Diverse Effects of BCL3 Phosphorylation on Its Modulation of NF-κB p52 Homodimer Binding to DNA*

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IκB proteins control the subcellular localization and DNA binding activity of NF-κB transcription factors. BCL3 is a nuclear IκB that can inhibit or enhance the binding of NF-κB p50 or p52 homodimers to consensus DNA-binding (κB) sequences or form a κB-binding complex with homodimers. To study BCL3 function, we have used gel shift analysis and tagged protein and tagged DNA coprecipitation analyses. Our results show that at intermediate ratios of BCL3 to p52 all observed phosphoforms of BCL3 are able to form a κB-binding complex with p52 homodimers. At low BCL3/p52 ratios, BCL3 increases the rate of p52 homodimer binding to κB sites in the presence of nonconsensus DNA and dissociates from the complex. At high BCL3/p52 ratios, BCL3 forms a higher order inhibitory complex with p52 homodimers. All of these effects depend on BCL3 phosphorylation and relative concentration. These results indicate that BCL3 phosphorylation may affect its regulation of NF-κB-dependent transcription in vitro.

BCL3 is a member of the IκB family of inhibitors of NF-κB transcription factors (1–3). IκB proteins control NF-κB activity in at least two ways: IκB can anchor NF-κB in the cytoplasm of resting cells by blocking its nuclear localization signal and can inhibit NF-κB binding to consensus DNA-binding (κB) sites. However, under some circumstances IκB proteins can associate with NF-κB without blocking the nuclear localization signal (4–6); they can form an IκB-NF-κB-κB complex (6–8), and they can enhance NF-κB binding to κB sites (9–11). BCL3 has been demonstrated to have each of these “nonclassical” IκB activities, but the mechanisms of BCL3 action have not been elucidated.

Whether or not all IκB proteins regulate NF-κB by common modes of interaction is not known. The interaction between IκB and NF-κB is mediated, at least in part, by domains that are conserved among family members. The IκB family, which includes BCL3 (12), IκBα (13), IκBβ (14), IκBγ (15, 16), and IκBe (17), contains a centrally located ankyrin repeat domain (ARD), 1 which is sufficient for association with NF-κB (4, 5, 10, 18). NF-κB proteins, including RelA (19–21), c-Rel (22), RelB (23), p50 (24, 25), and p52 (26, 27), are characterized by a N-terminal 300–330 amino acid Rel homology domain, which mediates IκB binding, DNA binding, and subunit dimerization and contains the nuclear localization signal. NF-κB subunits can form heterodimers or homodimers, and dimerization is prerequisite for binding to DNA.

BCL3 is unique among IκB proteins in that it is a nuclear protein (4, 5, 7) that specifically associates with homodimers of p50 or p52 subunits (4, 28–32) and that contains N- and C-terminal regions that can act as transactivation domains (7). p50 and p52 are similar in their primary structures, and they are unique among NF-κB family members in that they are processed from precursors (NF-κB1 p105 and NF-κB2 p100) with C-terminal IκB regions, have no defined transactivation domains, and are found in homodimeric form in both the cytosol and the nucleus of resting cells. These homodimers may function to competitively inhibit κB binding by transactivating NF-κB dimers. BCL3 can 1) remove homodimers from κB sites so that transactivating NF-κB dimers can bind and act as an antirepressor (28, 33); 2) form a complex with homodimers at κB sites and act as a transactivator (7, 8); or 3) enhance homodimer binding to κB sites (11). Thus, BCL3 may repress transcription indirectly by increasing κB-site occupancy by p50 or p52 homodimers or it can directly or indirectly activate transcription. Whether or not BCL3 activity is regulated in the cell is not known.

In this work, we have studied the mechanisms by which BCL3 modulates DNA binding by p52 homodimers. We have asked the following questions: 1) which regions of BCL3 are phosphorylated; 2) how does BCL3 phosphorylation affect its ability to form a BCL3-(p52)2-κB complex; 3) which region(s) of BCL3 are necessary to inhibit p52 homodimer binding to DNA; 4) what is the mechanism of BCL3 inhibition of homodimer binding to DNA; and 5) what is the mechanism of BCL3 enhancement of the formation of (p52)2-κB complexes? Our results show that BCL3 can have a variety of effects on p52 homodimer binding to DNA, depending on BCL3 concentration and phosphorylation. The results are consistent with a model in which BCL3 has a versatile role in the regulation of NF-κB-dependent gene expression.

MATERIALS AND METHODS

Plasmids, Antibodies, and κB Probe—H4IκB3 and H5IκB3ΔN plasmids have been described previously (5), and the plasmids pMT2TIP62, pMT2TBC13 (wtBCL3), and pMT2TBC13ΔC were a gift from U. Siebenlist (7). Antibodies to p52 (I-18) or to the C terminus of BCL3 were purchased from Santa Cruz. Antiserum to the N terminus of BCL3 has been described previously (5); rabbit polyclonal BCL3 ARD antiserum was made by immunizing rabbits with a glutathione S-transferase-ARD fusion protein (Josman Laboratories, Napa, CA). The κB probe used for all studies was prepared by annealing a 27-mer κB oligonucleotide (5′-CAACGGCAGGGGAATTCCCCTCTCCTT-3′) to a 5′-biotinylated 21-mer primer oligonucleotide (5′-bixin-AAGGAGAGGAATTTCCCTTCTCCTT-3′) and filling with dNTPs and Klenow enzyme. For labeled probe, [α-32P]dCTP was substituted for dCTP.

