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Article

Proteasome-Mediated Processing of Nrf1 Is Essential for Coordinate Induction of All Proteasome Subunits and p97

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Summary

Background: Proteasome inhibitors are widely used in the treatment of multiple myeloma and as research tools. Additionally, diminished proteasome function may contribute to neuronal dysfunction. In response to these inhibitors, cells enhance the expression of proteasome subunits by the transcription factor Nrf1. Here, we investigate the mechanisms by which decreased proteasome function triggers production of new proteasomes via Nrf1.

Results: Exposure of myeloma or neuronal cells to proteasome inhibitors (bortezomib, epoxomicin, and MG132), but not to proteotoxic or ER stress, caused a 2- to 4-fold increase within 4 hr in mRNAs for all 26S subunits. In addition, p97 and its cofactors (Npl4, Ufd1, and p47), PA200, and USP14 were induced, but expression of immunoproteasome-specific subunits was suppressed. Nrf1 mediates this induction of proteasomes and p97, but only upon exposure to low concentrations of inhibitors that partially inhibit proteolysis. Surprisingly, high concentrations of these inhibitors prevent this compensatory response. Nrf1 is normally ER-bound, and its release requires its deglycosylation and ubiquitination. Normally ubiquitinated Nrf1 is rapidly degraded, but when partially inhibited, proteasomes carry out limited proteolysis and release the processed Nrf1 (lacking its N-terminal region) from the ER, which allows it to enter the nucleus and promote gene expression.

Conclusions: When fully active, proteasomes degrade Nrf1, but when partially inhibited, they perform limited proteolysis that generates the active form of Nrf1. This elegant mechanism allows cells to compensate for reduced proteasome function by enhancing production of 26S subunits and p97.

Introduction

The ubiquitin-proteasome system (UPS) catalyzes the degradation of most proteins in eukaryotic cells. In the UPS, substrates are targeted for degradation by 26S proteasomes by attachment of a chain of ubiquitins (Ubs). Most ubiquitinated proteins are then rapidly degraded by the 26S proteasome. This ATP-dependent proteolytic complex consists of the 20S proteolytic particle capped by one or two 19S regulatory particles, which bind polyubiquitinated proteins and catalyze their unfolding and translocation into the 20S particle [1]. Proteasome function is also regulated by the association of the 20S with additional regulatory complexes [2], whose precise physiological importance is still unclear (e.g., PA28$\alpha\beta$, the γ-interferon-induced complex that functions in antigen presentation; PA28γ; and PA200/Blm10). Also associated with the 26S proteasome are the deubiquitinating enzymes (DUBs) USP14 and Uch37/UCHL5, which help recycle Ub, but also regulate the particle's (proteolytic and ATPase) activities [3]. The degradation of many Ub conjugates also requires the p97/VCP/Cdc48 ATPase complex, which functions with cofactors to extract ubiquitinated proteins from larger structures to facilitate their degradation by proteasomes [4]. The best-characterized role of p97 is in ER-associated degradation (ERAD) [5], through which misfolded proteins in the ER are ubiquitinated and extracted by p97 in complex with Npl4/NPLOC4, Ufd1/UFD1L, and p47/NSFL1C [6].

Proteasomes are essential for cell viability but are especially important in multiple myeloma cells [7]. Consequently, proteasome inhibitors such as bortezomib (BTZ) or carfilzomib have become the preferred therapy for this cancer. One challenge in their use is the occurrence of drug resistance, but the responsible mechanisms are largely unclear [8]. Therefore, information on how cells compensate for proteasome inhibition is of appreciable interest. Upon proteasome inhibition, mammalian cells show increased expression of multiple 26S subunits, which elevate proteasome content and promote survival [9–11]. This response limits the ability of proteasome inhibitors to kill myeloma cells. Thus, blocking this compensatory response may enhance the efficacy of this treatment. Conversely, decreased proteasome function due to the accumulation of aggregation-prone proteins seems to be important in the pathogenesis of neurodegenerative diseases [12], and pharmacological induction of proteasomes may enhance the cells' degradative capacity and prevent the accumulation of toxic proteins.

In mammals, upon proteasome inhibition, the transcription factor Nrf1/NFE2L1 mediates the induction of genes encoding many 26S subunits [9, 10]. Loss of Nrf1 makes cells more sensitive to killing by proteasome inhibitors. Nrf1, like its homolog Nrf2/NFE2L2, recognizes antioxidant response elements (AREs) in the promoters of many proteasome genes [9, 10]. However, Nrf2 induces 26S subunits only during oxidative stress, but not upon proteasome inhibition. Nrf1 is degraded by the UPS, with a half-life of only ~12 min [10]. Upon proteasome inhibition, ubiquitinated Nrf1 is readily detected, and several Ub ligases (HRD1, Fbw7/FBXW7, and β-TRCP) have been implicated in Nrf1 ubiquitination [10, 13, 14]. It is currently believed that proteasome inhibitors activate Nrf1 by blocking its rapid degradation [10, 14]. One basic problem with this simple mechanism is that it does not explain why Nrf2 does not play a similar role, given that it is also degraded by the UPS, with a half-life of ~13 min [15]. An unusual characteristic of Nrf1 is that it is associated with the ER. Therefore, activation of Nrf1 requires its release from the ER via proteolytic processing by an unidentified protease [10], which presumably is activated upon proteasome inhibition.

It is also unclear whether proteasome inhibition coordinately induces the expression of other components of the UPS, such as PA28, immunoproteasome subunits, proteasome assembly chaperones (POMP, p27/PSMD9, S5b/PSMD5, and gankyrin/PSMD10) [16, 17], 26S-associated DUBs (USP14 and Uch37), and p97 plus its major cofactors. Here, we investigated whether exposure to proteasome inhibitors causes coordinate induction of all 26S subunits and these related factors. Our study has focused on neuroblastoma and myeloma cells,

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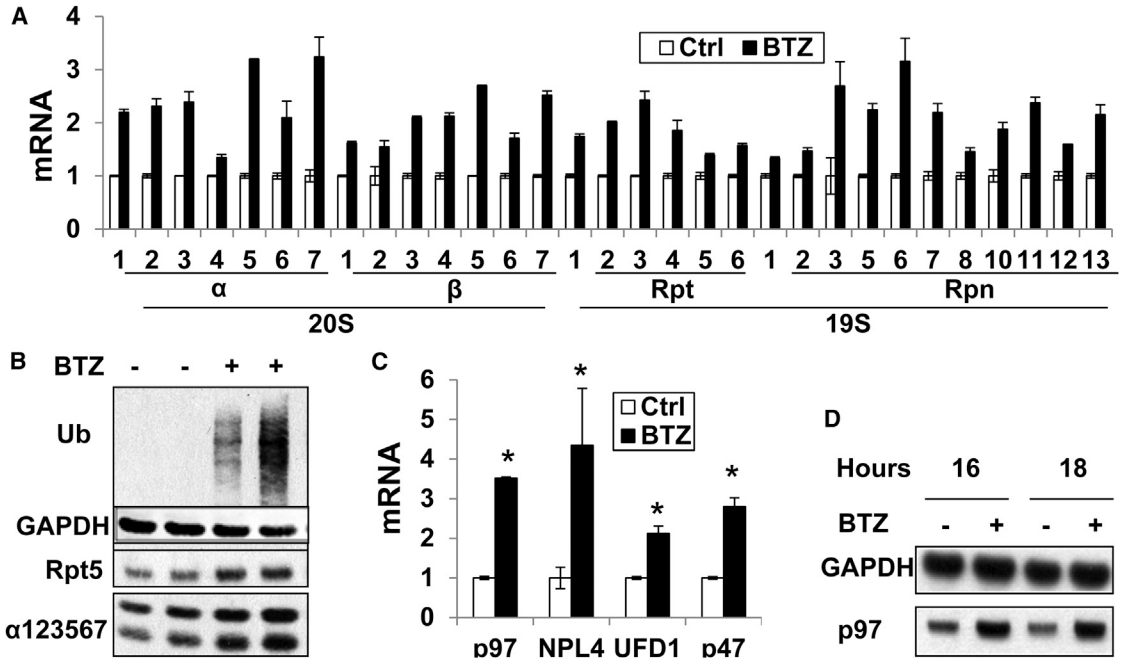

