

The prosurvival Bcl-2 homolog Bfl-1/A1 is a direct transcriptional target of NF- κ B that blocks TNF α -induced apoptosis

Wei-Xing Zong,^{1,2} Leonard C. Edelstein,^{1,2}
Cailin Chen,¹ Judy Bash,^{1,3,5}
and Céline Gélinas^{1,4,6}

¹Center for Advanced Biotechnology and Medicine, ²Graduate Program in Biochemistry and Molecular Biology, University of Medicine and Dentistry of New Jersey–Robert Wood Johnson Medical School (UMDNJ–RWJMS), ³Graduate Program in Microbiology and Molecular Genetics, Rutgers University, ⁴Department of Biochemistry, UMDNJ–RWJMS, Piscataway New Jersey 08854-5638 USA

Bcl-2-family proteins are key regulators of the apoptotic response. Here, we demonstrate that the pro-survival Bcl-2 homolog Bfl-1/A1 is a direct transcriptional target of NF- κ B. We show that *bfl-1* gene expression is dependent on NF- κ B activity and that it can substitute for NF- κ B to suppress TNF α -induced apoptosis. *bfl-1* promoter analysis identified an NF- κ B site responsible for its Rel/NF- κ B-dependent induction. The expression of *bfl-1* in immune tissues supports the protective role of NF- κ B in the immune system. The activation of Bfl-1 may be the means by which NF- κ B functions in oncogenesis and promotes cell resistance to anti-cancer therapy.

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Binding of the proinflammatory cytokine tumor necrosis factor α (TNF α) to its receptor triggers competing signaling pathways that determine whether a cell lives or dies. Whereas one pathway is conducive to cell death, the other leads to activation of Rel/NF- κ B transcription factors and the coincident inhibition of apoptosis (for review, see Nagata 1997). Accumulating evidence supports a proactive role for NF- κ B in the inhibition of cell death induced by TNF α and other death-causing agents (for review, see Van Antwerp et al. 1998). Whereas the activation of NF- κ B blocks cell killing, its inhibition enhances the cytotoxicity of TNF α and promotes apoptosis in various cell systems, demonstrating the need for NF- κ B function for cell survival (Beg et al. 1995; Beg and Baltimore 1996; White et al. 1995; Liu et al. 1996; Van Antwerp et al. 1996; Wang et al. 1996; Wu et al. 1996; Cai

et al. 1997; Zong et al. 1997). The protective effect of NF- κ B is dependent on RNA and protein synthesis, suggesting that it regulates the expression of genes that confer resistance to death-inducing signals (for review, see Nagata, 1997). The finding that an intact transactivation domain is required for Rel/NF- κ B factors to block cell death agrees with this hypothesis (Zong et al. 1998).

Proteins of the Bcl-2 family play critical roles in determining cell fate in the apoptotic pathway. Although some members antagonize cell death, others exhibit a proapoptotic activity (for reviews, see Reed 1996; Adams and Cory 1998). By subtractive cDNA cloning, we identified the prosurvival Bcl-2-homolog Bfl-1/A1 as a direct transcriptional target of NF- κ B. We show that ectopically expressed Rel proteins and stimuli that activate endogenous NF- κ B factors up-regulate *bfl-1* gene expression and that this is inhibited by a dominant I κ B α Δ N transgene. Expression of Bfl-1 alone conferred resistance to TNF α cytotoxicity, indicating that it can substitute for NF- κ B to suppress apoptosis. *bfl-1* promoter analysis identified a consensus NF- κ B site responsible for its Rel-dependent induction. Together, these results demonstrate that NF- κ B directly activates Bfl-1/A1 to inhibit programmed cell death. The preferential expression of *bfl-1* in immune tissues supports the protective role of NF- κ B in the immune system.

