Cell Cycle, Cell Death, and Senescence

Molecular Cancer Research

Bortezomib Induces Nuclear Translocation of IκBα Resulting in Gene-Specific Suppression of NF-κB–Dependent Transcription and Induction of Apoptosis in CTCL

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Abstract

Cutaneous T-cell lymphoma (CTCL) is characterized by constitutive activation of nuclear factor κB (NF-κB), which plays a crucial role in the survival of CTCL cells and their resistance to apoptosis. NF-κB activity in CTCL is inhibited by the proteasome inhibitor bortezomib; however, the mechanisms remained unknown. In this study, we investigated mechanisms by which bortezomib suppresses NF-κB activity in CTCL Hut-78 cells. We demonstrate that bortezomib and MG132 suppress NF-κB activity in Hut-78 cells by a novel mechanism that consists of inducing nuclear translocation and accumulation of IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha), which then associates with NF-κB p65 and p50 in the nucleus and inhibits NF-κB DNA binding activity. Surprisingly, however, while expression of NF-κB–dependent anti-apoptotic genes *cIAP1* and *cIAP2* is inhibited by bortezomib, expression of *Bcl-2* is not suppressed. Chromatin immunoprecipitation indicated that *cIAP1* and *cIAP2* promoters are occupied by NF-κB p65/50 heterodimers, whereas *Bcl-2* promoter is occupied predominantly by p50/50 homodimers. Collectively, our data reveal a novel mechanism of bortezomib function in CTCL and suggest that the inhibition of NF-κB–dependent gene expression by bortezomib is gene specific and depends on the subunit composition of NF-κB dimers recruited to NF-κB–responsive promoters. *Mol Cancer Res; 9(2); 183–94. ©2011 AACR.*

Introduction

Nuclear factor κB (NF-κB) is a dimeric transcription factor that plays a key role in the expression of genes involved in cell survival, proliferation, and immune responses (1–3). Because NF-κB transcriptional activity is increased in many types of cancer and leukemia, inhibition of NF-κB represents an important therapeutic target (4–8). In most unstimulated cells, NF-κB proteins are localized in the cytoplasm bound to the inhibitory protein IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha). Upon stimulation, IκBα is phosphorylated on Ser-32 and Ser-36 by the enzymes of IκB kinase (IKK) complex, ubiquitinated, and selectively degraded by the 26S proteasome (9,10). This results in the release of NF-κB dimers from the inhibitory complex and in the translocation of NF-κB to the nucleus, where it stimulates transcription of NF-κB–dependent antiapoptotic and proinflammatory genes.

The constitutive activation of NF-κB observed in many types of cancer including different types of leukemia and lymphoma has been associated with increased cytoplasmic degradation of IκBα, resulting in the increased nuclear translocation of NF-κB proteins and high levels of NF-κB DNA binding activity (11–13). Proteasome inhibition results in the blockage of the cytoplasmic IκBα degradation, concomitant with the inhibition of NF-κB nuclear translocation (14). Specifically, the 26S proteasome inhibitor bortezomib has been demonstrated to have antiproliferative and proapoptotic properties in a wide range of hematologic malignancies and has been widely used in the treatment of patients with multiple myeloma (15,16). In addition, it has shown promising results in cutaneous T-cell lymphoma (CTCL), non–Hodgkin's lymphoma, and other types of cancer and leukemia (17–20). It has been proposed that the proapoptotic and antiproliferative effects of bortezomib on cancer cells result from the inhibition of the cytoplasmic IκBα degradation and inhibition of NF-κB DNA binding activity (14). In cutaneous CTCL cells, where the constitutive activation of NF-κB plays a crucial role in their survival and resistance to apoptosis, bortezomib inhibited the *in vitro* NF-κB DNA binding activity and induced apoptosis (21–25). However, the molecular mechanisms of

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doi: 10.1158/1541-7786.MCR-10-0368

NF-κB inhibition by bortezomib in CTCL have not been investigated.

We have recently demonstrated that in solid tumors such as the metastatic prostate cancer cells, the proteasome inhibitors MG132 and MG115 block NF-κB activity by a novel mechanism that consists of inducing the nuclear translocation of IκBα (26). In this study, we tested the hypothesis that the clinically used proteasome inhibitor bortezomib induces the nuclear translocation of IκBα in CTCL Hut-78 cells, thus inhibiting NF-κB transcriptional activity and inducing apoptosis. Our results show that bortezomib induces the nuclear translocation and accumulation of IκBα, which then inhibits NF-κB activity in CTLC cells. Surprisingly, however, our data indicate that the regulation of NF-κB–dependent transcription by nuclear IκBα in CTCL is gene specific and depends on the subunit composition of NF-κB dimers recruited to the NF-κB–responsive promoters.

Materials and Methods

Antibodies and reagents

Purified polyclonal antibodies against human IκBα (sc-371), NF-κB p65(sc-372), NF-κB p50 (sc-7178), Bcl-2 (sc-492), and lamin B (sc-6216) were purchased from Santa Cruz Biotechnology. Purified polyclonal antibody against lactate dehydrogenase (LDH; 20-LG22) was from Fitzgerald Industries International, and actin antibody was from Sigma. cIAP1 (ab2399) and cIAP2 (ab32059) antibodies were from Abcam. Horseradish peroxidase (HRP)-conjugated anti-rabbit, anti-mouse, and anti-goat secondary antibodies were from Santa Cruz Biotechnology.

T4 polynucleotide kinase, poly(deoxyinosinic deoxycytidylic acid), and Sephadex G25 spin columns were purchased from Pharmacia. CREB (sc-2504, sc-2517) and NF-κB (sc-2505, sc-2511) gel shift oligonucleotides were from Santa Cruz Biotechnology. [^{32}P]-γ-ATP was purchased from Perkin Elmer. Proteasome inhibitor MG132 was purchased from EMD Chemicals and bortezomib was from ChemieTek. All other reagents were molecular biology grade and were purchased from Sigma.

Cell culture

Hut-78 human CTCL cells were obtained from American Type Culture Collection (ATCC). The cells were maintained at 37°C in RPMI 1640 medium, supplemented with 10% heat-inactivated FBS and 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin, in a humidified atmosphere with 5% CO_2.

Transfection with siRNA and proteasome inhibition

Human IκBα (sc-29360) and nonsilencing (sc-37007) siRNAs were obtained from Santa Cruz Biotechnology. Prior to transfection, Hut-78 cells were seeded into a 12-well plate and incubated in a humidified 5% CO_2 atmosphere at 37°C in antibiotic-free RPMI medium supplement with 10% FBS for 24 hour to 80% confluence. For each transfection, 60 μmol of either nonsilencing siRNA-A control or IκBα siRNA (Santa Cruz Biotechnology) were used. The cells were transfected for 6 hours in transfection medium with siRNA transfection reagent according to manufacturer's instructions (Santa Cruz Biotechnology). After transfection, fresh RPMI medium supplemented with FBS and antibiotics was added and the cells were treated with proteasome inhibitors for 24 hours.