Figure 1. Proteasome Inhibitors Cause Induction of All 26S Proteasome Subunits, p97, and Its Cofactors in ERAD

(A and B) Treatment of neuroblastoma SH-SY5Y cells with BTZ (10 nM, 16 hr) induced mRNAs for all 26S subunits, measured by RT-PCR ($p < 0.05$ for all changes) (A), and increased levels of Rpt5 and 20S subunits, measured by western blot (WB) (B). Biological duplicates were assayed, and levels of Ub conjugates were measured to prove proteasome inhibition. Ctrl, control.

(C and D) Treatment of SH-SY5Y cells with BTZ (10 nM, 16 hr) also induced mRNAs for p97 and its cofactors, Npl4, Ufd1, and p47 (C) and increased content of p97 protein (*$p < 0.05$) (D).

Error bars represent SD. See also Figure S1.

due to the importance of proteasome inhibitors in myeloma therapy and the apparent decreased proteasome function in neurodegenerative disease. These two types of cells are also interesting to study because myeloma cells are especially sensitive to killing by proteasome inhibitors, but neurons are relatively insensitive. We also attempted to determine how quickly these genes are induced after proteasome inhibition, whether all proteasome inhibitors have similar effects, and how the degree of inhibition of proteolysis influences this response. Our study uncovered the surprising finding that high concentrations of these agents were less effective than low ones in inducing this Nrf1-dependent response. In studies to understand this unusual concentration dependence, we found that some proteasome function is necessary for catalyzing the proteolytic processing of Nrf1 on the ER, which allows the processed Nrf1 (lacking its N-terminal region) to enter the nucleus and enhance expression of 26S subunits and p97.

Results

Proteasome Inhibition Rapidly Induces 26S Subunits Independently of the Unfolded Protein Response

In the neuroblastoma line SH-SY5Y, treatment with a low concentration (10 nM) of BTZ for 16 hr caused a maximal (2- to 4-fold) increase in mRNAs for all 33 proteasome subunits (Figure 1A; Figure S1A available online) and about a 50% increase in their protein levels (Figure 1B). The magnitude of this induction was similar for both 19S and 20S subunits, including Rpn6 [18] and Rpn11 [19], that were reported to be regulated independently, and the loosely associated subunit Ecm29/KIAA0368 (Figure S1B). At higher concentrations (1 μM), BTZ caused a clear induction of these mRNAs at 4 hr (Figure S1C), although the magnitude was much smaller than at 16 hr. A similar induction was detected in another neuroblastoma line, M17; the myeloma line MM1.S; and HEK293A cells (Figure S1D), although these lines differed in the degree of induction. SH-SY5Y cells induced 26S subunits much more strongly than others. Therefore, we mainly used SH-SY5Y cells in subsequent experiments. Several other proteasome inhibitors, including MG262 (Z-LLL-boronate) and epoxomicin (Epox), caused a similar induction of 26S subunits (Figure S1E). Thus, their induction appears to be a general cellular mechanism that compensates for reduced proteasome function.

The unfolded protein response (UPR) is triggered by the accumulation of misfolded proteins in the ER and is strongly induced by proteasome inhibitors [20]. Therefore, we tested whether the UPR per se induces 26S expression. Unlike BTZ, the UPR inducers, tunicamycin and thapsigargin, did not stimulate 26S induction (Figures S1E–S1H). Also, low concentrations of BTZ induced 26S subunits without causing the UPR (i.e., eIF2α phosphorylation; Figure S1F). Thus, proteasome induction is not mediated by the UPR.

Components of the UPS that Are Induced with 26S Subunits

We next determined whether these inhibitors also stimulated expression of several proteasome-associated proteins. In response to γ-interferon, cells induce three distinct catalytic subunits (immunoproteasome subunits), β1i/PSMB9, β2i/PSMB10, and β5i/PSMB8, which are more efficient in generating peptides suitable for antigen presentation on major histocompatibility complex class I molecules [21]. Unlike the

standard subunits, expression of immunoproteasome subunits decreased by 50%–70% (Figure S1I). PA28α and PA28β are also induced by γ-interferon, and together this complex can also enhance antigen presentation [22]. BTZ treatment caused a small decrease in PA28$\alpha\beta$ mRNA expression. BTZ did not alter the expression of the nuclear homolog PA28γ (Figure S1I). However, BTZ treatment did induce the other nuclear activator, PA200 (Figure S1I).

The assembly of newly synthesized subunits into mature proteasomes requires assembly chaperones. BTZ treatment induced the 20S chaperone POMP [17], but not the chaperones involved in assembly of the 19S base, S5b, p27, or Gankyrin [16] (Figure S1J). The 26S-associated DUBs, USP14 and Uch37, catalyze disassembly of Ub chains on substrates and can promote protein deubiquitination without degradation [3], but they also allosterically regulate 20S gate opening and ATP hydrolysis [23]. Treatment with BTZ or MG132 for 16 hr induced the mRNA for *USP14* 2-fold, but surprisingly did not affect Uch37 expression (Figure S1K). p97 and its cofactors Npl4, Ufd1, and p47 are essential for the degradation of many proteins, especially by ERAD. These genes were induced 2- to 5-fold by BTZ treatment coordinately with 26S subunits (Figures 1C, 1D, and S1C). However, BTZ did not induce RAD23A or RAD23B, both of which can shuttle Ub conjugates to proteasomes in ERAD (Figure S1L).

Thus, upon proteasome inhibition, mammalian cells induce all 26S components, USP14, PA200, and the p97 complex, which together should enhance the cell's capacity for proteolysis. Furthermore, BTZ treatment increased expression of the polyubiquitin gene *UBB* (2- to 3-fold; Figure S1M). Given that Ub conjugates accumulate upon proteasome inhibition and free Ub can be depleted [24], its increased production presumably compensates for decreased Ub recycling and enhances the cell's capacity for proteolysis.

High Concentrations of Proteasome Inhibitors Inhibit the Induction of 26S Subunits

All the agents used in this study preferentially inhibit the proteasome's chymotrypsin-like activity but vary in their affinities for this site. MG132 is commonly used at much higher concentrations than BTZ, Epox, and MG262. Treatment of SH-SY5Y cells with 10 μM MG132 for 16 hr blocked the chymotrypsin-like site without causing substantial cell death (Figures S2A–S2C), but surprisingly did not induce any of the α subunits, six of the β subunits, or many of the 19S subunits (Rpt4, Rpt5, Rpn3, Rpn8, Rpn10, and Rpn12) (Figure 2A). In addition, MG132 caused a smaller induction of Rpt3, Rpn3, Rpn6, and Rpn14 than did BTZ, though it caused a similar induction of Rpt2 Rpn1, Rpn2, Rpn5, Rpn7, and Rpn13. Strikingly, when cells were exposed to both MG132 (10 μM) and BTZ (10 nM) (Figures S2D and S2E), the inhibitory effect of MG132 was dominant over the induction of 26S and p97 by low concentrations of BTZ or other inhibitors. Furthermore, in contrast to 10 μM, lower MG132 concentrations (1–2.5 μM) did induce the expression of 26S subunits (Figure S2F), as reported previously [9, 10].