Results and Discussion

We used a PCR-selected subtractive cDNA cloning approach to identify anti-apoptotic genes under Rel/NF- κ B control. The HeLa-derived HtTA–CCR43 cell line, which conditionally expresses c-Rel under the control of a tetracycline-regulated promoter, is resistant to TNF α -induced cell death upon induction of c-Rel (Bash et al. 1997; Zong et al. 1998). mRNA from HtTA–CCR43 cells conditionally expressing c-Rel was reverse transcribed and subjected to subtraction and PCR amplification. A subtracted cDNA fragment of ~700 bp was found to be identical to *bfl-1*, a member of the Bcl-2 family of apoptosis inhibitors. Bfl-1 was originally isolated from fetal liver and from cytokine-treated endothelial cells (Choi et al. 1995; Karsan et al. 1996) and shares 72% amino acid identity with its mouse homolog A1 (Lin et al. 1993).

Northern blot analysis during a time course of c-Rel induction confirmed the up-regulation of *bfl-1* transcripts in HtTA–CCR43 cells, with kinetics that paralleled those of *c-rel* and of the Rel/NF- κ B target gene *ikba* (Fig. 1a). Induction of the transactivation-competent RelA subunit of NF- κ B also led to a sharp increase in *bfl-1* mRNA levels in the tetracycline-regulated HtTA–RelA cell line (Fig. 1b, lanes 4–6; Zong et al. 1998). In contrast no expression was detected in response to the p50/NF- κ B1 protein, which lacks a defined transcription activation domain (Fig. 1b, lanes 1–3). Thus, *bfl-1* gene expression was specifically up-regulated upon ectopic expression of transcriptionally active Rel/NF- κ B subunits.

[Key Words: Rel; NF- κ B; Bfl-1; A1; TNF α ; apoptosis]

⁵Present address: Cancer Institute of New Jersey, New Brunswick, New Jersey 08854 USA.

⁶Corresponding author.

E-MAIL gelinasc@mbcl.rutgers.edu; FAX (732) 235-5289.

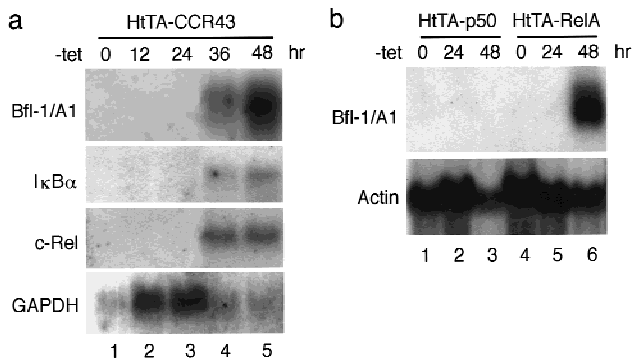


Figure 1. *bfl-1* gene expression is activated by c-Rel and RelA but not by p50/NF- κ B1. (a) Expression of *bfl-1* transcripts in HtTA-CCR43 cells maintained in the presence (lane 1) or absence of tetracycline for 12, 24, 36, or 48 hr to induce *c-rel* expression (lanes 2–5). The blot was successively hybridized to 32 P-labeled probes for *bfl-1*, *ikb α* , *c-rel*, and *gapdh*. (b) *bfl-1* gene expression in HtTA-p50 and HtTA-RelA cells maintained in the presence (lanes 1,4) or absence of tetracycline for 24 or 48 hr to induce the expression of *p50* or *relA* (lanes 2,3,5,6). The blot was hybridized to *bfl-1* and *actin* probes.

Next, we verified the ability of endogenous NF- κ B factors to activate *bfl-1* gene expression in response to various stimuli and in different cells. TNF α strongly induced *bfl-1* transcripts in human HT1080 fibrosarcoma cells (Fig. 2a, lanes 1,2). *bfl-1* mRNA levels were also strongly elevated in human Jurkat T-cells stimulated with phorbol 12-myristate 13-acetate (PMA) plus ionomycin (lanes 3,4). Likewise, the treatment of mouse 70Z/3 pre-B cells with bacterial lipopolysaccharides (LPS) promoted the accumulation of *bfl-1/a1* transcripts (lanes 5,6). Consistent with the nuclear NF- κ B activity found in mouse WEHI-231 B cells (Liou et al. 1994; Miyamoto et al. 1994), basal *bfl-1/a1* expression was observed in these cells (lanes 7,9,11). *bfl-1/a1* mRNAs were further induced by different NF- κ B-inducing agents, including TNF α , LPS, or PMA (lanes 8,10,12).

