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Proteasome Inhibitors Induce Inhibitory κB (IκB) Kinase Activation, IκBα Degradation, and Nuclear Factor κB Activation in HT-29 Cells

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ABSTRACT

The transcription factor nuclear factor κB (NF-κB) is activated and seems to promote oncogenesis in certain cancers. A major mechanism of NF-κB activation in cells involves cytoplasm-to-nucleus translocation of this transcription factor after hydrolysis of the cytoplasmic inhibitor inhibitory κB (IκB) by the 26S proteasome. Because selective proteasome inhibitors have been shown to block IκB degradation; consequently, NF-κB activation in a variety of cellular systems, proteasome inhibitors were proposed as potential therapeutic agents for the treatment of cancer. However, under certain conditions, IκB degradation and NF-κB activation are not mediated by the proteasome system. We investigated how proteasome inhibitors affected NF-κB activation in the intestinal epithelial cancer cell line HT-29, which has been documented to have an atypical NF-κB regulation. Treatment of cells with the selective proteasome inhibitors carbobenzoxy-L-leucyl-L-leucyl-L-norvalinal (MG-115), carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG-132), or lactacystin induced NF-κB activation as indicated by both an increase in NF-κB DNA binding and transcriptional activity. This increase in NF-κB activation caused by proteasome inhibitors was accompanied by an increase in IκB kinase activation and a degradation of IκBα but not IκBβ. Furthermore, proteasome inhibitors induced the expression of NF-κB target genes. In summary, these results demonstrate a unique effect of proteasome inhibitors on the IκB–NF-κB systems in HT-29 cells, in which proteasome inhibitors activate rather than deactivate the NF-κB system. We conclude that the use of proteasome inhibitors to block NF-κB activation in cancer cells may not always be a viable approach.

The transcription factor nuclear factor κB (NF-κB) has been linked recently to the control of cell growth, apoptosis, and cell-cycle progression (Sonenshein, 1997; Baeuerle, 1998; Mayo and Baldwin, 2000; Ghosh and Karin, 2002; Hoffmann et al., 2002). NF-κB is activated in many tumors, including breast cancer cells (Sovak et al., 1997), liver epithelial tumors (Arsura et al., 2000), and melanoma (Shattuck et al., 1994), and in a number of hematological malignancies (Garg and Aggarwal, 2002). Because NF-κB blockade has been documented to induce apoptosis of cancer cells, NF-κB was suggested as a potential therapeutic target in cancer (Baeuerle, 1998; Mayo and Baldwin, 2000; Ghosh and Karin, 2002; Hoffmann et al., 2002). NF-κB is a dimer composed of various combinations of members of the NF-κB/Rel family, which are NF-κB1 (p50), NF-κB2 (p52), c-Rel, RelA (p65), and RelB. The generally accepted model for NF-κB function postulates that the transcription factor is anchored and inactivated in the cytoplasm by association with members of the IκB family of proteins in quiescent cells. Under certain conditions during malignant transformation, IκB is degraded, which then allows nuclear translocation and DNA binding of NF-κB (Sonenshein, 1997). The most prominent member of the IκB family is IκBα, the degradation of which is usually initiated by phosphorylation on serine residues 32 and 36 by the IκB kinase (IKK) complex. This phosphorylation of IκBα is followed by ubiquitination and degradation by the 26S proteasome complex, which can be blocked by a variety of protea-

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ABBREVIATIONS: NF-κB, nuclear factor κB; IKK, inhibitory κB kinase; IκB, inhibitory κB; IL, interleukin; MG-115, carbobenzoxy-L-leucyl-L-leucyl-L-norvalinal; MG-132, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; PD 150606, 3-(4-iodophenyl)-2-mercapto-(*Z*)-2-propenoic acid; DMSO, dimethyl sulfoxide; EMSA, electromobility shift assay; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; RT, reverse transcriptase; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GRO-α, growth-related oncogene-α; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium.

some inhibitors (Kisselev and Goldberg, 2001; Goldberg and Rock, 2002). Taken from the ability of proteasome inhibitors to block NF-κB activation, these agents have been proposed and tested for the treatment of cancer in both laboratory animals and humans (Mayo and Baldwin, 2000).

In addition to the proteasome system, recent studies have provided evidence for alternative pathways of IκB degradation. One of these alternative proteolytic pathways is the caspase system, whose activation has been documented to induce IκB degradation both in vitro (Barkett et al., 1997) and in vivo (Reuther and Baldwin, 1999; Qin et al., 2000). The calpain system may serve as another means of IκB degradation under certain conditions. For example, whereas lipopolysaccharide-induced IκBα degradation in RAW 264.7 mouse macrophages is mediated by the proteasome pathway, silica induces IκBα degradation via a calpain-mediated mechanism (Chen et al., 1997). Similarly, IκBα can be degraded by both a signal-inducible proteasome-dependent and a constitutive proteasome-independent, calpain-dependent mechanism in WEHI 231 B cells (Miyamoto et al., 1998; Shen et al., 2001). In HepG2 liver cells, both the proteasome and calpain pathways participate in the tumor necrosis factor-α–induced degradation of IκB (Han et al., 1999). Mammary tumor cells overexpressing the oncogene Her-2/Neu exhibit an increased basal turnover of IκBα, which is mediated by the calpain and not the proteasome system (Pianetti et al., 2001; Romieu-Mourez et al., 2002). These examples of atypical, proteasome-independent NF-κB pathways raise the possibility that proteasome inhibition may have limitations as a therapeutic approach for tumors.

