Bcl-2 Down-regulates the Activity of Transcription Factor NF-κB Induced upon Apoptosis

Stefan Grimm, Manuel K.A. Bauer, Patrick A. Baeuerle, and Klaus Schulze-Osthoff

Institute of Biochemistry and Molecular Biology, Albert-Ludwigs-University, D-79104 Freiburg, Germany

Abstract. Among the many target genes of the transcription factor NF-κB are p53 and c-myc, both of which are involved in apoptosis. This prompted us to investigate the role of NF-κB in this process. We report that NF-κB is potently activated upon serum starvation, a condition leading to apoptosis in 293 cells. Similar to Bcl-2, a transdominant-negative mutant of the NF-κB p65 subunit partially inhibited apoptosis, indicating a direct involvement of the transcription factor in induction of cell death. As expected, the p65 mutant suppressed κB-dependent gene expression. Surprisingly, transiently or stably overexpressed Bcl-2 had the same effect. The transcription inhibitory activity of the two proteins correlated with their cell death protective potential. Like Bcl-2, the related protein Bcl-x_L but not Bcl-x_S was able to suppress κB-dependent transcription. Bcl-2 inhibited NF-κB activity by an unusual mechanism. It did not prevent the release of IκB in the cytoplasm but down-modulated the transactivating potential of nuclear p65. These data show that NF-κB can participate in apoptosis. We suggest that at least part of the anti-apoptotic potential of Bcl-2 may be explained from a hitherto undiscovered activity of Bcl-2 in controlling nuclear gene expression.

Apoptosis is a highly controlled form of cell death that involves an altruistic genetic program for the benefit of the organism. Recently, this process has gained considerable interest because of its involvement in embryonic development, tissue homeostasis, autoimmune diseases, T cell depletion in AIDS, chemotherapeutic drug-induced killing of cancer cells, and neurodegeneration (Kerr et al., 1994; Raff, 1992; Cohen, 1991; Ameisen, 1994; McConkey and Orrenius, 1989). The molecular mechanisms underlying programmed cell death are under intense investigation. Beginning with blebbing of the plasma membrane, endonucleolytic degradation of genomic DNA occurs, which culminates in the condensation and fragmentation of the nucleus (Arends and Wyllie, 1991). Ultimately, the cells are removed by phagocytes without provoking an inflammatory response. Because apoptosis is an active process often requiring protein synthesis, transcriptional control of specific "death genes" is likely to play a crucial regulatory role (Schwartz and Osborne, 1993). In the past years, several gene products have been identified that either promote or inhibit apoptotic processes.

Important gene products that interfere with apoptosis include Bcl-2 and related members of the Bcl-2 family (for reviews see Korsmeyer, 1992; Reed, 1994; Vaux, 1993; Nunez and Clarke, 1994). Experimental overexpression of Bcl-2 has been shown to protect cells from apoptosis in a great number of different systems. Aberrant overexpression of Bcl-2 as often observed in tumors might rescue precancerous cell clones from apoptotic elimination. This facilitates the accumulation of additional mutations that might ultimately lead to overt malignant transformation. In that way, anti-apoptotic proteins, such as Bcl-2, constitute a new class of oncogenes (Korsmeyer, 1992). The subcellular distribution of Bcl-2 is unusual for an oncogene. It is present in the nuclear membrane, the ER, as well as in mitochondria (Hockenbery et al., 1990; Krajewski et al., 1993; de Jong et al., 1994). Human Bcl-2 can functionally substitute for ced-9, an apoptosis-preventing gene in *Caenorhabditis elegans*, showing a high functional and structural conservation of the apoptotic pathway during evolution (Vaux et al., 1994).

Despite intense investigations, however, the mode of action of Bcl-2 remains enigmatic. Interactions of Bcl-2 with related proteins such as Bax, Bcl-x, and others, which either suppress or promote cell death, may determine the sensibility of cells to undergo apoptosis (Oltvai et al., 1993; Boise et al., 1993). By coimmunoprecipitation and transfection studies, it has recently been reported that Bcl-2 associates with components of signal transduction cascades, such as the GTPase protein R-Ras, the functionally associated protein kinase Raf-1, and E1B-associated molecules (Fernandez-Sarabia and Bischoff, 1993; Wang et al., 1994, 1995; Boyd et al., 1994). This suggests that Bcl-2 may interfere with events of signal transduction and gene expres-

Address all correspondence to Klaus Schulze-Osthoff, Institute of Biochemistry and Molecular Biology, Albert-Ludwigs-University, Hermann-Herder-Str. 7, D-79104 Freiburg, Germany. Tel.: (49) 761-203-5222. Fax: (49) 761-203-5257.

P.A. Baeuerle's present address is Tularik Inc., 270 East Grand Avenue, South San Francisco, CA 94080.

sion that may otherwise program cells for apoptosis. Other studies have suggested that Bcl-2 may directly interfere with processes of the execution phase of cell death (Hockenbery et al., 1993; Kane et al., 1993; Jacobson et al., 1994). The distribution of Bcl-2 in intracellular membranes has led to speculations that Bcl-2 interacts with electron transfer reactions and the formation of reactive oxygen intermediates (ROIs).[1] It has been suggested that Bcl-2 has antioxidant properties and directly impairs oxidative stress, which is often observed in response to a variety of apoptotic stimuli (Hockenbery et al., 1993; Kane et al., 1993). Recent studies, however, show that Bcl-2 may be also protective against apoptosis in hypoxic conditions where no ROI formation occurs (Jacobson and Raff, 1995; Shimizu et al., 1995).

The important role of oxidative events has been further emphasized for a variety of biological processes, such as signal transduction and gene expression. Numerous studies have demonstrated that in particular the activation of NF-κB, an important transcription factor involved in cellular defense mechanisms requires oxidative signaling (for review see Baeuerle and Henkel, 1994; Schulze-Osthoff et al., 1995). NF-κB is activated in response to a great number of stimuli, most of which represent pathogenic stresses (Baeuerle, 1991). A common intracellular reaction elicited by most, if not all, stimuli activating NF-κB is an increased production of ROIs leading to oxidative stress. Treatment of several cell lines with H_2O_2 has indeed been shown to activate NF-κB (Schreck et al., 1991; Meyer et al., 1993), and activation of the factor in response to all stimuli tested so far could be blocked by a variety of structurally unrelated antioxidative compounds (for review see Meyer et al., 1994). In the course of its cytoplasmic activation, the specific inhibitor of NF-κB, IκB-α, is phosphorylated and proteolytically degraded (Sun et al., 1993; Beg et al., 1993; Henkel et al., 1993; Traenckner et al., 1994). This exposes nuclear localization sequences in the remaining NF-κB heterodimer (Beg and Baldwin, 1993; Zabel et al., 1993), leading to nuclear translocation and subsequent binding of NF-κB to DNA regulatory elements within NF-κB target genes.

Because most NF-κB stimuli represent cellular stresses, some of which induce apoptosis, and because some of the NF-κB target genes, such as p53 (Wu and Lozano, 1994) and c-myc (La Rosa et al., 1994), have been implicated in apoptosis, we investigated whether a classical apoptotic stimulus would also activate NF-κB. We report here that NF-κB is specifically activated in the course of apoptosis induced by serum withdrawal. This activation of NF-κB was demonstrated to be necessary for the execution of the apoptotic program. Overexpression of Bcl-2, which was able to prevent apoptosis, specifically repressed NF-κB–dependent transactivation by attenuating the transactivation potential of its p65 subunit. Our data indicate that activation of NF-κB and κB-dependent gene transcription is involved in induction of apoptosis after serum deprivation.

1. *Abbreviations used in this paper*: CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; EMSA, electrophoretic mobility shift assay; HIV, human immunodeficiency virus; ICE, interleukin-1β converting enzyme; IL, interleukin; Luc, luciferase; LTR, long terminal repeat; PMA, phorbol 12-myristate 13-acetate; ROI, reactive oxygen intermediate; RSV, Rous sarcoma virus; TNF, tumor necrosis factor.

Materials and Methods

Cell Culture

The human embryonic kidney cell line 293 and COS-7 cells were maintained in DME medium supplemented with 10% FCS and antibiotics. The interleukin (IL)-2–dependent mouse T cell line Clone29 (Krammer et al., 1983) was grown in RPMI 1640 medium supplemented with 10% FCS, 50 μM 2-mercaptoethanol, and 50 IU/ml recombinant IL-2. For apoptosis assays, 2×10^5 cells were seeded onto 60-mm tissue-culture plates (Nunc GmbH, Wiesbaden, Germany) and grown on coverslips for 48 h until 60% confluency was reached. After serum withdrawal, cells were stained with propidium iodide as described (Jacobson et al., 1993), except that 5 ng/ml propidium iodide was used. Coverslips were mounted and photographed with a fluorescence microscope (Axiovert; Zeiss, Oberkochen, Germany).

Gel Electrophoresis of Fragmented DNA

Cells (2×10^6) were seeded on 10-cm petri dishes and incubated for 24 h before serum was withdrawn. For analysis of genomic DNA, cells were gently scraped off after the indicated time points and collected together with the nonattached cells in the supernatant. Cells were resuspended in 0.25 ml TBE (45 mM Tris-borate, 1 mM EDTA, pH 8.0) containing 0.25% NP-40 and 0.1 mg/ml RNAse A. After incubation at 37°C for 30 min, extracts were treated with 1 mg/ml proteinase K for an additional 30 min at 37°C. Then, 30 μl of the extracts were loaded on a 1.7% agarose gel and run in the presence of 0.5 μg/ml ethidium bromide.

