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ORIGINAL PAPER

Order of the Proteasomal ATPases and Eukaryotic Proteasome Assembly

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Abstract The 26S proteasome is responsible for a large fraction of the regulated protein degradation in eukaryotic cells. The enzyme complex is composed of a 20S proteolytic core particle (CP) capped on one or both ends with a 19S regulatory particle (RP). The RP recognizes and unfolds substrates and translocates them into the CP. The RP can be further divided into lid and base subcomplexes. The base contains a ring of six AAA+ ATPases (Rpts) that directly abuts the CP and is responsible for unfolding substrates and driving them into the CP for proteolysis. Although 120 arrangements of the six different ATPases within the ring are possible in principle, they array themselves in one specific order. The high sequence and structural similarity between the Rpt subunits presents special challenges for their ordered association and incorporation into the assembling proteasome. In this review, we discuss recent advances in our understanding of proteasomal RP base biogenesis, with emphasis on potential specificity determinants in ring arrangement, and the implications of the ATPase ring arrangement for proteasome assembly.

Keywords Proteasome · ATPase · Multiprotein complex assembly · Proteolysis · Ubiquitin · RP assembly chaperone

Introduction

In eukaryotes, nearly every aspect of cell biology is dependent on the regulated degradation of one or more proteins. The vast majority of this degradation is mediated by the 26S proteasome, a highly conserved 2.5 MDa multicatalytic protease complex, which cleaves substrate proteins into small peptides [1]. While substantial progress has been made in understanding the basic biochemistry of the proteasome in the years since its discovery, several recent studies have highlighted the challenge of assembling with high efficiency and fidelity its 33 individual and often highly similar subunits into a single complex structure. In this review, we discuss our current understanding of proteasome assembly with specific emphasis on the regulatory particle (RP) base subcomplex.

The biology of the ubiquitin–proteasome system has been reviewed extensively in recent years [1–3]. For degradation by this pathway, a protein substrate is typically first post-translationally modified on one or more lysine residues with the small protein ubiquitin. Additional ubiquitin molecules can be ligated onto the original ubiquitin to form a ubiquitin chain that ultimately targets the protein to the 26S proteasome. Following substrate binding, the proteasome removes the ubiquitin molecules, unfolds the substrate, and translocates it into the interior of the catalytic chamber where it is degraded into short peptides.

Proteasome Structure and Function

The eukaryotic 26S proteasome is one of the largest and most complicated of the chambered proteases [4, 5]. It is composed of a cylindrical 20S core particle (CP) that houses the protease active sites in its center and a 19S RP that interfaces directly with the ends of the CP, thereby controlling access to the narrow channels that lead to the CP interior (Fig. 1a). The RP confers substrate specificity

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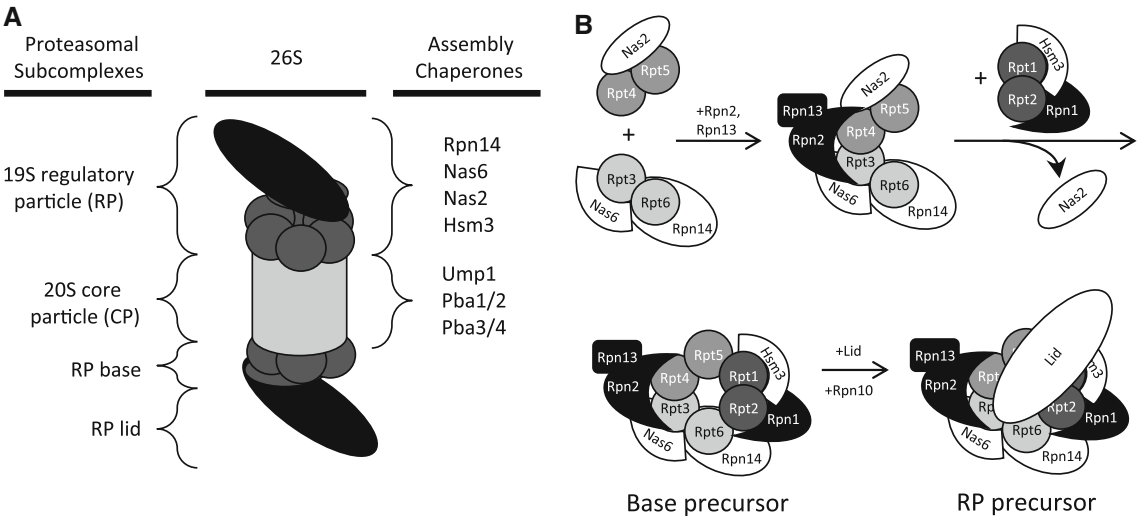

