MG-132 Sensitizes TRAIL-Resistant Prostate Cancer Cells by Activating c-Fos/c-Jun Heterodimers and Repressing c-FLIP(L)

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Abstract

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) is a promising anticancer agent because it induces apoptosis in cancer cells but not in normal cells. Unfortunately, some cancer cells develop resistance to TRAIL-induced apoptosis. Therefore, it is clinically relevant to determine the molecular mechanisms that differentiate between TRAIL-sensitive and TRAIL-resistant tumors. Previously, we have shown that the antiapoptotic molecule cellular-FLICE-inhibitory protein long isoform [c-FLIP(L)] is necessary and sufficient to maintain resistance to TRAIL-induced apoptosis. We have found that c-FLIP(L) is transcriptionally regulated by the activator protein-1 (AP-1) family member protein c-Fos. Here, we report that MG-132, a small-molecule inhibitor of the proteasome, sensitizes TRAIL-resistant prostate cancer cells by inducing c-Fos and repressing c-FLIP(L). c-Fos, which is activated by MG-132, negatively regulates c-FLIP(L) by direct binding to the putative promoter region of the c-FLIP(L) gene. In addition to activating c-Fos, MG-132 activates another AP-1 family member, c-Jun. We show that c-Fos heterodimerizes with c-Jun to repress transcription of c-FLIP(L). Therefore, MG-132 sensitizes TRAIL-resistant prostate cancer cells by activating the AP-1 family members c-Fos and c-Jun, which, in turn, repress the antiapoptotic molecule c-FLIP(L). [Cancer Res 2007;67(5):2247–55]

Introduction

Prostate cancer is the second leading cause of cancer death in American men accounting for 232,900 new cases and 30,350 deaths annually (1). In a majority of cases, early-stage prostate cancer can be treated effectively with surgery or radiotherapy. However, advanced hormone refractory metastatic prostate cancer can be a fatal disease without effective treatment.

Cell surface death receptor ligand, tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), has attracted attention to cancer therapy not only because of its ability to effectively kill cancer cells but also because it has little effect on normal cells; therefore, TRAIL has minimal cytotoxicity (2). TRAIL induces apoptosis by binding to DR4 and DR5, two related death receptors, causing the formation of a death-inducing signaling complex, which includes the receptors, the adaptor protein FADD, and caspase-8 (3, 4). Autoactivated caspase-8 initiates the apoptotic executing caspase cascade and subsequent programmed cell death (extrinsic cell death pathway). Activation of Bid to its truncated form, tBid, leads to the release of cytochrome c from the mitochondria, which then activates the mitochondrial-mediated proapoptotic pathway (intrinsic cell death pathway; ref. 5).

Although many cancers undergo TRAIL-induced apoptosis, some develop resistance (6). Cellular sensitivity for TRAIL-induced apoptosis can be modulated at several levels. Inducing the expression of DR5 can enhance TRAIL signaling and overcome TRAIL resistance in cancer cells (7, 8). TRAIL-induced apoptosis can also be modulated at the mitochondrial level by the proapoptotic molecules Bax and Bak and the antiapoptotic molecule Bcl-2 (9). Cellular-FLICE-inhibitory protein (c-FLIP) is another class of important intracellular antiapoptotic molecules, which can block the apoptotic signaling pathway of TRAIL-induced apoptosis. c-FLIP protein homologues interrupt apoptotic signaling by competing with caspase-8 for binding to the DED domains of FADD and also regulate apoptosis through their interference with the recruitment of caspase-8 to FADD (4, 10, 11). The levels of intracellular c-FLIP, therefore, may determine the sensitivity of cancer cells to apoptosis triggered by TRAIL (12, 13). The c-FLIP family of proteins, c-FLIP(L), c-FLIP(S), and perhaps the newly detected c-FLIP(r) (14), can bind to the DED domains of FADD and caspase-8 and regulate apoptosis through their interference with the recruitment of caspase-8 to FADD.

We have shown in the past that persistent expression of c-FLIP(L) is necessary and sufficient to maintain resistance to TRAIL-induced apoptosis (6). Intracellular c-FLIP(L) can be regulated at either the transcriptional, translational, or posttranslational levels. Expression of c-FLIP(L) has been shown to be modulated by nuclear factor-κB (NF-κB; refs. 15, 16), Akt (17, 18), c-Myc (13), p53 (19), and E3-ubiquitin ligase (20). We have found that transcriptional repression of c-FLIP(L) by the AP-1 family member protein c-Fos is critical in modulating resistance and sensitivity of cells in TRAIL-induced apoptosis.

