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Constitutive Expression of Bcl-3 in Thymocytes Increases the DNA Binding of NF-κB1 (p50) Homodimers In Vivo

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Previous studies have indicated that Bcl-3 interacts through its ankyrin repeats with the transcriptional factors NF-κB1 (p50) and NF-κB2 (p52), affecting their biological activities. To further investigate the role of Bcl-3 in vivo and its association with the NF-κB proteins, we have generated transgenic mice constitutively expressing Bcl-3 in thymocytes. The results indicate that Bcl-3 is associated with endogenous p50 and p52 in nuclear extracts from transgenic animals. Remarkably, constitutive expression of Bcl-3 in these cells augments the DNA binding activity of endogenous p50 homodimers more than 10-fold but does not significantly increase the activity of p52 homodimers. This effect could be reproduced in vitro and is blocked by anti-Bcl-3 antibodies. We have also shown that Bcl-3 is phosphorylated in thymocytes and that its dephosphorylation greatly decreases the effect on p50 homodimers.

The *bcl*-3 gene was cloned from a recurrent chromosomal translocation, t(14;19)(q32;q13.1), present in human B-cell chronic lymphocytic leukemias (39, 40, 60, 65). It is remarkable that all the rearrangements in this locus occur in the 5′ regulatory region of the gene and result in increased expression of wild-type Bcl-3 (44, 45, 65). This protein contains seven ankyrin repeats and shares structural features with IκBα, IκBβ, IκBγ, and the C terminus of NF-κB2 (5, 24, 57).

Members of the Rel/NF-κB family of transcription factors play a major role as early mediators of immune and inflammatory responses (4, 37, 54). Members of this family are differentially expressed during mouse embryonic development and in adult lymphoid tissues (13, 14, 62). Each of these proteins contains a highly conserved region of approximately 300 amino acids called the Rel homology domain which is responsible for both DNA binding and dimerization. In mammalian cells, this protein family can be divided into two classes. One class includes the precursors NF-κB1 (p105/p50) and NF-κB2 (p100/p52), which are synthesized as inactive molecules and remain in the cytoplasm. These precursors (p105 and p100), upon proteolytic processing, will generate the DNA-binding subunits p50 and p52, respectively. The other class of proteins comprises RelA (p65), c-Rel, and RelB. These proteins do not undergo proteolytic processing, and they harbor transcriptional activation domains (36). The biological functions of p50, RelA, RelB, and c-Rel have recently been addressed by the generation of null mice (7, 11, 30, 53, 63).

The primary Rel/NF-κB transactivating complexes in cells appear to be homo- and heterodimers involving members of each subclass that bind to specific κB sites and differ in their transcriptional activities. In unstimulated cells the Rel/NF-κB dimers are associated with the inhibitor IκB proteins and remain as an inactive pool in the cytoplasm. Upon stimulation by different agents, IκB molecules are rapidly phosphorylated and degraded, allowing the NF-κB dimers to translocate into the nucleus and regulate transcription through binding to the κB sites (2, 5, 10, 18, 19, 24, 36, 41, 46, 56–59).

The biological role of p50 homodimers is unclear, since several studies have generated ambiguous results. These dimers are present in both the nuclei and cytoplasms of unstimulated cells, and some experiments indicate that they are unable to activate transcription in vivo (6, 21, 28, 47, 50). However, other reports showed that p50 homodimers have the capacity to activate in vivo and in vitro a promoter containing κB sites (22, 23, 31). Moreover, other studies have postulated that the p50 homodimers are involved in regulating the expression of tumor necrosis factor alpha, NF-κB1 (p105), and major histocompatibility complex (MHC) class I genes (17, 51, 55, 61), although the surface expression levels of MHC class I molecules in cells of p50 null mice are normal (53).

Previous in vitro studies and transient-transfection experiments have shown that Bcl-3 is able to remove p50 homodimers from DNA, thus allowing transcriptional activation to occur through the potent p50-RelA heterodimers (20, 21, 26, 27, 34, 42, 43, 64). In contrast, Fujita et al. (23) have demonstrated that Bcl-3 associated with p50 homodimers acts as a transcriptional activation complex. One potential explanation for such contrasting results is the nature of the p50 protein used in these studies, as the precise in vivo cleavage site of the p105 precursor is yet unknown (23). Bcl-3 has also been postulated to function as a transcriptional coactivator when associated with p52 homodimers (9).

To further understand the interactions of Bcl-3 with the members of the Rel/NF-κB family of transcription factors in vivo, we have generated transgenic mice constitutively expressing Bcl-3 in thymocytes. Here we show that Bcl-3-expressing transgenic thymocytes present a dramatic increase in DNA binding activity of p50. Although Bcl-3 interacts with both p50 and p52 homodimers, no increase in the DNA binding of the latter was detected. Such effects are not altered upon cellular stimulation with a phorbol ester (phorbol 12-myristate 13-acetate [PMA]) and phytohemagglutinin (PHA). We also demonstrate that Bcl-3 needs to be phosphorylated to increase the DNA binding of the p50 homodimers.

