Linkage of the BH4 Domain of Bcl-2 and the Nuclear Factor κB Signaling Pathway for Suppression of Apoptosis*

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Nuclear factor (NF) κB is a ubiquitously expressed transcription factor whose function is regulated by the cytoplasmic inhibitor protein, IκBα. We have previously shown that IκBα activity is diminished in ventricular myocytes expressing Bcl-2. (de Moissac, D., Mustapha, S., Greenberg, A. H., and Kirshenbaum, L. A. (1998) J. Biol. Chem. 273, 23946–23951). In view of the growing evidence that the conserved N-terminal BH4 domain of Bcl-2 plays a critical role in suppressing apoptosis, we ascertained whether this region accounts for the underlying effects of Bcl-2 on IκBα activity. Transfection of human embryonic 293 cells with full length Bcl-2 resulted in a significant 1.9-fold reduction in IκBα activity (p < 0.006) with a concomitant increase in DNA binding and 3.4-fold increase in NFκB-dependent gene transcription (p < 0.022) compared with vector transfected control cells. In contrast, no significant change in IκBα activity was detected with either a BH4 domain deletion mutant (residues 10–30) or BH4 domain point substitution mutants, I14G, V15G, Y18G, K22G, and L23G (p = 2.77). However, a small 0.6-fold decrease (p < 0.04) in IκBα activity was noted with the BH4 mutant I19G, suggesting that this residue may not be critical for IκBα regulation. Furthermore, adenovirus-mediated delivery of an IκBα mutant to prevent NFκB activity impaired the ability of Bcl-2 to suppress apoptosis provoked by TNFα plus cycloheximide in ventricular myocytes. The data provide the first evidence for the regulation of IκBα by Bcl-2 through a mechanism that requires the conserved BH4 domain that links Bcl-2 to the NFκB signaling pathway for suppression of apoptosis.

Apoptosis or programmed cell death is a highly regulated event crucial for normal development and homeostasis. Deregulated cell death has been associated with disease entities such as cancer (1, 2), HIV (3), Huntington’s disease (4), and more recently cardiovascular disease (5, 6). Although our understanding of the molecular mechanisms that underlie programmed cell death in mammalian cells is poorly defined, there is considerable evidence that the bcl-2 gene family may play a critical role in this process (reviewed in Refs. 7–9). Bcl-2 can delay or prevent apoptosis provoked by a variety of death-promoting signals, suggesting that it likely impinges on more than one component of the death signaling pathway.

Structural analysis studies of Bcl-2 have identified several key domains with putative anti-apoptotic properties (10–12). In particular, the N terminus of Bcl-2, which encompasses an amphipathic α-helical loop designated the BH4 domain, has been suggested to play a crucial role in the prevention of apoptosis. This is substantiated by studies in which the deletion or mutation of this region was shown to render Bcl-2 defective for suppression of apoptosis (10, 11, 13, 14), independent of its ability to dimerize with the pro-apoptotic factors Bax, Bak, or Bad (11). The mechanism by which the BH4 domain confers protection against apoptosis is unknown but may reside in its ability to modulate the activity of certain factors involved in the apoptotic process. The BH4 domain of Bcl-2 has been shown to bind to and sequester the calcium-activated phosphatase calcineurin (15) crucial for the nuclear import of NF-AT4 and signal-induced apoptosis in T-cells (16). Moreover, the BH4 domain has been deemed critical for the interaction with Raf-1 and the Caenorhabditis elegans CED 4 homologue, Apaf-1 (13, 17–19). In this regard, Apaf-1, in association with cytochrome c, dATP, and pro-caspase 9, has been implicated in a mitochondrial-dependent pathway for caspase activation and apoptosis (18, 19). Thus, the BH4 domain, through its ability to interact with potentially pro-apoptotic factors, represents a critical region within the Bcl-2 molecule for the prevention of apoptosis.

In mammalian cells, the transcription factor NFκB1 is comprised of 50-kDa and 65-kDa protein subunits (20–22) bound to the cytoplasmic inhibitor protein IκBα (23–26). Activation of NFκB by agents such as TNFα or interleukin-1β involves the N-terminal phosphorylation and degradation of IκBα by the ubiquitin-proteasome pathway (27, 28). Owing to the ubiquitin-proteasome pathway (27, 28), IκBα unmaskes the NFκB nuclear localization motif, permitting NFκB to translocate to the nucleus and direct gene transcription (29).

