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Structure
Review

PACemakers of Proteasome Core Particle Assembly

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The 26S proteasome mediates ubiquitin-dependent proteolysis in eukaryotic cells. A number of studies including very recent ones have revealed that assembly of its 20S catalytic core particle is an ordered process that involves several conserved proteasome assembly chaperones (PACs). Two heterodimeric chaperones, PAC1-PAC2 and PAC3-PAC4, promote the assembly of rings composed of seven α subunits. Subsequently, β subunits join to form half-proteasome precursor complexes containing all but one of the 14 subunits. These complexes lack the β7 subunit but contain UMP1, another assembly chaperone, and in yeast, at least to some degree, the activator protein Blm10. Dimerization of two such complexes is triggered by incorporation of β7, whose C-terminal extension reaches out into the other half to stabilize the newly formed 20S particle. The process is completed by the maturation of active sites and subsequent degradation of UMP1 and PAC1-PAC2.

Introduction

The 26S proteasome is an ∼2.5 MDa protease complex that provides the central and essential proteolytic activity in the cytoplasm and the nucleus of eukaryotic cells (Baumeister et al., 1998). The main function of this protease is to degrade polyubiquitylated substrates including abnormal as well as regulatory proteins (Hershko and Ciechanover, 1998). Because its substrates include cell division cycle regulators and proapoptotic factors, the proteasome has emerged as an interesting drug target in the treatment of cancer (Adams, 2004; Goldberg, 2007). The 26S proteasome is composed of two 19S regulatory particles (RP) and the barrel-shaped catalytic core particle (CP), termed the ''20S proteasome'' (Hanna and Finley, 2007). The eukaryotic 20S CP and 20S proteasomes found in eubacteria or archaebacteria share a common $\alpha_7\beta_7\beta_7\alpha_7$ architecture, as was revealed by crystal structures of the 20S proteasome of the archaebacteria *Thermoplasma acidophilum* and *Archaeoglobus fulgidus*, the eubacteria *Rhodococcus* and *Mycobacterium tuberculosis*, as well as from *Saccharomyces cerevisiae* and *Bos taurus* (Löwe et al., 1995; Groll et al., 1997, 2003; Kwon et al., 2004; Hu et al., 2006; Unno et al., 2002). These complexes are composed of four stacked rings each composed of seven subunits either of the α or the β type, which share a β sandwich fold. Whereas archaebacterial 20S proteasomes, in most cases, are made of homo-oligomeric rings, the α and β rings in the eukaryotic 20S CP are composed of seven different α and seven different β subunits, respectively (Figure 1). The two inner β rings form the catalytic chamber, in which three subunits, β1, β2, and β5, provide the postacidic, tryptic, and chymotryptic activities, respectively (Arendt and Hochstrasser, 1997; Heinemeyer et al., 1997). The two outer α rings make contact with the 19S RPs, which recognize and unfold ubiquitylated proteins and translocate them into the 20S CP, or to alternative activator complexes such as PA28α/β, PA28γ, or PA200 (reviewed in Rechsteiner and Hill, 2005; DeMartino and Gillette, 2007). Biogenesis of the 20S CP appears to occur in an ordered set of steps starting with the formation of α rings, which form a platform onto which β subunits can assemble to form precursor complexes. Dimerization of such complexes leads to the formation of 20S particles and triggers active site maturation. Whereas prokaryotic 20S proteasomes apparently do not require ''help'' to assemble from their subunits, many steps in the biogenesis of the more complex eukaryotic 20S CPs are promoted by dedicated proteasome assembly chaperones (Figure 2). In this review, we summarize our current understanding of the function of these chaperones.

The Role of PACs in the Assembly of α Rings: A Question of Complexity

Studies with α subunits from archaebacterial proteasomes expressed in *Escherichia coli* showed that, in the absence of β subunits, they self-assemble into heptameric rings (Groll et al., 2003; Zwickl et al., 1994). Rings of α subunits alone have nearly the same structure as α rings in the assembled proteasome, except for their N-terminal regions (Groll et al., 2003). In the absence of β subunits, such α rings tend to dimerize. β subunits alone, in contrast, do not assemble into ring structures. When coexpressed, α and β subunits assemble into functional proteasomes, apparently without assistance from additional factors (Zwickl et al., 1994).

Assembly of ring structures during proteasome biogenesis is far more complex for the eukaryotic proteasomes, because all subunits within a ring are different and need to find and occupy a defined position. Several eukaryotic α subunits have retained the ability to form homomeric rings. It was reported, for example, that the human α7 subunit upon expression in *E. coli* forms double ring structures (Gerards et al., 1997), whereas α6 and α1 were unable to do so. Curiously, when expressed together with α7, these subunits were also incorporated into the double ring assemblies, although in variable positions, suggesting that α subunits may not possess enough structural information to self-guide them into the right place within an α ring (Gerards et al., 1998). Consistent with this notion, recent studies have revealed that correct positioning of α subunits and the prevention

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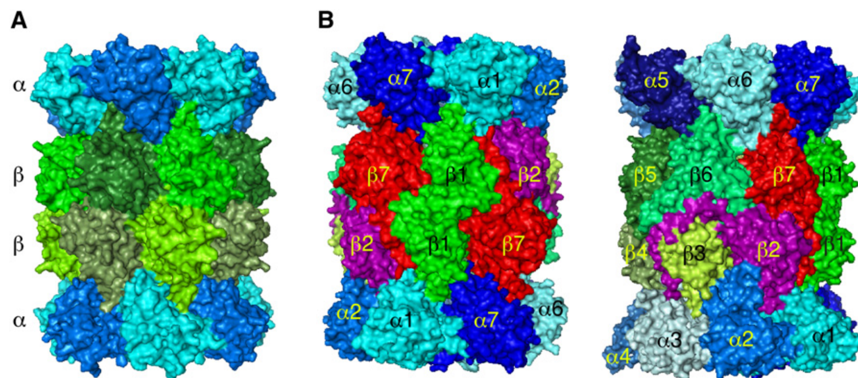

Figure 1. Comparison of Archaeal and Eukaryotic 20S Proteasomes

(A and B) Surface structure representation of the *T. acidophilum* (A) and *S. cerevisiae* (B) 20S proteasomes (Löwe et al., 1995; Groll et al., 1997).