MATERIALS AND METHODS

Plasmid construction and generation of transgenic mice. The full-length human *bcl*-3 cDNA (nucleotides 1 to 1338) was subcloned into the *Bam*HI site of the p*lck*-GH vector (15). This vector contains a 3.2-kb fragment of the mouse proximal *lck* promoter and a 2.1-kb genomic fragment of the human growth hormone gene that provides introns, exons, and polyadenylation sequences. The

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transgene was released from the vector sequences by *Sfi*I digestion and purified by zonal sucrose gradient centrifugation as previously described (38).

The microinjection of the transgene and the screening of the transgenic mice by PCR analysis were performed as previously described (46).

The results shown in this report were obtained with transgenic line 14. Identical results were obtained for transgenic line 18.

These studies were performed according to guidelines established by the Bristol-Myers Squibb Animal Care and Use Control Committee.

Northern (RNA) blot assays. Total RNA from the thymuses of 4- to 5-week-old mice was isolated with RNAzol according to the manufacturer's instructions (Cinna/Biotecx). Total RNA (20 μg) was denatured and separated by electrophoresis in a 0.8% agarose gel containing 2.2 M formaldehyde according to standard procedures (49). The gel was blotted onto Nytran membranes and hybridized to a full-length *bcl*-3 cDNA and a 1.2-kb cDNA fragment of the rat glyceraldehyde-3-phosphate dehydrogenase gene. Probes were labeled with [^{32}P]dCTP by the random priming technique (49).

In situ hybridization. In situ hybridization was performed as previously described (13). The sequences of *bcl*-3 used to generate the sense and antisense probes correspond to nucleotides 885 to 1362, which span the 3′ region of the gene.

Western blotting (immunoblotting) and immunoprecipitation assays. Thymic whole-cell extracts from 3- to 4-week-old mice were prepared as previously described (33). Cytoplasmic and nuclear extracts from isolated thymocytes were prepared as previously described (52). Aliquots of nuclear and cytoplasmic extracts (30 μg) were boiled in Laemmli buffer and separated on a 12.5% acrylamide-bisacrylamide (200:1) gel at 12 mA for 16 h. Western blotting was performed as previously described (62). The nuclear extracts were checked for cytoplasmic contamination by incubating the membranes with an antiserum specific for the cytosolic enzyme lactate dehydrogenase.

For immunoprecipitations, 70 μg of each nuclear extract was diluted to a volume of 300 μl with buffer C [20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.9), 25% glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA)] (52), cleared for 3 h with a preimmune serum (3 μl), immunoprecipitated with specific antiserum (3 μl) overnight, and then incubated with 30 μl of protein A-Sepharose CL-4B (Pharmacia) for 3 h on a roller system at 4°C. The immunocomplexes were washed three times with 1 ml of buffer C, boiled in 30 μl of Laemmli buffer, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described above.

The generation of p50, p52, and RelA antisera has previously been described (62). The Bcl-3 antiserum was generated with an MS2 polymerase fusion protein containing amino acids 163 to 454 of the human Bcl-3 protein. The p50 and p52 monoclonal antibodies were generated as previously described (25) with an MS2 polymerase fusion protein containing amino acids 1 to 128 and 1 to 82 of the mouse p50 and p52 proteins, respectively.

Cell culture. Single-cell suspensions from the thymus were prepared as previously described (16) and incubated at 37°C in RPMI 1640 with 10% heat-inactivated fetal calf serum. Cells were stimulated with PHA and PMA at final concentrations of 1 μg/ml and 50 ng/ml, respectively.

Electrophoretic mobility shift assays (EMSAs). Nuclear extracts (3 μg) were incubated with 50,000 cpm of ^{32}P-labeled probe and 3 μg of poly(dI-dC) in buffer containing 20 mM HEPES (pH 7.9), 100 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 17% glycerol in a final volume of 25 μl, for 15 min at 20°C. Complexes were analyzed on 5.5% native polyacrylamide gels run in 0.25× Tris-borate-EDTA buffer, dried, and exposed to Kodak X-Omat AR film at −70°C. The κB sites used for these assays were the following: H_2κB, PD (palindromic site), immunoglobulin kappa light chain (Igκ), and interleukin 2 (IL-2) (37). Quantitation was performed on a PhosphorImager according to the manufacturer's instructions (Molecular Dynamics).