Plasmid Constructs

The vector RcCMV-Bcl-2, which directs the synthesis of the human Bcl-2 protein from the cytomegalovirus (CMV) enhancer, was obtained by subcloning a PCR-generated cDNA fragment containing the full coding region of bcl-2 into the mammalian expression vector RcCMV (Invitrogen BV, De Schelp, The Netherlands). The insert was sequenced and found to contain no mutation relative to the published sequence (Cleary et al., 1986). Similar CMV enhancer–driven constructs were generated for Bcl-x_L and Bcl-x_S expression plasmids (Boise et al., 1993). The constructs Gal4-$p65^{286\text{-}550}$ and Gal4-VP16 have been described (Schmitz and Baeuerle, 1991) and contained the DNA-binding and dimerization domain of the yeast transcription factor Gal4 fused to the COOH-terminal transactivation domain of p65 and the herpes simplex virus transactivator VP16, respectively. The NF-κB reporter gene plasmid J16 contained two κB sites upstream from a truncated c-*fos* promoter controlling chloramphenicol acetyltransferase (CAT) expression, and the construct J32 had two mutated κB sites (Pierce et al., 1988). The mutant p65Sal lacking the transactivating COOH-terminus of p65 was generated by deleting the XbaI/SalI fragment from the parental vector RcCMVp65 (Schmitz and Baeuerle, 1991), followed by a fill-in with Klenow polymerase and insertion into the expression vector RcCMV. The expression vector encoding vaccinia virus–derived CrmA protein has been described (Los et al., 1995).

Transfections and CAT Assays

Cells were transfected by the calcium phosphate coprecipitation method in 60-mm plates as described (Roussel et al., 1984) with 2 μg reporter plasmid: J16, its mutant derivative J32, or RSV-CAT, which served as an NF-κB noninducible construct. Where indicated, 2 μg Bcl-2 expression plasmid was cotransfected. To adjust for equal amounts of transfected DNA, appropriate amounts of RcCMV–luciferase (Luc) (Pahl and Baeuerle, 1995) were added. After 4 h, the medium was changed, and the cells were stimulated with tumor necrosis factor (TNF) (200 U/ml; Boehringer Mannheim GmbH, Mannheim, Germany) and/or phorbol 12-myristate 13-acetate (PMA) (50 ng/ml; Sigma Chemical Co., Deisenhofen, Germany) for an additional 4 h. Transfections with the human immunodeficiency virus (HIV)–long terminal repeat (LTR)-Luc (Schwartz et al., 1990) were performed with 500 ng reporter plasmid and 6 μg expression plasmid. Here, the medium was changed after overnight incubation. Cells were harvested, and protein extracts were prepared by three freeze–thaw cycles. Equal amounts of protein were used in a CAT assay (Gorman et al., 1982) or luciferase assay (Pahl and Baeuerle, 1995).

Clones stably expressing the Gal4 fusion proteins were obtained by cotransfecting 2 μg of the hygromycin resistance plasmid pMEP4 (Invitrogen BV) together with either the expression vector Gal4-$p65^{286\text{-}550}$ or Gal4-VP16 (Schmitz and Baeuerle, 1991). Selection was performed in 100 μg/ml

hygromycin (Sigma Chemical Co.) for 2 wk. Cell lines expressing Bcl-2 and CrmA were generated by selection in 200 μg/ml G-418 (GIBCO BRL, Eggenstein, Germany) for 2 wk, after which individual clones were isolated. Bcl-2 expression was confirmed by immunofluorescence as described (Zabel et al., 1993) with an mAb against Bcl-2 (Cambridge Research Biochemicals, Northwick, UK).

Cell Extracts and Mobility Shift Assays

Analysis of DNA-binding activities by electrophoretic mobility assays (EMSAs) was performed as described (Zabel et al., 1991) using 2 μg poly(dI-dC) (Sigma Chemical Co.) as nonspecific competitor DNA. Binding reactions were electrophoresed on a native 4% polyacrylamide gels in 0.5× TBE buffer to separate bound and unbound DNA probe. The binding reaction contained 10,000 cpm. of ^{32}P-labeled double-stranded oligonucleotides with a high affinity NF-κB binding or an octamer-binding motif (Promega, Heidelberg, Germany). Nuclear extracts of cells were prepared as described (Schreiber et al., 1989). The protein content was measured by the method of Bradford (1976).

Western Blotting

IκB-α was detected in total cell extracts by Western blotting as described (Traenckner et al., 1994). Briefly, proteins were separated by a reducing 10% SDS-PAGE, transferred to Immobilon P membranes (Millipore GmbH, Eschborn, Germany), and stained with an affinity-purified rabbit serum directed against human IκB-α. Bound antibody was decorated with goat anti-rabbit/HRP conjugate (Bio-Rad Laboratories GmbH, Munich, Germany) followed by enhanced chemoluminescent staining using ECL reagents (Amersham Buchler GmbH & Co KG, Braunschweig, Germany).

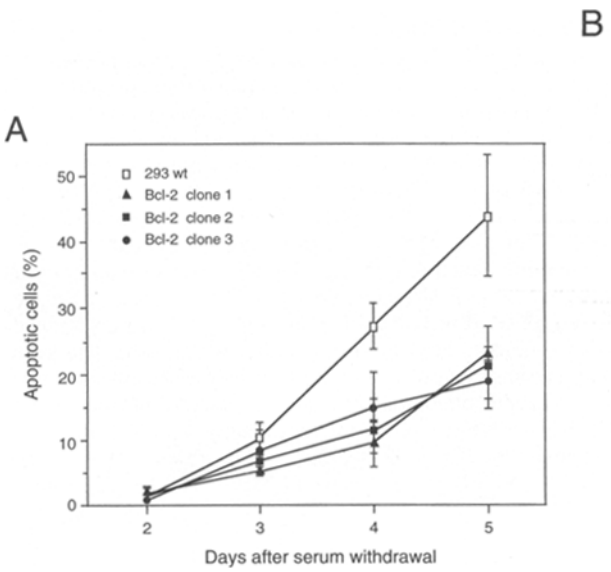
Results

Serum Withdrawal Causes Apoptosis in 293 Cells, Which Is Attenuated by Bcl-2

We studied programmed cell death in the human kidney epithelial carcinoma cell line 293. These cells underwent apoptosis after serum withdrawal (Fig. 1 *A*). 3 d after omission of serum, nuclear uptake of propidium iodide was apparent in ~10% of cells. On day 5, 50% of cell nuclei were stained. On day 6, almost all cells were apoptotic (data not shown). The stained cells displayed disintegrated nuclei and nonrandom DNA fragmentation as assessed by agarose gel electrophoresis of genomic DNA (Fig. 1 *B*). Bcl-2 was able to significantly suppress apoptosis in 293 cells (Fig. 1 *A*). In three cell clones stably overexpressing Bcl-2, apoptosis measured at day 5 was reduced to about threefold as compared to the parental line. While Bcl-2 was barely detected in parental 293 cells, the three clones showed a strong perinuclear and faint cytoplasmic Bcl-2–specific immunofluorescent staining, similar to what has been reported in other systems (Chen-Levy et al., 1989; Hockenbery et al., 1990; Krajewski et al., 1993). These data establish 293 cells as a suitable model to investigate apoptotic cell death after serum withdrawal.

Induction of Apoptosis Correlates with Activation of NF-κB

Using the EMSA, we analysed the DNA-binding activity of NF-κB in 293 cells after serum starvation. Equal amounts of protein from nuclear extracts were analyzed with a ^{32}P-labeled DNA probe encompassing one NF-κB–binding motif from the HIV-1 enhancer. As shown in Fig. 2 *A*, apoptosis in serum-starved 293 cells was accompanied by a very strong induction of a nuclear κB-binding activity starting at day 3. Two other faster-migrating binding activities were nonspecific and remained unaffected between days 1 and 5 after serum withdrawal. To analyze the specificity of the induced DNA-binding complex, competition and supershift analyses were performed. Formation of the slowly migrating complex was competed by an 100-fold excess of unlabeled κB oligonucleotide (Fig. 2 *B*, lane *3*) but not by an oligonucleotide mutated in the κB-binding site (lane *2*). As demonstrated by immunoreactivity with specific antisera in supershift assays, the induced protein–DNA complex of the slowest mobility contained the p50



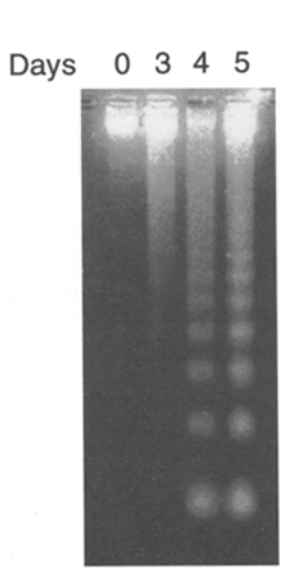


Figure 1. Induction of apoptosis in 293 cells by serum withdrawal. (*A*) The effect of serum starvation and Bcl-2 overexpression on the survival of 293 cells. Parental 293 cells, as well as three cell lines stably overexpressing Bcl-2 (clones 1-3), were cultured for the indicated times under serum-free conditions. Apoptotic cells were visualized by propidium iodide staining, and at least 500 cells were counted for each condition. The mean value and SD from three independent measurements of each cell line are shown. (*B*) Induction of internucleosomal DNA fragmentation by serum withdrawal. Parental 293 cells were cultured in serum-free medium. After the indicated time points, DNA was extracted and analyzed by agarose gel electrophoresis in the presence of ethidium bromide.

