Review

The proteasome: molecular machinery and pathophysiological roles

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Abstract

The 26S proteasome, in collaboration with ubiquitin, operates the energy-dependent regulated proteolysis process in eukaryotic cells. Over the past 30 years, several studies have comprehensively characterized the structure and molecular/physiological functions of the 26S proteasome. It is a sophisticated 2.5-MDa protein degradation machine comprising a proteolytic core particle (CP) and one or two terminal regulatory particle(s) (RP). The CP consists of two outer α rings and two inner β rings, which are made up of seven structurally similar α and β subunits, respectively. The CP contains catalytic threonine residues (β1, β2, and β5; caspase-like, trypsin-like, and chymotrypsin-like activities, respectively) on the inner surface of the chamber formed by two abutting β rings. Intriguingly, the immunotype proteasomes, named ‘immunoproteasome’ and ‘thymoproteasome’, whose catalytic subunits are replaced by the related counterparts, were discovered lately. Both unique isoenzymes essentially contribute to the acquisition of adaptive immunity in vertebrates. The RP, which serves to recognize polyubiquitylated substrate proteins and plays a role in their deubiquitylating, unfolding, and translocation into the interior of the CP for destruction, forms two subcomplexes: the base and the lid. On another front, the PA28 and PA200, alternative CP activator proteins discovered biochemically, both play independent roles in proteolysis of the 26S proteasome. Several studies have highlighted the importance of the proteasome in various intractable diseases that have been increasing in the aged society of the 21st century.

Keywords: 20S and 26S proteasomes; immunoproteasome; RP; thymoproteasome; ubiquitin.

Introduction

The proteasome is a gigantic and complex proteolytic machinery that regulates a variety of physiologically and pathologically important cellular processes by selective breakdown of short-lived and abnormal proteins in eukaryotes [reviewed in (Baumeister et al., 1998; Hershko and Ciechanover, 1998; Schwartz and Ciechanover, 2009)]. The nomenclature referred to 20S and 26S proteasomes as the ubiquitous and predominant forms in the cell, based on the sedimentation coefficient (i.e., S-value) determined by sucrose-density gradient centrifugation, have been used to date (Coux et al., 1996; Pickart and Cohen, 2004; Tanaka, 2009). However, it is widely recognized that their S-values lack accuracy and thereby may be misleading. The so-called 26S proteasome consists of two subcomplexes: a catalytic core particle (CP; also known as the 20S proteasome) and a terminal 19S regulatory particle (RP). However, in the late 1980s, many researchers working in the field noticed that the RP is detected in the higher-density fractions compared with the 20S CP when cell extracts were analyzed by density-gradient centrifugation. These findings suggested that the value of 19S of the RP was profoundly underestimated. In addition, the term 26S proteasome remains also vague because the cells usually contain two forms of the proteasomal complexes: i.e., the RP-CP and the RP-CP-RP. The weight of the 26S proteasome is also underestimated because the poor isolation procedure might enrich the RP-CP complex, as the CP and RP complexes tend to dissociate in high salt conditions and/or chromatographic operations. When using the highly purified RP-CP-RP complex (i.e., >90%), physicochemical analysis has demonstrated that the true S-value is approximately ‘30S’ (Yoshimura et al., 1993). Therefore, the difference in the size is assumed to be due to the attachment of one RP to the CP to form the so-called 26S proteasome, whereas the 30S molecule contains two RPs attached to both ends of the CP (Figure 1, left and middle left). Although it is still unclear whether there are functional differences between the 26S (RP1CP) and 30S (RP2CP) proteasomes, the latter is the most abundant proteasome species in the cell and is believed to be the physiologically functional unit (Voges et al., 1999).

Intriguingly, recent follow-up studies using more refined mass technology to characterize the intact yeast proteasomes, e.g., electrospray ionization mass spectrometry (ESI-MS) of whole complexes, revealed that molecular sizes of the CP, RP, RP-CP, and RP-CP-RP are ~730, ~930, ~1660, and ~2590 kDa, respectively (Sakata et al., 2011). The results of
subsequent extensive studies indicate that the numbers of subunits in the CP and RP are 14 and 19, respectively. Considered together, the results determined by the new MS techniques match those of the molecular masses calculated by the composition of the amino acids, confirming the accuracy of the aforementioned assumptions. In this article, however, we will primarily use the term ‘26S proteasome’ without distinguishing between these two forms of the proteasome. Over the past 5 years, there has been an explosion in our understanding of the ultrastructure of the 20S and 26S proteasomes. We also review the structure and function of other proteasome activators, such as PA28 and PA200.