Proteasome inhibitors MG132 and bortezomib were dissolved in DMSO and stored at −80°C. An equivalent volume of DMSO was used in all experiments as a solvent control.

Preparation of cytoplasmic and nuclear extracts

Nuclear (NE) and cytoplasmic extracts (CE) were prepared as described previously (27,28). Contamination of nuclear and cytoplasmic fractions by cytoplasmic and nuclear proteins, respectively, was determined by Western blot analysis, using LDH and lamin B as specific markers as described (27,28).

Electrophoretic mobility shift assay

Electrophoretic mobility shift assays (EMSA) of NF-κB and CREB DNA binding protein complexes were performed in NEs as described (27–29). For competition or supershift experiments, binding reactions were performed in the presence of 30 mol/L excess of unlabeled wild-type or mutant oligonucleotide or 1 μg of specific polyclonal antibody. The resulting complexes were resolved on 7.5% nondenaturing polyacrylamide gels that had been prerun at 150 V for 1 hour in 0.5× TBE buffer. Electrophoresis was conducted at 150 V for 3 hours. After electrophoresis, gels were transferred to a Whatman DE-81 paper, dried, and analyzed on Perkin-Elmer phosphoimager.

Immunoprecipitation

NEs were prepared with the Active Motif Nuclear Complex Co-IP Kit (catalogue no. 54001). The NEs were incubated (4°C, overnight) with IκBα antibody (sc-371) or control rabbit preimmune IgG (sc-2027) as described (26). The immune complexes were immunoprecipitated on A/G Plus Agarose (sc-2003), washed 4 times with PBS buffer, resolved on 10% SDS gel, and detected with IκBα and antibodies of NF-κB p65 and p50.

Apoptosis assay

Apoptosis was quantified with a cell death detection ELISA kit that quantifies release of nucleosomes into the cytoplasm (Cell Death Detection ELISA^{PLUS}; Roche) as described (26). The assay was performed at the indicated time points as per the manufacturer's instructions.

Real-time PCR

Total RNA was isolated by using RNeasy mini-kit (Qiagen). The iScript one-step RT-PCR kit with SYBR Green (Bio-Rad) was used as a supermix, and 20 ng of RNA

was used as template on a Bio-Rad MyIQ Single Color Real-Time PCR Detection System (Bio-Rad). The primers used for quantification of cIAP1, cIAP2, and Bcl-2 mRNA were purchased from SA Biosciences.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) analyses were performed by using the protocol from Upstate Biotechnology Inc. Proteins and DNA were cross-linked by adding formaldehyde to the growth medium to a final concentration of 1% for 10 minutes at 37°C and glycine was added at a final concentration of 0.125 mol/L to neutralize formaldehyde. Cells were washed with PBS containing protease inhibitors and collected by centrifugation. Cells were then resuspended in SDS lysis buffer, incubated at 4°C for 10 minutes, and sonicated. The lysates were centrifuged at 15,000 × *g* for 10 minutes at 4°C, and the supernatant extracts were incubated (4°C, overnight) with ChIP dilution buffer and precleared with Protein A/G Agarose (Santa Cruz Biotechnology) for 30 minutes at 4°C. Immunoprecipitation was performed overnight at 4°C, with p65 or p50 antibodies. Following immunoprecipitation, the samples were incubated with Protein A/G Agarose for 1 hour, and the immune complexes were collected by centrifugation (150 × *g* at 4°C), washed, and extracted with 1% SDS–0.1 mol/L $NaHCO_3$. The cross-linking was reversed by heating with 5 mol/L NaCl at 65°C for 4 hours. Proteins were digested with proteinase K, and the samples were extracted with phenol/chloroform, followed by precipitation with ethanol. The pellets were resuspended in nuclease-free water and subjected to real-time PCR. Immunoprecipitated DNA was analyzed by real-time PCR (25 μL reaction mixture), using the iQ SYBR Green Supermix and the Bio-Rad MyIQ Single Color Real-Time PCR Detection System. Each immunoprecipitation was performed 5 times, using different chromatin samples, and the occupancy was calculated by the ChIP-qPCR Human IGX1A Negative Control Assay (SA Biosciences) as a negative control and corrected for the efficiency of the primers, which detect specific genomic DNA sequences within open reading frame–free intergenic regions or "promoter deserts" lacking any known or predicted structural genes. The primers used for real-time PCR were the following: cIAP1, forward 5′-TGACTGGCAGGCAGAAATGA-3′ and reverse 5′-TTTGCCCGTTGAATCCGAT-3′; cIAP2, forward 5′-TTCAGTAAATGCCGCGAAGAT-3′ and reverse 5′-TGGTTTGCATGTGCACTGGT-3′; and Bcl-2, forward 5′-TGCATCTCAT-GCCAAGGG-3′ and reverse 5′-CCCCAGAGAAAGAAGAGGAGTT-3′.

Statistical analysis

The results represent at least 3 independent experiments. Numerical results are presented as means ± SE. Data were analyzed by using an InStat software package (GraphPAD). Statistical significance was evaluated by using Mann–Whitney *U* test with Bonferroni correction for multiple comparisons, and $P < 0.05$ was considered significant.

Results

Bortezomib and MG132 induce nuclear translocation of IκBα in leukemia Hut-78 cells, resulting in the inhibition of the constitutive NF-κB DNA binding activity

The CTCL Hut-78 cells are characterized by high levels of nuclear expression of NF-κB p65 and p50 proteins, resulting in the constitutive NF-κB DNA binding activity (21–24). To test the hypothesis that the proteasome inhibition induces nuclear IκBα translocation in these cells, Hut-78 cells were treated for 24 hours with increasing concentrations of bortezomib or MG132, and the cytoplasmic and nuclear fractions were prepared and analyzed by Western blotting. As a control of the purity of CE and NE fractions, we used LDH and lamin B as specific cytoplasmic and nuclear markers, respectively. In untreated Hut-78 cells, IκBα was localized predominantly in the cytoplasm, whereas NF-κB p65 and p50 proteins were both in the cytoplasm and in the nucleus (Fig. 1). Cell treatment with bortezomib (Fig. 1A) or MG132 (Fig. 1B) decreased IκBα levels in the cytoplasm and induced its dose-dependent translocation to the nucleus; the nuclear translocation of IκBα was induced by 10 nmol/L bortezomib (Fig. 1A) and 5 μmol/L MG132 (Fig. 1B). Figure 1C and D illustrate the densitometric evaluation of Western blot analysis of the nuclear IκBα levels induced by bortezomib and MG132, respectively. The nucleocytoplasmic distribution of NF-κB p50 and p65 proteins was not changed by 1 to 100 nmol/L bortezomib (Fig. 1A) or 1 to 10 μmol/L MG132 (Fig. 1B), and there was no pronounced effect on the nuclear levels of NF-κB p50 and p65 proteins within these concentrations.

To determine whether the nuclear translocation of IκBα in response to bortezomib and MG132 is time dependent, we analyzed IκBα levels in CE and NE of Hut-78 cells treated with 10 nmol/L bortezomib or 5 μmol/L MG132 for 0 to 24 hours. In bortezomib-treated cells, the nuclear IκBα translocation appeared 4 hours after incubation (Fig. 1E), whereas in MG132-treated cells, IκBα translocated to the nucleus 8 to 24 hours after treatment with MG132 (Fig. 1F).