The activity of NF- κ B is controlled by its association with I κ B factors in the cytoplasm. In contrast to wild-type I κ B α , which undergoes proteasome-mediated degradation in response to stimuli, an amino-terminally deleted I κ B α transgene is resistant to signal-induced degradation and acts as a constitutive repressor of NF- κ B in Jurkat-I κ B α Δ N cells (Chu et al. 1997). We investigated whether NF- κ B was necessary for the stimuli-induced activation of *bfl-1* by characterizing the effects of wild-type I κ B α and a trans-dominant I κ B α Δ N inhibitor on *bfl-1* gene expression. The levels of 28S rRNA and of mRNA for the NF- κ B-regulated gene *interleukin-8* (*IL-8*) were monitored as controls. Similar to the induction of *bfl-1* in the parental Jurkat T-cell line (Fig. 2a, lanes 3,4), *bfl-1* transcripts were sharply elevated by PMA plus ionomycin treatment of T cells expressing a wild-type I κ B α transgene (Fig. 2b, lanes 1,2). In contrast, the induction of *bfl-1* was reduced by 83% and that of *IL-8* was decreased by 65% in cells expressing I κ B α Δ N (lanes 3,4). This indicated that nuclear NF- κ B activity is important for *bfl-1* gene expression.

Similar to *c-rel*, *relA*, and their target *ikb α* , *bfl-1* was found to be abundantly expressed in discrete immune tissues. A Northern blot survey showed high levels of *bfl-1* transcripts in human spleen, lymph nodes, peripheral blood leukocytes and bone marrow (Fig. 3, lanes 6,7,9,10). Little expression was seen in thymus, fetal liver, ovary, and small intestine, whereas none was detected in prostate, testis, or colon (lanes 1–5,8,11). These results suggest a physiological role for *bfl-1* in promoting the survival of defined cell lineages in the immune system.

In agreement with the anti-apoptotic activity of NF- κ B toward TNF α , the transient cotransfection of a CMV-*bfl-1* expression vector with a CMV- β -galactosidase reporter plasmid significantly suppressed the TNF α -induced killing of human HeLa cells in the presence of the protein synthesis inhibitor cycloheximide (CHX; Fig. 4a). Quantitation of cell survival showed that the transient expression of *bfl-1* increased the viability of HeLa cells 8.5-fold in comparison to cells transfected with the control pCMV plasmid (Fig. 4b). Cell protection was also observed in the human HtTA-1 and HT1080 cell lines (Fig. 4b). Moreover, Bfl-1 also suppressed TNF α -induced apoptosis under conditions in which endogenous NF- κ B activity was inhibited by a serine-to-alanine mutant of

