Blm10 promotes proteasomal substrate turnover by an active gating mechanism

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Running Title: Protein turnover by Blm10-proteasomes

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Background: Association of the proteasome core with activators regulates proteasome activity.

Results: Blm10 association increases proteasome activity towards peptides and the unstructured proteasome substrate tau-441. This process is mediated by the C-terminus of Blm10.

Conclusion: C-terminal docking-mediated proteasome activation by Blm10 facilitates the turnover of peptide and protein substrates.

Significance: Blm10 contributes to the regulation of proteasome activity.

SUMMARY

For optimal proteolytic function the central core of the proteasome (CP or 20S) has to associate with activators. Here we investigate the impact of the yeast activator Blm10 on proteasomal peptide and protein degradation. We find enhanced degradation of peptide substrates in the presence of Blm10 and demonstrate that Blm10 has the capacity to accelerate proteasomal turnover of the unstructured protein tau-441 in vitro. Mechanistically, proteasome activation requires the opening of a closed gate, which allows passage of unfolded proteins into the catalytic chamber. Our data indicate that gate opening by Blm10 is achieved via engagement of its C-terminal segment with the CP. Crucial for this activity is a conserved C-terminal YYX motif, with the penultimate tyrosine playing a preeminent role. Thus, Blm10 utilizes a gate opening strategy analogous to the proteasomal ATPases HbYX-dependent mechanism. Since gating incompetent Blm10 C-terminal
point mutants confer a loss of function phenotype, we propose that the cellular function of Blm10 is based on CP association and activation to promote the degradation of proteasome substrates.

INTRODUCTION
Regulated protein degradation by the ubiquitin-proteasome system (UPS) is essential for the maintenance of eukaryotic protein homeostasis (1). The central component of the UPS is the 26S proteasome, a 2.5-MDa protease present in the cytoplasm and nuclei of eukaryotic cells (2). The proteasome is crucial for the removal of misfolded or damaged proteins, for providing building blocks for protein synthesis during nutrient limitation, and for the timed degradation of most short-lived proteins (3). The latter function is essential for the correct execution of many cellular processes including cell cycle control, transcription, translation, DNA repair, apoptosis, and antigen presentation (4). To execute its function as a universal protein degradation machinery, the proteolytic capacity of the proteasome is broad and for the most part indiscriminate. However, many control mechanisms regulate and limit proteasome function to prevent non-specific protein degradation (5).

The 26S proteasome consists of two modules: a proteolytic core cylinder and a regulatory/activating module that binds to the core (2). The core (termed core particle (CP) or 20S proteasome) is a barrel-shaped cylinder formed by four stacked rings (6). The two inner rings consist of seven homologous β-subunits each. Three of the β-subunits contain active sites with different substrate side chain preference (7). The active sites face the interior of the core particle, thus creating a sequestered proteolytic chamber. The two outer rings are composed of seven homologous α-subunits, which provide docking sites for proteasome activators. To enter the internal degradation chamber, unfolded substrates must pass through the center of the α-ring. This pore is gated by the N-termini of the α-subunits (8), which must be displaced by proteasome activators (9).

The proteasome core interacts with three activator families: a) complexes composed of or containing AAA+ ATPases (the eukaryotic regulatory particle (RP/19S/PA700) or the archaeal proteasome activating nucleotidase PAN) b) the PA28/11S protein family, with PA28αβ hetero-heptamers and the homo-heptameric PA28γ present in higher eukaryotes, as well as PA26 complexes found in Trypanosoma brucei (10,11), and c) the conserved Blm10/PA200 proteins found in all eukaryotes (9).

Yeast Blm10 and its human ortholog PA200 (for Proteasome Activator of molecular mass 200 kDa) are monomeric proteasome activators, which have been identified in hybrid complexes, where Blm10/PA200 occupies one end and the RP/19S the other end of the core cylinder (12-14). The same proteasome topology has been reported for PA28 family proteasome activators (15-18). Blm10/PA200 proteins are composed of HEAT repeats and display an elongated, curved tertiary structure (13, 19, 20). Similar to RP or PA28 binding, Blm10 or PA200 association with the CP enhances proteasomal peptidase activity (12, 13, 21), suggesting that proteasome activation is a conserved function for this class of proteins.

As the 26S proteasome and PA28-20S, Blm10- and PA200-proteasomes exhibit an open axial channel into the CP as demonstrated by cryo-electron microscopic studies (21, 22). These findings are supported by a recent X-ray structure of doubly capped reconstituted Blm10-CP complexes, which features a partially open, disordered CP gate (20). The structure also revealed that Blm10 binds to the CP via its C-terminus, which occupies the same binding sites as PA26 and the proteasomal ATPases. From the position of the Blm10 C-terminus within the binding pocket and its effect on the conformation of the outer surface of the α-subunit ring, it was proposed that gate opening by Blm10 might be achieved in a similar mechanism as suggested for the proteasomal ATPases (20).

