Proteasome Inhibitor MG132 Induces Death Receptor 5 through CCAAT/Enhancer-Binding Protein Homologous Protein

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Abstract

Combined treatment with a proteasome inhibitor and tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) is a promising strategy for cancer therapy. Proteasome inhibitors induce the expression of death receptor 5 (DR5), a receptor for TRAIL, and sensitize cancer cells to TRAIL-induced apoptosis; however, the molecular mechanism of DR5 up-regulation has not been elucidated. In this study, we report that CCAAT/enhancer-binding protein homologous protein (CHOP) is a regulator of DR5 induction by proteasome inhibitor MG132. MG132 induced DR5 expression at a protein and mRNA level in prostate cancer DU145 cells. Furthermore, MG132 increased DR5 promoter activity. Using a series of deletion mutant plasmids containing DR5 promoters of various sizes, we found that MG132 stimulated the promoter activity via the region of −289 to −253. This region contained a CHOP-binding site. Site-directed mutagenesis of the site abrogated the promoter activity enhanced by MG132. An electrophoretic mobility shift assay showed that CHOP directly bound to the MG132-responsive site on the DR5 promoter. Expression of the CHOP protein was increased with MG132 along with DR5 up-regulation. Furthermore, CHOP small interfering RNA attenuated the DR5 up-regulation due to MG132. These results indicate that the proteasome inhibitor MG132 induces DR5 expression through CHOP up-regulation. (Cancer Res 2005; 65(13): 5662-7)

Introduction

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL; refs. 1, 2) induces apoptosis selectively in cancer cells in vitro and in vivo, with little or no toxicity in normal cells (3–5). Therefore, TRAIL is one of the most promising new candidates for cancer therapeutics. Death receptor 5 (DR5) also called TRAIL-R2, Apo2, TRICK2, or KILLER) is a receptor for TRAIL (6–10). DR5 mediates TRAIL-induced apoptosis through the interaction with adapter proteins such as FADD and through the activation of caspases (11). However, some tumor types exhibit resistance to TRAIL (12). Thus, it is important to overcome this resistance.

Note: T. Yoshida and T. Shiraishi contributed equally to this work.

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Materials and Methods

Reagents. MG132 (Calbiochem, La Jolla, CA) was dissolved in DMSO. Soluble recombinant human TRAIL/Apo2L was purchased from PeproTech (London, United Kingdom).

Cell culture. Human prostate cancer DU145 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin and incubated at 37°C in a humidified atmosphere of 5% CO2.

Detection of apoptosis. Cells were treated with the agent and harvested from culture dishes. After washing with PBS, cells were fixed with 70% ethanol and treated with RNase A (Sigma, St. Louis, MO). The nuclei were stained with propidium iodide before the DNA content was measured using FACScalibur (Becton Dickinson, Franklin Lakes, NJ). For each experiment, 10,000 events were collected. The Cell Quest software (Becton Dickinson, Mountain View, CA) was used to analyze the data.

Northern blot analysis. Total RNA from the cells was extracted using the Sepasol-RNA I (Nacalai Tesque, Kyoto, Japan), according to the manufacturer’s instructions. Total RNA (10 μg) was separated with electrophoreses on a 1% agarose gel and transferred to a nylon membrane (BIOXYNE B, Pall, Pensacola, FL). A full-length DR5 or CHOP cDNA was used as a probe for Northern blot analysis. Hybridization was carried out with a 32P-labeled probe in PerfectHyb PLUS Hybridization buffer (TOYOBO, Osaka, Japan) at 68°C for 16 hours and the membrane was washed at 68°C in 2× SSC containing 0.1% SDS. The blot was exposed to X-ray Films (KODAK, Chalon-sur-Saone, France).

