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PROTEASOME INHIBITORS INDUCE APOPTOSIS OF PROSTATE CANCER CELLS BY INDUCING NUCLEAR TRANSLOCATION OF I κ B α

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Abstract

Proteasome inhibitors are known to suppress the proteasome-mediated degradation of I κ B α in stimulated cells. This results in the cytoplasmic retention of NF κ B and its reduced nuclear transcriptional activity. In this study, we show that in the metastatic prostate cancer cells, the proteasome inhibitors exhibit a novel, previously unrecognized effect: they increase the cellular levels of I κ B α , which then translocates to the nucleus, associates with the nuclear p65 NF κ B, thus inhibiting the constitutive NF κ B DNA binding activity and inducing apoptosis. The proteasome inhibition-induced nuclear translocation of I κ B α is dependent on *de novo* protein synthesis, occurs also in other cell types, and does not require I κ B α phosphorylation on Ser-32. Since NF κ B activity is constitutively increased in many human cancers as well as in inflammatory disorders, the proteasome inhibition-induced nuclear translocation of I κ B α could thus provide a new therapeutic strategy aimed at the specific inhibition of NF κ B activity by the nuclear I κ B α .

Keywords

Proteasome inhibition; I κ B α ; nuclear translocation; prostate cancer; NF κ B; apoptosis

INTRODUCTION

NF κ B is a key regulator of genes involved in immune and inflammatory responses, as well as genes regulating cell proliferation and survival [1–4]. In most unstimulated resting cells, NF κ B is localized in the cytoplasm bound to the inhibitory protein I κ B α . Following cell stimulation by extracellular stimuli, including apoptotic signals, inflammatory cytokines, and other forms of cellular stress, I κ B α is phosphorylated on Ser-32 and Ser-36 by the enzymes of I κ B kinase complex (IKK), ubiquitinated, and selectively degraded by the 26S proteasome [5]. This releases NF κ B proteins from the inhibitory complex, and NF κ B then translocates to the nucleus and stimulates transcription of NF κ B-dependent anti-apoptotic and pro-inflammatory genes [6,7].

In addition to many inflammatory disorders, NF κ B is constitutively activated in a variety of human cancers, including prostate cancer [8–11]. Prostate cancer is the third most common cause of death from cancer in men of all ages [12,13]. It proceeds from a localized, curable, androgen dependent disease to an invasive, metastatic, and always fatal, androgen-independent disease [14,15]. The metastatic, androgen-independent prostate cancer is characterized by a

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constitutive activation of NF κ B that induces synthesis of pro-survival genes Bcl-2 and Bcl-xL [16–18]. Thus, inhibition of NF κ B activity and induction of apoptosis represent an attractive therapeutic approach for prostate cancer as well as other cancers characterized by the high constitutive NF κ B activation [19–22].

We have previously shown that NF κ B activity can be inhibited by nuclear accumulation of I κ B α , and this is associated with the induction of apoptosis [23,24]. We have searched for experimental treatment, or conditions, that would induce the nuclear translocation of I κ B α , thus inhibiting NF κ B activity and inducing apoptosis. The 26S proteasome inhibitors such as MG132 and MG115 are known to suppress NF κ B activation and induce apoptosis by inhibiting the inducible degradation of phosphorylated I κ B α in stimulated cells [3,4]. However, several studies indicated that proteasome inhibitors can also induce nuclear translocation and accumulation of transcriptional regulators such as aryl hydrocarbon receptor (ACH)[25], glucocorticoid receptor (GR)[26] and varicella-zoster virus DNA binding protein ORF29p [27] in unstimulated resting cells.

In this study, we considered the possibility that proteasome inhibitors may increase the cellular levels of I κ B α , which may then translocate to the nucleus and inhibit the NF κ B DNA binding activity, thus inducing apoptosis. To this end, we selected the androgen independent metastatic prostate cancer PC-3 cells that are characterized by nuclear localization of NF κ B proteins and high levels of constitutive NF κ B DNA binding activity [28–30]. We found that in these cancer cells, as well as in HeLa cells, proteasome inhibition by MG132 and MG115 leads to the nuclear translocation of I κ B α . The nuclear accumulation of I κ B α then results in the nuclear I κ B α -p65 NF κ B association, inhibition of NF κ B DNA binding activity and induction of apoptosis. Our data show that proteasome inhibitors suppress NF κ B activity, and induce apoptosis, by a previously unrecognized mechanism that consists of inducing the nuclear translocation and accumulation of I κ B α .

