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The C-terminal Extension of the β7 Subunit and Activator Complexes Stabilize Nascent 20 S Proteasomes and Promote Their Maturation*

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António J. Marques[‡1], Christoph Glanemann[‡2], Paula C. Ramos[§], and R. Jürgen Dohmen[‡3]

From the [‡]Institute for Genetics, University of Cologne, Zülpicher Strasse 47, D-50674 Cologne, Germany and [§]Departamento de Química, Bioquímica e Farmácia, Faculdade de Ciências e Tecnologia, Universidade do Algarve, Campus de Gambelas, 8000-117 Faro, Portugal

The eukaryotic 20 S proteasome is formed by dimerization of two precursor complexes containing the maturation factor Ump1. β7/Pre4 is the only one of the 14 subunits forming the 20 S proteasome that is absent from these precursor complexes in *Saccharomyces cerevisiae*. Increased expression of Pre4 leads to a reduction in the level of precursor complex, indicating that Pre4 incorporation into these complexes is rate-limiting for their dimerization. When we purified these precursor complexes, we observed co-purification of Blm10, a large protein known to attach to the α ring surface of proteasomes. In contrast to single mutants lacking either Blm10 or the C-terminal extension of Pre4, a mutant lacking both grew extremely poorly, accumulated very high levels of precursor complexes, and was impaired in β subunit maturation. The effect of *blm10*Δ on proteasome biogenesis is modest, apparently because the 19 S regulatory particle is capable of substituting for Blm10, as long as precursor complex dimers are stabilized by the Pre4 C terminus. We found that a mutation (*sen3/rpn2*) affecting the Rpn2 subunit inhibits attachment of the 19 S activator to the 20 S particle or its precursors. Although the *sen3* mutation alone had no apparent effect on precursor complex dimerization and active site maturation, the *sen3 blm10* double mutant was impaired in these processes. Together these data demonstrate that Blm10 and the 19 S activator have a partially redundant function in stabilizing nascent 20 S proteasomes and in promoting their activation.

The multicatalytic protease, termed "26 S proteasome," provides the main and essential proteolytic activity in the cytoplasm and the nucleus of a eukaryotic cell (1). The main function of this protease is to degrade polyubiquitylated substrates, which include abnormal as well as regulatory proteins. Because of these properties, the proteasome has become an interesting drug target with potential in the treatment of cancer (2). The 26 S proteasome is composed of the catalytic core particle (CP),[4] termed the "20 S proteasome," and two 19 S regulatory particles (RPs), also known as PA700 activator complexes (3). The eukaryotic 20 S proteasome is assembled from 14 distinct subunits, 7 of the α type and 7 of the β type. The overall $\alpha_7\beta_7\beta_7\alpha_7$ structure of this particle is characterized by the presence of two identical halves, each comprising a ring of seven α subunits and a ring of seven β subunits (4, 5). The two inner β rings contact each other and form the catalytic chamber of the CP. Five of the β subunits are synthesized as precursors with N-terminal propeptides (6). Three of these subunits, β1, β2, and β5, provide the postacidic, the tryptic, and the chymotryptic activities, respectively (7, 8). The two outer α rings contact the β rings on one side, and the base of the 19 S RP or alternative activator complexes on the other side. The 19 S RP is composed of two subcomplexes, the base and the lid, that are linked by the Rpn10 subunit (9). The base is composed of six ATPase subunits (Rpt1–Rpt6) and three non-ATPase subunits (Rpn1, Rpn2, and Rpn13) (3). The 19 S RP has multiple roles in proteasome function. It recognizes ubiquitylated proteins either directly or indirectly and mediates their unfolding and translocation into the CP. In addition, the 19 S RP serves as an interaction platform for numerous other proteins, including ubiquitin ligases and deubiquitylating enzymes (3, 10). Aside from the 19 S RP, several activator complexes have been described that can alternatively attach to the 20 S proteasome and activate its proteolytic function. However, only the 19 S RP appears to promote the degradation of ubiquitylated proteins. In *Saccharomyces cerevisiae*, the HEAT repeat protein Blm10, a homologue of mammalian PA200 (11), has been reported to attach to 20 S CPs and to activate its peptide cleavage activity *in vitro* (12, 13). Blm10 (called Blm3 in some publications), however, has also been detected in association with proteasome precursor complexes (14, 15). The physiological function of Blm10 has remained unclear.

20 S CPs are assembled from "half-proteasome precursor complexes" containing unprocessed β subunits and maturation factor Ump1 (underpinning maturation of proteasome) (6, 16).

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[1] Supported by a predoctoral fellowship of the Deutsche Forschungsgemeinschaft graduate program "Genetics of Cellular Systems."

[2] Supported by a predoctoral fellowship of the North Rhine-Westphalia graduate school in "Genetics and Functional Genomics." Present address: Center for Neurologic Diseases, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115.

[3] To whom correspondence should be addressed. Tel.: 49-221-470-4862; Fax: 49-221-470-1631; E-mail: j.dohmen@uni-koeln.de.

[4] The abbreviations used are: CP, core particle; RP, regulatory particle; CTE, C-terminal extension; HA, hemagglutinin; FH-Ump1, FLAG-His_6-tagged Ump1.

Following dimerization of such complexes, autocatalytic processing and thereby activation of β1, β2, and β5 as well as degradation of Ump1 take place (17, 18). We have reported recently that a C-terminal extension (CTE) of *S. cerevisiae* β7/Pre4 serves two functions in 20 S CP biogenesis (19). This extension emanates from one half of the CP to the other, where it intercalates between subunits β1/Pre3 and β2/Pup1, thereby promoting formation and stabilization of nascent CPs. Residues within the Pre4 CTE, in addition, contact residues in β1/Pre3 to stabilize a conformation that is required to promote β1/Pre3 processing and activity (4, 19).