Recent studies have demonstrated that the colon cancer cell line HT-29 is unique in its regulation of NF-κB responses (Jobin et al., 1997, 1998; Elewaut et al., 1999; Böcker et al., 2000; Jobin and Sartor, 2000). For example, although HT-29 cells display an NF-κB–dependent inflammatory response to bacteria and the cytokine interleukin (IL)-1 (Jobin et al., 1997, 1998; Elewaut et al., 1999; Böcker et al., 2000; Jobin and Sartor, 2000), these cells fail to degrade IκB in response to these stimuli (Jobin et al., 1997, 1998; Elewaut et al., 1999; Böcker et al., 2000; Jobin and Sartor, 2000). Jobin and Sartor (2000) recently raised the possibility that the lack of IκB degradation in HT-29 cells may be caused by a defective proteasome system. This idea was derived from the observation that IL-1 receptor-associated kinase, another protein that is typically degraded by the proteasome upon IL-1 receptor stimulation also failed to undergo degradation in HT-29 cells (Böcker et al., 2000). In an attempt to further characterize the role of the proteasome in the HT-29 cell NF-κB response, we examined whether proteasome inhibition could prevent IL-1β–induced NF-κB activation in HT-29 cells. We found that the proteasome did not play a predominant role in IL-1β–induced NF-κB activation in HT-29 cells. More interestingly, we made the unexpected observation that proteasome inhibition alone induced a substantial activation of the IKK–IκB–NF-κB system in these cells.

Materials and Methods

Cell Culture. The human colon cancer cell lines HT-29 and Caco-2 were obtained from American Type Culture Collection (Manassas, VA). HT-29 cells were grown in modified McCoy's 5A medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). Caco-2 cells were grown in Dulbecco's modified Eagle's medium with high glucose containing 10% fetal bovine serum as well as 0.1 mM nonessential amino acids and 1 mM sodium pyruvate (Németh et al., 2002a,b,c).

Drugs. The selective proteasome inhibitors MG-115, MG-132, and lactacystin were purchased from Calbiochem (San Diego, CA). The selective calpain inhibitors calpeptin and PD 150606 were from Calbiochem. Stock solutions (50 mM) were made by dissolving the agents in DMSO. The final DMSO concentration in the treatment wells resulting from these agents was 0.02%. Control wells not receiving these agents also contained 0.02% DMSO (vehicle). Actinomycin D was obtained from Sigma (St. Louis, MO). Human IL-1β was obtained from R&D Systems (Minneapolis, MN).

NF-κB Electromobility Shift Assay and Supershift Assay. HT-29 or Caco-2 cells were stimulated with proteasome inhibitors for varying lengths of time, and nuclear protein extracts were prepared as described previously (Németh et al., 2002a,b,c). All nuclear extraction procedures were performed on ice with ice-cold reagents. Cells were washed twice with phosphate-buffered saline and harvested by scraping into 1.5 ml of phosphate-buffered saline and pelleted at 1500*g* for 10 min. The pellet was resuspended in 60 μl of cytosolic lysis buffer [20% (v/v) glycerol, 10 mM HEPES, pH 8.0, 10 mM KCl, 0.5 mM EDTA, pH 8.0, 1.5 mM $MgCl_2$, 0.5% (v/v) Nonidet P-40, 0.5 mM DTT, 0.2 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A] and incubated for 15 min on ice with occasional vortexing. After centrifugation at 4500*g* for 10 min, supernatants (cytosolic extracts) were removed and saved, whereas the pellet was further processed to obtain nuclear extracts. Two-cell pellet volumes of nuclear extraction buffer [20% (v/v) glycerol, 20 mM HEPES, pH 8.0, 420 mM NaCl, 0.5 mM EDTA, pH 8.0, 1.5 mM $MgCl_2$, 50 mM glycerol phosphate, 0.5 mM DTT, 0.2 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A] were added to the nuclear pellet and incubated on ice for 30 min with occasional vortexing. Nuclear proteins were isolated by centrifugation at 14,000*g* for 15 min. Protein concentrations were determined using a protein assay (Bio-Rad, Hercules, CA). Nuclear extracts were aliquoted and stored at −80°C until used for EMSA. The NF-κB consensus oligonucleotide probe used for the EMSA was purchased from Promega (Madison, WI). Oligonucleotide probes were labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Invitrogen) and purified in MicroSpin G-50 columns (Amersham Biosciences Inc., Piscataway, NJ). For the EMSA analysis, 8 to 12 μg of nuclear proteins were preincubated with EMSA-binding buffer [8% (v/v) glycerol, 10 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$, 0.5 mM EDTA, pH 8.0, and 0.5 mM DTT] as well as 15 ng/μl poly(dI)-poly(dC), 0.4 ng/μl single-stranded DNA, and 0.2 mg/ml bovine serum albumin at room temperature for 10 min before addition of the radiolabeled oligonucleotide for an additional 25 min. The final NaCl concentration in the binding reaction was 50.4 mM. The specificities of the binding reactions were tested by incubating samples with 50-fold molar excess of the unlabeled oligonucleotide probe. Protein-nucleic acid complexes were resolved using a nondenaturing polyacrylamide gel consisting of 4% acrylamide (29:1 ratio of acrylamide/bisacrylamide) and run in 0.5× Tris-borate/EDTA buffer (44.5 mM Tris-base, 44.5 mM boric acid, and 1 mM EDTA) for approximately 2.5 h at constant current (35 mA). Gels were transferred to 3M paper (Whatman, Clifton, NJ), dried under vacuum at 80°C for 40 min, and exposed to photographic film at −80°C with an intensifying screen. For supershift studies, before the addition of the radiolabeled probe, samples were incubated for 30 min with 2 μg of isotype control (rabbit polyclonal IgG Mad 1 antibody, sc-222X), RelB (sc-226X), c-Rel (sc-70x), p52 (sc-298x), p65 (sc-109X), or p50 (sc-114X) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Western Blot Analysis for the Assessment of IκB Protein Levels. IκB degradation was measured from the cytosolic extracts prepared during the nuclear extraction procedure (see above). Protein concentrations were determined using the Bio-Rad Protein Assay. Samples (20–30 μg) were separated on a 12% Tris-glycine gel

(Invitrogen) and transferred to a nitrocellulose membrane. The membranes were probed with anti-IκBα antibody (Cell Signaling Technology Inc., Beverly, MA) or anti-IκBβ antibody (Santa Cruz Biotechnology) and subsequently incubated with a secondary horseradish peroxidase-conjugated donkey anti-rabbit antibody (Santa Cruz Biotechnology). Bands were detected using enhanced chemiluminescence Western Blotting Detection Reagent (Amersham Biosciences).

