The proto-oncogene *BCL-3* encodes an I κ B protein

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The *bcl-3* gene product, overexpressed in chronic lymphocytic leukemia (CLL) patients with the translocation t(14;19), is a member of the I κ B family. The bcl-3 protein is able to inhibit the DNA binding and *trans*-activation of authentic NF- κ B heterodimers p50-p65 and p49-p65, as well as p50 and p49 homodimers. The bcl-3 protein does not inhibit either the DNA-binding activity of the Rel protein or its ability to *trans*-activate genes linked to the κ B site. A human 37-kD protein (I κ B α), identified previously as a member of the I κ B family, is also unable to inhibit DNA-binding activity of the Rel protein. However, unlike bcl-3, the 37-kD (I κ B α) protein has no effect on the DNA-binding activity of p50 or p49 homodimers. Two dimensional phosphotryptic peptide maps of the human bcl-3 and the human 37-kD (I κ B α) proteins reveal that the phosphopeptides from the 37-kD (I κ B α) protein are nested within the bcl-3 protein. Furthermore, bcl-3 antisera immunoprecipitates an in vitro-radiolabeled 37-kD (I κ B α) protein. Proteins of 56 and 38 kD can be identified in HeLa cells stimulated with PMA and immunoprecipitated with bcl-3 antisera. Comparison of tryptic peptide maps of the bcl-3 protein synthesized in vitro, and p56 and p38 from HeLa cells, shows that they are all structurally related. Removal of the amino-terminal sequences of the bcl-3 protein generates a protein that inhibits the DNA binding of the p50-p65 heterodimer but, like the 37-kD (I κ B α) protein, is no longer able to inhibit the binding of the p50 and p49 homodimers with κ B DNA. We propose that the bcl-3 and 37-kD (I κ B α) proteins are related and are members of the I κ B family.

[Key Words: NF- κ B; c-Rel; DNA binding; *trans*-activation]

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The molecular cloning of the gene located at the breakpoint junction in the t(14;19) translocation observed in several cases of human B-cell chronic lymphocytic leukemia (CLL) revealed that it can encode a protein of 446 amino acids (McKeithan et al. 1987, 1990; Ohno et al. 1990). The t(14;19) translocation in CLL patients juxtaposes the *IgH* gene on chromosome 14 to the *bcl-3* gene on chromosome 19 in a head-to-head manner (McKeithan et al. 1990). Although the transcriptional integrity of *bcl-3* appears uninterrupted by t(14;19), CLL patients with the translocation express the *bcl-3* mRNA in peripheral blood cells at least 3.5 times the levels of that of CLL patients without the translocation (Ohno et al. 1990). The predicted amino acid sequence of this basic protein contains a proline-rich amino terminus, a series of seven tandem ankyrin repeats, and a proline- and serine-rich carboxyl terminus. Comprising the entire central portion of the protein are seven 33- to 37-amino-

acid repeats first recognized in the yeast mating-type switching protein (SWI6) and later characterized in a number of other proteins (Breedon and Nasmyth 1987). Although the precise tertiary structure or function of these repeats is not well characterized, the ankyrin motif is present in numerous gene products involved in transcriptional regulation (Michaely and Bennett 1992), which include NF- κ B p105 (Ghosh et al. 1990; Kieran et al. 1990), NF- κ B p100 (Schmid et al. 1991; Bours et al. 1992), I κ B β /pp40 (Davis et al. 1991), MAD3 (Haskill et al. 1991), the GA-binding protein β (GABP β) (LaMarco et al. 1991; Thompson et al. 1991), and *cdc10* (*Schizosaccharomyces pombe*)/SWI4, SWI6 (*Saccharomyces cerevisiae*) (Aves et al. 1985; Breedon and Nasmyth 1987), and it is also found in several growth factor receptors [i.e., the *Drosophila melanogaster* Notch protein (Wharton et al. 1985) and the *Caenorhabditis elegans* proteins Lin-12 (Yochem et al. 1988) and glp-1] and in several vaccinia viral proteins.

The I κ B protein was first described as a cytoplasmic protein that inhibits the DNA-binding activity of the

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transcription factor NF- κ B heterodimeric complex (Sen and Baltimore 1986a,b). In the cytosolic fraction of many cells, the I κ B protein forms a complex with p50–p65 subunits of the NF- κ B heterodimeric complex and can be inactivated following the activation of cells with cytokines, TPA, and many other agents (Baeuerle 1991). It was proposed that following stimulation of cells with phorbol ester (PMA), the I κ B protein is phosphorylated, allowing the p50–p65 complex to migrate to the nucleus and bind to DNA (Baeuerle and Baltimore 1988a,b). A 40-kD phosphoprotein (pp40), which associates with the viral and cellular Rel protein, was shown to inhibit *in vitro* the DNA-binding activity of the NF- κ B/Rel protein (Kerr et al. 1991). Nucleotide sequence analysis of a cDNA encoding avian pp40 revealed that it contains five ankyrin-repeat motifs identified previously in a number of proteins, including the bcl-3 protein, a candidate proto-oncogene (Ohno et al. 1990; Davis et al. 1991). Interestingly, the carboxy-terminal half of p105, the precursor of p50, one of the subunits of the NF- κ B heterodimer, also contains ankyrin-repeat motifs (Ghosh et al. 1990; Kieran et al. 1990). A shorter 2.4-kb alternatively spliced mRNA encoding a 70-kD (I κ B γ) protein originating from the carboxy-terminal region of p105, and thus containing ankyrin repeats, was also identified (Inoue et al. 1992a).

