Leukemia (2003) 00, 1–8

www.nature.com/leu

NF-κB2/p100 induces Bcl-2 expression

P Viatour[1], M Bentires-Alj[1], A Chariot[1], V Deregowski[1], L de Leval[2], M-P Merville[1] and V Bours[1]

[1]*Center for Cellular and Molecular Therapy, University of Liège, Liège, Belgium; and* [2]*Experimental Cancerology Research Center, University of Liège, Liège, Belgium*

The *NF-κB2/p100* and *bcl-3* genes are involved in chromosomal translocations described in chronic lymphocytic leukemias (CLL) and non-Hodgkin's lymphomas, and nuclear factor kappaB (NF-κB) protects cancer cells against apoptosis. Therefore, we investigated whether this transcription factor could modulate the expression of the Bcl-2 antiapoptotic protein. *Bcl-2* promoter analysis showed multiple putative NF-κB binding sites. Transfection assays of *bcl-2* promoter constructs in HCT116 cells showed that NF-κB can indeed transactivate *bcl-2*. We identified a κB site located at position −180 that can only be bound and transactivated by p50 or p52 homodimers. As p50 and p52 homodimers are devoid of any transactivating domains, we showed that they can transactivate the *bcl-2* promoter through association with Bcl-3. We also observed that stable overexpression of p100 and its processed product p52 can induce endogenous Bcl-2 expression in MCF7AZ breast cancer cells. Finally, we demonstrated that, in breast cancer and leukemic cells (CLL), high NF-κB2/p100 expression was associated with high Bcl-2 expression. Our data suggest that Bcl-2 could be an *in vivo* target gene for NF-κB2/p100.
Leukemia (2003) **00,** 000–000. doi:10.1038/sj.leu.2402982
Keywords: NF-κB; Bcl-3; Bcl-2; oncogene; apoptosis; cancer

Introduction

Apoptosis or programmed cell death is a vital process that controls cell number in multicellular organisms. Mechanisms regulating apoptosis are complex and their dysfunction represents a central step in tumorigenesis and conditions cancer cell response to radiotherapy and chemotherapy.[1] Members of the Bcl-2 protein family are key regulators of apoptosis and their implication in cancer development has been largely documented.[2] At least 15 proteins of this family have been identified in mammalian cells. They are divided into proapoptotic (Bax, Bak, Bad, Bid and others) and antiapoptotic proteins (Bcl-2, Bcl-XL, A1/Bfl-1 and others).

Nuclear factor kappaB (NF-κB) is a ubiquitous transcription factor involved in a large variety of events such as inflammation, immune response and cell survival.[3–7] NF-κB consists of dimers of subunits (RelA, RelB, c-Rel, p50, p52) that all contain a Rel homology domain. Among the Rel-related proteins, p65 (RelA), RelB and c-Rel contain one or two transactivating domains, while p50 and p52 are derived from large cytoplasmic precursors, p105 and p100, respectively, and do not possess any transactivating domain. NF-κB is sequestered in the cytoplasm of resting cells through binding with inhibitors belonging to the IκB family (IκBα, IκBβ, IκBε, p100 and p105). Following several stimuli, the ubiquitous IκB-α inhibitor is degraded and NF-κB translocates to the nucleus.[8]

Several NF-κB proteins are found to be overexpressed in several cancers, following chromosomal translocation or gene amplification.[9,10] The rearrangement affecting the *NF-κB2* locus at 10q24, found in many lymphomas, results in the deletion of the C-terminal part of p100, thus enhancing p52 expression, notably in the nucleus.[11] Mice lacking NF-κB2/p100 develop normally, but exhibit impaired immune response and disruption in splenic and lymph node architecture.[12]

Bcl-3 is a member of the IκB protein family. Unlike the other IκB proteins, which are mostly localized in the cytoplasm, Bcl-3 is predominantly present in the nucleus where it forms transactivating complexes with p50 or p52 homodimers.[13,14] *bcl-3* gene was originally identified at the breakpoint in the t(14;19) translocation in chronic lymphocytic leukemias (CLL),[15] resulting in a constitutive enhanced Bcl-3 protein expression and a bad prognosis. *bcl-3* knockout (KO) mice develop normally, but exhibit severe defects in humoral immune response and impaired germinal center reaction.[16,17] Interestingly, KO mice for p52 or Bcl-3 show similar phenotypes, suggesting common *in vivo* target genes. On the contrary, *bcl-3* transgenic animals develop splenomegaly and an accumulation of mature B cells in lymph nodes, bone marrow and peritoneal cavity.[18] Despite these *in vivo* models, Bcl-3 biological functions and target genes remain largely unknown. An *in vitro* effect on the human P-selectin promoter has been reported[19] and, recently, Westerheide *et al*[20] showed that Bcl-3, in association with p52, could enhance cyclin D1 expression in breast cancer cells. Finally, Mitchell *et al*[21] demonstrated that immunological adjuvant-induced Bcl-3 could promote T-cell survival.

Similar to *bcl-3*, the *bcl-2* gene was identified in a t(14;18) translocation in B-cell lymphomas.[22] It codes for the Bcl-2 antiapoptotic protein, a key factor in the regulation of apoptosis. The *bcl-2* gene comprises three exons with a very long second intron.[23] The promoter is composed of two distinct regions (P1 and P2). P1 is located 1400 bp upstream of the translation initiation site and is a TATA less, GC-rich promoter that displays multiple initiation sites. Several regulatory regions have already been identified in this promoter and are bound by the transcription factors CREB, Π1, WT1, E2, etc.[24–27] The second promoter (P2) is located 1.3 kb downstream to P1. It contains a CCAAT box, a TATA element and a negative responsive element.[28]

In this paper, we show that (i) NF-κB transactivates the *bcl-2* promoter; (ii) p50 and p52 homodimers, in collaboration with Bcl-3, bind to and transactivate the *bcl-2* promoter, and (iii) p100 stable overexpression in a breast cancer cell line enhances endogenous Bcl-2 expression. In addition, breast cancer tissue and B cells from leukemic patients (CLL) showed an association between NF-κB2/p100 expression and enhanced Bcl-2 protein level.

Materials and methods

Cell culture and biological samples

The HCT116 human colon carcinoma cells (ATCC CCL 247) were grown in McCoy's 5A modified medium supplemented

Correspondence: Dr V Bours, Department of Genetics, CHU B35, University of Liège, Liège 4000 Belgium; Fax: +32 4 3668 146
Received 20 January 2003; accepted 14 March 2003

with 1% L-glutamine 200 mM, 10% (v/v) fetal bovine serum (FCS, Life Technologies, Grand Island, NY, USA), penicillin (100 IU/ml) and streptomycin (100 μg/ml). The MCF7AZ breast cancer cell line (from Professor M Mareel, University of Gent, Belgium) was maintained in RPMI 1640 supplemented with 10% FCS, 1% L-glutamine 200 mM, penicillin (100 IU/ml) and streptomycin (100 μg/ml).

The stably transfected cell lines were cultivated in the same medium as parental cells plus geneticin (500 μg/ml of G418 active concentration, Roche, Belgium).