Thus, MG132 inhibits proteasome induction only at high concentrations, at which this tripeptide aldehyde can also inhibit several cellular serine and cysteine proteases. Therefore, at such concentrations, MG132's ability to block induction of 26S subunits and p97 might be due to an effect on these other proteases (see Figures S3O–S3W) or its causing a greater inhibition of proteasome function by inhibiting both the caspase-like and the chymotrypsin-like activities [25]. To test these possibilities, we investigated the effects of higher concentrations of the much more specific peptide boronate proteasome inhibitors, BTZ and MG262 [26], and Epox, an epoxyketone that specifically inactivates the 20S threonine proteases [27]. At high concentrations (10 μM), BTZ, MG262, and Epox all suppressed the induction of 26S subunits and p97 (Figures 2B and 2C) without causing detectable cytotoxicity at 16 hr (Figures S2B and S2C), in sharp contrast to the increased expression of these genes with low concentrations (10 nM BTZ, 50 nM Epox, 1 μM MG262; Figures 2B, 2C, and S3L). Similarly, in HEK293A cells, high concentrations of BTZ inhibited the expression of 26S subunits (Figure 2F). In addition, the levels of 26S and p97 protein, as well as the amount of assembled 26S particles, were increased by low, but not high concentrations of BTZ (Figures 2D and 2E).

At these high concentrations, the inhibitors all block multiple peptidase activities and thus markedly reduce cellular proteolysis [25]. Most likely, these high concentrations prevent induction of 26S subunits by causing a greater inhibition of the proteasome. Therefore, we compared in HEK293A cells the effects of increasing BTZ concentrations on 26S expression, the extent of inhibition of the degradation of long-lived cell proteins labeled with [^{3}H]-phenylalanine, and the degree of inhibition of the 26S subunit's chymotrypsin-like and caspase-like activities in cell lysates (tested with specific fluorescent peptide substrates [28]). At 20 nM, BTZ inhibits the chymotrypsin-like site >50%, but 50 nM is required to inhibit it by 100% and to inhibit the caspase-like activity and protein-degradation rate by >50% (Figures 2G and 2H). Intriguingly, in these cells, maximal induction of 26S subunits β3, Rpt3, and Rpt5 occurred with 20 nM BTZ, but at 50 nM and higher, BTZ progressively inhibited the expression of these genes (Figure 2F). Therefore, greater inhibition of protein degradation (e.g., with high concentrations that block the chymotrypsin-like activity completely and also inhibit the caspase-like activity), suppressed the expression of 26S subunits and p97.

The Induction of 26S Subunits Requires Cleavage of the ER-Bound Transcription Factor Nrf1 and Some Proteasome Function

We confirmed the prior finding [9] that Nrf1 is the critical factor inducing 26S subunits after BTZ treatment by showing that stable Nrf1 knockdown in SH-SY5Y cells suppressed the induction of many 26S subunits' mRNAs and decreased their basal mRNA levels (Figures S3A and S3B). Thus, Nrf1 appears to be important in determining proteasome content under normal conditions. By contrast, knockdown or overexpression of Nrf2 did not affect 26S induction upon BTZ treatment (Figures S3C–S3F). Nrf1 is normally associated with the ER through its N terminus, and deletion of the 30 N-terminal residues allows its translocation into the nucleus and the stimulation of gene expression [14, 29, 30]. Therefore, it was hypothesized that a proteolytic cleavage of Nrf1 near its N terminus releases it from the ER [10], and during preparation of this manuscript, Nrf1 was reported to be cleaved before Leu104 [31]. However, no specific protease has been implicated in this step. As discussed above, the most likely explanation for the ability of high concentrations of proteasome inhibitors to suppress induction is that the 26S itself processes Nrf1 and high concentrations of inhibitors block its capacity for limited proteolysis.

We therefore tested whether Nrf1 was proteolytically processed upon exposure to low, but not high concentrations of

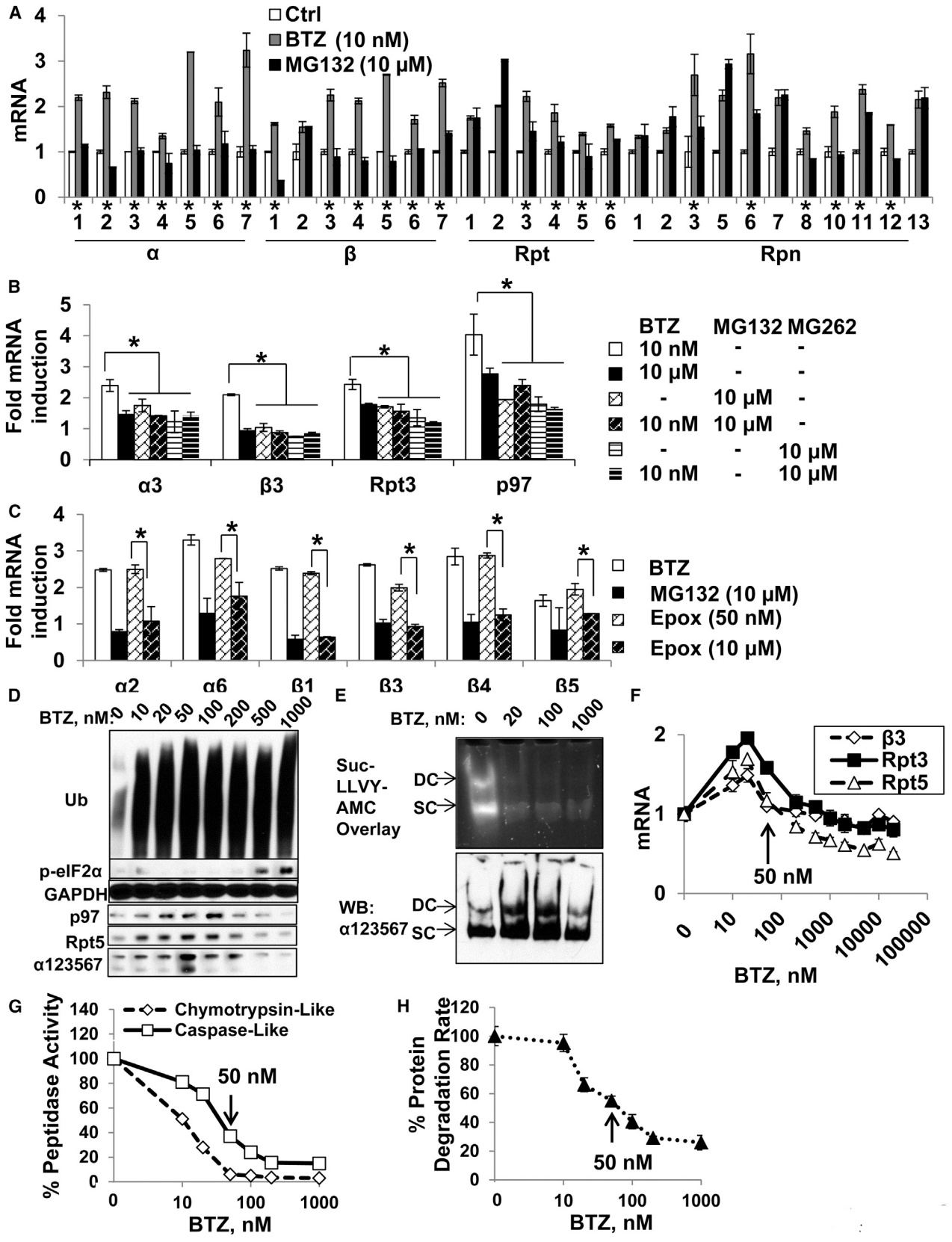

Figure 2. High Concentrations of Proteasome Inhibitors Block the Induction of 26S Subunits

(A) Unlike the treatment of SH-SY5Y cells with low concentrations of BTZ (10 nM), 10 μM MG132 for 16 hr did not induce the mRNA for 20S subunits and many of the 19S subunits. (*, genes whose mRNA levels are lower [$p < 0.05$] in MG132-treated than in BTZ-treated cells.)

