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The oncoprotein Bcl-3 can facilitate NF-κB-mediated transactivation by removing inhibiting p50 homodimers from select κB sites

Guido Franzoso[1], Vincent Bours[2], Viola Azarenko, Sun Park, Michiyo Tomita-Yamaguchi, Tomohiko Kanno, Keith Brown and Ulrich Siebenlist[3]

Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA

[1]Permanent address: Servizio Di Microbiologia, Complesso Convenzionato Universita-ULSS21, Via Ospedale 2, 35100 Padua, Italy

[2]Present address: Laboratoire d'Etude des Métastases et de l'Invasion Tumorale, Pathologie B23, Domaine du Sart-Tilman, Université de Liège, 4000 Liege, Belgium

[3]Corresponding author

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Previously we have proposed a role for Bcl-3 in facilitating transactivation through κB sites by counteracting the inhibitory effects of bound, non-transactivating homodimers of the p50 subunit of NF-κB. Such homodimers are abundant for example in nuclei of unstimulated primary T cells. Here we extend the model and provide new evidence which fulfills a number of predictions. (i) Bcl-3 preferentially targets p50 homodimers over NF-κB heterodimers since the homodimers are completely dissociated from κB sites at concentrations of Bcl-3 which do not affect NF-κB. (ii) Select κB sites associate very strongly and stably with p50 homodimers, completely preventing binding by NF-κB. Such κB sites are likely candidates for regulation by p50 homodimers and Bcl-3. (iii) Bcl-3 and p50 can be co-localized in the nucleus, a requirement for active removal of homodimers from their binding sites *in vivo*. (iv) The ankyrin repeat domain of Bcl-3 is sufficient for the reversal of p50 homodimer-mediated inhibition, correlating with the ability of this domain alone to inhibit p50 binding to κB sites *in vitro*. Our data support the model that induction of nuclear Bcl-3 may be required during cellular stimulation to actively remove stably bound p50 homodimers from certain κB sites in order to allow transactivating NF-κB complexes to engage. This exact mechanism is demonstrated with *in vitro* experiments.

Key words: Bcl-3/NF-κB/oncoprotein/p50 homodimers/transactivation

Introduction

The NF-κB/Rel family of transcription factors is involved in the regulated expression of a large number of genes containing *cis*-acting κB binding motifs, particularly those genes determining immune functions and acute phase reactions. In addition, several viruses including HIV are regulated, at least in part, by NF-κB/Rel. A variety of cellular stimuli cause the translocation of cytosolic NF-κB/Rel factors into nuclei where they transactivate κB-dependent genes (for reviews see Baeuerle, 1991; Grilli *et al.*, 1993). Cytoplasmic retention of these factors in unstimulated cells is due to association with the inhibitory IκB proteins, which have been shown to shield the nuclear translocation signal present within the transcription factors (Blank *et al.*, 1991; Beg *et al.*, 1992; Henkel *et al.*, 1992; Zabel *et al.*, 1993). Recently it has been demonstrated that the signal-dependent activation of NF-κB proceeds via proteolytic digestion of IκB-α and that the activated nuclear NF-κB then induces the synthesis of its own inhibitor IκB-α (Brown *et al.*, 1993; Sun *et al.*, 1993).

The family of dimeric NF-κB/Rel transcription factors share a conserved Rel homology domain of ~300 amino acids. These Rel-related proteins are p50 (NFKB1) (Bours *et al.*, 1990; Ghosh *et al.*, 1990; Kieran *et al.*, 1990; Meyer *et al.*, 1991), p52 (NFKB2, p50B, p49, lyt-10) (Bours *et al.*, 1992; Schmid *et al.*, 1991; Neri *et al.*, 1991; Mercurio *et al.*, 1992, respectively), RelA (p65) (Nolan *et al.*, 1991; Ballard *et al.*, 1992; Ruben *et al.*, 1991), RelB (Ruben *et al.*, 1992; Ryseck *et al.*, 1992), c-Rel (Brownell *et al.*, 1989), v-Rel (Stephens *et al.*, 1983; Wilhemsen *et al.*, 1984) and the *Drosophila* protein Dorsal (Steward, 1987). The Rel homology domain determines dimerization and DNA binding, dimerization being a prerequisite for DNA binding (Logeat *et al.*, 1991; Bressler *et al.*, 1993). Many different dimeric combinations are possible, generating by combinatorial power a large array of different factors (Molitor *et al.*, 1990; Bours *et al.*, 1992; Hansen *et al.*, 1992; Perkins *et al.*, 1992; Rice *et al.*, 1992; Ryseck *et al.*, 1992; Mercurio *et al.*, 1993). The p50-p65 heterodimer is usually the most abundant of the transactivating complexes and has been traditionally referred to as NF-κB primarily because other subunit combinations escaped initial detection. p50 homodimers are also present in many cells but these are located constitutively in the nucleus (Franzoso *et al.*, 1992). IκB-α has been shown to bind and retain in the cytoplasm all dimeric factors containing p65, plus those containing RelB or c-Rel (Haskill *et al.*, 1991; Davis *et al.*, 1992; Inoue *et al.*, 1992a; Tewari *et al.*, 1992). Unlike these IκB-α-inhibitable transactivating dimers, homodimers of p50 or p50B do not transactivate (Schmitz and Baeuerle, 1991; Schmid *et al.*, 1991; Ballard *et al.*, 1992; Bours *et al.*, 1992, 1993; Franzoso *et al.*, 1992; Kunsch *et al.*, 1992; Mercurio *et al.*, 1992; Ryseck *et al.*, 1992), though there is some evidence to the contrary (Fujita *et al.*, 1992; Kretzschmar *et al.*, 1992; Moore *et al.*, 1993) (see Discussion). We reported recently that p50 homodimers compete for κB sites with transactivating p50-p65 heterodimers and in this way inhibit transactivation, a role consistent with their presence in nuclei of unstimulated cells (Franzoso *et al.*, 1992).