To sensitize TRAIL-resistant cancer cells, proteosome inhibitors have been combined with TRAIL in a variety of different cancer models. For example, the proteosome inhibitor PS-341 has been shown to help overcome TRAIL resistance in colon and bladder cancer cells (21–25). Another proteosome inhibitor, MG-132, has a potent antitumor function and has been shown to help overcome TRAIL resistance in cancer models. For example, the proteasome inhibitor PS-341 has been shown to help overcome TRAIL resistance in colon and bladder cancer cells (21–25). Another proteosome inhibitor, MG-132, has a potent antitumor function and has been shown to sensitize resistant cancer cells to the proapoptotic effects of TRAIL (7, 8, 26, 27). In this study, we examined the mechanism that MG-132 sensitizes prostate cancer cells to TRAIL-induced apoptosis. We show that MG-132 sensitizes TRAIL-resistant...
prostate cancer cells by up-regulating the AP-1 family proteins c-Fos and c-Jun, which, in turn, repress the antiapoptotic molecule c-FLIP(L). c-Fos/c-Jun heterodimers bind to the c-FLIP(L) promoter, repress its transcriptional activity, and reduce c-FLIP(L) mRNA and protein levels. These findings suggest that elevated c-Fos and c-Jun can play an important role in determining whether a cell is responsive or resistant to the proapoptotic effects of TRAIL.

Materials and Methods

Chemicals and antibodies. Recombinant human TRAIL/TNFSF10 was obtained from R&D Systems, Inc. (Minneapolis, MN). Proteasome inhibitor MG-132 was obtained from EMD Calbiochem (La Jolla, CA). Antibodies were obtained from the following sources: horseradish peroxidase–conjugated secondary antibody (goat anti-mouse, goat anti-rabbit, and goat anti-rat antibodies), Oct-1 (C-21), c-Fos (D1), Fos B (C-11), Fra-1 (N-17), Fra-2 (L-15), JunB (N-17), Jun D (329), and c-Fos small interfering RNA (siRNA) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), c-Jun, phospho-c-Jun (Thr91), and c-Fos antibodies were obtained from Cell Signaling (Beverly, MA). Monoclonal c-FLIP(L) antibody (Dava II) was obtained from Apotech Corp. (San Diego, CA). Phospho-c-Fos (T232) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were obtained from Abcam, Inc. (Cambridge, MA).

Cell lines. PC3 and LNCaP prostate cancer cell lines and HEK 293T cells were from the American Type Culture Collection (Manassas, VA). BPH-1 (benign prostatic hyperplasia cells immortalized with SV40 large T antigen) cells were provided by Dr. Simon Hayward (Vanderbilt University, Nashville, TN; ref. 28). PC3-TR was a TRAIL-resistant subline established from parental PC3 cells by TRAIL treatment selection (6).

Cell viability and apoptosis assays. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) method (Cell TITER 96 Aqueous Assay, Promega, Madison, WI). Cells cultured in 96-well plates were treated with TRAIL and/or MG-132 for 24 h. MTS substrates were added and incubated for 2 h at 37°C. Absorbance was measured at 490 nm. Viability of control cells treated with DMSO was set at 100%, and absorbance of wells with medium and without cells was set at zero.

For apoptosis assays, cells were washed with PBS and resuspended in binding buffer from Sigma Chemical Co. (St. Louis, MO), and stained with FITC-conjugated Annexin V (Roche Diagnostic Co., Indianapolis, IN) and propidium iodide for 15 min at room temperature. Annexin V fluorescence was determined with a FACScan flow cytometer, and the membrane integrity of the cells was simultaneously assessed by the propidium iodide exclusion method.

Cell extracts and Western blot analysis. Cells were harvested for total cell lysates with radioimmunoprecipitation assay buffer (RIPA; 1% NP40, 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.5% deoxycholate, and 0.1% SDS) containing a mixture of protease inhibitors [cocktail 1×, 1 mmol/L phenylmethylsulfonil fluoride (PMSF), 20 mmol/L sodium fluoride (NaF), and 3 mmol/L Na3VO4]. After sonication for 15 s, cell debris was discarded by centrifugation at 12,000 × g for 10 min at 4°C, and the protein concentration was determined by bichinchoninic acid (BCA) protein assay reagent (Pierce Biotechnology, Rockford, IL). The procedure for the nuclear protein extraction was carried out according to the manufacturer’s instructions (NE-PER nuclear and cytoplasmic extraction reagents kit, Pierce Biotechnology). Cells were swollen with hypotonic buffer and then disrupted. The cytoplasmic fraction was removed, and the nuclear protein was released from the nuclei by a high-salt buffer. The lysate was boiled for 10 min and frozen at −80°C. Western blot was carried out as previously described (6).

