International Immunology, Vol. 7, No. 11, pp. 1709–1720

Pleiotropic effects of Bcl-2 on transcription factors in T cells: potential role of NF-κB p50–p50 for the anti-apoptotic function of Bcl-2

Vladimir N. Ivanov[1,2], Ge Deng[1], Eckhard R. Podack[1] and Thomas R. Malek[1]

[1]Department of Microbiology and Immunology, University of Miami School of Medicine, Miami, FL 33101, USA
[2]Institute of Gene Biology, Russian Academy of Science, Moscow, Russia

Keywords: AP-1, apoptosis, dexamethasone, IL-2, NF-AT, NF-κB/Rel

Abstract

Bcl-2 functions to repress apoptosis by regulation of genes which encode proteins required for programmed cell death and by interference with peroxidative damage. We investigated the interrelationship between expression of *bcl-2* and regulation of transcription factor DNA binding activities in the 2B4 T cell hybridoma and IL-2-dependent CTLL T cell line. Over-expression of *bcl-2* in 2B4 resulted in enhanced basal levels of activator protein (AP)-1, octamer binding factor (Oct)-1, lymphoid enhancer binding factor (LEF)-1, RelA–p50 and NF-κB p50–p50 DNA binding activities. After apoptotic signaling, down-regulation of AP-1, NF-AT and Oct-1 binding activities was observed in control 2B4 and CTLL, whereas suboptimal, but higher, levels of these transcription factors were found in *bcl-2*-transfected cells, potentially promoting cell survival. Furthermore, after apoptotic signaling, expression of *bcl-2* led to differential changes of NF-κB levels, resulting in a decrease in RelA–p50 and an increase in NF-κB p50–p50, altering the ratio of these DNA binding activities such that now p50–p50 markedly predominated in both 2B4-Bcl-2 and CTLL-Bcl-2. Apoptotic signaling in the presence or absence of Bcl-2 resulted in induction of the RelB–p50 heterodimer in 2B4. The changes in NF-κB/Rel levels raise the possibility that this family of transcription factors may play an important role in the regulation of apoptosis.

Introduction

Antigen activation of T lymphocytes is accompanied by clonal expansion of antigen-specific T cells and their differentiation into effector cells, which are characterized by secretion of cytokines or cytotoxic T lymphocyte activity. The production of cytokines is dependent upon activation of T cell signaling cascades, which in turn activate distinct transcription factors that control cytokine gene expression. One well characterized example is the IL-2 gene whose expression is controlled by coordinate action of several transcription factors including activator protein (AP)-1, NF-κB, NF-AT and octamer binding protein (Oct)-1 (1,2).

The regulation of the NF-κB/Rel family of transcription factors is of particular interest as these factors have been implicated in the regulation of many genes after T cell activation (3). Nuclear NF-κB is found as heterodimers of p50 or p52 subunits with three different members of the Rel family: RelA (p65), RelB and c-Rel (4). The activation of NF-κB p65–p50 (or RelA–p50) is dependent upon release of p65–p50 from a complex with cytoplasmic inhibitor IκB (MAD-3) with subsequent translocation of p65–p50 into the nucleus (5,6). RelA is an extremely potent transcription activation subunit, while the p50 and p52 subunits have a DNA binding domain but no apparent *trans*-activator domain (3). NF-κB p50 may form the homodimer complex (7), which only slightly activates NF-κB-dependent transcription *in vitro* (8,9), whereas both p50–p50 and p52–p52 homodimers may function as negative regulators *in vivo* (4,10–12). Two variables that are likely to control the overall level of NF-κB-dependent transcription *in vivo* are the nuclear ratio of NF-κB *trans*-activators (RelA–p50, RelB–p50, c-Rel–p50) to NF-κB *trans*-inhibitors (p50–

Correspondence to: V. N. Ivanov, Laboratory of T Cell Development, Sloan-Kettering Cancer Center, Box 98, 1275 York Avenue, New York, NY 10021, USA

Transmitting editor: D. Mathis

Received 16 May 1995, *accepted* 3 July 1995

p50 and p52–p52) and the relative affinity of distinct NF-κB binding sites for these transcription factors.

Another outcome of T cell activation is the induction of apoptosis which may function to limit an antigen-specific T cell response. It is likely that some of the same signaling molecules and transcription factors that control T cell differentiation and activation are also important participants in the induction of the genes required for apoptosis. Several genes have been implicated in regulation of apoptosis, some of which encode cell surface receptors, e.g. the TNF receptor and Fas, intracellular signaling molecules, or transcription factors, e.g. c-Myc, p53 and Nur 77 (13–16). The protooncogene *bcl-2* encodes a 26 kDa membrane protein that functions to inhibit many types of apoptotic cell death (17). The molecular mechanism, however, by which Bcl-2 inhibits apoptosis is poorly understood. This protein may also function as an intracellular antioxidant which prevents cell damage by oxygen free radicals (18,19). It is conceivable that these two functions of Bcl-2 are interrelated and the intracellular targets for the antioxidant effect of Bcl-2 might also play a pivotal role in the regulation of apoptosis. Two potential candidates are transcription factors NF-κB and TCF1(α)/lymphoid enhancer binding factor (LEF)-1 as a number of agents with antioxidant activity effectively inhibit activation of these transcription factors (3,20).

In the present study we have evaluated the interrelationship between T cell activation and apoptosis by examining the effect of Bcl-2 on several transcription factors that are normally up-regulated upon T cell activation. We show that Bcl-2 has pleiotropic effects on transcription factor binding activities, including NF-κB, AP-1, Oct and LEF-1. After receiving apoptotic signals, one target of Bcl-2 in T cells is the induction of enhanced levels of NF-κB p50–p50 homodimer which inhibits NF-κB-dependent transcription.

Methods

Cell culture

The 2B4 T cell hybridoma was cultured in RPMI 1640 medium supplemented with 5% FCS, 50 mM 2-mercaptoethanol and antibiotics. IL-2-dependent CTLL2 cells were cultured in the same medium containing IL-2 (100 U/ml).

Transfected cell lines

CTLL2 cells were stably transfected with mouse *bcl-2* cDNA in expression vector pBMGneo (21) (CTLL-Bcl-2 cells) or with the unmodified vector (CTLL-neo cells) as previously described (22). 2B4 cells were transfected with *bcl-2* cDNA in the same vector by electroporation and selected for G418 resistance. A cell line with high-level expression of bcl-2 mRNA was established on the base of Northern blot analysis (2B4-Bcl-2). Control cells were 2B4 transfected with the vector pBMGneo (2B4-neo). 2B4 clones were also stably transfected with pRc/CMV–p50, a NF-κB p50 expression vector. This construct was kindly provided by Dr A. Israel (Paris, France).

Cell cycle analysis

After fixation with 70% ethanol in PBS, cells were pelleted and resuspended in 0.5 ml of PBS containing propidium iodide (40 μg/ml) and DNase-free RNase A (100 μg/ml). Cells were incubated at 37°C for 30 min and then analyzed in a FACScan (Becton Dickinson, Mountain View, CA).

Antibodies

The mAb to the ε chain of the TCR complex, anti-CD3ε (145-2C11), has been previously characterized (23). Polyclonal rabbit antisera to the p50 subunit of NF-κB (10), to a N-terminal peptide of the p65 subunit of NF-κB (24), to a C-terminal peptide of c-Rel (25), to mouse RelB (26) and to mouse LEF-1 (27) were kindly provided by Drs M. Lenardo (Bethesda, MD), W. Greene (San Francisco, CA), N. Rice (Frederick, MD), R. Bravo (Princeton, NJ) and R. Grosschedl (San-Francisco, CA) respectively.

Oligonucleotides and electrophoretic mobility shift assay (EMSA)

The following double-stranded oligonucleotides were used in this study as specific probes for transcription factors (only one strand of a double-stranded oligonucleotide is shown, binding sites of transcription factors are underlined):

agctTGGGGACTTTCCAGCCG	for NF-κB
agctTGATGAGTCAGCCG	for AP-1
agctCCATGACGTCATGG	for CREB
AAAGAGGAAAATTTGTTTCATACAGAA	for NF-AT
GAAAATATGTGTAATATGTAAAACATTTTG	for Oct
AAAAAAGAACAAAGGGCCTAGATT	for LEF-1
GTCTGAAACATTTTTCTGATTGGTTAAAAGTTGAGTGCT	for NF-Y

These oligonucleotides were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Nuclear extracts were prepared from 5–15×10^6 T cells. Binding reactions were carried out by incubating the end-labeled DNA (50,000 c.p.m.) with 5 μg of nuclear proteins and 2 μg of poly(dI–dC) as described previously (20). For identification of transcription factors, nuclear extracts were preincubated with 1.0 μl of specific antiserum for 15 min at 20°C before addition of the labeled oligonucleotide probes. The incubation was continued for a further 30 min followed by EMSA.

Transfection and chloramphenicol acetyltransferase (CAT) assay

The following reporter CAT constructs were used in this study: pIL2CAT (original name pILCAT2/1+) which contained the regulatory region -293/–7 of mouse IL-2 gene (28); –121-HIV–CAT which contained the region –121/+232 from the HIV long terminal repeat with 2×NF-κB and 3×SP1 binding sites, and –76-HIV–CAT with deleted NF-κB sites (29). T cells growing in log phase were transfected by the DEAE–dextran method. Reference plasmid pRSVβgal was used as a control for transfection efficiency. For each transfection experiment 5×10^7 cells were treated with 50 μg reporter plasmid DNA, 10 μg pRSVβgal and 250 μg/ml DEAE–dextran. At 20 h after transfection each cell culture was split into five 100 mm tissue plates and stimulated with PMA (10 ng/ml) plus ionomycin (1 μM), dexamethasone (Dex; 1μM) or anti-CD3ε as previously described (30). After an additional 20 h of culture, transfected cells were harvested and used for isolation of total cell extracts. Protein concentration of cell extracts was normalized on the basis of β-galactosidase activity of transfected unstimulated cells to correct for transfection efficiency. Quantitation

of CAT assays was performed by liquid scintillation counting of the appropriate area from chromatography plates. Relative CAT activity (or fold of induction) was determined as a ratio of percentage of acetylated chloramphenicol for activated cells to that percentage for control unstimulated cells.