The new analyses, especially murine genetic analysis, have also added valuable information on the physiology and pathology of the proteasome in vivo. This review presents a comprehensive overview of the proteasome, including a summary of the basic structures and molecular functions, and insight into the possible biological and pathological roles, especially in mammals. Other excellent review articles have been published recently on the structure, functions, and assembly of the proteasome (Finley, 2009; Murata et al., 2009; Bedford et al., 2010; Park et al., 2010; Stadtmueller and Hill, 2011; Tomko and Hochstrasser, 2011; Weissman et al., 2011; Saeki and Tanaka, 2012).

Structure and mechanistic actions

Standard proteasome

The 20S proteasome (CP) is a well-organized protein complex of ~730 kDa. The barrel-shaped overall structure is highly conserved evolutionarily in various eukaryotes, ranging from yeast to mammal. In vertebrates, the isosforms of the CP complex are uniquely expressed in the immune tissue. To distinguish them, the predominant form present in many cells and tissues is often called the standard or constitutive proteasome, whereas the immune tissue-specific forms are called immunotype proteasomes (see below). Based on its ubiquitous distribution, the standard proteasome (simply referred to as ‘proteasome’ thereafter, unless otherwise specified) is believed to have multifaceted and housekeeping roles within the cell. Indeed, X-ray crystallographic examination revealed highly similar quaternary structure of the yeast (Saccharomyces cerevisiae) and mammalian (bovine) CPs (Groll et al., 1997; Unno et al., 2002). The CP consists of four heptameric rings (two outer α rings and two inner β rings), which are made up of seven structurally related, but not identical, α and β subunits, respectively, displaying an α₇₋β₇₋α₇₋β₇ organization (Figure 2, left), in which all individual subunits are ordered in the same orientations irrespective of the species.

In addition, the CP plays essentially the same proteolytic roles in all eukaryotes, constituting three β-type subunits of the inner β rings that contain catalytically active threonine residues at their N-termini and show N-terminal nucleophile (NtM) hydrolyase activity for peptide-bond cleavage. The β₁, β₂, and β₅ subunits are associated with caspase-like/post-acidic hydrolyzing, trypsin-like, and chymotrypsin-like activities, respectively, which confer cleavage of peptide bonds at the C-terminal side of acidic, basic, and hydrophobic amino acid residues, respectively (Figure 2, right). Two pairs of these three active sites face the interior of the cylinder and reside in a chamber formed by the two β rings. The crystal structures of the CPs reveal that the center of the α ring is almost completely closed, preventing substrate proteins from penetrating into the inner cavity of the β ring, in which the proteolytically active sites are located. Indeed, the N-termini of the α subunits form an additional physical barrier for access to the active sites, indicating that the CP exists as a latent form in cells. Thus, substrates are able to access the active sites only after passing through the narrow opening at the center of the α rings, and accordingly, there are multiple ways to open the closed gate (see below).

Immunoproteasome

It is still a mystery why and how the CP processively degrades substrate proteins so as to generate oligopeptides ranging in length from 3 to 15 amino acid residues, but not, even occasionally, to amino acids as the ultimate products. However, the generated small peptide products are subsequently hydrolyzed
The proteasome machinery and roles

Proteasome complexes are responsible for the degradation of proteins, primarily by cleaving proteins into oligopeptides. These oligopeptides are then further processed by various peptidases to yield amino acids, which are abundantly present in the cytosol compartment of the cell. Intriguingly, the oligopeptides produced by the proteasome in vertebrates can be used for antigen presentation by the major histocompatibility complex (MHC) class I molecules to cytotoxic T lymphocytes (CTLs) that express CD8 (Tanaka and Kasahara, 1998; Kloetzel, 2001; Rock et al., 2002; Kloetzel and Ossendorp, 2004; Groettrup et al., 2010). T cell receptor (TCR) recognizes the peptide-MHC class I complexes at high fidelity, activating those T cells and initiating the main pathway in cell-mediated immunity, a fundamental and elegant mechanism in the body defense systems.

In contrast to lower eukaryotes such as single cell organisms, higher organisms such as vertebrates have alternative catalytic β-type subunits. The γ-type interferon (IFN-γ), an immunocytokine, induces the expression of β1i, β2i, and β5i catalytic subunits, which are structurally highly similar to β1, β2, and β5 of the standard proteasome (Figure 3, left), with more than 50% identity, respectively. The expression of these three unique subunits causes the formation of CP isoforms in which the catalytic subunits are replaced with β1i, β2i, and β5i, respectively. Accordingly, we proposed in 1994 that the IFN-γ-inducible proteasome should be called ‘immunoproteasome’ to emphasize their specialized roles as

Figure 2 The crystal structure of the 20S proteasome from bovine liver. Top view (top left) and side view (bottom left) ribbon representations of the bovine CP (PDB: 1IRU). Top view ribbon representation of the β rings (top right). Cutaway ribbon representation of the CP (bottom right). Active threonine residues of β1, β2, and β5 appear in blue, green, and red, respectively. For details, see Unno et al. (2002).

