Molecular and Cellular Roles of PI31 (PSMF1) Protein in Regulation of Proteasome Function

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Background: PI31 is an HbYX-motif protein that inhibits 20 S proteasome activity in vitro.

Results: PI31 exerts multiple HbYX-independent effects on proteasome function in vitro, but has no effect on overall proteasome function or content in cells.

Conclusion: PI31 is an in vitro proteasome regulator whose physiologic roles remain to be defined.

Significance: PI31 regulation of cellular proteasome function may be limited to specific physiologic conditions and/or to selective proteasome pools.

We investigated molecular features and cellular roles of PI31 (PSMF1) on regulation of proteasome function. PI31 has a C-terminal HbXY (where Hb is a hydrophobic amino acid, Y is tyrosine, and X is any amino acid) motif characteristic of several proteasome activators. Peptides corresponding to the PI31 C terminus also bind to and activate the 20 S proteasome in an HbXY-dependent manner, but intact PI31 protein inhibits in vitro 20 S activity. Binding to and inhibition of the proteasome by PI31 are conferred by the HbXY-containing proline-rich C-terminal domain but do not require HbXY residues. Thus, multiple regions of PI31 bind independently to the proteasome and collectively determine effects on activity. PI31 blocks the ATP-dependent in vitro assembly of 26 S proteasome from 20 S proteasome and PA700 subcomplexes but has no effect on in vitro activity of the intact 26 S proteasome. To determine the physiologic significance of these in vitro effects, we assessed multiple aspects of cellular proteasome content and function after altering PI31 levels. We detected no change in overall cellular proteasome content or function when PI31 levels were either increased by moderate ectopic overexpression or decreased by RNA interference (RNAi). We also failed to identify a role of PI31 ADP-ribosylation as a mechanism for regulation of overall 26 S proteasome content and function, as recently proposed. Thus, despite its in vitro effects on various proteasome activities and its structural relationship to established proteasome regulators, cellular roles and mechanisms of PI31 in regulation of proteasome function remain unclear and require future definition.

The eukaryotic proteasome is a modular protease system in which multiple holoenzyme complexes are formed from multiple, interchangeable protease and regulatory components (1).

The protease component, called 20 S proteasome or core particle, is a cylinder-shaped complex composed of four axially stacked hetero-heptameric rings with an α7β7β7α7 architecture (2, 3). Three different β subunits in each of the two inner rings catalyze peptide bond hydrolysis with unique specificities (2, 4). Higher eukaryotes have at least three different 20 S proteasome subtypes featuring genetically distinct and differentially expressed complements of catalytic β subunits (1, 5–9). The catalytic residues of these subunits line the surface of a central luminal chamber of the 20 S cylinder (3, 4). Narrow, gated pores in the center of the outer α-subunit rings govern access of substrates to this chamber (10, 11). In the absence of regulatory proteins, the pore gates are constitutively closed, rendering isolated 20 S proteasomes catalytically inert (3, 4). However, binding of proteasome regulators to the apical face of the α-subunit rings induces gate opening, thereby licensing the resulting 20 S-regulator holoenzyme for proteolysis (11–17). Despite this common general theme of regulator function, various regulators have distinct molecular mechanisms for proteasome binding and activation (1, 11, 17). For example, 11 S regulators (PA28αβ, PA28ɣ, and PA26) utilize separate C-terminal and internal structural elements for proteasome binding and activation, respectively (16, 18, 19). In contrast, PA700 (also known as 19 S regulatory particle) and PA200/Blm10 as well as the archaean regulator, PAN, employ a single structural element, the HbXY motif, to mechanistically link their proteasome binding and activation functions (20–26). In each case, interactions of C-terminal HbXY residues with cognate sites between adjacent α subunits provide both binding energy for complex formation and induction of conformational changes that result in gate opening. The importance of the HbXY motif in proteasome binding and activation is highlighted by the ability of 7–10 residue peptides corresponding the HbXY-containing C termini of proteasome regulators to activate the 20 S proteasome (20, 23, 24, 26). C-terminal HbXY motifs also are found in several related heterodimeric complexes from different species. These complexes (Pba1-Pba2 in yeast, PAC1-PAC2 in mammals, and PbaA-PbaB in archaea) appear to function as

The abbreviations used are: Hb, hydrophobic amino acid; Ub, ubiquitin; AMC, amidomethylcoumarin; SUMO, small ubiquitin-like modifier.

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20 S proteasome assembly chaperones by organizing α subunits into rings during early phases of 20 S assembly (27–30). Pba1-Pba2, however, also binds to mature 20 S proteasomes, suggesting that it may have post-assembly regulatory roles (31).

PI31 is the least studied and most poorly characterized proteasome regulator. PI31 contains two domains: an N-terminal globular domain likely responsible for PI31 homodimerization and a proline-rich C-terminal domain with an intrinsically disordered structure (32, 33). We originally identified and characterized PI31 as an in vitro inhibitor of 20 S proteasome activity and showed that this function was localized to the C-terminal domain (32, 34). Recently, however, we and others have recognized that the PI31 C-terminal residues conform to an HbY motif characteristic of multiple proteasome activators (35). Moreover, recent findings provided evidence that PI31 activated the 26 S proteasome in vitro and exerted a positive effect on proteasome function in intact cells (35). These various contradictory findings have led us to extend earlier studies and reexamine biochemical and cellular features of PI31.