(A) The identical α subunits are shown in different shades of blue, and the identical β subunits in shades of green, to visualize their $\alpha_7\beta_7\beta_7\alpha_7$ arrangement.

(B) The *S. cerevisiae* proteasome is shown from two sides to allow a full view of the β7 (shown in red) and β2 subunits (shown in purple) and their C-terminal extensions. The figure was prepared using PyMOL (http://pymol.sourceforge.net/).

of α ring dimerization is promoted by two specific dimeric chaperones (PAC1-PAC2 and PAC3-PAC4).

PAC1-PAC2

PAC1 and PAC2 are two proteins of 33 and 29 kDa, respectively, that were found in human cells in association with proteasome assembly intermediates (Hirano et al., 2005). The *Pac1* gene is also known as *Dscr2* (down syndrome critical region 2) (Vidal-Taboada et al., 2000). Human *Pac2* was described earlier as hepatocellular carcinoma associated gene 3 (*Hcca3*) (Wang et al., 2001), its mouse ortholog as *Clast3* (Bahar et al., 2002). The smallest assembly intermediates containing PAC1 and PAC2 harbored a subset of α subunits. PAC1 and PAC2, however, were also found in nearly stoichiometric amounts in complexes containing all seven α subunits but lacking Ump1 or β subunits (Figure 2) (Hirano et al., 2005). These assemblies are therefore thought to be α rings. In vitro studies showed that PAC1 and PAC2 form heterodimers that bind α5 directly as well as α7 and promote formation of complexes containing all seven α subunits (Hirano et al., 2005). siRNA-mediated depletion of PAC1 or PAC2 from human cells resulted in the formation of off-pathway assemblies containing all seven α subunits but lacking β subunits. Because of their fractionation behavior, which was similar to that of half-proteasome precursor complexes, these assemblies were assumed to represent dimers of α rings. This finding indicated that, in addition to promoting the assembly of α rings, PAC1-PAC2 prevents the spontaneous dimerization of α rings (Hirano et al., 2005). Treatment with the proteasome inhibitor MG132 resulted in an accumulation of PAC1-PAC2 in fractions with fully assembled proteasomes, indicating that this chaperone remains associated with assembly intermediates until it is degraded upon formation of the mature proteasome (Hirano et al., 2005). It remains unclear whether PAC1-PAC2 is enclosed into the nascent proteasome, as shown for yeast Ump1 (see below), or remains associated at the surface. Structural information on the PAC1-PAC2 chaperone and assembly intermediates containing it is not yet available. It is therefore unclear which surface of the α ring is contacted by PAC1-PAC2.

Several recent studies have led to the identification of yeast proteins with weak sequence similarity to mammalian PAC1 and PAC2, which were termed Pba1/Poc1 and Pba2/Poc2/Add66, respectively (Li et al., 2007; Le Tallec et al., 2007; Scott et al., 2007). Experimental evidence characterized these proteins as orthologs of human PAC1-PAC2 because they form similar dimeric chaperones involved in proteasome assembly. Whereas knockdown of PAC1-PAC2 in human cells resulted in a significant reduction in cellular proteasome activity, deletion of the corresponding yeast genes had only very modest phenotypic