Blood (samples 1–6) or bone marrow (samples 7, 8) primary samples identified as CLL according to the REAL classification and with (samples 5–8) or without (samples 1–4) a t(14;19) chromosomal translocation were obtained from Professor A Hagemeijer (University of Leuven, Belgium) or from Dr F Lambert (Laboratory of Medical Chemistry, CHU Liège, Belgium). Breast cancer samples were obtained from the Department of Surgery, CHU Liège. The samples were immediately frozen in liquid nitrogen and reduced to powder. Total cellular extracts were made by using SDS 1%.

Sequence analysis

Analysis of the *bcl-2* promoter sequence was performed with the Ben (Belgian Embnet Node) software using the mapping program and the TF sites database.

Immunoblots

Whole-cell protein extracts (10 μg) obtained by SDS lysis were separated on a 14% SDS-PAGE gel. After transfer to a nylon membrane (Immobilon-P, Millipore, Bedford, MA, USA) and overnight blocking at 4°C in Tris-buffered saline Tween-20 (20 mM Tris pH 7.5, 500 mM NaCl, 0.2% Tween-20 plus 5% dry milk), the membranes were incubated for 1 h with antibodies directed against human actin, Bcl-2, Bcl-3 (Santa Cruz, CA, USA), p50 or p52 (UBI, France), washed and then incubated with the second peroxidase-conjugated antibody. The immunocomplexes were revealed with the enhanced chemiluminescence detection method (ECL kit, Amersham, UK).

Plasmids

The *bcl-2* Luc-reporter plasmids containing the fragments from −3934 to −8, −751 to −8 and −178 to −8 from the *bcl-2* promoter cloned upstream of the luciferase-reporter gene were generous gifts from Dr L Boxer (Stanford University Medical Center, Stanford, CA, USA). The *bcl-2* β-Gal-reporter plasmids containing the fragments from −760 to −8, −178 to −8 and −182 to −8 were constructed by insertion of PCR products corresponding to these fragments in a pBlue-TOPO TA Cloning vector (Invitrogen, Groningen, The Netherlands). The PMT2T-Bcl-3, PMT2T-p100, PMT2T-p50, PMT2T-p52 and PMT2T-RelA expression vectors have been previously described.[14,29]

Transient tranfections

Expression vectors and Luc- or β-Gal-reporter plasmids were transfected into HCT116 cells using the FuGENE transfection reagent (Roche, Mannheim, Germany). Cellular extracts were prepared 24 h later and the luciferase or β-galactosidase activities were measured with the Luciferase-reporter gene assay (Roche) or the β-Gal-reporter gene assay (Roche). Enzyme activities were normalized to the protein concentration of the extracts.

Nuclear protein extraction and electrophoretic mobility shift assay

Nuclear protein extraction for electrophoretic mobility shift assay (EMSA) has been previously described.[7] Briefly, the pelleted nuclei were resuspended in nuclear buffer (20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.63 M NaCl, 25% glycerol, protease inhibitors (Protease inhibitor kit, Roche), incubated for 20 min at 4°C and centrifuged for 30 min at 14 000 *g*. Protein amounts were quantified with the Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA). EMSAs were performed as described[7] with 5 μg of nuclear protein extracts. The prototypical palindromic κB probe was (upper strand) 5′- TTGGCAACGGCAGGGGAATTCCCCTCTCCTTA-3′ and (lower strand) TTGGTAAGGAGAGGGGAATTCCCC-TGCCGTTG.[29] Sequences for the κBI and κBII probes from the *bcl-2* promoter were 5′-TTGGCGCTCGGGGCGTCCCTAGAC-3′ and 5′-TTGGTCCCCGGGAGCCCCCACCCC-3′, respectively. Sequences for probes 1–7 were 5′-TTGGATCAGTCTGGGAAAATCCATAGAGTG-3′ (probe 1), 5′-TTGGGCTGAGGTGGGAGAATCCTTCGAGCC-3′ (probe 2), 5′-TTGGGACCTTTAGGGAGCCCACCCACCCCA-3′ (probe 3), 5′-TTGGCCCCCGGCGGGACGCGCCACTCCCGG-3′ (probe 4), 5′-TTGGGGGGCTCCGGGCCCTCCCTGCCGGCG-3′ (probe 5), 5′-TTGGGATTCATTGGGAAGTTTCAAATCAGC-3′ (probe 6) and 5′-TTGGTGCCAAGRGGGAAACACCAGAATCAA-3′ (probe 7). The sequence for probe 7 mut was 5′-TTGGTGCCAAGR**TTT**AAACACCAGAATCAA-3′ where κB7 is mutated (**TTT** instead of GGG). The double-stranded oligonucleotides probes were annealed and end labeled with ^{32}P-ATP and CTP by filling 5′ overhangs with the Klenow fragment.[29] p50, p52 and RelA polyclonal antibodies used for the supershifting experiment were provided by UBI (Lake Placid, NY, USA) and Santa Cruz (CA, USA), respectively; the c-Rel polyclonal antibody was a gift from Dr Nancy Rice (NCI, Frederick, MD, USA).

Results

Analysis of the bcl-2 *promoter and identification of κB sites*

Analysis of the P1 *bcl-2* promoter sequence revealed two putative NF-κB binding sites that we named κBI (GGGGCGTCCC) and κBII (GGGAGCCCCC), located at positions −2306 and −1896, respectively, as well as five other putative κB sites, numbered 1–5, that all harbor one mismatched

Table 1 Alignment of κB binding sites from the ***bcl-2*** gene promoter and comparison with a consensus (CS) binding site

κBI	GGGGCGTCCC
κBII	GGGAGCCCCC
κB1	GGGAAAATCC
κB2	GGGAGAATCC
κB3	GGGAGCCCAC
κB4	GGGACGCGCC
κB5	GGGCCCTCCC
κB6	GGGAAAGTTTC
κB7	GGGAAACACC
CS	GGGRNNYYCC

Mismatched nucleotides are underlined.

