Regulation of NF- κ B1 proteins by the candidate oncoprotein BCL-3: generation of NF- κ B homodimers from the cytoplasmic pool of p50–p105 and nuclear translocation

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The candidate oncoprotein BCL-3 has been shown to function as a transcriptional co-activator for homodimers of NF- κ B p50 and p50B. We expressed BCL-3 ectopically in pro-B cell lines and found that these cells exhibited a dramatic increase in nuclear κ B motif binding activity of p50 homodimers containing BCL-3 in the complex. Co-transfection and *in vitro* reconstitution experiments revealed that the complex of p50 with its precursor p105 (p50–p105), which was shown to accumulate in the cytoplasm of the pro-B cell lines, is required for induction of DNA binding of p50 homodimers by BCL-3. However, we could see no *in vivo* or *in vitro* evidence of a BCL-3-induced increase in proteolytic processing. Instead, BCL-3-mediated reorganization of NF- κ B1 subunits was demonstrated *in vitro*. Immunofluorescence staining clearly demonstrated that the transition from cytoplasmic p50–p105 to nuclear p50 homodimers was induced by BCL-3 expression. Thus BCL-3 has versatile functions: cytoplasmic activation of p50 homodimers, their nuclear translocation and, as previously shown, modulation of the transcriptional machinery in the nucleus.
Keywords: BCL-3/NF- κ B/nuclear translocation/transcription factor

Introduction

bcl-3 was originally identified at the breakpoint in the recurring chromosome translocation t(14;19), which is found in some cases of B cell chronic lymphocytic leukemia (McKeithan *et al.*, 1990; Ohno *et al.*, 1990). Involvement of *bcl-3* in leukemogenesis is suggested since its expression is constitutively activated in leukemic cells with the t(14;19) translocation (Ohno *et al.*, 1990). However, no experimental evidence has been presented showing transforming capacity of the gene. *bcl-3* mRNA is expressed in spleen and other lymphoid organs and can be highly induced by mitogen treatment (Ohno *et al.*, 1990; Bhatia *et al.*, 1991; Nolan *et al.*, 1993). These observations suggest that the product of the gene, BCL-3, has a functional role in the lymphoid cell lineage. Structural analyses of BCL-3 have revealed that it contains seven ankyrin-like repeats with close homology to that of NF- κ B1

p105, I κ B- α and I κ B- β , all of which function as negative regulators of NF- κ B (Blank *et al.*, 1992; Nolan and Baltimore, 1992; Verma *et al.*, 1995). Thus it is suggested that BCL-3 may have a role in regulation of the NF- κ B system.

Many cellular genes, including those involved in the immune response, and some viral genes are regulated by the transcription factor NF- κ B through interaction with the promoter sequences called the κ B motif. NF- κ B consists of a dimer of subunits which constitute a family of related proteins, including c-Rel, p65 (RelA), RelB, NF- κ B1 and NF- κ B2, each of which contains a conserved domain for DNA binding and dimerization (Rel homology domain) (Blank *et al.*, 1992; Nolan and Baltimore, 1992; Baeuerle and Henkel, 1994; Verma *et al.*, 1995). The major form of NF- κ B is a heterodimer of NF- κ B1 p50 and RelA. In some cells, the p50 homodimer, (p50)₂, which is also referred to as KBF1 (Kieran *et al.*, 1990), is constitutively present in the nucleus. One of the most important features of the NF- κ B system is that the function of NF- κ B is strictly regulated by its subcellular localization, i.e. various extracellular stimuli induce translocation of NF- κ B into the nucleus, otherwise it is retained in the cytoplasm as an inert complex by interaction with the I κ B family of proteins. I κ B- α and I κ B- β specifically interact with NF- κ B containing RelA or c-Rel (Zabel and Baeuerle, 1990; Haskill *et al.*, 1991; Thompson *et al.*, 1995). In contrast, BCL-3 specifically interacts with homodimers of NF- κ B1 p50 and NF- κ B2 p50B (Franzoso *et al.*, 1992; Bours *et al.*, 1993; Fujita *et al.*, 1993; Nolan *et al.*, 1993). Another feature which distinguishes BCL-3 and I κ B is that BCL-3 localizes predominantly to the nucleus (Franzoso *et al.*, 1993; Nolan *et al.*, 1993; Zhang *et al.*, 1994), whereas I κ B resides exclusively in the cytoplasm (Zabel and Baeuerle, 1990; Zhang *et al.*, 1994; Thompson *et al.*, 1995). Furthermore, it has been demonstrated that BCL-3 is directly involved in activation of κ B motif-dependent transcription through its association with p50 or p50B homodimers, which have otherwise weak transcriptional activity (Bours *et al.*, 1993; Fujita *et al.*, 1993).

The precise function of BCL-3 in the NF- κ B signaling system is still not well elucidated and there remain certain controversies. One potential source of confusion has been the use of artificially truncated p50 or p50B constructs in these studies. p50 and p50B are first synthesized as precursors, p105 and p100 respectively, both of which have an ankyrin-like repeat in their C-terminal region. The glycine-rich region of p105 is specifically recognized by cellular processing machinery and cleavage takes place by an unknown endopeptidase activity (Lin and Ghosh, 1996). This is followed by degradation of the C-terminal region, presumably by the action of a proteasome (Palombella *et al.*, 1994). Furthermore, several studies have provided evidence indicating that the

C-terminal region of the precursor has a functional role similar to I κ B, namely the C-terminal region retains not only p105 itself but also the p50 subunit or other Rel proteins in the cytoplasm through intra- or inter-molecular interaction (Blank *et al.*, 1991; Henkel *et al.*, 1992; Rice *et al.*, 1992; Hatada *et al.*, 1993; Mercurio *et al.*, 1993; Naumann *et al.*, 1993a,b). However, it is still ambiguous whether p105-containing complexes can be directly activated by proteolytic cleavage following cellular stimulation. It is also possible that it is a transient form that facilitates formation of NF- κ B-I κ B or other related complexes.

In this study we demonstrate that BCL-3 expression leads to nuclear translocation of the NFKB1 p50 subunit, which would otherwise be retained in the cytoplasm through interaction with its precursor p105. This process results in strong DNA binding activity of (p50)₂ complexed with BCL-3. This is consistent with a recent report showing that ectopic expression of *bcl-3* in thymocytes resulted in induction of DNA binding activity composed of (p50)₂ (Caamano *et al.*, 1996). Furthermore, we provide evidence indicating that BCL-3 releases (p50)₂ from the inactive p50-p105 complex without proteolytic processing, presumably by affecting the equilibrium between the complexes. These findings also suggest that p105-containing complexes in the cytoplasm function as a reservoir to provide active transcription factors through a non-proteolytic process. Thus BCL-3 facilitates the formation of free (p50)₂ and might further function to chaperone p50 into the nucleus.

