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 restrains 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.
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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-x$_L$ (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-x$_L$) 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-x$_L$) 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-x$_L$ 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-x$_L$ 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-x_L 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 G_0 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 α-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-x_L 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 con-
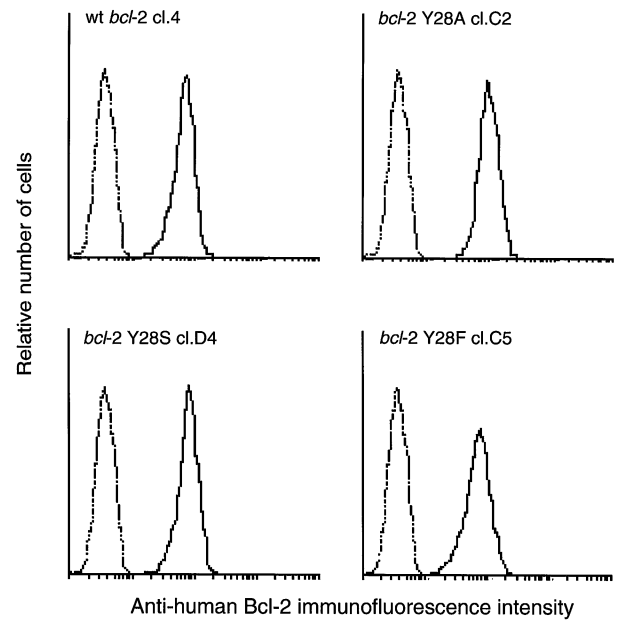
A

B

Fig. 1. Generation and expression of Bcl-2 Y28 mutants. (**A**) Comparison of amino acid sequence of the BH4 region of Bcl-2, Bcl-x_L and Bcl-w with the N-terminal sequence of Bax and Bak 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 in black. (**B**) Expression of wild-type (wt) or Y28A, Y28S or Y28F mutant Bcl-2 protein in FDC-P1 cells. Stably transfected clones (see Materials and methods) were fixed, permeabilized and stained with an anti-human Bcl-2 monoclonal antibody and analyzed by flow cytometry. Clones used in subsequent experiments expressed wild-type or Y28 mutant Bcl-2 at similar levels, as in the examples shown. Staining of the control (parental) cells is indicated by the dotted lines.

firmed 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, γ-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 γ-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).
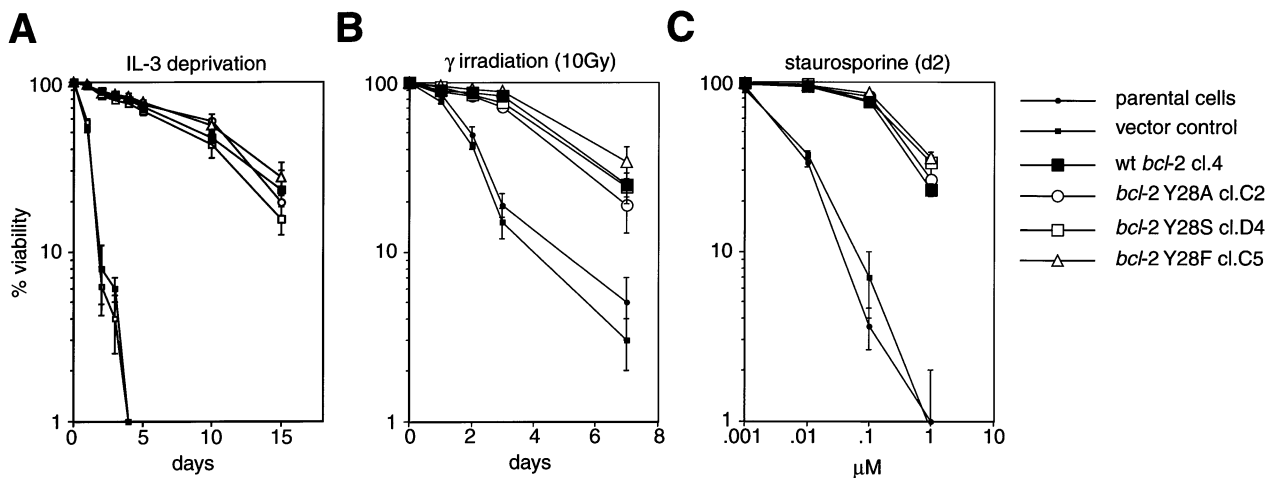
Fig. 2. Y28 mutant Bcl-2 proteins are as effective as wild-type Bcl-2 in inhibiting apoptosis of FDC-P1 cells. Parental FDC-P1 cells, clones transfected with an empty vector and clones expressing wild-type or Y28 mutant Bcl-2 were cultured in the absence of IL-3 (**A**), exposed to γ-irradiation (10 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.
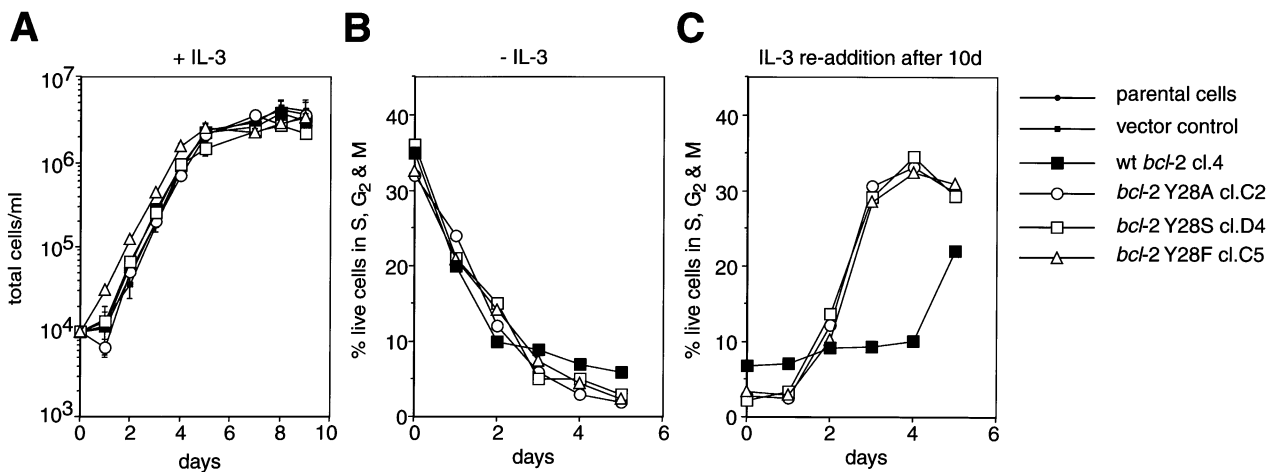