**EXPERIMENTAL PROCEDURES**

**Proteins and Peptides**—Wild-type and various mutant recombinant human PI31 proteins and SUMO-PI31 peptide fusion proteins were prepared as N-terminal His-tagged proteins using standard recombinant DNA methodologies. After expression in *Escherichia coli*, proteins were affinity-purified on Ni²⁺ beads as described previously (36). Latent 20 S proteasome, active 20 S proteasome, PA700, PA700 subcomplexes (PS-1, PS-2, and PS-3), and 26 S proteasome were purified from bovine red blood cells as described previously (37–40). 26 S proteasome was also affinity-purified from HEK293 cells stably expressing FLAG-Uch37 subunit (41). All peptides were synthesized using Fmoc (N-(9-fluorenylmethoxycarbonyl) chemistry and purified by HPLC by the Protein Core Facility at UT Southwestern Medical Center. Sequences of all peptides are listed in Table I and assay legends. Control centrifugations established the sedimentation positions of purified bovine 26 S proteasome, PA700, and 20 S proteasome.

For native PAGE, 20 S proteasome (0.8 μM) was incubated alone or with His-PI31 proteins (1.6 μM) in 50 mM Tris-HCl, pH 7.6, for 30 min at 4 °C. Samples were then subjected to native PAGE, as described below. Gels were either stained with Ponceau S or subjected to Western blotting with anti-His antibodies.

**Measurement of Proteasome Activity in Vitro**—Proteasome activity was measured in vitro by determining the rate of hydrolysis of peptide and protein substrates as described previously (41). Rates of production of 7-amino-4-methylcoumarin (AMC) from Suc-Leu-Leu-Val-Tyr-AMC were determined by continuous monitoring of fluorescence using a BioTek Synergy plate reader and were expressed as arbitrary fluorescent units produced per min of incubation. Proteasome-catalyzed hydrolysis of [methyl-14C]casein was determined as described previously (42). All reported data represent initial steady state rates of hydrolysis. All assays were conducted under conditions for a linear relationship between proteasome content and enzymatic activity. Assays were performed in triplicate and repeated at least three times. For assays with purified 20 S and 26 S proteasomes, protein content was determined by Bradford assays. For assays involving comparative activities of different cell extracts, activities were normalized to total extract protein, determined by the Bradford assay, and/or to actin, determined by Western blotting. Semiquantitative measures of proteasome activity were determined by zymography after native PAGE using Suc-Leu-Leu-Val-Tyr-AMC substrate (38, 41).

26 S Proteasome Assembly in Vitro—In vitro assembly of 26 S proteasome from purified 20 S proteasome and PA700 was determined as described previously (38). 20 S proteasome (29 nm) and PA700 (114 nm) were preincubated at 37 °C for 30 min in the presence of 200 μM ATP, 10 mM MgCl₂, and 1 mM dithiothreitol (38). Proteasome activity was measured after the addition of peptide substrate (Suc-Leu-Leu-Val-Tyr-AMC). Routine control reactions included preincubation and assay of 20 S proteasome and PA700 in the absence of ATP and preincubation and assay of 20 S proteasome and PA700 in the absence of the other. PA700 has no detectable peptidase activity in the absence of 20 S proteasome. 20 S proteasome activity is typically stimulated 10–20 fold by PA700 under conditions used in these assays, and stimulation is completely dependent on ATP during preincubation.

**Native PAGE**—Native PAGE was conducted at 4 °C in 3–8% Bis-acetate gels (NuPAGE, Invitrogen). After electrophoresis for 2.5 h at 150 V, gels were either stained for protein, subjected to zymography, or processed for Western blotting as described for individual experiments.

**Cell Culture and Preparation of Cell Extracts**—HEK293 cells and HeLa cells were cultured in the presence of 5% CO₂ at 37 °C in Dulbecco’s modified Eagle’s medium containing high glucose and glutamine supplemented with 10% fetal bovine serum.
**Drosophila** S2 cells were cultured at 25 °C in Schneider’s *Drosophila* medium supplemented with 10% fetal bovine serum. Cells were harvested, washed twice with PBS, and disrupted in ice-cold buffer consisting of 50 mM Tris-HCl, pH 7.5, 1 mM ATP, 5 mM MgCl₂, and 1 mM β-mercaptoethanol by 15 passages through a 27-gauge needle. In some experiments the lysis buffer contained 0.1% Nonidet P-40, which had no effect on any results. The lysates were centrifuged at 14,000 rpm in an Eppendorf microcentrifuge for 20 min to obtain a crude soluble cell extract.

**Cellular Degradation of Ub⁷⁷⁶-GFP and Ub⁸-R-GFP—Proteasomal degradation of GFP variants in intact cells was determined as described previously using HeLa cells stably expressing either ubiquitin-R-GFP or ubiquitin⁷⁷⁶-GFP. After treatments described in legends to specific experiments, cellular GFP content was determined by Western blotting (43).**

**Stable Overexpression of PI31 in HEK293 Cells—**HEK293 cells lines stably expressing FLAG-tagged human PI31⁷⁷⁶ or FLAG-tagged human PI31⁻¹⁻⁷⁷⁶ were prepared by standard methods. cDNA encoding either PI31⁷⁷⁶ or PI31⁻¹⁻⁷⁷⁶ was subcloned into pIRESpuro3 expression vector (Clontech). The sequence of each construct was verified by DNA sequencing. HEK293 cells were transfected with respective expression vectors at ~60% confluence using FuGENE 6 (Roche Applied Science). After 48 h the medium was replaced with medium containing puromycin (5 μg/ml), and cells were grown for 4 weeks for selection of clones exhibiting stable expression of respective
proteins. Additional rounds of selection were performed to assure consistent stable expression of the PI31 proteins.