Results

Constitutive expression of BCL-3 induced κ B motif binding activity and nuclear translocation of p50 in mouse pro-B cell lines

The high expression of *bcl-3* mRNA in spleen and other lymphoid organs (Ohno *et al.*, 1990; Bhatia *et al.*, 1991; Nolan *et al.*, 1993) and the possible involvement of *bcl-3* in certain B cell leukemias (Ohno *et al.*, 1990) prompted us to investigate the function of the gene product (BCL-3) in hematopoietic cells, especially of the B cell lineage. To investigate the effect of BCL-3 expression on regulation of the NF- κ B system, we ectopically expressed BCL-3 in LyH7 and BAF-B03 cells, both of which are IL-3-dependent mouse pro-B cell lines (Palacios *et al.*, 1987; Shibuya *et al.*, 1992). BAF-B03 cells were transfected with a BCL-3 expression plasmid and a hygromycin resistance gene by electroporation, and drug-resistant cell clones were selected and BCL-3 expression ascertained by immunoblot assay (Figure 1A). LyH7 cells were infected with a recombinant retrovirus which encodes BCL-3 in addition to the neomycin resistance gene. Drug-resistant cell populations were obtained and BCL-3 expression was confirmed (Figure 1A).

We first examined κ B element binding activity in BCL-3-expressing cells by electrophoretic mobility shift assay (EMSA). We detected very low κ B motif binding activity in whole cell extracts prepared from the control BAF-B03 and LyH7 cells. BCL-3 expression led to a significant increase in κ B element binding activity in these cell lines (Figure 1B). To identify the components of the κ B binding complex, the lysates were incubated with specific antisera prior to EMSA (Figure 1C). The anti-p50 antibody strongly

reacted with the induced complex. In contrast, neither anti-RelA antiserum nor antibodies specific for other Rel family proteins (c-Rel, RelB and NFKB2; data not shown) affected formation of the DNA-protein complex. The results indicate that the complex is composed of a NFKB1 p50 subunit, most likely (p50)₂. The upper DNA-protein complex was supershifted by the addition of anti-BCL-3 antiserum, indicating that a portion of the DNA-protein complex contained BCL-3 (Figure 1C, lanes 8–10). This is consistent with previous findings that BCL-3 preferentially interacts with (p50)₂ rather than a p50-RelA heterodimer (Franzoso *et al.*, 1992, 1993; Hatada *et al.*, 1992; Kerr *et al.*, 1992; Wulczyn *et al.*, 1992; Fujita *et al.*, 1993; Naumann *et al.*, 1993b; Nolan *et al.*, 1993; Zhang *et al.*, 1994) and that BCL-3 associates with p50 bound to DNA (Fujita *et al.*, 1993). The relative amount of the DNA-bound complex with or without BCL-3 varied between experiments and was dependent on the relative level of BCL-3 protein and κ B motif sequence (see Figure 2A; our unpublished observation). RNA blot analysis demonstrated that expression of the *NFKB1* gene was increased by BCL-3 expression, particularly in BAF-B03 clones (Figure 1D). This is likely because of activation by (p50)₂-BCL-3 of an endogenous *NFKB1* promoter which contains functional κ B motifs (Ten *et al.*, 1992). We do not strictly rule out the possibility that up-regulation of the *NFKB1* gene makes some contribution to DNA binding. However, immunoprecipitation with anti-p50 revealed that comparable amounts of p50 or p105 were accumulated in control and BCL-3-expressing BAF-B03 cells (Figure 1E). Therefore, the dramatic increase in κ B element binding (Figure 1B) could not be explained solely by the increase in p50 protein.

To investigate the subcellular localization of each subunit, control BAF-B03 cells and a BCL-3-expressing clone were metabolically labeled with ³⁵S-labeled amino acids followed by preparation of cytoplasmic and nuclear extracts. The amount of p50 and p105 was determined by immunoprecipitation with anti-p50 antibody (Figure 1F). The results show that p105 was present exclusively in the cytoplasm, irrespective of BCL-3 expression, and that a portion of the p50 was transported to the nucleus in BCL-3-expressing cells. The nuclear p50 is likely present as homodimers because κ B binding activity was specifically present in the nuclear fraction (Figure 1G). These results indicate that constitutive expression of BCL-3 leads to nuclear translocation of the p50 subunit in pro-B cell lines.

Expression of p105 precursor is required for BCL-3-mediated induction of p50 DNA binding activity

The above observation was unexpected because previous reports suggested that BCL-3 expression did not enhance DNA binding of p50 expressed from the truncated cDNA (Franzoso *et al.*, 1992, 1993; Hatada *et al.*, 1992; Wulczyn *et al.*, 1992; Bours *et al.*, 1993; Fujita *et al.*, 1993; Naumann *et al.*, 1993b; Nolan *et al.*, 1993; Zhang *et al.*, 1994). Detection of a significant amount of p105 in the cytoplasm of BAF-B03 cells prompted us to explore the role of p105 in BCL-3-mediated induction of κ B binding activity. BCL-3 and the *NFKB1* gene products were expressed in 293T (human embryonic kidney) cells by the transient transfection protocol and whole cell extracts were prepared for analysis. The cell line normally exhibits