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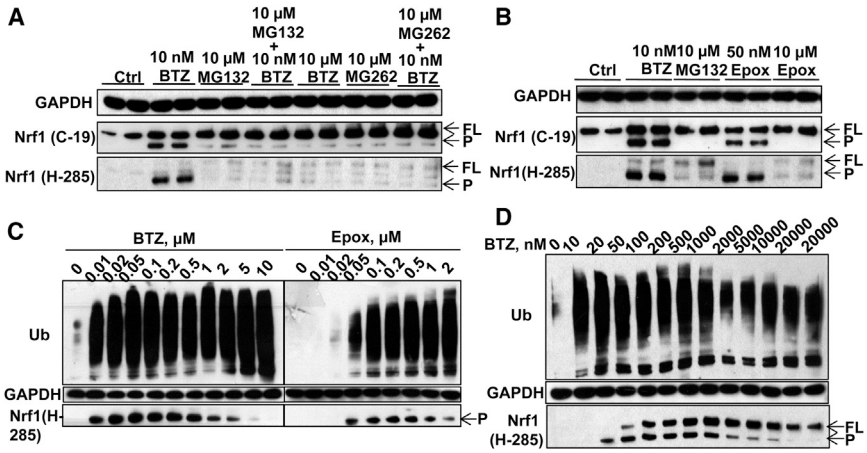

Figure 3. High Concentrations of Proteasome Inhibitors Block the Proteolytic Processing of Nrf1

(A) Treatment of SH-SY5Y cells with high concentrations (10 μM) of MG132, BTZ, or MG262 for 16 hr, alone or with 10 nM BTZ, suppressed the processing of Nrf1. FL, full-length Nrf1; P, processed Nrf1.

(B) Epox treatment for 16 hr at 10 μM, but not 50 nM, suppressed Nrf1 processing.

(C) BTZ or Epox treatment (SH-SY5Y cells) for 16 hr at concentrations higher than 0.5 μM decreases the processing Nrf1.

(D) Treatment of HEK293A cells with BTZ for 16 hr at >20 nM causes the accumulation of FL Nrf1 and >1 μM decreases the level of P Nrf1.

See also Figure S3.

proteasome inhibitors. Using an antibody that recognizes the C terminus of Nrf1 (C-19), we could detect the full-length (FL) Nrf1 (~85 kDa) plus a smaller form (~75 kDa) in cells treated with 10 nM BTZ (Figures S3B, S3G, and S3H). These two bands also reacted with another Nrf1 antibody (H-285, which recognizes residues 191–475), and levels of both were diminished by the Nrf1 small hairpin RNA (shRNA) (Figure S3B). The appearance of this smaller form upon BTZ treatment strongly suggests that it is a processed (P) form of Nrf1. Inhibition of protein synthesis by cycloheximide (CHX) after 4 hr of BTZ (20 nM) treatment caused a rapid decline in the levels of both FL and P forms (Figure S3J). Thus, both forms of Nrf1 have short half-lives even in the presence of 10 nM BTZ. Upon treatment with 20 nM BTZ, the P form gradually accumulated, and its level was much higher at 8–12 hr than at 4 hr (Figure S3J). However, ATP depletion during proteasome-inhibitor treatment completely abolished Nrf1 processing (Figure S3K), implying that generation of the P form involves an ATP-dependent process, such as the UPS.

We next tested whether Nrf1 processing is blocked by high concentrations of proteasome inhibitors. Nrf1 was processed in both SH-SY5Y cells (Figure 3A) and MM1.S cells (Figure S3I) treated with 10 nM BTZ, which caused 26S subunits' induction (Figure S1D), but not with 10 μM MG132, which failed to stimulate their expression. Similarly, high concentrations (10 μM) of BTZ, Epox, or MG262 also suppressed Nrf1 processing (Figures 3A and 3B), just as they prevent proteasome induction, whereas at low concentrations that permitted induction, these inhibitors caused Nrf1 processing (Figures 3A, 3B, and S3L–S3N). Furthermore, treatment with 10 μM MG132 or MG262 together with 10 nM BTZ suppressed the processing of Nrf1 (Figure 3A), just as these combinations repressed 26S expression (Figure 2B). We confirmed these observations using both C-19 and H-285 antibodies to monitor Nrf1 processing. Because the H-285 antibody recognizes the processed Nrf1 much more strongly than C-19, H-285 was used in most subsequent experiments.

To determine the minimal concentrations at which proteasome inhibitors begin to block Nrf1 processing, we compared the effects of increasing concentrations of BTZ, Epox, and MG132 in SH-SY5Y cells. MG132 was able to enhance Nrf1 processing (Figure S3N) and to induce subunit expression (Figure S2F) at 1 or 2.5 μM, but at higher concentrations (5 or 10 μM), both Nrf1 processing and proteasome gene expression were suppressed. When the concentrations of the more potent and specific inhibitors BTZ and Epox were raised above 0.5 μM, the level of the P form started to decrease in SH-SY5Y (Figure 3C) and HEK293A cells (Figure 3D). A more sensitive indicator of the incomplete processing of Nrf1 was the accumulation of FL Nrf1. In SH-SY5Y cells, FL Nrf1 is present at very low concentrations (probably due to its very rapid degradation) and was barely detectable by the H-285 antibody. However, in HEK293A cells, BTZ (≥50 nM) was able to cause FL Nrf1 accumulation (Figure 3D), and thus, at these concentrations, BTZ must already inhibit Nrf1 processing. Remarkably, 50 nM is exactly the concentration at which BTZ suppressed the expression of 26S subunits (Figure 2F). Also, 50 nM is the concentration at which BTZ causes complete inhibition of the chymotrypsin-like activity and also inhibits the caspase-like activity and cellular protein degradation >50% (Figures 2G and 2H). These extensive correlations make it very likely that the proteasome is responsible for Nrf1 processing to the active form. Therefore, only when proteasomes are partially compromised by low inhibitor concentrations that only partially block the chymotrypsin-like site will there be sufficient 26S function for limited proteolysis of Nrf1. However, when multiple active sites are inhibited and protein degradation is reduced >50%, Nrf1 processing cannot take place.

Although the proteasome is the only known enzyme in mammalian cells sensitive to epoxyketones [27], we carried out extensive tests of the unlikely possibility that Nrf1 is processed by an additional protease that is inhibited by peptide aldehydes and/or peptide boronates or an ER-associated intramembrane protease. By inhibiting these proteases with

(B) High concentrations (10 μM) of BTZ or MG262, alone or in combination with 10 nM BTZ, suppressed the induction of α3, β3, Rpt3, and p97.

(C) Treatment with Epox at 10 μM, but not 50 nM, suppressed the induction of 20S subunit mRNAs. *$p < 0.05$.

(D and E) SH-SY5Y cells were treated with BTZ for 16 hr. (D) The level of 26S subunits and p97 were determined by WB. (E) Next, singly or doubly capped (SC or DC) 26S proteasomes were separated by native PAGE, and their activity and amounts were determined by Suc-LLVY-AMC overlay assay or WB with the α123567 antibody.