Blm10 has been implicated in the final phase of CP maturation. It was identified in late stage CP assembly intermediates, yet deletion of BLM10 does not result in a pronounced CP maturation defect (23). Loss of
BLM10, however, in a mutant, which abrogates the association of the RP with CP (sen3-1) produced a marked proteasome maturation defect. These data suggest that the function of Blm10 during the final steps of proteasome maturation can be compensated by the RP and that activator binding to the CP in general is required for the final activation of the CP (23). After completion of proteasome biogenesis Blm10 remains stably bound to the CP and affects the turnover of proteasome substrates (24).

We demonstrated recently that Blm10 is required for proteasome-mediated turnover of the transcriptional activator Sfp1. (24). While Sfp1 protein levels increase upon loss of BLM10 in vivo, the turnover of Ubc6, a ubiquitin-dependent proteasome substrate, is not affected. These findings argue for a model in which Blm10 mediates the degradation of a specific subset of proteasome targets.

Sfp1 is a transcription factor required for the correct adaptation of ribosome biogenesis to the metabolic status of the cell and the absence of Blm10 results in dysregulated ribosome biosynthesis. BLM10 is strongly induced during oxidative growth or in the presence of rapamycin (24), which specifically inhibits TORC1 signaling, the major nutrient-sensitive signaling pathway. Deletion of BLM10 furthermore causes partial mitochondrial dysfunction (20). These observations suggest that Blm10-proteasomes are involved in the regulation of metabolism.

Mechanistically, it is unclear whether these cellular functions are related to the physical interaction of Blm10 with the CP to activate proteasomal degradation of Blm10-specific substrates. Here we explore whether the described cellular functions of Blm10 could be tied to regulation of proteasome gating by studying the molecular details of proteasome activation by Blm10.

As described for PA26 and proteasomal ATPase binding, Blm10 association with the CP is mediated by its C-terminus (20,24). The C-termini of Blm10 proteins contain a conserved motif, YYX or YFX, which is analogous to the C-terminal HbYX motif (Hb-Hydrophobic, Y-Tyr (sometimes Phe), X-variable) in PAN and three (Rpt2, Rpt3 and Rpt5) of the six eukaryotic proteasomal ATPases (Figure 1, 25,26). Biochemical and structural studies indicate that this motif is essential for ATPase-induced gate opening (25,26, 29-31).

We show here that mutagenesis in that region of Blm10 produces a loss of function phenotype. Interestingly, a peptide derived from the last seven residues of Blm10 is sufficient to activate the CP peptidase activity, indicating that Blm10 opens the CP gate via a C-terminal docking mechanism, analogous to proteasomal ATPases. A peptide where the penultimate tyrosine was exchanged against an alanine, however, failed to activate the CP. Thus, this residue is crucial for Blm10-mediated proteasome activation.

An enzymatic characterization of proteasomal peptidase stimulation by Blm10 suggests that similar to its mammalian ortholog PA200 (12, 14), Blm10 activates the caspase-like and tryptic-like activity of the proteasome more strongly than the chymotrypsin-like activity. Lastly, we provide evidence that Blm10-proteasomes are competent to contribute to the turnover of cellular proteasome targets by demonstrating that Blm10 enhances proteasome-mediated degradation in vitro utilizing hypophosphorylated unstructured tau-441 as a substrate.

EXPERIMENTAL PROCEDURES

Strains - The strains used in this study are listed in Table 1. Genomically integrated C-terminal mutants were constructed by homologous recombination using standard techniques (27,28) and were verified by sequencing. Primer sequences are available upon request.

Assay of proteasomal activity by C-terminal peptides-Gate opening in yeast CP/20S proteasome (0.2µg/100ml reaction) by C-terminal peptides was assayed in 50mM Tris-HCl, 1mM DTT and 5% glycerol using the indicated fluorogenic substrates (LLVY 100µM, nLPnLD 50µM, LRR 10µM) at 32°C. The peptides derived from the C-terminus of Blm10 (NH2-VLWRSYYA-COOH), from the C-terminus of PA26 (NH2-GTDHMVS-COOH), and from the C-terminus of PAN...

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were synthesized by EZ Biolabs and were HPLC purified to 98% purity. The peptides were dissolved in DMSO prior to addition to the reaction. The rate of substrate hydrolysis (AMC-liberation) was monitored in a Spectramax M5 spectrophotometric plate reader by exciting at 380 nm, and measuring emission at 460 nm.