Recently, an anti-apoptotic function for NFκB has been described (30–32). This is substantiated by studies in which cells defective for NFκB were found to be more sensitive to pro-apoptotic signals than NFκB expressing cells (31, 32). Although TNFα leads to NFκB activation, there is emerging evidence that TNFα predominately triggers apoptosis in cells that are either deficient or defective for NFκB (30, 31). This has led to the suggestion that TNFα is sufficient to activate both pro- and anti-apoptotic pathways with the anti-apoptotic signals, mediated through the NFκB, dominating to suppress death-promoting signals and apoptosis (30–32).

We have recently shown that adenoviral-mediated gene delivery of Bcl-2 to ventricular myocytes increased NFκB activity and prevented apoptosis mediated by TNFα plus cycloheximide (33). This was attributed to Bcl-2-mediated phosphorylation of

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1 The abbreviations used are: NFκB, nuclear factor κB; TNFα, tumor necrosis factor α; CMV, cytomegalovirus; TUNEL, terminal deoxynucleotidyl transferase nick end labeling.
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**Experimental Procedures**

**Cell Culture and Transfection**—Human embryonic kidney 293 cells (American Tissue Type Collection) were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Life Technologies, Inc.) as previously reported (33). For transfection experiments, cells were transfected for 3 h with Dulbecco's modified Eagle's medium containing Superfect (Qiagen) and 1–5 μg of CMV-driven eukaryotic expression plasmids. Data are expressed as the means ± S.E. (p < 0.05). Experiments were repeated at least three times with independent culture conditions with three replicates for each condition.

**Western Blot Analysis**—For immunodetection of IκBα protein, 293 cells were harvested in 1% Triton X-100, 1% sodium deoxycholate, 140 mM NaCl, 10 mM Tris-HCl, pH 8.0 (RIPA buffer). Cell lysates (50 μg) were resolved on a 10% sodium deoxycholate-polyacrylamide gel at 140 V for 4 h and electrophoretically transferred to polyvinylidene difluoride membrane (Roche Diagnostics). For detection of IκBα protein, the polyvinylidene difluoride filter was incubated for 3 h with a mouse monoclonal antibody directed toward human IκBα/MAD-3 protein clone C21 (1 μg/ml Santa Cruz Biotechnology) in 150 mM NaCl, 50 mM Tris-HCl, pH 7.4. O.3% Tween-20, 0.1% bovine serum albumin. Expression of wild type and ΔBH4 deletion mutant forms of Bcl-2 proteins were detected using a hamster monoclonal antibody directed toward Bcl-2 clone 6C8 (kindly provided by S. Korsmeyer). For detection of transfected IκBα/FLAG-tagged proteins, cell lysates were incubated with 1 μg of murine anti-FLAG antibody (Kodak) and immunoprecipitated with 25 μl of protein G agarose beads (Amersham Pharmacia Biotech) at 4 °C for 4 h (27). Immunoprecipitates were washed twice and mixed with 2X SDS Laemmli loading buffer, boiled, and subjected to gel electrophoresis as described above. Bound proteins were detected by chemiluminescence reaction with horseradish peroxidase-conjugated anti-rabbit IgG.

**Electromobility Gel Shift Assay**—Nuclear extracts of cells were prepared as described previously by McKinsey et al. (28). A 32P-radiolabeled duplex oligonucleotide probe with NFκB binding sites was used as a template for the gel shift experiments (27). DNA binding reaction mixtures (20 μl) were carried out on ice and contained 10 μg of nuclear extract, 2 μg of double-stranded probe, poly(dI-dC), (Amersham Pharmacia Biotech),
RESULTS AND DISCUSSION

To establish whether Bcl-2 could lead to activation of NFκB, 293 cells were transfected with a luciferase reporter gene containing putative binding sites for NFκB (27, 39) in the presence and absence of Bcl-2. A 3.4-fold increase ($p < 0.022$) in luciferase reporter gene activity was observed in cells expressing Bcl-2 compared with control cells transfected with the eukaryotic expression vector pcDNA3 alone (Fig. 1). Similar effects were observed in TNFα-stimulated cells that served as a positive control for induction of NFκB gene activation. In contrast, however, cells transfected with a Bcl-2 cDNA lacking the N-terminal BH4 domain failed to activate NFκB-dependent gene transcription ($p = 0.31$) compared with cells transfected with wild type Bcl-2.