To determine whether the nuclear translocation of IκBα induced by proteasome inhibition is associated with the inhibition of NF-κB activity, we measured NF-κB DNA binding activity in NEs prepared from Hut-78 cells treated 24 hours with increasing concentrations of bortezomib or MG132. As shown in Figure 2, the constitutive NF-κB DNA binding activity in Hut-78 cells was significantly reduced by 10 nmol/L bortezomib (Fig. 2A and C) or 5 μmol/L MG132 (Fig. 2B and D), which also induced the nuclear translocation and accumulation of IκBα (Fig. 1A–D). Supershift analysis using NF-κB p65 and p50 antibodies indicated that the NF-κB DNA binding complex in Hut-78 cells contained both p65 and p50 NF-κB proteins (Fig. 2E). In contrast to NF-κB, DNA binding activity of another IκBα-independent

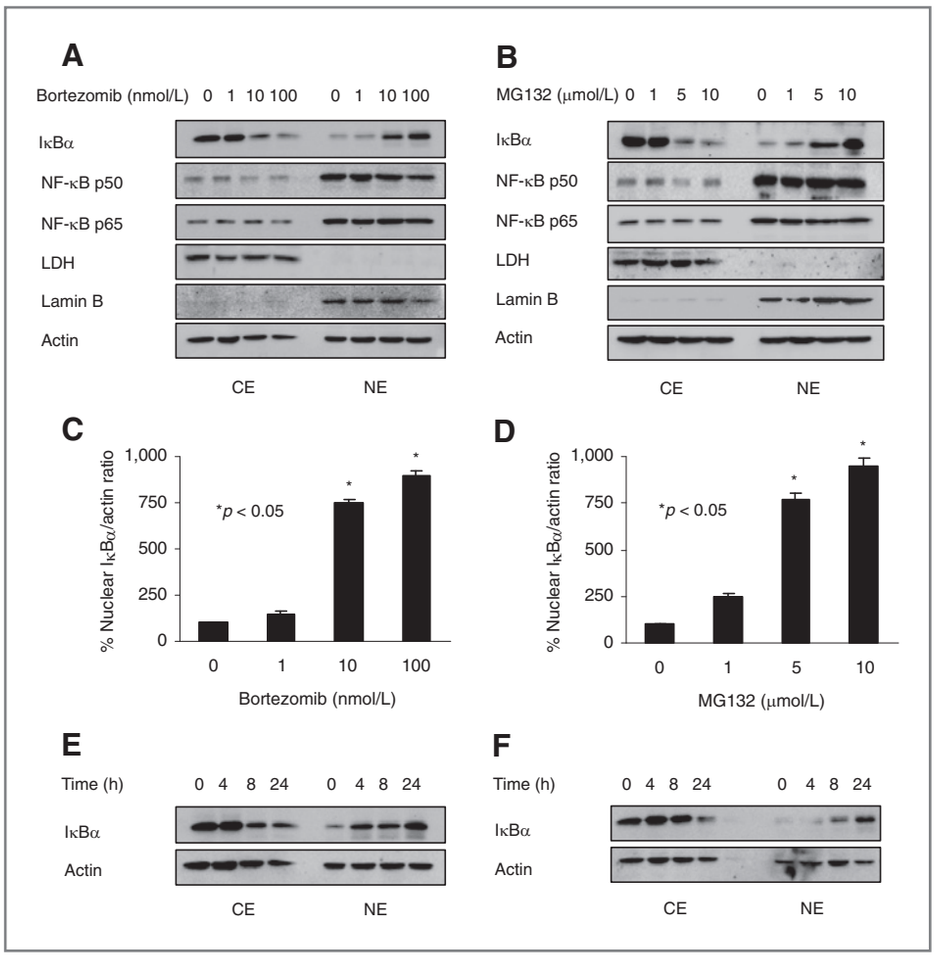

Figure 1. Proteasome inhibitors bortezomib and MG132 induce nuclear translocation of IκBα in Hut-78 cells. Hut-78 cells were treated with increasing concentrations of bortezomib (A) or MG132 (B) for 24 hours; CE and NE were prepared and analyzed by Western blotting with IκBα and NF-κB p50 and p65 antibodies. To confirm equal protein loading, the membranes were stripped and reprobed with actin antibody. The purity of cytoplasmic and nuclear fractions was monitored using LDH and lamin B antibodies. The nuclear IκBα bands in cells treated with bortezomib (C) or MG132 (D) were scanned and the densities were normalized to densities of nuclear actin. The value corresponding to zero proteasome inhibitor concentration was arbitrarily set to 100%, and the other values are presented relative to this value; *, statistically significant ($P < 0.05$) change compared with 0 nmol/L inhibitor. E and F, Western blotting of CE and NE prepared from Hut-78 cells treated with 10 nmol/L bortezomib or 10 μmol/L MG132 for 0 to 24 hours, respectively. Each lane corresponds to approximately 5×10^4 cells.

transcription factor, CREB, was not significantly affected by increasing concentrations of bortezomib (Fig. 2A and C) or MG132 (Fig. 2B and D), indicating specificity for NF-κB. Figure 2F confirms the CREB DNA binding specificity.

The bortezomib-induced nuclear IκBα accumulation is irreversible and is caused by the nuclear association of IκBα with NF-κB p65 and p50

To determine whether the bortezomib-induced nuclear translocation of IκBα is reversible, or whether IκBα remains bound in the nucleus even after bortezomib is removed, Hut-78 cells were incubated for 24 hours with control DMSO (Fig. 3A) or 10 nmol/L bortezomib (Fig. 3B), washed extensively, and incubated for another 0 to 48 hours in the medium. The cytoplasmic and nuclear levels of IκBα were then analyzed by Western blotting. Interestingly, once IκBα translocated to the nucleus in response to bortezomib treatment, it stayed there regardless of bortezomib removal (Fig. 3B). As expected, DMSO itself did not induce nuclear IκBα accumulation and IκBα remained in the cytoplasm (Fig. 3A). These data indicate that the bortezomib-induced nuclear accumulation of IκBα is irreversible and once IκBα translocates to the nucleus in Hut-78 cells, it binds to intranuclear proteins or structures.

