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# Proteasome Inhibitors Induce Death but Activate NF-κB on Endometrial Carcinoma Cell Lines and Primary Culture Explants*[S]

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**Proteasome inhibitors are currently used as chemotherapeutic drugs because of their ability to block NF-κB, a transcription factor constitutively activated in many different types of human cancer. In the present study, we demonstrate that proteasome inhibitors induce cell death in endometrial carcinoma cell lines and primary explants but, instead of blocking NF-κB, they increase its transcriptional activity. Proteasome inhibitors induce phosphorylation of IKKα/β, phosphorylation and degradation of IκBα, and phosphorylation of the p65 NF-κB subunit on serine 536. Proteasome inhibitor-induced NF-κB activity can be blocked by a non-degradable form of IκBα or dominant negative forms of either IKKα or IKKβ. Lentiviral delivery of shRNAs to either IKKα or IKKβ cause blockade of NF-κB transcriptional activity and inhibit phosphorylation of p65 on serine 536, but has no effect on IκBa degradation. These results suggest a role for p65 phosphorylation in proteasome inhibitor-induced NF-κB activation. Accordingly, siRNA knockdown of p65 inhibits proteasome inhibitor-induced NF-κB transcriptional activity. Our results demonstrate that proteasome inhibitors, including bortezomib, induce cell death on endometrial carcinoma cells and primary explants. However, they activate NF-κB instead of blocking its transcriptional potential. Therefore, the concept that proteasome inhibitors are blockers of NF-κB activation should be carefully examined in particular cell types.**

The proteasome represents a novel putative target for cancer therapy. PS-341 (Velcade/bortezomib) is a dipeptidyl boronic acid inhibitor with high specificity for the proteasome (1, 2). It is currently used in the treatment of patients with multiple myeloma (3–7). Preclinical studies have suggested that proteasome inhibitors show antitumor activity against solid tumors, including carcinomas of the breast (8), lung (9), colon (10), bladder (11), ovary, prostate (12), pancreas (13), and glioblastoma multiforme. Furthermore, evidence has shown that transformed cells appear to be more susceptible to proteasome inhibitor-induced apoptosis than nontransformed cells. Finally, these inhibitors can sensitize cancer cells to death induced by members of the tumor necrosis family (TNF)[5] family such as TRAIL.

The antitumoral effects of bortezomib have been extensively studied in multiple myeloma, inactivation of NF-κB being one of the proposed mechanisms of action. NF-κB is a pleiotropic transcription factor, which is activated by a broad variety of stimuli such as growth factors, cytokines, ionizing radiation, ultraviolet light, or chemotherapeutic drugs (14, 15). NF-κB regulates the expression of a large number of genes, which carry important functions in inflammation, apoptosis, proliferation, and angiogenesis. NF-κB shows constitutive or increased activity in a wide variety of tumors (16, 17), including endometrial carcinoma (18) and plays a crucial role in neoplastic transformation (16, 19). In resting cells, NF-κB is held inactive in the cytoplasm, bound to the inhibitors of NF-κB (IκB). Activation of the canonical NF-κB pathway depends on stimuli-induced phosphorylation of the IκB kinase (IKK) complex, which includes the kinases IKKα, IKKβ, and the regulatory subunit IKKγ. Activated IKKs induce phosphorylation of IκBα on serines 32 and 36, and the subsequent ubiquitination and degradation by a proteasome-dependent pathway. Free NF-κB is then translocated to the nucleus where it regulates the transcription of several sets of genes. Optimal activation of NF-κB transcriptional activity requires phosphorylation of NF-κB subunits such as p65. In addition, NF-κB activity can be further modulated by phosphorylation of the p65 subunit. p65 sequence contains several serine residues that can be phosphorylated (20). One such residues is serine 536, which can be

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This work is dedicated to Eric and Laura.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

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[5] The abbreviations used are: TNF, tumor necrosis factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; shRNA, short hairpin RNA; siRNA, small interfering RNA; fmk, fluoromethyl ketone; DMEM, Dulbecco's modified Eagle's medium; BAF, Boc-D-fmk; DN, dominant negative; ER, endoplasmic reticulum.

phosphorylated by several kinases in different signaling pathways. Phosphorylation of p65 at serine 536 is accomplished by several different stimuli such as lymphotoxin β (21), TNF (22), lipopolysaccharide (23), or interleukin 1 (24).

The main goal of the present study was to demonstrate that bortezomib and other three proteasome inhibitors induce apoptosis in endometrial carcinoma cell lines and primary culture explants obtained from endometrial carcinoma tumor samples. However, we have found that such inhibitors activate NF-κB. Proteasome inhibitors induced phosphorylation and reduction of the levels of IκBα protein and phosphorylation of p65 subunit on serine 536, leading to increased NF-κB transcriptional activity. p65 phosphorylation depended on either IKKα and IKKβ. Proteasome inhibitor-induced activation of NF-κB required degradation of IκBα, and also functional expression of IKKα, IKKβ, and the p65 NF-κB subunit. Proteasome inhibitors have been widely used as pharmacological inhibitors of NF-κB. However, our results demonstrate that this may not always be the case. Therefore, an accurate study of the effects of proteasome inhibitors in signaling pathways may be needed in specific cell types. More importantly, our results may also have clinical relevance because proteasome inhibitors are currently used as anticancer drugs.

## EXPERIMENTAL PROCEDURES

*Reagents, Plasmids, and Antibodies*—3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) and monoclonal antibody to tubulin were from Sigma. Proteasome inhibitors MG-132, epoxomicin, and ALLN and antibodies to IKKα and IKKβ were from Calbiochem (La Jolla, CA). Bortezomib (Millenium Pharmaceuticals, Cambridge, MA) was obtained from the Department of Pharmacy (Hospital Arnau de Vilanova, Lleida). The broad specificity caspase inhibitor Boc-D-fmk (BAF) was purchased from Calbiochem (La Jolla, CA). Antibodies to anti-p65-phosphoserine 536, anti-phospho-IκBα, and active caspase-3 were obtained from Cell Signaling (Beverly, MA). Antibody to anti-pantothenate-cytokeratin and cytokeratin-7 were from DAKO (Glostrup, Denmark). Antibody to IκBα, p65, and siRNA targeting p65 or c-Rel were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibody to β was from BD Biosciences. Peroxidase-conjugated anti-mouse and anti-rabbit antibodies were from Amersham Biosciences.

Dominant negative forms of IKKα and IKKβ were a generous gift from Dr. Alun M. Davies. Plasmid containing 5 NF-κB sites and the luciferase reporter gene (NFκB-LUC) was a gift from Dr. Giles Hardingham. Plasmid encoding β-galactosidase was a gift from Mari Carmen Ruiz Ruiz.

*Cell lines, Culture Conditions, and Transfection*—The Ishikawa 3-H-12 cell line was obtained from the American Type Culture Collection (Manassas, VA). KLE cells were a gift from Dr. Palacios (Centro Nacional de Investigaciones Oncológicas, CNIO, Madrid). RL-95 and HEC-1-A cells were a gift from Dr. Reventos (Hospital Vall d'Hebron, Barcelona). All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum (Invitrogen), 1 mM HEPES (Sigma), 1 mM sodium pyruvate (Sigma), 2 mM L-glutamine (Sigma), and 1% of penicillin/streptomycin (Sigma) at 37 °C with saturating humidity and 5% $CO_2$. Transfections of both plasmid constructs and siRNAs were performed by calcium phosphate or Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's instructions.

