl-x$_L$ (Chinnaiyan *et al*, 1997; Spector *et al*, 1997; Wu *et al*, 1997). In cells, this association can lead to the sequestration of CED-4 to the intracellular membrane compartments to which CED-9 is localized (Wu *et al*, 1997). In one report, CED-4 over-expression led to the activation of programmed cell death, an effect which was abrogated by co-expression of CED-9 or Bcl-x$_L$. Death induced by CED-4 overexpression was also inhibited by treatment of cells with the caspase inhibitor zVAD-fmk or co-expression of the viral encoded caspase inhibitors crmA or p35 (Chinnaiyan *et al*, 1997). These data suggested that the CED-4 function is upstream of caspase activation and that CED-9, via direct interaction, may inhibit the ability of CED-4 to act on a target effector. This model was further supported by the observation that CED-4 can directly associate with CED-3 and several mammalian caspase homologues (Chinnaiyan *et al*, 1997), suggesting that direct interaction of CED-4 with CED-3 may lead to protease activation and subsequent cell death.

In the present report we further characterize the association between CED-4 and CED-9. We show that CED-4S associates with CED-9, while CED-4L possesses greatly reduced binding activity. Deletion analysis of CED-4 and CED-9 suggests that their interaction requires nearly full length partners. We demonstrate that CED-4 binds to Bcl-2 and Bcl-x$_L$, the mammalian CED-9 homologues and that the CED-4/Bcl-x$_L$ interaction can be specifically blocked by a Bak BH3-derived peptide. Furthermore we demonstrate that a deletion of the unstructured 'loop'-region of Bcl-x$_L$ (Muchmore *et al*, 1996) which enhances its anti-apoptotic activity, also enhances its binding to CED-4. These data are discussed in the context of a molecular framework for apoptosis.
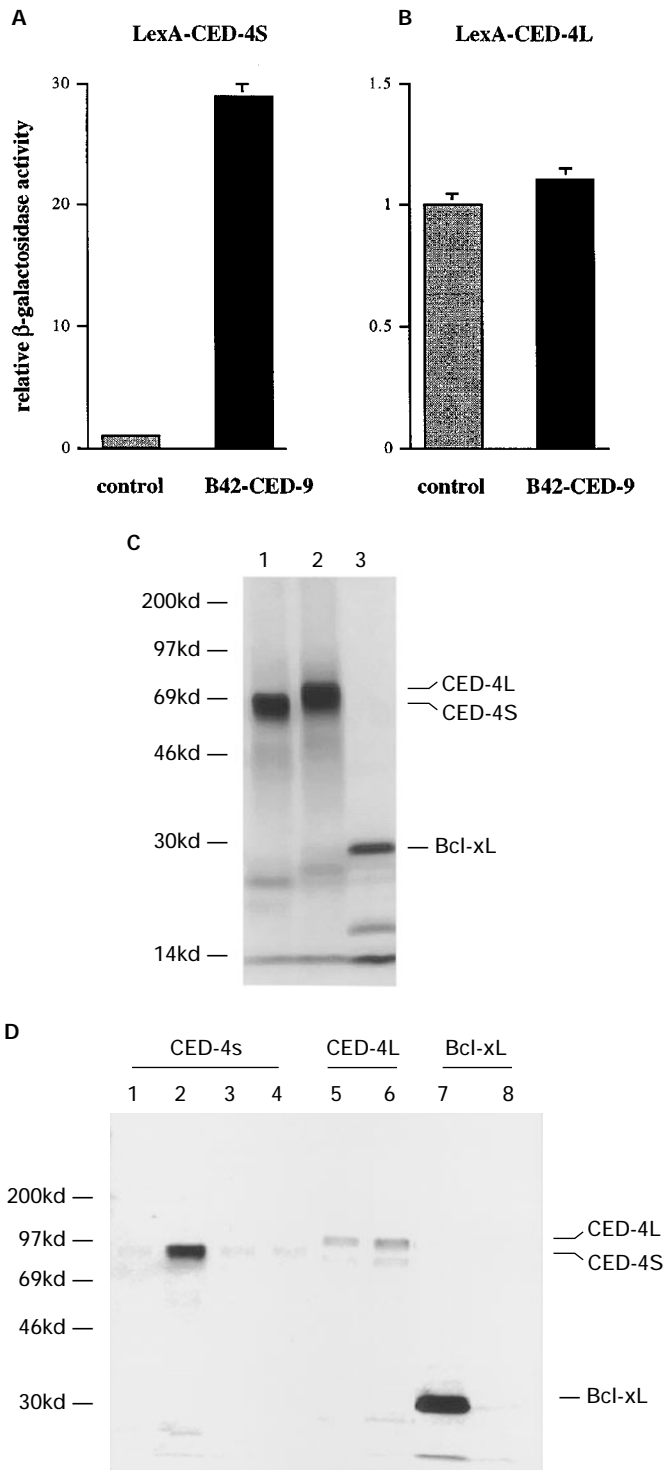
A