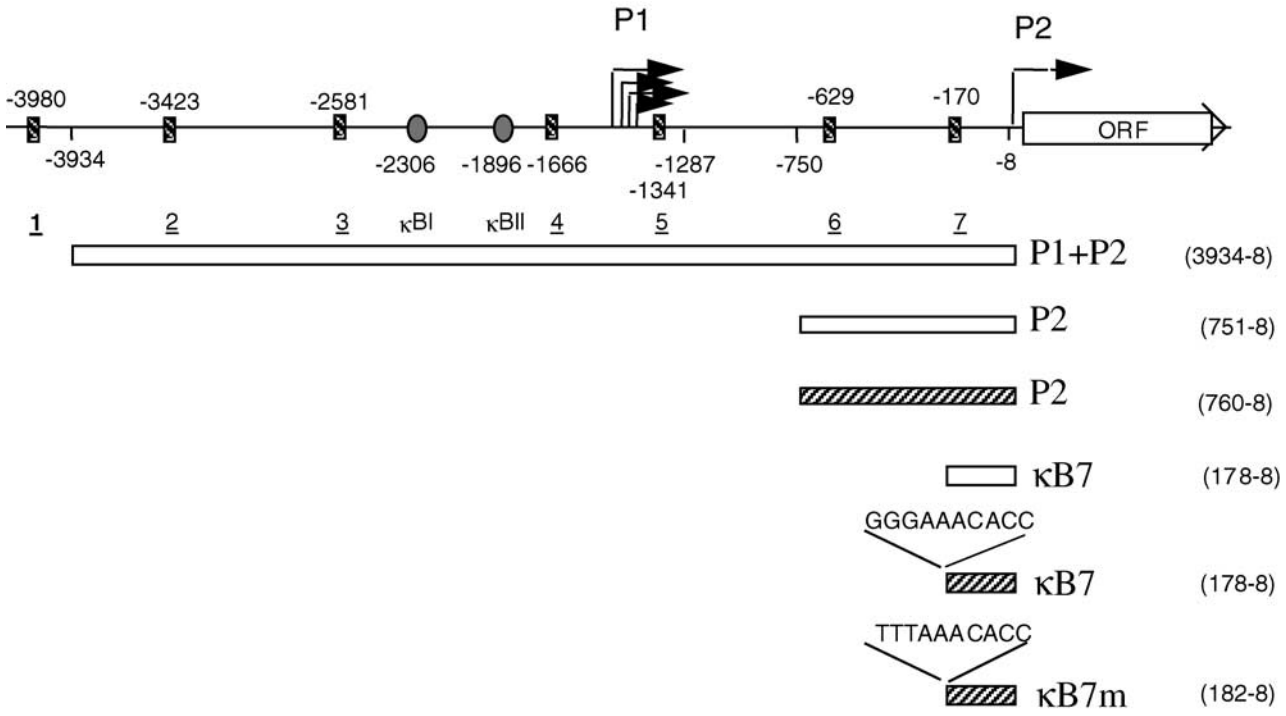

Figure 1 *bcl-2* promoter constructs. The human *bcl-2* upstream region contains two promoters: P1 and P2. P1, 1400 bp upstream of the open reading frame (ORF), displays multiple initiation sites (black arrows); P2 is located just upstream of the ORF (from −750 to −8) and displays one initiation site. Two κB consensus sites are located at −2306 (κBI) and -1896 (κBII) (black circles). The seven sites presenting one mismatch (1–7) are located at positions −3980, −3423, −2581, −1666, −1341, −629 and −170 and are represented as gray boxes. Plasmid constructs are illustrated as follows: P1+P2Luc, P2Luc and κB7Luc (white boxes) were cloned upstream of a Luc gene (a generous gift from L Boxer). P2Gal, κB7Gal and κB7mGal were cloned upstream of a β-gal-reporter gene (gray boxes). κB7m contains a PCR-generated mutated κB7 site (**TTT**AAACACC instead of **GGG**AAACACC).

nucleotide when compared to the consensus sequence (Figure 1). Several double-stranded oligonucleotides containing these sequences were synthesized and used to detect NF-κB binding in nuclear extracts from unstimulated and TNF-α-treated HCT116 cells. Stimulation of these cells rapidly induced a strong DNA binding to a control palindromic κB probe, and supershifting experiments showed that the main shifted band contained p50 and p65 proteins (Figure 2a, lanes 7–11). However, under the same experimental conditions, we could not detect any NF-κB DNA binding to the seven κB probes from the *bcl-2* P1 promoter (Figure 2a, lanes 1–6, and data not shown). This was surprising, as another study characterized a p50/p50 binding in the P1.[30] These results indicate that, in our cell system, NF-κB dimers do not bind to the P1 promoter through these identified κB sites.

While the P2 promoter does not harbor any perfect NF-κB binding site, it contains two sites presenting one mismatch with the NF-κB consensus sequence. To test whether NF-κB could bind one of these sites, EMSAs were performed with probes κB6 and κB7 harboring these sites using HCT116-derived nuclear extracts. As shown in Figure 2b and c, an NF-κB binding activity to probe 7 was observed and the shifted complex comigrated with the lower complex observed using the palindromic consensus κB probe (Figure 2c, left and middle panel), and thus contained probably p50 homodimers as this palindromic probe is very efficiently bound by p50 or p52 homodimers.[29] Supershifting experiments confirmed that only p50 binds to probe 7, while we could not detect any supershift by the p65/RelA antibody (Figure 2c, middle panel and data not shown). Under the same conditions, we showed that NF-κB cannot bind probe 7 where the κB site is mutated (TTTAAACACC instead of GGGAAACACC) (Figure 2c, right panel). Moreover, supershifting experiments performed with nuclear extracts from MCF7AZ cells (which express p52) showed that p52 homodimers are also able to bind to probe 7 (Figure 2d). Indeed, we have reproducibly observed that the p52 antibody could reduce the intensity of a p52-containing retarded complex, without inducing a 'supershift' (data not shown).

Regulation of the bcl-2 *gene promoter by NF-κB*

To further address the control of the *bcl-2* gene transcription by NF-κB, HCT116 cells were transiently transfected with several reporter plasmid constructs corresponding to the P2 (from −750 to −8) or the P1+P2 (from −3934 to −8) promoters (see Figure 1) as well as one construct containing only the κB7 site. The P1 promoter was weakly transactivated in response to p50/p65 overexpression and the deletion of the two consensus κB sites did not modify this activity (data not shown), confirming that NF-κB could not bind to these sites and suggesting an indirect effect. The P2-reporter and the κB7-reporter constructs were transcriptionally activated by p50/p65 complexes but also following transfection of p50 alone (Figure 3a and b). Mutation of the κB7 site suppressed transcription in the presence of transfected p50 (Figure 3b), thus demonstrating that the p50 effect on this fragment relies on the integrity of this degenerated κB response element. Finally, we showed that p52 or p100 was also able to transactivate the κB7 promoter (Figure 3c).

