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BCL3 Encodes a Nuclear Protein Which Can Alter the Subcellular Location of NF-κB Proteins

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***BCL3* is a candidate proto-oncogene involved in the recurring translocation t(14;19) found in some patients with chronic lymphocytic leukemia. BCL3 protein acts as an IκB in that it can specifically inhibit the DNA binding of NF-κB factors. Here, we demonstrate that BCL3 is predominantly a nuclear protein and provide evidence that its N terminus is necessary to direct the protein into the nucleus. In contrast to IκBα (MAD3), BCL3 does not cause NF-κB p50 to be retained in the cytoplasm; instead, in cotransfection assays, it alters the subnuclear localization of p50. The two proteins colocalize, suggesting that they interact in vivo. Further immunofluorescence experiments showed that a mutant p50, lacking a nuclear localization signal and restricted to the cytoplasm, is brought into the nucleus in the presence of BCL3. Correspondingly, a wild-type p50 directs into the nucleus a truncated BCL3, which, when transfected alone, is found in the cytoplasm. We tested whether BCL3 could overcome the cytoplasmic retention of p50 by IκBα. Results from triple cotransfection experiments with BCL3, IκBα, and p50 implied that BCL3 can successfully compete with IκBα and bring p50 into the nucleus; thus, localization of NF-κB factors may be affected by differential expression of IκB proteins. These novel properties of BCL3 protein further establish BCL3 as a distinctive member of the IκB family.**

NF-κB designates a group of transcription factors defined in part by their ability to bind a specific 10- or 11-bp DNA sequence first identified in the enhancer of the immunoglobulin κ light-chain gene (58). Similar sequences were later found in many genes involved in immune responses or acute-phase reactions, as well as in human immunodeficiency virus and other viruses (1). NF-κB is such a widely expressed transcriptional regulator that its actions have been likened to those of an intracellular second messenger. The prototypical NF-κB is a heterodimer composed of p50 and p65 subunits (3, 34), but many closely related transcription factors have been identified, encoded by a family of genes. In most cell types, the major form of NF-κB resides in the cytoplasm in an inactive form complexed with another class of proteins called IκBs (2). In response to a variety of extracellular stimuli, including pathogens and cytokines, NF-κB can be activated and transported into the nucleus.

Molecular cloning of the genes encoding several NF-κB subunits has led to elucidation of their structure and functional mechanisms (7, 22, 48). All have an N-terminal conserved domain of about 300 amino acids, the Rel homology domain, mediating dimerization and DNA binding activity. A functional nuclear localization signal (NLS) lies immediately C terminal to the Rel homology domain in all family members (5–7, 20, 26). For some of the NF-κB subunits, e.g., Rel and p65 (RelA), potent transactivation domains have been identified within their C-terminal domains (9, 55, 57). In contrast, two other NF-κB subunits (p50 and p52) do not seem to contain such potent transactivation domains, despite similar dimerization and DNA binding activity (42, 57). Moreover, they are translated as precursors, which can be processed into the mature forms by removal of their C-terminal domains, which largely consist of ankyrin repeats (13, 26, 43, 53).

Interaction with IκB proteins regulates the DNA binding activity and subcellular localization of the NF-κB proteins. Originally, only two forms of IκBs, IκBα (37 kDa) and IκBβ (42 kDa), were defined by column chromatography and gel shift assays (63). Besides differing in molecular weight, they also differ in specificity as inhibitors of NF-κB (35). While the molecular structure of IκBβ has not yet been reported, IκBα has been cloned and its structure and function have been investigated in detail. MAD3, the mammalian form of IκBα (23), effectively inhibits the DNA binding activity of NF-κB p50-p65, homodimeric p65, and homodimeric c-Rel. IκBα (MAD3) directly interacts with both p50 and p65 subunits and masks their nuclear localization signals. Coexpression of NF-κB and IκBα results in cytoplasmic sequestration of NF-κB proteins and inhibition of their transactivation activity (5, 16, 64).

So far, in addition to IκBα, several homologous and functionally similar proteins have been molecularly cloned (11, 19, 23, 27, 36, 50, 60). All of this family of IκB proteins contain six or seven tandem ankyrin repeats (7, 22, 48), which have been shown to be essential for interaction with NF-κB and probably determine the specificity of the interactions (24, 28, 61). It was surprising that the C-terminal domain of NFKB1 p105, the p50 precursor, also contains seven ankyrin repeats. This domain has been shown to have IκB-like activity but with specificity differing from that of IκBα. This C-terminal domain (IκBγ) can actually be encoded by an alternative mRNA (24, 25, 27, 39). Recent studies have also shown that the precursor proteins p105 and NFKB2 p100, the precursor of p52, have distinctive functional properties of their own. They not only inhibit the DNA binding activity of some NF-κB subunits but also can sequester them in the cytoplasm (43, 46, 47, 52). As

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mentioned above, some of the IκB proteins are located in the cytoplasm and act as inhibitors of NF-κB nuclear mobilization and DNA binding. However, studies of interleukin 2 gene regulation by NF-κB in T-cell clones (32) strongly suggested that a nuclear IκB-like activity is required.

BCL3 is a candidate proto-oncogene located adjacent to the breakpoint junction of the t(14;19) translocation in some patients with chronic lymphocytic leukemia. In these patients, *BCL3* mRNA is overexpressed (50). The central portion of the predicted protein comprises seven ankyrin repeats of 33 to 37 amino acids. The high degree of homology between the ankyrin repeat domain of BCL3 and the C-terminal domain of p105 (and other IκBs, reported later) suggested a possible IκB-like activity. We and others have shown that BCL3 protein can specifically inhibit the DNA binding activity of NF-κB p50 and p52 (25, 35). Here, we present data demonstrating that BCL3 is a nuclear protein whose N-terminal portion is required for effective nuclear localization. We show subnuclear colocalization of BCL3 and NF-κB p50 proteins after coexpression. Neither BCL3 nor NF-κB p50 appears to mask the nuclear localization signal of the other protein. Furthermore, we demonstrate that BCL3 can overcome the sequestering effect of IκBα and bring NF-κB p50 into the nucleus.

MATERIALS AND METHODS

Cell culture. COS-1 cells were grown in Dulbecco's modified Eagle's medium (GIBCO) supplemented with penicillin-streptomycin, HEPES (*N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) buffer, and 10% heat-inactivated fetal calf serum.

Expression plasmids. In preparing bacterial expression constructs 250 and 295, the first or second *Nco*I site of *BCL3* was blunt-end ligated to the *Bam*HI site of pET-3a, with recreation of the sites. The pETp50 construct was previously described (25). Constructs 314 and 316 were prepared in several steps. The N-terminal fusion sites resulted from filling in and blunt-end ligation of the *Bam*HI site of clones derived from construct 295 into the blunted *Xba*I site of pGEX-KG (a gift from Jack Dixon), while the C-terminal fusion sites of constructs 314 and 313 resulted from blunt-end ligation at the unique *Bsu*36I site and the *Hin*dIII vector site. The C termini of constructs 316, 318, and 320 were derived from a *Bal*31 deletion of the cDNA cloned into the *Sma*I and *Eco*RI sites of M13mp19 and ending at bp 1140 of the published sequence (50). Construct 320 is similar to construct 316 but begins at the fourth *Nco*I site of *BCL3*. Constructs 313 and 318 result from fusion at the first *BCL3 Sac*I site into the corresponding site in pGEX-KG.

Constructs FLAG-p50 and FLAG-p65 were previously described (49, 50). The FLAG-p50 NLS mutant was prepared by ligating the appropriate *Stu*I fragments of FLAG-p50 and the p50 NLS mutant (26). RcCMV-BCL3 was prepared by ligating the *BCL3* cDNA, removed from the Bluescript KSII vector with *Hin*dIII-*Xba*I, into the corresponding sites in RcCMV (Invitrogen). RcCMV-IκBα was similarly prepared by ligating the MAD3 insert (Cetus Corp. [23]) into *Xba*I-*Apa*I sites. To prepare the mammalian expression vector pEVH6, a double-stranded oligonucleotide, consisting of the complementary sequences GA TCT CAC CAT CAC CAT CAC CAT GGA TCC GGT ACC C and G GGT ACC GGA TCC ATG GTG ATG GTG ATG GTG A, was inserted into pEVRF0 (40), digested with *Bam*HI and *Sma*I. pEVH6-BCL3 was prepared by isolating the complete coding region of *BCL3* from construct 250 by *Bam*HI-*Bgl*II digestion and inserting it into the *Bam*HI site of pEVH6. BCL3ΔSmaI resulted from *Bam*HI-partial *Sma*I digestion, filling in, and religation. BCL3ΔNcoI was derived from pEVH6-BCL3 by replacing its *Bam*HI-*Not*I fragment with the corresponding fragment from a derivative of construct 295.

