The conserved N-terminal BH4 domain of Bcl-2 homologues is essential for inhibition of apoptosis and interaction with CED-4

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Bcl-2 and close homologues such as Bcl-x_L promote cell survival, while other relatives such as Bax antagonize this function. Since only the pro-survival family members possess a conserved N-terminal region denoted BH4, we have explored the role of this amphipathic helix for their survival function and for interactions with several agonists of apoptosis, including Bax and CED-4, an essential regulator in the nematode *Caenorhabditis elegans*. BH4 of Bcl-2 could be replaced by that of Bcl-x without perturbing function but not by a somewhat similar region near the N-terminus of Bax. Bcl-2 cell survival activity was reduced by substitutions in two of ten conserved BH4 residues. Deletion of BH4 rendered Bcl-2 (and Bcl-x_L) inactive but did not impair either Bcl-2 homodimerization or ability to bind to Bax or five other pro-apoptotic relatives (Bak, Bad, Bik, Bid or Bim). Hence, association with these death agonists is not sufficient to promote cell survival. Significantly, however, Bcl-x_L lacking BH4 lost the ability both to bind CED-4 and antagonize its pro-apoptotic activity. These results favour the hypothesis that the BH4 domain of pro-survival Bcl-2 family members allows them to sequester CED-4 relatives and thereby prevent apoptosis.

Keywords: apoptosis/Bax/Bcl-2/BH4/CED-4/mutagenesis

Introduction

Programmed cell death or apoptosis plays a major role in normal development and physiology as well as in many pathological states. The process is highly conserved between organisms as diverse as man and the nematode *Caenorhabditis elegans* (Hengartner and Horvitz, 1994b; Vaux *et al.*, 1994) and its underlying control mechanisms are under intense scrutiny. The effectors are a set of novel proteases, now termed caspases, that cleave vital cellular substrates (Nicholson and Thornberry, 1997). Much remains to be clarified about the regulation of caspase activation, but key players include members of the Bcl-2 family (Cory, 1995; White, 1996; Yang and Korsmeyer, 1996). Bcl-2 promotes the survival of many cell types against diverse cytotoxic affronts (Vaux *et al.*, 1988; Strasser *et al.*, 1991; Reed, 1994), as do its two closest homologues, Bcl-x_L and Bcl-w (Boise *et al.*, 1993; Gibson *et al.*, 1996). In contrast, more distant relatives such as

Bax and Bak (Oltvai *et al.*, 1993; Chittenden *et al.*, 1995b; Farrow *et al.*, 1995; Kiefer *et al.*, 1995) antagonize this anti-apoptotic function, so the relative abundance of these competing molecules may determine whether the cell lives or dies (Oltvai *et al.*, 1993; Oltvai and Korsmeyer, 1994). Pro- and anti-apoptotic family members heterodimerize (Oltvai *et al.*, 1993; Oltvai and Korsmeyer, 1994; Sato *et al.*, 1994; Sedlak *et al.*, 1995), potentially accounting for their ability to work in opposition.

Sequence comparison of Bcl-2 family members has revealed four recurring motifs, commonly denoted Bcl-2 Homology domains (BH1 to BH4) (Yin *et al.*, 1994; Chittenden *et al.*, 1995a; Gibson *et al.*, 1996; Zha *et al.*, 1996). The five most closely related mammalian homologues, Bcl-2, Bcl-x_L, Bcl-w, Bax and Bak, possess the BH1, BH2 and BH3 domains but only the first three, which inhibit apoptosis, also bear the N-terminal BH4 domain. BH1 and BH2 of Bcl-2 and Bcl-x_L are critical for heterodimerization with Bax and for promoting cell survival (Yin *et al.*, 1994). In contrast, the small BH3 domain of the pro-apoptotic molecules appears to be essential for their interaction with Bcl-2 and its functional homologues and for acceleration of apoptosis (Chittenden *et al.*, 1995b; Simonian *et al.*, 1996; Zha *et al.*, 1996). This domain is also found in several distantly related proteins that promote apoptosis: Bik/Nbk (Boyd *et al.*, 1995; Han *et al.*, 1996), Bid (Wang, K. *et al.*, 1996), Hrk (Inohara *et al.*, 1997), Bad (Yang *et al.*, 1995; Zha *et al.*, 1997) and Bim (O'Connor *et al.*, 1998). Recent structural studies on Bcl-x_L have revealed that its BH1, BH2 and BH3 domains form an elongated hydrophobic cleft which can bind the BH3-containing peptides of the death promoters (Muchmore *et al.*, 1996; Sattler *et al.*, 1997).

