Proteasome Structures Affected by Ionizing Radiation

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Abstract

Exposure of cells to ionizing radiation slows the rate of degradation of substrates through the proteasome. Because the 26S proteasome degrades most short-lived cellular proteins, changes in its activity might significantly, and selectively, alter the life span of many signaling proteins and play a role in promoting the biological consequences of radiation exposure, such as cell cycle arrest, DNA repair, and apoptosis. Experiments were therefore undertaken to identify the radiation target that is associated with the proteasome. Regardless of whether they were irradiated before or after extraction and purification from human prostate cancer PC3 cells, 26S proteasomes remained intact but showed a rapid 30% to 50% dose-independent decrease in their three major enzymatic activities following exposure to 1 to 20 Gy. There was no effect on 20S proteasomes, suggesting that the radiation-sensitive target is located in the 19S cap of the 26S proteasome, rather than in the enzymatically active core. Because the base of the 19S cap contains an ATPase ring that mediates substrate unfolding, pore opening, and translocation of substrates into the catalytic chamber, we examined whether the ATPase activity of purified 26S proteasomes was affected. In fact, in vitro irradiation of proteasomes enhanced their ATPase activity. Furthermore, pretreatment with low concentrations of the free radical scavenger tempol was able to prevent both the radiation-induced decrease in proteolytic activity and the increase in ATP utilization, indicating that free radicals are mediators of these radiation-induced phenomena. Finally, we have shown that cell irradiation results in the accumulation of proteasome substrates: polyubiquitinated proteins and ornithine decarboxylase, indicating that the observed decrease in proteasome function is physiologically relevant. (Mol Cancer Res 2005;3(7):381–90)

Introduction

The levels at which a protein is expressed in a cell is determined by a balance between rate of production and rate of degradation, alterations in either of which, if it is a regulatory protein, could critically affect cellular physiological processes. In recent years, proteasomes, which are large, cylindrical, multicatalytic enzymatic complexes that are ubiquitous in eukaryotes (1-3), have taken center stage in intracellular proteolysis as they have increasingly been shown to be responsible for degradation of most native regulatory proteins, as well as those misfolded and damaged. Proteasomes can degrade proteins in an ATP-independent manner or an ATP-dependent manner. The former primarily targets damaged proteins and peptides, whereas the latter is part of the ubiquitin-dependent 26S proteasome pathway that functions to control expression of most native proteins. The 26S proteasome therefore closely regulates short-lived proteins that mediate basic physiological processes such as cell cycle progression (cyclins A, D, and E, p53, p21, p27 mdm2, HIF-1α), DNA transcription (IκB/nuclear factor κB, c-myc, c-Jun, c-fos, AP-1, STAT-1), DNA repair (DNA-PKcs, rad 23), apoptosis (p53, p21, mdm2, bcl2, bax, caspase-3), inflammation and immunity (IκB/nuclear factor κB, tumor necrosis factor-R1), and cell growth (epidermal growth factor receptor, insulin-like growth factor receptor, and platelet-derived growth factor receptor; refs. 4-13). Proteasomes have therefore evolved to perform not only catabolic but also regulatory functions.

All proteasomes have a common 20S core that has the proteolytically active sites internally sequestered. Four septa-mer rings are arranged symmetrically so that two outer rings of α-type subunits form an antechamber and two inner rings of β-type subunits form the proteolytic chamber. Only three, β5 (X), β1 (Y) and β2 (Z), of the seven different β-type subunits are proteolytically active, being associated with the three major activities of the proteasome, namely chymotryp- sin-like activity (cleaving after the COOH-terminal of hydrophobic residues), trypsin-like activity (cleaving after basic residues), and peptidylglutamyl-hydrolyzing activity (PGPH, cleaving after acidic residues), respectively. Core proteasomes exist only in a latent state and need to be “activated” by binding either a 19S regulatory complex or an 11S activator to the α-type rings (14, 15).