Bacterial protein expression, purification, and antibody production. The constructs derived from ligation of various fragments of *BCL3* cDNA into the bacterial expression plasmid pGEX-KG expression vector were transformed into *Escherichia coli* XL-1 Blue (Stratagene) to express glutathione *S*-transferase (GST)-BCL3 fusion protein. An overnight culture of the transformed bacteria was diluted 100-fold in Luria-Bertani medium and incubated at 37°C to an optical density of 0.6 to 0.8. Addition of IPTG (isopropyl-β-D-thiogalactopyranoside [0.2 mM]) was followed by 3 h of incubation. The bacteria were pelleted, resuspended in a buffer containing 1% Triton X-100, 1% Tween 20, and 10 mM dithiothreitol in phosphate-buffered saline ([PBS pH 7.4]), disrupted by sonication, and centrifuged to remove debris. GST-BCL3 was isolated from the supernatant on glutathione-agarose beads (Sigma). For some GST fusion proteins, which were predominantly insoluble, an alternative purification method was used (21).

Rabbit polyclonal anti-BCL3 antibody was made by immunizing rabbits with the GST-BCL3 fusion protein (Josman Laboratories, Napa, Calif.). The titer of the antibody was monitored, and the specificity was tested by Western blot (immunoblot).

Anti-IκBα antibody is a polyclonal rabbit antiserum against the human IκBα (MAD3) N-terminal peptide, a kind gift from Nancy Rice. Anti-FLAG antibody is a mouse monoclonal antibody (M2) from IBI. Anti-NLS antibody is an affinity-purified rabbit antibody against the NLS peptide of NF-κB p50 (from Santa Cruz Biotechnology, Inc). The fluorochrome-labeled goat antibodies were purchased from Protos Immunoresearch.

EMSA. The electrophoretic mobility shift assay (EMSA) was performed as follows. The ^{32}P-labeled probe used is a double-stranded oligonucleotide containing an interleukin 2Rα κB site prepared as described in reference 4. p50 (from pETp50) and GST-BCL3 proteins were prepared as described above and incubated in the binding buffer (10 mM Tris [pH 7.5], 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol) for 30 min at room temperature. The probe (10,000 cpm) and 0.1 μg of poly(dI-dC) were then added to a final volume of 20 μl. After another 30-min incubation at room temperature, the DNA binding complexes were analyzed with a 4% nondenaturing polyacrylamide gel in TGE buffer (25 mM Tris base, 190 mM glycine, 1 mM EDTA).

Transient transfection and copurification of H6-BCL3 and FLAG-p50. Transfection of COS cells was performed by the DEAE-dextran method (33, 56). Briefly, COS cells on p100 plates were transfected 48 h after attaining about 80% confluence. For each plate, 10 μg of supercoiled plasmid, purified by QIAGEN column (Qiagen Inc.), was mixed with DEAE-dextran (0.25 mg/ml, [pH 7.3]). The mixture was then incubated with COS cells for 6 to 8 h. After removal of the DEAE-dextran mixture, cells were dimethyl sulfoxide shocked for 2 to 3 min at room temperature, followed by a 2-h incubation with Dulbecco's modified Eagle's medium containing 0.1 mM chloroquine. Cells were then left with fresh culture medium without chloroquine for 48 to 60 h before harvest.

For coisolation of the BCL3-p50 complexes, COS cells were cotransfected by pEVH6-BCL3 and FLAG-p50 as described above. After washing twice with PBS, the transfected cells were collected by scraping. The published procedure (30), with minor modifications, was used for the affinity purification of native protein on Ni^{2+} beads. The purified proteins were eluted from the beads by boiling in 2× sample buffer for 5 min

before loading for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Immunofluorescence. COS cells, transfected as described above, were plated on glass coverslips. At 48 to 60 h after transfection, cells were washed twice in PBS and fixed with methanol-acetone (1:1) at room temperature for 3 min with gentle shaking. The fixed cells were then washed twice in TBS (50 mM Tris [pH 7.4], 150 mM NaCl) and incubated with blocking solution (2% normal goat serum, 0.1% bovine serum albumin, 0.1% Nonidet P-40 in TBS) for 30 to 60 min. Incubation with primary antibody (anti-BCL3 at 1:2,000, anti-IκBα at 1:500, anti-FLAG at 5 μg/ml) was performed for 1 h at room temperature, followed by extensive TBS washing. Incubation with secondary antibody (fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G or rhodamine-conjugated goat anti-rabbit immunoglobulin G from Protos Immunoresearch) was performed for 1 h at room temperature, followed by extensive TBS washing in the dark. The cells were then counterstained with 4′, 6-diamidino-2-phenylindole (DAPI [0.5 μg/ml]) for 5 min, followed again by TBS washing. Coverslips with stained cells were mounted in 90% glycerol with 1 mg of *p*-phenylenediamine per ml (Sigma) and photographed with a Zeiss Photoscope III.

Western blotting. The protein samples were affinity purified on Ni^{2+} beads as described above. A whole-cell lysate from COS cells transfected with FLAG-p50 was prepared by boiling in sample buffer and clearing by centrifugation at about 12,000 × *g* for 10 min. The protein samples were resolved by SDS-PAGE (12% polyacrylamide). Before transfer, gels were equilibrated with ice-cold blotting buffer (39 mM glycine, 48 mM Tris base, 0.0375% SDS, 20% methanol) for 10 min. The protein transfer was performed with 2.5 mA/cm^2 for 1 h onto a nitrocellulose membrane (Schleicher & Schuell, Keene, N.H.) by using a semidry blotting apparatus (Pharmacia). Blots were blocked overnight at 4°C in Tris-buffered saline (TBST; 10 mM Tris [pH 8.0], 150 mM NaCl, 0.05% Tween 20) containing 5% nonfat milk powder (blocking buffer) with constant shaking. The primary antibody was diluted in the blocking buffer (anti-BCL3 at 1:1,000 to 1:2,000; anti-NLS of p50 at 1 μg/ml) and incubated with blots for 1 h at room temperature, followed by extensive washing with TBST. The horseradish peroxidase-labeled goat anti-rabbit secondary antibody was incubated for another hour at room temperature. After extensive washing in TBST, the antigen-antibody complexes were revealed by enhanced chemiluminescence according to the manufacturer's instructions (Amersham).