Fig. 3. Y28 mutant Bcl-2 does not affect the growth rate of FDC-P1 cells but accelerates entry of quiescent cells into the cell cycle. (**A**) Neither wild-type nor Y28 mutant Bcl-2 affects the rate of proliferation of FDC-P1 cells in IL-3. Parental FDC-P1 cells and clones bearing a control vector, wild-type 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, G_2 or M phases versus G_0/G_1 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
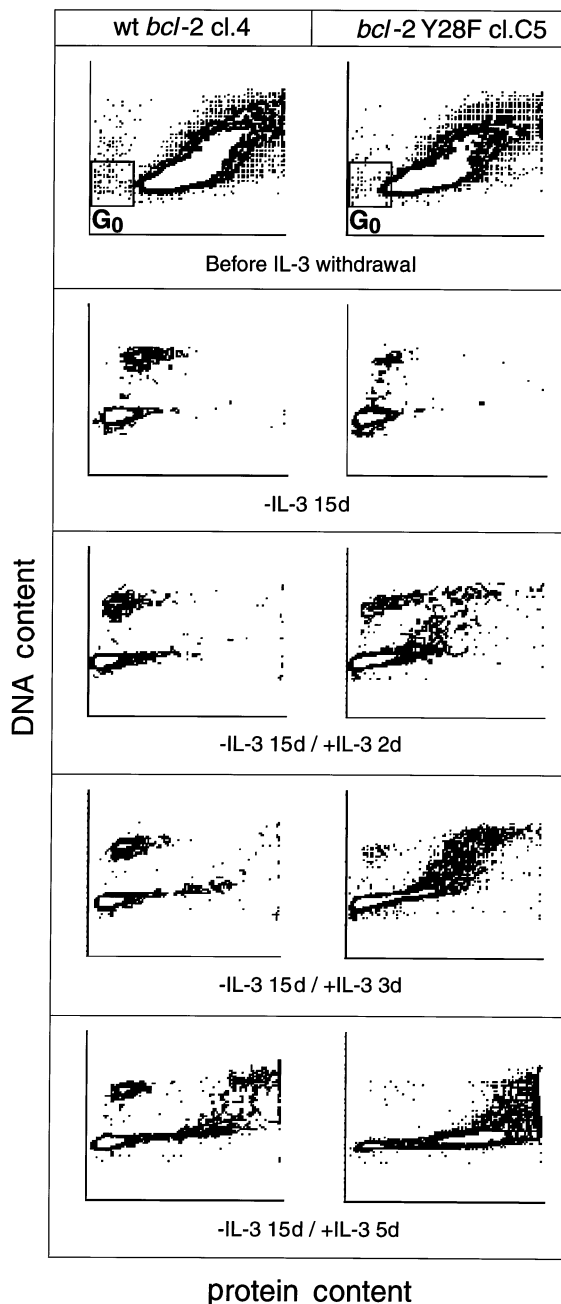
Fig. 4. Wild-type Bcl-2 retards entry of quiescent FDC-P1 cells into the cell cycle. Cells expressing wild-type Bcl-2 (left panels) or Y28F mutant Bcl-2 (right panels) were cultured for 15 days in medium containing 10% FCS but no IL-3, after which the cytokine was added back and samples were taken daily 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 G_0 (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 G_1 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 G_0 (>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 G_0 to G_1 (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 human Bcl-2 (Figure 6B). Transfectants expressing both wild-type Bcl-2 proteins were also refractory to re-stimulation. However, transfectants expressing Y28F human Bcl-2 as well as the wild-type murine protein entered the cycle substantially sooner than those expressing only wild-type Bcl-2 and their growth was indistinguishable from that of those expressing only the mutant protein (Figure 6B). Thus, Y28F mutant Bcl-2 is dominant and abrogates the cell cycle inhibitory effect of wild-type Bcl-2.

BH4 of Bcl-x$_L$ and Bcl-w contains a tyrosine residue functionally homologous to Y28 of Bcl-2