Fig. 1 Proteasome structure and assembly. **a** The 26S proteasome structure is shown with biochemically defined subcomplexes listed on the *left*. Assembly chaperones are listed to the *right* of the subcomplex on which they primarily act. **b** A current model for proteasomal RP assembly is shown, with emphasis on the putative steps in base assembly. The exact placement of Rpn2 and Rpn13 within the base is unknown. They are displayed at the interface between the Nas2 and Rpn14–Nas6 assembly modules based on reported interactions between Rpn2 and Rpt4 [44] and their presence in mammalian complexes containing Rpt3 and Rpt6, but not the other ATPases [33]

and energy dependence on the degradation process. It is also responsible for removing ubiquitin chains from substrates, which facilitates their degradation and allows reuse of the ubiquitin tag.

The eukaryotic 20S CP is composed of four axially stacked heteroheptameric rings; the two outer rings each contain seven different alpha subunits (α1–α7) present in single copy, while each of the two inner rings has seven different beta subunits (β1–β7). The β1, β2, and β5 subunits harbor the catalytic sites of the proteasome, which are sequestered in the central cavity formed by the β rings. The 19S RP includes six distinct AAA+ family ATPases named *r*egulatory *p*article *t*riphosphatase (Rpt) subunits 1–6 and 13 *r*egulatory *p*article *n*on-ATPase (Rpn) subunits. The RP can be biochemically separated into two subcomplexes, the lid and the base, at least when isolated from certain organisms such as the yeast *Saccharomyces cerevisiae* [6]. The lid contains nine of the Rpn subunits: Rpn3, 5–9, 11, 12, and Sem1/Rpn15. The base contains the two largest subunits of the proteasome, Rpn1 and Rpn2, the ubiquitin receptor Rpn13, and the six ATPases, Rpt1–Rpt6. Recent work from our lab has shown that these six Rpt subunits form a uniquely ordered heterohexameric ring [7].

Proteasome Assembly: A Complex Problem

Proper in vivo assembly of a protein complex with many different subunits poses several challenges, and this is especially true for a complicated, multi-tiered structure such as the proteasome. One such challenge is the need to control the relative timing of subunit incorporation to prevent inappropriate subunit interactions that could stall assembly into a full, functional complex. It is often also necessary to prevent enzymatic activities of the complex from being activated prematurely. Multisubunit ring structures pose several additional challenges. These include the need to assure that rings contain the appropriate number of subunits to allow ring closure. If the ring contains many highly similar but non-identical subunits, cells face the additional challenge of seating each subunit in its appropriate position(s). Formation of multi-ring structures such as the proteasome may also require linking multiple rings together properly and prevention of inappropriate ring pairings.

Despite all of these potential difficulties, proteasomes are rapidly and faithfully assembled in the dense and complex intracellular environment from 33 different and often highly homologous subunits. Only recently have the mechanisms that control eukaryotic 26S proteasome biogenesis begun to be unearthed, and they remain far from being completely understood. Assembly involves features of both stochastic self-assembly as well as assisted assembly from proteasome-specific assembly chaperones.