RESULTS

BCL3 is a nuclear protein, requiring its N terminus for efficient nuclear localization. Early subcellular fractionation and enucleation experiments suggested that IκBα resides in the cytoplasm in a complex with NF-κB (2). Recent immunofluorescence analysis with transfection or microinjection of expression constructs has confirmed this localization (59, 64). While BCL3 is clearly a member of the IκB family, it has unique structural characteristics. The amino terminus of BCL3 is proline rich, and the carboxyl terminus is proline and serine rich. A proline-rich amino acid sequence characterizes the transactivation domain of certain transcription factors (44). Moreover, there are two clusters of basic amino acids located near the N terminus of BCL3 (Fig. 1) which resemble nuclear localization signals, although the amino acid sequence does not precisely match known consensus sequences for

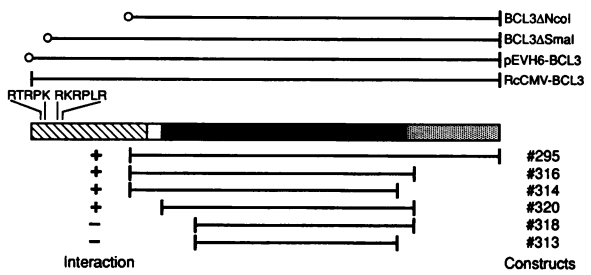

FIG. 1. Structural features of BCL3 protein and mammalian and bacterial expression constructs. The domain structure of BCL3 is illustrated. The central portion, containing seven ankyrin repeats, is flanked by N-terminal proline-rich and C-terminal proline- and serine-rich domains. The positions of the two clusters of basic amino acids on the N terminus are also shown. The mammalian expression constructs are shown at the top. The six-histidine tag is illustrated by an open circle. The bacterial expression constructs are shown at the bottom. The presence or absence of interaction between NF-κB p50 and various forms of BCL3 is also shown.

these signals (12, 54). These structural features suggested that BCL3 protein might be a nuclear protein. Since endogenous BCL3 protein is apparently expressed at a very low level in the cell lines tested (data not shown), we carried out immunofluorescence assays with BCL3-transfected COS cells by using a specific anti-BCL3 antibody. As shown in Fig. 2, both the wild-type BCL3 protein (expressed by RcCMV-BCL3) and an N-terminally tagged BCL3 (expressed by pEVH6-BCL3) were predominantly located in the nucleus. In the course of this work, similar results have been reported by others (8, 49). Moreover, in the cotransfection experiment, BCL3 has been shown to act as a coactivator with p52 or p50 (8, 15).

To test the role played by the clusters of the basic amino acid at the BCL3 N terminus, two N-terminal truncation mutants (Fig. 1) were tested. Interestingly, removal of these basic amino acids altered the pattern of intracellular localization. As shown in Fig. 3A, after removal of the first cluster (BCL3ΔSmaI), BCL3 was distributed to both cytoplasm and nucleus. BCL3 became predominantly cytoplasmic in many of the transfected cells (Fig. 3B) after removal of both clusters (BCL3ΔNcoI); in other cells, overlapping cytoplasm made it

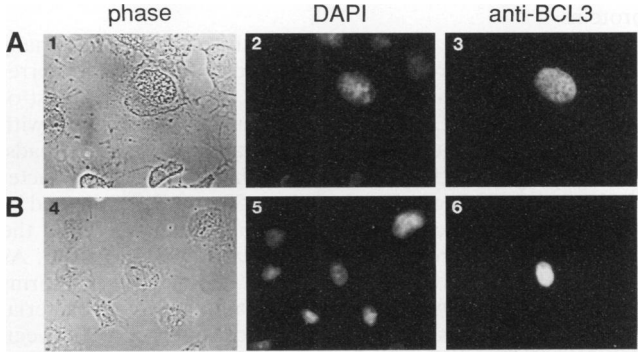

FIG. 2. Subcellular localization of BCL3 protein. Panels: 1 and 4, phase-contrast image; 2 and 5, DAPI staining; 3 and 6, immunofluorescence image of rhodamine-labeled goat anti-rabbit secondary antibody. (A) Indirect immunostaining of COS cells transfected by pEVH6-BCL3 with anti-BCL3 polyclonal antibody. (B) COS cells transfected with the RcCMV-BCL3 construct.

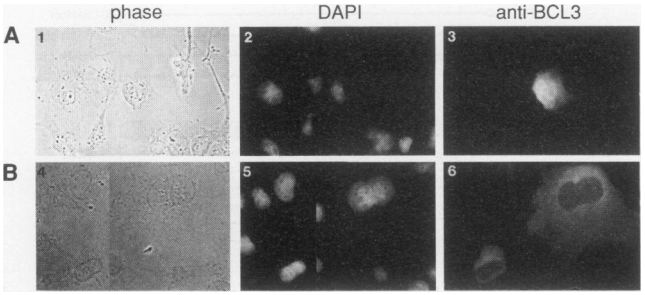

FIG. 3. Subcellular distribution of BCL3 N-terminal deletion mutants. Immunostaining of BCL3 protein in COS cells transfected with BCL3 deletion constructs with anti-BCL3 polyclonal antibody. (A) COS cells transfected with BCL3ΔSmaI, showing BCL3 in both nucleus and cytoplasm. (B) COS cells transfected with BCL3ΔNcoI, showing cytoplasmic localization of BCL3. Panels: 1 and 4, phase-contrast image; 2 and 5, DAPI staining; 3 and 6; immunofluorescence image.

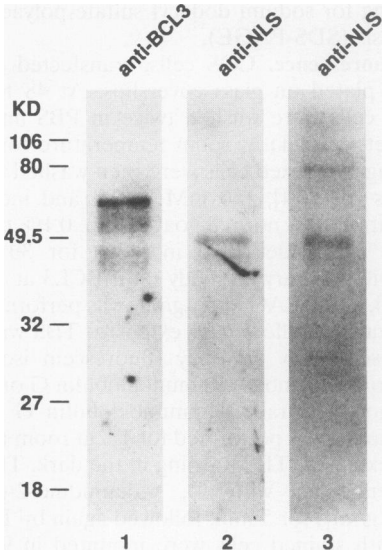

FIG. 4. BCL3 and NF-κB p50 associate with each other. From COS cells cotransfected with pEVH6-BCL3 and FLAG-p50, BCL3-p50 complexes can be coisolated through binding of the six-histidine tag of the BCL3 to Ni^{2+} beads. Protein samples were resolved by SDS-PAGE and detected by Western blot. Lanes 1 and 2 were loaded with the copurified proteins eluted from Ni^{2+} beads, with lane 1 stained by anti-BCL3 polyclonal antibody and lane 2 stained by affinity-purified antibody raised against the NLS peptide of NF-κB p50. Lane 3 (control) was loaded with a crude lysate of COS cells transfected with FLAG-p50 and stained with anti-NLS antibody.

impossible to evaluate whether some of the BCL3 was nuclear. These results indicate that these basic amino acid residues may act as a nuclear localization signal for BCL3.

Interaction of BCL3 protein with p50 protein. Before testing whether BCL3, as an IκB-like protein, affects NF-κB localization, it was necessary to test whether the proteins expressed by our constructs can interact with each other. On the basis of previous experiments (8, 25, 35), we expected BCL3 and NF-κB p50 to copurify after cotransfection if BCL3 and p50 proteins were functionally active. The BCL3 protein used in these experiments was tagged with six histidine residues on its N terminus to allow purification by Ni^{2+} beads. COS cells cotransfected with the FLAG-p50 and pEVH6-BCL3 constructs were lysed under mild conditions so that the proteins stably complexed with BCL3 could be copurified. Western blot analysis demonstrated that p50 could be specifically brought down together with BCL3. In Fig. 4, lane 3 shows the crude cell lysate of cells transfected by the FLAG-p50 construct probed with affinity-purified rabbit antibody against the NLS peptide of p50. At least two bands are visible: the 50-kDa band is the NF-κB p50 protein (confirmed by anti-FLAG monoclonal antibody [data not shown]), while the larger protein band is a cross-reacting protein. However, only the p50 band was detected in the eluted protein (lane 2). The specific copurification of the tagged BCL3 and p50 proteins from the transfected cells indicated that these constructs produced functionally active proteins.

To obtain some idea of the activity of the N-terminally truncated BCL3 proteins, we took advantage of the corresponding constructs used for bacterial expression. In most of the constructs, BCL3 was expressed as a fusion protein with GST, which can be purified on glutathione-agarose beads. Immobilized GST-BCL3 protein was incubated with a bacterial lysate containing induced soluble p50 protein, expressed by the pET vector; the beads were washed extensively, and the bound proteins were eluted and analyzed by SDS-PAGE. As shown in Fig. 1 and 5A, p50 was coisolated with several forms of BCL3 protein with minimal contamination by bacterial proteins. This indicated a direct physical interaction between BCL3 and p50. Moreover, the ankyrin repeat domain alone of BCL3 is sufficient to mediate the interaction, which, however, can tolerate limited C-terminal truncation of this domain (summarized in Fig. 1).