MATERIALS AND METHODS

Antibodies and Reagents

Purified polyclonal antibodies against human I κ B α (sc-371), NF κ B-p65 (sc-372), NF κ B-p50 (sc-7178), Bcl-2 (sc-492), Bcl-xL (sc-7195), and lamin B (sc-6216), as well as monoclonal antibody against Ser-32 phosphorylated I κ B α (sc-8404) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Purified polyclonal antibody against lactate dehydrogenase (LDH; 20-LG22) was from Fitzgerald Industries International (Concord, MA, USA), and actin antibody was from Sigma (St Louis, MO). Horseradish peroxidase (HRP)-conjugated anti-rabbit, anti-mouse and anti-goat secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

T4 polynucleotide kinase, poly (dI-dC), and Sephadex G25 spin columns were purchased from Pharmacia (Piscataway, NJ). CREB (sc-2504, sc-2517) and NF κ B (sc-2505, sc-2511) gel shift oligonucleotides were from Santa Cruz Biotechnology (Santa Cruz, CA). [32 P]- γ -ATP was purchased from Perkin Elmer (Boston, MA). Proteasome inhibitors MG132, MG115 and PSI, and I κ B kinase inhibitor VII (401486) were purchased from EMD Chemicals (San Diego, CA). Cycloheximide, recombinant human tumor necrosis factor- α (TNF) and all other reagents were molecular biology grade and were purchased from Sigma (St Louis, MO).

Cell Culture

All cell lines were obtained from American Type Culture Collection (ATCC; Rockville, MD). Human prostate cancer PC-3 cells were cultured in Ham's F12K medium (ATCC; Rockville, MD) supplemented with 2 mM L-glutamine, 1% penicillin and streptomycin, and 10% fetal

bovine serum (Invitrogen, Grand Island, NY). Epithelial cancer HeLa cells and leukemia HL-60 cells were cultured in Dulbecco's modified Eagle's medium and RPMI 1640 medium, respectively, supplemented with 10% fetal bovine serum and antibiotics. Prior to cell treatment, cells were seeded (10^6 cells/ml) for 24 hours in 6-well plates and grown at 37°C with 5% CO₂.

Transfection with siRNA and Proteasome Inhibition

Human IκBα (sc-29360) and control (sc-37007) small interfering RNAs (siRNAs) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The day before transfection, PC-3 cells were seeded into a 24-well plate and incubated in a humidified 5% CO₂ incubator at 37°C in antibiotic-free F12K medium supplemented with 10% FBS for 24 h to 80% confluence. For each transfection, 60 μmol of either control siRNA-A (sc-37007) or IκBα siRNA (sc-29360) were used. The cells were transfected for 6 hours in transfection medium with siRNA-transfection reagent according to manufacturer's instructions (Santa Cruz Biotechnology; Santa Cruz, CA). After transfection, fresh F12K medium supplemented with FBS and antibiotics was added, and the cells were treated with proteasome inhibitors for 24 hours.

Proteasome inhibitors MG132, MG115 and PSI were dissolved in DMSO and stored at -20°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 [23,24]. Contamination of nuclear and cytoplasmic fractions by cytoplasmic and nuclear proteins, respectively, was determined by Western analysis using LDH and lamin B as specific markers as described [23,24].

Electrophoretic Mobility Shift Assay (EMSA)

DNA binding activity of NFκB and CREB was measured by using specific NFκB and CREB double-stranded oligonucleotides as described [23,31].

Immunoprecipitation

Nuclear extracts were prepared by using the Active Motif's Nuclear Complex Co-IP Kit (Active Motif, 54001; Carlsbad, CA). The nuclear extracts were incubated (4°C, overnight) with IκBα antibody (sc-371) or control rabbit pre-immune IgG (sc-2027). The immune complexes were immunoprecipitated on A/G Plus Agarose (sc-2003), washed four times with PBS buffer, resolved on 10% SDS gel and detected with IκBα and p65 antibodies as described [32].

Proteasome Activity Assay

Activity of the 20S catalytic proteasomal core unit was measured in whole cell extracts by using the 20S Proteasome Activity Assay Kit (Chemicon, APT280; Temecula, CA) as described by the manufacturer. Briefly, cells were lysed in a lysis buffer (50 mM Hepes, pH 7.5; 5 mM EDTA; 150 mM NaCl; 1% Triton X-100) for 30 minutes on ice, and the lysates were collected by centrifugation (10,000g, 15 min, 4°C). The cell lysates were incubated (2h, 37°C) with a labeled substrate, LLVY-7-amino-4-methylcoumarin, and the cleavage activity was monitored by detection of the free fluorophore 7-amino-4-methylcoumarin using a fluorescence plate reader (Berthold Mithras LB940, Berthold Technologies) at 355/460 nm.

