Apoptosis is a fundamental process that is essential for normal tissue homeostasis and development (31, 40, 56). Hence, the dysregulation of apoptosis has been implicated in several human diseases, including autoimmune disorders and malignancies. Apoptosis can be instigated via two principal pathways, both of which result in the activation of a class of aspartate-specific cysteine-dependent proteases, called caspases (4), that lead to the demise of the cell via the limited proteolysis of a multitude of cellular substrates (10). In the extrinsic pathway, ligand-induced oligomerization of death receptors, such as TRAIL-R1, TRAIL-R2, or CD95, results in the direct recruitment of the adapter molecule Fas-associated death domain (FADD) and the initiator caspase -8 or -10 that, together, form the death-inducing signaling complex (DISC) (49). Signaling through tumor necrosis factor receptor 1 (TNF-R1), in contrast, appears to be more complex (59) and is proposed to proceed via two sequential complexes, in which the death-inducing signaling complex II containing FADD and an initiator caspase is formed only in the cytosol (39). Nevertheless, despite the fact that procaspase-8 can also be activated independently of DISC formation (51), receptor-triggered dimerization of initiator caspases represents a crucial event in this pathway that results in their autocatalytic processing and activation. In most cell types, activated caspase-8 stimulates the second principal death route, the mitochondrial or intrinsic death pathway, by cleaving Bid, a BH3-only proapoptotic member of the Bcl-2 family (32).

The caspase-generated truncated Bid (tBid) fragment promotes the release of the proapoptotic factors cytochrome c and Smac from the mitochondria. Once released into the cytoplasm, cytochrome c associates with apoptotic protease-activating factor 1 and procaspase-9 to form the apoptosome, which leads to the activation of caspase-9 and the effector caspase-3 (17, 28).

Both death pathways are tightly controlled by multiple mechanisms that efficiently prevent caspase activation (50). For example, the generation of active caspase-8 is blocked by the recruitment of the cellular FLICE-like inhibitory protein (c-FLIP) into the DISC (29). Two isoforms, c-FLIP-long and c-FLIP-short, are major detectable splice variants that have been the subjects of several studies so far. While c-FLIP-short consists of mainly one major detectable splice variant that has been the subject of several studies so far. While c-FLIP-short consists of mainly two death effector domains that are required for the interaction with FADD, c-FLIP-long is structurally more closely related to caspase-8 but lacks key residues that are necessary to form an active caspase and is therefore completely devoid of any catalytic activity. Due to their homologies, both c-FLIP isoforms are able to form heterodimers with caspase-8 at the DISC and thereby block the processing and activation of this initiator caspase, which is crucial for the induction of death receptor-induced apoptosis (33, 48). On the other hand, antiapoptotic members of the Bcl-2 family indirectly inhibit caspase-9 activation by preventing the release of proapoptotic factors from the mitochondria. Further complexity is added to the regulatory pathways involved in apoptosis signaling by the inhibitor-of-apoptosis proteins (IAPs) that include X-linked IAP (XIAP), cIAP1, and cIAP2, all of which are potent inhibitors of the active caspase-9, -7, and -3 (6, 47). IAPs are characterized by two distinct motifs, the baculovirus IAP repeats that are crucial for caspase inhibition and a carboxyl-terminal

**The Proteasome Is Required for Rapid Initiation of Death Receptor-Induced Apoptosis**

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Due to their tremendous apoptosis-inducing potential, proteasomal inhibitors (PIs) have recently entered clinical trials. Here we show, however, that various PIs rescued proliferating tumor cells from death receptor-induced apoptosis. This protection correlated with the stabilization of X-linked IAP (XIAP) and c-FLIP and the inhibition of caspase activation. Together with the observation that PIs could not protect cells expressing XIAP or c-FLIP short interfering RNAs (siRNAs) from death receptor-induced apoptosis, our results demonstrate that PIs mediate their protective effect via the stabilization of these antiapoptotic proteins. Furthermore, we show that once these proteins were eliminated, either by long-term treatment with death receptor ligands or by siRNA-mediated suppression, active caspses accumulated to an even larger extent in the presence of PIs. Together, our data support a biphasic role for the proteasome in apoptosis, as they show that its constitutive activity is crucial for the rapid initiation of the death program by eliminating antiapoptotic proteins, whereas at later stages, the proteasome acts in an antiapoptotic manner due to the proteolysis of caspases. Thus, for a successful PI-based tumor therapy, it is crucial to carefully evaluate basal proteasomal activity and the status of antiapoptotic proteins, as their PI-mediated prolonged stability might even cause adverse effects, leading to the survival of a tumor.

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RING domain that enables IAPs to catalyze the degradation of both themselves and selected target proteins via the ubiquitin-proteasome pathway (57).

Relative to all the death receptor ligands studied, TRAIL has attracted immense attention due to its ability to preferentially kill tumor cells while leaving most normal cells unharmed in vitro (61) and in vivo (1). However, clinical trials are hampered by the fact that more than 50% of all human cancers are resistant to the cytotoxic activity of TRAIL, demonstrating the necessity for alternative treatment modalities. Promising results were obtained from studies showing that the cytotoxic potential of TRAIL was synergistically enhanced by a combined treatment with chemotherapeutics (16) or radiation (36). Also, the simultaneous treatment with proteasomal inhibitors (PIs), such as bortezomib (PS-341), lactacystin, or MG-132, yielded encouraging results, as all of these compounds synergistically accelerated and enhanced TRAIL- or TNF-induced apoptosis in a variety of tumor cells (11, 19, 30, 60). In view of this success, the proteasome inhibitor bortezomib has recently entered clinical practice as a treatment for multiple myeloma and is also undergoing clinical trials for other types of cancer (45, 58).

The ubiquitin-proteasome pathway plays a central role in the regulation of cell cycle control, transcription, signal transduction, and apoptosis (27). In eukaryotes, it is the major machinery that mediates the targeted degradation of many key regulatory proteins, including p53, cyclins, and cyclin-dependent kinase inhibitors, as well as IkBα (41). Although many diverse mechanisms have been proposed regarding which inhibition of the proteasome sensitizes cells for apoptosis, the precise mode of action remains elusive. The observations that many proapoptotic proteins, such as TRAIL-R2, Smac, Bax, tBid, Bik, and Bim, as well as various caspases, are degraded by the proteasome explain, at least partially, the apoptotic function of proteasomal inhibitors (35, 41, 42). However, several antiapoptotic proteins, such as c-FLIP, Bcl-2, and IAPs, also represent prominent targets of the proteasome (7, 12, 44, 63).