Besides the binding activity tested above, we also tested the inhibitory activity of these proteins by EMSAs (Fig. 5B). With one exception, truncated BCL3 forms which bound p50 were also able to inhibit its binding to DNA. For example, increasing amounts of BCL3 derivative 320, which binds NF-κB (Fig. 5A), also inhibit the binding of p50 to a κB DNA site, as shown in Fig. 5B, lanes 1 to 4. In particular, construct 295, corresponding to the mammalian expression construct BCL3ΔNcoI, showed strong inhibitory activity by EMSA. Therefore, the N-terminally truncated mutants of BCL3 protein were thought likely to interact with NF-κB p50 functionally in cotransfected cells as well.

The exceptional BCL3 deletion derivative, construct 314, did not effectively inhibit p50 binding to κB sites, but increasing amounts of construct 314 (lanes 5 to 7) resulted in a supershifted complex under the same conditions. Since the consistent difference between construct 314 and the other derivatives capable of binding to p50 is the absence of part of the last ankyrin repeat, these results suggest the importance of that repeat in inhibition (see Discussion).

Change in localization pattern after cotransfection of BCL3 and NF-κB. IκBα has been shown to sequester NF-κB in the cytoplasm after cotransfection, apparently by masking its NLS (5, 16, 64). Therefore, it was of obvious interest to determine whether BCL3, a nuclear IκB, has a similar effect on NF-κB location. On the basis of in vitro experiments, BCL3 protein appears to have a strong preference for interaction with NF-κB p50 and p52 subunits (35, 49, 61); thus, we first tested whether BCL3 affects the localization of p50. In singly transfected cells, p50, like BCL3 (Fig. 2), was distributed diffusely throughout the nucleus, excluding the nucleoli (Fig. 6, panel 1), but it was sequestered in the cytoplasm when cotransfected with IκBα (Fig. 6, panel 2), as previously described by others (5). Upon cotransfection with BCL3, p50 was still found in the nucleus (Fig. 6, panel 3) but showed a dramatic alteration in sub-

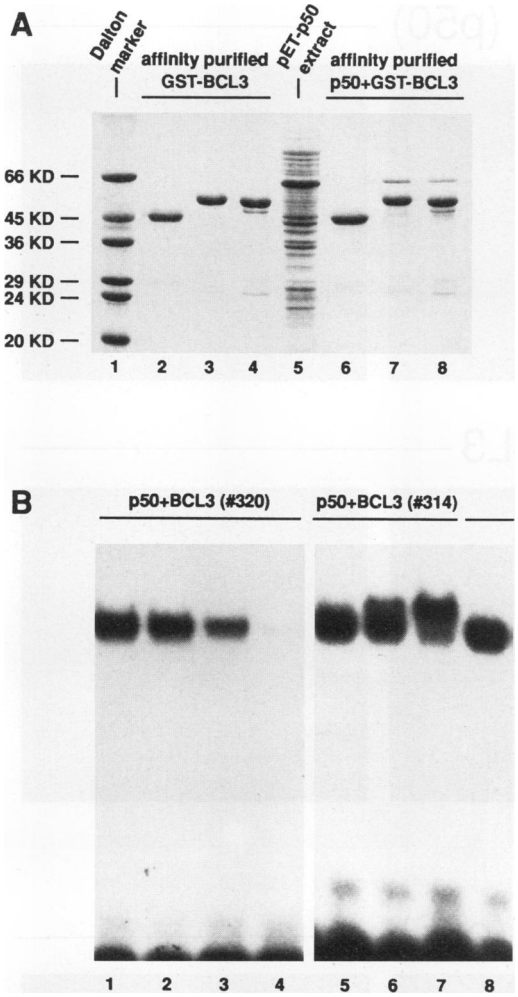

FIG. 5. Interaction between NF-κB p50 and BCL3 derivatives. (A) BCL3 and p50 physically associate with each other to form stable complexes in vitro. Bacterially expressed NF-κB p50 can be copurified with various forms of GST-BCL3 fusion protein. A lysate containing induced NF-κB p50 expressed in the pET vector (lane 5) was incubated with various forms of GST-BCL3 proteins immobilized on agarose beads (lane 2, construct 313; lane 3, construct 314; lane 4, construct 320 [see Fig. 1]). After extensive washing, the retained proteins were eluted from the beads, resolved by SDS-PAGE, and stained with Coomassie blue. Of the constructs shown, 314 and 320 bind to p50 in this assay. (B) EMSA showing the different effects of BCL3 derivatives on the DNA binding activity of p50. Approximately 5 ng of bacterial p50 protein was used in each lane (lanes 1 to 8). A radiolabeled double-stranded oligonucleotide containing the interleukin 2Rα κB site was used as a probe. Left panel, dose-dependent inhibitory effect of GST-BCL3 encoded by construct 320 on the DNA binding activity of NF-κB p50. Lanes 1 to 4, 0, 5, 15, and 30 ng of GST-BCL3 protein, respectively. Right panel, dose-dependent formation of a supershifted complex by GST-BCL3 encoded by construct 314. Lanes 5 to 7, 10, 20, and 40 ng of GST-BCL3 encoded by construct 314, respectively. Lane 8, control lane indicating the position of p50-DNA complex without bound BCL3.

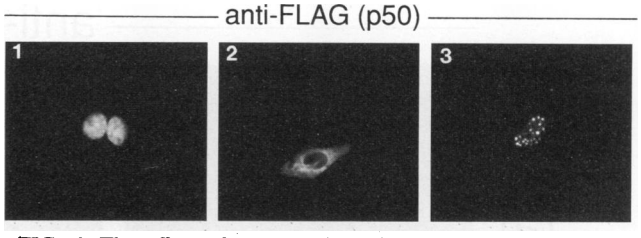

FIG. 6. The effect of coexpression of NF-κB p50 with IκBα or BCL3 on subcellular distribution of these proteins. Panels 1, 2, and 3 show indirect immunostaining of COS cells transfected with FLAG-p50 alone, IκBα plus FLAG-p50, and pEVH6-BCL3 plus FLAG-p50, respectively. The primary antibody used was anti-FLAG mouse monoclonal antibody M2, which was visualized by fluorescein isothiocyanate-labeled goat anti-mouse antibody (as the secondary antibody). Panel 1 shows diffuse nuclear staining of p50, panel 2 shows p50 sequestered in the cytoplasm by IκBα, and panel 3 shows punctate nuclear staining of p50 in cells cotransfected with BCL3.

nuclear localization. While in singly transfected cells, BCL3 and p50 were each distributed diffusely throughout the nucleus excluding the nucleoli, in two-thirds or more of the cotransfected cells, each showed a nuclear speckled pattern not seen in singly transfected cells (Fig. 6, panel 3). This speckled pattern is reminiscent of the localization pattern of another NF-κB family member, p65 (6) (Fig. 7B). Under certain conditions, p65 and the combination of BCL3 and p50 may be functionally similar in that they both have a DNA binding domain and a transactivation domain (8, 15).

To gain more information about the change in localization pattern after cotransfection, two-color immunofluorescence analysis was performed. Figure 7A demonstrates that BCL3 colocalized with p50 in distinct spots, but BCL3 tended to have a faint diffuse nuclear signal as well. The staining pattern was somewhat variable. In some cells, numerous minute spots were stained; in others, several medium-sized spots or large spots were stained (Fig. 7A). However, in virtually all cases, BCL3 colocalized with p50. The same colocalization patterns were observed with another fixation method (data not shown). In addition, no cross-reactivity of the various antibodies used in the double immunofluorescence was detected in control experiments. Cells singly transfected with a p65 expression construct showed a similarly variable pattern of immunofluorescent staining (Fig. 7B).