(F–H) BTZ inhibits 26S subunit expression in HEK293A cells at 50 nM or higher. (G) Peptidase activities were measured in cell lysates with fluorogenic peptide substrates. (H) The protein degradation rate was measured by assaying hydrolysis of radiolabeled long-lived cell proteins to acid-soluble products. (F) β3, Rpt3, and Rpt5 mRNAs were measured.

Arrows indicate 50 nM, above which BTZ blocks protein degradation and 26S induction. Error bar represents SD. See also Figure S2.

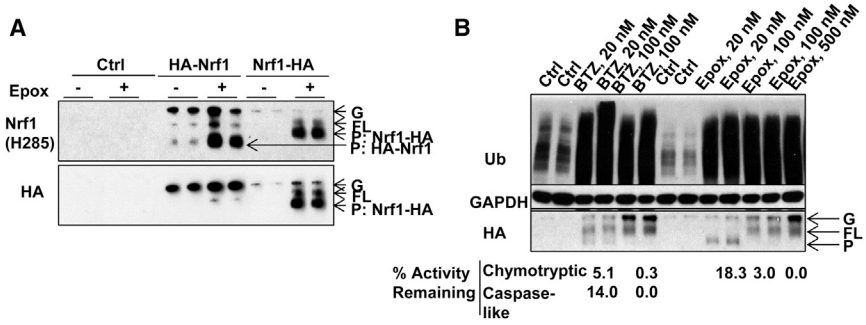

Figure 4. Low, but Not High Concentrations of Proteasome Inhibitors Cause the Processing of Nrf1 near Its N Terminus

(A) Nrf1 tagged with HA at its N terminus (HA-Nrf1) or C terminus (Nrf1-HA) was expressed in HEK293A cells. Nrf1-HA and HA-Nrf1 exist in both a glycosylated form (G, which is hardly detectable for the endogenous Nrf1 under most conditions) and a deglycosylated form (FL, because both full-length forms retain the HA epitope regardless of whether Nrf1 was tagged at its N or C termini). After treatment with Epox (20 nM, 16 hr), the HA tag still remained on the processed form of Nrf1-HA, but not on the product of HA-Nrf1. (Biological duplicates were assayed.)

(B) The processing of Nrf1-HA is most efficient with 20 nM Epox treatment that retains 18.3% chymotrypsin-like activity, whereas 20 nM BTZ treatment (that retains only 5.1% chymotrypsin-like activity and 14.0% caspase-like activity) partially suppressed Nrf1-HA processing. Higher concentrations of BTZ or Epox blocked Nrf1-HA processing.

See also Figure S4.

chemical inhibitors or small interfering RNAs, we obtained strong data (Figures S3O–S3W) indicating that the various potential candidates, including MG132-sensitive proteases (calpain and lysosomal cathepsins) or ER-associated intramembrane proteases (γ-secretase, signal peptidase, and ER-associated rhomboid family intramembrane proteases) do not play a role in processing Nrf1 upon proteasome inhibition and expression of 26S subunits (See the legend of Figures S3O–S3W for detailed discussion). Together, these various negative observations and the extensive correlations shown above make it very likely that Nrf1 processing is mediated by the proteasome itself.

Proteolytic Removal of Nrf1's N Terminus Is Essential for Nuclear Entry

Nrf1 contains several ER transmembrane domains, but the topology of FL Nrf1 has not been resolved. However, the N-terminal transmembrane domain (residues 2–30) is required for its ER association, and deletion of this region allows nuclear translocation [29]. To test whether Nrf1 processing involves proteolytic removal of this N-terminal region, we expressed FL Nrf1 tagged with hemagglutinin (HA) at its N or C terminus. FL Nrf1 initially exists as a glycosylated form (G) [10, 30]. After Epox treatment, the G species was first converted to the FL form, whose molecular weight decreased due to deglycosylation (Figure 4A), as reported recently [10]. The identity of the G and FL species was clear because both retained the HA tag, whether it was on the N or C terminus (Figure 4A), and the FL sequence of the G form was confirmed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Figure S4). After deglycosylation, Nrf1 was proteolytically processed, as shown by LC-MS/MS analysis of the shorter form (Figure S4). When Nrf1 was tagged at its N terminus (HA-Nrf1), the HA tag was lost during Nrf1 processing, but not when it was tagged on its C terminus (Nrf1-HA). The HA antibody therefore no longer reacted with processed HA-Nrf1 (Figure 4A) even though Epox at low concentrations still induced processing of HA-Nrf1, which still reacted with the Nrf1 antibody. As expected, the processed Nrf1-HA was larger than the processed HA-Nrf1, which no longer contained the HA tag. However, we could not detect the N-terminal HA tag after it was cleaved off HA-Nrf1. Like the processing of endogenous Nrf1, Nrf1-HA processing was very sensitive to proteasome inhibition. An 80% inhibition of the chymotrypsin-like activity by 20 nM Epox promoted Nrf1 processing, but a greater inhibition of the proteasome with 100 nM Epox or with 20 nM BTZ, which inhibits the chymotrypsin-like site completely as well as the caspase-like site, resulted in a decline in the P form and an accumulation of the FL form (Figure 4B).

To test whether the loss of the N terminus actually allows Nrf1 entry into the nucleus [14, 29], we treated HEK293A cells overexpressing Nrf1-HA with proteasome inhibitors and localized Nrf1-HA with an HA antibody. Nrf1-HA was initially cytoplasmic, as expected from its reported ER association, but entered the nucleus after treatment with BTZ or Epox (Figure 5B). Although the H-285 antibody detected this nuclear translocation of HA-Nrf1 (Figure 5C), the HA antibody could not detect any HA-Nrf1 in the nucleus (Figures 5B and 5C). Thus, Nrf1 could not enter the nucleus unless its N terminus was removed. However, as expected, high inhibitor concentrations (10 μM MG132, Figure 5A; or 500 nM Epox, Figure 5C) blocked Nrf1 accumulation in the nucleus. Thus, some proteasome activity is essential for Nrf1 release from the ER and nuclear entry.

Nrf1 Processing Requires Ubiquitination, but Is Not Activated by Heat Shock

Nrf1 is normally polyubiquitinated and rapidly degraded by proteasomes [10, 13, 14]. It was therefore assumed that proteasome inhibitors cause gene induction simply by blocking Nrf1 degradation [10]. However, this model is inconsistent with our finding that high concentrations of these inhibitors prevent Nrf1 degradation but do not induce 26S subunit expression. Instead, Nrf1 activation and induction of 26S subunits and p97 require some proteasome activity to process Nrf1 and allow nuclear entry. To determine whether the processing of Nrf1 by the 26S also requires the ubiquitination of Nrf1, we treated cells for 16 hr with BTZ (10/100 nM) and the specific inhibitor of ubiquitination, ML00603997 [32]. This treatment almost completely depleted various cell lines of ubiquitinated proteins within 1 hr (Figures S5A and S5C) and was not toxic to the cells during 24 hr. Blocking ubiquitination dramatically suppressed BTZ-induced expression of 26S subunits and p97 below their basal mRNA level (Figures 6A and S5B) and also completely suppressed the processing of endogenous Nrf1 induced by BTZ (Figures 6B, S5A, and S5C). Similarly, inhibiting ubiquitination prevented the processing of overexpressed Nrf1-HA in HEK293A cells and caused it to accumulate in its FL form (Figure 6C). Furthermore, this inhibitor alone caused the accumulation of FL Nrf1. Thus, ubiquitination is required for BTZ-induced Nrf1 processing and the induction of 26S subunits and p97.