Cell Culture, Transfections, and Metabolic Labeling—COS7 cells

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1 The abbreviations used are: ARD, ankyrin repeat domain; CIP, calf intestinal phosphatase; DTT, dithiothreitol; NTA, nitritriacetic acid; PAGE, polyacrylamide gel electrophoresis; wt, wild type; PVDF, polyvinylidene difluoride.
(ATCC) were maintained as described (5) and transfected using a Eu-
rogenecell Electrorator with pulse values set at 300 V, 960 microfarads, and infinite resistance. The transfection buffer consisted of protein-free Dulbecco’s modified Eagle’s medium (Sigma), 5–10 µg of plasmid, and 4 µg/ml DEAE-dextran (34). Typically, 5 × 10⁶ cells were electroporated in 1 ml of medium. BCL3-transfected cells were lifted 2 days after transfection, washed twice in phosphate-buffered saline, washed once in serum-free, phosphate-free Dul-
beco’s modified Eagle’s medium (Sigma), and then incubated in 1 ml of this medium for 10 min prior to the addition of 0.3–0.6 mCi/ml [³²P]orthophosphate (NEO Life Science Products). Cells were incubated in this medium for 0–2–5 h, washed twice in medium, and then used. Nonidet P-40 lysis buffer as described below. ³²P-H₆BCL3 was purified with nickel-nitrilotriacetic acid (NTA) agarose beads as described below.

Cell Extract Preparation—All centrifugations were performed in an
Eppendorf microcentrifuge (5415 C) unless otherwise indicated. Cells were washed 4 times in phosphate-buffered saline prior to lysis. BCL3 was prepared in whole cell extracts; cells were lysed in modified Non-
idet P-40 lysis buffer (buffer L) (20 mM Hepes, pH 8.0, 200 mM NaCl, 0.2% Nonidet P-40, 20% glycerol, 1 mM DTT, protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 10 mM leupeptin, 1 mM pepstatin, 1 mM aprotinin)); debris was pelleted at 15,000 rpm for 20 min, and extracts were stored under liquid nitrogen. For initial phosphorylation studies, BCL3 lysis buffer was supplemented with phosphatase inhib-
itors (1 mM Na₃VO₄, 50 mM NaF, 2 mM MgCl₂, 0.5 mM glycerophosphate, 5 mM EDTA). p52 was prepared as a nuclear extract; cytosol was solubilized in hypotonic buffer (20 mM Hepes, pH 8.0, 15 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, protease inhibitors); nuclei were pelleted at 15,000 rpm for 15 min, resuspended in hypotonic buffer (20 mM Hepes, pH 8.0, 0.420 mM NaCl, 0.2 mM EDTA, 1.5 mM MgCl₂, 20% glycerol, 1 mM DTT, protease inhibitors), and incubated on ice for 40 min; and nuclear debris was pelleted at 15,000 rpm for 30 min. Typical whole cell and nuclear extracts contained 5 µg/ml total protein.

Western Blotting—SDS-PAGE gels were electrotransferred to PVDF membranes (Promega). Western blotting and chemiluminescent detection were performed per manufacturer’s instructions (Promega, Tropix).

Graded Dephosphorylation of BCL3—Typically, 1 unit of calf intestinal phosphatase (CIP) (Sigma) per mg of total protein in control or
BCL3-transfected cellular extract was incubated in CIP reaction buffer (60 mM NaCl, 2 mM MgCl₂, 2 mM DTT, 20 mM Hepes, pH 8.0, protease inhibitors, 10 mM EDTA, 10 mM NaF) for 1 h at room temperature. EDTA and NaF were added at different time points to inhibit CIP activity. For exhaustive protease digestions, total protein in BCL3-transfected cell extracts was diluted to 1 µg/ml in buffer L and then boiled in 1% SDS and 5 mM DTT (puriﬁed CIP was not diluted). After cooling to room temperature, 5 µg of LysC (Sigma) or GluC (Sigma) per µg of substrate protein was added. Digestion reac-
tions were allowed to incubate at 37 °C for 2 h before the addition of 0.1 × volume of 4 × sample loading buffer, boiling, separation by SDS-PAGE, electrotransfer to a PVDF membrane, autoradiography of ³²P-labeled peptides, and Western blotting. For protease digestion of native BCL3, purified BCL3 was incubated in digestion buffer (70 mM Tris, pH 7.5, 100 mM NaCl, 5 mM EDTA) and titrated with LysC protease (0.5–5 µg of LysC per µg of purified BCL3). Digestion reactions were stopped after 2 h by boiling in sample loading buffer, and peptides were analyzed by SDS-PAGE and Western blotting.

Gel Shift Assays—These were performed as described previously (5), except that sonicated placental DNA or salmon testes DNA was used as nontarget competitor DNA to reduce probe binding by nonspeciﬁc DNA–protein binding proteins, the final concentration of NaCl was 60 mM, and EDTA was added to 1 mM before adding buffer to 4 mM when CIP-treated BCL3 was added to the binding reaction. For a standard gel shift assay, proteins were preincubated with competitor DNA in bind-
ing buffer (60 mM NaCl, 1–4 mM EDTA, 5% glycerol, 1 mM DTT, 40 mM Tris, pH 7.4) for 30 min before the addition of an excess (1 pmol/sample) of ³²P-labeled x probe and then allowed to equilibrate for 40 min before electrophoresis was performed in a 4.5% nondenaturing poly-

acrylamide gel in 25 mM Tris, 190 mM glycine, 1 mM EDTA.

Purification of BCL3—Binding complexes with biotinylated x probe—All reactions were performed at room temperature. 0.8 pmol of 5’-biotinylated x probe per sample was incubated and rocked in 100 µl of binding buffer (60 mM NaCl, 1 mM DTT, 5 mM EDTA, 10% glycerol, 0.01 mg/ml sonicated placental DNA, and 0.01 mg/ml of BSA) for 1 h. BCL3-transfected cells were lifted 2 days after transfection, washed twice in medium, and then used. Nonidet P-40 lysis buffer as described below. ³²P-H₆BCL3 was purified with nickel-nitrilotriacetic acid (NTA) agarose beads as described below.