One model for the action of Bcl-2 and its closest homologues is that they sequester their pro-apoptotic relatives, preventing them from conveying a death signal. This model is supported by data from some mutants (Yin *et al.*, 1994) but not others (Cheng *et al.*, 1996). An alternative model derives from genetic studies of cell death in *C.elegans*, where activation of the caspase CED-3 requires CED-4 (Yuan and Horvitz, 1992), which is normally held in check by CED-9, the nematode counterpart of Bcl-2 (Vaux *et al.*, 1992; Hengartner and Horvitz, 1994a). Evidence has emerged recently for direct association of CED-9 with CED-4 and of CED-4 with CED-3 (Chinnaiyan *et al.*, 1997; Irmeler *et al.*, 1997; Seshagiri and Miller, 1997; Spector *et al.*, 1997; Wu *et al.*, 1997b). Like CED-9, Bcl-x_L can bind to CED-4 and prevent its ability (in concert with CED-3) to kill mammalian cells (Chinnaiyan *et al.*, 1997). These findings have raised the possibility that the pro-survival molecules function by sequestering CED-4-like molecules, thereby preventing activation of the caspases (Chinnaiyan *et al.*, 1997; James *et al.*, 1997; Wu *et al.*, 1997b).

Fig. 1. Comparison of human Bcl-2 family members and *C.elegans* CED-9 showing the BH4 homology region. The sequences were compared using the GCG 'PILEUP' program. Residues which are identical or very similar (K~R; V~I) in at least three of the family members are shaded. The black line indicates residues that form the first α -helix in the NMR and X-ray structure of Bcl-x_L (Muchmore *et al.*, 1996). Indicated by asterisks are two critical residues (R12 and L23) in the BH4 homology region of Bcl-2 identified in this paper.

As the conserved N-terminal sequence specific to the three most closely related pro-survival Bcl-2 homologues is likely to have an essential role in cell survival (Borner *et al.*, 1994; Hanada *et al.*, 1995; Hunter *et al.*, 1996; Lee *et al.*, 1996), we have undertaken a systematic mutational analysis of that region in Bcl-2. We report here that the BH4 region is dispensable for both the homodimerization of Bcl-2 and its interaction with Bax and other antagonists. Nevertheless, deletion of this region or its replacement by an N-terminal region of Bax completely abrogated the ability of Bcl-2 to promote cell survival. We present evidence suggesting that BH4 is critical for interaction of Bcl-2 family members with CED-4, favouring the view that CED-4 homologues will be critical effectors of apoptosis in mammalian cells.

Results

The BH4 region is indispensable for the anti-apoptotic function of Bcl-2 and Bcl-x_L

Figure 1 depicts the BH4 region near the N-termini of the human Bcl-2-like proteins. The sequence similarity of Bcl-2, Bcl-x_L and Bcl-w is readily apparent, but there is very little homology with Bax or Bak. To determine whether the BH4 region of Bcl-2 is essential for its anti-apoptosis function, we deleted the region encoding amino acids 10–30 and used a transient transfection assay to assess whether the mutant gene could still protect 293 human embryonal kidney cells from apoptosis induced by the protein kinase inhibitor staurosporine. Transfectants were identified by co-transfection with a plasmid expressing a mutant CD4 protein which appears on the cell surface but lacks signalling capability (see Materials and methods). The cells were exposed for 6 h to graded levels of staurosporine and the viability of transfected cells was then assessed by determining the proportion of CD4⁺ cells excluding propidium iodide. Transfectants expressing wild-type (wt) Bcl-2 were relatively resistant to staurosporine, but those expressing Bcl-2 Δ BH4 (Figure 2A) were almost as sensitive as cells transfected with the vector control (Figure 2B).

We also assessed the survival capability of cells stably expressing the mutant Bcl-2 protein. FDC-P1 myeloid cells transfected with vectors co-expressing puromycin resistance and either wt or mutant Bcl-2 were selected by growth in medium containing interleukin-3 (IL-3) and

Fig. 2. Bcl-2 Δ BH4 fails to protect transiently transfected 293 cells from killing by staurosporine. (A) Lysates from 293 cells transiently transfected with the parental vector (control) and vectors encoding human Bcl-2 or Bcl-2 Δ BH4 were resolved by SDS-PAGE electrophoresis and the filter was blotted with an anti-human Bcl-2 antibody. (B) 293 cells were transiently co-transfected with the parental vector (control) and vectors encoding human Bcl-2 or Bcl-2 Δ BH4 and, 24 h later, staurosporine (1 to 1000 nM) was added to the medium. Flow cytometric analysis (see Materials and methods) was performed after 6 h to determine the proportion of CD4⁺ (transfected) cells that were propidium iodide-negative (live). Data shown are arithmetic means $\pm$ SD of at least six independent experiments.

puromycin. Clones expressing high levels of Bcl-2 Δ BH4 protein were readily isolated, indicating that the BH4 region is not essential for protein stability. Since the degree of protection afforded by Bcl-2 is proportional to its intracellular concentration (Huang *et al.*, 1997a), the clones chosen for functional comparisons had comparable levels of wt or mutant Bcl-2, as assessed by flow cytometry (e.g. Figure 3A) and Western blotting (not shown). FDC-P1 cells normally require IL-3 for both survival and proliferation (Dexter *et al.*, 1980), but enforced Bcl-2 expression allows prolonged survival in the absence of cytokine (Vaux *et al.*, 1988; Figure 3B). Clones expressing Bcl-2 Δ BH4 were just as sensitive to IL-3 deprivation as the parental cell line or clones transfected with an empty vector (Figure 3B). They also remained sensitive to γ -irradiation and staurosporine (Figure 3C and D), while those expressing wt Bcl-2 were impressively resistant, as shown previously (Huang *et al.*, 1997a).