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, Indianapolis, IN). The assay was performed at the indicated time points as per the manufacturer's instructions.

Statistical Analysis

The results represent at least three independent experiments. Numerical results are presented as means $\pm$ S.E. Data were analyzed by using an InStat software package (GraphPAD, San Diego, CA). Statistical significance was evaluated by using Mann-Whitney *U* test with Bonferroni correction for multiple comparisons, and $p < 0.05$ was considered significant.

RESULTS

Proteasome Inhibitor MG132 Induces Nuclear Translocation of I κ B α in Prostate Cancer Cells

The metastatic androgen-independent prostate cancer PC-3 cells are characterized by high levels of constitutive NF κ B activity that consists of the NF κ B p50 and p65 subunits [16,17]. To analyze the nucleo-cytoplasmic distribution of I κ B α and NF κ B proteins in these cells, and determine whether proteasome inhibition induces I κ B α translocation to the nucleus, PC-3 cells were treated with increasing concentrations of MG132 for 24 hours, and the cytoplasmic and nuclear fractions were prepared and analyzed by western blotting. As a control of the purity of cytoplasmic and nuclear extract preparations, we used lactate dehydrogenase (LDH) and lamin B as specific cytoplasmic and nuclear markers, respectively. Interestingly, in the resting unstimulated PC-3 cells, I κ B α was localized predominantly in the cytoplasm, while NF κ B p65 and p50 proteins were found mainly in the nuclear fraction (Fig. 1A). Cell treatment with MG132 remarkably decreased I κ B α levels in the cytoplasm and induced its dose-dependent translocation to the nucleus. The nuclear translocation of I κ B α was induced by 10 μ M MG132, and 25 μ M and higher concentrations of MG132 resulted in almost complete nuclear localization of I κ B α . The nucleo-cytoplasmic distribution of NF κ B p50 and p65 proteins was not changed by 1–100 μ M MG132, and there was no pronounced effect on the nuclear levels of p50 and p65 NF κ B proteins within these MG132 concentrations (Fig. 1A).

To determine whether the nuclear translocation of I κ B α in response to MG132 is time dependent, we analyzed I κ B α levels in cytoplasmic and nuclear extracts of PC-3 cells treated with 50 μ M MG132 for 0 to 24 hours. As shown in Fig. 1B, while the nuclear I κ B α translocation occurred 4 to 8 h after treatment with MG132, substantial accumulation of I κ B α in the nucleus was observed after 24 hour treatment with MG132.

However, since we observed that in addition to inducing the nuclear translocation of I κ B α , 4 and 8 h treatment with MG132 also somewhat increased the cytoplasmic I κ B α levels (Fig. 1B), and because I κ B α in PC-3 cells was shown to have a rapid metabolic turnover attributed to its phosphorylation on Ser-32 and subsequent degradation by the proteasome [16,17], we investigated whether MG132 treatment increases the total cellular levels of I κ B α in PC-3 cells. As shown in Fig. 1C, in untreated cells, the total cellular levels of I κ B α , analyzed in whole cell extracts, gradually decreased during 24 hour incubation. In contrast, 8 and 24 h treatment with 50 μ M MG132 increased the total cellular levels of I κ B α compared to the whole cell extracts of untreated cells (Fig. 1C). These data suggested that the nuclear translocation of I κ B α induced by proteasome inhibition in PC-3 cells is associated with an increase of the total cellular I κ B α levels, and indicated that the proteasome is constitutively active in these cells.

To demonstrate the proteasome activity in PC-3 cells, and to verify that MG132 indeed inhibits the proteasome activity, we measured activity of the 20S proteasome catalytic subunit in the whole cells extracts of PC-3 cells incubated 24 hours with increasing concentrations of MG132.

As shown in Fig. 1D, 1 μ M MG132 inhibited about 70% of the proteasome activity in PC-3 cells, and about 90% inhibition was achieved by 10 μ M MG132. Thus, these data indicate that a substantial inhibition of the proteasome is required to induce the nuclear accumulation of I κ B α , and suggest that even a small residual proteasomal activity in PC-3 cells is sufficient to control the level of I κ B α in the cytoplasm.