Semi quantitative reverse transcription-PCR analysis. Total RNA was isolated with the RNeasy Mini kit (Qiagen, Valencia, CA). The RNA yield and purity were evaluated by measuring A260/A280 and agarose gel electrophoresis. Reverse transcription-PCR (RT-PCR) was done using a Superscript One-Step RT-PCR kit (Invitrogen Life Technologies, Carlsbad, CA). The total

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AP-1-(f) site was designed as 5’-ATACCTGGAGGATCAGGATTACCT-3’

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assay was done using the ChIP Assay kit (Upstate Cell Signaling Solutions, Lake Placid, NY). PC3-TR cells were cultured in 10-cm dishes and treated with TRAIL and/or MG-132 for 4 h. Cross-linking of DNA and proteins were fixed by adding formaldehyde directly to the culture medium to a final concentration of 1% and incubated for 10 min at 37°C. Cells were collected and washed with PBS that contained protease inhibitors. Harvested cells were resuspended in 200 μl of SDS lysis buffer for 10 min. Cell lysates were sonicated and samples were centrifuged at 12,000 × g for 10 min at 4°C, and the supernatant was harvested. Concentration of each sample was quantitated by the BCA technique (Pierce Biotechnology). Positive controls were 10% of each DNA sample, which did not include the immunoprecipitation step. The remainder of the samples was divided equally into two groups. The experimental group was immunoprecipitated with specific c-Fos (D-1) antibody, whereas the negative control group was immunoprecipitated with the general IgG antibody. After eluting protein-DNA from antibody, protein-DNA cross-linking was reversed by heating at 65°C for 4 h. The isolated genomic DNA was first purified by phenol/chloroform extraction and ethanol precipitation. Then, the DNA was amplified by PCR, using specific primers encompassing the region containing the AP-1-(f) binding site according to the human c-FLIP(L) sequence (GenBank). The conditions were as follows: primers 5’-CTGCTGATCCCAGCACTTTG-3’ (forward) and 5’-CAGCCTATCTAGTATTTT-3’ (reverse); denaturation at 94°C for 30 seconds; annealing at 56°C for 30 seconds; polymerization at 72°C for 60 seconds, for 30 cycles. Finally, PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining.

Results

MG-132 sensitizes TRAIL-resistant prostate cancer cells to undergo apoptosis. Although PC3 cells are sensitive to TRAIL-induced apoptosis, PC3-TR and LNCaP cells are resistant to the proapoptotic effects of TRAIL (Fig. 1A; ref. 6). Combination of TRAIL with MG-132 sensitizes resistant prostate cancer cells, PC3-TR, and LNCaP, to undergo apoptosis (Fig. 1B and C). Because TRAIL is more effective against cancer cells than benign immortalized cells (2), we wished to determine whether the effect of MG-132 + TRAIL is specific to cancer cells or whether immortalized but nontumorigenic cells undergo cell death. Nontumorigenic and immortalized 293T (human embryonic kidney) and BPH-1 (benign prostatic hyperplasia) cells were treated with MG-132, TRAIL, or in combination with MG-132 + TRAIL. We found that neither treatment as single agents nor combination of treatments promoted cell death in the immortalized nontumorous cells (Fig. 1D). These data suggest that MG-132 is capable of sensitizing cancerous cells, but not benign transformed cells, to undergo TRAIL-induced apoptosis.

Combination of TRAIL and MG-132 represses c-FLIP(L) and induces c-Fos. The antiapoptotic protein c-FLIP(L) plays an important role in TRAIL sensitivity of cancer cells. We have shown in the past that persistent expression of c-FLIP(L) is necessary and sufficient to maintain resistance to TRAIL-induced apoptosis (6). In addition, we have found that in TRAIL-sensitive cancer cells, the antiapoptotic molecule c-FLIP(L) is repressed by the AP-1 family protein c-Fos, a mechanism that is lacking in TRAIL-resistant cancer cells.7 Because MG-132 sensitizes resistant cancer cells to undergo TRAIL-induced apoptosis, we wished to determine whether the ability of c-Fos to repress c-FLIP(L) is restored in the presence of MG-132.

c-FLIP(L) protein and mRNA levels were maintained when the resistant PC3-TR cells were treated with either TRAIL or MG-132 alone (Fig. 2A). However, the combination of MG-132 + TRAIL led to reduction of the antiapoptotic molecule c-FLIP(L) at the mRNA level and protein levels, as shown by the semiquantitative RT-PCR and Western blot analyses (Fig. 2B). The c-FLIP(L) mRNA level was noticeably reduced 12 h after treatment with MG-132 and TRAIL.