Some basic lessons regarding proteasome assembly can be inferred from the simpler proteasomes of certain eubacteria and archaea. Like those of eukaryotes, these proteasomes contain seven-membered α and β rings bound on the axial ends by hexameric ATPase activator complexes. However, these proteasome complexes often have just a

single subunit of each type (α, β, and ATPase), and the activator complexes lack the non-ATPase subunits characteristic of the eukaryotic RP. Archaeal 20S proteasomes appear to assemble by first forming a homoheptameric α ring, which serves as a template for the addition of seven β-subunit precursors to form a half-proteasome [8]. Two half-proteasomes then dimerize, bringing together the β-subunit precursor rings, followed by autocatalytic removal of the N-terminal β-subunit propeptides to complete assembly and activate the catalytic sites [9, 10]. These sites utilize the free N-terminal Thr residues as nucleophilic centers during proteolysis. Consistent with this simple mode of assembly, the α and β subunits of several archaea species can be coexpressed in *E. coli*¸ and they self-assemble into proteolytically active proteasomes.

Proteasomes are not found in most bacteria, but they are present in the actinomycete lineage of bacteria. Actinomycete proteasomes are also simple in composition, with one or sometimes two very similar subunits of each type. Their assembly mechanism differs significantly from what has been documented to date in archaea. The α subunits in these organisms have limited interfaces with their α-ring neighbors and therefore cannot assemble stably into α rings by themselves [11]. Instead, the basic assembly unit is an α–β-subunit precursor heterodimer [12, 13]. The β-subunit precursor has a long (∼60 residue) propeptide that extends to the interface that normally forms only transiently between neighboring α subunits, thereby stabilizing the interaction between each pair of α subunits [11, 12]. This allows the build-up of the α–β-subunit precursors into the paired rings of the half-proteasome. Subsequent steps are similar to those described for archaeal 20S proteasome assembly [9].

Assembly of the homohexameric ATPase complexes from both archaea and actinomycetes can occur without the CP/20S proteasome [14–17]. Interaction between the regulatory and core complexes is weak but involves specific interactions. The weak or transient nature of these associations may relate to the symmetry mismatch between the hexameric ATPase and heptameric α-subunit rings. Premature interaction between free α subunits and the activator ring appears to be limited by the fact that the C-termini of the activator subunits dock into pockets formed by the interfaces of neighboring α subunits [18]. The archaeal homohexameric ATPase complex, proteasome-activating nucleotidase (PAN), also self-assembles when expressed in *E. coli* [16, 17, 19]. This heterologously expressed PAN is a functional ATPase, and it allows the CP to degrade structured substrates in a nucleotide-dependent manner [17].

In eukaryotic 20S proteasomes, there are seven different and specific interfaces within each α and β ring since each subunit is unique. Subunit-specific associations between the α and β rings and between the two central β rings must also form. Proper positioning of each subunit and control of the register of the α, β, and Rpt rings with respect to one another would be expected to be more difficult in eukaryotic proteasomes compared to the much simpler prokaryotic ones discussed above.

Several lines of evidence suggest that eukaryotes do not rely solely on stochastic self-assembly for proper proteasome biogenesis. First, recombinant human α7 subunit, which normally occupies only a single position in the α ring, forms dimeric homoheptameric rings in vitro [20]. Its two neighbors, α1 and α6, do not form rings on their own, but will each form mixed rings with α7 if coexpressed [21]. The ratios of different subunits in these rings vary widely. Therefore, the α subunits by themselves seem to have insufficient information to find their correct positions in the ring. Nevertheless, data from mammalian cells indicate that correct heteroheptameric α rings do form in vivo.