We report here that purified half-proteasome precursor complexes lack β7 but contain Blm10. Our results show that β7/Pre4 incorporation completes half-proteasome assembly and is rate-limiting for its dimerization. Whereas deletion of the *BLM10* gene alone has little effect on 20 S CP formation, it causes growth inhibition, accumulation of half-proteasome precursor complexes, and β subunit processing defects when combined either with a deletion of the CTE of Pre4 or with a mutation in the *SEN3/RPN2* gene. The strong synthetic effects of these mutations indicate that Blm10, the 19 S RP, and the Pre4 CTE have partly redundant functions in 20 S CP formation and maturation.

EXPERIMENTAL PROCEDURES

Yeast Media and Strains—Yeast rich (YPD) and synthetic (SD) minimal media with 2% dextrose were prepared as described (19). All strains are derivatives of JD47-13C (19). Strains JD59 (*ump1*Δ) and JD139 (*PUP1-HA*$_2$) have been described previously (18). A strain (AM19) expressing truncated Pre4 protein lacking its C-terminal 19 residues (Pre4-ΔCTE) was generated by two-step gene replacement. A similar two-step strategy was used to generate AM22, a derivative of AM19 that stably expressed N-terminally FLAG-His$_6$-tagged Ump1 (FH-Ump1). Strains AM29 and AM47, which expressed HA$_2$-tagged Blm10 were derived from JD47-13C and AM22, respectively, using a plasmid integration strategy as described (18). Similar integrative strategies were used to generate strains AM75 (*PRE4-HA*$_2$) and AM128 (*pre4-ΔCTE-HA*$_2$). Strains AM36, AM74, and AM31 (all *blm10*Δ::*KanMX4*) were generated by amplifying the deletion cassette from the corresponding strain obtained from the EUROSCARF collection and introducing it into JD47-13C (wild type), AM19 (*pre4-ΔCTE*), or AM22, respectively. Strain DDY112-15a (*sen3-1*) (kindly provided by Dr. Mark Hochstrasser) was crossed to AM36 (*blm10*Δ) to produce *sen3-1 blm10*Δ double mutants. Identical results were obtained for several independent spore clones of this cross (data not shown). For overexpression of *PRE4*, *PRE2*, or *UMP1* from the copper-inducible P$_{CUP1}$, a set of 2μ/*LEU2*-based plasmids was generated. The parental plasmid pJDCEX1 was used as an empty vector control.

Fractionation of Whole Cell Extracts by Gel Filtration, Electrophoresis, and Immunoblotting—*S. cerevisiae* cells were grown at 30 °C in SD or YPD medium to A_{600} of 1.0 ± 0.2. Protein extraction and fractionation on a Superose 6 column coupled to an ÄCTA fast protein liquid chromatography (GE Healthcare) as well as SDS-PAGE and immunoblots were performed as described previously (18). The volume of a fraction was 600 μl, of which 40 μl were analyzed by SDS-PAGE.

Proteasomal Peptidase Activity Assays and Steady-state Immunoblots—Yeast cells were grown at 30 °C in YPD media to A_{600} of 1.0 ± 0.2 and frozen. Cells were lysed with glass beads in extraction buffer (50 mM Tris-HCl, pH 7.5, 2 mM ATP, 5 mM $MgCl_2$, 1 mM dithiothreitol, 15% glycerol). Cell debris was removed at 16,100 × *g*, and the total protein content in the supernatant was determined using the Bio-Rad protein assay. For the chymotryptic activity, 5 μl of each crude extract were diluted to a final volume of 50 μl in extraction buffer containing 25 ng/μl of succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Bachem). The hydrolysis of the peptide with release of the fluorophore was monitored at 60-s intervals for 30 min at 37 °C. For SDS-PAGE, 10 μg of total protein from each extract were loaded per lane. Immunoblot analysis and quantification of signals using the Odyssey infrared imaging system were performed as described (20). The primary antibodies used were monoclonal anti-HA (Covance), polyclonal anti-Pup1, or anti-Cim5/Rpt1.

Purification of the "Half-proteasome" Complex and Characterization of Subunit Composition—Yeast cells (strain AM22) were grown in 1 liter of YPD at 30 °C until A_{600} = 3.0 and harvested at 3500 × *g*, washed with cold water, frozen in liquid nitrogen, and stored at −80 °C. Cells were resuspended in cold FLAG buffer (50 mM Tris, pH 7.5, 5 mM $MgCl_2$, 2 mM ATP, 150 mM NaCl, and 15% glycerol) and lysed with the EmulsiFlex-C5 (Avestin) at 25,000 p.s.i. for 5 min. Cell debris was removed at 30,000 × *g* for 15 min at 4 °C. The supernatant was incubated with a 500-μl bed volume of anti-FLAG M2 EZview affinity resin (Sigma) for 90 min at 4 °C. The resin was then washed three times for 5 min each with 10 ml of FLAG buffer, and the bound material was eluted at 4 °C for 60 min in FLAG buffer containing 200 ng/ml FLAG peptide (Sigma). The eluate was loaded on a 12% SDS-polyacrylamide gel and stained with colloidal Coomassie as described (21). Protein bands were excised from the gel, and the proteins were eluted and trypsinated. The resulting peptides were used for protein mass fingerprint analysis.

