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Review

Some assembly required: dedicated chaperones in eukaryotic proteasome biogenesis*

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Abstract

The 26S proteasome is the key eukaryotic protease responsible for the degradation of intracellular proteins. Protein degradation by the 26S proteasome plays important roles in numerous cellular processes, including the cell cycle, differentiation, apoptosis, and the removal of damaged or misfolded proteins. How this 2.5-MDa complex, composed of at least 32 different polypeptides, is assembled in the first place is not well understood. However, it has become evident that this complicated task is facilitated by a framework of protein factors that chaperone the nascent proteasome through its various stages of assembly. We review here the known proteasome-specific assembly factors, most only recently discovered, and describe their potential roles in proteasome assembly, with an emphasis on the many remaining unanswered questions about this intricate process of assisted self-assembly.

Keywords: assembly factor; maturation; protein complex; yeast.

Introduction

The 26S proteasome is responsible for a large fraction of intracellular protein degradation in eukaryotes (Hochstrasser, 1996). Most proteins destined for degradation by the proteasome are initially marked with polyubiquitin chains, which serve to target the protein to the proteasome. The 26S proteasome comprises two major subassemblies. One is the 20S proteasome or core particle, which bears the actual protease active sites. These are housed in a central chamber within the four coaxially stacked heteroheptameric rings that make up the 20S proteasome (Groll et al., 1997). The second subassembly of the 26S proteasome is the 19S activator or regulatory particle (RP; also called PA700 for proteasome activator of 700 kDa). Polyubiquitinated substrate binding, substrate unfolding and deubiquitination, and substrate translocation into the degradative chamber are executed by the RP, which can occupy one or both ends of the 20S proteasome (Walz et al., 1998). The RP is a conglomerate of at least 19 proteins whose precise quaternary structure is not yet known (Glickman et al., 1998; Schmidt et al., 2005b).

This review will focus on 20S proteasome biogenesis because much more is known about its structure and assembly than is known for RP assembly. As shown schematically in Figure 1, the outer rings of the 20S proteasome each comprise seven different non-catalytic α subunits, while each inner ring consists of seven distinct β subunits. A dyad symmetry axis relates one pair of α and β rings to the other pair. In eukaryotes, only three of the seven β subunits carry the N-terminal threonine protease active sites (Chen and Hochstrasser, 1996; Arendt and Hochstrasser, 1997; Heinemeyer et al., 1997). Although detailed structural information for the 20S proteasome is available (Groll et al., 1997; Unno et al., 2002), and much is known about the overall function of the proteasome, a basic understanding of how such an intricate molecular machine is assembled is still lacking. A number of observations strongly suggest that proteasome assembly *in vivo* is a highly regulated process that goes beyond simple self-assembly. First, several proteasome β subunits are synthesized as precursors that bear N-terminal propeptides not present in the final assembled 20S proteasome (Chen and Hochstrasser, 1996; Arendt and Hochstrasser, 1997). The propeptide of the yeast $\beta5$ subunit is essential for viability and normal proteasome assembly, and it can also function *in trans*, suggesting a chaperone-like role in proteasome biogenesis (Chen and Hochstrasser, 1996). Second, mammals express three additional β subunits ($\beta1i$, $\beta2i$, and $\beta5i$) that are inducible by γ-interferon and can take the place of the constitutively expressed $\beta1$, $\beta2$, and $\beta5$ subunits within the β rings (Monaco and Nandi, 1995). The resulting 'immunoproteasomes' help process antigens for eventual presentation by MHC class I molecules as part of the cellular immune response. Third, additional subunit isoforms are found in many species. For example, *Arabidopsis thaliana* has 13 α-type and 10 β-type proteasome subunit genes, though the potential functional specializations of individual paralogs are not known (Fu et al., 1998). In *Drosophila melanogaster*, several of the subunit paralogs are expressed specifically in the testes and can alter proteasomes in a way that is important for spermatogenesis (Yuan et al., 1996; Zhong and Belote, 2007). Fourth, one of the 14 proteasomal core subunit genes in yeast, $\alpha3$, is not essential and can be replaced in the ring by another α subunit, raising the possibility of mixed proteasome populations with potentially different functional specificities (Velichutina et al., 2004). Fifth, almost all of the pro-

