The anti-apoptosis function of Bcl-2 can be genetically separated from its inhibitory effect on cell cycle entry

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The Bcl-2 family of proteins regulate apoptosis, some antagonizing cell death and others facilitating it. It has recently been demonstrated that Bcl-2 not only inhibits apoptosis but also restricts cell cycle entry. We show here that these two functions can be genetically dissociated. Mutation of a tyrosine residue within the conserved N-terminal BH4 region had no effect on the ability of Bcl-2 or its closest homologs to enhance cell survival and did not prevent heterodimerization with death-enhancing family members Bax, Bak, Bad and Bik. Neither did this mutation override the growth-inhibitory effect of p53. However, on stimulation with cytokine or serum, starved quiescent cells expressing the mutant proteins re-entered the cell cycle much faster than those expressing comparable levels of wild-type proteins. When wild-type and Y28 mutant Bcl-2 were co-expressed, the mutant was dominant. Although R-Ras p23 has been reported to bind to Bcl-2, no interaction was detectable in transfected cells and R-Ras p23 did not interfere with the ability of Bcl-2 to inhibit apoptosis or cell cycle entry. These observations provide evidence that the anti-apoptotic function of Bcl-2 is mechanistically distinct from its inhibitory influence on cell cycle entry.

Keywords: apoptosis/Bcl-2/Bcl-w/Bcl-x1/cell cycle control

Introduction

Physiological cell death (apoptosis) (Kerr et al., 1972) is controlled by an intrinsic genetic program remarkably conserved in evolution (Ellis et al., 1991) and is essential for the proper development of the embryo, for maintaining tissue homeostasis and for host defense mechanisms. The first intracellular regulator of apoptosis to be identified was bcl-2 (Vaux et al., 1988), the proto-oncogene commonly translocated to the Igh locus in human follicular lymphoma (Tsujimoto et al., 1984). The Bcl-2 protein, which is located on the cytosolic aspect of the nuclear envelope, the endoplasmic reticulum and the outer membrane of mitochondria, protects cells against diverse cytotoxic insults (reviewed by Reed, 1994; Cory, 1995; Yang and Korsmeyer, 1996). Two close homologs, Bcl-xL (Boise et al., 1993) and Bcl-w (Gibson et al., 1996), also promote cell survival, but more divergent members of the family, such as Bax (Oltvai et al., 1993) and Bak (Chittenden et al., 1995b; Farrow et al., 1995; Kiefer et al., 1995), antagonize this protective function.

Genetic studies in Caenorhabditis elegans have defined three central regulators of apoptosis (Hengartner and Horvitz, 1994). Ced-9 is the structural and functional homolog of Bcl-2, whereas Ced-3 and Ced-4 are essential for all developmentally programmed deaths. While the biochemical function of Ced-4 remains unknown, Ced-3 is the precursor form of a cysteine protease (Yuan et al., 1993; Xue et al., 1996). Mammalian cells express at least 10 such proteases, which cleave after aspartate residues and were recently designated caspases (Alnemri et al., 1996). Apoptosis appears to be initiated by a cascade of protease activation, followed by cleavage of a vital cellular substrate(s) (Kumar, 1995; Fraser and Evan, 1996). How Ced-9 and its mammalian counterparts inhibit apoptosis remains to be fully elucidated, but the recent demonstration that Ced-4 complexes with Ced-9 (or Bcl-xL) and Ced-3 (or certain pro-caspases) (Chinnaiyan et al., 1997; Spector et al., 1997; Wu et al., 1997) favors a model in which binding of Ced-9 (or Bcl-2) to Ced-4 prevents Ced-4 from activating Ced-3 (or the equivalent mammalian pro-caspases). In mammalian cells, additional regulation is afforded by Bax (or Bak) binding to Bcl-2 (or Bcl-xL) and neutralizing its survival function (Yin et al., 1994; Hanada et al., 1995), probably by preventing its association with mammalian Ced-4 homologs (Chinnaiyan et al., 1997).

Recent structural analysis has clarified how Bcl-2-related proteins heterodimerize. Sequence comparisons (Yin et al., 1994; Chittenden et al., 1995a; Gibson et al., 1996; Zha et al., 1996) had revealed four important motifs: BH1, BH2 and BH3, present in both the pro- and anti-survival subfamilies, and BH4 (also known as S1), present only in the former. BH3 is also found in several otherwise unrelated proteins, such as Bik (also known as Nbk) and Bid (Boyd et al., 1995; Han et al., 1996; Wang, K. et al., 1996), which interact with Bcl-2 and antagonize its survival function. BH1 and BH2 of Bcl-2 are critical for heterodimerization with Bax and for promoting cell survival (Yin et al., 1994). In contrast, the BH3 domain of Bax, Bak, Bik and Bid is essential for their interaction with Bcl-2 and acceleration of apoptosis (Chittenden et al., 1995a; Hunter and Parslow, 1996; Wang, K. et al., 1996). It is now apparent that the BH1, BH2 and BH3 domains of Bcl-xL form an elongated hydrophobic cleft which can bind BH3-containing peptides (Muchmore et al., 1996; Sattler et al., 1997). Similarity between the three dimensional structure of Bcl-xL and that of the pore-forming domains of certain bacterial toxins (Muchmore et al., 1996) has suggested that Bcl-2 proteins may form pores in cytoplasmic membranes and regulate ion flux across them (Muchmore et al., 1996; Minn et al., 1997).