We have found previously that four of the five ankyrin repeats within I κ B β /pp40 were necessary for its inhibitory function. However, the central domain containing all five ankyrin repeats alone is insufficient to mediate inhibition of c-Rel and the association of NF- κ B with DNA (Inoue et al. 1992b). The presence of the ankyrin repeats and the resemblance of its prototypic structure to I κ B β /pp40, a member of the I κ B family of proteins, suggested that the *bcl-3* gene might encode a protein that can act as an inhibitor of κ B-binding proteins. We report the following. (1) The full-length bcl-3 protein synthesized in bacteria inhibits the DNA-binding activity of the NF- κ B heterodimeric complexes p50–p65 and p49–p65, as well as p50 and p49 homodimers. (2) The bcl-3 protein does not inhibit the κ B DNA-binding activity of the Rel protein. (3) The bcl-3 protein inhibits the *trans*-activation of reporter genes linked to the κ B site. (4) bcl-3 antisera immunoprecipitate a 56-kD protein in the cytoplasm and an additional 38-kD protein in the nucleus of HeLa cells treated with PMA. The tryptic peptide maps of p56 and p38 indicate that p38 is a subset of p56. (5) bcl-3 antisera immunoprecipitate the *in vitro*-labeled human 37-kD (I κ B α) protein capable of inhibiting the DNA-binding activity of p50–p65 NF- κ B heterodimers but not that of the Rel protein to κ B DNA. (6) The phosphotryptic peptide maps of *in vitro*-labeled 37-kD (I κ B α) and bacterial bcl-3 proteins show that the human 37-kD protein is nested within the human bcl-3 protein. (7) Amino-terminal truncation of bcl-3 yields a protein with functional properties similar to that of the 37-kD (I κ B α) protein. We conclude that the bcl-3 protein is a member of the I κ B family of proteins; furthermore, the previously identified human 37-kD (I κ B α) protein is a truncated form of the bcl-3 protein.

Results

The bcl-3 protein inhibits the DNA binding of NF- κ B, p50, and p49 homodimers, but not c-Rel

The human bcl-3 protein synthesized in bacteria was examined for its ability to inhibit the binding of the NF- κ B/Rel proteins to their cognate DNA binding site by electrophoretic mobility-shift assays (EMSAs). In addition, several of the previously characterized I κ B proteins were examined in the same assay to standardize conditions and to control for protein concentrations. Figure 1A demonstrates that the bcl-3 protein does not inhibit the binding of the murine c-Rel protein to DNA (cf. lanes 1 and 7). As reported previously, the human 37-kD protein (I κ B α) also has no effect on the binding of the c-Rel protein to the κ B site (lane 2), whereas human 43-kD (I κ B β), chicken pp40, murine I κ B γ , and murine MAD3 proteins are efficient inhibitors (Fig. 1A, lanes 3–6). The bcl-3 protein is, however, able to inhibit the DNA-binding activity of the p50–p65 NF- κ B complex (Fig. 1B, lane 8), p50–p50 homodimers (Fig. 1C, lane 8), and p49–p49 homodimers (Fig. 1D, lane 8). In contrast, the human 37-kD (I κ B α) protein, which like bcl-3 is unable to inhibit the DNA-binding activity of the c-Rel protein (Fig. 1A, lane 2), has no effect on the binding of either p50 or p49 homodimers to the κ B site (Fig. 1C,D, lane 3). A mutant bcl-3 protein lacking the carboxy-terminal 140 amino acids [bcl-3(Δ PstI)] was unable to inhibit the DNA-binding activity of the p50–p65 heterodimeric complex (Fig. 1B, lane 9). Thus, the bcl-3 protein appears to function like pp40/I κ B β in that both the ankyrin repeats and the carboxyl terminus are necessary for complete inhibitory activity (Inoue et al. 1992b). On the basis of the data in Figure 1, we conclude that the bcl-3 protein is a member of the I κ B family.

The bcl-3 protein is able to repress κ B-mediated transcription in vivo

We then addressed whether the bcl-3 gene product can inhibit endogenous NF- κ B activity. Mouse 3T3 fibroblasts or human 293 adenovirus-transformed kidney cells were transfected with a reporter plasmid, human immunodeficiency virus–long terminal repeat chloramphenicol acetyltransferase (HIV–LTR CAT) (Rosen et al. 1985). Figure 2A demonstrates that *trans*-activation is dependent on the κ B site, as little or no activity is detected with a reporter construct containing mutated κ B sites (cf. lanes 1 and 2). Cotransfection of the bcl-3 cDNA expression vector caused a marked decrease in endogenous κ B activity (Fig. 2A, lane 3). The residual activity observed in both cell types might be the result of endogenous c-Rel activity, which may not be affected by the presence of the bcl-3 protein. A mutant bcl-3 cDNA that generates a truncated protein [bcl-3(Δ PstI)], unable to inhibit κ B DNA-binding activity of the NF- κ B heterodimer (Fig. 1B, lane 9), had little or no inhibitory activity when cotransfected in either cell type (Fig. 2A, lanes 3,4).

The direct inhibitory activity of the bcl-3 protein on individual members of the κ B-binding family was deter-

Figure 1. The effect of the 37-kD (IκBα), 43-kD (IκBβ), pp40, IκBγ, murine MAD3 (mMAD3), and bcl-3 protein on the κB-binding activity of bacterial c-Rel, purified NF-κB, and bacterial p50 and p49. (A) DNA-binding inhibition of Rel. A radiolabeled κB oligonucleotide (0.5 ng, 30,000 cpm) was incubated with bacterial c-Rel (~200 ng) that had been pretreated for 30 min at 0°C with purified IκBα (4 μl, ~20 ng), IκBβ (3 μl, ~10 ng), pp40 (3 μl, ~7.5 ng), bacterial IκBγ (200 ng), bacterial mMAD3 (50 ng), or bacterial bcl-3 (25 ng). Reactions were incubated at ambient temperature for 30 min before nondenaturing polyacrylamide gel analysis. The autoradiograph was exposed at -70°C for 6 hr. (B) Inhibition of human NF-κB p50-p65 DNA-binding activity. Purified human NF-κB (1 μl of 1 : 30) was preincubated in the absence of inhibitors (lane 1), 50× (25 ng) unlabeled κB oligonucleotide (lane 2), 50× (25 ng) unlabeled mutant κB oligonucleotide (lane 4), IκBα (lane 5), IκBβ (lane 6), mMAD3 (lane 7), bcl-3 (lane 8), or bcl-3(gDPstI) (lane 9), at concentrations used in A for 30 min on ice before the addition of radiolabeled κB DNA. Samples were analyzed as described previously, and autoradiography was for 4.5 hr. (*) Protein-DNA complexes not specifically competed by a 50-fold excess of κB oligonucleotide. (C) DNA-binding inhibitors of p50 homodimers. Radiolabeled DNA (3 × 10⁵ cpm) was combined with purified bacterial human p50 that had been pretreated with IκBα (lane 3), IκBβ (lane 4), pp40 (lane 5), IκBγ (lane 6), mMAD3 (lane 7), or bcl-3 (lane 8) at the concentrations described in A. Analysis of protein-DNA complexes was resolved as described previously, and autoradiography was for 8 hr. (D) DNA-binding inhibition of p49-DNA complexes. p49 generated in bacteria was preincubated in the absence (lane 2) or presence of IκBα (lane 3), IκBβ (lane 4), pp40 (lane 5), IκBγ (lane 6), mMAD3 (lane 7), or bcl-3 (lane 8) at concentrations shown in A, kept on ice before the addition of radiolabeled probe, and analyzed as described previously. The autoradiograph was exposed at -70°C for 2.5 hr.

