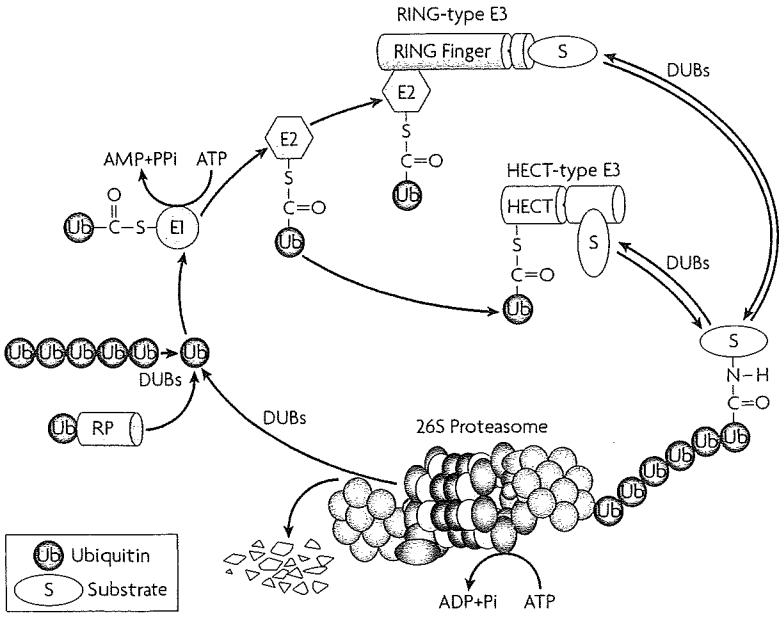
Box 1 | **The ubiquitin–proteasome system**



Ubiquitin (Ub) is translated as a tandemly fused polyubiquitin or as a fusion protein with the ribosomal protein (RP; see the figure). De-ubiquitylating enzymes (DUBs) hydrolyse the isopeptide bond between Ub molecules or Ub and the RP to produce free Ub. The Ub-conjugation pathway consists of three kinds of enzymes: E1 (Ub-activation enzyme), E2 (Ub-conjugation enzyme) and E3 (Ub ligase). Ub is activated by E1, forming a high-energy thiol ester bond between the carboxy-terminal Gly residue of Ub and the active site Cys of E1 in an ATP-dependent reaction. The activated Ub is transferred to E2, and E3 then attaches Ub to a specific substrate protein. The E3 enzymes are classified into two major classes. RING-type E3s bind to both E2 and a substrate and help E2 to transfer Ub to a substrate, whereas HECT-type E3s form a thiol ester with the activated Ub from E2 before transfer of Ub to a substrate. Ub is covalently attached to Lys residues of a substrate. Polyubiquitylated substrates are recognized and degraded by the 26S proteasome in an ATP-dependent manner. DUBs remove polyubiquitin from proteasome substrates before substrates are translocated into the 20S proteasome and regenerate free Ub from unanchored polyubiquitin chains.

the recognition of polyubiquitin chains, which are then excised coincident with unfolding and translocation into the proteolytic 20S proteasome using energy liberated from ATP hydrolysis[1,9].

Defining the molecular mechanism that is involved in the assembly of this huge and highly sophisticated protease machinery is important for our understanding of the process of protein degradation, as well as regulation of proteasome activity, and has been a major research challenge. Here, we describe the assembly mechanism of the 20S proteasome, our understanding of which has greatly advanced in the past decade[26–30]. We also discuss the formation of the 19S RP, although the available information on this process is fragmentary and much remains a mystery.

Assembly of prokaryotic 20S proteasome

The 20S proteasome was originally isolated from eukaryotes, but subsequent studies documented that this large protease complex is conserved in all three domains of life[31]. The basic understanding of the assembly of 20S proteasomes came from observations in archaeal 20S proteasomes. Each of the α- and β-subunits of the 20S proteasome of the archaebacteria *Thermoplasma acidophilum* is encoded by a single-copy gene and forms a homoheptameric ring[32,33] (BOX 2). Coexpression of *T. acidophilum* α- and β-subunits in *Escherichia coli* resulted in mature and functional 20S proteasomes. Expression of the α-subunit alone produced heptameric α-rings, whereas that of the β-subunit could not assemble and folded incompletely in the absence of α-subunits[34]. Thus, the assembly of the *T. acidophilum* 20S proteasome is probably initiated by the formation of an α-ring, to which β-subunits are attached subsequently. The ability to form the α-ring depends on the amino-terminal H0 helix in the α-subunit, which contacts the loop preceding the H0 helix of the next α-subunit[33,34]. This helix does not exist in the β-subunit, the tertiary structure of which otherwise resembles the α-subunit. The eight residue propeptide of the β-subunit is cleaved off during the assembly of the 20S proteasome, although it is dispensable for the assembly because its deletion did not affect the incorporation of the β-subunit.

In bacteria, the 20S proteasome is found only in actinomycetales. Other bacteria, such as *E. coli*, have a structurally related Thr protease called heat-shock locus gene V (HslV)[35,36]. The 20S proteasome of the actinobacterium *Rhodococcus erythropolis* is composed of two different α-subunits and two different β-subunits. These are encoded by two operons, each containing a pair of α- and β-subunits[37] (BOX 2). The two α-subunits and the two β-subunits are over 80% identical, which suggests a fairly recent duplication of the operon. Therefore, it is not surprising that the expression of any combination of one α-subunit and one β-subunit (for example, α1β1, α1β2, α2β1 and α2β2) in *E. coli* and *in vitro* yielded active and mature 20S proteasomes[38]. However, the *R. erythropolis* α-subunits could not form a ring-like structure by itself, in contrast to *T. acidophilum* α-subunits, presumably because of a smaller contact region between α-subunits[38,39]. Heterodimer formation of one α-subunit and one β-subunit precedes the assembly of *R. erythropolis* half-proteasomes, which are composed of one α-ring and one β-ring. The crystal structures of the *R. erythropolis* proteasome revealed that the β-propeptide acts as an assembly promoting factor by fitting in the interface of two adjacent α-subunits to help hold them together[39].

Collectively, archaebacterial and eubacterial proteasome subunits can assemble autonomously into functional mature proteasomes without the help of any other chaperone proteins.

Assembly of eukaryotic 20S proteasome

The eukaryotic 20S proteasome has a more complex subunit composition compared with prokaryotic counterparts. It is composed of seven different α-subunits and seven different β-subunits, each of which occupies a defined position within the 20S proteasome (FIG. 2; BOX 2).

Trypsin-like activity
β2 cleaves after the basic amino acids Lys and Arg. As this activity resembles that of trypsin, a Ser protease that is secreted in pancreatic juice, it is referred to as 'trypsin-like' activity.

Chymotrypsin-like activity
β5 cleaves after hydrophobic amino acids. This activity resembles that of chymotrypsin, a Ser protease that is secreted in pancreatic juice, and is thus referred to as chymotrypsin-like activity.

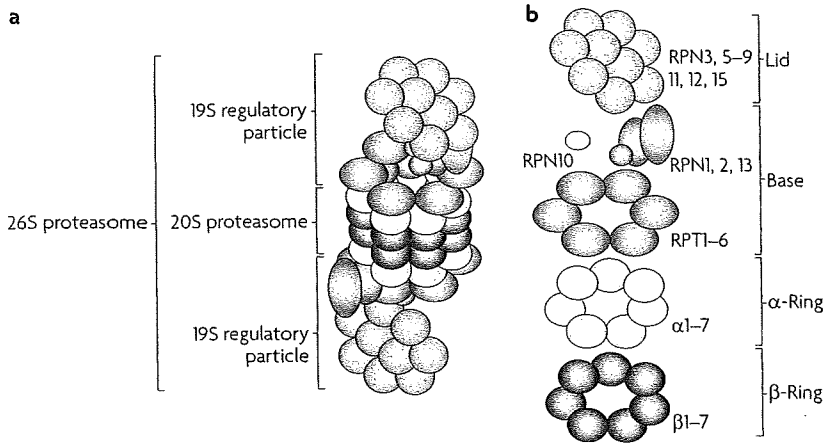


Figure 1 | **Schematic diagram of the 26S proteasome. a** | The 26S proteasome consists of the catalytic 20S proteasome (a barrel of four stacked rings: two outer α-rings and two inner β-rings) and the 19S regulatory particle (RP, also known as PA700). **b** | Subunit composition of the 26S proteasome. The regulatory particle is further divided into the base and the lid subcomplexes, which are composed of regulatory particle triple-A (RPT) and regulatory particle non-ATPase (RPN) subunits. RPN10 is coloured yellow because it is supposed to be located at the base–lid interface.

AAA+ ATPase
(ATPase associated with diverse cellular activities). ATP-hydrolysing enzyme that contains one or two conserved ATP-binding domains, which are in turn comprised of conserved A and B motifs. AAA+ ATPases assemble into oligomeric assemblies (often hexamers) that form a ring-shaped structure with a central pore.

UBL
(Ubiquitin-like). A protein domain with motifs that have significant sequence and structural similarity to ubiquitin. Type 1 UBLs are comprised solely of this motif and generally work as modifiers that are covalently attached to target proteins, in a fashion similar to the ubiquitin. Type 2 UBLs are larger proteins that contain this motif as a part of the protein.

UBA
(Ubiquitin-association). A domain of ~45 amino acids that adopts a structure that comprises a three α-helix bundle. The UBA domain binds to ubiquitin through a conserved hydrophobic surface patch.

Assembly of the α-ring. The assembly of the eukaryotic 20S proteasome is assumed to start with α-ring formation. This assumption is based on observations that the expression of *Trypanosoma brucei* α5 subunit or human α7 in *E. coli* results in the formation of four stacked or double homoheptameric rings, respectively[40,41]. However, neither homoheptameric rings nor stacked α-rings occur *in vivo*. Moreover, the α-ring is usually composed of seven different α-subunits that occupy defined positions in the α-ring, which suggests the existence of mechanisms that correctly arrange the seven α-subunits into a heteroheptamer and prevent oligomerization of α-rings. Recent studies identified two dimeric complexes that are dedicated to proteasome assembly both in human cells (proteasome assembling chaperone 1 (PAC1)–PAC2 and PAC3–PAC4)[42,43] and in yeast (proteasome biogenesis-associated 1 (Pba1; also known as Poc1), Pba2 (also known as Add66 and Poc2), Pba3 (also known as Poc3, Dmp2 and Irc25) and Pba4 (also known as Poc4 and Dmp1), which are orthologues of human PAC1, PAC2, PAC3 and PAC4, respectively)[44–49] (TABLE 1). This demonstrates a chaperone-assisted mechanism for the efficient assembly of eukaryotic 20S proteasomes.

The PAC1–PAC2 heterodimer was originally identified in a search for proteasome-associated proteins in human cells[42]. PAC1–PAC2 was found to be mainly associated with a proteasome assembly intermediate with a major peak of α-subunits in glycerol gradient centrifugation analysis. Purification of this intermediate revealed that this complex includes all seven α-subunits and PAC1–PAC2 but no β-subunits, and has a size of approximately 230 kDa. This is most likely to be the size of the α-ring[42]. The PAC1–PAC2 complex could bind directly to α5 and α7 *in vitro*, and indeed it is associated *in vivo* with a subset of α-subunits, including α5 and α7, that is presumably an intermediate on the way to the α-ring[42].