An intriguing aspect of Bcl-2 receiving increased atten-
tion is its influence on the cell cycle. An early hint of a connection to cell cycle control was the quiescence of Bcl-2-expressing cells that survived cytokine withdrawal (Vaux et al., 1988). It was subsequently noted that growth factor-starved cells were refractory to IL-3 stimulation of proliferation (Marvel et al., 1994). More recently it has been shown by BrdU labeling that thymocyte turnover is slower in bcl-2 transgenic mice than in their littermate controls and that B and T cells expressing the transgene enter into the cell cycle more slowly than normal lymphocytes when stimulated with mitogens in vitro (Linette et al., 1996; Mazel et al., 1996; O’Reilly et al., 1996, 1997a,b). In addition, quiescent NIH 3T3 fibroblasts expressing Bcl-2, Bcl-xL or their adenovirus counterpart, the E1B 19K protein, responded more slowly to serum stimulation (O’Reilly et al., 1996). Bcl-2 has also been observed to hasten withdrawal from the cell cycle. Constitutive expression of Bcl-2 in HL60 cells had little effect on their proliferation or differentiation, but significantly accelerated their exit into G0 in response to agents inducing differentiation (Vairo et al., 1996). Thus, under certain conditions Bcl-2 favors quiescence over the cycling state.

To further understanding of Bcl-2 function, we have undertaken an extensive mutational analysis, focusing particularly on the conserved N-terminal BH4 region, which contains an amphipathic $\alpha$-helix (Muchmore et al., 1996; Lee et al., 1996). We report here that a conserved tyrosine residue in this domain is required for Bcl-2 to inhibit cell cycle entry but is dispensable for its anti-apoptotic activity. Mutation of the corresponding residue in Bcl-xL or Bcl-w had the same effect. This evidence raises the possibility that Bcl-2 and its close relatives exert at least two distinct functions in normal cellular physiology and in neoplastic transformation.

**Results**

**Conserved Tyr28 is not essential for Bcl-2 survival function**

The sequence of the N-terminal BH4 region is highly conserved in most Bcl-2 homologs that promote cell survival, but not in those that enhance apoptosis (Figure 1A), raising the possibility that BH4 plays an important role in promoting cell survival. Consistent with this hypothesis, Bcl-2 deletion mutants lacking BH4 are unable to block apoptosis (Borner et al., 1994; Hunter et al., 1996; Lee et al., 1996; D.C.S.Huang, J.M.Adams and S.Cory, in preparation). As part of a mutagenesis screen of the BH4 region of Bcl-2 (D.C.S.Huang, J.M.Adams and S.Cory, in preparation), we mutated the conserved Tyr28 to alanine, serine or phenylalanine. To test the functional consequence of these mutations, IL-3-dependent promyelomonocytic FDC-P1 cells and B6.2.16BW2 T hybridoma cells were transfected with a vector co-expressing either wild-type or mutant human bcl-2 cDNA and a puromycin resistance gene. Since the degree of protection from apoptosis afforded by Bcl-2 is proportional to its intracellular concentration (Huang et al., 1997), the puromycin-resistant clones were stained with anti-human Bcl-2 antibody and screened by flow cytometry to select those expressing similar levels of wild-type or mutant Bcl-2 (see for example Figure 1B). Immunoblotting confirmed that the drug-resistant clones synthesized Bcl-2 proteins of the expected size (see below).

Multiple clones of transfected FDC-P1 cells were compared for their ability to withstand three types of insult: IL-3 deprivation, $\gamma$-irradiation or exposure to staurosporine, a broad spectrum protein kinase inhibitor. Those expressing Y28 mutant Bcl-2 proteins were found to be as resistant to these cytotoxic conditions as those expressing wild-type Bcl-2 (Figure 2A–C). Likewise, B6.2.16BW2 T hybridoma cells expressing the wild-type or mutant Bcl-2 proteins were equally protected from dexamethasone or $\gamma$-irradiation (not shown). We conclude that, while the BH4 region is essential for inhibiting apoptosis (Borner et al., 1994; Hunter et al., 1996; D.C.S.Huang, J.M.Adams and S.Cory, in preparation), its conserved Y28 residue is not critical for this function, in agreement with recent observations (Lee et al., 1996).
Y28 mutant Bcl-2 proteins are as effective as wild-type Bcl-2 in inhibiting apoptosis of FDC-P1 cells. Parental FDC-P1 cells, clones transfected with an empty vector and clones expressing wild-type or Y28 mutant Bcl-2 were cultured in the absence of IL-3 (A), exposed to γ-irradiation (10 Gy) (B) or incubated in medium containing graded doses of staurosporine (1 nM–1 μM) (C). Cell viability was assessed by vital dye exclusion over a 15 A) or 8 day (B) period or 48 h after adding the cytotoxic agent (C). 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.