The NF-κB/Rel family of factors is regulated not only by IκB-α but also by several proteins structurally related to

I$\varkappa$B-α: the precursor proteins for p50 and p52/p50B (p105 and p100, respectively) (Rice *et al.*, 1992; Mercurio *et al.*, 1993); I$\varkappa$B-γ, the separately expressed C-terminal part of p105 detected in some mouse preB cell lines (Inoue *et al.*, 1992b; Liou *et al.*, 1992); Bcl-3, a proto-oncogene product first cloned by way of its translocation into the immunoglobulin locus in some chronic lymphocytic leukemias (Ohno *et al.*, 1990; Franzoso *et al.*, 1992; Hatada *et al.*, 1992; Wulczyn *et al.*, 1992; Bours *et al.*, 1993); and the *Drosophila* protein Cactus, an inhibitor of Dorsal (Geisler *et al.*, 1992; Kidd, 1992). All contain between five and seven ankyrin or cell cycle repeats which mediate protein–protein interactions with members of the Rel-related gene family (Blank *et al.*, 1991; Bours *et al.*, 1992, 1993; Inoue *et al.*, 1992a,b; Wulczyn *et al.*, 1992). Bcl-3 can act positively in regulating transactivation (Franzoso *et al.*, 1992; Bours *et al.*, 1993). It forms transient ternary complexes with p52 (p50B) on $\varkappa$B DNA binding sites, causing direct transcriptional transactivation due to activation domains located on Bcl-3 outside the ankyrin domain (Bours *et al.*, 1993). p50B homodimers are restricted in their expression and to date have been detected in appreciable amounts only in a few mature hematopoietic cell lines (Bours *et al.*, 1993; G.Franzoso and U.Siebenlist, unpublished observations). Bcl-3 also strongly associates with the ubiquitously expressed p50 homodimers, interfering with their DNA binding (Franzoso *et al.*, 1992; Hatada *et al.*, 1992; Wulczyn *et al.*, 1992). Previously we have reported that Bcl-3 can in this way counteract the inhibitory effects of non-transactivating p50 homodimers and allow transactivating NF-$\varkappa$B complexes to engage (Franzoso *et al.*, 1992). Here we report new evidence in support of a mechanism by which induced Bcl-3 could facilitate transactivation during cellular stimulation, including a recreation of the envisioned cellular scenario with *in vitro* experiments. Alternative or additional roles for Bcl-3 are critically considered as well. Finally, a careful investigation of the primary targets of Bcl-3 is performed since such targets ultimately define the biological role of this regulator. In previous reports from other laboratories the primary targets were not defined, contributing to various speculations on the role of Bcl-3 (Kerr *et al.*, 1992; Wulczyn *et al.*, 1992; Naumann *et al.*, 1993).

Results

Bcl-3 binds p50 but not p65

p50 fused to glutathione S-transferase (p50–GST) bound to *in vitro* translated, radioactively labelled Bcl-3 as shown by their co-precipitation with glutathione-coated beads (Figure 1A, lane 2). An N-terminally deleted Bcl-3 and a combined N- and C-terminally deleted Bcl-3 also bound the p50–GST (lanes 5 and 8, respectively). The latter deleted form of Bcl-3 contains only the ankyrin repeats (see Figure 1B), indicating that this domain is sufficient for binding p50. This result is in agreement with a prior report (Wulczyn *et al.*, 1993) but differs from another (Kerr *et al.*, 1992). The ankryin domain of Bcl-3 is sufficient for binding to p50B also (Bours *et al.*, 1993).

Bcl-3 bound to p50 *in vivo*. NTera-2 embryonal carcinoma cells contain no measurable amounts of NF-$\varkappa$B activity or Bcl-3 (Bours *et al.*, 1992, 1993; Franzoso *et al.*, 1992; Segars *et al.*, 1993; Figure 1C, lane 1). These cells were co-transfected with expression vectors encoding p50 and Bcl-3 and labelled with [^{35}S]cysteine and [^{35}S]methionine. Antibodies to Bcl-3 co-immunoprecipitated Bcl-3 and p50 (lane 5) and this was blocked by inclusion of the Bcl-3 peptide to which the antibody was made (lane 6). The faster migrating series of closely spaced bands represented Bcl-3 as demonstrated with cells transfected with Bcl-3 only (lane 2). The identity of the co-immunoprecipitated p50 in lane 5 was confirmed by its comigration with the p50 protein

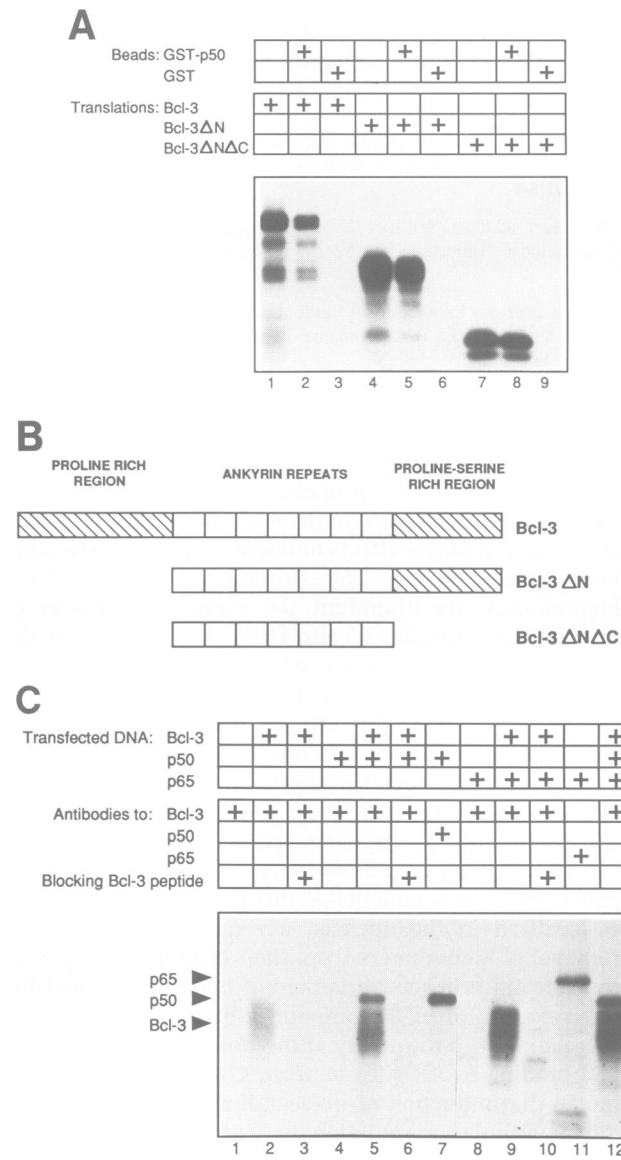

Fig. 1. (**A**) The ankyrin repeat domain of Bcl-3 is sufficient to bind p50. ^{35}S-labelled Bcl-3 wild-type (lanes 1–3) or deletion mutants Bcl-3ΔN (lanes 4–6) and Bcl-3ΔNΔC (lanes 7–9) are shown as *in vitro* translated products (2 μl) (lanes 1, 4 and 7) and after precipitations (of 5 μl) with GST–p50 fusion protein attached to glutathione–Sepharose beads (lanes 2, 4 and 6) or with GST alone attached to beads (lanes 3, 6 and 9). (**B**) Bcl-3 deletion mutants, (see Materials and methods). (**C**) p50 and Bcl-3 associate *in vivo*. NTera-2 cells were transfected with 3 μg (per plate of cells) of expression vectors for p50, p65 or Bcl-3, as indicated. Whole cell extracts of the transfected and metabolically labelled cells were used for immunoprecipitations with α-Bcl-3, α-p50 or α-p65 antibodies, as indicated (~1/3 of a harvested plate of cells was used per precipitation). α-Bcl-3 immunoprecipitations were blocked with 400 ng of the Bcl-3 peptide used to generate the antibody. The arrows point to immunoprecipitated proteins.

immunoprecipitated with p50 antibodies (lane 7) and by immunoprecipitation from unlabelled extracts followed by Western blotting with p50 antibodies (data not shown). Immunoprecipitation with Bcl-3 antibody of extracts from cells co-transfected with Bcl-3 and p65 or with Bcl-3, p50 and p65 resulted only in the appearance of Bcl-3 (lane 9) or Bcl-3 and p50 (lane 12), respectively (lane 11 shows the p65-specific protein). It was concluded that Bcl-3 was bound in these cells to p50 but not to p65. Bcl-3 could similarly associate with p50B (Bours *et al.*, 1993).