Knockdown of PAC1 or PAC2 by short interfering RNA (siRNA) decreases normal α-ring assembly and results in the accumulation of off-pathway products — presumably α-ring dimers[42]. Interestingly, knockdown of PAC1 results in loss of PAC2, and vice versa, indicating that PAC1 and PAC2 are stable only when they form a heterodimer. These findings show that PAC1–PAC2 does not only assist α-ring formation but also prevents aberrant dimerization of α-rings[42]. PAC1–PAC2 sticks to proteasome precursors until the 20S proteasome is completely formed. It is then degraded by the newly formed 20S proteasome and is therefore short-lived, with a half-life of approximately 30–40 minutes, which is consistent with the estimated maturation period of the mammalian 20S proteasome from 30 minutes to 2 hours[42,50] (FIG. 3a).

In the budding yeast, Pba1–Pba2 is probably the counterpart of PAC1–PAC2 because Pba1 and Pba2 have weak sequence similarities to PAC1 and PAC2, respectively, and form a heterodimer that binds to proteasome precursors. Similar to PAC1–PAC2, this heterodimer is degraded by newly formed 20S proteasomes[44,47,48]. However, yeast strains that lack Pba1–Pba2 grow normally and show only modest defects in proteasome assembly with slightly increased assembly intermediates, in contrast to the phenotypes that are observed in human cells with deleted PAC1–PAC2 (REFS 42,48).

However, several lines of evidence suggest that compromised proteasome function occurs in cells that lack Pba1 and Pba2. The loss of Pba2 causes accumulation of polyubiquitylated proteins in cells, stabilizes certain endoplasmic reticulum-associated degradation (ERAD) substrates and exhibits synthetic phenotypes when combined with the loss of inositol-requiring enzyme 1 (Ire1), a transducer of the unfolded protein response (UPR)[44,47,48,51]. Furthermore, deletion of Pba1 or Pba2 changes the phenotypes of mutants for proteasome-related genes; Δ*pba1* or Δ*pba2* mutants partially suppress the growth defect of ubiquitin-mediated proteolysis 1 (Δ*ump1*) mutants, another proteasome-dedicated chaperone (see below), whereas they worsen the phenotypes of cells that lack α3 (the only non-essential subunit of the yeast 20S proteasome) and Rpn4 (a transcription factor that positively regulates proteasome-related genes)[48]. The first observation suggests that slower proteasome formation owing to a lack of Pba1–Pba2 reduces the influence of Δ*ump1*, which causes premature dimerization of incomplete half-proteasomes[48,52] (see below). The genetic interaction with Δ*rpn4* is often observed in mutants that have potentially damaged proteasome activities.

PAC3 was identified as a 14 kDa protein co-purified with α-rings and PAC1–PAC2 in human cells[43]. As with PAC1–PAC2, most PAC3 is associated with α-rings in cells. However, in contrast to PAC1–PAC2, PAC3 is a long-lived protein and is released before the completion of 20S proteasome formation. Knockdown of PAC3 causes a reduction in α-rings and 20S proteasomes, an effect that is similar to PAC1–PAC2 knockdown[43]. However, ectopic expression of PAC3 does not compensate for the loss of PAC1–PAC2, suggesting that PAC1–PAC2 and PAC3 have roles in proteasome assembly at different steps[43]. In support of this conclusion, PAC3 and

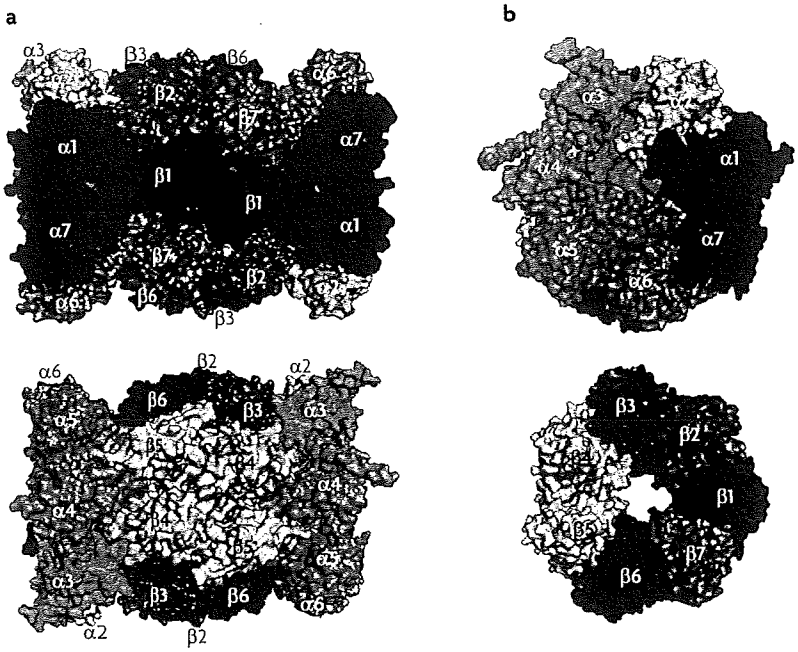


Figure 2 | **Molecular structures of eukaryotic 20S proteasomes. a** | Side views of the bovine 20S proteasome. The overall shape of the bovine 20S proteasome is an elongated cylinder with large central cavities and narrow constrictions. The approximate length and diameter are 150 Å and 115 Å, respectively. The α-subunits are located at the ends, whereas the β-subunits form the two inner rings. The carboxy-terminal extension of β2 embraces adjacent β3, and the C-terminal extension of β7 intercalates between β1 and β2 in the opposing β-ring. **b** | Top views of the α-ring (upper panel) and the β-ring (lower panel). The C-terminal helices of the α3 and α4 subunits protrude from the core with high flexibility. Whereas the β-ring has an open space in its centre, the α-ring is closed by the amino-terminal extensions of the α-subunits.

PAC1 knockdown have additive effects on proteasome assembly; simultaneous depletion of PAC1 and PAC3 causes a severe reduction in α-rings and the 20S proteasome compared with depletion of PAC1 or PAC3 alone. Interestingly, whereas the knockdown of only PAC3 is not associated with the accumulation of off-pathway products, such as α-ring dimers that are observed in PAC1-depleted cells, simultaneous knockdown of PAC1 and PAC3 causes the accumulation of half-proteasomes that lack β5 (REF. 43). These findings suggest that PAC1–PAC2 and PAC3 work differently but cooperate in the assembly of α-rings and the correct formation of half-proteasomes.

Pba3 and Pba4, the budding yeast orthologues of human PAC3 and PAC4, were identified independently through bioinformatics searches[45] and genetic screens of mutations that suppress lethal DNA damage[44], confer sensitivity to amino acid analogues[46] or stabilize ornithine decarboxylase protein[49]. Pba3 and Pba4 form a heterodimer complex, and this complex can be co-purified with proteasome precursors that consist of all seven α-subunits and an unprocessed β2 subunit *in vivo*[46]. Pba3–Pba4 directly interacts with α5 *in vitro*, and the affinity-purified complex with tagged Pba3–Pba4 includes a large amount of α4, α5, α6 and α7 compared with other α-subunits[45,46]. These observations suggest that Pba3–Pba4 has a role at an early stage of α-ring assembly, starting with the formation of a Pba3–Pba4–α5 tertiary complex, which in turn recruits neighbouring α-subunits, with Pba3–Pba4 remaining bound until the incorporation of β2.

Deletion of Pba3 or Pba4 in cells markedly decreases 20S proteasomes and causes the accumulation of assembly intermediates. These cells show accumulation of dead-end complexes that comprise β2 and all α-subunits except α4 (REF. 46). However, Δ*pba4* cells produce an alternative proteasome that is found in Δα3 cells; 20–50% of the assembled 20S proteasomes in Δ*pba4* cells contained a second copy of α4 in place of α3 (REF. 45). Such alternative proteasomes confer resistance to heavy metal stress on Δ*pba3-pba4* cells as well as Δα3 cells, suggesting that proteasome-dedicated chaperones provide structural flexibility of the proteasome depending on the cellular environment[45]. Whether such alternative proteasomes would be observed in the absence of PAC3–PAC4 in mammalian cells remains to be elucidated. Taken together, these studies show that Pba3–Pba4 catalyses correct subunit orientation of the α-ring, presumably by facilitating the recruitment of α4 to α5, which might prevent incorporation of a second copy of α4 in the position that is normally occupied by α3 in the α-ring.

β-ring formation on the α-ring. The α-ring serves as a scaffold for the assembly of β-subunits. Catalytic β-subunits and non-catalytic β6 and β7 are synthesized with N-terminal propeptides, which are removed at the final step of assembly to expose the catalytic Thr residues of β1, β2 and β5. The N-terminal active sites of β subunits are on the inner surface of the β-rings, whereas the C termini of β subunits are on the outer surface of the 20S proteasome. During the assembly pathway from the α-ring through the half-proteasome, each β subunit assembles on the α-ring. An intermediate called the 13S complex, which is composed of one α-ring and unprocessed β2, β3 and β4, has been identified both in yeast and mammals, indicating the rank order of β-subunit positioning onto the α-ring[48,53,54].

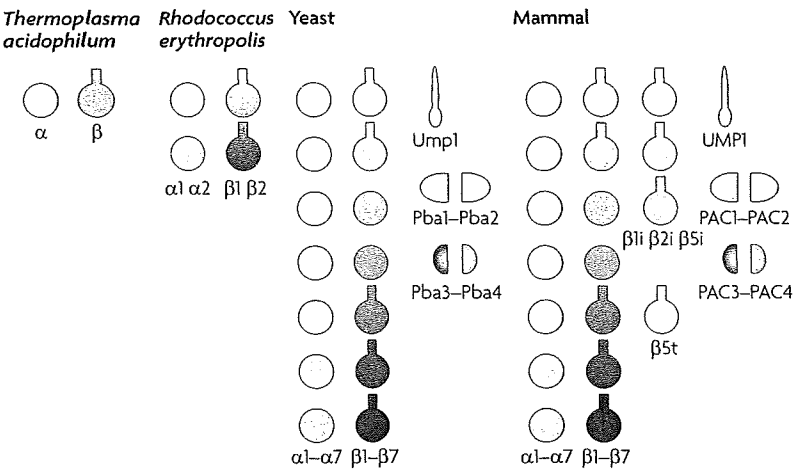
Recent studies in yeast showed that the addition of other β subunits, except β7, form another intermediate that is referred to as the half-mer (–β7) precursor complex[48,55]. siRNA-mediated silencing of each β-subunit in mammalian cells caused an accumulation of a specific 'assembly-arrested' intermediate just before incorporation of the knocked-down β-subunit[56]. This approach suggests the defined order of β-subunit assembly on the α-ring: assembly begins with β2, followed by β3, β4, β5, β6, β1 and finally β7 (REF. 56) (FIG. 3a). During β-ring assembly, release of PAC3 is coupled to β3 incorporation in human cells[43,56] (FIG. 3a). This is consistent with the observation that Pba3–Pba4 is selectively co-purified with β2 but not with other β-subunits in yeast[44,46], which indicates a conserved mechanism in the proteasome assembly between PAC3–PAC4 and Pba3–Pba4. As PAC3 directly binds to β3 *in vitro*, β3 might be recruited to the assembly intermediate by transient interaction with PAC3, whose release is an obligatory step in the incorporation of β3 (REFS 43,56).