Y28 mutant Bcl-2 does not affect the growth rate of FDC-P1 cells but accelerates entry of quiescent cells into the cell cycle. (A) Neither wild-type nor Y28 mutant Bcl-2 affects the rate of proliferation of FDC-P1 cells in IL-3. Parental FDC-P1 cells and clones bearing a control vector, wild-type Bcl-2 or Y28 mutant Bcl-2 were seeded into medium (10^4 cells/ml) containing IL-3 and cell counts were performed daily using a hemocytometer. The data shown are arithmetic means ± SD of triplicate samples of a representative line of each genotype; data from at least four other independent clones showed similar kinetics. (B) FDC-P1 cells expressing wild-type or Y28 mutant Bcl-2 survive equally well when deprived of IL-3. Cells were washed three times and plated in medium containing 10% FCS but lacking IL-3. The proportion of cells in the S, G2 or M phases versus G0/G1 was determined daily by flow cytometry (see Materials and methods). (C) Cells expressing Y28 mutant Bcl-2 re-enter the cell cycle more rapidly than those expressing wild-type Bcl-2. After 10 days of cytokine deprivation, IL-3 was added back to the cultures shown in (B) and samples were collected daily for cell cycle analysis by flow cytometry. The data shown in (B) and (C) are representative of multiple experiments (>3) performed with at least four independently derived clones transfected with each construct.

**Mutation of Y28 interferes with the ability of Bcl-2 to restrain entry into the cell cycle**

We next addressed the effect of the Y28 mutation on the ability of Bcl-2 to influence the cell cycle. We and others have previously shown that Bcl-2 and its functional homologs do not affect the growth rate of continuously cycling cells (Fanidi et al., 1992; O’Reilly et al., 1996), although others have reported contrary findings (Borner, 1996; Mazel et al., 1996). Multiple independent FDC-P1 clones were analyzed to determine whether the Y28 mutation had any effect on cell growth. No significant difference could be discerned in either the proliferation rate or growth plateau of cells expressing Y28 mutant Bcl-2 compared with parental cells or those expressing wild-type Bcl-2 (Figure 3A and data not shown).

The cell lines were then analyzed in more detail for their response to cytokine deprivation. In the absence of IL-3, FDC-P1 cells expressing high levels of Bcl-2 cease proliferating and enter a quiescent state in which they can remain viable for a prolonged period (Vaux et al., 1988). Flow-cytometric analysis of cellular DNA content showed that the rate of exit from the cell cycle was comparable, irrespective of whether the cells expressed wild-type or Y28 mutant Bcl-2 (Figure 3B). A very significant difference became apparent, however, when we re-introduced IL-3 into the cultures and compared their rates of re-entry into the cell cycle (Figure 3C). The cells expressing wild-type Bcl-2 only entered the cycle after 5 days, but those expressing Y28 mutant Bcl-2 required only 2–3 days (Figure 3C). This difference was readily apparent for
During exponential growth in the presence of IL-3, no differences could be discerned in the relative proportions of cells in different phases of the cell cycle for the parental line, transfectants expressing only the puromycin resistance gene and lines expressing either wild-type or Y28F Bcl-2 (see for example Figure 4, top panels). When deprived of IL-3, only cells expressing wild-type or Y28 mutant Bcl-2 survived (Figure 2A) and, within 7 days, all of these had ceased cycling (Figure 3B) and entered G0 (see for example Figure 4, second panels). After re-addition of IL-3, cycling eventually resumed, but cells expressing wild-type Bcl-2 took on average 2–3 days longer to enter G1 and thence S phase than those expressing Y28F Bcl-2 (compare Figure 4, third and fifth panels).

To extend these observations to a different cell type, we generated NIH 3T3 fibroblast lines expressing wild-type Bcl-2 or Y28 mutants. No difference could be detected in their proliferation rate in medium containing 10% serum (not shown). In medium lacking serum, the mutant proteins were just as effective at blocking apoptosis as wild-type Bcl-2 (Figure 5A). The cells ceased proliferating with the same kinetics (Figure 5B) and both arrested primarily in G0 (>90%; not shown). When serum was added back after 7 days, cells expressing Y28 mutant Bcl-2 started cycling significantly sooner than those expressing wild-type Bcl-2 (Figure 5C). Three-color fluorescent staining for DNA, protein and RNA, performed as for FDC-P1 cells (Figure 4), suggests that this acceleration is from G0 to G1 (not shown). Taken together, these results show that Bcl-2 impedes entry into the cell cycle and that Y28 is required for this function.