In vitro interaction was determined by mixing different amounts of Bcl-3/p50$^{-/-}$ nuclear extract (from Bcl-3-expressing transgenic mice in a p50 null background, created by crossing Bcl-3-expressing transgenic mice with mice lacking p50 [p50$^{-/-}$] [53]) with a constant amount of control thymocyte extract, incubating the mixtures at room temperature for 30 min, and performing EMSAs.

Dephosphorylation of the nuclear extracts was performed with alkaline phosphatase (Boehringer Mannheim) or protein phosphatase 2A (Upstate Biotechnology Inc.) according to the manufacturer's instructions. The alkaline phosphatase was inactivated by the addition of β-glycerophosphate, sodium fluoride, and sodium orthovanadate to the Bcl-3/p50$^{-/-}$ mixture after treatment but before incubation with the control extract.

Flow cytometric analysis. Cells were resuspended in phosphate-buffered saline containing 1% bovine serum albumin before being stained with the following conjugated antibodies: CD3, CD4, CD8, CD25, CD44, CD45R (B220), αβ T-cell receptor, Thy 1.2, and intracellular adhesion molecule 1. The antibodies were obtained from GIBCO-BRL and Pharmingen. Flow cytometry was performed with a Coulter Epics Profile II flow cytometer and cell sorter, and 10,000 events were recorded in each case.

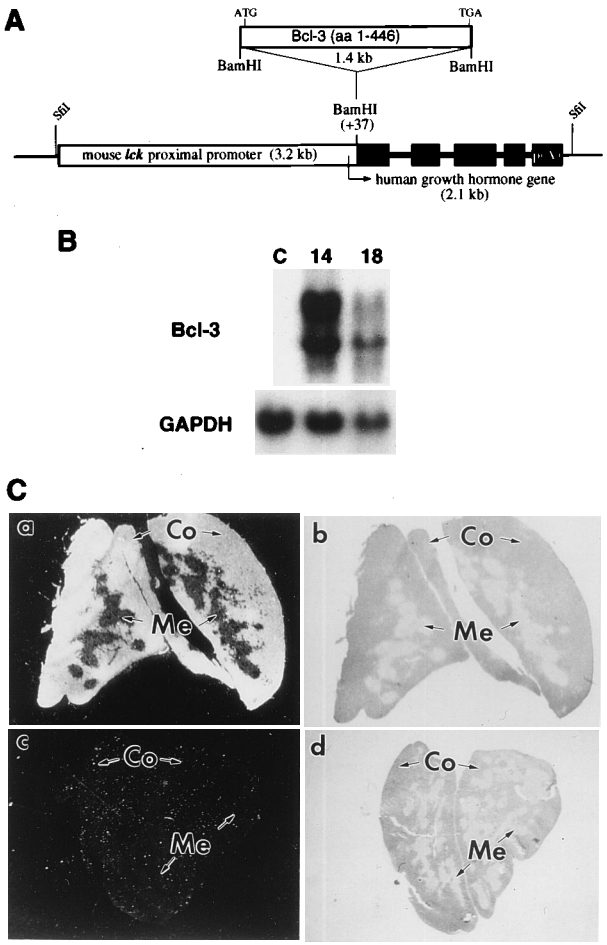

FIG. 1. Expression of *bcl*-3 in the thymuses of transgenic mice. (A) Schematic representation of the *bcl*-3 transgene. aa, amino acids. (B) Northern blot analysis showing the expression of *bcl*-3 in control (C) and two independent transgenic lines (14 and 18). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was used as a loading control. (C) In situ localization of *bcl*-3 in thymuses of transgenic (a and b) and control (c and d) adult mice. Tissue sections from 6-week-old mice were hybridized with a specific antisense ^{35}S-riboprobe and photographed under dark-field illumination (a and c). Sections were stained with hematoxylin and photographed under bright-field illumination (b and d). Co, cortex; Me, medulla. Magnification, ×10.

RESULTS

Generation of transgenic mice constitutively expressing Bcl-3 in the thymus. To study the interactions of Bcl-3 with different members of the Rel/NF-κB family of transcription factors in a physiological environment, we have generated several independent transgenic lines of mice constitutively expressing *bcl*-3 under the control of the mouse *lck* proximal promoter (Fig. 1A). This promoter directs the expression of the transgene predominantly in the thymic cortex (3).

The expression of the *bcl*-3 transgene was assessed by Northern blot analysis (Fig. 1B), and its highest level of expression was detected in lines 14 and 18. No mRNA corresponding to endogenous *bcl*-3 was detected in the thymuses of control mice. In situ hybridization studies were performed in order to characterize the expression of *bcl*-3 in the thymuses of the transgenic animals (Fig. 1C). Transgene mRNA was detected as a strong signal present in the thymic cortex.