RESULTS

The C-terminal Domain of BCL3 Is Extensively Phosphorylated—BCL3, with a predicted molecular mass of 48 kDa, migrates in SDS-PAGE as a major band with an apparent molecular mass of approximately 60 kDa and two minor bands of approximately 55 and 65 kDa, when derived from transiently transfected COS7 cells (Fig. 1A). COS7 cell-derived BCL3 comi-
grates with BCL3 derived from a variety of cell lines (not shown). Graded dephosphorylation of BCL3 with CIP, as de-
scribed under “Materials and Methods,” results in a shift of the 65- and 60-kDa forms to more rapidly migrating forms, indi-
cating that BCL3 is constitutively phosphorylated, consistent with previous results (8, 11, 32). Only the 60-kDa phosphoform of BCL3 is detectable in most preparations, as shown in lane 4, and only preparations with low levels of 55- and 65-kDa forms were used for functional studies, unless otherwise indicated. BCL3 subjected to graded dephosphorylation has several inter-
mediate phosphoforms, which indicates that BCL3 is exten-
sively phosphorylated. We will refer to the phosphorylated 60-kDa form of BCL3 as “BCL3⁵” and to the CIP-dephospho-
ylated 55-kDa form of BCL3 as “BCL3⁶.”

The BCL3 protein is composed of 16% (72 of 446 amino acids) serine, threonine, or tyrosine residues; 11 are in the N-terminal domain, 33 in the ARD, and 22 in the C-terminal domain (12). Serine alone represents 26% of C-terminal domain amino acids. To determine which regions of BCL3 are phosphorylated, we used one-dimensional peptide mapping of proteolytically cleaved proteins. The BCL3 C-terminal peptide product of LysC protease digestion, Lys33–Ser446, is predicted to have a molecular mass of approximately 18 kDa. Glutamic acid residue Glu336 was not vulnerable to GluC digestion and in any of our studies. BCL3⁵ in whole cell extracts was subjected to graded CIP
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**FIG. 1.** The C-terminal domain of BCL3 is extensively phosphorylated. Graded phosphatase treatment and proteolytic digestion reactions were performed as described under "Materials and Methods." A, BCL3 in whole cell extracts was subjected to graded dephosphorylation, and Western blotting was performed with antiserum to the BCL3 ARD. Lanes 1–3 show wild type (wtBCL3), and lanes 4–7 show H6BCL3. B, BCL3 in whole cell extracts was subjected to graded dephosphorylation and exhaustive digestion with LysC or GluC. C-terminal peptides were analyzed with 14% SDS-PAGE and Western blotting using antibodies to BCL3 C terminus. Under digestion with LysC or GluC, the C-terminal peptides were detected by autoradiography (Fig. 1 right panel). The autoradiographs are overexposed to detect light bands. The right panel is a Western blot of the C-terminal peptides of BCL3 samples. The same membrane was then subjected to Western blotting with antibodies to BCL3 C terminus (left panel) or the BCL3 ARD (right panel). D, 32P-labeled and nickel-NTA bead-purified H6BCL3 (lanes 1–3), which contains a small amount of the 55-kDa phosphoform, and H6BCL3ΔN (lanes 4 and 5), which has multiple phosphoforms, were subjected to exhaustive digestion with LysC or GluC. The left panel shows a Western blot, blotted with antibody to the BCL3 C terminus. The right panel is an autoradiograph of the same membrane, performed prior to Western blotting. The autoradiographs are overexposed to detect light bands.

Dephosphorylation and exhaustive digestion with proteases. Fig. 1B shows a Western blot of C-terminal peptides of these samples. Dephosphorylated C-terminal peptides are not easily detected; they may be poorly retained by PVDF membranes. The results show that the intermediate phosphoforms of BCL3 C-terminal peptides have distinctive mobilities, and the effect of dephosphorylation on peptide migration is similar to that seen with full-length BCL3 (compare Fig. 1, A and B). We conclude that the C-terminal domain of BCL3, like the C-terminal domains of IκBα (10, 18) and IκBβ (35), is extensively and constitutively phosphorylated.

To determine whether or not all of BCL3 phosphorylation is at the C-terminal domain, 32P-labeled, nickel-NTA bead-purified BCL3 was subjected to exhaustive protease digestion. In native form, BCL3 is highly resistant to LysC and GluC proteases, and complete digestion was not observed. The major labeled peptides detected by autoradiography (Fig. 1C, center panel) comigrate with BCL3 C-terminal peptides detected by Western blotting (left panel). No labeled bands are detectable which comigrate with ARD peptides (right panel). To ensure complete proteolysis of BCL3, 32P-labeled purified BCL3 samples were boiled in 1% SDS prior to being subjected to exhaustive protease digestion. In Fig. 1D, the left panel shows a Western blot with undigested (lanes 1 and 4), LysC-digested (lanes 2 and 5), or GluC-digested (lane 3) BCL3 proteins, blotted with antibody to the BCL3 C terminus. 32P-labeled peptides were detected by autoradiography of the same membrane, shown in the right panel. A sample containing predominantly BCL3 is shown in lane 1; there is a low but detectable amount of BCL3D. A histidine-tagged BCL3 construct lacking the N-terminal domain (H6BCL3ΔN) is shown in lane 4. The C-terminal peptide seen in lane 5 is the unphosphorylated H6BCL3ΔN C-terminal peptide; H6BCL3ΔN is poorly phosphorylated, and phosphorylated peptides are not detectable in reconstitutions of this Western blot, although they are detectable by autoradiography of 32P-labeled peptides.

The results show that the major 32P-labeled peptides visualized by autoradiography comigrate with C-terminal peptides detected by Western blotting, for both full-length and N-terminally truncated BCL3. In some experiments but not others, faint 32P-labeled peptides are detectable that do not comigrate with C-terminal peptides. Their source is unknown. We conclude that most of BCL3 phosphorylation is at the C-terminal domain.

**BCL3 Activity Is Phosphorylation- and Concentration-dependent—**Previous results have indicated that BCL3 inhibition of p50 or p52 homodimer binding to xB sites may be phosphorylation- and concentration-dependent. In gel shift assays, mammalian cell-derived BCL3 can form a BCL3-homodimer-xB complex (7, 8), but bacterially derived BCL3 inhibits p50 homodimer binding to xB sites (4, 5, 29), which suggests that BCL3 phosphorylation may affect the formation of a BCL3-homodimer-xB complex.

To study the concentration and phosphorylation dependence of the formation of a BCL3-(p52)2-xB complex, we performed gel shift assays in which p52 was titrated with control or CIP-treated BCL3. B, p52 was titrated with control or CIP-treated BCL3ΔC. The autoradiograph is overexposed to detect BCL3-(p55)2-xB complexes.