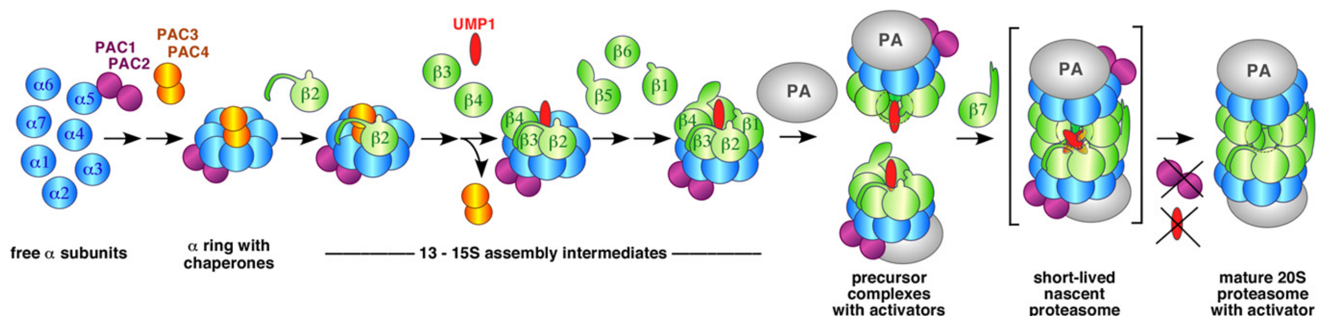

Figure 2. Assembly Pathway of Eukaryotic 20S Proteasomes

Shown is a schematic model that summarizes the order of events in the assembly of 20S proteasomes and the involvement of dedicated proteasome assembly chaperones. In a first step that is promoted by the two chaperone complexes PAC1-PAC2 and PAC3-PAC4, the subunits α1–α7 assemble into heptameric rings. Dimerization of such rings is prevented by PAC1-PAC2 (not shown). The first β subunit to enter the complex is β2, followed by β3, β4, and UMP1. In the course of these events, PAC3-PAC4 leave the complex. Subsequently β5, β6, and β1 join the complex to form a half-proteasome precursor complex that only lacks β7. In yeast, this complex is found in association with proteasome activators (PA), either the large Blm10 protein, an ortholog of human PA200, or in its absence with the 19S RP (PA700). Dimerization of such precursor complexes is triggered by the binding of β7, which stabilizes nascent proteasomes via its long C-terminal extension together with Blm10. The short-lived (therefore bracketed) nascent proteasome is activated by autocatalytic maturation of its β subunits. The activated proteasome then degrades Ump1 as well as PAC1-PAC2. The mature 20S proteasome is preferentially associated with the 19S RP, whereas PA200 is found preferentially on precursor complexes. Some of the β subunits are drawn with N-terminal propeptides, which are cleaved upon 20S CP formation.

consequences, which include the stabilization of certain ERAD substrates and an increased resistance to DNA damage (Scott et al., 2007; Le Tallec et al., 2007). Deletion of yeast *PAC1* or *PAC2* orthologs, however, resulted in strains that require the transcription factor Rpn4 for normal growth and temperature resistance (Le Tallec et al., 2007). Rpn4 controls the expression of proteasome subunits and is at the same time a substrate of the proteasome (Mannhaupt et al., 1999; Xie and Varshavsky, 2001). Mutations that cause an impairment of proteasome function therefore lead to a stabilization of Rpn4 and thus to an enhanced synthesis of proteasome components (Xie and Varshavsky, 2001; London et al., 2004). The observed synthetic phenotypes indicate that strains lacking the PAC1-PAC2 complex are impaired in the generation of functional proteasomes. Consistent with this notion, slightly increased amounts of precursor complexes and a reduction of the chymotryptic activity were detected in such strains (Li et al., 2007; Scott et al., 2007). Because the tryptic and postacidic activities appeared to be unaffected, the latter result is consistent with the possibility that impairing the function of yeast PAC1-PAC2 contributes to the formation of unusual proteasomes similar to what was observed upon deletion of the yeast PAC3-PAC4 complex (discussed below).

PAC3-PAC4

Affinity purifications that used a tagged version of PAC1 led to the identification of another small 14 kDa protein, termed PAC3, from human cells. PAC3 was detected in fractions containing α subunits and the PAC1-PAC2 dimer, but was absent from complexes containing Ump1. In contrast to PAC1-PAC2, PAC3 is not degraded by the proteasome. Instead, it is released from precursor complexes before half-proteasome assembly is accomplished (Hirano et al., 2006). Because various genetic screens as well as a bioinformatics approach led to the identification of what appears to be the yeast ortholog of PAC3, this 20 kDa protein received several names including Poc3, Pba3, or Dmp2 (Le Tallec et al., 2007; Kusmierczyk et al., 2008; Yashiroda et al., 2008; Hoyt et al., 2008). These studies revealed that the phenotypes of strains lacking yeast PAC3 are similar to those of strains lacking Poc4/Pba4/Dmp1. The latter 17 kDa protein forms stoichiometric complexes with yeast PAC3. These observations led to the identification of human PAC4, which coimmunoprecipitated with PAC3. Knockdown of PAC3 resulted in the degradation of PAC4, and vice versa, indicating that these two proteins form stable complexes (Le Tallec et al., 2007). Together, these studies identified PAC3-PAC4 as a functionally conserved second pair of chaperones involved in early steps of proteasome biogenesis (Figure 2).

siRNA-mediated knockdown of PAC3 in human cells resulted in a reduction of proteasomal chymotryptic activity and accumulation of ubiquitin conjugates, indicating that proteasome function was impaired. Simultaneous depletion of PAC1, PAC2, and PAC3 enhanced these effects to an extent that they were nearly as severe as observed for the knockdown of UMP1 (Hirano et al., 2006). PAC3 knockdown furthermore resulted in an accumulation of free forms of α subunits and PAC1-PAC2 complexes. These findings indicated that PAC1-PAC2 and PAC3-PAC4 have distinct functions in α ring formation. Consistent with this notion, an accumulation of larger assemblies of α subunits (presumptive α ring dimers), as observed upon depletion of PAC1 or PAC2, was not detected upon PAC3 knockdown. This result suggested that PAC3 is not required to prevent α ring dimerization. Interestingly, PAC3 itself was present in such α subunit complexes when PAC1-PAC2 was depleted (Hirano et al., 2006).

The analysis of *S. cerevisiae* mutants lacking the PAC3-PAC4 complex (called Pba3-Pba4 from here on) revealed specific defects or changes in the assembly of proteasomal α rings. In one study, an accumulation of complexes containing all but one α subunit and the β2 subunit was observed by native gel analyses. The missing subunit was α4 (Yashiroda et al., 2008). The authors concluded that the Pba3-Pba4 might be required to prevent α3 from binding to α5, or for the incorporation of α4 into α rings. In contrast to these results, genetic evidence suggested that Pba3-Pba4 might function in the incorporation of α3 during α ring assembly. When the genes encoding Pba3 or Pba4 were deleted in a strain lacking the only nonessential 20S subunit, namely α3, no phenotypic enhancement was detected (Kusmierczyk et al., 2008). Additional experiments confirmed that in a mutant lacking this chaperone, the same proteasome configuration is found as in mutants lacking α3. In these complexes, which make up 20%–50% of the total proteasome population in the chaperone mutants, a second α4 subunit is incorporated instead of α3 (Kusmierczyk et al., 2008). Together, these findings established that the Pba3-Pba4 complex, similar to its human counterpart, functions in the proper assembly of α rings. Proteasomes lacking the α3 subunit formed in the absence of Pba3-Pba4 conferred increased resistance to cadmium stress. Therefore, the possibility has been discussed that such alternative proteasomes might be produced as a physiological response to certain conditions by a differential regulation of Pba3-Pba4 and proteasome subunit synthesis (Kusmierczyk et al., 2008).