LexA-CED-4S

B

LexA-CED-4L

Results

CED-9 interacts directly with CED-4

We used the yeast 2-hybrid system to determine whether CED-9 can directly interact with the two alternatively spliced forms of CED-4. We found that CED-9 (expressed in a soluble form without its C-terminal membrane spanning domain) binds strongly to the death effector CED-4S (Figure 1A) but does not bind to CED-4L, the death-inhibitory isoform containing an additional 24 amino acid insertion (Shaham and Horvitz, 1996) (Figure 1B). Expression levels of these two versions of CED-4 proteins were comparable in Western Blot analysis (data not shown), excluding the possibility that differences in protein levels would account for the differences in interaction. The interaction of CED-4S and CED-9 can be detected by either using CED-9 as a LexA-DNA-binding-protein fusion (bait) or a B42-transcriptional-activator-protein fusion (prey) and with CED-4S expressed as the complementary fusion protein. However the interaction between LexA-CED-4 and B42-CED-9 (Figure 1A) is approximately tenfold stronger than between LexA-CED-9 and B42-CED-4 (data not shown).

To confirm the interaction of CED-9 and CED-4, we performed co-precipitation experiments with *in vitro* translated ^{35}S-CED-4 and purified glutathione-S-transferase-CED-9 (GST-CED-9) fusion protein. The data demonstrate a strong interaction between CED-4S and GST-CED-9 (Figure 1C and D), but only a weak interaction above background was detected between CED-4L and GST-CED-9.

Mapping of binding domains in CED-9

CED-9 is an anti-apoptotic member of the Bcl-2 family and shares sequence similarity with Bcl-2 and Bcl-x_L. The similarities are clustered in several domains, termed BH1−4 domains (Yin *et al*, 1994; Chittenden *et al*, 1995; Zha *et al*, 1996). The recently described NMR and X-ray diffraction structure of Bcl-x_L suggest a hydrophobic groove formed by the BH1, BH2 and BH3 domains (Muchmore *et al*, 1996) into which the BH3 domain of a dimerization partner can insert (Sattler *et al*, 1997). The importance of these domains for dimer formation amongst mammalian family members is well documented (Yin *et al*, 1994; Sedlak *et al*, 1995; Diaz *et al*, 1997). We were similarly interested to analyze the structural requirements for CED-9 interaction with CED-4. We made a series of deletion constructs in CED-9 (all without membrane spanning domains) and assayed their ability to bind to CED-4 in the yeast 2-hybrid system (Figure 2). Our mapping analysis indicates that only small deletions of the N-terminus are

Figure 1 Interaction of CED-9 with CED-4. (**A** and **B**) Yeast 2-hybrid analysis. *S. cerevisiae* EGY191 cells were transformed with the pGilda expression plasmids producing the LexA DNA-binding domain fused to either CED-4S or CED-4L and the pJG4-5 plasmid expressing the B42-activation domain fused to CED-9 with the empty vector (control). The data are plotted as relative β-galactosidase activity and are based on β-galactosidase activity in cells expressing the respective bait and the control prey, which is defined as a value of 1.0. Results represent the means ± the standard deviations of three different transformants assayed in duplicates. (**C**) Input of *in vitro* transcription/translation. ^{35}S-methionine labeled CED-4S (lane 1), CED-4L (lane 2) and Bcl-x_L (lane 3) were separated by SDS−PAGE and analyzed by autoradiography and by the Storm system (Molecular Dynamics). The amounts of various proteins loaded were one-fifth of the amount used in the co-precipitation experiments shown in **D**. (**D**) CED-4S binds to CED-9 *in vitro*. ^{35}S-methionine labeled CED-4S (lanes 1−4), CED-4L (lanes 5 and 6) and Bcl-x_L (lanes 7 and 8) were incubated with either glutathione beads (lanes 1 and 5) or with various immobilized GST-tagged proteins (GST-CED9: lanes 2 and 6; GST-Bax: lanes 3 and 7; GST-Ras: lanes 4 and 8). The bound proteins were separated by SDS−PAGE and analyzed by autoradiography

tolerated without a complete loss of interaction with CED-4. The ΔN-construct (aa 70–254), containing the putative BH1, 2, 3 and 4 domains, still interacts with CED-4, albeit more weakly than the CED-9 wild-type construct. To rule out that the deletion mutants of CED-9 do not interact with CED-4 due to low levels of protein expression we performed Western blot analyses and detected essentially equal amounts of protein (data not shown). These results suggest that the structure of CED-9 needs to be largely intact to preserve its ability to bind to CED-4.