RESULTS

Purification and Characterization of Half-proteasome Precursor Complexes—Our previous studies have implicated the Pre4 CTE and maturation factor Ump1 in 20 S CP assembly and maturation (18, 19). Seeking further insights regarding the details of these processes, we purified the Ump1-containing proteasome precursor complex from *S. cerevisiae* cells. Because this complex is of low abundance in wild-type cells, we took advantage of our observation that the *pre4-ΔCTE* mutation results in a severalfold increase of its levels. Since Ump1 is unique to the half-proteasome precursor (*i.e.* it is absent from mature 20 and 26 S particles), we chose to link this maturation factor to a tag for affinity purification. Because Ump1 becomes enclosed during the dimerization of these precursor complexes (18), this strategy, in addition, has the advantage that binding of Ump1 to an affinity matrix should prevent dimerization. Since C-terminal tags of Ump1 are buried within the precursor complex (18), we fused a FLAG-His$_6$ tag to its N terminus (FH-

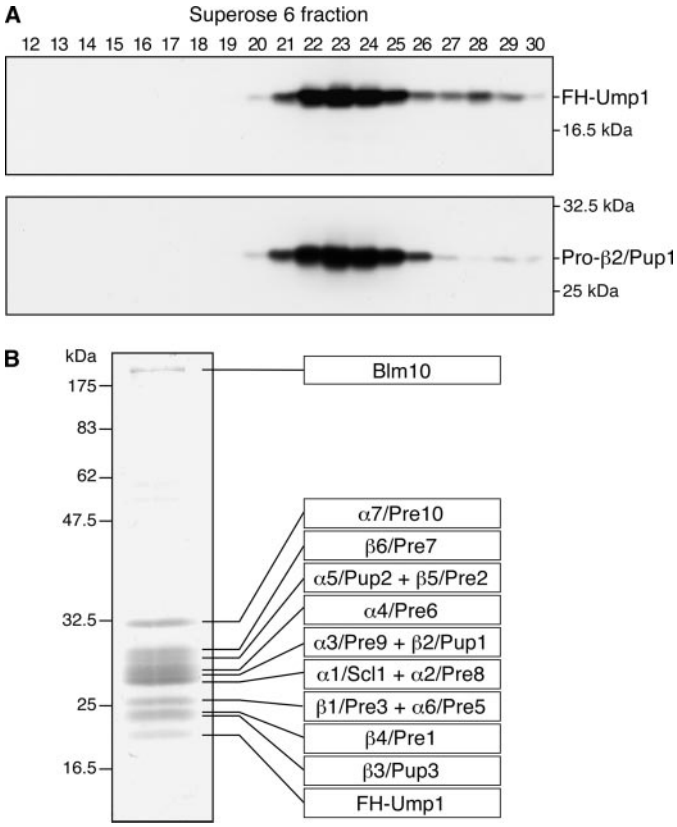

FIGURE 1. **Purification and characterization of proteasomal precursor complexes.** Complexes containing FH-Ump1 were purified from AM22 (*pre4-ΔCTE FH-Ump1*) by anti-FLAG affinity chromatography. *A*, analysis of purified Ump1-containing precursor complexes by Superose 6 gel filtration chromatography. *Top*, FH-Ump1 was detected with M2 anti-FLAG antibody. *Bottom*, Pup1 was detected with a polyclonal antibody raised against Pup1. *B*, colloidal Coomassie-stained polyacrylamide gel and assignment of the bands based upon peptide mass fingerprinting. The positions of molecular size markers are indicated.

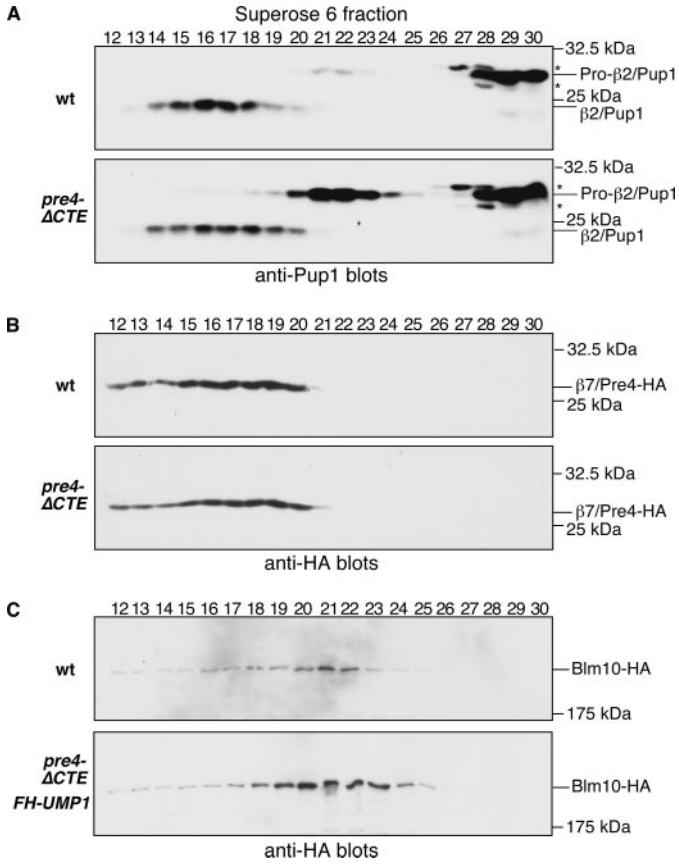

FIGURE 2. **β7/Pre4 is absent from Ump1-containing precursor complexes.** Proteasomal complexes from the wild type (*wt*) and the *pre4-ΔCTE* mutant were analyzed by gel filtration of crude extracts and detection of β2/Pup1 (*A*), of Pre4-HA_2 (*B*), or of Blm10-HA_2 (*C*). The *asterisks* in *A* indicate the positions of proteins that cross-reacted with the anti-Pup1 antibody. The positions of molecular size markers are indicated.