Figure 3 Functions of proteasome isoforms. Standard proteasome (left), immunoproteasome (middle), and thymoproteasome (right) are depicted. Note that the display of the position of the β subunits is simplified. See text for details.
a professional antigen-processing enzyme in cell-mediated immunity (Figure 3, middle) (Akiyama et al., 1994; Tanaka, 1994; Hisamatsu et al., 1996). The unique proteasome isoform can be generated by the preferential assembly mechanisms, but not replacement between mature proteasomes, in which peptidyls of immunoproteasomal subunits contribute to their preferentially fast incorporation into β ring as intramolecular chaperons (Groettrup et al., 1997; Griffin et al., 1998; Heinik et al., 2005). The chymotrypsin-like activity of the proteasome is thought to be important for production of antigenic peptides with high affinities for MHC class I clefts, which serve as peptide-binding pockets. Consistently, the immunoproteasome has higher chymotrypsin-like activity than the standard proteasome (Gaczynska et al., 1993; Aki et al., 1994). Collectively, the proteasome system in vertebrates has acquired considerable diversity among the catalytic subunits, which have evolved during the acquisition of adaptive immunity (Tanaka and Kasahara, 1998).

Thymoproteasome

In 2007, while searching the expressed sequence tag (EST) expression profile database, we accidentally identified a previously unrecognized catalytic subunit with an overall sequence highly similar to β5 and β5i, suggesting that the subunit belongs to the same β5 family proteins (Murata et al., 2007). Quite interestingly, the subunit was expressed exclusively in the mouse thymic cortex, particularly in cortical thymic epithelial cells (cTECs). Owing to this unique expression, the subunit was designated as β5t whose thymus-specific expression was reminiscent of human case (Tomaru et al., 2011). Intriguingly, β5t expression profi le database, we accidentally identifi ed a unique expression because transcriptional factor Foxn1 is responsible for the expression of Foxn1-deficient nude mouse embryo (Ripen et al., 2011). Intriguingly, β5t and the immunosubunits β1i and β2i, but not their standard counterparts, are incorporated into the vertebrate-specific alternative 20S proteasome, which we named the ‘thymoproteasome’ (Figure 3, right) (Murata et al., 2007). As mentioned above, although the chymotrypsin-like activity of proteasomes is important for the immune response, the incorporation of β5t into the proteasome, instead of β5 or β5i, selectively reduces this activity, strongly indicating that thymoproteasome may alter logically the quality of the product peptide repertoire. Indeed, mass spectrometric analysis of the MHC class I-loaded peptides from the cells showed the generation of considerably different peptides by immunoproteasomes and thymoproteasomes (K. Sasaki et al., unpublished results). Importantly, β5t-deficient mice displayed defective development of CD8+ T cells in the thymus, suggesting a key role for β5t in the generation of MHC class I-restricted CD8+ T cell repertoire during thymic selection (for details, see next section).

Sequence comparison of β-type subunit genes of the standard and immunoproteasome indicates that each subunit pair is derived from a common ancestor gene. We previously proposed a chromosomal duplication hypothesis to explain the emergence of the IFN-γ-regulated subunits (Tanaka and Kasahara, 1998). The basic assumption in this model is that all of the IFN-γ-regulated immunosubunits emerged simultaneously as a result of duplication of the MHC region during genome-wide duplication. Like the immunoproteasome genes, the β5t gene appears to result from modification and duplication of existing nonimmune genes, such as β5, and may have been instrumental in the emergence of the adaptive immune system. Intriguingly, β5t is encoded by an intronless gene, in contrast to the β5 and β5i genes, which comprise three exons. Curiously, the β5t gene (offi cially named PSMB11) is located next to the PSMB5 gene encoding the β5 subunit of the standard proteasome in mammals and other lower vertebrates, such as reptiles, amphibians, and teleost fish. This suggests that the PSMB11 gene arose by tandem duplication from the evolutionarily more ancient PSMB5 gene and lost introns evolutionarily by an as-yet-unknown mechanism (Sutoh et al., 2012).

Regulatory particles

The RP was initially found biochemically to serve as a proteasome activator of approximately 700 kDa (thereby often called PA700), though its correct size was more than 900 kDa, as described in the ‘Introduction’ section. The pair of symmetrically disposed RPs is attached to both ends of the central CP to form the holo proteasome, which is likely the functional unit for selective digestion of a wide array of polyubiquitylated substrate proteins in the cell. As mentioned in a previous section, we need to examine the functional differences in active proteasomes, CP-CP and RP-CP-RP, in the cell. To date, all efforts to define the atomic structures of the RP and the 26S holo proteasome, in contrast to yeast and mammalian 20S CPs, have failed, presumably because of their fragile nature and dynamics. Nevertheless, recent EM studies of the intact 26S proteasome provided valuable insights into its molecular architecture. Specifically, Baumeister and colleagues determined the structure of the 26S proteasome in Schizosaccharomyces pombe at 9.1 Å by cryo-EM and single particle analysis (Bohn et al., 2010).