Transient Overexpression of PI31—FLAG-tagged PI31 proteins were expressed transiently in HeLa or HEK293 cells using respective expression vectors and Lipofectamine 2000 (Invitrogen). Cells were harvested 48 h after transfection and analyzed as described in corresponding figure legends.

RNAi by siRNA—RNAi of PI31 was conducted by transfection of double-stranded siRNA oligonucleotides (Trilencer-27), designed and synthesized by Origene. Control transfections included both a proven non-targeting siRNA (SR30004 control) provided by Origene and transfection reagent only without oligonucleotides. Preliminary experiments established 72-h post-transfection as the time for optimal reduction of PI31 content.

Quantification of Relative Cellular Content of PI31, 20 S Proteasome, and PA700 Proteins—The relative cellular content of proteasome system components was estimated by Western blotting using antibodies against representative subunits of 20 S proteasome (α2 and β5), PA700 (Rpt2 and Rpn12), and PI31. Purified 20 S proteasome, PA700, and PI31 were used as standards. Protein concentrations were determined by Bradford assay and by OD280, which gave similar values. Immuno-reactive bands for both protein standards and corresponding proteins in cell extracts were determined at multiple concentrations, quantified by densitometry, and processed using Image J software. To estimate the cellular content of various proteasome complexes from the content of individual proteins or constituent subunits of proteasome complexes, we relied on established biochemical analysis of complex composition and on analysis of these complexes in cell extracts by native PAGE and glycerol density gradient centrifugation (44–46). We assumed that two copies of both α2 and β5 were present in each 20 S proteasome complex, one copy each of Rpt2 and Rpn12 was present in each PA700 complex, and that PI31 was a homodimer. Based on Western blotting of cell extracts fractionated by glycerol density gradient centrifugation and native PAGE, we estimated that >95% of cellular α2 and β5 subunits are present in assembled 20 S proteasome complexes and that >90% of cellular Rpt2 and Rpn12 subunits are present in assembled PA700. We also estimated that (i) ~50% of total 20 S proteasome is in the form of 26 S proteasome containing one or two copies of PA700 in equal proportion, (ii) that the remaining 20 S proteasome is either “free” or associated with regulatory complexes such as PA28 and PA200, and (iii) that >50% of PA700 is in 26 S proteasome complexes. To convert concentrations of complexes into numbers of molecules per cell we estimated cell volume to be 4000 μm³, derived as an average of literature values.

RESULTS

The C-terminal HbYX Motif of PI3 Is Sufficient for Binding to the 20 S Proteasome—We demonstrated previously that in vitro proteasome inhibitory activity of PI31 was conferred by the C-terminal proline-rich domain (32). The recent recognition that the last three residues of PI31 constitute an HbYX motif found in multiple proteasome activators prompted us to refine the structure-function analysis of PI31 with respect to proteasome binding, inhibition, and activation. To evaluate the role of the C-terminal HbYX motif of PI31 for 20 S proteasome binding, we utilized pulldown assays previously developed to analyze proteasome binding by HbYX motifs of Rpt subunits of PA700 (36). We expressed and purified recombinant proteins in which the last 10 residues of human PI31, either with or without the terminal HbYX residues, were fused to the 20 S proteasome. These proteins were immobilized on Ni²⁺ beads and analyzed for their ability to specifically bind purified 20 S proteasome. The SUMO fusion protein containing the HbYX residues of PI31 bound to 20 S proteasome, whereas an otherwise identical protein lacking only the HbYX residues did not (Fig. 1A, lanes 2 and 6, and B, lanes 10 and 11). Fusion proteins lacking the terminal one or two residues of the HbYX motif also had greatly reduced proteasome binding (Fig. 1B, lanes 12 and 13). 20 S proteasome binding was attenuated by an excess of free HbYX-containing PI31 peptide but not by an HbYX peptide corresponding to the C terminus of the
PA700 subunits Rpt5 or Rpt3 or by irrelevant peptides (Fig. 1, lanes 4 and 5, and C, lanes 18–23, and data not shown). These results show that the HbYX motif of PI31 is sufficient for 20 S proteasome binding. They also suggest that the binding site for the PI31 HbYX residues is distinct from that for HbYX motifs of the other proteasome regulators tested here. To determine the specificity of HbYX-dependent binding of PI31 to the 20 S proteasome, we repeated this analysis by testing the ability of isolated PA700 or intact double-capped 26 S proteasome to bind to His-SUMO containing the C-terminal PI31 peptide. No detectable binding of either protein was observed by this method (data not shown). These results indicate the HbYX motif of PI31 interacts selectively with 20 S proteasome and that this interaction is not manifested when the 20 S proteasome is bound to PA700.

The C-terminal HbYX Motif of PI31 Is Not Essential for PI31 Binding to, or Inhibition of 20 S Proteasome—To determine whether the HbYX motif is both sufficient and necessary for PI31 binding to the 20 S proteasome, we expressed and purified wild-type and various recombinant mutant PI31 proteins. Binding of these proteins to the 20 S proteasome was evaluated by multiple methods including pulldown assays, co-migration during native PAGE, and co-sedimentation during glycerol density gradient centrifugation. As expected, intact wild-type PI31 (PI31WT) bound to the 20 S proteasome as detected by each method (Fig. 2). The isolated C-terminal proline-rich domain (PI31152–271) also bound to 20 S proteasome, whereas the isolated N-terminal domain (PI311–151) had no detectable binding. These results are consistent with previously established effects of these mutant proteins on in vitro inhibition of 20 S proteasome activity. Surprisingly, however, PI31 lacking only the HbYX residues (PI31−HbYX) or the C-terminal domain lacking only the HbYX residues (PI31152–266) also bound to the 20 S proteasome. PI31 lacking the last 10 or 27 residues also bound to the 20 S proteasome (Fig. 2). Collectively, these results indicate that multiple structural elements of PI31 contribute to 20 S proteasome binding and that the HbYX motif is not essential for this interaction.