To determine whether the nuclear IκBα binds to NF-κB p65 and p50 proteins in the nucleus of Hut-78 cells, we performed a coimmunoprecipitation experiment with IκBα antibody and NEs prepared from untreated and bortezomib-treated (10 nmol/L, 24 hours) Hut-78 cells. As shown in Figure 3C (top), IκBα was immunoprecipitated from the nuclear extracts of bortezomib-treated cells, but not from the NEs of untreated cells, or from bortezomib-treated cells immunoprecipitated with control preimmune IgG. Immunoblotting using NF-κB p65 antibody revealed the presence of NF-κB p65 in the NEs of bortezomib-treated cells immunoprecipitated with IκBα antibody but not with preimmune IgG (Fig. 3C, middle). Similarly, immunoblotting using NF-κB p50

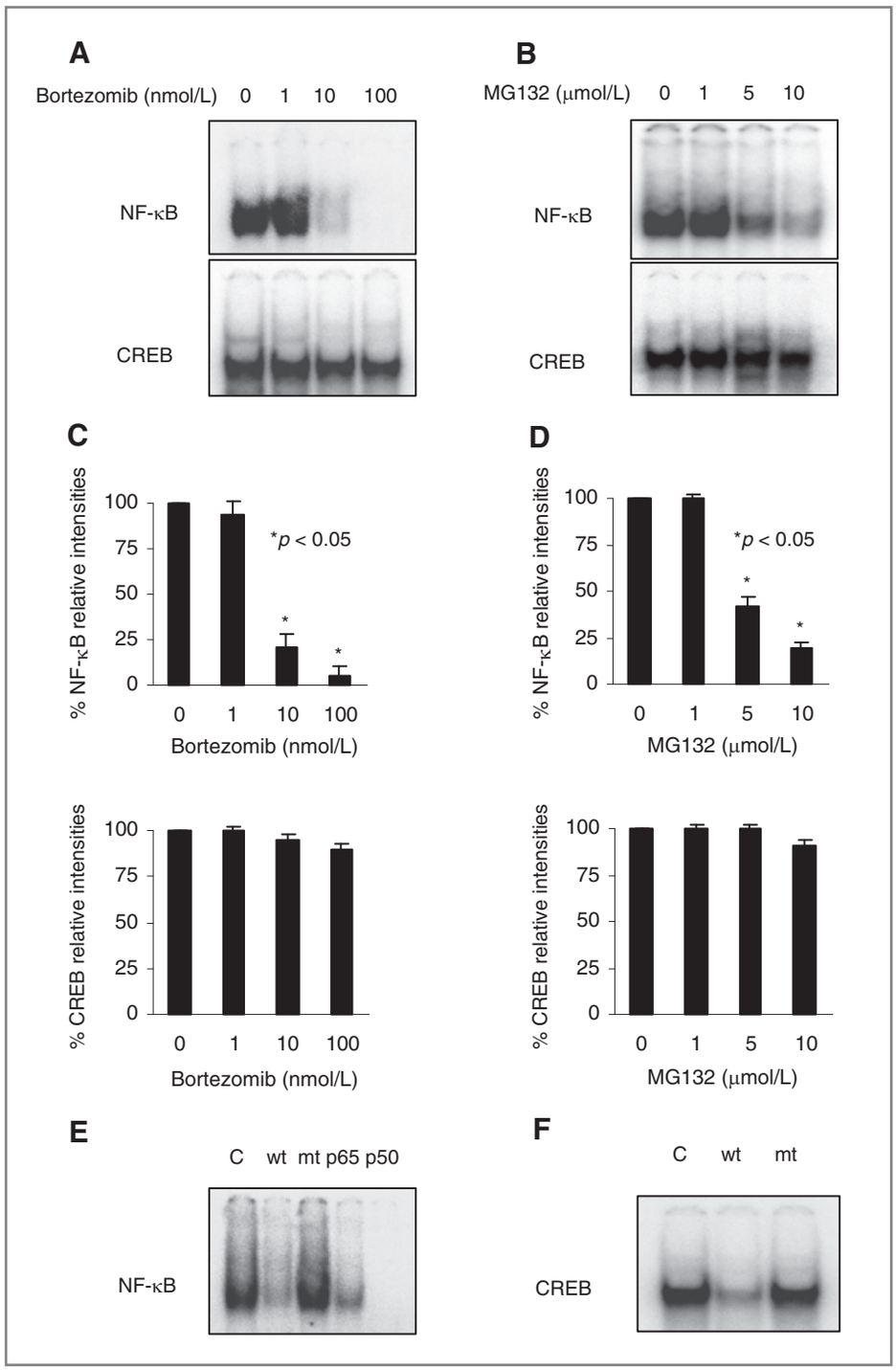

Figure 2. The bortezomib- and MG132-induced nuclear translocation of IκBα is associated with the inhibition of NFκB activity. EMSA of NF-κB and CREB DNA binding activities analyzed in NEs from Hut-78 cells treated for 24 hours with increasing concentrations of bortezomib (A) or MG132 (B). C and D, densitometric evaluation of EMSA of NF-κB (top) and CREB (bottom) DNA binding activities analyzed in NEs from Hut-78 cells treated 24 hours with increasing concentrations of bortezomib or MG132, respectively. The value corresponding to zero proteasome inhibitor concentration was arbitrarily set to 100%, and the other values are presented relative to this value; *, statistically significant ($P < 0.05$) change compared with no inhibitor. E and F, subunit and specificity characterization of the constitutive NF-κB and CREB DNA binding activities in Hut-78 cells, as described in the Materials and Methods section. wt, wild-type; mt, mutant.

antibody demonstrated the presence of NF-κB p50 in bortezomib-treated NEs immunoprecipitated with IκBα but not in control preimmune IgG antibody (Fig. 3C, bottom). Low levels of NF-κB p65 and p50 signals were detected in the IκBα immunoprecipitates prepared from the NEs of untreated cells, even though both NF-κB proteins are highly expressed in the nucleus of untreated Hut-78 cells (Fig. 1), demonstrating specificity for the IκBα binding proteins. Together, these data demonstrate that the bortezomib-induced nuclear trans-

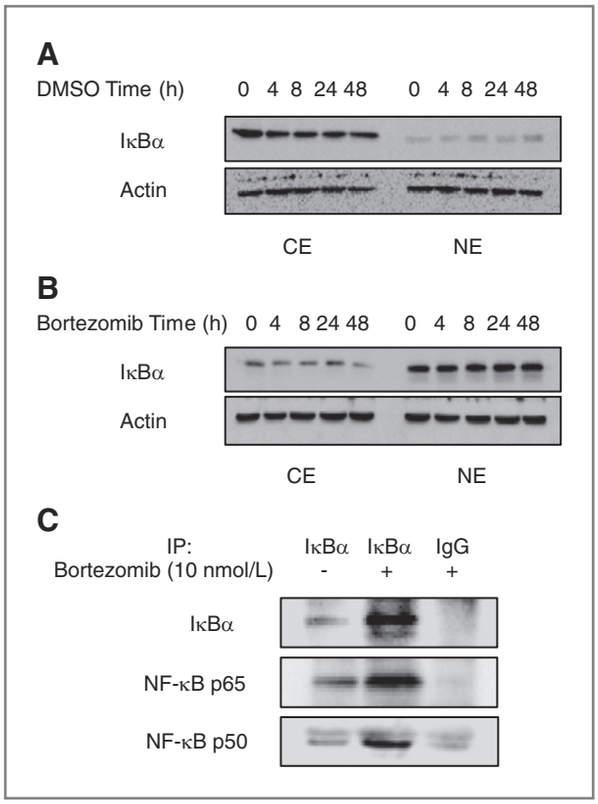

Figure 3. Irreversibility of the nuclear translocation of IκBα and its association with the nuclear NF-κB p65 and p50 in Hut-78 cells. A, Western blotting of CE and NE prepared from Hut-78 cells treated with DMSO (A) or 10 nmol/L bortezomib (B) for 24 hours, washed, incubated for another 0 to 48 hours in medium with FBS, and analyzed with IκBα and actin antibodies. Each lane corresponds approximately to 5×10^4 cells. C, coimmunoprecipitation experiment from NEs of untreated or bortezomib-treated (10 nmol/L, 24 hours) Hut-78 cells with preimmune IgG or IκBα-specific antibody. The Western blots were analyzed with IκBα and NF-κB p65 and p50 antibodies. IP, immunoprecipitation.

location of IκBα is irreversible and that the nuclear IκBα binds to NF-κB p65 and p50 proteins present in the nucleus.