Because we have found that up-regulation of the AP-1 family protein c-Fos is necessary for TRAIL-induced apoptosis, we examined whether c-Fos levels are up-regulated in the presence of MG-132. We found that c-Fos protein and mRNA levels were increased in the presence of MG-132 alone (Fig. 2B), a condition that does not promote cell death in resistant prostate cancer cells (Fig. 1B and D). However, combination of MG-132 and TRAIL sensitizes prostate cancer cells to undergo cell death while promoting c-Fos levels at the mRNA and protein levels (Fig. 2B). Decrease in c-FLIP(L) and increase in c-Fos protein levels are observed in a time-dependent (Fig. 2C) and dose-dependent (data not shown) manner. Because c-Fos is a well established transcription factor (30–32), we determined whether there is any significant change in nuclear c-Fos levels in the presence of MG-132. We found that nuclear c-Fos, and more specifically phosphorylated nuclear c-Fos, was increased when treated with MG-132 or MG-132 + TRAIL (Fig. 2D). This result shows that MG-132 sensitizes resistant prostate cancer cancers to undergo apoptosis (Fig. 1) by repressing expression of c-FLIP(L) and promoting expression of c-Fos. Similar to our previous results, increased expression of c-Fos in response to MG-132 does not induce cell death; it only primes resistant prostate cancer cells to undergo apoptosis (Figs. 1 and 2).

Combination of TRAIL and MG-132 increases AP-1 activity and decreases c-FLIP(L) promoter activity. Because the combination of MG-132 and TRAIL reduces the expression of c-FLIP(L) and enhances the expression of the AP-1 family member c-Fos (Fig. 2), we wished to determine whether there is any direct interaction between the transcription factor AP-1/c-Fos and the antiapoptotic molecule c-FLIP(L). First, we examined the luciferase AP-1 activity in the resistant PC3-TR cells. We found that MG-132 alone or combination of MG-132 + TRAIL significantly enhanced the AP-1 activity in the resistant PC3-TR cells (Fig. 3A). Enhancement of AP-1 activity was particularly pronounced when MG-132 was combined with TRAIL (Fig. 3A). c-FLIP(L) promoter activity was not significantly changed in the presence of MG-132; however, the combination of MG-132 + TRAIL led to significant reduction of the c-FLIP(L) promoter activity (Fig. 3B). This result, again, suggests that the proteasome inhibitor, MG-132 alone, sensitizes resistant prostate cancer cells to undergo apoptosis by enhancing AP-1 activity, which only in the presence of the proapoptotic agent TRAIL will lead to repression of the c-FLIP(L) antiapoptotic molecule (Fig. 2).

Next, we examined whether inhibition of c-Fos by siRNA can affect c-FLIP(L) promoter activity. To ensure that our siRNA was functioning as expected, AP-1 activity and c-Fos protein levels were assessed in the presence or absence of c-Fos siRNA. We found that AP-1 activity and c-Fos protein levels were reduced in the presence of c-Fos siRNA (Fig. 3C). In addition, inhibition of c-Fos by siRNA led to increased c-FLIP(L) promoter activity (Fig. 3C).

Next, we wished to examine the effect of inhibiting c-Fos by siRNA on c-FLIP(L) promoter activity when treated with MG-132 and TRAIL. The luciferase activity in the control groups were normalized (Fig. 3D). Then, we examined the luciferase activity when the cells were treated with MG-132, TRAIL, or MG-132 alone (Fig. 2A). However, the combination of MG-132 + TRAIL led to reduction of the antiapoptotic molecule c-FLIP(L) at the mRNA level and protein levels, as shown by the semiquantitative RT-PCR and Western blot analyses (Fig. 2A). The c-FLIP(L) mRNA level was noticeably reduced 12 h after treatment with MG-132 and TRAIL.
We found that c-FLIP(L) promoter luciferase activity did not differ significantly from the controls when the cells were treated with MG-132 or TRAIL alone. c-FLIP(L) promoter activity decreased in the cells that were treated with MG-132 + TRAIL (Fig. 3D, last two columns). However, c-Fos siRNA rescued and promoted c-FLIP(L) promoter activity when the cells were treated with MG-132 + TRAIL (Fig. 3D, last two columns). In addition, c-Fos siRNA helped maintain the expression of c-FLIP(L) protein (Fig. 4A).