Western blot analysis

Equal amounts of nuclear extract proteins (5 μg) were separated by SDS–PAGE in 10% gels. After transferral to nitrocellulose, the blots were blocked with 3% non-fat milk for 2 h and incubated with specific antibody at a dilution 1.100 for an additional 2 h at room temperature. After three washes with TBST (10 mM Tris–HCl, pH 7.5, 140 mM NaCl, 0.1% Tween 20), filters were incubated with the secondary antibody (goat anti-rabbit IgG, dilution 1:2500) for 1 h, then were washed with TBST and finally incubated with ^{125}I-labeled Protein G (Sigma, St Louis, MO) for 1 h. After several washes with TBS, filters were dried and exposed for 2–18 h at –80°C with intensifying screens.

Northern hybridization analysis

Cytoplasmic cell extracts were used for isolation and purification of total RNA. RNA was separated on formaldehyde/MOPS agarose gel and blotted on Hybond N membranes (Amersham, Arlington Heights, IL) which were hybridized with ^{32}P-labeled DNA probes. NF-κB p105/p50 cDNA (7) was kindly provided by Dr A. Israel.

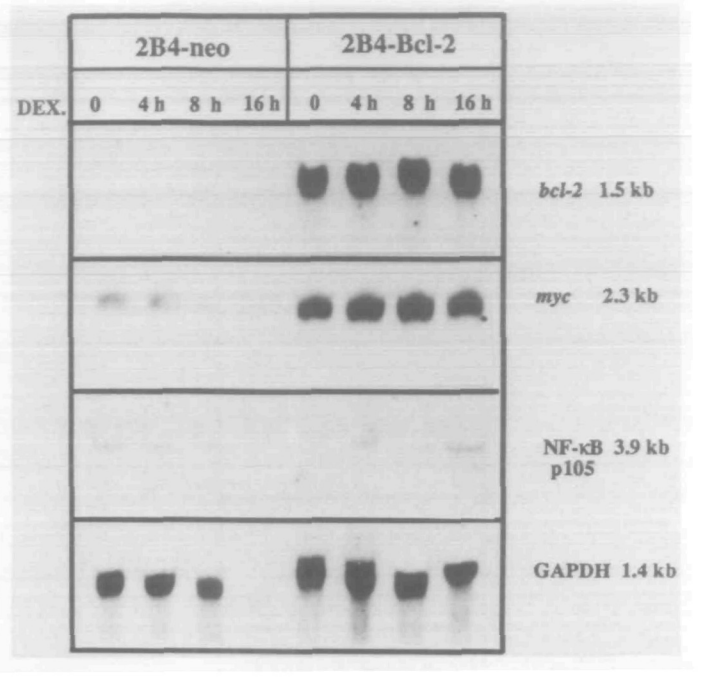


Fig. 1. Time-course analysis of specific mRNA levels in 2B4-neo and 2B4-Bcl-2 cells after Dex treatment. Northern hybridization with labeled probes for *bcl-2*, c-*myc*, NF-κB p105 and GAPDH mRNA. Total mRNA was isolated from 2×10^6 cells which were untreated (0) or stimulated with 1 μM Dex for 4–16 h.

Results

Transcription factor activities in 2B4 cells transfected with bcl-2 cDNA

To begin to determine the molecular basis by which Bcl-2 prevents apoptotic cell death, the 2B4 T cell hybridoma was stably transfected with *bcl-2* cDNA in the pBMGneo expression vector. Northern blot analysis of resulting drug-resistant cells (2B4-Bcl-2) demonstrated high levels of expression of transfected *bcl-2* mRNA when compared with 2B4 cells transfected only with the unmodified vector pBMGneo (2B4-neo) that served as control cells in this study. The levels of GAPDH mRNA were similar for both untreated cells, indicating that electrophoretic lanes contained similar quantities of RNA. Interestingly, the levels of c-*myc* mRNA were selectively enhanced in 2B4-Bcl-2 while levels of NF-κB p105 mRNA were similar for both cells (Fig. 1).

To determine the extent by which Bcl-2 affects gene expression, we examined DNA binding activity of several transcription factors important for T cell activation in nuclear extracts from 2B4-Bcl-2 and 2B4-neo by EMSA. Over-expression of *bcl-2* in unstimulated 2B4 cells enhanced the background levels of AP-1, Oct-1, LEF-1, the upper and lower NF-κB binding complexes, which were identified with specific antisera as p65–p50 (RelA–p50) and p50–p50 complexes, whereas cAMP response element binding protein (CREB) and NF-Y levels were relatively stable (Fig. 2). Although the basal levels of NF-κB p65–p50 were quite variable for 2B4-neo cells, 2B4-Bcl-2 always contained higher basal levels of this heterodimer. The second band detected with the NF-Y probe in extracts from 2B4-Bcl-2 cells comprises an additional CCAAT binding protein immunochemically distinct from NF-Y. The LEF-1 complex was identified by inhibition of DNA binding with a specific antibody. The upper AP-1 complex was identified by use of specific antibodies as a Jun–Fos heterodimer whereas the lower AP-1 complex was a Jun–Jun homodimer (data not shown). A 'non-canonical' octamer binding site from the IL-2 promoter was used because this probe may reveal more subtle differences in the regulation of Oct factors than intact Oct probe. Oct-1 was the only factor of the octamer binding protein family which was detected in 2B4-neo cells. For 2B4-Bcl-2 cells, a strong additional, but non-specific, activity was detected, as shown by the absence of competition with an unlabeled octamer oligonucleotide. The significant finding from EMSA experiments was that the over-expression of *bcl-2* led to enhanced nuclear activity for NF-κB p50–p50, p65–p50 and for several other factors without external cellular activation. Over-expression of *bcl-2* in the EL4 thymoma cell line also up-regulated the basal level of both NF-κB p50–p50 and p65–p50 (Fig. 2, lanes 5–7).

When compared with unstimulated cells, enhanced levels of nuclear NF-κB p65–p50, p50–p50, AP-1, LEF-1 and Oct-1 were observed 6 h after activation of 2B4-neo and 2B4-Bcl-2 by PMA and ionomycin. Activated 2B4-Bcl-2 cells contained the highest levels of these DNA binding activities. Similar results for NF-κB p65–p50 were observed after treatment of these cells with anti-CD3ε mAb (data not shown).

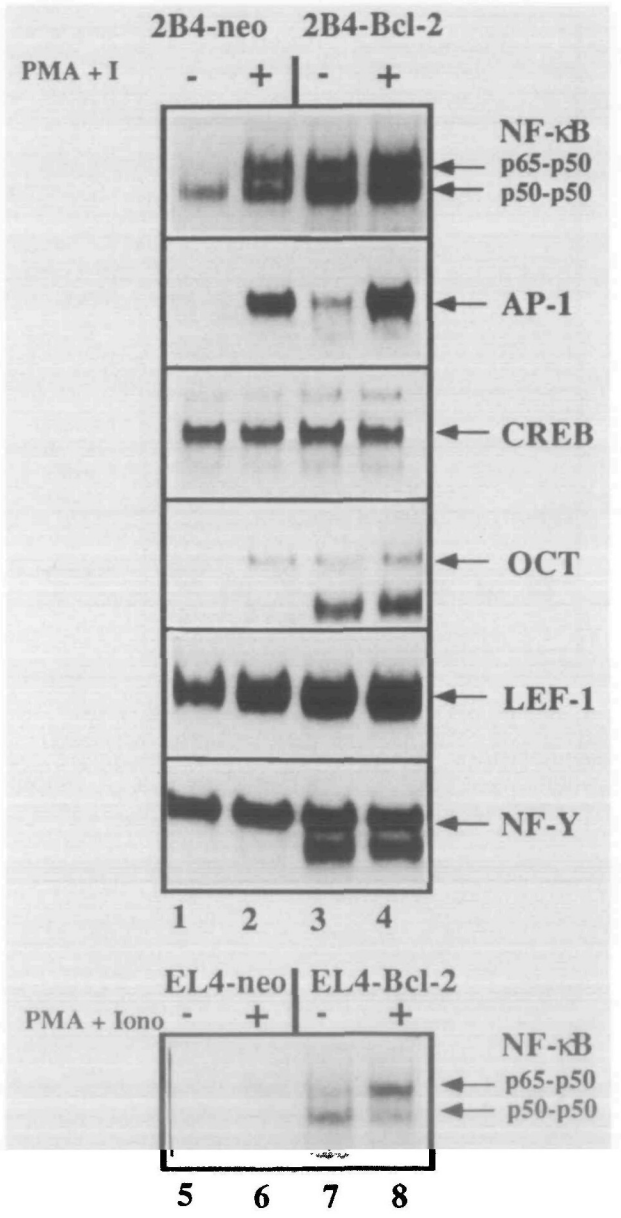


Fig. 2. Nuclear transcription factors in control and *bcl-2*-transfected 2B4 and EL-4. EMSA were performed with nuclear extract proteins (5 μg) from cells, that were not treated or stimulated for 6 h with PMA (10 ng/ml) plus ionomycin (1 μM), by use of labeled oligonucleotide probes for specific transcription factors. Specific DNA binding complexes are indicated by arrows. Bands of free labeled oligonucleotide probes are not shown.