A striking example of a proteasome subunit taking the "wrong" position in a eukaryotic cell is the finding that deletion of the gene encoding the α3 subunit in *S. cerevisiae* results in proteolytically active proteasomes that contain a second copy of α4 in the α3 position [22]. The α3 and α4 subunits are only ∼33% identical. Proteasomes with two α4 subunits per α ring have distinct properties from the standard proteasome, such as a constitutively open α ring pore. These properties could confer survival advantages under certain growth conditions [23]. Together, the data on eukaryotic proteasomal ring assembly indicate that high fidelity assembly requires more than simple co-evolution of uniquely complementary subunit interfaces. Similarly, yeast Rpt1 and Rpt2 or the Rpt4 protein alone expressed in *E. coli* form large oligomers that can activate the archaeal 20S, reminiscent of the homomeric PAN complex [24]. This is in contrast to the unique heterohexameric arrangement of ATPases in the RP base.

So how have eukaryotes managed the increased complexity of proteasome assembly? Over the past ten or so years, at least seven different proteins have been discovered in eukaryotes with dedicated roles as assembly chaperones for the proteasome. These assembly chaperones control assembly of either the CP or the RP base (Fig. 1a). For example, the CP chaperone Ump1 helps to ensure proper timing of half-proteasome dimerization and active subunit maturation, while the heterodimeric chaperone Pba3/Pba4 (PAC3/PAC4 in mammals) is responsible for ensuring the insertion of α3 rather than α4 next to the α2 subunit in the CP under normal circumstances [23, 25–27]. Deletion of these assembly chaperones either alone or in combination can cause various assembly defects, including altered subunit arrangements [23], incorrect timing of subunit incorporation or processing [26, 27], and accumulation of backlogged assembly intermediates [28–32]. These defects

highlight the importance of these assembly chaperones for efficient proteasome biogenesis in eukaryotes.

Assembly of the RP Base

Our understanding of proteasomal RP biogenesis is hampered by the lack of information on the locations of many subunits within the mature RP, in contrast to the high-resolution crystal structures we have for the CP. No atomic structures of the RP or any of its subcomplexes, such as the lid or base, are currently available. Although some RP subcomplexes containing both lid and base subunits have been reported [33, 34], evidence from yeast and, to a lesser extent, mammals suggests that the lid and base can form independently of one another [29, 35]. Using predicted homology to the archaeal PAN complex, we recently demonstrated by engineered disulfide crosslinking that the six ATPase subunits, Rpt1–Rpt6, form a uniquely ordered heterohexameric ring with the order Rpt1–Rpt2–Rpt6–Rpt3–Rpt4–Rpt5 within mature proteasomes [7]. Since no other arrangements of the ATPase ring appear to occur under normal growth conditions, base assembly requires the precise placement of each Rpt subunit into its position in the ring. It also requires the incorporation of three non-ATPase subunits, Rpn1, 2, and 13, whose precise positions in the RP are unknown. Efficient formation of the base depends upon four dedicated assembly chaperones, Rpn14, Nas6, Nas2, and Hsm3 (human PAAF1, p28/gankyrin, p27, and S5b, respectively) [28–32, 36]. These four conserved *R*P *a*ssembly *c*haperones (RACs) are not usually found associated with the mature 26S proteasome, consistent with roles as assembly chaperones.

Several subcomplexes of the base, assumed to be assembly intermediates, have been isolated from yeast and/or produced by in vitro cotranslation in mammalian lysates (Fig. 1b) [28–32]. Notably, each of these contains two of the six Rpt subunits, at least one RAC, and in some cases also an Rpn subunit. This suggests that one of the earliest steps in base assembly may be specific pairing of ATPases to form heterodimeric complexes containing Rpt1 and 2, Rpt3 and 6, or Rpt4 and 5. Current models of base assembly posit that following ATPase dimerization, the dimers associate with one another in an ordered fashion (Fig. 1b). In yeast, an apparent assembly intermediate containing Rpt5–Rpt4–Rpt3–Rpt6 and their associated chaperones (Nas2 with Rpt4–5 and Nas6/Rpn14 with Rpt3–6) as well as the non-ATPase subunits Rpn2 and Rpn13 (Fig. 2) have been identified [7]. This suggests that the Nas2 and Nas6/Rpn14 modules join together before the Hsm3 module, at least in yeast. In mammalian cells, there is evidence for RP species that specifically lack Rpt4–Rpt5–p27 [29], so it is possible that the order in which these ATPase modules associate is different from yeast or