Treatment with proteasome inhibitor results in protein stability and markedly increases a large number of cellular proteins such as cell cycle regulators, transcription factors, and apoptotic regulators (13). Proteasome inhibitors are attractive as potential cancer therapeutic agents. Bortezomib (also called PS-341 or Velcade) is a member of the proteasome inhibitors and had been approved by the U.S. Food and Drug Administration for the treatment of patients with multiple myeloma (14). Clinical trials are ongoing for the treatment of other types of malignancy (15). Furthermore, recent studies have shown that proteasome inhibitors, such as PS-341 and MG132, induce DR5 expression and sensitize tumor cells to TRAIL-induced apoptosis (16, 17). In particular, proteasome inhibitors even sensitize tumor cells resistant to TRAIL due to BAX deficiency or Bcl-XL overexpression to TRAIL-induced apoptosis. Thus, the combined treatment of proteasome inhibitors and TRAIL is a promising antitumor strategy. However, it remains unknown how proteasome inhibitors up-regulate DR5. To establish this combined treatment, it is required to identify the critical mediatory molecule for DR5 up-regulation by proteasome inhibitors.

Here, we report, for the first time, that CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP), a member of the C/EBPs and acting as a transcription factor (18), is a mediator for DR5 up-regulation caused by the proteasome inhibitor, MG132.
Western blot analysis. Western blotting was done as described previously (19). Rabbit polyclonal anti-DR5 antibody (Cayman Chemical, Ann Arbor, MI), rabbit polyclonal anti-GADD153 (CHOP), anti-C/EBPβ, anti-Sp1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), and mouse monoclonal anti-β-actin antibody (Sigma Chemical Co., St. Louis, MO) were used as the primary antibodies.

DNA transfection and luciferase assay. pDR5PF and deletion mutant plasmids containing DR5 promoter are previously described (20, 21). pDR5/mtCHOP was generated with a site-directed mutagenesis kit (Stratagene, La Jolla, CA) using synthesized oligonucleotides (5'-cgcttgcggaggattgcgttgacgagac and 5'-agagtctcgtcaactacctcctccgc). Reporter plasmid containing CHOP promoter, CHOP 3K, was described previously (22). DU145 cells (1 × 10^6 cells) were seeded in six-well plates, and 1.0 μg of plasmids were transfected with the DEAE-dextran method using a CellPhect transfection kit (Amersham Pharmacia Biotech., Piscataway, NJ). After 24 hours of incubation, the cells were treated with MG132 or solvent DMSO. Twenty-four hours after treatment with the agent, luciferase assay was carried out as described previously (20). Data were analyzed using Student’s t test and differences were considered significant compared with the control when P < 0.05.

Small interfering RNA. The DR5, CHOP, and LacZ small interfering RNA (siRNA) were described previously (23, 24) and synthesized by Proligo (Kyoto, Japan). One day before transfection, the cells were seeded into the medium without antibiotics at a density of 30% to 40%. The siRNA was transfected with Oligofectamine (Invitrogen, Carlsbad, CA). Twenty-four hours after transfection, the cells were treated with MG132 for 24 hours and cells were harvested.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assay (EMSA) was done as described previously (23) with nuclear extracts prepared from DU145 treated with DMSO or MG132. Double-stranded DNA probes containing synthetic oligonucleotides (5'-cgcttgcggaggattgcgttgacgagac and 5'-agagtctcgtcaactacctcctccgc) were labeled with [α-32P]dCTP and Klenow. Cold double-stranded nucleotides (wild type, 5'-cgcttgcggaggattgcgttgacgagac and 5'-agagtctcgtcaactacctcctccgc; mutant, 5'-cgcttgcggaggaggtagttgacgagac and 5'-agagtctcgtcaactacctcctccgc) were preincubated with the nuclear extracts for 10 minutes on ice before the addition of the radiolabeled probes. One microgram of rabbit polyclonal antibody against GADD153 (CHOP), C/EBPβ, or C/EBPα (Santa Cruz Biotechnology) was incubated with nuclear extracts for 30 minutes on ice before the addition of the

Figure 1. MG132 enhances TRAIL-induced apoptosis in DU145 cells. DU145 cells were treated with 0.5 μmol/L MG132 and/or 10 ng/mL TRAIL for 24 hours. Cells were analyzed for DNA content by propidium iodide staining (FL2-H) using flow cytometry. M1 shows populations of apoptotic cells with a sub-G1 DNA content. Columns, percentages of sub-G1.
radiolabeled probes. For immunodepletion, protein G-sepharose (Sigma) was incubated with 2 μg antibody against Bcl-X(s)l (Santa Cruz Biotechnology), GADD153 (CHOP), C/EBPα or C/EBPβ for 30 minutes at 4°C and washed with reaction buffer to remove the free antibodies. The antibody-conjugated Sepharose was added to 15 μg of nuclear extracts and rotated for 30 minutes at 4°C. The mixture was precipitated and the supernatants were collected. These immunodepleted supernatants were analyzed with EMSA as described above.