To determine whether inhibition of c-Fos by siRNA had any functional role, we examined the cell viability of PC3-TR cells. As previously shown, we found that combination of MG-132 + TRAIL sensitized prostate cancer cells to undergo apoptosis. However, when c-Fos was inhibited by siRNA, PC3-TR cells became more resistant to cell death than controls when treated with MG-132 + TRAIL (Fig. 4B, last two columns).

In contrast, ectopic expression of c-Fos (Fig. 4C, inset) increased AP-1 activity and c-Fos protein level as expected, but also led to reduction of c-FLIP(L) promoter activity (Fig. 4C). Ectopic expression of c-Fos increased c-FLIP(L) promoter activity and led to decreased cell viability.

**Figure 1.** Cell viability and apoptosis assays after treatment with TRAIL and/or MG-132. Points and columns, average of at least three independent experiments; bars, SD. A, sensitivity of prostate cancer cells to TRAIL. Cell viability was evaluated by MTS assay after treatment for 24 h with increasing concentrations of TRAIL. Viability of untreated cells was set at 100%. B, TRAIL-resistant prostate cancer cells, PC3-TR or LnCaP cells, were treated with MG-132, TRAIL, or TRAIL (100 ng/mL) + MG-132 (1 μM/L) for 24 h. Cell viability was evaluated by MTS. C, fluorescence-activated cell sorting analysis for apoptosis after treatment with MG-132 (1 μM/L), TRAIL (100 ng/mL), or combination of the two for 24 h. Apoptosis was assessed by FITC-conjugated Annexin V and propidium iodide staining for 15 min at room temperature. The percentage of apoptotic cells was determined by Annexin V–stained positive cells. D, cell viability after exposure of nonmalignant benign prostatic hyperplasia (BPH-1) or HEK 293T cells to MG-132 (1 μM/L), TRAIL (100 ng/mL), or combination of the two did not induce cell death after 24 h of treatment.

**Figure 2.** TRAIL combined with MG-132 represses c-FLIP(L) and induces c-Fos. A and B, c-FLIP(L) (A) and c-Fos (B) expression by Western blot (top) and semiquantitative RT-PCR analysis (bottom). PC3-TR cells were treated with MG-132 (1 μM/L), TRAIL (100 ng/mL), or MG-132 combined with TRAIL. Western blot results represent 24 h after treatment. GAPDH is used as loading control. C, protein expression of c-FLIP(L) and c-Fos. PC3-TR cells were treated with MG-132 (1 μM/L) + TRAIL (100 ng/mL) for different times (1, 4, 12, and 24 h). D, Western blot analysis of nuclear c-Fos and phosphorylated c-Fos (p-c-Fos) after treatment with MG-132 (1 μM/L), TRAIL (100 ng/mL), or MG-132 combined with TRAIL for 24 h. Oct-1 is used as loading control of nuclear extracts.
expression of c-Fos and reduced c-FLIP(L) promoter activity are associated with sensitizing resistant prostate cancer cells to undergo TRAIL-induced apoptosis (data not shown). Therefore, MG-132 enhances the c-Fos activity, reduces c-FLIP(L) promoter activity, and sensitizes prostate cancer cells to undergo apoptosis.

MG-132 up-regulates AP-1 activity by increasing nuclear translocation of c-Fos/c-Jun and their interaction. AP-1 family transcription factors are dimeric protein complexes composed of heterodimers between Fos (c-Fos, FosB, Fra-1, and Fra-2), Jun (c-Jun, JunB, and JunD), and activating transcription factor (ATF) family gene products, which convert extracellular signals into changes of specific target gene expression (31, 32). Because we found that the AP-1 activity of PC3-TR cells are increased in response to MG-132 (Fig. 3A), we wished to examine whether any other AP-1 family members, besides c-Fos (Fig. 2), plays a key role in sensitizing cancer cells and regulating promoter activity of c-FLIP(L) during TRAIL-induced apoptosis. We found that MG-132 increased levels of c-Jun protein in PC3-TR cells, whereas there was no significant change in the protein levels of other AP-1 members (FosB, JunB, JunD, and Fra-1, and Fra-2; Fig. 5A). In particular, nuclear levels of total c-Jun and phospho-c-Jun were significantly increased (Fig. 5B).

