cDNA Cloning, Expression, and Functional Characterization of PI31, a Proline-rich Inhibitor of the Proteasome*

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The primary structure of PI31, a protein inhibitor of the 20 S proteasome, was deduced by cDNA cloning and sequencing. The human protein has a calculated molecular weight of 29,792, a value in excellent accord with 31,000, as estimated by SDS-polyacrylamide gel electrophoresis for purified bovine PI31, and is not similar to any other protein in current data bases. PI31 is a proline-rich protein, particularly within its carboxy-terminal half where 26% of the amino acids are proline. Wild-type PI31 and various truncation mutants were expressed in Escherichia coli and purified to homogeneity. Recombinant wild-type PI31 displayed structural and functional properties similar to those of PI31 purified from bovine red blood cells and inhibited the hydrolysis of protein and peptide substrates by the 20 S proteasome. Analysis of truncation mutants demonstrated that proteasome inhibition was conferred by the carboxy-terminal proline-rich domain of PI31, which appears to have an extended secondary structure. Inhibition of the 20 S proteasome by PI31 involved formation a proteasome-PI31 complex. In addition to its direct inhibition of the 20 S proteasome, PI31 inhibited the activation of the proteasome by each of two proteasome regulatory proteins, PA700 and PA28. These results suggest that PI31 plays an important role in control of proteasome function, including that in ubiquitin-dependent pathways of protein degradation.

The proteasome is a 700,000-dalton protease found in all eukaryotes and in some bacteria and archaebacteria. The enzyme is composed of 28 subunits arranged in four heptameric rings stacked upon one another to form a cylinder-shaped particle (1–4). In proteasomes from the archaebacterium Thermoplasma acidophilum, the subunits represent two homologous gene products, α and β, each present in 14 copies per particle (5–7). In eukaryotes, proteasomes represent 14 different gene products, 7 of which are homologous to the archaeabacterial α subunit and 7 of which are homologous to the archaeabacterial β subunit (3, 8). Despite such differences in subunit diversity, all proteasomes have similar overall architecture and subunit topology; the two outer rings are composed of α subunits and the two inner rings are composed of β subunits (6, 9). Each α and β ring of eukaryotic proteasomes contains a complete complement of the seven respective gene products. Thus, all proteasomes are multisubunit dimers that exhibit C2 symmetry about an axis between the two inner rings.

The crystal structures of the proteasome from both archaeobacteria and yeast have been solved recently (10, 11). The structures have confirmed, clarified, and greatly extended knowledge of structural and functional features of the enzyme obtained previously from biochemical and molecular studies. For example, it is now clear that the β subunits contain the catalytic sites. These sites consist of amino-terminal threonine residues which act as the nucleophiles for peptide bond hydrolysis (10–12). In archaeabacteria, all 14 copies of the β subunit are probably active catalysts, whereas in yeast and mammals only six β subunits (i.e. two copies of three different subunits) are likely to be catalytically active. The active sites of the proteasome face an interior chamber formed by the center space of the two abutting β rings. This topology sequesters the active sites from proteins in bulk solution and may have evolved as a means of preventing inappropriate degradation of cellular proteins. The structure of the archaeabacterial proteasome suggests that access of substrates to the catalytic sites is through 13-Å diameter openings formed by the centers of the outer α rings. In eukaryotic proteasomes, this opening is blocked by amino-terminal portions of α subunits, suggesting that the portal may be gated (11).

A likely mechanism for control of substrate access to the proteasome involves binding of regulatory proteins to its terminal α rings. The binding of either of two such proteins, PA28 and PA700, greatly enhances the proteasome’s hydrolysis of substrates (1). Although the precise mechanisms of activation are unclear, these proteins probably induce conformational changes in the proteasome that open the blocked pores. PA28 is a ring-shaped molecule that activates the proteasome’s hydrolysis of short peptides (13–16). This effect may be part of the PA28-dependent mechanism of processing of antigens for presentation by class I major histocompatibility complexes (13, 17, 18). PA700 is a 700,000-dalton multisubunit complex that also activates the proteasome’s degradation of short peptides (1, 19, 20). PA700, however, also activates the proteasome’s hydroly-
sis of ubiquitinated proteins (19, 21, 22). For this activity, opening of pores in the proteasome structure is not sufficient for increased substrate access to catalytic sites because folded proteins are too large to pass through such narrow openings (23, 24). Thus, PA700 may unfold protein substrates prior to or in a mechanism linked with their translocation (25, 26). Because ubiquitin-dependent proteolysis is required for numerous cellular regulatory processes, the interaction between PA700 and the proteasome is of critical physiological importance (27, 28).