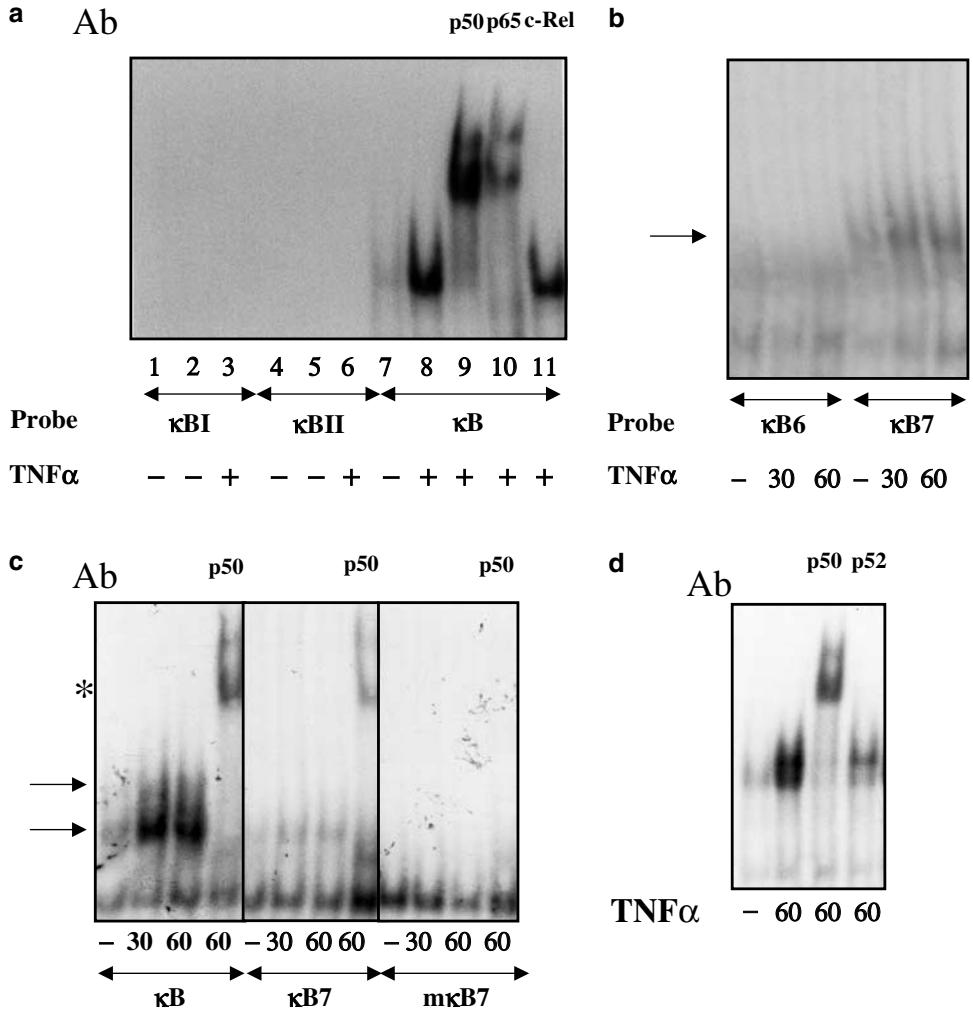

Figure 2 NF-κB binding to the *bcl-2* promoter. (a) NF-κB does not bind to κBI and κBII sites from the *bcl-2* promoter. Nuclear extracts were prepared from HCT116 cells either unstimulated or treated with TNF-α for 30 min. These extracts were analyzed for DNA-binding activity to a palindromic κB probe (κB) or to two κB probes derived from the *bcl-2* promoter (κBI and κBII). Supershift experiments were performed with antibodies directed against p50 (lane 9), p65 (lane 10) or c-Rel (lane 11), as indicated. Lanes 1–3: binding to the κBI probe from the Bcl-2 promoter; lanes 4–6: binding to the κBII probe from the Bcl-2 promoter; lanes 7–11: binding to a palindromic κB probe. (b) NF-κB binds the κB7 probe. Nuclear extracts were prepared from HCT116 cells either unstimulated (–) or treated with TNF-α for 30 min or 60 min. DNA binding was studied with κB probes 6 and 7 from the *bcl-2* promoter, as indicated. The arrow indicates p50 binding complexes. (c) NF-κB does not bind the mutated κB7 probe. Nuclear extracts binding was tested with a palindromic κB probe, a κB7 probe (GGGAAACACC) or a mutated κB7 probe (TTTAAACACC). Nuclear extracts from untreated cells (–) and cells treated with TNF-α for 30 or 60 min were tested, as indicated. Supershift experiments were performed with an antibody directed against p50. Upper and lower black arrows indicate p65/RelA and p50 complexes, respectively. The asterisk indicates the p50 supershift. (d) p52 also binds to the κB7 probe. Nuclear extracts from MCF7 AZ cells were tested with the κB7 probe. Supershift experiments were performed with antibodies directed against p50 and p52, as indicated.

Bcl-3 forms a transactivating complex with p50 or p52 homodimers on the bcl-2 *promoter*

As shown above, (i) p50 or p52 can bind to a site in the *bcl-2* promoter, and (ii) p50 or p52 can transactivate the *bcl-2* promoter in transient transfection assays. However, these two proteins are devoid of any transactivating domain and thus have to interact with an endogenous partner to exert these activities. Bcl-3 can interact with p50 or p52 to form a transcriptionally active complex (see Introduction) and Bcl-3 is expressed at low but detectable levels in HCT116 cells (Figure 4b, third panel from the top, left lane).

We therefore determined whether such complexes could influence the activity of the *bcl-2* promoter. We first cotransfected expression vectors for p50 and Bcl-3 together with the (−178/−8) reporter plasmid. As shown in Figure 4a, while p50 and Bcl-3 alone weakly transactivated the reporter plasmid, cotransfection of Bcl-3 expression vector resulted in a dose-dependent increase of luciferase activity. Moreover, immunoblots performed in parallel with nuclear extracts from HCT116 cells transfected with the p50 expression vector alone or together with increasing amounts of the Bcl-3 expression vector showed that Bcl-3 increasing presence in the nucleus (lower panel) resulted in a dose-dependent increase of p50 protein expression in the nucleus (middle panel) (Figure 4b). The enhanced p50 nuclear expression was also observed by EMSA performed with these same nuclear extracts and a κB7 probe, indicating that increased p50 nuclear expression in the presence of Bcl-3 could lead to increased DNA-binding activity (upper panel) (Figure 4b).

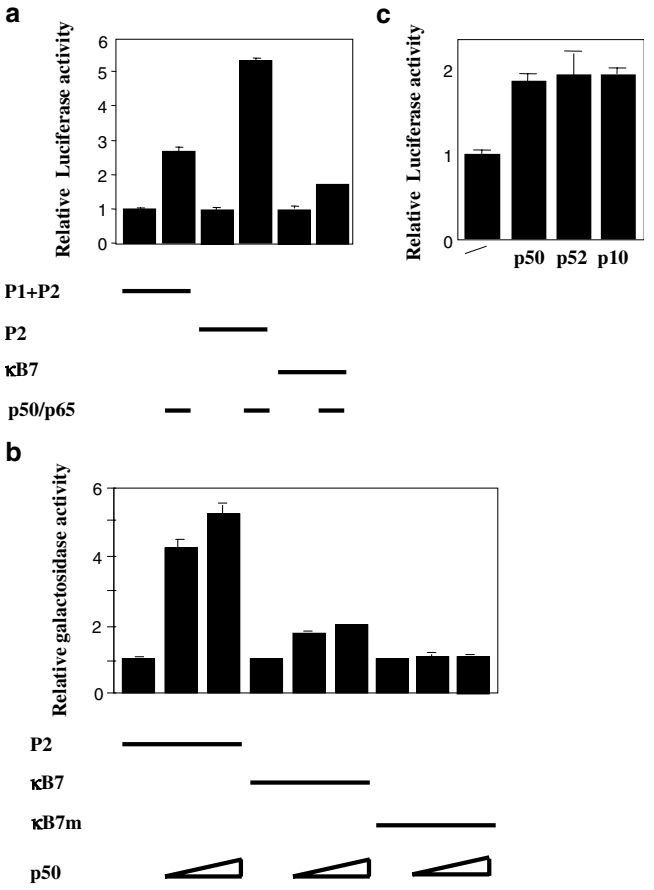

Figure 3 NF-κB subunits transactivate the *bcl-2* promoter. (a) NF-κB transactivates the P2 promoter. Reporter plasmid (1 μg) (P1+P2, P2, κB7) was transfected alone or together with 0.5 μg of expression vectors for p50 and p65 in the HCT116 cell line. Relative fold inductions were obtained by comparing basal luciferase activity *vs* p50/p65-induced activity for each promoter. The amount of transfected DNA was kept constant by the adddition of empty expression vector, when needed. (b) p50 homodimers transactivate through the κB7 site. The P2 or κB7 constructs (either wild-type or mutated) cloned upstream of a β-Gal gene were transfected alone or together with increasing amounts (0.5–1 μg) of expression vector for p50, and galactosidase activity was measured. (c) p100 and p52 transactivate through the κB7 site. A reporter plasmid (1 μg) containing the κB7 site was transfected alone or together with expression vectors for p50, p52 and p100 (1 μg each), as indicated, and luciferase activity was measured.