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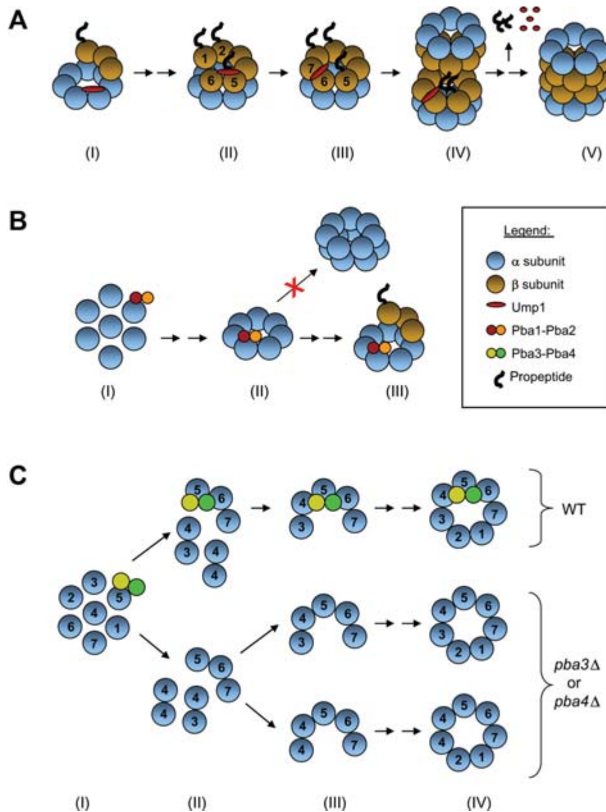

Figure 1 Assembly factors of the 20S proteasome.
(A) Ump1 functions as a checkpoint protein in 20S assembly. Ump1 associates with 15S intermediates (I) in yeast (13S–16S in mammals) consisting of a full complement of α subunits and a subset of β subunits (β2, β3, β4 in yeast). Subsequent entry of β subunits leads to a half-proteasome species that contains all subunits except β7 (II). During this time, Ump1 may prevent the premature dimerization (not shown) of any/all species along the assembly pathway from (I) through (II). Addition of β7 (III) represents the rate-limiting step of 20S assembly and may lead to a conformational change that relieves the inhibitory function of Ump1 (denoted by the shift in position towards β6). This is quickly followed by the β5-propeptide-mediated dimerization of two half-proteasomes (IV). Ump1 becomes encapsulated in the pre-holoproteasome and is degraded following the processing of the β subunit propeptides to yield the active 20S particle (V). Only some β subunits are specifically denoted. Other assembly factors and propeptides of non-catalytic β subunits are omitted for clarity. (B) Pba1-Pba2 complex maintains α rings competent for assembly. The Pba1-Pba2 complex can bind to select α subunits (I) and, via an unknown mechanism, participates in α ring assembly (II). The presence of Pba1-Pba2 bound to α rings ensures that they do not stray off-pathway into higher molecular weight complexes, such as α ring dimers (II, upper panel), but continue on-pathway towards the 15S intermediate (III). (C) Pba3-Pba4 complex promotes the assembly of wild-type 20S proteasomes. In wild-type cells (top panel), the Pba3-Pba4 complex can bind strongly to either free α5 (I) or α5 in complex with other subunits (II). The Pba3-Pba4 complex acts as a scaffold (III) that may stabilize the interaction between α5 and α4, which allows α3 to enter (either alone or in complex with α4 as shown). Subsequent binding events lead to the completion of the α ring (IV). The Pba3-Pba4 complex is then released and recycled during the transition to the 15S intermediate. In *pba3Δ* and/or *pba4Δ* cells (bottom panel), the absence of the Pba3-Pba4 scaffold (II) results in a second copy of α4 (either alone or in complex with α4 as shown) competing with the normal addition of α3 (III). This leads to the formation of two types of α ring (IV) which then become incorporated into 20S core particles, resulting in proteasomes of different subunit composition.

teasome genes are cotranscriptionally regulated by a feedback mechanism responsive to the proteolytic activity within a cell at any given time, ensuring a coordinated increase in the supply of the individual subunits when additional degradative capacity is needed (Mannhaupt et al., 1999; Xie and Varshavsky, 2001). Finally, a number of proteins that facilitate the assembly of the eukaryotic proteasome have been identified (Ramos et al., 1998; Fehlker et al., 2003; Hirano et al., 2005, 2006; Le Tallec et al., 2007; Marques et al., 2007; Kusmierczyk et al., 2008; Yashiroda et al., 2008). In this review, we focus on this expanding list of dedicated proteasome assembly factors/chaperones and summarize how they are thought to function in the assembly process. The assembly of 20S proteasomes has been addressed in a number of reviews, which provide more general discussion of assembly mechanisms (Zwickl et al., 2001; Heinemeyer et al., 2004; Hendil and Hartmann-Petersen, 2004).

