Molecular Architecture and Assembly of the Eukaryotic Proteasome

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Annu. Rev. Biochem. 2013. 82:415–45

First published online as a Review in Advance on March 13, 2013

The *Annual Review of Biochemistry* is online at biochem.annualreviews.org

This article's doi: 10.1146/annurev-biochem-060410-150257

Keywords

proteasome, ubiquitin, assembly, ATPase, deubiquitylation, proteolysis

Abstract

The eukaryotic ubiquitin-proteasome system is responsible for most aspects of regulatory and quality-control protein degradation in cells. Its substrates, which are usually modified by polymers of ubiquitin, are ultimately degraded by the 26S proteasome. This 2.6-MDa protein complex is separated into a barrel-shaped proteolytic 20S core particle (CP) of 28 subunits capped on one or both ends by a 19S regulatory particle (RP) comprising at least 19 subunits. The RP coordinates substrate recognition, removal of substrate polyubiquitin chains, and substrate unfolding and translocation into the CP for degradation. Although many atomic structures of the CP have been determined, the RP has resisted high-resolution analysis. Recently, however, a combination of cryo-electron microscopy, biochemical analysis, and crystal structure determination of several RP subunits has yielded a near-atomic-resolution view of much of the complex. Major new insights into chaperone-assisted proteasome assembly have also recently emerged. Here we review these novel findings.

Contents

INTRODUCTION

Ubiquitin (Ub): a 76-residue protein that is covalently conjugated to substrates, often in the form of polymers (polyUb)

26S proteasome: an ATP-dependent intracellular protease composed of a 20S proteolytic CP and 19S RP

The ubiquitin-proteasome system (UPS) is responsible for the majority of regulatory and quality-control protein degradation in eukaryotic cells. Nearly every cellular process is affected by the UPS (1, 2). The UPS utilizes ATP hydrolysis at several steps to mediate the selective destruction of its substrates. Degradation of a protein by the UPS is typically mediated by the energy-dependent covalent attachment of the small protein ubiquitin (Ub) to one or more lysines within the target protein via the concerted action of three enzymes: E1, E2, and E3 (**Figure 1*a***). Additional Ubs can also be ligated to the initial Ub via one of its seven lysines (or its N terminus), forming polyubiquitin (polyUb) chains. A chain of four or more Ubs is generally necessary and sufficient to create a targeting signal for delivery to the proteasome (3). The proteasome is responsible for recognizing the substrate, removing its polyUb tag, unfolding the substrate, and cleaving it into short peptides.

In this review, we focus on the structure and assembly of the proteasome, particularly the regulatory particle (RP), as numerous breakthroughs have occurred in these areas over the past several years. For recent, more general reviews of the UPS, we refer the reader to References 1 and 2 and references therein.

THE 26S PROTEASOME: ANATOMY OF A PROTEIN-DESTROYING MACHINE

The 26S proteasome is the largest and most complex member of an ancient superfamily of ATP-dependent chambered proteases found in all domains of life (4, 5). These proteases are characterized by an ATPases associated with various cellular activities (AAA)+-family ATPase ring responsible for unfolding substrates and threading them through a narrow central pore into an interior proteolytic chamber, which is usually formed by an associated multimeric protein complex (**Figure 1*b***). Sequestration of the proteolytic active sites within such a chamber prevents the unregulated destruction of folded cellular proteins. The ATPase ring converts the chemical energy of ATP hydrolysis into mechanical force for substrate unfolding, allowing a polypeptide end or interior loop to be translocated into the central chamber (**Figure 1*b***). In the eukaryotic 26S proteasome (**Figure 2*a***), the proteolytic chamber is referred to as the 20S core particle (CP), whereas the AAA+ ATPase ring is contained within a separable complex called the RP. In addition to the ATPase ring, the RP contains many additional specialized subunits

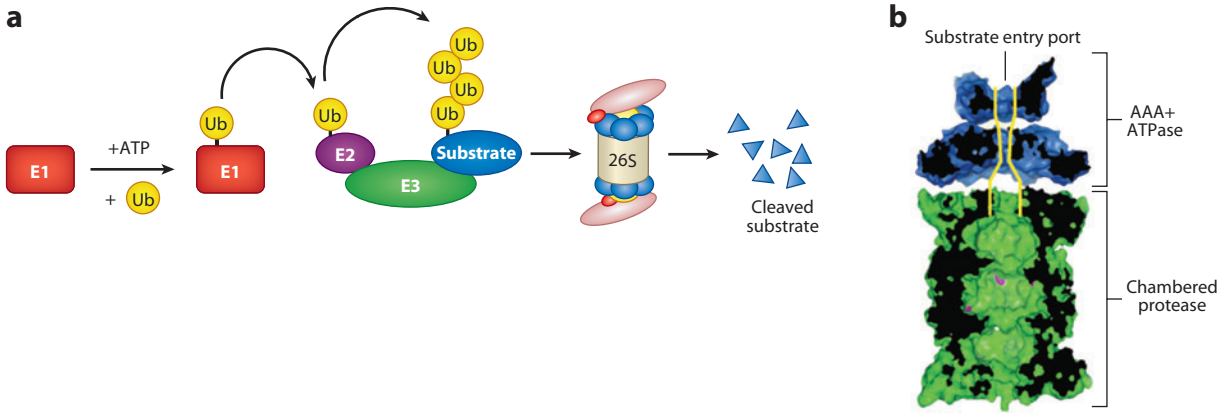

Figure 1

Schematics of the ubiquitin (Ub)-proteasome system and an AAA+ chambered protease. (*a*) Through the sequential actions of E1, E2, and E3 enzymes, a protein to be degraded is modified with a polyUb chain, which serves as a targeting signal for the proteasome. (*b*) A cutaway view of an AAA+ chambered protease that displays the path of substrates through the ATPase ring and into the proteolytic chamber. The width of the passage into the protease chamber is delineated by yellow lines, and the proteolytic active sites are shown as purple dots. In this protease, the catalytic chamber is bracketed by two antechambers. Panel *b* adapted with permission from Nature Publishing Group (142), copyright © 2012.

including ones that function as receptors for the polyUb tag and others that cleave the tag from substrates prior to their degradation.

The 26S proteasome is highly conserved throughout the Eukarya, where it is found in both the nucleus and cytoplasm. Simpler forms of the proteasome have been identified in archaea as well as in actinobacteria, although Ub has been found only in eukaryotes (6, 7). Several other protein complexes can bind the ends of the CP cylinder, but their precise contributions to the regulation of the CP remain less clear (**Table 1**). Because of space constraints, only RP-CP complexes, i.e., the 26S proteasome, are discussed in this review.

The 20S Core Particle

The twofold-symmetric CP comprises 28 related polypeptides encoded by 14 separate genes. Atomic structures of the CP from yeast and mammals have been solved (8–12), and their overall structures and subunit arrangements are virtually identical, despite millions of years of evolutionary divergence between these species. The CP consists of four axially stacked heteroheptameric rings (**Figure 2*b***). The inner rings contain seven distinct β-subunits (β1–β7), whereas the outer rings consist of seven different α-subunits (α1-α7). The β1-, β2-, and β5-subunits contain the proteolytic active sites, and each site cleaves preferentially after particular amino acid residues (13). In mammals, four additional β-subunits have been discovered: β1i, β2i, β5i, and β5t, where "i" and "t" stand for immuno- and thymo-, respectively (14, 15). These subunits are highly expressed in certain immune system tissues or are induced by particular stimuli, such as interferon-γ exposure, and they replace the canonical active site–bearing β-subunits within the CP, altering CP proteolytic specificity. The β1i-, β2i-, β5i-substituted CP, called the immunoproteasome, generates substrate cleavage patterns that enhance loading of peptides onto the class I major histocompatibility complex for immune presentation to killer T cells (16). Thymoproteasomes, which contain β1i, β2i, and β5t, appear to increase the repertoire of self peptides for positive selection during T cell development in the thymus (15).

In the crystal structures of the isolated CP, the entrances into the internal proteolytic chamber are usually occluded by the

ATPases associated with various cellular activities (AAA)+: a family of ATPases forming oligomeric rings that unfold or remodel substrates