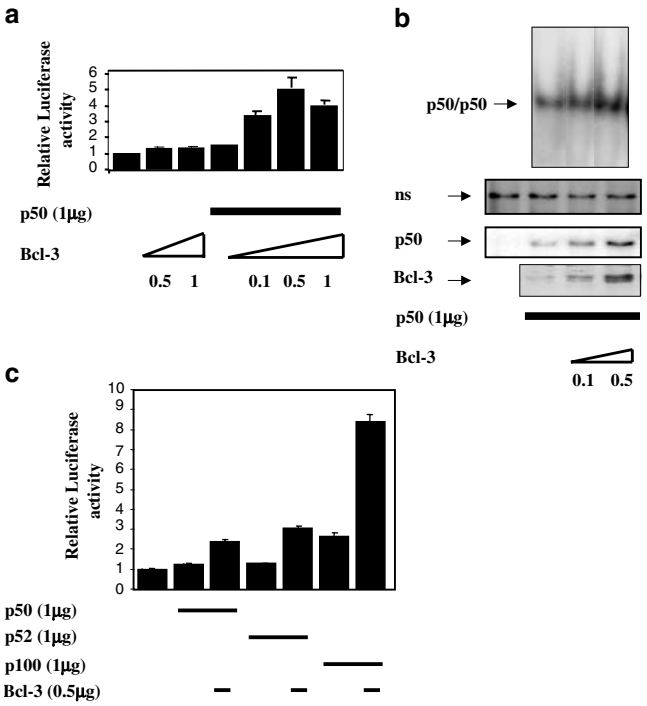

Figure 4 Bcl-3 cooperates with p50 or p52 homodimers to transactivate the *bcl-2* promoter. (a) Cotransfection of expression vectors for p50 and Bcl-3 potentiates p50 transactivation activity. A reporter plasmid (1 μg) containing the κB7 site was transfected alone or together with expression vectors for p50 (1 μg) or Bcl-3 (from 0.1 to 1 μg), as indicated. Luciferase activity was measured as in Figure 3. (b) Bcl-3 overexpression enhances p50 translocation to the nucleus. Nuclear extracts from HCT116 cells transiently transfected with expression vectors for p50 (1 μg) and Bcl-3 (0.1 or 0.5 μg) were studied by EMSA for DNA binding to a palindromic κB probe (upper panel, the arrow indicates p50 homodimer complexes). The same nuclear extracts were used for Western blotting revealed with Bcl-3 (lower panel) and p50 (upper panel) antibodies (NS: nonspecific). (c) Bcl-3 potentiates p100/p52 transactivation activity. Expression vectors for p50, p52, p100 or Bcl-3 were transfected with a κB7Luc-reporter plasmid. Luciferase activity was measured as in Figure 3.

Figure 4c shows that Bcl-3 also enhanced the transactivation activities of p52 and p100 on the κB7 fragment. The Bcl-3 synergistic effect was stronger with p100 than with p50 or p105 (data not shown).

Correlation between p52, Bcl-3 and Bcl-2 expression in human cancers

As we and others previously showed that NF-κB2/p100 is overexpressed in breast cancers as compared to normal adjacent tissue,[31,32] we investigated whether this expression could be correlated with Bcl-2 expression levels. We thus generated an MCF7AZ stable transfectant overexpressing NF-κB2/p100 and the p52 processed protein. This overexpression led to a clear enhanced Bcl-2 expression in p100-transfected cells compared to mock-transfected cells (Figure 5a).

We then studied the expression of p100/p52 and Bcl-2 in seven primary human breast cancers. Six out of seven samples showed a higher p100/p52 expression in the cancer tissue than in normal adjacent tissue (Figure 5b, upper panel), while p50 expression is similar (data not shown). This p100/p52 expression was associated, in five samples (sample numbers 1–6), with a significant Bcl-2 expression (Figure 5b, lower panel). Meanwhile, Bcl-3 expression was shown to be unmodified between adjacent or tumoral tissues (data not shown).

As previously mentioned, the *bcl-3* gene was cloned at the breakpoint of the t(14;19) translocation in CLL, resulting in a high Bcl-3 expression.[15] Western blotting analyses of eight CLL samples showed that samples bearing a t(14;19) translocation expressed high amounts of Bcl-3 (Figure 5c, samples 5–8). Another sample (lane 2) also showed a high Bcl-3 expression. Three out of eight samples (numbers 2, 5 and 6) also showed a significant p100/p52 expression. Interestingly, Bcl-2 expression was easily detectable in all samples where p100/p52 was observed. Since the amount of cells collected was very low, we were only able to obtain nuclear extracts from the four t(14;19)

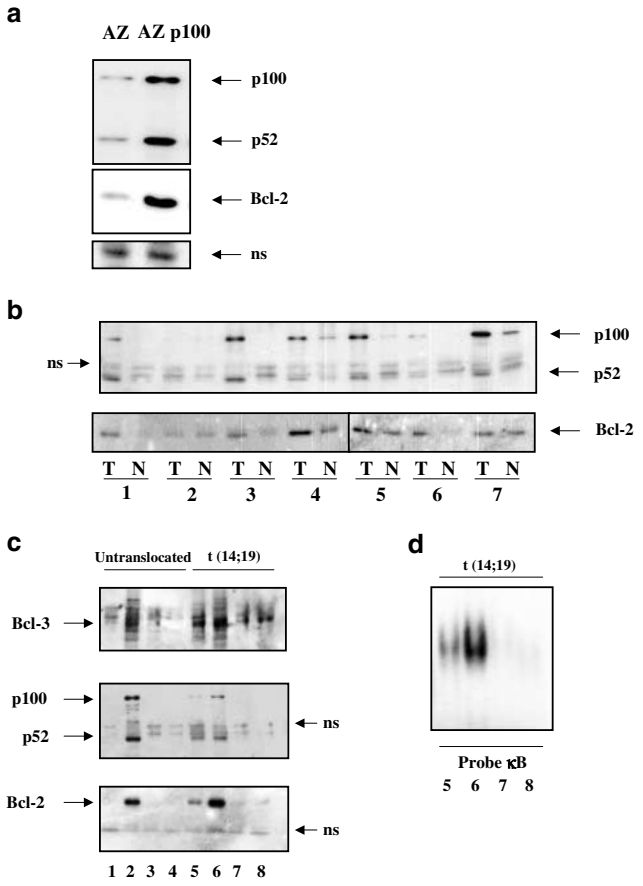

Figure 5 p100/p52 and Bcl-3 expression correlates with Bcl-2 expression in human cancer cells and primary tumors. (a) p100 stable overexpression enhances endogenous Bcl-2 expression. Upper panel: p100/p52 expression in transfected (AZ p100) *vs* control MCF7A/Z cells. Lower panel: basal Bcl-2 expression was examined by Western blotting in the same control and stably transfected cells. As a control for equivalent loadings, we showed a nonspecific band observed on the same gel (NS). (b) Bcl-2 expression correlates with p52 expression in breast cancer samples. Total cell lysates from tumor (T) or adjacent (N) breast tissue were assayed by Western blotting with specific antibodies against NF-κB2/p100 (upper panel) or Bcl-2 (lower panel). (c) Bcl-2 expression correlates with p52 expression in CLL. Total cell lysates from blood or bone marrow samples from eight CLL patients were assayed by Western blotting with specific antibodies against Bcl-2 (lower panel), Bcl-3 (upper panel) and p100/p52 (middle panel). Lanes 1–4: CLL patients; lanes 5–8 CLL patients with a t(14;19) translocation. (d) Binding to a κB probe: Nuclear extracts from t(14;19) CLLs (samples 5–8) were analyzed for DNA binding to a palindromic κB probe.