mined by transient transfections in the *Drosophila* Schneider cell system, shown previously to be devoid of endogenous κB activity (Inoue et al. 1992a). The κBAdh-CAT plasmid (Inoue et al. 1992a) was transfected in Schneider cells in the presence or absence of pA5c/c-Rel, a c-rel expression vector in which the expression of murine c-rel cDNA is under the control of the actin 5C promoter. c-Rel efficiently trans-activates the κB-containing promoter in a site-specific manner (Inoue et al. 1992a), and as expected, cotransfection with the A5c/bcl-3 expression plasmid has little effect on this activation (Fig. 2B, cf. lanes 2 and 3). In similar experiments, the expression vector A5c/IκBβ completely suppresses c-Rel-mediated CAT activity (see also Inoue et al. 1992a). When expression vectors A5c-p49 and A5c-p65, encoding the NF-κB p49 and p65 subunits, were transfected, a high level of κB-specific trans-activation was observed (Fig. 2B, lane 5); the additional cotransfection of A5c/bcl-3 results in marked suppression of the reporter

activity (Fig. 2B, lane 6). Further support for the inhibitory role of bcl-3 is observed in the cotransfection of Jurkat T cells with expression vectors for bcl-3, p49, and p65. Over 70% of the κB activity owing to reconstituted NF-κB p49-p65 is inhibited by bcl-3 (Fig. 2C, lane 3). The transient transfection data, along with the results obtained with EMSA, support the notion that the bcl-3 protein functions as a specific inhibitor of the NF-κB family of trans-activator proteins.

Identification of the bcl-3 protein in HeLa cells

To investigate the nature of the bcl-3 protein synthesized in vivo, we generated antibodies to bcl-3 made in bacteria. Figure 3A (lane 1) shows that 46- and 36-kD ³⁵S-labeled in vitro-translated bcl-3 proteins can be immunoprecipitated with bcl-3 antisera. The bcl-3 protein, however, does not cross-react with chicken pp40 (Fig. 3A, lane 3) or IκBγ antisera (lane 4), suggesting that it is

Figure 2. The effect of the Bcl-3 protein on κ B-mediated promoter activity owing to endogenous factors or to transfected c-Rel or NF- κ B p49-p65. (A) The bcl-3 protein is able to suppress endogenous κ B-mediated CAT activity. BALB/c-3T3 mouse fibroblasts or human adenovirus-transformed 293 kidney cells were transfected by calcium-phosphate precipitation with reporter DNA bearing either HIV-LTR CAT containing mutated κ B sites (4 μ g; lane 1) or wild-type HIV-LTR CAT (4 μ g; lanes 2-4). Ten micrograms of pSVK-bcl-3, a construct in which the human wild-type bcl-3 cDNA is driven by the SV40 promoter, was cotransfected (lane 3). Ten micrograms of pSVK-bcl-3(Δ PstI), in which a human carboxy-terminally truncated bcl-3 cDNA is expressed under the SV40 promoter, was cotransfected (lane 4). As a control, cells in lanes 1 and 2 received 10 μ g of the pSVK3 parental vector. All cells were transfected with 2 μ g of SV/ β -gal as a control for transfection efficiency. Cells were transfected, glycerol shocked, and refed in serum-containing medium. Two days post-transfection, cell lysates were made and assayed for β -galactosidase activity, and normalized amounts of lysate were analyzed for CAT activity. (B) bcl-3 inhibits NF- κ B-mediated CAT transcription but not that of c-Rel. *Drosophila* Schneider S2 cells (3×10^6 cells/60-mm dish) were transfected with 2 μ g of either the 6 $\times$ κ BAdhCAT (lanes 2,3,5,6) or the mutant κ B 6 $\times$ M κ BAdhCAT (lanes 1,4) (Inoue et al. 1992a). Cells in lanes 1-3 received 4 μ g of A5c/c-Rel (murine c-rel cDNA under the actin 5c promoter); lanes 4-6 received 2 μ g each of A5c/p49 and A5c/p65. Specific κ B-mediated activity is observed for both κ B trans-activators (cf. lane 1 with 2 and lane 4 with 5). The addition of 4 μ g of A5c/bcl-3 had no effect on the c-Rel-mediated CAT activity (lane 3), whereas reconstituted NF- κ B activity was inhibited by 70% on average in four independent transfection experiments. All transfections were normalized with 2 μ g of A5c/ β -gal and brought to a total DNA concentration of 12 μ g with the A5c parental vector. (C) bcl-3 inhibits NF- κ B activity in the Jurkat human T-cell line. Jurkat T cells were transfected with 10 μ g of 4 $\times$ κ B CAT in the absence (lane 1) or presence (lanes 2,3) of 0.2 μ g each of Rous sarcoma virus (RSV)/p49 and RSV/p65. Cells assayed in lane 3 received 0.5 μ g of RSV/bcl-3. Approximately 50-100 μ g of total protein from each cell lysate was analyzed for CAT activity for 2 hr at 37°C.

a unique I κ B protein. We consistently observe a full-length (46 kD) and truncated (36 kD) protein when bcl-3 mRNA is translated in vitro. Figure 3B shows that in HeLa cells stimulated with phorbol ester (PMA), a 56-kD protein can be identified in both the cytoplasm and the nucleus; however, in the nucleus an additional protein of 38 kD can also be observed to immunoprecipitate with bcl-3 antisera (Fig. 3B, lane 2). To investigate whether bcl-3 protein translated in vitro (Fig. 3A, lane 1) and p56 and p38 synthesized in vivo (Fig. 2B, lanes 1 and 2) are related, we performed two-dimensional tryptic peptide analysis. The data (Fig. 3C-E) show that (1) the in vitro-translated 46-kD bcl-3 protein and p56 immunoprecipitated from HeLa cells display nearly identical tryptic peptide maps (Fig. 3, cf. C and D) and (2) in vivo-labeled p56 and p38 proteins share extensive structural homology. We conclude that p56 immunoprecipitated in vivo

is the bcl-3 protein. Furthermore, p38 appears to be the truncated form of p56.