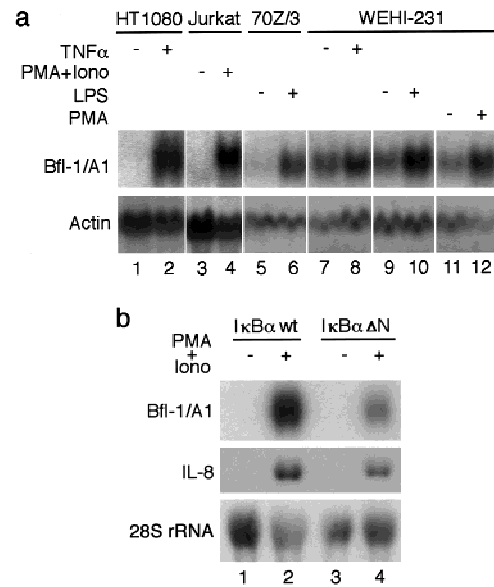


Figure 2. The expression of *bfl-1* is dependent on endogenous Rel/NF- κ B activity. (a) The activation of NF- κ B induces *bfl-1* gene expression. Human HT1080 cells untreated or treated with TNF α (lanes 1,2), human Jurkat T cells treated with DMSO as a control (lane 3) or with PMA plus ionomycin (lane 4), mouse 70Z/3 pre-B cells untreated or treated with LPS (lanes 5,6), mouse WEHI-231 B cells untreated or treated with TNF α (lanes 7,8), LPS (lanes 9,10), or PMA (lanes 11,12). Total RNA (20 μ g) was hybridized to *bfl-1* and *actin* probes. (b) The induction of *bfl-1* gene expression is repressed by a dominant I κ B α Δ N inhibitor. Jurkat T cells expressing wild-type I κ B α or an I κ B α Δ N transgene were treated with DMSO alone (lanes 1,3) or with PMA plus ionomycin (lanes 2,4). Total RNA (20 μ g) was hybridized to 32 P-labeled probes for *bfl-1*, *il-8*, and 28S rRNA.

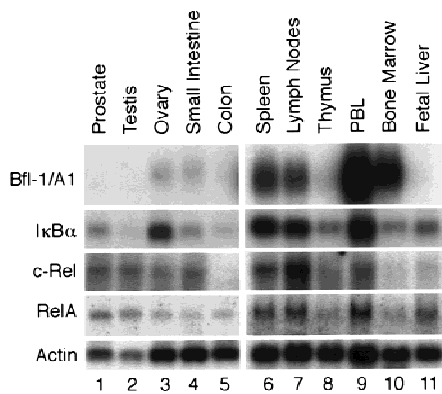


Figure 3. *bfl-1* is highly expressed in human immune tissues and correlates with endogenous Rel/NF- κ B activity. Multiple tissue Northern blots (Clontech) human II (lanes 1–5) and human immune system II (lanes 6–11) were successively hybridized to 32 P-labeled probes for *bfl-1*, *ikba*, *c-rel*, *rela*, and *actin*.

I κ B α that is resistant to signal-induced degradation (I κ B α M; Van Antwerp et al. 1996). Whereas HeLa cells transfected with I κ B α M were sensitized to TNF α alone, the cotransfection of Bfl-1 rescued the cells from cytolysis (Fig. 4c). These results indicated that Bfl-1 can substitute for NF- κ B to block TNF α -induced cytolysis. These data agree with the protective effect of Bfl-1 and its homolog A1 toward various inducers of cell death (D'Sa-Eipper et al. 1996; Karsan et al. 1996; Lin et al. 1996). Together, our findings support a role for Bfl-1 as a survival factor in the NF- κ B-signaling pathway that confers resistance to TNF α -induced apoptosis.

Immunofluorescence studies localized Bfl-1 to the cytoplasm (data not shown). This is compatible with the subcellular localization of its homolog Bcl-2 (Krajewski et al. 1993). Consistent with this observation, the inhibitory activity of Bfl-1 toward TNF α -induced cell death was similar to that of Bcl-2 in HeLa cells. Whereas Bfl-1 enabled ~45%–50% of the cells to survive treatment with TNF α plus CHX, Bcl-2 enabled ~50%–60% of the cells to escape cytolysis in transient transfection assays (data not shown). However, further studies are needed to determine whether Bfl-1 utilizes the same mechanisms as Bcl-2 to inhibit cell death.

Analysis of the *bfl-1* promoter region identified sequence elements responsible for its Rel-dependent induction. Nested PCR amplification of adaptor-ligated human genomic DNA libraries generated products of ~1.4, 1.3, 0.4, and 0.2 kb (GenomeWalker-kit, Clontech). All shared a common 3' end derived from the 5' end of the *bfl-1* cDNA and extended 5' into adjacent genomic sequences. The purified products were directionally cloned into a promoterless reporter plasmid for analysis (Fig. 5a). The detailed characterization of the promoter will be described elsewhere (L.C. Edelstein and C. G elinas, in prep.).

bfl-1 promoter activity was assayed by transient transfection of HeLa cells in the presence or absence of a CMV-*c-rel* vector. An IL6 κ BCAT reporter plasmid containing three κ B DNA sites served as a positive control.