CLL samples (5–8). We performed EMSA with these nuclear extracts and showed that binding to a palindromic κB probe was stronger with samples 5 and 6, which precisely showed the highest Bcl-2 expression, whereas samples 7 and 8 that do not express high levels of Bcl-2 did not show detectable NF-κB binding activity in similar experiments (Figure 5d). Taken together, our results suggest a potential link between expression levels of p52 and Bcl-2, therefore providing evidence for *bcl-2* being a target gene of this NF-κB protein.

Discussion

The role of NF-κB in the process of apoptosis has been vastly debated these last years. NF-κB has already been reported to regulate the expression of several genes controlling apoptosis.[33–36] Bcl-2-like proteins are important regulators of programmed cell death and the expression of Bcl-XL and A1/Bfl-1, two antiapoptotic members of this family, is regulated by NF-κB,[37–43] while NF-κB downregulates the expression of the proapoptotic Bax protein.[44] Although several authors failed to see any regulation of Bcl-2 expression by NF-κB, a few studies recently showed that in some circumstances, Bcl-2 could indeed be induced by NF-κB.[30,40,45–51] These studies are quite important in the context of CLL. Indeed, Bcl-2 expression in cells from CLL patients has been frequently observed and it has been reported that Bcl-2 expression correlates with prognosis, stage of the disease and resistance to chlorambucil.[52–54] Moreover, constitutive and CD40-induced NF-κB activity has also been described in CLLs and could inhibit apoptosis and participate in resistance to chemotherapy.[55,56] This last point is very relevant as CD40 induces p100 processing into p52.[57] Therefore, if a relation between these two events could be established, it would provide a new insight into molecular mechanisms leading to CLL progression and resistance to treatment and a new rationale for NF-κB targeting therapeutic agents.

The present report identifies a κB site in the P2 *bcl-2* promoter that had already been identified in another study.[49] However, in our hands, this κB site was found to be specifically bound by p50 and p52 homodimers and could be transactivated following exogenous expression of these complexes. These DNA-binding and transactivating activities are further increased by simultaneous Bcl-3 expression, an oncoprotein known to interact with p50 and p52 in the nucleus and to contain transactivating domains.[13,14] These data therefore provide a first insight into a putative biological role for p52 complexes, probably in association with Bcl-3. This is supported by several observations: (i) NF-κB2 stable overexpression led to an enhanced Bcl-2 level (Figure 5a), (ii) NF-κB2 and Bcl-2 expression levels correlate in two types of cancer (Figure 5b and c), and (iii) BAFF, probably by enhancing p100 processing, enhances Bcl-2 expression in transitional B cells.[51]

There is a clear redundancy between p50 and p52 biological roles but also some clear overlap between p52 and Bcl-3 functions, as indicated by the phenotypes of the KO mice. Simultaneous inactivation of the NF-κB1/p105/p50 and NF-κB2/p100/p52 genes generates mice with a decreased Bcl-2 expression in early B cells.[51] Meanwhile, Bcl-2 expression is unmodified in NF-κB1/p105/p50[39] and NF-κB2/p100/p52 (U Siebenlist, personal communication) KO mice. These data suggest that p52 and p50 proteins regulate the *bcl-2* gene *in vivo* in a redundant but essential way. Our data demonstrating an increased Bcl-2 expression in p100/p52 overexpressing MCF7 A/Z cells also indicate that p100/p52 regulates Bcl-2 expression.

The p50 and p52 interacting proteins that confer transactivation abilities to the homodimers for upregulation of Bcl-2 are currently unknown. According to our data, it could be p65 or Bcl-3. Indeed, Bcl-3 expression increases p50 and p52 nuclear translocation and their transactivating properties on the Bcl-2 promoter. As Bcl-3 and p52 are both required for a proper development of the splenic microarchitecture and germinal centers, it is likely that they form complexes that control the expression of survival and developmental genes.

The present data are also important for understanding the NF-κB biochemical function in cancer cells. Indeed, NF-κB is constitutively active in various cancers, such as multiple myelomas, CLL, or breast, ovarian, colon and skin cancers.[48,55,56,58–60] Such activity was also found in Hodgkin's

lymphomas, probably as a consequence of a loss of IκBα activity[61] or constitutive IKK activity.[62] In some of these cancers, NF-κB inhibition by an IκB-α super-repressor is sufficient to induce apoptosis,[6,63] thus demonstrating an antiapoptotic role for NF-κB in these cell systems. Moreover, in two different types of cancers, we observed a putative correlation between levels of p52 and Bcl-2 expression. If confirmed on larger series, this observation will demonstrate that p52, possibly in association with Bcl-3, plays a major role in the progression of very different cancers. Therefore, besides the ubiquitous NF-κB complexes, there exist other proteins from the same family that will have to be considered in our understanding of the cancer antiapoptotic pathways and in our design of specific treatments.

Indeed, as NF-κB is considered as a target for anticancer therapy and novel therapeutic agents are currently being developed in this prospect, a precise understanding of the regulation of NF-κB antiapoptotic target genes in cancer cells will be very helpful to delineate the optimal targets for these treatments and to understand their successes and failures. It is indeed possible that proteasome or IKK inhibitors will not be as efficient on cells expressing high levels of p52 and Bcl-3 than on cells constitutively expressing nuclear p50/p65 complexes. The exact activity of these novel inhibitors and their effect on distinct NF-κB complexes and on target gene expression, including Bcl-2, will thus have to be studied in various cell types expressing different NF-κB-related complexes.

Acknowledgements

We thank Dr U Siebenlist (NIH, Bethesda, MD, USA) for his helpful comments on the manuscript, Dr L Boxer (Stanford, CA, USA) for the Bcl-2 Luc plasmids, Dr Hagemeijer (Leuven, Belgium) and Dr Lambert (CHU Liège, Belgium) for CLL samples. We are most thankful to Professor J Gielen (University of Liège, Belgium) for his continuous support during this work. M-P Merville, A Chariot and L de Leval are Research Associates at the National Foundation for Scientific Research (FNRS, Belgium). P Viatour, M Bentires-Alj and V Deregowski are supported by Télévie fellowships (FNRS, Belgium). This research was supported by grants from the 'Centre Anti-Cancéreux' (University of Liège, Belgium) and the FRSM (FNRS, Belgium).