**Phenotypic analysis** - Strains were grown overnight in YPD and diluted in 96-well plates to a density of $6 \times 10^6$ cells per well followed by five-fold serial dilutions. They were spotted onto YPD plates in the absence or presence of CHX or on different carbon sources and incubated at the temperature indicated.

**Analysis of proteasome complex composition in unfractionated lysates** - Proteasomal complexes in lysates were detected as described previously (13), with the exception that the lysis was performed by cryogenic disruption using a Retsch MM301 grinder mill. Equal total protein (200 µg) was loaded in each well.

**Bml10-proteasome complex purification** - For the purification of WT and mutant Bml10-CP complexes, cells from yMS122 or yMS476 were collected and lysed by cryogenic disruption with a Retsch MM301 grinder mill. Purification was performed as described previously (13). Briefly, the cleared lysate was batch incubated with IgG Affinity gel (Cappel, USA), the beads were collected and washed. Proteasome complexes were eluted after cleavage with tobacco etch viral (TEV) protease (Invitrogen, USA). Subsequently, the purified complexes were first resolved on 3.5% acrylamide native gels (13), followed by an in-gel activity assay with the fluorogenic proteasome substrate Suc-LLVY-AMC as described previously (13) and SDS-PAGE electrophoresis followed by Sypro Ruby staining to assess complex composition and purity of the sample.

**Proteasome Activity Assay** - Three different fluorogenic peptide substrates were used to analyze proteasomal activity: Suc-LLVY-AMC (12.5 - 200μM), Ac-RLR-AMC (12.5 - 350 μM) and Ac-nLPnLD-AMC (12.5 - 350 μM). All substrates were purchased from BACHEM and prepared as 40x stock solutions in DMSO. Activity was assessed in 96-well assay plates (Costar 3631) by monitoring the kinetics of fluorescence increase of generated free AMC in a plate reader (Spectramax M5, excitation: 380 nm, emission: 460 nm). The assay buffer contained 50 mM Tris, pH7.5, 5 mM MgCl$_2$, 0.5 mM EDTA. Purified CP and Bml10-CP were used at a final concentration of 1 nM. The assay mixture was pre-incubated for 15 min at 30 °C. Fluorescence increase was recorded at an interval of 30 s for 30 min with 5 s of mixing prior to each cycle. Data were analyzed with GraphPad Prism 5 to calculate the corresponding values of $K_{\text{half}}$, $v_{\text{max}}$ and $R^2$. The equation was adapted from R. A. Copeland as provided on the GraphPad-webpage.

**In situ analysis of Bml10-CP complex activity** - 5 µg purified CP and Bml10-CP were separated on a 3.5% poly-acrylamide native gel. For the in-gel activity assay the gel was incubated in 50mM Tris, pH 7.4, 5mM MgCl$_2$, 1mM ATP, 100 µM Suc-LLVY-AMC (Bachem) for 10 min at 30ºC. Activity was assessed by visualizing AMC fluorescence after proteasomal cleavage of LLVY-AMC on an ultraviolet screen. Subsequently, the total protein concentration was assessed using SYPRO Ruby Protein stain (Invitrogen, USA) following manufacturer instructions. The same samples were analyzed by immuno-blotting using anti-Bml10 (this study) and anti-proteasome 20S core subunits (BioMol, USA) antibodies. Images were taken on an EL Logic 100 Imaging system (Kodak, USA). For signal quantification, analysis was performed using the software ImageJ 1.41o. CP activity was corrected for the protein abundance in each individual band.

**In vitro degradation of Tau** - Reaction mixtures contained 0.5 mM EDTA, 5 mM MgCl$_2$, 50 mM Tris-HCl pH7.4, and 600 nM recombinant tau-441 (recombinant human tau variant 2N4R, Enzo Life Sciences) in a final volume of 15µl. The reactions were supplemented with 1 nM of CP or Bml10-CP in the absence or presence of 40 μM MG132 (CalBiochem) as indicated. The reactions were incubated at room temperature, and samples were taken at the indicated time points, boiled for 5 min with ELPHO sample buffer to terminate the reactions, followed by...
immunoblot analysis with anti-tau antibody (Biosource). The signals were recorded using an ImageQuant LAS 4000 and analyzed by densitometry with the Image Quant software.