Transient Transfection and Dual Luciferase Activity. For transient transfections, 2 to 4 × 10^5 HT-29 cells were seeded per well of a 24-well tissue culture dish 1 day before transient transfection (Németh et al., 2002a,b,c). Cells were transfected with 30 μl/ml of FuGENE 6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN) in 160 μl of medium per well. This medium also contained 5 μg/ml NF-κB luciferase (pNFκB-luc) promoter construct (BD Biosciences Clontech, San Diego, CA) and 0.3 μg/ml of control *Renilla reniformis* luciferase (pRL-CMV) plasmid. After an overnight transfection, the cells were exposed to proteasome inhibitors or their vehicle for 6 to 7 h. Luciferase activity was measured with use of the Luciferase Reporter Assay System (Promega, Madison, WI).

IKK Assay. Treated cells were washed with phosphate-buffered saline containing 1 mM PMSF, 100 mM Na_3VO_4, 2 mM *p*-nitrophenylphosphate, and 210 mU/ml aprotinin (Sigma). Cells were scraped and centrifuged, and the cellular pellet was resuspended in lysis buffer containing 50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 1 mM PMSF, 100 μM Na_3VO_4, 2 mM *p*-nitrophenylphosphate, and 210 mU/ml aprotinin. Immunoprecipitation of the cell extract was carried out using anti-IKKγ antibody (Santa Cruz Biotechnology). Protein A/G agarose beads (Santa Cruz Biotechnology) were added to the cell extract-antibody mixture to pull down the immunoprecipitated IKK. After several washes, the pellet was resuspended in kinase assay buffer, to which was added 100 μM ATP, 6 μg of GST-IκBα as substrate (Chen et al., 2002; Malhotra et al., 2002), and 0.5 μl of [γ-^{32}P]ATP, and incubated for 30 min at 30°C. The reaction was stopped in an ice bath, and electrophoresis was carried out in a Mini-Cell System (Novex, San Diego, CA) for 90 min at 140 V. After several washes, the gel was dried on a gel-drying apparatus (Bio-Rad). Dried gels were exposed overnight on a PhosphorImager screen, scanned on a Storm system, and visualized using ImageQuant software (all from Amersham Biosciences).

RNA Isolation and RT-PCR. Total RNA was isolated from HT-29 cells using TRIzol reagent (Invitrogen). Reverse transcription of the RNA was performed using murine leukemia virus reverse transcriptase from Roche Diagnostics. RNA (5 μg) was transcribed in a 20-μl reaction containing 2 μl of 10× PCR buffer, 2 μl of 10 μM dNTP mix, 2 μl of 25 mM $MgCl_2$, 2 μl of 100 mM DTT, 1 μl of 50 μM oligo$(dT)_{16}$, and 0.3 μl of murine leukemia virus reverse transcriptase (50 U/μl). The reaction mix was incubated at 42°C for 15 min for reverse transcription. Thereafter, the reverse transcriptase was inactivated at 99°C for 5 min. RT-generated DNA was amplified using Expand High-Fidelity PCR System (Roche). The PCR reaction mix (25 μl) contained 2 μl of cDNA, water, 2.5 μl of 10× PCR buffer, 1.5 μl of 25 mM $MgCl_2$, 1 μl of 10 mM dNTP mix, 0.5 μl of 10 μM oligonucleotide primer (each), and 0.2 μl of DNA polymerase. cDNA was amplified using the following primers and conditions: IL-8 (Nilsen et al., 1998), 5′-ATGACTTCCAAGCTGGCCGTGGCT-3′ (sense) and 5′-TCTCAGCCCTCTTCAAAAACTTCTC-3′ (antisense); GAPDH, 5′-CGGAGTCAACGGATTTGGTCGTAT-3′ (sense) and 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′ (antisense); an initial denaturation at 94°C for 5 min; 27 and 23 cycles for IL-8 and GAPDH, respectively, of 94°C for 30 s, 58°C for 45 s, and 72°C for 45 s; and a final dwell at 72°C for 7 min. The expected PCR products were 289 base pairs (IL-8) and 306 base pairs (GAPDH). PCR products were resolved on a 1.5% agarose gel and stained with ethidium bromide (Németh et al., 2002b).

IL-8 and GRO-α Measurement. Cells were incubated in 96-well plates with proteasome inhibitors or calpain inhibitors for 16 to 18 h. Human IL-8 and GRO-α levels were determined from the cell supernatants using commercially available enzyme-linked immunosorbent assay kits (R&D Systems) and according to the manufacturer's instructions (Németh et al., 2002b).

Measurement of Mitochondrial Respiration. Mitochondrial respiration, an indicator of cell viability, was assessed by the mitochondria-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) to formazan (Németh et al., 2002b). Cells in 96-well plates were incubated with MTT (0.5 mg/ml) for 10 to 15 min at 37°C. Culture medium was removed by aspiration, and the cells were solubilized in DMSO (100 μl). The extent of reduction of MTT to formazan within cells was quantified by measurement of optical density at 550 nm using a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, CA).