In addition to proteasome activators, several protein inhibitors of the proteasome have been described. For example, Etlinger and colleagues have identified and purified two proteins that inhibit the proteasome (29, 30). These proteins have native molecular weights of 240,000 and 200,000 and are homomultimers of 40,000-dalton and 50,000-dalton subunits, respectively. We recently, we identified and purified a 31,000-dalton protein that we now call PI31 (for Proteasome Inhibitor of 31,000 daltons, Ref. 31). This protein inhibits the hydrolysis of small synthetic substrates and large unfolded proteins by the 20 S proteasome. The possible relationships among these various inhibitor proteins, all of which have been isolated from red blood cells by relatively similar methods, have been unclear. We report here the primary structure of PI31, deduced from the sequence of a human cDNA clone, and demonstrate that it is a novel protein with an unusual domain structure. We also report structure-function analysis of PI31 by expression of the wild-type and mutant forms of the protein in Escherichia coli. Finally, we demonstrate that PI31 affects the interaction of the proteasome with PA28 and PA700.

EXPERIMENTAL PROCEDURES

Protein Purification and Sequencing—PI31 (31), 20 S proteasome (32), PA28 (14), and PA700 (20) were purified from bovine red blood cells as described previously. Internal amino acid sequence of bovine PI31 was obtained by automated Edman degradation of tryptic peptides isolated by HPLC,1 as described previously (33).

Isolation of cDNA Clone and DNA Sequencing—A cDNA library was constructed from the poly(A) RNA of human hepatoblastoma HepG2 cells. cDNA was synthesized with a cDNA synthesis kit (Amersham Pharmacia Biotech) using oligo(dT)15 as a primer for synthesis of the first strand of cDNA. The cDNAs were ligated with EcoRI adapter containing a NotI site and then inserted into the EcoRI site of a ZAPII vector (Stratagene) for the construction of the phage library. On the basis of partial amino acid sequences of four tryptic peptides of purified bovine PI31 (see Fig. 1), we selected regions of the sequences (underlined) containing Nde I and HindIII restriction sites. This vector also contains an amino-terminal His6 sequence (His6Tag, MGGSSHHHHHHHSGLYPGSH), Novagen) for affinity purification and a thrombin cleavage site for removal of the His6Tag sequence from the purified fusion protein. The vector also contains a kanamycin resistance marker. Expression of recombinant PI31, E. coli BL21 (DE3) cells (Novagen) were grown overnight in LBkan medium at 37 °C. When the cultures reached an A 600 of 0.6, induction was initiated with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). After three hours cells were harvested by centrifugation, lysed, and sonicated. A soluble extract was prepared by centrifugation at 14,000 × g for 30 min and applied to a nickel-agarose column equilibrated with binding buffer. Bound protein was eluted with a 0–250 mM gradient of imidazole. Eluted fractions were assayed for PI31 activity and immunoreactivity to a PI31 antibody. Fractions containing PI31 were pooled and dialyzed extensively against Buffer H (20 mM Tris-HCl, pH 7.6, 20 mM NaCl, 1 mM EDTA, 5 mM β-mercaptoethanol). In this expression protocol, approximately 12 mg of PI31 were produced per 100 ml of bacterial culture. Typical yields of 95% (10 mg/100 ml of bacterial culture) of purified PI31 were obtained. The PI31 was stored at −20 °C where it was stable indefinitely. As described previously, PI31 protein reacted weakly in a Bradford protein assay, resulting in underestimates of protein concentration by this method (31). Protein concentration was therefore determined by UV absorbance at 280 nm and by quantitative densitometry of samples subjected to SDS-PAGE, stained with Coomasie Blue, and compared with standard proteins of known concentration (Mark 12 standards, Novex).