Nuclear transport of the NF-κB–BCL3 complex only requires karyophilicity from either NF-κB or BCL3. IκBα retains NF-κB p50-p65 in the cell cytoplasm by masking its NLS. Despite lower affinity for the NF-κB p50 homodimer, it is capable of retaining it in the cytoplasm as well (5, 16, 64) (Fig. 6, panel 2). Given the similarity in structure between BCL3 and IκBα, it is possible that BCL3 may also mask the NLS of p50. As previously reported, we found, on the basis of in vitro binding assays, that the ankyrin repeat domain of BCL3 was sufficient to mediate the interaction between BCL3 and NF-κB (see above). Thus, N-terminal truncation generates a cytoplasmic form of BCL3 which still can bind to p50. If BCL3 protein can mask the NLS on p50, cotransfection of the cytoplasmic form of BCL3 with p50 should result in retention of the p50 in the cytoplasm. Instead, however, we found that in some cells, p50 and the truncated BCL3 exclusively colocalized in the nucleus (Fig. 8Ca). The punctate pattern was seen much less frequently and prominently than in cotransfections with full-length BCL3. Other transfected cells expressed a different staining pattern, as shown in Fig. 8Cb, in which p50 was located in the cell nucleus as speckles (weakly stained) and the mutant BCL3 was distributed to both the nucleus and cytoplasm. These different localization patterns may be explained by variability in the ratio of BCL3 protein to p50 protein. Nevertheless, significant cytoplasmic p50 immunostaining was never seen. Therefore, the cytoplasmic form of BCL3 cannot seques-

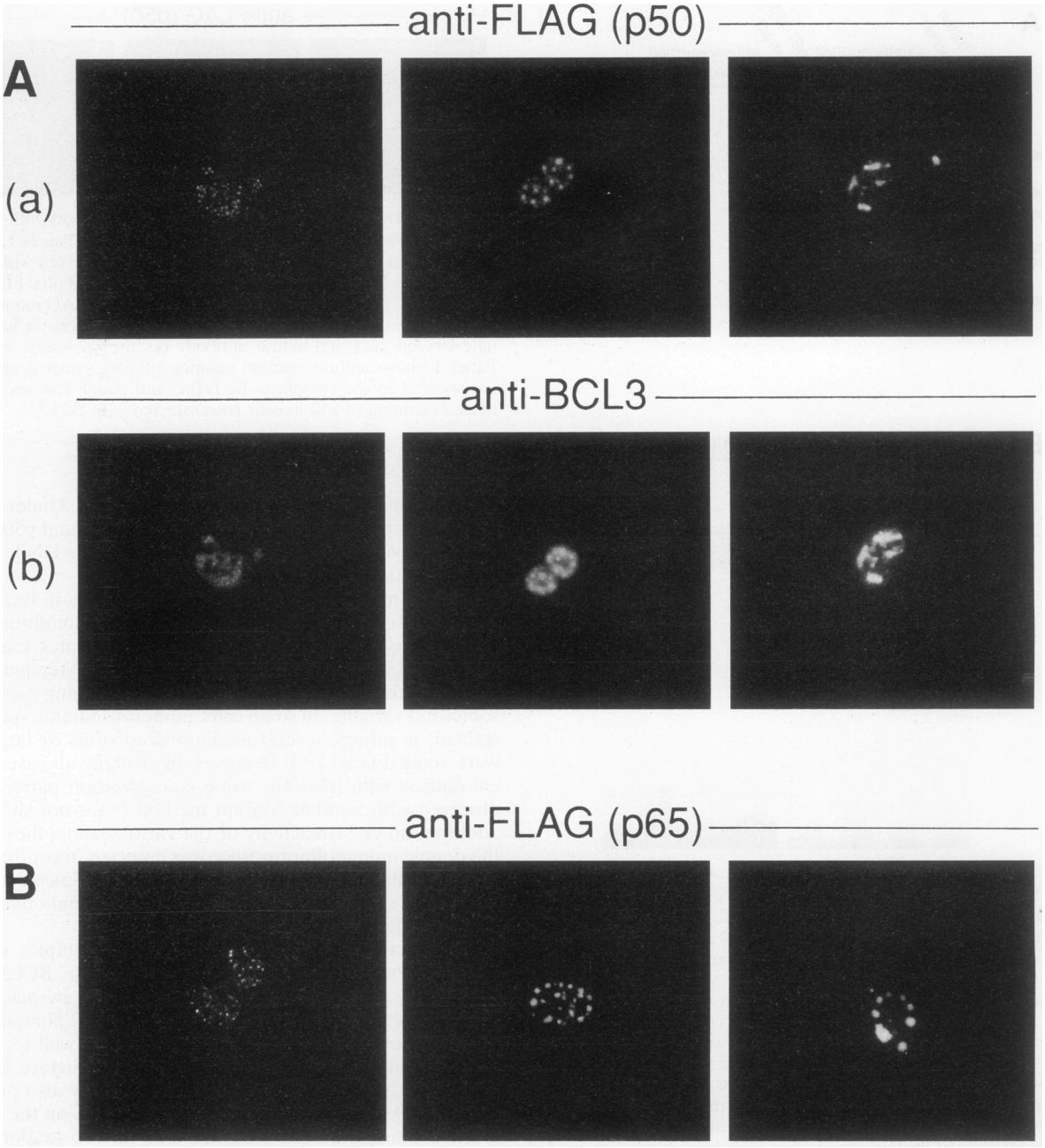

FIG. 7. The punctate nuclear staining of NF-κB p65 resembles that of cotransfected NF-κB p50 plus BCL3. (A) Double indirect immunostaining of COS cells transfected with pEVH6-BCL3 plus FLAG-p50 showing three representative staining patterns of these colocalization proteins. (a) Primary antibody, anti-FLAG mouse monoclonal antibody (M2); secondary antibody, fluorescein isothiocyanate-labeled goat anti-mouse antibody. (b) Primary antibody, anti-BCL3 rabbit polyclonal antibody; secondary antibody, rhodamine-labeled goat anti-rabbit antibody. (B) Three representative punctate staining patterns of p65 shown by indirect immunostaining of COS cells transfected with FLAG-p65. The primary antibody was anti-FLAG mouse monoclonal antibody (M2), and the secondary antibody was fluorescein isothiocyanate-labeled goat anti-mouse antibody.

ter p50 in the cytoplasm. This implies that BCL3 protein interacts with p50 without masking its NLS.

These results suggested that BCL3, unlike IκBα, might interact with a form of p50 lacking a functional NLS (5, 61 [but see reference 29]). We therefore investigated whether BCL3 could bring into the nucleus an otherwise cytoplasmic p50 with site-specific mutations in its NLS (FLAG-p50 NLS mutant). We confirmed that the mutant protein could bind specifically to BCL3 by incubating BCL3-loaded glutathione beads with a lysate from cells transfected with FLAG-p50 NLS mutant. After extensive washing, the retained proteins were tested by Western blotting with anti-FLAG monoclonal antibody. Clear signal was observed at the expected size for p50 when the incubation was performed with a form of BCL3 (construct 316) which can bind wild-type p50 but not when a form (construct 313) incapable of binding to wild-type p50 was used (results