Statistical Evaluation. Values in the figures and in the text are expressed as mean ± S.E.M. of *n* observations. Statistical analysis of the data was performed by one-way analysis of variance followed by Dunnett's test, as appropriate.

Results

Proteasome Inhibitors Induce NF-κB DNA Binding in HT-29 Cells. Because HT-29 cells were originally demonstrated to have an atypical NF-κB response after IL-1 treatment (Jobin et al., 1997), we first examined the effect of proteasome inhibitors on IL-1β–induced NF-κB DNA binding in these cells. As shown in Fig. 1A, IL-1β–stimulated cells exhibited a substantial increase in NF-κB DNA binding compared with unstimulated cells, and the proteasome inhibitor MG-115 (10 μM) further increased this response.

To study whether proteasome inhibitors alone affected NF-κB activation, we first exposed cells to MG-115 alone (10 μM). Figure 1B demonstrates that MG-115 induced enhanced NF-κB DNA binding of a slowly migrating complex, which was most evident 2, 3, and 4 h after MG-115 administration. In the next set of experiments, we examined the effect of MG-132 and lactacystin, which are also selective proteasome inhibitors (Kisselev and Goldberg, 2001; Goldberg and Rock, 2002) on NF-κB DNA binding in HT-29 cells. Treatment of the cells for 2 h with either of these agents (10 μM) induced enhanced NF-κB DNA binding (Fig. 1, C and D). Similar to MG-115, both MG-132 (Fig. 1C) and lactacystin (Fig. 1D) caused the augmentation of a slower migrating complex, which was similar in its position to that one induced by MG-115. In some of the experiments, there was an increase in the intensity of one of the faster migrating complexes; however, this was not a consistent observation (data not shown).

To identify the composition of the various NF-κB DNA-binding complexes, we next performed supershift studies. The results of these experiments indicated that the complex induced by MG-115 (Fig. 2A) or MG-132 (Fig. 2B) contained both p50 and p52, as well as p65, because antibodies against these proteins abrogated (for p50 and p52) or caused a clear supershift (for p65) of this complex. The faster migrating middle complex consisted of p50 and p52, because both the p50 and p52 antibodies reduced the intensity of this complex. This complex also had a nonspecific component, because a 50-fold molar excess of radiolabeled oligonucleotides failed to completely abrogate this complex. The lowermost complex was shifted only by the p52 antibody, suggesting that this complex contained p52. Interestingly, the addition of the p52 antibody resulted in the appearance of an even faster migrating complex. These results showing that p52 is a component

of NF-κB DNA binding complexes in HT-29 cells confirm similar observations obtained in a recent study (Dejardin et al., 2002). Because a RelB or c-Rel antibody failed to alter the appearance of any of the NF-κB DNA-binding complexes, it seems that none of these DNA-binding complexes contained either RelB or c-Rel (Fig. 2).

Finally, we examined whether the induction of NF-κB DNA binding caused by proteasome inhibitors in HT-29 cells could be reproduced using another intestinal epithelial cancer cell line. Caco-2 cells seemed to be a proper control cell line, because this tumor cell line has been shown to possess normal NF-κB regulatory mechanisms (Jobin et al., 1997, 1998; Elewaut et al., 1999). Exposure of Caco-2 cells to MG-132 (10 μM) for 2 h failed to augment NF-κB DNA binding, indicating that the stimulation of NF-κB activity by proteasome inhibitors is not a general characteristic of intestinal epithelial tumor cells (Fig. 2C). Furthermore, MG-115 (10 μM) also failed to induce NF-κB DNA binding in Caco-2 cells, as determined 30 min to 6 h after MG-115 administration (data not shown).

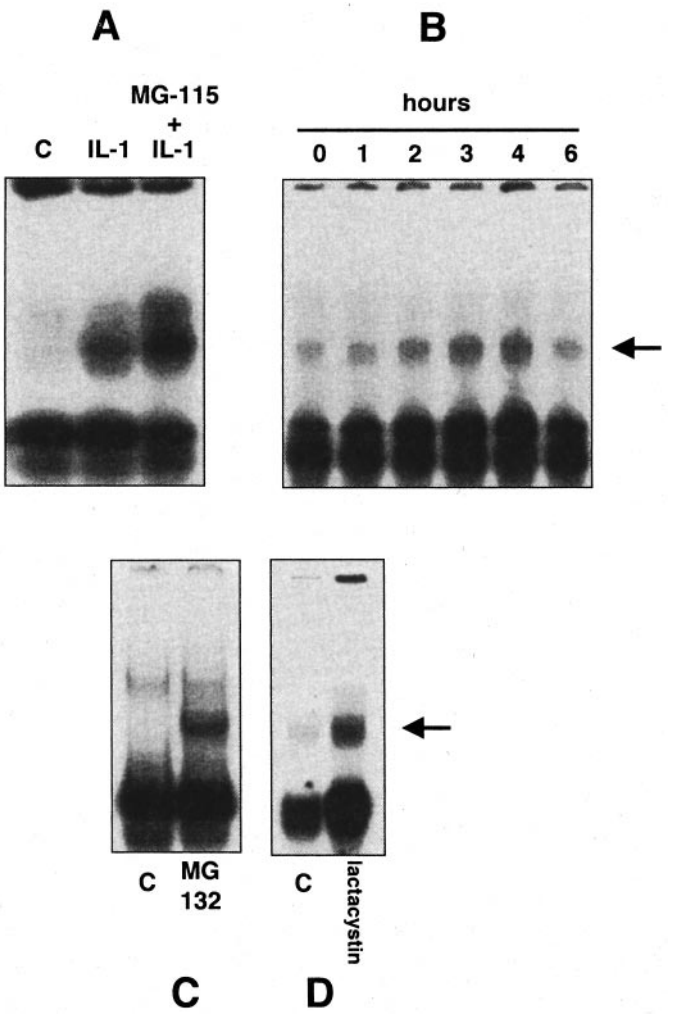

Fig. 1. Effect of proteasome inhibitors on NF-κB DNA binding in HT-29 cells. A, IL-1β and MG-115 additively stimulate NF-κB DNA binding. Cells were pretreated with MG-115 (10 μM) for 30 min and then stimulated with IL-1β (2 ng/ml) for an additional 45 min. B, MG-115 (10 μM) alone enhances NF-κB DNA binding. Cells were incubated with MG-115 for the indicated time periods. Treatment of HT-29 cells with MG-132 (C) or lactacystin (D) for 2 h augmented NF-κB DNA binding. This figure is representative of three to four separate experiments.