Biochemical and structural studies on yeast Pba3-Pba4 provided important insight into how this chaperone might operate. This complex binds directly to the α5 subunit (Kusmierczyk et al., 2008; Yashiroda et al., 2008). The interaction is also observed when α5 is complexed with α6 and α7 (Kusmierczyk et al., 2008). The Pba3-Pba4-α5 complex is thought to serve as a starting point for α ring assembly (Figure 2). The crystal structure of this complex has recently been solved (Figure 3A). The fact that the α5 structure in this complex is nearly the same as in the mature 20S proteasome allowed a modeling of Pba3-Pba4 onto an α ring (Yashiroda et al., 2008). The resulting picture suggested that Pba3-Pba4 is located on the "inner" surface of the ring that will eventually bind to the β ring (Figures 3B and 3C). In this model, when compared to β subunits in mature 20S CPs, Pba3-Pba4 binds more toward the center of the ring occupying areas on α5 and its neighboring subunits α4 and α6. This position of the chaperone complex would apparently be incompatible with a binding of β4 on top of α3 and α4. This model therefore suggested that Pba3-Pba4 is released from the complex before or during incorporation of β4, which is consistent with the absence of Pba3-Pba4 in later proteasome assembly intermediates (Hirano et al., 2006; Yashiroda et al., 2008).

Pba3 and Pba4 are structurally similar, with each consisting of a six-stranded β sheet and two α helices. In the dimer, the two β sheets form a β sandwich structure, which is flanked by two α helices on each side (Figure 4). This overall structure of the

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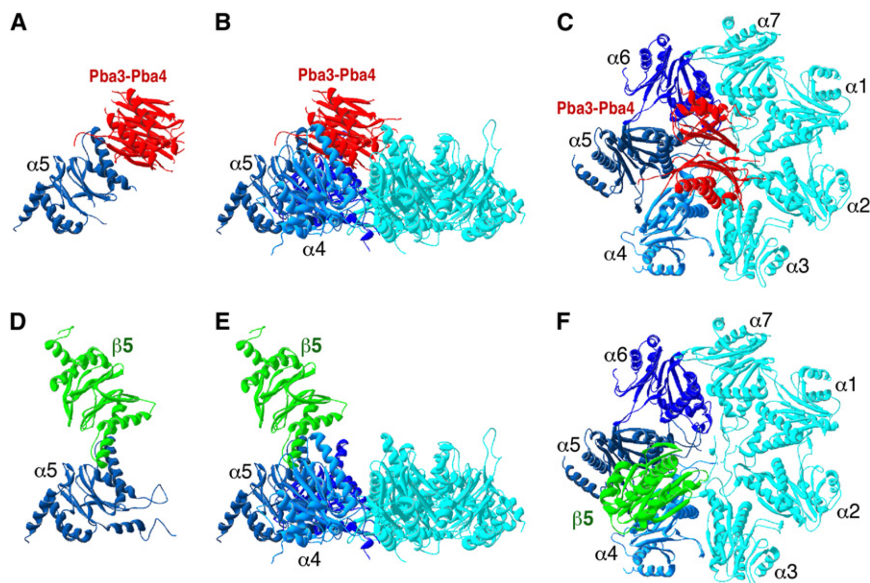

Figure 3. Structural Models of Proteasome Assembly Intermediates

(A) Shown is the cocrystal structure of yeast Pba3-Pba4 and α5 (Yashiroda et al., 2008).
(B) Using the structure shown in (A), the Pba3-Pba4 complex was modeled onto an α ring as it is present in the 20S CP.
(C) Top view of the model shown in (B).
(D–F) For comparison, parts of the yeast 20S core particle (CP) are shown (Groll et al., 1997).
(D) Arrangement of the α5 and β5 subunits.
(E) Side view of an α ring bound to the β5 subunit.
(F) Top view of the model shown in (E). The figure was prepared using Swiss PDB viewer (http://www.expasy.org/spdbv/).

dimer resembles those of proteasomal α or β subunits (Figure 4) (Yashiroda et al., 2008). The model described above, however, suggests that the Pba3-Pba4 complex does not occupy a similar position in assembling proteasomes as any of these subunits, even though this cannot be entirely excluded because real structures of such intermediates are not yet available. Tanaka and colleagues also reported the structure of a human PAC3 homodimer, which is very similar to the structure of yeast Pba3-Pba4 (Figure 4) (Yashiroda et al., 2008). It remains to be determined whether such homodimers, or the heterodimer with PAC4, or both, are physiologically relevant entities. In any case, the observation that human PAC3 also directly interacted with several β subunits in vitro, together with its orientation on the inner surface of the α ring (suggested by structural studies on its yeast ortholog), is consistent with an additional function of this chaperone in the initiation of β ring assembly (Hirano et al., 2006). How the two chaperones PAC1-PAC2 and PAC3-PAC4 and their orthologs cooperate in the formation of α rings and whether they interact directly remains unclear, although it was shown that the PAC3 homodimer does not bind to PAC1-PAC2 in vitro (Hirano et al., 2006).