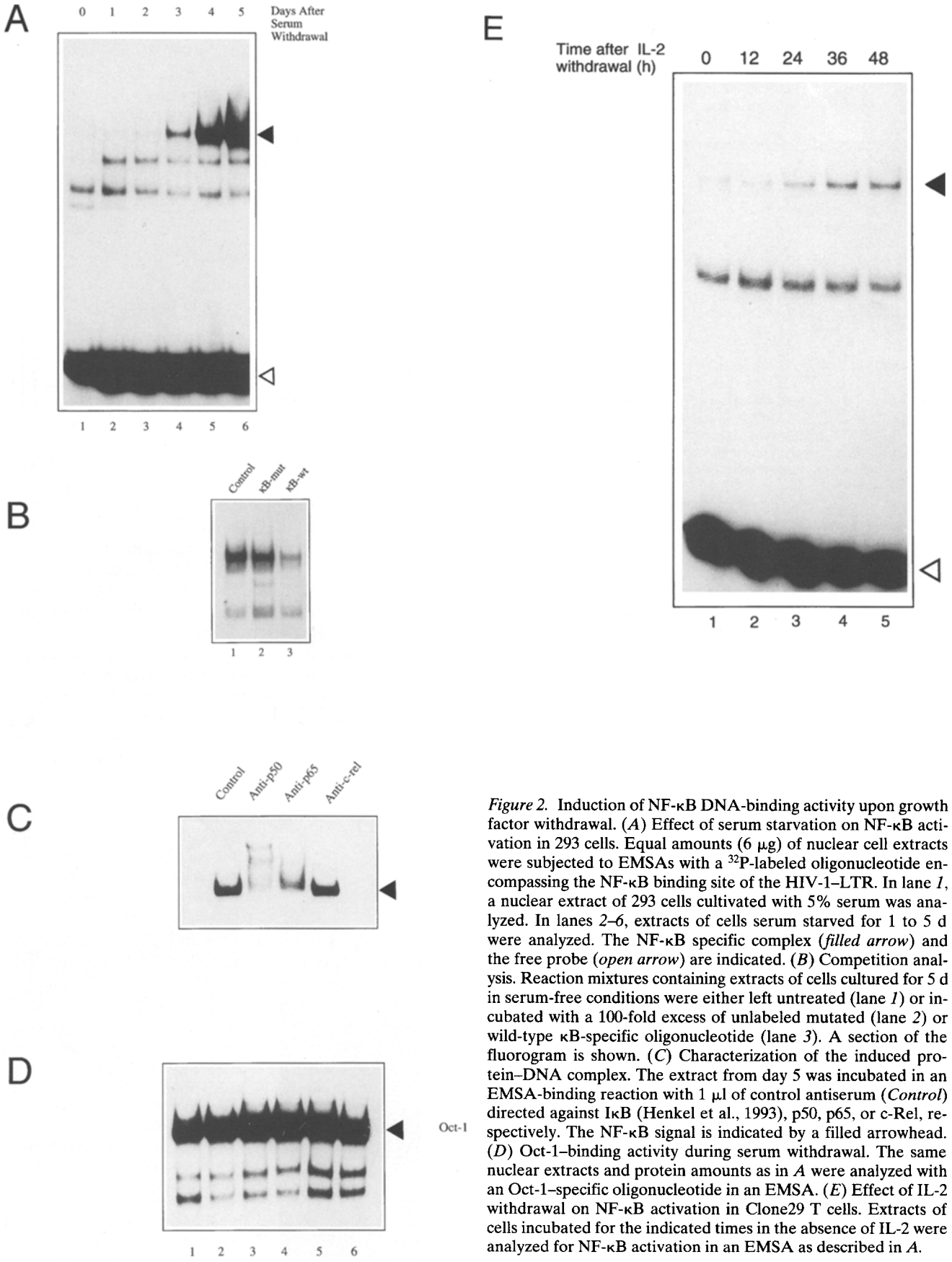


Figure 2. Induction of NF-κB DNA-binding activity upon growth factor withdrawal. (*A*) Effect of serum starvation on NF-κB activation in 293 cells. Equal amounts (6 μg) of nuclear cell extracts were subjected to EMSAs with a ^{32}P-labeled oligonucleotide encompassing the NF-κB binding site of the HIV-1–LTR. In lane *1*, a nuclear extract of 293 cells cultivated with 5% serum was analyzed. In lanes *2–6*, extracts of cells serum starved for 1 to 5 d were analyzed. The NF-κB specific complex (*filled arrow*) and the free probe (*open arrow*) are indicated. (*B*) Competition analysis. Reaction mixtures containing extracts of cells cultured for 5 d in serum-free conditions were either left untreated (lane *1*) or incubated with a 100-fold excess of unlabeled mutated (lane *2*) or wild-type κB-specific oligonucleotide (lane *3*). A section of the fluorogram is shown. (*C*) Characterization of the induced protein–DNA complex. The extract from day 5 was incubated in an EMSA-binding reaction with 1 μl of control antiserum (*Control*) directed against IκB (Henkel et al., 1993), p50, p65, or c-Rel, respectively. The NF-κB signal is indicated by a filled arrowhead. (*D*) Oct-1–binding activity during serum withdrawal. The same nuclear extracts and protein amounts as in *A* were analyzed with an Oct-1–specific oligonucleotide in an EMSA. (*E*) Effect of IL-2 withdrawal on NF-κB activation in Clone29 T cells. Extracts of cells incubated for the indicated times in the absence of IL-2 were analyzed for NF-κB activation in an EMSA as described in *A*.

and p65 DNA-binding subunits of NF-κB (Fig. 2 *C*). Both anti-p50 and anti-p65 sera impaired formation of the protein–DNA complex, while it did not react with anti-c-Rel nor with an irrelevant control serum (Fig. 2 *C*). In contrast, the DNA-binding activity of the ubiquitous factor Oct-1 was not affected during serum withdrawal (Fig. 2 *D*). In the same nuclear extracts, which were assayed for NF-κB activity, Oct-1–binding activity was present at very similar levels at all time points. In addition, we could not observe induction of the DNA binding activity of AP-1 upon serum withdrawal (data not shown), although c-*fos* or c-jun gene induction has been shown to correlate with apoptosis (Colotta et al., 1992; Smeyne et al., 1993; Estus et al., 1994; Ham et al., 1995).

To analyze whether activation of NF-κB during apoptosis was restriced to 293 cells, we furthermore performed experiments in the IL-2–dependent T cell line Clone29. When deprived of IL-2, these cells undergo apoptosis within 48 h. As in 293 cells, growth factor withdrawal also led to a significant activation of NF-κB in Clone29 cells (Fig. 2 *E*).

These results show a striking temporal correlation between the onset and progression of apoptosis and the activation of NF-κB. In contrast to other stimuli, such as phorbol esters or cytokines, which rapidly activate NF-κB for less than a few hours (data not shown), growth factor withdrawal caused a very persistent activation of NF-κB. In accordance, serum starvation led to a rather slow proteolytic degradation of the inhibitory subunit IκB-α, as shown by Western blotting of total cell extracts (Fig. 3 *A*). To analyze whether serum starvation was able to induce functional NF-κB–controlled gene expression, reporter gene assays were performed. A stably integrated luciferase reporter gene under the control of the HIV-LTR, which harbors two essential NF-κB–binding sites, was strongly activated upon serum starvation of 293 cells (Fig. 3 *B*). These results show that NF-κB induced upon apoptosis was transcriptionally active.

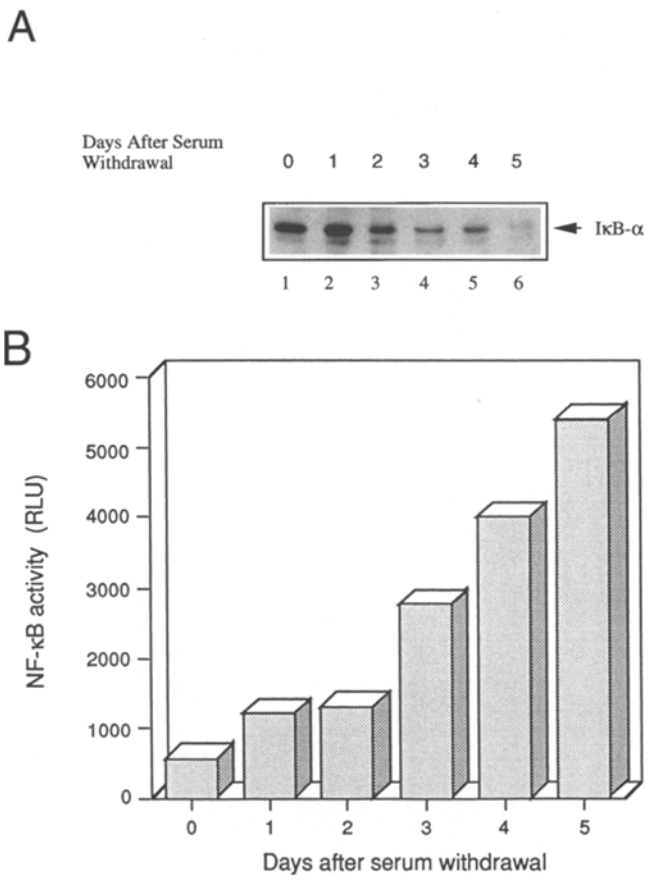


Figure 3. (*A*) Effect of serum withdrawal on the degradation of IκB-α. 293 cells were kept serum free for the indicated periods of time. Total cell extracts were electrophoresed under reducing, denaturating conditions, stained with affinity-purified anti-IκB-α, and visualized by enhanced chemoluminescent staining. (*B*) Effect of serum withdrawal on the activity of a κB-dependent reporter gene. 293 cells stably transfected with the luciferase gene under control of the HIV-LTR were cultivated without serum. NF-κB activity indicated as relative light units (*RLU*) was measured every day and normalized according to protein content. A representative experiment is shown.

NF-κB Activation Is Required for the Induction of Apoptosis

To investigate the significance of NF-κB activation for the induction and progression of apoptosis, we constructed a dominant-negative mutant of the p65 NF-κB subunit lacking the transactivating COOH-terminal 81 amino acids. This mutant, called p65Sal, was still able to heterodimerize with p50 and to bind to κB motifs, but was unable to transactivate κB-dependent gene expression (data not shown; compare Fig. 4 *B*). When p65Sal was stably overexpressed in 293 cells, a partial but significant reduction of apoptotic death was observed in three independent clones (Fig. 4 *A*). In comparison to Bcl-2, however, protection was less efficient. These results indicate that the activation of NF-κB observed after growth factor deprivation was involved in inducing apoptosis.