c-Fos functions as a transcription factor by heterodimerizing with c-Jun and other AP-1 family members (31, 32). To determine whether the increased c-Fos and c-Jun nuclear levels after MG-132 treatment are associated with direct interactions between c-Fos and c-Jun, immunoprecipitation experiments between c-Fos and c-Jun were done. We found that direct interactions between c-Fos and c-Jun were increased in PC3-TR cells when the cells were exposed to MG-132. Similar results were obtained when MG-132 was combined with TRAIL. However, TRAIL alone did not enhance c-Fos/c-Jun interactions (Fig. 5C). Similar results were obtained when c-Jun antibody was used for the immunoprecipitation experiments (Fig. 5C). Therefore, the proteasome inhibitor MG-132 enhances c-Fos and c-Jun levels, enhances direct interactions between c-Fos and c-Jun, and presumably promotes heterodimerization and transcriptional activity.

c-Fos and c-Jun bind to the c-FLIP(L) promoter region. To determine whether increased protein levels of c-Fos and c-Jun in response to MG-132 is associated with increased DNA binding, electrophoretic mobility shift assay (EMSA) and EMSA supershift assays were done. We found that AP-1 DNA binding is increased in the presence of MG-132, TRAIL, or MG-132 + TRAIL. However, we observed supershift bands for c-Fos and c-Jun particularly when the cells were treated with MG-132, demonstrating the specificity of binding of these AP-1 family member proteins in response to MG-132 (Fig. 6A).

Because c-Fos and c-Jun DNA binding is increased in response to treatment of cells with MG-132 and c-Fos represses the antiapoptotic molecule c-FLIP(L), we wished to determine if c-Fos and c-Jun specifically bind to the c-FLIP(L) putative promoter region. Previously, we examined 14 potential AP-1 binding sites upstream and within the first intron of c-FLIP(L) coding region (Fig. 6B). We found binding of c-Fos only to the AP-1-(f) site (see Fig. 6B) in the putative promoter region of c-FLIP(L).1 We have found that in prostate cancer cells that are sensitive to TRAIL-induced apoptosis, mutations or deletions to the AP-1-(f) site abrogates binding of c-Fos, increases c-FLIP(L) promoter activity, and converts the phenotype of TRAIL-sensitive prostate cancer cells to become TRAIL resistant. Therefore, in our current model, with TRAIL-resistant prostate cancer cells that are sensitized by MG-132, we wished to determine whether there is increased binding of c-Fos and/or c-Jun at the AP-1-(f) site of c-FLIP(L). ChIP experiments were done to determine direct binding of c-Fos and c-Jun at the AP-1-(f) site. There was no significant binding of either c-Fos or c-Jun to the AP-1-(f) site of c-FLIP(L) without treatment or with TRAIL treatment alone. However, in the presence of MG-132, both c-Fos and c-Jun showed enhanced binding to the c-FLIP(L) AP-1-(f) site (Fig. 6C). These data show that MG-132 sensitizes resistant prostate cancer cells...
to proapoptotic effects of TRAIL by enhancing c-Fos and c-Jun interactions and transcriptionally repressing the expression of c-FLIP(L) by binding to the AP-1-(f) site of c-FLIP(L).

Discussion

TRAIL has great potential as an antitumor agent because it can selectively induce apoptosis in cancer cells, yet spare most normal cells. Although many cancer cells are sensitive to TRAIL-induced apoptosis, some develop resistance. Many groups have been investigating the synergistic effects of different drugs in combination with TRAIL to overcome the resistance developed by cancer cells (8, 20, 21, 33–38). In the present study, we showed that TRAIL combined with the proteasome inhibitor MG-132 could effectively sensitize TRAIL-resistant prostate cancer cells to undergo apoptosis. Moreover, this combined treatment did not induce death in nonmalignant cell (BPH-1 and HEK 293T; Fig. 1). MG-132 sensitizes TRAIL-resistant prostate cancer cells by up-regulating the AP-1 family proteins c-Fos and c-Jun, which, in turn, repress the antiapoptotic molecule c-FLIP(L). As for the other well studied c-FLIP isoform, c-FLIP(s), we have not found it to be expressed in our prostate cancer cells. Therefore, the effects of MG-132 on c-FLIP(s) was not examined in our study.