Operon
A unit of genes in prokaryotes that is expressed as a single messenger RNA.

ERAD
(Endoplasmic reticulum-associated degradation). A system that ubiquitylates misfolded proteins in the endoplasmic reticulum for degradation by the 26S proteasome.

Unfolded protein response
A cellular stress response that is induced by the accumulation of unfolded proteins in the endoplasmic reticulum.

Box 2 | **Evolution of proteasome assembly in different organisms**



Most archaea and actinobacteria have one α- and one β-type subunit (see the figure), but their composition varies from one α-type and two β-type, two α-type and two β-type, and two α-type and one β-type subunits. Among the species that synthesize two β-type subunits, one of two β-type subunits is thought to be inactive in the *Sulfolobus*, *Pyrobaculum* and *Aeropyrum* species. Contrary to the diversity of the composition observed in prokaryotes, all eukaryotic 20S proteasomes consist of seven different α- and seven different β-type subunits. Of the seven β-type subunits, only three β-subunits, β1, β2 and β5, have hydrolytic activities. Primitive eukaryotes, such as yeast, have a single kind of 20S proteasome composed of seven distinct α- and seven distinct β-type subunits. However, many higher eukaryotes have paralogous genes. For example, all but α7, β1, β6 and β7 are duplicated in *Arabidopsis thaliana* and the paralogous α3, α4, α6, β2, β4 and β5 genes in *Drosophila melanogaster* are expressed in a male-specific manner, although the functions of these duplicated genes are unknown. In jawed vertebrates including mammals, the constitutive catalytic subunits β1, β2 and β5 are replaced with β1i, β2i and β5i by interferon (IFN)γ stimulation, which results in formation of the immunoproteasome. The immunoproteasomes, which have higher trypsin-like and chymotrypsin-like activities than standard proteasomes, are thought to have a role in better presentation of antigenic peptides on major histocompatibility complex (MHC) class I molecules. In the thymus, another catalytic subunit, β5t, is incorporated in the 20S proteasomes instead of β5 or β5i along with β1i and β2i. This thymus-specific proteasome is called thymoproteasome and is thought to have an essential role in positive selection of MHC class I restricted T cells, a process that increases useful T cell repertoire. Eukaryotes have acquired proteasome-dedicated extrinsic chaperones, namely ubiquitin-mediated proteolysis 1 (Ump1) or UMP1 and proteasome biogenesis-associated 1 (Pba1)–4 or proteasome assembling chaperone 1 (PAC1)–4, which coincides with the acquisition of subunit complexity.

Insight from the Pba3–Pba4–α5 complex structure. Crystal structure analysis has revealed that the tertiary structure of the Pba3–Pba4 heterodimer closely resembles that of the PAC3 homodimer, despite low sequence similarities[46] (FIG. 4a). However, it is uncertain whether the PAC3 homodimers exist *in vivo*. Intriguingly, Pba3–Pba4 and PAC3 have a β-sandwich structure that is formed by two six-stranded β-sheets surrounded by two helices on each side. This structure resembles those of α- and β-subunits, although the two β-sheets in α- and β-subunits are made up of five β-strands[46] (FIG. 4b). Analysis of the Pba3–Pba4–α5 complex revealed that Pba3–Pba4 is attached to the surface of the α-ring where β-subunits are assembled (FIG. 4c). However, the binding mode of Pba3–Pba4 is different from that of β-subunits; Pba3–Pba4 is located at the more inner space of the α-ring, which enables Pba3–Pba4 to interact with three different α-subunits (α4, α5 and α6), compared with β-subunits that interact with two neighbouring α-subunits (FIG. 4c). This feature of Pba3–Pba4 might be useful in initiating α-ring assembly. This location of Pba3–Pba4 is consistent with biochemical analyses that indicate that Pba3–Pba4 or PAC3 detach from α-rings during β-ring formation (FIG. 3a).

Structural analyses of PAC1–PAC2 and Pba1–Pba2 are not available, but the observation that the binding of PAC1–PAC2 or proteasome activator 28 kDa (PA28; also known as PSME1) to assembly intermediates is mutually exclusive suggests that the binding surface of PAC1–PAC2 on the α-ring is opposite to that of PAC3 (REF. 56). PA28 is an activator complex that is known to bind to the outer surface of the α-ring in place of 19S RP and is involved in major histocompatibility complex (MHC) class I antigen presentation[57,58].

Dual role of Ump1 in β-ring formation and dimerization. Before the discovery of Ump1, the 20S proteasome was presumed to assemble autonomously. In yeast, *ump1* mutants are defective in ubiquitin-mediated proteolysis, and Ump1 was the first identified extrinsic assembly factor for 20S proteasomes[52]. Ump1 is not essential for cell viability, but Δ*ump1* cells exhibit significant growth defects and hypersensitivity to various stresses. Ump1 is specifically associated with assembly intermediates of 20S proteasomes and seems to enter the assembly pathway after association of β2, β3 and β4 in yeast[48]. Following dimerization of half-proteasomes, Ump1 is encapsulated and degraded within the newly formed 20S proteasome[52]. Loss of Ump1 causes the accumulation of assembly intermediates and 20S proteasomes that contain unprocessed β-subunits, which indicates that Ump1 coordinates the processing of β-subunits and dimerization of half-proteasomes.

The human orthologue of Ump1 was identified by a database search and an amino acid sequence analysis of a small precursor proteasome protein and was named UMP1 (REF. 59), proteassemblin[60] or proteasome maturation protein (POMP)[61]. UMP1 is included in precursor proteasomes with unprocessed β-subunits and is degraded on the completion of proteasome assembly with a similar half-life to PAC1–PAC2 (REF. 42). However, knockdown of UMP1 does not cause the accumulation of assembly intermediates that contain unprocessed β-subunits, which contrasts with Δ*ump1* cells, which accumulate such intermediates[42,43,52,56]. Furthermore, knockdown of UMP1 impairs β5 recruitment[62], and UMP1 can bind to the α-ring in the absence of any β-subunits *in vitro*[63], suggesting that it is incorporated into proteasome precursors earlier than yeast Ump1 and that UMP1 is required for β-ring formation.

Subsequent studies showed that UMP1 knockdown results in the accumulation of α-rings that do not contain β-subunits, and that incorporation of UMP1 is coupled with β2 incorporation *in vivo*: UMP1 is required for β2 incorporation, and vice versa[56] (FIG. 3a). Therefore, UMP1 is required for the initiation of β-ring formation, a role that has not been observed for yeast Ump1. Furthermore, UMP1 also regulates

Table 1 | **Proteasome assembling chaperones in humans and yeast**

Humans				Yeast				
Name	**Alias**	**HUGO**	**Length (aa)**	**Name**	**Alias**	**Systematic gene name**	**Length (aa)**	**Sequence identity**
Proteasome assembling chaperones								
PAC1	DSCR2	PSMG1	288	Pba1	Poc1	YLR199C	276	<10%
PAC2	HCCA3	PSMG2	264	Pba2	Add66 and Poc2	YKL206C	267	19%
PAC3	MGC10911	PSMG3	122	Pba3	Poc3, Dmp2 and Irc25	YLR021W	179	<10%
PAC4	C6orf86	PSMG4	123	Pba4	Poc4 and Dmp1	YPL144W	148	<10%
UMP1	Proteassemblin	POMP	141	Ump1	Rns2	YBR173C	148	24%
α-subunit								
α5	Zeta	PSMA5	241	α5	Pup2 and Doa5	YGR253C	260	60%

For comparison, we provide information on the α5 subunit. aa, amino acids; Doa5, degradation of α2; DSCR2, down syndrome critical region gene 2; HCCA3, hepatocellular carcinoma associated gene 3; MGC10911, mammalian gene collection 10911; PAC, proteasome assembling chaperone; Pba, proteasome biogenesis-associated; POMP, proteasome maturation protein; Rns, RNase hypersensitive; UMP1, ubiquitin–mediated proteolysis 1.

the recruitment of precursor proteasome at the endoplasmic reticulum, which seems to be the main location of proteasome assembly in mammalian cells[63].

Intramolecular chaperones promote proteasome assembly. Propeptides of some proteases are known to facilitate their own folding or molecular assembly, working as intramolecular chaperones[64]. The propeptides and tails of 20S proteasome β-subunits have such roles. Bacterial *R. erythropolis* β-subunit propeptides promote subunit folding as well as proteasome assembly[38,39]. The roles of N-terminal propeptides and C-terminal tails of β-subunits have been studied in yeast and humans and these C-terminal tails have been shown to have important roles in proteasome assembly by providing specific interactions with *cis*- and *trans*-β-rings. The propeptide of β5 facilitates its incorporation into the 20S proteasome and is essential for yeast viability[65]. In human cells, β5 propeptide is not required for its own incorporation but rather for β6 recruitment[56]. The propeptides of β1 and β2 are dispensable for cell viability but are known to protect the N-terminal catalytic Thr residue of β1 and β2 against N^{α}-acetylation, and mutants lacking these two propeptides display modest defects in proteasome biogenesis[66]. In human cells, loss of β2 propeptide leads to failure of β3 recruitment and is therefore fatal[56]. The C-terminal tail of β2, which wraps around β3 in the same β-ring, is also essential for proteasome biogenesis both in yeast and human cells[6,7,56,67] (FIG. 2). The C-terminal tail of β7, which is inserted into a groove between β1 and β2 in the opposite ring, also has an important role in the dimerization of half-proteasomes[6,7,55,56,67] (FIG. 2) as well as stabilization of the active conformation of β1 (REF. 67).

Interestingly, the propeptides of human β-subunits are different from those of yeast counterparts, compared with the mature β-subunits, which are well conserved between yeast and humans (Supplementary information S1 (figure)). Such differences are also noted in the proteasome-dedicated chaperones (TABLE 1). It might be assumed that non-essential propeptides and chaperones do not need to be highly conserved during evolution, whereas their basic functions and tertiary structures are maintained: PAC3–PAC4 and Pba3–Pba4 are good examples (see above). However, the C-terminal extension of β2, which has an essential role by directly associating with β3, is highly conserved (Supplementary information S1 (figure)). In this sense, it is curious that the essential β5 propeptide is poorly conserved (Supplementary information S1 (figure)). As the β5 propeptide associates both physically and functionally with Ump1 or UMP1 (see below), the β5 propeptide might have evolved to work cooperatively with Ump1 and its orthologues, the sequences of which have significantly changed during evolution.