Bcl-2 Supresses the Transcriptional Potential of NF-κB

As shown in Fig. 4 *B*, p65Sal partially suppressed the transactivation of a κB-dependent CAT reporter construct after stimulation with the phorbolester PMA. Surprisingly, not only the transdominant-negative mutant of p65 but also Bcl-2 was able to suppress NF-κB–dependent transcriptional activation in 293 cells (Fig. 4 *B*). This was not due to differences in transfection efficiency, since a Rous sarcoma virus (RSV) LTR-controlled CAT reporter construct, which is independent of NF-κB, showed equal activity in all cell clones when compared to the parental cell line (data not shown). In addition, 293 cells expressing solely the neomycin resistance gene revealed no differences in NF-κB activation. Fig. 4 further shows that the ability of Bcl-2 to suppress κB-dependent transactivation correlated with its superior capability to repress apoptosis (compare Fig. 4, *A* and *B*).

The inhibitory effect of transiently overexpressed Bcl-2 on NF-κB activation was also observed in response to other potent NF-κB–activating stimuli, such as a combination of TNF and PMA (Fig. 5 *A*). Likewise, NF-κB activation by stimulation with PMA alone was inhibited in a dose-dependent manner by Bcl-2 expression (Fig. 5 *A*). Transient transfection of a CMV enhancer–controlled luciferase expression vector (RcCMV-Luc), which was used

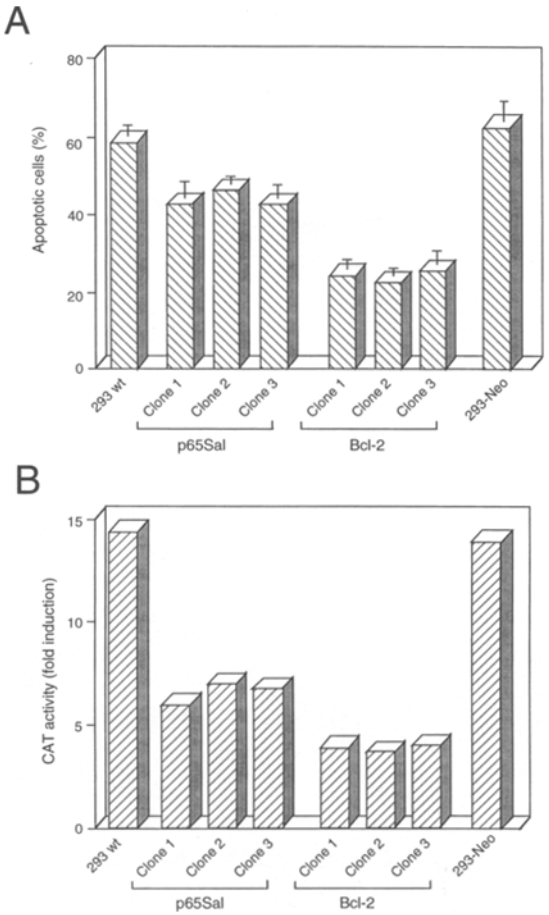


Figure 4. The effect of p65Sal and Bcl-2 on the rate of apoptosis and on the transactivation of NF-κB. (*A*) The effect of overexpression of the transdominant-negative mutant p65Sal and of Bcl-2 on the survival of 293 cells after serum withdrawal. Parental 293 cells, as well as each three independent cell clones stably overexpressing Bcl-2 or p65Sal (clones 1–3), were cultured for 5 d under serum-free conditions. As a control, cells stably expressing the neomycin resistance gene (*293-Neo*) were used. Apoptotic cells were visualized by propidium iodide staining and counted. The mean value and SD from three independent measurements of apoptotic cells are shown. (*B*) Repression of NF-κB transactivation in cell clones overexpressing p65Sal or Bcl-2. Cell lines stably expressing p65Sal or Bcl-2 were transiently transfected with a NF-κB–dependent CAT construct. After 4 h of stimulation with PMA, cells were harvested and CAT assays were performed. Mean values of duplicate experiments are shown relative to the uninduced level.

to adjust for equal amounts of transfected DNA, had no effect. The inhibitory effect of Bcl-2 relied on intact NF-κB–binding motifs, as it was neither observed with the CAT construct harboring two mutated inactive κB sites nor with an RSV enhancer–controlled CAT reporter gene (Fig. 5 *B*).

We further examined the effect of Bcl-2 on NF-κB activation in serum-starved cultures. Fig. 5 *C* shows that NF-κB–mediated transactivation in response to serum deprivation was dose dependently inhibited in Bcl-2–overexpressing cells. Hence, in a fashion similar to the rapid NF-κB inducers TNF and PMA, Bcl-2 was able to repress NF-κB in response to a slow but more persistent stimulus of NF-κB activation. Inhibition of NF-κB by Bcl-2 was also observed in COS-7 cells (Fig. 5 *D*), showing that the effects were not restricted to a single cell type.

Furthermore, we found that the Bcl-2–related protein, Bcl-x_L, which also prevents apoptosis (Boise et al., 1993), was equally capable to suppress κB-dependent transactivation (Fig. 6). Its splice variant, Bcl-x_S, which is devoid of an apoptosis-suppressing function, however, was unable to inhibit NF-κB activation. These data show that Bcl-2 and related proteins can specifically influence gene expression in the nucleus by attenuating the expression of NF-κB–controlled genes. These findings further support a correlation between anti-apoptotic and NF-κB–inhibiting activities of Bcl-2 proteins.

Recently, proteases of the interleukin-1β converting enzyme (ICE) family have been implicated in apoptosis induced by a variety of stimuli, such as Fas and TNF receptor ligation, and growth factor withdrawal (Los et al., 1995; Tewari and Dixit, 1995; Hsu et al., 1995; Gagliardini et al., 1994). The poxvirus protein CrmA encodes a specific serpin class inhibitor of ICE proteases (Ray et al., 1992; Schulze-Osthoff et al., 1996). To investigate whether ICE proteases were involved in apoptosis and NF-κB activation, we therefore stably expressed CrmA in 293 cells. Two clones were selected and found to overexpress CrmA (data not shown). As shown in Fig. 7, CrmA neither affected induction of apoptosis nor activation of NF-κB in serum-starved 293 cells. Although we cannot exclude that the level of CrmA expression was insufficient, the results indicate that induction of apoptosis and NF-κB activation do presumably not involve ICE proteases in 293 cells.

Bcl-2 Does Not Interfere with DNA Binding and Nuclear Transport of NF-κB

Bcl-2 has been reported to counteract apoptosis by inducing an antioxidant pathway (Hockenbery et al., 1993). Because the dissociation of the cytoplasmic NF-κB–IκB complex is induced by ROIs and inhibited by antioxidants (Schreck et al., 1992*b*), we investigated whether Bcl-2 suppresses the activation of NF-κB by interfering with the release of IκB in the cytoplasm. As shown in Fig. 8 *A*, 293 cells overexpressing Bcl-2 showed the same level of nuclear NF-κB DNA-binding activity after TNF stimulation as the parental cell line. The kinetics of activation of NF-κB DNA binding were also not significantly altered by Bcl-2 overexpression (data not shown). Hence, Bcl-2 overexpression presumably does not interfere with the redox-controlled release of IκB in the cytoplasm, but must affect the activity of IκB-released, nuclear NF-κB.

Bcl-2 Represses the Activity of the COOH-terminal Transactivation Domain of p65

The strong transcriptional activation by NF-κB is mediated by transactivating sequences in the COOH-terminal portion of the p65 subunit (Schmitz and Baeuerle, 1991). We therefore investigated the possibility that Bcl-2 modulates the activity of the COOH-terminal transactivating domain of p65. To this end, Gal4-$p65^{286\text{-}550}$, a fusion plasmid encoding the DNA-binding domain of the yeast transcription factor Gal4 and the transactivation domain of p65, was stably expressed in 293 cells. Six independent clones selected