Mapping of binding domains in CED-4

In order to analyze the structural requirements for CED-4 to bind to CED-9 we created a series of truncation mutants in CED-4 and assayed their interaction with CED-9 in the yeast 2-hybrid system. A fragment lacking the first 90 amino acids interacts very strongly with CED-9 while a fragment lacking the C-terminal 159 amino acids binds only weakly (Figure 3). Further truncation of the N-terminus or C-terminus abolishes interaction with CED-9 (Figure 3). A set of overlapping 120 amino acid fragments was also tested by yeast 2-hybrid analysis but none of them interacted with CED-9.

We correlated genetic analysis with direct protein-protein interaction studies, by analyzing loss-of-function mutations in CED-4 for their ability to bind to CED-9 in the yeast 2-hybrid system. We introduced two loss-of-function CED-4 point mutations (Yuan and Horvitz, 1992 and H.R. Horvitz, pers. comm.) into the yeast CED-4 expression vector and assayed for binding to CED-9. The CED-4 (P23L) mutant loses about 80% of its activity when compared to wild-type CED4 (Figure 3). The mutant CED-4 (I258N) protein does not show any detectable interaction with CED-9 by either

filter assay or in a quantitative liquid assay. To rule out that the lack of interaction of any of the CED4 point mutants merely reflects decreased protein expression levels we performed Western blot analysis with an anti-LexA-antibody and detected essentially equal amounts of proteins (data not shown).

Bcl-2 and Bcl-x$_L$ interact directly with CED-4

We next determined whether Bcl-2 and Bcl-x$_L$, the mammalian homologues of CED-9, interact with CED-4. Using the yeast 2-hybrid system and Bcl-2 family proteins expressed without transmembrane domains, we detected a reproducible but weak interaction between Bcl-2 and CED-4 but no detectable interaction between Bcl-x$_L$ and CED-4 (Figure 4A). Deletion mutants of Bcl-2 and Bcl-x$_L$ which remove their unstructured 'loop' domains were recently reported to increase their anti-apoptotic activities (Muchmore *et al*, 1996; Chang *et al*, 1997). We tested these loop-deleted mutants for interaction with CED-4. The results indicate that deletion of the loop-domain in Bcl-2 enhances slightly its interaction with CED-4 and a similar deletion in Bcl-x$_L$ leads to a strong interaction with CED-4 (Figure 4A), comparable to the strength of interaction of CED-4 with CED-9 (see Figure 1A). To confirm the interaction of Bcl-2 and Bcl-x$_L$ with CED-4 we made *in vitro* ^{35}S-labeled Bcl-2 and Bcl-x$_L$ full length proteins and incubated them with purified CED-4-6His protein immobilized on protein A-sepharose. Immuno-precipitations of CED-4-6His co-precipitated ^{35}S-labeled Bcl-2 and Bcl-x$_L$ but did not show binding to Bax (Figure 4B and 4C). In this co-precipitation assay Bcl-x$_L$ contained the unstructured loop but still showed robust binding to CED-4 in contrast to the yeast 2-hybrid results.
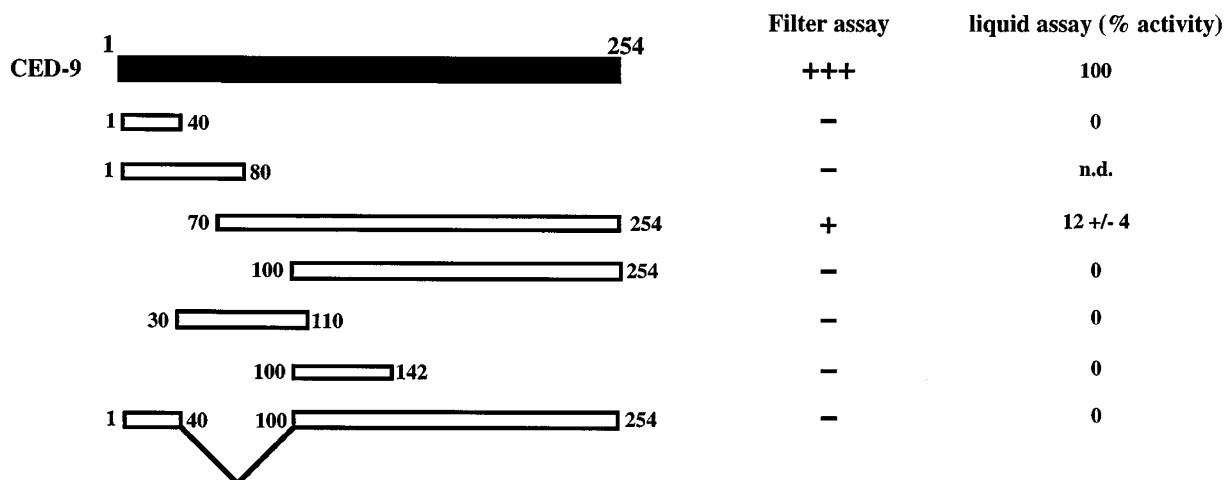
Interaction with CED-4