Fig. 1. Constitutive expression of BCL-3 induced κ B motif binding activity and nuclear translocation of p50 in mouse pro-B cell lines. LyH7 cells were incubated with recombinant retroviruses and the cell populations infected with control (LyH7-) or BCL-3-encoding virus (LyH7+) were selected (Materials and methods). BCL-3-expressing BAF-B03 cell clones (B1-B4) and control clones (C1 and C2) were obtained as described in Materials and methods. (A) BCL-3 expression in various cell clones and cell populations. Whole cell lysates (5 μ g protein) of BCL-3 expressing cells (LyH7+, lane 2; B1-B4, lanes 3-6) and control cells (LyH7-, lane 1; C1, lane 7; C2, lane 8) were subjected to immunoblot analysis using anti-BCL-3 antiserum. Brackets show ectopically expressed BCL-3. (B) Induction of κ B motif binding activity by BCL-3 expression. The whole cell lysates (10 μ g protein) used in (A) were subjected to EMSA using a 32 P-labeled IFN- β κ B probe. Lanes correspond to those in (A). Specific protein-probe complexes are indicated by brackets. (C) Identification of the κ B motif binding complexes. Whole cell lysate (10 μ g protein) of LyH7+ cells was incubated in the absence (lane 1) or presence of 0.1 (lanes 2, 5, 8 and 11), 0.3 (lanes 3, 6, 9 and 12) or 0.5 μ l (lanes 4, 7, 10 and 13) of the indicated antiserum (normal rabbit serum, lanes 2-4; anti-p50, lanes 5-7; anti-BCL-3, lanes 8-10; anti-RelA, lanes 11-13) on ice for 60 min, followed by EMSA. Supershifts are shown by brackets. (D) Analysis of *NFKB1* mRNA expression. Ten micrograms of total RNA prepared from the indicated cells (BCL-3-expressing BAF-B03 clones B1-B4, lanes 1-4; control clones C1 and C2, lanes 5 and 6; LyH7-, lanes 7 and 9; LyH7+, lanes 8 and 10) were subjected to Northern blot analysis using a mouse *NFKB1* cDNA as probe (upper panel). Lower panel: control hybridization of the same filter using an actin probe. Specific activities of the probes were 1×10^9 c.p.m./ μ g. Lanes 9 and 10 show short exposures of the *NFKB1* signals of lanes 7 and 8 respectively. Bars indicate the positions of 18S and 28S rRNA, determined by methylene blue staining of the filter. The radioactivity was quantitated by Image Analyzer (Fuji Inc.). Normalized relative *NFKB1* mRNA levels are: 2.3, 1.3, 2.3, 1.4, 0.6 and 1.0 for lanes 1-6 and 1.0 and 1.2 for lanes 7 and 8 respectively. (E) Analysis of the *NFKB1* gene products. BCL-3-expressing BAF-B03 clones B1-B4 (lanes 1-4) and control clones C1 and C2 (lanes 5 and 6) (2×10^6 cells) were metabolically labeled with 35 S-labeled amino acids (0.5 mCi) for 12 h. The whole cell lysates were subjected to immunoprecipitation using anti-p50 antiserum (Materials and methods) followed by re-precipitation with the same antiserum in order to reduce the background. The samples were analyzed by 10% SDS-PAGE and fluorography. Molecular weight markers are indicated on the left of the panel and the positions of p105 and p50 are shown by arrows. (F) Nuclear translocation of p50 induced by BCL-3 expression. B3 (lanes 1 and 3) and C2 (lanes 2 and 4) cells (5×10^6 cells) were metabolically labeled with 35 S-labeled amino acids (1 mCi) for 8 h and cytoplasmic and nuclear extracts were prepared as described in Materials and methods. The cells incorporated comparable radioactivity and extracts corresponding to equivalent numbers of cells were subjected to immunoprecipitation as in (E). Cytoplasmic extract, lanes 1 and 2; nuclear extract, lanes 3 and 4. Molecular weight markers are indicated on the left of the panel and the positions of p105 and p50 are shown by arrows. (G) Recovery of the κ B binding activity from the nucleus. Cytoplasmic and nuclear extracts were prepared from B3 (lanes 1 and 3) and C2 (lanes 2 and 4) cells and dialyzed against NP-40 lysis buffer. Equivalent amounts of protein fractions (3 μ g) were subjected to EMSA. Cytoplasmic extract, lanes 1 and 2; nuclear extract, lanes 3 and 4. Specific binding to the κ B motif is indicated by brackets.

a very low basal level of κ B binding activity, and BCL-3 expression alone did not induce DNA binding activity, in contrast to LyH7 or BAF-B03 cells (Figure 2A, lanes 1-4). Transfection of an expression vector encoding full-length p105 resulted in moderate κ B motif binding activity (Figure 2A, lane 5). However, co-transfection of expression vectors encoding BCL-3 and p105 resulted in a

dramatic increase in DNA binding activity (Figure 2A, lanes 6-8). In contrast, when an artificially truncated p50 was expressed by transfection (see Materials and methods) the generated κ B motif binding activity was not significantly augmented by BCL-3 (Figure 2A, lanes 9-12). Further increase in the amount of BCL-3 relative to p50 gave rise to a supershift corresponding to the ternary

Fig. 2. Expression of the p105 precursor is required for BCL-3-mediated induction of p50 DNA binding activity. (A) Induction of DNA binding activity by co-transfection of p105 and BCL-3 expression vectors in 293T cells. 293T cells (2×10^6) were transfected with a total of 6.0 μ g expression plasmids: pEF-BOS (6.0 μ g), lane 1; pEF-bcl-3 (0.1 μ g), lanes 2, 6 and 10; pEF-bcl-3 (1.0 μ g), lanes 3, 7 and 11; pEF-bcl-3 (5.0 μ g), lanes 4, 8 and 12; pEF-p105 (1.0 μ g), lanes 5–8; pEF-p50 (0.1 μ g), lanes 9–12. Whole cell lysates were prepared at 36 h after transfection and a portion (5 μ g protein) subjected to EMSA. κ B-specific binding activity derived from the expression constructs is indicated by brackets and endogenous non-specific DNA–protein complex by an asterisk. (B) Induction of κ B binding activity in cells co-expressing BCL-3 with either HA-tagged p105 or p100. 293T cells were transfected with a total of 4.0 μ g expression plasmids: pEF-BOS (4.0 μ g), lane 1; pEF-bcl-3 (0.2 μ g), lanes 2, 5 and 8; pEF-bcl-3 (2.0 μ g), lanes 3, 6 and 9; pEF-p105N-HA (2.0 μ g), lanes 4–6; pEF-p100N-HA (2.0 μ g), lanes 7–9. Portions (5 μ g protein) of the whole cell lysates were subjected to EMSA with IFN- β κ B as probe. The specific and non-specific probe–protein complexes are indicated by brackets and an asterisk respectively. (C) BCL-3 expression does not stimulate proteolytic processing of p105. The whole cell lysates (5 μ g protein) used in (B) were subjected to immunoblot analysis using anti-HA antibody. Lanes 1–6 correspond to the samples in lanes 1–6 in (B). Molecular weight markers are indicated on the left of the panel and the positions of p105 and p50 are shown by arrows. (D) Nuclear translocation of p50 induced by BCL-3 expression. 293T cells were transfected with pEF-p105N-HA (3 μ g) and pEF-BOS (6 μ g) (lanes 1 and 3) or with pEF-p105N-HA (3 μ g) and pEF-bcl-3 (6 μ g) (lanes 2 and 4). Cells were harvested at 36 h after transfection and then cytoplasmic (lanes 1 and 2) and nuclear (lanes 3 and 4) fractions prepared. The lysates were analyzed by immunoblotting using anti-HA antibody. Molecular weight markers are indicated on the left of the panel and the positions of p105 and p50 are shown by arrows. (E) The κ B DNA binding complex induced by expression of BCL-3 contains p50 but not the p105 precursor. 293T cells were transfected with a total of 22 μ g expression plasmids: pEF-BOS (22 μ g), lane 1; pEF-bcl-3 (6 μ g), lanes 2 and 4–10; pEF-p105C-HA (16 μ g), lanes 3–10. Whole cell lysates prepared from the transfected cells were directly analyzed by EMSA (lanes 1–4) or reacted with antibodies then subjected to EMSA: normal rabbit serum, lane 5; anti-p50 antiserum, lane 6; anti-RelA antiserum, lane 7; anti-BCL-3 antiserum, lane 8; anti-HA antibody, lane 9; anti-CTR antiserum, lane 10. The lysate derived from co-transfection of pEF-p105N-HA and pEF-bcl-3 was incubated in the absence (lane 11) or presence (lane 12) of anti-HA antibody (1.7 μ g IgG) and then subjected to EMSA. The open circle shows the DNA–protein complex supershifted by anti-HA antibody.

complex; however, no significant increase in DNA binding was observed (Figure 2A, lane 12). Co-expression of another ankyrin protein, IkB- α , and p105 did not induce κ B binding activity (data not shown).