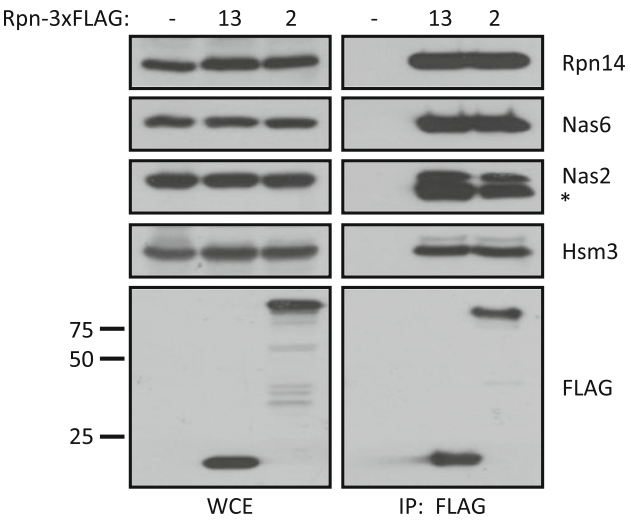

Fig. 2 Antibodies to FLAG-tagged Rpn2 or Rpn13 efficiently co-precipitate Nas2. Strains expressing the indicated triply FLAG-tagged proteasome subunits from their normal chromosomal loci were immunoprecipitated with anti-FLAG agarose, and copurifying proteins were eluted from the resin with 200 μg/ml 3× FLAG peptide. The eluates were then probed for the presence of the indicated RACs by Western blotting. The presence of Nas2 in the eluates of both Rpn2 and Rpn13 precipitates indicates that both of these subunits coexist in assembly intermediates containing Nas2. *Asterisk*, a cleavage product of Nas2 formed during immunoprecipitation

that multiple pathways lead to full ATPase ring formation. Mammalian Rpt4–Rpt5 may also be prone to dissociate at some stage later in assembly, yielding the observed complex lacking these ATPases.

The primary observation supporting late entry of the Hsm3 module (Rpn1–Rpt1–Rpt2–Hsm3) is that Nas2 and Hsm3 appear to exist in mutually exclusive assembly intermediates in yeast [7]. Specifically, antibodies to epitope-tagged Nas2 can co-immunoprecipitate all base subunits except those of the Hsm3 module; in contrast, Hsm3 is part of the fully assembled RP precursor, yet neither RAC can efficiently co-precipitate the other. Thus, it appears that Nas2 exits the assembling base shortly before, or immediately after, docking of the Hsm3-containing intermediate (Fig. 1b). How the ordered association of these proteins is carried out in vivo is still unknown, as are the precise roles of the individual RACs in RP assembly. It is important to emphasize that the data on which this model is based are static snapshots of RP subcomplexes that are sufficiently stable to isolate and characterize. Experiments exploring the kinetics of subunit association in vivo will be needed to verify or modify this provisional view.

Explaining Specificity in Ring Formation

Sequence identity among the six different proteasomal ATPases is very high, averaging ~40% in yeast, for

example [37]. Medium-resolution electron microscopy of the proteasomal RP together with our structure-based crosslinking studies suggests that the structures of these ATPases are highly similar not only to each other but also to the simpler homohexameric ATPases from prokaryotes for which crystallographic data are available [38–41]. These similarities in structure raise the question of how each RP ATPase finds its correct position in the heterohexameric ring. Several possible sources of positional specificity may exist.