From α Rings to Half-Proteasome Precursor Complexes

Studies described above on PAC1–4 during proteasome assembly have indicated their roles in the assembly of α rings and established these structures as intermediates in the assembly of eukaryotic 20S proteasomes (Figure 2). Intermediates containing all seven α subunits and PACs but lacking β subunits were observed in human cells (Hirano et al., 2005). Such complexes were apparently too unstable, however, to be detected in lysates from wild-type yeast strains (Yashiroda et al., 2008). In the absence of the Pba3-Pba4 complex, however, a structure accumulated that contained all α subunits except α4 plus the β2 subunit (Yashiroda et al., 2008). Also consistent with the idea that β2 is the first β subunit to bind to the α ring is the observation that Pba3-Pba4 selectively coimmunoprecipitated, in addition to all seven α subunits, the precursor form of only this β subunit (Le Tallec et al., 2007; Yashiroda et al., 2008). The β2 subunit carries a conserved C-terminal extension that in the structure of the mature proteasome embraces the neighboring β3 subunits (Figure 1). This extension is essential for the assembly of functional proteasomes, probably because it is required for stable incorporation of the β3 subunit (Ramos et al., 2004). The next characterized intermediate that has been termed the 13S complex in mammals contains all α subunits as well as β2, β3, and β4 (Nandi et al., 1997). Some of these complexes in addition contain the β6 subunit (Fricke et al., 2007). Related intermediates (here termed the 15S precursor) have been characterized as intermediates detectable upon native gel fractionation of proteasomal complexes found in several yeast mutants. Besides the same subunits observed for the mammalian 13S complex (except β6), these intermediates in addition contained Pba1-Pba2, one type of intermediate moreover contained Ump1, and a third type also contained Blm10. Pba3-Pba4 was not detected in any of these complexes (Li et al., 2007). The absence of the latter chaperone complex is consistent with its release from precursor complexes during the assembly of such intermediates (see above). A possible step that might coincide with the release of Pba3-Pba4 is the incorporation of the β3 subunits, because this subunit did not coprecipitate with Pba3-Pba4 (Yashiroda et al., 2008). Integrating β3 into the above-mentioned model of an α ring in association with Pba3-Pba4 (Figures 3B and 3C), however, does not indicate any spatial overlap between β3 and this chaperone (data not shown), indicating that this model may not reflect the actual structure of such intermediates. Potentially relevant to an understanding of this step in proteasome assembly might be the observation that PAC3 binds to β3 in vitro (Hirano et al., 2006). Initial binding of β3 to a PAC3-PAC4 complex might facilitate its binding to assembly intermediates, which subsequently results in a release of the chaperone complex. A later detectable intermediate in the assembly of yeast proteasomes is a half-proteasome precursor lacking the β7 subunit (Li et al., 2007; Marques et al., 2007). This complex also contains Ump1, Pba1-Pba2, and at least to some extend Blm10 (see below) (Fehlker et al., 2003; Li et al., 2007; Marques et al., 2007).

UMP1

Ump1 was identified as the ∼17 kDa product of a gene affected in a budding yeast mutant defective in ubiquitin-mediated

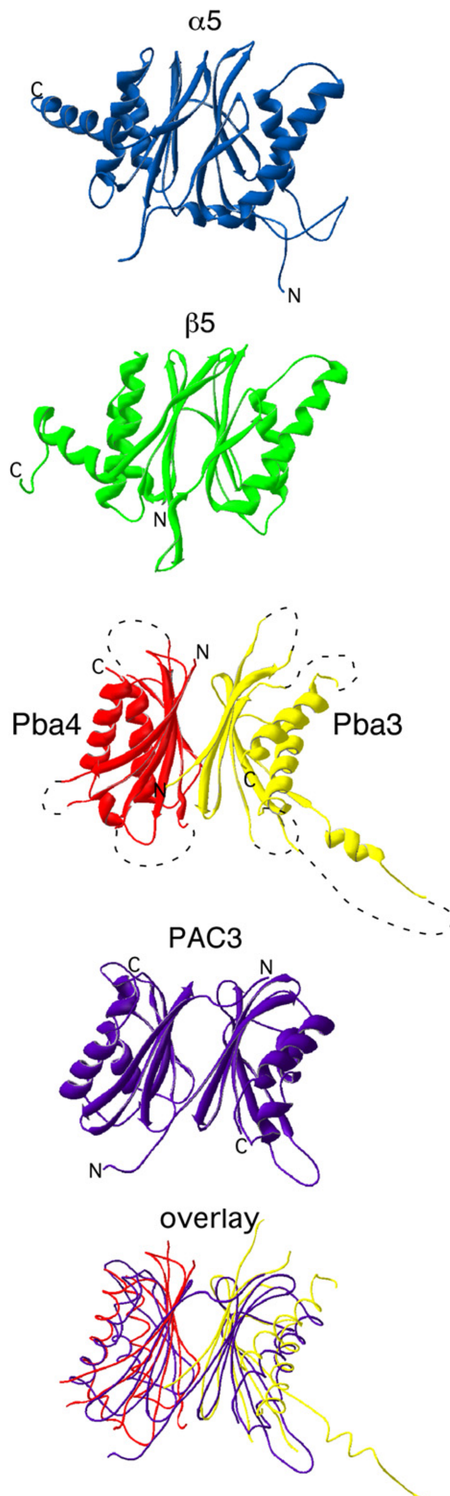

Figure 4. Structure Comparison of Yeast Pba3-Pba4 and Human PAC3 Dimer

Shown are ribbon diagrams of *S. cerevisiae* α5, β5 (both taken from the structure of the 20S proteasome), Pba3-Pba4, and human PAC3 homodimer, as well as an overlay of the backbones of the latter two structures (Groll et al., 1997; Yashiroda et al., 2008). Dotted lines were added manually to represent disordered regions. The figure was prepared using Swiss PDB viewer.