PCR-based Mutagenesis and Expression in E. coli—PCR methodology was used to produce deletion mutants of PI31 using wild-type PI31 cDNA. Primers were designed to introduce the NdeI and HindIII restriction sites flanking the PI31 deletion sequences for subcloning into the pET-28a (+) expression vector at the NdeI and HindIII restriction sites. Expression of mutant PI31 proteins in E. coli and protein purifications were performed as described above for wild-type PI31.

Production of Antibodies against PI31—Antibodies against bovine PI31 were produced in rabbits. Purified PI31 was subjected to SDS-PAGE, and the protein was visualized by soaking the gel in cold KCl. This region of the gel was isolated, dialyzed extensively against 150 mM NaCl, and then crushed in a small volume of 150 mM NaCl. The crushed material was mixed with an equal volume of Freund’s adjuvant and homogenized with a Polytron® homogenizer (Brinkman Instruments). Initial subcutaneous injections contained 200 μg of PI31 (with complete Freund’s adjuvant) per rabbit. Two booster injections, each consisting of 100 μg of PI31 (with Freund’s incomplete adjuvant) per rabbit, were given 4 and 8 weeks after the initial injections. The rabbits were bled 2 weeks after the second booster injection.

SDS-PAGE and Immunoblotting—SDS-PAGE and immunoblotting were conducted as described previously (31, 32). Immunoblots were developed with an enhanced chemiluminescence kit purchased from Amersham Pharmacia Biotech.

Assay for Proteasome Inhibitory activity by PI31—Functional activity of PI31 was demonstrated by quantitative inhibition of proteasome hydrolysis of either [methyl-14C]casein or small synthetic peptides as described previously (14, 31). Other details for specific assays are described in the figure legends of Figs. 3, 4, 5, and 9. Assays for PA28 and PA700 were as described previously (14, 20).

Glycerol Density Gradient Centrifugation—Glycerol density gradient centrifugation was conducted as described previously with minor modifications (15). Samples were centrifuged at 30,000 rpm for 16 h at 4 °C in a Beckman SW Ti55 rotor. The gradient solution ranged from 10 to 34% glycerol and contained 50 mM Tris-HCl, pH 7.6, 1 mM β-mercaptoethanol in a volume of 4.55 ml. Each tube was divided into 24, 200 μl fractions for subsequent assays.

Circular Dichroism—Circular dichroism spectra of wild-type and mutant forms of recombinant PI31 were recorded from 190 to 260 nm every 1 nm on an Aviv model 62DS spectropolarimeter at 25 °C using a 2.0-mm path length cell. Samples were prepared in 20 mM Tris-HCl, pH 7.6, 20 mM NaCl and 1 mM EDTA at a concentration of 7.8 μM. At this concentration approximately 50% of wild-type PI31 is dimeric.

Analytical Ultracentrifugation—All analytical ultracentrifugation experiments were performed in a Beckman XLI analytical ultracentrifuge. Samples obtained by absorbance measurement using an 86 Ti rotor. Data analysis was carried out using the Beckman Optima XL/XL software, version 4.0 (based on the Origin software, Microlab, Inc.).

Sedimentation equilibrium centrifugation of PI31 was conducted in buffer consisting of 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 1 mM EDTA. Data were collected at 280 nm, 22,000 rpm at 4 °C. Three samples were analyzed with initial absorbances at 280 nm of 0.32

1 The abbreviations used are: HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; Suc, succinyl; AMC, 7-aminomethylcoumarin; IPTG, isopropyl-1-thio-β-D-galactopyranoside.
(channel A), 0.18 (channel B), and 0.10 (channel C). The background absorbances were estimated by overspeeding at 42,000 rpm until a flat baseline was obtained. The baseline values (in optical density) were: 0.047 (channel A); 0.038 (channel B); 0.005 (channel C), and these values were used in the fitting. The data sets were edited according to the intensity scan taken at the start of the centrifugation. The data were analyzed using the Beckman software for various models (monomer alone, monomer/dimer, monomer/trimer, etc.). The equilibrium constants were calculated as monomer/n-mer constants. The quality of the fit was determined from the variance and from the distribution of the residuals. The best fits were those with the most random residuals.