To determine the relationship between binding and proteasome activity, we determined the effect of these various PI31 proteins on in vitro activity of 20 S proteasome. As expected, wild-type PI31 inhibited proteasome hydrolysis of both a short peptide substrate, Suc-Leu-Leu-Val-Tyr-AMC, and a structurally disordered protein, casein (Fig. 3). Proteasome inhibitory activity was retained by the intact C-terminal domain (PI31152–271) and by a truncated C-terminal domain (PI31192–271) but not by the N-terminal domain (PI311–151). PI31 lacking only the HbYX residues (PI31−HbYX) exerted sig-
significant inhibitory activity, as did PI31 mutants with progressively larger (10- and 27-residue) C-terminal deletions (Fig. 3). These functional effects mirror the binding features of the PI31 mutant proteins and demonstrate that HbYX motif is not essential for PI31 inhibition of 20 S proteasome. 20 S proteasome can be purified in both “latent” and “active” forms that differ by >10-fold in their ability to hydrolyze peptide and natively disordered protein substrates. This difference likely reflects different states of the substrate access gate as a consequence of different purification procedures for the two enzymes, as described previously (39). Data presented in Fig. 3 were obtained using active 20 S proteasomes to highlight and facilitate analysis of inhibition of the high constitutive proteasome activity. Nevertheless, qualitatively similar effects were obtained using latent 20 S proteasomes in analogous experiments (data not shown).

A C-terminal PI31 HbYX-motif Peptide Activates 20 S Proteasome but Not 26 S Proteasome—In contrast to PI31, other HbYX-containing proteins function as proteasome activators (11). The HbYX residues of these proteins bind to pockets between adjacent α subunits of 20 S proteasome and induce gate opening. To investigate the functional relationship between the HbYX motif of PI31 and those of other proteins, we synthesized short peptides corresponding to the C terminus of PI31. Analogous peptides for HbYX-containing proteins such as PA200, PAN, and Rpt subunits of PA700 are sufficient to activate 20 S proteasome hydrolysis of peptide substrates and structurally disordered proteins (20, 23, 24). Surprisingly, C-terminal PI31 peptides of 10 or 22 residues also activated the latent 20 S proteasome hydrolysis of these substrates in an HbYX-dependent manner (Fig. 4, upper). Thus, these PI31 HbYX peptides exerted functionally different effects than intact PI31 protein on proteasome activity. These results indicate that the HbYX residues of PI31 function similarly to the HbYX motifs of established proteasome activators by promoting gating of the 20 S proteasome. They also suggest that other structural features of the PI31 C-terminal domain must impose inhibitory effects that negate HbYX-mediated activation.

PI31 Binds to but Does Not Directly Regulate Activity of Intact 26 S Proteasome—PI31 was identified and characterized by its in vitro inhibition of 20 S proteasome activity. Recent reports presented evidence that intact PI31 also activated the in vitro hydrolysis of peptide substrates by the 26 S proteasome (35). To explore the basis of this surprising distinction and its possible relationship to the HbYX-activating function of PI31 C-terminal peptides described above, we directly compared the effects of intact PI31 and PI31 peptides on in vitro activity of purified 20 S and 26 S proteasomes. As presented above, PI31 protein inhibited the hydrolysis of short peptides and unstructured proteins, such as casein, by the 20 S proteasome. In contrast, however, we detected no effect of PI31 on 26 S proteasome activity against either class of these substrates or against the ATP-dependent degradation of polyubiquitylated proteins (Fig. 4, lower). Moreover, PI31 C-terminal peptides that activated 20 S proteasome activity had no effect on 26 S proteasome activity. To determine whether the lack of effect of PI31 on 26 S proteasome activity reflects an inability of these proteins to physically interact, we repeated in vitro PI31 binding experiments using purified 26 S proteasome and PA700. Surprisingly, PI31 bound to both 26 S proteasome and PA700 in an HbYX-independent manner (Fig. 5 and data not shown).

PI31 Blocks Assembly of 26 S Proteasome in an HbYX-independent Manner—We previously showed that PI31 blocked PA700-dependent activation of 20 S proteasome in vitro (32). We interpreted this result as an effect of PI31 on blocking ATP-dependent PA700 binding to the 20 S proteasome but made no direct assessment of this putative mechanism. Although the lack of PI31 inhibition on activity of intact 26 S proteasome described above supports our original interpretation, we sought to obtain direct evidence for PI31 inhibition of 26 S proteasome assembly from 20 S proteasome and PA700 subcomplexes and to evaluate the role of the PI31 HbYX motif in this process. The content and activity of reconstituted 26 S proteasomes can be determined by protein staining and zymography, respectively, after native PAGE. We incubated purified 20 S proteasome and PA700 in the presence and absence of wild-type and various PI31 mutant proteins. Wild-type PI31 blocked the ATP-dependent formation of 26 S proteasome when present during the assembly reaction but had no effect on 26 S proteasome content or activity after assembly was complete (Fig. 6A). Thus, PI31 inhibition of proteasome activation results from inhibition of 20 S and PA700 binding rather than from inhibited activity of the assembled 20 S-PA700 complex and is consistent with the lack of effect of PI31 on activity of purified 26 S proteasome. PI311–151 had no effect on 26 S proteasome formation, whereas PI31152–271 blocked assembly (Fig. 6B). PI31HbYX blocked