Ump1

Although a 16-kDa protein had been observed to associate with proteasome precursor complexes in mammalian cells (Frentzel et al., 1994), its identity remained a mystery until 1998 when Ramos et al. characterized what eventually turned out to be its yeast ortholog, Ump1 (Ramos et al., 1998). Ump1 was identified in a genetic screen for yeast mutants defective in the ubiquitin-mediated proteolysis of several test substrates. Its human ortholog, hUmp1 (also called POMP or proteassemblin), was subsequently identified based on weak sequence similarity (Burri et al., 2000; Griffin et al., 2000; Witt et al., 2000). Although mammalian Ump1 cannot functionally replace its yeast counterpart *in vivo* (Burri et al., 2000), there is evidence suggesting that Ump1-related proteins function in a similar manner in all eukaryotes. During assembly, Ump1 is encapsulated by the dimerization of two half-proteasome precursors and is degraded upon active-site maturation (Ramos et al., 1998).

Ump1 is found exclusively in proteasome precursor complexes consisting of a full complement of α subunits and at least three of the seven β subunits (Burri et al., 2000; Griffin et al., 2000; Witt et al., 2000; Li et al., 2007). Deletion of Ump1 in yeast causes the accumulation of proteasome precursors and results in impaired processing of β subunit propeptides, leading to a significant decrease in the proteolytic activity of the 20S and 26S proteasomes. Strikingly, deletion of Ump1 in yeast renders the propeptide of β5 dispensable for viability (Ramos et al., 1998). The bypass of the essential activity of the β5 propeptide by loss of Ump1 suggests that the β5 propeptide might normally overcome an inhibitory activity of Ump1 in proteasome assembly.

Recently, we proposed that the β5 propeptide helps drive the dimerization of two half-proteasomes and that this process is antagonized by Ump1 (Li et al., 2007). This hypothesis arose from the observation that bypass of the essential β5 propeptide function can also be achieved by overexpression of the β7 subunit. This depends on a unique β7 C-terminal extension, which intercalates in a surface groove between the β1 and β2 subunits of the *trans* β ring (Ramos et al., 2004; Li et al.,

2007). β7 is the last β subunit to insert into half-proteasomes before their dimerization, and Ump1 appears to provide a checkpoint that prevents half-proteasomes from dimerizing before all the β subunits have incorporated. According to this model, Ump1 facilitates assembly by helping to ensure the proper order of proteasome assembly events (Figure 1A).

Our understanding of Ump1 function is far from complete. Its roles in assembly have so far been linked specifically to N-terminal or C-terminal extensions of the late subunits (namely, β5, β6, β7), yet Ump1 can enter proteasome precursors before the incorporation of these subunits. Conceivably then, Ump1 may contribute to proteasome assembly at steps prior to late subunit addition. A very recent report proposes that mammalian Ump1 interacts with the endoplasmic reticulum (ER) membrane and recruits proteasome precursors as early as at the α ring stage to the ER, where incorporation of β subunits and subsequent assembly presumably occurs (Fricke et al., 2007). Whether such localization is necessary for mammalian proteasome assembly is not yet known; in the yeast *Saccharomyces cerevisiae*, Ump1 is concentrated inside the nucleus rather than at the ER (Lehmann et al., 2002). Localizing proteasome assembly to the ER could have the potential advantage of directing assembly to a site where substantial proteasome activity is required. This might be especially important for generating peptides presented by MHC I class molecules, which enter the secretory pathway at the ER.

Pba1-Pba2/PAC1-PAC2

Although an understanding of later events in proteasome assembly has begun to crystallize over the past decade or so (Heinemeyer et al., 2004; Li et al., 2007), early events remain more obscure. One reason for this is the difficulty in isolating intermediates that form prior to the 13S–16S/15S intermediate complexes, which already have a full complement of α subunits and at least three β subunits. Early assembly events, particularly in yeast, occur very quickly, which might be due to the tendency of α subunits to self-organize into ring-shaped complexes (Zwickl et al., 1994; Gerards et al., 1997, 1998). Importantly, these homomeric and heteromeric rings, which were observed when the subunits were expressed recombinantly in bacteria, have intersubunit contacts that do not occur in mature proteasomes, suggesting that in eukaryotic cells, additional factors might be required to limit non-native interactions. An indication that this might be the case came from the discovery of a heterodimeric protein complex in mammals, called PAC1-PAC2 (for proteasome assembling chaperone 1 and 2; Hirano et al., 2005). These proteins had been identified previously, but no connection to proteasome function was made (Vidal-Taboada et al., 2000; Bahar et al., 2002; Possik et al., 2004). PAC1 and PAC2 were identified as proteins coprecipitating with an epitope-tagged proteasome subunit (Hirano et al., 2005). In cell lysates fractionated by glycerol gradient centrifugation, the peak of PAC1 and PAC2 was found in fractions lighter than those containing hUmp1, suggesting an early role in assembly. Specifically, exhaustive immunoblot analyses of the fractionated proteins strongly suggested that PAC1 and PAC2, which form a heterodimer, associate with α subunit rings. PAC1-PAC2 also associates with subsets of α subunits prior to their assembly into rings, suggesting that PAC1-PAC2 could promote α ring assembly.