Hence, stabilization of these potent caspase antagonists by PIs should confer resistance to apoptotic stimuli; this is a hypothesis that, surprisingly, was not thoroughly investigated before. Therefore, we analyzed such a putative mechanism in more detail in HeLa cells and found that death receptor-induced apoptosis was indeed significantly blocked in the presence of various proteasome inhibitors. The inhibition of apoptosis induction correlated well with the stabilization of XIAP and c-FLIP, and the latter protein was efficiently recruited to the DISC (thereby preventing caspase-8 processing) only in the presence of PIs. The suppression of XIAP and c-FLIP expression by short interfering RNA (siRNA) technology not only abrogated the protective effect of proteasome inhibitors but also accelerated their cytotoxic potential in combination with death receptor ligands. Thus, our data demonstrate a biphasic role for the proteasome in apoptosis in which the degradation of antiapoptotic proteins, such as XIAP and c-FLIP, is necessary for a rapid initiation of the death receptor pathway.

**Materials and Methods**

**Cell lines, reagents, and antibodies.** HeLa D98 and H21 cells (24), MCF-7/casp-3 cells (26), and the KB cell line were maintained in RPMI 1640, whereas HCT116 cells were cultured in McCoy’s medium. Both media were supplemented with 10% heat-inactivated fetal calf serum, 10 mM glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (all from PAA Laboratories, Linz, Austria). The polyclonal goat antibody directed against caspase-3, the polyclonal caspase-8 antibody, and the monoclonal caspase-8 antibody were from R&D Systems (Wiesbaden, Germany), Santa Cruz Biotechnology, and BioCheck (Münster, Germany), respectively. The monoclonal actin antibody, cyclineximide (Chx), and the protease inhibitors phenylmethylsulfonyl fluoride, aprotinin, leupeptin, and pepstatin were from Sigma (Deisenhofen, Germany). The monoclonal antibodies against FADD and XIAP were purchased from BD Biosciences (Heidelberg, Germany). From Biomol (Hamburg, Germany) were the fluorogenic substrates DEVD-AMC (N-acetyl-Asp-Glu-Val-Asp-aminoethylcoumarin) for caspase-3, Suc-LLVY-AMC (N-succinyl-Leu-Leu-Val-Tyr-AMC) for 20S proteasomal activity, and Z-LRGG-AMC (carbobenzoxy-Leu-Gly-Gly-AMC) for ubiquitin C-terminal hydrolases, as well as the protease inhibitors MG-132, clasto-lactacystin (CLC), calpain inhibitor I (ALLN), and calcium/calmodulin inactivator II (ALLM). Human recombinant TNF with a specific activity of 10^9 U/mg of protein was obtained from Knoll AG (Ludwigshafen, Germany). The monoclonal c-FLIP antibody (NF6) and the His-tagged Killer-TRAIL, preparation were from Alexis (Lausen, Switzerland). If not otherwise indicated, all assays were performed in the presence of cycloheximide (10 μg/ml).

**Construction of small interfering RNAs and stable transfection.** For the suppression of c-FLIP and XIAP expression, siRNAs were designed using the Invitrogen siDESIGN Center. The selected sense sequences were 5′-TGTCTCAAGGAGCA-3′ for c-FLIP and 5′-AATAGTGCACACGACATCTA-3′ for XIAP. Complementary oligonucleotides consisting of sense, hairpin loop, and antisense sequences were annealed and ligated into the plasmid pSilencer siRNA expression vector according to the manufacturer’s instructions (Ambion, United Kingdom). HeLa D98 cells were stably transected by electroporation using the Bio-Rad gene pulsar (500 μA, 160 V). After hygromycin selection, several clones were obtained and the successful reduction of c-FLIP and XIAP expression was controlled by Western blot analysis.

**Preparation of cell extracts and Western blotting.** Cell extracts were prepared as described previously (26). Briefly, cells were lysed for 30 min at 4°C in lysis buffer A containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM dithiothreitol (DTT), 1% NP-40, and a protease inhibitor cocktail. After removing nuclei and cell debris by centrifugation (10,000 g, 4°C, 1 h), the resulting lysates were incubated for 1 h at 4°C, the resulting lysates were incubated for 1 h at 4°C, the protein concentration was determined by the Bio-Rad protein assay, and 10 μg of protein was loaded per lane. Proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Amersham, Braunschweig, Germany). Following incubation with the various antibodies, the proteins were visualized by enhanced chemiluminescent staining using ECL reagents (Amersham).

**Measurement of cell death.** Cell death determinations were done with the standard TNF cytotoxicity assay (crystal violet assay) that is based on the staining of viable cells (24). Briefly, cells (2 × 10^5) were seeded into 96-well microtiter plates in 100 μl culture medium. Cells were incubated with the death stimuli for the indicated times at 37°C, and viable cells were stained with 20% methanol containing 0.5% crystal violet and solubilized in 33% acetic acid. The absorbance was measured at an optical density of 590 nm (A_{590}). Percent specific cell death was defined as 100 – (A_{590} of test well × 100/A_{590} of untreated well). Each experiment was performed independently at least three times, and an individual experiment was carried out in triplicate. The percent inhibition of cell death by MG-132 (see Fig. 5 and 8) is defined as 100 – (percent specific cell death with MG-132)/percent specific cell death without MG-132 × 100. Please note that negative numbers indicate an increased cell death rate due to the cotreatment with MG-132.

**Fluorometric substrate assays.** Caspase-3 activities were expressed as arbitrary units that were determined by the incubation of cell extracts for 2 h with 50 μM of the fluorogenic caspase-3 substrate DEVD-AMC in 200 μl buffer containing 50 mM HEPES (pH 7.4), 100 mM NaCl, 0.1% CHAPS, 5 μM calpain inhibitor II (ALLN), and 10 mM DTT. The release of aminomethylcoumarin was measured by fluorometry by an excitation wavelength of 360 nm and an emission wavelength of 475 nm. For the determination of proteasomal and ubiquitin hydrolyse activity, cells were broken in lysis buffer B containing 30 mM Tris-HCl (pH 7.6), 2 mM MgCl2, 1 mM DTT, 0.1 mM EDTA, and 0.2% Triton X-100 by four freeze-thaw cycles. Following centrifugation (10,000 × g) at 4°C, the resulting lysates were incubated for 1 h with 100 μM of the fluorogenic substrates LLVY-AMC and Z-Arg-AMC in 200 μl assay buffer containing 30 mM Tris-HCl (pH 7.6), 10 mM KCl, 10 mM NaCl, 2 MgCl2, and 0.5 mM DTT. The release of aminomethylcoumarin was measured by fluorometry by an excitation wavelength of 360 nm and an emission wavelength of 475 nm. To ensure that the observed activity was indeed...
proteasome derived, the lysates that were analyzed were incubated for 30 min with the fluorogenic LLVY-AMC substrate in the presence of 10 μM MG-132.

Immunoprecipitation. Immunoprecipitation reactions were performed as described previously (51). Briefly, cell extracts were prepared from 1 × 10⁷ cells in 500 μl lysis buffer without DTT. For the precipitation, 1 μg polyclonal caspase-8 antibody and 30 μl protein G-Sepharose (Sigma) were added to the extracts and rotated for 4 h or overnight at 4°C. The Sepharose beads were extensively washed in lysis buffer and then analyzed by Western blotting.