RESULTS

The role of the C-terminal residues of Blm10 in CP binding and activation - Sequence identity among Blm10 orthologs is as low as 17 % (human vs. yeast) (12). The physico-chemical features of the C-terminal residues on the other hand are highly conserved (Figure 1A). The last residue is always hydrophobic (Ala, Val, Ile), the penultimate is always aromatic (Phe or Tyr), while the preceding residue is always a Tyr. This motif thus resembles the HbYX motif in proteasomal ATPases and in the archaeal PAN (Figure 1A) (29), which has been demonstrated to trigger gate opening. We have shown previously that the last three residues of Blm10 are required for CP binding and its function in vivo (24). Since CP gate opening can be induced by peptides derived from the eukaryotic ATPases Rpt2 and Rpt5 or from the archael ATPase PAN (29-31), we speculated that the C-terminal peptide of Blm10 (ct-Blm10) might be sufficient to induce gate opening and thus to stimulate CP peptidase activity.

We tested this hypothesis with a WT Blm10 C-terminal peptide encompassing the last seven residues (ct-Blm10) and a control peptide where the penultimate tyrosine was substituted with alanine (ct-Blm10Y-A). This conserved residue is critical for RP and PAN induced gate opening (29). We found that the C-terminal peptide stimulated markedly the hydrolysis of fluorogenic peptide substrates of the proteasome’s trypsin-like (LRR-AMC) and the caspase-like (nLpnLD-AMC) activity (Figure 1B). Since these substrates are cleaved by different sites and since their hydrolysis is limited by their entry rate, these findings suggest that Blm10 activates gate opening. Accordingly, the activity of a mutant CP with a constitutively open gate due to N-terminal truncations at two of the α-subunits involved in the gating process (8) was not stimulated by ct-Blm10 (Figure 1B). These data strongly suggest that the increased peptide hydrolysis by the CP, observed in the presence of ct-Blm10 is caused by gate opening and not direct stimulation of CP active sites. Furthermore, an exchange of the penultimate tyrosine in the peptide (ct-Blm10 Y-A) abrogated CP activation, confirming a crucial function either in CP binding or gate opening for this residue. The overall capacity of ct-Blm10 to stimulate yeast CP activity was comparable to that of the C-terminal peptide of PAN (ct-PAN), while the gating-incompetent C-terminus of PA26 (ct-PA26) was ineffective in stimulating yeast CP (Figure 1C), in accordance with results obtained for mammalian CP (29). In contrast to the trypsin-like or caspase-like activity, we did not observe stimulation of the chymotrypsin-like activity with ct-Blm10, which is consistent with data reported for the C-termini of the proteasomal ATPases (29).

To further characterize the role of the C-terminal residues, we constructed genomically integrated point mutations where the last 5 residues were substituted with the residues corresponding to the same position in PA26. Since the PA26 C-terminus binds to the same pockets as Blm10, this strategy is expected to minimize structural disturbances during CP docking. We have shown previously that Blm10 is required for correct repression of ribosome biogenesis and that BLM10 deleted cells exhibit cycloheximide (CHX) resistance at low concentrations of this translation inhibitor (24). We therefore tested whether the C-terminal mutants produce a loss of function phenotype and found that an exchange of the penultimate tyrosine (Y2142V) indeed mimicked BLM10 deletion showing increased viability with CHX present (Figure 2A). A similar increase in viability was observed for S2140H, while a partial loss of function phenotype is observed for R2139D (Figure 2A). Mutation of the last residue (A2143S) or Y2141M had no effect on viability in the presence of CHX. To explore whether the phenotypes were caused by disrupted Blm10-CP interaction, we studied the relative distribution of proteasomal complexes in unfractionated lysates after native gel separation followed by an in-gel activity assay. Y2142V and S2140H mutations
abrogated binding, which was preserved in R2139D (Figure 2B). We speculate that the partial loss of function phenotype of the latter mutant is caused by an impaired gating mechanism. These findings are in agreement with previously published data, which revealed that the deletion of the last residue of Blm10 or the substitution of the last seven residues with the gating incompetent PA26 C-terminal peptides, allowed CP binding yet produced a loss of function phenotype (24).

The impact of Blm10 on enzymatic activities of the proteasome - The C-terminal Blm10 peptide impacted the different proteasomal peptidase activities of the CP differentially. While the chymotrypsin-like activity remained largely unaffected, the trypsin-like and the caspase-like activities were elevated (Figure 3). Differential stimulation has also been observed for purified PA200-20S complexes (12). To test whether the effects obtained with ct-Blm10 are also observed with the endogenous Blm10-CP complex, they were purified and subjected to a detailed enzymatic analysis. We obtained essentially the same results as for the ct-Blm10 stimulated CP peptidase activities: stimulation of trypsin-like and caspase-like activities, but unaffected chymotrypsin-like activity (Figure 3A-C). Prior studies have in fact shown that chymotrypsin-like fluorogenic peptide substrates can induce gate-opening (32) and because of their very rapid cleavage by the chymotrypsin-like-site, their hydrolysis is less dependent on gate-opening than the basic and acidic substrates, whose entry is rate-limiting. Although we were unable to determine the half-maximal binding constant or a proper V_max for the trypsin-like activity since saturation could not be reached, we did not observe a change in binding affinity for the caspase or the chymotrypsin substrate, yet observed changes in V_max for the caspase substrate, arguing for a model in which Blm10-mediated stimulation of peptide hydrolysis does not originate from an allosteric effect on the active sites. These results are consistent with an active gate-opening mechanism induced by Blm10 and are corroborated by the observation that ct-Blm10 could not activate peptide hydrolysis in open-gated CP mutants (Figure 1B).