Bcl-3 preferentially inhibits the DNA binding of p50 homodimers relative to p50–p65 heterodimers (NF-κB)

Extracts were made of NTera-2 cells transfected with Bcl-3 and of cells transfected with p50 and p65. The latter cells gave rise to both p50 homodimers and p50–p65 heterodimers (NF-κB) as judged by electrophoretic mobility shift assay (EMSA) (Figure 2, lane 1) and confirmed by supershifting antibodies (Franzoso *et al.*, 1992). Addition of Bcl-3 eliminated DNA binding by p50 homodimers but had little or no effect on binding by p50–p65 NF-κB (lanes 2 and 3).

In analogous experiments, the N-terminally deleted Bcl-3 (lanes 6 and 7) or the combined N- and C-terminally deleted Bcl-3 (lanes 4 and 5) exhibited the same properties as the wild-type protein. Therefore, the ankyrin domain of Bcl-3 was sufficient both for binding to p50 proteins (see above) and for selectively inhibiting DNA binding. In contrast to Bcl-3, IκB-α specifically inhibited binding by NF-κB but had no effect on binding by the p50 homodimer (lanes 8 and 9). Bcl-3 is the only protein known to specifically target p50 homodimers over p50–p65 heterodimers. As demonstrated in prior reports by us (Franzoso *et al.*, 1992) and others

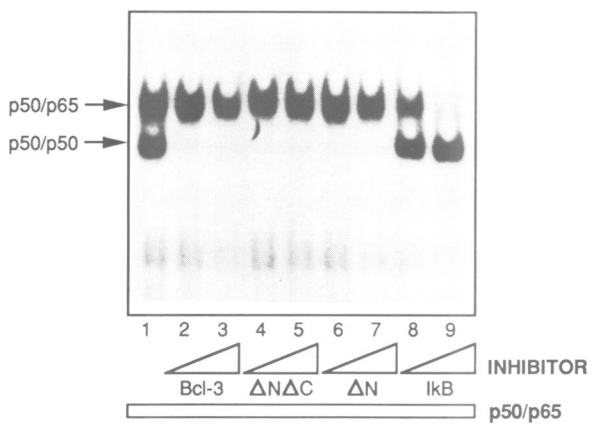

Fig. 2. Bcl-3 specifically inhibits the DNA binding of p50 homodimers. EMSA was performed with nuclear extracts (0.5 μl) from NTera-2 cells transfected with the p50 and p65 expression vectors (4 μg each per plate). Prior to adding the IL-2R κB probe the nuclear extracts were premixed with 1 μl (lanes 2, 4, 6 and 8) or 2 μl (lanes 3, 5; 7 and 9) of whole cell extracts obtained from cells transfected with 8 μg (per plate) of expression vector encoding Bcl-3 (lanes 2–3), Bcl-3ΔNΔC (lanes 4–5), Bcl-3ΔN (lanes 6–7) or IκB-α (lanes 8–9). The total amount of whole cell extract was kept constant throughout by adding compensating amounts of an extract obtained from NTera-2 cells transfected with 8 μg (per plate) of the expression vector PMT2T without any insert (e.g. for lane 1, 2 μl of this whole cell extract was premixed with the nuclear extract). The identity of the DNA binding complexes, which are indicated by arrows, was confirmed also by specific supershifting antibodies (data not shown; see Franzoso *et al.*, 1992).

(Kerr *et al.*, 1992; Wulczyn *et al.*, 1992), increased amounts of Bcl-3 well above those used here partially reduced NF-κB binding as well (G.Franzoso and U.Siebenlist, unpublished observations).

NF-κB-mediated transactivation of particular κB sites is susceptible to inhibition by p50 homodimers and the ankyrin domain of Bcl-3 is sufficient to relieve inhibition

p50 homodimers competitively inhibited NF-κB-mediated transactivation of an HIV-κB site-driven CAT reporter (Franzoso *et al.*, 1992). A relatively stronger p50 homodimer-mediated inhibition was observed with an IL-2 receptor alpha-derived κB site (IL-2R-κB; Figure 3A, stippled bars). As little as a 3-fold excess of transfected p50 DNA over p65 markedly inhibited transactivation relative to optimal transactivation (compare solid bars 5 and 4). In contrast to the IL-2R-κB site, the immunoglobulin κ light chain derived κB site (Ig-κLC-κB) was noticeably less sensitive to inhibition by p50 homodimers (Figure 3A, hatched bars). These experiments suggest a hierarchy of sensitivity of κB sites for inhibition by p50 homodimers. The observed *in vivo* differences between the CAT reporters based on the IL-2R-κB site and the Ig-κLC-κB site correlated well with these sites' relative binding affinities for p50 homodimers versus NF-κB (see Figure 4, below).

A dramatic reversal of the p50 homodimer-mediated inhibition of the IL-2R-κB-driven reporter was observed with co-transfected Bcl-3 (Figure 3B). Previously we reported a less dramatic reversal by Bcl-3 of inhibition of the HIV-κB site based CAT vector (Franzoso *et al.*, 1992). Both the wild-type Bcl-3 (data not shown) and the ankyrin domain of Bcl-3 [Bcl-3ΔNΔC, lacking transactivation domains (Bours *et al.*, 1993)] counteracted the p50-mediated inhibition, correlating with the ability of both proteins to specifically inhibit DNA binding of p50 homodimers *in vitro* (see Figure 2 above). Either form of Bcl-3 also reversed the inhibition of the Ig-κLC-κB driven reporter, albeit less obviously because the p50 homodimers did not fully inhibit transactivation to begin with (data not shown). Larger amounts of transfected Bcl-3 did not completely restore optimal transactivation levels (cf. Figure 3A and B) most likely due to the lower amounts of CAT reporter used and/or possibly to some inhibitory effects on heterodimers at these higher levels (see above).