RESULTS

Proteasome inhibition blocks TNF-induced caspase activation and apoptosis. In an attempt to better understand the mode of action of PIs on death receptor-induced apoptosis, we treated two HeLa cell lines that display different apoptosis susceptibilities toward death receptor ligands (24) with increasing concentrations of TNF in the absence or presence of the peptide aldehyde inhibitor MG-132. Surprisingly, we found that MG-132 protected both cell lines very efficiently from TNF-induced apoptosis even when TNF concentrations as high as 100 ng/ml were used (Fig. 1A). This effect not only was determined using the crystal violet assay (Fig. 1A) but also was visualized by micrographs taken from HeLa D98 cells that were incubated for 2.5 or 5 h with TNF in the absence or presence of MG-132 (Fig. 1D). MG-132-mediated protection was caused by the inhibition of caspases, as processing and activity of both caspase-8 and -3 were significantly delayed by this compound in both cell lines (Fig. 1B and C). Although this was clearly evidenced by the diminished generation of active caspase-8 and caspase-3 fragments, the inhibitory effect of MG-132 was most obvious with regard to the unprocessed proforms of both caspases. Depending on the apoptosis susceptibility of the cell line, TNF treatment resulted either in an almost complete loss of both caspase proforms, as evidenced in TNF-sensitive HeLa D98 cells (Fig. 1B, left panel), or in an at least significant reduction of the proforms, as was observed in the more-resistant H21 cell line (Fig. 1B, right panel). In the presence of MG-132, however, TNF induced only a marginal decrease of both proforms in both cell lines, clearly demonstrating that the inhibition of the proteasome blocks TNF-induced caspase processing. Note that protection by MG-132 was much more pronounced in the resistant HeLa H21 cells as the generation of active caspase fragments became almost completely inhibited even after a 20-h incubation with TNF.
These results are consistent with a marked delay in caspase-8 (IETDase) (data not shown) and caspase-3 (DEVDase) (Fig. 1C) activities that were observed in both cell lines and also with the cleavage of α-fodrin into the caspase-3-dependent 120-kDa fragment (25) that was accordingly delayed when the cells were treated with TNF in the presence of MG-132 (data not shown). Interestingly, the incubation of the cells with and without MG-132 in the absence of TNF for up to 20 h did not reveal any significant differences in the amount of procaspase-8 and -3 (data not shown), suggesting that these procaspases are not direct targets of the proteasome. In contrast, after the initial delay in caspase activation, we even observed enhanced caspase-8 and caspase-3 (Fig. 1C) cleavage activities when the cells were treated with TNF in the presence of MG-132. Thus, in contrast to the procaspases, active caspases are degraded by the proteasome, which so far was shown for only caspase-3 (53).

Inhibition of the proteasome transiently blocks TNF-induced apoptosis in a dose- and time-dependent manner. The activity of the peptide aldehyde inhibitor MG-132 appears to be not exclusively specific for the proteasome (9). Therefore, we also performed the experiments in the presence of various other compounds, such as ALLN and ALLM, that are effective and weak inhibitors of the proteasome, respectively, as well as with CLC, which is among the most selective PIs known (9). Except for ALLM, which did not interfere with TNF signaling at any concentration tested, all the other compounds reproducibly blocked TNF-induced apoptosis of both HeLa cell lines in a dose- and time-dependent manner (Fig. 2A and B). The protective effect was of a transient nature, as all of the various PIs efficiently delayed TNF-induced apoptosis of HeLa D98 cells up to 6 h, but they could not protect these cells at later time points (Fig. 2B, left panel). This is consistent with the caspase activation profiles shown in Fig. 1C. On the other hand, more than 80% of HeLa H21 cells survived even a 20-h TNF treatment in the presence of either of these compounds (Fig. 2B, right panel). However, these cells also eventually succumbed to apoptosis, even in the presence of PIs, when they were treated with TNF for up to 36 h (data not shown). Together, these data not only suggest that the protective effect of these inhibitors is indeed caused by their ability to block the 26S proteasome but also demonstrate that the efficiency of this effect critically depends on the strength of the individual apoptotic response. Furthermore, and more intriguingly, they indicate that proteasomal activity might be required for the efficient initiation of the death receptor pathway.

To further analyze the conditions required for a maximal protective effect, we have also investigated the necessity to preincubate the cells with these PIs. Relative to a 1-h preincubation time, the simultaneous addition of the proteasome inhibitors, together with TNF, resulted in a weaker protection, whereas preincubation for more than 1 h did not further increase their protective effect. In contrast, no protection was observed when the PIs were added 1 h or 2 h after TNF treatment (data not shown). Thus, the preincubation of HeLa D98 cells with either MG-132 or CLC for 1 h before the addition of TNF was both necessary and sufficient to achieve optimal protection against the cytotoxic activity of this cytokine (Fig. 2C). Hence, subsequent experiments were conducted following a 1-h preincubation period with the PIs.

Inhibition of the proteasome also blocks TRAIL- and anti-CD95-induced apoptosis and caspase activation. Next we investigated whether the observed effect is restricted to the TNF system or whether PIs would generally protect cells from death.

FIG. 2. Dose- and time-dependent protection from TNF-induced apoptosis specifically by proteasomal inhibitors. (A) HeLa D98 and HeLa H21 cells were preincubated with or without (no inhibitor) the indicated concentrations of MG-132, CLC, calpain inhibitor I (ALLN), and the calpain inhibitor II (ALLM), which does not interfere with proteasomal activity before the addition of TNF (10 ng/ml) and Chx. Cell death was assessed after 4 h (HeLa D98) and 8 h (HeLa H21) by the crystal violet assay. (B) HeLa D98 and HeLa H21 cells were preincubated with or without (no inhibitor) MG-132 (10 μM), CLC (10 μM), ALLN (50 μM), or ALLM (50 μM), followed by the addition of TNF (10 ng/ml) and Chx. After the indicated times, cell death was assessed by the crystal violet assay. (C) Before the treatment with TNF (10 ng/ml) and Chx, HeLa D98 cells were preincubated for the indicated times with MG-132 (10 μM), CLC (10 μM), or ALLM (50 μM). After the indicated times, cell death was assessed by the crystal violet assay. One representative experiment out of four performed in triplicate is shown.
receptor-induced apoptosis. To this end, we analyzed TRAIL and CD95 sensitivities of HeLa D98 cells that either were left untreated or were preincubated for 1 h with MG-132. Similar to the results obtained so far with TNF, inhibition of the proteasome by MG-132 also transiently protected the cells against the cytotoxic activities of these two death receptor ligands (Fig. 3A). However, the protection was clearly more pronounced in the CD95 signaling pathway, which induces a more pronounced in the CD95 signaling pathway, which induces a more pronounced apoptotic response in these cells than TRAIL. This is reminiscent of the scenario observed with the less sensitive HeLa H21 cells, which are also more efficiently protected from TNF-induced apoptosis by PIs than are the TNF-sensitive HeLa D98 cells. Nevertheless, depending on the dose and time that the two death receptor ligands were applied, the cells also eventually succumbed to apoptosis in the presence of MG-132 (Fig. 3A and data not shown). Similar to TNF-induced apoptosis, MG-132-mediated protection from TRAIL- and anti-CD95-induced killing correlated closely with a significant delay of processing caspase-8 and caspase-3 (Fig. 3B) and the corresponding activities of these caspases (Fig. 3C and data not shown). In addition, MG-132 also significantly delayed TRAIL-induced apoptosis and caspases-8 and -3 activation in HeLa D98 cells in the absence of cycloheximide (data not shown), indicating that the PI-mediated protection also occurs under more physiological conditions, which might be especially relevant for TRAIL-mediated signaling in vivo.