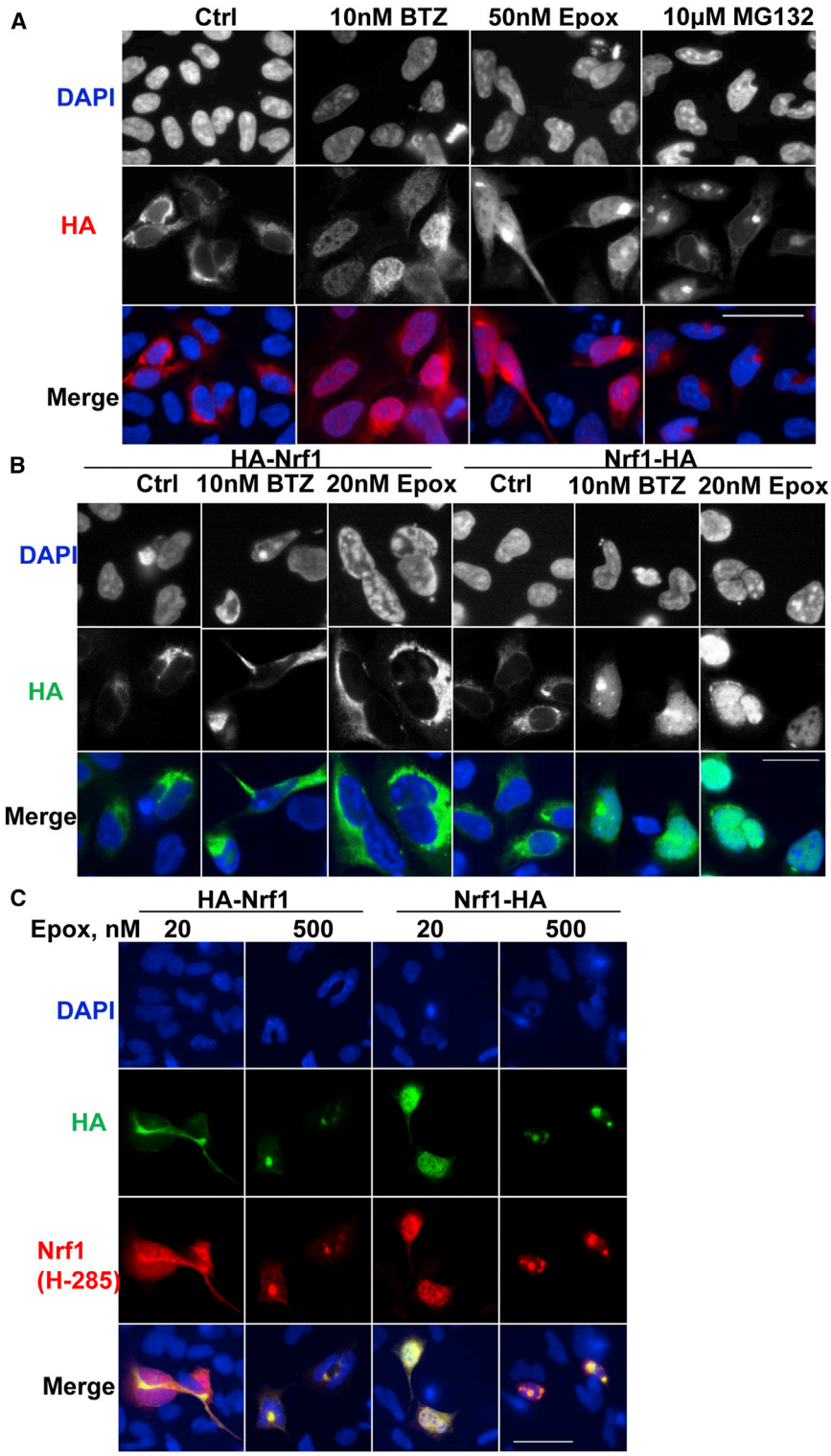

Figure 5. Nrf1 Nuclear Translocation with Low, but Not High Concentrations of Proteasome Inhibitors Requires Its N-Terminal Processing

(A) HEK293A cells overexpressing Nrf1-HA were treated with BTZ (10 nM), Epox (50 nM), or MG132 (10 μM) for 16 hr, and Nrf1-HA localization was detected by HA immunostaining. Nuclear translocation of Nrf1-HA occurred after treatment with BTZ and Epox, but not MG132.

(B) After HEK293A cells overexpressing Nrf1-HA or HA-Nrf1 were treated with BTZ (10 nM) or Epox (20 nM) for 16 hr, HA immunostaining detected Nrf1-HA, but not HA-Nrf1, in the nuclei of the BTZ- or Epox-treated cells.

(C) HEK293A cells overexpressing Nrf1-HA or HA-Nrf1 were treated with Epox (20 or 500 nM) for 16 hr. Nrf1 antibody, but not the HA antibody, could detect nuclear HA-Nrf1 upon 20 nM Epox treatment. Like 10 μM MG132, 500 nM Epox did not induce Nrf1 nuclear translocation.

All scale bars represent 50 μm.

A possible explanation for why blocking ubiquitination reduces Nrf1 processing and proteasome production could be that reduced proteasome function activates Nrf1-mediated gene induction by causing an accumulation of misfolded or ubiquitinated proteins. However, merely increasing protein misfolding and the level of Ub conjugates in the cytosol by treating SH-SY5Y cells with arsenite or heat shock not only failed to promote Nrf1 processing, but surprisingly, even blocked this process and the induction of 26S subunits and p97 by BTZ (Figures S5D–S5G). Therefore, Nrf1 processing and production of new proteasomes is not signaled by an accumulation of misfolded or ubiquitinated proteins, which actually reduces these processes. Alternatively, Nrf1 may need to be ubiquitinated in order to be processed by the proteasome. We tested this possibility using RA190 [33], an inhibitor of Rpn13, one of two proteasome subunits that bind Ub chains. RA190 binds covalently to Rpn13's Ub-binding domain and thus seems to block the association of conjugates with the 26S [33]. Rpn13 inhibition blocked the accumulation of processed Nrf1 in HEK293A and SH-SY5Y cells and reduced the induction of 26S expression by 20 nM of BTZ (Figures S5H–S5L). Therefore, Nrf1 processing requires recognition of ubiquitinated species by the 26S. Most likely, E1 inhibition stops Nrf1 processing by blocking its ubiquitination and binding to the 26S and not by reducing the cell's content of Ub conjugates.

Nrf1 Processing Requires p97 Function for Its Deglycosylation

This ubiquitination and proteasome-mediated limited proteolysis of Nrf1 resembles the processing of three other transcription factors, NF-κB [34], Gli [35], and Spt23 [36]. The processing of Spt23, which is also ER associated, requires p97 to extract the processed Spt23 from the ER [36]. To test whether p97 is also essential for Nrf1 processing, we expressed a dominant-negative mouse p97K524A (DN-p97) that cannot bind ATP [37] or treated cells with the p97 inhibitor NMS859, which also blocks ATP binding [38]. As expected,

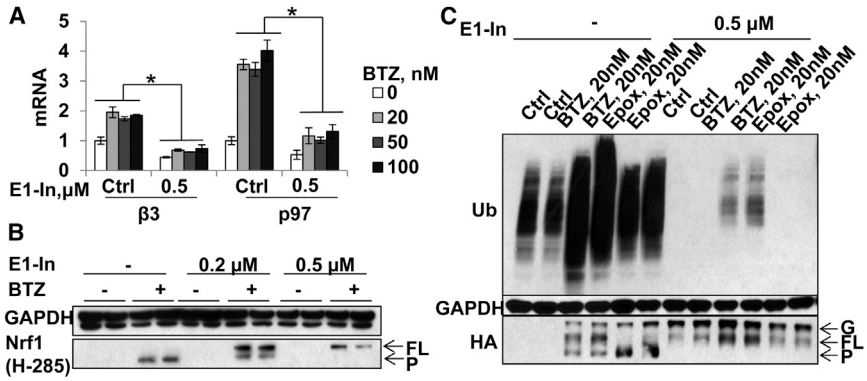

Figure 6. Nrf1 Processing and the Expression of 26S Subunits and p97 Require Nrf1 Ubiquitination

(A and B) SH-SY5Y cells were treated with the E1 inhibitor ML00603997 (E1-In, 0.5 μM) for 1 hr, then treated with different concentrations of BTZ together with 0.5 μM ML00603997 for 16 hr. E1-In suppressed both (A) expression of mRNA for p97 and the 26S subunit β3 and (B) the processing of Nrf1. (Biological duplicates were assayed.) (C) HEK293A cells expressing Nrf1-HA were treated with E1-In as in (A) and (B) and different concentrations of proteasome inhibitors. E1-In caused the accumulation of full-length Nrf1-HA by itself or in the presence of proteasome inhibitors. Error bar represents SD. See also Figure S5.