**lane 4.** BCL3 activity is dependent on BCL3 phosphorylation and concentration. Gel mobility shift analyses were performed as described under "Materials and Methods." Each sample has an equal volume of p52-transfected or untransfected nuclear extract and antiserum to the BCL3 N terminus, and each sample is filled to equal volumes of cell extract with untransfected, mock-treated extracts. A, p52 was titrated with control or CIP-treated full-length BCL3. B, p52 was titrated with control or CIP-treated BCL3ΔC. The autoradiograph is overexposed to detect BCL3-(p55)2-xB complexes.
stances. The C-terminal domain of mammalian IκBα (18) and of the avian IκBα homolog p40 (10) is required for these proteins to inhibit κB binding by NF-κB. In contrast, the ARD of the Drosophila IκB is sufficient for inhibition of the Drosophila NF-κB Dorsal (36), and the ARD of bacterially derived BCL3 is sufficient for inhibition of p50 homodimer binding to DNA (4, 5). This suggests that different IκB proteins may inhibit by distinct mechanisms.

To determine whether or not the C-terminal domain of mammalian cell-derived BCL3 affects its ability to inhibit κB binding by (p52)2, we performed a gel shift assay in which p52 was titrated with a BCL3 protein truncated at the C-terminal domain (BCL3ΔC) (Fig. 2B). The BCL3ΔC protein contains the entire 7th ankyrin repeat domain and no C-terminal domain residues (7). Full-length BCL3 was incubated with CIP in parallel reactions, and Western blotting was performed to ensure that CIP was active, because CIP treatment does not affect the mobility of BCL3ΔC in SDS-PAGE (not shown).

The results show that BCL3ΔC activity is not affected by CIP treatment and that, at high BCL3ΔC/p52 ratios, BCL3ΔC is an efficient inhibitor. This pattern of concentration-dependent inhibition is similar to that seen with full-length BCL3D (Fig. 2A) and suggests that BCL3ΔC and BCL3D may have similar mechanisms of inhibition. It has been suggested that the C-terminal domain is required for BCL3 to inhibit homodimer κB binding, but truncated constructs used in the previous study lacked the 7th ankyrin repeat as well as the C-terminal domain (30). The combined results suggest that phosphorylation at the C-terminal domain of BCL3 impairs its ability to inhibit p52 homodimer binding to κB sites.

**All Observed Phosphoforms of BCL3 Can Form a BCL3-(p52)2−κB Complex**—There are at least two possible models to explain the mechanism of BCL3 modulation of homodimer binding to DNA that are consistent with results seen in our gel shift assays (Fig. 2A). One possibility is that only BCL3P (or certain phosphoisoforms of BCL3P) can form a BCL3-(p52)2−κB complex. Then high concentrations of samples containing predominantly BCL3P may be required to inhibit homodimer binding to κB probe because different BCL3 phosphoforms have different binding affinities for p52 homodimers or other complexes in the binding reaction. In this case, the major species of interest that contain p52 at equilibrium are BCL3P-(p52)2κB, (p52)2−κB, and BCL3D-(p52)2. A second possibility is that all phosphoforms of BCL3 can form a BCL3-(p52)2−κB complex, but high BCL3/p52 ratios a higher order complex is formed that cannot bind to DNA. This higher order BCL3-p52 complex would be predicted to be destabilized by BCL3 phosphorylation, because BCL3P does not inhibit homodimer binding to DNA. In this case, the major species of interest are BCL3-(p52)2−κB, (p52)2−κB, and (BCL3)2−(p52)2.

To distinguish between these possibilities, it is necessary to show whether 1) BCL3D is able to form a BCL3D-(p52)2−κB complex and 2) BCL3 can form a higher order complex with p52. We studied the formation of a BCL3D-(p52)2−κB complex with gel shift analysis and by coprecipitation with tagged κB probe and detection by Western blotting. An analysis of the formation of a (BCL3)2−(p52)2 complex is described below (Fig. 4).

First, different phosphoforms of BCL3 were titrated with p52 in a gel shift assay, as shown in Fig. 3A. In the presence of excess p52, BCL3D forms a BCL3D-(p52)2−κB complex which migrates more slowly than the BCL3D-(p52)2−κB complex, perhaps due to its decreased net negative charge or a difference in the conformation of the complexes. Under these conditions the BCL3D-(p52)2−κB complex is more abundant than the BCL3P-(p52)2−κB complex, which suggests that BCL3P binds relatively weakly to (p52)2−κB.

To compare the abilities of different phosphoforms of BCL3 to form a BCL3-(p52)2−κB complex in solution, BCL3P was subjected to graded dephosphorylation, and mixed phosphoforms of BCL3 were coprecipitated with p52 and a 5′-biotinylated κB probe on avidin-coated Dynal beads. Immobilized complexes were suspended in binding buffer, aliquoted into sample tubes, and titrated with heterogeneous BCL3 phosphoforms, shown as form A or form B. Complexes were pelleted, and precipitated proteins were eluted by boiling in sample buffer and detected by Western blotting with antibodies to the BCL3 C terminus (above) or p52 (below).

**BCL3 Can Associate with p52 in a Higher Order Complex**—Because all observed phosphoforms of BCL3 can form a BCL3-(p52)2−κB complex in solution, BCL3D was subjected to gel shift analysis, and glutaraldehyde cross-linking, bacterially derived BCL3 and p50 can form a 1:2 or a 3:1 complex with (p52)2−κB. Thus, BCL3P associates with (p52)2−κB relatively weakly, both in solution and in gel shift assays. These results also imply that all observed phosphoforms of BCL3 can form a BCL3-(p52)2−κB complex.