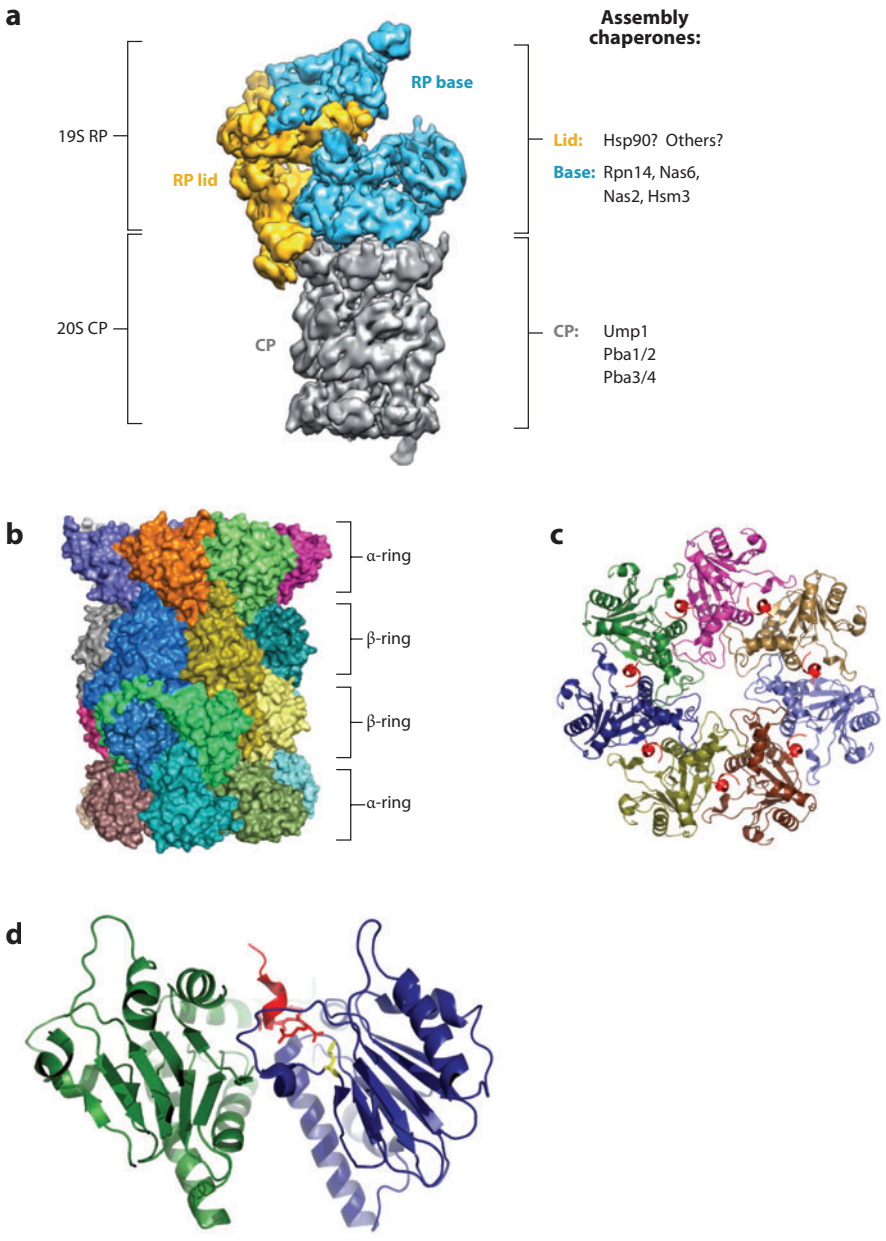

Figure 2

Structure of the 26S proteasome. (*a*) The cryoelectron microscopy density of the 26S proteasome is shown. The 19S regulatory particle (RP) lid subcomplex is displayed in yellow, the RP base subcomplex in blue, and the 20S core particle (CP) in gray. Known and putative assembly chaperones for each subcomplex are displayed to the right. Figure adapted with permission from Nature Publishing Group (46), copyright © 2011. (*b*) Space-filling model of the 20S CP atomic structure from yeast [Protein Data Bank (PDB) ID: 1RYP]. (*c*) A view into the axial pore of the 20S CP from *Thermoplasma acidophilum*. In this model (PDB ID: 3IPM), the archaeal ATPase HbYX motif (*red ribbons*) is inserted into the pockets formed at the interfaces of two adjacent α-subunits, which helps open the CP gate. (*d*) The HbYX motif binds a critical lysine residue (*yellow*) in the α-pocket.

Table 1 20S CP Subunits and Associated Proteins

Subparticle	Standardized name	Yeast gene name(s)	Human standardized name	Function
α-ring	α1	*SCL1*	PSMA6	
	α2	*PRE8*	PSMA2	
	α3	*PRE9*	PSMA4	
	α4	*PRE6*	PSMA7	
	α5	*DOA5; PUP2*	PSMA5	
	α6	*PRE5*	PSMA1	
	α7	*PRE10*	PSMA3	
β-ring	β1	*PRE3*	PSMB6	Postacidic protease activity
	β2	*PUP1*	PSMB7	Trypsin-like protease activity
	β3	*PUP3*	PSMB3	
	β4	*PRE1*	PSMB2	
	β5	*PRE2*	PSMB5	Chymotrypsin-like protease activity
	β6	*PRE7*	PSMB1	
	β7	*PRE4*	PSMB4	
20S-associated proteins	Pba1	*PBA1; POC1*	PAC1	α-ring chaperone
	Pba2	*PBA2; POC2; ADD66*	PAC2	α-ring chaperone
	Pba3	*PBA3; POC3; DMP2; IRC25*	PAC3	α-ring chaperone
	Pba4	*PBA4; POC4; DMP1*	PAC4	α-ring chaperone
	Ump1	*UMP1; RNS2*	POMP; hUMP1	β-ring chaperone
	Blm10	*BLM10*	PA200	CP regulator

N-terminal tails of specific α-subunits (8, 9). These tails form an ordered gate that must be opened for substrate entry. Thus, free CP, which is detectable in cell extracts, usually has low activity by itself, at least against folded proteins (17). Atomic force microscopy analysis indicates that the gate exists in a dynamic equilibrium between open and closed states that is biased toward the latter (18). Many proteasomal activators, such as the RP, and proteins known to bind the α-ring surface contain a conserved HbYX (hydrophobic, tyrosine, any amino acid) motif at their very C terminus (19–22). The HbYX motif inserts into a pocket formed at the interface of adjacent α-subunits (**Figure 2*c***), where the C-terminal carboxylate of the activator subunit forms a salt bridge with the ε-amine of a conserved lysine residue in the pocket (**Figure 2*d***) (23, 24). These interactions help to maintain contact with the CP, and in vitro studies of CP activation by synthetic HbYX peptides suggest that their insertion into the α-ring pockets induces conformational changes in the α-ring that displace (at least in part) the α-ring N-terminal tails from the pore of the CP, allowing substrate entry (25).

The 19S Regulatory Particle

The RP is responsible for the binding, deubiquitylation, unfolding, and translocation of substrates into the CP as well as the opening of the CP α-ring gate. As such, the RP functions as a highly regulated gatekeeper for the majority of proteasome substrates. It contains at least 19 subunits, each usually present in a single copy. The RP can split under certain in vitro conditions into two subcomplexes, termed the lid and base (**Figure 2*a***). The base consists of nine subunits: six RP triphosphatases, Rpt1–6, and three RP non-ATPases, Rpn1, 2, and 13. Rpt1–6 are paralogous AAA+

HbYX motif: a hydrophobic residue–tyrosine–any amino acid tripeptide motif commonly found at the C terminus of proteins that bind the CP α-ring surface

Proteasome/cyclosome (PC) repeats: α-helical repeats found in proteasome subunits Rpn1 and Rpn2 and in the APC/cyclosome that fold into a toroid or superhelix

Ubiquitin-associated (UBA) domain: a conserved domain found in many Ub-binding proteins that directly recognizes ubiquitin

ATPases that form a heterohexameric ring (26). The ATPase ring directly contacts the surface of the CP α-ring and exerts upon substrates the ATP-dependent unfolding force that is required for their translocation into the CP for degradation. Rpn1 and Rpn2 are the two largest subunits of the proteasome. The central portions of these subunits are composed of 11 α-helical proteasome/cyclosome (PC) repeats (27), which are thought to form scaffolds onto which substrates and other factors dock (**Figure 3**). Rpn13, as well as the Rpn10 protein, directly binds Ub, and both proteins function as polyUb-substrate receptors (28–32). In addition to Rpn10 and Rpn13, several extrinsic Ub receptors have been identified, including the UBA-UBL proteins Rad23, Dsk2, and Ddi1 (33–36). The Ub-associated (UBA) domains of these proteins bind the polyUb chains on substrates and shuttle them to the proteasome through interactions of their Ub-like (UBL) domains with the Rpn1 subunit (37, 38).

The lid consists of nine different Rpn subunits, Rpn3, 5–9, 11, 12, and Rpn15 (Sem1). The Rpn11 subunit is a deubiquitylating

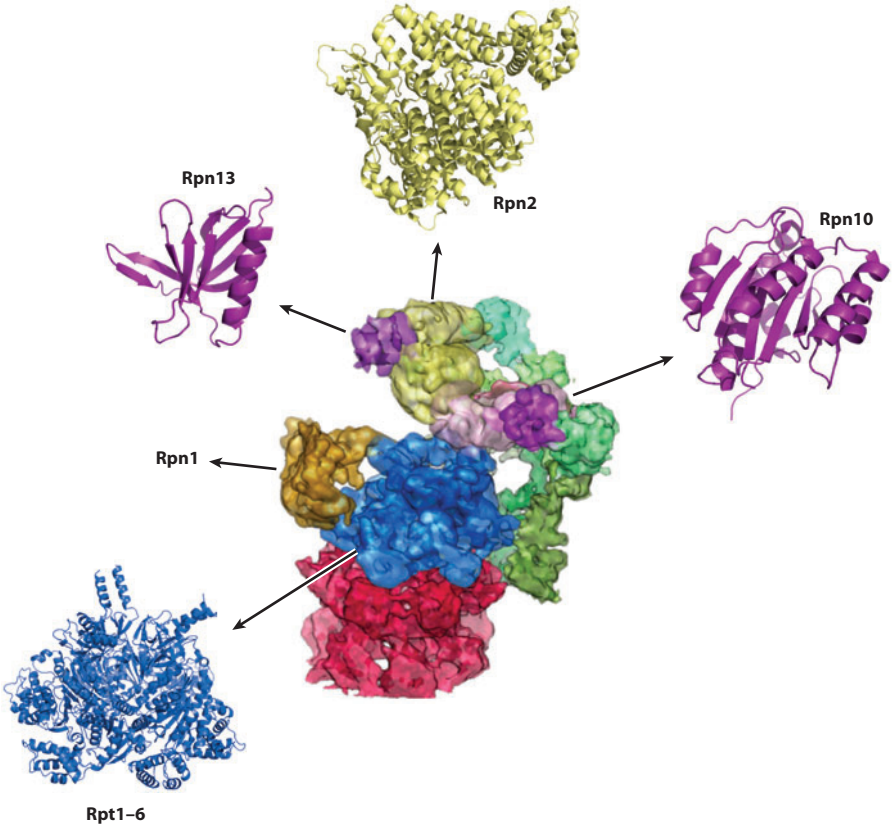

Figure 3

Organization of the regulatory particle, including atomic and pseudoatomic models of base subunits. A bird's-eye view of the 26S proteasome electron microscopy (EM) density is shown. Where available, the atomic or pseudoatomic models of Rpt1–6 [modeled using Protein Data Bank (PDB) ID: 3H4M, 3H43], Rpn2 (PDB ID: 4ADY), Rpn10 von Willebrand domain (PDB ID: 2X5N), and Rpn13 pleckstrin-like receptor for ubiquitin domain (PDB ID: 2R2Y) are shown and colored according to their respective EM densities. Figure adapted with permission from *Proceedings of the National Academy of Science of the U.S.A.* (47), copyright © 2012.

enzyme (DUB) (39, 40). The lid is structurally related to the COP9 signalosome (CSN) and the eIF3 translation initiation complexes (41, 42). Although the compositions of the CSN and eIF3 vary somewhat across the Eukarya (43, 44), the canonical form of each of these complexes contains six subunits with proteasome/CSN/initiation complex (PCI) domains and two subunits with Mpr1/Pad1/N-terminal (MPN) domains. The composition of the lid appears invariant in eukaryotes, consisting of the canonical six PCI proteins (Rpn3, 5, 6, 7, 9, and 12) and two MPN subunits (Rpn8 and Rpn11) as well as Sem1, a small acidic protein with neither PCI nor MPN domains.

ARCHITECTURE OF THE 19S REGULATORY PARTICLE

An unprecedented view of RP structure and subunit architecture has recently emerged. Although the overall shape of the RP had been observed long ago by electron microscopy (EM) (45), the positions of its subunits and their high-resolution structures were unknown. Over the past three years, a series of high-resolution cryo-EM-based reconstructions, along with biochemical experiments and crystal-structure determinations for several isolated subunits, has yielded a much clearer picture of RP architecture (**Figure 3**).

Surprisingly, the RP lid and base terminology (41) turns out to be somewhat misleading. The base includes subunits positioned farther from the CP than any lid subunit (46–48). Rather than the lid capping the base, it instead straddles the long edge of the RP, contacting both the base and the CP α-ring (**Figure 2*a***). Nevertheless, to avoid confusion, we continue to use the lid and base terms here. We describe the new proteasome structural data in the following sections.