The bcl-3 protein is related to the human 37-kD (I κ B α) protein

The inability of both the human 37-kD (I κ B α) and bcl-3 proteins to inhibit DNA-binding activity of the Rel protein (Fig. 1A, lanes 2,7) suggested some functional and perhaps structural relatedness. The human 37-kD (I κ B α) and in vitro-translated bcl-3 proteins were labeled with [32 P]ATP, using purified casein kinase II (CKII), followed by immunoprecipitation with bcl-3 antisera. As expected, 43- and 38-kD proteins can be immunoprecipitated from in vitro-translated bcl-3 mRNA (Fig. 4A, lane 2). More importantly, the in vitro-labeled 37-kD (I κ B α) protein is also immunoprecipitated with bcl-3 antisera

Figure 3. Characterization of bcl-3 antiserum and comparison of bcl-3 protein synthesized in vitro and in vivo. (A) Specific immunoprecipitation of ^{35}S -labeled in vitro-translated bcl-3 proteins. bcl-3 Protein was synthesized in vitro by transcription-translation of the human *bcl-3* cDNA in the presence of [^{35}S]methionine. The resulting radiolabeled bcl-3 protein (5 of 50 μl) was immunoprecipitated in the presence of either the anti-bcl-3 serum (5 μl , lane 1), preimmune serum (5 μl , lane 2), anti-pp40 serum (7 μl , lane 3), or anti-I κ B γ (10 μl , anti-5177, lane 4; Inoue et al. 1992a). Immune complexes were collected on protein A-Sepharose and analyzed by SDS-PAGE. Slight distortion of the proteins occurred during transfer to nitrocellulose for two-dimensional mapping. The autoradiograph of the wet nitrocellulose filter was for 14 hr at -70°C . The rabbit reticulocyte lysate has been shown to contain I κ B-like activity; thus, the other bands in lanes 3 and 4 may represent bona fide I κ B proteins. (B) HeLa cells express bcl-3 protein. HeLa S2 cells (1×10^7 cells) were deprived of methionine for 3 hr before the addition of 1 mCi/ml of Expre ^{35}S (New England Nuclear-Dupont) and 100 ng/ml of PMA. Three hr poststimulation, cells were collected, washed in PBS, and separated into cytoplasmic and nuclear fractions. Equal TCA-precipitable counts were analyzed for anti-bcl-3 immune complexes under denaturing conditions, and the resulting proteins were visualized by SDS-PAGE. The autoradiograph of the wet nitrocellulose filter was exposed for 30 hr at -70°C . (C) Two-dimensional tryptic peptide map of the in vitro-translated human bcl-3 protein. The 46-kD human bcl-3 protein translated in vitro was subjected to complete digestion with trypsin, oxidation of soluble peptides, and lyophilization before high-voltage electrophoresis at pH 8.9 and subsequent resolution by thin layer chromatography. The amount of 8000 cpm was analyzed, and the resulting autoradiograph represents a 5-day exposure at -70°C with Enhance. (D) Two-dimensional analysis of the 56-kD protein from HeLa nuclear extracts. The radiolabeled tryptic peptides generated from the 56-kD protein from HeLa cells were subjected to two-dimensional electrophoretic analysis. The amount of 5000 cpm was analyzed for seven days at -70°C with Enhance. (E) Two-dimensional analysis of the 38-kD protein from HeLa nuclear extracts. Radiolabeled p38 was digested with trypsin as described above, and the resulting peptides were analyzed. The amount of 5000 cpm was analyzed for 7 days at -70°C with Enhance. Thick arrows indicate the presence of at least two peptides absent in the smaller protein (p38). Through use of the Peptidesort program of the Wisconsin GCG program, these peptides are likely to represent two amino-terminal, methionine-containing peptides of the human bcl-3 protein. Thin arrows indicate the directions of first-dimensional electrophoresis (rightward) and second-dimensional chromatography (upward); (O) the spot where samples were applied.

Figure 4. Anti-bcl-3 serum recognizes the previously identified 37-kD (I κ B α) protein. (A) Anti-bcl-3 serum immunoprecipitates the 32 P-labeled bcl-3 and 32 P-labeled 37-kD (I κ B α) proteins but not 32 P-labeled pp40. The bacterial bcl-3, purified human 37-kD (I κ B α), and purified pp40 proteins were phosphorylated in vitro in the presence of [γ - 32 P]ATP and CKII for 30 min at 30°C. Reactions were terminated by the addition of SDS, and samples were boiled before immunoprecipitation using anti-bcl-3 serum. Immune complexes were resolved by SDS-PAGE and processed for autoradiography. The pp40 protein has been shown previously to be efficiently phosphorylated by CKII (Kerr et al. 1991). (B) Anti-bcl-3 serum recognizes the immobilized 37-kD (I κ B α) protein but not the 43-kD (I κ B β) or pp40 proteins. The 37-kD (I κ B α) (10 ng), 43-kD (I κ B β) (10 ng), and pp40 (15 ng) proteins were resolved by SDS-PAGE and transferred to Immobilon; nonspecific proteins were blocked; and the filter was incubated with anti-bcl-3 (1 : 250) overnight at 4°C. Immune complexes were recognized using 125 I-labeled protein A-Sepharose, and the resulting autoradiograph was exposed for 4 days at -70°C.