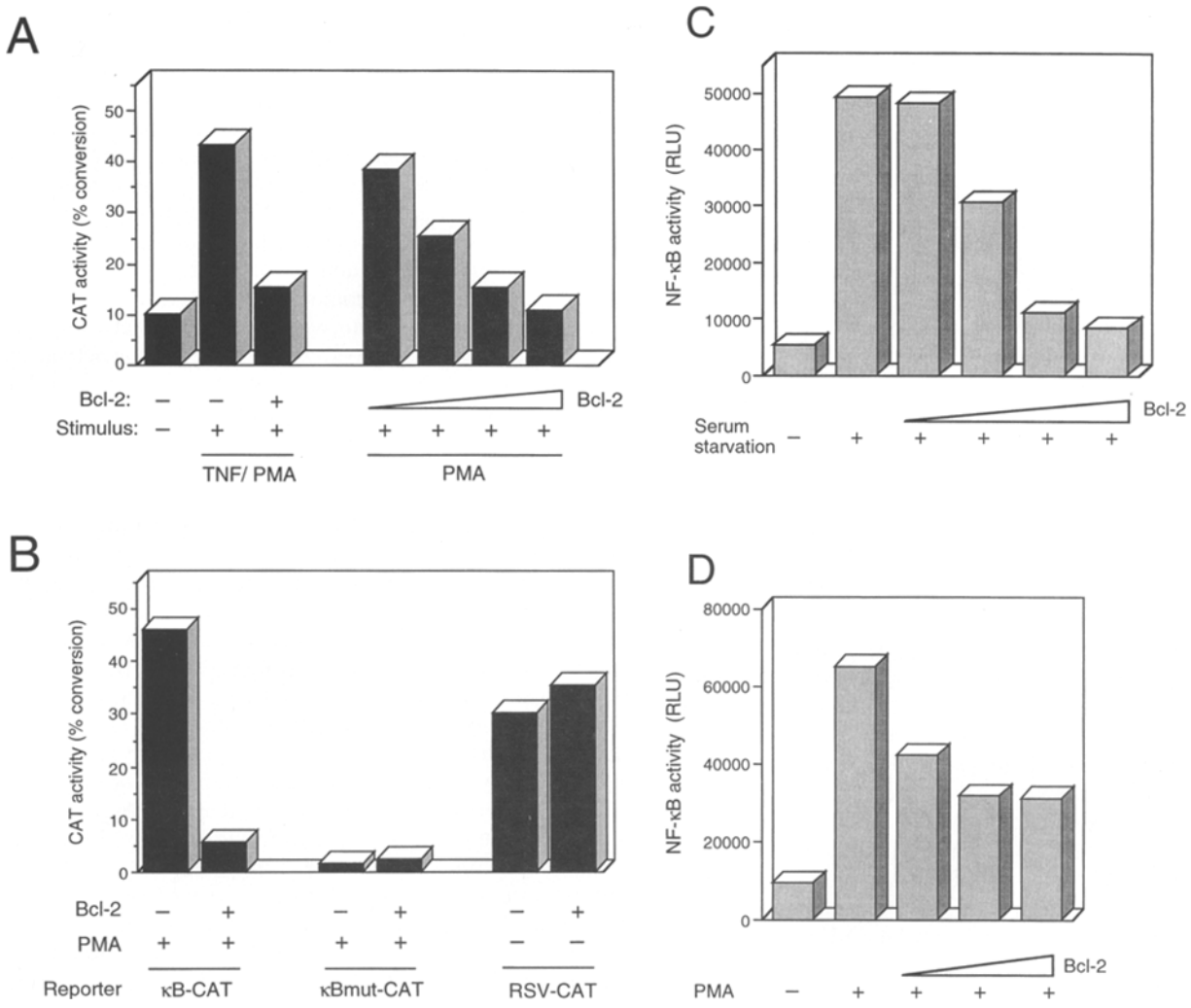


Figure 5. The effect of overexpression of Bcl-2 on the transcriptional activity of NF-κB. (*A*) The effect of Bcl-2 expression on NF-κB–mediated transactivation in 293 cells. Cells were cotransfected with the NF-κB CAT reporter gene plasmid J16 and 1,000 ng of either a Bcl-2 expression plasmid (*Bcl-2*) or the control vector RcCMV-Luc (−) (*left*). In the titration experiment (*right*), 100, 200, 500, and 1,000 ng of Bcl-2 expression plasmid were used, and the total amount of DNA was adjusted to 2 μg with the control plasmid. After stimulation for 4 h with PMA and/or TNF, CAT assays were performed. The mean from two duplicate experiments is shown. (*B*) Specificity of Bcl-2–mediated repression. 293 cells were treated as in *A* except that two other reporter plasmids were tested. In addition to the NF-κB CAT plasmid (κB-CAT), a homologous reporter plasmid with two mutated NF-κB binding sites (*κBmut-CAT*) was used, as well as RSV-CAT, which directs the synthesis of the CAT gene under control of the NF-κB–independent RSV long terminal repeat. (*C*) The effect of Bcl-2 overexpression on NF-κB activity in response to serum starvation. 293 cells stably expressing an HIV-LTR controlled luciferase construct were transiently transfected with increasing concentrations (0, 100, 200, 500, and 1,000 ng) of the Bcl-2 expression plasmid. The total amount of transfected DNA was adjusted to 2 μg with a control plasmid. 12 h after transfection, cells were serum starved for an additional 72 h or left in the presence of serum. Mean values of NF-κB activity indicated as relative light units (*RLU*) from duplicate experiments are shown. (*D*) The effect of Bcl-2 overexpression on NF-kB activity in COS-7 cells. Cells were cotransfected with an HIV-LTR luciferase construct and increasing amounts of the Bcl-2 expression plasmid as described in *C*. 12 h after transfection, cells were stimulated with PMA and harvested after an additional 4 h. Mean values of NF-κB activity indicated as relative light units (*RLU*) from triplicate experiments are shown.

for stable Gal4-p65 expression were then transiently transfected with a Gal4-controlled reporter gene construct. As a control, cells overexpressing Gal4-VP16, a fusion protein containing an NF-κB–independent, constitutively active transactivation domain, were analyzed. Fig. 8 *B* shows that in the individual clones, Gal4-dependent reporter gene expression was activated to distinct levels. In all Gal4-p65–expressing clones, cotransfection of Bcl-2 attenuated the Gal4-dependent activity between 56% and 83% (Fig. 8 *B*). This inhibitory effect of Bcl-2 was stronger in cell lines expressing low levels of Gal4-p65 than in those expressing higher levels (compare, for instance, clones 2 and 4). No inhibition, however, was observed in cells expressing Gal4-VP16, demonstrating that the effects of Bcl-2 were specific to NF-κB (Fig. 8 *C*). Thus, the strong inhibitory effect of Bcl-2 on Gal4-p65 indicates that the COOH-terminus of p65 NF-κB is the primary target for the gene regulatory activity of Bcl-2.

Since Bcl-2 has been found to be associated in part with the nuclear pore complex (Krajewski et al., 1993; de Jong

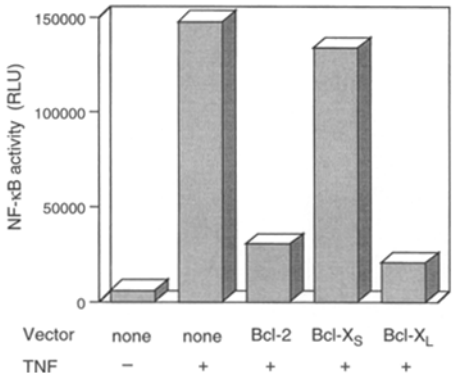


Figure 6. The effect of Bcl-2–related proteins on the transcriptional activity of NF-κB. Luciferase activity of the NF-κB–specific reporter plasmid HIV-LTR-Luc was measured in 293 cells after cotransfection of expression plasmids for Bcl-2, Bcl-x_s, and Bcl-x_L, as indicated, and stimulation with TNF to activate endogenous NF-κB. The means of doubly performed experiments are shown.

et al., 1994), we also investigated whether Bcl-2 altered the translocation of NF-κB to the nucleus. In immunocytological analyses, we could not detect changes in the intracellular localization or expression of NF-κB subunits (data not shown). We could neither obtain evidence for a physical interaction between Bcl-2 and p65 upon immunoprecipitation of proteins from lysates of transfected cells using either Bcl-2– or p65-specific antibodies (data not shown). From these observations, we consider it unlikely that Bcl-2 interacts directly with the COOH terminus of p65, but rather it controls a signaling pathway, causing modulation of the transactivating potential of nuclear NF-κB.

Discussion

Bcl-2: A Regulator of Gene Expression

The molecular mechanism by which Bcl-2 protects cells from apoptosis is poorly understood. Recent studies have suggested that Bcl-2 is a component of a signal transduction pathway that counteracts the effects of oxidative stress (Hockenbery et al., 1993; Kane et al., 1993). This signaling pathway controlled by Bcl-2 may be related to growth factor–dependent pathways because Bcl-2 can associate with the R-Ras protein and Raf-1 kinase (Fernandez-Sarabia and Bischoff, 1993; Wang et al., 1994, 1995). However, no further component has been identified that could help to unravel the activity of Bcl-2.

The potential involvement of ROIs in Bcl-2 action prompted us to investigate its effect on the activation of NF-κB, which might be involved in transcriptional activation of "death genes." Numerous reports have documented a requirement of ROIs for NF-κB activation. This event involves the release of the inhibitory subunit IκB from the latent NF-κB complex in the cytoplasm (for review see Schreck et al., 1992*a*; Meyer et al., 1994; Baeuerle and Henkel, 1994; Schulze-Osthoff et al., 1995). In the present study, we found that Bcl-2 could indeed attenuate the activity of NF-κB, indicating that the transcription factor might be a potential target for the action of a Bcl-2–controlled pathway. Further support for this came from our finding that the Bcl-2–related protein, Bcl-x_L (Boise et al., 1993), down-regulated NF-κB activity, whereas the nonprotective splice variant Bcl-x_s was not able to display this effect. This suggests that the signaling pathway that leads to NF-κB down-regulation is mediated by one of the Bcl-2 homology domains that are conserved among members of the Bcl-2 gene family (Hengartner and Horvitz, 1994; Yin et al., 1994; Nunez and Clarke, 1994). Our data therefore demonstrate a hitherto unknown activity of Bcl-2 in controlling nuclear gene expression. A recent study showed that overexpressed Bcl-2 was able to inhibit apoptosis even in enucleated cells (Jacobson et al., 1994). However, it cannot be excluded that, in this case, Bcl-2 was protective by preventing gene expression before the enucleation of cells.

Oxidative stress interferes with the activation of NF-κB by inducing the release of IκB in the cytoplasm. Thus, we were surprised that Bcl-2 did not inhibit this step of NF-κB activation nor did it affect DNA binding of NF-κB. This finding therefore argues against an antioxidant effect of Bcl-2 during NF-κB activation. Instead, we observed that Bcl-2 acted on the transactivation domain of the p65 NF-κB subunit and thereby repressed κB-dependent transactivation in the nucleus. This represents a novel posttranslational level to control the activity of NF-κB. We have recently observed that, in intact cells, the transactivation domain is phosphorylated on serine residues (Schmitz, M.L., and P.A. Baeuerle, manuscript in preparation). Future studies are required to investigate whether Bcl-2 overexpression induces changes in the phosphorylation status of p65 and whether these allow for regulation of the transactivation potential of NF-κB. The level of Bcl-2 expression and its ratio to the negative regulatory proteins such as Bax (Oltvai et al., 1993) might determine its interaction with R-Ras at intracellular membranes. Analogous to growth factor–controlled pathways, the Bcl-2/Ras complex might then recruite Raf-1 or another protein kinase, which directly or indirectly leads to modification of nuclear NF-κB. Recently, a protein kinase of 43 kD was

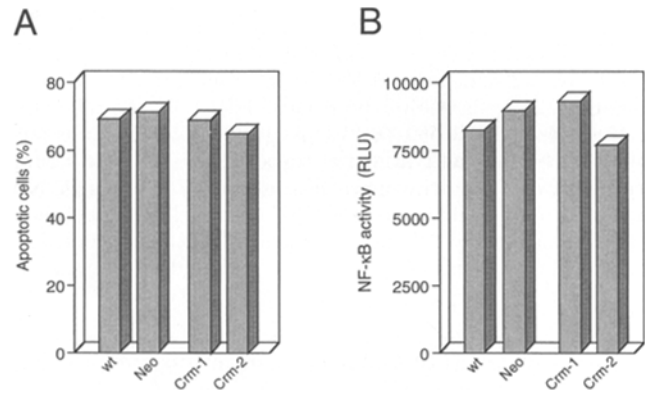


Figure 7. The effect of the ICE inhibitor CrmA on apoptosis (*A*) and NF-κB activity (*B*). 293 cells, either untransfected (*wt*) or stably transfected with the neomycin resistance gene (*Neo*) and two CrmA expressing clones (*Crm-1, Crm-2*), were incubated for 5 d in the absence of serum. Apoptotic cells (*A*) were visualized as described in Fig. 1 *A*. NF-κB activity (*B*) was measured after transient transfection with an HIV-LTR controlled luciferase construct as described in Fig. 2 *A*.