Notably, when levels of PAC1-PAC2 protein were knocked down in cells using siRNA, the putative α rings disappeared and were replaced by a higher molecular mass complex, which the authors suggested was a dimer of two α rings (Hirano et al., 2005). Formation of α ring dimers (and higher-order structures) had previously been observed for archaeal proteasome α subunits and certain eukaryotic α subunits when expressed in *Escherichia coli*, suggesting a certain proclivity of α subunits to form these structures (Zwickl et al., 1994; Gerards et al., 1997, 1998). Such structures presumably represent off-pathway complexes that do not lead to proteasome formation. Regardless of whether these complexes turn out to be α ring dimers or some other higher molecular mass complex, a role for PAC1-PAC2 in preventing the formation of non-productive, or dead-end, complexes is likely to be correct. A function for this assembly factor in the formation of α rings is less clear.

PAC2 was noted to have weak homology to a yeast protein encoded by the *YKL206C* gene, although no putative homolog to PAC1 outside of vertebrates was reported (Hirano et al., 2005). Thus, whether or not the PAC1-PAC2 complex was specific to higher eukaryotes, or was present in all taxa, remained an open question. Loss of *YKL206C* (also named *ADD66*) causes a very weak defect in ER-associated protein degradation, suggesting a potential link to the proteasome (Palmer et al., 2003). Intriguingly, Add66 protein was found to associate with several proteasome subunits and an uncharacterized polypeptide, YLR199C, in a proteomic analysis of affinity purified protein complexes in yeast (Krogan et al., 2006). In a recent analysis of yeast 20S proteasome assembly, our group identified a number of new potential assembly intermediates, all of which contained Add66 and the product of the *YLR199C* gene (Li et al., 2007). We proposed the names *PBA1* and *PBA2* (for proteasome biogenesis associated) for the *YLR199C* and *YKL206C* genes, respectively. Pba1 and Pba2 were found in proteasome subcomplexes lacking Ump1, suggesting an earlier role in assembly, just as in the case of PAC1-PAC2. Pba1 and Pba2 were not found in mature 20S proteasomes. Like PAC1-PAC2, Pba1 and Pba2 form a heterodimer (Li et al., 2007), and they also bind subsets of α subunits *in vitro* (A.K. and M.H., unpublished data). Moreover, we detected weak sequence similarity between yeast Pba1 and human PAC1. Thus, Pba1-Pba2 is likely to be the yeast ortholog of PAC1-PAC2, suggesting that the function of this assembly factor (Figure 1B) is conserved throughout the Eukarya. As is true for PAC1-PAC2, the exact role(s) of Pba1-Pba2 in proteasome assembly remains to be deciphered.

Pba3-Pba4/PAC3-PAC4

Following up on the discovery of PAC1-PAC2, Hirano et al. reported the existence of a third putative 20S proteasome assembly chaperone, which they called PAC3

(Hirano et al., 2006). PAC3, which reportedly forms a homodimer, also associates with early 20S assembly intermediates, notably apparent α ring complexes, but not with late assembly intermediates, suggesting a role in the early stages of 20S biogenesis. PAC3 is not a short-lived protein, unlike PAC1-PAC2 and hUmp1, suggesting that PAC3 dissociates from proteasomes before assembly is completed. Knockdown of PAC3 by RNA interference implied that PAC3 function is distinct from PAC1-PAC2, because knockdown of PAC3 did not lead to the accumulation of α ring dimer-like structures, and because a triple knockdown exacerbated the assembly defects relative to PAC3 or PAC1-PAC2 knockdowns alone. The exact role of PAC3 in α subunit assembly, however, remains undefined.