Ump1). We found this tag in combination with the *pre4-ΔCTE* mutation to further enhance accumulation of the precursor complex. Complexes containing FH-Ump1 were purified by a one-step protocol on a resin containing anti-FLAG antibody. Gel filtration analysis of the purified material confirmed that it exclusively contained proteasomal precursor complexes and no 20 or 26 S particles (Fig. 1*A*). Subunits of the purified precursor complex were separated by SDS-PAGE and identified by peptide mass fingerprint analysis (Fig. 1*B*). This analysis revealed the presence of 13 of the 14 CP subunits. Remarkably, the missing subunit was β7/Pre4. In addition, we detected Ump1 and Blm10. Previous studies that used Ump1 fused at its C terminus with protein A for affinity purification similarly yielded complexes containing Blm10. These complexes, however, lacked β6 and β7 and contained undisclosed chaperones (14, 22). Based upon our experience, the differences between these preparations are probably due to the bulky C-terminal protein A domain, which may result in an incompletely assembled aberrant complex associated with molecular chaperones. As pointed out above, a short peptide tag at the C terminus of Ump1 was previously shown not to be accessible to anti-tag antibody in the context of the precursor complex, indicating that the C terminus of the protein points into the interior of the complex (18).

β7/Pre4 Incorporation Is a Rate-limiting Step in 20 S CP Formation—Our finding that β7/Pre4 is the only subunit present in mature 20 S proteasomes that is absent from purified half-proteasome precursor complexes suggested that it is the last subunit to be incorporated into such complexes, triggering their fast dimerization. Consistent with this assumption, in contrast to Pro-Pup1 (Fig. 2A), no significant amounts of β7/Pre4-HA were detected in fractions of a gel filtration that contained the half-proteasome precursor complex (Fig. 2*B*, fractions 21 and 22). Similar results were obtained with extracts derived from an otherwise identical untagged wild-type strain and detection with an anti-Pre4 antiserum (data not shown). Remarkably, aside from the absence of Pre4 in fractions 21 and 22, we also did not detect unassembled free Pre4 in these fractionation experiments. The latter finding suggested that Pre4 is a rate-limiting subunit that, following its synthesis, is rapidly incorporated into precursor complexes and drives their dimerization. Since the *pre4-ΔCTE* mutant accumulated higher levels of the precursor complex, we asked whether the truncated Pre4, which is impaired in 20 S CP formation, would be detectable in these complexes. This was not the case (Fig. 2*B*). We conclude that stable incorporation of Pre4 and 20 S CP formation mutually depend on each other. In contrast to what was observed for Pre4, we detected increased amounts of Blm10 in fractions containing proteasomal precursor complexes in the *pre4-ΔCTE*

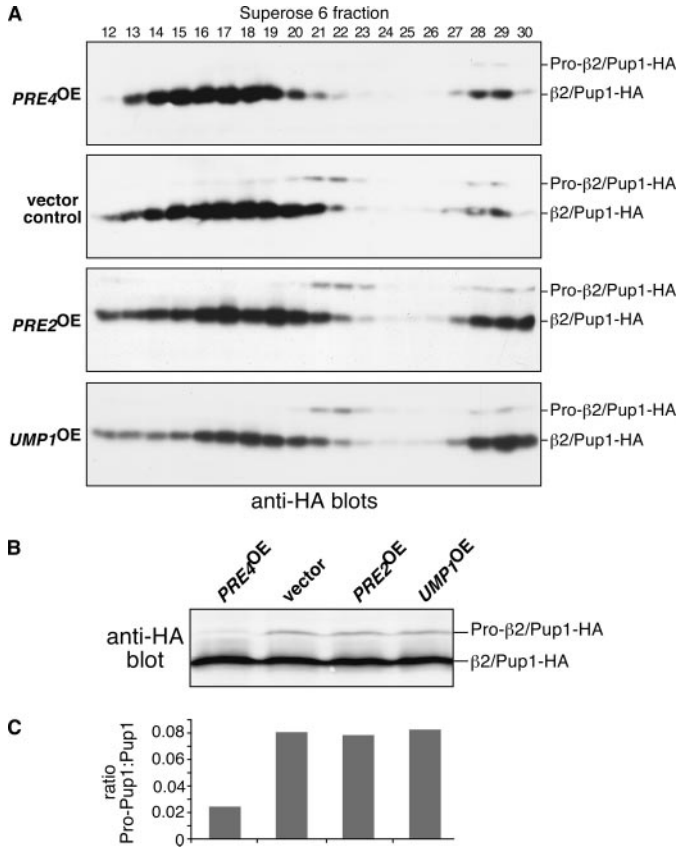

FIGURE 3. ***β*7/Pre4 incorporation is rate-limiting for dimerization of proteasomal precursor complexes.** *A*, proteasomal complexes from strain JD139 (*PUP1-HA*$_2$) transformed either with an empty vector or with plasmids overexpressing *PRE4*, *PRE2*, or *UMP1* were analyzed by gel filtration of crude extracts and detection of *β*2/Pup1-HA$_2$ by immunoblotting. The positions of the propeptide-bearing precursor form of Pup1 and of mature Pup1 are indicated. *B*, comparison of the ratio of Pro-Pup1 to mature Pup1 in crude extracts of the same strains as in *A*. *C*, quantification of the immunoblot data shown in *B*.