Results

MG132 enhances tumor necrosis factor–related apoptosis-inducing ligand–induced apoptosis in DU145 cells. We examined the effect of combined treatment with proteasome inhibitor MG132 and TRAIL on apoptosis by measuring the sub-G1 population. MG132 or TRAIL slightly induced apoptosis as single agents in prostate cancer DU145 cells; however, combined treatment with MG132 and TRAIL markedly induced apoptosis (Fig. 1).

MG132 induces death receptor 5 expression in DU145 cells. Next, we examined DR5 up-regulation by MG132. First, we carried out Western blotting to investigate the induction of DR5 protein by MG132. MG132 increased DR5 protein in a dose-dependent manner (Fig. 2A). We examined whether MG132 regulated DR5 expression at an mRNA level. DR5 mRNA was also remarkably increased by MG132 treatment (Fig. 2B). These results indicated that MG132 up-regulates DR5 expression at an mRNA and protein level in DU145 cells.
Identification of MG132-responsive elements in the death receptor 5 promoter. To elucidate the mechanism of the DR5 up-regulation by MG132, we carried out a luciferase assay using reporter plasmids containing the DR5 promoter. MG132 increased the promoter activity of pDR5PF, a luciferase reporter plasmid containing an ~2.5-kbp fragment of the DR5 promoter region (Fig. 3A). This result indicated that MG132 regulates DR5 expression through transcription. Using a series of 5′-deletion mutants, we investigated the MG132-responsive elements on the DR5 promoter. pDR5/−448 but not pDR5/−198 was activated by MG132 (Fig. 3A). Therefore, we generated 5′-deletion mutants between −448 and −198 and did the luciferase assay. As shown in Fig. 3B, the MG132-responsive element seemed located in a 37-bp region between −289 and −253 in the DR5 promoter, although we can not rule out the possibility that other responsive regions might also exist.

Mutation of the CCAAT/enhancer-binding protein homologous protein–binding site attenuates activation of the death receptor 5 promoter by MG132. This MG132-responsive site contains a CHOP-binding site. Therefore, we introduced a site-directed mutation into the site to generate pDR5/mtCHOP (Fig. 3A). This result indicates that MG132-responsive element seemed located in a 37-bp region between −289 and −253 in the DR5 promoter. pDR5/−448 but not pDR5/−198 was activated by MG132 (Fig. 3A). Therefore, we generated 5′-deletion mutants between −448 and −198 and did the luciferase assay. As shown in Fig. 3B, the MG132-responsive element seemed located in a 37-bp region between −289 and −253 in the DR5 promoter, although we can not rule out the possibility that other responsive regions might also exist.

Analysis of proteins binding to the MG132-responsive site. We used EMSA to see whether CHOP bound directly to the MG132-responsive site (Fig. 5A). Competition assays using cold competitors showed that the proteins bound specifically to the site (Fig. 5B). Next, we did EMSA with antibodies against the C/EBP family containing CHOP. As shown in Fig. 5C, antibodies against CHOP or C/EBPβ diminished the signal of the protein-DNA complex. Moreover, we did EMSA using immunodepleted nuclear extracts. The immunodepletion of nuclear extracts with the anti-CHOP or anti-C/EBPβ antibody resulted in the depletion of the complex (Fig. 5D). These results suggested that CHOP and C/EBPβ bind directly to the MG132-responsive site.