**Transient inhibition of death receptor-induced apoptosis and caspase activation by PIs is a common event observed in several cell lines.** Next we analyzed whether our findings can also be applied to other experimental cell systems. To this end, we compared caspase-3 (DEVD) activities in several tumor cell lines of various origins that were treated with TNF in the absence or presence of MG-132. Similar to the data obtained with HeLa D98 cells, the inhibition of the proteasome by MG-132 also transiently blocked TNF-induced caspase-3 activation of MCF-7/casp-3 breast carcinoma cells, KB cervical carcinoma cells, and HCT116 colon carcinoma cells (Fig. 4A). Consistent with the transient nature of this effect, we also found that MG-132 efficiently protected the various tumor cell lines from death receptor-induced apoptosis in a short-term (4 h to 8 h) assay but failed to do so following a longer (24 h) stimulation time (Fig. 4B). Similar results were obtained with the lung carcinoma cell line A549, albeit not to the same extent (data not shown). Although MCF-7/casp-3 cells that do not express the CD95 receptor were treated with only TNF and TRAIL, our data clearly show the general protective impact of PIs on death receptor-induced apoptosis.

**Proteasomal activity correlates with apoptosis susceptibility.** Our experiments performed so far indicate that the proteasome might be required for the initiation of the apoptotic cascade induced by death receptors. Thus, diminished proteasomal activity might be one mechanism contributing to the apoptosis-resistance phenotype of HeLa H21 cells. To investigate this hypothesis, we compared proteasomal activities in cellular extracts of untreated and TNF-stimulated HeLa D98 and H21 cells over a period of 20 h by determining their capabilities to cleave the fluorogenic peptide LLVY-AMC that represents a substrate for the 20S proteasome. Indeed, extracts of untreated HeLa D98 cells reproducibly displayed a proteasomal activity that was on average approximately 1.5-fold higher than that observed in extracts of untreated HeLa H21 cells, a difference that was even further enhanced when the cells were stimulated with TNF (Fig. 5A, left panel). When the cell extracts were analyzed in the presence of MG-132, no LLVY-AMC cleavage could be observed, demonstrating that the cleavage was mediated by the proteasome (data not shown). In contrast, regardless of whether the cells were stimulated with TNF, both cell lines displayed similar ubiquitin C-terminal hydrolase activities, as determined by cleavage of the fluorogenic substrate LRGG-AMC (Fig. 5A, right panel). These results show that apoptosis-sensitive HeLa D98 cells exhibit a more constitutive proteasomal activity than HeLa H21 cells that are less sensitive toward death receptor-induced cell death. Although the reasons for the differential proteasomal
activities in these two cell lines are unknown, this finding is in agreement with our data, demonstrating an important role for the proteasome in the initiation of the death receptor program.

In the search for the cellular target(s) that is responsible for the observed PI-mediated resistance toward death receptor-induced apoptosis, we analyzed the expression of several cell cycle and/or apoptosis regulatory proteins. As expected, MG-132 prevented the TNF-induced degradation of the proteasomal targets p21, p27, and cyclin A (41) but had no influence on unrelated proteins, such as RIP and FADD (data not shown). For our study, however, we analyzed the status of XIAP and c-FLIP in more detail because they represent two proteasomal targets that are well-known antiapoptotic proteins which are interfering with the initial stages of caspase activation (12, 44, 47, 63). In addition to their degradation by the proteasome, both proteins are also specifically proteolysed during death receptor-induced apoptosis by caspase-3 and caspase-8, respectively (5, 33). Interestingly, although TNF treatment resulted in comparable amounts of caspase-generated XIAP fragments in both cell lines, a significant reduction of full-length XIAP protein was observed only in HeLa D98 cells (Fig. 5B, left panel). In contrast, the levels of uncleaved XIAP remained almost unchanged during apoptosis of HeLa H21 cells (Fig. 5B, right panel), a finding that is consistent with their lower proteasomal activity (Fig. 5A). Similar results were obtained when we analyzed the status of c-FLIP during apoptosis in these cells. In both cell lines, treatment with TNF induced a rapid proteasome-mediated degradation of c-FLIP-short that was efficiently prevented in the presence of MG-132 (Fig. 5B). In addition to being a proteasomal target, c-FLIP-long was also cleaved within 1 h in both cell lines by DISC-bound caspase-8 generating a p43 fragment. Also, this process was inhibited by MG-132 much more efficiently in HeLa H21 cells, as uncleaved c-FLIP-long remained detectable in these cells up to 8 h, whereas it was cleaved in HeLa D98 cells even in the presence of MG-132 following a 3-h TNF treatment (Fig. 5B). As it is known that c-FLIP-long is only cleaved by caspase-8 when both proteins heterodimerize at the DISC (33, 48), this finding strongly indicates substantially reduced caspase-8 activation at the DISC in both cell lines treated with TNF in the presence of MG-132. Other antiapoptotic proteins, including IAP1, IAP2, and survivin, were not differentially expressed in the two HeLa cell lines, nor were they affected by the individual treatments (TNF in the absence or presence of various PIs) (Fig. 5C) as was observed for XIAP and c-FLIP (Fig. 5B). Together, these data strongly support our hypothesis that the proteasome is an important component required for the rapid initiation of death.
receptor-induced cell death via the degradation of antiapoptotic proteins, such as XIAP and c-FLIP.

Inhibition of the proteasome fails to protect c-FLIP knockdown cells from death receptor-induced apoptosis. If the protective effect of PIs is mediated at least partially by the stabilization of XIAP protein levels. Our finding that suppression of XIAP expression abrogated the protective effect of MG-132 was further verified by Western blot analyses demonstrating that MG-132 was not capable of preventing TNF-induced caspase processing in XIAP siRNA clones as efficiently as in parental HeLa D98 cells (Fig. 7). Whereas the decrease of both caspase-8 and caspase-3 proforms, as well as the generation of the active caspase fragments, was strongly inhibited by MG-132 in D98 cells, no such effect was observed in similarly treated XIAP siRNA clones. Hence, our results suggest that the observed PI-mediated protection against death receptor-induced apoptosis is brought about at least partially by stabilizing XIAP protein levels.