For the purposes of data analysis, the molecular weight was calculated from the sequence 31,575 (for the recombinant human PI31 with His\textsuperscript{z}Tag\textsuperscript{TM}). Confirmation of this value was obtained by mass spectral analysis of the protein. The partial specific volume was calculated to be: 0.725 ml/g at 4 °C and 0.732 ml/g at 20 °C based upon amino acid composition using the program Sedterp, version 1.0. The solvent density was calculated as 1.008 g/ml at 4 °C and 1.006 g/ml at 20 °C, and the solvent viscosity was 1.60 cP at 4 °C and 1.02 cP at 20 °C using Sednterp 1.0. All sedimentation coefficient values were corrected to the

FIG. 1. Nucleotide and deduced amino acid sequences of a PI31 cDNA clone. A human cDNA clone of PI31 was identified and sequenced as described under "Experimental Procedures." The upper portion of the figure diagrammatically indicates features of the clone and the sequencing strategy. The bottom portion of the figure shows the sequence. Continuous underlines denote the amino acid sequences of peptides from bovine PI31 obtained by Edman degradation. The amino acids underlined with dashed lines represent residues for which there was a discrepancy between the deduced amino acids from human PI31 and the amino acids sequenced from bovine PI31. The boxed sequence, AATAAA, indicates the putative polyadenylation signal. Proline residues are highlighted with darkened backgrounds. The asterisk denotes the stop codon.
Proline-rich Proteasome Inhibitor

**RESULTS**

***cDNA Cloning and Sequencing of PI31***—To determine the primary structure of the proteasome inhibitor, PI31, we identified and sequenced cDNA clones of this protein. Purified PI31 from bovine red blood cells was digested with trypsin, and the resulting peptides were separated by HPLC. Four selected peptides were subjected to automated Edman degradation resulting in four selected peptides. The four peptides were subjected to automated Edman degradation and sequenced to determine the primary structure of PI31. The resulting amino acid sequences were used to construct corresponding oligonucleotide probes.

**Computer-assisted comparison of the deduced amino acid sequence of human PI31 with amino acid sequences in current data bases failed to identify proteins with significant similarity.**

**Identification of a proteasome-PI31 complex by glycerol density gradient centrifugation.** Purified bovine proteasome (40 μg) was incubated for 5 min with control buffer or recombinant PI31 (4 μg). Recombinant PI31 (4 μg) was also incubated under the same conditions without the proteasome. Samples were subjected to glycerol density gradient centrifugation as described under “Experimental Procedures.”

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Expression of PI31 in E. coli—The cDNA encoding human PI31 was subcloned into a pET-28a(+) expression vector for bacterial expression as described under “Experimental Procedures.” Upon induction with IPTG, E. coli produced a protein of Mr 53,000 (as estimated by SDS-PAGE), the size expected for a fusion protein consisting of PI31 and the amino-terminal His\textsuperscript{z}Tag\textsuperscript{TM} (Fig. 2). This protein reacted specifically with a polyclonal antibody produced against purified bovine PI31 (Fig. 2). The expressed protein was purified to homogeneity from the soluble extracts using nickel-agarose affinity chromatography, as described under “Experimental Procedures” (Fig. 2). At concentrations of greater than 1 mg/ml, recombinant PI31 tended to aggregate. This property was also a feature of the native bovine PI31 (data not shown).