The Nuclear I κ B α , Induced upon Proteasome Inhibition, Associates with p65 NF κ B in the Nucleus, Thus Inhibiting the Constitutive NF κ B DNA Binding Activity

To determine whether the nuclear translocation of I κ B α , induced by proteasome inhibition, is associated with the inhibition of NF κ B activity, we measured NF κ B DNA binding activity in nuclear extracts prepared from PC-3 cells treated with 0–50 μ M MG132. As shown in Fig. 2A, the constitutive nuclear NF κ B DNA-binding activity in PC-3 cells was drastically reduced by 24 hour treatment with 10 μ M MG132, which also induced the nuclear translocation of I κ B α (Fig. 1A), and completely inhibited by 25 and 50 μ M MG132 concentrations, which resulted in the predominantly nuclear localization of I κ B α (Fig. 1A, 2A). Using supershift characterization, we confirmed that this activity consisted of p50/p65 NF κ B heterodimers (Fig. 2B). In contrast to NF κ B, DNA binding activity of another, I κ B α -independent transcription factor, CREB, was not affected by 1–50 μ M concentrations of MG132, indicating specificity for NF κ B (Fig. 2A). Fig. 2C shows a time course of 50 μ M MG132 effect on the NF κ B and CREB DNA binding activities. While the CREB activity was not markedly changed, 24 hour treatment with 50 μ M MG132 resulted in a pronounced inhibition of NF κ B DNA binding (Fig. 2C), consistent with the increased nuclear levels of I κ B α (Fig. 1B).

To support the data that the NF κ B DNA binding activity is inhibited by I κ B α that translocates to the nucleus as a result of the proteasome inhibition, we performed a co-immunoprecipitation experiment using I κ B α antibody and nuclear extracts prepared from untreated as well as MG132-treated (50 μ M, 24h) PC-3 cells. As shown in Fig. 2D (top panel), I κ B α was immunoprecipitated from the nuclear extracts of MG132-treated cells, but not from the nuclear extracts of untreated cells, or from MG132-treated cells immunoprecipitated by using control pre-immune IgG. Immunoblotting by using p65 NF κ B antibody revealed the presence of p65 NF κ B in the nuclear extracts of MG132-treated cells immunoprecipitated with I κ B α antibody, but not with pre-immune IgG (Fig. 2D, lower panel). Importantly, no p65 NF κ B signal was detected in the I κ B α -immunoprecipitates prepared from the nuclear extracts of untreated cells (Fig. 2D, lower panel), even though p65 NF κ B is present in the nucleus of untreated PC-3 cell (Fig. 1A, B), demonstrating specificity for the I κ B α -binding proteins. Together, these data show that I κ B α that translocates to the nucleus after the proteasome inhibition, associates with p65 NF κ B in the nucleus, and provide the mechanism by which the nuclear I κ B α inhibits NF κ B DNA binding activity.

The Nuclear Translocation of I κ B α Is Induced by Other Proteasome Inhibitors As Well

To ascertain that the nuclear translocation of I κ B α is not restricted only to MG132, but can be induced by other proteasome inhibitors as well, we tested effects of other reversible proteasome inhibitors, MG115 (Z-Leu-Leu-Nva-CHO) and PSI (Z-Ile-Glu(OtBu)-Ala-Leu-CHO). Both MG115 and PSI induced a dose-dependent translocation of I κ B α from the cytoplasm to the nucleus, similarly as in the cells treated with MG132, and this was associated with the inhibition of NF κ B DNA binding activity (data not shown).

The MG132-Induced Nuclear Translocation of I κ B α Results in the Induction of Apoptosis

To determine whether the MG132-induced nuclear translocation of I κ B α and inhibition of NF κ B activity are associated with the induction of apoptosis, we analyzed the protein levels of two NF κ B-regulated anti-apoptotic proteins, Bcl-2 and Bcl-xL, and measured apoptosis by a quantitative ELISA assay based on the detection of nucleosome release into the cytoplasm.

As shown in Fig. 3A and B, Bcl-2 and Bcl-xL expression were pronouncedly reduced by 25 and 50 μ M MG132, and the cytoplasmic nucleosome release was increased by 10–50 μ M MG132. Altogether, these data indicated that the 26S proteasome inhibitors suppress NF κ B activity and induce apoptosis, at least partly, by inducing translocation of I κ B α from the cytoplasm to the nucleus.