Dimerization of the half-proteasome. Incorporation of β7 into half-mers (–β7) and intercalation of its C-terminal tail between β1 and β2 of the opposing β-ring triggers dimerization of the half-proteasomes in yeast and human[48,55,56,67]. Overexpression of β7 suppresses the lethality of a β5 mutant that lacks its propeptide (β5Δpro), which suggests that β5 propeptide has a partially redundant role for half-mer dimerization. Intriguingly, Δ*ump1* and β6Δpro also rescued the lethality of β5Δpro, and Δ*ump1* rescued the lethality of β6ΔNTE (NTE stands for N-terminal extension; a unique N-terminal nine residue sequence in mature β6). These observations suggest that β7, β5 propeptide and β6 NTE promote proteasome assembly, whereas Ump1 and β6 propeptide have an inhibitory role on dimerization of half-mers. Ump1 could be an assembly checkpoint factor that inhibits this dimerization until a full set of β-subunits are recruited on to the α-ring, and thus mediates productive proteasome assembly[48].

Bleomycin-sensitive 10 (Blm10) was identified as a component of proteasome precursors that have been purified with Ump1 in yeast[68]. Δ*blm10* cells grow apparently normally under normal conditions but show mild temperature sensitivity. The turnover of Ump1 and the processing of β5 are accelerated in Δ*blm10* cells, which suggests a role for Blm10 in preventing the premature formation of 20S proteasomes[68].

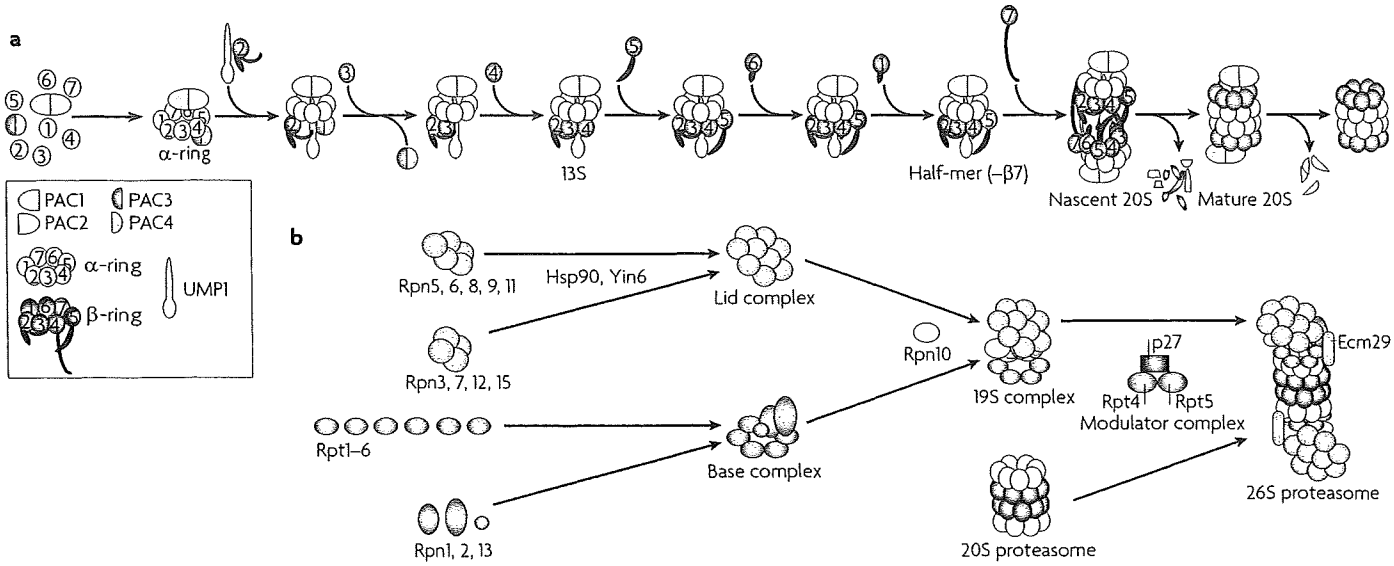


Figure 3 | **Model for proteasome assembly. a** | The assembly of human 20S proteasomes. Proteasome assembling chaperone 1 (PAC1)–PAC2 heterodimers and PAC3–PAC4 complexes assist α-ring formation. PAC1–PAC2 suppresses off-pathway aggregation of α-rings. Sequential incorporation of β-subunits starts from the association of β2 and ubiquitin-mediated proteolysis 1 (UMP1) on the α-ring. UMP1 is required for association of β2 in the early assembly intermediates. PAC3–PAC4 is released following the association of β3 and is recycled. Subsequent orderly incorporation of other β-subunits is assisted by intramolecular chaperones such as the propeptides of β2 and β5 and the carboxy-terminal tail of β2. Dimerization of half-proteasomes without β7, also called half-mers (–β7), is triggered by incorporation of β7 and intercalation of its C-terminal tail into the groove between β1 and β2 of the opposing β-ring. This is followed by removal of β-subunit propeptides (β1, β2, β5, β6 and β7) and UMP1 degradation. PAC1–PAC2 is subsequently degraded by the newly formed active 20S proteasomes. **b** | The assembly of 19S regulatory particle (RP) is largely unknown, but it is suggested that the base and the lid are assembled independently. In yeast, Yin6 and heat-shock protein 90 (Hsp90) seem to be involved in the assembly of the lid, which is thought to be formed from two different subclusters. Regulatory particle non-ATPase 10 (Rpn10) stabilizes the connection between the lid and the base. In yeast, extracellular matrix 29 (Ecm29) has a role in tethering the 20S to the RP. In mammals, the so-called modulator complex facilitates the association of the RP with the 20S by an unknown mechanism. Rpt, regulatory particle triple-A.

However, the combination of Blm10 deletion and β7 C-terminal truncation results in severe impairment of proteasome activity and β2 processing, indicating that Blm10 promotes proteasome maturation, presumably by stabilizing nascent 20S proteasomes[55]. A single model that can account for these two conflicting roles of Blm10 remains elusive at this stage. Additional functions beyond 20S maturation have been reported for Blm10 and its mammalian homologue PA200 (also known as PSME4). Blm10 and PA200 bind to the ends of mature 20S proteasomes and open the axial channel, thus enhancing peptide hydrolysis, most notably after acidic residues, which is responsible for a role for Blm10 and PA200 in maintaining genomic stability[69–74].

The 19S RP might also participate in stabilizing nascent 20S proteasomes, because the RP associates with an Ump1-containing complex in the absence of Blm10, and an *rpn2* mutant that is defective in the 19S RP–20S interaction shows a large accumulation of precursor complexes that contain unprocessed β2 when combined with *Δblm10* (REF. 55).

The correct dimerization of half-proteasomes is followed by the removal of β-propeptides and degradation of Ump1 coincides with the completion of proteasome maturation, followed by degradation of PAC1–PAC2 (REFS 42,52) (FIG. 3a).

Tissue specific proteasomes

Vertebrates encode four additional catalytic β-subunits: three interferon (IFN)γ-inducible β1i, β2i, β5i immunosubunits and one thymus-specific β5t subunit, which are incorporated in the place of their most closely related β-subunits, thus forming distinct subtypes of proteasomes with altered catalytic activities. These are called immunoproteasomes and thymoproteasomes[75–77]. These alternative proteasomes have key roles in acquired immunity by altering antigen processing. The immunoproteasome has increased chymotrypsin-like and trypsin-like activities, which are favourable for the production of antigenic peptides that bind to the groove of MHC class I molecules[76,78]. The thymoproteasome has reduced chymotrypsin-like activity, which is thought to be important for the production of a unique peptide repertoire in the thymus[77,79,80]. By comparison, the 20S proteasome, including constitutively expressed catalytic subunits β1, β2 and β5, is often called the standard or constitutive proteasome.

In *Drosophila melanogaster*, approximately one-third of the proteasome subunits are found to have testes-specific isoforms[81]. One of these, proteasome subunit α6 testis-specific (PROSα6T), is required for spermatogenesis[82]. However, whether there are specific mechanisms for the assembly of such testes-specific subtypes has not been explored.

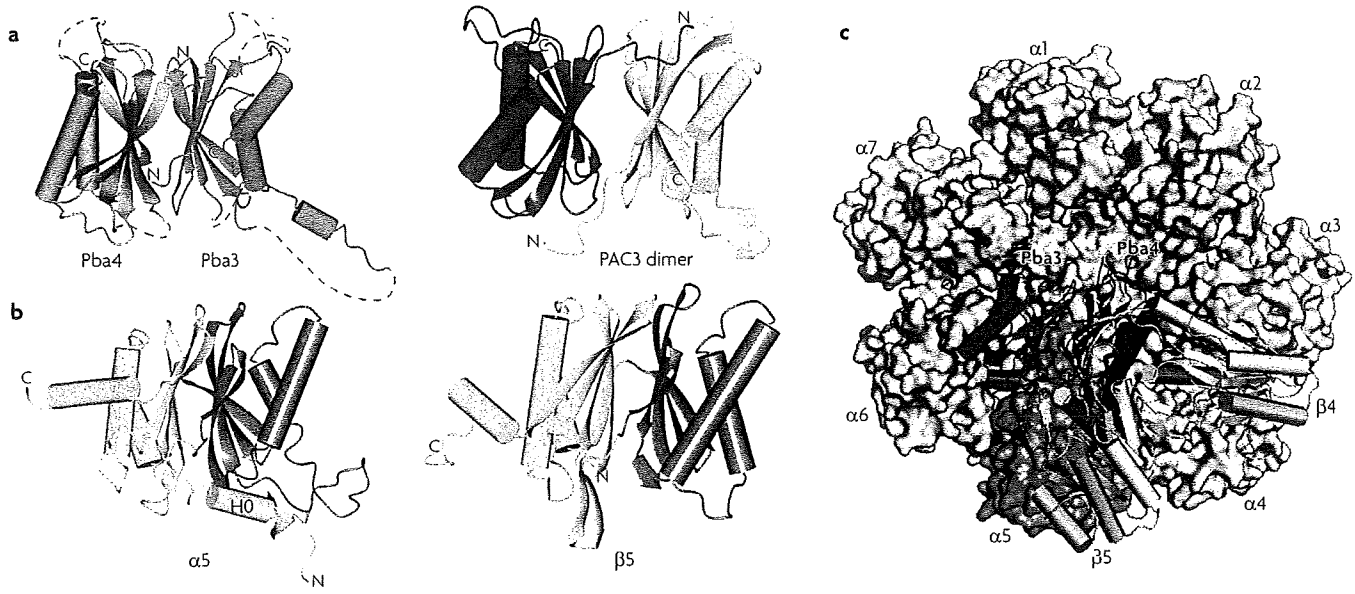


Figure 4 | **Structural basis of Pba3–Pba4 and PAC3. a** | Ribbon diagrams of the proteasome biogenesis-associated 3 (Pba3)–Pba4 heterodimer (in yeast) and the proteasome assembling chaperone 3 (PAC3) homodimer (in mammals). Both complexes have β-sandwich structures that are formed by two six-stranded β-sheets, and these sandwich structures are surrounded by four helices. α-helices and β-strands are shown by cylinders and arrows, respectively. **b** | Ribbon diagrams of the α5 and β5 subunits. All α- and β-subunits have the same basic structures, except the helical structure of H0 at the amino terminus of the α-subunits. **c** | Model of the Pba3–Pba4–α-ring–β4–β5 complex. Pba3–Pba4, β4 and β5 are shown in ribbon presentations. Pab3–Pba4 is located deeper in the α-ring compared with β-subunits and thus interacts with three α-subunits, α4, α5 and α6, whereas β5 interacts with only two α-subunits, α4 and α5. This model also shows that the presence of Pba3–Pba4 on the α-ring causes steric hindrance with β4.