Electromobility shift analysis of nuclear extract prepared from 293 cells revealed a significant increase in DNA binding activity of NFκB in cells expressing the wild type Bcl-2 but not the ΔBH4 deletion mutant of Bcl-2 (Fig. 2, lane 3 versus lane 4) compared with control cells (lanes 1 and 6). A similar increase

FIG. 4. The effect of BH4 domain point mutants on IκBα activity. A, cell lysates from 293 cells transfected with wild type Bcl-2 (Bcl-2) or ΔBH4 point substitution mutants were prepared for Western blot analysis for detection of IκBα protein as shown in Fig. 3. The six BH4 domain point mutants utilized are indicated by the amino acid substitution at the designated positions: I14G, V15G, Y18G, I19G, K22G, and L23G. pcDNA3, control cells transfected with equivalent amounts of the CMV-driven eukaryotic expression vector lacking Bcl-2 cDNA inset. B, Ponceau S stain of membrane to demonstrate equivalent loading of protein samples. C, cell lysate from A was subjected to Western blot analysis for detection of Bcl-2 proteins using a hamster monoclonal antibody directed toward human Bcl-2. The figure verifies that Bcl-2 proteins were expressed to comparable levels. D, histogram represents densitometric analysis of three Western blots for IκBα activity in the presence of wild type (WT) Bcl-2 and BH4 domain mutants. Data are expressed as fold reduction relative to vector transfected control cells. IκBα activity is repressed by 1.9-fold ($p < 0.006$) by wild type Bcl-2. The BH4 domain point mutants I14G, V15G, Y18G, K22G, and L23G had no apparent effect on IκBα activity ($p = 2.77$) with the exception of the I19G, which repressed IκBα by 0.6-fold ($p < 0.04$) compared with vector control cells.

FIG. 5. Effects of BH4 domain mutants on NFκB nuclear DNA binding. Equivalent amounts of nuclear extract from 293 cells were prepared following transfection with either wild type or BH4 domain mutants detailed in Fig. 4 and analyzed for NFκB DNA binding activity. Lane 1, free probe; lane 2, control cells (CNTL); lane 3, wild type Bcl-2 (Bcl-2); lanes 4–9, BH4 point substitution mutants; lane 10, pcDNA3 transfected cells. Wild type Bcl-2 but not the BH4 domain point mutants increase NFκB nuclear DNA binding, compared with controls. Arrow indicates NFκB complex.
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In the nuclear binding activity of NFκB was also observed in cells stimulated by TNFα (lane 5). Competition binding assays with 100-fold excess cold probe (lane 7) as well as supershift experiments with antibodies directed toward the p65 subunit (lane 8) confirmed that the migrating complex contained NFκB/p65.

Because NFκB activity is largely influenced by IκBα, which sequesters NFκB in the cytoplasm, we determined whether the observed increase in nuclear NFκB binding activity and gene expression in the presence of Bcl-2 was due to a reduction in IκBα protein levels. Protein extracts of 293 cells expressing Bcl-2 and FLAG-tagged IκBα proteins were subjected to Western blot analysis and probed with a murine antibody directed toward IκBα. As shown in Fig. 3, IκBα levels were significantly suppressed in cells expressing the wild type Bcl-2 but not the ΔBH4 mutant of Bcl-2 compared with vector transfected control cells.

To confirm the notion that the conserved N-terminal BH4 domain of Bcl-2 is responsible for the underlying effects on IκBα activity, we utilized point substitution mutations of the BH4 domain that had been previously shown to disrupt the anti-apoptotic function of Bcl-2 (14). In contrast to cells transfected with wild type Bcl-2 that displayed a 1.9-fold reduction (p < 0.006) in IκBα activity compared with vector transfected control cells, cells transfected with the BH4 domain point mutants, with the exception of the I19G mutant, were not statistically different from vector transfected control cells (p = 0.77; Fig. 4, A and D). Interestingly, a small 0.60-fold reduction (p < 0.04) in IκBα activity was observed with the I19G BH4 mutant.