Y28 mutant Bcl-2 has a dominant effect over wild-type Bcl-2

It was important to determine whether the Y28 mutant was dominant or recessive to wild-type Bcl-2 in its influence on cell cycle entry. To allow independent monitoring of mutant and wild-type Bcl-2 within the same cells, we utilized vectors expressing wild-type or mutant human Bcl-2 and FLAG-tagged wild-type mouse Bcl-2. FDC-P1 cells were transfected with each vector alone or in pairwise combinations and clones checked for appropriate expression of human or mouse Bcl-2 (Figure 6A). We first ascertained that FDC-P1 clones expressing FLAG-tagged wild-type mouse Bcl-2 were resistant to apoptosis when deprived of IL-3 (not shown) and exhibited the same delayed cell cycle re-entry as those expressing the cell cycle. Cells expressing wild-type Bcl-2 (left panels) or Y28F mutant Bcl-2 (right panels) were cultured for 15 days in medium containing 10% FCS but no IL-3, after which the cytokine was added back and samples were taken daily for cell cycle analysis. Cellular DNA content was determined by staining with Hoechst 33342 and protein content by staining with fluorescein isothiocyanate. The results are representative of those obtained from at least four clones analyzed similarly on at least three occasions.

multiple independent FDC-P1 clones expressing Y28A, Y28S or Y28F Bcl-2 (at least four of each) and also in polyclonal lines expressing these mutants.

In order to achieve better resolution of the different phases of the cycle, the kinetic analysis was repeated using three-color fluorescent staining for DNA, protein and RNA content. Figure 4 displays the results for DNA versus protein content; equivalent results were apparent when DNA was plotted versus RNA content (not shown).

During exponential growth in the presence of IL-3, no differences could be discerned in the relative proportions of cells in different phases of the cell cycle for the parental line, transfectants expressing only the puromycin resistance gene and lines expressing either wild-type or Y28F Bcl-2 (see for example Figure 4, top panels). When deprived of IL-3, only cells expressing wild-type or Y28 mutant Bcl-2 survived (Figure 2A) and, within 7 days, all of these had ceased cycling (Figure 3B) and entered G0 (see for example Figure 4, second panels). After re-addition of IL-3, cycling eventually resumed, but cells expressing wild-type Bcl-2 took on average 2–3 days longer to enter G1 and thence S phase than those expressing Y28F Bcl-2 (compare Figure 4, third and fifth panels).
homologs with phenylalanine. When deprived of IL-3, FDC-P1 clones expressing Y22F Bcl-xL or Y27F Bcl-w were as resistant to apoptosis as those expressing the corresponding wild-type protein (Table I). However, when IL-3 was added back to the cultures, cells expressing the mutant proteins resumed cycling much faster than those expressing comparable levels of the wild-type protein (Table I). The fact that a conserved tyrosine residue in BH4 displays comparable function for the three pro-survival proteins argues that its inhibitory effect on the cell cycle is of physiological significance.

**Y28 Bcl-2 blocks apoptosis of p53-expressing cells but does not permit their proliferation**

We and others have previously shown that Bcl-2 blocks p53-induced apoptosis and that the surviving cells undergo growth arrest (Selvakumaran et al., 1994; Strasser et al., 1994; Sabbatini et al., 1995; Wang Y. et al., 1996). Recently it has been reported that baby rat kidney (BRK) cells expressing a mutant human Bcl-2 protein lacking residues 51–85 of the non-conserved loop between BH4 and BH3 are not only resistant to p53-induced apoptosis but, unexpectedly, continue to proliferate (Uhlmann et al., 1996). To assess whether Y28 mutant Bcl-2 might have a similar effect, we transfected BRK cells bearing a temperature-sensitive mutant of p53 (Debbas and White, 1993) with wild-type or Y28F Bcl-2 expression vectors and analyzed the proliferation and survival of cells at the permissive (32°C) and restrictive (38°C) temperatures. When p53 was in the mutant conformation (38°C), the parental cells and Bcl-2-expressing transfectants proliferated at the same rate and cell viability was high (Figure 7C and D). At 32°C, when p53 assumes its normal conformation, the parental cells died rapidly (Figure 7A). Bcl-2 Y28F inhibited p53-induced apoptosis as efficiently as wild-type Bcl-2 but, in contrast to the Bcl-2Δ51–85 mutant (Uhlmann et al., 1996), the Y28F mutant did not override the growth-inhibitory effect of p53 (Figure 7B). Thus, Y28 does not mediate the same function as the region encompassed by amino acids 51–85, which forms part of the unstructured loop in Bcl-xL (Muchmore et al., 1996).

**Tyr28 mutation does not prevent Bcl-2 association with known protein partners**

In an attempt to gain some insight into how Bcl-2 might influence cell cycle re-entry, we compared the ability of wild-type and Y28 mutant Bcl-2 to associate with other members of the wider Bcl-2 family. Wild-type or mutant Bcl-2 was co-expressed with FLAG-tagged Bax or GluGlu-tagged Bak, Bad (Yang et al., 1995) and Bik protein in L929 mouse fibroblast cells. Immune complexes were then isolated from cell lysates using anti-human Bcl-2 antibody. Immunoblotting of immunoprecipitated material with anti-FLAG and anti-GluGlu antibodies demonstrated that the Y28 mutant was indistinguishable from wild-type Bcl-2 in its ability to associate with Bax, Bak, Bad or Bik (Figure 8). Mutation of Y28 was also shown not to affect the ability of Bcl-2 to homodimerize (not shown). Thus, the inability of Y28 mutant Bcl-2 proteins to inhibit cell cycle re-entry is not due to an inability to homodimerize or to interact with several family members known to promote apoptosis.