No obvious orthologs of human PAC3 were detected in yeast species by Hirano et al., (2006). However, in the past year, a series of independent studies by several groups, including ours, identified what turns out to be the *S. cerevisiae* ortholog of PAC3 (called Poc3, Dmp2, or Pba3) and a partner protein, encoded by the *YPL144W* gene (called Poc4, Dmp1, or Pba4). Here, we will use the Pba3-Pba4 name for the complex.

In the first of these reports, the Pba3 and Pba4 proteins were identified in a genetic screen targeting the DNA damage response and were shown to form a complex (Le Tallec et al., 2007). Inactivation of either subunit of the complex resulted in identical defects that correlated with impaired proteasome assembly. Interestingly, just like the proposed PAC3 homodimer, the Pba3-Pba4 complex was metabolically stable. Immunoprecipitation experiments further suggested that Pba3-Pba4 associated with a spectrum of assembly intermediates distinct from those bound by Pba1-Pba2 or Ump1. Pba3-Pba4 was found in immunoprecipitates containing primarily the unprocessed precursor form of β2 and was not recovered efficiently from immunoprecipitates containing Ump1. These results imply that the Pba3-Pba4 complex is associated with early intermediates during yeast 20S assembly, just like PAC3. Congruent with this, we had not detected Pba3-Pba4 in purified late assembly intermediates that contained the full α subunit ring plus β2-β3-β4 and Ump1 (the '15S intermediate') or additional subunits (Li et al., 2007). Notably, Le Tallec et al. (2007) identified a mammalian ortholog of Pba4, which they dubbed PAC4, and demonstrated that PAC4 forms a complex with the previously identified PAC3 protein. They also observed that the metabolic stability of PAC3 depends on the presence of PAC4, and vice versa, suggesting that the functional assembly factor in mammals, as in yeast, is a complex of both proteins (although see below). Knockdown of PAC3 or PAC4 in HEK293T cells impaired their proliferation, consistent with the known requirement for proteasomes in mammalian cell growth.

Our group came across Pba3-Pba4 in a targeted bioinformatic search that sought uncharacterized yeast proteins with mutational evidence from genomics studies suggesting a link to proteasome function (Kusmierczyk et al., 2008). Consistent with the Le Tallec et al. study, our data indicated that Pba3 and Pba4 form a complex involved in 20S proteasome biogenesis. More interestingly, we uncovered a surprising connection between Pba3-Pba4 and α3/Pre9, the only non-essential subunit of the yeast 20S proteasome. Specifically, in the absence of the Pba3-Pba4 assembly chaperone, yeast synthesizes an alternative 20S proteasome containing a second copy of the α4/Pre6 subunit in the place of α3. Proteasomes of exactly the same composition are assembled in the *α3Δ* yeast mutant (Velichutina et al., 2004). We estimated that these alternative proteasomes containing at least one α4-α4 α subunit ring comprise anywhere from 20% to 50% of the total proteasome pool in *pba4*Δ cells. In other words, the Pba3-Pba4 assembly factor functions to ensure entry of the α3 subunit between α2 and α4 in the α ring, thereby generating 'normal' 20S proteasomes. Although the exact mechanism of Pba3-Pba4 action remains to be determined, we postulated that it could act as a scaffold for α ring assembly (Figure 1C). Pba3-Pba4 binds tightly to the α5/Doa5 proteasome subunit; this might stabilize the interaction of α5 with its neighboring subunits, channeling α ring assembly along a pathway that brings in α3 next to α4 rather than a second copy of α4. Whatever the precise mechanism, the Pba3-Pba4 complex provides the first example of a chaperone that directly regulates the composition of the 20S proteasome.

The concept that Pba3-Pba4 can serve as a scaffold for α ring assembly is supported by a co-crystal structure of Pba3-Pba4 in complex with α5, recently solved by Tanaka and colleagues, who independently identified Pba3-Pba4 in a genetic screen for yeast mutants unable to grow in the presence of certain amino acid analogs (Yashiroda et al., 2008). In this structure, the H1 and H2 helices of α5 bind to both Pba3 and Pba4, burying 984 $Å^2$ of surface area. The α5 subunit has essentially the same structure as when it is part of the mature 20S proteasome. When Yashiroda et al. modeled the Pba3-Pba4-α5 complex in the context of a full α ring taken from the known structure of the mature 20S, Pba3-Pba4 could be seen to bind near the central axis of the ring on what will become the antechamber surface inside the proteasome and to make contacts with the neighboring α6 and α4 subunits. The β4 subunit, which is part of the 15S intermediate, would clash sterically with Pba3-Pba4, explaining why Pba3-Pba4 is not found in this or any later assembly intermediate.