An examination of the archaeal PAN activator complex, which contains six identical ATPase subunits and no orthologs to Rpn1, 2, or 13, suggests that the N-terminal regions of the Rpts could be a significant source of pairing specificity. The N-terminal portion of each PAN subunit (and, by homology, each Rpt) contains a domain that can form a coiled coil (CC), followed by an oligonucleotide/oligosaccharide-binding (OB) domain (Fig. 3a) [14, 16]. The remainder of the protein consists of a highly conserved AAA+ family ATPase domain, which includes a helical C-terminal domain (CTD). The CC and OB domains bury nearly 9100 $Å^2$ of total surface area within PAN, while the ATPase domains appear to make less stable contacts, probably because dynamic changes induced by ATP binding and hydrolysis at the active sites between subunits are integral to PAN function. Thus, it is likely that inter-subunit contacts formed by the CC and OB domains of the eukaryotic RP ATPases make a major contribution to the stable, subunit-specific placement of these subunits in the ATPase ring.

Notably, the six subunits of PAN form a trimer of dimers. The dimers are defined by the formation of CCs between two successive subunits within the ring [14, 16]. The three dimers contact one another via their OB and ATPase domains to form a hexameric ring. Shortly after the CC domain, the PAN subunit bears a pivotal proline residue (Fig. 3a) that can assume either a *cis* or *trans* configuration with the preceding residue in the polypeptide [14, 16]. Proline is unique among the twenty natural amino acids in its capacity to assume a *cis* peptide bond configuration. In the PAN crystal structure, each dimer within the hexamer contains a subunit in which its CC domain is followed by the proline in the *cis* configuration and a second in which the proline is in the *trans* configuration.