The immunoproteasome. The mechanism of immunoproteasome assembly has been well studied. The propeptides of the immunosubunits and UMP1 have key roles in this process. Similar to the standard proteasome, the immunoproteasome is assembled in a stepwise manner, but one remarkable difference is that β1i enters the assembly pathway of the immunoproteasome earlier than in standard proteasome assembly, forming an assembly intermediate containing an α-ring, β1i, β2i, β3 and β4 (REF. 54). In this intermediate, the incorporation of β2i depends on β1i, and the incorporation of β1i is in turn facilitated by β2i, although β1i can be incorporated in the absence of β2i[83–85]. β5i is incorporated preferentially over β5 into the intermediates that contain β1i and β2i and is required for the processing of β1i and β2i, which is dependent on the β5i propeptide but not on β5i catalytic activity[84,85]. Such interdependency provides homogenous formation of immunoproteasomes that contain all three inducible subunits. β5i can also be incorporated into the standard proteasome. Consistent with these observations, β2i processing and incorporation is severely impaired in β1i-deficient cells, and β1i incorporation is partially impaired in β2i-deficient cells, whereas β5i incorporation is not affected in both cells[86,87]. β5i-deficient cells that have been treated with concanavalin-A, a lectin that is known to activate lymphocytes, exhibit significant retardation of proteasome assembly and accumulation of proteasome precursors containing unprocessed β1i and β2i[86].

Intriguingly, IFNγ stimulation increased the transcription of *UMP1* mRNA along with that of immunosubunits[59–61], but it decreased UMP1 protein levels, thereby accelerating its turnover by approximately fourfold[62]. This rapid turnover was coupled with the maturation of active immunoproteasomes, which indicates that the generation of immunoproteasomes is fourfold faster than that of standard proteasomes[62]. The higher affinity of UMP1 to β5i compared with β5 is likely to contribute to the rapid maturation of immunoproteasomes[62]. Through these mechanisms, immunoproteasomes are preferentially assembled over standard proteasomes, despite the coexistence of both immunosubunits and standard subunits.

The thymoproteasome. Another vertebrate-specific 20S proteasome is the thymoproteasome, which is exclusively expressed in cortical thymic epithelial cells (cTECs). cTECs are responsible for a process that is referred to as positive selection, which enriches the useful repertoire of developing T cells in the thymus[77]. The catalytic subunits of the thymoproteasome are composed of β1i, β2i and β5t. Ectopically expressed β5t in a human cell line that does not express immunosubunits can be readily processed and incorporated into the proteasome[77], suggesting that β5t is preferentially incorporated over β5 and that β1i and β2i are not prerequisites for its incorporation. As most proteasomes in cTECs are thymoproteasomes, it is presumed that β5i is also expressed in cTECs and that β5t is preferentially incorporated over β5i.

The propeptide or exceptionally extended C-terminal tail (51 amino acids) of β5t might be involved in the assembly of thymoproteasomes, but there is no evidence in support of this.

Assembly of the 19S RP

The 19S RP is found only in eukaryotes. Compared with the 20S proteasome, the mechanism involved in the assembly of the 19S RP is mostly unknown. In an N-terminally truncated mutant of Rpn2, a base subunit, 26S proteasomes were almost completely abolished at the restrictive temperature, but the complete lid complex was observed in yeast[88]. Conversely, the base complex was found in the *rpn5-1* lid mutant[88]. These findings suggest that the base and the lid are assembled independently, after which these two subcomplexes are joined (FIG. 3b). However, accumulation of Rpt5-containing particles of various sizes was detected in cells with impaired 20S proteasome formation (Δ*ump1*, Δ*pba3* and Δ*pba4*) or Δα3 cells[45]. This indicates erroneous RP formation in the presence of defective 20S proteasomes, raising the possibility that the 20S proteasome functions as an assembly factor for the RP[45].

Putting together the base. The base is composed of six related AAA$^+$ ATPase and three non-ATPase subunits. The six ATPase subunits are thought to form a ring-like structure by analogy to other AAA$^+$ ATPase complexes, such as cell division cycle 48 (Cdc48) and proteasome-activating nucleotidase, which are successfully assembled into homohexamers when expressed in *E. coli*[34,89]. However, coexpression of the six proteasome ATPases in *E. coli* failed to yield a heterohexameric complex[90]. Rather, coexpression of Rpt1 and Rpt2 or solo expression of Rpt4 yielded high molecular weight complexes[90]. These results suggest the existence of chaperones that discriminate and arrange the six homologous ATPase subunits in a defined order, as is observed in the assembly of 20S α-ring. Recently, a model in which the RPN1–RPN2 complex is located as the central unit of the base, around which the six ATPase subunits wrap to complete the base, has been presented. This might suggest a role of RPN1–RPN2 as a platform for base assembly[91].

Several extrinsic factors of unknown functions that associate with certain base subunits have been identified: p27 forms a complex with RPT4 and RPT5 (REF. 92), p28 (also known as gankyrin) with RPT3 (REFS 93–95), and subunit 5b (S5b) with RPT1, RPT2 and RPN1 (REF. 19). These subcomplexes might be assembly intermediates of the base complex, and these non-proteasomal factors might act as chaperones to help assemble the base complex correctly.

Assembling the lid. Based on the detection of partially assembled lid subcomplexes in yeast lid mutants[96] and results of mass spectrometry analysis of intact lid[97], two clusters in the lid complex have been proposed: one is composed of Rpn5, Rpn6, Rpn8, Rpn9 and Rpn11 and the other of Rpn3, Rpn7, Rpn12 and Rpn15, in which the interaction of Rpn3 and Rpn5 connects the two clusters (FIG. 3b). These observations are consistent with the reported subunit interaction maps that are based on yeast two-hybrid analysis[98,99]. However, whether these clusters are authentic assembly intermediates and, if so, how these clusters are assembled, remains elusive.

Hsp90, the most abundant molecular chaperone known to assist in protein folding and cell signalling[100], is thought to have a role in both the assembly and maintenance of the lid in yeast[101]. Inactivation of Hsp90 in yeast led to disassembly of the lid complex, which was then reassembled into the 26S proteasome by reactivation of Hsp90 *in vivo* or by adding normal Hsp90 and ATP *in vitro*[101]. These findings suggest that the ATP-dependent chaperone activity of Hsp90 contributes to the assembly of the lid as well as the 26S proteasome. However, the molecular role of Hsp90 in the 26S proteasome assembly remains to be identified.

Schizosaccaromyces pombe Yin6 is the homologue of the mammalian oncoprotein INT6. It has a proteasome–COP9–initiation factor domain (PCI domain) and is known to interact with the 26S proteasome through Rpn5 (REF. 102). The lid itself also contains four PCI domain proteins (Rpn3, Rpn5, Rpn6 and Rpn9) and the PCI domain is suggested to function in protein–protein interaction. In Δ*yin6* cells, Rpn5 does not efficiently incorporate into 26S proteasomes owing to mislocalization of Rpn5, which results in defective 26S proteasome formation[102]. Overexpression of Rpn7 rescues proteasome mislocalization or disassembly in Δ*yin6* cells, suggesting further functional interaction between Yin6 and the 26S proteasome[103]. However, there is no evidence for mammalian INT6 being involved in the assembly of 26S proteasomes. Furthermore, INT6 binds not only to the lid but also to the COP9 signalosome and eukaryotic translation initiation factor 3 (eIF3)[104], and therefore the role of INT6 and Yin6 might not necessarily reflect a role in proteasome function.

Regulation of 20S–RP interactions

The eukaryotic 20S proteasome is a latent protease on its own because the centre of the α-ring, through which unfolded polypeptides enter the chamber of the 20S proteasome, is gated by the N termini of the α-subunits[6,105]. Association of the C termini of RPT2 and RPT5 of the base is proposed to open the gate to the chamber of the 20S and enables the 20S proteasome to degrade proteins by sticking the conserved C-terminal residues of RPT2 and RPT5 into pockets between α-subunits[106–108]. ATP binding to the ATPase subunits is required for this interaction[107–109]. Thus, regulation of 20S–RP interactions is a crucial step in proteasome function.

Intriguingly, in a yeast lid mutant (*rpn5-1*) that exhibits defective lid assembly but normal base and 20S assembly, the base and the 20S proteasome could not form a stable complex, suggesting that the association of the lid with the base induces a conformational change of the base that is necessary for the stable association of the RP with the 20S proteasome[88]. Inhibition of proteasome active sites also stabilized 26S proteasomes, suggesting that the interface between the RP and the 20S proteasome dynamically changes depending on the activities of the 20S proteasome[110]. However, it has been reported that the disassembly of the 26S proteasome

Cdc48
A chaperone-like AAA$^+$ ATPase that is required for various cellular processes, such as cell cycle progression, homotypic membrane fusion and ERAD. The human counterpart of Cdc48 is valosin-containing protein.

Proteasome-activating nucleotidase
An AAA$^+$ ATPase ring complex and archaeal regulatory particle triple-A homologue that opens the gate of 20S proteasomes and activates protein degradation.

PCI domain
The homology domain of unclear function that is present in several components of the proteasome, the COP9 signalosome and eukaryotic translation initiation factor 3.

COP9 signalosome
An eight-subunit protein complex that regulates protein ubiquitylation and turnover in various developmental and physiological contexts. Extensively characterized in plants but fundamental to all eukaryotes, this complex post-translationally modifies the cullin subunit of E3 ubiquitin ligases by cleaving the covalently coupled polypeptide Nedd8.

and even dissociation of the RP into subcomplexes or subunits are induced following ATP-dependent degradation of a substrate protein in yeast[111]. By contrast, mammalian 26S proteasomes can degrade substrate proteins without any release of subunits or subcomplexes[112], and therefore this 'chew and spew' model observed in yeast is still controversial[112–114].

Some auxiliary factors have been shown to regulate RP association with the 20S proteasome. Extracellular matrix 29 (Ecm29) is a protein of approximately 200 kDa and can bind to both the RP and the 20S proteasome in yeast[115]. Purified 26S proteasomes from Δ*ecm29* cells tend to dissociate into RPs and 20S proteasomes. Together with the findings of electron micrographs of Ecm29–20S proteasome complexes, it is likely that Ecm29 stabilizes 26S proteasomes by tethering the 20S proteasome to the RP[115], a function that can overcome even the absence of ATP[110]. The so-called modulator complex, which is a complex of p27, RPT4 and RPT5 and is found in mammalian cells, enhanced the proteolytic activity of the proteasome by promoting the association of RPs with 20S proteasomes[116,117] (FIG. 3b). However, the mechanism of this action remains unknown. Nob1, which was identified as a binding partner of Rpn12 and Pno1, was reported to interact with the RP–half-proteasome precursor that contains Ump1, and is required for this precursor maturation, resulting in both 20S and 26S proteasome formation in yeast[118]. However, Nob1 was also reported as an endonuclease for processing of the 20S pre-rRNA to the mature 18S rRNA, and its role in proteasome biogenesis has been challenged[5,119]. Discrepancies between these results remain to be reconciled.