(Fig. 4A, lane 3). Radiolabeled pp40 was not immunoprecipitated with bcl-3 antisera (Fig. 4A, lane 4). The 37-kD (I κ B α) protein is also recognized by bcl-3 antibodies using Western blotting analysis (Fig. 4B, lane 3). Other polypeptides recognized by bcl-3 antisera probably represent degraded bcl-3 products in the preparation. Neither pp40 nor I κ B β reacted with bcl-3 antisera (Fig. 4B, lanes 1,2). Thus, it appears that the human 37-kD (I κ B α) protein is functionally and immunologically related to the bcl-3 protein.

We then compared the phosphotryptic peptide maps of the partially purified 37-kD (I κ B α) protein obtained from human placenta and bacterially produced human bcl-3 following in vitro phosphorylation with either cAMP-dependent protein kinase (PKA) or CKII in the presence of [32 P]ATP (Fig. 5A). In lanes 1 and 2, in addition to the

prominent 37-kD-labeled band, there are a number of other labeled proteins that most likely represent autophosphorylation of CKII and phosphorylation of contaminating proteins in CKII and the 37-kD (I κ B α) preparation. A mutant bcl-3 protein made in bacteria is slightly smaller in size (~38 kD) because it lacks the carboxy-terminal 140 amino acids [bcl-3(Δ PstI)]. The labeled bands indicated with arrowheads were isolated and subjected to two-dimensional tryptic peptide analysis. A comparison of the tryptic peptide maps of the 37-kD protein (I κ B α) and the carboxy-terminally truncated bcl-3 protein reveals a high degree of structural similarity (Fig. 5B). A mixture of 37-kD and bcl-3(Δ PstI) peptides reveals no additional tryptic peptides. One explanation of structural similarities between the bcl-3 and the 37-kD proteins is that the 37-kD protein is a truncated form of bcl-3 (Fig. 3A). We therefore conclude that the 37-kD protein (I κ B α) is embedded within the bcl-3 protein.

The human 37-kD (I κ B α) protein is a truncated form of bcl-3

To address the possibility that the 37-kD protein (I κ B α) is generated by proteolysis of the larger bcl-3 protein, deletion mutants of the bcl-3 protein that selectively encode either the amino-terminal, ankyrin-repeat region, or ankyrin-repeat carboxy-terminal residues of the bcl-3 protein were generated by linking them to glutathione S-transferase (GST). Mutant bcl-3 proteins were expressed in bacteria, and their effects on the DNA-binding activity of p50, p49, and NF- κ B p50-p65 were analyzed by EMSA. Figure 6A schematizes the structure of bcl-3 and mutant proteins. The ability of the bacterially produced bcl-3 proteins to bind in vitro with members of the κ B-binding family were examined in an in vitro association assay. As a representative of the κ B family, [35 S]methionine-labeled NF- κ B p49 translated in vitro was incubated with wild-type and mutant bcl-3 proteins coupled to glutathione-Sepharose and washed extensively. Examination by polyacrylamide gel electrophoresis and autoradiography shows that p49 is selectively capable of binding to both the wild-type and the bcl-3 mutant protein expressing the ankyrin and carboxy-terminal domains (Fig. 6B, lanes 2,5). Little or no association was observed with the bcl-3 protein containing either the amino-terminal or the ankyrin-repeat domain (Fig. 6B, lanes 3,4), even though the truncated proteins could be synthesized efficiently in vitro.

We then addressed whether various truncated forms of the bcl-3 protein can inhibit the DNA-binding activity of NF- κ B proteins. As expected, the full-length bcl-3 protein inhibited the binding of p50 and p49 homodimers (Fig. 6C, lanes 3,9), as well as the p50-p65 heterodimer (lane 15). The amino-terminal-deleted bcl-3 protein (ANK-C) was able to inhibit DNA binding of the p50-p65 heterodimer (Fig. 6C, lane 8) but had no effect on the DNA-binding ability of either p50 (lane 6) or p49 (lane 12) homodimers. Thus, amino-terminal truncation of the bcl-3 protein generates a protein with properties like that of the 37-kD (I κ B α) protein in that it can inhibit the

Figure 5. Direct structural comparison of the ^{32}P -labeled bcl-3 and ^{32}P -labeled 37-kD ($\text{I}\kappa\text{B}\alpha$) proteins by two-dimensional phosphotryptic mapping. (A) Autoradiograph of proteins phosphorylated by PKA or CKII and the resulting proteins used for mapping. The purified human 37-kD ($\text{I}\kappa\text{B}\alpha$) protein and the bacterially produced human bcl-3 protein were phosphorylated in the presence of [^{32}P]ATP by either purified cAMP-dependent PKA or CKII, as described in Materials and methods, analyzed by SDS-PAGE, and transferred to nitrocellulose before autoradiography. Arrowheads indicate the radiolabeled proteins that were excised for mapping. (B) Two-dimensional analysis of phosphotryptic peptides of the bcl-3(ΔPstI) and 37-kD ($\text{I}\kappa\text{B}\alpha$) proteins. Phosphorylated proteins (arrowheads in A) were excised from the nitrocellulose filter and subjected to exhaustive digestion with trypsin before analysis by two-dimensional mapping. The amount of 1000 cpm from each protein was analyzed in individual maps, whereas 500 cpm was mixed for the combination maps. All thin-layer plates were exposed for 7 days at -70°C using intensifying screens. The arrows indicate the direction of first-dimensional electrophoresis (rightward) and second-dimensional chromatography (upward); (○) the spot where samples were applied. (*) In vitro-phosphorylated form of the protein.

DNA-binding activity of the p50–p65 heterodimer but not that of p50 or p49 homodimers (Fig. 1). Therefore, it appears that truncation of the amino-terminal domain of bcl-3 generates a protein that has a function similar to the previously identified 37-kD ($\text{I}\kappa\text{B}\alpha$) protein.