Inhibition of the proteasome fails to protect XIAP knockdown cells from death receptor-induced apoptosis. With regard to the role of c-FLIP in this process, we observed that MG-132 prevented TNF-induced cleavage of c-FLIP-long in both cell lines (Fig. 5B). As c-FLIP-long is predominantly cleaved by DISC-bound caspase-8, this result suggested that PIs prevent the recruitment of this initiator caspase into the DISC. This hypothesis would also be consistent with our findings that treatment with MG-132 not only led to a massive accumulation of c-FLIP-short (that actively prevents caspase-8 processing and activation (Fig. 1 and 3). To verify this scenario, we analyzed caspase-8 immunoprecipitates of HeLa D98 cells that were treated with or without MG-132 for up to 3 h with TNF for the presence of both forms of c-FLIP. Western blot analysis of these precipitates not only verified our previous findings that MG-132 efficiently prevents activation of caspase-8 but also confirmed a successful and efficient caspase-8 precipitation (Fig. 8A). More importantly, however, whereas the adaptor molecule FADD as well as the cleaved p43 fragment of c-FLIP-long was coprecipitated with caspase-8 regardless of whether the TNF treatment occurred in the presence of MG-132, c-FLIP-short was only found in these immunoprecipitates when MG-132 was present during TNF stimulation (Fig. 8B). As both c-FLIP isoforms bind to caspase-8 only within the DISC via interaction with FADD (23), these findings imply that the levels of both c-FLIP isoforms found in the caspase-8 precipitates are constituents of the TNF-R signaling complex.
II (39) and thus explain how MG-132 is able to inhibit caspase-8 activation. MG-132-mediated inhibition of caspase-8 recruitment (due to stabilization and accumulation of c-FLIP-short at the DISC) was also observed with regard to the CD95 DISC or when clasto-lactacystin was used to block TNF-induced cell death (data not shown).

To unambiguously determine the role of c-FLIP in the PI-mediated protection from death receptor-induced apoptosis, we finally generated HeLa D98 transfectants in which c-FLIP expression is suppressed due to the stable expression of a c-FLIP siRNA. As both c-FLIP forms are expressed only very weakly in HeLa D98 cells but accumulate significantly following proteasome inhibition, we analyzed the efficiency of the siRNA-mediated c-FLIP knockdown in the absence and presence of MG-132. As shown in Fig. 9A, the expression of both c-FLIP-long (upper panel) and c-FLIP-short (lower panel) was almost completely suppressed in all three clones examined, whereas a representative D98 clone that was transfected with the empty vector displayed c-FLIP levels comparable to those observed in wild-type D98 cells. All clones, including the vector cells, displayed no obvious alterations in death receptor surface expression (data not shown). Similar to the results obtained with the XIAP siRNA clones (Fig. 6), MG-132 protected only the parental HeLa D98 and vector control cells from death receptor-induced apoptosis but completely failed to do so with regard to the c-FLIP siRNA clones (Fig. 9B through D). In fact, MG-132 treatment even further accelerated and enhanced TNF- and TRAIL-induced apoptosis of the three c-FLIP siRNA clones (Fig. 9B and C). This intriguing finding was substantiated even further when we analyzed TNF-induced caspase processing in these clones. Whereas process-

FIG. 6. Inhibition of the proteasome does not protect XIAP knockdown cells from death receptor-induced apoptosis. (A) HeLa D98 cells (wild type [WT]) and four HeLa D98 clones stably transfected with the XIAP siRNA construct were analyzed for XIAP expression. As a control, the blot was reprobed with an actin antibody. (B through D) Following preincubation for 1 h with or without MG-132 (10 μM), HeLa D98 cells and the four XIAP siRNA clones were incubated with 100 ng/ml TNF (B), 30 ng/ml TRAIL (C), or 300 ng/ml of the antagonistic CD95 antibody (D) in the presence of Chx. Cell death was assessed with the crystal violet assay after 4 h (B and C) or 8 h (D). The graphs show percent inhibition of cell death achieved in the presence of MG-132. One representative experiment out of six performed in triplicate is shown.

FIG. 7. Proteasome inhibition does not prevent TNF-induced caspase activation in XIAP knockdown cells. Following a 1-h preincubation with or without MG-132 (10 μM), HeLa D98 cells (wild type [WT]), and the four XIAP siRNA clones either were left untreated or were incubated with 10 ng/ml TNF and Chx. After 3 h, cell extracts were prepared and analyzed for the status of XIAP expression and for processing of caspases-8 and -3. The actin blot serves as a loading control. +, with indicated substance.
ing and thereby activation of caspase-8 and caspase-3 were significantly blocked when HeLa D98 cells were treated with TNF in the presence of MG-132, both caspases were processed much more efficiently and with an accelerated kinetic in similarly treated c-FLIP siRNA clones (Fig. 10). Similar results were obtained when the c-FLIP siRNA clones were treated with TRAIL (data not shown). Interestingly, compared to parental HeLa D98 cells, TNF-induced caspase processing was slightly delayed in the three c-FLIP siRNA clones, which might be explained by a recently proposed hypothesis that c-FLIP-long is required for caspase-8 activation at the DISC (3). Collectively, our data clearly demonstrate that proteasomal inhibitors transiently protect cells from death receptor-induced apoptosis and that they exert this protective effect via stabilization of XIAP and c-FLIP.

DISCUSSION

Two complicated proteolytic systems are involved in the control of cell death: the caspase family of cysteine proteases and the ubiquitin-proteasome degradation system. Whereas the function of caspases is well defined, the exact role of the ubiquitin-proteasome system in apoptosis is far from being elucidated. Several studies showed that the inhibition of the proteasome blocked apoptosis in various settings (18, 20, 46), whereas others demonstrated efficient induction of apoptosis when cells were treated with PIs either alone or in combination with various death-inducing stimuli (8, 11, 19, 30, 60). As the antiapoptotic activities of PIs were described for resting thymocytes and differentiated neuronal cells and the proapoptotic effects were described for various tumor cell lines, it was postulated that the requirement of proteasomal activity for the

FIG. 8. TNF induces the association of c-FLIP-short with caspase-8 and FADD only in the presence of MG-132. Following a 1-h preincubation with or without MG-132 (10 μM), HeLa D98 cells either were left untreated or were treated with TNF (10 ng/ml) and Chx. After the indicated times, cell extracts were prepared and subjected to immunoprecipitation (IP) by using a polyclonal caspase-8 antibody. To verify an efficient immunoprecipitation reaction, Western blot (WB) analysis was performed with a monoclonal caspase-8 antibody (A), whereas the immunoprecipitates were analyzed for the presence of c-FLIP-short, the cleaved p43 fragment of c-FLIP-long, and FADD (B). +, with indicated substance. Asterisks indicate the light chain of the antibody used for precipitation.