Since the Bcl-2 homologs Bcl-x$_L$ and Bcl-w can also delay entry into the cell cycle (O'Reilly *et al.*, 1996; Table I), we examined the consequence of replacing the equivalent BH4 tyrosine residue within these Bcl-2
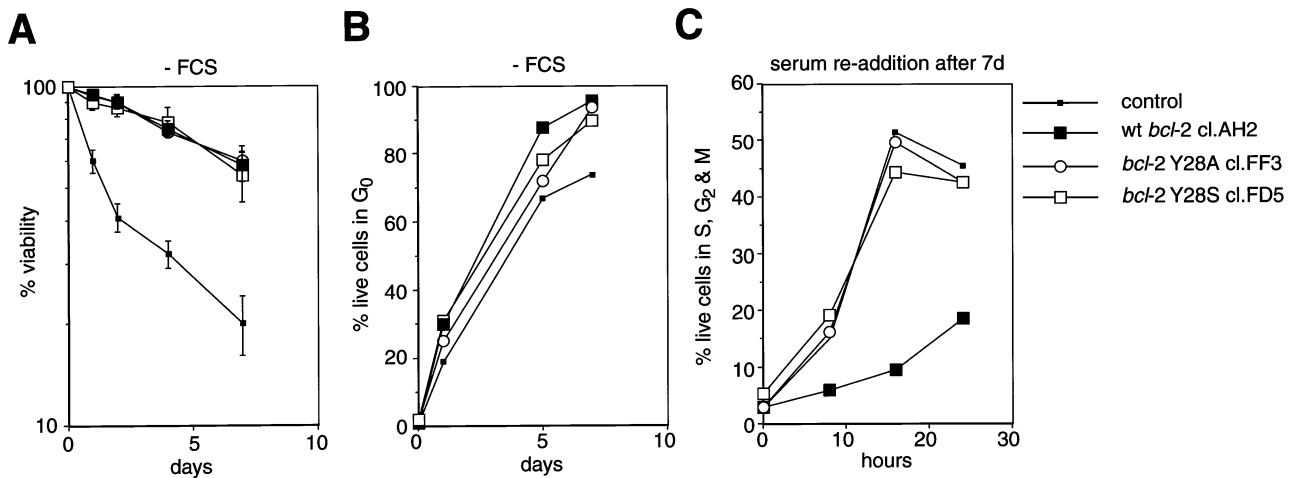
Fig. 5. Wild-type, but not Y28 mutant, Bcl-2 delays entry of quiescent NIH 3T3 fibroblasts into the cell cycle. (**A**) Y28 mutant Bcl-2 proteins are as effective as wild-type Bcl-2 in inhibiting apoptosis of NIH 3T3 fibroblasts deprived of serum. Puromycin-resistant control transfected NIH 3T3 fibroblasts and clones expressing either wild-type or Y28 mutant Bcl-2 were cultured for 7 days in DME medium lacking serum and cell survival was determined by visual inspection under phase contrast. Data shown are arithmetic means ± SD of at least three independent clones of each genotype representative of at least three experiments. (**B**) NIH 3T3 cells expressing wild-type and Y28 mutant Bcl-2 cease proliferating with similar kinetics when cultured in medium lacking serum. Entry into quiescence was determined by DNA and protein staining followed by flow-cytometric analysis. (**C**) NIH 3T3 cells expressing wild-type Bcl-2 are slower to re-enter the cell cycle than those expressing Y28 mutant Bcl-2. After 7 days of deprivation, 10% serum was added back to the cultures shown in (B) and cell cycle analysis performed at regular intervals by flow cytometry. The data shown are representative of experiments performed at least twice for more than three lines transfected with each construct.

homologs with phenylalanine. When deprived of IL-3, FDC-P1 clones expressing Y22F Bcl-x$_L$ 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-x$_L$ (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 BHRF-1, the Epstein–Barr homolog of Bcl-2 (Theodorakis *et al.*, 1996). Furthermore, a BHRF-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
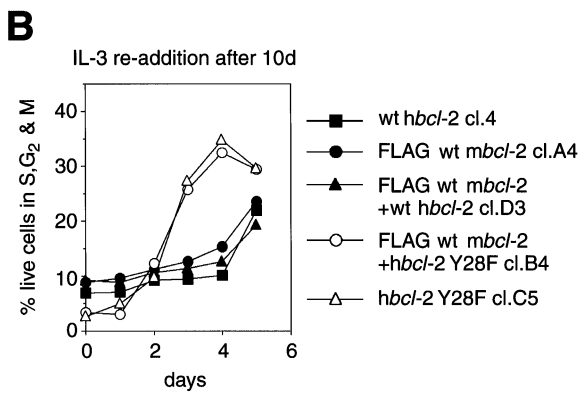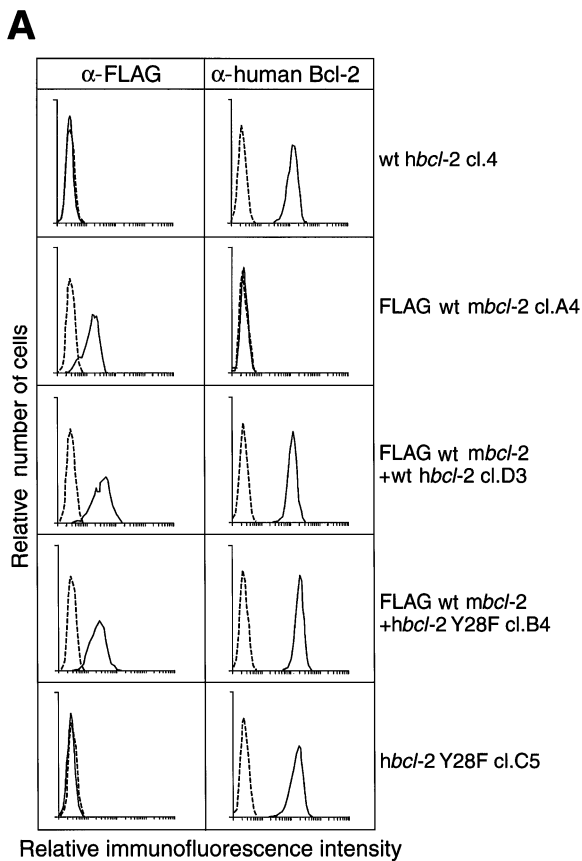
A

B

IL-3 re-addition after 10d

Fig. 6. Mutant Y28F Bcl-2 is dominant over wild-type Bcl-2. (**A**) Expression of wild-type or Y28 mutant human Bcl-2 and FLAG-tagged wild-type mouse Bcl-2 in FDC-P1 transfectants, revealed by anti-human Bcl-2 or anti-FLAG monoclonal antibodies. Staining of untransfected parental cells is indicated by dotted lines. (**B**) Kinetics of entry of quiescent cells into the cell cycle after re-addition of cytokine. FDC-P1 clones expressing wild-type or Y28 mutant human or mouse Bcl-2, as indicated, were cultured for 10 days in medium lacking IL-3, then re-stimulated by addition of IL-3. Samples were collected daily for cell cycle analysis by flow cytometry. Data shown are from representative experiments that were repeated at least twice; two other lines of the same genotype showed the same result.

mutant R-Ras p23. FDC-P1 cells expressing wild-type or Y28 mutant Bcl-2 were transfected with vectors encoding GluGlu-tagged wild-type R-*ras* p23, G38V R-*ras* p23 (an activated mutant) (Cox *et al.*, 1994; Saez *et al.*, 1994) or S43N R-*ras* p23 (a dominant negative mutant). Expression