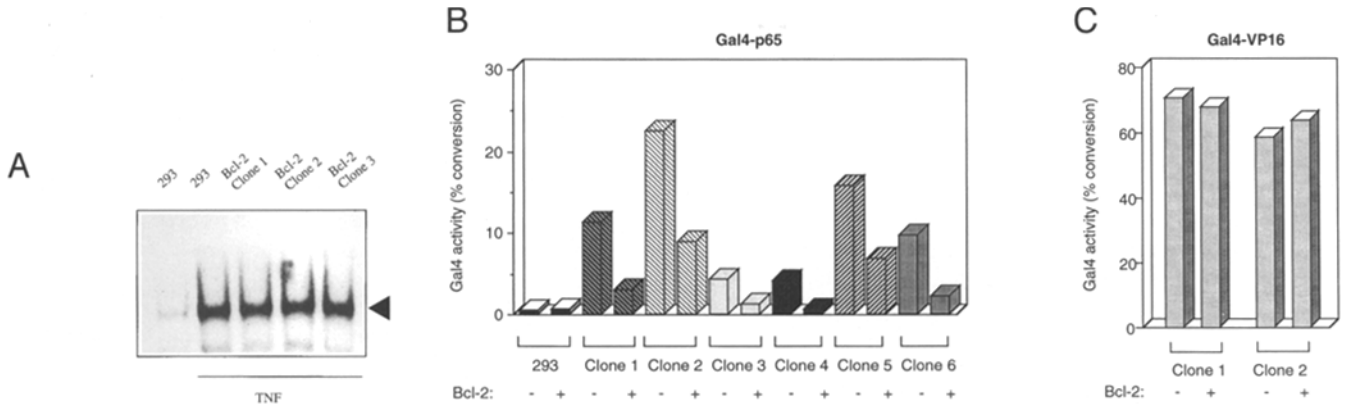


Figure 8. The effect of Bcl-2 on the activation and transcriptional activity of NF-κB. (*A*) Inducibility of NF-κB DNA-binding activity in cell lines stably overexpressing Bcl-2. Three Bcl-2 expressing cell clones (see Fig. 1) and the parental cell line were stimulated for 4 h with TNF (200 U/ml). Nuclear cell extracts were prepared and analyzed by EMSA with an NF-κB–specific oligonucleotide (see Fig. 2 *A*). (*B*) The effect of Bcl-2 overexpression on the transactivation potential of the COOH-terminal part of p65 NF-κB. Six 293 cell clones stably expressing Gal4–p65$^{286\text{-}550}$ fusion protein were cotransfected with a Gal4-dependent CAT reporter plasmid together with either 2 μg RcCMV-Bcl-2 (*Bcl-2*) or the control plasmid RcCMV-Luc (−). CAT assays from one series of a representative experiment are shown. (*C*) The effect of Bcl-2 overexpression on the activity of the NF-κB–independent and constitutively active transactivation domain VP16. Two 293 clones stably expressing a Gal4–VP16 fusion protein were treated as described in *B*. Mean values of two assays of Gal4-dependent CAT activity are shown.

found associated with the cytoplasmic NF-κB–IκB complex (Hayashi et al., 1994). This kinase could be a candidate for a protein controlling the transcriptional activity of p65 because it specifically phosphorylates p65 on serine residues.

A Potential Role of NF-κB in Apoptosis

There is increasing evidence that transcription factors of the NF-κB/Rel family are involved in programmed cell death. First, many potent NF-κB–activating stimuli induce apoptosis, such as TNF (Osborn et al., 1989), ceramide (Schütze et al., 1992; Obeid et al., 1993), H_2O_2 (Schreck et al., 1991; Hockenbery et al., 1993), and, as shown here for the first time, serum withdrawal. Second, apoptosis can be prevented by conditions that at the same time suppress NF-κB activation. This has been shown for the antioxidant *N*-acetyl-L-cysteine and other compounds (Staal et al., 1990; Schreck et al., 1991; Schulze-Osthoff et al., 1994). Third, among the genes induced upon apoptosis are potential target genes for NF-κB. Those include the Fas/APO-1 ligand (Suda et al., 1993; Takahashi et al., 1994), c-myc (La Rosa et al., 1994), p53 (Wu and Lozano, 1994), and ICE (Casano et al., 1994). These death genes are transcriptionally induced by stimuli activating NF-κB, and, where analyzed, their upstream promoter regions harbor potential NF-κB–binding motifs. Another target gene for NF-κB is encoding its alternative DNA-binding subunit c-Rel. A recent report has shown that c-Rel expression is induced in apoptotic cells in the chicken embryo (Abbadie et al., 1993). Here, we could not observe that c-Rel is a major component of the NF-κB activity induced upon apoptosis in 293 cells. It is possible that c-Rel expression is preferentially activated in lymphoid cells under such conditions. From all these observations, it appears that NF-κB participates as a signal transducer and gene activator in the process of programmed cell death: apoptotic signals lead to its activation, and the activated transcription factor then turns on the transcription of genes whose products are required to execute the apoptotic program. In this scenario, NF-κB only indirectly functions as a "death factor" in that it prepares cells for apoptotic stimulation by expression of a receptor, a ligand, or other genes with apoptotic activity. It should be noted that other apoptotic pathways exist that do not require de novo protein synthesis. One example is Fas/APO-1–mediated cell death that does not involve NF-κB activation (Schulze-Osthoff et al., 1994).

Because it is clear from many studies that activation of NF-κB will not cause cell death in each case, additional factors might determine the effect of NF-κB in apoptosis. It is well established that NF-κB has to synergize with other transcription factors in transcriptional regulation (for review see Baeuerle and Henkel, 1994). Those unrelated factors might include the steroid receptor Nur77 (Liu et al., 1994; Woronicz et al., 1994) or AP-1 that may synergize with NF-κB in the regulation of apoptotic genes and could specify, for instance, cell type–specific differences. Another determinant for the involvement of NF-κB in apoptosis could be how long the factor is activated. In many systems, NF-κB activation in response to TNF or PMA stimulation is very transient, lasting only a few hours. No apoptotic death of such cell cultures has been reported. The down-regulation of NF-κB activity is most likely mediated by transcriptional activation of inhibitory proteins, such as IκB-α, p105, and p100 genes (Sun et al., 1993, 1994). In the present study, we were intrigued to observe that a p50-p65 NF-κB complex was activated for several days after serum withdrawal, suggesting that prolonged activation of the transcription factor may be required to induce an apoptotic program. There are B and T lymphoma cell lines containing constitutive NF-κB activity in their nuclei; however, a recent report showed that their nuclear NF-κB contained other members of the family, predominantly RelB and c-Rel (Lernbecher et al., 1993). The subunit composition of NF-κB, which influences DNA-binding specificity and transactivating potential (Hansen et al.,

1992; Perkins et al., 1992), might thus be another important determinant controlling the role of NF-κB in apoptosis. Recently, some circumstantial evidence for this notion has been presented (Ivanov et al., 1995).

Our present data might be of relevance for a variety of pathological conditions in which apoptosis occurs. In fact, NF-κB is activated by a large number of stimuli that induce apoptosis (for review see Baeuerle and Henkel, 1994). For instance, infection of T cells with the HIV-1 or T cell activation with HIV-encoded proteins, such as gp120 or Tat, result in their apoptotic death (Meyaard et al., 1992; Ameisen, 1994; Westendorp et al., 1995*a*,*b*; Li et al., 1995). Under the same conditions, NF-κB is activated (Hazan et al., 1990; Westendorp et al., 1995*a*). Since the expression of HIV-1 is also mediated by NF-κB via two binding sites in the long terminal repeat (Nabel and Baltimore, 1987), NF-κB could at the same time mediate apoptosis and allow the HIV-1 genome to be expressed and the virus escape from dying cells. This would represent a novel strategy of HIV to escape cellular defense mechanisms. Another example is p65Δ, a naturally occurring splice variant of the p65 NF-κB subunit that acts as a transdominant-negative mutant (Narayanan et al., 1992). Expression of p65Δ has been implicated in tumorigenesis, which could be explained by an anti-apoptotic activity (Grimm and Baeuerle, 1994). Conditions inhibiting or stably activating NF-κB may therefore provide a means to control apoptotic processes. Future experiments will clarify under which cellular conditions NF-κB activation is sufficient or necessary for apoptosis.

We thank Lino Schmitz for providing expression plasmids, and Marek Los for helpful advice.

This work was supported by grants from the Deutsche Forschungsgemeinschaft (SFB 217) and the Bundesministerium für Forschung und Wissenschaft awarded to P.A. Baeuerle and is a partial fulfillment of the doctoral thesis of S. Grimm and M.K.A. Bauer.

Received for publication 7 September 1995 and in revised form 26 February 1996.

Note added in proof. While this manuscript was under revision, a study (Lin et al., 1995) appeared demonstrating that Sindbis virus–induced apoptosis also requires NF-κB activation.