To trace the ectopically expressed *NFKB1* gene products unambiguously, an expression vector encoding p105 with a hemagglutinin epitope tag at the N-terminus (p105N-HA) was transfected with or without BCL-3 expression vector and the lysates were subjected to EMSA. Augmentation of κ B binding activity was reproduced with p105N-HA (Figure 2B) and the presence of the tag in the binding complex was confirmed by supershift with anti-HA (Figure

2E, lane 12). When p100N-HA was transiently expressed, virtually no κ B binding was detected; however, co-expression of BCL-3 resulted in strong κ B binding activity (Figure 2B, lanes 7–9), which contained BCL-3 (data not shown). The level of p100N-HA expressed was comparable with that of p105; however, the level of p50B was ~20% of p50 (anti-HA immunoblotting; data not shown). This may explain the lower level of κ B binding generated from p100N-HA compared with p105N-HA (Figure 2B, lanes 6 and 9). Next, the products of the p105 expression vector were analyzed by anti-HA immunoblotting. The precursor p105 and its processed form, p50,

were detected in the lysate (Figure 2C) and expression of BCL-3 did not affect the gross amount of p50 or p105, suggesting that BCL-3 expression does not lead to stimulation of p105 processing. Cell fractionation confirmed translocation of the tagged p50 to the nucleus in the BCL-3-expressing but not in control cells (Figure 2D). These results suggest that expression of p105 is a prerequisite for the increase in DNA binding activity in response to BCL-3 expression. To examine whether p105 itself is a component of the DNA binding complex, we constructed an expression plasmid encoding p105 HA-tagged at its C-terminus (p105C-HA). Again, expression of p105C-HA and BCL-3 resulted in increased κ B binding activity (Figure 2E). Analysis using specific antibodies confirmed that the complex contained p50 and BCL-3 but not RelA (Figure 2E). However, addition of the anti-HA antibody did not affect formation of a DNA-protein complex (Figure 2E, lane 9), although the same amount of antibody was sufficient to completely supershift the DNA binding activity produced from p105N-HA (Figure 2E, lane 12). The presence of the tag on p105 was confirmed by immunoblotting and immunoprecipitation (data not shown; see also below). Furthermore, anti-CTR antibody, which specifically recognizes p105 (Liou *et al.*, 1992), did not affect formation of the DNA-protein complex (Figure 2E, lane 10). These results strongly suggest that the p105 precursor is required for induction of κ B binding but it is not included in the DNA-protein complex.

Nuclear translocation of (p50)₂ induced by BCL-3

The cell fractionation results indicate that translocation of p50 is facilitated by BCL-3 in BAF-B03 and 293T cells (Figure 1F and 2D). To ascertain this function of BCL-3 in individual cells, indirect immunofluorescent staining was performed. The p105N-HA and/or BCL-3 expression plasmid was transfected into COS cells (Figure 3A) or 293T cells (Figure 3B) and the ectopically expressed product was detected using specific antibodies. In cells transfected with p105N-HA expression vector alone, the tag was detected in the cytoplasmic region (Figure 3Aa, Ba and Bc). This is consistent with previous reports and considered to be due to the I κ B-like activity of the C-terminal region of the precursor, which retains not only p105 itself, but also p50 in the cytoplasm through intra- and intermolecular interactions. An immunofluorescent signal derived from BCL-3 was detected within the nucleus (Figure 3Ab, Ac, Ae, Be and Bh), as previously reported. In contrast, co-expression of BCL-3 with p105N-HA led to accumulation of the anti-HA signal within the nucleus (Figure 3Ac, Bb and Bd). Considering the biochemical subcellular fractionation results (Figures 1F and 2D), this nuclear staining is likely derived from the p50 subunit rather than the p105 precursor. However, in this assay we cannot distinguish between the anti-HA immunofluorescent signals derived from p105 and p50. Therefore, to examine more strictly the localization of p105 in BCL-3-expressing cells, anti-CTR was used. Anti-CTR detected cytoplasmic p105N-HA with similar sensitivity to anti-HA (Figure 3Bc). In cells expressing both p105N-HA and BCL-3 significant staining was detected in the nucleus by anti-HA, but anti-CTR gave only cytoplasmic signals (Figure 3Bd). Similarly, p105C-HA-

expressing cells exhibited no nuclear signal with anti-HA, irrespective of BCL-3 expression (Figure 3Ae, Be and Bf). These and previous results obtained from subcellular fractionation indicate that p105 is exclusively cytoplasmic and that p50 is translocated to the nucleus in response to BCL-3 co-expression. Essentially similar results were obtained when p100 was expressed (Figure 3Bg and Bh), i.e. p100 and p50B are restricted to the cytoplasm when expressed alone and co-expression with BCL-3 results in nuclear detection of the HA epitope, which most likely corresponds to p50B.

To investigate the involvement of proteolytic processing, we constructed an expression vector for a p105 derivative, p105 Δ N-Myc, which contains a Myc epitope at its N-terminus and has the glycine-rich region deleted and is therefore severely impaired for proteolytic processing (Lin and Ghosh, 1996; Figure 3C) and is localized in the cytoplasm. Expression of p105 Δ N-Myc with p50N-HA, an artificially designed p50 with an HA tag at its N-terminus, resulted in cytoplasmic retention of p50N-HA (Figure 3Dc and Dd), which otherwise translocates to the nucleus (Figure 3Da and Db). Additional expression of BCL-3 resulted in nuclear translocation of p50N-HA but not p105 Δ N-Myc (Figure 3De and Df). These results suggest that BCL-3 selectively translocates p50 from the p50-p105 complex without proteolytic generation of p50 from p105.

BCL-3 augments DNA binding of the p50 complex *in vitro* by transition from p50-p105 to (p50)₂ without proteolytic processing

It has been shown that thymocytes ectopically expressing BCL-3 exhibit elevated p50 DNA binding activity and that this enhancement can be reproduced *in vitro* by mixing the lysates derived from normal and *NFKB1*(-/-), BCL-3-expressing thymocytes (Caamano *et al.*, 1996). We tested whether induction of κ B binding activity would be reproduced by mixing 293T cell lysates containing BCL-3 and *NFKB1* products respectively. Whole cell lysates were prepared from 293T cells transfected with either p105N-HA or BCL-3 expression plasmids. Mixing the two lysates resulted in a dramatic increase in κ B binding activity, similar to the co-transfection experiments (Figure 4, lanes 1-3). Also, the complex formed was indistinguishable from that observed in the co-transfection as judged by reactivities to the specific antibodies (Figure 4, lanes 3-7; data not shown). Thus BCL-3-mediated activation of a complex with κ B can be reproduced *in vitro*.