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**Fig. 3.** All observed BCL3 phosphoforms can form a BCL3-(p52)2−κB complex. A, for this gel shift assay, equal amounts of different phosphoforms of BCL3, shown below, were incubated with a 1 μl of p52 (lanes 1–3) or 3 μl of p52 (lanes 4–6). Electrophoresis was performed for 6 h to ensure separation of complexes, because no antiserum was added to supershift BCL3 complexes. B, p52-transfected or untransfected nuclear extract was immobilized with 5′-biotinylated κB probe on avidin-coated Dynal beads. Immobilized complexes were suspended in binding buffer, aliquoted into sample tubes, and titrated with heterogeneous BCL3 phosphoforms, shown as form A or form B. Complexes were pelleted, and precipitated proteins were eluted by boiling in sample buffer and detected by Western blotting.
was added in control for the requirement for p52 in a higher order complex, no p52 immunoblotted H6BCL3, no wtBCL3 was added in elutable wtBCL3D and immobilized H6BCL3D migrate at approximately 55 kDa and thus cannot be distinguished; however, the bands are much darker than background bands, so it is likely that higher order complexes have formed. In lanes 13–15, untreated wtBCL3 was incubated with immobilized H6BCL3D in the presence of p52: these lanes show that some H6BCL3 elutes from beads, and wtBCL3 was detected by Western blotting.

The results of the sandwich coprecipitation assay are shown in Fig. 4. Lanes 4, 8, and 12, with no wtBCL3 present, show that some H6BCL3 elutes from beads, but this background is low. In lanes 5–7, immobilized H6BCL3D was incubated with increasing amounts of wtBCL3 in the absence of p52: here, a low level of background wtBCL3 binding to immobilized H6BCL3D is detectable. Lanes 9–11, wtBCL3D was incubated with immobilized H6BCL3D in the presence of p52: here, both elutable wtBCL3D and immobilized H6BCL3D migrate at approximately 55 kDa and thus cannot be distinguished; however, the bands are much darker than background bands, so it is likely that higher order complexes have formed. In lanes 13–15, untreated wtBCL3 was incubated with immobilized H6BCL3D in the presence of p52: these lanes show that wtBCL3 coprecipitates with H6BCL3 in the presence of p52. Note that the relative proportion of BCL3 phosphoforms precipitated varied with BCL3 concentration. In particular, the minor 65-kDa form may preferentially form a (BCL3)2-p52 complex.

These results show that BCL3 can associate with p52 in a higher order complex. Previous work has shown that bacterially derived BCL3 can form a 1:2 or a 2:2 complex with p50 proteins by the technique of glutaraldehyde cross-linking, which indicates that direct interactions between the proteins are likely to be responsible for the higher order complex. This indicates that BCL3 inhibits NF-kB binding by a mechanism that is unique among IκB proteins (see "Discussion").

BCL3p accelerates p52 homodimer binding to κB sites in the presence of excess nontarget DNA. 30 min before the addition of 32P-labeled κB probe, proteins were prequilluclated with non-specific DNA (sonicated salmon sperm DNA) at either 6- or 120-fold molar excess. Standard gel shift assays were performed except that probe was added at different time points before samples were loaded onto the gel. A, each sample contained equal aliquots of BCL3-transfected or untransfected whole cell extracts, p52-transfected nuclear extracts, and BCL3 N-terminal antisera. B, a gel shift assay with purified control- or CIP-treated BCL3, or purified untransfected extracts, was performed with 30-fold molar excess of nonspecific DNA, as above, except that no BCL3 N-terminal antisera was added. Samples were incubated with 32P-labeled κB probe for 3 min prior to loading the gel.

BCL3p can enhance (p50)2 binding to a variety of κB sites (11). The mechanism of this enhancement has not been elucidated. One possibility is that BCL3p enhances κB binding by p52 homodimers, at least in part, by increasing the overall rate of their binding to κB sites. NF-κB proteins have a high nonspecific DNA binding activity, and it has been shown that double-stranded oligonucleotides, such as poly(dI-dC), can reduce the overall rate of NF-κB binding to κB sites (37). For standard gel shift assays, proteins in cellular extracts are preincubated with nonspecific DNA to reduce probe binding by nonspecific DNA-binding proteins. Homodimers bound to nonspecific DNA must dissociate before they can bind to κB probe: (preincubated (p52)2-DNA) + κB → DNA + κB + free (p52)2 → DNA + (p52)2-κB.

The (p52)2-κB complex forms more slowly in the presence of higher concentrations of nonspecific DNA because there is less free (p52)2 available to rapidly associate with κB probe. We predicted that two conditions are required for BCL3p to increase the overall rate of homodimer binding to κB probe in the presence of excess nonspecific DNA. First, BCL3p must associate with homodimers and reduce their affinity for DNA, so that there is more free (p52)2 available to rapidly associate with κB probe. Second, BCL3 must dissociate from the BCL3-(p52)2-κB complex: (preincubated BCL3p-(p52)2-DNA) + κB → DNA + BCL3p + (p52)2-κB.

High BCL3p/p52 ratios in binding reactions would be expected to favor the formation of a BCL3p-(p52)2-κB complex and make the formation of a (p52)2-κB complex difficult to study. Thus, to study the effect of BCL3p on the formation of a (p52)2-κB complex, we used low BCL3p/p52 ratios.

Gel shift assays were performed to determine the effect of low concentrations of BCL3p on the ratio of (p52)2 binding to κB sites in the presence of excess nonspecific DNA as shown in Fig. 5A. Proteins were prequilluclated with a 6-fold or a 120-fold molar excess of nonspecific DNA over probe, and the κB probe was added and allowed to incubate for decreasing periods before the binding reactions were loaded on the gel. Under these conditions, BCL3p enhanced κB binding in a variety of κB sites (11). The mechanism of this enhancement has not been elucidated. One possibility is that BCL3p enhances κB binding by p52 homodimers, at least in part, by increasing the overall rate of their binding to κB sites. NF-κB proteins have a high nonspecific DNA binding activity, and it has been shown that double-stranded oligonucleotides, such as poly(dI-dC), can reduce the overall rate of NF-κB binding to κB sites (37). For standard gel shift assays, proteins in cellular extracts are preincubated with nonspecific DNA to reduce probe binding by nonspecific DNA-binding proteins. Homodimers bound to nonspecific DNA must dissociate before they can bind to κB probe: (preincubated (p52)2-DNA) + κB → DNA + κB + free (p52)2 → DNA + (p52)2-κB.