A number of questions are raised by these new data. First, is there any selective advantage to synthesizing 20S proteasomes of alternative composition, in particular ones with α rings carrying two copies of α4, and does this occur under any conditions in wild-type cells? We found that yeast cells which accumulate proteasomes with α4-α4 rings are more resistant to the heavy metal cadmium (Kusmierczyk et al., 2008). Cadmium is known to cause oxidative stress in growing yeast cells (Brennan and Schiestl, 1996). Moreover, global transcriptional profiling studies consistently show that proteasome genes are strongly upregulated by oxidative stress-inducing conditions, such as growth in the presence of diamide, hydrogen peroxide, arsenic, dithiothreitol, and heat shock (Gasch et al., 2000). Conversely, the *PBA3* and *PBA4* genes are either not induced, or even mildly repressed, under the same conditions (Gasch et al., 2000). This raises the potential scenario wherein

increased synthesis of 20S subunits, relative to Pba3-Pba4, in response to such stresses, drives the assembly of alternative 20S proteasomes by virtue of exceeding the chaperoning capacity of Pba3-Pba4. Second, what properties of the 'α4-α4 proteasome' might allow greater resistance to the heavy metal? A number of possibilities exist. Since α4-α4 proteasomes lack α3, a subunit required for sealing the α ring pore that provides access to the core particle interior, they are predicted to be in a constitutively open conformation (Groll et al., 2000). Perhaps such a constitutively open proteasome is especially suited for the degradation of oxidatively damaged proteins, which studies suggest are preferentially degraded by 20S proteasomes (rather than 26S proteasomes) in an ubiquitin-independent manner (reviewed in Davies, 2001). Moreover, the top surface of the α ring provides the 20S proteasome with the interface to all its known regulators; thus, α4-α4 proteasomes will present a structurally distinct surface to regulatory partners, such as the RP or Blm10. Such structural changes will very likely alter binding and regulation by these different proteasomal cofactors.

Another obvious question is whether the ability to synthesize the α4-α4 proteasome is conserved. The existence of a mammalian ortholog for Pba3-Pba4, the ability of α4 from other species (*Arabidopsis*) to occupy both the α3 and α4 position within the α rings of *α3Δ* yeast (Velichutina et al., 2004), and the observation that the non-essential nature of α3 is probably not unique to *S. cerevisiae* (Lee and Shaw, 2007), all support the case, albeit circumstantially, that α4-α4 proteasomes will be found in all eukaryotic taxa. Such conservation would imply that this variant 20S proteasome has an important physiological function(s) common to many or all eukaryotes. This in turn would explain why α3 and α4 subunits did not simply evolve to the point where each of them could only insert into a single unique position in the α ring, as appears to be true for all the other proteasome subunits, but rather require a dedicated chaperone for this.

Blm10/PA200

20S proteasomes can be activated by regulatory factors other than the RP. In mammalian cells, these include the two isoforms of the PA28 activator, PA28αβ and PA28γ, and PA200; only the latter has orthologs in plants and yeast. PA28 stimulates peptide hydrolysis by the proteasome and modulates MHC class I antigen processing (Hill et al., 2002). Interestingly, mice lacking PA28αβ show greatly impaired assembly of immunoproteasomes (Preckel et al., 1999). Therefore, incorporation of the alternative β1i, β2i, and β5i subunits is regulated by PA28, even though the β subunits do not directly contact PA28. PA28 is known to associate with 15S assembly precursors, leading to the hypothesis that PA28 alters the conformation of the α ring in a way that facilitates selective β1i, β2i, and β5i incorporation at the expense of the constitutive subunits (Preckel et al., 1999).