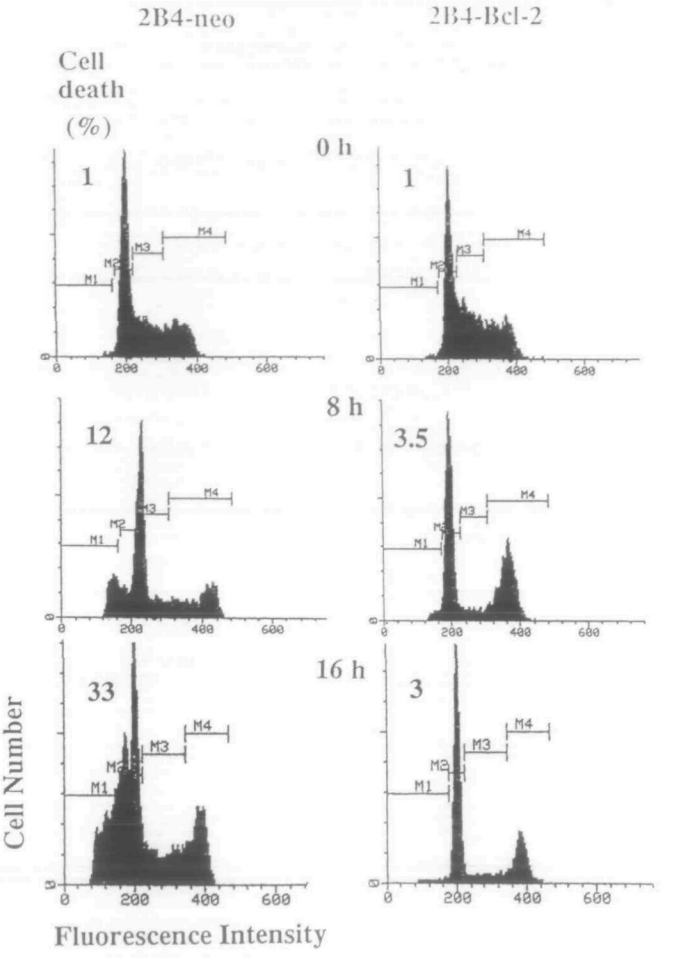


Fig. 3. Cell cycle analysis of 2B4-neo and 2B4-Bcl-2 cells after Dex treatment. The DNA content in the cells was determined 0, 8 and 16 h after Dex treatment by propidium iodide staining and flow cytometry. Gates M1, M2, M3 and M4 identify apoptotic cells with hypodiploid DNA content, stages G_1, S and G_2/M of the cell cycle respectively.

Selective regulation of transcription factor levels in 2B4-neo and 2B4-Bcl-2 by Dex

Previous studies have demonstrated that Dex induces apoptosis of 2B4 (31). Over-expression of *bcl-2* in 2B4 inhibits this apoptosis (18). Cell cycle analysis revealed that the number of both 2B4-neo and 2B4-Bcl-2 cells in the S phase was significantly reduced, whereas increased level of apoptotic 2B4-neo with hypodiploid DNA content was detected (Fig. 3, gate M1). At 16 h after treatment, 2B4-Bcl-2 were viable but with cells arrested at G_1 and G_2/M phases, while >30% of 2B4-neo were detected in the area of dead cells with decreased DNA content (Fig. 3). Finally, 24 h after 1 μM Dex treatment, 80–90% of 2B4-neo underwent DNA fragmentation and apoptosis, whereas the majority of 2B4-Bcl-2 (85–95%) were viable (Fig. 4A, lanes 3 and 7). 2B4-Bcl-2 remained in a growth arrested state until 48 h after Dex treatment; thereafter cell proliferation ensued at a minimal level (data not shown). In contrast to Dex treatment, DNA fragmentation and apoptosis of both 2B4-neo and 2B4-Bcl-2 were induced 24 h after stimulation by anti-CD3ε (Fig. 4A, lanes 2 and 6).

To determine whether transcription factors were selectively regulated by Bcl-2 after apoptotic signaling, we examined binding activity of several transcription factors 24 h after Dex treatment in 2B4-neo and 2B4-Bcl-2. EMSA revealed that AP-1, LEF-1 and Oct-1 almost completely disappeared from

nuclear extracts of dying 2B4-neo, whereas NF-Y and CREB remained at relatively high levels (Fig. 4B). By contrast, the regulation of NF-κB was more complex after Dex treatment. For 2B4-neo, a strong down-regulation of both NF-κB p65–p50 and p50–p50 activities was noted, while a new NF-κB-related band with the intermediate mobility was detected (Fig. 4B, center arrow).

Similar experiments with nuclear extracts from 2B4-Bcl-2 revealed down-regulation of AP-1, Oct-1 and LEF-1, although changes of these transcription factor levels were not so dramatic when compared with 2B4-neo. Very low levels of NF-AT activity were detected in 2B4-neo and this binding activity completely disappeared after Dex treatment. By contrast, NF-AT activity was readily detected in untreated 2B4-Bcl-2 and remained at decreased, but detectable, levels after Dex treatment (Fig. 4B). The most remarkable changes were revealed for NF-κB complexes in 2B4-Bcl-2. Similar to 2B4-neo, the p65–p50 complex was almost undetectable 24 h after Dex treatment of 2B4-Bcl-2, whereas a band with intermediate mobility was observed. Furthermore, compared with untreated cells, the level of p50–p50 complex in Dex treated 2B4-Bcl-2 was obviously up-regulated (Fig. 4B).

The nuclear extracts were pretreated with NF-κB subunit-specific antibodies prior to EMSA to identify the constituents of NF-κB complexes. Based on detection of either inhibition and/or supershift of bands, the upper band mainly was composed of RelA–p50, the lower complex was p50–p50 and the intermediate band was primarily RelB–p50; also there may be a minor combination of RelB with p52 subunit (Fig. 4C). When RelA–p50 was present at a high level, both RelA–p50 and RelB–p50 complexes migrate as a single band and their unambiguous presence was determined by detection of specific supershifted bands. By this approach, RelB–p50 binding activity was higher for untreated 2B4-Bcl-2 compared with 2B4-neo. The anti-p50 serum had a pronounced, but not complete, effect on the inhibition/supershift of the upper band. This minor resting activity may represent the p65–p52 heterodimer (Fig. 4C).

Time-course studies revealed that down-regulation of the levels of NF-κB and AP-1 transcription factors were detected 4 h after Dex treatment of 2B4-neo (Fig. 4D) when 90% of the cells were viable. At 8 h after Dex treatment, low levels of these factors were observed that correlated with inhibition of cell growth. By contrast, between 8 and 16 h after Dex treatment, when 2B4-neo viability dropped dramatically, increased levels of RelB–p50 activity were detected. For Dex-treated 2B4-Bcl-2, a decrease in the level of RelA–p50 occurred over time. However, an increase of p50–p50 as well as RelB–p50 activity was noted between 8 and 16 h (Fig. 3C).

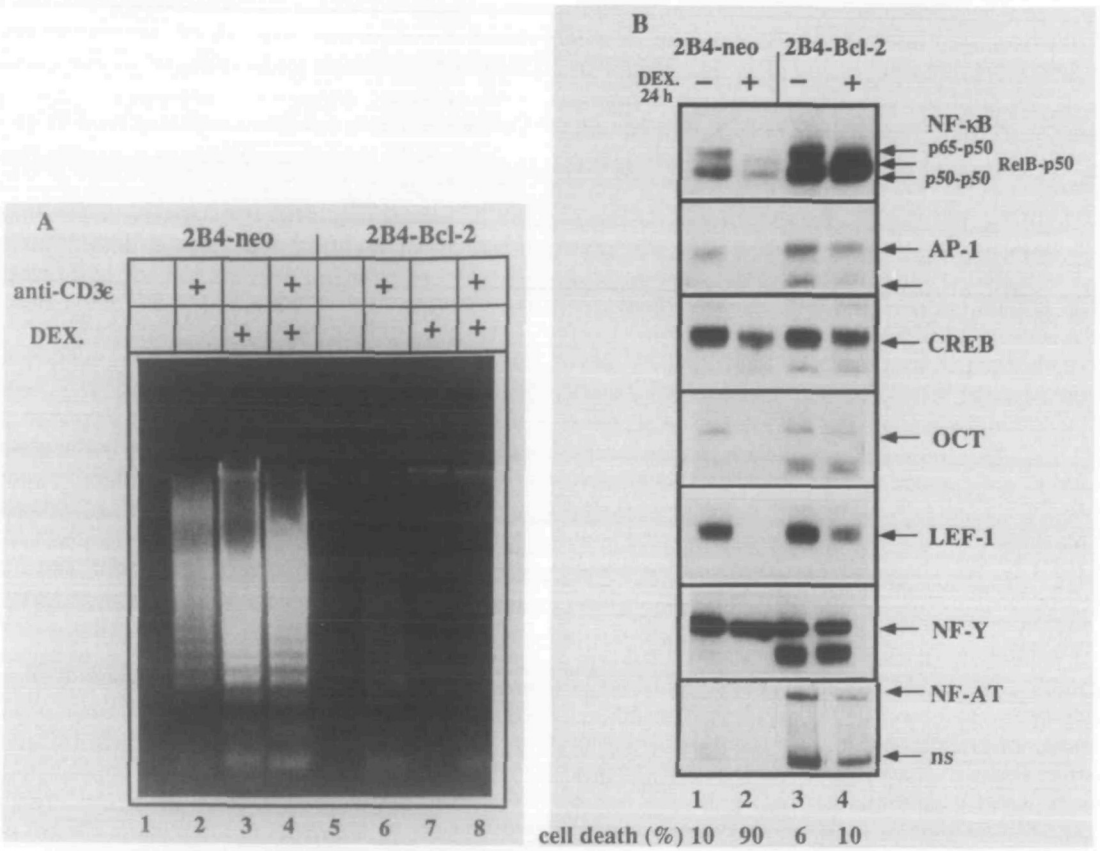
The effects of Bcl-2 on the steady-state levels of NF-κB p50 and RelB proteins and mRNAs

