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Research Article

Ubiquitin-proteasome pathway as a primary defender against TRAIL-mediated cell death

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Abstract. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptotic cell death as well as expression of proinflammatory genes such as CXCL8 in malignant human astrocytoma cells. However, the molecular mechanisms that determine the fate of cells are not yet understood. The ubiquitin (Ub)-proteasome pathway regulates a wide range of cellular functions through degradation of various regulatory proteins; given this, we hypothesized that this pathway may play a central role in TRAIL-mediated signaling. We demonstrate here that inhibition of the Ub-proteasome pathway enhanced TRAIL-mediated cell death of human astrocytoma CRT-MG cells within hours by blocking degradation of active caspase-8 and -3. Proteasome inhibitors suppressed TRAIL-mediated activation of NF-κB; however, inhibition of the NF-κB pathway alone was not sufficient to enhance TRAIL-mediated cell death. Collectively, these results suggest that the Ub-proteasome pathway may play an important role as an antiapoptotic surveillance system by eliminating activated caspases as well as mediating NF-κB-dependent signals.

Key words. Proteasome; caspase; apoptosis, TRAIL; astrocytoma; ubiquitin.

Introduction

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of the TNF family of cytokines, has a selective cytotoxicity against transformed tumor cells in vitro and in vivo [1, 2]. Although TRAIL has been considered as a therapeutic agent for treatment of malignant astrocytomas [3], we have recently shown that human astrocytoma cells can utilize this death ligand to transduce inflammatory and angiogenic signals [4]. Like other members of the TNF family of cytokines, TRAIL activates two distinct signaling pathways, the NF-κB and caspase cascades; however, the molecular mechanisms which determine whether cells induce proinflammatory responses or undergo apoptosis are not yet fully understood [4]. These observations prompted us to investigate the molecular mechanisms by which the fate of the cell is determined upon TRAIL ligation.

The ubiquitin (Ub)-proteasome pathway regulates a wide range of essential cellular functions including intracellular protein turnover, cell cycle progression, immune responses, apoptosis and cell proliferation through degradation of various vital regulatory proteins such as cyclins, transcription factors and oncoproteins [5]. In addition, this pathway plays a key role in activation of the NF-κB transcription factor, which transactivates a series of inflammatory and antiapoptotic genes [6]. Therefore, inhibition of the NF-κB pathway has been thought to be the molecular basis by which proteasome inhibitors enhance receptor-mediated cell death [7–9].

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Given the vital cellular functions of the Ub-proteasome pathway, we hypothesize that this pathway plays a central role in determining the fate of cells upon TRAIL ligation. In this study, we demonstrate that the Ub-proteasome pathway promotes survival of cells by enhancing inflammatory/survival signals (NF-κB pathway) and suppressing apoptotic cascades (caspase pathway). Thus, inhibition of this pathway renders cells more susceptible to TRAIL-mediated cytotoxicity and can be used to potentiate the efficacy of TRAIL in treatment of malignant brain tumors.

Materials and methods

Cells and reagents

Human astrocytoma CRT-MG and U87-MG cells were grown as described previously [4, 10]. Human recombinant TRAIL was generously provided by Dr. K. Kim (Yonsei University College of Medicine, Seoul, Korea). Proteosome inhibitors, MG-132, lactacystin and proteosome inhibitor II, were purchased from Calbiochem (La Jolla, Calif.) as was a nonspecific caspase inhibitor, Boc-D-Fmk. The cell-permeable NF-κB inhibitory peptide, SN-50, was purchased from Biomol (Plymouth Meeting, Pa). Mouse polyclonal anti-human caspase-3 and caspase-8 antibodies were obtained from Cell Signaling Technology (Beverly, Mass.).

Detection of cell death

Cell death was determined by staining with Annexin V-FITC (PharMingen, San Diego, Calif.), a 35.8-kDa protein that has a strong affinity for phosphatidylserine, and propidium iodide (PI) as previously described [4]. Ten thousand cells were analyzed on a FACStar within 30 min after staining.

Measurement of NF-κB binding activity in nuclear extracts

Nuclear extracts were prepared as described previously [4]. Lysates (4 µg total protein) were assayed for DNA-binding activity of p65/c-Rel using the TransAM NF-κB assay kit (ActiveMotif, Carlsbad, Calif.) according to the manufacturer's manual [4]. The same extracts were measured for NF-κB-binding activity by electrophoretic mobility shift assay (EMSA) as described previously [4].