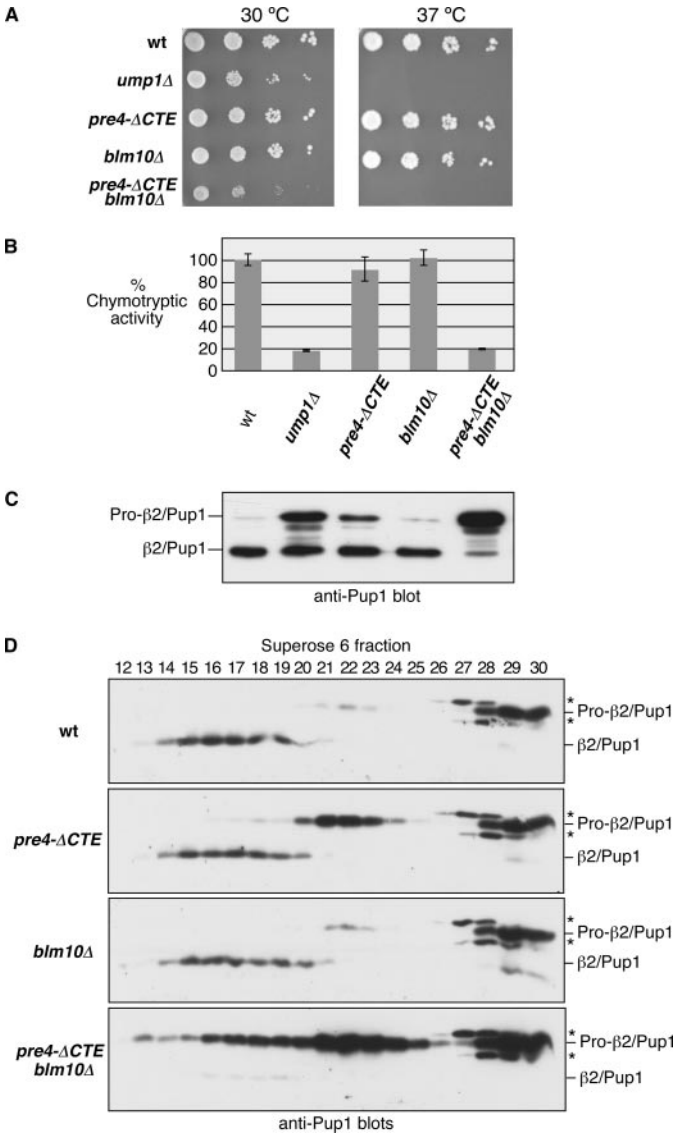

FIGURE 4. **Synthetic effects of *β*7/Pre4 C-terminal truncation and *blm10*Δ.** Comparison of various properties of a congenic set of strains with the indicated genotypes. *A*, growth properties of the indicated strains were compared by spotting serial dilutions of yeast cultures onto YPD media and incubation at 30 or 37 °C for 2 days. *B*, comparison of proteasomal chymotryptic activities in yeast cell extracts. *C*, comparison of steady state levels of unprocessed Pup1 precursor (Pro-Pup1) and mature Pup1. *D*, comparison of proteasomal complexes by Superose 6 gel filtration analysis. The *asterisks* indicate the positions of cross-reactive bands. *wt*, wild type.

mutant in comparison with the wild type (Fig. 2*C*). These data are consistent with the results obtained after purification of precursor complexes using FH-Ump1 (Fig. 1) and indicate that Blm10 binds to precursor complexes lacking Pre4.

Incorporation of β7/Pre4 Drives Dimerization of Proteasomal Precursor Complexes—The apparent lack of free Pre4 subunit and its absence from the precursor complex suggested that its incorporation coincides or is closely followed by precursor complex dimerization and that Pre4 availability should thus be rate-limiting. A comparison of proteasomal complexes in a wild-type strain with those in a strain overexpressing PRE4 supported this assumption (Fig. 3). Compared with the wild-type control, a strain overexpressing *β*7/Pre4 contained significantly lower amounts of the precursor complex bearing unprocessed *β*2/Pup1 (Fig. 3*A*). A quantitative immunoblot analysis of the relative amounts of unprocessed *versus* processed forms of Pup1 in unfractionated crude extracts corroborated this result (Fig. 3, *B* and *C*). Overexpression of *β*5/Pre2 or Ump1, in contrast, had no detectable effect on the relative amounts of proteasomal complexes. These data support the notion that the observed rate-limiting role for dimerization of precursor complexes is a specific function of Pre4.

BLM10 Is Important for 20 S CP Formation and Maturation in the Pre4-ΔCTE Mutant—The presence of Blm10, a large protein reported to function as a regulatory complex that attaches to *α* rings of 20 S CPs (see Introduction), in our proteasome precursor preparations prompted us to ask whether its absence has an impact on proteasome biogenesis. The *blm10*Δ mutation had a very modest effect on this process, with relative amounts of unprocessed *β*2/Pup1 increased by ~20% when cells were grown at a low temperature (15 °C; data not shown). No apparent effects were detectable on Pup1 processing or peptidase activity at 30 or 37 °C (Fig. 4) (data not shown). In comparison, already significantly higher levels of proteasome precursor were detected in the *pre4-ΔCTE* mutant. Both single mutants, moreover, did not display any detectable growth phe-

notype at 30 or 37 °C (Fig. 4*A*). The *blm10*Δ *pre4*-Δ*CTE* double mutant, in contrast, displayed a striking synthetic growth defect at 30 °C and was inviable at 37 °C (Fig. 4*A*). Biochemical analysis revealed that this mutant showed also dramatically reduced peptidase activity and was severely impaired in β2/Pup1 processing (Fig. 4, *B* and *C*). To investigate further which steps in proteasome biogenesis are affected, we compared the distribution of proteasomal complexes in this mutant with those of wild type and the single mutants (Fig. 4*D*). In the double mutant, we observed a striking accumulation of proteasomal precursor complexes, much stronger than in the *pre4*-Δ*CTE* single mutant. In contrast to the latter mutant, the double mutant moreover displayed a profound defect in 20 S CP maturation. Unprocessed β2/Pup1 was detected in fractions 13–21 containing the 20 and 26 S particles. Together these results indicate that Blm10 has a role in proteasome assembly and maturation. In the absence of Blm10, these processes appear to occur nearly normally, but destabilizing the nascent dimer by deletion of the CTE from Pre4 makes this role of Blm10 apparent. Similarly, the lack of the Pre4 CTE, although impairing precursor dimerization, has no apparent effect on β2/Pup1 maturation. Loss of the CTE, however, results in a dramatic impairment of β2/Pup1 processing in a strain lacking Blm10. Together, these observations indicate that both Blm10 and the Pre4 CTE stabilize nascent 20 S CPs, thereby promoting their maturation.