Proteasome Inhibitors Induce NF-κB–Dependent Transcriptional Activity in HT-29 Cells. In the next step, we explored whether the increase in NF-κB DNA binding caused by proteasome inhibitors translated into an enhanced NF-κB–dependent transcriptional activity. To this end, HT-29 cells were transiently transfected with a NF-κB–firefly-luciferase reporter construct and a control *R. reniformis*-luciferase vector. Exposure of the cells to either MG-115 (10 μM) or MG-132 (10 μM) for 7 h induced a significant increase in the firefly/*R. reniformis* ratio compared with vehicle-treated cells, which indicates that proteasome inhibitors augment NF-κB–dependent transcriptional activity in HT-29 cells (Fig. 3).

Proteasome Inhibitors Trigger IκBα Degradation in HT-29 Cells. To determine whether the activation of NF-κB after proteasome inhibitor treatment was caused by an effect on IκB, we examined the effect of proteasome inhibitors on IκB protein levels in HT-29 cells. Exposure of the cells to MG-115 (Fig. 4A) or MG-132 (Fig. 4B) for 2 h induced the degradation of cytosolic IκBα. Because IκBβ also plays an important role in controlling NF-κB activation in HT-29 cells (Inan et al., 2000), we also determined the effect of proteasome inhibition on IκBβ. However, proteasome inhibition failed to cause degradation of IκBβ (Fig. 4C). Furthermore, proteasome inhibition using MG-132 did not induce IκBα degradation in Caco-2 cells (Fig. 4D).

MG-132 Increases IKK Activity in HT-29 Cells. The primary signal leading to the degradation of IκB is IKK-dependent phosphorylation of IκB (Ghosh and Karin, 2002). Having demonstrated that proteasome inhibitors cause degradation of IκB, we next determined the effect of MG-132 on IKK activity. Treatment with MG-132 (10 μM) increased IKK activity as determined 45 and 90 min after MG-132 treatment (Fig. 5).

Proteasome Inhibitors Induce NF-κB–Dependent Gene Expression. We next investigated whether the NF-κB

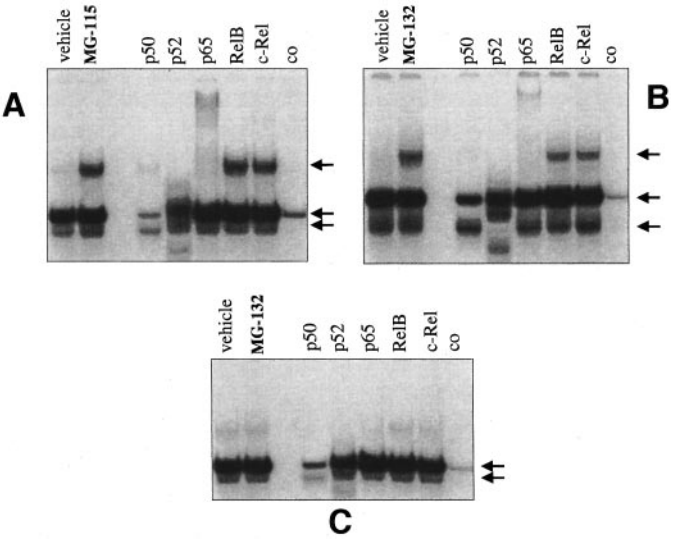

Fig. 2. Proteasome inhibitors activate distinct members of the NF-κB family. These supershift studies were performed using nuclear extracts harvested 2 h after treatment of cells with proteasome inhibitors (10 μM). Nuclear extracts were preincubated for 30 min with antibodies to Rel family members. Arrows, specific NF-κB DNA-binding complexes. A and B, supershift studies using HT-29 cells. C, results with Caco-2 cells. co, 50× molar excess of radiolabeled oligonucleotides.

activation induced by proteasome inhibitors correlated with an increased expression of NF-κB–dependent target genes. Because the induction of IL-8 gene expression is largely dependent on NF-κB (Mukaida et al., 1994), we examined the effect of MG-115 on IL-8 mRNA levels. As shown in Fig. 6A, treatment of HT-29 cells with MG-115 for 3 h yielded a substantial accumulation of IL-8 mRNA levels. Because MG-115 did not affect mRNA levels of the housekeeping gene GAPDH (Fig. 6A), we can conclude that the effect of MG-115 was not caused by a general facilitation of gene transcription. Furthermore, the stimulatory effect of MG-115 was completely prevented by pretreatment of the cells with the transcription inhibitor actinomycin D (Fig. 6B), which excludes the possibility that the stimulatory effect of MG-115 on IL-8 mRNA accumulation was caused by an increase in IL-8 mRNA stability. Similar to MG-115, MG-132 also increased the accumulation of IL-8 mRNA (data not shown).