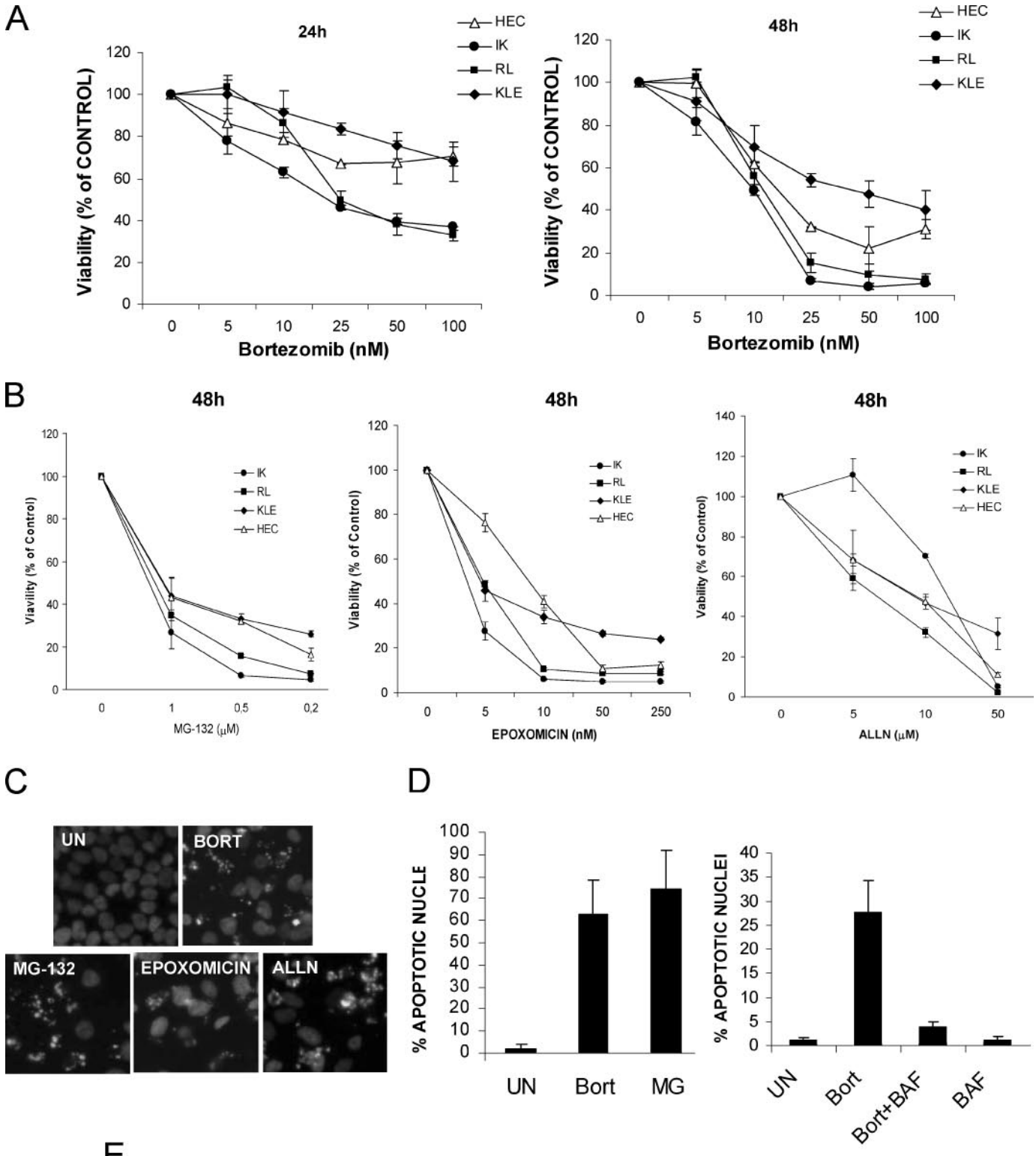
*Explant Culture of Endometrial Adenocarcinoma*—Endometrial carcinoma samples were collected in the operating room of the Department of Gynecology, Hospital Universitari Arnau de Vilanova of Lleida, by a pathologist (J.P.). A specific informed consent was obtained from each patient, and the study was approved by the local Ethics Committee. Tissue were collected in DMEM, minced in 1-mm pieces, and incubated with collagenase in DMEM for 1.5 h at 37 °C with periodic mixing. Digested tissue was mechanically dissociated through a 10-ml pipette and a 1-ml blue tip and resuspended in 2 ml of fresh DMEM. To separate endometrial epithelial cells from the stromal fraction, the dissociated tissue was seeded on top of 8 ml of DMEM and tissue was allowed to sediment by gravity for 5 min. This step was repeated three times. Finally, tissue explants were resuspended in DMEM supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 2 mM L-glutamine, and 1% of penicillin/streptomycin (Sigma) and seeded on M24 multiwell plates. Explant cultures were incubated at 37 °C with saturating humidity and 5% $CO_2$. After 2 days in culture, explants were treated with the indicated concentrations of proteasome inhibitors.

*Lentiviral Production and Infection*—Oligonucleotides to produce plasmid-based shRNA were cloned into the FSFsi vector using AgeI-BamHI restriction sites. shRNA target sequence to IKKα was GCAGGCTCTTTCAGGGACA and target sequence to IKKβ was AAAGTGTCAGCTGTATCCT. To produce infective lentiviral particles, 293T cells were co-transfected by the calcium phosphate method with the virion packaging elements (VSV-G and D8.9) and the shRNA producing vector (FSPsi) or the expression vector (FCIV) on 293T human embryonic kidney. 293T cells were allowed to produce lentiviral particles during 3–4 days in the same culture medium used for endometrial cell lines and explants. Culture medium was collected, centrifuged for 5 min at 1000 × *g*, and filtered through a 0.45-μm filter (Millipore). The medium was diluted 1:2 to 1:4 with fresh medium, and added to growing cell lines or primary explants. Cells were incubated for 24–48 h in the presence of medium containing lentiviral particles. After this period, medium was replaced for fresh medium and cells were incubated for two additional days to allow endogenous protein knockdown or protein overexpression.

*Cell Viability Assays and Assessment of Apoptosis*—Cell viability was determined by the MTT assay. Endometrial adenocarcinoma cells were plated on M96-well plates at $15 \times 10^3$ cells per well. After the indicated treatments, the cells were incubated for 2–3 h with 0.5 mg/ml of MTT reagent and lysed with $Me_2SO$. Absorbance was measured at 595 nm in a microplate reader (Bio-Rad).

Hoechst staining was performed by adding Hoechst dye to a final concentration of 0.5 mg/ml to each M96 well. Cells were counted under epifluorescence microscope (Leica Microsystems).

*Western Blot Analysis*—Endometrial adenocarcinoma cell lines were washed with cold phosphate-buffered saline and


A
24h
48h
HEC
IK
RL
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Viability (% of CONTROL)
Bortezomib (nM)
B
48h
Viavility (% of Control)
MG-132 (μM)
Viability (% of Control)
EPOXOMICIN (nM)
Vability (% of Control)
ALLN (μM)
C
UN
BORT
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Bort+BAF
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E
IK
HEC
RL
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MG-132
0,5 μM
Pro-Caspase-9
CF
Active
Caspase-3

lysed with lysis buffer (2% SDS, 125 mM Tris-HCl, pH 6.8). Protein concentrations were determined with the protein assay kit (Bio-Rad). Equal amounts of proteins were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Nonspecific binding was blocked by incubation with TBST (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) plus 5% nonfat milk. Membranes were incubated with primary antibodies overnight at 4 °C. Signal was detected with ECL Advance (Amersham Biosciences).

*Luciferase Assays*—Endometrial carcinoma cell lines were plated in M24 multiwell plates and transfected using either calcium phosphate or Lipofectamine 2000 following the manufacturer's instructions, with the reporter NFκB-LUC construct together with a plasmid encoding β-galactosidase. After 24 h, cells were treated as indicated in each experiment and cells were lysed with 60 ml of luciferase lysis buffer (25 mM glycylglycine, pH 7.8, 15 mM $Mg_2SO_4$, 1% Triton X-100, 5 mM EGTA) and rocked on ice for 15 min. 30 ml of lysates were transferred to M96 multiwell plates and 30 ml of luciferase assay buffer was added to a final concentration of (25 mM glycylglycine, 15 mM $KHPO_4$, pH 7.8, 15 mM $Mg_2SO_4$, 1% Triton X-100, 5 mM EGTA, 1 mM dithiothreitol containing, 2 mM ATP, 100 mM acetyl-coenzyme A, and 100 mM luciferine). Luciferase was measured using a microplate luminometer. After luciferase measuring 60 ml of 2× β-galactosidase buffer (200 mM $NaPO_4$, 20 mM KCl, 2 mM $MgSO_4$, 4 mg/ml *o*-nitrophenyl β-D-galactopyranoside) was added to each well and measured on a microplate reader at 415 nm.

*Electrophoretic Motility Shift Assay*—After appropriate treatment, nuclear and cytoplasmic extracts were obtained from $2 \times 10^6$ IK and HEC cells using the NE-PER nuclear and cytoplasmic extraction kit (Pierce). Electrophoretic mobility shift assay was carried out using the Light-Shift Chemiluminiscent kit (Pierce), 5–10 μg of nuclear protein extracts were incubated with 20 fmol of 5′ biotin-labeled NF-κB consensus oligonucleotide (MWG-Biotech, Ebersberg, Germany) for 20 min at room temperature. DNA complexes were electrophoresed on a 5% acrylamide gel, and transferred to a nylon membrane (Amersham Biosciences). Light signal was developed following the manufacturer's instructions. For supershift experiments, 2 μg of p65 antibody was added to the binding reaction and incubated for additional 45 min.