Bcl-3 efficiently dissociates prebound p50 homodimers from DNA

Unstimulated cells, including primary T lymphocytes, contain abundant amounts of p50 homodimers in their nuclei (Franzoso *et al.*, 1992). If Bcl-3 were to play an anti-inhibitory role during cellular activation, then Bcl-3 should be able to remove actively already prebound homodimers from their κB sites, allowing access by heterodimers. EMSA experiments were performed as in Figure 2, except that Bcl-3 was added after the p50 homodimers and the p50–p65 heterodimers were bound to κB sites (Figure 4). Increasing amounts of Bcl-3 specifically displaced the prebound p50 homodimers from several different κB sites (in agreement with Hatada *et al.*, 1992) while having only marginal effects on binding by heterodimers.

The κB sites displayed very different binding affinities for homodimers versus heterodimers, under identical

experimental conditions. The Ig-κLC-κB site preferentially associated with heterodimers while the IL-2R site and especially the IL-2 site preferentially bound homodomers (Figure 4). Heterodimer binding to the IL-2 site was evident only with relatively high amounts of NF-κB. The stronger binding by p50 homodimers to the IL-2R-κB site as opposed to the immunoglobulin site is most likely responsible also for the stronger p50-mediated inhibition *in vivo* of the IL-2 receptor driven reporter (see above).

p50 homodimers can stably associate with κB sites

A synthetic palindromic κB (PD-κB) site was bound efficently by both p50 homodimers and NF-κB in EMSAs (Figure 5, lanes 1 and 6). Following the association of these factors with radioactively labelled PD-κB DNA a 3000-fold excess of unlabelled probe was added and aliquots were removed at the times indicated and immediately analyzed by electrophoresis on a continuously running gel (lanes 1−5). Binding by p50 homodimers was barely diminished even after 4 h of competition by unlabelled probe, while binding by NF-κB proved to be relatively unstable. In similar experiments, the addition of Bcl-3 shortly after addition of unlabelled competitor removed the p50 homodimers, as expected (lanes 6−10). The IL-2R-κB site also exhibited stable binding with p50 homodimers, albeit somewhat less than that observed with the PD-κB probe (data not shown). If similarly stable binding existed *in vivo*, the activation-dependent influx of NF-κB into the nucleus would not be able to transactivate through these p50 homodimer-occupied sites. Bcl-3 may then be required to facilitate transactivation by NF-κB.

Saturating amounts of p50 homodimers block binding of NF-κB to κB sites and Bcl-3 reverses this block

To test whether Bcl-3 could facilitate transactivation during cellular activation, we performed the following experiment *in vitro*. Increasing amounts of p50 homodimers were added to limiting amounts of the IL-2 receptor κB probe to determine the point of saturation (Figure 6A, lanes 1−6). Addition of increasing amounts of NF-κB probe prebound with near-saturating p50 homodimers (simulating nuclear entry of NF-κB) resulted in barely detectable binding of NF-κB (lanes 7−9; the amount of p50 homodimers corresponds to that in lane 4; lanes 10−12 show the binding of NF-κB in the absence of prebound p50 homodimers). When Bcl-3 was titrated into a mixture of near-saturating amounts of prebound p50 homodimers and non-bound NF-κB heterodimers, a dose-dependent decrease of p50 homodimer binding was observed as well as a corresponding increase in binding by NF-κB (lanes 13−16).

When the Ig-κLC-κB probe was used a similar but less dramatic Bcl-3-mediated reversal was observed. This is because NF-κB competed well and was only partially inhibited from binding this site by prebound p50 homodimers (Figure 6B). The results correlate with the transfection experiments described above in which expression from the

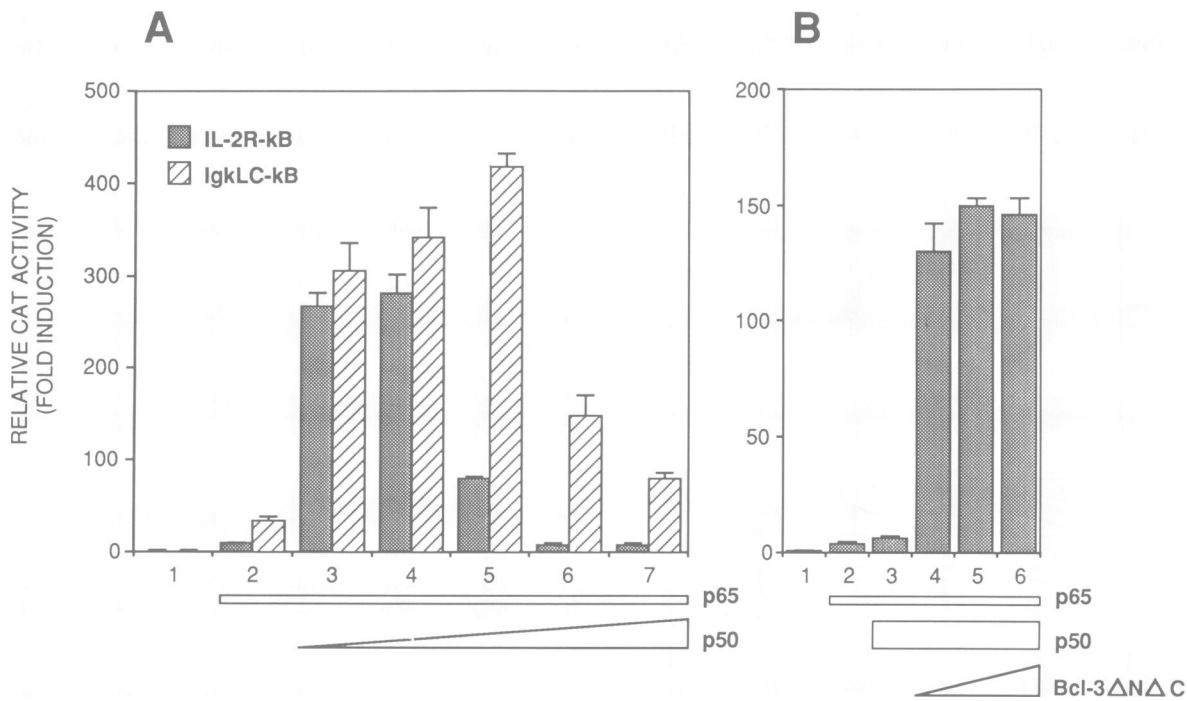

Fig. 3. (**A**) p50 homodimers strongly inhibit p50−p65-dependent transactivation of an IL-2R-κB site-driven CAT reporter. NTera-2 cells were transfected with a constant amount of the p65 expression vector (columns 2−7; 0.3 μg) plus increasing amounts of the p50 expression vector (columns 3−7; 0.1, 0.3, 1, 3 and 6 μg, respectively) and 6 μg of CAT reporter plasmid (columns 1−7) driven by the IL-2R-κB site (stippled columns) or the Ig-κLC-κB site (hatched columns) (transfected amounts per plate of cells). Shown is the relative CAT activity over the activity observed with transfection of the reporter plasmid alone (fold induction). Each column represents the mean of three or more independent experiments after normalization to the protein concentration of the cellular extracts. The total amount of transfected DNA and expression vector was kept constant throughout by adding appropriate amounts of expression vector without insert. (**B**) The ankyrin repeat domain of Bcl-3 is sufficient to reverse p50 homodimer-mediated inhibition. Experiments were performed as in panel A except that 3 μg of the IL-2R-κB-dependent reporter were used (columns 1−6) and a constant amount of both p65 (0.3 μg; columns 2−6) and p50 expression vectors (3 μg; columns 3−6) were transfected as indicated (this ratio of p50 to p65 corresponds to column 6 in A). In addition, increasing amounts of Bcl-3ΔNΔC were transfected (columns 4−6; 1, 1.5 and 2 μg, respectively). As before the total amount of DNA/expression vector was kept constant.