DN-p97 and NMS859 both raised cellular levels of Ub conjugates (Figure S6). Upon BTZ treatment (50 nM, 4 hr), DN-p97 also caused an accumulation of the glycosylated form of Nrf1 but decreased the levels of both the FL and the P forms of Nrf1 (Figures 7A and S6B). The same effects were observed in HEK293A cells and SH-SY5Y cells treated with the p97 inhibitor (Figures S6E and S6F) at concentrations that do not cause cell death (Figure S6D). The DN-p97 also blocked the formation of both the FL and P forms of ectopically expressed Nrf1-HA (Figure 7B). Therefore, p97 function is necessary for the production of the deglycosylated FL form. As these findings predict, the DN-p97 also prevented Nrf1 entry into the nucleus upon treatment with 20 nM BTZ and reduced the expression of 26S subunits (Figures S6C and S6H). Similarly, the p97 inhibitor also abolished induction of 26S subunits and p97 in HEK293A and SH-SY5Y cells (Figures S6G and S6I).

Because FL Nrf1 accumulated upon E1 inhibition, whereas G Nrf1 accumulated when p97 was inhibited, Nrf1 must be ubiquitinated after its deglycosylation. To test this conclusion, we treated HEK293A cells coexpressing Nrf1-HA and DN-p97 with the E1 inhibitor. Although blocking ubiquitination alone caused a buildup of Nrf1 in the FL form, inhibition of both E1 and p97 led to an accumulation of Nrf1 in the G form (Figure 7B). Therefore, Nrf1 must be ubiquitinated again after its deglycosylation.

Discussion

The increased production of new proteasomes after treatment with proteasome inhibitors is an important compensatory response that promotes cell survival and is likely to reduce therapeutic efficacy. As shown here, this response involves coordinate induction of all the standard proteasome subunits via Nrf1 as well as several other key UPS components. The 20S assembly chaperone POMP, but not chaperones involved in 19S base assembly (S5b, p27, and Gankyrin) were induced, presumably because POMP is destroyed during 20S assembly. The simultaneous induction of PA200 is interesting given that PA200 can promote Ub-independent proteasomal degradation of certain proteins [39]. On the other hand, the lack of induction of immunoproteasome subunits and PA28αβ correlates with their functioning predominantly in antigen presentation and regulation by γ-interferon, rather than in protein degradation. In fact, expression of immunoproteasomes was suppressed by BTZ, as was also noted previously [10, 11], and none of these genes contains an ARE sequence in its promoters. Clearly, their production is regulated differently from standard proteasomes. Proteasome inhibition induced Usp14, but surprisingly not the other 26S-associated DUB, Uch37, even though it, like Usp14, activates gate opening and ATP hydrolysis upon binding of Ub conjugates [23]. Nevertheless, the finding that Uch37 expression is not coordinately regulated suggests that these two 26S-associated DUBs serve distinct roles. Lastly, our finding that p97 and its cofactors (Ufd1, Npl4, and p47) were strongly induced together with proteasome subunits is consistent with their important general role in the UPS.

This simultaneous induction of UBB, PA200, USP14, and p97 plus its ERAD cofactors suggests strongly that these proteins are all important in enhancing the cell's proteolytic capacity. However, simultaneous induction does not prove that the mechanisms are the same. In fact, high concentrations of proteasome inhibitors inhibited the induction of p97 together with 26S subunits, but not the induction of USP14, Ecm29, and UBB, which thus must not require Nrf1. Nrf1-dependent induction of all proteasome subunits and p97 differs sharply from the selective induction of Rpn6 by FOXO4 [40]. This response to proteasome inhibition also clearly differs from the heat-shock response and the UPR [20, 41], both of which are caused by the buildup of misfolded proteins and therefore can also be induced by proteasome inhibitors. Neither the UPR nor heat shock leads to Nrf1 processing or induction of 26S subunits. In fact, surprisingly, heat shock suppresses this response to proteasome inhibition (Figures S5D–S5G). Among four cell lines tested, the induction of 26S subunits was strongest in SH-SY5Y cells, but this property is not general for all neuronal cells because another neuroblastoma line, M17, induced the expression of 26S subunits much more weakly (Figure S1D) despite being more resistant to killing by BTZ than SH-SY5Y cells (data not shown). Therefore, although both neuroblastoma lines were much more resistant to killing by BTZ than the myeloma line MM1.S, this greater resistance cannot be attributed to a greater ability to induce the expression of 26S subunits.

Because Nrf1 is a short-lived protein, it was believed that upon proteasome inhibition, Nrf1 is activated simply because its degradation is prevented [10, 13, 14]. Such a mechanism is unable to explain our finding that 26S subunits and p97 are induced upon treatment with low, but not high concentrations of proteasome inhibitors. In fact, this mechanism would predict the exact opposite result, i.e., greater Nrf1 activation at higher concentrations. Our results instead support the type of mechanism illustrated in Figure 7C, in which 26S proteasomes catalyze not only the complete degradation of Nrf1 [14], but also limited proteolysis to release Nrf1's active region from its N-terminal transmembrane domain. Because Nrf1 also

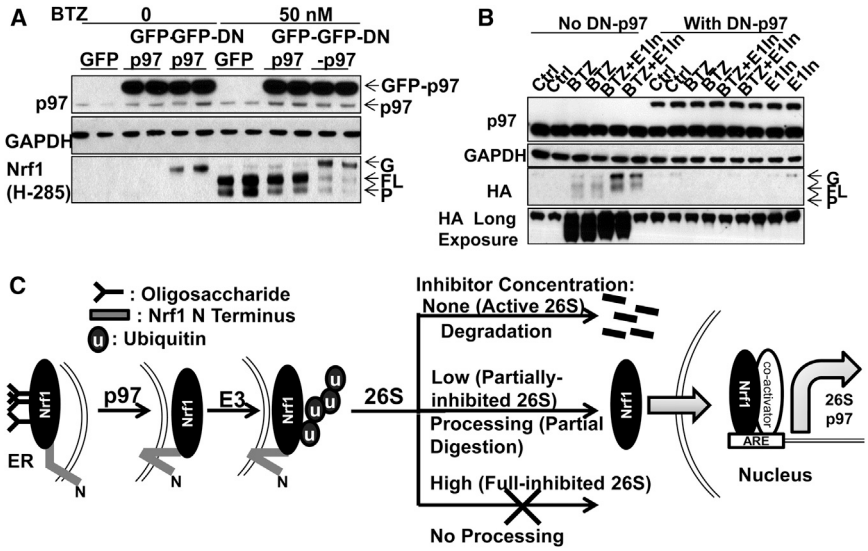

Figure 7. Nrf1 Processing and the Expression of 26S Subunits and p97 Require Nrf1 Deglycosylation and p97 Activity

(A) To test whether p97 function is essential for Nrf1 processing, we incubated HEK293A cells overexpressing GFP-p97, GFP-DN-p97, or GFP (control) with or without 50 nM BTZ for 4 hr. GFP-DN-p97 (but not GFP-p97) reduced the levels of full-length and processed Nrf1 but caused the accumulation of a 100 kDa glycosylated form of Nrf1.