PA200, a large, HEAT-repeat containing protein, also stimulates the peptidase activity of the 20S proteasome and has been proposed to function in DNA repair and spermatogenesis (Ustrell et al., 2002; Khor et al., 2006). Most germane for the present discussion, the yeast PA200 ortholog, Blm10, has been proposed to contribute to 20S proteasome assembly or maturation (Fehlker et al., 2003; Marques et al., 2007), although this remains a matter of some debate (Schmidt et al., 2005a). In freshly lysed yeast, Blm10 was found predominantly in mature RP-20S-Blm10 hybrid particles, which apparently give rise to Blm10-20S particles upon prolonged incubation and/or purification (Schmidt et al., 2005a). Others have reported Blm10 to be associated with proteasome precursors, including incomplete half-proteasomes, suggesting that Blm10 may function in 20S assembly (Fehlker et al., 2003; Li et al., 2007). Indeed, when a deletion of Blm10 was combined with a deletion of the β7 (Pre4) C-terminal tail, which plays a role in half-proteasome dimerization, it resulted in accumulation of 20S precursors and a considerable maturation defect, characterized by a profound defect in β-propeptide processing (Marques et al., 2007). This suggests a positive role for Blm10 in the late stages of 20S assembly, possibly by stabilizing nascent dimerized half-proteasomes. Analogous to the effect of mammalian PA28 on immunoproteasome assembly, Blm10 could presumably regulate yeast 20S maturation through conformational alterations of the intervening α ring(s). PA28 forms a heptameric ring that binds the surface of the α ring and stabilizes the open state of the entry pore (Whitby et al., 2000). Similarly, Blm10, with its extended α-solenoid structure, also forms an extensive binding surface that contacts all the α subunits and appears to open the α ring channel as well (Ustrell et al., 2005; Iwanczyk et al., 2006). Intriguingly, the RP can apparently substitute, at least in small part, for Blm10 in the late stages of 20S maturation (Marques et al., 2007).

Regulatory particle (RP) assembly

Our knowledge of RP assembly mechanisms lags considerably behind that of its 20S core particle partner. Lack of high resolution structural information for the RP has meant that our understanding of RP subunit interactions and assembly derives from lower resolution methods, and no RP assembly factors are known (Davy et al., 2001; Fu et al., 2001; Isono et al., 2004, 2005, 2007; Sharon et al., 2006). The RP is composed of two major subcomplexes, the base and lid (Glickman et al., 1998). The base consists of the six ATPase subunits (Rpt1–6) and three non-ATPase subunits (Rpn1, 2, 13); the lid is made up of nine non-ATPase subunits (Rpn3, 5–9, 11, 12, and Sem1/Rpn15). Base-lid association is stabilized by another subunit, Rpn10. How each of these subcomplexes is assembled from its individual constituents is not understood, though the lid appears to be able to assemble independently of the base (Isono et al., 2007).

Whether dedicated chaperones exist for the assembly of the RP is an open question. Nob1 has been proposed as an assembly factor for the yeast 26S proteasome (Tone and Toh, 2002), but its significance in this role has

been challenged (Heinemeyer et al., 2004). Certain proteins, perhaps best referred to as accessory factors, have been shown to stabilize the interaction between the RP and 20S proteasome. These include Ecm29 and Sem1 (Leggett et al., 2002; Funakoshi et al., 2004; Sone et al., 2004; Kleijnen et al., 2007). Insofar as they are found in association with the mature 26S proteasome, rather than being degraded upon completion of assembly (like Pba1-Pba2 and Ump1) or recycled during assembly (like Pba3-Pba4), these factors do not readily fit the mold of an assembly chaperone.

RP assembly has generally been assumed to occur independently of 20S assembly based on a number of observations. The two complexes can be isolated individually from cells and can be made to associate and dissociate repeatedly *in vitro* in forming 26S complexes (Saeki et al., 2000; Verma et al., 2000). Moreover, the RP or RP subcomplexes appear to function in certain processes without the 20S proteasome (Russell et al., 1999a; Lee et al., 2005). Finally, RP complexes can apparently be imported into the nucleus separately from 20S particles (Wendler et al., 2004; Isono et al., 2007).

Surprisingly, however, recent results from our group indicate that yeast mutants with variant 20S proteasomes or reduced amounts of normal 20S proteasomes accumulate RP subparticles rather than just excess free RP (Kusmierczyk et al., 2008). All the tested 20S assembly mutants, including *pba3Δ*, *pba4Δ*, *pba1Δ pba2Δ*, *α3Δ*, and *ump1Δ*, accumulated excess free lid subcomplex and various subcomplexes of the base. The data strongly suggest that the 20S proteasome is required for normal RP base assembly and that the assembled lid subcomplex does not associate stably with incomplete base subassemblies. To our knowledge, this is the first evidence of 20S proteasome mutants causing defects in RP assembly and opens up the exciting possibility that the 20S proteasome also serves as an RP assembly factor. The six paralogous ATPases of the base are thought to form a toroid that stacks coaxially with the heptameric rings of the 20S proteasome. It is tempting to speculate that the α rings of the 20S proteasome (and possibly 20S precursor particles) provide a platform upon which the RP base is assembled. We are currently designing experiments to test these concepts.