## RESULTS

*Proteasome Inhibitors Induce Cell Death on Endometrial Adenocarcinoma Cell Lines*—Bortezomib is currently used as a chemotherapeutic agent in patients with relapsed multiple myeloma. It is under ongoing clinical trials for evaluation of efficacy in the treatment of some solid tumors. However, some tumoral cell types undergo apoptosis or cell growth arrest after proteasome inhibitor treatment, whereas others are insensitive to them, or require co-treatment with other drugs or factors. To investigate whether bortezomib and the other three proteasome inhibitors were able to induce cell death on endometrial adenocarcinoma cells, we treated four endometrial carcinoma (EC) cell lines, Ishikawa (IK), KLE, RL-95 (RL), and HEC-1-A (HEC) with bortezomib and three different proteasome inhibitors (MG-132, epoxomicin, or ALLN). All proteasome inhibitors tested caused a dose-dependent decrease on cell viability as assessed by MTT (Fig. 1, *A* and *B*). The reduction on viability was accompanied by an increase of apoptotic nuclei as seen by Hoechst staining (Fig. 1*C*) suggesting apoptotic cell death. Treatment with MG-132 resulted in processing of caspase-3 and caspase-9. (Fig. 1*D*). Co-treatment of IK with bortezomib or MG-132 plus BAF, a broad specificity caspase inhibitor almost completely abolished cell death (Fig. 1*E*). Altogether these data indicate that proteasome inhibitors are effective in inducing apoptotic cell death on endometrial carcinoma cell lines.

*Proteasome Inhibitors Induce Phosphorylation and Degradation of IκBα*—Inhibition of proteasome prevents NF-κB activation and causes cell cycle arrest or cell death on many different types of cell. The antitumoral effects of bortezomib have been proposed to involve NF-κB inhibition as a main mechanism of action. To investigate whether this was the case in our cell lines, we assessed the levels of IκBα, after treatment with proteasome inhibitors. Time course treatment of IK, HEC, or RL cells with 25 nM bortezomib resulted in a marked increase on phosphorylation of serine 32, followed by reduction of IκBα protein levels over time (Fig. 2*A*). To rule out the possibility that IκBα phosphorylation and reduction were nonspecific effects of bortezomib in our particular cell lines, we treated IK cells with the other three proteasome inhibitors. Treatment of IK cells with MG-132 resulted in phosphorylation and reduction of IκBα, similar to that observed with bortezomib (Fig. 2*B*). Although each cell line showed different basal levels of IκBα, treatment with MG-132 resulted in a similar reduction on IκBα levels in all of them (Fig. 2*C*). We also observed a significant reduction of IκBα with epoxomicin or ALLN (Fig. 2*D*).

*Proteasome Inhibitors Induce NF-κB Nuclear Translocation, DNA Binding, Transcriptional Activity, and Phosphorylation of p65 on Serine 536*—The results described above stimulated us to investigate whether the reduction of IκBα resulted in an activation of NF-κB transcriptional activity. To address this point, we used different experimental approaches. We carried out a transcriptional activity assay of NF-κB by luciferase reporter assay. Cells were transfected with NF-κB-dependent luciferase reporter construct and treated with the indicated doses of proteasome inhibitors for 14–16 h. IK cells treated with 10 or 25 nM bortezomib displayed a marked increase on the basal luciferase activity (Fig. 3*A*). Such an increase was also observed

FIGURE 1. **Proteasome inhibitors trigger apoptosis on endometrial carcinoma cell lines.** *A*, Ishikawa (*IK*), KLE, RL-95 (*RL*), and HEC-1-A (*HEC*) cells were treated with increasing doses of bortezomib for 24 or 48 h and cell viability was assessed by MTT. Results are expressed as percent of the control values. *B*, Ishikawa (*IK*), KLE, RL-95 (*RL*), and HEC-1-A (*HEC*) cells were treated for 48 h with the indicated doses of MG-132, epoxomicin, or ALLN and cell viability was assessed by MTT. Results are expressed as percent of the control values. *C*, micrographs of IK cells left untreated (*UN*) or treated for 36 h with 25 nM bortezomib, 1 μM MG-132, 50 nM epoxomicin, or 50 μM ALLN and stained with Hoechst dye. *D*, quantification of apoptotic nuclei after 36 h treatment with 25 nM bortezomib (*Bort*) or 0.5 μM MG-132 (*left graph*). Quantification of Hoechst-stained apoptotic nuclei of IK cells treated with 25 nM bortezomib alone (*Bort*), 100 μM BAF, or a combination (*Bort* + *BAF*) (*right graph*). *E*, IK, RL, KLE, or HEC cells were treated with 0.5 μM MG-132 for 24 h and lysates were subjected to Western blot with antibodies to active caspase-3 or antibody to caspase-9.

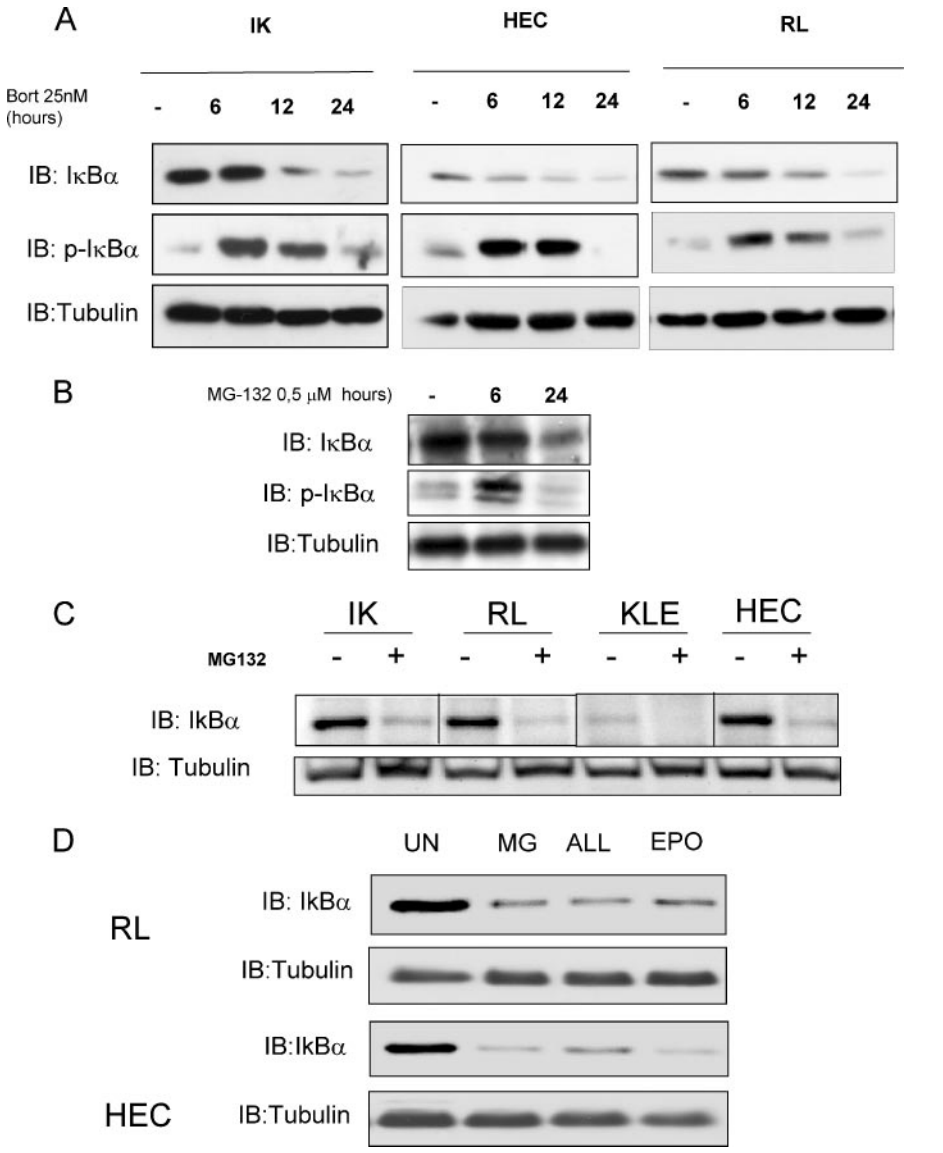



FIGURE 2. **Proteasome inhibitors induce phosphorylation and degradation of IκBα.** *A*, Ishikawa (*IK*), HEC-1-A (*HEC*), and RL-95 (*RL*) cells were treated for the indicated times with 25 nM bortezomib and cell lysates were subjected to Western immunoblot (*IB*) with antibodies to phosphorylated IκBα, IκBα, or tubulin. *B*, IK cells were treated for 6 or 24 h with 0.5 mM MG-132 and cell lysates were analyzed by Western blot with antibodies to phosphorylated IκBα, IκBα, or tubulin. *C*, IK, RL, KLE, or HEC cells were treated with 0.5 μM MG-132 or left untreated for 24 h and cell lysates were subjected to Western blot with antibodies to IκBα (*top panel*) or tubulin (*bottom panel*). *D*, RL and HEC cells were treated for 24 h with 0.5 μM MG-132 (*MG*), 50 nM epoxomycin (*EPO*), or 50 μM ALLN (*ALL*) and cell lysates were subjected to Western blot antibodies to IκBα (*top panel*) or tubulin (*bottom panel*).