As shown in Figure 5b, all four clones showed minimal basal activity on their own. The cotransfection of pCMV-*c-rel* enhanced expression from the -1374/+81 and -1240/+81 promoter constructs by 12- and 9-fold, respectively. In contrast, the activity of the -367/+81 and -129/+81 constructs was only marginally increased by *c-rel*. Our mapping of a consensus NF- κ B DNA site at position -833 relative to the transcription start site of *bfl-1* agreed with these results (GGGGATTACC; Fig. 5a). Consistent with these findings, cell treatment with a physiological inducer of NF- κ B also activated the *bfl-1* promoter. As shown in Figure 5c, TNF α stimulated CAT expression from the *bfl-1* promoter, similar to its effect on the control IL6 κ BCAT reporter plasmid. Inactivation of the consensus NF- κ B motif in the context of the *bfl-1* promoter region provided direct evidence that *bfl-1* is under Rel/NF- κ B control (GTTTATTACC; -1374/+81m κ B). Mutation of this NF- κ B site decreased gene expression significantly in the presence of c-Rel (Fig. 5d).

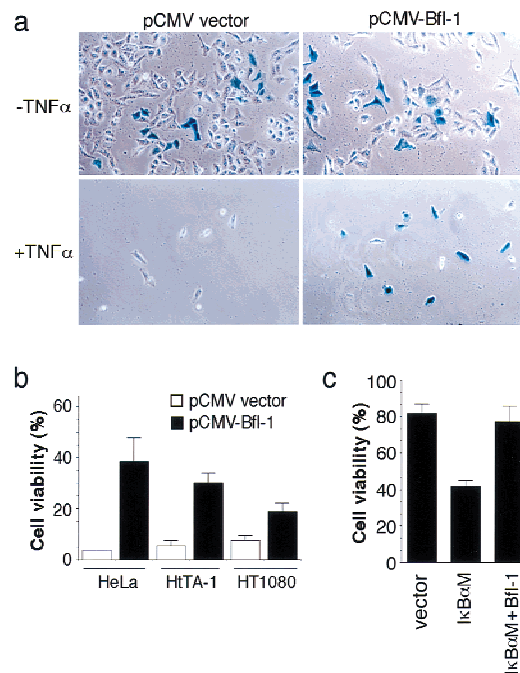


Figure 4. Bfl-1 suppresses TNF α -induced cell death. (a) HeLa cells were cotransfected with pCMV- β -gal, together with an empty pCMV vector or pCMV-*bfl-1*. The cells were treated with CHX alone or together with TNF α for 16 hr, stained with X-gal, and photographed. (b) Quantitation of cell survival upon expression of *bfl-1*. The viability of HeLa, HtTA-1, and HT1080 cells transfected as described in a represents the ratio of cells expressing β -gal in wells treated with TNF α plus CHX over that in wells treated with CHX alone. Cells from a minimum of 10 randomly chosen fields were counted. The average survival from three independent experiments is shown. (c) HeLa cells were cotransfected with pCMV- β -gal and an empty pCMV vector or pCMV-I κ B α M, alone or together with pCMV-*bfl-1*. The cells were treated with TNF α alone for 16 hr and stained with X-gal. Cell survival represents the ratio of cells expressing β -gal in wells treated with TNF α over that in wells left untreated. The average survival from three experiments is shown.