Discussion

We have characterized the bcl-3 protein, the product of the gene affected by the translocation t(14;19) found in some CLL patients. Because the bcl-3 mRNA is overexpressed in CLL patients, we undertook a study to determine the molecular mechanism that may implicate the bcl-3 protein in neoplastic growth. We have demonstrated that the bcl-3 protein synthesized in bacteria is able to inhibit the DNA-binding activity of many members of the NF- κB family. The bcl-3 protein is also able to inhibit *trans*-activation by the NF- κB heterodimeric complex of reporter genes linked to the κB site (Fig. 2). Thus, on the basis of its function, we propose that bcl-3 is a member of the $\text{I}\kappa\text{B}$ family. Our results are in agreement with those of Hatada et al. (1992) and Wulczyn et al. (1992), who have shown that the bacterially expressed human bcl-3 protein inhibits the DNA binding of the p50 homodimer to DNA.

Relationship with the human 37-kD ($\text{I}\kappa\text{B}\alpha$) protein

Zabel and Baeuerle (1990) purified both a 37-kD ($\text{I}\kappa\text{B}\alpha$) and a 43-kD ($\text{I}\kappa\text{B}\beta$) protein from human placenta. These

two chromatographically distinct forms of proteins inhibited binding of the p50–p65 heterodimer to the κB site. The 43-kD ($\text{I}\kappa\text{B}\beta$) protein has been shown to be structurally, functionally, and immunologically related to chicken pp40 [Kerr et al. 1991]. The deduced sequence of human MAD3 protein, an immediate-early gene product in monocytes, also showed extensive homology with the chicken pp40 protein [Haskill et al. 1991]. In early growth response during hepatic regeneration, an $\text{I}\kappa\text{B}$ -like protein, RL/IF-1 (regenerating liver inhibitory factor) was identified as the rat homolog of MAD3. It inhibits κB -binding site of p50–p65 NF- κB , c-Rel/p50, and Rel B/p50, but not p50 homodimers [Tewari et al. 1992]. On the basis of extensive structural and functional homology, the chicken pp40, the human or mouse MAD3, the RL/IF-1, and the human 43-kD ($\text{I}\kappa\text{B}\beta$) proteins are highly related and perhaps identical proteins. We have shown previously that unlike pp40/ $\text{I}\kappa\text{B}\beta$, the human 37-kD ($\text{I}\kappa\text{B}\alpha$) protein was unable to inhibit the binding of Rel to DNA; however, like pp40/ $\text{I}\kappa\text{B}\beta$, it inhibited the binding of the p50–p65 heterodimer to the κB site [Davis et al. 1991; Kerr et al. 1991]. Neither the 37-kD ($\text{I}\kappa\text{B}\alpha$) nor pp40/ $\text{I}\kappa\text{B}\beta$ inhibited binding of the p50 homodimer to DNA. Tryptic peptide maps of the 37-kD ($\text{I}\kappa\text{B}\alpha$) and 43-kD ($\text{I}\kappa\text{B}\beta$) proteins were shown to be unrelated [Kerr et al. 1991]. More recently, we have described the identification of a 70-kD ($\text{I}\kappa\text{B}\gamma$) protein that originates from the carboxy-terminal portion of the p105 protein and has the ability to inhibit DNA-binding activity of the p50–p65

ambient temperature before analysis by nondenaturing polyacrylamide gel electrophoresis. Autoradiographs were exposed for 8, 6, and 10 hr, respectively. The probe in the p49 + bcl-3 lane was run off the gel.

heterodimer, and Rel, as well as the p50 homodimer to DNA (Inoue et al. 1992a). Thus, on the basis of functional and structural homologies, there are at least three distinct I κ B family members, namely p37 (I κ B α), pp40 (I κ B β), and p70 (I κ B γ).

The inability of the bcl-3 protein to inhibit DNA binding of Rel prompted us to consider that it may be related to the previously identified human 37-kD (I κ B α) protein. Here, we have demonstrated that the human 37-kD (I κ B α) protein represents a subset of the bcl-3 protein for the following reasons. (1) The in vitro-radiolabeled 37-kD (I κ B α) protein can be immunoprecipitated with bcl-3 antisera (Fig. 4). (2) Comparison of phosphotryptic peptide maps of the 37-kD (I κ B α) and bcl-3 proteins reveal extensive structural homology (Fig. 5). The phosphotryptic peptides of the 37-kD (I κ B α) protein are nested within the bcl-3 protein. (3) The removal of the amino-terminal 125 amino acids from the bcl-3 protein compromises its ability to inhibit DNA-binding activity of the p50 or p49 homodimers (Fig. 1), a property similar to that of the 37-kD (I κ B α) protein (Fig. 1). On the basis of the structural, functional and immunological relationship, we conclude that the 37-kD (I κ B α) species of the I κ B protein identified in human placenta is a truncated form of the bcl-3 protein. The mechanism by which the 37-kD (I κ B α) protein originates from the full-length bcl-3 protein is not known but could have been generated by alternative

splicing, as a number of bcl-3-specific mRNA species can be identified (L. Kerr, unpubl.). Furthermore, two forms of the bcl-3 protein can be identified in vivo (Fig. 3). The smaller 38-kD form, which is present only in the nucleus following PMA stimulation, may be the equivalent of the 37-kD (I κ B α) protein. At least three bcl-3-specific mRNA transcripts of 5.1, 2.0, and 1.8 kb have been identified in HeLa cells (L. Kerr, unpubl.), one of which may code for the truncated I κ B α protein. The mechanism by which the 38-kD bcl-3-related protein is generated in the nucleus also remains obscure.