References

Abbadie, C., N. Kabrun, F. Bouali, J. Smardova, D. Stehelin, D.B. Vandenbunder, and P.J. Enrietto. 1993. High levels of c-rel expression are associated with programmed cell death in the developing avian embryo and in the bone marrow cells in vitro. *Cell.* 75:899–912.

Ameisen, J.C. 1994. Programmed cell death (apoptosis) and cell survival regulation: relevance to AIDS and cancer. *AIDS (Phila.).* 8:1197–1213.

Arends, M.J., and A.H. Wyllie. 1991. Apoptosis: mechanisms and roles in pathology. *Int. Rev. Exp. Pathol.* 32:223–254.

Baeuerle, P.A. 1991. The inducible transcription factor NF-κB: regulation by distinct protein subunits. *Biochim. Biophys. Acta.* 1077:63–80.

Baeuerle, P.A., and T. Henkel. 1994. Function and activation of NF-κB in the immune system. *Annu. Rev. Immunol.* 12:141–179.

Beg, A.A., and A.S. Baldwin, Jr. 1993. The IκB proteins: multifunctional regulators of Rel/NF-κB transcription factors. *Genes & Dev.* 7:2064–2070.

Beg, A.A., T.S. Finco, P.V. Nantermet, and A.S. Baldwin, Jr. 1993. Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of IκBα: a mechanism for NF-κB activation. *Mol. Cell. Biol.* 13:3301–3310.

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

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

Bradford, M. 1976. A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of dye-binding. *Anal. Biochem.* 72:248–254.

Casano, F.J., A.M. Rolando, J.S. Mudgett, and S.M. Molineaux. 1994. The structure and complete nucleotide sequence of the murine gene encoding interleukin-1β converting enzyme (ICE). *Genomics.* 20:474–481.

Chen-Levy, Z., J. Nourse, and M.L. Cleary. 1989. The bcl-2 candidate protein is a 24 kilodalton integral membrane protein highly expressed in lymphoid cell lines and lymphomas carrying the t(14;18) translocation. *Mol. Cell. Biol.* 9: 701–710.

Cleary, M.L., S.D. Smith, and J. Sklar. 1986. Cloning and structural analysis of cDNAs for bcl-2 and a hybrid bcl-2 immunoglobulin transcript resulting from the t(14;18) translocation. *Cell.* 47:19–28.

Cohen, J.J. 1991. Programmed cell death in the immune system. *Adv. Immunol.* 50:55–85.

Colotta, F., N. Polentarutti, M. Sironi, and A. Mantovani. 1992. Expression and involvement of c-fos and c-jun protooncogenes in programmed cell death induced by growth factor deprivation in lymphoid cell lines. *J. Biol. Chem.* 267: 18278–18283.

de Jong, D., F.A. Prins, D.Y. Mason, J.C. Reed, G.B. van Ommen, and P.M. Kluin. 1994. Subcellular localization of the *bcl-2* protein in malignant and normal lymphoid cells. *Cancer Res.* 54:256–260.

Estus, S., W.J. Zaks, R.S. Freeman, M. Gruda, R. Bravo, and E.M. Johnson, Jr. 1994. Altered gene expression in neurons during programmed cell death: identification of c-jun as necessary for neuronal apoptosis. *J. Cell Biol.* 127: 1717–1727.

Fernandez-Sarabia, M.J., and J.R. Bischoff. 1993. Bcl-2 associates with the ras-related protein R-ras p23. *Nature (Lond.).* 266:274–275.

Gagliardini, V., P.A. Fernandez, R.K.K. Lee, H.C.A. Drexler, R.J. Rotello, M.C. Fishman, and J. Yuan. 1994. Prevention of vertebrate neuronal death by the crmA gene. *Science(Wash. DC).* 263:826–828.

Gorman, C.M., L.F. Moffat, and B.H. Howard. 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* 2:1044–1051.

Grimm, S., and P. A. Baeuerle. 1994. Failure of splicing variant p65Δ of the NF-κB subunit to transform fibroblasts. *Oncogene.* 9:2391–2398.

Ham, J., C. Babij, J. Whitfield, C.M. Pfarr, D. Lallemand, M. Yaniv, and L.L. Rubin. 1995. A c-Jun dominant-negative mutant protects sympathetic neurons against programmed cell death. *Neuron.* 14:927–939.

Hansen, S.K., C. Nerlov, U. Zabel, P. Verde, M. Johnsen, P.A. Baeuerle, and F. Blasi. 1992. A novel complex between the p65 subunit of NF-κB and c-Rel binds to a DNA element involved in the phorbol ester induction of the human urokinase gene. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:205–213.

Hayashi, T., T. Sekine, and T. Okamoto. 1994. Identification of a new serine kinase that activates NF-κB by direct phosphorylation. *J. Biol. Chem.* 268: 26790–26795.

Hazan, U., D. Thomas, J. Alcami, F. Bachelerie, N. Israel, H. Yssel, J.-L. Virelizier, and F. Arenzana-Seisdedos. 1990. Stimulation of a human T cell clone with anti-CD3 or tumor necrosis factor induces NF-κB activation but not immunodeficiency 1 enhancer-dependent transcription. *Proc. Natl. Acad. Sci. USA.* 87:7861–7865.

Hengartner, M.O., and H.R. Horvitz. 1994. Activation of *C. elegans* cell death protein Ced-9 by an amino acid substitution in a domain conserved in Bcl-2. *Nature (Lond.).* 369:318–320.

Henkel, T., T. Machleidt, I. Alkalay, M. Krönke, Y. Ben-Neriah, and P.A. Baeuerle. 1993. Rapid proteolysis of IκB-α is necessary for activation of transcription factor NF-κB. *Nature (Lond.)* 365:182–185.

Hockenbery, D., G. Nunez, C.L. Milliman, R.D. Schreiber, and S.J. Korsmeyer. 1990. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature (Lond.).* 348:334–336.

Hockenbery, D.M., Z.N. Oltvai, X.-M. Yin, C.L. Milliman, and S.J. Korsmeyer. 1993. Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell.* 75:241–251.

Hsu, H., J. Xiong, and D.V. Goeddel. 1995. The TNF receptor 1-associated protein TRADD signals cell death and NF-κB activation. *Cell.* 81:495–504.

Ivanov, V.L., G. Deng, E.R. Podack and T. Malek. 1995. 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. *Int. Immunol.* 7:1709–1720.

Jacobson, M.D., and M.C. Raff. 1995. Programmed cell death and Bcl-2 protection in very low oxygen. *Nature (Lond.).* 374:814–816.

Jacobson, M.J., J.F. Burne, M.P. King, T. Miyashita, J.C. Reed, and M.C. Raff. 1993. Bcl-2 blocks apoptosis in cells lacking mitochondrial DNA. *Nature (Lond.).* 361:365–369.

Jacobson, M.D., J.F. Burne, and M.C. Raff. 1994. Programmed cell death and Bcl-2 protection in the absence of a nucleus. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:1899–1910.

Kane, D.J., T.A. Sarafian, R. Anton, H. Hahn, E.B. Gralla, J.S. Valentine, T. Örd, and D.E. Bredesen. 1993. Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. *Science (Wash. DC).* 262:1274–1277.

Kerr, J.F.R., C.M. Winterford, and B.V. Harmon. 1994. Apoptosis: its significance in cancer and cancer therapy. *Cancer.* 73:2013–2026.

Korsmeyer, S.J. 1992. Bcl-2 initiates a new category of oncogenes: regulators of cell death. *Blood.* 80:879–886.

Krajewski, S., S. Tanaka, S. Takayama, M.J. Schibler, W. Fenton, and J.C. Reed. 1993. Investigation on the subcellular distribution of the bcl-2 onco-

protein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. *Cancer Res.* 53:4701–4714.

Krammer, P.H., B. Echtenacher, D. Gemsa, U. Hamann, L. Hültner, B. Kaltmann, U. Kees, C. Kubelka, and F. Marcucci. 1983. Immune-interferon (IFN-γ), macrophage-activating factors (MAFs), and colony-stimulating factors (CSFs) secreted by T cell clones in limiting dilution microcultures, long-term cultures, and by T cell hybridomas. *Immunol. Rev.* 76:5–28.

La Rosa, F.A., J.W. Pierce, and G.E. Sonenshein. 1994. Differential regulation of the c-myc oncogene promoter by the NF-κB Rel family of transcription factors. *Mol. Cell. Biol.* 14:1039–1044.

Lernbecher, T., U. Müller, and T. Wirth. 1993. Distinct NF-κB/Rel transcription factors are responsible for tissue-specific and inducible gene activation. *Nature (Lond.).* 365:767–770.

Li, C.J., D.J. Friedman, C. Wang, V. Metelev, and A.B. Pardee. 1995. Induction of apoptosis in uninfected lymphocytes by HIV-1 Tat protein. *Science (Wash. DC).* 268:429–431.

Lin, K.-I., S.-H. Lee, R. Narayanan, J.M. Baraban, J.M. Hardwick, and R.R. Ratan. 1995. Thiol agents and Bcl-2 identify an alphavirus-induced apoptotic pathway that requires activation of the transcription factor NF-kappa B. *J. Cell Biol.* 131:1149–1161.

Liu, Z.G., S.W. Smith, K.A. McLaughlin, L.M. Schwartz, and B.A. Osborne. 1994. Apoptotic signals delivered through the T-cell receptor of a T-cell hybrid require the immediate-early gene *nur 77*. *Nature (Lond.).* 367:281–284.

Los, M., M. van de Craen, L. Penning, H. Schenk, M. Westendorp, P.A. Baeuerle, W. Dröge, P.H. Krammer, W. Fiers, and K. Schulze-Osthoff. 1995. Requirement of an ICE/Ced-3 protease for Fas/APO-1-mediated apoptosis. *Nature (Lond.).* 375:81–83.