when IK cells were treated with MG-132, epoxomicin, or ALLN. Consistent with this result, we also observed that treatment with MG-132 or bortezomib resulted in the formation of a DNA·NF-κB complex as assessed by electrophoretic mobility shift assay (Fig. 3*B*). This complex showed a similar pattern of migration to that observed with TNF. To provide a control of proteasome inhibitor action, we stimulated a melanoma cell line (M16) and endometrial carcinoma cell line RL-95 with TNF. TNF treatment triggers the canonical, proteasome-dependent, IκBα degradation and p65 NF-κB binding activity in both cell lines. As expected, both bortezomib and MG-132 reduce NF-κB binding to DNA in M16 cells. Similar results are obtained in RL cells with MG-132 (Fig. 3*C*). A supershift analysis using a p65 antibody caused a marked reduction on the NF-κB complex and the appearance of a supershifted band in both MG and TNF stimulated cells (Fig. 3*B*). Accordingly, Western blot analysis on nuclear and cytoplasmic extracts revealed that treatment with either MG-132 or bortezomib induced p65 nuclear translocation on IK cells. As control we treated HEC cells with TNF, which is known to induce p65 nuclear translocation (Fig. 3, *D* and *E*). Finally, we also analyzed phosphorylation of the p65 subunit of NF-κB on serine 536, which has been associated with increased transactivation potential. Treatment with bortezomib or other proteasome inhibitors resulted in increased phosphorylation on serine 536 of p65 in all the cell lines that were tested (Fig. 3*F*).

*SR-IκBα Blocks Proteasome Inhibitor-induced NF-κB Activity* —To ascertain whether IκBα degradation was dependent on serine phosphorylation, we transfected IK and HEC cells with a construct encoding a form of IκBα carrying serine to alanine mutations at residues 32 and 36, named SR-IκBα. These mutations prevent IκBα phosphorylation and its subsequent proteasome-mediated degradation, thereby preventing release and nuclear translocation of NF-κB (46). Expression of SR-IκBα caused a marked reduction on the activation of NF-κB transcriptional activity by either bortezomib or MG-132 in HEC and IK cells (Fig. 4). This data supports the hypothesis that proteasome inhibitors require phosphorylation and degradation of IκBα.

*NF-κB Activity Induced by Proteasome Inhibitors Requires Functional IKKα and IKKβ*—The major upstream kinases involved in NF-κB activation are IKKα and IKKβ. We investigated whether these kinases were involved in activation of NF-κB by proteasome inhibitors. Treatment of IK, HEC, or RL cells with bortezomib caused increased phosphorylation of IKKα/β, as assessed by Western blot analysis of lysates with an antibody that specifically recognizes the phosphorylated forms of both proteins (Fig. 5*A*).

To determine the requirement of either or both IKKα or IKKβ subunits, we co-transfected IK or HEC cells with the NF-κB-luciferase reporter construct plus dominant negative forms of either IKKα (DN-IKKα) or IKKβ (DN-IKKβ). Both DN-IKKα and DN-IKKβ blocked NF-κB transcriptional activ-

ity on IK cells treated with 25 nM bortezomib (Fig. 5*B*, *left*) and in IK and HEC cells treated with MG132 (Fig. 5*B*, *right*).

To further demonstrate the role of IKKα or IKKβ subunits, we designed lentiviral shRNAs targeting each subunit to knockdown endogenous expression. We designed shRNAs targeting two sequences for each kinase. Infection of IK cells with lentiviruses carrying shRNAs to either IKKα or IKKβ subunits revealed that each shRNA selectively knocked down the expression of the corresponding protein (Fig. 5*C*). These two shRNAs were selected for subsequent experiments. We infected IK cells with the functional IKKα or IKKβ shRNAs for 3 days to allow protein knockdown, and cells were subsequently treated with bortezomib. As shown in Fig. 5*D*, both IKKα shRNA and IKKβ shRNA blocked NF-κB activity with similar efficiency to the dominant negative forms. Our data suggest that phosphorylation and expression of both IKKα and IKKβ subunits are required for proteasome inhibitor-induced NF-κB activity. Neither IKKα nor IKKβ knockdown increased the cell viability in proteasome inhibitor-treated cells (supplemental Fig. 1).

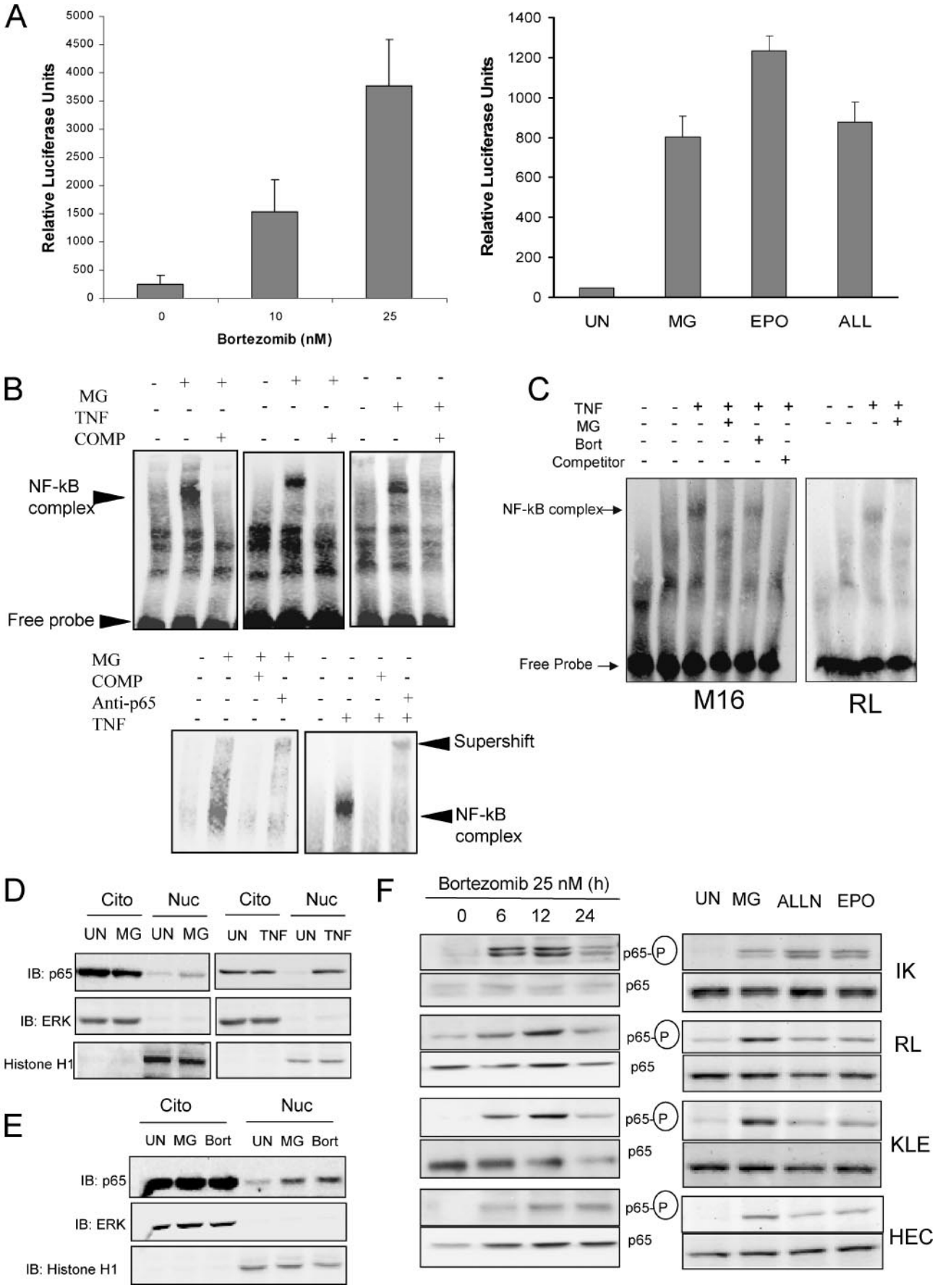
*IKKα and IKKβ Are Required for p65 Phosphorylation but Not for IκBa Degradation after Proteasome Inhibitor Treatment*—To determine whether IKK was responsible for proteasome inhibitor-induced p65 phosphorylation, HEC cells were infected for 3 days with lentiviruses carrying either IKKα or IKKβ shRNAs. HEC cells were treated for 6 or 24 h with 25 nM bortezomib and cell lysates were analyzed by Western blot (Fig. 6*A*). As shown in Fig. 6, both IKKα and IKKβ shRNA strongly inhibited p65 phosphorylation. The cells transfected with IKKβ shRNA showed a reduction of phosphorylated IκBα, but also a decrease on total IκBα protein. However, the molecular weight shift on migration of IκBα and degradation of IκBα by bortezomib remained unaffected (Fig. 6*A*). Our findings suggest that p65 phosphorylation is mediated by either IKKα or IKKβ, but also show that IκBα degradation is independent of IKKs. Similar results were obtained with IK cells (data not shown).