I κ B family

The NF- κ B/Rel family of transcription factors is growing and includes p105 (p50), p100 (p49/50 B), p65, Rel, and Rel B. The p105 protein, which is a precursor for the NF- κ B p50-binding subunits, contains ankyrin repeats in its carboxy-terminal region, whereas p65 and Rel have a trans-activation domain. In addition, Rel B may have an inhibitory domain (Ruben et al. 1992). Because there are so many members of the NF- κ B/Rel family, it is possible that they require multiple members of the I κ B family as selective inhibitors. Table 1 summarizes the properties of various I κ B family members. It is not clear whether there is a temporal induction of the specific members of the I κ B proteins in response to selective inducers. Fur-

Figure 6. Amino-terminal truncation of the bcl-3 cDNA alters its substrate specificity and inhibitory activity in vitro. (A) Schematic representation of the inhibitory effects of the 37-kD (I κ B α) and bcl-3 wild-type and mutant proteins on the in vitro DNA-binding potential of NF- κ B, p50, and p49. (B) In vitro association assay of in vitro-translated 35 S-labeled p49 with glutathione-Sepharose-coupled bcl-3 and deletion mutants. 35 S-labeled p49 was synthesized in vitro and combined in the presence of equal amounts of Sepharose-GST-bcl-3 wild type (wt), amino terminus (N), ankyrin repeats (ANK), or ankyrin repeats and carboxyl terminus (ANK-C). After several washings, specific complexes were resolved by SDS-PAGE and processed for autoradiography. The arrowhead indicates the position of full-length p49. (C) The effect of the bacterially produced bcl-3 deletion proteins on the DNA binding of p50 and p49 homodimers and human NF- κ B. Purified bacterially produced human p50 (lanes 1–7), p49 (lanes 8–13), or purified human NF- κ B (lanes 14–19) were pretreated with 50 ng of bacterially produced bcl-3, N, ANK, ANK-C, or GST for 30 min on ice before the addition of radiolabeled κ B DNA (0.5 ng, 3×10^5 cpm). Samples were incubated for an additional 30 min at

Table 1. Properties of I κ B proteins

Sources	37 kD/I κ B α (human/ rabbit protein)	bcl-3 (human/ mouse cDNA)	43 kD/ I κ B β (human protein)	pp40 (chicken protein/ chicken cDNA)	MAD3 (human/ mouse cDNA)	RL/IF-1 (rat cDNA)	I κ B γ (mouse cDNA)
Molecular mass (kD)	37	56	43	40	38	37	70
Number of ankyrin repeats	ND	7	ND	5	5	5	8
Inhibition of NF- κ B DNA binding							
p50-p65	+	+	+	+	+	+	+
p49-p65	+	+	+	+	+	ND	+
p50-p50	-	+	-	-	-	-	+
p49-p49	-	+	-	-	-	ND	+
Inhibition of Rel DNA binding							
c-Rel	-	-	+	+	+	+	+
v-Rel	-	-	+	+	+	ND	+
Rel B	ND	ND	ND	ND	ND	+	ND
Immunoreactivity with anti-pp40	-	-	+	+	-	ND	-
Immunoreactivity with anti-bcl-3	+	+	-	-	-	ND	-

(ND) Not determined.

thermore, modification of the I κ B family members may also dictate their functions and, perhaps, their partners. We have shown previously that either phosphorylation or dephosphorylation of pp40/I κ B β compromises its activity as an inhibitor of DNA-binding activity of NF- κ B/Rel proteins (Kerr et al. 1991). The mechanism by which I κ B proteins prevent DNA binding remains unclear, but it involves direct association with NF- κ B/Rel proteins through ankyrin-repeat elements (Inoue et al. 1992b).

Mechanisms of oncogenesis by bcl-3

How can overproduction of bcl-3 induce tumorigenesis? One scenario hypothesizes that the bcl-3 protein will "tie up" the NF- κ B proteins in the cytoplasm, thus preventing their translocation to the nucleus. Some NF- κ B proteins may be required for the transcription of genes whose product is necessary for normal cell growth. In CLL cells, where there is overproduction of the bcl-3 gene product owing to chromosomal translocation t(14;19), the normal cell growth function is compromised because of a lack of an appropriate NF- κ B transcription factor in the nucleus. Alternatively, the bcl-3 protein in the nucleus, either in free form or in association with the NF- κ B protein, may also prevent the activation of specific growth-control genes. In B-cell lymphoma-associated chromosomal translocation t(10;14)(q24;q32), the ankyrin-repeat-containing domain of p100 (precursor of p49) is deleted to allow DNA binding to the κ B site (Neri et al. 1991). Recently, in the human lymphoma cell line RC-K8, a chromosomal translocation t(2;2)(p13;p11.2-14) was reported in which the amino-terminal half of the coding region of Rel is fused with the carboxy-terminal-coding region of an unrelated gene, *nrg*, to form a Rel-Nrg fusion protein (Lu et al. 1991). The Rel-Nrg fusion protein has no *trans*-activation ac-

tivity, as it is lacking the carboxy-terminal Rel *trans*-activation domain (J. Chen, pers. comm.). Thus, Rel-Nrg can act as a dominant-negative protein to inhibit the transcription of genes containing the κ B site in a manner analogous to that shown for v-Rel. Once again, it appears that blocking the normal function of NF- κ B/Rel proteins may induce neoplastic transformation. It will be interesting to see whether overproduction of bcl-3 or Rel-Nrg proteins in normal cells can lead to cellular transformation.

Materials and methods

EMSA

EMSAs were performed as described previously (Kerr et al. 1991). The mouse κ -light-chain enhancer element double-stranded oligonucleotide [AB site; 5'-AGCTTCAGAGGG-GACTTTCCGAGG-3' and 5'-TCGACCTCTCGAAAGTC-CCCTCTGA-3' described in Urban and Baeuerle (1990)] was phosphorylated with polynucleotide kinase in the presence of [³²P]ATP (New England Nuclear-Dupont, >3000 Ci/mmol) and purified by two passes through G50 spin columns.

Bacterial protein preparations

The preparation of bacterial GST fusion proteins was as described previously (Inoue et al. 1992b), with the exception that the vector utilized was pGM-GST in place of pT7GT. Details of pGM-GST will be described elsewhere (L. Ransone, pers. comm.). The preparation of bacterial p50 and p49 were as described previously (Perkins et al. 1992).

Plasmid construction

The murine *c-rel* cDNA (Bull et al. 1990) was cloned in-frame into the *Bam*HI site of pGM-GST for expression in *Escherichia coli*. The bcl-3 cDNA (McKeithan et al. 1987) was subcloned

into the Δp expression vector [Inoue et al. 1992a] for expression in *Drosophila* Schneider cells. *bcl-3*(Δ PstI) was constructed by deleting the 914-bp PstI fragment from the 3' of the *bcl-3* cDNA. p49 [Perkins et al. 1992] and p65 [Nolan et al. 1991] were subcloned into Δp for use in Schneider cell transfections.