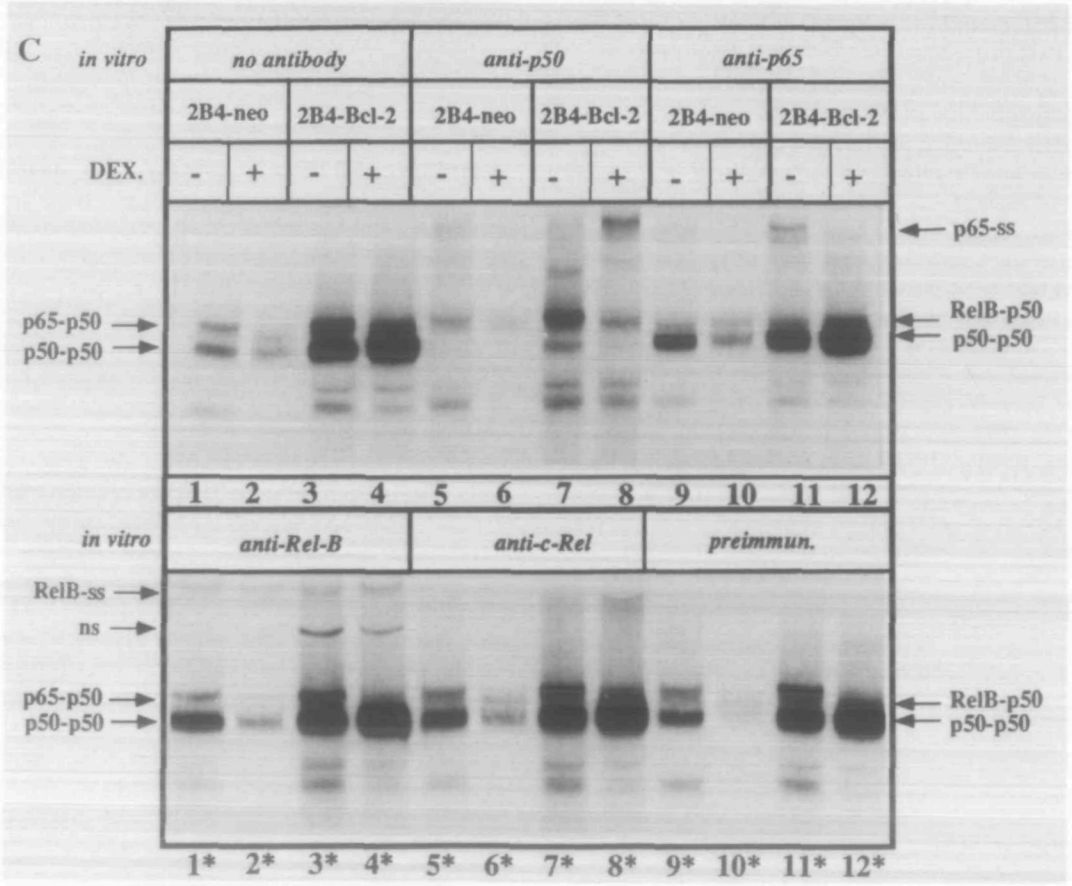
It was somewhat surprising that over-expression of *bcl-2* in 2B4 cells resulted in a large increase of NF-κB p50–p50 and RelB–p50 binding activity considering that the levels of p105 mRNA, that encodes the precursor for NF-κB p50, were similar for both untreated 2B4-neo and 2B4-Bcl-2. Furthermore, the levels of NF-κB p105, c-*myc*, *bcl-2* and GAPDH mRNAs remained constant in 2B4-Bcl-2 up to 16 h after Dex treatment, whereas c-*myc* mRNA levels strongly decreased 8 h after Dex treatment in control 2B4-neo (see Fig. 1). To determine whether the increased binding activity of NF-κB p50–p50 and RelB–p50 reflected an increase in protein levels of p50 and RelB subunits, Western blot analysis of nuclear proteins was performed using specific antibodies (Fig. 5). Higher levels of nuclear RelB protein were detected for 2B4-Bcl-2 than 2B4-neo with the highest levels seen 8–24 h after Dex treatment for 2B4-Bcl-2 (Fig. 5A). This increase in RelB nuclear protein essentially paralleled the binding activity of RelB–p50. By contrast, the nuclear levels of NF-κB p50 protein detectable by Western blotting were similar for both 2B4-neo and 2B4-Bcl-2 prior to Dex treatment (Fig. 5B), although an enhanced level of NF-κB p50–p50 DNA binding activity was detected by EMSA for unstimulated 2B4-Bcl-2. This finding suggests that the high basal level of NF-κB p50–p50 DNA binding activity in 2B4-Bcl-2 cells may be the result of releasing p50–p50 from a complex with a nuclear inhibitor without a change in the total nuclear levels of p50 protein. However, 24 h after treatment of 2B4-Bcl-2 with Dex, enhanced protein levels of NF-κB p50 were observed, consistent with increased p50 protein levels accounting for the highest levels of p50–p50 DNA binding activity (Fig. 5B).

NF-κB-dependent transcription in 2B4-neo and 2B4-Bcl-2

Since the EMSA experiments revealed not only up-regulation of basal levels of RelA–p50 and NF-κB p50–p50 complexes in 2B4-Bcl-2 cells but also selective regulation of distinct NF-κB DNA binding complexes after Dex treatment, it was of interest to examine the net effect on NF-κB-dependent transcription in these cells. After transient transfection with the –121-HIV–CAT construct (Fig. 6A), that contained two NF-κB binding sites, an ~2-fold higher basal level of NF-κB-dependent transcription occurred for 2B4-Bcl-2 when compared with 2B4-neo. This result correlated with the enhanced levels of both NF-κB complexes from nuclear extracts of unstimulated 2B4-Bcl-2, although the magnitude of this increase was not as substantial as noted for NF-κB DNA binding activity. Furthermore, after Dex stimulation of 2B4-neo, a somewhat increased level of NF-κB-dependent transcription was noted, whereas for 2B4-Bcl-2, Dex treatment resulted in ~2-fold decrease in CAT activity when compared with unstimulated cells. This result also correlated with the selective decrease in RelA–p50 and the increase in NF-κB p50–p50 binding activity, which acts as a negative regulator of NF-κB-dependent transcription. As a positive control for NF-κB-dependent transcription, anti-CD3ε stimulation enhanced CAT activity for both 2B4-neo and 2B4-Bcl-2 (Fig. 6A). In addition, no induction of CAT activity was detected for cells transfected with the –76-HIV–CAT construct, that lacks the NF-κB binding sites, demonstrating specificity of the assay (not shown).

To further examine the effect of Bcl-2 on regulation of gene transcription, 2B4-neo and 2B4-Bcl-2 were transiently transfected with the IL-2–CAT construct (original name pIL-CAT2/1+) which contains the promoter/enhancer region of the mouse IL-2 gene, while the variant of this construct pILCAT2/1+(205/6A) had mutations in the NF-κB binding site (32). These constructs were chosen because CAT activity directed by the IL-2 enhancer is dependent upon binding of several transcription factors, including NF-κB, NF-AT, AP-1 and Oct (28,33). The inducibility of this enhancer is very


A
2B4-neo
2B4-Bcl-2
anti-CD3ε
DEX.
1 2 3 4 5 6 7 8
B
2B4-neo
2B4-Bcl-2
DEX. 24 h
NF-κB
p65-p50
p50-p50
RelB-p50
AP-1
CREB
OCT
LEF-1
NF-Y
NF-AT
ns
1 2 3 4
cell death (%) 10 90 6 10


C
in vitro
no antibody
anti-p50
anti-p65
2B4-neo
2B4-Bcl-2
DEX.
p65-ss
p65-p50
p50-p50
RelB-p50
1 2 3 4 5 6 7 8 9 10 11 12
in vitro
anti-Rel-B
anti-c-Rel
preimmun.
RelB-ss
ns
1* 2* 3* 4* 5* 6* 7* 8* 9* 10* 11* 12*

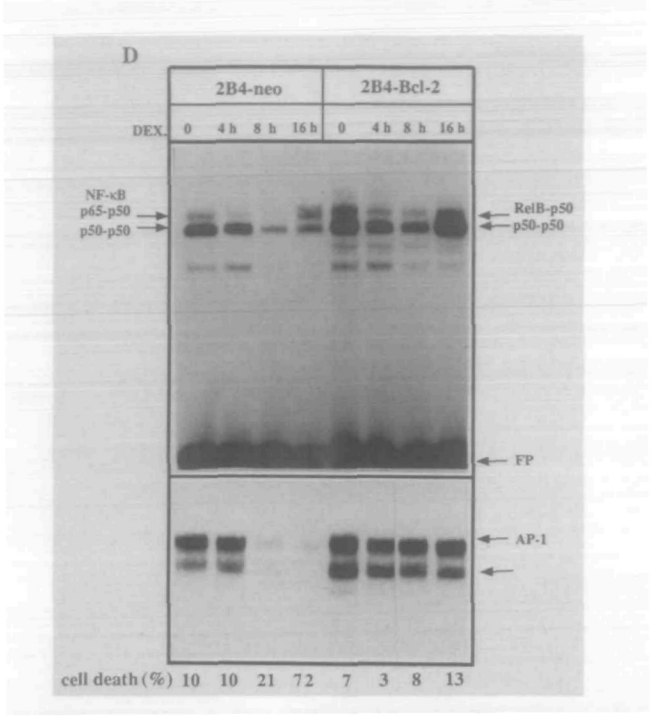


Fig. 4. Changes in the levels of nuclear transcription factors after treatment of 2B4-neo and 2B4-Bcl-2 cells with 1 μM Dex. (A) DNA fragmentation analysis of cytosolic extracts of control and Dex-treated cells by gel electrophoresis in 1% agarose gel (B) EMSA of nuclear extracts from indicated cells 24 h after Dex treatment. Specific DNA binding complexes are shown by arrows. The percentage of viable cells was determined by Trypan blue exclusion. (C) Identification of NF-κB DNA binding complexes from untreated or Dex-stimulated 2B4-neo and 2B4-Bcl-2. Nuclear extracts were preincubated before EMSA with antisera to NF-κB p50, NF-κB p65 (RelA), RelB, c-Rel or preimmune serum, as indicated above the lanes Bands which were supershifted with anti-p65 or anti-RelB are design as RelA-ss and RelB-ss respectively. (D) Time-course analysis of nuclear transcription factor NF-κB and AP-1 levels by EMSA after Dex treatment