**R-Ras p23 does not mediate the effects of Bcl-2 on cell survival or the cell cycle**

R-Ras p23 is reported to bind to Bcl-2 (Fernandez-Sarabia and Bischoff, 1993) and BHFR-1, the Epstein–Barr homolog of Bcl-2 (Theodorakis et al., 1996). Furthermore, a BHFR-1 mutant permissive for proliferation of cells expressing wild-type p53 had reduced capacity to bind to R-Ras p23 (Theodorakis et al., 1996). These observations led us to assess the capacity of wild-type and Y28 mutant Bcl-2 to interact with wild-type and
Bcl-2 effects on cell cycle and apoptosis

Table I. Functional activity of BH4 mutants of Bcl-2, Bcl-xL and Bcl-w

<table>
<thead>
<tr>
<th>Protein</th>
<th>Inhibition of apoptosis</th>
<th>Inhibition of entry into cell cycle</th>
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<tbody>
<tr>
<td>Bcl-2</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Bcl-2 ΔBH4</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Bcl-2 Y28A, S or F</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Bcl-xL</td>
<td>+</td>
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<td>Bcl-xL Y22F</td>
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<td>Bcl-w</td>
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<td>Bcl-w Y22F</td>
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Cell survival and cell cycle analysis were performed as described in Materials and methods on parental FDC-P1 cells and multiple (at least four) independent cell lines stably expressing each of the indicated proteins: wild-type Bcl-2; a Bcl-2 mutant lacking amino acids 10–30 (Bcl-2ΔBH4); three Y28 mutants of Bcl-2; wild-type Bcl-xL; Y22F mutant Bcl-xL; wild-type Bcl-w; and Y27F mutant Bcl-w. Comparable results were obtained in NIH 3T3 fibroblasts (see text).

Discussion

Genetic separation of two functions of Bcl-2

The ability of Bcl-2 to promote cell survival is well established (Vaux et al., 1988; Cory, 1995; Korsmeyer, 1995; White, 1996), but only recently has its influence on the cell cycle begun to attract scrutiny. In vitro Bcl-2-overexpressing cells that resist apoptosis when deprived of an obligate growth factor cease cycling (Vaux et al., 1988; Nunez et al., 1990) and are surprisingly slow to start proliferating again upon stimulation with growth factor (Marvel et al., 1994; O'Reilly et al., 1996). In vivo the B cells that accumulate in greatly increased numbers in Eμ-bcl-2 transgenic mice are smaller than normal resting B cells (Strasser et al., 1990), and both B and T lymphoid cells expressing a bcl-2 transgene respond more slowly to activation in vitro by mitogens and/or cytokines (Linette et al., 1996; Mazel et al., 1996; O'Reilly et al., 1996). Bcl-xL, Bcl-w and the adenovirus E1B 19K protein repress the re-activation of quiescent cells just as effectively as Bcl-2 (O’Reilly et al., 1996; this paper), so the growth-inhibitory function of Bcl-2 may prove to be a general feature of the pro-survival subfamily of Bcl-2-related proteins.

The experiments described here demonstrate that the
Fig. 7. Wild-type and Y28 mutant Bcl-2 can inhibit p53-induced cell death. BRK cells expressing temperature-sensitive p53 (Debbas and White, 1993) were transfected with vectors encoding either wild-type or Y28F mutant Bcl-2; drug-resistant clones selected for comparable levels of Bcl-2 were cultured at the permissive (32°C) (A and B) or restrictive temperature (38°C) (C and D) and cell viability (A and C) and cell number (B and D) determined at regular intervals by phase contrast microscopy. The data shown are from a single experiment; a second experiment gave comparable results.

Fig. 8. Wild-type and Y28 mutant Bcl-2 can interact with Bax, Bak, Bad and Bik. Lysates containing similar levels of wild-type or Y28F mutant Bcl-2 as shown by immunoblotting (top) were immunoprecipitated with anti-human Bcl-2 monoclonal antibody. The immune complexes were resolved by SDS–PAGE and blotted using mouse anti-FLAG and anti-GluGlu antibodies followed by a rabbit anti-mouse IgG antibody and then probed with 125I-conjugated staphylococcal protein A (bottom).