For construction of the *bcl-3* deletion constructs utilized in Figure 6, BamHI sites were inserted by oligonucleotide-directed site mutagenesis into the *bcl-3* cDNA at positions 39 (B1), 402 (B2), and 938 bp (B3). pGM-GST-*bcl-3* was constructed by subcloning the BamHI fragment of pBS*bcl-3* into pGM-GST so that the resulting fusion protein (448 amino acids) was in-frame with GST. *bcl-3*-N (amino terminus) was constructed by cloning the 363-bp fragment between B1 and B2 into pGM-GST so the resulting protein product (122 amino acids) was in-frame with GST. *bcl-3*-ANK was constructed by cloning the 536-bp fragment between B2 and B3 into pGM-GST, resulting in the expression of the 178 amino acids of the ankyrin domain. The *bcl-3*-ANK-C plasmid was constructed by cloning the 1422-bp B2 insert into pGM-GST for expression in *E. coli* of the 326 amino acids of the ankyrin and carboxy-terminal domains. All constructs were confirmed by sequence and restriction enzyme analysis. Chicken pp40 [Davis et al. 1991], murine MAD3 [P. Chou, in prep.], and I κ B γ [Inoue et al. 1992a] were subcloned into pGM-GST for expression in *E. coli*.

Transfections of eukaryotic cells and CAT assays

Transfection on BALB/c-3T3 and 293 cells was performed as described previously [Kerr et al. 1990]. *Drosophila* Schneider S2 cells were seeded at 3×10^6 cells/60-mm dish in 5 ml of complete medium (GIBCO Schneider medium, 12% fetal bovine serum, nonessential amino acids, 25 U/ml of penicillin, 25 μ g/ml of streptomycin) 6 hr before transfection. Calcium-phosphate precipitates were prepared in Hank's buffered saline (pH 7.14) and incubated for 20 min on ice before dropwise addition onto cells. Transfections into Jurkat cells were performed as described [Schmid et al. 1991]. Cell lysates were prepared 48 hr later in 0.25 M Tris-HCl (pH 8.0) by freeze-thaw cycles, assayed for β -galactosidase activity, and analyzed for CAT activity as described previously [Gorman et al. 1982; Hall et al. 1983]. The percentage of [14 C]chloramphenicol converted to acetylated forms was determined by cutting out the appropriate areas of thin layer chromatography sheets and assaying for 14 C radioactivity by scintillation counting.

In vitro transcription and translation

[35 S]Methionine-labeled proteins were generated from double-stranded, supercoiled DNA templates using TNT (Promega) in the presence of Expre 35 S 35 S (New England Nuclear-Dupont) using either T3 or T7 RNA polymerase. Translated proteins were visualized by SDS-PAGE before use and were used to quantitate synthesis.

In vivo metabolic labeling and immunoprecipitation

HeLa spinner 2 (S2) cells (1×10^7 cells/ml) were starved for methionine in methionine minus DMEM for 3 hr before the addition of radiolabel. Expre 35 S 35 S was added to a final concentration of 1 mCi/ml for 3 hr at 37°C in humidified atmosphere containing 10% CO $_2$. Cells were collected by centrifugation and washed twice in PBS. Cytoplasmic and nuclear proteins were isolated by the protocol of Bos et al. [1988], and equal trichloroacetic acid (TCA)-precipitable counts (5×10^5 cpm) were used. Total volume was adjusted to 200 μ l in 1% SDS, and samples were boiled and diluted to 0.2% SDS final concentra-

tion. Lysates were precleared using protein A-Sepharose before the addition of 10 μ l of anti-*bcl-3* serum and incubation at 4°C overnight. Immune complexes were precipitated with protein A-Sepharose and washed four times in immunoprecipitation buffer containing 0.2% SDS. Radiolabeled proteins were diluted in Laemmli sample buffer, boiled, and separated by SDS-PAGE. If samples were to be processed further for two-dimensional mapping, the gel was transferred to Immobilon as described previously [Luo et al. 1990]. Otherwise, gels were fixed in acetic acid/methanol (7% : 40%), saturated in PPO, dried, and exposed for autoradiography.

Western blot analysis

Protein immune complexes were detected in Western blot analysis as described previously [McDonnell et al. 1990]. Anti-*bcl-3* serum was diluted 1 : 250 in blocking buffer [5% nonfat dried milk (Carnation) plus 0.2% NP-40 in Tris-buffered saline] and incubated overnight at 4°C. Filters were washed three times with blocking buffer and incubated for 1 hr with 125 I-labeled protein A at a concentration of 1 μ Ci/ml. Filters were then washed twice with blocking buffer and once with Tris-buffered saline, air-dried, and exposed to X-Omat film (Eastman Kodak Co.) at -70°C.

In vitro phosphorylation and two-dimensional tryptic mapping

In vitro phosphorylation of proteins using cAMP-dependent PKA and CKII was as described previously [Kerr et al. 1991]. Two-dimensional tryptic and phosphotryptic mapping of peptides was as described previously [Kerr et al. 1991].

In vitro association assay

The association between radiolabeled proteins and Sepharose-GST proteins was as described previously [Inoue et al. 1992b], with the exception that the binding and wash buffer used was NETN (0.5% NP-40, 20 μ M Tris-HCl at pH 8.0, 100 μ M NaCl, 1 μ M EDTA).

Antibody production and purification

Bacterially produced *bcl-3* protein (500 μ g) was injected in Freund's complete adjuvant into New Zealand white rabbits with two subsequent injections of 500 μ g in Freund's incomplete adjuvant before the first bleed. Serum was collected and immunoglobulins were purified using the Affi-gel protein A purification system (Bio-Rad Laboratories).

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Note added in proof

At a recent meeting, new nomenclature for NF- κ B/Rel/I κ B protein was proposed (details to follow). Accordingly, the 37-kD (I κ B α) protein in this manuscript should be referred to as Δ Bcl3; pp40/43 kD I κ B β /MAD-3 and RL/IF-1 should be referred to as I κ B α ; p65 as RelA; and p49/p50B as p52.

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