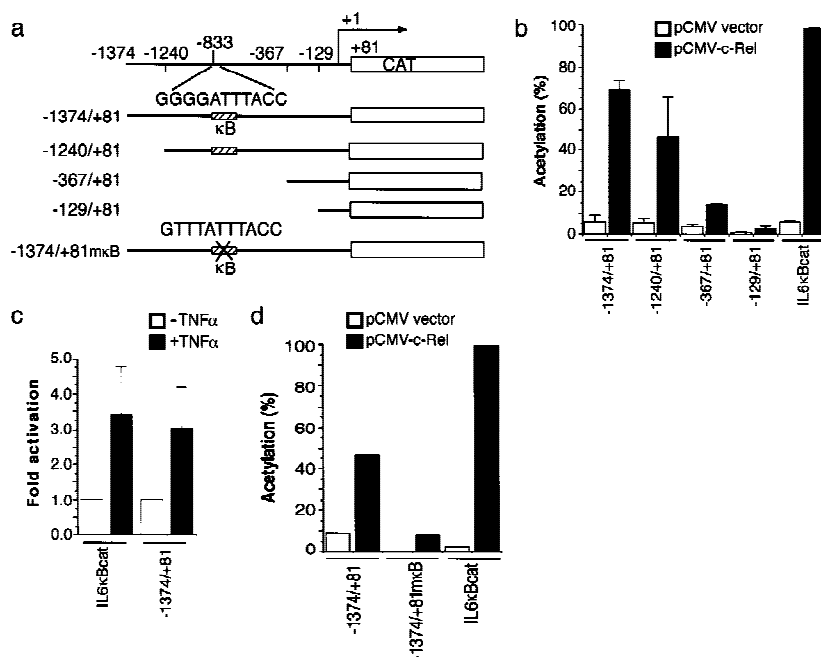


Figure 5. The human *bfl-1* promoter contains a consensus NF- κ B site responsible for its Rel-dependent induction. (a) Schematic representation of CAT reporter gene constructs driven by various regions of the human *bfl-1* promoter. (b) c-Rel-dependent transactivation of the *bfl-1* promoter. HtTA-1 cells were cotransfected with *bfl-1*-CAT reporter plasmids together with pCMV-*c-rel* or an empty pCMV vector as a control. The IL6 κ BCAT plasmid containing three NF- κ B DNA sites derived from the IL6 promoter was used as a positive control. The average CAT activity from three independent experiments is shown. (c) TNF α -inducible activation of the *bfl-1* promoter. HtTA-1 cells were transfected with -1374/+81 *bfl-1*-CAT or the control IL6 κ BCAT reporter plasmid. Where indicated, cells were stimulated with TNF α for 6 hr prior to harvest. The average fold activation from three independent experiments is shown. (d) κ B site-dependent activation of the *bfl-1* promoter. Cells were cotransfected with wild-type -1374/+81 *bfl-1*-CAT or the mutant -1374/+81m κ B-CAT reporter plasmid, with a mutated NF- κ B site at position -833 (GTTTATTACC), together with pCMV-*c-rel* or an empty CMV vector as a control. IL6 κ BCAT was used as a control.

Together, these findings demonstrate that *bfl-1* is a pro-survival gene under direct Rel/NF- κ B control.

Prosurvival members of the Bcl-2 family have been shown to block apoptosis in lymphoid cells under conditions in which NF- κ B activity was inhibited (Wu et al. 1996; for review, see Sonenshein 1997). This raised the possibility that some members of the Bcl-2 family may lie downstream of NF- κ B in the survival cascade. Our demonstration that *bfl-1/a1* is a transcriptional target of NF- κ B provides the first direct evidence that a Bcl-2-family member is controlled by NF- κ B proteins. These findings are consistent with previous reports indicating that *bfl-1/a1* gene expression is induced by proinflammatory cytokines in endothelial, leukemic, and hemopoietic cells (Moreb and Schweder 1997; Lin et al. 1993; Karsan et al. 1996b).