The Regulatory Particle Base

As noted earlier, the RP base includes a heterohexamer of AAA+ ATPases and three non-ATPase subunits. The new structural data have revealed important details about the three-dimensional structures and interactions of many of these subunits. The data also show unexpected features regarding how the base interacts with the CP and RP lid.

The Rpt ATPases. As expected from structures of other AAA+ chambered proteases (5), the Rpt ATPase ring directly abuts the ends of the CP cylinder. However, the new EM models unexpectedly indicate that the pore of the ATPase ring does not align perfectly with the pore of the CP, as it is axially offset by as much as 30 Å and tilted on its vertical axis by approximately 5–10° (49, 50). The functional significance of this remains unknown. The six ATPases adopt a single arrangement in normal cells: Rpt1-Rpt2-Rpt6-Rpt3-Rpt4-Rpt5 (26). Although this arrangement was originally predicted on the basis of sequence divergence among the proteasomal ATPases (51) and later by molecular modeling approaches (52), several alternative arrangements had been proposed on the basis of yeast two-hybrid (53), chemical cross-linking (54), and other (55) studies. Further, whether the ATPases normally adopt multiple arrangements had been unclear. The Rpt1-2-6-3-4-5 arrangement was unambiguously established only recently using engineered disulfide cross-linking (26); this analysis was guided by the structure of an archaeal homohexameric ATPase ring, proteasome-activating nucleotidase (PAN) (56, 57). The success of this approach implies a close evolutionary conservation of the core ATPase ring architecture.

The Rpts, PAN, and the actinobacterial ortholog ATPase forming ring-shaped complexes (ARC) share a similar domain organization (**Figure 4*a***) (56, 57). Each contains an N-terminal region that includes a coiled-coil domain (CC), an oligonucleotide/oligosaccharide-binding (OB) domain, the AAA+ ATPase domain, and a C-terminal α-helical domain that sits on the outer surface of the ATPase ring. In AAA+ ATPases, the protein sequence N-terminal to the ATPase domain is typically specialized for its particular

Ubiquitin-like (UBL) domain: a protein domain that adopts a β-grasp fold similar to ubiquitin; this domain often mediates interaction with Ub-binding proteins

Deubiquitylating enzyme (DUB): a specialized protease that cleaves Ub from substrates such as other proteins and Ub precursors

Proteasome/COP9 signalosome/initiation complex (PCI) domain: a conserved domain with an N-terminal α-helical region followed by a winged-helix fold that is found in six RP lid subunits

Mpr1/Pad1/N-terminal (MPN) domain: a conserved fold found in subunits of the RP lid and COP9 signalosome; the MPN+/JAMM variant has metalloprotease activity

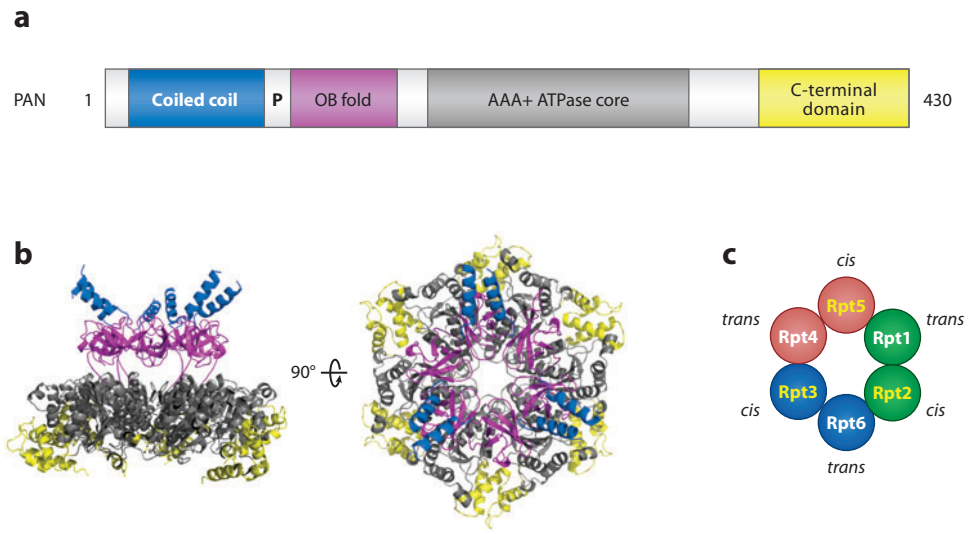

Figure 4

Proteasomal AAA+ ATPase structure and hexameric ring organization. (*a*) The domain architecture of the *Methanocaldococcus jannaschii* proteasome-activating nucleotidase (PAN) is shown. The eukaryotic ATPases Rpt1–6 share a similar domain organization as PAN. (*b*) A pseudoatomic model of the proteasomal ATPase ring (modeled as in **Figure 3**) is shown. Domains are colored as in panel *a*. (*c*) Arrangement of the eukaryotic ATPases in the heterohexameric ring. The inferred proline *cis/trans* isomerism of each Rpt subunit is listed. Subunits forming pairs in the trimer of dimers model are similarly colored. Abbreviations: OB, oligonucleotide/oligosaccharide-binding; P, proline.

function, and the combination of N-terminal CC and OB domains appears unique to the proteasomal ATPases. In PAN and ARC, there is a critical and highly conserved proline residue at the junction between the N-terminal CC region and the OB domain (**Figure 4*a***) (56, 57). Proline is unusual in that the peptide bond it forms with the preceding residue can adopt either a *cis* or *trans* configuration. In the crystal structures of PAN N domains, this peptide bond alternates between *cis* and *trans* conformations in adjacent subunits, allowing their N-terminal helices to form pairwise CCs. The ATPase subunits in the PAN crystal structure thus form a trimer of dimers (**Figure 4*b***) (56, 57). This *cis-trans* pairing of ATPases appears to be conserved in the eukaryotic ATPase ring: Rpt2, Rpt3, and Rpt5 contain highly conserved prolines that align with the critical PAN proline (26, 56, 57) and take up alternating places within the ATPase ring (**Figure 4*c***). In agreement, many isolated base subcomplexes, which are thought to be assembly intermediates, contain pairs of Rpt subunits, and in each case they are a predicted *cis-trans* pair.

Rpn1 and Rpn2. Until recently, the structures and positions of Rpn1 and Rpn2 within the RP remained unclear and have been subject to extensive debate. Most investigators expected the PC repeats of these proteins to take on toroidal or horseshoe-like superfolds (58, 59); some further proposed that these subunits were nestled inside the pore of the ATPase ring (or perhaps stacked on top) (58, 60). However, the strong structural similarity of the eukaryotic ATPase ring compared with PAN argued against this latter possibility because the central pore of PAN is extremely narrow and unable to accommodate a large folded domain even at its widest point (26, 52, 56, 57).

He et al. (27) recently determined the crystal structure of yeast Rpn2. It resembles a tobacco pipe (**Figure 5*a***), with a central region of 11 PC repeats that make up the pipe's barrel that is bracketed by an extended N-terminal α-helical domain comprising the stem and a small C-terminal globular domain that packs against the barrel. Although the PC repeats of Rpn2 form a toroid as predicted, their detailed arrangement was quite unexpected: They adopt a tightly packed shape in which the pore formed by the PC repeats is plugged by two α-helices extending into the toroid (**Figure 5*b***). This packing explains the highly hydrophobic character of these two central helices, as they are largely sequestered from solvent. Thus, the toroidal PC repeat domain of Rpn2 is very densely packed and is likely quite rigid. The molecular model of Rpn2 is readily docked into the RP EM density owing to its large size and distinctive shape (**Figure 3**). Rpn2 is one of the most distally positioned subunits along the proteasomal long axis. Both its N-terminal stem-like extension and part of the toroidal domain interact with the N termini of Rpt3 and Rpt6, whereas the center of the toroid and the C-terminal domain (CTD) are positioned over the pore of the ATPase ring (48).

Comparison of purified Rpn2 and Rpn1 by EM indicates that these two proteins adopt very similar folds, consistent with their ∼20% sequence identity (27, 58). In the 26S proteasome cryo-EM structures, Rpn1 also harbors a toroidal domain, presumably encoded by its PC repeats. Notably, all known docking sites of the extrinsic Ub receptors lie within this toroid (37, 38). Thus, the Rpn1 (and perhaps Rpn2) toroids likely serve as loading platforms for incoming substrates or other proteins. In contrast to Rpn2, Rpn1 makes extensive contacts with the outside face of the ATPase ring in the 26S holoenzyme EM structures (**Figure 3**) (46–48), and its density is somewhat variable, suggesting it may undergo movement. The close association of Rpn1 with the ATPase ring may help to control substrate docking by coupling conformational changes in Rpn1 with the binding and/or hydrolysis of ATP by the ATPases.

Rpn10, Rpn13, and the extrinsic shuttle factors. Two general pathways exist for the delivery of ubiquitylated substrates to the proteasome: Ub recognition by the intrinsic receptors Rpn10 and Rpn13 or shuttling to the proteasome via the extrinsic receptors, which include Rad23, Dsk2, and Ddi1 (**Figure 5*c***). Whereas the cellular protein levels of the extrinsic and intrinsic receptors are very similar, at least in yeast (61), the extrinsic receptors are generally substoichiometric on purified proteasomes. In contrast, the occupancy of the intrinsic receptors Rpn10 and Rpn13 is typically greater than 50% (62). As the extrinsic receptors may bind the proteasome transiently, this low apparent occupancy could potentially reflect their steady-state binding levels in vivo. Rpn13 interacts with a short peptide segment in the CTD of Rpn2, positioning it far from the CP (**Figure 3**) (27, 63). Rpn13 adopts a pleckstrin homology domain–like fold (**Figure 3**) named the pleckstrin-like receptor for Ub (PRU) (28). Rpn13 binds Ub chains with high affinity (∼90 nM K_D for Lys48-linked diUb) via loops extending from the β-strands comprising the PRU domain (**Figure 5*d***). Like Rpn13, Rpn10 directly binds Ub, but in this case via its α-helical Ub-interacting motif (UIM) (**Figure 5*d***). Rpn10 contains one to three UIMs at its C terminus depending on the species, but even Rpn10 orthologs with a single UIM prefer polyUb chains over monoUb (64, 65). Solution-structure studies indicate that the UIM region of Rpn10 is highly flexible (64), and this domain appears as a diffuse, poorly resolved density in EM structures of the 26S proteasome (62). In contrast, the N-terminal region of Rpn10 consists of a tightly folded von Willebrand factor A (vWA) domain that is readily resolved in these structures (**Figure 3**) (62, 66). Despite findings that loss of Rpn10 or disruption of its vWA domain destabilizes lid-base association within the RP (41, 53, 67), the vWA domain makes extensive contact only with subunits in the lid (46–48), suggesting it functions indirectly to promote or maintain RP stability (see Lid-base association and Rpn10 incorporation, below).