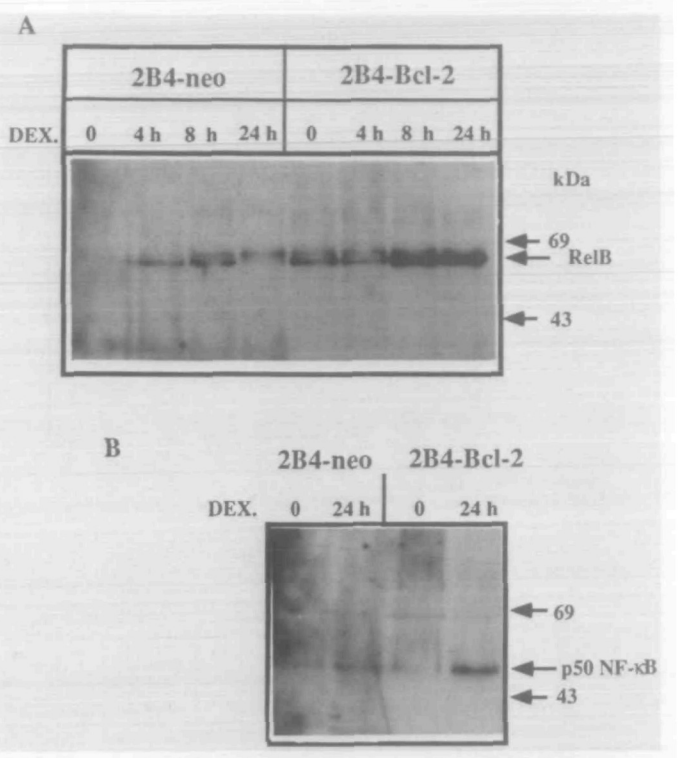


Fig. 5. Western blot analysis of RelB (A) and NF-κB p50 proteins (B) in 2B4-neo and 2B4-Bcl-2 cells after Dex treatment. Nuclear extracts were prepared at the different time points after Dex treatment of 2B4-neo and 2B4-Bcl-2 cells as indicated above the lanes. Molecular weights of the protein markers are shown at the right side.

sensitive to the increase in binding activity of nuclear NF-κB p50–p50 as the NF-κB binding site of the IL-2 promoter has a very high affinity for NF-κB p50–p50 and low affinity for RelA–p50 (3,10). For the IL-2–CAT construct (Fig. 6B) CAT activity was up-regulated in 2B4-neo after anti-CD3ε treatment while co-stimulation with Dex somewhat down-regulated this induced level. Dex alone slightly increased the basal level of CAT activity. By contrast, unaltered minimal levels of CAT activity were detected for 2B4-Bcl-2 after Dex treatment or anti-CD3ε stimulation, probably due to high levels of NF-κB p50–p50 in the nuclear fraction from these cells. The control construct with a mutated NF-κB binding site (pILCAT2/1+(205/6A) was not inducible in both 2B4-neo and 2B4-Bcl-2 (data not shown). Furthermore, the level of IL-2 secretion detected by a standard bioassay was 45–50% less for anti-CD3ε-activated 2B4-Bcl-2 compared with similarly activated 2B4-neo.

Cell death levels of 2B4 clones stably transfected with a NF-κB p50 expression vector after dexamethasone treatment

To determine whether Bcl-2 regulation of NF-κB p50–p50 levels is directly related to protection of cells from Dex-induced apoptosis, 2B4 clones were stably transfected with pRc/CMV-p50, a NF-κB p50 expression vector. Transfected cells were selected for G418 resistance and expression of NF-κB p50 mRNA, which was distinguishable from endogenous p105 precursor mRNA by Northern blot analysis. As shown by EMSA, two clones, 2B4-p50(A9) and 2B4-p50(B4), were characterized by a 2-fold increase in the nuclear p50–p50/p65–p50 ratio compared with control 2B4-neo(2) and 2B4-neo(3) cells, which were transfected by vector alone (Fig. 7A). Cell cycle analysis 16 h after Dex treatment revealed that the percentage of apoptotic cells with hypodiploid DNA content was somewhat decreased for 2B4-p50(A9) and 2B4-p50(B4) compared with control cells, although cell death levels were higher than observed for 2B4-Bcl-2 cells (Fig. 7B). The average percentage of apoptotic cells from three experiments was 14 ± 8, 31 ± 10, 42 ± 4 and 53 ± 3% respectively. These results are consistent with a potential role of NF-κB p50 in the down-regulation of cell death levels.

Regulation of transcription factor levels in CTLL-2 over-expressing bcl-2

Previous studies have shown that over-expression of *bcl-2* in the IL-2-dependent CTLL-2 cell line substantially delayed apoptosis induced by IL-2 withdrawal from culture (22). To determine whether Bcl-2 also affected transcription factor binding activities in a cell system other than 2B4, we examined levels of transcription factors by EMSA for control (CTLL-

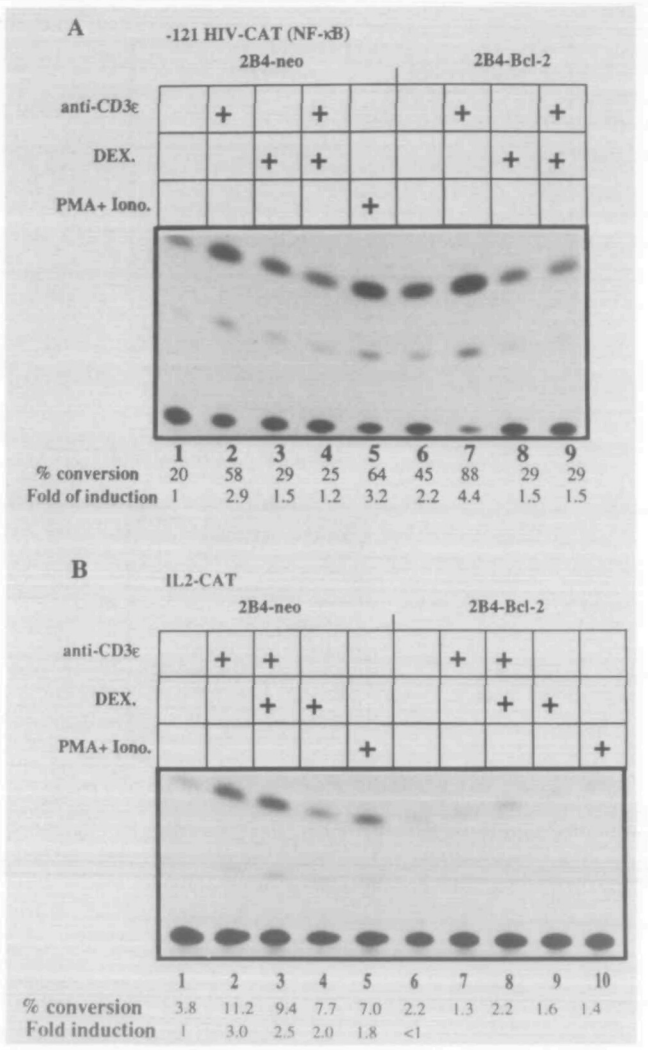


Fig. 6. CAT activity directed by NF-κB-dependent CAT construct (A) and IL-2–CAT (B) in 2B4-neo and 2B4-Bcl-2. Cells were transfected with –121-HIV–CAT construct containing two NF-κB binding sites (A) or with IL-2–CAT (B), and then were stimulated with anti-CD3ε, Dex or PMA plus ionomycin as indicated above the lanes. Rsv-β-gal was used as an internal standard to normalize efficiency of transfection. One representative experiment of three is shown Average induction level of CAT activity from three experiments based on the ratio of conversion of chloramphenicol to its acetylated form is shown at the bottom

neo) or *bcl-2*-transfected CTLL-2 (CTLL-Bcl-2). At 24 h after removal of IL-2 from culture, DNA fragmentation and apoptotic death of ~50% CTLL-neo occurred, whereas the CTLL-Bcl-2 survived and were arrested in the G_0/G_1 and the G_2/M phases of the cell cycle (22). Down-regulation of several transcription factors, including RelA–p50, NF-κB p50–p50, AP-1, NF-AT and Oct-1, was observed for CTLL-neo upon induction of apoptosis, whereas CREB and NF-Y were readily detected. The changes in the levels of AP-1 and NF-AT were less pronounced for CTLL-Bcl-2. Furthermore, RelA–p50 and Oct-1 were down-regulated while NF-κB p50–p50 and Oct-2 were substantially up-regulated in CTLL-Bcl-2 (Fig. 8A).

Time-course analysis of transcription factor levels demonstrated detectable down-regulation of RelA–p50 and NF-κB p50–p50 levels as early as 2 h after IL-2 withdrawal from culture when most of the CTLL-neo (95–97%) were viable (Fig. 8B). The levels of RelA–p50 and AP-1 levels gradually continued to decline, whereas NF-κB p50–p50 almost disappeared 4 h after IL-2 withdrawal from culture. By contrast, NF-κB p50–p50 levels began to increase for CTLL-Bcl-2 cells 16 h after IL-2 withdrawal from culture. These experiments indicate that Bcl-2 caused pleiotropic effects on transcription factors in CTLL-Bcl-2 after apoptotic signaling. Nevertheless, the common feature of the over-expression of *bcl-2* for both 2B4 and CTLL2 was up-regulation of NF-κB p50–p50 levels after apoptotic signaling that reduced the RelA–p50/p50–p50 ratio, and maintenance of suboptimal levels AP-1, NF-AT and Oct that probably rescued 2B4-Bcl-2 from 'transcription factor exhaustion'

Discussion

Recent studies have established that Bcl-2 functions as an important factor that represses cell death (13,17) The anti-apoptotic effect of Bcl-2 has been suggested to ultimately affect gene regulation in cells by preventing activation of a key gene(s) that encodes proteins required for programmed cell death. As a first approximation of this notion, in this study we have examined the extent by which the expression of *bcl-2* regulates the levels of several transcription factors that are known to function in T cell activation. Two important points emerge from this study. First, over-expression of *bcl-2* resulted in pleiotropic effects on transcription factors as the levels of several transcription factors were enhanced in the untreated 2B4 T cell hybridoma. Second, transcription factors of NF-κB/Rel family as well as AP-1, NF-AT and Oct were differentially regulated after 2B4 and CTLL2 received an apoptotic signal.