proteolysis, hence the name. This mutant was deficient in the degradation of proteasome substrates, was growing poorly, and was hypersensitive to various stresses. Biochemical studies characterized Ump1 as a component of proteasome precursor complexes containing unprocessed β subunits. In wild-type cells, Ump1 is absent from mature 20S proteasomes. However, Ump1 was detectable in a mutated 20S proteasome impaired in proteolytic activity. In this complex, Ump1 was protected from binding to polyclonal antibodies or against tryptic digestion, indicating that it had been encased in the newly formed proteasome. Consistent with this idea was the observation that Ump1 is an unstable protein whose degradation by the proteasome coincided with the maturation of its active subunits (Ramos et al., 1998). Yeast *ump1Δ* mutants are viable but accumulate proteasomal precursor complexes and proteasomes with incompletely processed β subunits. These findings characterized Ump1 as a proteasome assembly chaperone that promotes dimerization of precursor complexes and maturation of active sites. At which step Ump1 enters the assembly line is not exactly clear. Its presence or absence from complexes containing β2, β3, and β4 suggests that in *S. cerevisiae*, it follows the incorporation of these subunits into proteasome precursors (Li et al., 2007). Ump1 apparently stabilizes such complexes because, even though in its absence such precursor complexes accumulate (Hirano et al., 2005; Ramos et al., 1998), they could not be detected after affinity purification and native gel analysis (Li et al., 2007).

Genes encoding proteins with significant sequence similarity to *S. cerevisiae* Ump1 are ubiquitously found in the genomes of eukaryotes. The human ortholog, hUMP1 (also called POMP or proteassemblin), has been found as a part of proteasomal precursor complexes containing unprocessed β subunits and to be degraded upon generation of the proteasome (Witt et al., 2000; Burri et al., 2000; Griffin et al., 2000; Heink et al., 2005; Hirano et al., 2005). siRNA knockdown of hUMP1 in human cells suggested that this chaperone is essential for proteasome formation and viability (Heink et al., 2005; Le Tallec et al., 2007). In vitro experiments showed that hUmp1 binds directly to the β5 subunit. Support for an in vivo relevance of this interaction came from siRNA experiments, in which depletion of hUmp1 prevented the incorporation of β5 into nascent proteasome precursor complexes (Heink et al., 2005). The presence of hUMP1 in the 13S precursor complex thus seems to be critical for downstream assembly steps that will eventually lead to the formation of half-proteasome precursor complexes. When such complexes were immunoprecipitated from human cells with an antibody that recognized a precursor complex-specific conformation of the α6 subunit, all 14 20S proteasome subunits were detected, including β7. Yeast two-hybrid interaction studies revealed that hUMP1 interacted with several α subunits (α3, α4, and α7). In vitro and in vivo experiments confirmed that hUMP1 can associate with α rings (Fricke et al., 2007). These data indicated that hUMP1 enters the assembly pathway earlier, as assumed for its yeast counterpart (see above) by binding to α rings. The fact that hUMP1 binds to all β subunits in two-hybrid assays is consistent with it playing an important role in coordinating the assembly of a β ring on an α ring platform (Fricke et al., 2007). In the same study, it was, in addition, observed that hUMP1 mediates binding of precursor complexes to the membrane of the

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endoplasmic reticulum (ER). Based on these findings, it was proposed that most if not all proteasomes in mammalian cells assemble at the ER (Fricke et al., 2007). Previous fractionation experiments had indicated that proteasomal precursor complexes containing Ump1 in *S. cerevisiae* are also mainly detected in the nucleus/ER fractions (Lehmann et al., 2002). But in this case, additional experiments led to the conclusion that precursor complexes are targeted to the nuclear envelope, which is continuous with the ER, by binding to nuclear import factors (karyopherins). These factors were shown to be required for nuclear import of proteasome precursor complexes, which were proposed to assemble into 20S CPs inside the nucleus (Lehmann et al., 2002).

Dimerization of Proteasome Precursor Complexes and Active Site Maturation

20S CPs are formed upon dimerization of two precursor complexes containing unprocessed β subunits and UMP1 (Figure 2). Studies in *S. cerevisiae* revealed that dimerization is triggered by the rate-limiting incorporation of the β7 subunit (Li et al., 2007; Marques et al., 2007). A C-terminal extension (CTE) of *S. cerevisiae* β7 serves two functions in 20S CP biogenesis (Ramos et al., 2004). This extension reaches from one half of the CP to the other where it intercalates between subunits β1 and β2, thereby promoting formation and stabilization of nascent CPs (Figures 1 and 2). Residues within this extension, in addition, contact residues in β1 to stabilize a conformation that is required to promote β1 maturation and activity (Groll et al., 1997; Ramos et al., 2004). Deletion of this extension results in the accumulation of half-proteasome precursor complexes lacking the β7 subunit (Marques et al., 2007). Another structural feature of β subunit precursors that is important for dimerization is the unusually long propeptide of β5. In the presence of Ump1, this propeptide is essential for viability (Chen and Hochstrasser, 1996). Like a deletion of Ump1, however, overexpression of β7 was shown to suppress the lethality of a deletion of the β5 propeptide (Li et al., 2007; Ramos et al., 1998). The CTE of β7 was essential for this suppressive effect. Based on these and similar findings for the role of an N-terminal extension of the β6 subunit, it was suggested that Ump1 provides a checkpoint function that prevents dimerization of precursor complexes until their assembly is completed (Li et al., 2007). The propeptides of β5 and β6 as well as the β7 CTE might contribute to overcome this checkpoint upon incorporation of β7 by moving Ump1 or changing its conformation. In yeast, half-proteasome precursor complexes containing β7 are apparently very short lived, as they have not been detected thus far. This suggests that productive precursor complex dimerization occurs rapidly after incorporation of β7. Its CTE appears to be important to stabilize the nascent dimer (Ramos et al., 2004; Li et al., 2007; Marques et al., 2007). In the absence of the β7 CTE, however, mature proteasomes are still formed albeit with a reduced efficiency. What other features could help to stabilize the nascent 20S proteasome? Another protein that was found in association with proteasome precursor complexes is Blm10, a large 246 kDa HEAT repeat protein related to mammalian proteasome activator PA200 (Fehlker et al., 2003; Li et al., 2007; Marques et al., 2007; Iwanczyk et al., 2006). Strong synthetic effects of a β7 CTE truncation and deletion of the *BLM10* gene indicated that Blm10 contributes to the stabilization of nascent 20S particles. In the absence of Blm10, the 19S RP can bind to proteasome precursor complexes and appears to substitute partially for Blm10 in stabilizing native CPs (Figure 2) (Marques et al., 2007).