References

1 Johnstone RW, Ruefli AA, Lowe SW. Apoptosis: a link between cancer genetics and chemotherapy. *Cell* 2002; **108**: 153–164.
2 Reed JC. Dysregulation of apoptosis in cancer. *J Clin Oncol* 1999; **17**: 2941–2953.
3 Beg AA, Baltimore D. An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. *Science* 1996; **274**: 782–784.
4 Van Antwerp DJ, Martin SJ, Kafri T, Green DR, Verma IM. Suppression of TNF-alpha-induced apoptosis by NF-kappaB. *Science* 1996; **274**: 787–789.
5 Wang CY, Mayo MW, Baldwin AS. TNF- and cancer therapy induced apoptosis: potentiation by inhibition of NF-kappaB. *Science* 1996; **274**: 784–787.
6 Bargou RC, Emmerich F, Krappmann D, Bommert K, Mapara MY, Arnold W *et al*. Constitutive nuclear factor-kappaB-RelA activation is required for proliferation and survival of Hodgkin's disease tumor cells. *J Clin Invest* 1997; **100**: 2961–2969.
7 Bentires-Alj M, Hellin AC, Ameyar M, Chouaib S, Merville MP, Bours V. Stable inhibition of nuclear factor kappaB in cancer cells does not increase sensitivity to cytotoxic drugs. *Cancer Res* 1999; **59**: 811–815.
8 Baldwin AS. The NF-kappaB and IkappaB proteins: new discoveries and insights. *Annu Rev Immunol* 1996; **14**: 649–681.
9 Rayet B, Gélinas C. Aberrant rel/nfkb genes and activity in human cancer. *Oncogene* 1999; **18**: 6938–6947.
10 Garg A, Aggarwal BB. Nuclear transcription factor-kappaB as a target for cancer drug development. *Leukemia* 2002; **16**: 1053–1068.
11 Neri A, Chang CC, Lombardi L, Salina M, Corradini P, Maiolo AT *et al*. B cell lymphoma-associated chromosomal translocation involves candidate oncogene lyt-10, homologous to NF-kappaB p50. *Cell* 1991; **67**: 1075–1087.
12 Franzoso G, Carlson L, Poljak L, Shores EW, Epstein S, Leonardi A *et al*. Mice deficient in nuclear factor (NF)-kappa B/p52 present with defects in humoral responses, germinal center reactions, and splenic microarchitecture. *J Exp Med* 1998; **187**: 147–159.
13 Fujita T, Nolan GP, Liou HC, Scott ML, Baltimore D. The candidate proto-oncogene bcl-3 encodes a transcriptional coactivator that activates through NF-kappaB p50 homodimers. *Genes Dev* 1993; **7**: 1354–1363.
14 Bours V, Franzoso G, Azarenko V, Park S, Kanno T, Brown K *et al*. The oncoprotein Bcl-3 directly transactivates through kappaB motifs via association with DNA binding p50B homodimers. *Cell* 1993; **72**: 729–739.
15 Ohno H, Takimoto G, Mc Keithan T. The candidate proto oncogene bcl-3 is related to genes implicated in cell lineage determination and cell cycle control. *Cell* 1990; **60**: 991–997.
16 Franzoso G, Carlson L, Scharton-Kersten T, Shores EW, Epstein S, Grinberg A *et al*. Critical role for the Bcl-3 oncoprotein in T-cell mediated immunity, splenic microarchitecture, and germinal center reactions. *Immunity* 1997; **6**: 479–490.
17 Schwarz EM, Krimpenfort P, Berns A, Verma IM. Immunological defects in mice with a targeted disruption in Bcl-3. *Genes Dev* 1997; **11**: 187–197.
18 Tiong Ong S, Hackbarth ML, Degenstein LC, Baunoch DA, Anastasi J, McKeithan T. Lymphadenopathy, splenomegaly, and altered immunoglobulin production in Bcl-3 transgenic mice. *Oncogene* 1998; **16**: 2333–2433.
19 Pan J, McEver RP. Regulation of the human P-selectin promoter by Bcl-3 and specific homodimeric members of the NF-kappa B/Rel family. *J Biol Chem* 1995; **270**: 23077–23083.
20 Westerheide SD, Mayo MW, Anest V, Hanson JL, Baldwin AS. The putative oncoprotein Bcl-3 induces Cyclin D1 to stimulate G1 transition. *Mol Cell Biol* 2001; **21**: 8428–8436.
21 Mitchell TC, Hildeman D, Kedl RM, Teague TK, Schaefer BC, White J *et al*. Immunological adjuvants promote activated T cell survival via induction of Bcl-3. *Nat Immunol* 2001; **2**: 397–402.
22 Tsujimoto Y, Croce CM. Analysis of the structure, transcripts, and protein products of bcl-2, the gene involved in human follicular lymphoma. *Proc Natl Acad Sci USA* 1986; **83**: 5214–5218.
23 Seto M, Jaeger U, Hockett RD, Graninger W, Bennett S, Goldman P *et al*. Alternative promoters and exons, somatic mutation and deregulation of the Bcl-2-Ig fusion gene in lymphoma. *EMBO J* 1988; **7**: 123–131.
24 Chen HM, Boxer LM. Pi 1 binding sites are negative regulators of bcl-2 expression in pre-B cells. *Mol Cell Biol* 1995; **15**: 3840–3847.
25 Heckman C, Mochon E, Arcinas M, Boxer LM. The WT1 protein is a negative regulator of the normal bcl-2 allele in t(14;18) lymphomas. *J Biol Chem* 1997; **272**: 19609–19614.
26 Dong L, Wang W, Wang F, Stoner M, Reed JC, Harigai M *et al*. Mechanisms of transcriptional activation of bcl-2 gene expression by 17beta-estradiol in breast cancer cells. *J Biol Chem* 1999; **274**: 32099–32107.
27 Mayo MW, Wang CY, Drouin SS, Madrid LV, Marshall AF, Reed JC *et al*. WT1 modulates apoptosis by transcriptionally upregulating the bcl-2 proto-oncogene. *EMBO J* 1999; **18**: 3990–4003.
28 Young RL, Korsmeyer SJ. A negative regulatory element in the bcl-2 5′-untranslated region inhibits expression from an upstream promoter. *Mol Cell Biol* 1993; **13**: 3686–3697.
29 Bours V, Burd PR, Brown K, Villalobos J, Park S, Ryseck RP *et al*. A novel mitogen inducible gene product related to p50/p105-NF-kappaB participates in transactivation through a kappaB site. *Mol Cell Biol* 1992; **12**: 685–695.
30 Kurland JF, Kodym R, Story MD, Spurgers KB, McDonnell TJ, Meyn RE. NF-*k*B1 (p50) homodimers contribute to transcription of the bcl-2 oncogene. *J Biol Chem* 2001; **276**: 45380–45386.