To verify that the observed differences between wild type Bcl-2 and BH4 domain mutants on IκBα activity were not due
to discrepancies in protein loading, the filter was stained with Ponceau S dye, which confirmed equivalent protein loading (Fig. 4B). In addition, Western blot analysis of these samples revealed that the wild type and BH4 domain mutants were expressed to comparable levels (Fig. 4C), ruling out the possibility that the noted differences in IxBa activity were due to discrepancies in Bcl-2 protein expression.

Furthermore, consistent with our Western blot data for IxBa, electromobility shift analysis for NFkB revealed that each of the point mutants tested with the exception of the I19G mutant were defective for directing NFkB-dependent DNA binding and were not significantly different from vector-transfected control cells (Fig. 5). The fact that the I19G BH4 mutant had an intermediate effect on NFkB DNA binding compared with wild type Bcl-2 suggests that this residue may not be critical for directing IxBa degradation.

Previously, we demonstrated that Bcl-2 activated NFkB and suppressed apoptosis of ventricular myocytes provoked by TNFa plus cycloheximide (33). Because NFkB has been reported to be important for suppressing apoptosis in mammalian cells, we tested functional significance of our observations by determining whether a block to NFkB activation, would impair the ability of Bcl-2 to rescue TNFa-mediated apoptosis. For these studies we generated a recombinant adenovirus that encodes a mutant version of the IxBa molecule that contains serine to alanine point substitutions at amino acids 32 and 36, respectively. This renders IxBa defective for phosphorylation and degradation, thereby preventing NFkB activation (27). As shown by gel shift analysis (Fig. 6), the IxBa mutant prevented the increase in NFkB nuclear DNA binding activity mediated by Bcl-2, confirming that the IxBa mutant was functionally active in these cells. Furthermore, expression of the IxBa mutant impaired the ability of Bcl-2 to suppress apoptosis triggered by TNFa plus cycloheximide, demonstrated by the increased TUNEL positive nuclei (Fig. 7A) and nucleosomal DNA laddering (Fig. 7B).

The mechanism by which Bcl-2 mediates NFkB activation is unknown but may involve the inactivation of IxBa. In the present study, we provide evidence for the regulation of IxBa activity by Bcl-2 through a mechanism that requires the BH4 domain of Bcl-2. Precedence for cellular factors other than NFkB to be regulated by Bcl-2 has been documented (40). Although the mode by which the BH4 domain modulates IxBa activity is unknown, we have previously demonstrated that Bcl-2 leads to the phosphorylation of IxBa and degradation by the proteasome (33). However, our studies indicate that Bcl-2 does not directly interact with IxBa. Therefore, it is tempting to speculate that Bcl-2 modulates IxBa activity by interacting with one or more cellular factors that directly or indirectly activate NFkB. Alternatively, the BH4 domain could influence the activity of IxBa by interacting with one of the IxB kinases (41, 42). Nevertheless, in view of the growing evidence that the BH4 domain of Bcl-2 is critical for the prevention of apoptosis, our finding that inhibition of NFkB activation impairs the anti-apoptotic properties of Bcl-2 provides compelling evidence that links Bcl-2 to the NFkB signaling pathway for the suppression of apoptosis. A current model for the operation of Bcl-2 proposes that the BH4 domain binds to and sequesters factors leading to caspase activation and apoptosis. In this regard, the physical interaction of BH4 domain with mitochrondrial Apaf-1 has reportedly been shown to inhibit association of Apaf-1 with cytochrome c and caspase 9, preventing the subsequent processing of caspase 3 (15, 19). Furthermore, the relationship between Bcl-2 and the NFkB signaling pathway becomes even more profound, given that activated caspase 3 can directly cleave the N-terminal segment of IxBa, resulting in a peptide fragment that inhibits NFkB activation (31, 35). Thus, Bcl-2 may in part operate through a mechanism that intersects the activation of caspases and of NFkB for the suppression of apoptosis.

Our data provide the first direct evidence for the regulation of IxBa by Bcl-2 through a mechanism that requires the conserved BH4 domain and links Bcl-2 to the NFkB signaling pathway for the suppression of apoptosis.

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REFERENCES

[References are omitted to save space.]

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