Mitochondria related phenotypes of Blm10 C-terminal mutants and of Blm10 overexpression - We reported recently that a chimeric Blm10 protein, where the last seven residues were exchanged against the corresponding residues in PA26 (Blm10PA26C) allowed complex formation with the CP, yet cells expressing this protein are characterized by a loss of function phenotype (24). Purification resulted in low yields of intact Blm10PA26C-CP complexes, which might be indicative of reduced binding affinity (24). To improve the purification yield, we substituted the endogenous promoter of the mutant with the overexpressing promoter of GAL1, however the yield of intact complexes remained low (Supplemental Figure 1). Since therefore a biochemical characterization was impeded, we studied potential effects of this mutation in vivo by comparing growth related phenotypes of cells overexpressing BLM10 or the BLM10PA26C chimera.

To investigate potential epistatic effects with proteasome hypomorphs, we generated the same strains deleted for the proteasome-related transcription factor Rpn4. rpn4Δ cells are characterized by a reduced proteasome pool (34,35). If the overexpression of BLM10 or BLM10PA26C causes proteasome dysfunction the phenotype should be exacerbated in cells deleted for RPN4.

Blm10 has recently been implicated in mitochondrial function and oxidative metabolism since loss of BLM10 results in a high frequency of cells with dysfunctional mitochondria (20). We observed decreased viability of cells overexpressing BLM10 during growth on non-fermentable carbon sources at higher temperature and in the absence of RPN4 (Figure 4, lower panel). The data support a role of Blm10 in correct maintenance of mitochondrial activity and indicate that this function is linked to the proteasome. Loss of BLM10 on the other hand did not result in significant growth defects under these conditions.

The C-terminus of PA26 is unable to induce gate opening. Thus, replacing the last seven residues of Blm10 with the
corresponding residues in PA26 should abrogate the gate-opening function of Blm10. Consistent with this hypothesis we observed a gain-of-function phenotype in cells overexpressing the Blm10PA26 chimera. After induction of the gene in the presence of galactose, the cells were not viable at higher temperature or on non-fermentable carbon sources (YPGly). Furthermore, co-deletion of RPN4 aggravated the phenotype resulting in lethality under inducing conditions (YPGal) already at normal growth temperatures (Figure 4, upper panel). In summary, while cells tolerate Blm10 overexpression unless oxidative metabolism is induced at higher growth temperature, elevated expression of a gating incompetent Blm10 version is toxic at higher temperature or in cells with reduced proteasome capacity as in cells deleted for RPN4.

Although previous reports suggested that plasmid derived overexpression of WT Blm10 might be lethal (33) or might result in a slow growth phenotype (21) overexpression from the genomic locus (GAL1pBLM10) did not produce a visible phenotype at normal growth temperature under the conditions chosen (Figure 4A, upper panel). Since the lethal phenotype was previously speculated to be caused by blocking RP binding sites in the presence of elevated Blm10 levels (33), we investigated proteasomal complex formation in cells overexpressing BLM10 via native gel electrophoresis in unfractionated lysates. Although we observed a partial reduction in RP2-CP complexes, the dominant change upon Blm10 overexpression was a complete shift of the RP-CP band to the hybrid Blm10-CP-RP complex (Figure 4B). This finding argues for a model in which Blm10 preferentially interacts with the unoccupied surface of RP-CP complexes, rather than competing for RP binding. A significant population of Blm10-CP species without co-associated RP was not observed (Figure 4B).