Inhibitory action of Bcl-2 on cell cycle entry can be genetically separated from its ability to promote cell survival. Mutation of a conserved tyrosine residue (Y28) at the C-terminal end of the BH4 homology region to alanine, serine or phenylalanine did not affect the anti-apoptotic activity of Bcl-2 but markedly reduced its ability to restrain re-entry of quiescent FDC-P1 myeloid cells or NIH 3T3 fibroblasts into the cell cycle upon exposure to...
growth factors (Figures 3 and 5). Similar observations were made for analogous mutants of Bcl-xL (Y22F) and Bcl-w (Y27F).

The effect of Bcl-2 on the cell cycle appears to be tightly linked to its anti-apoptotic activity, since three inactivating mutations of Bcl-2 (G145E, W188A and deletion of BH4) abrogate both activities and Bax, an antagonist of the anti-apoptotic activity of Bcl-2, can also counteract the cell cycle effect imposed by Bcl-2 (Borner, 1996; Brady et al., 1996; O’Reilly et al., 1996). Thus the cell cycle control function of Bcl-2 appears to be distinct from, but dependent on, its anti-apoptotic function.

It has been suggested that Bcl-2 inhibits cell cycle progression by lengthening the G1 phase (Borner, 1996; Mazel et al., 1996). However, we and others (Fanidi et al., 1992) have consistently found that overexpression of Bcl-2 and its functional homologs does not affect the growth rate of cell lines cultured under optimal conditions but only the rate at which cells transit from the quiescent G0 state into the first S phase (O’Reilly et al., 1996; Figure 3).

How might the C-terminal conserved tyrosine in the BH4 homology region of Bcl-2 and its homologs modulate cell cycle activity? Post-translational modification of this residue may be essential for Bcl-2 to interact with a protein that regulates the G0/G1 transition and inability to form this complex may permit quiescent cells to enter the cell cycle more rapidly. Since phenylalanine could not replace tyrosine at this position, an interesting possibility is phosphorylation at this site. To date, only serine phosphorylation of Bcl-2 has been reported (Haldar et al., 1995; Chen and Faller, 1996), but tyrosine phosphorylation of Bcl-2 may occur when cells are quiescent or transiting from G0 to G1. We are currently investigating post-translational modification of Bcl-2 at different stages of the cell cycle.

Several proteins that bind to Bcl-2 and are therefore potential modulators of its cell cycle function have been screened for ability to interact with the Y28 mutants. Bax, Bak, Bad and Bik, all of which interfere with the anti-apoptosis function of Bcl-2, bound in vivo to both the mutant and the wild-type protein (Figure 8). By this criterion, none of these appears to be the putative protein partner that regulates cell cycle entry. R-Ras p23 seemed to be a particularly attractive candidate, since it has been reported to bind Bcl-2 in vitro (Fernandez-Sarabia and Bischoff, 1993) and to promote cellular transformation when overexpressed (Cox et al., 1994; Saez et al., 1994). Furthermore, R-Ras p23 has been shown to bind in vivo to BHRF1, the Epstein–Barr virus homolog of Bcl-2, and mutant BHRF1 proteins defective in restraining cell cycling (see below) displayed reduced binding (Theodorakis et al., 1996). However, we were unable to detect any physical interaction between Bcl-2 and R-Ras p23 in FDC-P1 cells. Furthermore, activating or dominant interfering mutations of R-Ras p23 did not interfere with the ability of Bcl-2 to inhibit either apoptosis or cell cycle entry (Figure 9). Other protein partners that remain to be tested include Bid (Wang, K. et al., 1996), Raf-1 kinase (Wang, H.G. et al., 1994), Nip-1, Nip-2, Nip-3 (Boyd et al., 1994), Bag-1 (Takayama et al., 1995) and 53BP2 (Naumovski and Cleary, 1996).

The phenotype of the Y28 mutant is distinct from that of other recently reported mutants of Bcl-2 and BHRF1 which exhibit reduced proliferation restraining function (Theodorakis et al., 1996; Uhlmann et al., 1996). Expression of Bcl-2 or BHRF1 in BRK cells transformed with a temperature-sensitive mutant of p53 and adenovirus E1A (Debbas and White, 1993) inhibits apoptosis normally provoked by incubation at the temperature permissive for wild-type p53 (Chiou et al., 1994; Theodorakis et al., 1996). Bcl-2 lacking much of the non-conserved loop (amino acids 51–85) located between BH4 and BH3 was an effective inhibitor of p53-induced apoptosis of the transformed BRK cells, but rather than arresting their growth, permitted their continued proliferating (Chennadurai and colleagues (Theodorakis et al., 1996; Uhlmann et al., 1996), implicating the region encompassing BH4–BH3 in the proliferation restraining function of Bcl-2 and its homologs.