The (p52)2-κB complex forms more slowly in the presence of higher concentrations of nonspecific DNA because there is less free (p52)2 available to rapidly associate with κB probe. We predicted that two conditions are required for BCL3p to increase the overall rate of homodimer binding to κB probe in the presence of excess nonspecific DNA. First, BCL3p must associate with homodimers and reduce their affinity for DNA, so that there is more free (p52)2 available to rapidly associate with κB probe. Second, BCL3 must dissociate from the BCL3-(p52)2-κB complex: (preincubated BCL3p-(p52)2-DNA) + κB → DNA + BCL3p + (p52)2-κB.

High BCL3p/p52 ratios in binding reactions would be expected to favor the formation of a BCL3p-(p52)2-κB complex and make the formation of a (p52)2-κB complex difficult to study. Thus, to study the effect of BCL3p on the formation of a (p52)2-κB complex, we used low BCL3p/p52 ratios.

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conditions, both the off-rate of protein bound to nonspecific DNA and the on-rate of protein binding to κB probe are expected to affect the time required for the formation of (p52)_{2}-κB and BCL3-(p52)_{2}-κB complexes.

The rate of (p52)_{2} binding to the κB probe, with 6-fold excess of nonspecific DNA, compared with the rate with 120-fold excess, shows that nonspecific DNA significantly retards κB binding by (p52)_{2}, consistent with previous results. BCL3\textsuperscript{p} has little detectable effect in the presence of the lower concentration of nonspecific DNA, and equilibrium is apparently approached after 35 min in both the presence and the absence of BCL3\textsuperscript{p}.

With the higher concentration of nonspecific DNA, however, BCL3\textsuperscript{p} significantly increases the rate of κB binding by (p52)_{2}. Quantitative analyses measuring the rate of homodimer binding to κB probe in the presence of increasing nonspecific DNA confirmed these results (not shown). Dephosphorylated BCL3, which has not been observed to enhance (p52)_{2} binding to κB sites in standard gel shift assays, does not accelerate κB binding by (p52)_{2} in the presence of excess nonspecific DNA (Fig. 5B).

These results indicate that BCL3\textsuperscript{p} enhances κB binding by homodimers, at least in part, by impairing their ability to bind to nontarget DNA. Thus, more of a (p52)_{2}-κB complex is observed in the presence of BCL3\textsuperscript{p} in some standard gel shift assays because the binding reactions have approached equilibrium only in the presence of BCL3\textsuperscript{p}.

**DISCUSSION**

In this work, we elucidate the mechanisms of BCL3 modulation of κB binding by p52 homodimers. All observed phosphoforms of BCL3 can form a BCL3-(p52)_{2}-κB complex, but whether BCL3 enhances or inhibits DNA binding by p52 homodimers depends on the concentration and phosphorylation state of BCL3. At high BCL3/p52 ratios, phosphorylated BCL3 has little or no inhibitory activity, whereas dephosphorylated BCL3 is an efficient inhibitor. This suggests that the formation of a higher order BCL3-p52 inhibitory complex depends on BCL3 concentration and phosphorylation. We show that BCL3 coprecipitates with p52 in a higher order complex. Our results support a model in which constitutively phosphorylated BCL3 enhances p52 homodimer binding to κB sites, at least in part, first by impairing its binding to DNA and then by forming a weak BCL3-(p52)_{2}-κB complex from which BCL3 is released.

The physiological role of BCL3 is not understood. BCL3 may activate gene expression either directly by forming a BCL3-homodimer-κB complex or indirectly by acting as an antirepressor. Our data indicate that the formation of a BCL3-homodimer-DNA complex depends on BCL3 concentration and phosphorylation. BCL3 activity in vivo may potentially be controlled by many factors, including the specific NF-κB homodimer, the κB sequence, protein phosphorylation, and interactions with other transcription factors. Thus, the physiological role of BCL3 may be highly dependent on local influences.

**A Model for the Mechanism of BCL3 Action**—Based on our results, we propose a model for the mechanism of BCL3 action which is outlined in Fig. 6. Different aspects of the model are discussed in detail below. Fig. 6A illustrates the key complexes formed in the presence of BCL3. The abundance of each of these complexes at equilibrium is dependent both on BCL3 concentration and phosphorylation. At high ratios of BCL3 to p52, BCL3 phosphorylation destabilizes a higher order complex (reaction 5), whereas BCL3\textsuperscript{p} inhibits p52 homodimer DNA binding by forming a relatively stable higher order BCL3-p52 complex. At low ratios of BCL3 to p52, BCL3 phosphorylation destabilizes the BCL3-(p52)_{2}-κB complex (reaction 4). However, BCL3\textsuperscript{p} does not effectively inhibit p52 homodimer DNA binding in a 1:2 stoichiometry because BCL3\textsuperscript{p} dissociates from a relatively weak BCL3\textsuperscript{p}-(p52)_{2}-κB complex (reaction 3). In summary, BCL3 phosphorylation destabilizes both a BCL3-(p52)_{2}-κB complex and a (BCL3)_{2}-(p52)_{2}-κB complex.

Fig. 6B illustrates a model for how these combined phosphorylation effects result in BCL3\textsuperscript{p}-mediated enhancement of homodimer κB binding. Because a BCL3\textsuperscript{p}-(p52)_{2}-nonspecific DNA complex is weak, more p52 homodimers are free to associate rapidly with κB probe in the presence of BCL3\textsuperscript{p}. Similarly, a BCL3\textsuperscript{p}-(p52)_{2}-κB complex is weak, but because BCL3\textsuperscript{p} dissociates from the complex, the quantity of (p52)_{2}-κB is only minimally reduced at equilibrium.

**BCL3 Phosphorylation**—Whether or not BCL3 phosphorylation is regulated in the cell or regulates the ability of BCL3 to act as a transactivator is not known. We and others (11, 32) have shown that BCL3 regulation of p50 or p52 homodimer binding to κB sites is phosphorylation-dependent. We have shown that all observed phosphoforms of BCL3 are able to form a BCL3-homodimer-κB complex (Fig. 3) and that BCL3 phosphorylation has at least two effects: it abrogates the ability of BCL3 to inhibit p52 homodimer κB binding (Fig. 2A), and it impairs BCL3 association in a BCL3-homodimer-κB complex (Figs. 2A, 3, and 5A).