Caspase-3 activity measurement

Soluble lysates (20 µg total protein) were incubated with 5 µg of the caspase-3/7 substrate Ac-DEVD-AMC (Calbiochem) in reaction buffer (100 mM NaCl, 50 mM HEPES, 10 mM DTT, 1 mM EDTA, 10% glycerol) for 2 h at 37 °C. Samples were read on a fluorescence plate reader (Labtech, Lewes, UK) at wavelengths of 360 nm for excitation and 460 nm for emission, as described previously [4]. Intracellular caspase-3/7-like activity was determined using a caspase-3 intracellular activity assay kit II (Calbiochem) according to the manufacturer's instructions. Intracellular fluorescence was measured using flow cytometry.

Immunoprecipitation and Western blot analysis

Total cell extracts were prepared as previously described [4], and lysates (500 µg of total protein) were used for analysis of caspases. Immunoprecipitation was performed using anti-human caspase-3 antibodies (Cell Signaling Technology). Immunoprecipitated proteins or total cell extracts (50 µg of total protein) were electrophoresed in 12% SDS gels. Proteins were then transferred to nitrocellulose and probed with anti-human caspase-3 or -8 antibodies. Enhanced chemiluminescence was used for detection of bound antibody.

Pulse chase experiment

Cells were metabolically labeled with [^{35}S]-methionine in methionine-free medium for 4 h and were incubated in the absence or presence of MG-132 and/or hrTRAIL for an additional 3 h. Soluble lysates (500 µg of total protein) were incubated with Biotin-x-DEVD (Calbiochem) at 37 °C for 1 h, and were precipitated with avidin-conjugated agarose beads (Pierce, Rockford, Ill.). Precipitated proteins were electrophoresed in 12% SDS-PAGE gels. Proteins were then transferred to nitrocellulose and autoradiographed.

Statistical analysis

Data are presented as the mean ± SD. Levels of significance for comparisons between samples were determined using Student's t test distribution.

Results

Proteasome inhibitors augment TRAIL-mediated apoptosis in a NF-κB-independent manner

To determine the role of the Ub-proteasome pathway in TRAIL-mediated apoptosis, human astrocytoma CRT-MG cells were treated with hrTRAIL in the absence or presence of various pharmacological proteasome inhibitors, and analyzed for apoptotic cell death. Human astrocytoma cells express functional TRAIL receptors and are susceptible to TRAIL-mediated cell death in a time-dependent manner [4]; however, significant cell death was not observed at 3 h after TRAIL ligation alone (fig. 1 A, control). Incubation with MG-132, a proteasome inhibitor, increased cell death of human astrocytoma cells when treated with hrTRAIL in a dose-dependent manner, while MG-132 alone did not induce cell death at this time point (fig. 1 A). Similar results were obtained using other proteasome inhibitors such as lactacystin and proteasome