Fig. 9. Expression of constitutively active or dominant negative R-Ras p23 does not affect the ability of Bcl-2 to inhibit apoptosis (A) or delay cell cycle entry (B). FDC-P1 clones expressing vector control, wild-type Bcl-2, R-Ras p23 mutants or Bcl-2 plus R-Ras p23 were cultured in the absence of IL-3 for 5 days and cell viability was assessed at daily intervals by visual inspection and vital dye exclusion. After 10 days, IL-3 was added to the cultures (B) and cell cycle analysis was performed at daily intervals. The data shown are representative of triplicate experiments; equivalent results were obtained with three other lines of each genotype.
Implications for the role of Bcl-2 in normal physiology and in neoplastic transformation

The inhibitory effect of Bcl-2 on entry into the cell cycle may contribute to the indolent nature of lymphomas associated with Bcl-2 overexpression (Cleary and Rosenberg, 1990) and the better prognosis of patients whose breast cancer tissue shows abnormally high levels of Bcl-2 expression (Lipponen et al., 1995). It may also account for the relatively low incidence of lymphomas in mice expressing a bcl-2 transgene in B and T lymphocytes (McDonnell et al., Korsmeyer, 1991; Strasser et al., 1993; Linette et al., 1995), since lymphocytes from these animals showed a slower turnover compared with control animals (Linette et al., 1996; Mazel et al., 1996; O’Reilly et al., 1996, 1997a,b).

The demonstration that the Y28 mutant is dominant over wild-type Bcl-2 in cell lines (Figure 6) encourages the prediction that constitutive expression of Y28F Bcl-2 in vivo would overcome the restraint on cycling normally exercised by Bcl-2, while still inhibiting apoptosis. If so, Y28 mutant bcl-2 might be a more aggressive oncogene than bcl-2, and we are currently testing this possibility. Somatic mutations of the translocated bcl-2 gene have been described (Tanaka et al., 1992; Matolesy et al., 1996) and, intriguingly, in three out four cases of morphologically transformed follicular lymphoma, the mutations involve amino acids (29, 46 and 59) within or very close to those regions of Bcl-2 that we (Y28) and others (amino acids 51–85; Uhlmann et al., 1996) have implicated in cell cycle control. Furthermore, BHRF1 mutant 50-1 was consistently more effective as wild-type BHRF1 in collaborating with adenovirus E1A to transform primary BRK cells (Theodorakis et al., 1996). Thus somatic mutation of survival promoting genes may contribute to tumor progression.

The realization that Bcl-2 can retard entry into the cell cycle also has implications for anti-cancer therapy. Since most chemotherapeutic drugs target dividing cells, malignant cells expressing Bcl-2 are ‘doubly’ protected: they are refractory to apoptosis and more likely to be quiescent. Therefore, any agents that could overcome the inhibitory effects of Bcl-2 on cell cycle entry should prove useful adjuncts to currently available chemotherapeutic drugs.

Materials and methods

Expression constructs and site-directed mutagenesis
cDNAs were cloned into the expression vectors pEF PGKpurO (Huang et al., 1997) or pEF PGKhygro (D.C.S.Huang, unpublished), both of which were developed from pEF BOS (Mizushima and Nagata, 1990) or derivatives thereof incorporating N-terminal FLAG (DYKDDDK) (Hopp et al., 1988) or GluGlu (EYPMEE) (Grussmeyer et al., 1985) tags. The cDNAs for bcl-2, bcl-xL, bcl-w, bax, bak and R-ras p23 were gifts from M.Cleary, C.Thompson, L.Gibson, S.Korsmeyer, G.Evan and A.Hall respectively. The other cDNAs were generated by PCR from cDNA libraries and their sequence verified by automated sequencing. Mutations of bcl-2, bcl-xL, bcl-w and R-ras p23 were generated by PCR 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. The sequences of derived clones were all verified by automated sequencing (ABI Perkin Elmer) prior to function analysis.

Tissue culture and cell death analysis

Cell lines used were FDC-P1, a mouse IL-3-dependent promyelomonocytic cell line; B6.216BW2, a mouse T cell hybridoma; mouse fibroblastoid lines L929 [subline LMs-TK] and NIH 3T3 (for references see Lithgow et al., 1994; Strasser et al., 1994, 1995); p53A (BRK-p53val135-E1A), a baby rat kidney cell line that expresses a temperature-sensitive mutant of p53 (p53val135) (Debass and White, 1993; a kind gift of E.White). All cell lines were cultured in the high glucose version of Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS), 50 μM 2-mercaptoethanol, 13 mM folic acid and 100 μg/ml L-asparaginase. Media for FDC-P1 cells contained 1000 U/ml IL-3, unless stated otherwise.

Cell lines were transfected by electroporation using a Gene-pulsor (BioRad) and selected by growth in puromycin (2–5 μg/ml) (Sigma), bygromycin (1–2 mg/ml) (Boehringer Mannheim) or both, depending on the construct(s) transfected. Drug-resistant cell lines were cloned using the cell deposition unit of a FACStarPlus (Becton Dickinson) or by limiting dilution culture. Clones expressing high levels of the protein of interest were identified by immunofluorescence staining of fixed and permeabilized cells followed by flow-cytometric analysis (see below).