To further elucidate the target molecule for BCL-3, the *NFKB1* gene products were fractionated. The lysate prepared from 293T cells transfected with p105C-HA was subjected to Mono Q chromatography and the fractions containing exclusively p50 (p50 fraction) and a mixture of p50 and p105 (p50/p105) were obtained (Figure 5A). The p50 fraction exhibited intrinsic κ B motif binding activity, corresponding most likely to naturally processed (p50)₂ (Figure 5B, lane 3). Mixing the fraction with BCL-3 resulted in a supershift in EMSA, but the intensity of the band was not significantly altered (Figure 5B, lane 4). The p50/p105 fraction exhibited no DNA binding activity (Figure 5B, lane 5) and p50 and p105 were complexed, most likely as a heterodimer (see below). When the p50/

p105 fraction was mixed with BCL-3, a κ B binding activity similar to that obtained from the p50 fraction and BCL-3 emerged (Figure 5B, lane 6). Immunoblotting showed no significant change in the total amount of p50 or p105 after incubation with BCL-3 (Figure 5C, lanes 1–4). We determined the amount of p50 associated with p105 by immunoprecipitation with anti-HA followed by immunoblotting with anti-p50. Because the C-terminal HA tag was present only on p105 and not on processed

p50, only p50 associated with p105 could be detected. Incubation with BCL-3, but not with control extract, dramatically reduced the amount of p50 associated with p105; however, again, the amount of precipitated p105 was unchanged (Figure 5C, lanes 5 and 6). The results suggest that in the presence of BCL-3, p50 is released from the p50–p105 complex and that the increased (p50)₂ is unlikely to be generated by proteolytic processing of p50–p105.

The p50–p105 complex is present as a physiological reservoir to generate (p50)₂ in pro-B cells

The above findings suggest that p50–p105, which is normally retained in the cytoplasm, could function as a potential reservoir to generate (p50)₂ in response to BCL-3. Therefore, accumulation of p50–p105 was examined under physiological conditions in two representative mouse cell lines: LyH7 cells, which induce (p50)₂ DNA binding activity in response to ectopic expression of BCL-3, and L929 cells, which require expression of p105 in addition to BCL-3 for induction (Figure 6A; our unpublished observation). LyH7 and L929 cells were metabolically labeled with ³⁵S-labeled amino acids for 6 h and whole cell lysates were prepared in non-denaturing lysis buffer. The lysates corresponding to the equivalent cell number were precipitated first with anti-CTR under non-denaturing conditions. The precipitates were denatured by boiling in SDS sample buffer, then re-precipitated with anti-p50. A significant level of p50 associated with p105 was detected in LyH7 cells, but the complex was barely detectable in L929 cells, suggesting that LyH7 physiologically contains a larger pool of p50–p105 (Figure 6B).

Discussion

In this paper we show that the cytoplasmic p50–p105 complex is a target molecule for BCL-3 and that BCL-3 can release (p50)₂ from p50–p105 by a non-proteolytic mechanism and then translocate the homodimer into the nucleus. This novel activation of κ B binding and nuclear translocation is based on specific, exchangeable interactions among the Rel and ankyrin proteins.

Cell type-specific effect of BCL-3 on κ B binding activity

We show that expression of BCL-3 protein in pro-B cell lines LyH7 and BAF-B03 resulted in a dramatic increase in κ B motif binding activity which was composed of p50 and BCL-3. Similar observations were made in primary mouse thymocytes expressing human BCL-3 (Caamano *et al.*, 1996). However, expression of BCL-3 did not induce κ B binding in non-hematopoietic cell lines such as mouse L929 and human 293T, suggesting that the effect of BCL-3 is different depending on the cell type. It has been shown that phosphorylation of BCL-3 is required for enhancement of DNA binding by p50 in thymocytes (Caamano *et al.*, 1996). This argues for a tissue-specific phosphorylation which accounts for the cell type-specific effect of BCL-3. However, since BCL-3 derived from 293T or insect cells similarly enhanced DNA binding *in vitro* (Figure 6A; our unpublished observation), cell-specific phosphorylation is an unlikely explanation, although phosphorylation itself is required (Nolan *et al.*,

Fig. 3. Nuclear translocation of the p50 subunit induced by BCL-3. COS (A) or 293T cells (B–D) were transiently transfected by expression vectors, either alone or in combination as indicated on the left of each panel. p105N-HA and p105C-HA indicate p105 tagged with an HA epitope at its N- and C-terminus respectively. Similarly, p105N-Myc contains a Myc tag at its N-terminus. p105ΔN-Myc is similar to p105N-Myc but with its glycine-rich region deleted. Cells were fixed after transfection (36 h for COS cells and 24 h for 293T cells) and stained with the indicated primary antibodies, visualized with TRITC-labeled goat anti-mouse Ig antibody or FITC-labeled goat anti-rabbit Ig antibody. Phase contrast images are presented. (A) Double immunostaining of COS cells. COS cells (1×10^6) were transfected with 5 μ g pEF-p105N-HA (a), pEF-bcl-3 (b) or pEF-p105C-HA (d) alone or co-transfected with 5 μ g pEF-p105N-HA and pEF-bcl-3 (c) or pEF-p105C-HA and pEF-bcl-3 (e) by the DEAE-dextran method. Total amount of transfected DNA was kept at 10 μ g by adding pEF-BOS. (B) Double immunostaining of 293T cells. 293T cells (2×10^6) were transfected with 3 μ g pEF-p105N-HA (a and c), 3 μ g pEF-p105N-HA (g), 3 μ g pEF-p105N-HA and 6 μ g pEF-bcl-3 (b and d), 3 μ g pEF-p105C-HA and 6 μ g pEF-bcl-3 (e and f) or 3 μ g pEF-p105N-HA and 6 μ g pEF-bcl-3 (h). Total amount of transfected DNA was kept at 9 μ g by adding pEF-BOS. (C) p105Δ, which lacks the glycine-rich region, is deficient in proteolytic processing. 293T cells (2×10^6) were transfected with 2 μ g plasmids: pEF-BOS, lane 1; pEF-p105N-Myc, lane 2; pEF-p105ΔN-Myc, lane 3. Alternatively, cells were transfected as in (D): pEF-p50N-HA (0.2 μ g), pEF-p105N-Myc (4 μ g) and pEF-BOS (4 μ g), lane 4; pEF-p50N-HA (0.2 μ g), pEF-p105ΔN-Myc (4 μ g) and pEF-bcl-3 (4 μ g), lane 5. Whole cell lysates were prepared 24 h post-transfection and subjected to immunoblot analysis using anti-Myc monoclonal antibody. Molecular weight markers are indicated on the left of the panel and the positions of p105 and p50 are shown by arrows. Endogenous c-myc product is shown by an asterisk. (D) Cytoplasmic retention of p50 by p105Δ and its liberation into the nucleus by BCL-3. 293T cells (2×10^6) were transfected with 0.2 μ g pEF-p50N-HA (a), 0.2 μ g pEF-p50N-HA and 4 μ g pEF-bcl-3 (b), 4 μ g pEF-p105ΔN-Myc and 0.2 μ g pEF-p50N-HA (c and d) or 0.2 μ g pEF-p50N-HA, 4 μ g pEF-p105ΔN-Myc and 4 μ g pEF-bcl-3 (e and f). Total amount of transfected DNA was kept to 8.2 μ g by adding pEF-BOS.