Pleckstrin-like receptor for ubiquitin (PRU) domain: domain found in the proteasome Ub receptor Rpn13; in many species, Rpn13 has a domain that binds the UCH37 DUB

Ubiquitin-interacting motif (UIM): a hydrophobic, α-helical motif that binds Ub and was originally identified in the proteasome Ub receptor Rpn10

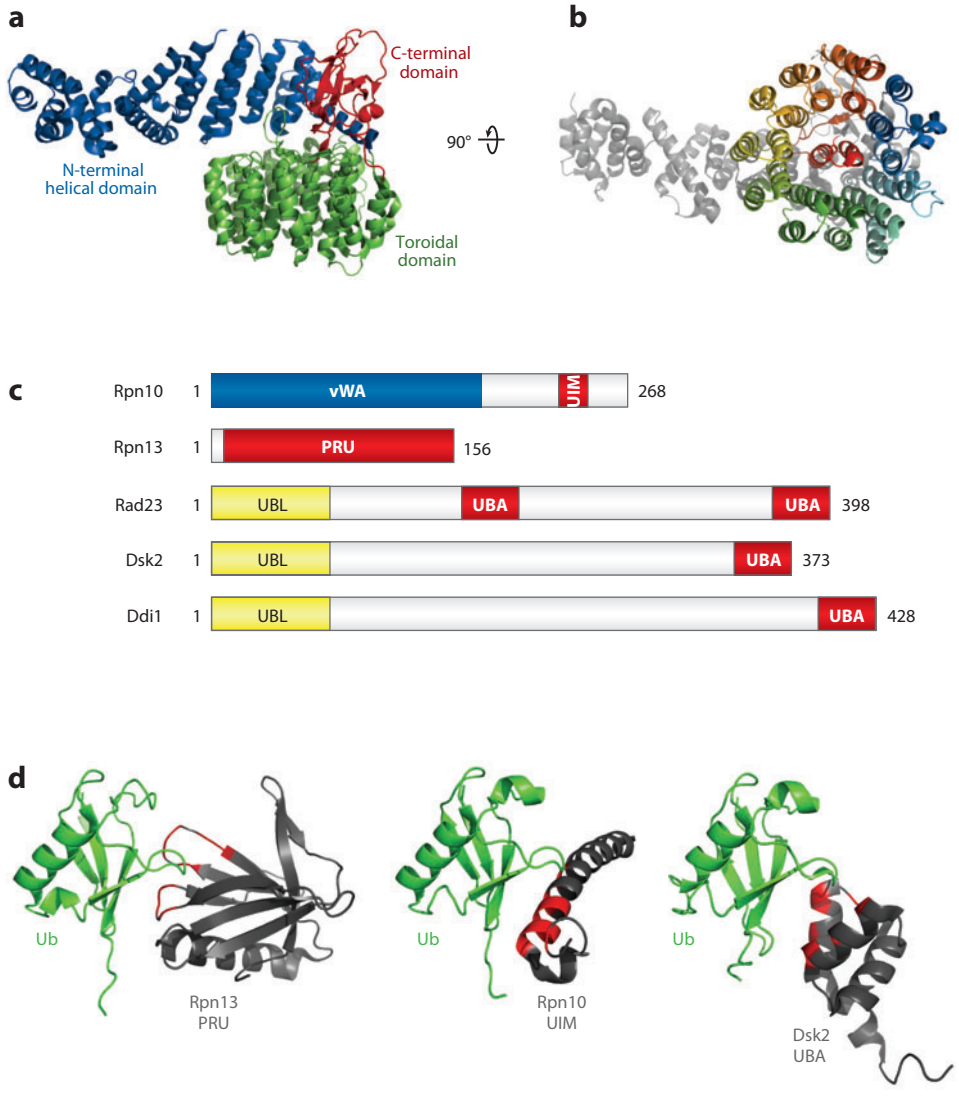

Figure 5

The architecture of the 19S base scaffold subunit Rpn2 and ubiquitin (Ub) receptor subunits. (*a*) Ribbon structure of Rpn2 from *Saccharomyces cerevisiae* [Protein Data Bank (PDB) ID: 4ADY]. The N-terminal helical domain is colored blue, the toroidal domain green, and the C-terminal domain red. (*b*) An axial view of the Rpn2 toroid illustrating the central α-helices. Proteasome/cyclosome repeats are colored blue (N-terminal) through orange (C-terminal) with the central helices in red. (*c*) Domain organization of the intrinsic Ub receptors Rpn10 and Rpn13 and the extrinsic receptors Rad23, Dsk2, and Ddi1. Domains and amino acid numbering are according to the *S. cerevisiae* gene products. (*d*) Distinct modes of interaction with Ub are utilized by each Ub receptor. Amino acids of each Ub-binding domain (*gray*) that contact Ub (*green*) are highlighted in red (PDB ID: 2Z59, 2KDE, and 1WR1 for PRU, UIM, and UBA, respectively). Abbreviations: PRU, Pleckstrin-like receptor for ubiquitin domain; UBA, ubiquitin-associated domain; UBL, ubiquitin-like domain; UIM, ubiquitin-interacting motif; vWA, von Willebrand factor A domain.

The Regulatory Particle Lid

EM structures are now available for the RP lid as an isolated complex (46) and in the context of the 26S holoenzyme (46–48). The lid appears to act, in part, as a clamp to keep the RP and CP together (68). The lid looks similar to a horseshoe, with the arc formed by the PCI domains of (from end to end) Rpn9, 5, 6, 7, 3, and 12 (**Figure 6**). The N-terminal helical extensions of each PCI subunit extend radially away from the horseshoe, much like fingers on a hand. Within the 26S proteasome, the Rpn5 and Rpn6 N termini extend toward the CP and make extensive contact with the outer flanks of both the RP base and the CP (46, 68), suggesting the potential for coupling between the enzymatic activities of the lid and the rest of the proteasome. In the center of the horseshoe are the two MPN subunits, Rpn8 and Rpn11, which sit side by side to form a heterodimer positioned over the ATPase ring in the 26S holoenzyme (46, 69). Each MPN subunit makes extensive contact with several PCI subunits. The MPN subunit N termini appear to be flexible or disordered in the EM density of the isolated lid but become more ordered in the 26S proteasome (46), suggesting that their positioning is constrained upon their incorporation into the RP. Similarly, large movements in Rpn5 and Rpn3 likely occur as the lid is integrated into the RP (or 26S); these movements may be important for accommodation of base subunits upon RP assembly (46). Although the exact position of Sem1 in the lid has not been determined, it has been found in a complex with Rpn3 and Rpn7 (67), indicating it directly binds one or both of these subunits.

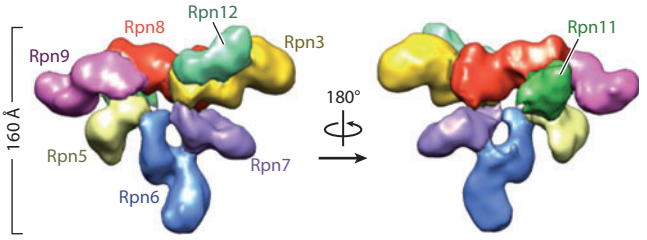

Figure 6

Molecular architecture of the proteasome lid. The electron microscopy structure of the purified lid complex and the densities contributed by each subunit are shown. Figure adapted with permission from Nature Publishing Group (46), copyright © 2012.

STRUCTURAL INSIGHTS INTO SUBSTRATE SELECTION AND DEGRADATION

Substrate Recognition and Positioning for Degradation

With a nearly complete three-dimensional model of the RP at subnanometer resolution, it now becomes apparent that the placement of subunits and proteasome-associated factors within the RP is likely organized in a way that facilitates movement of the substrate from the outer edges of the RP toward the CP for degradation. The subunits responsible for each successive step in substrate manipulation (i.e., recognition, deubiquitylation, unfolding, and translocation into the CP) are positioned sequentially closer to the CP pore. At the periphery of the RP are the Ub receptors Rpn10 and Rpn13. Both are located above the ATPase ring (below the ring is defined as the CP-binding surface) with an unobstructed potential substrate path toward the ATPase pore. Rpn13 is positioned above Rpt1 and Rpt2, whereas affinity labeling of the Rpn10 UIM domain indicates this region projects internally toward the Rpt4/Rpt5 CC (62). Given the placement of the Ub receptors, one might hypothesize that the CCs of specific pairs of Rpt subunits are responsible for binding substrates that dock at the proteasome via particular Ub receptors.

In contrast to Rpn10 and Rpn13, the positions and orientations of the shuttle factors upon binding the proteasome are unknown. Interpretation of how they dock onto the proteasome is complicated by their interaction with multiple subunits within the RP and the variability of Rpn1 density within EM images (37, 64, 65, 70). Although Rpn1 is considered the major binding site for Rad23, Dsk2, and Ddi1, the UBL domains of the human orthologs of Rad23, Dsk2, and Ddi1 can bind the UIM and PRU domains of hsRpn10 and

Shuttle factor: a polyUb-binding protein that also binds the proteasome RP and functions to deliver polyUb-modified substrates to the proteasome

hsRpn13 (29, 65). The physiological significance of such shuttle factor UBL binding to Rpn10 and Rpn13 and whether these receptors cooperate with Rpn1 to process substrates bound to the shuttle factors remain to be explored. Deletions of the shuttle factor genes display synthetic genetic defects when combined with *RPN10* or *RPN13* deletions (29), which argues against a model in which the shuttle factors function exclusively by handing off substrates to the intrinsic receptors.

Substrate Deubiquitylation

After a substrate binds the proteasome, its polyUb tag must be removed either prior to or potentially during substrate degradation. The proteasome harbors several DUBs: two less tightly associated DUBs [Ubp6 (Usp14 in humans) and Uch37 (not in all species)] and the Rpn11 subunit of the RP lid (**Table 2**) (39, 40). Additionally, several Ub ligases, most notably Hul5, are found associated with proteasomes, strongly suggesting that the polyUb chains of substrates can undergo remodeling on the proteasome, perhaps as a substrate triage or quality-control step (71). Whereas the nonintegral proteasomal DUBs trim polyUb chains from the distal end, Rpn11 instead removes whole chains at their point of attachment to the substrate. This observation and the severe growth phenotypes associated with attenuation of Rpn11 catalytic activity strongly suggest it is the primary DUB responsible for removal of polyUb chains immediately prior to or concomitant with the initiation of protein unfolding and degradation by the proteasome.