CCAAT/enhancer-binding protein homologous protein is increased by MG132 treatment and CCAAT/enhancer-binding protein homologous protein small interfering RNA reduces death receptor 5 induction by MG132. As described above, CHOP and C/EBPβ were suggested to bind directly to the MG132-responsive site on the DR5 promoter. To investigate the behavior of CHOP and C/EBPβ in nuclei, we carried out Western blotting for nuclear extracts. Western blotting showed CHOP protein was markedly increased in nuclei by MG132 treatment (Fig. 6A). On the other hand, C/EBPβ increased only slightly. Another nuclear transcription factor, Sp1, was not altered by MG132. Next, we examined CHOP expression in whole cells. MG132 treatment induced CHOP protein along with DR5 expression (Fig. 6B). MG132 also up-regulated CHOP mRNA (Fig. 6C) and enhanced CHOP promoter activity (Fig. 6D). These results indicate that CHOP is also induced by MG132 at a transcriptional level through the promoter. To confirm that CHOP played a role in DR5 up-regulation by MG132, we employed CHOP siRNA. CHOP siRNA markedly reduced the CHOP protein (Fig. 6E). Furthermore, CHOP siRNA prevented MG132 from inducing DR5 expression.

**Figure 5.** Identification of proteins binding to the MG132-responsive site. A, nuclear extracts from cells treated with 0.5 μM MG132 or DMSO for 24 hours were analyzed with EMSA, as described in Materials and Methods. B, increased amounts of the indicated unlabeled oligonucleotides (~5× and ~10-fold) were added to the EMSA. wt, wild-type CHOP-like site in DR5 promoter. mt, mutant CHOP-like site in DR5 promoter. C, anti-CHOP, anti-C/EBPβ or anti-C/EBPα monoclonal antibody (1 μg) was incubated with nuclear extracts for 30 minutes before the addition of the radiolabeled probes. D, nuclear extracts from cells treated with 0.5 μM MG132 were immunodepleted with antibodies against the indicated proteins. Anti-Bcl-Xl monoclonal antibody was used as a control antibody (CT). The immunodepleted nuclear extracts were analyzed with EMSA.
Discussion

Combined treatment of a proteasome inhibitor and TRAIL is a promising strategy against tumors (16, 17). The proteasome inhibitor, MG132, can induce the expression of DR5, a receptor for TRAIL, and sensitizes cells to TRAIL-induced apoptosis; however, the molecular mechanism of DR5 up-regulation has not been elucidated. Here, we have for the first time shown a regulatory mechanism by which MG132 up-regulates DR5 expression.

Using a series of reporter plasmids containing DR5 promoters of various sizes, we identified that a CHOP-binding site is crucial as the MG132-responsive element (Fig. 4). Furthermore, EMSA suggested that CHOP binds directly to the site on the DR5 promoter (Fig. 5). The EMSA also showed that C/EBPβ binds rather weakly to the site. Because CHOP acts as a dimer with other C/EBP proteins (18), CHOP may form a heterodimer with C/EBPβ on the DR5 promoter and thereby induce DR5 expression. Moreover, CHOP siRNA prevented MG132 from inducing DR5 expression (Fig. 6E). Together, these results indicate that CHOP is involved in DR5 up-regulation due to MG132.

Proteasome inhibitors regulate the stability of various target proteins (13). However, interestingly, we showed that MG132 enhances DR5 and CHOP expression at a transcriptional level, indicating that proteasome inhibitors regulate the transcriptional network as well as post-translation.

Recently, other research groups have shown that thapsigargin, an inducer of ER stress, up-regulates DR5 expression through CHOP (23). Thus, this and our findings suggest that CHOP is an important regulator of DR5 and one of the good targets for new anticancer agents that cooperate with TRAIL.

Our findings provide useful information regarding the combined treatment of TRAIL and a proteasome inhibitor. DR5 is a downstream gene of the tumor suppressor p53 (25–27). Furthermore, p53 is known to be activated by a proteasome inhibitor (28, 29). However, it has not been addressed as to whether DR5 up-regulation by MG132 is independent of p53. This problem is important for the clinical use of the combined treatment with MG132 and TRAIL, because more than half of malignant tumors possess an inactivating mutation in the p53 gene (30, 31). Instead of the model related to p53, we showed that MG132 up-regulates DR5 expression through CHOP. In addition, the DU145 cells we used in this study have mutations in the p53 gene, indicating that MG132 up-regulates DR5 in a p53-independent manner. Furthermore, in Fig. 1, we showed that the combination of MG132 and TRAIL synergistically induces apoptosis, even in DU145 harboring p53 mutations. These results suggest that the combined treatment with TRAIL and MG132 is a promising strategy against tumors carrying a p53 mutation.

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