Bortezomib-induced nuclear accumulation of IκBα results in the induction of apoptosis in leukemia Hut-78 cells

To determine whether the inhibition of NF-κB DNA binding by bortezomib is directly caused by the bortezomib-induced nuclear IκBα, we hypothesized that suppression of IκBα nuclear levels should increase the NF-κB DNA binding in Hut-78 cells. To test this, we suppressed IκBα expression by IκBα-specific siRNA and then treated cells with increasing concentrations of bortezomib for 24 hours. As expected, IκBα siRNA greatly reduced the cellular protein levels of IκBα compared with cells transfected with nonsilencing siRNA, resulting in barely detectable IκBα in the nucleus of bortezomib-treated cells (Fig. 4A). The decreased nuclear levels of IκBα in cells transfected with IκBα siRNA, compared with cells transfected with nonsilencing siRNA, resulted in a substantially increased NF-κB DNA binding activity, both in untreated Hut-78 cells and in cells treated with increasing concentrations of bortezomib (Fig. 4B and C).

Next, we investigated whether the increased NF-κB DNA binding activity in Hut-78 cells transfected with IκBα siRNA would translate into an increased resistance to apoptosis in response to bortezomib treatment. To this end, Hut-78 cells were transfected with nonsilencing or IκBα siRNA, treated with increasing concentrations of bortezomib as described previously, and apoptosis was measured by a quantitative ELISA based on the detection of nucleosome release into the cytoplasm. As shown in Figure 4D, the decreased nuclear expression of IκBα in cells treated with 10 and 100 nmol/L bortezomib and transfected with IκBα siRNA, compared with cells transfected with nonsilencing siRNA, resulted in a significantly reduced apoptosis ($P < 0.05$). Thus, these results show that the increased apoptosis observed in bortezomib-treated Hut-78 cells is directly caused by the increased nuclear levels of IκBα.

Bortezomib-induced nuclear IκBα differentially regulates NF-κB–dependent antiapoptotic gene expression in Hut-78 cells

Because the NF-κB–regulated antiapoptotic genes involve *Bcl-2* as well as *cIAP1* and *cIAP2*, we speculated that all these NF-κB–responsive genes would be inhibited by the nuclear IκBα in bortezomib-treated Hut-78 cells. To test this, Hut-78 cells were treated 24 hours with 10 nmol/L bortezomib or control DMSO and mRNA levels were analyzed by quantitative real-time RT-PCR. Surprisingly, however, while expression of *cIAP1* and *cIAP2* was significantly reduced by 10 nmol/L bortezomib, expression of *Bcl-2* was not suppressed (Fig. 5A). To confirm these results also on a protein level, we analyzed the total cellular protein levels of Bcl-2, cIAP1, cIAP2, as well as IκBα and control actin, in whole-cell extracts prepared from Hut-78 cells treated 24 hours with increasing concentrations of bortezomib (Fig. 5B). Similar to mRNA expression, protein levels of cIAP1 and cIAP2 were decreased by 10 and 100 nmol/L bortezomib whereas Bcl-2 levels were not changed. These data suggested that the bortezomib-induced nuclear IκBα might regulate NF-κB–dependent transcription in a gene-specific manner.

Of note, increased concentrations of bortezomib did not change the total cellular levels of IκBα (Fig. 5B), which was in a good agreement with the data illustrated in Figure 1, showing that the net gain of IκBα in the nucleus equals its net loss in the cytoplasm in cells treated with proteasome inhibitors. These results indicate that the rate of IκBα degradation in Hut-78 cells equals the rate of IκBα resynthesis, which is regulated by NF-κB. However, as NFκB activity is inhibited by the bortezomib-induced nuclear IκBα, IκBα resynthesis is suppressed, and thus