Following β7-induced dimerization of half-proteasome precursor complexes, autocatalytic processing and thereby activation of β1, β2, and β5 takes place (Chen and Hochstrasser, 1996). This process might be kicked off by the β7 CTE, whose insertion between β1 and β2 promotes processing of proβ1 (Ramos et al., 2004). The analysis of proteasomal complexes by gel filtration indicated that the presence of Ump1 is required for efficient maturation of active site β subunits (Ramos et al., 1998). The Ump1 protein, which is encased during precursor dimerization, as well as the PAC1-PAC2 chaperone are degraded once the active sites have matured. It is plausible that active site maturation is accompanied by conformational changes that lead to a further stabilization of the 20S proteasome. Consistent with this notion is the observation that 20S proteasomes with immature β subunits are detected in extracts from yeast strains lacking Ump1 after gel filtration but are not detectable after affinity purification and native gel electrophoresis (Li et al., 2007). This concept is further supported by analyses of the stability and structure of maturation-impaired or -incompetent mutant proteasomes from *Rhodococcus* and *A. fulgidus* (Groll et al., 2003; Witt et al., 2006). An active site mutant of the *A. fulgidus* proteasome yielded two loosely associated half-proteasomes that are separated by a 4.5 Å gap between the β rings. The presence of this gap, which is apparently filled by disordered propeptides, indicates that β subunit maturation is required to pull the two halves together and to establish the full β-β interface (Groll et al., 2003). Based on structural analyses of the *Rhodococcus* proteasome, an assembly-dependent activation model was proposed in which interactions between β subunits of the opposing half-proteasomes promote a repositioning of the S2-S3 loop in a way that the active site threonine is appropriately oriented for propeptide cleavage (Witt et al., 2006). In order to understand whether and how the structural arrangements of the propeptide relative to the active site of a β subunit promotes its autocatalytic maturation, a mutant version of the yeast proteasome was analyzed that was impaired in activation of the proβ1 subunit. The crystal structure of this proteasome β1-Thr(+1)Ala mutant revealed that, in close proximity to the active site Thr(+1) residue, the β1 propeptide adopts a γ turn conformation extending from Leu(−2) to Thr(+1). This structural information enabled a modeling of the autolysis reaction according to which the Thr(+1) hydroxyl group is appropriately positioned to perform a nucleophilic attack onto the Gly(−1) carbonyl carbon atom located at the inner side of the γ turn (Ditzel et al., 1998). How Ump1 might promote the maturation of active sites of eukaryotic 20S proteasomes remains unclear. One idea is that, upon dimerization of precursor complexes, it helps to induce conformational changes in the β subunit or their propeptides that facilitate their autocatalytic processing (Heinemeyer et al., 2004).

Despite some apparent differences in the interactions between subunits and assembly chaperones and the composition of intermediates, the principle order of events in proteasome assembly and the roles of dedicated chaperones appear to be conserved among eukaryotes, as they are quite similar between yeast and humans.

Assembly of Alternative Proteasomes

Multicellular organisms often express different 20S proteasome subtypes. The best-studied subtype is the so-called immunoproteasome found in vertebrates. This subtype is characterized by the presence of alternative interferon-inducible β subunits, two of which are encoded in the MHC class II region (Kloetzel, 2004). The three inducible subunits (β1i, β2i, β5i) are assembled into a nascent proteasome instead of their counterparts of the constitutive proteasome subtype, whereas the other 11 subunits are the same in both types of proteasomes. As a result, the specificity of the peptidase activities in the immunoproteasome is changed in such a way that the generation of certain antigenic peptides is promoted (Kloetzel, 2004). A cooperative incorporation of the three immunosubunits occurs preferentially over the formation of mixed proteasomes, wherein only a subset of the immunosubunits is combined with constitutive ones. In contrast to β1 in the constitutive subtype, β1i enters the assembly pathway early and its presence positively influences the incorporation of β2i (Griffin et al., 1998; Groettrup et al., 1997; Nandi et al., 1997). The β5i subunit is preferentially incorporated over β5, when β1i and β2i are present (Kingsbury et al., 2000). The propeptides of these immunosubunits bear important information that favors their cooperative assembly (De et al., 2003; Kingsbury et al., 2000). Formation of immunoproteasomes is about 4-fold faster than that of constitutive proteasomes. Rapid maturation of immunoproteasomes in human cells depends on a stronger interaction of β5i with hUMP1 in comparison to β5, which in part depends on the β5i propeptide. To ensure efficient maturation of immunoproteasomes, hUMP1 expression is increased upon stimulation with type II interferon (Heink et al., 2005). The interferon-inducible alternative proteasome activator PA28α/β also contributes to the generation of antigenic peptides (reviewed in Kloetzel, 2004; Rechsteiner and Hill, 2005). It was reported that PA28α/β binds to proteasome precursor complexes and that ablation of PA28β in mice resulted in a loss of immunoproteasome assembly (Preckel et al., 1999). Other studies, in which PA28α/β was either overexpressed or inactivated, however, came to a different conclusion (Schwarz et al., 2000; Murata et al., 2001). In one of these studies, mice were analyzed in which the genes for both PA28α and β were ablated. These mice exhibited normal immunoproteasome assembly (Murata et al., 2001). A possible reason for the seemingly discrepant findings is that the PA28α subunits in the absence of PA28β might interfere with immunoproteasome assembly (Murata et al., 2001).