Constitutive expression of Bcl-3 in the thymus does not result in impaired T-cell development. Since *bcl*-3 expression is

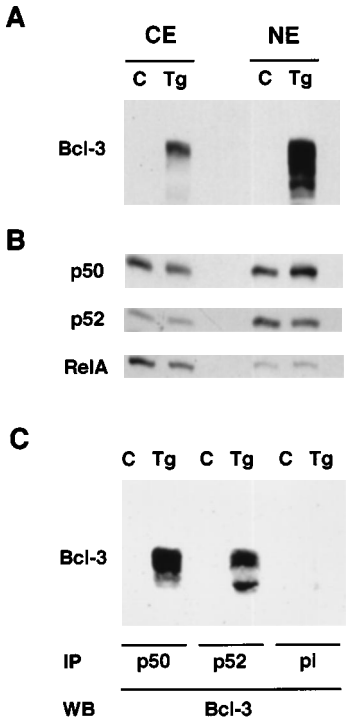

FIG. 2. Bcl-3 is predominantly located in the nucleus. (A) Western blot analysis of cytoplasmic extracts (CE) and nuclear extracts (NE) from thymocytes of control (C) and Bcl-3-expressing transgenic (Tg) animals with anti-Bcl-3 antiserum. (B) Expression of Bcl-3 does not affect the levels of other NF-κB proteins in thymocytes. Western blotting was performed as described for panel A with antiserum specific to p50, p52, and RelA. (C) Bcl-3 associates with p50 and p52 in thymocytes. Nuclear extracts from control and Bcl-3-overexpressing thymocytes were immunoprecipitated with monoclonal antibodies against p50 and p52 and immunoblotted with a polyclonal antibody against Bcl-3. pi, preimmune serum; IP, immunoprecipitation; WB, Western blot.

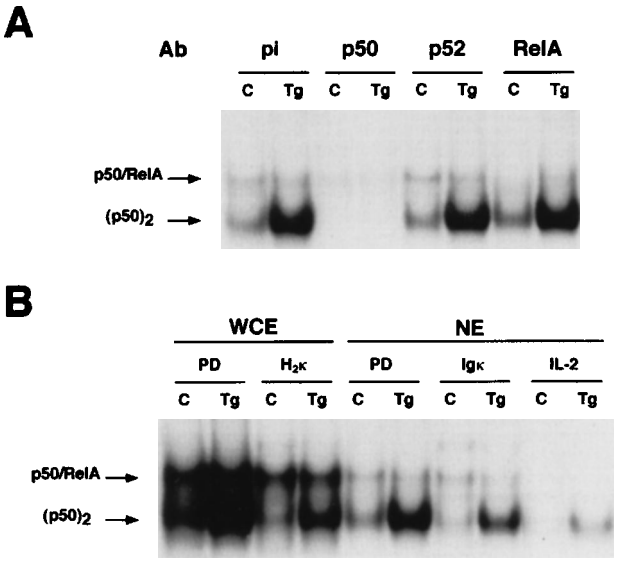

FIG. 3. Effect of Bcl-3 constitutive expression on κB binding activity in thymocytes. (A) Increased DNA binding of p50 in transgenic thymocytes. Nuclear extracts from thymocytes of control (C) and Bcl-3-expressing transgenic (Tg) mice were incubated with the palindromic κB site and analyzed by EMSA. The addition of antisera against p50, p52, and RelA is indicated. Ab, antibody; pi, preimmune serum. (B) κB binding activity of whole-cell extracts (WCE) and nuclear extracts (NE) from thymocytes of control (C) and transgenic (Tg) mice with different κB sites: PD (palindromic site), MHC class II H_2κB (H_2κ), Igκ, and IL-2. The κB binding complexes are indicated at the left of each panel.

induced upon cellular stimulation (45), we wanted to determine whether constitutive expression of Bcl-3 could affect T-cell development in these animals. Thymocytes from 3- to 4-week-old transgenic and control mice were analyzed by flow cytometry with the following markers: CD3, CD4, CD8, CD25, CD44, CD45, αβ T-cell receptor, and ICAM-1. These studies revealed no significant changes in the T-cell subpopulations defined by these markers between transgenic and control mice (data not shown).

The thymuses of Bcl-3-expressing transgenic animals were normal in size and showed no gross morphological alterations (Fig. 1C and data not shown). After a year, Bcl-3-expressing transgenic animals did not present an increased susceptibility to infections or tumor development compared with control littermates. These observations indicate that constitutive expression of Bcl-3 in the thymus neither affects T-cell maturation nor leads to the rapid development of lymphoid tumors.

Bcl-3 is predominantly located in the nucleus and is associated with p50 and p52. The expression levels and subcellular distribution of Bcl-3 in thymic cells were analyzed by Western blotting. A strong band of approximately 52 kDa, corresponding to Bcl-3, was detected in nuclear extracts from transgenic animals (Fig. 2A). Although present in both the nuclear and cytoplasmic extracts, Bcl-3 was found predominantly in the nuclear compartment. The Bcl-3 protein was detected as a broad band of multiple species, probably because of posttranslational modifications, including phosphorylation (see below).