Concluding remarks

The regulation of proteasome activity in tumour cells is clinically important[120]. Based on the successful clinical use of bortezomib, an inhibitor of proteasomal catalytic activity, in the treatment of multiple myeloma, attempts to broaden its application for other types of malignant neoplasms as well as development of new proteasome inhibitors are currently underway[120–122]. In contrast to increased proteasome activities in tumour cells, decreased proteasome activities have been suggested in neurodegenerative diseases and ageing[123–126]. In addition, it has been reported that a single nucleotide polymorphism in the *PSMA6* gene that increases the expression of the α1 subunit confers susceptibility to myocardial infarction[127], although the precise mechanism is unclear. Therefore, elucidating proteasome regulation at various steps, such as transcription, assembly, catalytic activity and localization, could provide clues to treatment of these disorders.

Among these potential regulatory processes, the assembly mechanism of the 20S proteasome is well studied. 20S proteasome assembly in yeast and human cells have been proven to closely resemble each other, sharing common assembly chaperones as well as intramolecular chaperones, although some of their roles are slightly different between yeast and humans. As the crystal structure of the mammalian 20S proteasome is known, it might be feasible to design drugs that inhibit contact surfaces that are important for the assembly between subunits or subunits and assembly chaperones. Furthermore, the mechanism of RP assembly should be studied more extensively. At present, we do not even know the initial events or the requirements for certain assembly chaperones in the RP assembly. Extensive genetic screening has been done to identify genes that modulate the ubiquitin–proteasome pathway, and numerous proteasome mutants and mutants that affect the 20S proteasome assembly have been identified. However, the exact molecules that are essential for RP assembly remain undiscovered at present. This could also suggest that the assembly of the 19S RP is autonomous, these molecules have been simply overlooked, or a novel and alternative screening approach is needed to identify such molecules.

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Entrez Gene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
ump1
UniProtKB: http://www.uniprot.org
DSS1 | PAC1 | PAC2 | PAC3 | PAC4 | Pba1 | Pba2 | Pba3 | Pba4 | RPN1 | RPN2 | RPN3 | RPN5 | RPN9 | RPN10 | RPN11 | RPN12 | RPN13 | RPT1 | RPT5 | RPT6 |

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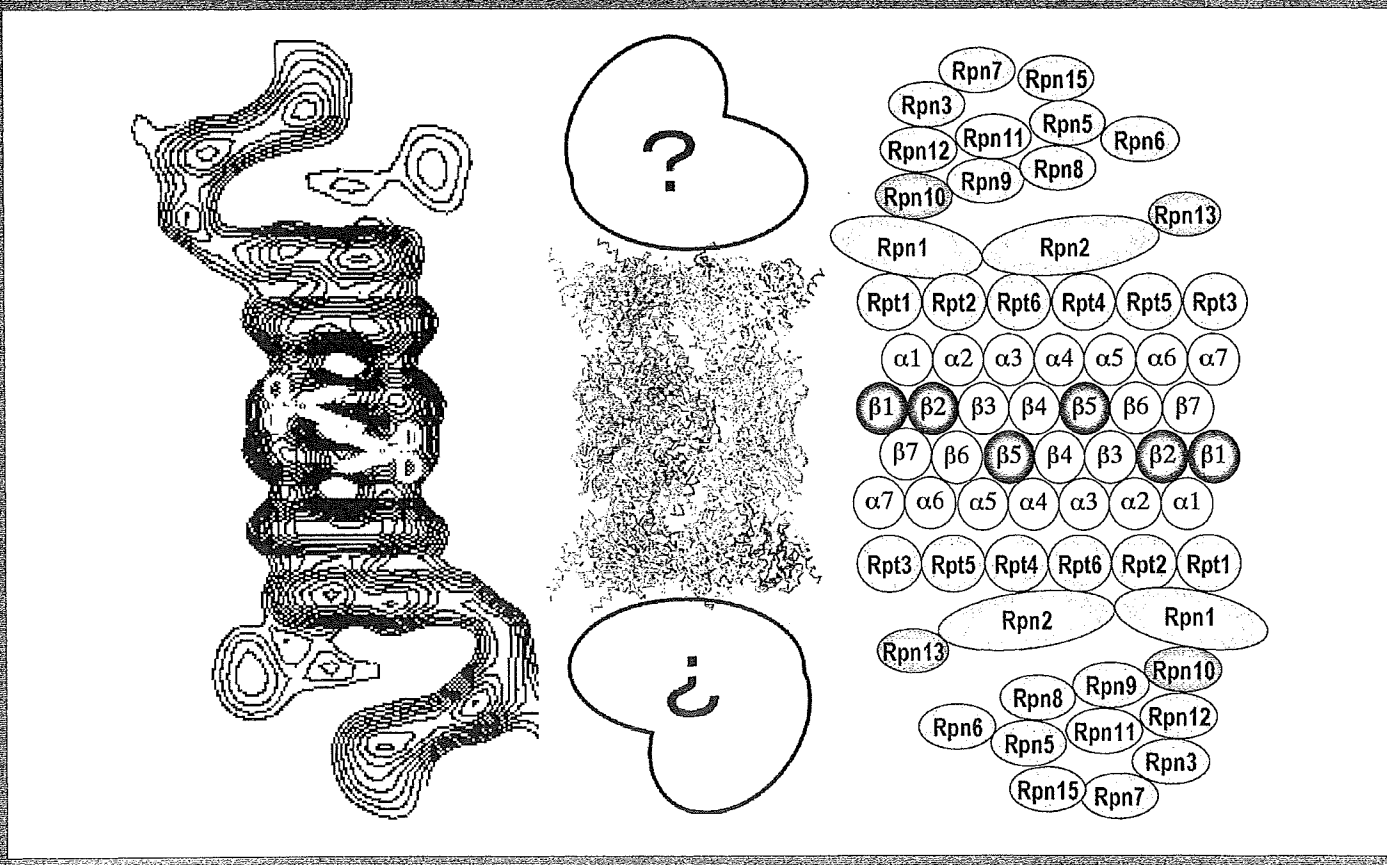

Molecular organization of the 26S proteasome

Cover Illustration: The structure and subunits of the 26S proteasome consisting of the 20S proteasome (the 20S core particle) and the 19S regulatory particle.

The 26S proteasome (left panel, its averaged image based on electron micrographs) is made up of two subcomplexes, the 20S core complex (CP) and the 19S regulatory particle (RP). Much attention is now focused on the structure and functions of the 26S proteasome because it functions as a protein-destruction machine in ubiquitin-dependent proteolysis, which plays essential roles in almost all cellular events such as cell cycle progression, signal transduction, cell death, immune responses, metabolism, protein quality control and development. The 20S CP (also known as the 20S proteasome) is classified as a threonine protease that contains two pairs of three different catalytic sites (see the left panel, the two scissors shown in yellow). The middle panel shows its overall tertiary structure, whereas the structures of the terminal 19S RP have not yet been determined and are depicted by question marks. The 26S proteasome consists of many different subunits (right panel). The central 20S CP forms an $\alpha_{1\text{-}7}\beta_{1\text{-}7}\beta_{1\text{-}7}\alpha_{1\text{-}7}$ structure with axial stacking of two outer α and two inner β rings, which are made up of seven similar α and β subunits, respectively: β1, β2 and β5 contain catalytic sites with caspase-like, trypsin-like and chymotrypsin-like activities, respectively. The 19S RP comprises approximately 20 different subunits that can be classified into Rpt and Rpn ones and comprises two subcomplexes, the lid and the base: The former subcomplex is composed of at least nine Rpn subunits (Rpn3, Rpn5, Rpn6, Rpn7, Rpn8, Rpn9, Rpn11, Rpn12 and Rpn15), while the latter is composed of six Rpt (Rpt1-Rpt6) and four Rpn (Rpn1, Rpn2, Rpn10 and Rpn13) subunits. Rpn10 and Rpn13 function as ubiquitin receptors that trap polyubiquityalted proteins, Rpn11 functions as a metalloprotease that cleaves the polyubiquitin chains tagged to client proteins at a proximal site and a hexameric ring containing Rpt1-Rpt6 promotes unfolding of client proteins and the gate opening of the 20S CP. There are several subtypes of the vertebrate proteasomes: The immunoproteasome and the thymoproteasome, which have different subunit structures and substrate specificities from those of the standard (or constitutive) proteasome, play important roles in antigen processing and positive selection of developing thymocytes, respectively. In addition, various proteasome inhibitors have been developed and one of the inhibitors has recently been approved for the treatment of multiple myeloma.

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Review

The proteasome: Overview of structure and functions

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(Communicated by Takao SEKIYA, M.J.A.)

Abstract: The proteasome is a highly sophisticated protease complex designed to carry out selective, efficient and processive hydrolysis of client proteins. It is known to collaborate with ubiquitin, which polymerizes to form a marker for regulated proteolysis in eukaryotic cells. The highly organized proteasome plays a prominent role in the control of a diverse array of basic cellular activities by rapidly and unidirectionally catalyzing biological reactions. Studies of the proteasome during the past quarter of a century have provided profound insights into its structure and functions, which has appreciably contributed to our understanding of cellular life. Many questions, however, remain to be elucidated.

Keywords: proteasome, ubiquitin, intracellular proteolysis, multisubunit complex, molecular chaperone

Introduction

The proteasome is a large protein complex responsible for degradation of intracellular proteins, a process that requires metabolic energy. Polymerization of ubiquitin, a key molecule known to work in concert with the proteasome, serves as a degradation signal for numerous target proteins; the destruction of a protein is initiated by covalent attachment of a chain consisting of several copies of ubiquitin (more than four ubiquitin molecules), through the concerted actions of a network of proteins, including the E1 (ubiquitin-activating), E2 (ubiquitin-conjugating) and E3 (ubiquitin-ligating) enzymes.[1),2)] The polymerized ubiquitin chain acts as a signal that shuttles the target proteins to the proteasome, where the substrate is proteolytically broken down. For accurate selection of the proteins, numerous enzymes (e.g., 2 E1 proteins, approximately 30 E2 proteins and more than 500 different species of E3 in humans) are mobilized with this cascade system. The set of E3 proteins is highly diverse, because each E3 enzyme usually selectively recognizes a protein substrate for ubiquitylation. Furthermore, it should be noted that ubiquitylation is a reversible reaction, because many cysteine-protease and metalloprotease deubiquitylating enzymes (DUBs) are present in the cell. Interestingly, the human genome encodes approximately 95 putative DUBs.[3)] Certain DUBs are responsible for the maturation of ubiquitin from its precursor proteins and products of genes that encode polyubiquitin or ubiquitin fused with ribosomal proteins. Other DUBs function at the initial stage during the breakdown of ubiquitin-tagged proteins to allow ubiquitins to be recycled. The ubiquitin–proteasome system (UPS) controls almost all basic cellular processes—such as progression through the cell cycle, signal transduction, cell death, immune responses, metabolism, protein quality control and development—by degrading short-lived regulatory or structurally aberrant proteins.[4)-6)] The divergent roles of the UPS have been reported in detail and reviewed comprehensively.[1)-6)] In this review, I provide an overview of the structure and functions of uniquely specified proteasomes. Due to space limitations, I have primarily cited review articles with the exception of particularly important or recently published papers.