Another vertebrate-specific proteasome subtype was found to be specifically expressed in the thymus and hence called "thymoproteasome." It is characterized by the presence of an alternative β5 subunit, termed β5t, which is preferentially combined with β1i and β2i. Thymoproteasomes, which display a reduced chymotryptic activity, are thought to function in the generation of MHC class I-restricted $CD8^+$ T cells because mice lacking β5t were defective in the development of these cells (Murata et al., 2007).

Multiple isoforms of 20S proteasome subunits are also found in other eukaryotes. The model plant *Arabidopsis thaliana* encodes 13 different α subunits and 10 different β subunits (Fu et al., 1998). It also encodes two UMP1 homologs, whereas only single gene copies for orthologs of PAC1–4 appear to be present (Müller et al., 2005; K. Hofmann, personal communication). These observations indicate that there might be functionally specialized alternative 20S proteasome subtypes in plants as well. In *Drosophila*, a testis-specific proteasome subtype is essential for spermatogenesis. Male flies lacking the testis-specifically expressed gene *Prosα6T* encoding an isoform of α6 are sterile (Zhong and Belote, 2007). Nothing is known about whether there are specific requirements for the assembly of these alternative 20S proteasome subtypes.

Evolution of Chaperone-Dependent Proteasome Assembly

The apparent absence of proteasome assembly chaperones from eubacteria and archaeons indicates that evolution of these factors coincided with the increased subunit complexity of 20S proteasomes acquired by eukaryotes. Much in contrast to subunits of mature 20S proteasomes, which are highly conserved between prokaryotes and eukaryotes, the eukaryotic proteasome assembly chaperones display comparably little sequence conservation. The α and β subunits of *Thermoplasma* share between 30% and 41% identity with the respective subunits from *S. cerevisiae* and humans. Sequence identity between the orthologous yeast and human proteasome chaperones for comparison are only about 7% for PAC1, 14% for PAC2, 10% for PAC3, 10% for PAC4, and 22% for UMP1. These numbers indicate that the selective pressure on conservation of their sequence is significantly lower than that on the mature subunits. This is consistent with the observation that none of these chaperones is strictly essential in *S. cerevisiae*. Together, these observations indicate that the eukaryotic proteasomal α and β subunits still have the ability to assemble autonomously into functional proteasomes. Unassisted assembly, however, is error prone, as is demonstrated by the increasing loss of efficiency of proteasome biogenesis in cells lacking PAC1-PAC2, PAC3-PAC4, or UMP1 (Le Tallec et al., 2007). The higher complexity of eukaryotic proteasomes therefore apparently requires additional factors to assure an efficient and correct assembly, probably by stabilizing intermediates, and by preventing formation of off-pathway products. It has been suggested that proteasome chaperones might ensure a "quality control" mechanism during the assembly of the more complex eukaryotic 20S proteasome (Le Tallec et al., 2007; Li et al., 2007).

Like the proteasome assembly chaperones, the propeptides and extensions of β subunits are highly divergent among the eukaryotes, and they are not essential for proteasome assembly in yeast (except for the propeptide of β5; see above). These appendages mediate specific interactions between subunits and between subunits and assembly chaperones, thereby facilitating assembly (Ramos et al., 2004; Heink et al., 2005). Even within a given vertebrate species, the propeptides of immunosubunits are very different from those of their corresponding constitutive subunits. The main significance of these distinct propeptides is that they promote assembly of the subunits carrying them into the respective alternative proteasome subtype (see above). The latter example illustrates that cells can adapt to changes in their physiological conditions and their environment by regulating the expression of proteasome subunits and by utilizing alternative assembly modes.

Outlook

After the groundbreaking determination of the structure of the mature 20S proteasome, it became a major challenge to

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understand how this fascinating complex is generated in living cells. We are beginning to appreciate that, in eukaryotes, this intricate process involves subunit-intrinsic targeting elements as well as a number of dedicated assembly chaperones. The first structural analysis of such a chaperone has illuminated how it promotes specific steps in the assembly or prevents off-pathway reactions. Future challenges include the definition of the exact order of events in the assembly pathway and structural analyses of its assembly intermediates. A largely unexplored area is the assembly of the 19S regulatory particle, a complex that in addition awaits full structural characterization. One important aspect of understanding the details of proteasome biogenesis is to determine which of its steps are subject to cellular regulation or might be a target for pharmacological intervention.

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Note Added in Proof

While this article was in press, a report was published (Hirano, Y., Kaneko, T., Okamoto, K., Bai, M., Yashiroda, H., Furuyama, K., Kato, K., Tanaka, K., and Murata, S. (2008). Dissecting β-ring assembly pathway of the mammalian 20S proteasome. EMBO J., in press. Published online July 24, 2008. 10.1038/emboj.2008.148), in which the order of events in the assembly of β rings in mammalian cells was studied by detecting assembly intermediates that accumulated upon knockdown of individual subunits or assembly chaperones in native acrylamide gels. The conclusions of this paper are by and large in agreement with the model presented in Figure 2 of this review. The data of this study indicated that, at least in mammals, β2 and UMP1 are the first polypeptides to assemble onto α rings complexed with the PAC1-PAC2 and PAC3-PAC4 chaperones.

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