Proteasome inhibitors are attractive cancer therapeutic agents because they can regulate apoptosis-related proteins (e.g., TRAF2, BAX, IAP, and p53 proteins; refs. 12, 23–25). PS-341 has been approved by the Food and Drug Administration for treatment of patients with multiple myeloma, and many clinical trials are ongoing to examine the efficacy of PS-341 for treatment of other malignancies (39, 40). MG-132 is another small-molecule proteasome inhibitor, and numerous reports have shown that MG-132 inhibits NF-κB activation through stabilization of the inhibitor of NF-κB complex, as well as prevention of nuclear translocation of NF-κB (41, 42). Other mechanisms that MG-132 sensitizes cancer cells include increased expression of mitogen-activated protein kinase and activation of c-Jun-NH2 kinase (26, 27) or up-regulation of death receptor DR5 and Bik accumulation (7, 8, 26, 27).

In the current study, we determined whether TRAIL-resistant prostate cancer cells, which are sensitized by MG-132, have changes in the AP-1/c-Fos and c-FLIP(L) signaling pathway. In the presence of MG-132, we found that inhibition of c-Fos by siRNA led to up-regulation of c-FLIP(L) promoter activity, and, conversely, ectopic expression of c-Fos reduced c-FLIP(L) promoter activity (Fig. 3). After priming the resistant prostate cancer cells by MG-132 to undergo apoptosis, we showed that the AP-1 family members c-Fos and c-Jun directly bind to the c-FLIP(L) AP-1(f) site (Fig. 6) after treatment with TRAIL.

In TRAIL-sensitive prostate cancer cells, we have found that the AP-1 family member proteins only bind to the AP-1-(f) site of the c-FLIP(L) promoter region and none of the other putative AP-1 binding sites in the putative promoter region of c-FLIP(L). Deletions and mutations at the c-FLIP(L) AP-1(f) site abrogate binding of c-Fos to the c-FLIP(L) promoter and maintain expression of c-FLIP(L) promoter activity. In the current study shows that treatment of resistant prostate cancer cells with MG-132 potentiates binding of c-Fos and c-Jun proteins to the c-FLIP(L) AP-1(f) site (Fig. 6B and C). Although binding of c-Fos/c-Jun to the putative promoter region of c-FLIP(L) after treatment with MG-132 may be necessary, it is not sufficient to reduce c-FLIP(L) mRNA and protein levels (Fig. 2). Therefore, addition of TRAIL to MG-132 induces other factors to repress c-FLIP(L) levels and potentiate cell death.

The AP-1 transcription factor is composed of protein dimers between the Jun, Fos, and ATF family members. The predominant forms of AP-1 in most cells are Fos/Jun heterodimers, which have a high affinity for binding to an AP-1 site. The regulation of these transcription factors is critical in determining the response to various physiologic and environmental stimuli (31, 32). In addition to c-Fos, we found that c-Jun was another AP-1 family protein that was activated by MG-132. c-Jun protein levels increased in the TRAIL-resistant cancer cells after treatment with MG-132. More specifically, MG-132 promoted expression of nuclear c-Jun protein and heterodimerization with c-Fos and binding to the c-FLIP(L) promoter (Fig. 5). These results suggest that c-Fos/c-Jun heterodimers may act concomitantly to down-regulate c-FLIP(L) expression and sensitize resistant cancer cells to undergo TRAIL-induced apoptosis.
Activation of AP-1 family members by other tumor necrosis factor (TNF) family signaling pathways, besides TRAIL, has been implicated. For example, the TNF receptor member receptor activator of NF-κB ligand (RANKL) is a key regulator of bone homeostasis. RANKL induces expression of c-Fos, an important step in bone development. To maintain proper balance in bone development, c-Fos activates its own inhibitor, IFN-β, to reduce RANKL signaling. Thus, an autoregulatory mechanism involving c-Fos, the TNF receptor family member RANKL, and IFN-β play a crucial role in bone development (43). In the present study, we identified a similar autoregulatory mechanism that involves c-Fos/c-Jun heterodimerization in TRAIL-resistant cancer cells.

We postulate that posttranslational modifications of AP-1 family member proteins, particularly c-Fos and c-Jun, play an important role in determining whether cancer cells are sensitive or resistant to TRAIL-induced apoptosis. Cellular localization and activation of c-Fos, the TNF receptor family member RANKL, and IFN-β play a crucial role in bone development (43). In the present study, we identified a similar autoregulatory mechanism that involves c-Fos/c-Jun heterodimerization in TRAIL-resistant cancer cells.