To determine whether the increased apoptosis observed in MG132-treated cells is caused by the increased nuclear levels of I κ B α , rather than by some side effects of MG132, we hypothesized that suppression of I κ B α nuclear levels should inhibit apoptosis in MG132-treated cells. To test this hypothesis, we silenced I κ B α expression by I κ B α -specific si RNA, and then treated cells with increasing concentrations of MG132 for 24 hours. As expected, I κ B α si RNA greatly reduced the cellular protein levels of I κ B α compared to cells transfected with control si RNA, resulting in barely detectable I κ B α in the nucleus of MG132-treated cells (Fig. 3C). The decreased nuclear expression of I κ B α in cells transfected with I κ B α si RNA, compared to cells transfected with control RNA, resulted in a significantly reduced apoptosis in response to treatment with 10–50 μ M MG132 ($P < 0.01$) (Fig. 3D), demonstrating that the increased nuclear I κ B α levels induce apoptosis.

New Protein Synthesis Is Required for the MG132-Induced Nuclear Translocation of I κ B α

The induced nuclear accumulation of I κ B α in response to proteasome inhibition raised a question whether *de novo* protein synthesis is required for the I κ B α nuclear translocation. To investigate this, PC-3 cells were treated with cycloheximide (CHX, 10 μ g/ml, 1h) to inhibit new protein synthesis, before 24 h incubation with increasing concentrations of MG132. Interestingly, CHX treatment completely prevented the MG132-induced nuclear I κ B α translocation, resulting in exclusively cytoplasmic localization of I κ B α (Fig. 4). These results suggest that *de novo* protein synthesis is required for the proteasome inhibition-induced nuclear translocation of I κ B α .

The MG132-Induced Nuclear Translocation of I κ B α Is Not Dependent on I κ B α Phosphorylation by I κ B Kinase and Occurs Also in Other Cell Types

Since the constitutive NF κ B activity in PC-3 cells is associated with high level of I κ B α that is phosphorylated by I κ B kinase (IKK) on Ser-32 [16,17], we investigated whether the phosphorylated I κ B α translocates to the nucleus after MG132 treatment. PC-3 cells were treated with control vehicle (DMSO) or 50 μ M MG132 for 0–24 hours, and the cytoplasmic and nuclear extracts were analyzed by immunoblotting using monoclonal antibody specific against I κ B α phosphorylated on Ser-32. As expected, in unstimulated cells and cells treated only with DMSO, I κ B α was phosphorylated, and was localized in the cytoplasm (Fig. 5A). However, during 4–24 h treatment with MG132, the phosphorylated form of I κ B α translocated from the cytoplasm to the nucleus (Fig. 5A). These results suggested that phosphorylation by IKK may be required for the proteasome inhibition-induced nuclear translocation of I κ B α .

To further examine the role of phosphorylation by IKK in the MG132-induced nuclear translocation of the endogenous I κ B α , we analyzed the nucleo-cytoplasmic localization of I κ B α in PC-3 cells treated with MG132 in the absence and presence of a specific inhibitor of IKK. As shown in Fig. 5B, 10 μ M IKK inhibitor did not prevent the MG132-induced nuclear translocation of I κ B α , even though it did inhibit the TNF-induced degradation of I κ B α in the cytoplasmic extracts of PC-3 cells (Fig. 5C), thus verifying the inhibitor's functionality.

To validate these results to be independent of a specific cancer cell line, we used additional well-characterized cancer cell lines, epithelial HeLa cells and leukemia HL-60 cells. Even though I κ B α is not constitutively phosphorylated on Ser-32 in these cells [33,34], MG132 induced a dose-dependent translocation of I κ B α from the cytoplasm to the nucleus (Fig. 5D).

Thus, even though the Ser-32 phosphorylated I κ B α translocates to the nucleus in response to proteasome inhibition in prostate cancer cells, this phosphorylation does not seem to be required for the nuclear translocation of I κ B α . Together, these data show that proteasome inhibition suppresses NF κ B activity by a novel mechanism that consists of inducing the nuclear translocation and accumulation of I κ B α .

DISCUSSION

Proteasome inhibitors are known to suppress NF κ B activity and induce apoptosis by inhibiting the 26S proteasome-mediated degradation of I κ B α in stimulated cells [1–4]. Thus, modulation of proteasome function has emerged as a novel target for the treatment of multiple cancers as well as inflammatory and immune disorders [35–37]. In this study, we have shown that proteasome inhibitors suppress the constitutive NF κ B DNA binding activity in metastatic prostate cancer cells by a novel mechanism, which consists of increasing the cellular levels of I κ B α , which then translocates from the cytoplasm to the nucleus (Fig. 6). The nuclear accumulation of I κ B α , induced by proteasome inhibition, results in the nuclear I κ B α -p65 NF κ B association, inhibition of NF κ B DNA binding activity and induction of apoptosis of prostate cancer cells. Since NF κ B activity is constitutively increased in many human cancers as well as in inflammatory disorders, the proteasome inhibition-induced nuclear accumulation of I κ B α could thus provide a new therapeutic strategy aimed at the specific inhibition of NF κ B activity by the nuclear I κ B α .