The pET-28a(+) expression vector contains a thrombin-susceptible cleavage site (LVPR\textsuperscript{2}GS) engineered for removal of the His\textsuperscript{z}Tag\textsuperscript{TM}. Although treatment of the expressed fusion protein with thrombin resulted in cleavage at the engineered site (as determined by mass spectrometry and Edman degradation of the cleavage products, data not shown), proteolysis occurred at additional sites within PI31 (as determined by SDS-PAGE and mass spectrometry, data not shown). To determine whether this effect was unique to the recombinant protein, purified PI31 from bovine red blood cells was also treated with thrombin. The same cleavage pattern was observed for native bovine PI31 (data not shown). Automated Edman degradation of the PI31 fragments produced by thrombin digestion demonstrated that the main cleavage point within the protein occurred at position 219 (GFPR\textsuperscript{2}AL). Other cleavage sites included position 177 (HHPH\textsuperscript{2}TS), position 205 (GPRR\textsuperscript{2}GG), and position 242 (PGAR\textsuperscript{2}FD). We attempted to achieve selective cleavage at the His\textsuperscript{z}Tag\textsuperscript{TM} site by systematically altering the pH, salt concentrations, digestion time, as well as thrombin source and concentration, for the cleavage reaction. However, no condition significantly reduced the extensive proteolysis of the PI31 protein. Expression of PI31 in other systems was not as successful as in the pET-28a(+) vector (data not shown). Therefore, characterization of expressed PI31 protein was performed with uncleaved fusion protein containing the His\textsuperscript{z}Tag\textsuperscript{TM}.

Functional Characterization of Recombinant PI31—Purified recombinant PI31 fusion protein inhibited 20 S proteasome-catalyzed hydrolysis of denatured proteins, such as [\textsuperscript{methyl-14}C]casein, and of short synthetic peptides. The recombinant human protein inhibited the proteasome with the same efficiency as that of native bovine PI31 (Fig. 3). Half-maximal inhibition was achieved at proteasome:PI31 molar ratios of approximately 1:1, suggesting that the proteasome could inter-
act with two molecules of PI31. As with native PI31, the recombinant protein was specific for the proteasome and had no inhibitory activity against other proteases such as trypsin, chymotrypsin, papain, and protease V8 (data not shown). These data indicate that recombinant PI31 functioned similarly to native PI31.

Previous kinetic analysis indicated that PI31 interacts with the proteasome by a tight-binding mechanism (31). To demonstrate directly the interaction between PI31 and the proteasome we identified proteasome-PI31 complexes by glycerol density gradient centrifugation. As shown in Fig. 4, purified PI31 sedimented very slowly in this gradient, consistent with a low native molecular weight, which cannot be estimated accurately by this method. However, after a brief preincubation with the proteasome, PI31 cosedimented with the proteasome at a position corresponding to a size of approximately 700,000 daltons, as detected by anti-PI31 antibodies (Fig. 4). Furthermore, proteasome activity of samples containing cosedimenting PI31 was reduced markedly compared with that of samples lacking PI31. These results demonstrate that PI31 forms a complex with the proteasome and that proteasome activity in this complex is inhibited. These results also demonstrate that PI31 is not proteolyzed by the proteasome. This conclusion was confirmed by additional experiments in which no cleavage of PI31 was detected after incubation with the proteasome for up to 3 h (data not shown). These results rule out the possibility that the inhibition measured in proteasome assays reflected competition between PI31 and the intended substrate for proteasome hydrolysis.

The Proline-rich Carboxyl Terminus of PI31 Is Responsible for Proteasome Inhibition—The primary structure of PI31 suggests that the protein contains at least two structural domains. To investigate the relative functions of these domains, several deletion mutants of PI31 were constructed and expressed in E. coli as described under “Experimental Procedures.” The data described above indicate that PI31 contains distinct structural and functional domains. To further define structure-function relationships for PI31, the secondary structures of wild-type PI31 and two mutant proteins were estimated by circular dichroism spectroscopy. The full-length protein displayed elements of both α-helical structure and random-coil structure (Fig. 6, top panel). A mutant consisting of the amino-terminal domain (residues 1–192) showed predominantly α-helical structure, as indicated by strong dichroic bands at about 207 and 222 nm (Fig. 6, middle panel). In contrast, a mutant consisting only of the proline-rich carboxy-terminal domain (residues 192–271) displayed a random coil structure, characterized by a single dichroic band at about 200 nm (Fig. 6, bottom panel). These results indicate that the functionally distinct domains of PI31 have distinctly different secondary structures.