Future prospects

The growing list of dedicated 20S proteasome assembly factors opens up a host of new avenues of research to pursue and raises a number of interesting questions in addition to the ones already mentioned above. Perhaps foremost is the question of whether or not dedicated assembly factors exist for the RP as well. Given that both the RP and 20S proteasome have comparable numbers of different subunits and that both appear to assemble via defined pathways, it is reasonable to suppose that specific factors – in addition to the 20S proteasome – may help chaperone the RP into its mature quaternary structure. With regards to the known 20S assembly chaperones, the obvious gaps in our understanding have to do with determining how exactly they carry out their functions. For instance, how does the Pba3-Pba4 complex promote α3 entry into the 20S proteasome if it does not appear to bind α3 directly? What is the role of the Pba1-Pba2 complex in α ring assembly? It is noteworthy that the Pba3-Pba4 complex binds strongly to α5, a subunit also strongly bound by both mammalian PAC1-PAC2 (Hirano et al., 2005) and yeast Pba1-Pba2 (A.K. and M.H., unpublished data). Given that Pba3-Pba4 exits the assembly pathway relatively early, could there be some sort of 'molecular hand-off' mechanism centered around α5 that also involves Pba1-Pba2? These sorts of questions would benefit greatly from the ability to reconstitute 20S assembly *in vitro* using purified components, although this is no simple undertaking.

Despite Ump1 being the first dedicated proteasome assembly chaperone identified, its mechanism of action is still poorly defined and it could turn out to have multiple roles. Its function as an assembly checkpoint protein that prevents premature dimerization of half-proteasomes is genetically linked to the N- and C-terminal extensions of the β5, β6, and β7 subunits, all subunits that are incorporated relatively late, but Ump1 enters the assembly pathway much earlier (Li et al., 2007). The recent report that hUmp1 localizes to ER membranes and might serve as a scaffold for α ring assembly and promote β subunit incorporation in mammalian cells suggests potential roles in earlier steps of proteasome assembly (Fricke et al., 2007). Whether this mechanism can be extended to all eukaryotes is an interesting question. These results highlight the issue of whether specific steps of proteasome assembly might be linked to particular subcellular sites.

Mature proteasomes are found in both the cytoplasmic and nuclear compartments in eukaryotic cells. However, the distribution can differ among species and even between cell cycle stages (Amsterdam et al., 1993). In interphase mammalian cells, proteasomes appear evenly distributed between the nucleus and cytoplasm (Reits et al., 1997), whereas they are concentrated in the nucleus and the nuclear envelope periphery in yeast (Enenkel et al., 1998; Wilkinson et al., 1998; Russell et al., 1999b). Debate continues as to how proteasomes are transported across the nuclear membrane, e.g., whether this occurs for mature 20S proteasomes or only precursor species (Reits et al., 1997; Wang et al., 1997; Lehmann et al., 2002). Perhaps more relevant to the discussion of proteasome assembly factors is the question of what role they might play in nuclear transport processes. Ump1 and Blm10 are primarily nuclear (Lehmann et al., 2002; Ustrell et al., 2002; Fehlker et al., 2003; Schmidt et al., 2005a), whereas Pba1-Pba2 and Pba3-Pba4 are found to be diffusely localized throughout the yeast cell (Huh et al., 2003; Le Tallec et al., 2007). Interestingly, both Pba3 and Pba4 seem to concentrate in the nucleus in *ump1Δ* cells (Le Tallec et al., 2007). In mammalian cells, PAC1 has been suggested to be associated with the ER (Possik et al., 2004), while PAC2 has been reported in nuclear foci (Bahar et al., 2002). The relevance to proteasome assembly of the localization of any proteasome assembly factor is in no case firmly established.

The study of factors that aid in the assembly of specific multi-protein complexes has featured prominently in

diverse fields of molecular biology ranging from virus assembly (Dokland, 1999) to the assembly of nucleosomes (Laskey et al., 1978). Moreover, despite being involved in disparate processes, dedicated assembly factors for different macromolecular complexes often fulfill analogous roles, from acting as potential scaffolds or nucleation sites (Ma et al., 2005) to preventing off-pathway interactions (Saschenbrecker et al., 2007). In this regard, understanding the functions of dedicated chaperones in the assembly of proteasomes bears relevance for the assembly of other large multi-protein complexes that carry out so many of the core functions of the eukaryotic cell.

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Note added in proof

While this manuscript was under review, a report by Hoyt et al. (2008) identified Pba3-Pba4 in a screen for yeast mutants affecting degradation of a ubiquitin-independent substrate. Notably, stable expression of Pba3 depended on expression of Pba4 (and vice versa), echoing the situation observed with PAC3 and PAC4 (Le Tallec et al., 2007).

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