IL-2R-κB site-driven CAT reporter was more sensitive to inhibition by p50 homodimers (see above) than reporters driven by the HIV- (Franzoso *et al.*, 1992) or Ig-κLC-κB sites. The *in vitro* data confirm a hierarchy of κB sites likely to be inhibited by p50 homodimers and consequently most likely subject to regulation by Bcl-3.

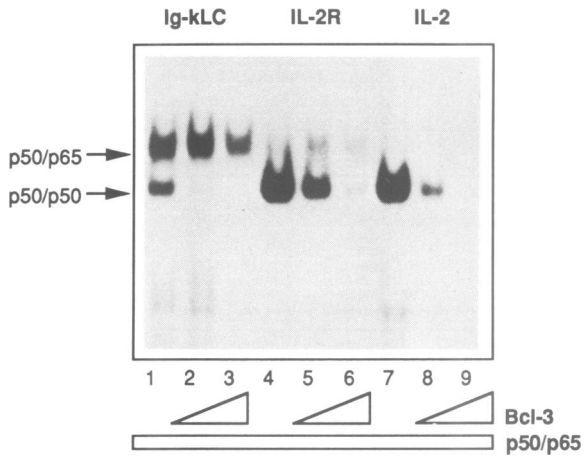

Fig. 4. Bcl-3 can actively displace p50 homodimers prebound to different κB sites. The EMSA was performed with nuclear extracts (0.5 μl) obtained from p50 plus p65 transfected NTera-2 cells as in Figure 2. The extract was preincubated for 30 min at room temperature with the Ig-κLC-κB probe (lanes 1–3), the IL-2R-κB probe (4–6) or the IL-2-κB probe (7–9) to allow binding of the p50–p50 and p50–p65 complexes to DNA. Subsequently, 1 μl (lanes 2, 5 and 8) or 2 μl (lanes 3, 6 and 9) of Bcl-3-containing whole cell extract was added for 20 min. The total amount of whole cell extract was kept constant throughout (see Figure 2 for further details).

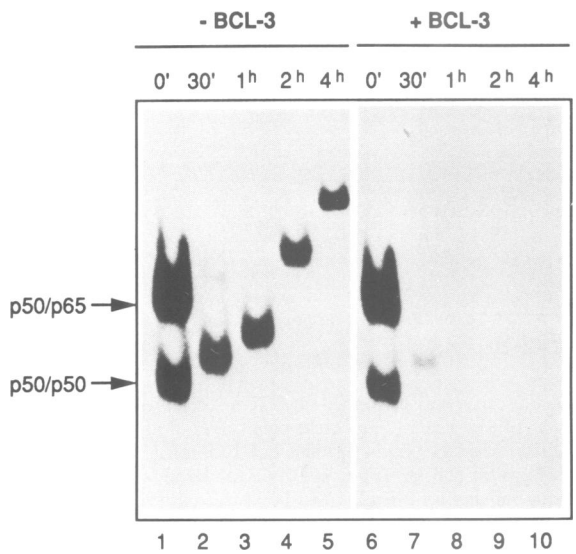

Fig. 5. p50 homodimers can form very stable complexes with κB sites. p50 homodimers and p50–p65 heterodimers (obtained as in Figures 2 and 4) were incubated with the PD-κB probe for 30 min at room temperature. Subsequently, whole cell extracts were added which did not (lanes 2–5) or which did (lanes 7–10) contain Bcl-3 (obtained as in Figures 2 and 4) immediately followed by the addition of a 3000-fold excess of unlabelled PD-κB probe. A 6 × reaction was initiated with 3 μl nuclear extract and 12 μl of whole cell extract and 1 × aliquots were taken at the times indicated and loaded onto a continuously running gel.

Bcl-3 and p50 co-localize to the nucleus

If induced Bcl-3 were to actively remove p50 homodimers from their DNA binding sites *in vivo* then Bcl-3 and p50 should localize to the nucleus. NTera-2 cells transfected with various expression vectors were analyzed by indirect immunofluorescence. Bcl-3 antibodies detected Bcl-3 in the nuclei of cells transfected with either Bcl-3 alone (Figure 7G; some cytoplasmic staining is evident there as well) (as