Hydrodynamic Properties of PI31—Our original report of PI31 purified from bovine red blood cells indicated that the native protein eluted on gel filtration chromatography with an apparent molecular weight of approximately 60,000, suggesting that PI31 might be a homodimer under nondenaturing conditions (31). However, because of the unusual primary and secondary structures of PI31, we reinvestigated the hydrodynamic properties of PI31 by analytical ultracentrifugation. The data from sedimentation equilibrium centrifugation best fit a model for an equilibrium between monomer and dimer (Fig. 7). Models of monomer alone, dimer alone, and trimer alone yielded very poor fits to the data and the residuals for these fits displayed distinctly nonrandom patterns (data not shown). The monomer/dimer equilibrium dissociation constant was 6.25 μM. This value was used to plot the species fraction for monomer and dimer forms of PI31 (Fig. 7). These data indicate that in intact cells nearly all PI31 should be monomeric (see “Discussion”).

Inhibition of Proteasome—PA28 and Proteasome—PA700 Complexes—PI31 is only one of several proteins that binds to the proteasome and regulates its catalytic functions (1). Because multiple proteasome regulators are located within the same subcellular compartments of individual cells, the relative interactions of these proteins with the proteasome are likely to represent important determinants of proteasome function. Therefore, we tested whether PI31 could block proteasome stimulation by either of two proteasome activators: PA700 and PA28. The proteasome was preincubated with increasing con-
centrations of PI31, including those sufficient to completely inhibit its activity. Then PA700 or PA28, at concentrations sufficient for maximal proteasome activation, was added to the proteasome-PI31 complexes, and the preincubations were continued under conditions normally appropriate to achieve the respective activator’s stimulatory effect. Proteasome activity was assessed at the end of the preincubation by addition of a synthetic peptide substrate. As shown in Fig. 6, PI31 markedly inhibited proteasome activation by each of the regulatory proteins. The maximum degree of inhibition reached approximately 50% and the concentration of PI31 required to attain this level of inhibition was similar to that required to attain 50% inhibition of unstimulated 20 S proteasome activity (Fig. 9). However, unlike the unstimulated proteasome activity, the PA28- and PA700-stimulated proteasome activities were not inhibited further by higher concentrations of PI31. These results demonstrate that PI31 can block stimulation of proteasome activity by both PA28 and PA700; however, approximately 50% of the stimulated activities are refractory to inhibition under the conditions of these assays.

DISCUSSION

The current work establishes the primary structure of the proteasome inhibitor, PI31, a novel protein characterized by an unusually high proline content. Proline residues occur in numerous structural motifs, many of which are involved in specific protein-protein interactions (34). Despite the abundance of proline in PI31, including several short repeated proline-containing sequences, the protein contains no previously recognized motifs of defined function involving proline residues. Analysis of mutant PI31 proteins expressed in E. coli demonstrated that proteasome inhibitory activity resides in the carboxyl-terminal portion of the molecule where the proline residues are concentrated. Thus, mutant forms of PI31 from which the proline-rich domain was deleted had no inhibitory activity. In contrast, PI31 consisting of only various portions of the proline-rich domain showed significant inhibitory activity. One such mutant consisting of only the last 80 residues (PI31192–271) demonstrated inhibitory activity indistinguishable from that of the wild-type protein. Secondary structure analysis of the full-length and mutant proteins by circular dichroism suggests that the carboxyl-terminal portion of the molecule conforms to a random coil. In contrast, the amino-terminal domain contains a high content of α-helical structure. These results suggest a “ball and string” model for PI31 structure in which the amino-terminal domain is globular, whereas the carboxyl-terminal domain is extended. Unfortunately analytical sedi-

![Species plot for PI31](http://www.jbc.org/)

Fig. 8. Species plot for PI31. Data from sedimentation equilibrium centrifugation (Fig. 7) were analyzed for a monomer/dimer model using a $K_d = 6.25 \mu M$.