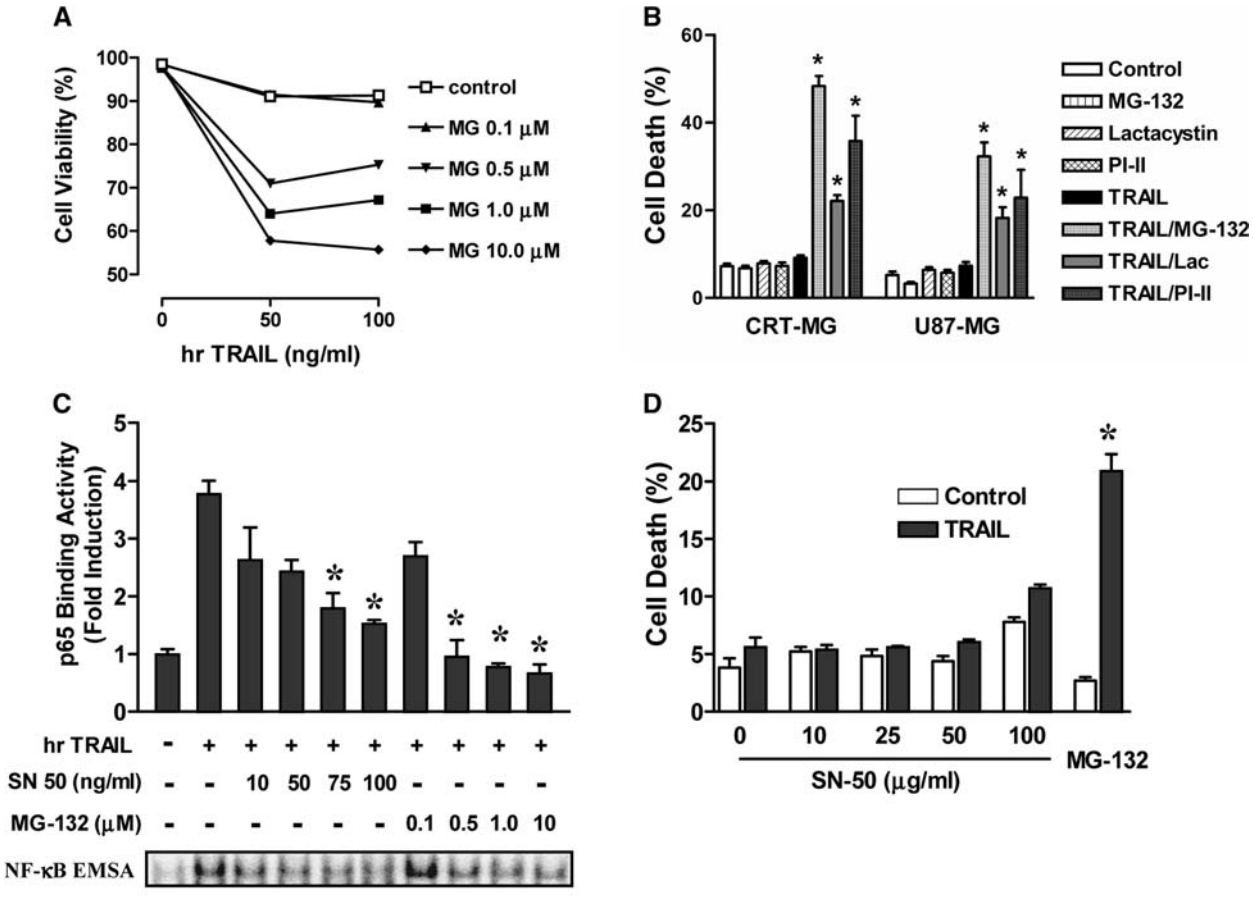

Figure 1. Inhibition of the proteasome pathway enhances TRAIL-mediated apoptosis by a NF-κB-independent mechanism. (*A*) CRT-MG cells were treated with varying concentrations of human recombinant (hr)TRAIL for 3 h in the absence or presence of increasing doses of MG-132, and analyzed for cell death by staining with annexin V-FITC and PI. (*B*) CRT-MG and U87-MG cells were treated with hrTRAIL for 3 h in the absence or presence of MG-132, lactacystin or proteasome inhibitor II (1 μM), and analyzed for cell death. Values significantly different from the value for the TRAIL-treated sample without inhibitor treatment are indicated by asterisks ($p \leq 0.05$). (*C*) Cells were incubated in the absence or presence of MG-132 or SN-50 for 1 h, treated with hrTRAIL for an additional 45 min, and then nuclear extracts (4 μg of total protein) were examined for NF-κB-binding activity by EMSA or a modified p65/c-Rel assay kit. Fold induction values are calculated as the ratio of the value of each sample with that of the control. Data are the mean ± SD for quadruplicate samples from one experiment, and are representative of three independent experiments. Values significantly different from the value for the TRAIL-treated sample without inhibitor treatment are indicated by asterisks ($p \leq 0.05$). (*D*) Cells were incubated with varying doses of SN-50 or MG-132 (1 μM) for 1 h, treated with hrTRAIL (100 ng/ml) for an additional 3 h, and cell death was determined. Data are the mean ± SD for quadruplicate samples from one experiment, and are representative of two independent experiments. Values significantly different from the value for the untreated sample are indicated by asterisks ($p \leq 0.05$).