Two lines of evidence suggest that phosphorylation within the C-terminal domain affects BCL3 activity. First, the vast majority of phosphorylation in COS cell-derived BCL3 is within its C-terminal domain (Fig. 1), and a graded change in phosphorylation corresponds to a graded change in BCL3 activity (Figs. 2A and 3A). Second, the C-terminal domain is not required for concentration-dependent inhibition of p52 homodimer binding to DNA (Fig. 2B). This suggests that C-terminal phosphorylation impairs inhibition by effects on conformation or charge. BCL3\textsuperscript{D} is sensitive to LysC digestion.
whereas BCL3\textsuperscript{P} is not, which suggests that the conformation of BCL3 is affected by phosphorylation (not shown). Our data do not rule out the possibility that phosphorylation of N-terminal or ARD residues may also affect BCL3 activity.

**BCL3 Inhibition of p52 Homodimer Binding to \(\kappa B\) Sites**—Gel shift analysis shows that BCL3 inhibition of p52 homodimer binding to \(\kappa B\) sites depends on BCL3 concentration and phosphorylation (Fig. 2A). Using tagged protein coprecipitation analysis, we have shown that BCL3 can form a higher order complex with p52 (Fig. 4). In combination with previous results that have shown that BCL3 forms a 2:2 complex with p50 (4), the results indicate that the mechanism of BCL3 inhibition of p52 homodimer binding to \(\kappa B\) sites is through the concentration- and phosphorylation-dependent formation of a 2:2 BCL3-p52 complex. This mechanism of inhibition of NF-\(\kappa B\) DNA binding is unique among I\(\kappa B\) proteins, as discussed below.

Mutational analyses have identified three I\(\kappa B\) contact sites within the Rel homology domain, including the nuclear localization signal. The crystallographic structure of p50 suggests that these sites form a composite surface or “I\(\kappa B\)-binding cleft” (36, 40–42). BCL3 may associate with homodimers at this region.

Our results show that, at high ratios of BCL3 to p52, at least one more BCL3 molecule can associate with (p52)\(_2\). Two rotationally symmetric sites may exist within the I\(\kappa B\)-binding cleft of p50 and p52 homodimers, and a BCL3 molecule may be able to bind at each site. BCL3 phosphorylation may increase steric hindrance or electrostatic repulsion and decrease the stability of a 2:2 complex, thereby reducing BCL3’s ability to inhibit p52 DNA binding.

Our results appear to conflict with previous work that has suggested that phosphorylation increases, rather than decreases, the ability of insect cell-derived BCL3 to inhibit p50 homodimer binding to \(\kappa B\) sites (32). This contrasting result may be due to differences in the \(\kappa B\) sequence, the specific NF-\(\kappa B\) homodimer, BCL3 phosphorylation in insect cells, or binding conditions. We have observed that BCL3\textsuperscript{P} is an efficient inhibitor in the presence of unphysiologically high concentrations of divalent cation, whereas the activity of BCL3\textsuperscript{P} is not affected (not shown). Divalent cation may stabilize the formation of a 2:2 BCL3-p52 complex or it may prevent BCL3\textsuperscript{P} dissociation from a relatively weak BCL3\textsuperscript{P}(p52)\(_2\)-DNA complex so that BCL3\textsuperscript{P} inhibits in a 1:2 stoichiometry.

**BCL3\textsuperscript{P} Enhancement of p52 Homodimer Binding to \(\kappa B\) Sites**—BCL3 has been shown to increase \(\kappa B\) binding by p50 or p52 homodimers in vitro. Our data show that BCL3\textsuperscript{P} increases the rate of p52 homodimer binding to \(\kappa B\) sites in the presence of excess nontarget DNA (Fig. 5). The simplest explanation is that BCL3\textsuperscript{P} impairs homodimer binding to both target and nontarget DNA. With excess nontarget DNA, most of the homodimer is bound to nontarget DNA and is not available to bind to \(\kappa B\) sites rapidly; in the presence of BCL3\textsuperscript{P}, more homodimer is free to bind rapidly to \(\kappa B\) sites, and the reaction is driven toward the formation of a homodimer-\(\kappa B\) complex with the release of BCL3\textsuperscript{P} (Fig. 6B).

Protease sensitivity and circular dichroism spectroscopy analyses indicate that p50 homodimers change their conformation upon binding to either specific or nonspecific DNA sequences (43, 44). It may be that association with BCL3\textsuperscript{P} impairs conformational adjustments required for (p50)\(_2\) or (p52)\(_2\) to bind DNA.

For BCL3\textsuperscript{P} to enhance homodimer binding to \(\kappa B\) sites, it must first associate with homodimers to increase their rate of \(\kappa B\) binding and then dissociate. Gel shift and tagged \(\kappa B\) coprecipitation analyses show that, compared with BCL3\textsuperscript{P}, BCL3\textsuperscript{P} associates weakly in a BCL3-(p52)\(_2\)-\(\kappa B\) complex (Fig. 3). High concentrations of BCL3\textsuperscript{P} favor the formation of a BCL3\textsuperscript{P}-homodimer-\(\kappa B\) complex in vitro; but in the nucleus the association of BCL3\textsuperscript{P} or (p52), with other proteins, or other local influences, may affect the retention of BCL3\textsuperscript{P} in the complex.

BCL3\textsuperscript{P} does not increase the overall rate of homodimer-\(\kappa B\) binding (Fig. 5B). Our data show that a BCL3-(p52)\(_2\)-\(\kappa B\) complex is stabilized by BCL3 dephosphorylation. This supports a model in which weak DNA binding and the release of BCL3 from a BCL3-(p52)\(_2\)-\(\kappa B\) complex are responsible for the ability of BCL3 to enhance homodimer-\(\kappa B\) binding.