Figure 2 Mapping of CED-9 domains. *S. cerevisiae* EGY191 cells were transformed with the pGilda expression plasmid producing the LexA DNA-binding domain fused to CED-4S and the pJG4-5 plasmid expresing the B42-activation domain fused to a set of CED-9 deletion constructs, or with the empty vector (control). The strength of interaction is based on a visual filter assay and by a quantitative β-galactosidase assay. The interaction of wild-type CED-9 with CED-4 is defined as 100%. Results represent the means ± the standard deviations of six different transformants assayed in duplicates. +++: strong blue color; +: light blue color; −: white

A BH3-peptide inhibits CED-4 binding to Bcl-x$_L$

It was recently shown that Bak binds into the Bcl-x$_L$ binding pocket via its BH3 domain (Chittenden *et al*, 1995; Sattler *et al*, 1997) and that BH3-derived peptides can block dimerization of Bcl-2 family members (Diaz *et al*, 1997). We were interested to analyze whether CED-4 binding to Bcl-x$_L$ can be blocked by a Bak BH3-peptide. We preincubated ^{35}S-labeled Bcl-x$_L$ with a 16 amino acid peptide encompassing the Bak BH3-domain or a control Bak BH3 peptide containing several point mutations. The Bak peptide has previously been shown to block the formation of Bax/Bcl-x$_L$ dimers whereas the mutant peptide was inert (Diaz *et al*, 1997, and data not shown). Our results demonstrate that only the peptide with the wild-type Bak BH3-domain can inhibit CED-4 binding to Bcl-x$_L$ (Figure 5). A peptide derived from the BH4-domain of Bcl-x$_L$ does not inhibit interaction (data not shown) further demonstrating the specificity of the BH3-domain peptides.

Discussion

The genetic relationship in *C. elegans* between the cell death regulators *ced-4* and *ced-9* suggested the possibility of direct biochemical interaction between the two encoded proteins. In this study we have confirmed such a direct biochemical link by two independent methods: yeast 2-hybrid analysis and *in vitro* co-precipitation assays, consistent with recently published studies (Chinnaiyan *et al*, 1997; Spector *et al*, 1997; Wu *et al*, 1997).

We found a significant difference in binding of CED-9 to the two described splice variants of CED-4. CED-4S bound strongly to CED-9 whereas CED4-L interacted only weakly with CED-9, both in the yeast 2-hybrid system and *in vitro* co-precipitation experiments. These results confirm the data of Spector *et al* (1997) who also showed preferential binding of CED-9 to CED-4S. CED-4S and CED-4L have opposite function in the regulation of programmed cell death, CED-4S inducing cell death, while CED-4L displays
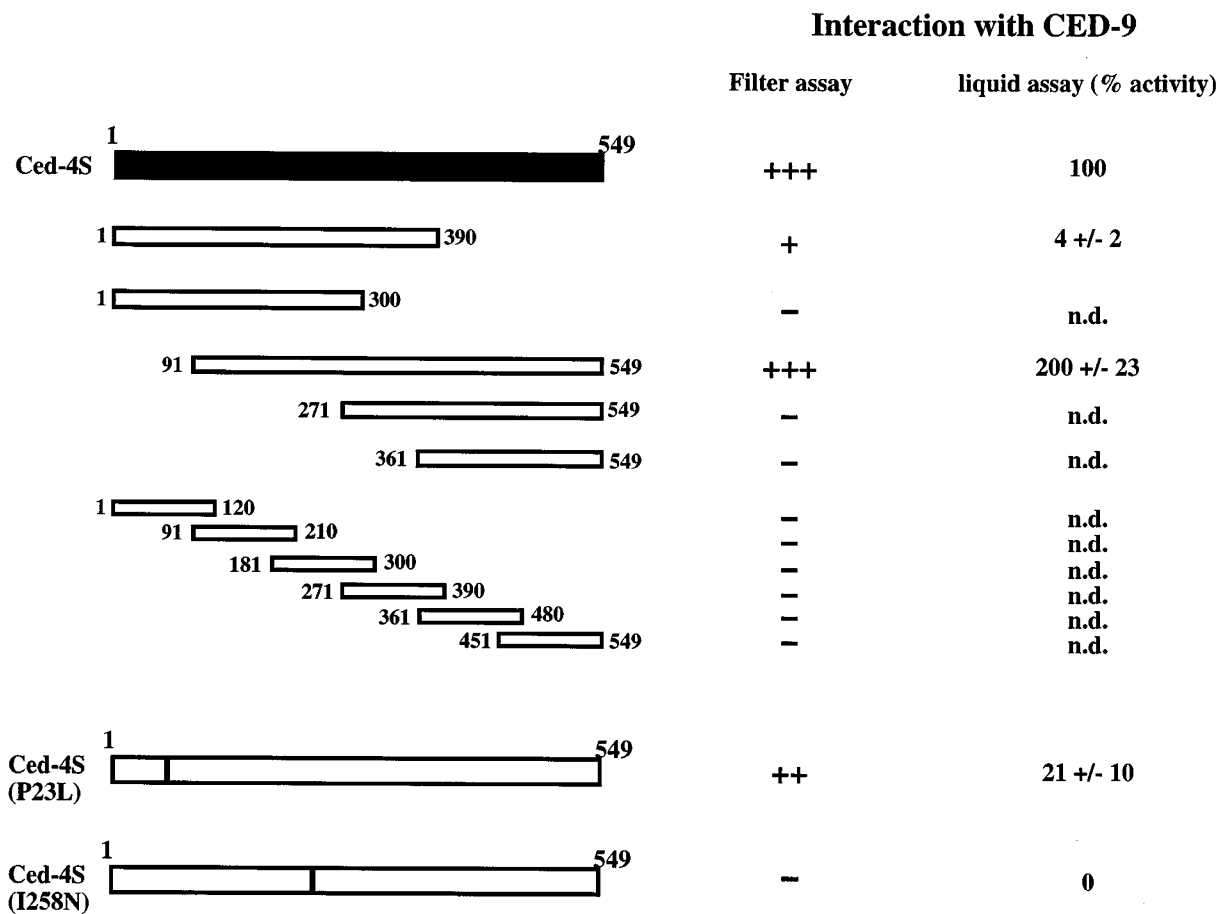