Table I. Functional activity of BH4 mutants of Bcl-2, Bcl-x$_L$ and Bcl-w

Protein	Inhibition of apoptosis	Inhibition of entry into cell cycle
Bcl-2	+	+
Bcl-2 ΔBH4	−	−
Bcl-2 Y28A, S or F	+	−
Bcl-x$_L$	+	+
Bcl-x$_L$ Y22F	+	−
Bcl-w	+	+
Bcl-w Y27F	+	−

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-x$_L$; Y22F mutant Bcl-x$_L$; wild-type Bcl-w; and Y27F mutant Bcl-w. Comparable results were obtained in NIH 3T3 fibroblasts (see text).

of the R-Ras p23 proteins was confirmed by flow-cytometric analysis of permeabilized cells stained with the anti-GluGlu antibody and by immunoblotting. R-Ras p23 weakly facilitated apoptosis of FDC-P1 cells induced by cytokine deprivation (Figure 9A), consistent with a previous report (Wang,H.G. *et al.*, 1995). The activated G38V R-Ras mutant caused FDC-P1 cells to proliferate faster but did not result in IL-3 independence (not shown). No association between wild-type or Y28 mutant Bcl-2 and any of the R-Ras p23 proteins could be detected in co-immunoprecipitation experiments (not shown). Furthermore, co-expression of Bcl-2 and wild-type (not shown), activating (G38V) or dominant negative (S43N) mutants of R-Ras p23 did not alter the anti-apoptotic activity of Bcl-2 (Figure 9A) or its effect in retarding entry into the cell cycle (Figure 9B). Therefore, at least in this cell type, it seems very unlikely that R-Ras p23 mediates the effect of Bcl-2 on either cell survival or cell cycle re-entry.

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-x$_L$, Bcl-w and the adenovirus E1B 19K protein restrain 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.

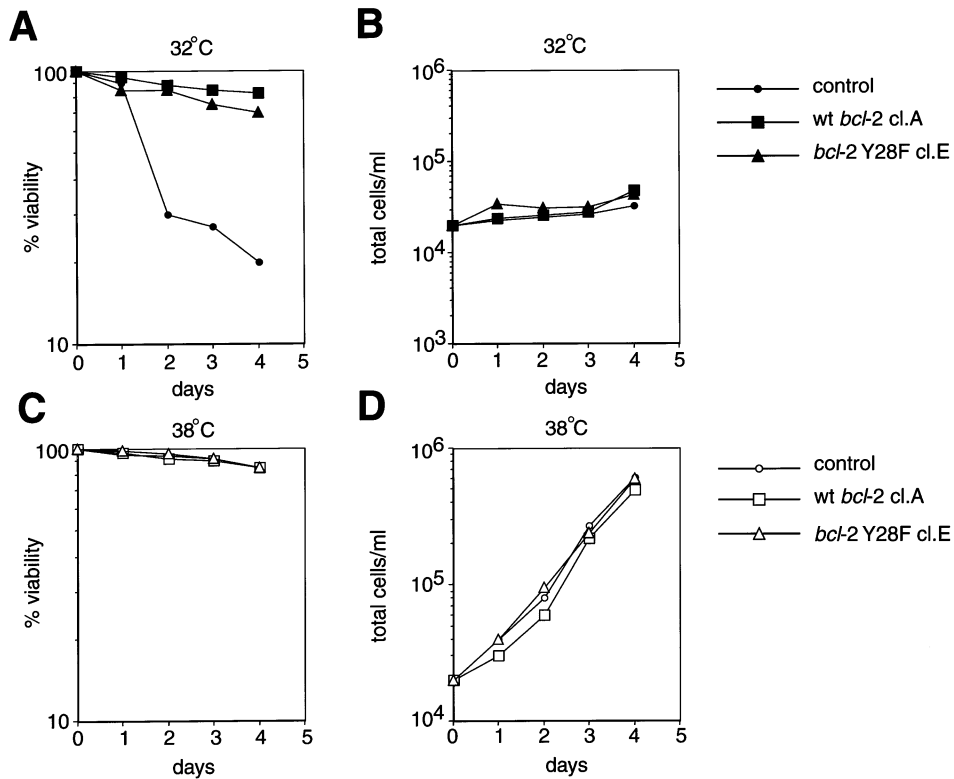The experiments described here demonstrate that the

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.
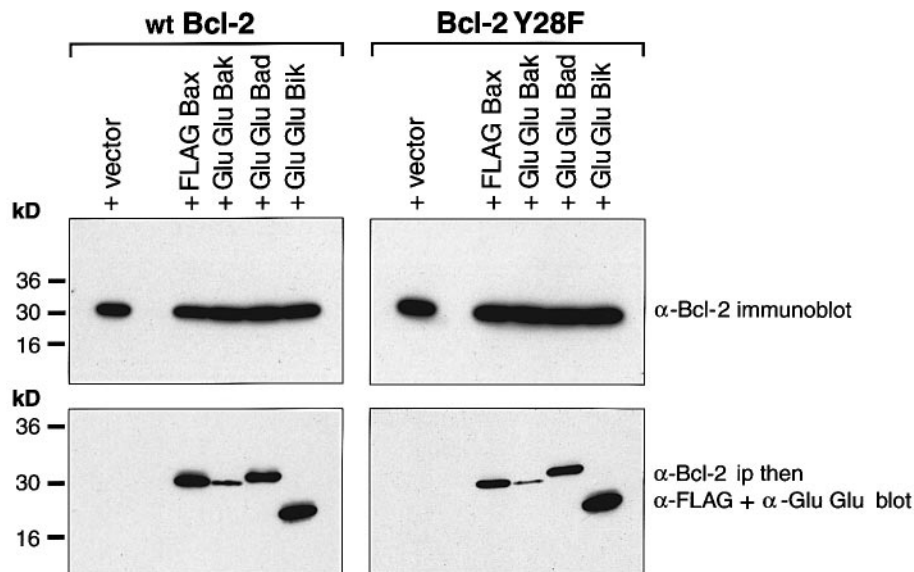