inhibitor II in both CRT-MG and U87-MG cells (fig. 1B). We also observed similar effects of proteasome inhibitors on TRAIL-mediated cell death in another human astrocytoma U251-MG cell line (data not shown). These results clearly indicate that proteasomal inhibition enhances TRAIL-mediated cell death in human astrocytoma cells. Since the Ub-proteasome pathway plays a critical role in the activation of the NF-κB transcription factor, which then transactivates a series of inflammatory and antiapoptotic genes [6], we next examined whether the effect of proteasome inhibitors was through suppression of TRAIL-induced NF-κB activation. Preincubation with MG-132 or SN-50, which can block nuclear translocation of NF-κB specifically [11], suppressed ~80% of TRAIL-induced NF-κB (p65)-binding activity in the nuclear extracts (fig. 1C). However, preincubation with SN-50 had no significant effect on TRAIL-mediated apoptosis (fig. 1D). Prolonged (~24 h) treatment with SN-50 eventually induced significant cell death (~40%), which was augmented in an additive manner by treatment with TRAIL (data not shown). These results clearly indicate that TRAIL-mediated NF-κB activation is not required to protect cells from early apoptotic events upon TRAIL ligation.

Active fragments of capase-8 and -3 are sensitive to proteosomal degradation

Since TRAIL-induced apoptosis is mediated by a proteolytic activation of caspases, especially caspase-8 and -3