Interaction with CED-9

	Filter assay	liquid assay (% activity)
Ced-4S (1–549)	+++	100
(1–390)	+	4 +/- 2
(1–300)	−	n.d.
(91–549)	+++	200 +/- 23
(271–549)	−	n.d.
(361–549)	−	n.d.
(1–120)	−	n.d.
(91–210)	−	n.d.
(181–300)	−	n.d.
(271–390)	−	n.d.
(361–480)	−	n.d.
(451–549)	−	n.d.
Ced-4S (P23L) (1–549)	++	21 +/- 10
Ced-4S (I258N) (1–549)	−	0

Figure 3 Mapping of CED-4S domains. *S. cerevisiae* EGY191 cells were transformed with the pGilda expression plasmids producing the LexA DNA-binding domain fused to a set of CED-4S constructs and the pJG4-5 plasmid expressing the B42-activation domain fused to CED-9, or with the empty vector (control). The strength of interaction is based on a visual filter assay and a quantitative β-galactosidase assay. The data are plotted as relative β-galactosidase activity and are based on β-galactosidase activity in cells expressing the respective bait and the control prey, which is defined as a value of 1.0. Results represent the means $\pm$ the standard deviations of at least six different transformants assayed in duplicates. +++: strong blue color; ++: medium blue color; +: light blue color; −: white

a cell protective function (Shaham and Horvitz, 1996). Our results are in accord with a model that CED-9 antagonizes CED-4S pro-apoptotic activity by direct biochemical interaction, but does not oppose the anti-apoptotic molecule CED-4L.

Structural analysis of Bcl-x$_L$ has revealed a hydrophobic binding groove formed by the BH-1, BH-2 and BH-3 homology domains, while the N-terminal BH4-domain was hypothesized to be important for stabilizing the Bcl-x$_L$