These assays on two different cell types exposed to diverse cytotoxic conditions establish that the conserved

Fig. 3. The BH4 homology region is essential for the pro-survival function of Bcl-2. **(A)** Expression of wild-type Bcl-2 and mutant Bcl-2 Δ BH4 in stably transfected FDC-P1 clones was determined by staining with an anti-human Bcl-2 monoclonal antibody (see Materials and methods). Clones compared in subsequent experiments expressed wt or mutant Bcl-2 at similar levels, as in the examples shown. Staining of the control (parental) cells is indicated by the dotted lines. **(B–D)** Parental FDC-P1 cells and clones transfected with the control vector or vectors encoding wt Bcl-2 or Bcl-2 Δ BH4 were cultured in medium lacking IL-3 **(B)**; in IL-3-containing medium after exposure to 10 Gy γ -irradiation **(C)**; or in IL-3-containing medium supplemented with 1 nM to 1 μ M staurosporine **(D)**. Cell viability was assessed by vital dye exclusion over 3–7 days **(B and C)** or 48 h after adding the cytotoxic agent **(D)**. Data shown are arithmetic means $\pm$ SD of at least three experiments and are representative of the results obtained with at least three independent clones of each genotype.

BH4 region is essential for the anti-apoptotic activity of Bcl-2, confirming and extending earlier studies (Borner *et al.*, 1994; Hanada *et al.*, 1995; Hunter *et al.*, 1996). Comparable results were obtained for a mutant Bcl- x_L protein lacking BH4 (residues 4–24) (data not shown).

Bcl-2 Δ BH4 is not a dominant-negative inhibitor

A deletion very similar to that studied here (Δ 6–31) has been reported to convert Bcl-2 into a dominant-negative molecule (Hunter *et al.*, 1996). To test whether the non-functional Bcl-2 Δ BH4 protein could suppress the activity of wt Bcl-2, we transfected FDC-P1 cells expressing FLAG-tagged wt Bcl-2 with a vector encoding HA-tagged Bcl-2 Δ BH4. As a control for a protein known to modulate Bcl-2 function, we also co-transfected FLAG wt Bcl-2 and HA Bax plasmids. Transfectants were cloned and co-expression confirmed by staining with anti-FLAG and anti-HA antibodies (Figure 4A). Cells expressing both wt Bcl-2 and Bcl-2 Δ BH4 survived just as well in the absence of IL-3 as those expressing wt Bcl-2 alone (Figure 4B), while those co-expressing HA Bax died considerably faster (Figure 4C). Thus, unlike Bax, the Bcl-2 Δ BH4 mutant does not interfere with the activity of wt Bcl-2.

We conclude that deletion of BH4 from Bcl-2 does not create a dominant-negative mutant.

BH4 regions of Bcl-2 and Bcl-x are interchangeable but Bax lacks an equivalent domain

The BH4 region of Bcl-x (residues 4–24) is 62% identical to that of Bcl-2 (residues 10–30) (Figure 1). When we replaced the BH4 region of Bcl-2 with that from Bcl- x_L , we found that the chimeric protein protected transiently transfected 293 cells from apoptosis induced by staurosporine just as effectively as wt Bcl-2 (data not shown). We also isolated FDC-P1 cell lines stably expressing Bcl-2 BH4_{Bcl-x} (Figure 5B) and showed that they were as resistant to cytokine withdrawal as cells expressing wt Bcl-2 (Figure 5C). Thus, the BH4 region of Bcl- x_L can substitute for that of Bcl-2.

It has been suggested that residues 22–42 of Bax form an amphipathic helical region equivalent to that in BH4 of Bcl- x_L (Muchmore *et al.*, 1996), although the three glycines in the Bax region (Figure 1) would be expected to reduce its helical character. We therefore replaced BH4 of Bcl-2 with the putative Bax helix and tested its function. Cells transfected with the chimeric construct were as

Fig. 4. Bcl-2 Δ BH4 is not a dominant-negative inhibitor. (A) Expression of FLAG-tagged Bcl-2 and/or HA-tagged Bax or Bcl-2 Δ BH4 in stably transfected FDC-P1 clones. (B,C) Survival of clones of FDC-P1 cells expressing FLAG-Bcl-2 either alone or together with HA-Bcl-2 Δ BH4 (B) or HA-Bax (C) when cultured in medium deprived of IL-3. Cell viability was assessed by vital dye exclusion; data shown are arithmetic means $\pm$ SD of at least three experiments and are representative of the results obtained with at least three independent clones of each genotype.

sensitive as the control cells (Figure 5C and data not shown). Their failure to survive was not due to protein instability, since immunoblotting of cell lysates (Figure 5B) and flow cytometric analysis of cells stained for intracellular Bcl-2 (data not shown) revealed equivalent levels of the wt and chimeric proteins. A Bcl-2 mutant bearing residues 15 to 33 of Bax was also stably expressed but inactive (data not shown). Thus, Bax does not contain a region functionally equivalent to BH4 of Bcl-2.