Doubly capped Blm10-CP complexes retain proteasomal peptidase activity. Recently, a study performed on Blm10-CP in unfractionated lysates or with reconstituted complexes suggested that while the binding of one Blm10 promotes the peptidase activity of the CP, Blm10 binding to both ends of the cylinder inhibits CP activity (36). This model is difficult to reconcile with a proposed function of Blm10 as a proteasome activator and is not supported by an x-ray crystallographic study, which demonstrates that both gates are partially open in CP flanked on both ends with Blm10 (20). To assess this model, we purified endogenous Blm10-CP complexes under conditions that yielded a high portion of doubly capped Blm10-CP (Figure 5A). The contribution of the different complexes towards CP peptidase stimulation was evaluated through an in situ analysis, where we determined the peptidase activity and the relative protein content after an in-gel activity assay (Figure 5, upper two panels). Due to its broad linear dynamic range, protein content was assessed with the fluorigenic dye SYPRO Ruby. To ascertain that all signals analyzed were in the linear range, we performed standard curves for fluorescence increase after cleavage of the fluorigenic peptide Suc-LLVY-AMC by the CP, as well as for protein stain using Sypro Ruby (Supplemental Figure 2). As shown in Figure 5B, doubly capped Blm10 proteasomes are fully active. To demonstrate that the slowest migrating complex indeed contains doubly capped Blm10-CP, we analyzed the Blm10 content in the bands with an anti-Blm10 antibody (Figure 5 B, second to last panel).

Blm10 promotes protein degradation in vitro. Cryo-EM (21) and x-ray crystallographic studies (20) revealed a fully or partially open gate upon Blm10 binding, respectively. Furthermore, the X-ray structure revealed a pore of 13 Å x 22 Å within the Blm10 dome. The largest opening in the PAN complex from Methanocaldococcus jannaschii has a diameter of 13 Å (37) and this ATPase complex promotes the degradation of unfolded proteins by the proteasome (38). Thus, we speculated that the overall topology of the Blm10-CP complex might accommodate the passage and subsequent degradation of an unstructured protein. To test this, we established an in vitro assay to investigate whether Blm10-CP complexes are able to promote the degradation of recombinant unstructured tau-441 (Supplemental Figure 3),
a substrate, which has been demonstrated to be degraded by the proteasome in vitro in the absence of ATP or ubiquitination (39). For this assay equal molar amounts of CP or Blm10-CP (Figure 6 A, B) were incubated with tau in the presence or absence of the proteasome inhibitor MG132. While in the presence of the proteasome inhibitor MG132 tau turnover was blocked, both CP and Blm10-CP complexes promoted tau degradation. Blm10-CP complexes exhibited an approximately two-fold accelerated turnover of the unstructured protein (Figure 6 C, D) as compared to CP alone. We conclude that Blm10-binding activates the proteolytic activity of the CP resulting in a more efficient turnover of unstructured proteins.

DISCUSSION

The cylindrical proteasome core particle is assembled in a latent form and has its active sites sequestered within the interior of the cylinder (6). Stimulation of the proteolytic capacity of the CP requires its association with a proteasome activator (9). These proteins mediate structural changes in the α-ring, opening the gate to allow substrate entry. The open and the closed conformations are distinguished by the positions of the N-termini of the CP α-subunits. While they form an interlaced layer in the closed form, thus occluding substrate entry, in the case of PA26 they rotate away from the body of the cylinder and adopt an ordered 7-fold symmetric conformation in the open form (40). The structural motifs in the N-terminal portion of the α-subunits, which are involved in the gating mechanism, are the rigid helix H0, a reverse turn, and the extreme N-termini of each α-subunit. Two mechanistically and structurally different strategies for proteasome gating have been reported or suggested so far. Biochemical, cryo-EM and X-ray diffraction studies indicate that the proteasomal ATPases induce gate opening through the docking of their C-termini but their final position in the CP binding pockets has not been resolved yet (25, 26, 29-31). The crystal structure of PA26, a homolog of the mammalian PA28 proteasome activator family (11), in a complex with yeast CP, revealed a different proteasome activation mechanism: while the heptomeric PA26 ring still associates with the CP α-ring through their extreme C-termini, activation is achieved via an internal structure, the activation loop (40, 41). It interacts with a conserved proline 13 (P13) within the reverse turn in the proteasome α-subunits. Displacement of P13 appears to drive the formation of the open pore, by dislocating the reverse turn by ~1 Å, a movement, which is propagated to the N-termini (40).

Blm10 orthologs contain a universally conserved motif at their C-termini, YYX or YFX, which is analogous to the HbYX motif found in proteasomal ATPases involved in CP gating. The data presented in this study demonstrate that this motif is actively involved in the CP gate-opening mechanism by Blm10 proteins. Mutagenesis in that region affects the function of Blm10. Two different phenotype classes were observed. Manipulating the penultimate tyrosine (Y2142) either through deletion of the last two residues (24) or exchange against a valine as shown in Figure 2 abrogates proteasome binding and produces a loss of function phenotype. The same observations were made for an exchange of S2140 against histidine. Exchange of R2139 against an aspartate on the other hand promotes binding. The cells however exhibit a partial loss of function phenotype, which we speculate to be caused by inefficient CP gating. Similar characteristics were observed for a Blm10 chimera, where the C-terminus of Blm10 was exchanged against the seven last residues of the gating-incompetent PA26 C-terminus (24).