We next studied whether constitutive expression of Bcl-3 altered the protein levels or localization of Rel/NF-κB family members by using antisera specific to p50, p52, and RelA (Fig. 2B). Our data show that neither the expression nor the cellular localization of p52 and Rel-A was altered in the transgenic thymocytes. In some cases, a small increase in the amount of nuclear p50 was observed in the cells constitutively expressing Bcl-3.

Previous in vitro and cotransfection experiments have indicated that Bcl-3 interacts with p50 and p52 (20, 21, 23, 43, 64). To investigate if these interactions occur in thymocytes constitutively expressing Bcl-3, nuclear extracts were immunoprecipitated with p50 or p52 antisera under nondenaturing conditions and the immunocomplexes were analyzed by Western blotting with antibodies specific to Bcl-3 (Fig. 2C). The results clearly indicate that Bcl-3 is associated with endogenous p50 and p52 in nuclear extracts from the transgenic animals.

Increased DNA binding of p50 in Bcl-3-expressing transgenic thymocytes. We investigated whether the DNA binding of p50 and p52 was modified upon interaction with Bcl-3. Two complexes with different mobilities were detected in nuclear extracts from both control and Bcl-3-expressing transgenic thymocytes by EMSAs (Fig. 3A). Anti-p50 antiserum eliminated both binding activities, while the anti-RelA antiserum displaced only the more slowly migrating band. These results indicate that the more slowly migrating complex corresponds to p50-RelA heterodimers and that the faster-migrating one corresponds to p50 homodimers. The addition of anti-p52 antiserum did not affect the mobilities of these complexes. Interestingly, the κB binding activity corresponding to the p50 homodimers was increased more than 10-fold in the nuclear extracts from thymocytes constitutively expressing Bcl-3 compared with control cells, as quantitated by PhosphorImager analysis (Fig. 3A and data not shown). We have obtained evidence that Bcl-3 increases the affinity of binding of p50 homodimers to κB sites (data not shown).

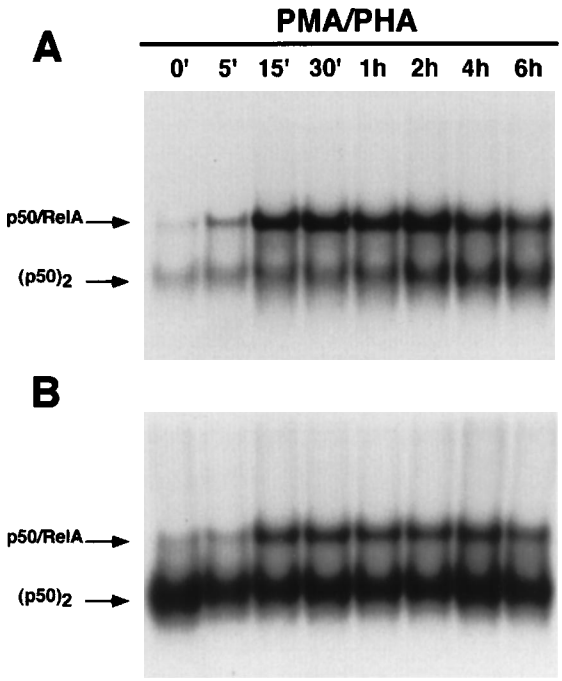

FIG. 4. κB binding activity in thymocytes from control (A) and Bcl-3-expressing transgenic (B) mice upon stimulation with PMA and PHA. Nuclear extracts from thymocytes stimulated for the indicated periods of time were incubated with the palindromic κB site and analyzed by EMSA.