Most of the conserved BH4 residues are dispensable for Bcl-2 anti-apoptotic function

The strong conservation of BH4 in Bcl-2, Bcl-x and Bcl-w suggested that its specific sequence might be critical to their ability to antagonize apoptosis. To investigate this directly, we engineered 19 replacements of the 10 most conserved residues, including alanine substitutions at nine of the positions (Table I). Western blots of transiently transfected 293 cells confirmed that the mutant proteins were expressed at the wild-type level (Figure 6A). When the transfectants were challenged with staurosporine, most of the mutant Bcl-2 proteins protected as effectively as wt Bcl-2 (Table I), as exemplified by Bcl-2 V15A in Figure 6B. The same conclusion was reached when stable transfectants of FDC-P1 cells expressing comparable levels of wt and mutant protein (e.g. Figure 6C) were deprived of IL-3 or treated with γ -irradiation or staurosporine (Figure 6D, Table I and data not shown). The fully functional BH4 mutants included all the alanine

substitutions, even the replacements of each of the three conserved basic residues (R12A, K22A, R26A), as well as replacements of an uncharged residue with a charged residue (G27E) or of another with a bulky heterocyclic (V15W). Even a mutant with two alanine substitutions (V15A and Y18A) retained full activity.

Particular substitutions at two positions did markedly impair function. Replacement of Arg12 by glutamine (R12Q), or Leu23 by tryptophan (L23W), led to a significant loss of function in both 293 and FDC-P1 cells (Figure 6B and D). Since alanine substitution at each of those positions did not diminish Bcl-2 activity (Table I), however, it appears that no single conserved residue in BH4 is essential for its function.

Deletion of BH4 from Bcl-2 does not prevent binding to Bax, Bad, Bak, Bik/Nbk, Bid or Bim, nor its homodimerization

Certain BH1 and BH2 mutations that inactivate Bcl-2 also abrogate its ability to bind to Bax (Yin *et al.*, 1994). We therefore explored whether deletion of the BH4 region altered the binding repertoire of Bcl-2. Immunoprecipitates prepared from lysates of FDC-P1 cells expressing wt and Bcl-2 Δ BH4 proteins using a monoclonal antibody specific for human Bcl-2 were fractionated electrophoretically and transferred to filters, which were then blotted with polyclonal antibody raised against mouse Bax. Bcl-2 Δ BH4 bound to Bax as avidly as wt Bcl-2 (Figure 7A), in contrast to the G145E mutation in BH1 and W188A

Fig. 5. The BH4 domain of Bcl-x, but not that of Bax, can substitute for the BH4 domain of Bcl-2. (A) The BH4 homology region of Bcl-2 (boxed) was either deleted (Bcl-2 ΔBH4), replaced with a similar region (aa 4 to 24) from Bcl-x (Bcl-2 BH4_{Bcl-x}) or Bax (aa 22 to 42) (Bcl-2 BH4_{Bax}) (see Materials and methods). Identical or very similar residues are shaded in dark grey (see Figure 1); the ID residues (light grey) encoded a *Clal* restriction site used to make the replacement constructs. (B) Lysates from parental FDC-P1 cells or representative clones stably expressing wt Bcl-2, Bcl-2 ΔBH4, Bcl-2 BH4_{Bcl-x} or Bcl-2 BH4_{Bax} were resolved by SDS-PAGE and immunoblotted using anti-human Bcl-2 antibody. Clones expressing comparable levels of wt or mutant Bcl-2 were used for subsequent analysis. (C) Parental FDC-P1 cells or clones stably expressing wt Bcl-2, Bcl-2 ΔBH4, Bcl-2 BH4_{Bcl-x} or Bcl-2 BH4_{Bax} were cultured for a week in medium lacking IL-3 and the cell viability was assessed by vital dye exclusion. Data shown are arithmetic means ± SD of at least three experiments and are representative of the results obtained with at least three independent clones of each genotype.

Table 1. Functional activity of BH4 mutants of Bcl-2		
Protein	Inhibition of apoptosis	
	Cytokine withdrawal	Treatment with staurosporine
Bcl-2	+	+
Bcl-2 ΔBH4	—	—
Bcl-2 R12A	+	+
Bcl-2 R12Q	±	±
Bcl-2 V15A/W	+	+
Bcl-2 V15A/Y18A	+	+
Bcl-2 Y21A	+	+
Bcl-2 K22A/Q	+	+
Bcl-2 L23A	+	+
Bcl-2 L23W	±	±
Bcl-2 Q25A	+	+
Bcl-2 R26A/Q	+	+
Bcl-2 G27E	+	+
Bcl-2 Y28A/S/F	+	+
Bcl-2 W30A	+	+

Cell survival analysis of cells transfected with vectors expressing comparable levels of wt Bcl-2 or the indicated mutant Bcl-2 proteins. Stably transfected FDC-P1 clones were tested by cytokine withdrawal and irradiation (not shown) assays and transiently transfected 293 cells were tested by treatment with staurosporine, as described in Materials and methods. At least four independent transfectants were tested for each of the proteins.

mutation in BH2 (Yin *et al.*, 1994; our unpublished results). Consistent with binding studies using the yeast two-hybrid system (Hanada *et al.*, 1995), equivalent results were obtained with lysates of L929 mouse fibroblasts transiently transfected with vectors expressing EE-tagged Bax and either wt Bcl-2 or Bcl-2 ΔBH4 (Figure 7B). Deletion of the BH4 region also had no effect on the ability of Bcl-2 to bind to EE-tagged Bak, Bad, Bik/Nbk or Bid (Figure 7B), nor in other experiments to Bim (data not shown). Thus, Bcl-2 ΔBH4 is not inactive because it cannot bind to homologues known to antagonize cell survival.