The catalytic metalloprotease domain of Rpn11 is related to the structurally determined MPN+/JAMM (JAB1/MPN/Mov34 metalloenzyme) domain of AMSH (associated molecule with SH3 domain of STAM), a functionally distinct DUB. Modeling of the atomic structure of the AMSH MPN+/JAMM domain into the EM density of Rpn11 in the 26S proteasome suggests that the Rpn11 active site is positioned 10–20 Å over the pore of the ATPase ring (46), and near-atomic-resolution EM recently confirmed this positioning (69). This proximity to the ATPase pore suggests a model in which substrates are unfolded until the polyUb chain–attachment site is pulled near the pore of the ATPase ring, bringing it into position for deubiquitylation by Rpn11. Conversely, positioning of the polyUb chain–attachment site and/or deubiquitylation may trigger the initiation of unfolding and threading through the ATPase ring. The first model is supported by the finding that unfolding generally requires an unstructured or structurally destabilized region for initiation (72). Furthermore, in the case of substrates with polyUb chains attached at multiple sites, Rpn11 may need to act multiple times on the substrate; in this case, the threading action of the ATPases on the substrate could pull successive polyUb attachment sites into the Rpn11 active site during unfolding and translocation.

Substrate Unfolding by the AAA+ ATPase Ring

Many inferences about the function and mechanism of the proteasomal ATPase ring have been drawn from the simpler homomeric ATPases from archaea and bacteria. Like the eukaryotic ATPases, PAN forms a hexameric ring that binds the archaeal CP in an ATP-dependent manner (73). Using PAN as a model, investigators deduced recently that the ATPase ring can bind a maximum of four nucleotides (ATP or ADP) at once, with the remaining two sites being empty owing to negative allostery (74). Based on this finding and information about the relative rates of ATP binding and hydrolysis, it appears that nucleotides tend to bind to the ATPase ring and be hydrolyzed in pairs by subunits *para* to one another in the six-membered ring.

Although PAN has been a powerful model for elucidating some of the basic features of the proteasomal ATPases, emerging structural, biochemical, and genetic data suggest that the eukaryotic ATPase ring diverges from PAN in several ways. Aside from the obvious diversification of eukaryotic Rpt sequences

compared with PAN, a recent EM analysis of the 26S holoenzyme suggests that the ATPases actually take up a corkscrew-like arrangement similar to that seen in some ATP-dependent helicases, in which the pore residues form a downward-spiraling staircase, whereas the N-terminal OB-fold ring and the small CTDs of the ATPases remain largely planar (46, 69). This observation raises the possibility that instead of, or in addition to, the mechanical forces from the ATPases being exerted through small, local movements of pore loops (as has been proposed for PAN) (75), substrate unfolding may be driven by movements of the entire large domains if these domains were able to proceed sequentially through the positions observed in the staircase arrangement (46). Biochemical and phenotypic assays comparing mutations at paralogous positions in each eukaryotic ATPase subunit have unveiled Rpt-specific defects in substrate unfolding, CP gate opening, and resistance to proteasome stresses (76, 77). We do not yet know whether these apparently specialized roles of the ATPases reflect a mechanism allowing processing of substrates with more diverse sequences and structures than those processed by the archaeal and bacterial ATPases, or if instead this functional diversification helps to couple unfolding and proteolysis to the activity of the proteasomal lid, which is not present in these simpler proteasomes.

ASSEMBLY OF THE 26S PROTEASOME

The proteasomes of archaea and actinobacteria are structurally much simpler than those of eukaryotes (78). In most species, a single type of α-subunit, β-subunit, and ATPase subunit is predicted. Thus, assembly of these compositionally simple proteasomes requires only that the correct number of subunits be placed in each ring and that the rings stack properly. In contrast, eukaryotic proteasomes contain seven distinct copies of each α- and each β-subunit, and there are six different ATPases. Each subunit shows high similarity to its paralogs, but it must typically occupy a single, defined site within the final structure. Thus, controlling the relative position of each paralogous subunit to form the appropriate subunit arrangement is necessary. Similarly, the task of finding the appropriate register between each ring and its neighboring rings is more complicated than in the simpler prokaryotic proteasomes, as the rotational symmetry within each ring is broken.

In many cases, a specific order of subunit incorporations into assembly intermediates may be important to prevent competition for similar binding sites among subunits or structural occlusion of binding sites during assembly. These alternative or incomplete arrangements could stall proteasome biogenesis or lead to dead-end assembly products. This may be especially true in the eukaryotic RP, which contains 13 non-ATPase subunits not present in simpler proteasomes. Finally, the high level of coordination of multiple enzymes within the proteasome requires that the subunits harboring enzymatic activity be restrained until the proteasome is fully assembled to prevent the decoupling of deubiquitylation, substrate unfolding, and proteolysis.

Eukaryotic cells have evolved several strategies to meet these demands. These generally fall into three categories: intrinsic regulatory elements such as certain CP β-subunit appendages, extrinsic assembly chaperones that control the assembly of specific proteasomal subcomplexes, and hierarchical assembly mechanisms that act to regulate the order in which specific subunits or subcomplexes of the proteasome associate (78, 79). We present an overview of 20S CP (**Figure 7**) and 19S RP (**Figures 8** and **9**) assembly and highlight examples of how these strategies are utilized in proteasome biogenesis.

20S Core Particle Assembly

Assembly of the eukaryotic CP initiates with the formation of the heptameric α-ring (**Figure 7**). The exact order of α-subunit incorporation is unknown, and the possibility of assembling α-rings with aberrant subunit arrangements (e.g., homoheptameric rings) is well known (80–82).

Table 2 19S RP Subunits and Associated Proteins

Subparticle	Standardized name	Yeast gene name(s)	Human standardized name	Other mammalian names	Activity/domains	Function	Other names
Base	Rpt1	*RPT1; CIM5; YTA3*	PSMC2	S7; p48	AAA+ ATPase	Unfoldase	*CIM5; S7; p48; PSMC2*
	Rpt2	*RPT2; YHS4; YTA5*	PSMC1	S4; p56	AAA+ ATPase	Unfoldase	S4; p56; PSMC1
	Rpt3	*RPT3; YNT1; YTA2*	PSMC4	S6; S6b; p47	AAA+ ATPase	Unfoldase	S6; S6b; p47; PSMC4
	Rpt4	*RPT4; CRL13; PCS1; SUG2*	PSMC6	S10b; p42	AAA+ ATPase	Unfoldase	S10b; p42; PSMC6
	Rpt5	*RPT5; YTA1*	PSMC3	S6′; s6a; p50	AAA+ ATPase	Unfoldase	S6′; S6a; p50; PSMC3
	Rpt6	*RPT6; CIM3; SUG1*	PSMC5	S8; p45	AAA+ ATPase	Unfoldase	S8; p45; PSMC5
	Rpn1	*RPN1; HRD2; NAS1*	PSMD2	S2; p112	Scaffold	Ub receptor docking	S2; p112; PSMD2
	Rpn2	*RPN2; SEN3*	PSMD1	S1; p97	Scaffold	Rpn13 docking	S1; p97; PSMD1
	Rpn10	*RPN10; MCB1; SUN1*	PSMD4	S5a; p54	vWA domain; UIM domain	Ub receptor	S5a; p54; PSMD4
	Rpn13	*RPN13*	ADRM1		PRU domain	Ub receptor; Uch37 receptor; Ub receptor docking	ADRM1
Lid	Rpn3	*RPN3; SUN2*	PSMD3	S3; p58	PCI domain	Scaffold?	S3; p58; PSMD3
	Rpn5	*RPN5; NAS5*	PSMD12	p55	PCI domain	Scaffold?	p55; PSMD12
	Rpn6	*RPN6; NAS4*	PSMD11	S9; p44.5	PCI domain	Scaffold?	S9; p44.5; PSMD11
	Rpn7	*RPN7*	PSMD6	S10a; p44	PCI domain	Scaffold?	S10a; p44; PSMD6
	Rpn8	*RPN8*	PSMD7	S12; p40; MOV34	MPN domain	Scaffold?	S12; p40; MOV34; PSMD7
	Rpn9	*RPN9; NAS7*	PSMD4	S11; p40.5	PCI domain	Scaffold?	S11; p40.5; PSMD4
	Rpn11	*RPN11; MPR1*	PSMD14	S13; Poh1	MPN+ domain; DUB activity	Substrate deubiquitylation	S13; PSMD14; Pad1; Poh1
	Rpn12	*RPN12; NIN1*	PSMD8	S14; p31	PCI domain	Scaffold?	S14; p31; PSMD8
	Sem1	*SEM1; HOD1*	PSMD9	S15; p27L; DSS1	Largely unstructured	Scaffold?	S15; p27L; Rpn15; Dss1; PSMD9

(*Continued*)

Table 2 (*Continued*)

RP-associated proteins	Dsk2	*DSK2*	PLIC-2		UBL domain; UBA domain	Extrinsic Ub receptor	
	Rad23	*RAD23*	hHR23b		UBL domain; UBA domain	Extrinsic Ub receptor	
	Ddi1	*DDI1; VSM1*	Ddi1		UBL domain; UBA domain	Extrinsic Ub receptor	
	Ubp6	*UBP6*	USP14		UBL domain; DUB activity	pUb chain editing	
	Uch37	No ortholog	UCH37		UCH domain; DUB activity	pUb chain editing	
	Hul5	*HUL5*	KIAA10/E3a		HECT family E3 ligase activity	E3 ligase; substrate triage?	
	Ecm29	*ECM29*	Ecm29		HEAT repeats	Proteasome stabilizer?	
	Spg5	*SPG5*	No obvious ortholog			Unknown	
	Rac1	*NAS6*	PSMD10	p28; gankyrin	Ankryin repeats	RP assembly chaperone	
	Rac2	*NAS2*	PSMD9	p27; BRIDGE	PDZ domain	RP assembly chaperone	
	Rac3	*HSM3*	S5b		HEAT repeats	RP assembly chaperone	
	Rac4	*RPN14*	PAAF1		WD-40 domain	RP assembly chaperone	

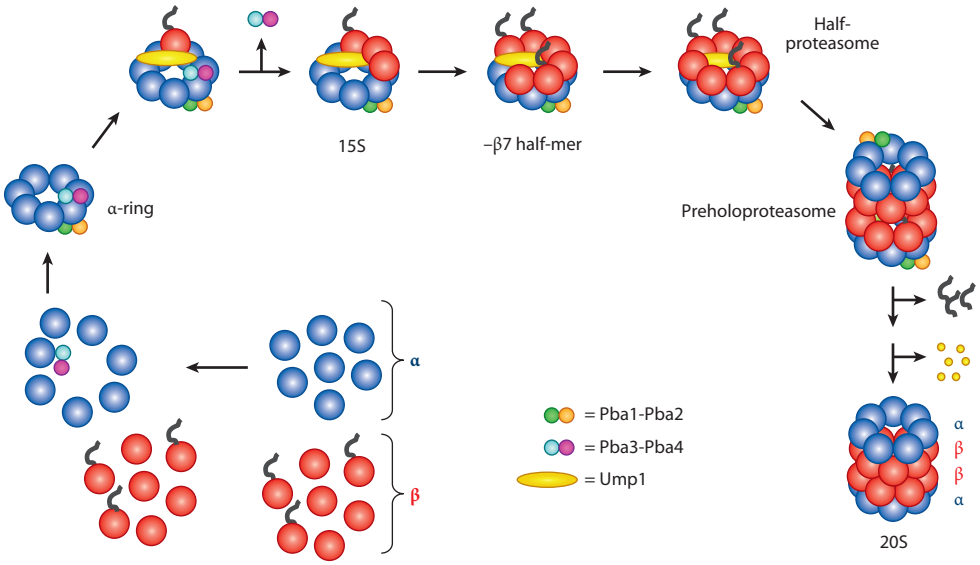

Figure 7

The 20S core particle assembly pathway.