FIG. 9. Inhibition of the proteasome does not protect c-FLIP knockdown cells from death receptor-induced apoptosis. (A) HeLa D98 cells and HeLa D98 clones stably transfected with an empty vector or with the c-FLIP siRNA construct either were left untreated or were incubated for 2 h with 10 μM MG-132 alone and analyzed for c-FLIP-long and c-FLIP-short expression. +, with indicated substance. The upper band marked with an asterisk served as a loading control. (B through D) Following a 1-h preincubation with or without MG-132 (10 μM), HeLa D98 cells and HeLa D98 clones stably transfected with an empty vector or with the c-FLIP siRNA construct were incubated for 4 h with 100 ng/ml TNF (B) or 30 ng/ml TRAIL (C) or for 8 h with 300 ng/ml of the antagonistic CD95 antibody in the presence of Chx (D). Cell death was assessed with the crystal violet assay. The graphs show percent inhibition of cell death achieved in the presence of MG-132. One representative experiment out of four performed in triplicate is shown.
activation of caspase-8, the most apical initiator caspase in caspase-3 but also with a marked delay in the processing and correlated well not only with the inhibition of the executioner TNF-, TRAIL- or CD95-induced apoptosis. This protection cathepsin inhibitor ALLM efficiently protected cells against demonstrated that specifically proteasomal inhibitors, such as based tumor therapy.

progression or inhibition of apoptosis critically depends on the proliferative status of the cells (9, 41). In an alternative model, it was suggested that the PI concentrations used determine whether such peptide aldehyde inhibitors function in a pro- or antiapoptotic manner (34). Here, however, we describe an antiapoptotic role of PIs in several proliferating tumor cells that was observed not only at low but also at high PI concentrations. In addition, although at first glance our study appears to be controversial to many other reports, it provides strong evidence that both the pro- and antiapoptotic functions of the proteasome have to be taken into account for efficient PI-based tumor therapy.

Using death receptor-induced apoptosis as a model system, we demonstrated that specifically proteasomal inhibitors, such as elasto-lacteasyn, MG-132, or ALN, but not the calpain and cathepsin inhibitor ALLM efficiently protected cells against TNF-, TRAIL- or CD95-induced apoptosis. This protection correlated well not only with the inhibition of the executioner caspase-3 but also with a marked delay in the processing and activation of caspase-8, the most apical initiator caspase in death receptor signaling. Hence, our results suggest that the PI-mediated protection is caused by inhibition of an early initiation event that most likely involves the stabilization of pre-existing antiapoptotic proteins, such as c-FLIP and XIAP, which are both known to efficiently counteract the initial stages of caspase activation (29, 47). Indeed, both proteins were stabilized by PIs in the two cell lines used. In addition, c-FLIP-short was found to be associated with the DISC only in the presence of PIs, clearly explaining the lack and delay of caspase-8 processing under these conditions. Finally, siRNA-mediated knockdown of either c-FLIP or XIAP expression completely abrogated the protective function of PIs and, in some instances, even reversed this effect, resulting in an increased apoptosis rate. Thus, from these results, it is obvious that PIs exert their antiapoptotic effect via the stabilization of XIAP and c-FLIP. This result is especially important with regard to the fact that many human tumors express high levels of these antiapoptotic proteins, rendering them rather resistant toward apoptosis induction by various agents (21, 37, 38, 47, 55). Based on our results, it is, however, also unlikely that a combined treatment of death receptor ligands (or perhaps drugs; see below) with PIs would result in a more beneficial outcome, as such a treatment might even cause antagonistic effects due to the PI-mediated prolonged survival of these tumors. For an efficient therapy, it is therefore important to thoroughly analyze every individual tumor for the expression levels of XIAP and c-FLIP as well as for the status of other antiapoptotic proteins, such as Bcl-2, that are known to be targets of the proteasome (7, 12, 44, 63). In addition, it is inevitable to also carefully evaluate the biochemical pathways involved, as PIs were recently shown to also inhibit apoptosis induced by retinoic acid, geldanamycin, and staurosporine and even the combined treatment of melanoma cells with TNF and PIs did not show the expected synergism of action (2, 43, 52, 62). The underlying mechanisms causing the resistance to these drugs remained, however, unknown.

Of note, however, is also our observation that although PI treatment significantly delayed caspase activation and cell death of various tumor cell lines, it did not result in a permanent protection from death receptor-induced apoptosis. In fact, when the initial PI-mediated roadblock of caspase processing was eventually overcome, active caspases accumulated to an even larger extent in cells treated with the individual death receptor ligands in the presence rather than in the absence of PIs. Such a scenario was found not only in cells that have been exposed to this treatment for longer terms but also in cells with suppressed c-FLIP or XIAP expressions. These observations clearly demonstrate a biphasic role for the proteasome in death receptor-induced apoptosis in which at first its activity is absolutely essential for the initiation phase by eliminating various roadblocks, including c-FLIP and XIAP. During later stages, however, the proteasome acts in an antiapoptotic manner, as it also promotes the degradation of proapoptotic proteins such as caspases. The proposed biphasic role of the proteasome would also imply that the outcome of a PI-based tumor therapy depends not only on the expression levels of antiapoptotic proteins but also on the time point at which the PIs are applied. With regard to this, we found that the protection was most pronounced when the cells were exposed to MG-132 1 h prior to the death stimulus, whereas the
and/or activity of antiapoptotic proteins (13). With various apoptosis-inducing drugs or death receptor ligands, when a PI-based tumor treatment is considered in combination with the proteasome. Hence, our data should be taken into serious account as the proteasome plays an important biphasic role in apoptosis. At proteolytic activity is essential for every proliferating cell, these tumors adapted to the low proteasomal activity by switching to another newly identified proteolytic system, the tripeptidyl peptidase II (15). Due to the hereby altered specificity of cytosolic proteolysis, however, these tumors were unable to efficiently degrade the various IAPs, including XIAP, resulting in rapidly growing tumors in vivo (22).

In summary, our data clearly support a model in which the proteasome plays an important biphasic role in apoptosis. At first, it is essential for the initiation of the cell death program by degrading antiapoptotic proteins but it acts at later stages in an antiapoptotic manner as it also proteolyses proapoptotic proteins. Hence, our data should be taken into serious account when a PI-based tumor treatment is considered in combination with various apoptosis-inducing drugs or death receptor ligands. However, they also encourage attempts to combine such treatments with small molecule inhibitors targeting expression and/or activity of antiapoptotic proteins (13).