The 19S regulator is comprised of about 17 distinct subunits with ATPase subunits in a base adjacent to the core and a lid comprised of non-ATPase subunits. The functions of the lid complex are not fully elucidated but it seems to be involved in substrate recognition and binding (16-18), whereas ATPases are, in addition, responsible for processes relating to pore opening, gating of the translocation channel, substrate unfolding and transfer of unfolded proteins to the enzymatically active sites (19-22). The 11S hexameric activator performs similar functions in an ATPase-independent manner. It is
composed of two homologous but not identical subunits, PA28α and PA28β (23) arranged in a ring (24). IFN-γ stimulates expression of the 11S activator (15, 25) and substitution of the X, Y and Z constitutive subunits of the 20S core by LMP7, LMP2 and MECL-1 (26). This substitution forms what is often called the “immunoproteasome” because it alters substrate cleavage specificity so as to enhance the production of antigenic peptides suitable for presentation by MHC class I molecules (27).

When making rapid responses to a changing environment or other external stresses, a cell initially relies on posttranscriptional control mechanisms. Because of its position as a “master controller” of protein degradation, the proteasome is a good candidate for affecting a switch of cells from nonstressed to stressed states. Reports that proteasome function is altered following exposure of cells to ionizing and UV radiation, heat shock, or oxidative stress are consistent with this hypothesis (28-30). We have shown that exposure of cells to doses of ionizing radiation ranging from 25 Gy to 20 Gy slows the rate at which the proteasomes degrade small fluorogenic peptides specific for chymotrypsin-like activity by 30% to 50% (31). Radiation is “alarming” to cells and activates mechanisms leading to rapid cell cycle arrest, DNA repair, and cell death or survival. At least initially it seems likely that these responses are controlled posttranscriptionally and involve alterations in mRNA and protein stability. This study was designed to identify the proteasomal targets that are affected by radiation and mediate the slowing of the rate of substrate degradation, which is critical for our understanding of how cells respond to radiation challenge.

Results

Pajonk et al. (31, 32) previously described slowing of the rate of degradation of substrates specific for proteasome chymotrypsin-like activity following exposure to ionizing radiation that was independent of dose between 25 cGy and 20 Gy. To determine if all major enzymatic activities were affected, the rate of degradation of fluorogenic peptides specific for chymotrypsin-like, trypsin-like, and PGPH, as well as for tripeptidyl peptidase II, activity was assessed in crude extracts 3 hours after radiation doses ranging from 0.5 to 20 Gy (Fig. 1A). A 30% to 50% slowing of the rate of degradation of substrates specific for the proteasome, but not for tripeptidyl peptidase II, indicated that inhibition was limited to the proteasome, but not to any specific enzymatic activity associated with the proteasome.

The dynamics and the duration of inhibition induced by ionizing radiation were assessed by measuring chymotrypsin-like activity of crude PC3 cellular extracts at different time

FIGURE 1. Radiation-induced inhibition of proteasome activity. A, PC3 cells were irradiated with different doses (0.5-20 Gy) and harvested at 3 hours postirradiation. Proteasome activities (chymotrypsin-like, trypsin-like, and PGPH) and tripeptidyl peptidase II activity were assayed using appropriate fluorogenic peptides as described in Materials and Methods. B, Time response: cells were irradiated with 10 Gy, harvested at different times thereafter and extracts were assayed for chymotrypsin-like activity. Asterisks, significant differences as calculated by Student’s t test; *, P < 0.025, n = 6. C, Proteasome content in PC3 cells following irradiation: crude extracts from irradiated (10 Gy) and control cells were harvested at indicated times, separated by 10% SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, and immunoblotted with monoclonal antibodies raised against 20S proteasome subunit α6, 11S activator subunit α (PA28 α), and 19S regulator non-ATPase subunit Rnp8 (S12). Equal loading was confirmed by reblotting with anti-α-tubulin.
Ionizing radiation generates free radicals that mediate many of its effects. To test if free radicals mediated radiation-induced proteasome inhibition and ATPase changes, two free radical scavengers were used—glutathione (GSH), an endogenous cellular antioxidant, and 4-hydroxy-tempo (tempol), a low-molecular weight nitroxide compound with antioxidant activity—both of which protect cells against oxidative stress caused by ionizing radiation (33, 34). Both, tempol and GSH, inhibited proteasome activity at high doses (Fig. 4A), but only minimally at 10 and 5 mmol/L, respectively. These concentrations were, however, able to prevent proteasome inhibition caused by irradiation (Fig. 4B) indicating involvement of free radicals in this radiation effect. Furthermore, 10 mmol/L tempol was able to prevent the increase in ATP usage by the 26S proteasome (Fig. 4C), suggesting that radiation-induced free radicals also affected ATPase activity. GSH was not tested because it interfered directly with luciferase activity (data not shown).