![FIGURE 5. PI31 binds to PA700 and 26 S proteasome in vitro.](image-url)
26 S proteasome assembly as effectively as PI31WT under the conditions of this assay. Likewise, PI31 variants lacking either the last 10 or 27 C-terminal residues also blocked 26 S proteasome assembly. PI31192–271, a mutant that effectively inhibited 20 S proteasome activity, had little effect on inhibition of assembly. Thus, with this latter exception, the relative effects of PI31 variants on 26 S proteasome assembly closely mirrored their relative abilities to bind to and inhibit the 20 S proteasome. Moreover, these results indicate that the HbYX motif is not required for the inhibition of 26 S assembly in this in vitro system.

We previously showed that 26 S proteasome also can be assembled in vitro from 20 S proteasome and three purified subcomplexes (PS-1, PS-2, and PS-3) that collectively compose PA700 (40). Moreover, PA700 itself can also be reassembled in vitro from these subcomplexes in the absence of 20 S proteasome (37, 40). Therefore, to further examine features of PI31 regulation on 26 S proteasome assembly, we tested the effects of PI31 on each of these processes. PI31 inhibited the assembly of both 26 S proteasome and PA700 in their respective in vitro assembly assays from PS-1, PS-2, and PS-3 (Fig. 6, C and D).}

**FIGURE 6.** PI31 blocks in vitro assembly of 26 S proteasome and PA700. Panels A–C, 26 S proteasome was assembled in vitro from purified 20 S proteasome and either PA700 (A and B) or PA700 subcomplexes, PS-1, PS-2, and PS-3 (40) (C) as described under “Experimental Procedures.” Panel A, assembly reactions were conducted under identical conditions in the absence (●) of PI31. After the assembly reaction, proteasome activity was determined by measuring the rate of hydrolysis of Suc-Leu-Leu-Val-Tyr-AMC (during). Assembly reactions were performed in the presence (○) of the indicated concentrations of PI31. After the assembly reaction, proteasome activity was measured as described above. The activity of 20 S proteasome added to each assay was assigned a value of 1.0 (dotted line), and other activities are expressed relative to that. Panel B, samples from 26 S assembly reactions containing either no PI31 (lanes 1 and 10) or 19.2 µM of the indicated PI31 variants (lanes 3–9) were subjected to native PAGE and either stained with Coomassie (upper) or overlaid with Suc-Leu-Leu-Val-Tyr-AMC for zymography (middle). Arrows indicate known migration positions for indicated proteins including 26 S proteasome with either one (26 S-1) or two (26 S-2) copies of PA700, PA700, and 20 S proteasome, respectively. The band denoted with an asterisk (*) in lane 9 represents a contaminant of the PI31192–271 protein. Samples from the reactions described above were also measured directly for proteasome activity using Suc-Leu-Leu-Val-Tyr-AMC substrate (lower). Panel C, assembly reactions were conducted in the presence (+) or absence (−) of PI31, and proteasome activity was measured by hydrolysis of Suc-Leu-Leu-Val-Tyr-AMC substrate. The activity of unassembled 20 S proteasome was assigned a value of 1.0 (dotted line), and other activities are expressed relative to that. Each bar represents the mean activity of triplicate assays (±S.D.), and similar results were obtained in three independent experiments. D, PA700 subcomplexes PS-1, PS-2, and PS-3 were preincubated (+) for 30 min at 37 °C or not (−) in the absence (−) or presence of indicated PI31 proteins and then subjected to native PAGE. Assembled PA700 was identified with an antibody against the Rpt2 subunit. The arrow indicates the established migration position of purified PA700. Panel E, recombinant His-PI31WT was bound to Ni2+ beads and used for pulldown assays with the indicated purified PA700 subcomplexes, PS-1, PS-2, and PS-3. Imidazole-eluted samples were Western-blotted with the indicated subunits of the respective subcomplexes.
and the subcomplexes of PA700 using pulldown assays between His-PI31 and purified PS-1, PS-2, and PS-3. Only PS-1 (composed of Rpt3, Rpt6, Rpn2, and all “lid” subunits) bound to PI31 (Fig. 6E). Collectively, these results suggest that PI31 may affect 26 S proteasome assembly by multiple mechanisms. Additional work will be required to dissect the relative importance of these interactions.