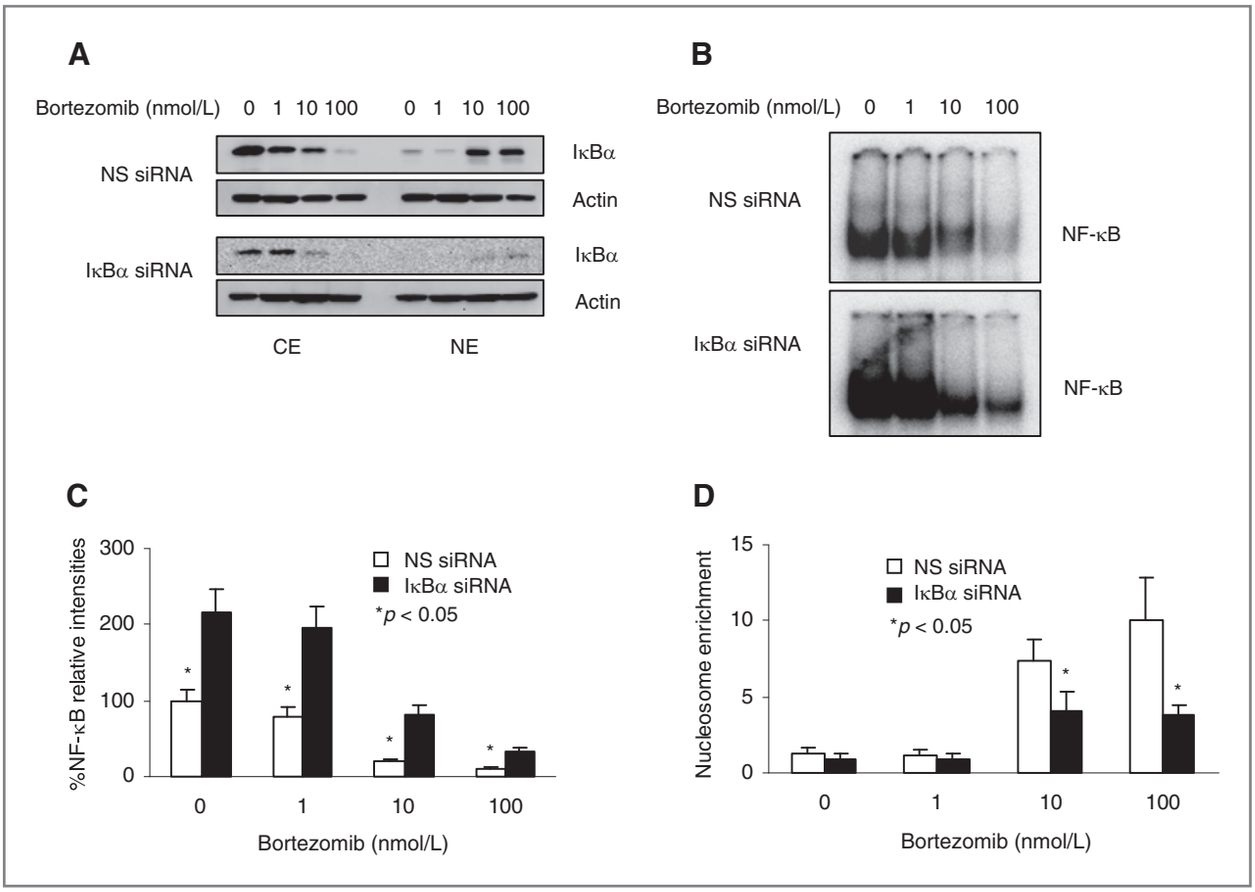

Figure 4. The bortezomib-induced nuclear translocation of IκBα results in the suppression of NF-κB DNA binding activity and induction of apoptosis in Hut-78 cells. A, Western blotting of CE and NE prepared from Hut-78 cells transfected with nonsilencing (NS) or IκBα siRNA, followed by treatment with increasing concentrations of bortezomib for 24 hours. The membranes were analyzed with IκBα and actin antibodies. B, representative EMSA of NF-κB DNA binding activity measured in Hut-78 cells transfected with NS (top) or IκBα (bottom) siRNA, followed by incubations with increasing concentrations of bortezomib for 24 hours. C, densitometric evaluation of the EMSAs of NF-κB DNA binding activity measured in Hut-78 cells transfected with NS (empty columns) or IκBα (full columns) siRNA as described previously in Figure 4B. D, apoptosis measured by the nucleosome enrichment assay in Hut-78 cells transfected with NS (empty columns) or IκBα (full columns) siRNA, followed by 24-hour treatment with increasing concentrations of bortezomib. C and D, the values represent the mean ± SE of 4 experiments; *, statistically significant ($P < 0.05$) change compared with control siRNA–transfected cells.

the total IκBα cellular levels remain constant, despite the inhibited IκBα degradation.

To confirm the cIAP1 and cIAP2 regulation by nuclear IκBα, we analyzed Bcl-2, cIAP1, and cIAP2 mRNA and protein levels in cells that were transfected with nonsilencing or IκBα siRNA and treated with increasing concentrations of bortezomib (Fig. 6) or MG132 (data not shown). As expected, Bcl-2 mRNA (Fig. 6A) and protein (Fig. 6B) levels did not change between cells transfected with nonsilencing and IκBα siRNAs, and there was no substantial change in response to bortezomib treatment. In contrast, both cIAP1 and cIAP2 mRNA and protein levels decreased with increasing bortezomib concentrations; however, transfection with IκBα siRNA reduced this bortezomib-induced decrease. Similar data were obtained when cells were treated with MG132 instead of bortezomib (data not shown). These data correlate well with the increased levels of NF-κB DNA binding activity in cells transfected with IκBα siRNA (Fig. 4) and indicate that the nuclear IκBα regulates transcription of cIAP1 and cIAP2, but not Bcl-2, in Hut-78 cells.

The gene-specific inhibition of NF-κB–dependent transcription by bortezomib in Hut-78 cells depends on the subunit composition of recruited NF-κB proteins

To analyze the mechanisms regulating transcription of NF-κB–responsive genes in Hut-78 cells, we used ChIP to measure the *in vivo* recruitment of NF-κB p65 and p50 subunits to promoters of *Bcl-2*, *cIAP1*, and *cIAP2* genes. Hut-78 cells were treated 24 hours with 10 nmol/L bortezomib or control DMSO, cells were cross-linked with formaldehyde, lysed, chromatin was sheared by sonication,

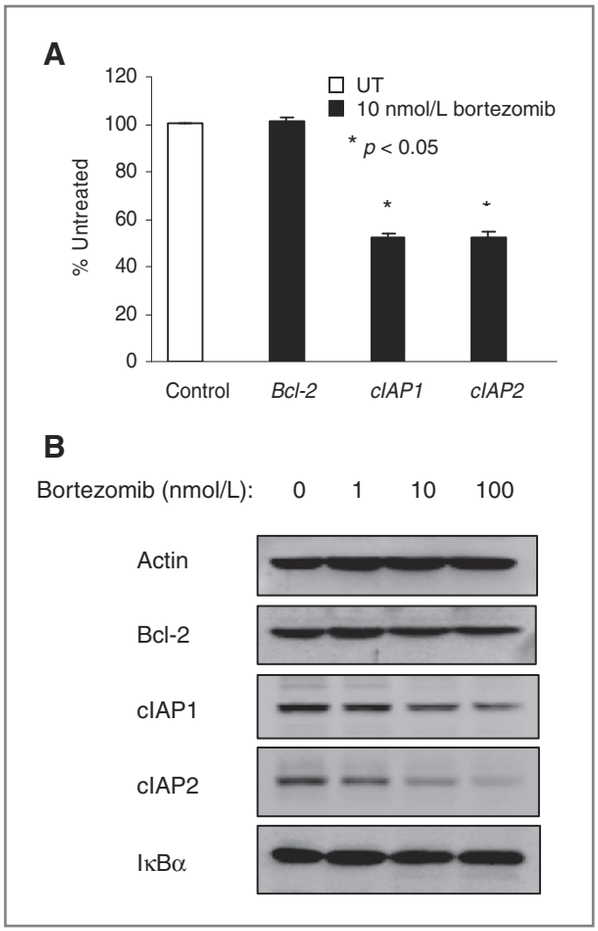

Figure 5. Bortezomib-induced nuclear IκBα differentially regulates NF-κB–dependent antiapoptotic gene expression in Hut-78 cells. A, real-time RT-PCR analysis of *Bcl-2*, *cIAP1*, and *cIAP2* mRNA levels in Hut-78 cells treated 24 hours with control DMSO (empty column) or 10 nmol/L bortezomib (full columns). The values represent the mean ± SE of 5 experiments; *, statistically significant ($P < 0.05$) inhibition compared with control untreated (UT) cells. B, Western blot analysis of actin, Bcl-2, cIAP1, cIAP2, and IκBα protein expression in whole-cell extracts of Hut-78 cells treated 24 hours with increasing concentrations of bortezomib.

and NF-κB p65 and p50 proteins were immunoprecipitated. The binding of NF-κB p65 and p50 proteins to promoter regions of *Bcl-2*, *cIAP1*, and *cIAP2* genes was measured by quantitative real-time PCR.