These results suggest that proteasome inhibitors increase NF-κB activity, by involving the p65 subunit of NF-κB. To determine whether transcriptional activity of NF-κB was really dependent of p65 subunit, we co-transfected p65 siRNA or c-Rel siRNA with the NF-κB reporter construct, and cells were exposed to bortezomib. Transfection of p65 siRNA resulted in a significant decrease of NF-κB transcriptional activity in both IK and HEC cells (Fig. 6*B*). Such a result was not observed when c-Rel siRNA was transfected.

*Proteasome Inhibitors Induce Cell Death and Activate NF-κB in Primary Culture Explants from Endometrial Adenocarcinoma Samples*—To further demonstrate the effect of proteasome inhibitors on endometrial carcinoma, we assessed the effects of such inhibitors on primary explants obtained from human endometrial carcinomas. After enzymatic digestion, glandular-like structures were plated on 24-well dishes and the epithelial origin of the samples was assessed by immunofluorescence to a wide spectrum cytokeratin, cytokeratin-7 and β-catenin, two proteins that are only expressed on endometrial cells of epithelial origin, but not on stromal cells (Fig. 7*A*). To ascertain whether proteasome inhibitors induced apoptosis on primary explants as well as on cell lines, we treated endometrial explant cultures with 0.5 μM MG-132 or 25 nM bortezomib and we assessed caspase-3 activation by immunofluorescence with an antibody that recognizes its active form (Fig. 7*B*). Both MG-132 and bortezomib increased the number of cytokeratin positive cells displaying active caspase-3. These results suggest that bortezomib and MG-132 induce apoptotic cell death on primary endometrial carcinoma explants. To determine whether MG-132 and bortezomib also were able to activate NF-κB, we analyzed IκBα phosphorylation and degradation after treatment with these two inhibitors at doses that induced IκBα degradation and phosphorylation on endometrial carcinoma cell lines. As shown in Fig. 7*C*, both MG-132 and bortezomib induced phosphorylation and degradation of IκBα and phosphorylation of p65 at serine 536 in a similar way to that observed in all cell lines tested. Altogether, these results demonstrate that proteasome inhibitors are able to activate NF-κB signaling in primary endometrial carcinoma explants.

## DISCUSSION

Bortezomib and other proteasome inhibitors trigger cell growth arrest or apoptosis on several tumors (2). Bortezomib is a modified dipeptidyl boronic acid that creates compounds that form covalent complexes with the proteasome. It is used as a chemotherapeutic agent for treatment of relapsed and refractory multiple myeloma (3–7). It is also a promising anti-cancer agent for treatment of solid tumors. In many different types of tumor cells, proteasome inhibition cause cell death by blocking NF-κB activity. In this article, we demonstrate for the first time that bortezomib and other three proteasome inhibitors (MG-132, epoxomicin, and ALLN) may induce apoptosis in endometrial cancer cell lines and primary culture explants from endometrial carcinomas. Cell death was accompanied by activation of caspases and apoptotic nuclear morphology. However, in contrast to that observed on other cancer cells, this cell death is not related with NF-κB blockage. In endometrial cancer cells, bortezomib and other proteasome inhibitors increase NF-κB activity rather than its inhibition. NF-κB is a family of transcription factors involved in the regulation of genes encoding cytokines, cytokine receptors, and cell adhesion molecules, which drive immune and inflammatory responses (14–16). However, NF-κB is also related to carcinogenesis, by regulating genes involved in apoptosis, the cell cycle, differentiation, invasion, and cell migration (16, 19). Therefore, inhibition of NF-κB is a promising target for treatment of cancer. Because of their ability to block IκBα degradation, proteasome inhibitors have been widely used as inhibitors of NF-κB. In fact, inhibition of NF-κB activity has been reported as the main mediator of cytotoxic effects of bortezomib. One of the main goals of this study was to investigate the effect of proteasome inhibition on NF-κB in endometrial carcinoma. Treatment of endometrial carcinoma cell lines with bortezomib did not stabilize or inhibit IκBα, but induced phosphorylation and reduction of IκBα levels. Similar results were obtained when the endometrial adenocarcinoma cell lines were exposed to the other three proteasome inhibitors (MG-132, epoxomicin, or ALLN) that inhibit the proteasome by other chemical mechanisms. Although these results seem to contradict the well established mechanism of IκBα degradation by proteasome inhibition, it is worth mentioning that some recent evidence suggests that proteasome inhibitors may acti-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IB: ERK
IB: Histone H1
E
Cito
Nuc
UN MG Bort UN MG Bort
IB: p65
IB: ERK
IB: Histone H1
F
Bortezomib 25 nM (h)
0 6 12 24
UN MG ALLN EPO
p65-P
p65
IK
RL
KLE
HEC

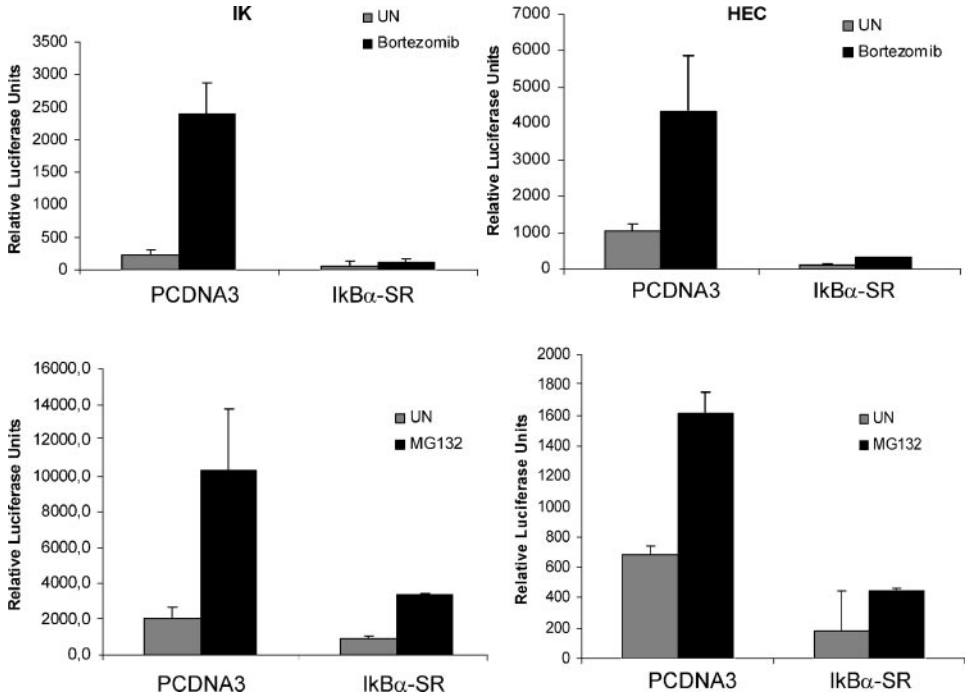



FIGURE 4. **IκBα superrepressor blocks bortezomib and MG132-induced NF-κB transcriptional activity.** IK or HEC cells were co-transfected with the NFκB-luciferase reporter construct and either the empty vector (PCDNA3), or a IκBα superrepressor, which cannot be phosphorylated on serines 32 and 36 (SR-IκBα). After 48 h, cells were stimulated with 25 nM bortezomib or 0.5 μM MG132 and luciferase activity was assayed 16 h later. Results are expressed in relative luciferase units.