(B) DN-p97 and Nrf1-HA were coexpressed in HEK293A cells. DN-p97 caused the sequestration of Nrf1-HA in its glycosylated form and blocked the formation of full-length and processed Nrf1 upon treatment (12 hr) with BTZ (20 nM) or E1-In (0.5 μM).

(C) Model: Upon partial inhibition of proteasomes with low concentrations of inhibitors, cells induce 26S subunits and p97 via Nrf1. Although Nrf1 is normally degraded completely, partial inhibition of proteasomes by low concentrations of inhibitors favors limited degradation of the N-terminal part of Nrf1. Consequently, the processed C-terminal portion of Nrf1 is released from the ER and enters the nucleus to transcribe 26S subunits and p97. Complete inhibition of proteasomes by high concentrations of inhibitors blocks Nrf1 processing and transcription. Both Nrf1 degradation and its proteolytic processing require first deglycosylation and extraction of Nrf1 from the ER via p97 activity, and second, ubiquitination of Nrf1.

See also Figure S6.

regulates basal expression of 26S subunits (Figure S3A), both Nrf1 degradation and processing probably also occur under normal conditions, but with reduced proteasome function, processing is favored.

A variety of observations support this model, especially our demonstration that some proteasome function is essential for Nrf1 processing and its translocation into the nucleus. Specifically, BTZ most efficiently induced Nrf1 processing and 26S subunit expression at low concentrations that only partially block the chymotrypsin-like site and cause only a minor (<40%) decrease in proteolysis (Figures 2F–2H and 3D). By contrast, higher concentrations that completely block this site and also cause a large (>50%) inhibition of the caspase-like site and protein breakdown inhibit Nrf1 processing, nuclear entry, and 26S induction. Thus, although active proteasomes tend to degrade Nrf1 completely, partially inhibited 26S subunits no longer digest ubiquitinated Nrf1 processively and tend to release partially digested Nrf1, which activates transcription. When 26S function is blocked to a greater extent, the inactive Nrf1 precursor is stabilized, and the expression of 26S subunits and p97 is blocked.

The precise mode of processing of ubiquitinated Nrf1 by the partially inhibited 26S subunit remains uncertain. Although the N-terminal HA tag on Nrf1 and the transmembrane segment were not detected after they were cleaved from HA-Nrf1, these observations do not distinguish whether Nrf1 is partially digested from its N terminus or, as seems more likely, proteasomal cleavage is initiated from an internal loop near the transmembrane segment, followed by degradation of the N-terminal part, perhaps by an ER-associated peptidase (e.g., signal peptidases). Whereas 26S generally degrade proteins in a processive manner, incomplete degradation beginning from one end or from an internal loop and release of large fragments are often observed with isolated proteasomes [42]. Perhaps simply the slowing of proteasomal function, as occurs with low concentrations of inhibitors, enhances the probability of release of Nrf1's C-terminal part. The lack of processivity leading to its release may depend on the tightness of folding of its different domains, the direction of translocation into 20S [42], the site(s) of ubiquitination, and the length or number of Ub chains on the substrate. Three E3s have been reported to act on Nrf1: HRD1, an ER-associated E3; β-TRCP, a nuclear E3 that probably catalyzes degradation of the mature Nrf1; and Fbw7, which acts on multiple growth-related proteins [13, 14]. Exactly how these E3s (or others) influence the complete degradation or processing of Nrf1 is an important issue for future research.

This mechanism (Figure 7C) for Nrf1 maturation resembles the 26S-catalyzed processing of inactive precursors by proteasomes and p97 to three other transcription factors: NF-κB [34], Gli [35], and the ER-bound Spt23 in yeast [36]. These other examples, like Nrf1 processing, require precursor ubiquitination and ATP. In these respects, Nrf1 resembles a typical ERAD substrate. Not surprisingly, p97 is important for Nrf1 degradation by fully active proteasomes [10], as well as its processing by partially inhibited 26S. Nrf1 may translocate across the ER membrane several times [29]. This complex topology should block the release of Nrf1 from the ER. Given that p97 activity allows removal of these glycosyl chains (Figures 7A and S6B), the p97 complex probably extracts most of Nrf1's C-terminal part from the ER, so that the subsequent N-terminal proteolytic processing can release Nrf1. After the present studies were completed, Deshaies and colleagues reported studies of Nrf1's ER topology based on susceptibility to protease K [31], and they also concluded that Nrf1's C-terminal part is initially localized in the ER lumen and was relocated into the cytosol by p97. We further demonstrated that after Nrf1 is deglycosylated and its C-terminal region relocated to the cytosol by p97, Nrf1 needs to be ubiquitinated again in order to be processed. Thus, p97 functions differently here than in Spt23 processing, wherein p97 seems to extract Spt23 from the ER after it has already been processed by the proteasome [36]. Because p97 is required for Nrf1 processing, p97 inhibition, like high concentrations of proteasome inhibitors, suppressed rather than activated the transcription of 26S genes. Because the p97 inhibitors have potential as

chemotherapeutic agents [38], it is noteworthy that they do not elicit the compensatory production of more 26S.

The finding that partial proteasome inhibition triggers the induction of new 26S has clear implications for multiple myeloma therapy. Because this compensatory response promotes cancer survival [9–11] and increases the chances of drug resistance, the optimal treatment regimen should be one that inhibits multiple active sites and achieves more complete inhibition of proteasomes for longer periods. For the optimization of drug administration, monitoring Nrf1 processing may be a useful biomarker for evaluating the efficacy of proteasome inhibition and rates of new particle production. Further understanding of the mechanisms for Nrf1 processing may also indicate ways to block new proteasome production for improvement of the treatment of cancer, or conversely, ways to enhance Nrf1 processing to promote clearance of misfolded proteins in neurodegenerative diseases.

Experimental Procedures

Detailed experimental procedures are presented in the Supplemental Information.

Gene Overexpression or Knockdown

Lipofectamine 2000 (Life Technologies) was used to transfect plasmids into HEK293A cells. Stable knockdowns in SH-SY5Y cells were performed by infection with lentiviral particles expressing shRNA and puromycin-resistant marker.

Immunostaining

Transfected cells were seeded on coverslips in 6-well plates. Treated cells were fixed with −20°C methanol. Images were taken at a Nikon Ti inverted fluorescence microscope.

Real-Time RT-PCR

mRNA was extracted via TRIzol Reagent (Life Technologies), and cDNA was synthesized using MultiScribe Reverse Transcriptase (Applied Biosystems). Real-time RT-PCR was performed using ABsolute Blue qPCR ROX Mix (Thermo Scientific) on a Bio-Rad C1000 thermal cycler.

Assay of Proteasome Function and Protein Degradation

Proteasomal peptidase activities were measured as described previously [28]. Native gels and overlay assay with Suc-LLVY-AMC were conducted as described [43]. The degradation rate of long-lived cell proteins was measured after labeling with L-Phe-[3,4,5-^{3}H] (American Radiolabeled Chemicals) as described [44].

Supplemental Information

Supplemental Information includes six figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2014.06.004.

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