To determine if the effect of radiation on the 26S proteasome had physiological relevance, we assayed levels of ubiquitinated proteins following cell irradiation. Because ATP-dependent 26S proteolysis in cells is coupled with the ubiquitination process through removal of ubiquitinated substrates (35), a decline in 26S proteasome function should impair degradation of ubiquitinated proteins. To test this, cellular extracts from irradiated and control PC3 cells were precipitated using agarose-immobilized p62-derived ubiquitin-binding domain, separated on SDS-PAGE (10-20% gradient) and immunoblotted with antitubulin antibody. As shown in Fig. 5A and B, ionizing radiation induced a 50% increase in the cytosolic levels of ubiquitinated proteins, suggesting that radiation directly affects processing of proteasome intracellular substrates through the ubiquitin/26S proteasome system.

We further tested the effect of irradiation on degradation of the 26S proteasome substrate ornithine decarboxylase (ODC), which does not require ubiquitination (36). This was done in living cells using an ODC-ZsGreen protein (ZsG) fusion protein reporter system. PC3 cells were transiently transfected with ODC-ZsG. Flow-cytometric analysis detected accumulation of the fusion protein 5 hours following irradiation.

**Discussion**

In recent years, a picture is emerging of the proteasome as a master controller of multiple signal transduction pathways whose activity is affected by a variety of agents including oxidative stress (37), heat shock (38), ionizing radiation (39), UV radiation (28), chemotherapeutic drugs (40), viruses (41-43), and carcinogens (44). This is replacing the previous held view that the proteasome was simply a disposal mechanism for unwanted proteins that played little role in cellular regulation. Here we confirm our previous observation that chymotrypsin-like activity of the proteasome is partially inhibited over a wide range of radiation doses and further show that trypsin-like and PGPH activities of the proteasome are similarly affected. This suggests a global effect on proteasome function in the cell, even though the points over a 24-hour period after exposure to 10 Gy. Radiation-induced slowing of the rate of substrate degradation occurred within 15 minutes postirradiation. The activity recovered slowly with time and was normal at 24 hours (Fig. 1B). To ensure that the proteasome content was not altered following cell irradiation, extracts of PC3 cells were separated by SDS-PAGE 1 and 3 hours following 10 Gy irradiation and immunoblotted with monoclonal antibodies against the 20S proteasome subunit α6, the 11S activator subunit α (PA28α), and the 19S proteasome regulator non-ATPase subunit Rnp8 (Fig. 1C). Irradiation did not alter proteasome expression levels over the 3-hour time period.