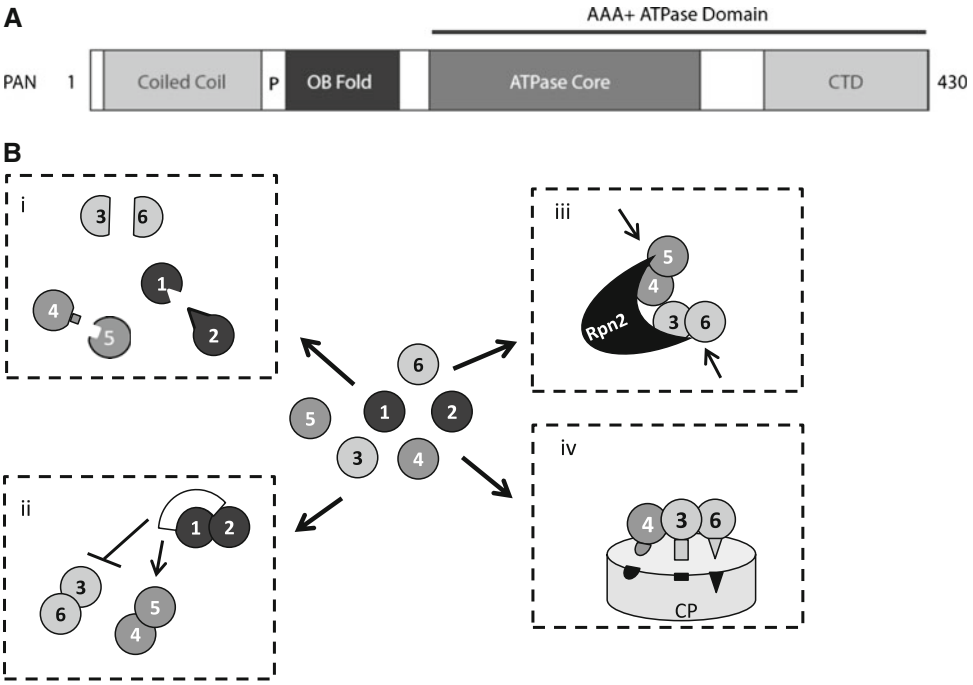

Fig. 3 Potential sources of specificity in eukaryotic proteasomal ATPase ring arrangement. **a** The domain structure of the *M. jannaschii* PAN subunit is shown. Based on high sequence homology of the eukaryotic ATPases to PAN, the Rpts are assumed to contain the same domains. The letter P between the coiled coil and OB fold domains indicates the position of a pivotal proline residue that can assume a *cis* or *trans* peptide bond with its preceding amino acid. *CTD*, C-terminal domain. **b** Potential sources of specificity in eukaryotic ATPase ring arrangement are shown. *i*, Protein sequences of each ATPase may encode complementary tertiary structures, promoting specific pairings between the Rpts. *ii*, The RACs may both positively and negatively influence interactions between ATPases. The strategic positioning of each RAC near the interface between Rpt subunits is consistent with a role in regulating heterodimer interactions within the assembling ATPase ring. *iii*, Other subunits within the base, such as Rpn2, may contain interaction sites with multiple base assembly intermediates and act as a scaffold to bring them together in the proper arrangement. *iv*, Analogous to (iii), the 20S CP may act as a template or scaffold to position the ATPase subunits appropriately within the forming ring

The alternating *cis* and *trans* proline configurations allow the N-terminal helices of each dimer to pair into a CC. The OB domains appear to make similar contacts with one another at all six interfaces in the ring, although the reported crystal structures differ somewhat in this regard [14, 16].

The critical proline between the CC and OB domains in PAN is conserved in a specific subset of the eukaryotic RP ATPases. Importantly, the ATPase heterodimers inferred from isolated base assembly intermediates each contain one subunit with a conserved proline and one without (or with a less conserved) proline at this position [28–30, 34]. This suggested that, as in PAN, these paired Rpt subunits would also assume *cis* and *trans* configurations at this site between CC and OB domains to allow CC formation and a trimer of dimers arrangement in the full ring. Our disulfide engineering experiments in yeast provided strong support for these predictions. In principle, stable pair-wise interaction of the RP ATPases will be greatly limited by the preservation of the pivotal proline in only half of the six ATPases, thereby preventing formation of the majority of inappropriate ATPase ring arrangements.

How the putative *cis* Rpt subunits select their appropriate *trans* partner awaits discovery. An examination of their sequences indicates that although the ATPase domains are highly conserved, the N-terminal portions containing the CC and OB domains are much more divergent. These differences might be sufficient to ensure the specificity in pairing of ATPases observed in purified base assembly intermediates (Fig. 3b, panel i). The large buried surface area predicted between ATPases within the dimers suggests that these pairings would be quite stable, and are unlikely to dissociate easily once formed. In support of this concept, the Rpt4–5 dimer (lacking its RAC, Nas2) can be readily detected in yeast cell extracts separated by native PAGE, indicating that while the specific pairing may not initiate exclusively between these two subunits, it can be stably maintained [28]. If more than one Rpt can pair with a given Rpt (appropriately or otherwise), a potential role for the RACs could be the discrimination of correct pairings of Rpt subunits or stabilization of interactions between the ATPase modules during assembly.

If the ATPase pairings are stringently encoded within the sequence of the interacting subunits, then it is likely the RACs perform a different function in base assembly (Fig. 3b, panel ii). Each of the four RACs binds to the CTD of a distinct ATPase [30, 32]. From modeling studies and a co-crystal structure of a complex between Nas6 and the Rpt3–CTD, it appears that the RAC–CTD interaction is on the outside face of the ring [32, 42]. These binding interactions are consistent with a role in modulating the interactions between ATPase domains rather than between CC/OB domains. With the subunit order within the ATPase ring now known, it has become clear that these chaperones are strategically positioned at each of the three junctions between ATPase dimers (Fig. 1b). Thus, it is possible that the assembly chaperones are positioned to coordinate association of the dimer modules to complete ring assembly. Although many groups have reported base subcomplexes that contain pairs of ATPases, no pairings aside from those shown in Fig. 1b have been identified. Disruption of RAC function, either individually or *en masse*, may lead to inappropriate pairings, and by extension, the inefficient formation of 26S proteasomes observed in RAC knockdown and knockout experiments.