**Altered Expression of PI31 Does Not Influence Cellular Proteasome Content or Function**—The various in vitro effects described above may reflect mechanisms by which PI31 regulates proteasome content and function in intact cells. To determine the possible physiologic significance of these effects, we examined and compared features of normal and altered cellular PI31 content on multiple aspects of cellular proteasome organization and function. First, we determined the association of PI31 with other proteasome system components by monitoring PI31 distribution in cell extracts fractioned by either native PAGE or glycerol density gradient centrifugation. Endogenous PI31 displayed a multimodal distribution pattern, as determined by each method. The majority of cellular PI31 behaved indistinguishably from isolated purified PI31, whereas lesser amounts were associated with gradient fractions or electrophoretically separated complexes characteristic of the 20 S proteasome, PA700, and 26 S proteasome (Fig. 7A and data not shown). These results indicate that PI31-proteasome complexes demonstrated by in vitro studies likely occur in intact cells but reveal that most endogenous cellular PI31 is not found in such complexes. Second, we prepared HEK293 cells that stably expressed either wild-type FLAG-PI31 (PI31WT) or FLAG-PI31 lacking the C-terminal three residues (PI31ΔHbYX) or from parent HEK293 cells expressing only endogenous PI31 (PI31Endo) and analyzed. Panel A, Western blotting (WB) with indicated antibodies after glycerol density gradient centrifugation. Panel B, Western blotting for the indicated proteins after SDS-PAGE. Control cells were treated with 20 μM MG132 for 8 h. Panel C, proteasome activity using Suc-Leu-Leu-Val-Tyr-AMC. Control cells were treated with 20 μM MG132 for 8 h and assayed similarly. Activity of control cells was assigned a value of 100, and other values are expressed relative to that. Each bar represents the mean of triplicate assays (±S.D.), and similar results were obtained in five independent experiments. Panel D, the indicated cell extracts were subjected to native PAGE and either assayed for proteasome activity by zymography (activity) or Western-blotted for the Rpt2 subunit of PA700.
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**FIGURE 8.** Transient overexpression of PI31 has no effect on overall proteasome content or function in mammalian cells. The indicated cell lines were transfected with expression vectors for FLAG-PI31\textsuperscript{WT} or FLAG-PI31\textsuperscript{HbYX}. After 48 h, cells were analyzed for proteasome function and content. Some cells were treated for 17 h with 10 \( \mu \text{M} \) MG132. Panel A, Western blotting (WB) for the indicated proteins after SDS-PAGE. Con, control. Panel B, the indicated cell extracts were subjected to native PAGE and either assayed for proteasome activity by zymography or Western-blotted for the Rpt2 subunit of PA700. Panel C, proteasome activity using Suc-Leu-Leu-Val-Tyr-AMC. The activity of control cells was assigned a value of 100, and other values are expressed relative to that. Each bar represents the mean of triplicate assays (± S.D.), and similar results were obtained in three independent experiments.

rect but diagnostic measure of altered cellular proteasome function, was also indistinguishable among these various cells (Fig. 7B). The lack of effect of PI31 overexpression on proteasome function was mirrored by the lack of effect on 26 S proteasome content (Fig. 7D). Thus, overexpression of PI31 proteins (FLAG-PI31\textsuperscript{WT} or FLAG-PI31\textsuperscript{HbYX}) that negatively regulated 20 S proteasome activity and 26 S proteasome assembly in vitro had no detectable effect on multiple measures of proteasome content and function in intact cells. To determine whether cells chronically overexpressing PI31 had compensated for this condition by adjusting proteasome function to normal levels, we repeated this analysis after transient overexpression of PI31. However, transient PI31 overexpression in HEK293 or HeLa cells also had no significant effect on any of the measures of proteasome function monitored above (Fig. 8). In a few experiments, as exemplified in Fig. 8A, we detected a modest increase in high molecular weight polyubiquitylated proteins of cells transiently overexpressing PI31, but this was not consistently reproducible and when observed was not associated with corresponding changes in other measures of proteasome content or function. We also tested the effects of transient PI31 overexpression on the cellular degradation Ub\textsuperscript{V76}-GFP, and Ub-R-GFP, two model substrates subject to constitutive proteasomal degradation. PI31 overexpression had no effect on the steady state level of either substrate (Fig. 8A).

To further examine the possible cellular roles of PI31, we analyzed proteasome function after reducing cellular PI31 content by RNAi. Reduction of PI31 protein levels by >85% had no detectable effect on cell growth or morphology (data not shown). Moreover, no measure of proteasome function, including hydrolysis of peptide substrates, total cellular content of 26 S proteasome, levels of polyubiquitylated cellular proteins, or the levels of Ub\textsuperscript{V76}-GFP and Ub-R-GFP was affected by RNAi of PI31 (Fig. 9). Thus, neither overexpression nor reduced expression of cellular PI31 significantly affected any measure of proteasome function tested here.

The failure to detect cellular effects of PI31 on proteasome activity or content indicates that most cellular proteasome is unaffected by PI31 and/or that the various in vitro effects of PI31 on proteasome function do not occur to appreciable extents in intact cells. To fully interpret these results, quantitative information is required about the relative cellular content of various proteasome complexes such as 20 S proteasome, PA700, and 26 S proteasome compared with PI31. Therefore, we quantified the levels of PI31 and proteasome complexes in HEK293 and HeLa cells by Western blotting using purified proteins as standards. We used two representative subunits of 20 S proteasome and of PA700 to estimate the respective content of these complexes, which were approximately similar in three different cell lines. As expected from previous studies, the relative levels of 20 S and PA700 subunits are consistent with their distribution in 20 S, PA700, and 26 S complexes (Tables 1 and 2). Molar levels of PI31 were approximately equal to those estimated for free 20 S proteasome and 26 S proteasome in each of the cell lines. These results indicate that endogenous PI31 levels are sufficient to interact with a quantitatively significant proportion of proteasome complexes. Moreover, PI31 levels in cells overexpressing this protein should exceed the levels of their putative corresponding targets. Thus, the lack of significant interaction between PI31 and proteasome complexes in cell extracts, as noted above, indicates substantial differences between PI31 action in vitro and PI31 action in intact cells.