1. 26S and 30S Proteasomes

The proteasome is made up of two subcom-

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Table 1. Subtypes of proteasomes and their regulators

	Other nomenclature
Catalytic 20S Proteasomes	
Standard (or Constitutive) Proteasome	20S Core Particle (CP)
Immunoproteasome	
Thymoproteasome	
Testis-specific Proteasome	
Regulators	
PA700	19S Regulatory Particle (RP)
PA200	Blm10
PA28$\alpha\beta$	11S Regulator (REG)
PA28γ	
Active Proteasomes	
PA700–CP–PA700 (19S–20S–19S)	30S Proteasome
PA700–CP (19S–20S)	26S Proteasome
PA200–CP–PA200	
PA200–CP	
PA28$\alpha\beta$–CP–PA28$\alpha\beta$ (11S–20S–11S)	
PA28$\alpha\beta$–CP*	
PA28$\alpha\beta$–CP–PA700	Hybrid Proteasome
PA28γ–CP–PA28γ	
PA28γ–CP*	
PA28γ–CP–PA700*	
PA200–CP–PA700**	
PA28$\alpha\beta$–CP–PA200*	
PA28γ–CP–PA200*	

*unidentified complex; **referred to as alternative hybrid proteasome

plexes: a catalytic core particle (CP; also known as the 20S proteasome) and one or two terminal 19S regulatory particle(s) (RP) that serves as a proteasome activator with a molecular mass of approximately 700 kDa (called PA700) (Table 1).[7)–9)] The 19S RP binds to one or both ends of the latent 20S proteasome to form an enzymatically active proteasome. The apparent sedimentation coefficient of the active proteasome as determined by density-gradient centrifugation analysis is 26S and accordingly the complex is usually referred to as the 26S proteasome. Physicochemical analysis, however, has revealed that the correct sedimentation coefficient is approximately 30S.[10)] The size difference is probably due to the attachment of one 19S RP to the 20S proteasome to form the so-called 26S proteasome, whereas the elongated 30S molecule, which is likely the functional unit in the cell, may include a pair of symmetrically disposed 19S RPs that are attached to both ends of the central portion of the complex (Fig. 1). In this article, however, I will primarily use 26S proteasome without distinguishing between these two forms of the proteasome, unless otherwise specified.

As mentioned above, the 26S proteasome is a 2.5-MDa multicatalytic degradation machine that contains a 20S CP and one or two 19S RPs, which associate with the termini of the barrel-shaped central particle. The 19S RP serves to recognize ubiquitylated client proteins and is thought to play a role in their unfolding and translocation into the interior of the 20S CP, which contains catalytic

Abbreviations:
aa: amino acids
AAA-ATPase: ATPase associated with diverse cellular activities
AIRAP: arsenite-inducible proteasomal 19S regulatory-associated protein
AIRAPL: AIRAP-like gene
CD: cluster of differentiation
CP: core particle
cTECs: cortical thymic epithelial cells
CTL: cytotoxic T lymphocyte
DSBs: double strand breaks
DUB: deubiquitylating enzyme
EM: electron microscopy
FDA: Food and drug administration
GFP: green fluorescent protein
HbYX: hydrophobic-tyrosine-X
HCV: hepatitis C virus
IFN: interferon
MG-132: *N*-carbobenzoxy-leu-leu-leucinal
MHC: major histocompatibility complex
NER: nuclear excision repair
Ntn: N-terminal nucleophile
PA: proteasome activator
PAC: proteasome assembling chaperone
PGPH: peptidylglutamyl-peptide hydrolyzing
PI: proteasome inhibitor
PIP: proteasome-interacting protein
POMP: proteasome maturation protein
PSI: *N*-carbobenzoxy-L-gamma-t-butyl-L-glutamyl-L-alanyl-L-leucinal
REG: 11S regulator
RP: regulatory particle
siRNA: small interfering RNA
TAP: transporter associated with antigen processing
TCR: T cell receptor
TOP: thimet oligopeptidase
UBA: ubiquitin-associated
UBL: ubiquitin-like
UPS: ubiquitin–proteasome system
Z-L_3VS: carboxybenzyl-leucyl-leucyl-leucine vinyl sulfone

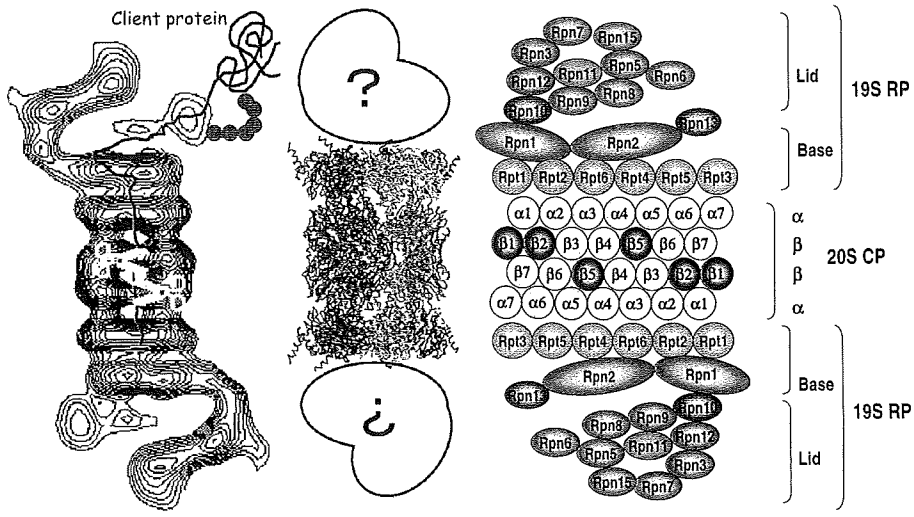

Fig. 1. Schematic diagram of the 26S proteasome. Left panel: Averaged image of the rat 26S proteasome complex based on electron micrographs. Photograph kindly provided by W. Baumeister. U, ubiquitin. Middle panel: The overall tertiary structure of the bovine 20S proteasome (central portion); the structures of the 19S RPs have not yet been determined (the pair of symmetrically disposed terminal structures depicted by question marks). Right panel: Schematic drawing of the subunit structure. CP, core particle (20S proteasome); RP, 19S regulatory particle consisting of the base and lid subcomplexes; Rpn, RP non-ATPase; Rpt, RP triple-ATPase.

threonine residues on the surface of a chamber formed by two β-rings.

2. The CP or 20S Proteasome

The 20S CP (alias 20S proteasome) is well characterized structurally (Fig. 1). It is a well-organized protein complex with a sedimentation coefficient of 20S and a molecular mass of approximately 750 kDa. When viewed electron microscopically, the 20S proteasome appears as a cylinder-like structure in various eukaryotes, including yeast and mammals. It forms a packed particle, a result of axial stacking of two outer α-rings and two inner β-rings, which are made up of seven structurally similar α and β subunits, respectively; the rings form an $\alpha_{1\text{-}7}\beta_{1\text{-}7}\beta_{1\text{-}7}\alpha_{1\text{-}7}$ structure. The 20S proteasome plays essentially the same proteolytic roles in all eukaryotes, differing from proteasomes in prokaryotes that mainly consists of homo-hepatmeric α- and β-rings of the same α and β subunits, respectively, i.e., the $\alpha\beta\beta\alpha$ structure.[8),11)] Accordingly, the overall structures and functions of the individual subunits are highly conserved among eukaryotic species, except for a specialized form(s) that is associated with adaptive immune responses, which will be described in a later section. Indeed, the yeast (*Saccharomyces cerevisiae*) and mammalian (bovine) 20S proteasomes are characterized by the same highly ordered, quaternary structures, as demonstrated by X-ray crystallography.[12),13)] The subunits of the 20S proteasome are specifically located within the complex with C2 symmetry. These subunits are listed in Table 2.

The three β-type subunits of each inner ring contain catalytically active threonine residues at their N termini and show N-terminal nucleophile (Ntn) hydrolase activity, indicating that the proteasome is a threonine protease that does not fall into the known seryl, thiol, carboxyl and metalloprotease families. The β1, β2 and β5 subunits are associated with caspase-like/PGPH (peptidylglutamyl-peptide hydrolyzing), trypsin-like and chymotrypsin-like activities, respectively, which confer the ability to cleave peptide bonds at the C-terminal side of acidic, basic and hydrophobic amino-acid residues, respectively. Two pairs of these three active sites face the interior of the cylinder and reside in a chamber formed by the centers of the abutting β-rings. The crystal structure of the 20S proteasome reveals that the center of the α-ring is almost completely closed, preventing proteins from penetrating into the inner chamber of the β-ring that contains the proteolytically active sites. Moreover, the N termini of the α subunits

Table 2. Proteasome subunits and proteasome-interacting proteins (PIPs) known to function as auxiliary factors

