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-xL 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-xL) 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-xL 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-xL 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-xL, 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-xL 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), Bik (Wang K.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-xL 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; Irmler et al., 1997; Seshagiri and Miller, 1997; Spector et al., 1997; Wu et al., 1997b). Like CED-9, Bcl-xL 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).
CD4 cells was then assessed by determining the proportion of levels of staurosporine and the viability of transfected and methods). The cells were exposed for 6 h to graded cell surface but lacks signalling capability (see Materials and methods). The cells were exposed for 6 h to graded expressing a mutant CD4 protein which appears on the human embryonal kidney cells from apoptosis induced by the protein kinase inhibitor staurosporine. Transfectants were identified by co-transfection with a plasmid amino acids 10–30 and used a transient transfection assay to assess whether the mutant gene could still protect 293 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-xL (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.

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-xL (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-xL

Figure 1 depicts the BH4 region near the N-termini of the human Bcl-2-like proteins. The sequence similarity of Bcl-2, Bcl-xL 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 ΔBH4 (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 puromycin. Clones expressing high levels of Bcl-2 ΔBH4 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 blots (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 ΔBH4 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
Bcl-2 function requires N-terminal BH4 region

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 ΔBH4 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 ΔBH4 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 μM 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 ± 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-xL protein lacking BH4 (data not shown).

Bcl-2 ΔBH4 is not a dominant-negative inhibitor

A deletion very similar to that studied here (Δ6-31) has been reported to convert Bcl-2 into a dominant-negative molecule (Hunter et al., 1996). To test whether the non-functional Bcl-2 ABH4 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 ABH4. 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 ABH4 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 ΔBH4 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-xL, 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 BH4Bcl-xL (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-xL 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-xL (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
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 ΔBH4 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 ΔBH4 bound to Bax as avidly as wt Bcl-2 (Figure 7A), in contrast to the G145E mutation in BH1 and W188A...
Bcl-2 function requires N-terminal BH4 region

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 BH4Bcl-x) or Bax (aa 22 to 42) (Bcl-2 BH4Bax) (see Materials and methods). Identical or very similar residues are shaded in dark grey (see Figure 1); the ID residues (light grey) encoded a ClaI 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 BH4Bcl-x or Bcl-2 BH4Bax 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 BH4Bcl-x or Bcl-2 BH4Bax 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 I. Functional activity of BH4 mutants of Bcl-2

<table>
<thead>
<tr>
<th>Protein</th>
<th>Inhibition of apoptosis</th>
<th>Cytokine treatment</th>
<th>Treatment with staurosporine</th>
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<tr>
<td>Bcl-2</td>
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<td>Bcl-2 ΔBH4</td>
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<td>Bcl-2 Y21A</td>
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<td>Bcl-2 K22A/Q</td>
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<td>Bcl-2 Y28A/S/F</td>
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<td>Bcl-2 W30A</td>
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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 ± 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 ± SD of at least three experiments and are representative of the results obtained with at least three independent clones of each genotype.

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

In view of recent reports that Bcl-xL 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 CED4-HA and wild-type or ΔBH4 mutant forms of Bcl-2 or Bcl-xL. Anti-HA immunoprecipitates were then resolved electrophoretically and blotted with antibodies reactive with human Bcl-2 or Bcl-xL. The results showed that CED-4 can interact with wt Bcl-xL 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-xL 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-xL but not by the ΔBH4 mutant (Figure 8B). Collectively, these results suggest that the BH4 region of Bcl-xL 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-xL 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-xL indicates that the BH4 region encompasses an amphipathic α-helical loop on the surface that forms extensive hydrophobic interactions with α2, α5 and α6 (Muchmore et al., 1996). Although the N-terminal regions of Bax and Bak may also contain an amphipathic helix (Muchmore et al., 1996), sequence
Bcl-2 function requires N-terminal BH4 region

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).
proteins interact with distinct downstream effectors. The BH4 motif. Their lack of an obvious BH4 region may be important for a particular conformation 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 7C 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-xL and CED-4 in mammalian cells supports such an idea (Chinnaiyan et al., 1997; Irmler et al., 1997; James et al., 1997; Spector et al., 1997; Wu et al., 1997b). We have confirmed the interaction between wild-type Bcl-xL and CED-4 of C.elegans and shown that a Bcl-xL 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-xL/CED-4 association. Presumably the interaction of Bcl-xL 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 (Ottilie 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-xL, 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.

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 (DYKDDDK) (Hopp et al., 1988), EE (EYMPME) (Grussenneyer 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 which incorporates a C-terminal HA or B-Tag (QYPALT) (Wang 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 (Strategen); details of oligonucleotides used will be supplied on request. In the cDNA encoding amino acids 10–30 was replaced by that of a human CD4 monoclonal antibody (Pharmigen) 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.

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References
Cheng, E.-H.Y., Nicholas, J., Bellows, D.S., Hayward, G.S., Guo, H.-G., Reitz, M.S. and Hardwick, J.M. (1997) A Bcl-2 homolog encoded by Kaposi sarcoma-associated virus, human herpesvirus 8, inhibits 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.
apoptosis but does not heterodimerize with Bax or Bak. Proc. Natl Acad. Sci. USA, 94, 690–694.


Bcl-2 function requires N-terminal BH4 region