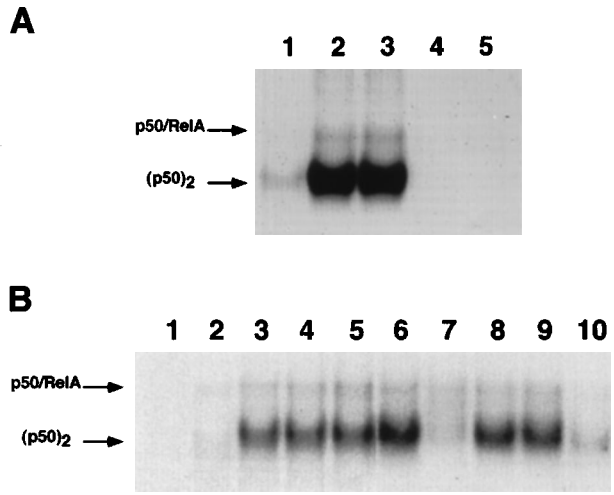

FIG. 5. Bcl-3 does not increase the DNA binding of p52 homodimers. (A) EMSA profile showing the κB binding complexes in nuclear extracts of thymocytes from control (lane 1), Bcl-3 (lane 2), Bcl-3/p50$^{+/-}$ (lane 3), p50$^{-/-}$ (lane 4), and Bcl-3/p50$^{-/-}$ (lane 5) mice. Three micrograms of nuclear extract was used per reaction. (B) Bcl-3 increases p50 homodimer binding activity in vitro. Shown are EMSA results of the κB binding activities in nuclear extracts of Bcl-3/p50$^{-/-}$ (lane 1), control (lane 2), and control plus Bcl-3/p50$^{-/-}$ (lanes 3 to 5) thymocytes. Lanes 1 and 2 each contain 1 μg of nuclear extract. Lanes 3, 4, and 5 each contain 1 μg of control nuclear extract together with 1, 1.5, and 2 μg of Bcl-3/p50$^{-/-}$ nuclear extract, respectively. Lanes 6 and 7 were like lane 3, except that preimmune or anti-Bcl-3 serum, respectively, was added at the time the extracts were mixed. Lanes 8 and 9 were also like lane 3, except that preimmune or anti-Bcl-3 serum, respectively, was added after the incubation of the extracts. Lane 10 contained control extract plus p50$^{-/-}$ extract (1 μg each).

We further verified the DNA binding activity of transgenic thymocytes by using the κB sites present in the promoters of the MHC class I gene H_2κB, IL-2, and Igκ (Fig. 3B). A significant increase in DNA binding of p50 was observed with each of the κB sites. It is noteworthy that the binding of the p50-RelA heterodimers is not modified in the presence of Bcl-3. No changes in p50 phosphorylation were detected upon its interaction with Bcl-3 (12).

Stimulation of Bcl-3-expressing transgenic thymocytes does not affect the increased binding of p50 homodimers. It has been well established that NF-κB activity rapidly increases following stimulation of T cells; therefore, we wanted to determine whether changes in NF-κB activity occurred in Bcl-3-expressing transgenic thymocytes upon stimulation. Thymic-cell suspensions were treated for different periods of time with PHA and PMA, and the DNA binding activity present in each nuclear extract was examined by EMSA (Fig. 4). Control and Bcl-3-expressing transgenic thymocytes showed strong increases in DNA binding activity corresponding to the p50-RelA heterodimers that reached maximum levels after 15 min of stimulation, remaining at high levels for at least 6 h. Although the DNA binding of the p50 homodimers in control thymocytes was also increased following stimulation (35), it never reached the levels present in the cells constitutively expressing Bcl-3 (Fig. 4A). Remarkably, no significant changes were seen in the strong binding corresponding to the p50 homodimers in the transgenic thymocytes (Fig. 4B).

DNA binding of p52 homodimers is not increased in Bcl-3-expressing transgenic thymocytes. Our results showed that although Bcl-3 is associated with both p50 and p52 (Fig. 2C), only an increase in the DNA binding of p50 homodimers was observed. One possible explanation may be that p50 homodimers bind more efficiently to DNA and effectively compete with the binding of p52 homodimers. Therefore, we determined whether Bcl-3 could modify the DNA binding properties of p52 in the absence of p50. To address this question we generated Bcl-3/p50$^{-/-}$ transgenic mice (see Materials and Methods).

Comparison of the EMSA results shown in Fig. 5A obtained with the nuclear extracts from p50$^{-/-}$ (lane 4) and Bcl-3/p50$^{-/-}$ (lane 5) thymocytes revealed no clear differences. These data indicate that, in contrast to what is observed for p50, constitutive expression of Bcl-3 in thymocytes does not result in a significant increase in the κB binding capacity of p52.

Bcl-3 increases the DNA binding of p50 in vitro. Previous reports indicate that Bcl-3 removes p50 homodimers from DNA in vitro (20, 21, 26, 27, 34, 42, 43, 64). We did similar assays with nuclear extracts from thymocytes from Bcl-3/p50$^{-/-}$ mice, as a source of Bcl-3 protein, mixed with nuclear extracts from control cells and then performed EMSAs. As shown in Fig. 5B, an increase in the binding of the p50 homodimers was readily detected when the nuclear extracts containing Bcl-3 were added to the reaction mixture at a 1:1 ratio (compare lanes 2 and 3). Higher concentrations of extracts containing Bcl-3 did not further augment the binding of the p50 homodimers. Moreover, the effect on DNA binding of p50 exerted by Bcl-3 was completely abolished upon addition of anti-Bcl-3 antiserum simultaneous with the mixing of the extracts (Fig. 5B; compare lanes 6 and 7). However, if the same antiserum was added after the mixing of Bcl-3 and p50, no effect could be seen (lanes 8 and 9). No changes in the binding of p50 homodimers in control extracts were detected by addition of nuclear extracts from p50$^{-/-}$ mice (lane 10), confirming that the effect is Bcl-3 dependent. Similar results were obtained when samples were incubated at 37°C (data not shown). These observations correlate with the in vivo data demonstrat-