The 2B4 T cell hybridoma was used as the primary cell model system since the activation of these cells is well characterized and 2B4 is susceptible to apoptosis after cross-linking the TCR or after treatment with PMA and ionomycin, glucocorticoids or γ-irradiation (31,34). Over-expression of *bcl-2* in 2B4 resulted in a large increase in c-*myc* mRNA and enhanced basal levels of AP-1, LEF-1, Oct-1 as well as RelA–p50 and NF-κB p50–p50 nuclear transcription factor binding activities. These data demonstrated that several transcription factors that are induced upon TCR stimulation are enhanced by Bcl-2, presumably due to effects on the intracellular signaling pathways of Bcl-2. It is clear from our data that these enhanced transcription factor levels are not a common property of all T cells that express high levels of Bcl-2. Although we have noted that untreated *bcl-2*-transfected EL4 also contained high constitutive levels of RelA–p50 and NF-κB p50–p50, the levels of RelA–p50, NF-κB p50–p50 and AP-1 are high in both control and *bcl-2*-transfected CTLL. This finding raises the possibility that the potential effects of Bcl-2 on preactivation of transcription factors may be related to a distinct subset of T cells or T cells in a particular phase of activation or differentiation.

We observed three instances of selective changes in NF-κB levels in 2B4 and CTLL that raise the possibility that the NF-κB/Rel family of transcription factors may play an important

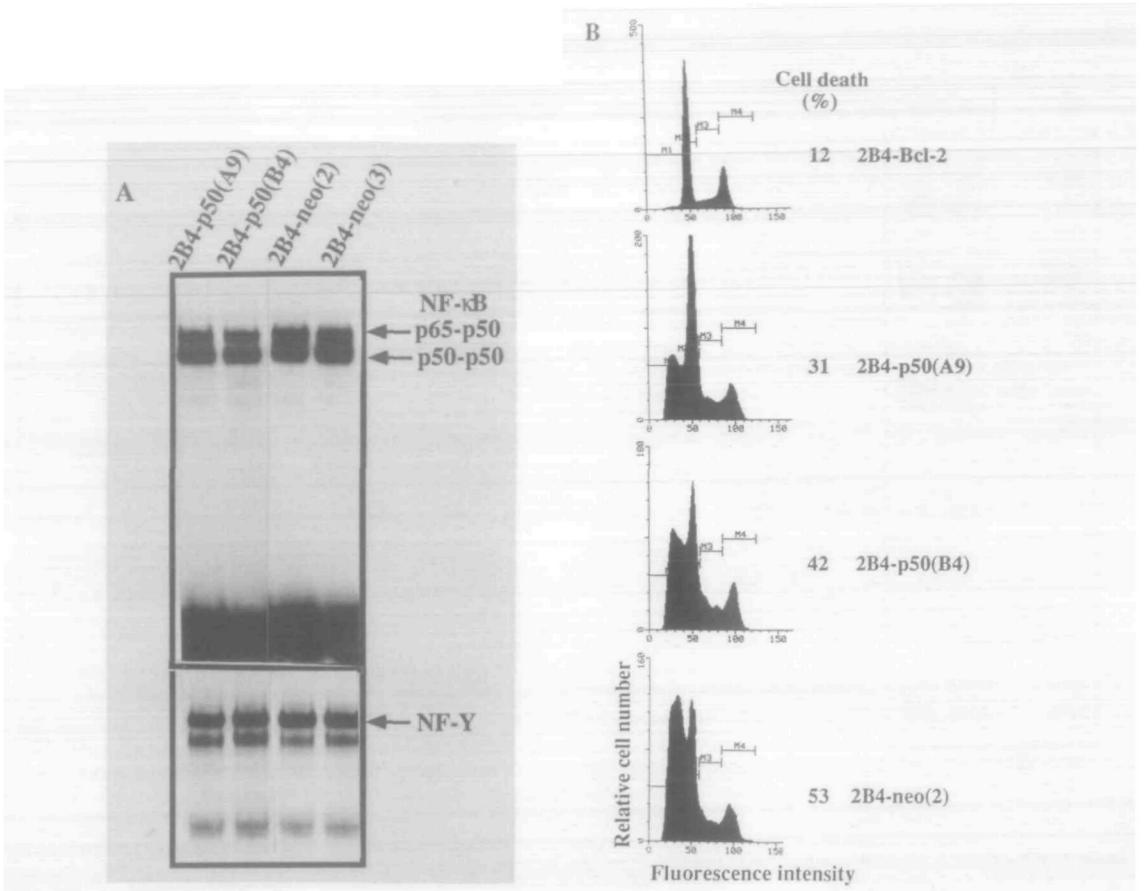


Fig. 7 Cell death levels 16 h after Dex treatment of 2B4 clones stably transfected with a NF-κB p50 expression vector. (A) EMSA of nuclear proteins from 2B4-p50 clones and control cell lines, 2B4-neo(2) and 2B4-neo(3), with labeled probes for NF-κB and NF-Y. (B) Cell cycle analysis of 2B4-p50 clones. The DNA content in the cells was determined by propidium iodide staining and flow cytometry. Gates M1, M2, M3 and M4 identify apoptotic cells with hypodiploid DNA content, stages G_1, S and G_2/M of the cell cycle respectively. One representative experiment is shown.

role in regulation of apoptosis First, after 2B4 received an apoptotic signal via the steroid hormone Dex, RelB DNA binding activity was induced while all other transcription factors tested decreased, including RelA–p50 and NF-κB p50–p50. The presence of RelB might contribute to induction of new gene transcription, perhaps including a cell death gene or may promote to the switch from proliferation to cell arrest. Second, the nuclear levels of RelA–p50 in 2B4-neo and 2B4-Bcl-2 as well as in CTLL-neo and CTLL-Bcl-2 substantially decreased after the cells received apoptotic signals, whereas most other transcription factors were maintained at relatively high levels in the presence of Bcl-2. Lastly, and perhaps most importantly, the levels of NF-κB p50–p50 binding activity substantially decreased in 2B4-neo and CTLL-neo shortly after receiving apoptotic signals. However, p50–p50 activity significantly increased in 2B4-Bcl-2 and CTLL-Bcl-2 16–24 h after apoptotic treatment. These latter two findings indicate that a consequence of high Bcl-2 expression in these cells is to alter the ratio of NF-κB *trans*-activators (RelA–p50 and RelB–p50) to NF-κB p50–p50 *trans*-inhibitor.

These Bcl-2-induced changes in nuclear factor levels are functionally relevant since they correlate with the extent of NF-κB-dependent transcription *in vivo*. The large basal increase in nuclear levels of NF-κB p50–p50 after transfection of 2B4 with *bcl-2* was not accompanied by increased levels of NF-κB p105 mRNA, that encodes the precursor for p50 (7), or increased amounts of nuclear p50 protein. After Dex stimulation of 2B4-Bcl-2, the levels of p105 mRNA were stable, although enhanced levels of p50 protein were detected in the nucleus, which correlated with increase DNA binding activity. These finding are consistent with a post-transcriptional mechanism that is primarily responsible for the regulation of NF-κB p50–p50 via Bcl-2. The increased levels of basal NF-κB p50–p50 binding activity in nuclear extracts of unstimulated 2B4-Bcl-2 before apoptotic commitment may be explained by Bcl-2-dependent down-regulation of the protooncogene *bcl-3* as this protein is primarily localized to the nucleus and functions as an IκB-like molecule with high affinity for NF-κB p50–p50 (11,35,36). Low levels of activity of Bcl-3 or a similar nuclear IκB-like inhibitor are predicted to lead to higher levels of uncomplexed nuclear p50–p50 that may result in higher binding of p50–p50 to DNA without need for increased nuclear p50 protein. By contrast, the increase in p50–p50 DNA binding activity upon Dex stimulation cannot be solely accounted for