Because all three proteasome enzymatic activities were diminished by irradiation, experiments were undertaken to determine if 20S core activity was affected. Cells were irradiated with 0 or 10 Gy and 20S and 26S proteasomes were separated from crude extracts by velocity sedimentation centrifugation using continuous 10% to 40% glycerol gradients. 26S proteasomes were detected in fractions 15 to 18 by degradation of fluorogenic substrates specific for chymotrypsin-like activity in the presence of 2 mmol/L ATP (Fig. 2A). 20S core particle activity was activated by the addition of 0.05% SDS, which also inactivates 26S proteasomes, and was found in fractions 4 to 7. To determine if irradiation directly influenced proteasome function, fractions containing the 26S and the 20S proteasomes were pooled, total protein concentration was equalized, and half the unirradiated samples were irradiated in vitro with 10 Gy. Irrespective of whether the proteasomes had been irradiated prior to or after extraction from cells, the rate of degradation of fluorogenic substrate by 26S proteasomes was slowed by the same extent, whereas 20S proteasome activity was not affected (Fig. 2B). Furthermore, in vitro irradiation of 26S proteasomes that were purified from irradiated cells did not further increase the extent of inhibition, which was similar over the dose range 1 to 20 Gy (Fig. 2C). The finding that the activity of the 20S enzymatic core particle was resistant to irradiation indicated that the radiation target(s) was not within proteolytically active sites but resided in the 19S cap associated with the 26S structure. To confirm that the 26S purified particles were intact after irradiation, they were resolved by native gel electrophoresis followed by functional staining with 100 mmol/L fluorogenic peptide LLVY-7-amido-4-methylcoumarin (AMC) and protein staining with Coomassie blue. There was no change in the protein level associated with the functional 26S proteasome band after direct proteasome irradiation and no evidence of the presence of free 19S or 20S fractions (Fig. 3A), indicating that the proteasome structure remained intact.

We next considered the possibility that radiation induced structural or functional changes within the subunits of the 19S regulator and specifically that the base of the 19S regulator consisting of six ATPase subunits, which is responsible for opening the 20S proteasome and translocation of substrates into the catalytically active core, might be a target. Proteasome ATP utilization was assessed following irradiation using a chemiluminescent luciferase assay to measure residual ATP. As shown in Fig. 3B, proteasome ATP utilization increased following irradiation, as proteolytic activity declined.
sites of the three enzymatic activities seem to operate fairly independently, as is seen by the finding that the proteasome inhibitor PS-341 completely inhibits chymotrypsin-like activity and only partially affects PGPH activity, whereas trypsin-like activity remains unaffected.1

We have shown that the radiation effect is limited to the 26S proteasome, whereas 20S structures remain active, indicating that the proteolytic sites are not targets for radiation. There are reports that the 26S proteasome is more sensitive than the 20S core to UV radiation, low dose H2O2, as well as to N-acetyl cysteine (28, 45, 46). It is tempting to hypothesize that slowing the rate of degradation of regulatory proteins through the 26S proteasome initiates a stress alarm in the cell, while continuing to

1 Pervan, unpublished observations.
allow removal of damaged, potentially toxic proteins through non-26S proteasomes, although this potential division of labor between different proteasome species may be an oversimplification of the situation as some proteasome functions overlap.

The effects of radiation on the activity of purified enzymes are well known and it requires massive doses well above those used in this study to inactivate them (47, 48). This is confirmed by the finding that we didn’t observe any significant alterations in tripeptidyl peptidase II/tricorn hydrolyzing activity. These giant self-compartmentalized proteases have been reported to substitute for loss of proteasome function in lymphoma cells adapted to grow in the presence of proteasome inhibitors (49, 50). The suggestion is therefore that the abnormally high sensitivity of the proteasome to even low doses of radiation is specific and unique.

As with the response to other stress stimuli, the inhibition of proteasome activity by ionizing irradiation is very rapid, probably immediate. Activity recovers within 24 hours. The duration of inhibition seems different for different stressors. For example, inhibition of proteasome activity in human keratinocytes induced by UVA- and UVB-irradiation was much more pronounced at 24 hours than 1 hour postirradiation and lasted at least for 96 hours (28). Proteasome activity in human leukemia K562 cells partially recovers 24 hours after treatment with 1 mmol/L H2O2 but not after 2 mmol/L H2O2 (38), suggesting that the level of oxidative stress may determine the rate of proteasome recovery. It may be particularly prolonged after exposure to agents that cause substantial damage to proteins. This makes even more striking the finding that doses of ionizing radiation that would be expected to cause only perhaps 10,000 ionization events in a cell are as effective as a dose 100-fold larger at causing proteasome inhibition.