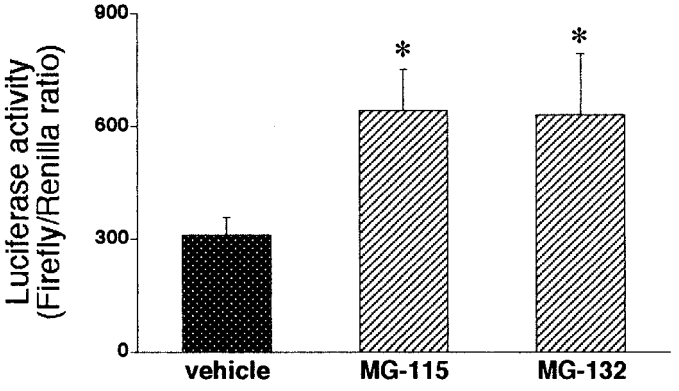

Fig. 3. Proteasome inhibition increases NF-κB–dependent transcriptional activity. HT-29 cells were transiently transfected with a firefly NF-κB–luciferase promoter construct and control *R. reniformis*-luciferase vector. Exposure of the cells to either MG-115 (10 μM) or MG-132 (10 μM) for 7 h induced a significant increase in the firefly/*R. reniformis* ratio. Data are mean ± S.E.M. of $n = 6$ to 8; *, $p < 0.05$. Shown are representative results of a single experiment of three experiments with similar results.

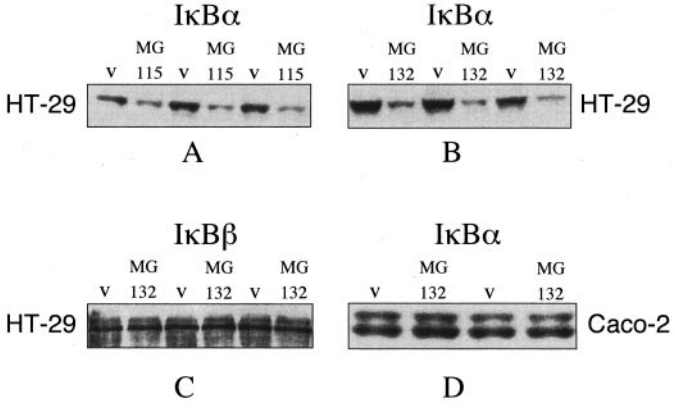

Fig. 4. Proteasome inhibition by either MG-115 (10 μM) (A) or MG-132 (10 μM) (B) induced IκBα degradation in HT-29 cells. C, MG-132 (10 μM) failed to induce IκBβ degradation in HT-29 cells. D, MG-132 fails to induce degradation of IκBα in Caco-2 cells. IκB protein levels were assessed from cytosolic extracts prepared 2 h after exposure of cells to proteasome inhibitors or vehicle (v). This figure is representative of three separate experiments with similar results.

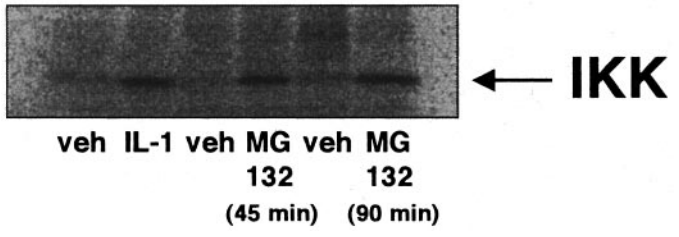

Fig. 5. Proteasome inhibition with MG-132 induces IKK activity in HT-29 cells. Representative gel of an in vitro kinase assay demonstrating an increase of IKK activity in HT-29 cells after treatment with 10 μM MG-132 for 45 or 90 min. Treatment with IL-1β (20 ng/ml) for 30 min was used as positive control. Three experiments with similar results were performed. veh, vehicle.

Proteasome Inhibitors Augment NF-κB–Dependent Protein Expression in HT-29 Cells. To further study the effect of proteasome inhibitors on NF-κB–dependent events, we next evaluated the effects of these agents on the production of IL-8. MG-115 and MG-132, as well as lactacystin, increased the production of IL-8 by HT-29 cells in a concentration-dependent manner (Fig. 7, A-C). Similar to its effect on IL-1β–induced NF-κB binding, MG-115 increased IL-1β–induced IL-8 production (Fig. 7D). Furthermore, similar to its effect on MG-115–induced IL-8 mRNA expression, actinomycin D (10 μg/ml) completely prevented MG-115–induced IL-8 production (data not shown).

Because the chemokine GRO-α is another NF-κB–dependent mediator, we examined whether proteasome inhibitors induced the production of this chemokine. Figure 8 shows that similar to results with IL-8, MG-115, MG-132, or lactacystin all induced the production of GRO-α (Fig. 8).

Because all of the proteasome inhibitors used in this study have been documented to be weak calpain blockers (Goldberg and Rock, 2002), the possibility existed that the stimulatory