β-subunits, several of which are synthesized as precursors with N-terminal propeptides, then add to the α-ring in an ordered fashion, creating a half-proteasome (83–86). Upon dimerization of two half-proteasomes to form the preholoproteasome (PHP), the active site–bearing β-subunits undergo autocatalytic cleavage of their propeptides, generating the mature catalytic sites and completing CP assembly (87).

At least three dedicated chaperone factors facilitate CP assembly. Two heterodimeric chaperone complexes participate in the assembly of the α-ring: proteasome biogenesis-associated 1/2 (Pba1/2) and Pba3/4 (PAC1/2 and PAC3/4 in mammals) (22, 88–92). Pba1/2 is a heterodimer that can bind isolated α-subunits (89) and associates with proteasomal precursors but not the mature CP in vivo, suggesting that it is released upon maturation of the catalytic active sites (22). Pba1 and Pba2 both contain HbYX motifs and bind to the α-ring similarly to proteasomal activators (22, 93). However, Pba1/2 does not enhance proteolytic activity (89). The interaction of Pba1/2 with the outer face of the α-ring suggests it may prevent premature docking of activators to the assembling CP. In mammalian cells, knockdown of PAC1/2 causes accumulation of large particles containing α-subunits but no β-subunits (89). This observation led to the proposal that Pba1/2 also functions to prevent the formation of aberrant α-ring dimers that might stall proper proteasome biogenesis.

Pba3 and Pba4 also form a heterodimer, which binds to α5 in vitro (88, 95). A crystal structure of Pba3/4 with α5 revealed that instead of binding to the outer α-ring surface, Pba3/4 instead binds to the inner surface recognized by the β-subunits (88). Modeling suggested that Pba3/4 would clash sterically with the incoming β4-subunit, indicating that Pba3/4 is likely released prior to or concomitant with β4 incorporation (**Figure 7**).

Whereas *PBA3/4* deletions enhanced the defects caused by a point mutation in α5, they did not exacerbate defects associated with loss of the α3-subunit (95). This suggested that Pba3/4 function was no longer required in cells lacking α3. In *α3Δ* yeast, a second copy of α4 fills the slot normally occupied by α3 (96). Deletion of *PBA4* resulted in a large fraction of CPs bearing the alternative α4-α4

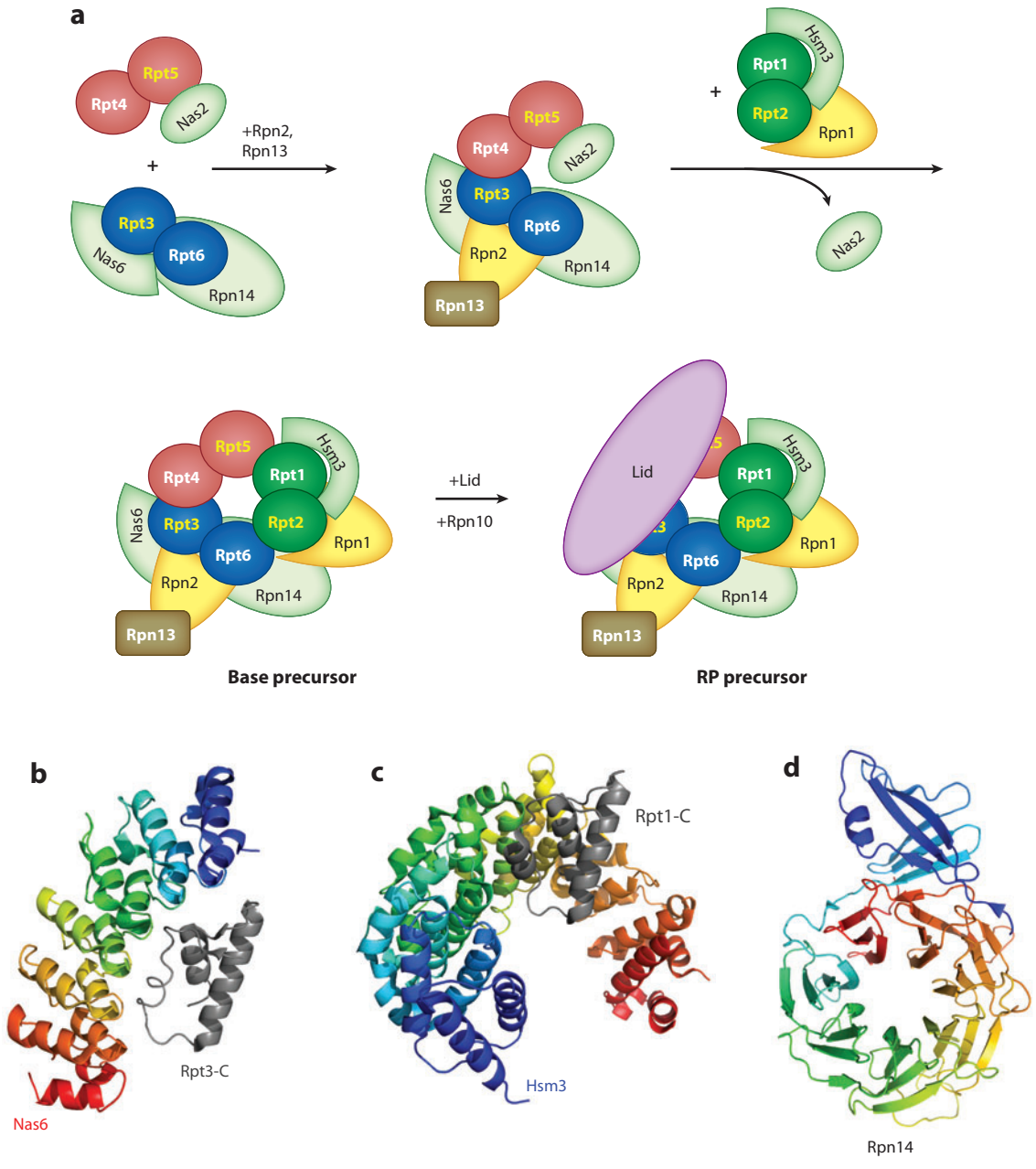

Figure 8

Assembly of the regulatory particle (RP) base is mediated by RP assembly chaperones. (*a*) The RP base assembly pathway in yeast. (*b*,*c*) Interaction between (*b*) *Saccharomyces cerevisiae* Nas6 and the C domain of Rpt3 [Protein Data Bank (PDB) ID: 2DZN] and (*c*) *S. cerevisiae* Hsm3 and the Rpt1 C domain (PDB ID: 3VLF). C domains are similarly oriented. (*d*) Atomic structure of *S. cerevisiae* Rpn14 (PDB: 3ACP).

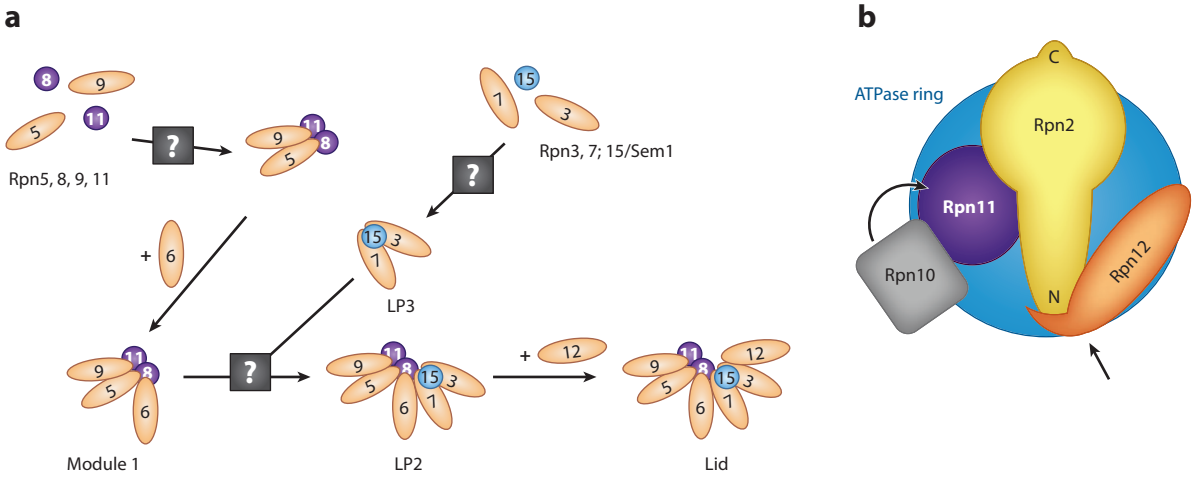

Figure 9

Assembly and attachment of the regulatory particle (RP) lid. (*a*) The RP lid assembly pathway inferred from studies in yeast. Mpr1/Pad1/N-terminal subunits are shown in purple, proteasome/CSN/initiation complex domain subunits are shown in peach, and Sem1 is shown in light blue. (*b*) A model for the stabilization of lid-base interaction by Rpn10 and the Rpn12 C-terminal tail via interaction with Rpn2 during RP assembly. In this model, Rpn10 positions Rpn11 to interact with Rpn2 (*curved arrow*), whereas the C-terminal tail of Rpn12 directly contacts the Rpn2 N-terminal extension (*straight arrow*). The N and C termini of Rpn2 are marked.

configuration. Thus, Pba3/4 functions, at least in part, to ensure that α3 is inserted into its appropriate place in the α-ring. Intriguingly, cells with increased amounts of α4-α4 proteasomes display a growth advantage under certain stressors, suggesting a potential physiological role for these alternative proteasomes.

Upon completion of the α-ring, β-subunits assemble onto the inner surface of the α-ring. Initiation of β-ring assembly is accompanied by the binding of Ump1 to the assembly intermediate, at least in mammalian cells (97). Ump1 may help prevent the premature dimerization of precursors containing incomplete sets of β-subunits (91, 98, 99). Completion of the β-ring overcomes this Ump1-dependent checkpoint (91, 99) and is followed closely by dimerization of half-proteasomes and degradation of Ump1 upon maturation of the CP catalytic sites (98). Systematic knockdown of β-subunits in mammalian cells suggested that β-subunit addition begins with β2 and proceeds in a defined order, although the timing of β1 addition was ambiguous (97). Ump1 was already associated with the α-ring in β3 knockdown cells in which the only β-subunit present on the α-ring is β2, suggesting Ump1 binds with or before β2. The ordered association of β-subunits is driven, at least in part, by the propeptides and peptide tails of certain β-subunits. Both the β2 propeptide and C-terminal tail (which wraps around β3) are required for efficient incorporation of the β3-subunit in mammals (and probably also yeast) (97, 100). Similarly, the β5 propeptide helps drive incorporation of β6, and the C-terminal tail of β7 is required for efficient β7 incorporation. The β7 tail in conjunction with the β5 propeptide also promotes the dimerization of fully assembled half-proteasomes to form the PHP.