The sen3 Mutation Inhibits Attachment of the 19 S RP to the 20 S Proteasome—The Rpn2/Sen3 protein is a subunit of the base subcomplex of the 19 S RP (9). We analyzed the effect of the *sen3-1* mutation (23) on proteasomal complexes by size fractionation (Fig. 5*A*). We observed a strikingly altered distribution of proteasomal complexes in fractions obtained by gel filtration. Although mature 20 S CP proteasomes, with or without attached 19 S RP, eluted in fractions 13–22 of wild-type extracts, they were detectable only in fractions 16–23 of *sen3-1* extracts. The latter fractions typically contain the 20 S proteasome, whereas 20 S proteasomes capped with one or two 19 S RPs are distributed through fractions 13–18 (24). This result indicated that *sen3-1* inhibits assembly of the 19 S complex with the 20 S CP.

Rpn2 and Blm10 Can Partially Substitute for Each Other in Stabilizing Nascent 20 S Proteasomes—The properties of the *sen3-1* mutant described above allowed us to address the question of whether the 19 S RP plays a role in the assembly and maturation of the 20 S CP. Similar to the *blm10*Δ mutant (see above), the *sen3-1* mutant alone, however, displayed neither an increase in half-proteasome precursors levels nor a defect in the processing of β2/Pup1. We therefore asked whether combining *sen3-1* with *blm10*Δ would result in such defects, since this mutant would lack Blm10 entirely and have reduced functional 19 S RP. This double mutant grew more poorly than the *sen3-1* single mutant and showed a strong accumulation of precursor complexes containing unprocessed β2/Pup1 (Fig. 5, *A* and *B*). We conclude that the 19 S RP can substitute for Blm10 in stabilizing nascent 20 S proteasomes and promoting their maturation. To verify this notion, we asked whether components of the 19 S RP could be detected in association with proteasome precursor complexes. Indeed, Rpt1, an ATPase subunit of the 19 S RP, reproducibly co-precipitated with FH-Ump1 from

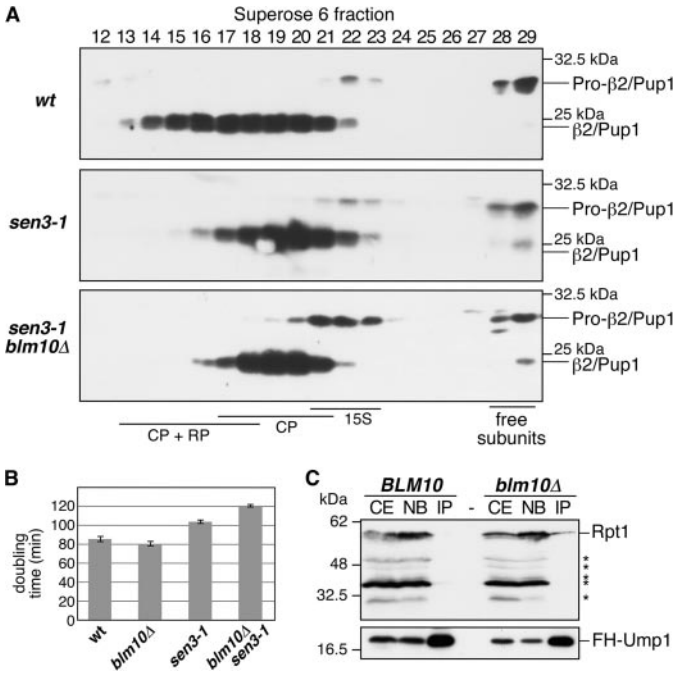

FIGURE 5. **The 19 S RP and Blm10 can functionally replace each other in promoting the formation of mature 20 S CP.** *A*, comparison of proteasomal complexes in the strains of the indicated genotypes by Superose 6 gel filtration analysis. The Western blots were probed with anti-β2/Pup1 antibody. The positions of the precursor form (Pro-Pup1) and the mature form of Pup1 are indicated. The *asterisks* indicate the positions of cross-reactive bands. *B*, growth rates of the indicated strains were compared by determining the doubling times in the exponential growth phase. Shown are the mean values obtained with three independent cultures for each genotype. *C*, the 19 S RP subunit Rpt1 is associated with half-proteasome precursor complexes in *blm10*Δ cells. Extracts from strain AM22 (*FH-Ump1 pre4*-Δ*CTE*) and from AM31 (*FH-Ump1 pre4*-Δ*CTE blm10*Δ) were subjected to anti-FLAG immunoprecipitation. Crude extracts (*CE*), the nonbinding material (*NB*), and the precipitates (*IP*) were analyzed by anti-Rpt1 and, after stripping, with anti-FLAG immunoblotting. The *asterisks* denote cross-reactive bands. *wt*, wild type.

extracts of cells lacking Blm10 but was not detected in the precipitates when extracts from cells containing Blm10 were used (Fig. 5*C*). We conclude that the 19 S RP binds to half-proteasome precursor complexes in the absence of Blm10.

DISCUSSION

In the present work, we have investigated the factors that control dimerization of proteasome precursor complexes and the maturation steps that go along with it in *S. cerevisiae*.