Fig. 4. BCL-3 augments DNA binding of the p50 complex *in vitro*. 293T cells (2×10^6) were transfected with either 20 μ g pEF-p105N-HA (p105N-HA) or 20 μ g pEF-bcl-3 (BCL-3). After 24 h, whole cell lysates were prepared. A portion of the whole cell lysate was directly subjected to EMSA or the lysates were mixed (0°C , 10 min) before assay. p105N-HA, lane 1; BCL-3, lane 2; p105N-HA plus BCL-3, lanes 3–7. The mixed lysate was also reacted simultaneously with antibodies (0.5 μ l normal rabbit serum, lane 4; 1.7 μ g IgG anti-HA, lane 5; 0.5 μ l anti-BCL-3 antiserum, lane 6; 0.5 μ l anti-CTR antiserum, lane 7) and analyzed by EMSA.

1993; our unpublished observation). Another explanation discussed by Caamano *et al.* (1996) is the use of artificially truncated constructs for p50 expression in previous studies. However, partially purified (p50)₂ physiologically processed in 293T cells bound to the kB motif with a comparable affinity in the presence or absence of BCL-3 (Figure 5B). This excludes the possibility that BCL-3 selectively increases DNA binding by correctly processed p50.

Our rationale for the cell type specificity is that the target molecule for BCL-3 is present only in responsive cells, and the following observations indicate that p50–p105 is the target. We have demonstrated that LyH7 cells contain relatively high levels of p50–p105 heterodimer compared with L929 fibroblasts (Figure 6B) and BCL-3 increased kB binding activity in the former but not in the latter *in vivo* and *in vitro* (Figure 6A; our unpublished observation). 293T cells, which normally contain low levels of p50–p105, accumulated a large pool of the heterocomplex by overexpression of p105. Concomitant with generation of the p50–p105 pool, the cells exhibited responsiveness to BCL-3. Finally, BCL-3 dramatically induced the DNA binding activity of (p50)₂ from partially purified p50–p105 *in vitro* (Figure 5B).

We tested a panel of cell lines for induction of kB binding activity by BCL-3 *in vitro*. Some cell lines in addition to LyH7 and BAF-B03 exhibited a significant induction. These cells included RS4;11 (human bone marrow leukemia) and Reh (human acute lymphocytic leukemia) (data not shown). Also, thymocytes have been shown to respond to BCL-3 by increasing p50-containing DNA binding activity (Caamano *et al.*, 1996). These results suggest that BCL-3 may play a physiological role in cells of hematopoietic lineage.

Interchangeable interactions among Rel and ankyrin proteins

One explanation for induction of p50 DNA binding from the p50–p105 complex is enhanced proteolytic processing

Fig. 5. BCL-3 releases p50 homodimer from the p105–p50 complex without proteolytic processing. Whole cell lysate was prepared from 293T cells transfected with pEF-p105C-HA and fractionated by Mono Q chromatography as described in Materials and methods. The two fractions containing exclusively p50 (eluted with 60 mM NaCl, p50/p50) and a mixture of p50 and p105 (eluted with 100 mM NaCl, p50/p105) were obtained and dialyzed against NP-40 lysis buffer. (A) Ten microliters of the p50/p50 fraction (lane 1) and p50/p105 (lane 2) fractions were subjected to immunoblot analysis using anti-p50 antiserum. Molecular weight markers are indicated on the left of the panel and the positions of p105 and p50 are shown by arrows. (B) Ten microliters of the p50/p50 (lanes 3 and 4) or p50/p105 (lanes 5 and 6) fractions were incubated (25°C , 20 min) with 1 μ l whole cell lysate from 293T cells transfected with pEF-BOS (control extract, lanes 3 and 5) or pEF-bcl-3 (BCL-3 extract, lanes 4 and 6) and analyzed by EMSA. Lane 1, control extract alone; lane 2, BCL-3 extract alone. Arrows indicate DNA–protein complexes composed of p50 homodimer and p50 plus BCL-3. Non-specific DNA–protein complex is shown by an asterisk. (C) Ten microliters of the p50/p105 fraction were incubated with 1 μ l control cell extract (lanes 3 and 5) or BCL-3 extract (lanes 4 and 6) as in (B) and subjected to immunoblot analysis using anti-p50 antiserum (lanes 3 and 4) or first precipitated with anti-HA then immunoblotted with anti-p50 (lanes 5 and 6). Lanes 1 and 2 correspond to immunoblotting of the control and BCL-3 lysates (1 μ l) respectively. Molecular weight markers are indicated on the left of the panel and the positions of p105, p50 and the heavy chain (IgH) of the anti-HA antibody are shown by arrows.

which results in conversion of p105 to p50. However, this is unlikely, for the following reasons. First, neither a gross increase in p50 nor a decrease in p105 was observed on expression of BCL-3 in BAF-B03 or p105-transfected 293T cells (Figures 1E and 2C). Second, *in vitro* mixing of lysates containing p50–p105 and BCL-3 rapidly generated DNA binding activity without detectable change in the amount of p50 and p105 (Figure 5). Third, similar effects were observed with purified recombinant p50–p105 and BCL-3 produced in insect cells (our unpublished observation). Thus we hypothesize an alternative model: (p50)₂ is generated from p50 subunits which are released from the p50–p105 complex without proteolytic processing

Fig. 6. The p50-p105 complex is present as a pool to generate p50 homodimer in LyH7 cells. **(A)** Whole cell lysates of L929 (lanes 1–3) or LyH7 (lanes 4–6) cells were mixed with lysates of 293T cells transfected with either pEF-BOS (control, lanes 2 and 5) or pEF-bcl-3 (BCL-3, lanes 3 and 6). After incubation at 25°C for 10 min, samples were subjected to EMSA. κ B-specific binding activity is indicated by brackets and non-specific DNA-protein complex by an asterisk. **(B)** L929 (lane 1) or LyH7 cells (lane 2) (2×10^6 cells) were metabolically labeled with 35 S-labeled amino acids (0.5 mCi) for 6 h and whole cell lysates were prepared. Incorporated radioactivity in the L929 lysate was six times as high as that of the LyH7 lysate, as determined by TCA precipitation. Lysates corresponding to an equivalent cell number were first precipitated using anti-CTR antiserum under non-denaturing conditions then denatured and re-precipitated using anti-p50 antiserum followed by SDS-PAGE (Materials and methods). Molecular weight markers are indicated on the left of the panel and the positions of p105 and p50 are shown by arrows.