Bcl-2 has also been reported to homodimerize (Sedlak *et al.*, 1995), although it is unclear whether homodimerization is essential for its biological function. To test whether Bcl-2 lacking BH4 can bind to wt Bcl-2, 293T human kidney cells were transiently transfected with vectors encoding FLAG-tagged wt Bcl-2, HA-tagged Bcl-2 ΔBH4 or both these constructs. Anti-FLAG immunoprecipitates of the cell lysates were then subjected to Western blotting with antibody specific for human Bcl-2. Cells expressing only FLAG-tagged wt Bcl-2 yielded two bands, corresponding to FLAG-Bcl-2 and endogenous Bcl-2 (Figure 7C), while cells transfected with both FLAG-tagged wt Bcl-2 and HA-tagged Bcl-2 ΔBH4 also yielded a band corresponding to HA Bcl-2 ΔBH4 (Figure 7C). Since the

Fig. 6. Mutation of R12 or L23 partially abrogates the pro-survival activity of Bcl-2. (A) Lysates of 293 cells transiently transfected with plasmids encoding wt or BH4 point mutants of human Bcl-2 were fractionated by SDS-PAGE and then immunoblotted using anti-human Bcl-2 monoclonal antibody. (B) 293 cells transiently co-transfected with a reporter plasmid (CD4) and plasmids encoding either wt Bcl-2 or the indicated Bcl-2 mutants were treated with staurosporine 24 h after transfection and viability of CD4⁺ cells was determined by flow cytometry after a further 6 h. The data shown are the arithmetic means $\pm$ SD of the percentage of CD4⁺ (transfected) cells that are propidium iodide-negative (live) and are derived from at least six independent experiments. (C) Expression of wt or R12Q, L23W, V15A mutant Bcl-2 proteins in cloned FDC-P1 lines, determined by flow cytometry after staining with anti-human Bcl-2 antibody. (D) FDC-P1 clones expressing comparable levels of wt or mutant Bcl-2 were cultured in the absence of IL-3 and cell viability was assessed. Data shown are arithmetic means $\pm$ SD of at least three experiments and are representative of the results obtained with at least three independent clones of each genotype.

mutant protein could bind to the wild-type molecule, deletion of BH4 did not affect ability to homodimerize.

Deletion of BH4 from Bcl-x_L abrogates its ability to interact with and antagonize CED-4

In view of recent reports that Bcl-x_L can bind to *C.elegans* CED-4 while Bax cannot (Chinnaiyan *et al.*, 1997), we speculated that BH4 might be critical for the interaction of Bcl-2 analogues with CED-4. To test this hypothesis, 293T cells were transiently co-transfected with CED-4-HA and wild-type or Δ BH4 mutant forms of Bcl-2 or Bcl-x_L. Anti-HA immunoprecipitates were then resolved electrophoretically and blotted with antibodies reactive with human Bcl-2 or Bcl-x_L. The results showed that CED-4 can interact with wt Bcl-x_L but not its BH4 deletion mutant (Figure 8A). Consistent with previous data (Chinnaiyan *et al.*, 1997) there was little, if any, binding of CED-4 to Bcl-2 (data not shown). Equivalent results were obtained when the immunoprecipitation was performed with the Bcl-2 or Bcl-x antibodies and the Western blot was incubated with anti-HA antibody (Figure 8A and data not shown). Thus, the BH4 region of Bcl-x_L appears to be required for binding to CED-4.

We also tested whether the BH4 mutant could antagonize the pro-apoptotic activity of CED-4. Consistent with a recent report (Wu *et al.*, 1997a), CED-4 significantly enhances killing of 293 cells by CED-3 (Figure 8B). This potent apoptotic stimulus was efficiently antagonized by Bcl-x_L but not by the Δ BH4 mutant (Figure 8B). Collectively, these results suggest that the BH4 region of Bcl-x_L is required for both its interaction with CED-4 and its ability to abrogate the cell death induced by CED-4 in concert with CED-3.

Discussion

The BH4 region is highly conserved in sequence among the three closest homologues in the mammalian Bcl-2 family, Bcl-2, Bcl-x_L and Bcl-w (Figure 1), and we have shown that BH4 of Bcl-x can substitute for that of Bcl-2 (Figure 4). The structure of Bcl-x_L indicates that the BH4 region encompasses an amphipathic α -helical loop on the surface that forms extensive hydrophobic interactions with α 2, α 5 and α 6 (Muchmore *et al.*, 1996). Although the N-terminal regions of Bax and Bak may also contain an amphipathic helix (Muchmore *et al.*, 1996), sequence