Assembly of the 19S Regulatory Particle

Like the CP, the RP also requires the assistance of dedicated chaperones for its efficient and accurate assembly in vivo. The lid and base subcomplexes of the RP appear to assemble independently and then associate with one another (and Rpn10) to complete the RP (67, 101). The

recent RP EM structures show a dense mesh of protein-protein interactions between lid and base subunits. Therefore, forming these subcomplexes prior to their association to limit assembly of RPs with aberrant compositions may be advantageous. This mode of assembly may also help restrict RP DUB and ATPase activities until assembly is completed, thereby ensuring the coupling of substrate deubiquitylation, unfolding, and degradation.

Base assembly. Although a single arrangement of the proteasomal ATPases is observed in vivo (26), the Rpt subunits do not seem to encode within their primary structure all of the information required for their appropriate arrangement. For example, heterologously expressed Rpt4 or coexpressed Rpt1 and Rpt2 subunits have a propensity to form nonnative structures capable of hydrolyzing ATP (102). To help guide the proper formation of the base, eukaryotes utilize at least four dedicated RP assembly chaperones (RACs): Rpn14, Nas6, Nas2, and Hsm3 (PAAF1, gankyrin/p28, p27, and S5b in mammals) (**Table 2**) (55, 103–107). Each RAC binds a distinct subset of base subunits (**Figure 8*a***) but is not present in the 26S holoenzyme, at least in yeast, consistent with roles as assembly chaperones. Deletion of each RAC either alone or in combination impairs base formation but does not affect assembly of the CP or lid. Thus, the known RACs act specifically at the base assembly stage, although a role in lid-base and/or initial RP-CP joining cannot be excluded.

Our current understanding of base assembly derives primarily from studies of relatively stable base subcomplexes isolated from yeast mutants or mammalian cells subjected to small interfering (si)RNA-mediated knockdowns. Similar to the trimer of dimers proposed for the archaeal PAN complex, two of the earliest steps in base assembly seem to be the formation of Rpt *cis-trans* dimers—Rpt3-Rpt6, Rpt5-Rpt4, and Rpt2-Rpt1 (**Figure 4*c***)—and their association with their specific RACs (and for Rpt1–2, association with Rpn1) (78). Whether ATPase dimerization precedes Rpt binding to RACs or non-ATPase subunits is unclear, although the RACs can bind their cognate ATPase (or a fragment thereof) in the absence of the paired ATPase subunit (106, 108). Although ATP binding may not be required for formation of Rpt *cis-trans* pairs, it does appear to be important for association of these pairs with other ATPase pairs (109).

Following formation of the Rpn14-Rpt6-Rpt3-Nas6, Rpt4-Rpt5-Nas2, and Hsm3-Rpt1-Rpt2-Rpn1 complexes (referred to as the Rpn14/Nas6, Nas2, and Hsm3 modules, respectively), they associate with one another, along with Rpn2 and Rpn13, to form the base (**Figure 8*a***). In yeast, the Rpn14/Nas6 and Nas2 modules appear to associate first, and upon incorporation of the Hsm3 module (or before), Nas2 is released from the complex (26). In mammalian cells, a complex between the Rpn14/Nas6 and Hsm3 modules has been observed that specifically lacks Rpt4 and Rpt5 (55), possibly indicating that the order in which these modules come together to form the base may vary among species. Alternatively, the modules may assemble in multiple orders. Rpn14, Nas6, and Hsm3 each can be found bound to the fully assembled RP but not to the 26S proteasome, suggesting that they are normally released prior to or during RP-CP binding.

Structure and function of the regulatory particle assembly chaperones. Each RAC contains distinct protein-protein interaction domains: Rpn14 contains seven WD40 repeats, Nas6 seven ankyrin repeats, Nas2 a PDZ domain, and Hsm3 11 HEAT repeats (108, 110–113). These domains mediate interaction of each RAC with its cognate ATPase: Rpn14-Rpt6, Nas6-Rpt3, Nas2-Rpt5, and Hsm3-Rpt1. Studies have determined the crystal structures of Nas6 and Hsm3 bound to fragments of their cognate ATPases (**Figure 8*b*,*c***) (111–113); the repeat domain in each recognizes the respective Rpt CTD. Rpn14 forms a β-propeller as expected (**Figure 8*d***) (110). Although Rpn14 has not been crystallized with its binding partner, yeast two-hybrid and protein pulldown assays

indicate it interacts with the Rpt6 CTD. In contrast, recognition of Rpt5 by Nas2 depends on the Rpt5 HbYX motif; deletion of these three C-terminal residues is sufficient to abolish Nas2 binding (108).

Despite this structural information, the molecular mechanisms by which the RACs control base assembly are still poorly understood. In some models, docking of Nas6 and Hsm3 onto the ATPase ring would clash sterically with the 20S CP (106), perhaps explaining why these RACs are absent in the full 26S proteasome. Rpn14, Nas6, and Hsm3 appear positioned either to mediate association of their cognate ATPases with their dimerization partners or perhaps to control the arrangement of the ATPase-heterodimer pairs in the ATPase ring. Hsm3 uses distinct elements to make contacts with both Rpt1 and Rpt2, and the integrity of both of these elements is required for efficient base assembly in vivo (112). Thus, Hsm3 appears to function, at least in part, by promoting the specific pairing of Rpt1 and Rpt2. In contrast, Rpt4 and Rpt5 appear to be quite stable together in the absence of their chaperone, Nas2 (105). The specific binding of Nas2 to the C-terminal tail of Rpt5 instead suggests a role in preventing the premature docking of this subunit, either alone or in complex with other base subunits, onto the CP (108).

Is the core particle a template for regulatory particle base assembly? In addition to the RACs, the CP may also function as a base assembly chaperone by providing a template or scaffold for RP base formation. This hypothesis was first proposed because base subparticles accumulated when subunits or assembly chaperones of the yeast CP were mutated (95). In human cells, a nascent RP subcomplex containing Rpn2, Rpn10, Rpn11, and Rpn13 (and the proteasome-associated protein Txnl1), but apparently lacking the remaining RP subunits, coimmunoprecipitates with the mature CP under some conditions (114). However, none of these subunits directly contacts the CP in the 26S holoenzyme, suggesting either that large-scale rearrangement of the RP-CP interface may occur during assembly or that the observed complex may be a dissociation product of the RP.

In yeast, deletion of the most C-terminal residue of specific ATPases greatly disrupts RP formation without significantly affecting their association with their cognate RACs (107, 108). As these mutations disrupt interaction of the RP with the CP, the CP was hypothesized to serve as a template for base formation via insertion of the C-terminal tails of the assembling ATPases into specific surface pockets of the α-ring. Further, as purified CPs could displace the RACs from their cognate ATPases, proper docking of ATPases onto the α-ring was proposed to be the trigger for release of bound assembly chaperones. In contrast, others have assembled functional RPs equally efficiently in the absence of the CP from RP intermediates purified from bovine erythrocytes, arguing against a stringent requirement for the CP in base assembly (115).

Although the data are clear that disruption of the CP or RP-CP interactions correlates with an accumulation of base assembly intermediates, whether this reflects a templating function for the CP in base assembly remains uncertain. Base intermediates might accumulate for other reasons. In yeast, proteasome gene expression is driven by the transcription factor Rpn4 (Nrf1 in humans), which is itself a substrate of the proteasome (116, 117). Thus, low proteasome activity will increase Rpn4 levels, which will in turn increase the cellular levels of proteasomes (and their assembly intermediates). Whereas nearly all proteasome subunit genes contain Rpn4 binding sites in their promoters, the RAC genes do not. Thus, the chaperoning capacity of the RACs may become limiting when proteasome synthesis rates are very high, e.g., owing to defective RP-CP association, leading to the accumulation of base assembly intermediates even though assembly per se is not compromised. Accordingly, deletion of *RPN4* in the context of specific ATPase C-terminal deletions reduced the levels of RP assembly intermediates without affecting the apparent levels of 26S proteasomes (107), although the levels of these intermediates were still higher than in wild-type cells. Lower

effective RAC levels may also be engendered by tight binding to accumulating base or RP intermediates that do not allow their ready release. This could occur either if the RP-CP interface is compromised or if defects in CP formation lead to an excess of free RP. Hence, the CP-templating model for RP assembly, though attractive, requires additional experiments to address these ambiguities.

Lid assembly. In contrast to the base and CP, much less is known about lid assembly; moreover, no lid-specific assembly chaperones have been identified to date. Although the general molecular chaperone Hsp90 has been implicated in RP assembly in yeast (118), whether it contributes directly or indirectly to RP biogenesis is unclear. Coexpression of the nine lid subunits with Hsp90 in *Escherichia coli* is sufficient to yield a particle containing at least eight of the nine subunits (Sem1 was not detected) (46). The recombinant lid incorporates Zn^{2+} into the Rpn11 active site and, when incorporated into the 26S proteasome, displays DUB activity comparable with proteasomes containing yeast-derived lid, strongly suggesting that, other than potentially Hsp90, no eukaryote-specific chaperones are required.

The high-resolution EM structures of the lid and the mapping of the subunits within it are sufficient to allow the unambiguous identification of the winged-helix domains and adjacent α-helices in each PCI subunit (27, 46, 47). Each PCI domain associates laterally with the PCI domain of its neighbor(s) to form a horseshoe-shaped lattice (**Figure 6**). Although this lateral association could in principle initiate with any PCI subunit and finish in any order, only specific subcomplexes of the lid are observed in vivo (67, 119–121), pointing to a potential hierarchical assembly mechanism in which subsequent subunit incorporations are triggered by the binding of the preceding subunits. In vitro binding studies, although still limited, support this idea (see below in this section) (65). Assembly of specific subcomplexes may cause conformational changes that favor binding of subsequent subunits or may provide additional binding sites that enhance the affinity for other subunits. The MPN domain subunits Rpn8 and Rpn11 appear to contact several PCI subunits and may help to stabilize specific interactions between PCI subunits or dictate the order in which the PCI subunits bind one another.