The RACs may exert both positive and negative influences on ring formation. By virtue of their presence near the interfaces between ATPase dimers, they are positioned both to facilitate interaction between appropriate ATPase dimer modules and to impair interaction between inappropriate ones. For example, Nas2 appears to exit the assembling ATPase ring before or immediately after the Hsm3 module binds [7]. Thus, Nas2 may help dock this final module to the intermediate, and once the Hsm3 module has bound, Nas2 is no longer needed and exits. A similar function has been proposed by Kaneko et al. [29] for Nas6/gankyrin and Hsm3/S5b, in which they function to bring their respective modules together. In their model, Nas2 may serve as a negative regulatory role by assuring that the Nas6 and Hsm3 modules unite before interaction with Rpt4–5 (unlike what was proposed for yeast RP assembly).

An alternative means of assuring that the appropriate ATPase heterodimers come together might involve mediation by the Rpn1 and Rpn2 base subunits (Fig. 3b, panel iii). With molecular masses greater than 100 kDa, these subunits are the two largest in the proteasome. Based on electron microscopy, these subunits form elongated, curved structures from the α-solenoid-like repeats that make up a large portion of each protein [43]. If these subunits directly interact with multiple base assembly modules, they could act either to guide the arrangement of the ring or to stabilize the more favorable ATPase junctions before completion of base assembly. Rpn2 has been reported to interact with both Rpt4 [44] and complexes containing Rpt3 and Rpt6 [33]; simultaneous interaction between these subunits and Rpn2 might enhance formation of the two-module intermediate containing these subunits. We found Rpn2 in such an apparent assembly intermediate, making this role for Rpn2 plausible [7].

It has been suggested, first by our group and later by others, that the surface of the 20S CP onto which the RP docks may serve as a template for the assembly of the RP base (Fig. 3b, panel iv) [23, 31, 32, 34]. The C-termini of certain Rpts dock into pockets formed at the interfaces of α subunits in the CP α ring. In some cases, the interacting pockets and Rpt tails have been mapped to one another

[45]. This interaction is known to coordinate the activities of the RP with the proteolytic activity of the CP [18, 45]. Unexpectedly, disruption of CP assembly or structure results in the accumulation of free lid and subcomplexes of the base, suggesting that base formation also depends upon an intact CP [23]. Moreover, deletion of the extreme C-terminal amino acid of certain Rpt subunits causes structural defects in the 26S proteasome and accumulation of what is likely to be the Hsm3 module [31]. These results together suggest that the pockets in the surface of the CP α ring may help select the appropriate Rpt C-terminal tails and thus guide ATPase ring assembly. Experiments in mammalian cells also support a templating function for the CP in RP assembly [34].

Conclusions and Future Directions

In the past few years, we have seen a major increase in our understanding of the assembly, arrangement, and function of the ATPase ring complexes of both prokaryotic and eukaryotic proteasomes. The atomic structures of the PAN and ARC complexes will undoubtedly continue to serve as guides in studies of the eukaryotic proteasomal base. These structures in combination with the definitive establishment of the arrangement of the different ATPases within the RP base should allow for a much deeper understanding of the exact processes that regulate the assembly and function of this complex structure in eukaryotes, and can highlight basic mechanisms regulating the assembly of other heteromeric ATPase rings. Regarding the proteasome, it should now be possible to explore potential cooperativity between specific ATPases during protein degradation and to determine the register of the RP with respect to the CP, which is currently uncertain. Addressing these questions will also further our understanding of how the RP and CP communicate with one another during proteolysis. Finally, the question of exactly how the RP ATPase ring is assembled with the help of the four RACs should become accessible to experimental dissection.

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