RNA polymerase II (45) and the lac repressor (46) have also been shown to have an impaired ability to bind to nontarget DNA as a result of their association with ligands. Whether or not this mode of controlling DNA binding activity is physiologically relevant is not known and is discussed below.

**BCL3 Regulation of p50 Homodimers**—BCL3 appears to be a more efficient inhibitor with p50 than with p52 homodimers (33), and a BCL3-(p50)\(_2\)-\(\kappa B\) complex is more difficult to detect than a BCL3-(p52)\(_2\)-\(\kappa B\) complex (28). Our preliminary results indicate that BCL3 has the same spectrum of activity with p50 homodimers as with p52 and that BCL3 phosphorylation reduces its inhibition of p50 homodimer binding to DNA (not shown). It may be that BCL3 mechanisms of enhancement and inhibition are similar for p50 and p52 homodimers, but a 2:2 complex is more stable or a BCL3-homodimer-\(\kappa B\) complex is less stable with p50 than with p52 homodimers.

**I\(\kappa B\) Activity in the Nucleus**—The possibility that other members of the I\(\kappa B\) family have a nuclear role in the regulation of NF-\(\kappa B\) activity is supported by recent studies demonstrating that I\(\kappa B\)\(\alpha\) and I\(\kappa B\)\(\beta\), which are primarily cytosolic proteins, can localize in the nucleus and affect NF-\(\kappa B\) activity (6, 51).

There are several lines of evidence that different I\(\kappa B\) proteins use distinct mechanisms to inhibit NF-\(\kappa B\) binding to \(\kappa B\) sites as follows: 1) only BCL3 has been shown to form a higher order (2:2) complex with NF-\(\kappa B\) subunits, whereas other I\(\kappa B\)s have been shown to associate with NF-\(\kappa B\) in a 1:2 stoichiometry; 2) although I\(\kappa B\)\(\alpha\) and p40 require acidic residues within their C-terminal domains to inhibit, BCL3 and cactus require only the ARD; and 3) although I\(\kappa B\)\(\alpha\) and BCL3 can dissociate preformed NF-\(\kappa B\)-\(\kappa B\) complexes, I\(\kappa B\)\(\gamma\) cannot (32, 52, 53).

The ability of I\(\kappa B\) proteins to form stable I\(\kappa B\)-NF-\(\kappa B\)-\(\kappa B\) complexes is also distinct for different I\(\kappa B\) proteins; all observed phosphoforms of BCL3 are able to form a BCL3-(p52)\(_2\)-\(\kappa B\) complex; only dephosphorylated I\(\kappa B\)\(\beta\) forms an I\(\kappa B\)\(\beta\)-p50p65-\(\kappa B\) complex (6), and mammalian I\(\kappa B\)\(\alpha\) is apparently unable to form a stable I\(\kappa B\)-NF-\(\kappa B\)-\(\kappa B\) complex under any circumstances.

This diversity in the activity of I\(\kappa B\) family members suggests that different I\(\kappa B\) proteins have distinct and synchronous roles in the regulation of nuclear NF-\(\kappa B\) activity. By enhancing or inhibiting nontransactivating homodimer binding to \(\kappa B\) sites, BCL3 may coordinate with other I\(\kappa B\) proteins to affect which \(\kappa B\) sites are bound (7). BCL3 may affect which \(\kappa B\) sites are bound to internal complexes, I\(\kappa B\)\(\alpha\) may affect which \(\kappa B\) sites are bound to external complexes, and BCL3 may affect external complexes as well.

The possibility that other \(\kappa B\) sites might be regulated by I\(\kappa B\) proteins in other ways is significant. For example, the specificity of NF-\(\kappa B\)-controlled gene expression is determined largely by which NF-\(\kappa B\) dimers are available to bind to \(\kappa B\) sites. If (p50)\(_2\) and (p52), are constitutively available to compete for the occupation of \(\kappa B\) sites, they may impair the ability of transactivating NF-\(\kappa B\) proteins to affect gene expression. Thus, it was originally proposed that a primary role for BCL3 may be to inhibit homodimer binding to \(\kappa B\) sites and act as an antirepressor. In light of this, it is surprising that thy-
mocytes that express a BCL3 transgene show an increase, rather than a decrease, in kB binding by p50 homodimers in vitro, with no change in the level of the p50 subunit in the nuclear extract (11). This is consistent with the notion that BCL3 enhancement of homodimer binding to kB sites may be a physiologically relevant activity.

Recently, it has been shown that BCL3 can increase the quantity of nuclear p50 homodimers by inducing a redistribution of the subunits of cytoplasmic heterodimers containing p50 and its precursor p105 to yield p50 homodimers that translocate to the nucleus; this occurs without an effect on processing of p105 (54). In combination with our results, this suggests that BCL3 may inhibit NF-kB-dependent gene expression by increasing nuclear levels of nontransactivating NF-kB homodimers as well as by increasing their ability to bind to kB sites.

There are several other mechanisms by which homodimer binding to available kB sites may be regulated in vitro. p50 and p52 subunits are apparently not phosphorylated in the cell (49), and there is no evidence that their activity is affected by phosphorylation. Homodimer kB binding may be affected by a monomer/dimer equilibrium, association with specific inhibitors, cooperative binding interactions with other transcription factors, or association with chromatin. The extent to which DNA is available to act as a nonspecific competitor for interphase chromatin (50). Thus, p50 or p52 homodimer binding to bulk chromatin may impair their binding to kB sites. Further studies must be performed to determine whether or not NF-kB associates nonspecifically with chromatin in the cell.

BCL3\(^{\text{nuc}}\) which remains associated in a BCL3\(^{\text{nuc}}\)-homodimer-kB complex may act as a transactivator, but BCL3\(^{\text{cyt}}\) which is released from the complex may function solely to escort (p50)(2) and (p52)(2) to kB sites. InkB, which has also been observed to enhance p50 or p52 homodimer binding to kB sites in vitro, may have an escort function as well. An InkB escort function would be predicted to increase the ability of p50 or p52 homodimers to compete for kB site occupancy and thus decrease the efficiency or the duration of kB binding by transactivating NF-kB dimers.

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