The 26S proteasome is a large, multi-subunit protein complex that selectively degrades cellular proteins, present both in the cytoplasm and in the nucleus. Our data show that although proteasome inhibition resulted in the translocation of I κ B α from the cytoplasm to the nucleus, it did not change the cellular localization on NF κ B p50 and p65 proteins, nor induced nuclear translocation of LDH (Fig. 1). Interestingly, however, proteasome inhibition has been also shown to induce the nuclear translocation of GR [26], aryl hydrocarbon receptor [25], and varicella zoster DNA-binding protein ORF29p [27]. All these proteins are transcriptional regulators that can be specifically degraded by the 26S proteasome. Although the exact mechanisms by which the proteasome inhibition induces their nuclear translocation have not been fully elucidated, studies suggest an involvement of protein stabilization resulting in increased total cellular levels, and post-translational modification by phosphorylation by protein kinase C [25–27,38].

Our data demonstrate that the nuclear translocation of I κ B α induced by proteasome inhibition in metastatic prostate cancer cells correlates with the stabilization of I κ B α , resulting in the increased total cellular levels of I κ B α (Fig. 1). Even though in prostate cancer cells, the proteasome inhibition induces nuclear translocation of I κ B α that is phosphorylated by IKK on Ser-32 (Fig. 5A), this phosphorylation does not seem to be necessary for the I κ B α nuclear translocation in response to proteasome inhibition. First, the nuclear translocation of the endogenous I κ B α is not prevented by the inhibition of IKK (Fig. 5B and C), and second, the nuclear translocation of I κ B α occurs also in cells that exhibit low levels of constitutive IKK activity and I κ B α Ser-32 phosphorylation: HeLa cells (Fig. 5D) and HL-60 cells (not shown). These data suggest that there might be an IKK-independent mechanism that targets I κ B α to the proteasome in these cells. In this context, several mechanisms have been described that lead to ubiquitin-mediated proteasome-dependent NF κ B activation that occurs in the absence of I κ B α phosphorylation on Ser-32 and Ser-36 [39–44]. These IKK-independent mechanisms include the UV-induced NF κ B activation [39–41], NF κ B activation induced by hepatitis B virus X protein [42], and the I κ B α degradation and NF κ B activation induced by prolonged cell treatment with a chemotherapeutic agent, doxorubicin [43]. Although the precise mechanisms by which these slow-activating agents induce the proteasomal I κ B α degradation have not been

identified, they seem to involve phosphatidylinositol 3-kinases, a serine-threonine kinase Akt, and the extracellular signal regulated kinase ERK [43,44].

Since I κ B α is a protein that has a high rate of metabolic turnover, our data indicate that proteasome inhibition prevents degradation of the cytoplasmic I κ B α , which then translocates to the nucleus, and by binding to p65 NF κ B inhibits NF κ B DNA binding activity. These data are also consistent with our results showing that new protein synthesis is required in order for I κ B α to translocate to the nucleus (Fig. 4). The lack of I κ B α nuclear translocation in response to proteasome inhibition in CHX-treated cells could be explained by two mutually non-exclusive mechanisms. In the first model, treatment with CHX might prevent resynthesis of a protein that is otherwise necessary for the nuclear translocation of I κ B α in MG132-treated cells, but has a short half-life; thus, treatment with CHX would significantly decrease its level. Alternatively, the nuclear translocation of I κ B α in MG132-treated cells may require that the cellular (cytoplasmic) level of I κ B α increases above certain threshold level. When the cells are treated with CHX, *de-novo* synthesis of I κ B α is inhibited, and I κ B α never reaches this threshold level, even after the degradation of I κ B α is blocked by MG132. Similar mechanism has been suggested to account for the nuclear accumulation of GR [26] and the varicella-zoster virus DNA binding protein ORF29p [27]. In this model, the nuclear translocation of I κ B α , triggered by the increased cytoplasmic level of I κ B α , could be explained by saturating a cytoplasmic protein, or a cellular structure, which binds and anchors I κ B α in the cytoplasm. This model is also supported by previous studies that used cells transfected with constructs expressing wild type (wt) I κ B α and demonstrated that when the wt I κ B α protein is over-expressed, it localizes in the nucleus [45–48]. These studies indicated that I κ B α is retained in the cytoplasm through its association with the NF κ B proteins; however, when not bound to NF κ B, the free I κ B α translocates to the nucleus [45–48].