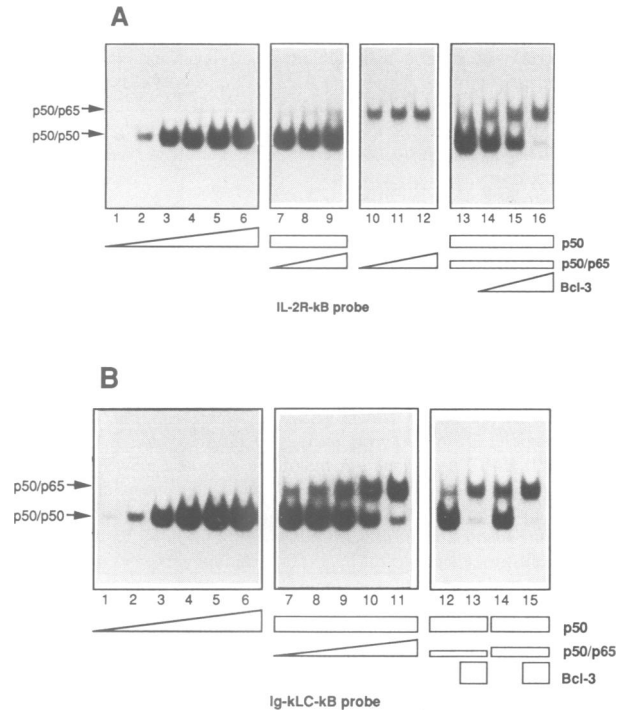

Fig. 6. Bcl-3 can free select κB sites for binding to p50–p65 NF-κB by dissociating prebound saturating amounts of p50 homodimers. The EMSA was performed with 8 pg of IL-2R-κB (**A**) or Ig-κLC-κB (**B**) radioactively labelled probe (8000 c.p.m.) to facilitate saturation of binding with p50–p50 homodimers. (A) Lanes 1–6, increasing amounts of a nuclear extract from NTera-2 cells transfected with 8 μg of the p50 expression vector (0.05, 0.1, 0.2, 0.3, 0.4 and 0.8 μl, respectively) incubated for 20 min with the IL-2R-κB probe; lanes 7–9, 0.3 μl of the p50 extract prebound for 20 min (as in lane 4) plus increasing amounts of a nuclear extract obtained from cells transfected with 3 μg of the p50 expression vector plus 6 μg of the p65 vector (0.25, 0.5 and 1 μl, respectively) incubated for an additional 20 min after the initial binding to p50 homodimers; lanes 10–12, as in lanes 7–9 except for the absence of the p50 homodimer extract; lane 13, probe prebound with 0.3 μl of p50 extract plus subsequent incubation for 20 min with 1 μl of the p50–p65 extract (as in lane 9); lanes 14–16, binding reaction as for lane 13 (or 9) plus increasing amounts of Bcl-3-containing extract (0.5, 1 and 2 μl, respectively; obtained as in Figures 2, 4 and 5) added immediately after addition of p50–p65. As before the total amount of extract was kept constant with the addition of extract from cells transfected with the expression vector without insert. Lanes 13–16 were adjusted separately to a slightly higher total amount of extract which may account for the somewhat better overall binding activity observed here and in lanes 12–15 in panel B. (B) Experiments analogous to those in panel A except that the Ig-κLC-κB site was used. Lanes 1–6, binding reaction with increasing amounts of a p50 extract (0.05, 0.1, 0.2, 0.4, 0.6 and 1 μl, respectively); lanes 7–11, 0.6 μl of p50 extract (as in lane 5) plus increasing amounts of the p50–p65 extract (see panel A) (0.025, 0.05, 0.1, 0.2 and 0.4 μl, respectively); lanes 12 and 13, binding reaction as for lane 8 except that lane 13 also received Bcl-3 (2 μl); lanes 14 and 15, as for lane 9 except that lane 15 also received Bcl-3 (2 μl); as for panel A the total amount of extract was kept constant throughout and lanes 12–15 were adjusted separately.

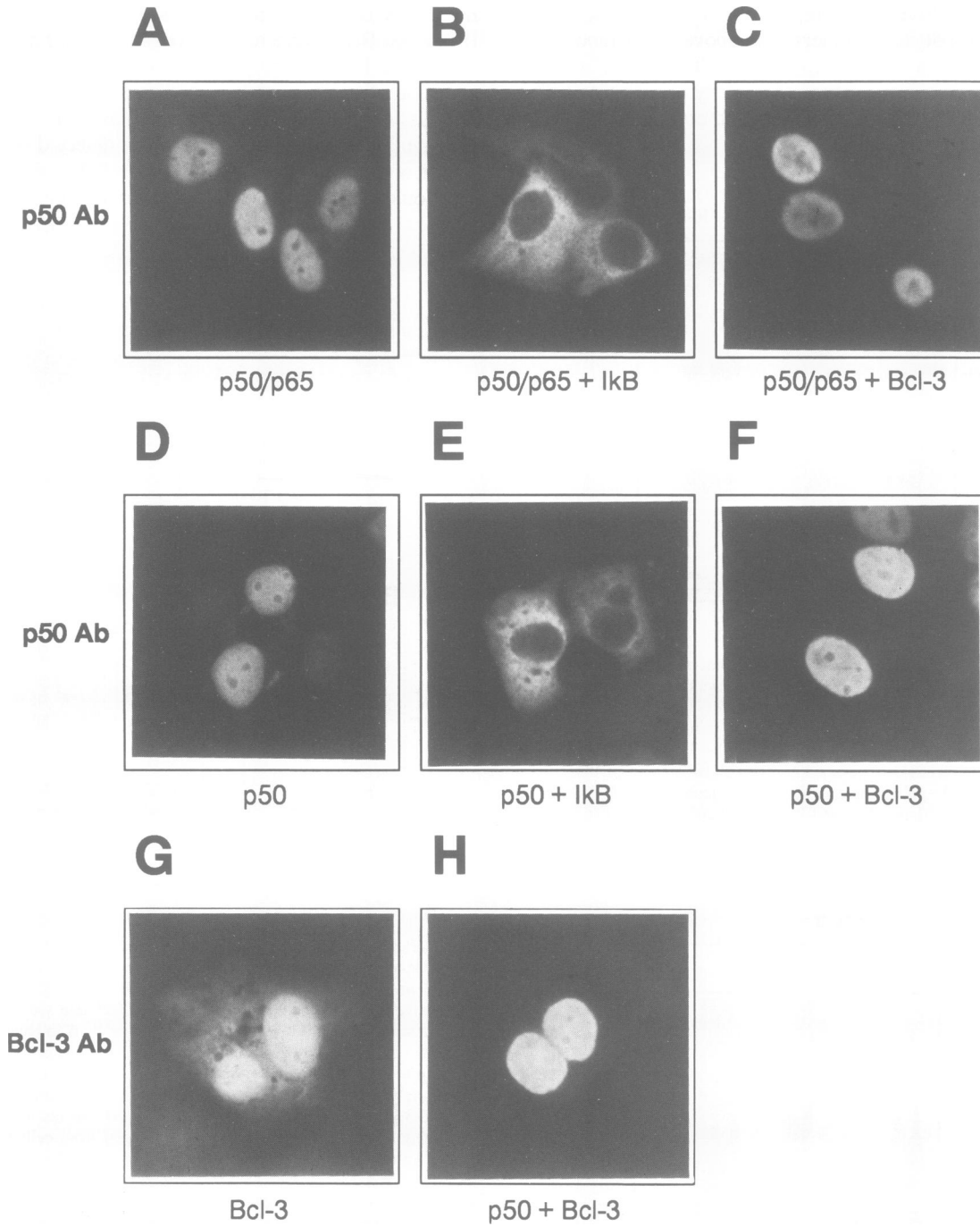

Fig. 7. Bcl-3 is located predominantly in nuclei of NTera-2 cells. Indirect immunofluorescence was performed on NTera-2 cells transfected with the following expression vectors: p50 plus p65 (2 μg, each, per plate of cells) in panel **A**; p50 plus p65 (2 μg, each) plus I$\varkappa$B-α (5 μg) or Bcl-3 (5 μg) in panels **B** and **C**, respectively; p50 (4 μg) in panel **D**; p50 (4 μg) plus I$\varkappa$B-α (5 μg) in panel **E**; p50 (4 μg) plus Bcl-3 (5 μg) in panels **F** and **H**; Bcl-3 alone (5 μg) in panel **G**. Transfected cells were stained with antibodies directed against p50 (A–F) or Bcl-3 (G and H). Transfections with Bcl-3 alone (panel G) resulted in generally low levels of expression. Some cytoplasmic staining for NF-$\varkappa$B could be visualized with longer exposures of panel A, presumably due to induction of some endogenous I$\varkappa$B-α (Brown *et al.*, 1993).

reported in Bours *et al.*, 1993) or with both p50 and Bcl-3 (panel H). Use of p50 antibodies confirmed the nuclear location for this protein when transfected alone or in combination with Bcl-3 (panels D and F, respectively). p50 and Bcl-3 thus co-localized to the nucleus. As a control, co-transfection of I$\varkappa$B-α and p50 led to cytoplasmic localization for p50 (panel E), as reported by Beg *et al.* (1992). Bcl-3 did not affect the nuclear localization of NF-$\varkappa$B (p50–p65) (panels A and C), while co-transfected I$\varkappa$B-α was very effective in retaining the transcription factor in the cytoplasm (panel B) (see also Beg *et al.*, 1992; Bours *et al.*, 1993). In a recent report Bcl-3 was primarily localized to the cytoplasm of Bcl-3-transfected Cos cells (Naumann *et al.*, 1993). This suggests the possibility that the cellular background may significantly affect localization of Bcl-3.