Even more surprising is the finding that we were able to observe a direct effect of radiation on purified proteasomes, suggesting a chemical rather than biological mechanism of inhibition. This chemical mechanism was mediated through free radicals, which was shown by the addition of free radical scavengers, tempol and GSH. Because these two drugs are often used in radiation experiments, it is noteworthy to mention that changes in redox status by addition of the higher doses of either of the two free radical scavengers used in this study also impaired proteasome activity. This observation was in agreement with previous evidence that alterations in intracellular glutathione metabolism affect proteasome function (51) as does N-acetyl cysteine (46). The implication is that proteasome function is highly sensitive to redox changes and that the proteasome is an exquisitely sensitive sensor of stress that responds rather like a switch to initiate rapid molecular reprogramming.

We present evidence that the targets for radiation are located in the 19S proteasome regulator or at the 19S and 20S junction. Lack of a radiation effect on the core particle excludes the possibility that proteolytically active sites are the sensitive proteasome components. Several years ago, Glickman showed that a complex formed by the 20S proteasome and the base of the 19S regulator is sufficient for ATP-dependent degradation of nonubiquitinated substrates, but it is insufficient for degradation of ubiquitinated proteins (52). The fluorogenic peptide substrates used in our experiments do not require ubiquitination; their degradation depends on activation of the core particle which is a role played by the ATPase subunits. We found that proteasome ATPase activity was increased following irradiation, an effect that was prevented by addition of free radical scavengers. Thus, redox changes in the proteasome ATPase environment modify ATPase activity in a manner that decreases proteolytic processing but increases ATP uptake. Recently, ATP hydrolysis has been linked to proteasome disassembly during substrate proteolysis (53). This does not seem to happen following irradiation in vitro, possibly because of the absence of substrate degradation in our experimental setup.

FIGURE 3. Radiation does not cause 26S proteasomes to dissociate but does affect proteasome ATPases. A. Glycerol gradient fractions containing 26S proteasome were irradiated with 0, 10, and 50 Gy, and separated by nondenaturing PAGE. The position of the 26S fraction was identified by overlaying the gel with fluorogenic substrate in 100 nmol/L in buffer I (right) and then the gel was stained with Coomassie blue (left) showing that the 26S proteasome did not disassociate. B. 26S gradient purified proteasomes were irradiated with 0 or 10 Gy and incubated at 37°C with varying concentrations of ATP. For the standard curve, the same volume of buffer I was added to each sample. The remaining, unused ATP was determined after 15 minutes by addition of luciferase/luciferin reaction. Emitted light was measured immediately using the chemoluminometer.
Presently, very little is known about proteasome ATPases. However, it seems likely that the three-dimensional structure is crucial for proper function of this dynamic complex. Parallel arguments can be drawn between the proteasome and other ATPase-regulated systems, such as the Na⁺/K⁺ pump, in which minor structural alterations cause functional inactivation of the pump (54). Our hypothesis is that small conformational changes in the proteasome subunit tertiary structure caused by redox imbalance inactivates a “clutch” mechanism(s) that couples ATP hydrolysis to substrate translocation or gate opening, resulting in a “revving engine” with no accomplishment of useful work. However, future studies are needed to test this hypothesis.