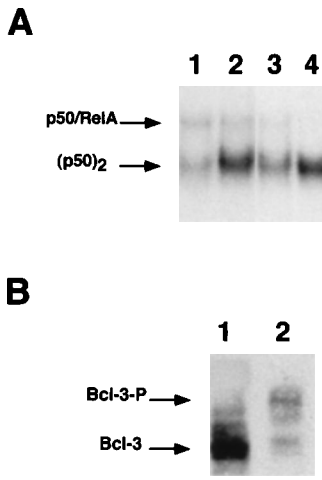

FIG. 6. (A) Increased DNA binding of p50 is dependent on Bcl-3 phosphorylation. Shown are EMSA profiles of the κB binding activities with the following thymocyte extracts: control (lane 1); control plus Bcl-3/p50$^{-/-}$ (lane 2); control plus Bcl-3/p50$^{-/-}$ treated with alkaline phosphatase, with the addition of phosphatase inhibitors in the mix (lane 3); Bcl-3-expressing transgenic (lane 4); and Bcl-3-expressing transgenic treated with phosphatase (lane 5). The amounts of nuclear extract used in these reactions were 1 μg of control extract (lanes 1 to 3), 1 μg of Bcl-3/p50$^{-/-}$ extract (lanes 2 and 3), and 0.3 μg of Bcl-3-expressing transgenic extract (lanes 4 and 5). (B) Bcl-3 is constitutively phosphorylated in thymocytes. A nuclear extract from Bcl-3-expressing transgenic thymocytes was either treated with alkaline phosphatase (lane 1) or not treated (lane 2), separated by SDS-PAGE, and transferred onto a nitrocellulose membrane. The membrane was probed with anti-Bcl-3 antiserum.

ing that Bcl-3 positively affects the DNA binding of p50 homodimers.

Increased DNA binding of p50 is dependent on Bcl-3 phosphorylation. Earlier studies have shown that Bcl-3 produced in either baculovirus or human 293 cells is phosphorylated and that its activity depends on the degree of phosphorylation (23, 43). Therefore, we investigated whether the effect of Bcl-3 on p50 is phosphorylation dependent. Nuclear extracts from Bcl-3/p50$^{-/-}$ mice were treated with phosphatase and then mixed with nuclear extracts from control thymocytes prior to the performance of EMSAs. The increase in p50 binding observed after addition of Bcl-3/p50$^{-/-}$ nuclear extracts was significantly reduced if the latter were first treated with phosphatase (Fig. 6A; compare lanes 2 and 3). Also, when nuclear extracts from the Bcl-3-expressing transgenic mice were treated with alkaline phosphatase, a reduction in binding corresponding to the p50 homodimers was detected (compare lanes 4 and 5). As a control, the nuclear extracts from transgenic animals treated with alkaline phosphatase were subjected to Western blot analysis. Figure 6B shows that most of the more slowly migrating bands of Bcl-3 (lane 2) could be converted to faster-migrating bands upon phosphatase treatment (lane 1), indicating that Bcl-3 is a phosphoprotein in thymocytes. These results show that only phosphorylated Bcl-3 exerts a full effect on the DNA binding properties of p50 homodimers.

DISCUSSION

There has been controversy about the function of the Bcl-3 protein; several groups have shown that Bcl-3 interacts, through its ankyrin repeats, with NF-κB1 (p50) and NF-κB2 (p52). The formation of ternary complexes including Bcl-3 and either p50 or p52 with DNA has been demonstrated in the cases of the PD, Igκ, and H2-κB binding sites (9, 22). While some researchers propose that Bcl-3 acts by removing p50 homodimers from the κB sites (9, 20, 21, 34, 42, 43, 64), thus allowing the p50-RelA complexes to bind DNA (9, 20, 21, 64), others suggest that the Bcl-3–p50 complex activates transcription through specific κB sites (23).

The focus of this study was to investigate in vivo the potential effects of the constitutive expression of Bcl-3 on T-cell development and the significance of the interactions of Bcl-3 with p50 and p52; for these purposes we have generated transgenic mice constitutively expressing Bcl-3 in the thymus.