As shown in Figure 7, although NF-κB p65 was heavily recruited to promoter regions of *cIAP1* and *cIAP2* genes, its recruitment to *Bcl-2* promoter was only marginal. The p65 recruitment to *cIAP1* and *cIAP2* promoters was significantly reduced by the bortezomib-induced nuclear IκBα, whereas its recruitment to *Bcl-2* promoter was not affected. As shown in Figure 7B, in contrast to NF-κB p65, the NF-κB p50 subunit was recruited to all tested promoters, including *Bcl-2*, and this recruitment was inhibited by the bortezomib-induced nuclear IκBα. Thus, these data indicate that in Hut-78 cells, the *cIAP1* and *cIAP2* promoters are occupied predominantly by NF-κB p65/50 heterodimers, whereas the promoter of *Bcl-2* is occupied mainly by NF-κB p50/50 homodimers (Table 1). However, while the bortezomib-induced nuclear IκBα removes both NF-κB p65 and p50 from the gene promoters (Fig. 7A and B), p65/50-regulated transcription of cIAP1 and cIAP2 is inhibited whereas the *Bcl-2* promoter occupied by p50/50 homodimers is not regulated by IκBα. Together, these data indicate that in Hut-78 cells, the inhibition of NF-κB–dependent transcription by bortezomib is gene specific and depends on the subunit composition of NF-κB proteins recruited to the gene promoters (Table 1).

Discussion

The proteasome inhibitor bortezomib, which is approved by the Food and Drug Administration for treatment of multiple myeloma and mantel cell lymphoma, acts by targeting the catalytic 20S core of the proteasome and induces apoptosis in cancer cells (15–20). One of the mechanisms consists of inhibiting the cytoplasmic degradation of IκBα, resulting in the suppression of NF-κB DNA binding activity and decreased expression of NF-κB–dependent antiapoptotic genes (14,30). NF-κB is constitutively activated in CTCL and many other forms of cancer and leukemia, in which it plays a crucial role in cell survival and resistance to apoptosis (21–23). Recently, bortezomib has been evaluated in CTCL and exhibited promising antitumor effects *in vitro* and *in vivo* (18,19).

In this study, we have shown that the proteasome inhibitors bortezomib and MG132 suppress the constitutive NF-κB DNA binding activity in CTCL Hut-78 cells by a new mechanism that consists of inducing the nuclear translocation and accumulation of IκBα. Once in the nucleus, the nuclear IκBα then binds to NF-κB p65 and 50 proteins and removes them from the promoters of NF-κB–dependent genes. Importantly, however, our data show that the ability of nuclear IκBα to inhibit NF-κB–dependent transcription in Hut-78 cells is gene specific. While expression of NF-κB–dependent antiapoptotic genes *cIAP1* and *cIAP2* is inhibited by bortezomib, expression of *Bcl-2* is not suppressed. Analysis of the *in vivo* binding of NF-κB proteins to *cIAP* and *Bcl-2* promoters by ChIP showed that NF-κB p65 and p50 are recruited to *cIAP1* and *cIAP2* promoters whereas the *Bcl-2* promoter is occupied only by NF-κB p50. Thus, these results indicate that the *cIAP1* and *cIAP2* promoters associate with NF-κB p65/50 heterodimers and this binding and transcription are inhibited by the bortezomib-induced nuclear IκBα. In contrast, *Bcl-2* promoter is occupied predominantly by NF-κB p50/50 homodimers and its transcription is not inhibited by the bortezomib-induced nuclear IκBα.

Compared with NF-κB p65, NF-κB p50 lacks the transactivation domain and therefore p50/50 homodimers,

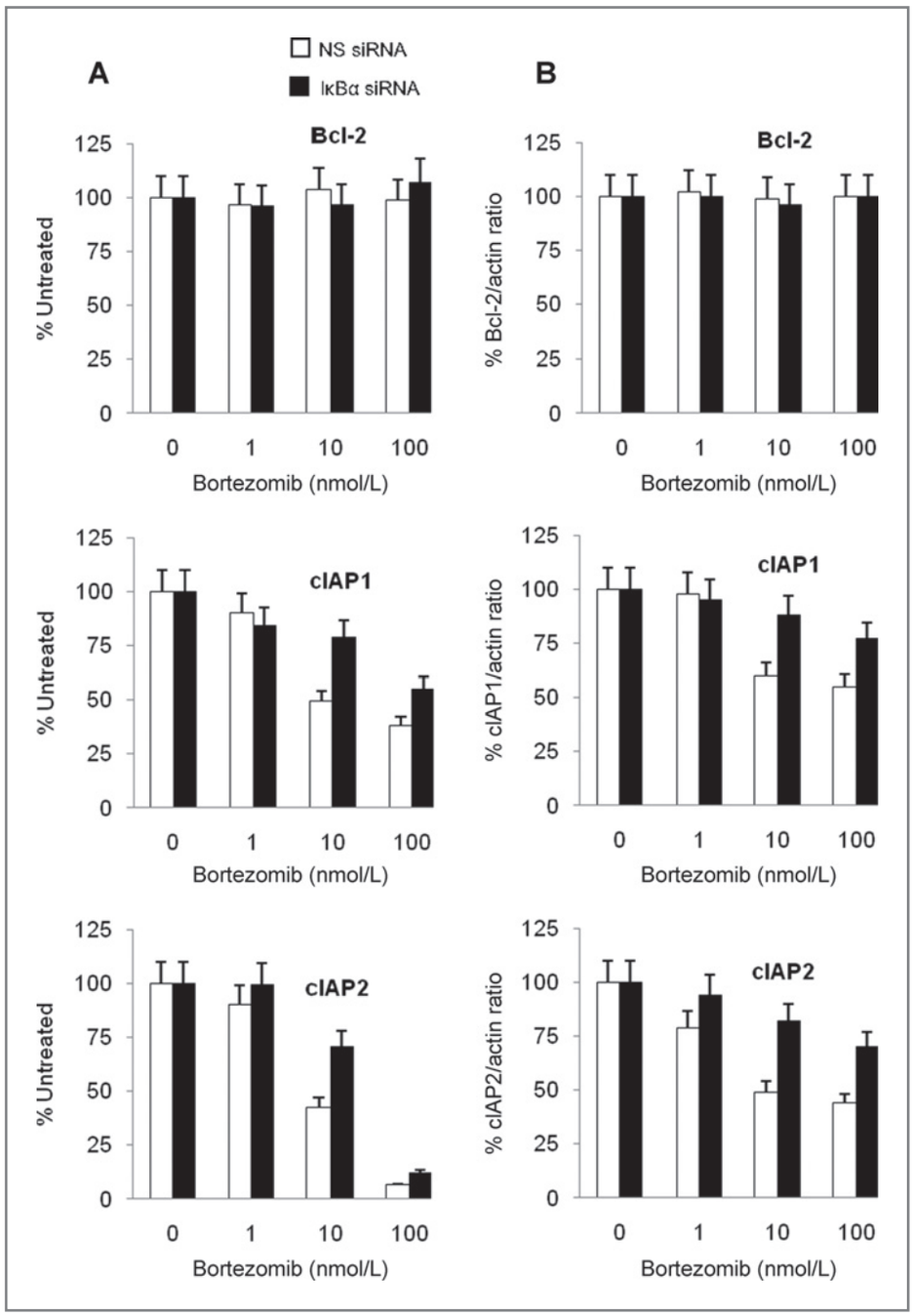

Figure 6. The analysis of Bcl-2, cIAP1, and cIAP2 expression in IκBα siRNA–transfected Hut-78 cells treated with increasing concentrations of bortezomib. *Bcl-2*, *cIAP1*, and *cIAP2* mRNA expression analyzed by real-time RT-PCR (A) and total cellular protein levels analyzed by Western blotting and densitometry normalized to actin (B) in Hut-78 cells transfected with NS (empty column) or IκBα siRNA (full columns) and treated 24 hours with increasing concentrations of bortezomib.