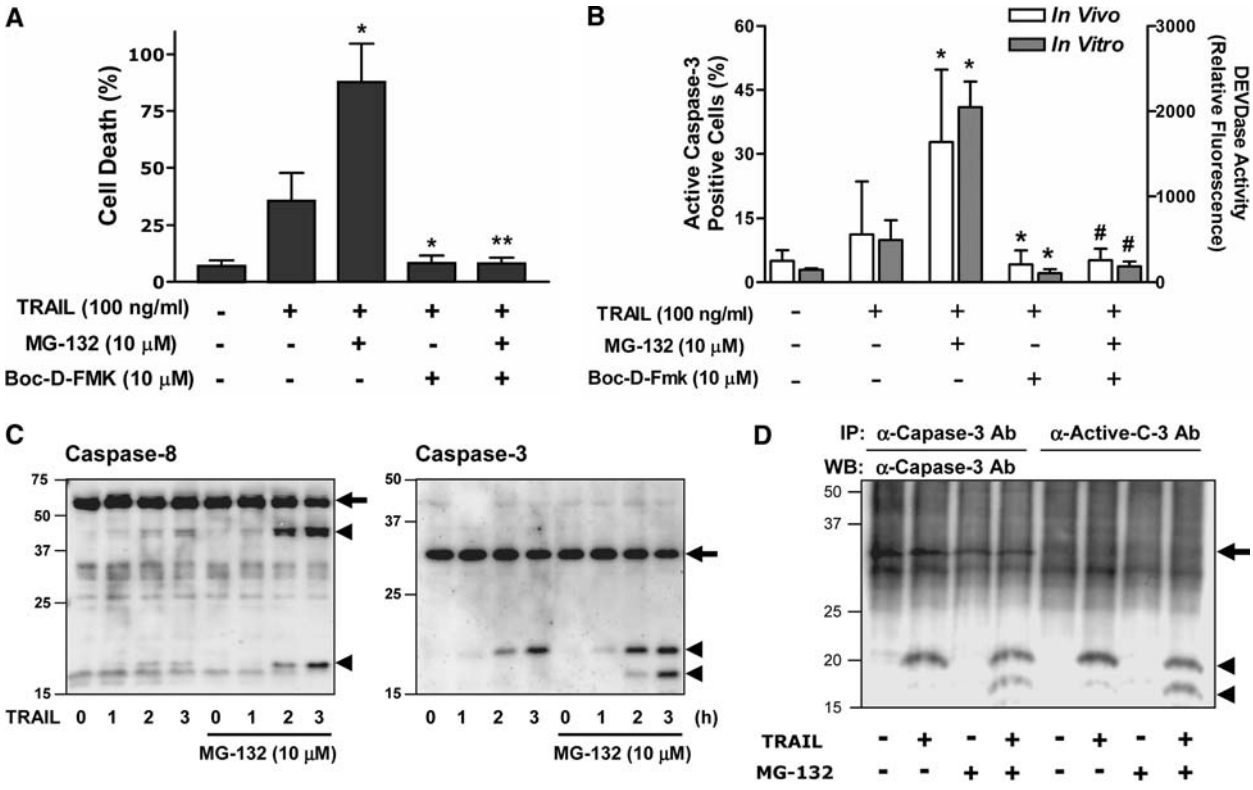

Figure 2. Proteasome inhibition stabilizes and increases active fragments of capase-8 and -3 cleaved upon TRAIL ligation. (*A*) Cells were incubated in the absence or presence of MG-132 and/or Boc-D-Fmk for 1 h, treated with hrTRAIL for an additional 24 h, and cell death determined after staining with annexin V-FITC and PI by FACS analysis. Data are the mean SD for five independent samples. Values significantly different from the value of the unstimulated sample are indicated by asterisks ($p \leq 0.05$). Values significantly different from the value of the sample treated with hrTRAIL alone are indicated by double asterisks ($p \leq 0.05$). (*B*) Cells were treated with hrTRAIL (100 ng/ml) for 2 h in the absence or presence of MG-132 (1 μM). In vivo caspase-3 activity was measured after incubating with a cell-permeable caspase-3 fluorescent substrate by FACS analysis, while soluble cell extracts (20 μg total protein) were analyzed for in vitro caspase-3 activity. Representative of two independent experiments. Values significantly different from the value of the unstimulated sample were indicated by asterisks ($p \leq 0.05$). Values significantly different from the value of the sample treated with hrTRAIL alone are indicated by # ($p \leq 0.05$). (*C*) Cells were treated with hrTRAIL (100 ng/ml) for varying time periods in the absence or presence of MG-132 (1 μM). Total cell extracts (50 μg total protein) were analyzed for caspase-8 or -3 by Western blot analysis. The arrows indicate the precursor forms of caspase-3 (32 kDa) and -8 (55 kDa), and the arrowheads indicate proteolytic products of caspase-3 (17/22 kDa) and -8 (18/40 kDa). Representative of five independent experiments. (*D*) Cells were incubated in the absence or presence of MG-132 (10 μM) for 1 h and treated with hrTRAIL (100 ng/ml) for an additional 3 h. Total lysates (500 μg total protein) were precipitated with anti-caspase-3 antibodies, and the precipitated complexes were analyzed for caspase-3 by immunoblot analysis. Arrow indicates procaspase-3, and arrowheads indicate proteolytic fragments of caspase-3 (p17/p22). Representative of three independent experiments.

[4], we further examined the role of caspases on MG-132-induced augmentation of TRAIL-mediated cell death. Pretreatment with a broad-spectrum caspase inhibitor Boc-D-Fmk completely suppressed not only TRAIL-mediated cell death but also inhibited cell death induced by combined treatment with MG-132 and hrTRAIL (fig. 2 A). These results indicate that the synergistic effect of MG-132 and TRAIL on cell death is also mediated by activation of caspases. To further investigate the involvement of caspases in MG-132-induced sensitization, we next examined the in vivo and in vitro enzymatic activity of caspase-3. Treatment with TRAIL alone for 2 h induced a minimal increase of DEVDase activities in vivo and in vitro, while preincubation with MG-132 significantly increased the enzymatic activity of caspase-3 (fig. 2 B). Preincubation with Boc-D-Fmk also suppressed not only TRAIL-mediated activation of caspase-3 but also abrogated DEVDase activity induced by combined treatment with MG-132 and hrTRAIL.

Next, we examined the effect of MG-132 on TRAIL-induced proteolytic cleavage of caspase-8 and -3. TRAIL ligation induced a proteolytic cleavage of procaspase-8 and -3 into active fragments (fig. 2 C) in a time-dependent manner as described previously [4]. TRAIL ligation induced a proteolytic cleavage of procaspase-8 into active fragments (arrowheads, p18/40), which increased dramatically after pretreatment with MG-132. TRAIL ligation also induced a proteolytic cleavage of procaspase-3 (p32) resulting in a slightly longer fragment (p22), which is cleaved by caspase-8. Combination of MG-132 and