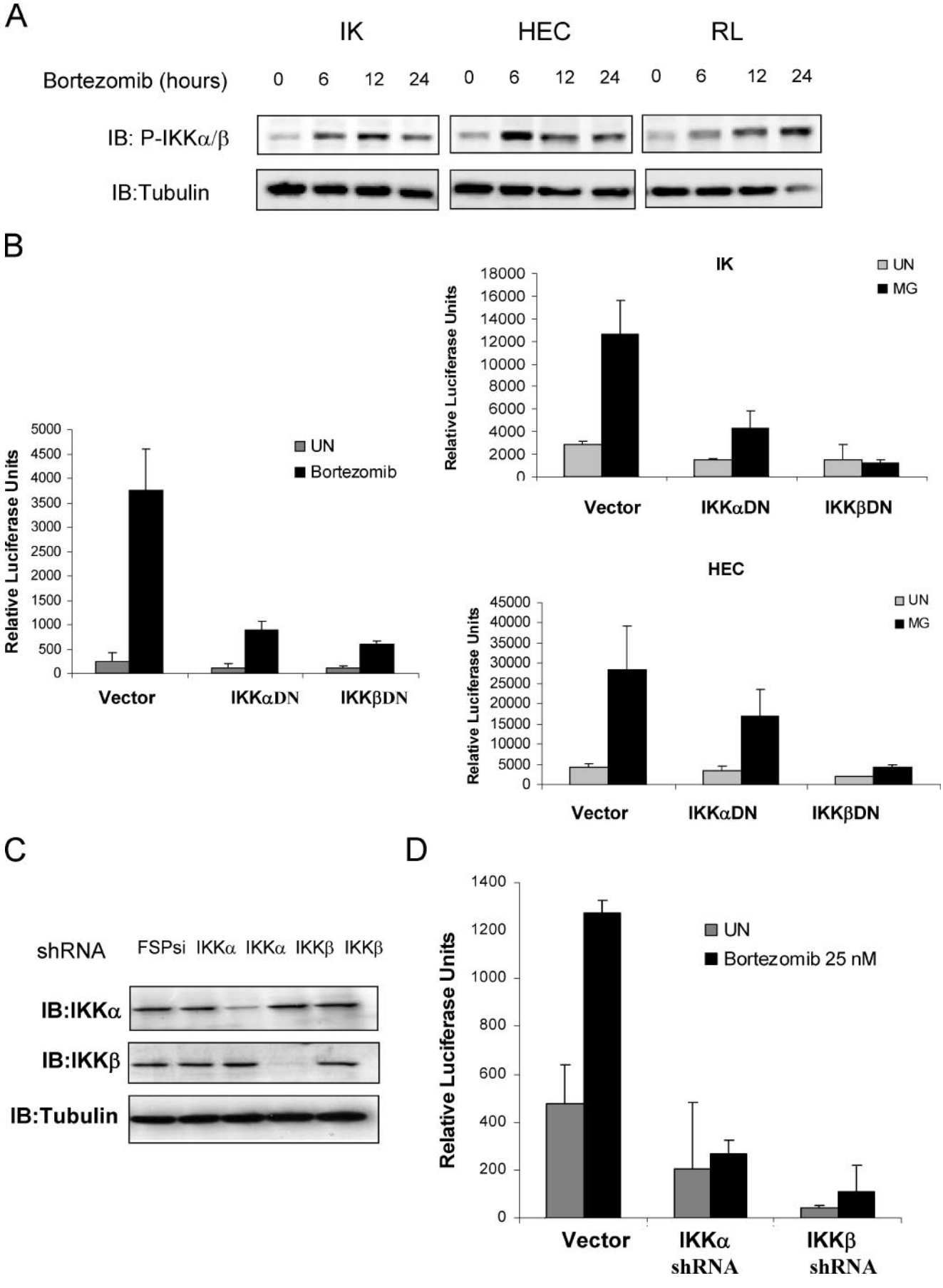
vate NF-κB in some cancer cell lines. This was the case of the colon adenocarcinoma cell line HT-29 after treatment with MG-132 (25). In agreement with this report, we have found IκBα degradation, IKK phosphorylation, and increased transcriptional activity of NF-κB, as a result of exposure to the four different proteasome inhibitors.

Phosphorylation and degradation of IκBα is followed by an increase of NF-κB activity. We found that proteasome inhibitors induced the formation of a DNA·NF-κB complex displaying similar migration properties than that observed after stimulation with TNF. TNF is a well known activator of the canonical NF-κB pathway that results in nuclear translocation of p65 subunits. Therefore, this result suggests that p65 may be involved in NF-κB activity induced by such inhibitors. Accordingly, we have found supershift of the NF-κB complex using p65 antibody, nuclear translocation of p65, and more importantly, p65 siRNA reduced the transcriptional activity induced by proteasome inhibitors. Moreover, we have found that all proteasome inhibitors tested, including bortezomib, increase p65 phosphorylation on serine 536. Recent evidence suggest that phosphorylation on this serine is critical for p65 transcriptional activity after different stimuli such as lymphotoxin β (21), TNF (22), lipopolysaccharide (23), or interleukin 1 (24) stimulation. Moreover, a recent report also shows that MG-132 can also enhance serine 536 phosphorylation on HeLa cells (24). Therefore, phosphorylation of p65 is consistent with the increased transcriptional activity observed after treatment with proteasome inhibitors.

Recent reports describe IKK-independent mechanisms that can also activate NF-κB upon certain stimuli. Recently, it has been shown that DNA damaging agents such as topoisomerase inhibitors require IKK complex to induce NF-κB (26, 27), whereas others suggest that NF-κB activation can take place independently of IKKs (28). To address the involvement of IKKα or IKKβ in proteasome inhibitor effects on endometrial carcinoma cell lines, we transfected IK or HEC cells with the dominant negative forms of IKKα or IKKβ or with lentiviral-transduced shRNAs, and we assessed NF-κB activity after bortezomib or MG-132 exposure. These experiments clearly demonstrated that, in contrast to topoisomerase inhibitors, NF-κB activation by proteasome inhibitors in endometrial carcinoma cell lines requires functional IKKs.

Reporter experiments performed with the IκBα supperrepressor indicate that IκBα phosphorylation and degradation are required for proteasome inhibitor-induced NF-κB activity. However, shRNA to either IKKα or IKKβ did not block IκBα degradation by proteasome inhibitors. IKKβ shRNA diminished IκBα phosphorylation but such reduction can be explained by the reduction of total IκBα protein levels.

FIGURE 3. **Proteasome inhibitors increase NF-κB nuclear translocation, DNA binding, transcriptional activity, and phosphorylate p65 subunit at serine 536.** *A*, IK cells were transfected with the NFκB-luciferase reporter construct and treated with 10 and 25 nM bortezomib (*left graph*), or treated with 0.5 μM MG-132 (*MG*), 50 nM epoxomycin (*EPO*), or 50 μM ALLN (*ALL*) (*right graph*) for 16 h, and cell lysates were assayed for luciferase activity. Results are expressed as relative luciferase units. *B*, IK cells were treated with bortezomib MG-132 (*MG*) for 16 h, and HEC cells were treated with MG-132 for 16 h or with TNF for 30 min, and nuclear lysates were incubated with 20 fmol of labeled probe. A 200 M excess of unlabeled competitor was included in the indicated lanes to demonstrate the specificity (*upper panel*). For supershift experiments HEC cells were treated with MG-132 or TNF and nuclear lysates were incubated with 200 M excess of the unlabeled competitor antibody to p65 as indicated (*lower panel*). *C*, RL endometrial carcinoma cells and the melanoma cell line M16 were treated for 30 min in the presence or absence of proteasome inhibitors and nuclear lysates were incubated with 20 fmol of biotin-labeled probe. *Arrows* indicate the proteasome inhibitor-induced NF-κB complex and the free probe. A 200 M excess of unlabeled competitor was included in the indicated lanes to demonstrate the specificity. *D*, HEC cells were treated with 50 ng/ml TNF for 30 min or with 0.5 mM MG-132 for 16 h. Cytoplasmic (*Cito*) and nuclear (*Nuc*) cell lysates were analyzed by Western blot with antibodies to p65 NF-κB subunit. To determine the purity of cytoplasmic and nuclear lysates, membranes were reprobed with anti-ERK antibody (marker of cytoplasmic fraction) or with histone H1 antibody (nuclear fraction). *IB*, immunoblot. *E*, IK cells were treated for 16 h with either 25 nM bortezomib (*Bort*) or 0.5 mM MG-132 and nuclear and cytoplasmic lysates were analyzed with p65 antibodies. Membranes were reprobed with antibodies to ERK and histone H1. *F*, IK cells were treated for 0, 6, 12, or 24 h with 25 nM bortezomib (*left panel*) and IK, RL, HEC, and KLE cells were treated for 16 h with 0.5 μM MG-132, 50 nM epoxomycin, or 50 μM ALLN (*right panel*) and cell lysates were subjected to Western blot with antibodies that specifically recognize serine 536 only when it is phosphorylated. Membranes were also incubated with antibodies to total p65 to ensure equal amounts of protein.