Fig. 7. Deletion of BH4 from Bcl-2 does not prevent binding to Bax, Bak, Bad, Bik/Nbk and Bid, or homodimerization. **(A)** Lysates from cloned FDC-P1 cells stably expressing either wt Bcl-2 or Bcl-2 ΔBH4 were analysed by Western blotting using an anti-Bcl-2 monoclonal antibody (top panel) or by immunoprecipitation using the anti-Bcl-2 antibody followed by Western blot analysis using polyclonal anti-murine Bax antibody (lower panel). **(B)** Lysates from L929 fibroblasts transiently expressing either wt Bcl-2 or Bcl-2 ΔBH4 plus EE-tagged Bax, Bak, Bad, Bik/Nbk or Bid, as indicated, were analysed by Western blotting using the anti-Bcl-2 (top panel) or anti-EE (middle panel) antibodies. Anti-Bcl-2 immunoprecipitates were analysed by Western blotting with the anti-EE antibody (bottom panel). **(C)** Lysates from transiently transfected 293T cells expressing FLAG Bcl-2 and HA Bcl-2 ΔBH4, either alone or together, were analysed by Western blotting using anti-Bcl-2 antibody (top panel). Anti-FLAG immunoprecipitates were blotted using the anti-Bcl-2 antibody (lower panel). The data shown are representative of at least three independent experiments.

homology is minimal (Figure 1) and replacement of the BH4 region of Bcl-2 with the putative Bax helix inactivated Bcl-2 (Figure 4).

Since the BH4 region is the only conserved domain that sets these pro-survival members of the Bcl-2 family apart from their pro-apoptosis relatives (Figure 1), we assessed how point mutations introduced into that region of Bcl-2 affected its survival function. We found that most single amino acid substitutions within BH4 were well tolerated. The exceptions were the reduced activity found with relatively drastic changes at position 12 (arginine → glutamine) and 23 (leucine → tryptophan). Both these residues reside on the outer face of the amphipathic α -helix that is formed by BH4. Others have previously reported—based on glycine substitutions—that I14, V15, Y18, I19 and L23 are indispensable for the anti-apoptosis activity of Bcl-2 (Lee *et al.*, 1996), although positions 14, 18 and 19 are not highly conserved (Figure 1). Our data supports the importance of L23, but substitution of alanine

or tryptophan for V15 was inconsequential, and even a double mutant where alanines replaced both V15 and Y18 was as active as wt Bcl-2 (Table I). The puzzling discrepancies between the two studies may reflect the use of glycine (which breaks α -helices) rather than alanine for the substitutions, the different cell types and assays used, or differences in the relative amounts of expression achieved for the mutants. Lee *et al.* (1996) analysed their mutants in a transient expression system in a human fibroblast cell line, while we utilized clones of stably transfected FDC-P1 myeloid cells expressing comparable levels of wild-type or mutant proteins.

Early mutagenesis analysis of Bcl-2 found a correlation between loss of survival function and loss of ability to bind to Bax (Yin *et al.*, 1994). An attractive model arising from these observations was that Bcl-2 acted by sequestering death-enhancing homologues and that cell fate was determined by the relative proportions of Bcl-2-like and Bax-like proteins (Oltvai and Korsmeyer, 1994).

Fig. 8. Deletion of BH4 from Bcl-x_L abrogates ability to interact with CED-4 and antagonize its pro-apoptotic activity. (A) 293T cells were transiently co-transfected with plasmids encoding C-terminally HA-tagged CED-4 and a plasmid encoding either wt Bcl-x_L or Bcl-x_L ΔBH4. The upper panels show Western blots of lysates using anti-HA monoclonal antibody HA.11 (top) and anti-Bcl-x polyclonal antiserum (second panel). The lysates were also immunoprecipitated using the anti-HA antibody and the filters probed with anti-Bcl-x antibodies (third panel), or immunoprecipitated using anti-Bcl-x antibody and then probed with anti-HA antibody (fourth panel). The asterisk marks immunoglobulin heavy chain, revealed by the secondary reagent. The data shown are representative of three independent experiments. (B) 293 cells were transiently co-transfected with a CD4 reporter plasmid (see Materials and methods) and CED-4, CED-3, Bcl-x_L or Bcl-x_L ΔBH4 expression constructs. Cell viability was determined 24 h after transfection as described in Materials and methods. Data shown are arithmetic means $\pm$ SD of at least three independent experiments.

More recent findings argue against this model. Certain BH1 mutants of Bcl-x_L that cannot bind to Bax or Bak retain significant anti-apoptotic activity (Cheng *et al.*, 1996). Conversely, Bcl-2 without the BH4 domain lacks survival function (Borner *et al.*, 1994; Hanada *et al.*, 1995; Hunter *et al.*, 1996; Lee *et al.*, 1996; this paper) but can bind as avidly as wild-type Bcl-2 to Bax, Bak, Bad, Bik/Nbk, Bid and Bim (Hanada *et al.*, 1995; Figure 7 and data not shown). Thus, Bcl-2 survival function cannot simply reflect its ability to titrate its pro-apoptotic relatives. Contrary to one previous report (Hunter *et al.*, 1996), deletion of BH4 did not produce a dominant-negative version of Bcl-2 (Figure 4). Since binding to the pro-apoptotic molecules was retained, this result argues against a model in which, say, a Bcl-2:Bax heterodimer is an active mediator of cell survival.