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REFERENCES

1. Ashkenazi, A., R. C. Pai, S. Fong, S. Leung, D. A. Lawrence, S. A. Marsters, C. Blackie, L. Chang, A. E. McMurray, A. Hebert, L. DeForge, I. L. Kounenis, D. Lewis, L. Harris, J. Busiere, H. Koeppen, Z. Shahrokh, and R. H. Schwall. 1999. Safety and antitumor activity of recombinant soluble Apo2 ligand. J. Clin. Investig. 104:155–162.
2. Brophy, V. A., J. M. Tavare, and A. J. Rivett. 2002. Treatment of COS-7 cells with protease inhibitors or γ-interferon reduces the increase in caspase 3 activity associated with staurosporine-induced apoptosis. Arch. Biochem. Biophys. 397:199–205.
3. Chang, D. W., Z. Xing, Y. Pan, A. Algeciras-Schimnich, B. C. Barnhart, S. Yaish-Olad, M. E. Peter, and X. Yang. 2002. c-FLIP(L) is a dual function regulator for caspase-8 activation and CD95-mediated apoptosis. EMBO J. 21:3704–3714.
4. Dekterev, A., M. Boyce, and J. Yuan. 2003. A decade of caspases. Oncogene 22:8543–8567.
5. Deveraux, Q. L., E. Leo, H. R. Stennicke, K. Welsch, G. S. Salvesen, and J. C. Reed. 1999. Cleavage of human inhibitor of apoptosis protein XIAP results in fragments with different specificities for caspases. EMBO J. 18:5242–5251.
6. Deveraux, Q. L., and J. C. Reed. 1999. IAP family proteins—suppressors of apoptosis. Genes Dev. 13:239–252.
7. Dimmeler, S., K. Breitschopf, J. Haendeler, and A. M. Zeiher. 1999. De-phosphorylation targets Bcl-2 for ubiquitin-dependent degradation: a link between the apoptosis and the proteasome pathway. J. Exp. Med. 189:1815–1822.
8. Drexler, H. C. 1997. Activation of the cell death program by inhibition of protease function. Proc. Natl. Acad. Sci. USA 94:855–860.
9. Drexler, H. C. 1996. Programmed cell death and the proteasome. Apoptosis 1:3–7.
10. Fischer, U., R. U. Jänicke, and K. Schulze-Osthoff. 2003. Many cuts to ruin: a comprehensive update of caspase substrates. Cell Death Differ. 10:76–100.
11. Fujita, E., T. Mukasa, T. Tsukahara, K. Arakata, S. Omura, and T. Monno. 1996. Enrichment of TNFα-like activity in the TNF-treated U937 cells by the proteasome inhibitors. Biochem. Biophys. Res. Commun. 224:74–79.
12. Fukazawa, T., T. Fujimura, F. Uno, F. Teraishi, Y. Kadowaki, T. I. Yoshima, Y. Takata, S. Kagawa, J. A. Roth, J. Tschopp, and N. Tanaka. 2001. Accelerated degradation of cellular FLIP protein through the ubiquitin-proteasome pathway in p53-mediated apoptosis of human cancer cells. Oncogene 20:5225–5231.
13. Garber, K. 2005. New apoptosis drugs face critical test. Nat. Biotechnol. 23:409–411.
14. Gavioli, R., T. Frisian, S. Vertuani, G. W. Bornkamm, and M. G. Masucci. 2001. c-myc overexpression activates alternative pathways for intracellular proteolysis in lymphoma cells. Nat. Cell. Biol. 3:283–288.
15. Glas, R., M. Bogos, J. S. McMaster, M. Gazdzynska, and H. L. Ploegh. 1998. A proteolytic system that compensates for loss of protease function. Nature 392:618–622.
16. Glimia, B., and T. Le. 1999. Tumor necrosis factor-related apoptosis-inducing ligand’s antitumor activity in vivo is enhanced by the chemotherapeutic agent CPT-11. Cancer Res. 59:653–658.
17. Green, D. R. 2000. Apoptotic pathways: paper wraps stone blunts scissors. Cell 102:1–4.
18. Grimm, L. M., A. L. Goldberg, G. P. Goirier, L. M. Schwartz, and B. A. Osborne. 1996. Proteasomes play an essential role in thymocyte apoptosis. EMBO J. 15:3835–3844.
19. He, Q., Y. Huang, and M. S. Sheikh. 2004. Proteasome inhibitor MG132 upregulates death receptor 5 and cooperates with Apo2L/TRAIL, to induce apoptosis in BxPC-1 pancreatic and −deficient cells. Oncogene 23:2554–2558.
20. Hirsh, T., B. Dallaporta, N. Zamzami, S. A. Susin, L. Ravagnan, I. Marzo, C. Brenner, and G. Kroemer. 1998. Proteasome activation occurs at an early, premitochondrial step of thymocyte apoptosis. J. Immunol. 161:35–40.
21. Hofmann, H. S., A. Simm, A. Hammer, R. E. Silber, and B. Bartling. 2002. Expression of inhibitors of apoptosis (IAP) proteins in non-small cell human lung cancer. J. Cancer Res. Clin. Oncol. 128:554–560.
22. Hong, X., L. Lei, and R. Glas. 2003. Tumors acquire inhibitor of apoptosis protein (IAP)-mediated apoptosis resistance through altered specificity of the proteasome. J. Biol. Chem. 278:1731–1743.
23. Irmr, M., M. Thome, M. Hahne, P. Schneider, K. Hofmann, V. Steiner, J. L. Bodmer, M. Schrotter, K. Burns, C. Mattmann, D. Rimoldi, L. E. French, and J. Tschopp. 1997. Inhibition of death receptor signals by cellular FLIP. J. Biol. Chem. 272:1901–1906.
24. Jänicke, R. U., F. H. Lee, and A. G. Porter. 1994. Nuclear c-Myc plays an important role in the cytositotoxicity of tumor necrosis factor alpha in tumor cells. Mol. Cell. Biol. 14:5661–5670.
25. Jänicke, R. U., P. Ng, M. L. Sprengart, and A. G. Porter. 1998. Caspase-3 is required for alpha-fodrin cleavage but dispensable for cleavage of other death substrates in apoptosis. J. Biol. Chem. 273:15540–15545.
26. Jänicke, R. U., M. L. Sprengart, M. R. Waiti, and A. G. Porter. 1998. Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. J. Biol. Chem. 273:9357–9360.
27. Jesenberger, V., and S. Jentsch. 2002. Deadly encounter: ubiquitin meets apoptosis. Nat. Rev. Mol. Cell Biol. 3:112–121.
28. Jiang, X., and X. Wang. 2004. Cytochrome C-mediated apoptosis. Annu. Rev. Biochem. 73:87–106.
29. Kataoka, T. 2005. The caspase-8 modulator c-FLIP. Crit. Rev. Immunol. 25:31–58.
30. Kim, S., K. Choi, D. Kwon, E. N. Benveniste, and C. Choi. 2004. Ubiquitinin-proteasome pathway as a primary defender against TRAIL-mediated cell death. Cell. Mol. Life Sci. 61:1075–1081.
31. Krammer, P. H. 2000. CD95’s deadly mission in the immune system. Nature 407:789–795.
32. Kroemer, G., and J. C. Reed. 2000. Mitochondrial control of cell death. Nat. Rev. Mol. Cell Biol. 1:513–519.
33. Krueger, A., I. Schmitz, S. Baumann, P. H. Krammer, and S. Kirchhoff. 2001. Cellular FLICE-inhibitory protein splice variants inhibit different steps of caspase-8 activation at the CD95 death-inducing signaling complex. J. Biol. Chem. 276:20633–20641.
34. Lin, K. J., J. M. Baraban, and R. R. Ratan. 1998. Inhibition versus induction of apoptosis by proteasome inhibitors depends on concentration. Cell Death Differ. 5:577–583.