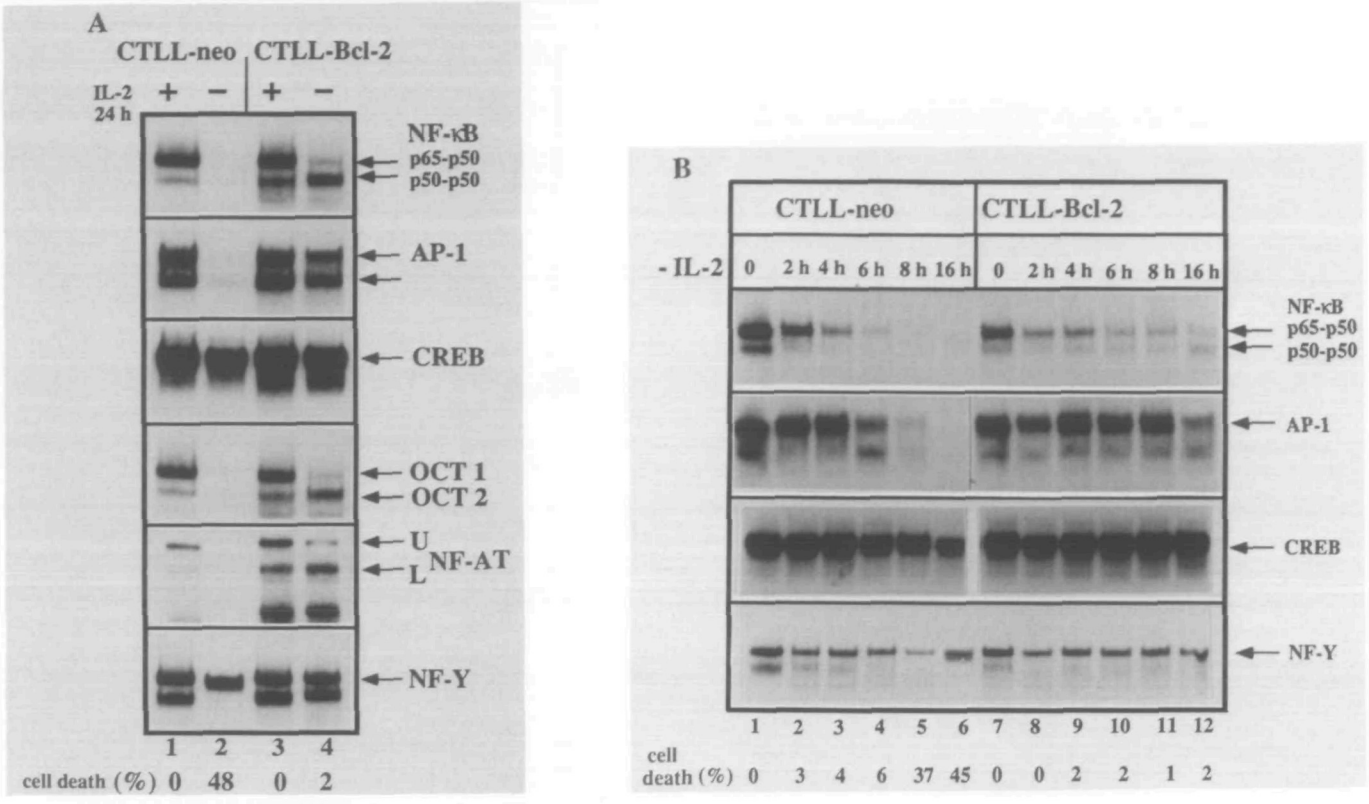


Fig. 8. Changes in the levels of nuclear transcription factors in CTLL-neo and CTLL-Bcl-2 after IL-2 withdrawal from cell culture. (A) EMSA of nuclear extracts from control cells and cells 24 h after IL-2 withdrawal from culture (IL-2, –). Specific DNA binding complexes are shown by arrows. (B) Time-course analysis of NF-κB and AP-1 nuclear transcription factor levels by EMSA after IL-2 withdrawal from culture The percentage of viable cells was determined by Trypan blue exclusion.

by such a mechanism and may be related to the capacity of Bcl-2 to increase the processing of the p105 precursor by the ubiquitin-proteasome pathway (37). Additionally, we generated 2B4 clones stably transfected with a NF-κB p50 expression vector. As the nuclear ratio of p50–p50/p65–p50 was increased in these cells, elevated levels of cell survival were noted after Dex treatment. These experiments suggest that regulation of NF-κB p50–p50 levels is directly related to cells surviving after apoptotic signaling.

It is highly likely that Bcl-2 interacts at several levels with the intracellular signaling pathways to regulate the levels of NF-κB and other transcription factors. Simply considering NF-κB, there are several examples whereby expression of *bcl-2* affects cellular activity that may be expected to lead to regulation of the levels of NF-κB. This includes the antioxidant effect of Bcl-2 (18,19) and the ability of Bcl-2 to associate with R-Ras (38) and Raf-1 (39), which are critical elements in several signaling pathways, including one that regulates activation of RelA–p50. Furthermore, Bcl-2 has been shown to repress apoptosis by inhibition of endoplasmic reticulum-associated Ca^{2+} fluxes (40), while the release RelA–p50 from IκB is partially dependent upon Ca^{2+} (41). It is obvious that the ability of Bcl-2 to affect an intracellular second messenger as Ca^{2+} or a down-stream signaling protein such as R-Ras and Raf-1 is almost certain to lead to regulation of multiple transcription factors in the cell. The pleiotropic effects on transcription factors we have noted here support this view. However, our data have not confirmed a direct antioxidant function of Bcl-2 on transcription factor regulation. In fact, we observed preactivation rather than inactivation of NF-κB and LEF-1, which is characteristic for antioxidant action (3,20). An observed preactivation of several transcription factors in the presence of Bcl-2 in 2B4 without any external stimulation appears to correspond to the recently identified pro-oxidant function of Bcl-2 (42).

Suppression of c-*myc* expression has been proposed as a critical step in glucocorticoid-induced apoptosis (43). The enhanced levels of c-*myc* mRNA in 2B4-Bcl-2 may be functionally relevant since in some cases Bcl-2 cooperates with c-Myc to overcome cell growth arrest (44). However, it should be stressed that c-Myc plays a distinct role in glucocorticoid-induced and activation-induced apoptosis. In the latter case, c-Myc promotes cell death (45). Furthermore, c-*myc* transcription is in part dependent upon NF-κB/Rel factors (46). Suppression of c-*myc* expression after Dex treatment of 2B4 and the absence of suppression in *bcl-2*-transfected cells may be regulated by differential effects of Bcl-2 on NF-κB/Rel factor activities after receiving apoptotic signals.

Our data has not established a direct role for NF-κB-dependent transcription in the regulation of cell death genes. However, the induction of RelB in 2B4 upon receiving an apoptotic signal and the selective inducible increase in NF-

κB p50–p50 by *bcl-2*-transfected 2B4 which was resistant to apoptosis strongly suggest that the NF-κB/Rel family is a prime candidate for transcription factors that participate in the regulation of apoptosis. Our working model is that upon receiving an apoptotic signal, NF-κB/Rel factors contribute to the induction of transcription of one or more key genes important for cell death of 2B4. One aspect of the anti-apoptotic function of Bcl-2 is to regulate NF-κB in a manner to inhibit expression of this gene by enhancing the nuclear levels of NF-κB p50–p50, which has been shown to represent DNA binding, but not *trans*-activating, function and sometimes serves as a very potent suppressor of NF-κB-dependent transcription (10–12). Although RelB was not detected in CTLL2 upon induction of apoptosis by IL-2 withdrawal, disbalance of the RelA–p50/p50–p50 ratio by increasing NF-κB p50–p50 levels in CTLL-Bcl-2 also correlated with suppression of apoptosis.

It is also clear from our data that the second aspect of anti-apoptotic effect of Bcl-2 is likely to involve other transcription factors, especially AP-1, that regulates genes which may not be dependent upon NF-κB. Transcription factors of the AP-1 family as well as c-Myc participate in the control of cell proliferation by an unknown mechanism (43,47,48). The observed down-regulation of AP-1 levels as a result of Dex treatment or absence of IL-2-dependent signals correlates with inhibition of cell proliferation. Others have shown a direct physical interaction between activated glucocorticoid receptor and the Jun subunit of AP-1 (49) or between glucocorticoid receptor and the NF-κB p65 and p50 subunits (50,51) that strongly inhibited specific AP-1 and NF-κB binding activities after Dex treatment. If such glucocorticoid receptor-mediated interaction in the absence of Bcl-2 caused cell death (14), over-expression of Bcl-2 promotes cell survival by the maintenance of minimal levels of several key transcription factors.

Acknowledgements

We thank Dr E. Serfling for reporter CAT plasmids, Dr M. J. Lenardo for anti-p50 NF-κB serum, Dr W. Greene for anti-p65 NF-κB sera, Dr N. Rice for anti-c-Rel serum, Dr R. Bravo for anti-RelB serum, Dr R. Grosschedl for anti-LEF-1 serum and Dr A. Israel for NF-κB p50 expression vector, and Dr E. Codias and Dr M. Lichtenheld for discussions. This work was supported by National Institute of Health grant RO1-CA46096.

Abbreviation

AP-1	activator protein-1
CAT	chloramphenicol acetyltransferase
CREB	cAMP response element binding protein
Dex	dexamethasone
EMSA	electrophoretic mobility shift assay
LEF-1	lymphoid enhancer binding factor-1
Oct	octamer binding factor
PMA	phorbol myristate acetate