A
IK
HEC
RL
Bortezomib (hours)
0 6 12 24 0 6 12 24 0 6 12 24
IB: P-IKKα/β
IB:Tubulin
B
IK
UN
MG
Relative Luciferase Units
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
Vector
IKKαDN
IKKβDN
UN
Bortezomib
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Vector
IKKαDN
IKKβDN
HEC
UN
MG
Relative Luciferase Units
45000
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25000
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IB:IKKα
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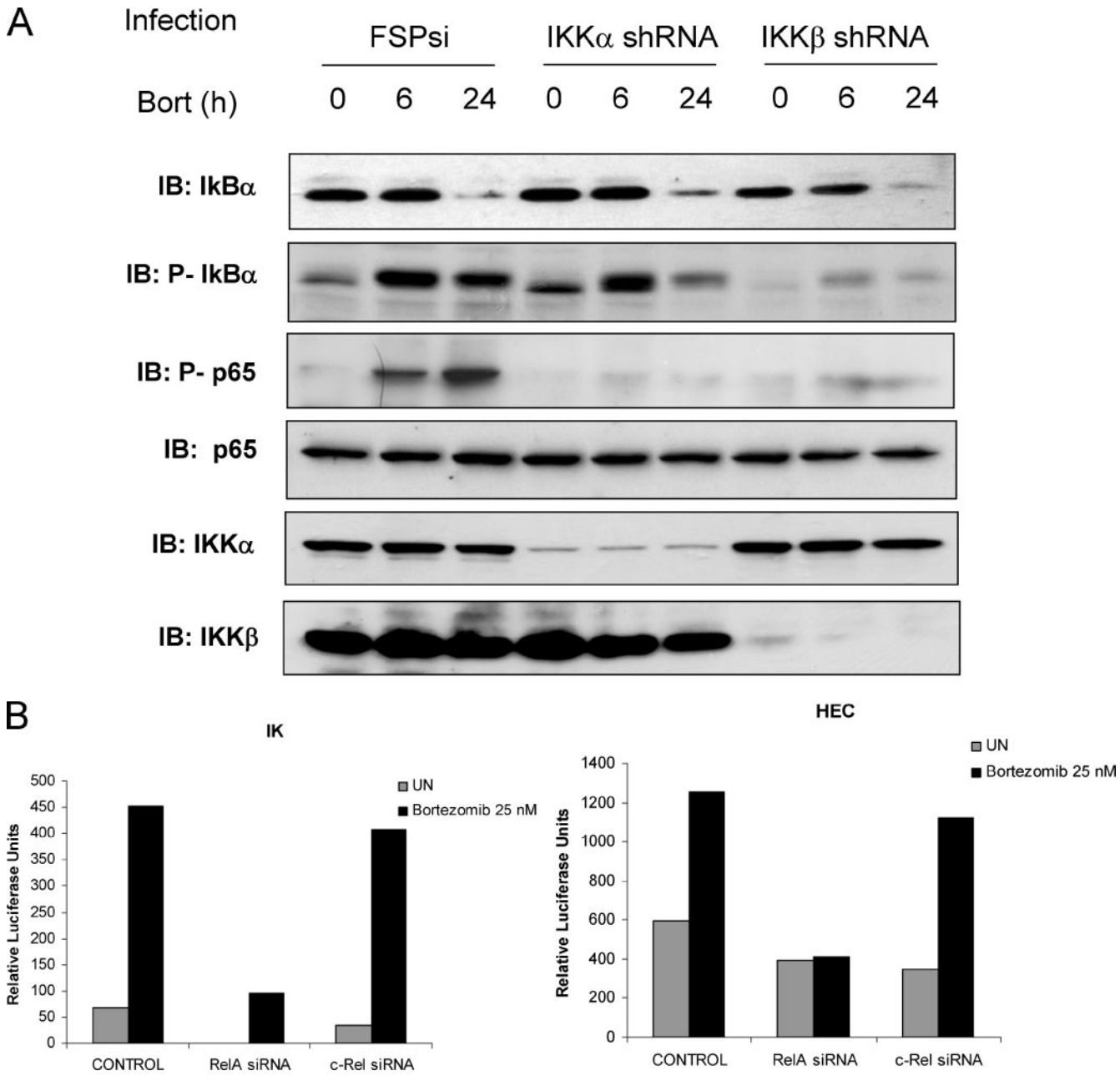


FIGURE 6. **Down-regulation of IKKα and IKKβ inhibit p65 phosphorylation and activity but not IκBα degradation.** *A*, HEC cells were infected with lentiviruses carrying functional shRNAs to IKKα or IKKβ to allow protein knockdown and then stimulated for 6 or 24 h with 25 nM bortezomib, and lysates were analyzed by Western blot. Blots were incubated with antibodies to IκBα, phospho-IκBα, and phospho-Ser$^{536}$-p65. Membranes were also blotted with antibodies to IKKα or IKKβ to ensure down-regulation of target proteins by shRNAs. *B*, *bar chart* showing HEC co-transfected with the NFκB-luciferase reporter construct and either siRNAs targeting p65 (RelA) or c-Rel subunits of NF-κB. After 48 h to allow siRNA to down-regulate protein expression, cells were stimulated with 25 nM bortezomib and luciferase activity was assayed 16 h later. Results are expressed in relative luciferase units. *IB*, immunoblot.

Although both IKKα and IKKβ shRNAs did not block IκBα degradation, they markedly reduced NF-κB activity. These results suggest that NF-κB activation by proteasome inhibitors is further regulated downstream of IκBα degradation. Increasing evidence supports that NF-κB phosphorylation may be an important mechanism of NF-κB regulation (20). As mentioned above, we found that all proteasome inhibitors phosphorylated p65 at serine 536. Such phosphorylation can be achieved by

FIGURE 5. **IKKα and IKKβ are required for bortezomib and MG132-induced NF-κB activity.** *A*, Western blot showing IK, HEC, and RL cell lines stimulated for 6, 12, or 24 h with 25 nM bortezomib and incubated with an antibody that recognizes IKKα/IKKβ phosphorylated or with tubulin to ensure equal protein loading (*bottom panel*). *B*, *bar charts* showing NF-κB transcriptional inhibition by either IKKα or IKKβ dominant negative forms on IK cells treated with bortezomib (*left chart*) or in IK and HEC cells treated with MG-132 (*right charts*). *C*, Western blot analysis of IKKα and IKKβ expression on IK cells infected for 3 days with lentiviruses carrying two different shRNAs targeting IKKα or IKKβ. *D*, IK cells infected with lentiviruses carrying functional shRNAs to IKKα or IKKβ for 3 days to allow protein knockdown and then transfected with the NFκB-luciferase reporter construct. 24 h later they were treated with 25 nM bortezomib and luciferase activity was assayed. *IB*, immunoblot.