Radiation slows degradative rate—it does not stop it completely, at least as assessed using fluorogenic substrates, and this opens the question of whether such an effect is biologically relevant. In support of the fact that this is relevant, we show that partial inhibition is sufficient to affect the intracellular molecular profile as there is a general increase in the level of ubiquitylated proteins and accumulation of the 26S proteasome substrate ODC. The contention is further supported by the findings of others showing rapid alterations in stability of many proteins that are cleared by the proteasome such as p53, p21, and IκB (55, 56) following irradiation, which has obvious implications for radiation-induced cellular responses. However, many of these stress proteins are controlled by complex molecular interactions and their expression may not depend only on proteasome degradation. For example, we have shown that expression of IκB follows a biphasic pattern (56). It was enhanced at low doses of radiation, as would be expected if proteasome function was inhibited, and was not decreased after higher doses which activate nuclear factor κB. Therefore, partial proteasome inhibition seems to be a state within which radiation-induced transcriptional responses take place in an interactive manner.

The longstanding concept of the proteasome as a robust nonspecific degradation machine is rapidly changing. Recent advances in the field imply critical involvement of proteasome in the regulation of many aspects of cellular physiology. Proteasomes show high sensitivity to minimal redox changes caused by irradiation (and other stress stimuli) implying that this pathway should be perceived as a sophisticated and highly sensitive stress sensing mechanism that can rapidly and simultaneously orchestrate multiple cellular processes following exposure to radiation. However, a number of important

**FIGURE 4.** Free radical scavengers prevent radiation-induced changes in proteasome activity. **A.** Effect of free radical scavengers on proteasome function. Dose-response curves for treatment of proteasomes with tempol and GSH were generated by measuring chymotrypsin-like activity with Suc-LLVY-AMC. **B.** 26S gradient purified proteasomes were irradiated (10 Gy) in the presence of 10 mmol/L tempol and 5 mmol/L GSH and chymotrypsin-like activity was determined. Asterisks, significant differences from control, as calculated by Student’s t test; *, P < 0.025; n = 3. **C.** 26S gradient purified proteasomes were irradiated with and without 10 mmol/L tempol, incubated 15 minutes with varying concentrations of ATP, and the amount of the remaining ATP was determined by the addition of luciferase/luciferin. Emitted light was measured immediately using the chemoluminometer.
structural and functional questions regarding the proteasome as a sensor of radiation effects remain unanswered and answers to some of these questions could reshape our current understanding of events involved in cellular radiobiology.

**Materials and Methods**

**Cell Culture**

Human prostate carcinoma PC3 cells (obtained from American Type Culture Collection, Manassas, VA) were maintained at 37°C and 5% CO₂ in DMEM (Mediatech, Inc., Herndon, VA) supplemented with 10% FCS (Sigma-Aldrich, Inc., St. Louis, MO) and 1% antibiotic-antimycotic (Mediatech). Cells were used at 70% confluence, with medium changed 1 day prior to experimentation.

**Drugs, Peptides, and Antibodies**

Fluorogenic peptides for proteasome chymotrypsin-like activity: Suc-LLVY-AMC (Sigma), trypsin-like activity: Z-ARR-AMC (Calbiochem, San Diego, CA), PGPH activity: Z-LLE-AMC (Sigma), and tricorn peptidase (tripeptidyl

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**FIGURE 5.** Levels of proteasome substrate changes following radiation. A. Ubiquitinated proteins from PC3 cells were purified by agarose-immobilized p62-derived ubiquitin-binding domain, separated by SDS-PAGE (10-20%), and immunoblotted with anti-ubiquitin antibody (clone FK1). Radiation increased the level of polyubiquitinated proteins. B. Quantified data from (A) are expressed as a percentage of control. C. Expression of ODC-ZsG in unirradiated and irradiated (2 Gy) transiently transfected PC3 cells after 5 hours as assayed by flow-cytometry. Radiation increased the level of ODC-ZsG protein.
peptidase II) activity: 2-acetylaminofluorene-AMC were dissolved in DMSO as 100 mmol/L stock solutions and stored at −20°C in 200 mL aliquots. Free radical scavengers tempol (Sigma) and GSH (Sigma) were freshly prepared for each experiment and diluted with DMEM. Monoclonal antibodies against the 20S proteasome subunit α6, the 11S activator subunit α (PA28 α) and the 19S proteasome regulator non-ATPase subunit Rnp8 (Affinity Research Products, Ltd., Exeter, United Kingdom) were aliquoted and stored at −20°C until used.