Our data also show clearly that *bfl-1* can suppress TNF α -induced cytolysis. The anti-apoptotic activity of Bfl-1 in this context agrees with work indicating that Bfl-1/A1 can confer resistance to a variety of death inducers in different cells (D'Sa-Eipper et al. 1996; Karsan

et al. 1996a; Lin et al. 1996). Thus, Bfl-1 may be viewed as an important player in the survival pathway. It remains possible that *bfl-1* may act in combination with other anti-apoptotic genes to block cell death efficiently in response to different stimuli and in different cells. For example, whereas NF- κ B was recently implicated in inducing expression of the death inhibitors c-IAP1, c-IAP2, and the TRAF1 (TNFR-associated factor 1) and TRAF2 factors, all four proteins must act in combination to efficiently block TNF-induced apoptosis in cells where NF- κ B is inactive (Chu et al. 1997; Wang et al. 1998; You et al. 1997). The presence of an NF- κ B site in the promoter region of the zinc finger protein A20 suggests that it may also be under NF- κ B control (Krikos et al. 1992), although A20 failed to rescue RelA^{-/-} cells from TNF α -induced cytolysis (Beg and Baltimore 1996). Similarly, the immediate-early response gene *IEX-1L* was shown to be involved in NF- κ B-mediated cell survival, but its mechanism of action remains to be clarified (Wu et al. 1998). Preliminary data from our laboratory agree with these reports and suggest that other anti-apoptotic factors are also regulated by Rel/NF- κ B (C. Chen and C. G elinas, in prep.). It will thus be important to evaluate how their activities are coordinated by the NF- κ B-signaling pathway and to determine whether they function individually or cooperatively in response to different stimuli and in different cellular environments.

The coinciding expression of *bfl-1* and *c-rel* in the white pulp of the spleen, the germinal centers of lymphatic tissues, and inflammatory cells (Carrasco et al. 1994; Jung-ha et al. 1998) supports a model whereby Bfl-1 may be a critical factor for carrying out the protective role of Rel/NF- κ B in the immune system and during the inflammatory response. Bfl-1 was shown previously to cooperate with the adenovirus E1A protein in inducing cell transformation and to be overexpressed in certain cancers (Choi et al. 1995; D'Sa-Eipper et al. 1996). Although the participation of Bfl-1 in oncogenesis is still a topic of controversy (Jung-ha et al. 1998), the activation of Bfl-1 by NF- κ B may also be a means by which NF- κ B functions in oncogenesis and promotes the resistance of tumor cells to anti-cancer therapy.

Materials and methods

Cells and endogenous NF- κ B activation

Parental HtTA-1 cells and the HtTA-1-derived HtTA-CCR43, HtTA-RelA, and HtTA-p50 cell clones that expressed *c-rel*, *relA*, and *p50*, respectively, under tetracycline-regulated control have been described

[Gossen and Bujard 1992; Bash et al. 1997; Zong et al. 1998]. Human HeLa cervical carcinoma cells, HT1080 fibrosarcoma cells and Jurkat T-lymphocytic leukemia cells, and mouse 70Z/3 pre-B cells and WEHI-231 mature B-cells were obtained from ATCC. Jurkat-IkBa Δ w and Jurkat-IkBa Δ N T cells were a gift of D.W. Ballard [Chu et al. 1997]. Endogenous NF- κ B activity was induced by treatment with TNF α (Sigma; 10 ng/ml) for 2 hr (WEHI-231) or 3 hr (HT1080), with PMA (50 ng per ml) plus ionomycin (1 μ M in DMSO) for 2 hr or DMSO alone as a control (0.05%), with LPS (10 μ g/ml) for 4 hr, or with PMA (100 nM) for 2 hr.