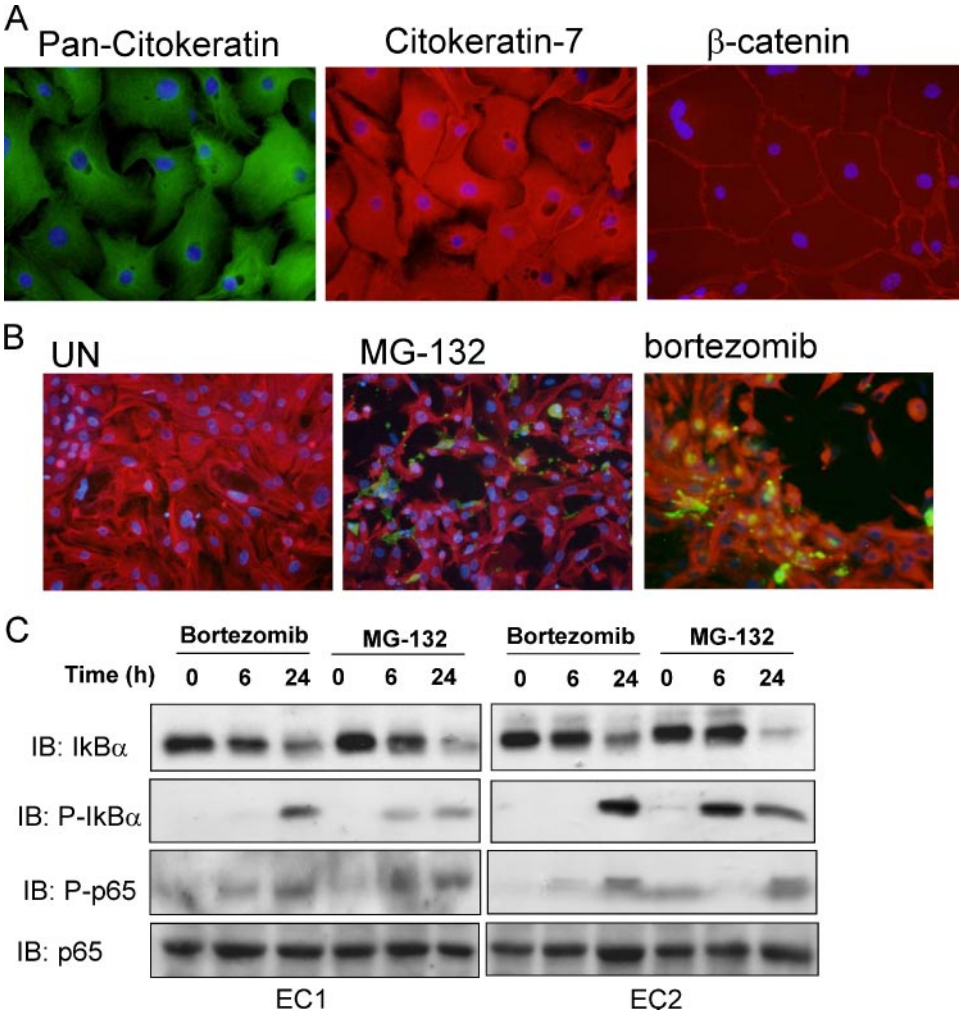


FIGURE 7. **Proteasome inhibitors induce cell death and activate NF-κB on primary endometrial carcinoma explants.** *A*, endometrial carcinoma explants after 3 days in culture stained with antibodies against pantothenate-cytokeratin, cytokeratin-7, or β-catenin. Nuclei were visualized by Hoechst staining. *B*, endometrial carcinoma explants were treated with 25 nM bortezomib, 0.5 mM MG-132, or left untreated for 24 h, fixed, and stained with anti-active caspase-3 antibody (*green*), citokeratin-7 (*red*), and Hoechst dye to visualize nuclei (*blue*). *C*, two different endometrial carcinoma explants were treated with 25 nM bortezomib or 0.5 μM MG-132 and cell lysates were analyzed by Western blot by IκBα, phosphorylated IκBα, phosphorylated p65, or tubulin. *IB*, immunoblot.

Despite the activation of NF-κB, proteasome inhibitors are able to induce cytotoxic effects on endometrial carcinoma cell lines. Although it may be surprising, these features are also observed after treatment of cancer cells with other stress signals such as ionizing radiation, hypoxia, or ultraviolet light and chemotherapeutic drugs such as vincristine, vinblastine, etoposide, adriamycin, cisplatin, daunorubicin, etc. (31). For example, topoisomerase inhibitors such as camptothecin or doxorubicin activate NF-κB but induce cell death (32–34). However, the NF-κB activation observed after proteasome inhibitor treatment has a particular relevance because they are widely used as pharmacological blockers of IκBα degradation and subsequent NF-κB inhibition. In fact, as a control of correct proteasome inhibition, we have found that such inhibitors are effective to block NF-κB activation by TNF in both a melanoma cell line and the RL-95 endometrial cell lines, indicating that such drugs are inhibiting proteasome and, as a consequence, the NF-κB activation by the canonical pathway. These results indicate that proteasome inhibitors are effective to block NF-κB by TNF, but long treatment (hours) lead to induction of NF-κB by themselves.

NF-κB inhibition has been proposed as the main mechanism by which proteasome inhibitors are used as anticancer drugs. Although in some cell types NF-κB activation trigger cell death (35, 36), NF-κB does not mediate cytotoxic effects of proteasome inhibition. To test this hypothesis endometrial cancer cell lines were infected with lentiviruses carrying shRNA either to IKKα or IKKβ and treated with cytotoxic doses of bortezomib or MG-132 and we assessed cell viability by MTT assay. Neither IKKα nor IKKβ shRNA increased cell viability after treatment with such inhibitors (supplementary Fig. 1). Rather, we observed a slight decrease. We observed that IKKα or IKKβ knockdown reduced basal proliferation and viability of endometrial cancer cells (data not shown), which is consistent with an anti-apoptotic role of NF-κB in endometrial carcinoma cell lines.

Because of the use of bortezomib on treatment of multiple myeloma and the promising effects on some solid tumors we decided to test the ability of this inhibitor to induce apoptosis on primary explants from tumor samples from patients with endometrial carcinoma. Both MG-132 and bortezomib

different upstream kinases. Phosphorylation on serine 536 is triggered by IKKα after TNFα (29) and lymphotoxin β (30) or by IKKβ after T cell co-stimulation (47) or lipopolysaccharide (30) but also by TBK1, Akt, or IKKε (20, 24). In this study, we have found that shRNA inhibition of either IKKα and IKKβ blocked p65 at serine 536 and NF-κB transcriptional activity, suggesting a major role for the p65 subunit in proteasome inhibitor-induced activation. Consistent with this hypothesis, we have found p65 nuclear translocation and reduced NF-κB transcriptional activity in p65 siRNA-transfected cells, indicating a main role for p65 in NF-κB activation. As discussed above, IκBα is degraded even after IKK knockdown by shRNA, but both serine phosphorylation of p65 and NF-κB transcriptional activity is inhibited. Altogether the mechanism by which proteasome inhibitors induce NF-κB activity seems to be tightly regulated. Such activation requires phosphorylation and degradation of IκBα but also an IKK-mediated phosphorylation of p65.

induced activation of caspase-3, suggesting that both inhibitors triggered apoptotic cell death. Similar to that observed on endometrial cancer cell lines, bortezomib and MG-132 caused IκBα phosphorylation and degradation, which suggests that such inhibitors also activate NF-κB on primary endometrial carcinoma explants.

During the last few years, other molecular mechanisms, not related to NF-κB, have been involved in proteasome inhibitor cell death. Among them, it has been reported an involvement of the endoplasmic reticulum stress (ER stress) (13, 37, 39) proteins or increases in expression of death receptors (40). Other recent findings suggest that proteasome inhibitors may induce apoptosis by up-regulating BH3 only family members such as Noxa (41–43), Bik (44, 45), or Puma (45). To determine whether proteasome inhibitors produce ER stress in endometrial cancer cells we have performed reverse transcriptase-PCR analysis of *GADD153* and *HOX-1* (heme oxygenase), two stress genes that have been previously demonstrated to increase after ER stress after bortezomib treatment (13, 37, 39). We have also analyzed Noxa, BIK, and Puma expression after treatment with bortezomib (supplementary Fig. 2). In agreement with previous reports, we have found up-regulation of both *GADD153* and *HOX-1* (heme oxygenase), suggesting that ER stress may be involved in apoptosis triggered by proteasome inhibitors. We have not found significant changes in either Puma or Noxa, but a slightly and transient increase in Bik levels. Future experiments will determine whether BH3 only proteins such as Bik and ER stress regulate apoptosis induced by proteasome in endometrial carcinomas.

In summary, we have demonstrated that bortezomib and other three proteasome inhibitors induce cell death in four endometrial carcinoma cell lines as well as in primary culture explants obtained from endometrial carcinoma samples. However, all proteasome inhibitors tested activated NF-κB, a signaling pathway strongly associated in oncogenesis in many types of cancer. Our findings may have important biochemical and clinical relevance. First, because proteasome inhibitors are widely used as pharmacological inhibitors of NF-κB on the basis of their ability to block IκBα degradation but, as we show here for endometrial carcinomas, they can rather lead to activation of NF-κB. Therefore, the general use of such inhibitors as NF-κB blockers has to be carefully analyzed for particular cell types. Second, if proteasome inhibitors activate NF-κB, administration of these drugs may increase the expression of genes involved with proliferation, apoptosis resistance, or angiogenesis. The activation of these genes could explain the development of adverse effects in patients under treatment with proteasome inhibitors. Additional studies should be performed to demonstrate that the NF-κB activation shown in this report in endometrial carcinoma cell lines, and in a previous study on colonic adenocarcinoma cell lines, is also taking place in other types of tumor cells.

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