β7/Pre4 Incorporation Triggers Dimerization of Half-proteasome Precursor Complexes—We found that purified half-proteasome precursor complexes contained Ump1, all seven α subunits, six of the seven β subunits (the missing one being β7/Pre4), and Blm10. These data are consistent with another report, which was published while our work was in the submission process (15). These authors also suggested that Pre4 incorporation is a rate-limiting step in dimerization of precursor complexes, based upon the finding that overexpression of Pre4 suppressed the defects of several proteasome assembly mutants. Our data provide direct evidence for Pre4 incorporation being a rate-limiting step in proteasome assembly by demonstrating that overexpression of Pre4 in wild-type cells results in a profound enhancement of precursor complex dimerization (Fig. 3).

Possible Regulatory Function of Pre4 in Activating Preassembled Precursor Complexes—The observation that β7/Pre4 is hardly detectable as a free subunit (Fig. 2) and that increased intracellular Pre4 concentration results in a strong reduction of the level of proteasome precursor complexes (Fig. 3) make this subunit an attractive candidate for regulatory responses. Similar to those of other proteasomal subunits, the promoter of the *PRE4* gene contains so-called PACE elements that are recognized by transcriptional activator Rpn4 (25, 26). Rpn4 is a central factor in an autoregulatory feedback loop that controls co-expression of proteasome genes in response to impairment or overloading of the proteasome (27, 28). A comparison of expression profiles of *PRE4* with those of other proteasome subunit genes revealed, aside from many similarities supporting their co-regulation, also a distinct strong response of *PRE4* to repression of *GLC7* (encoding a protein phosphatase) (available at the *Saccharomyces* Genome Database) (29). It is tempting to speculate that, under such conditions, *PRE4* expression might be selectively up-regulated, resulting in an increased mobilization of proteasomal precursor complexes and thereby in elevated levels of functional proteasome. Induction of proteasome levels by such a mechanism would not require an increased expression of all proteasome subunit genes. In this view, half-proteasome precursor complexes lacking Pre4 would represent a storage form of proteasomal complexes ready to be activated by incorporation of Pre4 in response to certain stimuli.

Blm10 Attaches to Proteasome Precursor Complexes and Stabilizes Nascent 20 S Proteasomes—Consistent with other reports (14, 15), we detected Blm10 in purified half-proteasome precursor complexes. A deletion of the gene encoding Blm10 (*blm10*Δ) had only a modest effect on proteasome maturation. This was judged by determining the ratio of Pro-Pup1 to mature Pup1 in wild-type and *blm10*Δ cells. Although at normal and higher growth temperatures (30 or 37 °C), no clear differences in these ratios were detectable, the relative amounts of Pro-Pup1 were slightly increased (by ~20%) in *blm10*Δ cells grown at 15 °C (Fig. 4*C*) (data not shown). Much more striking effects were observed when we combined *blm10*Δ with the *pre4-ΔCTE* mutation. Deletion of a CTE of Pre4 by itself causes increased levels of proteasome precursor, indicating that it has a role in stabilizing the nascent 20 S proteasome upon precursor complex dimerization (19). Processing of β2/Pup1 does not require the presence of the Pre4 CTE, since 20 and 26 S proteasome complexes formed in its absence contained mature Pup1. It is therefore remarkable that we did not observe significant amounts of processed Pup1 in fractions containing half-proteasome complexes in the *pre4-ΔCTE* mutant (Fig. 4*D*). This result indicated that, once β subunits have matured in the nascent proteasome, the 20 S CP is stable even without the Pre4 CTE. Although the effects of the lack of the Pre4 CTE or of Blm10 by themselves are relatively moderate, the double mutant lacking both grew even more poorly than the *ump1*Δ mutant, accumulated striking amounts of proteasome precursor complexes, and displayed impaired processing of Pup1 in 20 and 26 S complexes. This strong genetic interaction indicated that both the Pre4 CTE and Blm10 contribute to a stabilization and maturation of nascent 20S CP. As far as Blm10 is concerned, this conclusion appears to be in conflict with a previous report, in which Blm10 was suggested to negatively regulate proteasome maturation. This conclusion was based upon pulse-chase experiments that suggested a modest acceleration of β subunit processing and of Ump1 degradation in *blm10*Δ (14). Our results, in contrast, characterize Blm10 as a factor promoting proteasome maturation.

Role of Activators in the Proteasome Assembly Pathway—The weak effect of *blm10*Δ alone on proteasome assembly and maturation suggested that an alternative activator complex might take over this function in the absence of Blm10. We therefore asked whether the 19 S RP might do so. To address this question, we took advantage of our results indicating that a mutation (*sen3-1/rpn2*) affecting its Rpn2 subunit resulted in a drastic inhibition of 19 S RP attachment to the 20 S CP (Fig. 5*A*). Although the *sen3-1* mutation severely impaired formation of 26 S proteasomes, it had no detectable effect on the level of half-proteasome precursor complex or on β subunit processing (Fig. 5*A*). When we combined this mutation with *blm10*Δ, which by itself also did not show strong effects on proteasome maturation, we found the double mutant to be clearly impaired in 20 S CP formation. It is worth stressing that in this scenario all 20 S subunits and Ump1 are wild-type. Therefore, these data suggested that activator complexes are required to stabilize nascent 20 S CPs and to promote their activation by β subunit maturation. 19 S RP (PA700) or Blm10 (PA200) apparently can alternatively serve this function. The effect of inhibiting both activators on the maturation of 20 S CPs, however, was less severe than that observed in the *blm10*Δ *pre4-ΔCTE* double mutant. One possible explanation is that nascent 20 S CPs that are stabilized by the Pre4 CTE have the capacity to mature without activators. Another possibility is that proteins or complexes other than the 19 S RP or Blm10 may serve as alternative activators of nascent proteasomes. A candidate protein is Ecm29, which attaches to 20 S CP and promotes its binding to the 19 S RP (30). Deletion of the *ECM29* gene causes a synthetic growth inhibition in combination with *blm10*Δ (12).