structure (Muchmore *et al*, 1996). Sequence alignments of CED-9 with Bcl-2 family members demonstrate that CED-9 shows significant sequence conservation of these domains, suggesting a conserved three dimensional structure, possibly including a binding pocket for a dimerization partner. Our deletion mutant analysis of CED-9 reveals that only a small deletion of the N-terminus, corresponding to a part of the CED-9 that is not present in either Bcl-2 or Bcl-x$_L$, can be tolerated without leading to a loss of
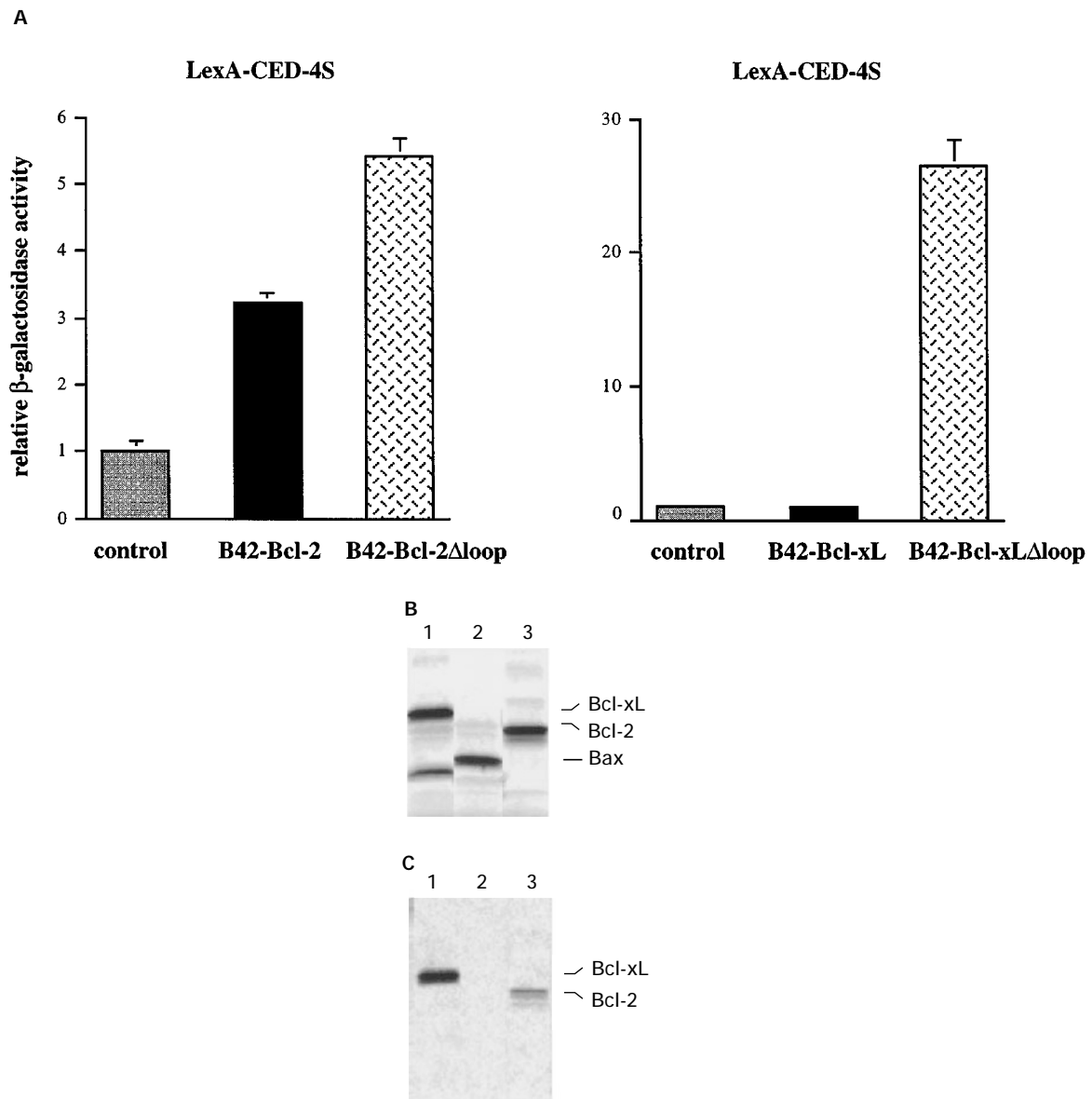

Figure 4 Interaction of Bcl-2 and Bcl-x$_L$ with CED-4. (**A**) Yeast 2-hybrid analysis. *S. cerevisiae* EGY191 cells were transformed with the pGilda expression plasmid producing the LexA DNA-binding domain fused to CED-4S and the pJG4-5 plasmid expressing the B42-activation domain fused to Bcl-2, Bcl-2Δloop, Bcl-x$_L$, Bcl-x$_L$Δloop or with the empty vector (control). The data are plotted as relative β-galactosidase activity and are based on β-galactosidase activity in cells expressing the respective bait and the control prey, which is defined as a value of 1.0. Results represent the means±the standard deviations of three different transformants assayed in duplicates. (**B**) Input of *in vitro* transcription/translation. ^{35}S-methionine labeled Bcl-x$_L$ (lane 1). Bax (lane 2) and Bcl-2 (lane 3) were seperated by SDS-PAGE and analyzed by autoradiography and by the Storm System (Molecular Dynamics). (**C**) CED-4 binds to Bcl-2 and Bcl-x$_L$ *in vitro*. ^{35}S-methionine labeled Bcl-x$_L$ (lane 1), Bax (lane 2) and Bcl-2 (lane 3) were incubated with CED-4-6His fusion protein immobilized on protein A-sepharose beads. The bound proteins were separated by SDS–PAGE and analyzed by autoradiography
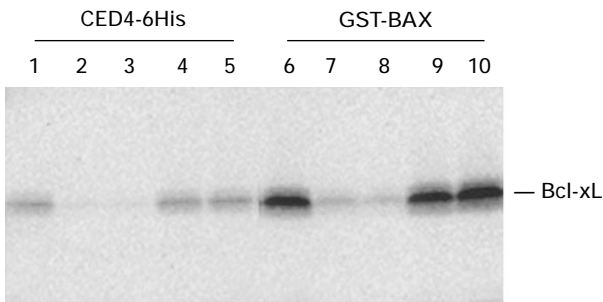
CED4-6His   GST-BAX

1   2   3   4   5   6   7   8   9   10

— Bcl-xL

Figure 5 A Bak BH3-peptide inhibits CED-4 binding to Bcl-x_L. ^{35}S-methionine labeled Bcl-x_L was preincubated with either no peptide (lanes 1 and 6) or with a Bak derived BH3-peptide in a final concentration of 10 μM (lanes 2 and 7) or 40 μM (lanes 3 and 8) or with a control Bak peptide (lanes 4 and 9, 10 μM; lanes 5 and 10, 40 μM) followed by an incubation with either immobilized CED-4-6His (lanes 1–5) or immobilized GST-Bax (lanes 6–10). The bound proteins were separated by SDS–PAGE and analyzed by autoradiography

interaction with CED-4. Further deletions of the N-terminus, including the putative BH4 domain lead to a complete loss of interaction. Our results suggest that CED-9 requires a correctly folded molecular structure consisting of intact BH1, 2, 3 and 4 domains.