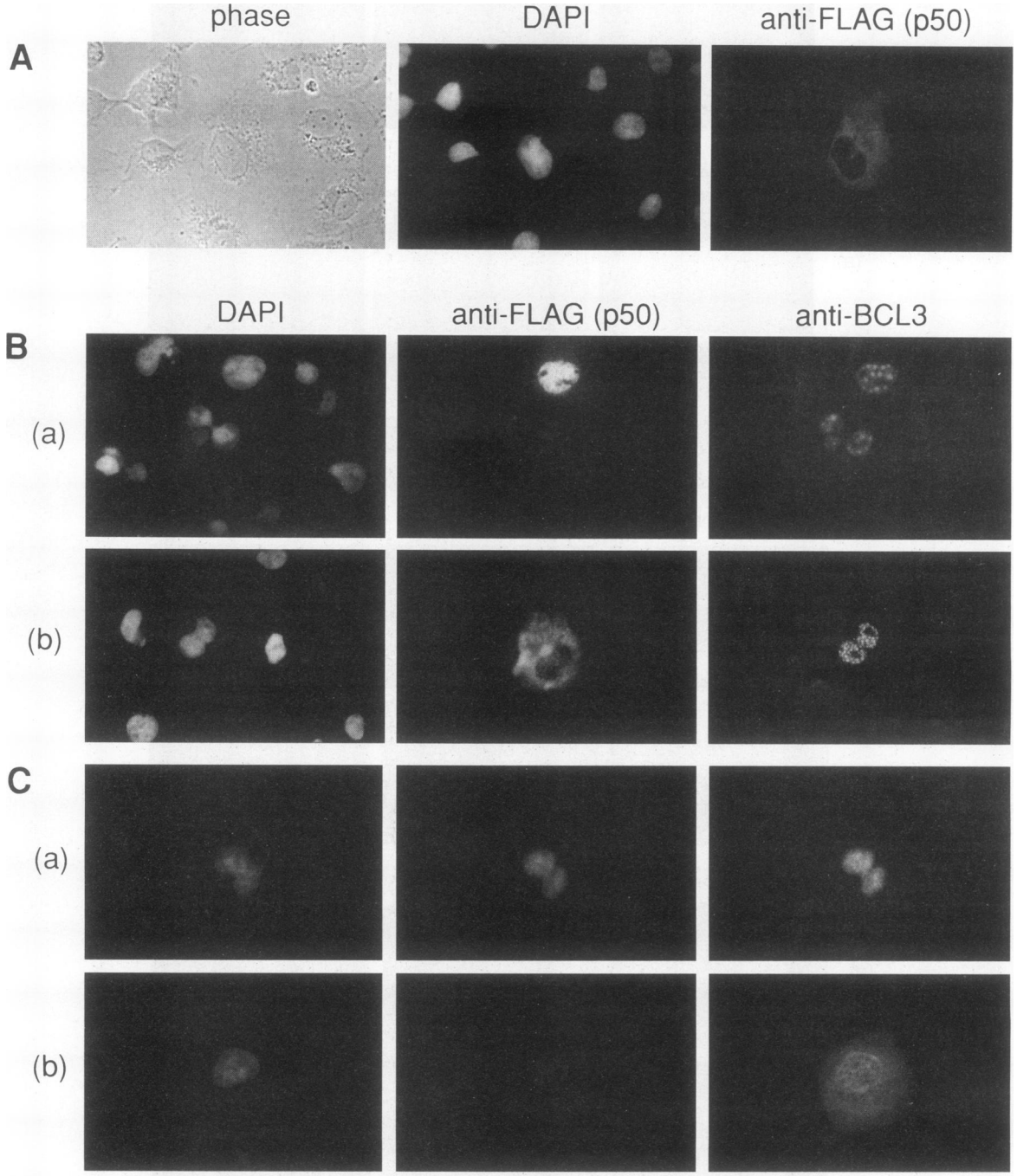

FIG. 8. BCL3 can bring a cytoplasmic mutant form of p50 into the nucleus, and p50 can also bring cytoplasmic BCL3 into the nucleus. (A) Subcellular distribution of FLAG-p50 NLS mutant shown by immunostaining of COS cells transfected by FLAG-p50 NLS mutant. The third panel gives the immunofluorescence image showing the exclusive cytoplasmic distribution of the FLAG-p50 NLS mutant. (B) Nuclear localization of FLAG-p50 NLS mutant in cotransfected cells shown by double immunostaining of COS cells cotransfected with pEVH6-BCL3 and the FLAG-p50 NLS mutant at a molar DNA ratio of 3:1 (a) or 1:3 (b). Additional ratios were tested as well. As the relative amount of the BCL3 construct was decreased, more of the mutant p50 remained in the cytoplasm, but there was no effect on the distribution of BCL3, which maintains a predominant nuclear location in all cases. The primary antibodies used are marked at the top of each panel. The same set of secondary antibodies in Fig. 7A was used, except that the secondary antibody in panel b used to visualize anti-BCL3 was Texas red-labeled goat anti-rabbit antibody. (C) Double immunostaining of COS cells cotransfected with BCL3ΔNcoI and FLAG-p50 showing that p50 can bring an otherwise cytoplasmic truncated BCL3 into the nucleus. Antibodies were the same as in Fig. 7A. Two patterns of nuclear localization of truncated BCL3 (BCL3ΔNcoI) in cotransfected cells are illustrated, reflecting the differing relative abundance of the two proteins.

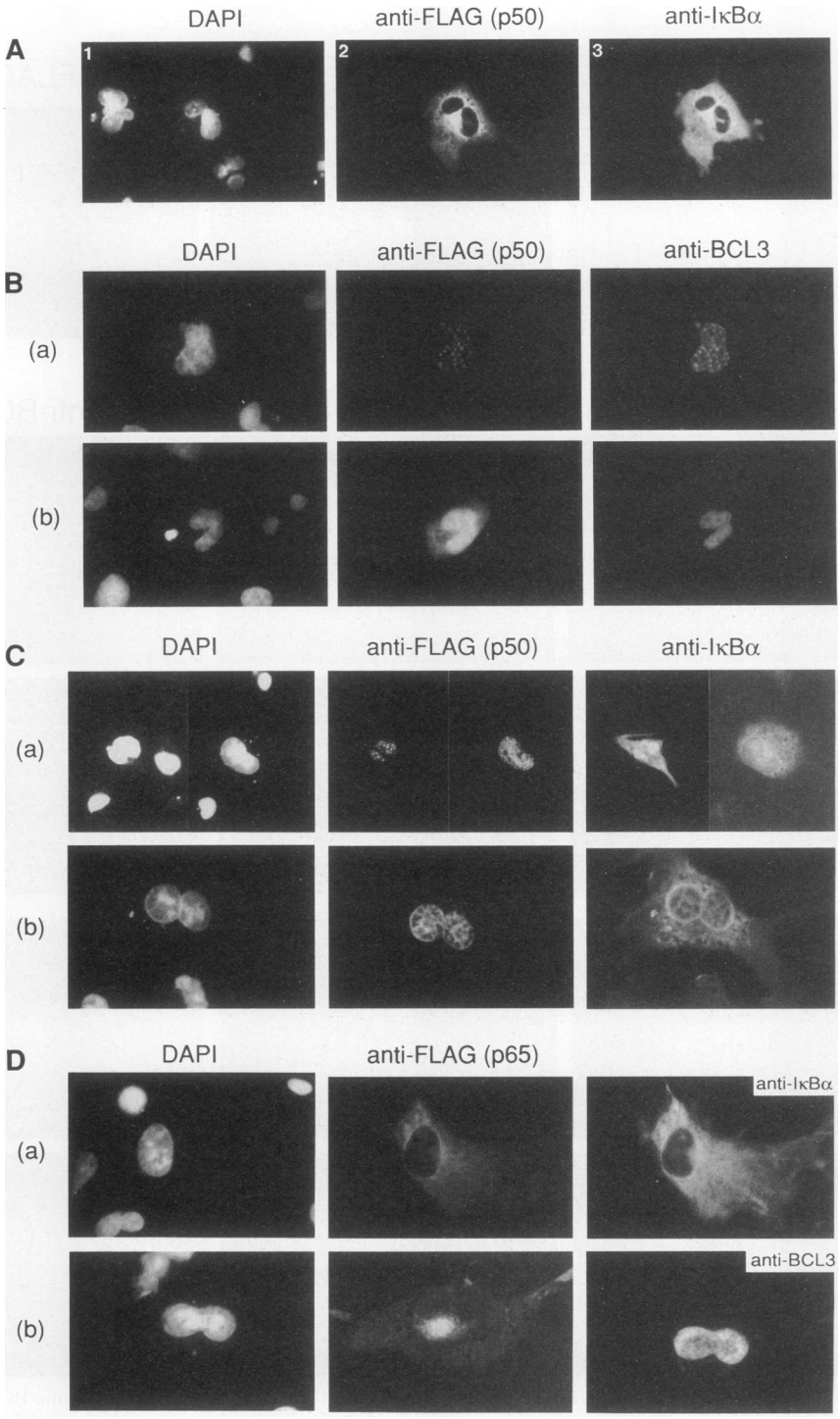

FIG. 9. Functional competition between BCL3 and IκBα in the regulation of NF-κB localization. (A) Subcellular location of p50 and IκBα proteins after coexpression shown by double immunostaining of COS cells cotransfected with IκBα and the FLAG-p50 construct (plasmid molar ratio at 2:1; at this ratio, IκBα completely sequestered p50 in the cytoplasm). The primary antibodies used are marked at the top of each panel. The bound anti-FLAG antibody was visualized by fluorescein isothiocyanate-labeled goat anti-mouse antibody; the bound anti-IκBα antibody was visualized by rhodamine-labeled goat anti-rabbit antibody. Panels 2 and 3 give an immunofluorescence image showing that p50 and IκBα are colocalized in the cytoplasm of cotransfected cells. (B) Subcellular localization of p50 and BCL3 proteins in COS cells cotransfected with pEVH6-BCL3, IκBα, and FLAG-p50 (plasmid molar ratio of 3:2:1). Double immunostaining was performed with anti-FLAG mouse monoclonal antibody M2 and anti-BCL3 rabbit polyclonal antibodies. Panels a and b show two different staining patterns: a, complete nuclear colocalization of BCL3 and p50 in the triply transfected cells; b, partial nuclear colocalization of p50 and BCL3 in the triply transfected cells. (C) Nuclear localization of p50 despite the presence of abundant IκBα protein. Double immunostaining of COS cells triply transfected as in panel B was performed with the primary antibodies marked. Panels a and b show two staining patterns: a, complete nuclear location of p50 in a punctate pattern (similar to that seen in the doubly transfected cells in Fig. 7A); b, complete nuclear localizaiton of p50, but in a more diffuse pattern. (D)

not shown). Thus, p50 does not require its NLS for interaction with BCL3 in vitro.