Category	Subclassification	Systematic nomenclature	HUGO	Miscellaneous nomenclature		human (yeast) amino acids	Motif	Lethality	Function
				Human	Yeast (budding/fission)				
20S CP	α type subunits	α1	PSMA6	iota	SCL1, YC7	(252) 246	NLS	+	
		α2	PSMA2	C3	PRE8, Y7	(250) 233	NLS	+	
		α3	PSMA4	C9	PRE9, Y13	(258) 261	NLS	−	
		α4	PSMA7	C6	PRE6	(254) 248	NLS	+	
		α5	PSMA5	zeta	PUP2, DOA5	(260) 241		+	
		α6	PSMA1	C2	PRE5	(234) 263		+	
		α7	PSMA3	C8	PRE10, YC1	(288) 254		+	
		α8	PSMA8		—	256			
	β type subunits	β1	PSMB6	Y, delta	PRE3	(19+196) 34+205	Ntn	+	Caspase-like
		β2	PSMB7	Z	PUP1	(29+232) 43+234	Ntn	+	Trypsin-like
		β3	PSMB3	C10	PUP3	(205) 205		+	
		β4	PSMB2	C7	PRE1	(198) 201		+	
		β5	PSMB5	X, MB1, epsilon	PRE2, DOA3	(75+212) 59+204	Ntn	+	Chymotrypsin-like
		β6	PSMB1	C5	PRE7	(19+222) 28+213		+	
		β7	PSMB4	N3, beta	PRE4	(33+233) 45+219		+	
		β1i	PSMB9	LMP2, RING12	—	20+199	Ntn	(−)	Caspase-like
		β2i	PSMB10	MECL1, LMP10	—	39+234	Ntn	(−)	Trypsin-like
		β5i	PSMB8	LMP7, RING10	—	72+204	Ntn	(−)	Chymotrypsin-like
		β5t	PSMB11		—	44+251	Ntn	(−)	Chymotrypsin-like
PA700 (19S RP)	ATPase subunits	Rpt1	PSMC2	S7, Mss1	YTA3, CIM5	(467) 433	AAA	+	ATPase
		Rpt2	PSMC1	S4, p56	YTA5/mts2	(437) 440	AAA, HbYX	+ (−)	ATPase, Gate-opning
		Rpt3	PSMC4	S6, Tbp7, P48	YTA2	(428) 418	AAA, HbYX	+ (+)	ATPase, Gate-opning
		Rpt4	PSMC6	S10b, p42	SUG2, PCS1, CRL13	(437) 389	AAA	+	ATPase
		Rpt5	PSMC3	S6′, Tbp1	YTA1	(434) 439	AAA, HbYX	+ (+)	ATPase, Gate-opning
		Rpt6	PSMC5	S8, p45, Trip1	SUG1, CRL3, CIM3/let1	(405) 406	AAA	+	ATPase
	non-ATPase subunits	Rpn1	PSMD2	S2, p97	HRD2, NAS1/mts4	(993) 908	PC	+	PIPs scaffold
		Rpn2	PSMD1	S1, p112	SEN3	(945) 953	PC, NLS	+	PIPs scaffold
		Rpn3	PSMD3	S3, p58	SUN2	(523) 534	PCI, PAM	+	
		Rpn5	PAMD12	p55	NAS5	(445) 456	PCI	+	
		Rpn6	PSMD11	S9, p44.5	NAS4	(434) 422	PCI, PAM	+	
		Rpn7	PSMD6	S10a, P44		(429) 389	PCI	+	
		Rpn8	PSMD7	S12, p40, MOV34	NAS3	(338) 324	MPN	+	
		Rpn9	PSMD13	S11, p40.5	NAS7/mts1	(393) 376	PCI	−	

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Category	Subclassification	Systematic nomenclature	HUGO	Miscellaneous nomenclature		human (yeast) amino acids	Motif	Lethality	Function
				Human	Yeast (budding/fission)				
		Rpn10	PSMD4	S5a, Mbp1	SUN1, MCB1/pus1	(268) 377	UIM, VWA	− (+)	Ub receptor
		Rpn11	PSMD14	S13, Poh1	MPR1/pad1, mts5	(306) 310	MPN, JAMM	+	DUB
		Rpn12	PSMD8	S14, p31	NIN1/mts3	(274) 257	PCI	+	
		Rpn13	ADRM1	ADRM1	DAQ1	(156) 407	Pru	−	Ub receptor, Uch37 recruit
		Rpn15	SHFM1	DSS1, SHFM1	SEM1	(89) 70		−	
PA28 (11S REG)			PSME1	PA28α, REGα	—	249		(−)	PSM activator
			PSME2	PA28β, REGβ	—	239		(−)	PSM activator
			PSME3	PA28γ, REGγ, Ki	—	254		(−)	PSM activator
PA200			PSME4	PA200, TEMO	BLM10	(2143) 1843	HEAT, ARM	(−)	PSM activator
PI31			PSMF1		—	271	Proline-rich		PSM inhibitor
Assembling chaperones		hUmp1	POMP	Proteassemblin	UMP1	(148) 141		−(+)	PSM formation
		PAC1	PSMG1		Pba1, Poc1	(276) 288		−(+)	PSM formation
		PAC2	PSMG2		Pba2, ADD66, Poc2	(267) 264		−	PSM formation
		PAC3	PSMG3		Pba3, Dmp2, Poc3	(179) 122		−	PSM formation
		PAC4	PSMG4		Pba4, Dmp1, Poc4	(148) 123		−	PSM formation
PIPs			PSMD5	S5b, p50.5	—	504	ARM		
		Rpn4		—	SON1, UFD5	(531)	Zn finger	−	PSM gene transcription
		Rpn14	PAAF1	FLJ11848	YGL004C	(417) 392	WD40, G-beta	−	PSM inhibitor
			PSMD9	p27	NAS2	(220) 223	PDZ	−	PSM modulator
			PSMD10	p28, gankyrin	NAS6	(228) 226	ANK	−	
		KIAA0368	KIAA0368	ECM29	Ecm29	(1868) 1870	HEAT	−	PSM stabilizer
		USP14	USP14		Ubp6/ubp6	(499) 494		−	DUB
		UCHL5	HCHL5	Uch37	—/uch2	329			DUB
		UBE3C	UBE3C	KIAA10	Hul5	(910) 1083	RING		Ub ligase
			UBE3A	E6AP	—	852	HECT		Ub ligase
			PARK2	Parkin	—	465	UBL, RING	(−)	Ub ligase
			RAD23A/B	hH23A/B	Rad23	(398) 363/409	UBL, UBA	−	Shuttling factor
			UBQLN1/2	hPLIC-1/2	DSK2	(373) 589/624	UBL, UBA	−	Shuttling factor

(+): Lethal (mouse), (−): Non-lethal (mouse), +: Lethal (yeast), −: Non-lethal (yeast), —: No orthologue, AAA: ATPase associated with diverse cellular activities, ANK: ankyrin repeats, ARM: Armadillo repeats, Amino acids (β subunit): Propeptide + mature protein, DUB: Deubiquitylating enzyme, HECT: a domain homologous to the E6-AP carboxyl terminus, HUGO: Human Genome Organization, MPN: Mpr1, Pad1 N-terminal, NLS: Nuclear localization signal, Ntn: N-terminal nucleophile hydrolase, PAC: Proteasome assembling chaperone, PAM: PCI associated module, PC: proteasome/cyclosome repeat, HbYX: hydrophobic-tyrosine-X, PCI: proteasome, COP9,eIF3, PDZ: PSD-95/DLG/ZO-1, PIPs: Proteasome interacting proteins, PSM: Proteasome, Pru: Pleckstrin-like receptor for ubiquitin, RING: Ring finger, UBA: Ubiquitin associated, UBL: Ubiquitin-like, UIM: Ubiquitin Interacting Motif, Ub: Ubiquitin, VWA: von Willebrand factor type A.

form an additional physical barrier for access to the active sites.[14] Thus, the 20S proteasome is latent in cells; substrates are able to access the active sites only after passing through the narrow opening at the center of the α-rings.

The 20S proteasome processively degrades client proteins, generating oligopeptides ranging in length from 3 to 15 amino-acid residues. The resulting peptide products are subsequently hydrolyzed to amino acids by oligopeptidases and/or amino-carboxyl peptidases. One such enzyme is the metalloendopeptidase thimet oligopeptidase (TOP), which associates with the 26S proteasome (our unpublished results) and displays an efficient hydrolytic activity in the soluble fraction of the cells.[15] Of note, there is evidence to suggest that unfolded proteins are generated in response to stressors. For example, proteins damaged by oxidation or intrinsically unstructured proteins (also known as natively unfolded proteins) are degraded directly by the 20S proteasome. The mechanisms that control the gate opening of the closed α-ring, however, are poorly understood, although the binding of denatured proteins to the α-ring seem to help open the gate.[16),17] While this process has been examined *in vitro*, it is not clear at this stage whether the 20S proteasome itself is responsible for proteolysis *in vivo* without facilitation by other activator protein(s).

3. The RP or PA700

The enzymatically active proteasome is generally capped on either or both ends of the central 20S proteasomal core by regulatory proteins (Table 1). The RP recognizes client proteins marked by polyubiquitin chains, removes the chain and entraps the protein moiety, unfolds the substrate proteins, opens the α-ring, and transfers the unfolded substrates into the CP for destruction (Fig. 2). The 19S RP comprises approximately 20 different subunits that can be subclassified into two groups: Regulatory particle of triple-ATPase (Rpt) subunits and Regulatory particle of non-ATPase (Rpn) subunits, both of which contain multiple proteins with molecular masses ranging from 10 to 110 kDa. The following is a brief description of the 19S RP (alias PA700), which comprises two subcomplexes: the lid and the base.[18),19]

3.1 The lid subcomplex. The lid complex is composed of at least nine non-ATPase subunits:

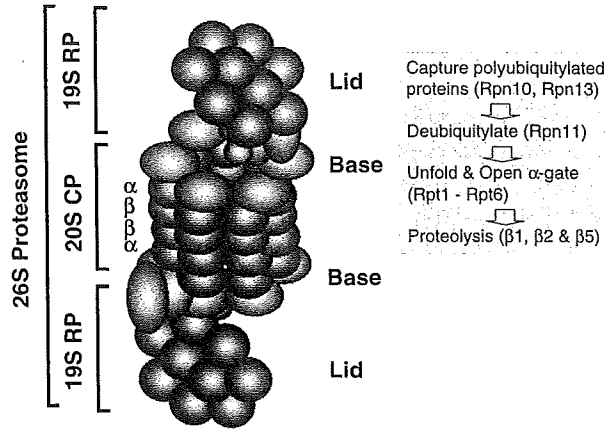

Fig. 2. Schematic diagram of proteolysis by the 26S proteasome. Positions of subunits indicated (i.e., Rpn10, Rpn13, Rpn11, Rpt1-Rpt6, β1, β2 and β5 are represented in Fig. 1. The β1, β2 and β5 subunits are associated with caspase-like, trypsin-like and chymotrypsin-like activities, respectively (for details, see text).

Rpn3, Rpn 5, Rpn6, Rpn 7, Rpn 8, Rpn 9, Rpn 11, Rpn 12 and Rpn 15 (Fig. 1). The main function of the lid is to deubiquitylate the captured substrates, a process in which the metalloisopeptidase Rpn11 functions to recycle the ubiquitins (Fig. 2).[20] Indeed, Rpn11 DUB cleaves the polyubiquitin chain at a proximal site; this chain is further cleaved into monomeric ubiquitins by other DUBs. In addition, in mammalian cells, two other DUBs that are physically associated with the base complex cleave the ubiquitin moiety at a distal site. Usp14 (yeast UBP6) is associated with Rpn1[21] and Uch37 binds to the C-terminal domain of Rpn2-bound Rpn13; i.e., Uch37 associates with the base via Rpn13.[22),23] Intriguingly, deubiquitylation by Uch37 is activated by proteasome binding, which is also involved in the editing of polyubiquitin chains. In addition, the yeast Ubp6 is induced by ubiquitin deficiency although ubiquitin stress does not upregulate proteasome abundance. Namely the enhanced loading of proteasomes with Ubp6 alters proteasome function, implying a dual role for Ubp6 in regulating ubiquitin levels and proteasome function.[24] The functions of most of the other subunits in the lid, however, have yet to be elucidated.

3.2 The base subcomplex. The base complex is composed of six homologous AAA-ATPase subunits, (Rpt1–Rpt6) and four non-ATPase subunits (Rpn1, Rpn2, Rpn10 and Rpn13 Fig. 1). The base complex of proteasomes has three functional