Fig. 8. Wild-type and Y28 mutant Bcl-2 can interact with Bax, Bak, Bad and Bik. Lysates containing similar levels of wild-type or Y28F mutant Bcl-2 as shown by immunoblotting (top) were immunoprecipitated with anti-human Bcl-2 monoclonal antibody. The immune complexes were resolved by SDS–PAGE and blotted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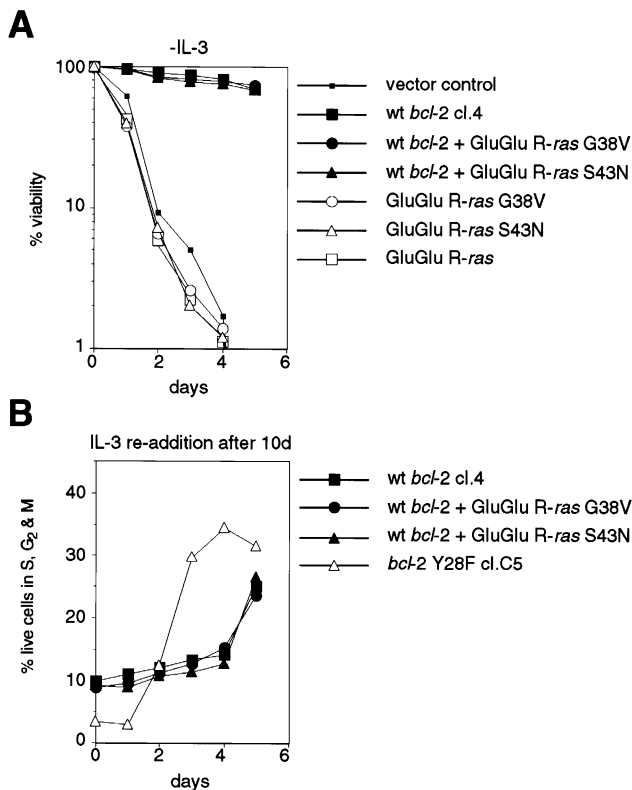
A

B

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 (A) 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.

growth factors (Figures 3 and 5). Similar observations were made for analogous mutants of Bcl-x_L (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 G_1 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 G_0 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 G_0/G_1 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 G_0 to G_1. 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 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 as effective as wild-type Bcl-2 in inhibiting p53-induced apoptosis of the transformed BRK cells, but rather than arresting their growth, permitted their continued proliferation (Uhlmann *et al.*, 1996). Similarly, a mutant BHRF1 protein in which the YH at the beginning of BH3 (residues 50 and 51) had been replaced by AS was an effective inhibitor of p53-induced apoptosis and, unlike wild-type BHRF1, permitted cells to continue proliferating (Theodorakis *et al.*, 1996). In contrast to these mutations, mutation of Bcl-2 at Y28 does not prevent cells leaving the cycle (Figures 3B and 5B); it merely attenuates the ability of Bcl-2 to slow cell cycle re-entry (Figures 3C and 5C). Nevertheless, our observations, taken together with those of Chinnadurai and colleagues (Theodorakis *et al.*, 1996; Uhlmann *et al.*, 1996), implicate the region encompassing BH4–BH3 in the proliferation restraining function of Bcl-2 and its homologs.

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 and 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; Matolcsy *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 than 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 (EYPME) (Grussenmeyer *et al.*, 1985) tags. The cDNAs for *bcl*-2, *bcl*-x$_L$, *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*-x$_L$, *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; B6.2.16BW2, a mouse T cell hybridoma; mouse fibroblastoid lines L929 [subline LM(–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) (Debbas 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 µM folic acid and 100 µM L-asparagine. Medium for FDC-P1 cells contained 1000 U/ml IL-3, unless stated otherwise.

Cell lines were transfected by electroporation using a Gene-pulser (BioRad) and selected by growth in puromycin (2–5 µg/ml) (Sigma), hygromycin (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-radiation (provided by a ^{60}Co 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 FACScan (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, ~10^5 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; Pezzella *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 Biotechnology) as the secondary reagent. Cells were analyzed in a 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 among Bcl-2 family members, mouse L929 fibroblasts (7.5×10^5) 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 Na$_3$VO$_4$; 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 (Pharmacia) 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, 5% 2-mercaptoethanol, 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 (Fcγ specific; Jackson Immuno Research). Membranes were then probed (1 h at room temperature) with ^{125}I-labeled staphylococcal protein A (~2×10^6 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 clones 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 (6×10^6) were washed twice in DMEM containing 10% FCS but no IL-3 and resuspended at a density of 2×10^5 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-

P1 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 in a FACScan II (Becton Dickinson).

Simultaneous analysis of DNA content, RNA content and protein content of the cells was performed as previously described (Crissman *et al.*, 1990). In brief, ~10^6 cells were fixed in 70% ethanol and stored protected from light at 4°C. 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 Cell-fit software (Becton Dickinson) or an in-house program (F.Battye, unpublished).

Acknowledgements

References

Alnemri,E.S., Livingston,D.J., Nicholson,D.W., Salvesen,G., Thornberry, N.A., Wong,W.W. and Yuan,J. (1996) Human ICE/CED-3 protease nomenclature. *Cell*, **87**, 171.

Boise,L.H., Gonzalez-Garcia,M., Postema,C.E., Ding,L., Lindsten,T., Turka,L.A., Mao,X., Nunez,G. and Thompson,C.B. (1993) *bcl-x*, a *bcl*-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell*, **74**, 597–608.

Borner,C. (1996) Diminished cell proliferation associated with the death-protective activity of Bcl-2. *J. Biol. Chem.*, **271**, 12695–12698.

Borner,C., Martinou,I., Mattmann,C., Irmler,M., Schaerer,E., Martinou, J.-C. and Tschopp,J. (1994) The protein bcl-2α does not require membrane attachment, but two conserved domains to suppress apoptosis. *J. Cell Biol.*, **126**, 1059–1068.

Boyd,J.M., Malstrom,S., Subramanian,T., Venkatesh,L.K., Schaeper,U., Elangovan,B., D'Sa-Eipper,C. and Chinnadurai,G. (1994) Adenovirus E1B 19 kDa and Bcl-2 proteins interact with a common set of cellular proteins. *Cell*, **79**, 341–351.