Subtractive hybridization and cloning of a *bfl-1* cDNA

Poly(A)⁺ RNA was isolated from HtTA-CCR43 cells using a QuickPrep RNA purification kit (Pharmacia). Purified mRNA (2 μ g) was reverse transcribed and subjected to subtractive hybridization with a PCR-Select cDNA subtraction kit (Clontech). Subtracted cDNA fragments were cloned in the pCRII vector (Invitrogen) and sequenced (Sequenase 2.0; U.S. Biochemical). A full-length HA-tagged *bfl-1* cDNA clone was obtained by RT-PCR of total RNA from human HeLa cells in two successive rounds of amplification. The 5' primer used in the first round was GCGTTCCAGATTACGCTAGCTTGATGACAGACTGTGAATTTGGA, and the 3' primer was CTGCTTAAGAGCTCTCAACATGATTGCTTCAGG. In the second round, the 5' primer containing an HA tag was GGATCCGCCATGGCATACCCATATGATGTTCCAGATTCGCT. The 3' *bfl-1*-specific primer was identical to that used in the first round. The amplification product was cloned in a pCMV vector for transient transfection assays (pCDNA3; Invitrogen). The *bfl-1* cDNA sequence was confirmed with a T7 sequencing kit (Pharmacia).

Northern blot analysis

Total RNA (20 μ g) extracted with RNAzol B (TEL-TEST) was fractionated in a 1% agarose-formaldehyde gel and transferred onto a Hybond-NX membrane (Amersham). The membrane was baked for 10 min at 80°C under vacuum and UV cross-linked with a Stratilinker (Stratagene). Multiple tissue Northern blots were purchased from Clontech (human, human II, human immune system II). Probes were generated by random priming with Klenow polymerase in the presence of [³²P]dCTP and [³²P] dGTP [Feinberg and Vogelstein 1983]. Membranes were hybridized in 5 \times SSC (0.75 M NaCl, 75 mM Na citrate at pH 7.0), 5 \times Denhardt's solution, 0.5% SDS, and 100 μ g/ml sheared salmon sperm DNA at 65°C overnight. Membranes were washed twice in 2 \times SSC, 0.1% SDS, and twice in 1 \times SSC, 0.1% SDS, at 65°C, followed by autoradiography.

TNF α -induced apoptosis

Cell resistance to TNF α -induced apoptosis was assayed as described [White et al. 1992]. Cells (3 \times 10⁶) were incubated with pCMV- β -gal (5 μ g), together with pCMV-*bfl-1* (15 μ g) or an empty pCMV vector as a control, and electroporated at 220 V, 960 μ F using a Bio-Rad Gene Pulser. The cells were then distributed equally into two 35-mm wells. After 24 hr, the cells were treated with CHX alone (30 μ g/ml) or together with TNF α (10 ng/ml) for 16 hr. The cells were fixed and stained with X-gal and photographed at a magnification of 200 \times . In assays of cell death performed in the absence of CHX, HeLa cells were coelectroporated with pCMV- β -gal (3 μ g), an empty CMV vector, or pCMV-IkBaM (12 μ g) to constitutively repress NF- κ B, alone or together with pCMV-*bfl-1* (6 μ g). Cells were then distributed equally into two 35-mm wells and treated 24 hr later with TNF α (10 ng/ml) for 16 hr.

Cloning of the human *bfl-1* promoter, transient CAT assays, and mutagenesis

The human *bfl-1* promoter region was isolated by nested PCR amplification with a GenomeWalker-PromoterFinder kit (Clontech) and cloned in a promoterless vector expressing a CAT reporter gene (pCAT-basic; Promega). *bfl-1* promoter activity was analyzed by transient transfection of HtTA-1 cells with *bfl-1*-CAT reporter plasmids (3 μ g) in the presence of a CMV-*c-rel* expression vector (1 μ g; Xu et al. 1993) or an empty pCMV vector as a control. Where indicated, cells were treated with TNF α (10 ng/ml) for 6 hr before harvest. An IL6 κ B/CAT reporter plasmid containing three NF- κ B DNA sites from the IL6 promoter was used as a positive control [Xu et al. 1993]. The -1374/+81 *bfl-1* promoter region cloned in pAlter-1 was subjected to site-directed mutagenesis to inactivate the consensus NF- κ B motif at position -833 (GTTTATTACC, -1374/+81m κ B; Altered Sites Mutagenesis System, Promega). Mutation of the consensus NF- κ B site was confirmed by sequencing.

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