In view of the strong evidence that BH4 is required for survival function in Bcl-2 and Bcl-x_L, it is puzzling that no readily recognisable BH4 sequence appears in two more distant mammalian Bcl-2 family members, A1 (Lin *et al.*, 1993) and Mcl-1 (Kozopas *et al.*, 1993), which also promote cell survival, albeit not as effectively as Bcl-2 (Lin *et al.*, 1996; Zhou *et al.*, 1997; our unpublished results). Similarly, most viral homologues, such as E1B19K (Rao *et al.*, 1992; White *et al.*, 1992), BHRF-1 (Henderson *et al.*, 1993) and KS-Bcl-2 (Cheng *et al.*, 1997) also apparently lack a BH4 region, although LMW5-HL has a weak region of homology (Neilan *et al.*, 1993). Further investigation is needed to determine whether these proteins do in fact harbour a region functionally equivalent to the BH4 motif. Their lack of an obvious BH4 region may mean that different sub-sets of the pro-survival proteins interact with distinct downstream effectors.

Why is BH4 critical to the pro-survival function of

Bcl-2 and its close homologues? It is not essential for overall protein stability, since Bcl-2 lacking the BH4 domain can be stably expressed in a number of cell types (Borner *et al.*, 1994; this paper), nor for homodimerization (Hanada *et al.*, 1995; Hunter *et al.*, 1996; Figure 7C). BH4 may be important for a particular conformation of Bcl-2, Bcl-x and Bcl-w. It may also facilitate their binding to another regulator of apoptosis. Our data excludes Bax, Bak, Bad, Bik/Nbk, Bid or Bim (Figure 7 and data not shown), but other potential candidates include the phosphatase calcineurin (Shibasaki *et al.*, 1997) and Raf-1 kinase (Wang, H.-G. *et al.*, 1994, 1996; Ali *et al.*, 1997), both of which reportedly bind to BH4, although binding to Raf-1 has been disputed (Olivier *et al.*, 1997).

An attractive candidate for the relevant BH4-binding regulator is a mammalian CED-4 homologue. The recent demonstration of direct association between CED-9 and CED-4 and of interactions between Bcl-x_L and CED-4 in mammalian cells supports such an idea (Chinnaiyan *et al.*, 1997; Irmeler *et al.*, 1997; James *et al.*, 1997; Spector *et al.*, 1997; Wu *et al.*, 1997b). We have confirmed the interaction between wild-type Bcl-x_L and CED-4 of *C.elegans* and shown that a Bcl-x_L mutant lacking the BH4 domain fails to bind (Figure 8A). Moreover, that mutant was unable to restrain the pro-apoptotic activity of CED-4 (Figure 8B), underlining the physiological significance of the Bcl-x_L/CED-4 association. Presumably the interaction of Bcl-x_L and CED-4 mirrors that of CED-9 with CED-4. Although one recent study (Spector *et al.*, 1997) reported that a CED-9 mutant lacking its N-terminal 97 amino acids could still bind CED-4, another study (Otilie *et al.*, 1997) found that a CED-9 mutant lacking residues 40 to 100 did not interact with CED-4. Hence it seems likely that the BH4 region of CED-9 (amino acids

79–99), like that of Bcl-x_L, is required for binding to CED-4. The BH4 region may be essential for the pro-survival molecules to assume a conformation allowing interaction with CED-4-like molecules.

In summary, our data, taken together with these other recent findings, favour the hypothesis that the BH4 domain allows the pro-survival molecules to sequester CED-4-like molecules, preventing them from activating the caspases. Our demonstration that Bcl-2 lacking BH4 still binds pro-apoptosis family members suggests that the binding sites for CED-4 and for the BH3-containing proteins such as Bax are structurally distinct. The recent identification of a mammalian CED-4 homologue, denoted Apaf-1 (Zou *et al.*, 1997), should facilitate further tests of this model. However, the multiple CED-9 and CED-3 homologues in mammals leads us to think that there will also be a number of CED-4 relatives. Whether a given Bcl-2 family member will pair with all mammalian CED-4 relatives or only particular ones remains to be determined.

Materials and methods

Expression constructs and site-directed mutagenesis

cDNAs were cloned into the expression vectors pEF PGKpuro and pEF PGKhygro (Huang *et al.*, 1997a), which were developed from pEF BOS (Mizushima and Nagata, 1990), or derivatives thereof incorporating the N-terminal epitope tags FLAG (DYKDDDDK) (Hopp *et al.*, 1988), EE (EYMPME) (Grussenmeyer *et al.*, 1985) or HA (YPYDVPDYA) (Wilson *et al.*, 1984). The *ced-4* and *ced-3* cDNAs were cloned into a derivative of the pcDNA3 (Invitrogen) mammalian expression vector that incorporates a C-terminal HA or B-tag (QYPALT) (Wang, L.-F. *et al.*, 1996) respectively.