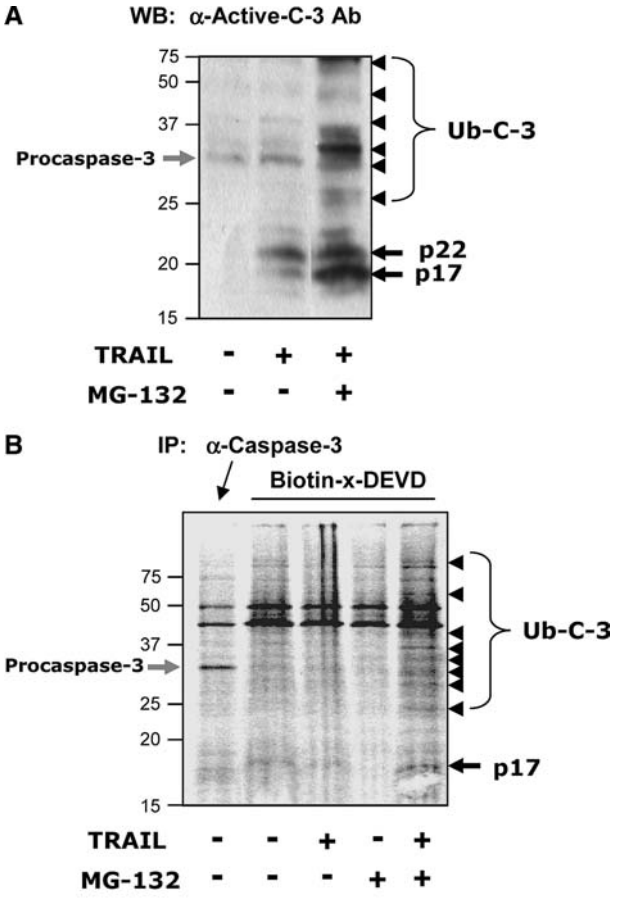

Figure 3. Modified caspases are stabilized by proteosomal inhibition. (*A*) Cells were incubated in the absence or presence of MG-132 for 1 h, treated with hrTRAIL for an additional 3 h, and cell lysates (50 µg total protein) were analyzed for caspase-3 using an antibody specific for active caspase-3. Arrows indicate proteolytic fragments of caspase-3 (p17/p22) and arrowheads indicate poly-ubiquitinated caspase-3. (*B*) Pulse-chase experiment for caspase-3. CRT-MG cells were metabolically labeled with [^{35}S]-methionine for 4 h, incubated in the absence or presence of MG-132 (10 µM) for 1 h, and then treated with hrTRAIL (100 ng/ml) for an additional 3 h. Cytosolic extracts (500 µg total protein) were incubated with an anti-caspase-3 antibody (lane 1) or biotin-x-DEVD (lanes 2–5), and precipitated with protein A/G-conjugated or avidin-conjugated agarose beads. Precipitated proteins were electrophoresed in 12% SDS-PAGE, transferred to nitrocellulose membrane, and analyzed after autoradiography. Arrow indicates a proteolytic fragment of caspase-3 (p17) and arrowheads indicate multiple ubiquitinated caspase-3. Representative of two independent experiments.

TRAIL markedly increased the level of a shorter fragment (p17) of caspase-3, which is believed to be generated by a further cleavage of p22 by activated caspase-3. These results suggest that the Ub-proteasome pathway is responsible for rapid degradation and elimination of cleaved caspases, so that inhibition of this pathway subsequently increases TRAIL-mediated cell death. To further investigate the proteolytic cleavage of caspase-3, cellular proteins (500 µg total protein) were precipitated with anti-caspase-3 antibodies that can recognize both pro- and active forms of caspase-3 or only activated caspase-3 fragments (fig. 2D). In concordance to previous results, TRAIL ligation induced a proteolytic cleavage of procaspase-3 into a p22 fragment (fig. 2D, lanes 2 and 6), and preincubation with MG-132 markedly increased a further cleavage of p22 into p17 fragment (fig. 2D, lanes 4 and 8).

Since proteosomal degradation of proteins requires poly-ubiquitination of target proteins, which is mediated by complex ubiquitination enzyme cascades [12], inhibition of proteasomal degradation might increase poly-ubiquitinated products of target proteins. Cells were treated with hrTRAIL in the absence or presence of MG-132 for 3 h, and lysates were analyzed for modification of caspase-3 using an antibody specific for active caspase-3 (fig. 3A). TRAIL ligation induced a cleavage of procaspae-3 mainly into a p22 fragment (lane 2), which was further cleaved into p17 by pretreatment with MG-132 (lane 3). As expected, treatment with MG-132 increased the amount of multiple ubiquitinated products of active caspase-3 (fig. 3A, lane 3). Identical results were obtained after metabolic labeling of cellular proteins and precipitation of functionally active caspase-3 complexes with a biotinated caspase-3-specific substrate (fig. 3B). These results collectively suggest that activated caspase-3 is rapidly ubiquitinated and degraded by the proteasome pathway.