Discussion

Based on our findings presented here and previously, we envision a role for Bcl-3 in markedly assisting transactivation

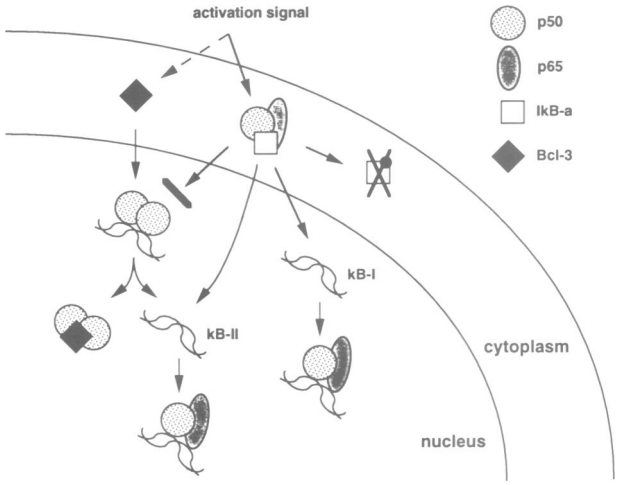

Fig. 8. Model: an indirect role for Bcl-3 in NF-κB-mediated transactivation through select κB sites. NF-κB can bind to one group of κB sites directly (κB-I) whereas binding to another select group of sites (κB-II) is strongly facilitated by Bcl-3-mediated removal of the previously stably bound p50 homodimers. κB-I sites may be partially inhibited by p50 homodimers.

of certain but not all κB-dependent genes during cellular activation (see model, Figure 8). This role is dependent on the ability of induced Bcl-3 to antagonize specifically the binding of p50 homodimers to κB target sequences. p50 homodimers are abundant in nuclei of several primary cell types analyzed to date, including primary human T cells (Franzoso *et al.*, 1992; G.Franzoso and U.Siebenlist, unpublished observations) and they generally do not transactivate (see below). Our *in vitro* data predict a very stable association of the p50 homodimers with a subset of κB sites in cells. NF-κB complexes that translocate into nuclei following cellular activation would be unable to displace the tightly bound p50 homodimers, thus preventing NF-κB from potently activating through the κB sites of these inhibited genes. Given that Bcl-3 is induced upon stimulation of T cells and other cells (Ohno *et al.*, 1990; M.Tamita-Yamaguchi, G.Franzoso and U.Siebenlist, unpublished observations) this protein could then actively remove p50 homodimers, allowing NF-κB to bind and transactivate. That Bcl-3 can carry out this function is shown most directly in experiments which recreate the envisioned scenario with binding studies *in vitro* (Figure 6). The transfection experiments (Figure 3B) strongly support this mechanism as well.

Alternative considerations regarding the biological role of Bcl-3

Direct proof of the *in vivo* function(s) of Bcl-3 remains to be obtained. Roles for Bcl-3 additional or alternative to that discussed above may be considered. Constitutive expression of Bcl-3 in particular cell types or stages of differentiation could any function of p50 homodimers in the absence of NF-κB. In this case the biological role of Bcl-3 would depend on the function(s) of p50 homodimers in the unstimulated cells; this probably depends on the promoter context of the target κB site (see below).

A different and intriguing role for Bcl-3 and p50 homodimers could be analogous to the previously reported interaction of Bcl-3 with p50B homodimers (Bours *et al.*, 1993). p50 is closely related to p50B (Neri *et al.*, 1991; Schmid *et al.*, 1991; Bours *et al.*, 1992; Mercurio *et al.*, 1992). p50B homodimers can associate with Bcl-3 on κB target sites, generating a ternary complex capable of direct transactivation due to the presence of activation domains on Bcl-3. Although the combination of p50 and Bcl-3 did not lead to significant transactivation in our experiments (Franzoso *et al.*, 1992; Bours *et al.*, 1993) (Figure 3), It is conceivable that in a different cellular milieu p50 and Bcl-3 could do so (see below). Importantly, however, in the experiments presented here it is clear that Bcl-3 acted only indirectly in transactivation since even the truncated Bcl-3 devoid of the activation domains effectively reversed p50 homodimer-mediated inhibition (Figure 3B).

Another potential physiological role for Bcl-3 could be based on its weak inhibition of NF-κB activity, as shown by us and others (Franzoso *et al.*, 1992; Kerr *et al.*, 1992; Wulczyn *et al.*, 1992). Such a role appears less likely since the levels of Bcl-3 required for inhibition of NF-κB far exceed those required for IκB-α (Franzoso *et al.*, 1992; this report), a very potent widely distributed and specific inhibitor (Baeuerle *et al.*, 1988a,b; Haskill *et al.*, 1991; Beg *et al.*, 1992; Brown *et al.*, 1993).

Function(s) of p50 homodimers, the targets of Bcl-3

The physiological role(s) of Bcl-3 depends on the precise function(s) of its target, in this context the widely distributed p50 homodimers. In NTera-2 transfection studies the p50 homodimers competively inhibited NF-κB mediated transactivation and exhibited no significant transactivation alone. These undifferentiated cells have no measurable NF-κB activity (Bours *et al.*, 1992, 1993; Franzoso *et al.*, 1992; Segars *et al.*, 1993) and thus permit an analysis not complicated by the presence of other Rel-related proteins which may complex with the exogenously introduced protein. While the function of p50 homodimers remains somewhat controversial due to reports of transactivation observed with *in vitro* transcription [especially with the MHC-derived κB site (Fujita *et al.*, 1992; Kretzschmar *et al.*, 1992] and transactivation observed in yeast (Moore *et al.*, 1993), the consensus from many mammalian transfection studies indicates only minimal or no transactivation by p50 homodimers (Schmitz and Baeuerle, 1991; Schmid *et al.*, 1991; Ballard *et al.*, 1992; Bours *et al.*, 1992, 1993; Franzoso *et al.*, 1992; Kunsch *et al.*, 1992; Mercurio *et al.*, 1992; Ryseck *et al.*, 1992). In addition, binding of p50 homodimers to the IL-2 κB site has been shown to correlate with inhibition of IL-2 expression in normal T cell clones, even in the presence of activated NF-κB (Kang *et al.*, 1992). Finally, the presence of p50 homodimers in nuclei of unstimulated primary T cells and other cells argues against general transactivation of κB sites (Franzoso *et al.*, 1992). Therefore, full NF-κB-mediated transactivation of at least that subset of κB-regulated genes which is blocked by tightly binding p50 homodimers may require Bcl-3 to first relieve this block upon cellular stimulation.