Altered ADP-ribosylation Has No Effect on Proteasome Function—Recently, Steller and co-workers (35, 47) presented evidence that PI31 acts as a positive regulator of proteasome function in Drosophila and mammalian cells. Their data also indicate that ADP-ribosylation of PI31 promotes 26 S proteasome assembly by two mechanisms that collectively favor PA700 binding to 20 S proteasome: (i) attenuation of PI31 binding to the 20 S proteasome and (ii) enhancement of PI31 binding to and sequestration of the 26 S assembly chaperones, p27 and S5b (47). This attractive model is consistent with a physiologic role for the in vitro inhibition of PI31 on 26 S proteasome assembly described above and provides a potential molecular mechanism for regulation of the relative interactions of PI31 and PA700 with the 20 S proteasome. To determine the possible relationship of this mechanism to the results described...
above, we repeated and extended published experiments using the pharmacologic ribosylation inhibitor, XAV939 (48). First, we determined the effect of XAV939, a potent inhibitor of tankyrase 1/2 ribosylation enzymes, on proteasome activity in extracts of HEK293 and *Drosophila* S2 cells. In contrast to the reported data for similar experiments, we observed no inhibition of proteasome activity by XAV939, which effectively inhibited *in vitro* autoribosylation of tankyrase (Fig. 10A and data not shown). We also failed to detect effects of XAV939 on increased 26 S proteasome content expected for the model (Fig. 10B).

Second, we modified this experiment by measuring proteasome content and activity in extracts from mammalian (HEK293 and HeLa) and *Drosophila* (S2) cells treated for various times and with different concentrations of XAV939. XAV939 exerted established cellular effects such as the stabilization of tankyrase and axin 1 (48) but had no effect on proteasome activity or 26 S proteasome content (Fig. 11). To evaluate whether XAV939 affected measures of cellular proteasome function, we also determined the steady state levels of polyubiquitylated cellular proteins and the UPS-dependent degradation of UbV76-GFP or Ub-R-GFP (43). XAV939 had no significant effect on any of these sensitive indicators of proteasome function (Fig. 11B).

Collectively, these results indicate that ADP-ribosylation of PI31 does not regulate overall cellular content or function of the 26 S proteasome in several cell types.

**DISCUSSION**

We originally identified PI31 in a biochemical screen for proteins that affected hydrolytic activity of purified mammalian 20 S proteasome *in vitro* (34). In contrast to other identified proteins that activated proteasome function (49, 50), we found PI31 as protein that inhibited the hydrolysis of peptide and protein substrates. Our previous work showed that proteasome inhibition was conferred by PI31 proline-rich C-terminal domain, but more detailed information about the structural
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**A.**

| Proteasome activity (% Control) |
|-------------------------------|
| HEK293 | dS2 |
| Con | 2 µM | 50 µM | MG132 |

**B.**

Panel A: Treatment of cell extracts with XAV939, an inhibitor of tankyrase 1 and 2, does not affect 26S proteasome content or activity. Cell-free extracts of HEK293 cells or *Drosophila* S2 cells were prepared as described under "Experimental Procedures." Panel A, extracts were preincubated with no additions (Con), the indicated concentrations of XAV939, or 100 µM MG132 for 30 min and then assayed for proteasome activity using Suc-leu-Leu-Val-Tyr-AMC. Proteasome activity in the absence of agents was assigned a value of 100, and other values are described relative to that. Data represent the mean values (±S.D.) of triplicate assays from each of three independent cell extracts. Panel B, extracts treated as indicated were subjected to native PAGE and either zymography (upper) or Western blotting (WB) for the Rpt2 subunit of 26S proteasome (lower).

PI31 bound to and inhibited either of two forms of purified 20S proteasome (term latent and active) that differ greatly in their hydrolytic activities, probably due to different constitutive states of gate openness (39). Although PI31 inhibition of 20S proteasome activity was proportionally similar for each type of proteasome, its absolute magnitude was more prominent against the active proteasome. In contrast, the stimulatory effect of PI31 HbYXX peptides was detected readily with latent 20S proteasomes but not with constitutively active 20S proteasome whose gate is presumably open even in the absence of activators. Nevertheless, HbYXX-dependent binding of these peptides was equivalent for each 20S proteasome type.

In addition to its interaction with 20S proteasome, PI31 also bound to 26S proteasome and PA700. Although the semiquantitative binding assays used here do not allow accurate comparison of the relative affinities of these interactions, PI31 binding to 26S and PA700 appeared weaker than to 20S proteasome. Because the natively disordered structure of the C terminus of PI31 is an element characteristic of many proteasome substrates, we considered the possibility that PI31 binding to 26S reflect an enzyme-substrate interaction. However, as discovered previously with 20S proteasome, PI31 and various PI31 fragments were resistant to detectable proteolysis after *in vitro* incubations with 26S proteasome under conditions used here (data not shown).