Cell death was induced by culturing the cells in the absence of cytokine (no IL-3 for FDC-P1 cells or no serum for NIH 3T3 fibroblasts) or by treatment with 10 Gy γ-irradiation (provided by a 60Co source at a rate of 3 Gy/min), 1 nM–1 μM staurosporine (Sigma) or 1 μM dexamethasone (Sigma). Cell viability was assessed by visual inspection in a hemocytometer by vital dye (0.4% eosin) exclusion or by flow-cytometric analysis on a FACSscan (Becton Dickinson) of cells which excluded propidium iodide (5 μg/ml; Sigma) (Nicoletti et al., 1991).

Immunofluorescence, immunoprecipitation and immunoblotting

Immunofluorescence staining of cytoplasmic proteins was performed as previously described (Strasser et al., 1995). Briefly, ~106 cells were fixed for 5 min in 80% methanol and permeabilized with 0.3% saponin (Sigma), which was included in all the subsequent steps. The cells were stained with ~1 μg/ml of the monoclonal antibodies Bcl-2-100 (mouse anti-human Bcl-2; Pezella et al., 1990), mouse anti-FLAG M2 (IBI) or mouse anti-GluGlu (BabCO) and then with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1 μg/ml; Southern Biotechnol.) as the secondary reagent. Cells were analyzed in a FACSScan (Becton Dickinson) after exclusion of dead cells on the basis of their forward and side scatter characteristics.

To test for protein–protein interactions among Bcl-2 family members, mouse L929 fibroblasts (7.5×105) were co-transfected by lipofection using Lipofectamine® (Gibco BRL) with vectors expressing wild-type bcl-2 or bcl-2 Y28F mutant and vectors expressing FLAG-tagged Bax or GluGlu-tagged Bak, Bad and Bik. After ~48 h the cells were harvested into 500 μl lysis buffer (20 mM Tris–HCl, pH 8, 135 mM NaCl, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 0.5 μg/ml Pefabloc, 1 μg/ml each leupeptin, aprotinin, soybean trypsin inhibitor and pepstatin, 5 mM NaF and 2 mM Na3VO4; reagents from Sigma or Boehringer Mannheim). Equivalent amounts of the lysates (~200 μg determined by bicinchoninic acid precipitation; Pierce) were precleared twice by rotating for ~1 h at 4°C with protein G-Sepharose beads (Phar macia) and then immunoprecipitated with 5 μg anti-human Bcl-2 monoclonal antibody. Immunoprecipitates were then collected by rotating with protein G-Sepharose for ~1 h at 4°C, washed five times with lysis buffer and once with phosphate-buffered saline, boiled in gel-running buffer (0.25 M Tris–HCl, pH 6.8, 1% SDS, 20% glycerol, 0.02% bromophenol blue), resolved on a 4–20% polyacrylamide gel (Novex) and transferred to nitrocellulose membranes by electroblotting. After incubation overnight at 4°C in 5% skimmed milk, 1% casein and 0.05% Tween-20 to prevent non-specific binding, the filter was incubated (~1 h at room temperature) with mouse anti-FLAG and anti-GluGlu antibodies (~1 μg/ml) followed by 5 μg/ml affinity-purified rabbit anti-mouse IgG (Fc specific; Jackson Immuno Research). Membranes were then probed (1 h at room temperature) with 125I-labeled staphylococcal protein A (~2×106 c.p.m./ml). Experiments to test for interactions between Bcl-2 and R-Ras p23 were similarly performed on lysates derived from FDC-P1 cells that stably co-expressed Bcl-2 and the different forms of R-Ras p23.

Cell cycle analysis

Prior to cell cycle analysis, FDC-P1 and NIH 3T3 cells were maintained in exponential growth by supplementing the cultures daily with excess culture medium. All cultures chosen for analysis were >95% viable immediately prior to withdrawal of IL-3 or serum. FDC-P1 cells (6×106) were washed twice in DMEM containing 10% FCS but no IL-3 and resuspended at a density of 2×105 cells/ml. The NIH 3T3 clones were cultured at low density (<30% confluence) in medium lacking FCS. Following 10–15 days growth factor deprivation, Bcl-2-expressing FDC-
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PI or NIH 3T3 clones were harvested, counted and re-seeded at 2 × 10^5 cells/ml in medium containing 10% FCS plus 1000 U/ml IL-3 (FDC-P1 cells) or 10% FCS (NIH 3T3 fibroblasts). Samples were then taken at appropriate time points for counting and cell cycle analysis by staining ethanol-fixed cells with propidium iodide (69 μM) in 38 mM sodium citrate, pH 7.4, containing 5 μg/ml RNase A (Boehringer Mannheim). The samples were analyzed by flow cytometry using a modified dual laser FACS II (Becton Dickinson) after staining the cells with 0.5 g/ml bisbenzimide H 33342 fluorochrome (Calbiochem), 1 μg/ml Pyronin Y (Polysciences) and 0.1 μg/ml fluorescein isothiocyanate (Molecular Probes) for 30 min at room temperature. Twenty-five thousand events were collected per sample and the data were analyzed using CellFit software (Becton Dickinson) or an in-house program (F.Battey, unpublished).

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