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35. MacFarlane, M. W., M. Harrison, S. B. Bratton, and G. M. Cohen. 2002. Proteasome-mediated degradation of Smac during apoptosis: XIAP promotes Smac ubiquitination in vitro. J. Biol. Chem. 277:36611–36616.
36. Marini, P., and C. Belka. 2003. Death receptor ligands: new strategies for combined treatment with ionizing radiation. Curr. Med. Chem. Anti-Canc. Agents 3:334–342.
37. Mathas, S., A. Lietz, I. Anagnostopoulou, F. Hummel, W. Wiesner, M. Janz, F. Jundt, B. Hirsh, K. Johrens-Leder, H. P. Vornlocher, K. Bommerl, H. Stein, and B. Durken. 2004. c-FLIP mediates resistance of Hodgkin/Reed-Sternberg cells to death receptor-induced apoptosis. J. Exp. Med. 199:1041–1052.
38. Medema, J. P., J. de Jong, T. van Hall, C. J. Melief, and R. Oeffinger. 1999. The role of Bcl-2 family members in protection from UV-induced apoptosis. J. Exp. Med. 190:1033–1038.
39. Micheau, O., and J. Tschopp. 2003. Induction of TNF receptor 1-mediated apoptosis via two sequential signaling complexes. Cell 114:181–190.
40. Nagata, S. 1997. Apoptosis by death factor. Cell 88:355–365.
41. Nanjokat, C., and S. Hoffmann. 2002. Role and function of the 26S proteasome in proliferation and apoptosis. Lab. Invest. 82:965–980.
42. Nikrad, M., T. Johnson, H. Puthalalath, L. Coultas, J. Adams, and A. S. Kraft. 2005. The proteasome inhibitor bortezomib sensitizes cells to killing by death receptor ligand TRAIL via BH3-only proteins Bik and Bim. Mol. Cell. Biol. 25:36611–36616.
43. Naujokat, C., and S. Hoffmann. 2002. Role and function of the 26S proteasome in response to apoptotic stimuli. Science 297:874–877.
44. Poukkula, M., A. Kaunisto, V. Hietakangas, K. Denessiouk, T. Katajamaki, M. S. Johnson, L. Sistonen, and J. E. Eriksson. 2005. Rapid turnover of c-FLIPshort is determined by its unique C-terminal tail. J. Biol. Chem. 280:27345–27355.
45. Richardson, P. G., B. Barlogie, J. Berenson, S. Singhal, S. Jagannath, D. P. Schenkein, and K. C. Anderson. 2003. A phase 2 study of bortezomib in relapsed, refractory myeloma. N. Engl. J. Med. 348:2609–2617.
46. Sadoul, R., I. Martinou, M. Maki, M. Schroter, J. D. Becherer, M. Irmler, J. Tschopp, and J. C. Martinou. 1996. Involvement of the proteasome in the programmed cell death of NGF-deprived sympathetic neurons. EMBO J. 15:3845–3852.
47. Salvesen, G. S., and C. S. Duckett. 2002. IAP proteins: blocking the road to death’s door. Nat. Rev. Mol. Cell Biol. 3:401–410.
48. Scaffidi, C., I. Schmitz, P. H. Krammer, and M. E. Peter. 1999. The role of c-FLIP in modulation of CD95-induced apoptosis. J. Biol. Chem. 274:1541–1548.
49. Schulze-Osthoff, K., D. Ferrari, M. Los, S. Wesselborg, and M. E. Peter. 1998. Apoptosis signaling by death receptors. Eur. J. Biochem. 254:439–459.
50. Shi, Y. 2002. Mechanisms of caspase activation and inhibition during apoptosis. Mol. Cell 9:459–470.
51. Sohn, D., K. Schulze-Osthoff, and R. U. Janicke. 2005. Caspase-8 can be activated by interchain proteolysis without receptor-triggered dimerization during drug-induced apoptosis. J. Biol. Chem. 280:5267–5273.
52. Stoklosa, T., C. Wojciech, J. Golab, A. Giermasz, and S. Wilk. 1999. Inhibition of apoptosis, proteasome and sensitization to tumour necrosis factor alpha: do they always go together? Br. J. Cancer 79:375–376.
53. Suzuki, Y., Y. Nakabayashi, and R. Takahashi. 2001. Ubiquitin-protein ligase activity of X-linked inhibitor of apoptosis protein promotes pro- 
54. Tabata, M., R. Tabata, D. R. Grabowski, R. M. Bukowski, M. K. Ganapathi, and R. Ganapathi. 2001. Roles of NF-κB and 26 S proteasome in apoptotic cell death induced by topoisomerase I and II poisons in human nonsmall cell lung carcinoma. J. Biol. Chem. 276:8029–8036.
55. Tam, L., S. M. Korahlan, H. Segall, S. Krajewski, K. Welsh, S. Kitada, D. A. Scudiero, G. Tudor, V. H. Qui, A. Monks, M. Andreeff, and J. C. Reed. 2000. Expression and prognostic significance of IAP-family genes in human cancers and myeloid leukemias. Clin. Cancer Res. 6:1796–1803.
56. Thompson, C. B. 1995. Apoptosis in the pathogenesis and treatment of disease. Science 267:1456–1458.
57. Vaux, D. L., and J. Silke. 2005. IAPs, RINGs and ubiquitylation. Nat. Rev. Mol. Cell. Biol. 6:287–297.
58. Voorhees, P. M., and R. Z. Orlovsksy. 2005. The proteasome and proteasome inhibitors in cancer therapy. Annu. Rev. Pharmacol. Toxicol. 45:784–787.
59. Wallach, D., E. E. Varfolomeev, N. L. Malinin, Y. V. Goltsnev, A. V. Kovalenko, and M. P. Boldin. 1999. Tumor necrosis factor receptor and Fas signaling mechanisms. Annu. Rev. Immunol. 17:331–367.
60. Wang, C. Y., M. W. Mayo, and A. S. Baldwin, Jr. 1996. TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-κB. Science 274:764–767.
61. Wiley, S. R., K. Schooley, P. J. Smolak, W. S. Din, C. P. Huang, J. K. Nicholl, G. R. Sutherland, T. D. Smith, C. Rauch, C. A. Smith, et al. 1995. Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity 3:673–682.
62. Wojcik, C., I. Mylnarczuk, G. Hoser, J. Kawiak, T. Stoklosa, J. Golab, and S. Wilk. 1999. A combination of retinoic acid and proteasome inhibitors for the treatment of leukemias is potentially dangerous. Blood 94:1827–1828.
63. Yang, Y., S. Fang, J. P. Jensen, A. M. Weissman, and J. D. Ashwell. 2000. Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. Science 280:874–877.