Boyd,J.M. *et al.* (1995) Bik, a novel death-inducing protein shares a distinct sequence motif with Bcl-2 family proteins and interacts with viral and cellular survival-promoting proteins. *Oncogene*, **11**, 1921–1928.

Brady,H.J.M., Gil-Gómez,G., Kirberg,J. and Berns,A.J.M. (1996) Baxα perturbs T cell development and affects cell cycle entry of T cells. *EMBO J.*, **15**, 6991–7001.

Chen,C.-Y. and Faller,D.V. (1996) Phosphorylation of Bcl-2 protein and association with p21^{Ras} in Ras-induced apoptosis. *J. Biol. Chem.*, **271**, 2376–2379.

Chinnaiyan,A.M., O'Rourke,K., Lane,B.R. and Dixit,V.M. (1997) Interaction of CED-4 with CED-3 and CED-9: a molecular framework for cell death. *Science*, **275**, 1122–1126.

Chiou,S.-K., Tseng,C.-C., Rao,L. and White,E. (1994) Functional complementation of the adenovirus E1B 19-kilodalton protein with Bcl-2 in the inhibition of apoptosis in infected cells. *J. Virol.*, **68**, 6553–6566.

Chittenden,T., Flemington,C., Houghton,A.B., Ebb,R.G., Gallo,G.J., Elangovan,B., Chinnadurai,G. and Lutz,R.J. (1995a) A conserved domain in Bak, distinct from BH1 and BH2, mediates cell death and protein binding functions. *EMBO J.*, **14**, 5589–5596.

Chittenden,T., Harrington,E.A., O'Connor,R., Flemington,C., Lutz,R.J., Evan,G.I. and Guild,B.C. (1995b) Induction of apoptosis by the Bcl-2 homologue Bak. *Nature*, **374**, 733–736.

Cleary,M. and Rosenberg,S.A. (1990) The *bcl*-2 gene, follicular lymphoma, and Hodgkin's disease. *J. Natl Cancer Inst.*, **82**, 808–809.

Cory,S. (1995) Regulation of lymphocyte survival by the *BCL*-2 gene family. *Annu. Rev. Immunol.*, **13**, 513–543.

Cox,A.D., Brtva,T.R., Lowe,D.G. and Der,C.J. (1994) R-Ras induces malignant, but not morphologic, transformation of NIH3T3 cells. *Oncogene*, **9**, 3281–3288.

Crissman,H.A., Darzynkiewicz,Z., Steinkamp,J.A. and Tobey,R.A. (1990) Simultaneous fluorescent labeling of DNA, RNA and protein. *Methods Cell Biol.*, **33**, 305–314.

Debbas,M. and White,E. (1993) Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. *Genes Dev.*, **7**, 546–554.

Ellis,R.E., Yuan,J. and Horvitz,H.R. (1991) Mechanisms and functions of cell death. *Annu. Rev. Cell Biol.*, **7**, 663–698.

Fanidi,A., Harrington,E.A. and Evan,G.I. (1992) Cooperative interaction between c-*myc* and *bcl*-2 proto-oncogenes. *Nature*, **359**, 554–556.

Farrow,S.N., White,J.H.M., Martinou,I., Raven,T., Pun,K.-T., Grinham, C.J., Martinou,J.-C. and Brown,R. (1995) Cloning of a *bcl*-2 homologue by interaction with adenovirus E1B 19K. *Nature*, **374**, 731–733.

Fernandez-Sarabia,M.J. and Bischoff,J.R. (1993) Bcl-2 associates with the *ras*-related protein R-ras p23. *Nature*, **366**, 274–275.

Fraser,A. and Evan,G. (1996) A license to kill. *Cell*, **85**, 781–784.

Gibson,L. *et al.* (1996) *bcl-w*, a novel member of the *bcl*-2 family, promotes cell survival. *Oncogene*, **13**, 665–675.

Grussenmeyer,T., Scheidtmann,K.H., Hutchinson,M.A., Eckhart,W. and Walter,G. (1985) Complexes of polyoma virus medium T antigen and cellular proteins. *Proc. Natl Acad. Sci. USA*, **82**, 7952–7954.

Haldar,S., Jena,N. and Croce,C.M. (1995) Inactivation of Bcl-2 by phosphorylation. *Proc. Natl Acad. Sci. USA*, **92**, 4507–4511.

Han,J., Sabbatini,P. and White,E. (1996) Induction of apoptosis by human Nbk/Bik, a BH3-containing protein that interacts with E1B 19K. *Mol. Cell. Biol.*, **16**, 5857–5864.

Hanada,M., Aime-Sempe,C., Sato,T. and Reed,J.C. (1995) Structure–function analysis of Bcl-2 protein. Identification of conserved domains important for homodimerization with Bcl-2 and heterodimerization with Bax. *J. Biol. Chem.*, **270**, 11962–11969.

Hengartner,M.O. and Horvitz,H.R. (1994) Programmed cell death in *Caenorhabditis elegans. Curr. Opin. Genet. Dev.*, **4**, 581–586.

Hopp,T.P., Prickett,K.S., Price,V.L., Libby,R.T., March,C.J., Cerretti,D.P., Urdal,D.L. and Conlon,P.J. (1988) A short polypeptide marker sequence useful for recombinant protein identification and purification. *Biotechnology*, **6**, 1204–1210.

Horton,R.M., Ho,S.N., Pullen,J.K., Hunt,H.D., Cai,Z. and Pease,L.R. (1993) Gene splicing by overlap extension. *Methods Enzymol.*, **217**, 270–279.

Huang,D.C.S., Cory,S. and Strasser,A. (1997) Bcl-2, Bcl-x$_L$ and adenovirus protein E1B19kD are functionally equivalent in their ability to inhibit cell death. *Oncogene*, **14**, 405–414.

Hunter,J.J. and Parslow,T.G. (1996) A peptide sequence from Bax that converts Bcl-2 into an activator of apoptosis. *J. Biol. Chem.*, **271**, 8521–8524.

Hunter,J.J., Bond,B.L. and Parslow,T.G. (1996) Functional dissection of the human Bcl2 protein: sequence requirements for inhibition of apoptosis. *Mol. Cell. Biol.*, **16**, 877–883.