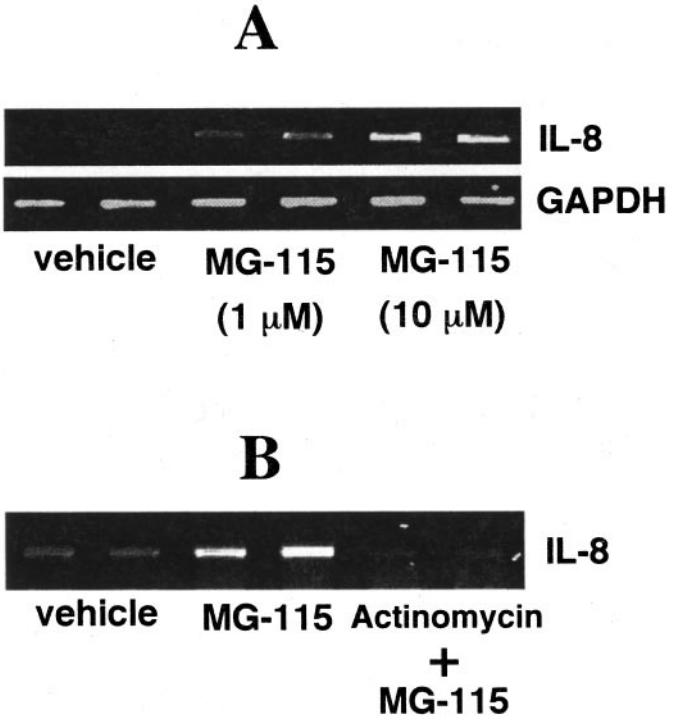

Fig. 6. MG-115 induces up-regulation of IL-8 mRNA levels in HT-29 cells. GAPDH levels were not affected by MG-115. A, IL-8 and GAPDH mRNA levels were quantified using RT-PCR. B, the stimulatory effect of MG-115 (10 μM) was completely prevented by pretreatment of the cells for 1 h with the transcription inhibitor actinomycin D (10 μg/ml). This figure is representative of three separate experiments.

effects of proteasome inhibitors on the HT-29 inflammatory response were caused by the blockade of calpain. However, because the selective calpain inhibitor calpeptin or PD 150606 was unable to induce IL-8 production (Fig. 9), it is unlikely that the NF-κB–activating activities of proteasome inhibitors were mediated by the inhibition of the calpain system. Finally, none of the drugs used had any effect on cell respiration as determined using the MTT assay (data not shown).

Discussion

In this study, we demonstrate that the proteasome inhibitors MG-115, MG-132, and lactacystin induce IKK activation, IκB degradation, and NF-κB transcriptional activation in HT-29 cells. These results describing a stimulatory effect of proteasome inhibitors on IκB degradation and NF-κB activity in an intestinal epithelial cell line contradict evidence obtained by most previous studies using other cell types (Kisselev and Goldberg, 2001; Goldberg and Rock, 2002) showing the blockade of IκB degradation and NF-κB activation by proteasome inhibitors. We found only one study in the literature reporting NF-κB activation after treatment with a proteasome inhibitor (Ferrari et al., 1997). In that study, Ferrari et al. (1997) showed that 2.5 μM lactacystin increased NF-κB DNA binding in N9 mouse microglial cells; however, there were no attempts to study the exact mechanisms leading to this effect. An important point that needs to be stressed is that in both our study and in the study of Ferrari et al. (1997), the proteasome inhibitor concentrations were in the 1 to 10 μM range. These, but not higher concentrations of MG-115, MG-132, and lactacystin are believed to selectively inhibit the proteasome (Kisselev and Goldberg, 2001; Goldberg and Rock, 2002). Although at this point it is not known whether the proteasome system was the only target of these low concentrations of proteasome inhibitors used in our study, it is clear that the chances of having nonselective effects are even greater using higher concentrations of these agents. In any case, to the best of our knowledge, our study is the first to demonstrate that proteasome inhibitors that are widely used to block the NF-κB transcription factor system can induce IKK activation, IκB degradation, and NF-κB–dependent transcriptional activity.

At this point, it is unclear what mechanisms lead to the activation of the IKK–IκB–NF-κB system after treatment with proteasome inhibitors in HT-29 cells. Proteasome inhibitors stimulate many pathways that have been shown to contribute to NF-κB activation in a variety of systems. Proteasome inhibitors have been demonstrated to activate stress kinases, including c-Jun terminal kinase (Meriin et al., 1998), p42/p44 mitogen-activated protein kinase (Hashimoto et al., 2000), and p38 (Madrid et al., 2001). All of these stress kinase pathways have been documented previously to mediate IKK activation, the degradation of IκB, and NF-κB activation (Lee et al., 1997; Zhao and Lee, 1999). Another possibility is that proteasome inhibition induces NF-κB activation in HT-29 cells by a mechanism involving the FasL-Fas pathway, because proteasome inhibitors have been demonstrated to stimulate this pathway (Tani et al., 2001) and FasL-Fas ligand interaction can activate IKK and consequently NF-κB (Russo et al., 2002). Whether these effects are caused by proteasome inhibition or whether they are nonselective effects on the various signaling pathways will have to be investigated in the future. Another important question is which proteolytic system mediates the degradation of IκBα after proteasome inhibitor treatment in HT-29 cells. One such proteolytic system may be the caspase system, because caspase-3 activation can result in IκB degradation (Barkett