We postulate that posttranslational modifications of AP-1 family member proteins, particularly c-Fos and c-Jun, play an important role in determining whether cancer cells are sensitive or resistant to TRAIL-induced apoptosis. Cellular localization and activation of

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**Figure 5.** MG-132 up-regulates AP-1 activity by increasing nuclear c-Fos and c-Jun and their heterodimerization. A, Western blot analysis of AP-1 member proteins (FosB, c-Jun, JunB, JunD, Fra-1, and Fra-2). PC3-TR cells were treated with MG-132 (1 μmol/L), TRAIL (100 ng/mL), or MG-132 combined with TRAIL for 24 h. B, Western blot analysis of nuclear protein for total c-Jun and phosphorylated c-Jun in PC3-TR cells. C, immunoprecipitation (IP) assay between c-Fos and c-Jun protein. PC3-TR cells were treated with MG-132 (1 μmol/L), TRAIL (100 ng/mL), or MG-132 combined with TRAIL for 4 h. Equal-quantity whole-cells lysates were immunoprecipitated using c-Fos or c-Jun antibody. The resulting immune complex was subjected to Western blot analysis. Input was 2% of the total from each sample of immunoprecipitation.

**Figure 6.** AP-1 binding to c-FLIP(L) promoter was analyzed by EMSA and ChIP assay. A, EMSA and EMSA supershift assay for c-Fos and c-Jun binding to DNA. B, AP-1 binding sites of the putative regulatory region of c-FLIP(L) before the ATG start codon. C, AP-1 binding to 5′ site of c-FLIP(L) putative promoter region was analyzed by ChIP assay. Negative controls are samples using nonspecific IgG. Positive controls are whole-cell lysates without the immunoprecipitation step, and experimental samples include the ChIP assay using c-Fos or c-Jun antibodies.
c-Fos and c-Jun can depend on their phosphorylation, protein stability, and other chaperone proteins. Recent work has suggested that phosphorylation of c-Fos, which is an important determinant of its activity and expression, is tightly regulated by a variety of kinases (20, 44–48). Protein stability of c-Fos, another regulator of its physiologic function, has been shown to be dependent on its COOH-terminal PEST3 domain, which modulates the proteasome-mediated degradation of c-Fos (49). Associated proteins in the form of chaperone proteins or heterodimers can also regulate c-Fos structure and function. Therefore, we believe that c-Fos and c-Jun posttranslational modifications can significantly affect its ability to regulate c-FLIP(L) gene expression and TRAIL-induced apoptosis, and it is an area under investigation in our laboratory.

One limitation of our study is that MG-132 is a general proteasome inhibitor and can affect many different molecular pathways. Noting this limitation, we focused our attention on the effect of MG-132 on the AP-1–related protein c-Fos. Because our prior work has suggested that c-Fos, and not other AP-1 protein family members, is an important modulator of c-FLIP(L) protein, we primarily focused our attention on the effects of c-Fos and c-FLIP(L). However, our present results suggest that sensitization of TRAIL-resistant cancer cells by MG-132 lead to increased levels of c-Jun, as well as to c-Fos, a finding not seen in TRAIL-sensitive cells. In particular, we showed that DNA binding of c-Jun to potential AP-1 sites after treatment with TRAIL may be more pronounced than binding of c-Fos to potential AP-1 sites (Fig. 6). Our future studies will determine whether c-Fos and c-Jun have an equal or disproportionate effect on transcriptional regulation of c-FLIP(L) and modulation of TRAIL-induced apoptosis in cancer cells that are sensitized by the proteasome inhibitor MG-132.

In summary, we show that MG-132 primes and sensitizes TRAIL-resistant prostate cancer cells to undergo apoptosis by activating the AP-1 family member proteins c-Fos and c-Jun (Fig. 7A). Combination of MG-132 with TRAIL in TRAIL-resistant prostate cancer cells promotes cell death by increased heterodimerization of c-Fos/c-Jun and direct repression of the c-FLIP(L) antiapoptotic molecule (Fig. 7B). Therefore, we report a new regulatory pathway by which MG-132 sensitizes cancer cells for apoptosis, and combination of TRAIL with proteasome inhibitors may be an effective strategy for treating TRAIL-refractory tumors.

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MG-132 Sensitizes TRAIL-Resistant Prostate Cancer Cells by Activating c-Fos/c-Jun Heterodimers and Repressing c-FLIP(L)

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