We furthermore report that a heptameric peptide derived from the Blm10 C-terminus is sufficient to stimulate the peptidase activity of WT CP but not of a CP mutant with a constitutively open gate. A Blm10 C-terminal peptide where the penultimate tyrosine has been replaced by an alanine looses the ability to stimulate. These data are in agreement with a model in which docking of Blm10’s C-terminus occurs in a similar fashion as the proteasomal ATPases C-termini and induce structural changes in the α-ring surface, which result in gate opening. Thus, our data demonstrate that the HbYX motif is crucial for
the Blm10-proteasome interaction as to mediate gate opening. It furthermore supports information obtained from a recent crystal structure, which demonstrates that the HbYX motif in Blm10 repositions crucial residues of the reverse turn within its binding pocket and induces a similar conformation as observed in the open conformation of the PA26-CP complex (9, 20).

Blm10 is an unusual proteasome activator since it is a monomeric protein of 246 kDa in contrast to PA28 activators or the proteasomal ATPases, which form oligomeric rings (42-44). Blm10 binding to the proteasome breaks the seven-fold pseudo-symmetry of the core cylinder, because it engages only with a single binding pocket. However, breaking the CP symmetry during activation is not without precedent. Biochemical analyses of the activation capacity of the RP ATPase C-termini revealed that only three of the six ATPases effectively stimulate CP activity (29) and it is likely that only one HbYX C-terminus may interact with the CP at any given time (45). This hypothesis is supported by the finding that although the C-terminus of Rpt3 does contain a HbYX motif its function appears to be restricted to CP docking rather than gating (46). These reports argue for a mechanism in which an asymmetric engagement of the activator binding pockets within the α-ring surface is sufficient for gate opening, although in the case of the RP, the binding of ubiquitin conjugates somehow further enhances gate opening and substrate entry (47).

In summary, while all proteasome activators bind via their C-termini to the same binding pockets located at the CP surface, two different gate opening mechanisms can be used: C-terminal-docking mediated activation by the proteasomal ATPases and Blm10 and activation loop mediated gate-opening by the PA26/PA28 family of proteasome activators. Mechanistically, proteasome activators can also be classified according to whether they possess ATPase activity or not. Even an open CP gate allows only restricted access to the internal degradation chamber of the proteasome and substrate proteins must be unfolded before they can enter (38, 43, 48). The proteasomal ATPases within the RP and PAN are able to perform this activity, while PA28 and Blm10 activators do not.

Very few targets for PA28 have been described (49), and there is little information on which substrates are specifically degraded by Blm10-proteasomes. The only protein so far that has been reported to require Blm10 for correct proteasome-mediated turnover is the ribosome-related transcription factor Sfp1 (24). Many transcription factors or transcriptional co-regulators are predicted to contain large intrinsically unstructured regions (50). The graphic webserver FoldIndex© (51) provides an algorithm to predict unstructured regions in proteins based on their primary sequence. Such an analysis of the Sfp1 sequence predicts a high portion of intrinsically unstructured regions (Supplemental Figure 3A). A preference for degrading unstructured proteins might explain the lack of ATPase activity in Blm10 activators. This hypothesis is corroborated by our findings that Blm10 is able to stimulate CP-mediated degradation of the unstructured proteasome substrate tau-441 (Supplemental Figure 3B) in vitro as shown in this report. We infer from these data that the structural change of the CP gate upon Blm10 binding is sufficient for passage of both peptides and unfolded protein substrates. We propose that the ability of Blm10-proteasomes to promote the degradation of unfolded proteasome substrates might represent a critical and perhaps principal function of Blm10 in vivo.

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41. Whitby, F. G., Masters, E. I., Kramer, L., Knowlton, J. R., Yao, Y., Wang, C. C., and...
Figure Legends