When this mutant p50 was tested in transfection experiments, as shown in Fig. 8A, it was exclusively localized to the cytoplasm; the same pattern was previously observed with anti-p50 peptide antibodies for the p50 NLS mutant without the FLAG epitope tag (26). However, after cotransfection with the tagged p50 NLS mutant and full-length BCL3, the two proteins colocalized to the nucleus (Fig. 8Ba). This colocalization pattern resembles that of the wild-type proteins. Therefore, mutation of the NLS of p50 did not significantly affect its interaction with BCL3, on the basis of this direct in vivo evidence. These experiments also revealed the potency of the karyophilicity of BCL3 protein. Experiments were also performed in which the FLAG-p50 NLS mutant protein was expressed in apparently great excess over the BCL3 protein (Fig. 8Bb). Even in such experiments, in which insufficient BCL3 is present to direct all of the mutant p50 to the nucleus, there is little detectable cytoplasmic BCL3 and no apparent increase over that found in singly transfected cells.

NF-κB p50 localization can be modulated by differential expression of IκBs. The evidence presented above strongly suggests that BCL3 can regulate NF-κB through direct physical interaction. The specificity of various IκB factors for inhibition of NF-κB proteins correlates with their affinity. For example, IκBα inhibits p65 more effectively than p50 and binds to p65 with much higher affinity than to p50 (5, 16, 64). In contrast, BCL3 appears to have higher affinity for p50 than for p65, and BCL3 inhibits the DNA binding activity of p50 more effectively than that of p65 (29, 35, 49, 61). As shown above, BCL3 and IκBα affected the localization of NF-κBs differently. IκBα sequestered p50 in the cytoplasm (further shown in Fig. 9A), but BCL3 brought p50 into the nucleus (Fig. 8B). Therefore, BCL3 and IκBα might be expected to regulate NF-κB in a competitive manner if they are simultaneously present. We tested this hypothesis by triple transfection with BCL3, IκBα, and p50. Immunofluorescence analysis of BCL3 and p50 displayed two major staining patterns (Fig. 9B). In most cells, BCL3 and p50 colocalized in the nucleus, often in the same speckled pattern seen in double transfection in the absence of IκBα (Fig. 9Ba). Other cells showed both nuclear and cytoplasmic staining of p50 (Fig. 9Bb); the BCL3 protein may have been less abundant than IκBα in these cells, so that some p50 could still be retained in the cytoplasm. Therefore, inclusion of BCL3 completely changed the pattern produced by cotransfection only of p50 and IκBα, which, with the plasmid ratio used here, led to complete cytoplasmic sequestration of p50. The most likely explanation is that BCL3, because of its higher affinity for p50, overcame the effect of IκBα on p50 localization. Regulation of subcellular location is a key mechanism in the control of NF-κB activity. Here, we present a new potential mechanism for modulation of the cellular localization and, therefore, activity of the NF-κB transcription factors.

To gain more information from the triple transfection experiments, we stained the cells with different antibodies. As shown in Fig. 9C and D, in many of the transfected cells p50 was localized exclusively to the nucleus. While in some cells, diffuse nuclear staining of p50 was present (Fig. 9Cb), in others a punctate pattern was seen (Fig. 9Ba and Ca), similar to that of cells transfected only with BCL3 and p50. This staining pattern suggests the presence of BCL3-p50 complex in the nucleus. When the same cells were stained by anti-IκBα (Fig. 9Ca), a high level of IκBα protein was detected. Therefore, even in the presence of abundant IκBα protein, BCL3 is still able to bring the p50 into the nucleus. On the other hand, BCL3 should not be able to overcome the effect of IκBα on p65, if the relative affinity is the determining factor for the competition. Following triple transfection with BCL3, IκBα, and p65, immunostaining revealed cytoplasmic colocalization of IκBα and p65 (Fig. 9D). In cells with strong nuclear staining of BCL3, the p65 still was exclusively located in the cytoplasm. Thus, as expected, IκB factors differentially control NF-κB localization on the basis of their relative binding affinities.

DISCUSSION

BCL3 encodes a multifunctional nuclear IκB. We and others have previously shown that BCL3 protein can inhibit the DNA binding activity of NF-κB p50 and p52. The specificity of BCL3 differs from that of another well-defined IκB, IκBα (MAD3), which more effectively inhibits forms of NF-κB containing the p65 subunit. However, BCL3 at a high concentration can also inhibit the DNA binding activity of other NF-κBs as revealed by gel shift assays; in addition, it inhibits transactivation activity in chloramphenicol acetyltransferase transfection assays (14, 61). This suggests that the binding affinity between IκB and NF-κB determines the specificity. Since IκBα is predominantly cytoplasmic, we were surprised that BCL3 was present in the nuclear pellet as determined by Western blot analysis of fractionated cells (reference 35 and unpublished data). Evidence shown here by immunostaining indicates that the BCL3 protein is predominantly nuclear.

A possible function for an IκB protein in the nucleus has been suggested (1, 64). In most cases, the induction of NF-κB activity is transient; the activated NF-κB activity in the nucleus must be turned off at an appropriate time after induction. If a form of IκB can migrate into the nucleus, it may be able to release NF-κB from the κB sites. The latter phenomenon has actually been demonstrated in vitro. Preformed NF-κB–DNA complexes can be effectively disrupted by IκBα, IκBβ, or BCL3 (references 49 and 63 and unpublished data). On the other hand, in the resting state of at least one cell type (T lymphocytes), there is abundant nuclear p50 homodimer, which can bind to κB sites and inhibit gene activation (14, 32). After stimulation, the bound p50 homodimer must be removed before the activated NF-κB p50-p65 can bind to the κB sites and activate gene expression. Interestingly, BCL3 mRNA can be induced as an immediate-early gene in T cells by a T-cell mitogen (reference 50 and unpublished data); the induced nuclear BCL3 protein may then remove the prebound p50 homodimer.

While the ankyrin repeat domain of BCL3 protein is responsible for its activity as an IκB, its N- and C-terminal regions are not homologous to those of the other IκB proteins but are very proline rich, suggesting a potential role as a transactivator. This is compatible with its nuclear localization, presented in this paper. Recently, Bours et al. (8) provided strong evidence that when coexpressed with p52, BCL3 can have potent

Predominant cytoplasmic retention of FLAG-p65 in the presence of abundant BCL3 protein in COS cells cotransfected with pEVH6-BCL3, IκBα, and FLAG-p65 (plasmid molar ratio of 3:2:1). Panel a shows double immunostaining with M2 (for p65) and anti-IκBα rabbit antibodies as the primary antibodies, and panel b shows double immunostaining with M2 and anti-BCL3 rabbit polyclonal antibodies as the primary antibodies. The cytoplasmic sequestration of p65 by IκBα was not affected by BCL3.

transactivation activity, which is dependent on both N- and C-terminal domains.

We have demonstrated that the N-terminal domain of BCL3, which contains two clusters of basic amino acids, is necessary for efficient nuclear localization of BCL3 protein (Fig. 3). So far, determination of the region sufficient for nuclear localization by fusion to heterologous proteins has been hampered by the functional inactivity of the resulting fusion proteins.