Here we show that constitutive expression of Bcl-3 in thymocytes does not affect the localization or the amounts of the Rel/NF-κB family members. Only in some cases did we observe a slight increase in the amount of nuclear p50. Also, no gross alterations in T-cell development or increases in tumor incidence were observed in the Bcl-3-expressing transgenic mice. These results suggest that additional genetic changes which must cooperate with Bcl-3 to transform T cells are required (1, 48). It is likely that Bcl-3 must interact with a B-cell-specific protein(s) before its transforming characteristics can be exhibited. The coimmunoprecipitation experiments have demonstrated that the physiological partners of Bcl-3 are endogenous NF-κB1 (p50) and NF-κB2 (p52) in thymocytes.

The interaction between Bcl-3 and p50 results in a strong increase in the nuclear DNA binding of p50 homodimers to several κB sites. In contrast, the interaction between Bcl-3 and p52 did not increase the DNA binding of p52 homodimers. Identical results were obtained with Bcl-3/p50$^{-/-}$ thymocytes, eliminating the possibility that DNA binding of p52 was displaced by DNA binding of p50. Thus, although Bcl-3 associates with both p50 and p52 in thymocytes, it increases only p50 DNA binding.

When primary thymocytes were stimulated with phorbol esters and lectins, which strongly induce NF-κB activity, we detected by EMSA an increased binding activity corresponding to p50-RelA heterodimers that correlates with the translocation of these complexes to the nucleus in both control and Bcl-3-expressing transgenic thymocytes. In addition, p50 homodimer binding is also increased in control cells but never reaches the high levels observed in the cells constitutively expressing Bcl-3. It is important to note that the strong p50 binding observed in Bcl-3-expressing transgenic thymocytes remains unchanged upon stimulation. These results are of particular significance since it has been proposed that Bcl-3 promotes transcription by removing p50 homodimers from κB sites upon T-cell stimulation (20, 32).

We also determined whether the changes in the NF-κB binding activity observed in Bcl-3-expressing transgenic thymocytes could have a direct effect on the expression of a set of genes (those for κ^b, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, tumor necrosis factor alpha, vascular cell adhesion molecule, intracellular adhesion molecule 1, E-selectin, granulocyte-macrophage colony-stimulating factor, transforming growth factor β1, monocyte inflammatory protein 1α, and alpha and gamma interferons) potentially regulated by NF-κB. No significant changes in the mRNA levels of these genes were observed in unstimulated transgenic thymocytes or in transgenic thymocytes stimulated with PMA and PHA compared with the mRNA levels in control cells (data not shown). We further investigated this by stimulating thymocytes with PMA and PHA in the presence of calpain inhibitor I, which is known to block the degradation of IκBα and therefore blocks the induction of NF-κB activity. Although the p50 binding activity present in Bcl-3-overexpressing thymocytes was unaffected by calpain inhibitor I in cells stimulated with PMA and PHA, it was not sufficient to induce the expression of the selected genes. Thus, at least in thymocytes, Bcl-3-induced changes in

p50 activity do not alter the normal expression of several NF-κB target genes.

The assays involving Bcl-3/p50$^{-/-}$ and control extracts demonstrated that the effect of Bcl-3 on p50 seen in vivo can be reproduced in vitro. The antibody to the amino-terminal portion of Bcl-3 was able to block the action of Bcl-3 on p50. Furthermore, we showed that Bcl-3 is constitutively phosphorylated in thymocytes and that dephosphorylation largely diminishes its ability to increase the DNA binding of the p50 homodimers. Sequence analysis of human Bcl-3 reveals four putative phosphoacceptor sites for protein kinase C (29). There are also four consensus sites for casein kinase I and II phosphorylation. All of them are highly conserved in human and mouse Bcl-3 (8, 45).

In contrast to our findings, previous observations have shown that Bcl-3 has an inhibitory effect on DNA binding of p50 (20, 21, 26, 27, 42, 43, 64). There are important differences between our system and those used previously that could explain this discrepancy. First, some of the previous work was done in vitro with Bcl-3 and p50 expressed in bacteria or insect cells. Second, previous studies used transient expression of Bcl-3 in cell lines. We have used long-term constitutive expression, which resembles the situation in tumor cells expressing Bcl-3. Third, previous work determined the effect of Bcl-3 on cotransfected p50. Furthermore, the p50 constructs used in those studies generated a p50 protein that was longer than the endogenous one. This is important since it has been demonstrated that the longer p50 protein fails to cooperate with Bcl-3 (23). We have assayed the action of Bcl-3 on endogenous p50, and the levels of Bcl-3 expression in transient-transfection experiments are probably significantly higher than those obtained in transgenic thymocytes. Fourth, cell-type-specific modifications of Bcl-3 or additional proteins could also be responsible for the diverse effects of Bcl-3 on NF-κB1 and NF-κB2 homodimers.

Our results demonstrate that when Bcl-3 is constitutively expressed in thymocytes, it plays a role as a regulatory subunit, with a cooperative effect on p50 binding to κB sites in a phosphorylation-dependent manner.

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