Discussion

Our results clearly indicate that the immediate effect of proteasome inhibitors on TRAIL-mediated apoptosis is not through inhibition of NF-κB activation but via stabilization of active caspases. These results are consistent with previous findings that XIAP and cIAP2 have an E3-ligase activity to facilitate ubiquitination and subsequent proteosomal degradation of caspase-3 and -7 [13–15]. To our knowledge, this is the first report demonstrating that active fragments of caspase-3 and -8 are readily ubiquitinated and degraded by the proteasome pathway in vivo. Apoptotic signaling through the family of TNF receptors such as TNFRp55, Fas and DR4/5 is evolutionarilly distinct from the classical apoptotic paradigm, mediated by rapid protein-protein interactions that take place within minutes upon receptor ligation, and thus does not require new RNA or protein synthesis. Another characteristic of signaling through these receptors is activation of the NF-κB pathway, which has been regarded as an antiapoptotic and proliferative arm to determine sensitivity of cells to receptor-mediated cytotoxicity [4, 16]. The mechanism by which proteasome inhibitors augment apoptotic cell death in various situations has been thought to be by inhibition of NF-κB activation [7–9]. However, an unsolved puzzle has been how the rapid receptor-mediated

activation of caspases can be affected by NF-κB activation, which mediates its antiapoptotic effects by transcriptional regulation of antiapoptotic genes such as c-FLIP, gadd45β and XIAP [17–19].

Altered expression of c-FLIP or XIAP may be responsible for MG-132-induced sensitization to TRAIL-mediated cell death [17–19]; however, this effect takes relatively longer to sensitize target cells to apoptotic signals. Altered expression of cFLIP or XIAP has been observed only after 18–24 h of MG-132 treatment [7–9]. We have clearly demonstrated that inhibition of NF-κB is not necessary for the earlier (within 4 h) phase of sensitization to TRAIL-mediated cell death (fig. 1C, D). Therefore, we could exclude altered expression of cFLIP or XIAP as possible molecular mechanisms responsible for MG-132-induced sensitization of TRAIL-mediated cell death in our system. Even though receptor-mediated activation of NF-κB was dispensable for immediate survival upon TRAIL ligation, prolonged inhibition of NF-κB activation by pharmacological inhibitors such as SN-50, PDTC or TPCK eventually augmented TRAIL-mediated cell death in an additive manner (data not shown), suggesting that this pathway is still necessary for sustained survival as well as receptor-mediated induction of proinflammatory and angiogenic mediators [4]. Based on these findings, we propose that the Ub-proteasome pathway plays a central role to determine the functional outcome of death receptor ligation through several distinct mechanisms: ubiquitination-dependent proteosomal degradation of caspases, NF-κB-mediated induction of proinflammatory mediators or induction of NF-κB-dependent antiapoptotic molecules such as XIAP, c-FLIP and gadd45β [4, 9, 17–19].

We have utilized various proteasome inhibitors including MG-132, lactacystin and proteasome inhibitor II (Calbiochem), to evaluate the effect of proteasome inhibition on TRAIL-mediated cell death. Proteasome inhibitors including clinically available PS-132 have been shown to sensitize tumor cells to apoptotic signals through inhibition of NF-κB-mediated gene transcription [7–9]. PS-132 is under clinical trial for malignant tumors, including glioblastoma multiforme; therefore, our results provide a novel molecular mechanism responsible for tumor sensitization to various apoptotic signals by proteasome inhibitors.

Recognition by specific E3 ligase is a rate-limiting step of ubiquitination and subsequent degradation by the proteasome pathway [12]. Previous studies indicate that conformational changes of caspases by cleavage into active subunits expose the recognition sites to E3 ligases such as XIAP and cIAP2 [13–15]. Even though knowledge about substrate-specific E3 ligases is growing [20], the E3 ligase specific for active caspase-8 is still not known. Given the critical role of ubiquitination of active caspases in TRAIL-mediated apoptosis, discovery of caspase-specific E3 ligases warrants further investigation and could be a novel target to modulate TRAIL signaling.