Previous studies suggested that BCL3 protein is poorly retained in the nucleus during biochemical fractionation (reference 47 and data not shown), so it is unlikely that the nuclear localization of BCL3 was due to the nuclear accumulation of protein passively diffusing into the nucleus. In addition, the fact that BCL3 efficiently brings the p50 NLS mutant into the nucleus also argues for an active transportation mechanism, since the complex between BCL3 and p50 is much larger than the cutoff size for proteins to diffuse into the nucleus (18).

BCL3 interacts with NF-κB factors in vitro and in vivo. Some biochemical studies have strongly implied that IκB proteins bind to NF-κB factors at a site distinct from the DNA binding site and function by changing the conformation of NF-κB into one with greatly reduced affinity for DNA (63). Recent studies showed that the NLS on the NF-κB is necessary for the binding to IκBα (MAD3), and the binding of IκBα blocked the accessibility of specific anti-NLS antibodies (5, 16, 64). Presumably, this masking also prevents the binding of NF-κB proteins to the NLS receptor on the nuclear envelope, leading to sequestration of NF-κB in the cytoplasm (54). However, this is not the case for the nuclear IκB BCL3. Clearly, BCL3 also binds to the Rel homology domain of NF-κB subunits, but binding is independent of the NLS (reference 61 and data not shown). Another NLS mutant of p50 fails to interact with BCL3 (29). While the reason for this is unclear, it is possible, for example, that the mutation affected the conformation of p50. As shown in Fig. 8A and B, BCL3 protein can bring into the nucleus a form of p50 with an inactivated NLS; the two proteins colocalized in subnuclear compartments. This is further supported by Fig. 8C, which shows that a truncated BCL3 which was cytoplasmic when expressed alone did not retain the p50 in the cytoplasm after coexpression. Thus, despite their structural similarity, BCL3, unlike IκBα, does not mask the NLS of NF-κB and does not require it for binding.

Our data differ somewhat from those of Nolan et al. (49), who also showed that the p50 NLS was not required for interaction with BCL3 but found a partial redistribution of BCL3 protein to the cytoplasm after coexpression with a mutant cytoplasmic p50 with deletion of the NLS region. In analogous experiments, we have never detected a redistribution of BCL3 into the cytoplasm even when a clear excess of mutant p50 is present (Fig. 8Bb). The different mutants and different cell systems used may have contributed to the different results.

As expected, IκBα colocalized with NF-κB in the cytoplasm after coexpression (64). In sharp contrast, BCL3 colocalized with NF-κB p50 in the nucleus in an immunostaining pattern different from that of either BCL3 protein or p50 alone. The colocalization of these two proteins in a variety of punctate or speckled patterns strongly implies direct physical interaction in vivo.

A number of functional activities appear to be compartmentalized in the nucleus (10, 31, 38, 62). Splicing components, sites of transcriptional activation, and sites of DNA replication all demonstrate punctate patterns by immunofluorescence. It is not clear at this time whether the punctate p50-BCL3 staining corresponds to any of the compartments. However, the patterns of transcriptional active sites in the transiently transfected COS cells reported by Jimenez-Garcia et al. (31) resemble the patterns presented here (Fig. 7). It is possible that, in these experiments, p50 and BCL3 cooperate to act as a potent transactivator and localize to sites of active transcription.

It has been shown that the ankyrin repeat domain of IκB is essential for interaction with NF-κBs. Site-directed mutagenesis studies suggested that some repeats are more important than others (28). As previously reported (61) (Fig. 5), the seven ankyrin repeats in BCL3 are actually sufficient for interaction with NF-κB and for inhibition of its DNA binding activity. Interestingly, BCL3 with partial truncation of the last repeat still binds to NF-κB p50 (Fig. 5A) but without inhibiting its DNA binding activity. Instead, in EMSA experiments, this form of BCL3 causes an additional retardation of the migration of the p50-DNA complex. These results imply that this truncated BCL3, NF-κB p50, and DNA containing a κB site can form a stable triple complex (Fig. 5B). This may suggest that the full-length BCL3 protein after proper modification can act as a transactivator in vivo through the formation of a similar complex. Recently, Bours et al. (8) and Fujita et al. (15) have presented results apparently demonstrating such an activity.

Although at moderate concentrations BCL3 can influence which NF-κB family member binds a κB site when different forms compete for binding, a strictly inhibitory function in mammalian cells appears to require a great excess of BCL3; in contrast, bacterially expressed BCL3 can readily be shown to inhibit the DNA binding activity of p50 and p52. This apparent discrepancy may result from the fact that mammalian BCL3 is phosphorylated and its activity is affected by phosphorylation. The ability of BCL3 to inhibit or to act instead as a coactivator may be regulated by this phosphorylation.

Functional competition between BCL3 and IκBα (MAD3). Almost from the time of its discovery, NF-κB activity has been thought to be regulated through reversible cytoplasmic sequestration by IκB factors. More recently, evidence has been presented for a second, but related, regulatory mechanism, the controlled proteolytic processing of the p105 and p100 precursors to their active forms (43, 52). Here, we show that BCL3, a nuclear IκB, can bring NF-κB p50, otherwise sequestered by IκBα, into the nucleus (Fig. 9). On the other hand, BCL3 was not able to overcome the cytoplasmic sequestration of p65 by IκBα (Fig. 9D), showing the specificity of this functional interference. Therefore, in the absence of a signal-dependent release of NF-κB factors from their specific cytoplasmic IκB, NF-κB proteins may move into the nucleus as a result of differential expression of functionally distinctive IκB proteins.

A surprising observation was the presence of IκBα in the nucleus of cells triply transfected with BCL3, IκBα, and p50 (Fig. 9C). In our experiments, IκBα could be visualized only in the cytoplasm in the double transfections. It is possible that BCL3 may have brought the NF-κB p50-IκBα complex into the nucleus.

Previous studies have shown that both p105 and p100 are cytoplasmic and can also sequester certain NF-κB subunits in the cytoplasm (43, 46, 47, 52). In addition, IκBγ can sequester Rel protein in the cytoplasm (27). It will be of interest to determine whether BCL3 protein can also interfere with the function of p105, p100, or IκBγ in regulating NF-κB localization.

The data presented here demonstrate a novel way in which BCL3 may regulate the localization of NF-κB. Whether this phenomenon is of significance in normal cells remains to be

determined. While the level of functionally active nuclear p50 homodimers is markedly stimulated or reduced by various stimuli, little is known about its regulation. Recent evidence suggests that some NF-κB p50 homodimers may be present in the cytoplasm of nontransfected cells, perhaps bound to IκBα (17, 51). While the cytoplasmic concentration of BCL3 is likely to be significantly smaller than that of IκBα, BCL3 may still be able to compete effectively for binding to those factors, such as p50, for which it has the higher affinity. Thus, the subcellular localization of p50 homodimers and perhaps certain other forms of NF-κB may be regulated in part through the relative level of expression of BCL3 compared with that of IκBα and other cytoplasmic IκBs.

BCL3 overexpression and tumorigenesis. The t(14;19) translocation in chronic lymphocytic leukemia patients, which juxtaposes the *IGH* locus on chromosome 14 with the *BCL3* gene on chromosome 19, does not interrupt the transcriptional integrity of *BCL3* but is associated with overexpression of the gene (41, 50). In these chronic lymphocytic leukemia patients, *BCL3* overexpression presumably results in dysregulation of a gene or genes normally regulated by NF-κB factors and important in some aspect of cell proliferation, differentiation, or survival. In several cases, aberrations in NF-κB activity have been found to cause neoplastic transformation (37, 45).

Whether the postulated target gene is over- or underexpressed consequent to the translocation is difficult to predict since, as mentioned above, BCL3 is capable of acting either as an inhibitor or an activator of transcription; the activating effect may be indirect or through coactivation. The results shown here suggest a potential additional mechanism through which BCL3, overexpressed as a result of the translocation, may affect the subcellular localization of NF-κB factors. Further analysis of these mechanisms and identification of the relevant affected genes in leukemia and lymphoma are important areas for investigation.

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