Cell Irradiation

Exponentially growing cells were washed with HBSS (Mediatech), detached from plates with trypsin-EDTA solution (Mediatech) at 37°C, washed in media, and counted in a hemocytometer. Irradiations were done in tubes using a 137Cs-iradiator at a dose rate of about 5 Gy/min. Purified proteasomes were irradiated on ice and cells at room temperature.

Preparation of Crude Extracts

Following drug and/or radiation treatment, cells were washed with ice-cold PBS, and buffer I [50 mmol/L Tris-HCl (pH 7.5), 5 mmol/L MgCl₂, 2 mmol/L DTT, 2 mmol/L ATP], gently scraped off the plates and lysed by three freeze-thaw cycles (using liquid nitrogen) in the presence of homogenization buffer [50 mmol/L Tris-HCl (pH 7.5), 5 mmol/L MgCl₂, 1 mmol/L DTT, 2 mmol/L ATP, 250 mmol/L sucrose]. Samples were clarified by centrifugation at 4°C for 5 minutes at 10,000 × g followed by 25 minutes at 10,000 × g. Supernatants were collected and kept on ice until used.

Proteasome Purification

20S and 26S proteasomes were separated using 10% to 40% glycerol gradients prepared with buffer I. Crude extract containing 1 mg of total protein was applied to the top of the gradient and centrifuged at 100,000 × g for 18 hours at 4°C. Fractions of 400 mL were taken starting from the top of gradient. 20S proteasomes were activated using 0.05% SDS. 26S proteasomes were maintained and assayed in the presence of 2 mmol/L ATP. The presence of proteasomes was indicated by fluorogenic assay using Suc-LLVY-AMC as described below. The protein concentration of each fraction was determined using micro-BCA kit (Pierce, Rockford, IL) according to the manufacturer’s protocol.

Fluorogenic Assay

Chymotryptsin-like, trypsin-like, and PGPH activity of the proteasome and tricorn protease activity were determined by their ability to degrade appropriate fluorogenic substrates, as described before (49). Briefly, serial dilutions of samples starting at 100 μg/mL were made in buffer I with a final volume of 100 mL and placed into 96-well black plate (Corning Inc., New York, NY). Fluorogenic substrate was added to a final concentration of 100 μmol/L and the fluorescence of the released AMC group was measured over a 30-minute period at 37°C at 360/430 nm in kinetic mode making one reading every minute.

Precipitation of Ubiquitinated Proteins

Ubiquitinated proteins were precipitated using agarose-immobilized, p62-derived ubiquitin-binding domain (Affinity Research Products). PC3 cells were lysed in ice-cold lysis buffer [20 mmol/L Tris-HCl (pH 7.4), 1% NP40, 137 mmol/L NaCl, 50 μmol/L EDTA, 1 mmol/L PMSF, 1 μmol/L pepstatin A, 1 μmol/L leupeptin, and 5 mmol/L N-ethylmaleimide] for 20 minutes at 4°C with occasional rocking. Cells were further disrupted by passage through a 21-gauge needle. Extracts were spun in the microcentrifuge at 20,000 × g for 10 minutes at 4°C and the supernatant was transferred into a fresh tube. Protein concentration was determined using the bicinchoninic acid kit, as described earlier. Cell lysates (protein, 1 mg/mL) were incubated with 25 μL of washed ubiquitin binding domain–agarose beads (peptide, 2 mg/mL). Following incubation at room temperature for 20 minutes with occasional agitation, unbound proteins were removed and beads were washed thrice with 50 mL of Tris, 0.1% bovine serum albumin (pH 7.5). Bound proteins were eluted in 50 μL of the sample buffer [62.5 mmol/L Tris-HCl (pH 6.8), 2% SDS, 25% glycerol, 0.01% bromophenol blue, 14.4 mmol/L β-mercaptoethanol] and heated to ≥95°C for 4 to 5 minutes. Samples were centrifuged at 12,000 × g for 20 seconds to pellet the beads and supernatants were carefully removed. If not used immediately, samples were frozen at −20°C for later use.