The nuclear translocation of I κ B α is mediated by the second ankyrin repeat of I κ B α that also mediates interaction with NF κ B proteins [49,50]. As a result, in most unstimulated cells, I κ B α is localized in the cytoplasm bound to NF κ B proteins, which can translocate to the nucleus only after I κ B α had been degraded by proteasome upon stimulation [1–4]. However, during post-induction repression in continuously stimulated cells, since the NF κ B proteins are in the nucleus, the newly synthesized I κ B α is not bound to NF κ B in the cytoplasm, resulting in I κ B α translocation to the nucleus and terminating NF κ B-dependent transcription [51]. Interestingly, however, we have found that in the metastatic prostate cancer PC-3 cells, I κ B α is localized in the cytoplasm, while the NF κ B proteins are predominantly in the nucleus (Fig. 1). Thus, the mechanisms that regulate the cytoplasmic localization of I κ B α in PC-3 cells, and its MG132-induced nuclear translocation, are clearly different from the I κ B α nuclear shuttling during post-induction repression.

In addition, the cytoplasmic localization of I κ B α in PC-3 cells raises a question of the mechanisms responsible for its cytoplasmic retention in these cells. Since I κ B α contains nuclear export sequences [52–54], one possibility might be that I κ B α continuously shuttles in the prostate cancer cells, and the nuclear export is dominant over the nuclear import, as has been described in most unstimulated cells [46,47]. However, this does not seem very likely, since our data indicate that once in the nucleus, I κ B α binds to p65 NF κ B, inhibits NF κ B DNA binding activity and induces apoptosis of the androgen-independent PC-3 cells (Figs. 2 and 3). Alternatively, the cytoplasmic retention of I κ B α in untreated PC-3 cells can be explained by binding of I κ B α to cytoplasmic proteins or components. This hypothesis seems to be supported by our CHX data (Fig. 4), suggesting that the nuclear translocation of I κ B α is regulated by its total cytoplasmic levels, as well as by previous study by Prigent *et al* demonstrating that in HeLa cells, I κ B α is retained in the cytoplasm by binding to protein G3BP2 [48]. In addition, even though the cytoplasmic level of p65 NF κ B in PC-3 cells is relatively low, it might be sufficient to retain I κ B α in the cytoplasm. However, if the concentration of free cytoplasmic

I κ B α is increased by blocking the proteasome-dependent I κ B α degradation, I κ B α translocates and accumulates in the nucleus.

Inhibitors of the 26S proteasome have been used in the treatment of patients with multiple myeloma, and are undergoing evaluation in clinical trials in a variety of malignancies, including the metastatic, androgen independent prostate cancer [55,56]. In summary, our results show that the proteasome inhibitors have a novel, previously unrecognized effect: they induce the nuclear translocation and accumulation of I κ B α . In the metastatic prostate cancer cells, this results in the inhibition of the constitutive NF κ B activity and induction of apoptosis. Future studies should address the specific mechanisms by which the increased stability of I κ B α leads to its translocation to the nucleus. The induction of nuclear accumulation of I κ B α could provide a basis for novel therapies in disorders characterized by high levels of constitutive NF κ B activity, such as certain cancers and inflammatory disorders.

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Figure 1. Proteasome inhibitor MG132 induces nuclear translocation of I κ B α in prostate cancer PC-3 cells

(A) Western blotting of cytoplasmic (CE) and nuclear extracts (NE) prepared from PC-3 cells treated with increasing concentrations of MG132 for 24 hours, and analyzed by using I κ B α , p65 and p50 NF κ B antibodies. To confirm equal protein loading, the membranes were stripped and re-probed with actin antibody. The presence of cytoplasmic proteins in nuclear fraction was evaluated by re-probing the membrane with LDH antibody. Nuclear contamination in the cytoplasmic fraction was assessed by using lamin B specific antibody. Each lane corresponds to approximately 5×10^4 cells. (B) Western blotting of CE and NE performed as described above, prepared from PC-3 cells treated with 50 μ M MG132 for 0–24 hours. (C) Western

blotting of whole cell extracts (WCE) prepared from unstimulated cells and cells treated with 50 μ M MG132 for 0–24 hours. Each lane corresponds to approximately 2×10^4 cells. **(D)** Proteasome activity measured in WCE prepared from PC-3 cells treated with increasing concentrations of MG132 for 24 hours. The 20S proteasomal activity is expressed as relative fluorescence units (RFU) of MG132-treated cells compared to untreated cells. The values represent the mean $\pm$ SE of three independent experiments measured in duplicates.