(Figure 7). The results obtained with processing-deficient p105 Δ (Figure 3C and D) also support this model.

It appears that proteolytic conversion of p105 to p50 occurs in an orderly manner, i.e. the intermediate p50-p105 accumulates to high levels in the cytoplasm of LyH7 and other lymphoid cells (Blank *et al.*, 1991), and further proteolytic processing of p50-p105 to (p50)₂ presumably occurs much less efficiently. In this regard, we observed that artificial formation of heterodimers between p50 and p105 generated by transfection of truncated and full-length cDNA respectively resulted in inhibition of processing of the p105 precursor (our unpublished observation). This is most likely because the processing machinery recognizes the higher order structure of the substrates.

The strict cytoplasmic localization of p105 (and thus

Fig. 7. A model for induction of nuclear (p50)₂ from cytoplasmic p50-p105 by BCL-3 expression. **(A)** In certain cells, although p50-p105 accumulates in the cytoplasm, the level of mature (p50)₂ is relatively low. **(B)** Expression of BCL-3 in the above cells results in the generation of (p50)₂ associated with BCL-3 in the nucleus (see text).

p50-p105; Figure 3) suggests that BCL-3 interacts with p50-p105 in the cytoplasm. Although BCL-3 localizes predominantly in the nucleus when expressed alone, an apparent cytoplasmic signal for BCL-3 is detected in cells co-expressing p105 or p105 Δ (Figure 3), suggesting that BCL-3 can exist in the cytoplasm, most likely as an intermediate complex. The intermediate complex might exist only transiently, because release of (p50)₂ occurs rapidly, as shown by the *in vitro* experiment (Figure 4).

Subunit exchange between NF- κ B dimers in solution has been shown to occur between homodimers of p50 and RelA and results in nearly complete conversion to p50-RelA heterodimer (Fujita *et al.*, 1992). It is worth noting that mixing p50-p105 and (RelA)₂ efficiently generated p50-RelA without proteolytic processing *in vitro* (our unpublished observation). These observations suggest a cascade of partner exchange between Rel and ankyrin proteins. p50-p105 can be converted to p50-RelA in the presence of sufficient amounts of RelA. p50-RelA efficiently associates with I κ B in the cytoplasm. Thus p50-RelA-I κ B, the reservoir for signal-dependent transient activation of NF- κ B, accumulates in the cytoplasm. In some cell types in which p50-p105 accumulates, presumably due to excess production of p50-p105 over the amount of RelA or other Rel proteins, BCL-3 expression results in persistent release of (p50)₂ and (p50)₂-BCL-3 translocation into the nucleus.

Function of the NFKB1 and NFKB2 gene products

Our results show that p50B-p100 can also be a target for BCL-3-mediated activation of κ B binding (Figure 2B). However, the κ B binding activity induced by BCL-3 in pro-B cell lines (data not shown) and thymocytes (Caamano *et al.*, 1996) did not contain detectable NFKB2 gene products. This is presumably because the levels of p50B-p100 in these cells are lower than those of p50-p105. It is worth noting that processing of p100 occurs much less efficiently than p105 in 293T cells (Betts and Nabel, 1996; our unpublished observation), suggesting that the levels of p50-p105 and p50B-p100 are differentially regulated post-translationally.

Co-transfection experiments using fibroblasts indicated that BCL-3 and (p50)₂ co-activate the reporter gene via tandemly repeated κ B motifs (Fujita *et al.*, 1993). This co-activation was also observed in cells overexpressing p105 (Fujita *et al.*, 1993). However, other reports have demonstrated that BCL-3 and (p50B)₂ co-activate, but BCL-3 and (p50)₂ do not (Schmid *et al.*, 1991; Schmitz and Baeuerle, 1991; Franzoso *et al.*, 1992, 1993). It is therefore possible that the activation/repression function of (p50)₂ or (p50)₂-BCL-3 depends on the promoter (Fujita *et al.*, 1992; Liou *et al.*, 1994) or other cellular conditions. In sum, the *NFKB1* and *NFKB2* gene products may function in an overlapping but distinct manner in terms of BCL-3-mediated gene regulation.

Physiological implications

So far the target genes for BCL-3-mediated co-activation or repression are yet to be identified. Recently the involvement of signal-dependent activation of NF- κ B in protection of cells from apoptosis induced by TNF- α has been reported (Beg and Baltimore, 1996; Liu *et al.*, 1996; Van Antwerp *et al.*, 1996; Wang *et al.*, 1996). In this regard it is interesting to speculate that abnormal expression of BCL-3 may result in persistent activation of a presumptive gene(s) required for prevention of programmed cell death and may lead to the generation of leukemia. This issue needs to be investigated further.

It has been reported that a predominant induction of (p50)₂ is observed in anergic T cells which are tolerant to stimulation by a specific antigen (Sundstedt *et al.*, 1996). A similar transition of NF- κ B subunits from p50-RelA to (p50)₂ is observed in monocytes with induced tolerance to lipopolysaccharide (Ziegler-Heitbrock *et al.*, 1994). It is worth noting that one of the characteristics of CLL, a malignancy with a suspected link to *bcl-3* gene activation, is reduced responsiveness to mitogenic stimuli (Lankester *et al.*, 1995). In this regard we observed a significant down-regulation of surface CD25 and CD43 in LyH7 cells by retrovirus-mediated BCL-3 expression (our unpublished observation). The promoter of the *CD25* gene contains a copy of the κ B motif (Baeuerle, 1991) and therefore it is possible that (p50)₂ or (p50)₂-BCL-3 mediates this down-regulation through interaction with the κ B motif. In sum, the involvement of BCL-3 in induction of immunological unresponsiveness is implied. Surely this issue needs to be investigated further.

Materials and methods

Cell culture and transfections

IL-3-dependent mouse pro-B cell lines BAF-B03 and LyH7 (Palacios *et al.*, 1987; Shibuya *et al.*, 1992) were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% (v/v) fetal bovine serum (FBS) and 10% (v/v) WEHI-3B conditioned medium as a source of IL-3. BAF-B03 cells (1×10^7) were co-transfected with 1 μ g *Bam*HI-digested pmiwhph (Shibuya *et al.*, 1992) and 36 μ g *Sca*I-digested pEF-bcl-3 or *Sca*I-digested pEF-BOS by electroporation. Transfected cells were seeded in 48-well plates and grown in selection medium containing 1 mg/ml hygromycin B. Recombinant retrovirus stocks were prepared according to the procedure described by Pear *et al.* (1993). LyH7 cells (1×10^6) were infected with the retrovirus for 24 h in the presence of 10 μ g/ml polybrene, followed by G418 selection (1 mg/ml).