Mutations of *bcl-2* were generated by polymerase chain reaction via splice overlap extension (Horton *et al.*, 1993) using the proof-reading *Pfu* DNA polymerase (Stratagene); details of oligonucleotides used will be supplied on request. In the cDNA encoding *bcl-2* ΔBH4, the sequence encoding amino acids 10–30 was replaced by that of a *Cla*I restriction site, which encodes two amino acids (ID) (Figure 5A). Sequence encoding amino acids 4–24 of *bcl-x_L* was similarly replaced by that of a *Cla*I restriction site to generate *bcl-x_L* ΔBH4. To swap BH4 domains, oligonucleotides corresponding to the BH4 region of Bcl-x (aa 4–24) or the putative BH4 regions of Bax (aa 22–42 or aa 15–33) were inserted into the *Cla*I site of the *bcl-2* ΔBH4 cDNA (Figure 5A). The sequences of all derived clones were verified by automated sequencing (ABI Perkin-Elmer) prior to function analysis.

Tissue culture and cell death analysis

All cell lines were cultured in the high glucose version of Dulbecco's modified Eagle's (DME) medium supplemented with 10% fetal calf serum (FCS), 50 μM 2-mercaptoethanol, 13 μM folic acid and 100 μM L-asparagine. Medium for FDC-P1 cells contained 1000 U/ml of IL-3 unless stated otherwise.

FDC-P1 cell lines were transfected by electroporation using a Gene-pulser (Bio-Rad) and transfectants selected by growth in puromycin (2–5 μg/ml) (Sigma) or hygromycin (2 mg/ml) (Boehringer-Mannheim). Cell lines were single cell-cloned using limiting dilution or the fluorescence activated cell deposition unit of the FACStarPlus (Becton Dickinson). Clones expressing high levels of the protein of interest were identified by immunofluorescence staining of permeabilized, fixed cells and flow cytometric analysis (Strasser *et al.*, 1995; Huang *et al.*, 1997a). To test their sensitivity to apoptosis, FDC-P1 lines were cultured in medium lacking IL-3; in IL-3-containing medium supplemented with 1 nM to 1 μM staurosporine (Sigma); or in IL-3-containing medium after exposure to 10 Gy γ-radiation (provided by a ⁶⁰Co source at a rate of 3 Gy/min). Cell viability was assessed by visual inspection in a haemocytometer by vital dye (0.4% eosin) exclusion or by flow cytometric analysis of cells which excluded propidium iodide (5 μg/ml; Sigma) using a FACScan (Becton Dickinson) (Nicoletti *et al.*, 1991).

Cell death assays were also performed by staurosporine treatment of transiently transfected 293 human embryonal kidney cells (ATCC CRL-1573) (Graham *et al.*, 1977). Cells (~5×10⁵) were transfected using 9 μl

Lipofectamine® (Gibco-BRL) and 1 μg of DNA:0.1 μg of plasmid DNA encoding a mutant human CD4 lacking the cytoplasmic domain (J.M.Adams, unpublished) and 0.9 μg of the construct(s) of interest. At 24 h after transfection, staurosporine was added at a concentration of 1–1000 nM and 6 h later the cells were harvested, stained for surface human CD4 expression using 1 μg/ml FITC-conjugated mouse anti-human CD4 monoclonal antibody (Pharmingen) and propidium iodide (5 μg/ml; Sigma) and analysed by flow cytometry. Viability of transfected cells was assessed by determining the proportion of CD4⁺ cells that had not taken up propidium iodide.

Immunofluorescence, immunoprecipitation and immunoblotting

Immunofluorescence staining of cytoplasmic proteins with the monoclonal antibodies Bcl-2-100 (mouse anti-human Bcl-2; Pezzella *et al.*, 1990), anti-FLAG M2 monoclonal antibody (IBI), anti-HA monoclonal antibody HA.11 (BabCO) or mouse anti-EE (BabCO) followed by FITC-conjugated goat anti-mouse IgG (Southern Biotechnology) was performed as previously described (Strasser *et al.*, 1995; Huang *et al.*, 1997a). Cells were analysed in the FACScan II (Becton Dickinson) after exclusion of dead cells on the basis of their forward and side scatter characteristics.

To test for protein–protein interactions *in vivo*, immunoblotting was performed on stably transfected FDC-P1 cells or transiently transfected L929 or 293T cells as described previously (Huang *et al.*, 1997a,b). Briefly, lysates prepared from 1–2×10⁶ cells were incubated with ~5 μg primary antibody followed by protein G- or protein A-Sepharose (Pharmacia) and then pelleted, washed, fractionated by SDS-PAGE and transferred to nitrocellulose membranes by electroblotting. The filters were then incubated with rabbit polyclonal anti-murine Bax (Huang *et al.*, 1997a), rabbit polyclonal anti-Bcl-x (Pharmingen) or mouse monoclonal antibodies anti-FLAG, anti-EE, anti-human Bcl-2 or anti-HA (~1 μg/ml). Blots with rabbit polyclonal anti-sera were incubated with ¹²⁵I-labelled staphylococcal protein A (~2×10⁶ c.p.m./ml) and the blots with mouse monoclonal antibodies were probed with by 5 μg/ml affinity-purified rabbit anti-mouse IgG (Fcγ-specific) (Jackson Immuno Research) followed by ¹²⁵I-labelled staphylococcal protein A (~2×10⁶ c.p.m./ml), as described elsewhere (Huang *et al.*, 1997a,b).

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