A requirement for activators in 20 S CP assembly and maturation could be explained by conformational changes that they induce upon binding to the α rings that lead to a stabilization of 20S CP either directly or indirectly by promoting β subunit maturation. The structural details of such conformational changes, which are most apparent in the α rings, have been well documented for the attachment of the trypanosome 11 S regulator (PA26) to the *S. cerevisiae* 20 S CP (31). No counterpart of the 11 S regulator, however, appears to exist in *S. cerevisiae*. A model, in which the activators Blm10 or 19 S RP promote β subunit maturation, is in line with the observation that the defects observed in the *sen3 blm10*Δ double mutant include accumulation of precursor complex and incomplete maturation of 20 S particles (Fig. 5*A*). Considering the idea that these activator complexes induce conformational changes to the 20 S CP that are transmitted to the β subunits, it is plausible to assume that they promote autocatalytic processing of β sub-

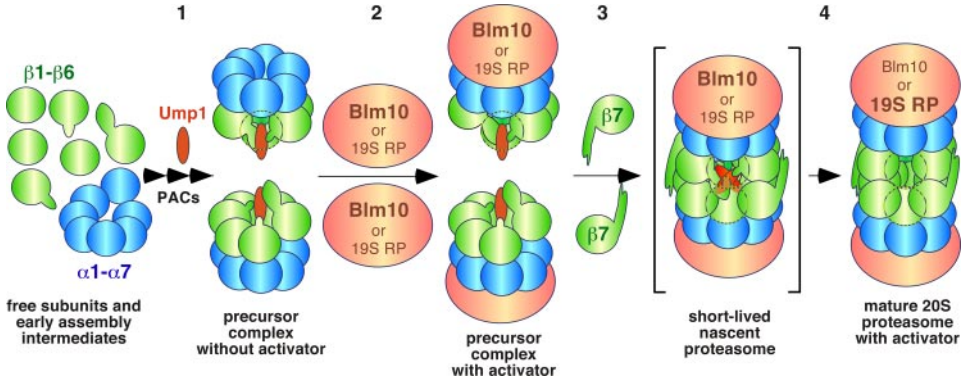

FIGURE 6. **Model illustrating the role of β7 and activator complexes in promoting 20 S proteasome assembly and maturation.** In step 1, subunits α1–α7 and β1–β6 (some of which bear propeptides) assemble via early assembly intermediates and with participation of Ump1 as well as other proteasome assembly chaperones (*PACs*) (15, 37, 38) into half-proteasome precursor complexes lacking β7. In step 2, Blm10 or with lower efficiency the 19 S RP attach to the half-proteasome precursor complex. In step 3, β7 with its long C-terminal extension promotes dimerization of half-proteasome precursor complexes that are bound to activator complexes, leading to the short lived (therefore *bracketed*) nascent proteasome. In step 4, conformational changes that depend on the activator complexes and that are triggered upon dimerization and β7 incorporation lead to the formation of mature proteasomes, a process that involves β subunit processing and degradation of Ump1.

units as well as cleavage of substrate peptides. This model provides a reasonable explanation for two distinct observations. On one side, there are reports that demonstrate an activation of *in vitro* peptidase activity of the 20 S CP by Blm10 (12, 13). On the other side, Blm10 is found in association with half-proteasome precursor complexes (14, 15) and promotes their dimerization and subsequent maturation of active sites. Studies both on bacterial and mammalian 20 S proteasomes and their precursor complexes have revealed that there are substantial structural changes occurring during precursor complex dimerization and proteasome maturation (32–34). It is plausible to assume that multiple events, including insertion of the Pre4 CTE clamp and of β subunit propeptides into the other half of the proteasome (15, 19), rearrangement and degradation of Ump1 enclosed in the nascent CP (18), β subunit maturation (17), and the attachment of activator complexes to the α rings (35), may contribute to such changes during the assembly of eukaryotic proteasomes.

Our model, in which Blm10 functions in promoting 20 S CP maturation (Fig. 6), is compatible with the possibility that Blm10, similar to the 19 S regulator, might also serve as a specialized activator of mature 20 S CPs. Our results, however, provide clear genetic evidence that implicates Blm10 in proteasome biogenesis, whereas genetic evidence pointing to functions as a specialized activator of mature 20 S CP is missing.

Several lines of evidence indicate that Blm10 is more efficient than the 19 S RP in binding to half-proteasome precursor complexes and in promoting their dimerization and subsequent 20 S particle maturation. First, Blm10 was found in relatively high amounts that were detectable in Coomassie-stained gels in purified precursor complexes (Fig. 1). 19 S RP subunits, in contrast, were only detected when such complexes derived from a strain lacking Blm10 were analyzed by immunoblotting (Fig. 5*C*). Second, Blm10 is still capable of promoting precursor complex dimerization and 20 S CP maturation relatively efficiently when the dimer is weakened by a deletion of the Pre4 C-terminal extension (*pre4-ΔCTE*) (Fig. 2). The striking synthetic effect of the *blm10* and *pre4-ΔCTE* mutations indicated that the 19 S RP cannot substitute efficiently for Blm10 in the proteasome assembly process if the precursor complex dimer interaction is weakened by the lack of the Pre4 CTE.

We propose a model (Fig. 6) in which proteasome activator complexes are required for efficient dimerization of half-proteasome precursor complexes and 20 S particle β subunit maturation. Our data indicate that there is a division of labor in which Blm10 functions mainly in proteasome biogenesis, whereas 19 S RP functions predominantly in association with mature 20 S particles or in 20 S CP-independent processes. The latter have been shown to include functions in DNA repair and the control of transcription (36).

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The C-terminal Extension of the β7 Subunit and Activator Complexes Stabilize Nascent 20 S Proteasomes and Promote Their Maturation

António J. Marques, Christoph Glanemann, Paula C. Ramos and R. Jürgen Dohmen

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