Kerr,J.F.R., Wyllie,A.H. and Currie,A.R. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer*, **26**, 239–257.

Kiefer,M.C., Brauer,M.J., Powers,V.C., Wu,J.J., Umansky,S.R., Tomei,L.D. and Barr,P.J. (1995) Modulation of apoptosis by the widely distributed Bcl-2 homologue Bak. *Nature*, **374**, 736–739.

Korsmeyer,S.J. (1995) Regulators of cell death. *Trends Genet.*, **11**, 101–105.

Kumar,S. (1995) ICE-like proteases in apoptosis. *Trends Biochem. Sci.*, **20**, 198–202.

Lee,L.C., Hunter,J.J., Mujeeb,A., Turck,C. and Parslow,T.G. (1996) Evidence for α-helical conformation of an essential N-terminal region in the human Bcl2 protein. *J. Biol. Chem.*, **271**, 23284–23288.

Linette,G.P., Hess,J.L., Sentman,C.L. and Korsmeyer,S.J. (1995) Peripheral T-cell lymphoma in lckpr-bcl-2 transgenic mice. *Blood*, **86**, 1255–1260.

Linette,G.P., Li,Y., Roth,K. and Korsmeyer,S.J. (1996) Cross talk between cell death and cell cycle progression: BCL-2 regulates NFAT-mediated activation. *Proc. Natl Acad. Sci. USA*, **93**, 9545–9552.

Lipponen,P., Pietilainen,T., Kosma,V.M., Aaltomaa,S., Eskelinen,M. and Syrjanen,K. (1995) Apoptosis suppressing protein bcl-2 is expressed in well-differentiated breast carcinomas with favourable prognosis. *J. Pathol.*, **177**, 49–55.

Lithgow,T., van Driel,R., Bertram,J.F. and Strasser,A. (1994) The protein product of the oncogene *bcl*-2 is a component of the nuclear envelope, the endoplasmic reticulum and the outer mitochondrial membrane. *Cell Growth Differ.*, **5**, 411–417.

Marvel,J., Perkins,G.R., Lopez-Rivas,A. and Collins,M.K.L. (1994) Growth factor starvation of bcl-2 overexpressing murine bone marrow cells induced refractoriness of IL-3 stimulation of proliferation. *Oncogene*, **9**, 1117–1122.

Matolcsy,A., Casali,P., Warnke,R.A. and Knowles,D.M. (1996) Morphologic transformation of follicular lymphoma is associated with somatic mutation of the translocated Bcl-2 gene. *Blood*, **88**, 3937–3944.

Mazel,S., Burtrum,D. and Petrie,H.T. (1996) Regulation of cell division cycle progression by *bcl*-2 expression: a potential mechanism for inhibition of programmed cell death. *J. Exp. Med.*, **183**, 2219–2226.

McDonnell,T.J. and Korsmeyer,S.J. (1991) Progression from lymphoid hyperplasia to high-grade malignant lymphoma in mice transgenic for the t(14;18). *Nature*, **349**, 254–256.

Minn,A.J., Vélez,P., Schendel,S.L., Liang,H., Muchmore,S.W., Fesik,S.W., Fill,M. and Thompson,C.B. (1997) Bcl-x$_L$ forms an ion channel in synthetic lipid membranes. *Nature*, **385**, 353–357.

Mizushima,J. and Nagata,S. (1990) pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res.*, **18**, 5322.

Muchmore,S.W. *et al.* (1996) X-ray and NMR structure of human Bcl-x$_L$, an inhibitor of programmed cell death. *Nature*, **381**, 335–341.

Naumovski,L. and Cleary,M.L. (1996) The p53-binding protein 53BP2 also interacts with Bcl2 and impedes cell cycle progression at G$_2$/M. *Mol. Cell. Biol.*, **16**, 3884–3892.

Nicoletti,I., Migliorati,G., Pagliacci,M.C., Grignani,F. and Riccardi,C. (1991) A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J. Immunol. Methods*, **139**, 271–279.

Nunez,G., London,L., Hockenbery,D., Alexander,M., McKearn,J.P. and Korsmeyer,S.J. (1990) Deregulated *Bcl*-2 gene expression selectively prolongs survival of growth factor-deprived hemopoietic cell lines. *J. Immunol.*, **144**, 3602–3610.

O'Reilly,L., Huang,D.C.S. and Strasser,A. (1996) The cell death inhibitor Bcl-2 and its homologues influence control of cell cycle entry. *EMBO J.*, **15**, 6979–6990.

O'Reilly,L.A., Harris,A.W. and Strasser,A. (1997a) A *bcl*-2 transgene expression promotes survival and reduces proliferation of CD3⁻CD4⁻ CD8⁻ T cell progenitors. *Int. Immunol.*, in press.

O'Reilly,L.A., Harris,A.W., Tarlinton,D.W., Corcoran,L.M. and Strasser,A. (1997b) Expression of a *bcl*-2 transgene reduces proliferation and shows turnover of developing B lymphocytes *in vivo*. *J. Immunol.*, in press.

Oltvai,Z.N., Milliman,C.L. and Korsmeyer,S.J. (1993) Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. *Cell*, **74**, 609–619.

Pezzella,F., Tse,A.G.D., Cordell,J.L., Pulford,K.A.F., Gatter,K.C. and Mason,D.Y. (1990) Expression of the *bcl*-2 oncogene protein is not specific for the 14;18 chromosomal translocation. *Am. J. Pathol.*, **137**, 225–232.

Reed,J.C. (1994) Bcl-2 and the regulation of programmed cell death. *J. Cell Biol.*, **124**, 1–6.

Sabbatini,P., Chiou,S.K., Rao,L. and White,E. (1995) Modulation of p53-mediated transcriptional repression and apoptosis by the adenovirus E1B 19K protein. *Mol. Cell. Biol.*, **15**, 1060–1070.

Saez,R., Chan,A.M., Miki,T. and Aaronson,S.A. (1994) Oncogenic activation of human R-*ras* by point mutations analogous to those of prototype H-*ras* oncogenes. *Oncogene*, **9**, 2977–2982.