As with base assembly, our understanding of lid assembly derives primarily from the cataloging of the contents of lid subparticles that accumulate in yeast strains harboring mutations in specific lid subunits (67, 119–121). Lid assembly appears to begin via formation of two nonoverlapping and complementary subcomplexes: Rpn5/6/8/9/11 [herein called module 1 (122)] and Rpn3/7/Sem1 [called lid particle 3 (LP3)] (**Figure 9*a***). The sequence(s) of subunit additions that form these two particles are unknown, although a module 1–related complex lacking Rpn6 has also been observed in an *rpn6* mutant (121). In *rpn12* mutants, a lid subcomplex (LP2) containing all subunits of module 1 and LP3 (but lacking Rpn12) accumulates (67, 119), and from this result, module 1 and LP3 were inferred to join one another to form LP2 (**Figure 9*a***). Notably, mutation of any lid subunit (besides Rpn12) leads to the accumulation of free Rpn12, suggesting that Rpn12 addition to the lid precursor is the final step in lid assembly (67). Consistent with this, purified Rpn12 and LP2 associate with one another in vitro to form a particle indistinguishable from the lid. Notably, purified LP2 added to Rpn12-deficient yeast extracts fails to incorporate into 26S proteasomes even after extended incubation. In contrast, LP2 addition to LP2-deficient yeast extracts that have ample free Rpn12 causes rapid incorporation of both LP2 and Rpn12 into 26S proteasomes. These results support the idea of a hierarchical or ordered assembly mechanism in which LP2 does not associate with the base and continue RP assembly unless Rpn12 first joins to complete lid formation.

Lid-base association and Rpn10 incorporation. The finding that LP2 cannot incorporate into the RP until Rpn12 has joined suggests an important role for this subunit in governing lid-base association. A highly conserved

Proteasome assembly chaperone: a dedicated assembly factor, not usually found in mature proteasomes, that assists in the biogenesis of the proteasome

C-terminal tail of Rpn12 is essential for efficient lid-base association in the absence of Rpn10, indicating that these two proteins have overlapping roles in securing the lid onto the base (67). Within the 26S proteasome, Rpn10 and Rpn12 are positioned on opposite sides of the Rpn2 structure (**Figure 9*b***), and whereas Rpn12 makes extensive contact with Rpn2, Rpn10 does not (46, 47). Instead, Rpn10 interacts with the MPN domain of Rpn11, which in turn binds Rpn2. Thus, Rpn2 may act like a saddle horn onto which the lid attaches in a manner aided by Rpn10-dependent stabilization of Rpn2-Rpn11 binding (**Figure 9*b***). Of course, there are many points of interaction between the lid and base subcomplexes, but these two interactions may be the first formed and may stabilize the lid until it can seat firmly onto the base. Further experiments are necessary to clarify the contributions of distinct subdomains of the lid and base subunits to this late stage of RP assembly.

Assembly of Alternative Proteasome Isoforms

As a detailed assembly map has developed over the past several years for the canonical 26S proteasome, an emerging question is how the assembly of the canonical proteasomes versus the noncanonical immuno-, thymo-, and in the case of yeast (and perhaps humans), α4-α4 proteasomes is governed in cells. In cells expressing alternative proteasome subunits, such as the cortical thymus, all isoforms of the variant subunits (i.e., β1, β1i, β2, β2i, β5, β5i, β5t) are readily detectable by immunoblot analysis of whole-cell extracts, but only certain configurations appear in mature CPs (15). For example, β5t-containing proteasomes seem to include only the β1i- and β2i-subunits and not β1 and β2. Although these subunits are expressed at higher levels than their canonical counterparts, this may not be sufficient to account for the specificity of proteasome formation observed. In support of this, immunoproteasome subunits help recruit other immunoproteasome subunits into assembling CPs (123–126). This is to some extent mediated by the different propeptides of these subunits (126), but other factors may also contribute. Isoform-specific assembly chaperones represent one intriguing but as yet untested possibility for such factors.

Besides the RP, the α-ring surface of CPs can bind at least three alternative regulators: PA200/Blm10, PA28, and REGγ (only Blm10 is found in yeast) (reviewed in Reference 127). A common function of these regulators is to open the gate in the α-subunit ring; each regulator also forms a structure that contains an opening that could potentially provide a channel for passage of substrates through it to the CP (20, 21, 24, 128–130). In addition, the homohexameric AAA+-family ATPase Cdc48 (p97 in mammals) from archaea was recently reported to bind and activate the archaeal CP (131, 132). As Cdc48 is highly conserved in eukaryotes, it may function as a proteasomal activator in eukaryotes as well. In principle, assembling different regulators onto the ends of distinct CPs could generate a multitude of proteasomal species, although the range of such potential regulator-CP complexes in vivo and the control of their assembly remain unknown.

Quality Control of Proteasome Assembly

The evolutionary conservation of proteasome assembly chaperones across eukaryotes (and in some cases extending to archaea) suggests a considerable investment in controlling proteasome assembly. Invariably, however, assembly errors will occur; what becomes of these misassembled proteasomes? Recent work suggests that in addition to stringent cellular control over the assembly process, post-assembly quality controls may exist as well. The highly conserved, proteasome-associated protein Ecm29 is enriched on proteasomes in mutant yeast bearing certain proteasomal defects (94, 108, 133). Disruption of efficient proteasome assembly by deletion of the CP assembly chaperone gene *UMP1* (133), mutation of the critical conserved lysine residues in the CP α-ring pockets (94), or deletion of the C-terminal residue of Rpt5 necessary for efficient docking into the

CP surface (108) resulted in enhanced Ecm29 association with RP-CP-related complexes. Deletion of *UMP1* resulted in the apparent absence of the β3-subunit in Ecm29-containing RP-CP complexes and failure to properly process the β5 propeptide from its precursor form (133). Each of the aforementioned mutations would be expected to affect the RP-CP interface (22, 134). From EM reconstructions, Ecm29 appears to contact both the RP and CP (135), and it is not known to bind either in the absence of the other (94, 108, 133–135). Thus, Ecm29 appears to specifically recognize proteasomes with an aberrant RP-CP interface, at least in yeast.

Exactly why Ecm29 is associated with these defective proteasomes is still unclear. Ecm29 was reported to inhibit the catalytic activity of RP-CP complexes with which it was associated based on reduced cleavage of a fluorogenic peptide substrate compared with *ecm29Δ* proteasomes (108). However, proteasomes to which Ecm29 is bound may be misassembled rather than simply less active. Similarly, addition of recombinant β3-subunit to Ecm29-bound proteasomes (which lacked β3) resulted in its incorporation into the CP and release of Ecm29 (133). However, whether Ecm29 simply sequesters defective proteasomes or facilitates their repair remains unclear.

In mammalian cells, Ecm29 interacts with several proteins of the microtubule and molecular motor machinery and decorates the surfaces of late endosomes presumably destined for the lysosome (136, 137). Normal proteasomes are slowly turned over by lysosomes in rat liver (138) and may associate with the vacuole in yeast (139). Thus, Ecm29 may promote the recruitment and degradation of malformed proteasomes via the lysosome/vacuole. Ecm29 is not required for gross localization of proteasomes to the vacuole in yeast (139), but whether a defective subpopulation of proteasomes is degraded, repaired, or instead sequestered from general circulation in some way remains to be determined.

CONCLUDING REMARKS

The ever-growing list of human diseases in which protein homeostasis is disrupted is a testament to the importance of the UPS for normal cellular function and its potential as a therapeutic target. Notably, both inhibition and augmentation of protein degradation may have clinical value. Proteasome inhibition has been an accepted antineoplastic strategy since the approval of bortezomib for treatment of multiple myeloma in 2003. In contrast, recent studies have indicated that enhancement of proteasome activity, typically by blocking proteasome-associated DUBs, may in principle be beneficial in protein misfolding disorders, such as many forms of neurodegeneration, and may block replication of some viruses (140, 141). Similarly, unique types of proteasomes in lymphoid tissues provide novel targets for immune modulation via proteasome inhibition (10). Thus, modulation of proteasome biogenesis may also provide opportunities to regulate proteolysis in cells for therapeutic gain.

Although the recent advances in our understanding of proteasome structure will undoubtedly serve as a template for improved understanding of its function and assembly, detailed structural and biochemical information on the proteasome during its various catalytic steps is necessary to more fully understand its mechanisms of action. Similarly, although knowledge of the architecture of subunits within the 26S holoenzyme provides a convenient end point for assessment of proteasome assembly, more detailed characterization of assembly intermediates is also needed. We remain far from understanding the basis for the impressive efficiency and specificity of proteasome assembly in vivo. Given the myriad factors involved in proteasomal assembly and proteolysis and the discovery of several uniquely configured proteasomes, the analysis of proteasome mechanism and assembly should continue to yield fundamental insights of both basic and clinical importance.

SUMMARY POINTS

1. Recent structural and biochemical analyses of the eukaryotic proteasome RP and its prokaryotic antecedents have provided a detailed view of RP subunit architecture.
2. The Rpt ATPase subunits, which are responsible for substrate unfolding for degradation, form a uniquely ordered heterohexameric ring that directly abuts the entrance to the CP.
3. The polyUb-substrate-binding sites of the RP are generally located most distally from the CP, whereas the subunits responsible for removing the polyUb tag and unfolding the substrate are poised over the entrances to the RP ATPase ring and CP ring, respectively.
4. Despite the availability of atomic structures of several RP assembly chaperones bound to domains of their cognate Rpt subunits, the mechanistic basis of chaperone function in RP biogenesis remains poorly understood.
5. No dedicated chaperones have been found for RP lid assembly, and a hierarchical mechanism of subunit addition may largely account for high-fidelity lid assembly and lid-base joining.
6. Compositional plasticity in the 20S CP provides an additional layer of complexity to proteasomal assembly, and CP assembly chaperones may have key roles in mediating biogenesis of these alternative forms.
7. Post-assembly proteasome quality controls may also be important in eukaryotes, as suggested by recent work on the proteasome-associated Ecm29 protein in yeast.

FUTURE ISSUES

1. How are the different enzymatic activities of the proteasome coordinated?
2. Why has the proteasome evolved six distinct ATPases whereas all other ATP-dependent proteases have homomeric rings? How does this heterogeneity contribute to substrate binding, unfolding, and translocation into the CP?
3. How do the multiple intrinsic and extrinsic Ub receptors contribute to substrate recognition and processing?
4. What are the relative contributions of the CP and the RACs to RP base assembly?
5. How is association of the CP with its multiple regulator complexes governed in cells?
6. How is post-assembly proteasome quality control orchestrated, and what are the pathways and mediators controlling it?
7. Where in the cell do proteasomal subparticles assemble, and how are the localization dynamics of assembly and disassembly regulated?

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

We thank Yanjie Li and Nicole R. Wilson for comments on the manuscript. Work on proteasomes in our lab is funded by National Institutes of Health grant GM83050 to M.H. R.J.T. Jr. is supported in part by an American Cancer Society New England Division—Massachusetts Biotech Council Cancer Research Challenge—Millennium: The Takeda Oncology Company Postdoctoral Fellowship.

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Volume 82, 2013

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Errata

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