COS7 and 293T cells were maintained in DMEM containing 10% (v/v) FBS and transfected by the DEAE-dextran and calcium phosphate methods respectively. In co-transfection experiments the total amount of

transfected DNA was kept constant by including the control vector pEF-BOS.

RNA blot analysis

RNA extraction and RNA blot analysis were performed as described by Shibuya *et al.* (1992). The probe DNAs for *NFKB1* and β -actin were prepared using a mouse *NFKB1* cDNA (Ghosh *et al.*, 1990) and a DNA fragment derived from a human β -actin pseudogene respectively.

Preparation of cell lysate and partial purification of factors

To prepare whole cell lysate, collected cells were suspended in NP-40 lysis buffer [20 mM HEPES, pH 7.9, 50 mM NaCl, 1 mM EDTA, 10% (v/v) glycerol, 0.1% (v/v) Nonidet P-40, 1 mM dithiothreitol (DTT), 10 mM sodium orthovanadate] supplemented with 100 μ g/ml leupeptin and 0.5 mM phenylmethylsulfonyl fluoride and allowed to stand on ice for 30 min. The suspension was clarified by centrifugation (356 000 g, 10 min) and the resulting supernatant was subjected to EMSA, immunoblot analysis and immunoprecipitation.

For partial purification of p50 and the p50-p105 complex, the whole cell lysate prepared from 293T cells transfected with pEF-p105C-HA was applied to a FPLC Mono Q column. The column was washed with buffer D' (Fujita *et al.*, 1993) and proteins were eluted by NaCl gradient (50 mM–1 M) in the same buffer. Each fraction was subjected to immunoblot analysis using anti-p50. Fractions containing p50 subunit alone or both p50 and p105 were separately collected and subjected to the experiments described in Figure 5 as p50/p50 and p50/p105 fractions respectively. Nuclear and cytoplasmic extracts were prepared as described in Schreiber *et al.* (1989).

Electrophoretic mobility shift assay (EMSA)

Oligonucleotide probes containing one copy of the IFN- β κ B motif or its palindromic derivative were as described previously (Fujita *et al.*, 1992). Protein fractions and DNA probes were incubated for 20 min at room temperature in 10 μ l binding mixture containing 20 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 1% (v/v) NP-40, 5% (v/v) glycerol and 0.35 μ g herring sperm DNA. The DNA-protein complexes were resolved by electrophoresis on 4% acrylamide gels in $1 \times$ TBE at 10 V/cm for 60 min at room temperature.

Immunological analyses

Antibodies. Rabbit antisera against BCL-3, NFKB1 and RelA have been described previously (Nolan *et al.*, 1993; Scott *et al.*, 1993). Anti-CTR antiserum, which was raised against the C-terminal region of NFKB1 p105 (Liou *et al.*, 1992), was kindly provided by H.-C.Liou. Anti-HA mouse monoclonal antibody 12CA5, anti-MYC mouse monoclonal antibody 9E10 and alkaline phosphatase- or fluorochrome-conjugated secondary antibodies were obtained commercially.

Immunoblot analysis. Immunoblot analyses were performed as described previously (Fujita *et al.*, 1993). The concentrations of the primary antibodies were a 1/2000 dilution for polyclonal antisera and 5 μ g IgG/ml for monoclonal antibodies. Secondary alkaline phosphatase-conjugated antibodies (Boehringer Mannheim) were used at a 1/2000 dilution and blots were developed with the BCIP/NBT system (Promega).

Immunoprecipitation. Whole cell lysate was incubated with primary antibody on ice for 60 min and immune complexes were precipitated with protein G-Sepharose (Pharmacia) and washed five times with NP-40 lysis buffer. The resins were boiled with Laemmli sample buffer and the supernatants were subjected to immunoblot analysis or sequential immunoprecipitation after adjusting the buffer composition to 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS.

Indirect immunofluorescence. Twenty-four or 36 h after transfection of 293T or COS cells respectively, cells plated on coverslips (coated with poly-L-lysine for 293T cells) were fixed with 4% (v/v) paraformaldehyde, freshly prepared as described (Harlow and Lane, 1988) and adjusted to pH 7.0 just before use, followed by a permeabilizing treatment using 0.2% (v/v) Triton X-100. Primary antibody treatment was performed with a 1/500 dilution of antiserum or 10 μ g IgG/ml monoclonal antibody for 1 h at room temperature in phosphate-buffered saline supplemented with 0.05% Tween 20 (PBST) containing 5 mg/ml bovine serum albumin (PBST/BSA). After washing four times with PBST, the cells were incubated with goat anti-rabbit or anti-mouse IgGs conjugated to fluorochrome at a concentration of 8 μ g IgG/ml in PBST/BSA for 30 min at room temperature. After washing four times with PBST, the slides were mounted in Mowiol.

Expression plasmids

pEF-bcl-3, pEF-p50, pEF-p105 and pEF-p100 were constructed by subcloning the cDNAs encoding mouse BCL-3, mouse NFKB1 p50 (amino acids 1–401; Fujita *et al.*, 1992), mouse NFKB1 p105 and human p100 (Schmid *et al.*, 1991) respectively into the pEF-BOS vector (Mizushima and Nagata, 1990). Epitope-tagged constructs were made by addition of double-stranded DNA linkers encoding a Myc or HA epitope. Resulting constructs encode the following sequences: at the N-terminus: p105N-Myc, MEKLISEEDLPG; p105N-HA, p50N-HA and p100N-HA, MGYPYDVPDYASLGGPA; at the C-terminus: p105C-HA, GPGYPYDVPDYASLGG. pEFp105 Δ N-Myc, which encodes p105N-Myc with an internal deletion of amino acids 372–394, was constructed using a synthetic DNA linker. pGDbcl-3 was constructed by subcloning the cDNA for mouse BCL-3 into the pGD vector (Pear *et al.*, 1993).

Acknowledgements

We thank Drs Shigekazu Nagata, Ronald Palacios and Hsiou-Chi Liou for providing pEF-BOS, LyH7 cells and the anti-CTR antibody respectively. We are especially grateful to Drs Edward Barsoumian, Garry P. Nolan and Mitsutoshi Yoneyama for critical reading of the manuscript. The work was supported by grants from the Ministry of Education, Science, Sports and Culture of Japan, Kato Memorial Bioscience Foundation, Nippon Boehringer Ingelheim Co. Ltd, Toray Industries Inc. and Ajinomoto Co. Inc.

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Received on July 30, 1996; revised on March 6, 1997