Sattler,M. *et al.* (1997) Structure of Bcl-x$_L$–Bak peptide complex: recognition between regulators of apoptosis. *Science*, **275**, 983–986.

Selvakumaran,M., Lin,H.-K., Miyashita,T., Wang,H.G., Krajewski,S., Reed,J.C., Hoffman,B. and Liebermann,D. (1994) Immediate early up-regulation of *bax* expression by p53 but not TGFβ1: a paradigm for distinct apoptotic pathways. *Oncogene*, **9**, 1791–1798.

Spector,M.S., Desnoyers,S., Hoeppner,D.J. and Hengartner,M.O. (1997) Interaction between the *C.elegans* cell-death regulators CED-9 and CED-4. *Nature*, **385**, 653–656.

Strasser,A., Harris,A.W., Bath,M.L. and Cory,S. (1990) Novel primitive lymphoid tumours induced in transgenic mice by cooperation between *myc* and *bcl*-2. *Nature*, **348**, 331–333.

Strasser,A., Harris,A.W. and Cory,S. (1993) Eμ-*bcl*-2 transgene facilitates spontaneous transformation of early pre-B and immunoglobulin-secreting cells but not T cells. *Oncogene*, **8**, 1–9.

Strasser,A., Harris,A.W., Jacks,T. and Cory,S. (1994) DNA damage can induce apoptosis in proliferating lymphoid cells via p53-independent mechanisms inhibitable by Bcl-2. *Cell*, **79**, 329–339.

Strasser,A., Harris,A.W., Huang,D.C.S., Krammer,P.H. and Cory,S. (1995) Bcl-2 and Fas/APO-1 regulate distinct pathways to lymphocyte apoptosis. *EMBO J.*, **14**, 6136–6147.

Takayama,S., Sato,T., Krajewski,S., Kochel,K., Irie,S., Millan,J.A. and Reed,J.C. (1995) Cloning and functional analysis of BAG-1: a novel Bcl-2-binding protein with anti-cell death activity. *Cell*, **80**, 279–284.

Tanaka,S., Louie,D.C., Kant,J.A. and Reed,J.C. (1992) Frequent incidence of somatic mutations in translocated BCL2 oncogenes of non-Hodgkin's lymphomas. *Blood*, **79**, 229–237.

Theodorakis,P., D'Sa-Eipper,C., Subramanian,T. and Chinnadurai,G. (1996) Unmasking of a proliferation-restraining activity of the anti-apoptosis protein EBV BHRF1. *Oncogene*, **12**, 1707–1713.

Tsujimoto,Y., Finger,L.R., Yunis,J., Nowell,P.C. and Croce,C.M. (1984) Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. *Science*, **226**, 1097–1099.

Uhlmann,E.J., D'Sa-Eipper,C., Subramanian,T., Wagner,A.J., Hay,N. and Chinnadurai,G. (1996) Deletion of a nonconserved region of Bcl-2 confers a novel gain of function: suppression of apoptosis with concomitant cell proliferation. *Cancer Res.*, **56**, 2506–2509.

Vairo,G., Innes,K.M. and Adams,J.M. (1996) Bcl-2 has a cell cycle inhibitory function separable from its enhancement of cell survival. *Oncogene*, **13**, 1511–1519.

Vaux,D.L., Cory,S. and Adams,J.M. (1988) *Bcl*-2 gene promotes haemopoietic cell survival and cooperates with c-*myc* to immortalize pre-B cells. *Nature*, **335**, 440–442.

Wang,H.-G. *et al.* (1994) Apoptosis regulation by interaction of Bcl-2 protein and Raf-1 kinase. *Oncogene*, **9**, 2751–2756.

Wang,H.-G., Millan,J.A., Cox,A.D., Der,C.J., Rapp,U.R., Beck,T., Zha,H. and Reed,J.C. (1995) R-ras promotes apoptosis caused by growth factor deprivation via a Bcl-2 suppressible mechanism. *J. Cell Biol.*, **129**, 1103–1114.

Wang,K., Yin,X.-M., Chao,D.T., Milliman,C.L. and Korsmeyer,S.J. (1996) BID: a novel BH3 domain-only death agonist. *Genes Dev.*, **10**, 2859–2869.

Wang,Y., Okan,I., Pokrovskaja,K. and Wiman,K.G. (1996) Abrogation of p53-induced G1 arrest by the HPV 16 E7 protein does not inhibit p53-induced apoptosis. *Oncogene*, **12**, 2731–2735.

White,E. (1996) Life, death, and the pursuit of apoptosis. *Genes Dev.*, **10**, 1–15.

Wu,D., Wallen,H.D. and Nuñez,G. (1997) Interaction and regulation of subcellular localization of CED-4 by CED-9. *Science*, **275**, 1126–1129.

Xue,D., Shaham,S. and Horvitz,H.R. (1996) The *Caenorhabditis elegans* cell-death protein CED-3 is a cysteine protease with substrate specificities similar to those of the human CPP32 protease. *Genes Dev.*, **10**, 1073–1083.

Yang,E. and Korsmeyer,S.J. (1996) Molecular thanatopsis: a discourse on the Bcl-2 family and cell death. *Blood*, **88**, 386–401.

Yang,E., Zha,J., Jockel,J., Boise,L.H., Thompson,C.B. and Korsmeyer,S.J. (1995) Bad, a heterodimeric partner for Bcl-x$_L$ and Bcl-2, displaces Bax and promotes cell death. *Cell*, **80**, 285–291.

Yin,X.-M., Oltvai,Z.N. and Korsmeyer,S.J. (1994) BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax. *Nature*, **369**, 321–323.

Yuan,J., Shaham,S., Ledoux,S., Ellis,H.M. and Horvitz,H.R. (1993) The *C.elegans* cell death gene *ced*-3 encodes a protein similar to mammalian interleukin-1β-converting enzyme. *Cell*, **75**, 641–652.

Zha,H., Aimé-Sempé,C., Sato,T. and Reed,J.C. (1996) Proapoptotic protein Bax heterodimerizes with Bcl-2 and homodimerizes with Bax via a novel domain (BH3) distinct from BH1 and BH2. *J. Biol. Chem.*, **271**, 7440–7444.