31 Cogswell PC, Guttridge DC, Funkhouser WK, Baldwin AS. Selective activation of NF-kappaB subunits in human breast cancer: potential roles for NF-kappaB2/p52 and for Bcl-3. *Oncogene* 2000; **19**: 1123–1131.
32 Dejardin E, Bonizzi G, Bellahcene A, Castronovo V, Merville M-P, Bours V. Highly expressed p100/p52 (NFKB2) sequesters other NF-kappaB related proteins in the cytoplasm of human breast cancer cells. *Oncogene* 1995; **11**: 1835–1841.
33 Stehlik C, de Martin R, Kumabashiri I, Schmid JA, Binder BR, Lipp J. Nuclear factor (NF)-kappaB-regulated X chromosome-linked iap gene expression protects endothelial cells from tumor necrosis factor alpha induced apoptosis. *J Exp Med* 1998; **188**: 211–216.
34 Wang CY, Mayo MW, Korneluk RG, Goeddel DV, Baldwin AS. NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase 8 activation. *Science* 1998; **281**: 1680–1683.
35 Krikos A, Laherty CD, Dixit VM. Transcriptional activation of the tumor necrosis factor alpha-inducible zinc finger protein, A20, is mediated by kappaB elements. *J Biol Chem* 1992; **267**: 17971–17976.
36 Jones PL, Ping D, Boss JM. Tumor necrosis factor alpha and interleukin-1beta regulate the murine manganese superoxide dismutase gene through a complex intronic enhancer involving C/EBP-beta and NF-kappaB. *Mol Cell Biol* 1997; **17**: 6970–6981.
37 Dixon EP, Stephenson DT, Clemens JA, Little SP. Bcl-Xshort is elevated following severe global ischemia in rat brains. *Brain Res* 1997; **776**: 222–229.
38 Chen F, Demers LM, Vallyathan V, Lu Y, Castranova V, Shi X. Involvement of 5′-flanking kappaB-like sites within bcl-x gene in silica-induced Bcl-x expression. *J Biol Chem* 1999; **274**: 35591–35595.
39 Grumont RJ, Rourke IJ, Gerondakis S. Rel-dependent induction of A1 transcription is required to protect B cells from antigen receptor ligation induced apoptosis. *Genes Dev* 1999; **13**: 400–411.
40 Tamatani M, Che YH, Matsuzaki H, Ogawa S, Okado H, Miyake S *et al*. Tumor necrosis factor induces Bcl-2 and Bcl-x expression through NFkappaB activation in primary hippocampal neurons. *J Biol Chem* 1999; **274**: 8531–8538.
41 Tsukahara T, Kannagi M, Ohashi T, Kato H, Arai M, Nunez G *et al*. Induction of Bcl-x(L) expression by human T-cell leukemia virus type 1 Tax through NF-kappaB in apoptosis-resistant T-cell transfectants with Tax. *J Virol* 1999; **73**: 7981–7987.
42 Wang CY, Guttridge DC, Mayo MW, Baldwin AS. NF-kappaB induces expression of the Bcl-2 homologue A1/Bfl1 to preferentially suppress chemotherapy-induced apoptosis. *Mol Cell Biol* 1999; **19**: 5923–5929.
43 Zong WX, Edelstein LC, Chen C, Bash J, Gelinas C. The prosurvival Bcl-2 homologue Bfl-1/A1 is a direct transcriptional target of NF-kappaB that blocks TNFalpha induced apoptosis. *Genes Dev* 1999; **13**: 382–387.
44 Bentires-Alj M, Dejardin E, Viatour P, Van Lint C, Froesch B, Reed JC *et al*. Inhibition of the NF-kappaB transcription factor increases Bax expression in cancer cell lines. *Oncogene* 2001; **20**: 2805–2813.
45 Rowe M, Peng-Pilon M, Huen DS, Hardy R, Croom-Carter D, Lundge-ren E *et al*. Upregulation of bcl-2 by the Epstein–Barr virus latent membrane protein LMP1: a B-cell-specific response that is delayed relative to NF-kappaB activation and to induction if cell surface markers. *J Virol* 1994; **68**: 5602–5612.
46 Feng Z, Porter AG. NF-kappaB/Rel proteins are required for neuronal differentiation of SH-SY5Y neuroblastoma cells. *J Biol Chem* 1999; **274**: 30341–30344.
47 Feuillard J, Schuhmacher M, Kohanna S, Asso-Bonnet M, Ledeur F, Joubert-Caron R *et al*. Inducible loss of NF-kappaB activity is associated with apoptosis and Bcl-2 down regulation in Epstein–Barr virus-transformed B lymphocytes. *Blood* 2000; **95**: 2068–2075.
48 Davis RE, Brown KD, Siebenlist U, Staudt LM. Constitutive nuclear factor kappaB activity is required for survival of activated B cell like diffuse large B cell lymphoma cells. *J Exp Med* 2001; **194**: 1861–1874.
49 Catz SD, Johnson JL. Transcriptional regulation of bcl-2 by nuclear factor kB and its significance in prostate cancer. *Oncogene* **20**: 7342–7351. Q1
50 Heckman CA, Mehew JW, Boxer LM. NF-kappaB activates Bcl-2 expression in t(14;18) lymphoma cells. *Oncogene* 2002; **21**: 3898–3908.
51 Claudio E, Brown K, Park S, Wang H, Siebenlist U. BAFF-induced NEMO independent processing of NF-kappaB2 in maturing B cells. *Nat Immunol* 2002; **3**: 958–965.
52 Aviram A, Rabizadeh E, Zimra Y, Yeshoron M, Shaklai M, Bairey O. Expression of bcl-2 and bax in cells isolated from B-chronic lymphocytic leukemia patients at different stages of the disease. *Eur J Haematol* 2000; **64**: 80–84.
53 Thomas A, Pepper C, Hoy T, Bentley P. Bcl-2 and bax expression and chlorambucil-induced apoptosis in the T-cells and leukaemic B-cells of untreated B-cell chronic lymphocytic leukaemia patients. *Leukemia Res* 2000; **24**: 813–821.
54 Faderl S, Keating MJ, Do KA, Kantarjan HM, O'Brien S, Garcia-Manero G *et al*. Expression profile of 11 proteins and their prognostic significance in patients with chronic lymphocytic leukemia (CLL). *Leukemia* 2002; **16**: 1045–1052.
55 Furman RR, Asgary Z, Mascarenhas JO, Liou HC, Schattner EJ. Modulation of NF-kappaB activity and apoptosis in chronic lymphocytic leukemia B cells. *J Immunol* 2000; **164**: 2200–2206.
56 Romano MF, Lamberti A, Turco MC, Venuta S. CD40 and B chronic lymphocytic leukemia cell response to fludarabine: the influence of NF-kappaB/Rel transcription factors on chemotherapy-induced apoptosis? *Leukemia Lymphoma* 2000; **36**: 255–262.
57 Coope HJ, Atkinson PG, Huhse B, Belich M, Janzen J, Holman MJ *et al*. CD40 regulates the processing of NF-kappaB2 to p52. *EMBO J* 2002; **21**: 5375–5385.
58 Munzert G, Kirchner D, Stobbe H, Bergmann L, Schmid RM, Dohner H *et al*. Tumor necrosis factor receptor-associated factor 1 gene overexpression in B-cell chronic lymphocytic leukemia: analysis of NF-kappaB/Rel-regulated inhibitors of apoptosis. *Blood* 2002; **100**: 3749–3756.
59 Sovak MA, Bellas RE, Kim DW, Zanieski GJ, Rogers AE, Traish AM *et al*. *J Clin Invest* 1997; **100**: 2952–2960. Q2
60 Nakshatri H, Bhat-Nakshatri P, Martin DA, Goulet RJ, Sledge GW. Constitutive activation of NF-kappaB during progression of breast cancer to hormone-independent growth. *Mol Cell Biol* 1997; **17**: 3629–3639.
61 Wood KM, Roff M, Hay RT. Defective IkappaBalpha in Hodgkin cell lines with constitutively active NF-kappaB. *Oncogene* 1998; **16**: 2131–2139.
62 Krappmann D, Emmerich F, Kordes U, Scharschmidt E, Dorken B, Scheidereit C. Molecular mechanisms of constitutive NF-kappaB/Rel activation in Hodgkin/Reed–Sternberg cells. *Oncogene* 1999; **18**: 943–953.
63 Wu M, Lee H, Bellas RE, Schauer SL, Arsura M, Katz D *et al*. Inhibition of NF-kappaB/Rel induces apoptosis of murine B cells. *EMBO J* 1996; **15**: 4682–4690.