which retain their ability to bind DNA, were thought to function only as transcriptional repressors (1–3). However, recent studies have shown that p50/50 homodimers may be transcriptionally active as well, especially if bound to transactivating elements (31–35). Indeed, increased constitutive DNA binding activity of p50/50 homodimers has been observed in several types of lymphoma and leukemia and has been associated with the increased expression of Bcl-2 (31–35). Interestingly, while Bcl-2 expression was shown to be suppressed by bortezomib in some solid tumors, such as lung or prostate cancer (36,37), its suppression in other cells has not been consistently observed (38–40). These differences in Bcl-2 regulation by bortezomib—and by the nuclear IκBα-–could be caused by a differential expression

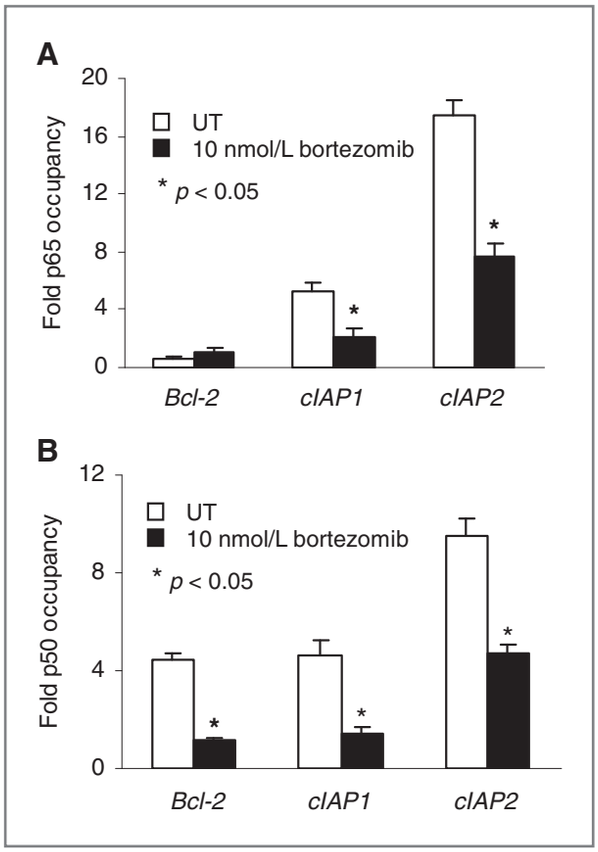

Figure 7. Recruitment of NF-κB p65 and p50 proteins to NF-κB–dependent promoters in Hut-78 cells. The recruitment of NF-κB p65 (A) and p50 (B) proteins to NF-κB–dependent promoters of *Bcl-2*, *cIAP1*, and *cIAP2* genes in Hut-78 cells treated 24 hours with DMSO (empty columns) or 10 nmol/L bortezomib (full columns) was analyzed by ChIP and quantified by real-time PCR. The data are presented as the change in occupancy over the human IGX1A (SA Biosciences) sequence control and represent the mean ± SE of 5 experiments. *, statistically significant ($P < 0.05$) inhibition compared with control untreated (UT) cells.

and regulation of NF-κB subunits. In this model, in cells expressing NF-κB p50, the *Bcl-2* promoter would be occupied predominantly by p50/50 homodimers, which would not regulate Bcl-2 transcription, and thus Bcl-2 expression would not be suppressed by the bortezomib-induced nuclear IκBα. Conversely, in cells that do not express NF-κB p50, the *Bcl-2* promoter might be occupied by other NF-κB dimers, which regulate Bcl-2 transcription, and thus in these cells, Bcl-2 transcription would be inhibited by bortezomib.

Alternatively, the regulation of Bcl-2 transcription by the bortezomib-induced nuclear IκBα might depend on a promoter-specific recruitment of other transcription factors, which might affect NF-κB affinity for IκBα. Interestingly, the Bcl-2 NF-κB binding site differs from *cIAP* promoters as well as from the oligonucleotide sequence used in EMSA by having A and C instead of G and T in the second and sixth position, respectively (Table 1). It might be possible that the *Bcl-2* promoter associates with transcriptional factors and/or regulators that decrease the *in vivo* NF-κB affinity for IκBα. Because Bcl-2 plays a crucial role in cell survival and drug resistance (41–44), future studies should determine the mechanisms that regulate its transcription both by bortezomib and by the bortezomib-induced nuclear IκBα.

In addition to IκBα, bortezomib controls the ubiquitin-proteasome–mediated cytoplasmic degradation of other short-lived proteins that include p53, c-myc, N-myc, cyclins, and the cyclin-dependent kinase inhibitors p21 and p27 (45). Intriguingly, all these proteins function as transcriptional regulators and/or tumor suppressors and their proapoptotic and cell-cycle regulatory function is controlled by their nucleocytoplasmic translocation. Because bortezomib is being evaluated for the treatment of a wide range of human malignancies (45), it will be interesting to determine whether it induces the nuclear translocation and accumulation of these proteins as well.

NF-κB activity and expression of NF-κB–dependent antiapoptotic genes are increased in many types of cancer and leukemia. Thus, the bortezomib-induced nuclear translocation of IκBα could provide a new therapeutic strategy aimed at the suppression of NF-κB activity by nuclear IκBα and induction of apoptosis. However, the regulation of NF-κB–dependent transcription by the bortezomib-induced nuclear IκBα is gene specific and, in CTCL Hut-78 cells, depends on the subunit composition of recruited NF-κB complexes. These differences in the transcriptional regulation by the bortezomib-induced nuclear IκBα might hold the key for development of more specific therapies for cancers characterized by increased NF-κB activity.

Table 1. NF-κB sequences in EMSA and antiapoptotic gene promoters, composition of the bound NF-κB dimers, and regulation by the nuclear IκBα

Gene	NF-κB site	NF-κB proteins	Inhibition by IκBα
EMSA consensus sequence	GGGACTTTCC	p50/65	+
cIAP1	GGAATTCCCC	p50/65	+
cIAP2	GGAAATCCCC	p50/65	+
Bcl-2	GAAATCCTCC	p50/50	–

Disclosure of Potential Conflicts of Interests

No potential conflicts of interest were disclosed.

Grant Support

This work was supported by the NIH research grants GM079581 and AI085497 to I. Vancurova.

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Received August 21, 2010; revised November 10, 2010; accepted December 19, 2010; published OnlineFirst January 11, 2011.

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