Figure 2. The MG132-induced nuclear translocation of I κ B α is associated with the inhibition of NF κ B activity

(A) EMSA of NF κ B and CREB DNA binding activities analyzed in nuclear extracts from PC-3 cells treated for 24 hours with increasing concentrations of MG132. (B) Specificity analysis of the constitutive NF κ B and CREB activities in PC-3 cells. Nuclear extracts from untreated PC-3 cells were incubated with NF κ B or CREB specific DNA probes alone or in the presence (+) of 30 molar excess of unlabeled wild type (wt) or mutant (mut) oligonucleotides. Antibodies used in supershift NF κ B assays included antibodies to p50 and p65. (C) EMSA of NF κ B and CREB activities analyzed in nuclear extracts from PC-3 cells treated with 50 μ M MG132 for 0–24 hours. (D) Co-immunoprecipitation experiment from nuclear extracts of untreated or

MG132-treated (50 μ M, 24h) cells by using pre-immune IgG or I κ B α -specific antibody. The western blots were analyzed with I κ B α and p65 NF κ B antibodies.

Figure 3. The MG132-Induced Nuclear Translocation of I κ B α Results in the Induction of Apoptosis (A) Western blotting of WCE prepared from PC-3 cells treated with increasing concentrations of MG132 for 24 hours, and analyzed by using Bcl-2, Bcl-xL, and actin antibodies. Each lane corresponds approximately to 2×10^4 cells. (B) Apoptosis measured by the quantitative nucleosome enrichment assay in PC-3 cells treated 24 hours with increasing concentrations of MG132. (C) Western blotting of CE and NE prepared from PC-3 cells transfected with control and I κ B α si RNA, followed by treatment with increasing concentrations of MG132 for 24 hours. The membranes were analyzed by using I κ B α and actin antibodies. Each lane corresponds approximately to 4×10^4 cells. (D) Apoptosis measured by the nucleosome enrichment assay in PC-3 cells transfected with control (full columns) and I κ B α (empty

columns) *si* RNA, followed by treatment with increasing concentrations of MG132 for 24 hours. The values represent the mean $\pm$ SE of four independent experiments. Asterisks denote a statistically significant ($P < 0.01$) inhibition.

Figure 4. *De novo* protein synthesis is required for the MG132-induced nuclear translocation of IκBα in PC-3 cells

Western blotting of CE and NE prepared from PC-3 cells treated with increasing concentrations of MG132 for 24 hours, in the absence and presence of 1h pre-treatment with CHX (10 μg/ml). The membranes were analyzed by using IκBα and actin antibodies. Each lane corresponds approximately to 5×10^4 cells.

Figure 5. The MG132-induced nuclear translocation of IκBα is not dependent on IκBα phosphorylation by IκB kinase

(A) Western blotting of CE and NE prepared from PC-3 cells treated for 0–24 hours with control DMSO or 50 μM MG132, and analyzed by using Ser-32-phospho-IκBα (pIκBα) and IκBα antibodies. Each lane corresponds approximately to 5×10^4 cells. (B) Western analysis of CE and NE prepared from PC-3 cells pre-treated for 1 hour with 10 μM IKK inhibitor VII (Calbiochem, 401486) or control DMSO, followed by 24 hour incubation with increasing concentrations of MG132. The samples were analyzed by using IκBα and actin antibodies; each lane corresponds approximately to 5×10^4 cells. (C) Western analysis of cytoplasmic extracts prepared from PC-3 cells treated for 1 hour with 10 μM IKK inhibitor VII or control

DMSO, followed by 0–30 minutes stimulation with 10 ng/ml TNF. The samples were analyzed by using I κ B α and actin antibodies, and each lane corresponds approximately to 5×10^4 cells.

(D) Western analysis of CE and NE prepared from HeLa cells treated for 24 hours with increasing concentrations of MG132, and analyzed by using I κ B α , Ser-32-phospho-I κ B α (pI κ B α) and actin antibodies. Each lane corresponds approximately to 5×10^4 cells.

Figure 6. Current model of the regulation of nuclear IκBα translocation by proteasome inhibition