Western Immunoblotting

Crude cell extracts were prepared in lysis buffer [20 mmol/L Tris-HCl (pH 7.4), 1% NP40, 137 mmol/L NaCl, 50 μmol/L EDTA, 1 mmol/L PMSF] supplemented with a cocktail of protease inhibitors (Sigma). Lysates were centrifuged at 20,000 × g for 10 minutes at 4°C and resulting supernatants were assayed for total protein using micro-BCA (Pierce). Twenty to 30 μg of total protein was diluted with Laemmli sample buffer (Bio-Rad, Richmond, CA) supplemented with 5% β-mercaptoethanol and separated by 10% SDS-PAGE. The proteins were transferred onto poly(vinylidene difluoride) membrane (Bio-Rad), blocked in 5% nonfat dry milk, and probed with appropriate primary and peroxidase-conjugated secondary antibodies. Protein bands were detected with enhanced ECL. Plus chemiluminescence detection solution (Amersham Life Science Inc., Piscataway, NJ) and blue chemiluminescence was detected using the dual-fluorescence PhosphorImager Storm 860 (Molecular Dynamics, Amersham).

ATPase Activity Assay

ATPase usage by purified 26S proteasomes was determined using an ATP determination kit (Molecular Probes, Eugene, OR). Ten microliters (1 μg) of irradiated and nonirradiated proteasomes were incubated for 15 minutes with 100 μL of varying concentrations of ATP diluted in modified buffer I [50 mmol/L Tris-HCl (pH 7.5), 5 mmol/L MgCl₂] and residual activity assayed. A standard curve was prepared using dilutions of ATP with 10 μL of buffer added in place of proteasomes. The reaction was started by mixing 80 μL solution [0.5 mmol/L α-luciferin, 1.25 μg/mL firefly luciferase, 25 mmol/L tricine buffer (pH 7.8), 5 mmol/L MgSO₄, 100 μmol/L EDTA and
1 mmol/L DTT] with 20 μL of proteasome/ATP preparations for 10 seconds followed by luminescence detection in a luminometer. Background luminescence was subtracted from all samples.

**ODC-ZsGreen Construct and Transfections**

PC3 cells were transiently transfected with pZsProsensor-1, a eukaryotic expression vector designed to express ZsGreen fused to the mouse ornithine decarboxylase degradation domain (BD Biosciences Clontech, Palo Alto, CA). For optimization of transfection conditions, the pEGFP-NI vector encoding a red-shifted variant of a wild-type Green Fluorescent Protein (BD Biosciences Clontech) was used. Expression of both vectors is driven by a CMV promoter.

Transfections were done using a lipid carrier, TFX-20 reagent (Promega, Madison, WI), according to the manufacturer’s protocol. Briefly, cells were plated 1 day before transfection at ~70% confluency. Optimization of the DNA/TFX ratio was done prior to the first transfection using pEGFP reporter gene and it was found to be 1:2 for PC3 cells. For each TFX ratio was done prior to the first transfection using pEGFP-NI vector encoding a red-shifted variant of a wild-type Green Fluorescent Protein (BD Biosciences Clontech) was used. Expression of both vectors is driven by a CMV promoter.

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**Flow Cytometry**

For flow cytometry, ZsGreen-producing or control cells were harvested by trypsinization, washed with PBS and fluorescence was detected with a FACSCalibur machine (Becton Dickinson, San Jose, CA). Data analysis was done using CellQuest software.

**References**


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