![Proteasome Activity vs. PI31 Concentration](http://www.jbc.org/)

Fig. 9. PI31 blocks activation of proteasome by PA28 and PA700. Left panel, PA28. 20 S proteasome (0.25 μg) was preincubated alone or with increasing concentrations of recombinant PI31 for 5 min at 37 °C. Purified PA28 was added to a final concentration of 110 nM (proteasome concentration, 7 nM), and the preincubation was continued at 37 °C for an additional 10 min in 40 mM Tris-HCl, pH 7.6 and 2 mM dithiothreitol. Proteasome activity was then assayed by measuring the rate of hydrolysis of the synthetic peptide Suc-Leu-Leu-Val-Tyr-AMC, as described previously (15). Control reactions were performed identically but contained no PA28. Proteasome activity in the absence of PA28 and PI31 was 0.75 unit. Right panel, PA700. 20 S proteasome (0.25 μg) was preincubated alone or with various concentrations of recombinant PI31 for 5 min at 37 °C. Purified PA700 was added to a final concentration of 34 nM, and preincubation was continued for an additional 45 min at 37 °C in the presence of 40 mM Tris-HCl, pH 7.6, 200 μM ATP, 10 mM Mg2+, 2 mM dithiothreitol, in a final volume of 50 μl. Proteasome activity was then assayed by measuring the rate of hydrolysis of the synthetic peptide Suc-Leu-Leu-Val-Tyr-AMC. Control reactions were performed identically but contained no PA700. Proteasome activity in the absence of PA700 and PI31 was 0.95 unit. Proteasome activity in the presence of PA700 but without PI31 had 44.5 units of activity. These activities were designated “control” for their respective conditions and assigned values of 100%. Activities in the respective conditions are reported as a percentage of this value. ▲, assays without PA700; ■, assays with PA700.
mentation velocity ultracentrifugation was not useful in providing definitive information about possible asymmetry of PI31, because the protein exists in an equilibrium between monomer and dimer forms; PI31 has very low optical absorbency at concentrations where it was predominantly a monomer but tends to aggregate at concentrations where it is predominantly a dimer. Thus, additional work will be required to define the shape of PI31.

The current data demonstrate directly that PI31 binds to the proteasome. This interaction, which seems to involve up to two molecules of PI31 per molecule of proteasome, may result in inhibition of proteasome activity by any of several related mechanisms. An attractive model is that PI31, like the other proteasome regulators, binds to the proteasome's terminal α rings. Such an interaction might directly block access of substrates to the proteasome's active sites. Thus, PI31 might act as a "cap," where it remains entirely outside of the central channel, or as a "plug," where a segment of PI31 enters the channel. The "cap," where it remains entirely outside of the central channel, or as a "plug," where a segment of PI31 enters the channel. The "cap," where it remains entirely outside of the central channel, is compatible with the latter model. In fact, recent studies or as a "plug," where a segment of PI31 enters the channel. The "cap," where it remains entirely outside of the central channel, or as a "plug," where a segment of PI31 enters the channel. The "cap," where it remains entirely outside of the central channel.

Recent mechanistic studies with proteasome inhibitors have suggested an active role for proteasome regulators in modulating the catalytic activity of the proteasome. For example, the inhibition of each regulatory protein such as PA28 and PA700. Thus, in cells that contain multiple inhibitors of the proteasome, which may regulate different aspects of proteasome function. The results presented here demonstrate that PI31 can block proteasome activation by both PA28 and PA700, but indicate that the mechanisms responsible for these effects are likely to be complex. For example, the inhibition of each regulator-stimulated proteasome activity attained a maximum of 50%. Although the inhibited portion of the regulator-stimulated activity was achieved by the same low PI31 concentrations required to inhibit the activity of isolated 26 S proteasome, higher concentrations of PI31 produced no additional inhibition. In this regard, PI31 concentrations in various mammalian cell lines under basal conditions are 3–10-fold lower than those of PA700 and PA28. Considerable additional work will be required to define the relative interactions of these proteins with the proteasome in vitro and in vivo. Nevertheless, because PA700 and PA28 mediate proteasome function in both ubiquitin-dependent and ubiquitin-independent pathways of protein degradation, the current results implicate PI31 as a potentially critical regulator of numerous and diverse cellular processes (28).

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