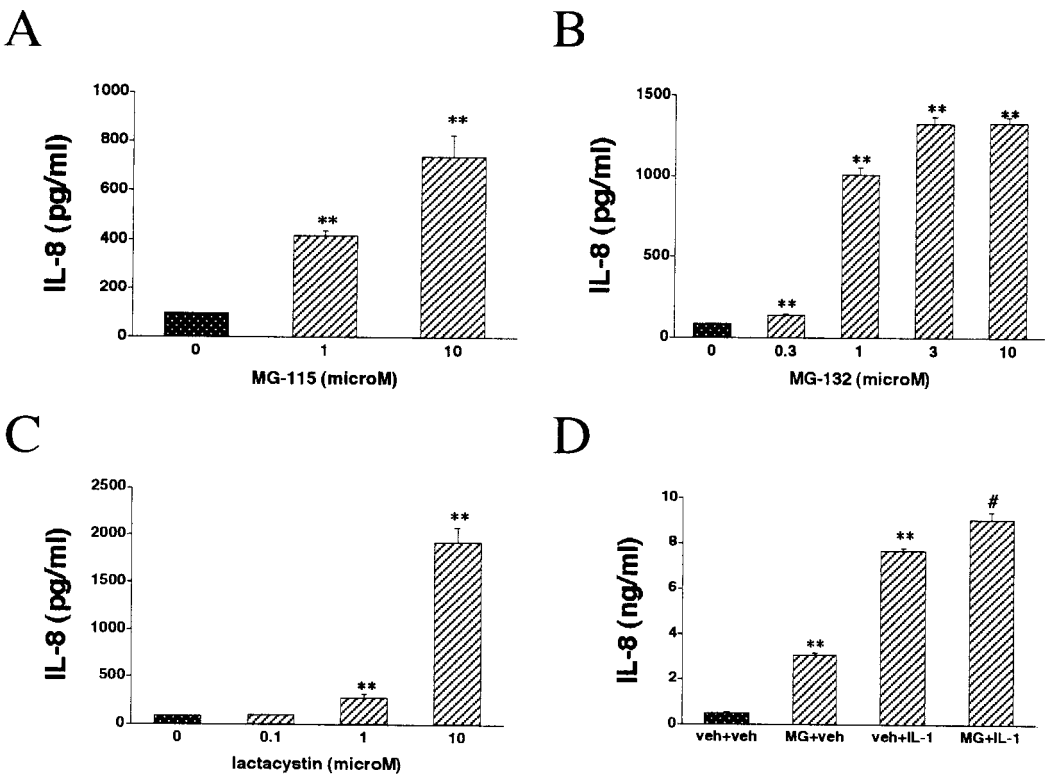

Fig. 7. A, B, and C, proteasome inhibitors stimulate IL-8 production by HT-29 cells. D, IL-1β (2 ng/ml)–induced IL-8 production is further augmented by cotreatment with MG-115 (3 μM). Supernatants for IL-8 measurement were taken 18 h after treatment with MG-115 or IL-1β. Data are mean ± S.E.M. of $n = 14$ to 16 wells from two separate experiments. **, $p < 0.01$; #, $p < 0.05$ versus the IL-1β–treated group.

et al., 1997; Reuther and Baldwin, 1999; Qin et al., 2000). Furthermore, proteasome inhibition stimulates caspase-3 activation (Zhang et al., 1999). In addition to the caspase system, calpains may also play a role in the degradation of IκBα, because calpains have been shown to represent an important alternative mechanism for IκB degradation in a variety of cell types (Chen et al., 1997; Miyamoto et al., 1998; Han et al., 1999; Pianetti et al., 2001; Shen et al., 2001; Romieu-Mourez et al., 2002).

Taken together, using an intestinal epithelial cell line, the current study demonstrates for the first time that proteasome inhibitors can induce IKK activation, IκB degradation, and NF-κB–dependent transcriptional activity. Clinical trials with proteasome inhibitors for the treatment of cancer are currently in progress (Orlowski and Baldwin, 2002). Our results underline the previous proposition that conclusions drawn with small-molecule, nonselective NF-κB inhibitors, such as proteasome inhibitors, must be interpreted carefully.

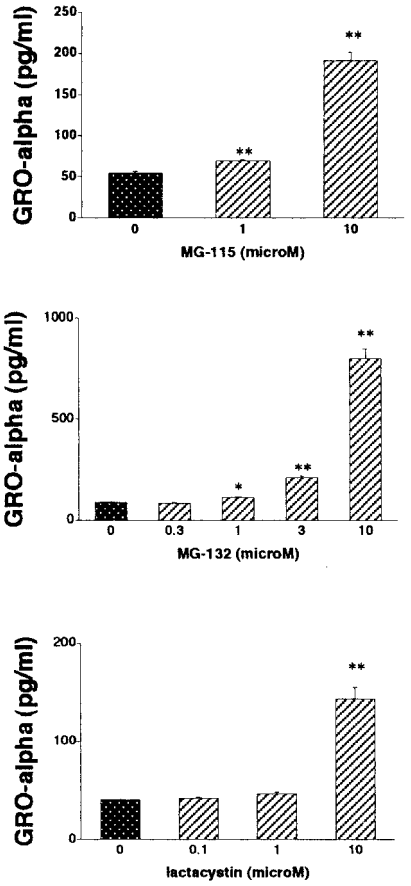

Fig. 8. Proteasome inhibitors stimulate GRO-α production by HT-29 cells. Supernatants for IL-8 measurement were taken 18 h after treatment with proteasome inhibitors. Data are mean ± S.E.M. of $n = 14$ to 16 wells from two separate experiments. **, $p < 0.01$.

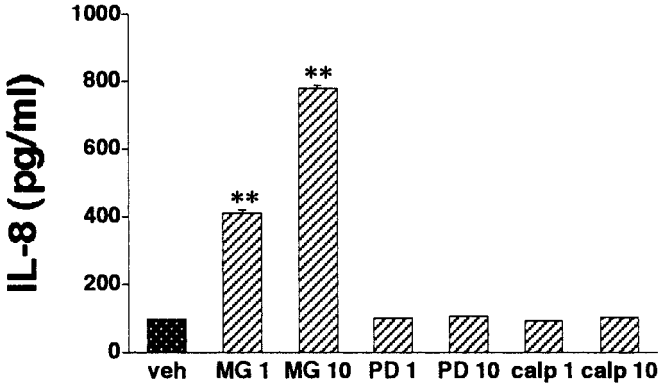

Fig. 9. The selective calpain inhibitors calpeptin (calp; 1 and 10 μM) and PD 150606 (PD; 1 and 10 μM) failed to induce IL-8 production. MG-115 (MG; 1 and 10 μM) was used as positive control. Supernatants for IL-8 measurement were taken 16 h after treatment with MG-115 or calpain inhibitors. Data are mean ± S.E.M. of $n = 14$ to 16 wells from two separate experiments. **, $p < 0.01$; veh, vehicle.

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