References

1 Crabtree, G. R. and Clipstone, N. A. 1994. Signal transmission between the plasma membrane and nucleus of T lymphocytes. *Annu. Rev. Immunol.* 63: 1045.
2 Chen, D. and Rothenberg, E. V. 1994. Interleukin 2 transcription factors as molecular targets of cAMP inhibition: delayed inhibition kinetics and combinatorial transcription roles. *J. Exp. Med.* 179:931.
3 Baeuerle, P. A. and Henkel, T. 1994. Function and activation of NF-κB in the immune system. *Annu. Rev. Immunol.* 12:141.
4 Liou, H.-C. and Baltimore, D. 1993. Regulation of the NF-κB/rel transcription factor and IκB inhibitor system. *Curr. Opin. Cell Biol.* 5: 477.
5 Henkel, T., Machleidt, T., Alkalay, I., Kronke, M., Ben-Neriah, Y. and Baeuerle, P. A. 1993. Rapid proteolysis of IκB-α is necessary in the activation of transcription factor NF-κB. *Nature* 365:182.
6 Beg, A. A. and Baldvin, A. S. 1993. The IκB proteins: multifunctional regulators of Rel/NF-κB transcription factors. *Genes Dev.* 7:2064.
7 Kieran, M., Blank, V., Logeat, F., Vandekerckhove, J., Lottspeich, F., Bail, O. L., Urban, M. B., Kourilsky, P., Baeuerle, P. A. and Israel, A. 1990. The DNA binding subunit of NF-κB is identical to factor KBF1 and homologous to the *rel* oncogene product. *Cell* 62:1007.
8 Fujita, T., Nolan G. P., Ghosh, S. and Baltimore, D. 1992. Independent modes of transcriptional activation by the p50 and p65 subunits of NF-κB. *Genes Dev.* 6:775.
9 Kretschmar, M., Meisterernst, M., Scheidereit, C., Li, G. and Roeder, R. G. 1992. Transcriptional regulation of the HIV-1 promoter by NF-κB *in vitro*. *Genes Dev.* 6:761.
10 Kang, S.-M., Tran, A.-C., Grilli, M. and Lenardo, M. J. 1992. NF-κB subunit regulation in nontransformed $CD4^+$ T lymphocytes. *Science* 256:1452.
11 Franzoso, G., Bours, V., Park, S., Tomita-Yamagushi, M., Kelly, K. and Sibenlist, U. 1992. The candidate oncoprotein Bcl-3 is an antagonist of p50/NF-κB-mediated inhibition. *Nature* 359:339.
12 Plaksin, D., Baeuerle, P. A. and Eisenbach, L. 1993. KBF1 (NF-κB homodimer) acts as a repressor of $H\text{-}2K^b$ gene expression in metastatic tumor cells. *J. Exp. Med.* 177:1651.
13 Krammer, P. H., Behrmann, I., Daniel, P., Dhein, J. and Debatin, K.-M. 1994. Regulation of apoptosis in the immune system. *Curr. Opin. Immunol.* 6:279.
14 King, L. B. and Ashwell, J. D. 1993. Signaling for death of lymphoid cells. *Curr. Opin. Immunol.* 5:368.
15 Suda, T., Takahashi, T., Golstein, P. and Nagata, S. 1993. Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. *Cell* 75:1169.
16 Vaux, D. L., Haecker, G. and Strasser, A. 1994. An evolutionary perspective of apoptosis. *Cell* 76:777.
17 Williams, G. T. and Smith, C. A. 1993. Molecular regulation of apoptosis: genetic control of cell death. *Cell* 74:777.
18 Hockenbery, D. H., Oltvai, Z. N., Yin, X.-M., Milliman, C. L. and Korsmeyer, S. J. 1993. Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell* 75:241.
19 Kane, D. J., Sarafian, T. A., Anton, R., Hahn, H., Gralla, E. B., Valentine, J. S., Ord, T. and Bredesent, D. E. 1993. Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. *Science* 262:1274.
20 Ivanov, V. N., Merkenschlager, M. and Ceredig, R. 1993. Antioxidant treatment of thymic organ cultures decreases NF-κB and TCF1(α) transcription factors activities and inhibits αβ T cell development. *J. Immunol.* 151:4699.
21 Karasuyama, H. and Melchers, F. 1988. Establishment of mouse cell lines which constitutively secrete large quantities of interleukin 2, 3, 4 or 5, with modified cDNA expression vector. *Eur. J. Immunol.* 18:97.
22 Deng, G., and. Podack, E. R. 1993. Suppression of apoptosis in cytoxic T-cell line by interleukin 2-mediated gene transcription and deregulated expression of the protooncogene *bcl-2*. *Proc. Natl Acad. Sci. USA* 90:2189.
23 Leo, O., Foo, M., Sachs, D. H., Samelson, L. E. and Bluestone, J. A. 1987. Identification of monoclonal antibody specific for murine T3 polypeptide. *Proc. Natl Acad. Sci. USA* 84:1374.
24 Ballard, D. W., Dixon, E. P., Peffer, N. P., Bogerd, H., Doerre, S., Stein, B. and Greene, W. 1992. The 65-kDa subunit of human NF-κB functions as a potent transcriptional activator and a target

for v-Rel-mediated repression. *Proc. Natl Acad. Sci. USA* 89:1875.
25 Rice, N. R., MacKichan, M. L. and Israel, A. 1992. The precursor of NF-κB p50 has IκB-like functions. *Cell* 71:243.
26 Ryseck, R.-P., Bull, P., Takamiya, M., Bours, V., Siebenlist, U., Dobrzanski, P. and Bravo, R. 1992. RelB, a new Rel family transcription activator that can interact with p50-NF-κB. *Mol. Cell. Biol.* 12:674.
27 Travis, A., Amsterdam, A., Belanger, C , and Grosschedl, R. 1991. LEF-1, a gene encoding lymphoid-specific with protein, an HMG domain, regulates T cell receptor α enhancer function. *Genes Dev.* 5:880.
28 Randak, C., Brabletz, T., Hergenroether, M., Sobotta, I. and Serfling, E. 1990. Cyclosporin A suppresses the expression of the interleukin-2 gene by inhibiting the binding of lymphocyte-specific factors to the IL-2 enhancer. *EMBO J.* 9:2529.
29 Ivanov, V., Stein, B., Baumann, I., Dobbelaere, D. A., Herrlich, P. and Williams, R. O. 1989. Infection with intracellular protozoan parasite *Theleria parva* induces constitutively high levels of NF-κB in bovine T lymphocytes. *Mol. Cell. Biol.* 9:4677.
30 Ivanov, V., Fleming, T. and Malek, T. R. 1994. Regulation of nuclear transcription factor κB and AP-1 activities after stimulation of T cells via GPI-anchored Ly-6A/E1. *J. Immunol.* 153:2394.
31 Zacharchuk, C. M., Mercep, M., Chakraborti, P. K., Simons, S. S. and Ashwell, J. D. 1990. Programmed T lymphocyte death. Cell activation- and steroid-induced pathways are mutually antagonistic. *J. Immunol.* 145:4037.
32 Briegel, K., Hentsch, B., Pfeuffer, I. and Serfling, E. 1991. One base change abolished the T cell-restricted activity of κB-like protoenhancer element from IL-2 promoter. *Nucleic Acids Res.* 21:5929.
33 Ullman, K. S., Notrhrop, J. P., Admon, A. and Crabtree, G. R. 1993. Jun family members are controlled by a calcium-regulated, cyclosporin A-sensitive signaling pathway in activated T lymphocytes. *Genes Dev.* 7:188.
34 Ishida, Y., Agata, Y., Shibahara, K. and Honjo, T. 1992. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *EMBO J.* 11:3887.
35 Nolan, G. P., Fujita, T., Bhatia, K., Huppi, C., Liou, H.-C., Scott, M. L. and Baltimore, D. 1993. The *bcl-3* proto-oncogene encodes a nuclear IκB-like molecule that preferentially interacts with NF-κB p50 and p52 in phosphorylation-dependent manner. *Mol. Cell. Biol.* 13:3557.
36 Lenardo, M. and Siebenlist, U. 1994. Bcl-3-mediated nuclear regulation of the NF-κB *trans*-activating factor. *Immunol. Today* 15:145.
37 Palombella, V. J., Rando, O. J., Goldberg, A. L. and Maniatis, T. 1994. The ubiquitin-proteasome pathway is required for processing of the NF-κB1 precursor protein and the activation of NF-κB. *Cell* 38:773.
38 Fernandez-Sarabia, M. J. and Biscoff, J. R. Bcl-2 associates with the *ras*-related protein R-*ras* p23. *Nature* 366:274.
39 Wang, H.-G., Miyashita, T., Takayama, S., Sato, T, Torigoe, T., Krajewski, S., Tanaka, S., Hovely, L., Troppmair, J., Rapp, U. R. and Reed, J. C. 1994. Apoptosis regulation by interaction of Bcl-2 protein and Raf-1 kinase. *Oncogene* 9:2751.
40 Lam, M., Dubyak, G., Chen, L., Nunez, G., Miesfeld, R. L. and Distelhorst, C. W. 1994. Evidence that Bcl-2 represses apoptosis by regulating endoplasmic reticulum-associated Ca^{2+} fluxes. *Proc. Natl Acad. Sci. USA* 91:6569.
41 Frantz, B., Nordby, E. C., Bren, G., Steffan, N., Paya, C. V., Kincaid, R. L., Tocci, M. J., O'Keefe, S. J. and O'Neill, E. A. 1994. Calcineurin acts in synergy with PMA to inactivate IκB/MAD3, an inhibitor of NF-κB. *EMBO J.* 13:861.
42 Steinman, H. M. 1995. The Bcl-2 oncoprotein functions as a pro-oxidant. *J. Biol. Chem.* 270:3491.
43 Thulasi, R.,. Harbour, D. V, and Thompson, E. B. 1993. Suppression of *c-myc* is a critical step in glucocorticoid-induced human leukemic cell lysis. *J. Biol. Chem.* 268: 18306.
44 Nunez, G. and Clarke, N. F. 1994. The Bcl-2 family of proteins: regulators of cell death and survival. *Trends Cell Biol.* 4:399.
45 Green, D., Mahboubi, A., Nishioka, W., Oja, S., Echeverri, F., Shi, Y., Glynn, J., Yang, Y., Ashwell, J. and Bissonnette, R. 1994. Promotion and inhibition of activation-induced apoptosis in T cell hybridomas by oncogenes and related signals. *Immunol. Rev.* 142:320.
46 La Rosa, F. A., Pierce, J. W. and Sonenshein, G. E. 1994. Differential regulation of the *c-myc* oncogene promoter by the NF-κB Rel family of transcription factors. *Mol. Cell. Biol.* 14:1039.
47 Angel, P. and Karin, M. 1991. The role of Jun, Fos and the AP-1 complex in cell proliferation and transformation. *Biochim. Biophys. Acta* 1072:129.
48 Helmberg, A., Auphan, N., Caelles, C. and Karin, M. 1995. Glucocorticoid-induced apoptosis of human leukemic cells is caused by the repressive function of the glucocorticoid receptor. *EMBO J.* 14:452.
49 Yang-Yen, H.-F., Chambard, J.-C., Sun, Y.-L., Smeal, T., Schmidt, T. J., Drouin, J. and Karin, M. 1990. Transcriptional interference between c-Jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein–protein interaction. *Cell* 62:1205.
50 Ray, A. and Prefontaine, K. E. 1994. Physical association and functional antagonism between the p65 subunit of transcription factor NF-κB and the glucocorticoid receptor. *Proc. Natl Acad. Sci. USA* 91:752.
51 Schenman, R. I., Gualberto, A., Jewell, C. M., Cidlowski, J. A. and Baldwin, A. S. 1995. Characterization of mechanisms involved in transrepression of NF-κB by activated glucocorticoid receptors. *Mol. Cell. Biol.* 15:943.