McConkey, D. S. J., and S. Orrenius. 1989. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) kills glucocorticoid-sensitive thymocytes in vivo. *Biochem. Biophys. Res. Commun.* 160:1003–1008.

Meyaard, L., S.A. Otto, R.R. Jonker, M.J. Mijnster, R.P. Keet, and F. Miedema. 1992. Programmed cell death of T cells in HIV infection. *Science (Wash. DC).* 257:217–219.

Meyer, M., R. Schreck, and P.A. Baeuerle. 1993. H_2O_2 and antioxidants have opposite effects on activation of NF-κB and AP-1 in intact cells: AP-1 as a secondary antioxidant-responsive factor. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:2005–2015.

Meyer, M., R. Schreck, J. Müller, and P.A. Baeuerle. 1994. Redox control of gene expression by eukaryotic transcription factors NF-κB, AP-1 and SRF/TCF. *In* Oxidative Stress, Cell Activation and Viral Infection. L. Packer and C. Pasquier, editors. Birkhäuser Verlag, Basel, Switzerland. 217–235.

Nabel, G., and D. Baltimore. 1987. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. *Nature (Lond.).* 326: 711–713.

Narayanan, R., J.F. Klement, S.M. Ruben, K.A. Higgins, and C.A. Rosen. 1992. Identification of a naturally occuring transforming variant of the p65 subunit of NF-κB. *Science (Wash. DC).* 256:367–370.

Nunez, G., and M.F. Clarke. 1994. The Bcl-2 family of proteins: regulators of cell death and survival. *Trends Cell Biol.* 4:399–403.

Obeid, L.M., C.M. Linardic, L.A. Karolak, and Y.A. Hannun. 1993. Programmed cell death induced by ceramide. *Science (Wash. DC).* 259:1769–1771.

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

Osborn, L., S. Kunkel, and G.J. Nabel. 1989. Tumor necrosis factor alpha and interleukin-1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor kappa B. *Proc. Natl. Acad. Sci. USA.* 86:2336–2340.

Pahl, H.L., and P.A. Baeuerle. 1995. Influenza hemagglutinin activates transcription factor NF-κB. *J. Virol.* 69:1480–1484.

Perkins, N.D., R.M. Schmid, C.S. Duckett, K. Leung, N.R. Rice, and G.J. Nabel. 1992. Distinct combinations of NF-κB subunits determine the specificity of transcriptional activation. *Proc. Natl. Acad. Sci. USA.* 89:1529–1533.

Pierce, J.W., M. Lenardo, and D. Baltimore. 1988. Oligonucleotide that binds nuclear factor NF-κB acts as a lymphoid-specific and inducible enhancer element. *Proc. Natl. Acad. Sci. USA.* 85:1482–1486.

Raff, M.C. 1992. Social controls of cell survival and cell death. *Nature (Lond.).* 356:397–400.

Ray, C.A., R.A. Black, S.R. Kronheim, T.A. Greenstreet, P.R. Sleath, G.S. Salvesen, and D.J. Pickup. 1992. Viral inhibition of inflammation: cowpox virus encodes an inhibitor of the interleukin-1β converting enzyme. *Cell.* 69: 597–604.

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

Roussel, M.F., C.W. Rettenmeier, A.T. Look, and C.T. Sherr. 1984. Cell surface expression of v-fms coded glycoprotein is required for transformation. *Mol. Cell. Biol.* 4:1999–2009.

Schmitz, M.L., and P.A. Baeuerle. 1991. The p65 subunit is responsible for the strong transcriptional activation potential of NF-κB. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:3805–3817.

Schreck, R., P. Rieber, and P.A. Baeuerle. 1991. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcription factor and HIV-1. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:2247–2258.

Schreck, R., K. Albermann, and P.A. Baeuerle. 1992*a*. Nuclear transcription factor NF-κB: an oxidative stress-responsive transcription factor of eukaryotic cells (a review). *Free Radical Res. Commun.* 17:221–237.

Schreck, R., B. Meier, D.N. Männel, W. Dröge, and P.A. Baeuerle. 1992*b*. Dithiocarbamates as potent inhibitors of nuclear factor κB activation in intact cells. *J. Exp. Med.* 175:1181–1194.

Schreiber, E., P. Matthias, M.M. Muller, and W. Schaffner. 1989. Rapid detection of octamer binding proteins with "mini-extracts" prepared from a small number of cells. *Nucleic Acids Res.* 17:6419.

Schulze-Osthoff, K., P.H. Krammer, and W. Dröge. 1994. Divergent signaling via APO-1/Fas and the TNF receptor, two homologous molecules involved in physiological cell death. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:4587–4596.

Schulze-Osthoff, K., M. Los, and P.A. Baeuerle. 1995. Redox signalling by transcription factors NF-κB and AP-1 in the immune system. *Biochem. Pharmacol.* 50:735–741.

Schulze-Osthoff, K., M.K.A. Bauer, M. Vogt, and M. Los. 1996. Involvement of ICE-related and other proteases in Fas-mediated apoptosis. *Cell Death Differ.* 3:177–184.

Schütze, S., K. Potthoff, T. Machleidt, D. Berkovic, K. Wiegmann, and M. Krönke. 1992. TNF activates NF-κB by phosphatidylcholine-specific phospholipase C-induced "acidic" sphingomyelin breakdown. *Cell.* 71:765–776.

Schwartz, L.M., and B.A. Osborne. 1993. Programmed cell death, apoptosis and killer genes. *Immunol. Today.* 14:582–590.

Schwartz, O., J.L. Virelizier, L. Montagnier, and U. Hazan. 1990. A microtransfection method using the luciferase-encoding reporter gene for the assay of human immunodeficiency virus LTR promoter activity. *Gene (Amst.).* 88: 197–205.

Shimizu, S., Y. Eguchi, H. Kosaka, W. Kamiike, H. Matsuda, and Y. Tsujimoto. 1995. Prevention of hypoxia-induced cell death by Bcl-2 and Bcl-x. *Nature (Lond.).* 374:811–813.

Smeyne, R.J., M. Vendrell, M. Hayward, S.J. Baker, G.G. Miao, K. Schilling, L.M. Robertson, T. Curran, and I. Morgan. 1993. Continous c-fos expression precedes programmed cell death in vivo. *Nature (Lond.).* 363:166–169.

Staal, F.J., M. Roederer, L.A. Herzenberg, and L.A. Herzenberg. 1990. Intracellular thiols regulate activation of nuclear factor κB and transcription of human immunodeficiency virus. *Proc. Natl. Acad. Sci. USA.* 87:9943–9947.

Suda, T., T. Takahashi, P. Golstein, and S. Nagata. 1993. Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. *Cell.* 75:1169–1178.

Sun, S.C., P.A. Ganchi, D.W. Ballard, and W.C. Greene. 1993. NF-κB controls expression of inhibitor IκBα: evidence for an inducible autoregulatory pathway. *Science (Wash. DC).* 259:1912–1915.

Sun, S.C., P.A. Ganchi, C. Beraud, D.W. Ballard, and W.C. Green. 1994. Autoregulation of the NF-κB transactivator RelA (p65) by multiple cytoplasmic inhibitors containing ankyrin motifs. *Proc. Natl. Acad. Sci. USA.* 91:1346–1350.

Takahashi, T., M. Tanaka, J. Inazawa, T. Abe, T. Suda, and S. Nagata. 1994. Human Fas ligand: gene structure, chromosomal localization and species specificity. *Int. Immunol.* 6:1567–1574.

Tewari, M., and V. Dixit. 1995. Fas and TNF-induced apoptosis is inhibited by the poxvirus crmA gene product. *J. Biol. Chem.* 270:3255–3260.

Traenckner, E.B.-M., S. Wilk, and P.A. Baeuerle. 1994. A proteasome inhibitor prevents activation of NF-κB and stabilizes a newly phosphorylated form of IκB-α that is still bound to NF-κB. *EMBO (Eur. Mol. Biol. Organ.) J.* 13: 5433–5441.

Vaux, D.L. 1993. Toward an understanding of the molecular mechanism of physiological cell death. *Proc. Natl. Acad. Sci. USA.* 90:786–789.

Vaux, D.L., G. Haecker, and A. Strasser. 1994. An evolutionary perspective on apoptosis. *Cell.* 76:777–779.

Wang, H.-G., T. Miyashita, S. Takayama, T. Sato, T. Torigoe, S. Krajewski, S. Tanaka, L. Hovey, J. Troppmair, U.R. Rapp, and J.C. Reed. 1994. Apoptosis regulation by interaction of Bcl-2 protein and Raf-1 kinase. *Oncogene.* 9: 2751–2756.

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

Westendorp, M.O., V.A. Shatrov, K. Schulze-Osthoff, R. Frank, M. Kraft, M. Los, P.H. Krammer, W. Dröge, and V. Lehmann. 1995*a*. HIV-1 Tat potentiates TNF-induced NF-κB activation and cytotoxicity by altering the cellular redox state. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:546–554.

Westendorp, M.O., R. Frank, C. Ochsenbauer, K. Stricker, J. Dhein, H. Walczak, K.M. Debatin, and P.H. Krammer. 1995*b*. Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp120. *Nature (Lond.).* 375: 497–500.

Woronicz, J.D., B. Calnan, V. Ngo, and A. Winoto. 1994. Requirement for the orphan steroid receptor Nur 77 in apoptosis of T-cell hybridomas. *Nature (Lond.).* 367:277–281.

Wu, H., and G. Lozano. 1994. NF-κB activation of p53. A potential mechanism for suppressing cell growth in response to stress. *J. Biol. Chem.* 269:20067–20074.

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

Zabel, U., R. Schreck, and P.A. Baeuerle. 1991. DNA binding of purified transcription factor NF-κB: affinity, specificity, Zn^{2+} dependence and differential half-site recognition. *J. Biol. Chem.* 266:252–260.

Zabel, U., T. Henkel, M.S. Silva, and P.A. Baeuerle. 1993. Nuclear uptake control of NF-κB by MAD-3, an IκB protein present in the nucleus. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:201–211.