Our analysis of CED-4 suggests that its interaction with CED-9 is also dependent on more than a short linear binding motif. First, deletion of the N-terminal 90 amino acids was tolerated, larger N-terminal or C-terminal truncations led to a loss of binding activity. Second, none of the overlapping 120 amino acid segments of CED-4 interacted with CED-9 in the yeast 2-hybrid assay. However, although full length CED-4 interacts with CED-9, we cannot rule out that the N-terminal fusion of the deletion mutants to LexA in the yeast 2-hybrid system had a negative influence on binding. And third, two loss-of-function mutations located in separate regions of the CED-4 sequence, P23L and I258N, have a major effect on the CED-9 interaction. The P23L mutation leads to an approximately 80% reduction in activity compared to wild-type CED-4, and the I258N mutation completely abolishes the interaction with CED-9. It was previously reported that the expression of CED-4S in 293 cells induces cell death, whereas the I258N mutant protein does not (Chinnaiyan *et al*, 1997). However, in that study the mutant protein was reported to be unstable, and thus it was not clear whether the mutation abolished the activity or whether lower protein concentrations accounted for the phenotype. Since in yeast the I258N mutant protein is as stable as the wild-type protein (data not shown), its failure to bind CED-9 is a true property of the mutant and not an artifact due to protein instability. Further work is required to determine whether the loss of function phenotype of these mutations is due to a loss of binding to CED-4 in *C. elegans*.

Our results demonstrate that the *C. elegans* CED-4 protein can interact not only with the *C. elegans* CED-9 protein but also with the mammalian homologues Bcl-2 and Bcl-x_L. Bcl-2 shows weak but consistent and reproducible interaction with CED-4S in yeast 2-hybrid analysis as well

as by *in vitro* coprecipitation. Bcl-x_L did not show interaction in yeast 2-hybrid analysis, however, a deletion mutant of Bcl-x_L without the unstructured loop region (Muchmore *et al*, 1996) strongly interacted with CED-4. A similar deletion in Bcl-2 also enhanced interaction with CED-4. It was recently shown that loop-deletion mutants of Bcl-x_L and Bcl-2 show an enhanced ability to inhibit apoptosis and it was speculated that the loop domains suppress the anti-apoptotic function of these molecules by being a target for post-translational modifications (Chang *et al*, 1997). The apparent discrepancy between the yeast 2-hybrid analysis and the *in vitro* precipitation assay for the Bcl-x_L - CED-4 interaction could be due to several factors. The N-terminal LexA-fusion used in the yeast 2-hybrid system may have an unpredictable effect on protein conformation and accessibility of binding domains. Alternatively, post-translational modifications of the loop-domain with known negative effects on the biological activity of Bcl-x_L (Chang *et al*, 1997) may negatively influence the binding to CED-4. Yeast cells are more efficient in the addition of some post-translational modifications than reticulocyte lysates. Our data are consistent with a model in which Bcl-2 and Bcl-x_L suppress the death promoting activity of a mammalian CED-4 homologue by direct biochemical interactions that are negatively regulated by the loop domain. We propose that the loop-deleted Bcl-2 and Bcl-x_L molecules have enhanced anti-apoptotic activity by virtue of their enhanced ability to bind a pro-apoptotic CED-4 homologue.

It was recently shown that short peptide sequences derived from the BH3-domains of Bcl-2 family members are sufficient to bind to the hydrophobic binding pocket of Bcl-x_L (Sattler *et al*, 1997) and that the dimerization of Bcl-2 family members can be blocked by peptides encoding BH3-domains (Diaz *et al*, 1997). We demonstrated that CED-4 binding to Bcl-x_L can be specifically blocked with a Bak-derived BH3-peptide. Our result is in accord with the observation that Bax expression inhibits the association of CED-4 with Bcl-x_L in transfected mammalian cells (Chinnaiyan *et al*, 1997), and further suggests that this Bax effect was due to a direct BH3 interaction with Bcl-x_L. Our data thus support the idea that BH3-containing proteins induce cell death in mammalian cells by directly displacing a CED-4 homologue from Bcl-2 and Bcl-x_L.