Despite the *in vitro* effects of PI31 on numerous aspects of proteasome function, we failed to observe the corresponding effects on multiple indicators of proteasome function in intact mammalian cells. Thus, neither stable nor transient PI31 overexpression at moderate levels nor reduction of PI31 by RNAi had detectable effects on total proteasome activity, 26S proteasome content, cellular degradation of model protein substrates, steady state levels of polyubiquitylated proteins, or overall cell growth. In contrast to these negative results, Steller and co-workers (35, 47) recently presented evidence for multiple roles of PI31 in regulating general proteasome function in *Drosophila* and mammalian cells. Based on various biochemical and cellular results, they have proposed a novel, multifaceted model in which PI31 acts as an HbYXX-dependent positive regulator of 26S proteasome activity *in vitro* and *in vivo* by increasing 26S content and by directly stimulating 26S activity. The basis for the conflicting conclusions between their studies and those presented here, including differing results for closely similar experiments, is not clear. For example, Steller and co-workers (35) reported *in vitro* activation of 26S proteasome peptidase activity by intact PI31, whereas we found no such effect in highly similar assays. Perhaps their use of heterologous proteins (*i.e.* mammalian 26S proteasome and *Drosophila* PI31) produces effects not normally achieved with proteins from the same or more closely related species, although most proteasome-regulator pairs function similarly regardless of species differences. Other facets of their model involve regulatory roles for PI31 in 26S proteasome assembly consistent with the *in vitro* effect of PI31 on this process described here and could potentially explain why we failed to detect an effect of PI31 in 26S assembly in intact cells. Specifically, their model proposes that ADP-ribosylation of PI31 promotes 26S proteasome assembly by two coordinated mechanisms; first, by attenuating
PI31 binding to the 20 S proteasome, thus favoring 26 S assembly by removing the negative influence of PI31 on 20 S-PA700 binding and, second, by enhancing PI31 binding to two 26 S assembly chaperones normally associated with PA700 (47). The latter effect is proposed to promote dissociation of the chaperones from PA700, thereby exposing structures of PA700 required for its binding to 20 S proteasome. Thus, discrepancies between in vitro and cellular effects of PI31 might be accounted for by different states of PI31 ribosylation in these settings. For example, extensive constitutive cellular PI31 ribosylation could explain why most endogenous PI31 is not associated with intact proteasome system components such as free 20 S proteasome or PA700 even though cellular PI31 levels are approximately similar to those of these binding targets (Tables 1 and 2). Likewise, the failure of cellular-overexpressed PI31 to mimic the strong negative effect of PI31 on in vitro 26 S proteasome assembly also might result from extensive cellular PI31 ribosylation.

Nevertheless, a number of other findings reported here lead us to question the overall compatibility of our data with these published models of PI31 action. For example, it is unclear why increased ribosylated PI31 would not positively influence 26 S proteasome assembly and content via the chaperone-sequestration mechanism, as predicted by this model. Moreover, we found that RNAi of PI31 had no significant effect on 26 S proteasome content even though reduced PI31 should promote 20 S-PA700 binding according to this model. On the other hand, if ribosylated PI31 enhances 26 S proteasome assembly via its action on assembly chaperones, RNAi of PI31 would be expected to reduce 26 S content, but it did not. In either case, our in vitro reconstitution of 26 S proteasomes from 20 S and either intact PA700 or PA700 subcomplexes (which contain assembly chaperones) in the absence of either ribosylated or non-ribosylated PI31 indicates that there is no obligatory role for PI31 in this process. In fact, we found that PI31 was inhibitory for in vitro 26 S assembly by each assembly route.

Although investigation of the role of ADP ribosylation on PI31 function was not an initial or primary objective of this study, we explored several aspects of this topic because of its potential to explain and reconcile disparities between in vitro and cellular effects of PI31. However, we were unable to observe key elements of this recently reported process. For example, we failed to detect the reported inhibitory effect of a ribosylation inhibitor on in vitro 26 S proteasome activity and content in mammalian and Drosophila cell extracts. Moreover, treatment of either mammalian or Drosophila cells with this inhibitor had no effect on overall 26 S proteasome content or function. Finally, we failed to obtain evidence of ADP-ribosylation of either bovine PI31 purified from red blood cells or human FLAG-PI31 affinity-purified human from HEK293 cells using several commercial antibodies directed against ADP-ribose.4

4 X. Li and G. N. DeMartino, unpublished results.
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Although the exact interpretation of such negative results is uncertain, we note that human PI31, unlike Drosophila PI31, lacks RXGXXGXX(E/D) or RXPDG motifs characteristic of tankyrase-binding proteins (51, 52).

Although our results do not support a role of either ribosylated or non-ribosylated PI31 in regulation of global proteasome function, it is possible that quantitatively small changes in proteasome content or activity directed by PI31 through mechanisms revealed here and elsewhere could be directed selectively to unique or limited functions. The importance of spatially localized changes in proteasome function in the absence of changes in global proteasome activity has been demonstrated in several examples, including the remodeling of dendritic spines in response to neuronal activity (53, 54). Moreover, disruption of the PI31 gene in Drosophila blocks embryonic development at an early stage by a mechanism that involves the proteasome (35). This effect may reflect PI31-regulated proteasome action against highly selective targets or in a spatially or temporally restricted manner. The interaction between the N terminus of PI31 and the F-box protein Fbxo7, a component of an SCF-type E3 ubiquitin ligase, further suggests a role for PI31 in selective aspects of proteasome function (33). Thus, physiologic roles of PI31 may be revealed by analysis of more specific processes than the assays of general proteasome function examined here. In this regard the dimeric nature of HbX-containing PI31 (32, 33) is physically similar to that of several 20 S proteasome assembly chaperones that interact with α-units of intermediate 20 S assembly complexes (27–30). Although we have no definitive evidence that PI31 might play a similar function, additional studies on that possible target are warranted. Notably, these chaperones provide examples of conditional proteasome regulation that may be relevant to PI31. For example, PAC1-null mice (like PI31-null Drosophila) die in early embryonic development even though PAC1-deficient mouse hepatocytes have normal levels of 26 S proteasome (30). Likewise, effects of yeast Pba1-Pba2 deletions are revealed only when another proteasome regulator, Rpn4, is also deleted (29). These and other findings, combined with those reported here, are consistent with the view that PI31 may play important roles in cellular proteasome regulation despite its lack of effect on global proteasome function.

In summary, the work presented here defines new biochemical features of PI31, including its inclusion in the family of HbX motif-containing proteasome regulators and its interactions with components of the proteasome system. It also reveals limitations on the roles of these interactions for overall proteasome function in cells and establishes a framework for future work directed toward identification of the physiologic role for PI31.

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