κB-dependent reporter constructs enable the assessment of direct transcriptional effects of p50 homodimers, but the exact function of the homodimers in the regulation of a given cellular gene is likely to depend also on the promoter context of the κB site. p50 homodimers may have negative or positive effects on transcription indirectly through yet to be defined interactions with other transcription factors and consequently the effects of Bcl-3 would change accordingly.

While such interactions are speculative, suggestive evidence exists in at least one case. C/EBP factors have been shown to associate physically with p50 homodimers and some promoters may even permit cooperative interactions due to the presence of binding sites for both these factors (LeClair *et al.*, 1992).

Bcl-3, a protein with two distinct functions

Experiments in our laboratory have established that p50 homodimers (this report and Franzoso *et al.*, 1992) and p50B homodimers (Bours *et al.*, 1993), but not NF-κB, are primary targets of Bcl-3. In other studies primary targets were not clearly defined, thus leading to differing proposed roles for Bcl-3 (Kerr *et al.*, 1992; Wulczyn *et al.*, 1992; Naumann *et al.*, 1993). Another source for conflict resides in the apparently opposing functions of the activation and ankyrin domains present in Bcl-3. The ankyrin domain has the intrinsic ability to dissociate targeted complexes from DNA (Figures 2 and 4; Franzoso *et al.*, 1992; Wulczyn *et al.*, 1992), whereas the activation domains can function only when complexes remain on DNA (Bours *et al.*, 1993). A resolution of this conflict may be indicated by how effectively Bcl-3 dissociates the p50 complex compared with the p50B (p52) complex from DNA. The p50B–Bcl-3 complex (but presumably not p50–Bcl-3) must survive on DNA long enough to allow transactivation (Bours *et al.*, 1993). Different post-translational modifications including phosphorylations may also regulate the two intrinsic functions of Bcl-3.

Bcl-3 could act as a facilitator of NF-κB activity during cellular activation, but its precise physiological role(s) may vary depending on the gene- and cell-specific functions of its targets (primarily p50 and p50B homodimers) and on possible post-translational modifications. Our research to date establishes a firm basis for exploring the *in vivo* role(s) of Bcl-3.

Materials and methods

Constructs

The mammalian PMT2T-based expression vectors for p50, p65, IκB-α, Bcl-3, Bcl-3ΔN (amino acids 119 to end) and Bcl-3ΔNΔC (amino acids 119–359), the Bluescript and the pMR-based vectors for *in vitro* transcription/translation of the wild-type and truncated Bcl-3 proteins, respectively and the glutathione S-transferase fusion construct of p50 (pGEX–p50 construct giving rise to GST–p50) have been described (Bours *et al.*, 1992, 1993; Franzoso *et al.*, 1992; Brown *et al.*, 1993).

The CAT reporter plasmids were driven transcriptionally by κB sites derived from (i) the mouse immunoglobulin κ light chain enhancer (Ig-κLC-κB–CAT) (Sen *et al.*, 1986) or (ii) the IL-2 receptor-alpha chain promoter (IL-2R-κB–CAT) (Boehnlein *et al.*, 1988) or (iii) the human immunodeficiency virus long terminal repeat (HIV-LTR) (HIV-κB–CAT; containing two κB sites) and these κB sites were inserted upstream of the minimal c-*fos* promoter (Bours *et al.*, 1992, 1993; Franzoso *et al.*, 1992).

Transfections and immunoprecipitations

Calcium phosphate-mediated transient DNA transfections of NTera-2 cells and CAT assays (involving scintillation vial counting) were performed as described (Newmann *et al.*, 1987; Bours *et al.*, 1992). About 1.8×10^6 cells were seeded per 100 mm plate 24 h prior to a 12 h transfection period and cells were harvested 24 h after the transfection. For immunoprecipitations, the transfected cells were incubated for 30 min in medium lacking cysteine and methionine, then metabolically labelled for 2 h with [^{35}S]cysteine and [^{35}S]methionine and immediately harvested. Whole cell extracts were prepared (see below) and immunoprecipitations performed in a buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 0.1% NP-40 and a cocktail of protease inhibitors (Boehringer, protease inhibitor kit without EDTA but plus PMSF). Antigen–antibody complexes were precipitated with protein A bound to Sepharose beads prior to SDS gel electrophoresis.

Glutathione S-transferase-based assays

GST–p50 fusion protein and GST protein were prepared by glutathione-coated bead purification from appropriate bacterial sonicates (Franzoso *et al.*, 1992; Bours *et al.*, 1993). The pMR-based Bcl-3ΔN and Bcl-3ΔNΔC plasmids, and wild-type Bcl-3 in a Bluescript vector (Ohno *et al.*, 1990) were transcribed and translated *in vitro* with [^{35}S]methionine (Bours *et al.*, 1992). The labelled products were assayed for interaction with the GST–p50 fusion protein attached to glutathione-coated beads as detailed (Franzoso *et al.*, 1992; Bours *et al.*, 1993).

Cell extracts and EMSAs

Cell extracts were prepared from NTera-2 cells 24 h post-transfection (100 μl of extract per harvested plate of cells) by repeated freeze–thawing, salt extraction in 0.5 M NaCl and ultracentrifugation as reported previously (Franzoso *et al.*, 1992; Bours *et al.*, 1993). Nuclear extracts were prepared from the transfected NTera-2 cells by Dounce homogenization followed by salt extraction of the resulting nuclei (20 μl of extract per harvested plate) (Franzoso *et al.*, 1992). κB DNA probes and methods for EMSA, gel electrophoresis (4% polyacrylamide in 0.25 × Tris–borate–EDTA), antibody-mediated supershift analyses of the protein–DNA complexes and radioactive labelling of the probes have been described (Bours *et al.*, 1990, 1992, 1993; Franzoso *et al.*, 1992). EMSAs were carried out with 100–150 pg of the labelled DNA probe (100 000–150 000 c.p.m.) unless otherwise stated.

Indirect immunofluorescence and antibodies

Indirect immunofluorescence was performed on transfected NTera-2 cells as described (Bours *et al.*, 1993). The antibodies used were: an α-Bcl-3 (N-terminal peptide) rabbit antibody (Figure 1C), an α-Bcl-3 rabbit polyclonal antibody (Figure 7), an α-p50 (N-terminal peptide) rabbit antibody (Figures 1C and 7) and an α-p65 rabbit polyclonal antibody (Figure 1C) (Bours *et al.*, 1992, 1993; Franzoso *et al.*, 1992).

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