Our BH3 peptide data suggest the possibility that CED-4 contains a BH3-like domain that binds directly to the hydrophobic groove of Bcl-x_L. If so, this domain lacks obvious sequence homology to the known BH3 domains. Alternatively, the binding of the BH3 peptide to the hydrophobic groove could induce a conformational change in the Bcl-x_L molecule that inhibits CED-4 binding to a separate site. Additional studies will be necessary to distinguish between these possibilities and to further characterize the relevant binding sites.

Materials and Methods

Plasmids

CED-9 without a C-terminal putative transmembrane domain (residues 1–254), CED-4S (residues 1–549) and CED4L (residues 1–573)

were PCR amplified and subcloned into the yeast 2-hybrid vectors pJG4-5 (Gyuris *et al*, 1993) and pGilda (C. Kaiser, Massachusetts Institute of Technology). Standard PCR procedures were used to generate the various deletion fragments of CED-4 and CED-9. The internal deletion construct of CED-9 was made by using a two-step PCR technique. The first step consisted of two PCR reactions generating two overlapping products which had the amino acids 40–100 deleted. Using these two overlapping PCR products as a template, the second PCR reaction was performed with the two outside primers to generate the final internal deletion fragment. All constructs were confirmed by sequencing. pJG4-5-Bcl-2 and pJG4-5-Bcl-x$_L$ constructs without C-terminal putative membrane spanning domains were described previously (Sato *et al*, 1994). Site-directed mutagenesis was performed to generate CED-4 (P23L) and CED-4 (I258N) by using the Quick-Change Kit (Stratagene) according to the manufacturer's protocol. To generate Bcl-2Δloop(aa 30–79) and Bcl-x$_L$Δloop(aa 25–88) the Muta-Gene M13 Kit (BioRad) and the following oligonucleotides were used: 5′-CGC AGG CCC CGC GGC GGC CTC GTA GCC CCT CTG CGA-3′ and 5′-CAG AAA GGA TAC AGC TGG GCC CTG AGG GAG GCA GGC-3′.

Yeast 2-hybrid analysis

A bait vector (LexA fusion protein), a prey vector (B42 fusion protein), and the β-galactosidase expression plasmid were transformed into the *S. cerevisiae* strain EGY191 (Gyuris *et al*, 1993). For filter assays and quantitative liquid assays at least three independent colonies were assayed in duplicates as described previously (Bertin *et al*, 1997).

In vitro protein interaction assay

For *in vitro* coprecipitation assays we employed the following two methods. *ced-4S* and *ced-4L* cDNAs in the Bluescript vector (Stratagene), and Bcl-x$_L$ cDNA in the pCIneo vector (Promega) were used as templates for an *in vitro* transcription/translation reaction (TNT kit, Promega) in the presence of ^{35}S-methionine. Various GST-tagged gene products (CED-9, Bcl-x$_L$, Bcl-2, Bax and Ras) were expressed from the pGEX-4T vector (Pharmacia) and purified by glutathione-Sepharose beads (Pharmacia). None of these proteins contains a putative transmembrane domain. Pre-cleared *in vitro* transcription/translation lysates were added to various GST fusion proteins immobilized on glutathione beads in the presence of 250 μl immunoprecipitation buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 2 mM EDTA, 5 mM DTT, 0.5% Nonidet P40). The reaction mixtures were incubated for 1.5 h at 4°C and then washed four times with the immunoprecipitation buffer. Bound proteins were separated by SDS–PAGE and analyzed by autoradiography and by the Storm system (Molecular Dynamics). To analyze CED-4 binding to Bcl-2 and Bcl-x$_L$ the following methods were employed. Bcl-x$_L$, Bax and Bcl-2 cDNAs in the pCIneo vector (Promega) were used as templates for *in vitro* transcription/translation reactions (TNT kit, Promega) in the presence of ^{35}S-methionine. The *ced-4* cDNA was cloned into the NcoI site of pET15B (Novagen) to generate CED4-6His protein. The expressed protein was purified through Ni-NTA beads. The *in vitro* transcription/translation lysates were added to the CED-4-6His fusion protein immobilized on protein-A sepharose beads through an anti-6His antibody (Santa Cruz, #SC804) in 250 μl immunoprecipitation buffer. The reaction mixtures were incubated for 1.5 h at 4°C and washed four times with the immunoprecipitation buffer. Bound proteins were separated by SDS–PAGE and analyzed by autoradiography and by the Storm system (Molecular Dynamics).

Peptide competition assay

Peptide competition experiments were performed using the same procedure as described above except that the 10 μM and 40 μM of the following peptides: Bak BH3 peptide: GQVGRLAIIGDDINR and control Bak peptide: GQDGRQEAIIRRLINR were preincubated with either immobilized CED-4-6His or GST-BAX at 4°C for 30 min before ^{35}S-labeled Bcl-x$_L$ was added. Bound proteins were separated by SDS–PAGE and analyzed by autoradiography and by the Storm system (Molecular Dynamics).

Acknowledgements

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