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1 French L. E. and Tschopp J. (1999) The TRAIL to selective tumor death. Nat. Med. **5:** 146–147
2 Gura T. (1997) How TRAIL kills cancer cells, but not normal cells. Science **277:** 768
3 Rieger J., Naumann U., Glaser T., Ashkenazi A. and Weller M. (1998) APO2 ligand: a novel lethal weapon against malignant glioma? FEBS Lett. **427:** 124–128
4 Choi C., Kutsch O., Park J., Zhou T., Seol D. W. and Benveniste E. N. (2002) Tumor necrosis factor-related apoptosis-inducing ligand induces caspase-dependent interleukin-8 expression and apoptosis in human astroglioma cells. Mol. Cell. Biol. **22:** 724–736
5 Lee D. H. and Goldberg A. L. (1998) Proteasome inhibitors: valuable new tools for cell biologists. Trends Cell Biol. **8:** 397–403
6 Karin M. and Ben-Neriah Y. (2000) Phosphorylation meets ubiquitination: the control of NF-κB activity. Annu. Rev. Immunol. **18:** 621–663
7 Cusack J. C. Jr, Liu R., Houston M., Abendroth K., Elliott P. J., Adams J. et al. (2001) Enhanced chemosensitivity to CPT-11 with proteasome inhibitor PS-341: implications for systemic nuclear factor-κB inhibition. Cancer Res. **61:** 3535–3540
8 Hideshima T., Richardson P., Chauhan D., Palombella V. J., Elliott P. J., Adams J. et al. (2001) The proteasome inhibitor PS-341 inhibits growth, induces apoptosis, and overcomes drug resistance in human multiple myeloma cells. Cancer Res. **61:** 3071–3076
9 Sayers T. J., Brooks A. D., Koh C. Y., Ma W., Seki N., Raziuddin A. et al. (2003) The proteasome inhibitor PS-341 sensitizes neoplastic cells to TRAIL-mediated apoptosis by reducing levels of c-FLIP. Blood **102:** 303–310
10 Choi C., Xu X., Oh J. W., Lee S. J., Gillespie G. Y., Park H. et al. (2001) Fas-induced expression of chemokines in human glioma cells: involvement of extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase. Cancer Res. **61:** 3084–3091
11 Zhang L., Torgerson T. R., Liu X. Y., Timmons S., Colosia A. D., Hawiger J. et al. (1998) Preparation of functionally active cell-permeable peptides by single-step ligation of two peptide modules. Proc. Natl. Acad. Sci. USA **95:** 9184–9189
12 Pickart C. M. (2001) Mechanisms underlying ubiquitination. Annu. Rev. Biochem **70:** 503–533
13 Huang H., Joazeiro C. A., Bonfoco E., Kamada S., Leverson J. D. and Hunter T. (2000) The inhibitor of apoptosis, cIAP2, functions as a ubiquitin-protein ligase and promotes in vitro monoubiquitination of caspases 3 and 7. J. Biol. Chem. **275:** 26661–26664
14 Yang Y., Fang S., Jensen J. P., Weissman A. M. and Ashwell J. D. (2000) Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. Science **288:** 874–877
15 Suzuki Y., Nakabayashi Y. and Takahashi R. (2001) Ubiquitin-protein ligase activity of X-linked inhibitor of apoptosis protein promotes proteasomal degradation of caspase-3 and enhances its anti-apoptotic effect in Fas-induced cell death. Proc. Natl. Acad. Sci. USA **98:** 8662–8667

16 Lin Y., Devin A., Cook A., Keane M. M., Kelliher M., Lipkowitz S. et al. (2000) The death domain kinase RIP is essential for TRAIL (Apo2L)-induced activation of IκB kinase and c-Jun N-terminal kinase. Mol. Cell. Biol. **20:** 6638–6645
17 Kreuz S., Siegmund D., Scheurich P. and Wajant H. (2001) NF-κB inducers upregulate cFLIP, a cycloheximide-sensitive inhibitor of death receptor signaling. Mol. Cell Biol. **21:** 3964–3973
18 Tang G., Minemoto Y., Dibling B., Purcell N. H., Li Z., Karin M. et al. (2001) Inhibition of JNK activation through NF-κB target genes. Nature **414:** 313–317
19 De Smaele E., Zazzeroni F., Papa S., Nguyen D. U., Jin R., Jones J. et al. (2001) Induction of gadd45β by NF-κB downregulates pro-apoptotic JNK signalling. Nature **414:** 308–313.
20 Pickart C. M. (2001) Ubiquitin enters the new millennium. Mol. Cell **8:** 499–504