FIGURE 1. The C-terminus of Blm10 stimulates the CP peptidase activity and thus mediates gate opening. (A) Left Panel: Sequence alignments of the C-terminal segments of S. cerevisiae (yeast), human and archaeal (Methanocaldococcus jannaschii, PAN) proteasomal ATPases. HbYX motifs are indicated in bold letters. Right panel: Sequence alignments of the C-terminal segments of Blm10 orthologs. S.c. Saccharomyces cerevisiae, C.a. Candida albicans, C.e. Coenorhabditis elegans, H.s. Homo sapiens, A. t. Arabidopsis thaliana (B) Heptapeptides derived from the wild-type Blm10 C-terminus (ct-Blm10) as well as a peptide where the penultimate tyrosine has been replaced by alanine (ct-Blm10 Y-A) were tested for their ability to stimulate CP peptidase activity against three fluorogenic peptides assaying the chymotryptic (Suc-GGL-AMC), the tryptic (Suc-LRR-AMC) and the caspase-like activity (Ac-nLPnLD-AMC) of the proteasome. The assays were performed with 0.2 µg purified wild-type CP and an open gate mutant, where the N-termini of α3 and α7 were deleted. The open gate mutant was purified from either WT cells or cells deleted for BLM10. The data are normalized for the activity of wild-type CP with the respective substrate. (C) To compare ct-Blm10 mediated stimulation with ct-PAN and ct-PA26 the same assay as in (A) was performed with Suc-LRR-AMC, the proteasome substrate that showed the highest stimulation for the C-terminal peptide of Blm10.

FIGURE 2. The penultimate tyrosine within the Blm10 YYX motif is required for proteasome binding. (A) Wild-type (WT), BLM10 deleted cells (blm10Δ) and genomically integrated point mutants within the last five residues of Blm10 as indicated by bold letters to the right were spotted on YPD plates in the absence (left panel) or in the presence of 0.3 µg cycloheximide (right panel) and were grown for 3-5 days at 30°C. The C-terminal sequences are indicated to the right. (B) The same strains as in (A) were tested for proteasome association in unfractionated lysates. Cell lysates were separated by native-gel electrophoresis. Active proteasomes are visualized by an in-gel activity assay using Suc-LLVY-AMC.

FIGURE 3. Impact of Blm10 on the different proteasomal peptidase activities. Substrate kinetics were determined using purified CP and Blm10-CP with varying concentrations of Suc-LLVY-AMC to determine the chymotrypsin-like activity (upper panel), Ac-RLR-AMC for trypsin-like activity (middle panel) and Ac-nLPnLD-AMC for caspase-like activity (lower panel). The kinetic parameters of each reaction are shown in the tables to the right.

FIGURE 4. Phenotypic characterization of cells overexpressing Blm10 or a gating incompetent Blm10PA26 chimera. (A) The strains indicated were grown in YPD and spotted on complete media with glucose (YPD) as control, galactose (YPGal) to induce Blm10 overexpression and glycerol as carbon source for analysis of non-fermentative growth. The plates were incubated either at 30°C (upper panel) or at 37°C (lower panel). (B) The strains indicated were subjected to the same analysis as described in Fig. 2B to visualize the different proteasome holocomplexes.

FIGURE 5. Proteasome peptidase activity in singly and doubly capped Blm10-CP. (A) Equal molar quantities of CP or Blm10 CP were separated by native-gel electrophoresis. Peptidase activity was visualized by an in-gel activity assay (upper panel) and protein concentration was assessed with Sypro Ruby (second panel). The picture of the Sypro Ruby stain is presented in a black-white inverted version. Parallel gels were subjected to immune-blotting using anti-Blm10 (third panel or anti-CP antibodies. (B) Signals obtained from (A) were quantified using ImageJ. The mean of three independent experiments is presented. The different values obtained for free CP present in lane 1 and 2 represent differences in the latency of the CP.

FIGURE 6. Accelerated in vitro degradation of tau-441 by Blm10-proteasomes. (A) Equal molar
amounts of Blm10-CP or CP were separated by SDS-PAGE and stained with Sypro Ruby to demonstrate that equal amounts of CP were present in the assay. (B) Proteasomal complex composition was assessed by native-gel electrophoresis followed by an in-gel activity assay. (C) tau-441 was incubated with equal molar amounts of CP or Blm10-CP in the absence or presence of MG132. Aliquots were taken at the times indicated, separated by SDS-PAGE. Tau-441 was detected by immuno-blotting with a tau-specific antibody. (D) The mean values of the densitometric analysis of three independent degradation assays are presented.
Table I: Strains used in this study

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Figure 1

### ATPase C-Termini

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### Blm10 C-Termini

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**Figure B**

- Bar graph showing fold stimulation over control for different samples.

**Figure C**

- Bar graph showing fold stimulation over control for different samples.
Figure 2

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![B lane](image)

RP-CP-RP

RP-CP-Blm10

RP-CP
Figure 3

A. Chymotryptic-like Activity

B. Trypsin-like Activity

C. Caspase-like Activity
Blm10 promotes proteasomal substrate turnover by an active gating mechanism
Thomas Dange, David Smith, Tahel Noy, Philipp C. Rommel, Lukas Jurzitza, Anne Legendre, Daniel Finley, Alfred L. Goldberg and Marion Schmidt

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