The RP serves to recognize polyubiquitylated substrate proteins followed by deubiquitylation for recycling of ubiquitin (Ub) moieties after trapping them, then to unfold and translocate them into the interior of the CP. Interestingly, the opening of the α ring closed gate of the CP is also involved in RP. The RP comprises a set of different integral subunits that can be subclassified into two groups: RP of 6 triple-ATPase (Rpt) subunits and RP of 13 non-ATPase (Rpn) subunits, which form two subcomplexes: the base includes six different AAA+ ATPase subunits (Rpt1-Rpt6) and three non-ATPase subunits (Rpn1, Rpn2, and Rpn13), while the lid comprises nine non-ATPase subunits (Rpn3, 5–9, 11, 12, and 15) (Finley, 2009; Tanaka, 2009; Forster et al., 2010). The subunit Rpn10 was previously thought to connect the base to the lid subcomplexes and stabilize them because deletion of Rpn10 tends to dissociate into the base and the lid in yeast by enzymological operations (Glickman et al., 1998). However, a recent single particle analysis by cryo-EM revealed that
Rpn10 and Rpn13 are located in a distant site on the apical part of the RP complex near the periphery, where the sites have been thought to be the lid (see for model in Figure 4) (Lander et al., 2012; Lasker et al., 2012; Sakata et al., 2012). Such orientation is rational because both Rpn10 and Rpn13 serve as Ub receptors, i.e., Rpn10 and Rpn13 function as integral Ub receptors to efficiently trap polyubiquitylated substrates, which is a prerequisite at the initial stage of degradation, during which both should be located on the outer positions of the RP. Rpn10 achieves this function via a C-terminal Ub-interacting motif (UIM) (Deveraux et al., 1994). Interestingly, the N-terminal domain of the Rpn13 shows no homology to known Ub-binding motifs, but instead contains the novel ‘pleckstrin-like receptor for Ub’ (Pru) domain (Husnjak et al., 2008; Schreiner et al., 2008). The Pru domain in human Rpn13 shows high affinity for diubiquitin (Zhang et al., 2009c). Interestingly, the C-terminal domain of Rpn13, which is found only in higher eukaryotes, stimulates the deubiquitylating activity of the proteasome-associated deubiquitylating enzyme Uch37 (also known as UCHL5), which seems to collaborate with the N-terminal Ub receptor to facilitate proteolysis (Hamazaki et al., 2006; Qiu et al., 2006; Yao et al., 2006; Chen et al., 2010). It is worth noting that Rpn1 is also responsible for acceptance of various Ub-like (UBL) proteins, similar to Rpn10, including shuttling factors for polyubiquitylated proteins (see below).

The main function of the lid is to deubiquitylate the captured substrates, a process in which the metalloisoenzyme Rpn11 functions to recycle the Ubs (Verna et al., 2002; Yao and Cohen, 2002). Importantly, Rpn11 deubiquitylating enzyme (DUB) cleaves the polyubiquitin chain at a proximal site to substrate, coupled with substrate degradation; this chain is further cleaved into monomeric Ubs by other DUBs. In addition, in mammalian cells, two DUBs, Usp14 and Uch37/UCHL5, which are physically associated with the base complex, sequentially cleave the Ub moiety at a distal site to the substrate and regulate the degradation of the substrate proteins (Lam et al., 1997; Leggett et al., 2002; Hu et al., 2005; Koulich et al., 2008). Usp14 (equivalent to yeast Ubp6) is directly associated with Rpn1, whereas Uch37 binds to the base Rpn2 via Rpn13 (Leggett et al., 2002; Hamazaki et al., 2006; Qiu et al., 2006; Yao et al., 2006; Chen et al., 2010) (see Figure 4). The functions of most other subunits of the lid remain to be elucidated.

The base complex is composed of six homologous AAA-ATPase subunits, (Rpt1–Rpt6), forming a crown-like shape, and three non-ATPase subunits (Rpn1, Rpn2, and Rpn13). The base serves three functions: promoting substrate unfolding, opening the channel in the closed α ring, and translocating their substrates to the CP, thus enhancing their destruction (see below). Recent studies have provided significant insights into the structures and functions of the ATPases in the base subcomplex (Forster et al., 2010; Stadtmueller and Hill, 2011). The Rpt ring is thought to pull substrates into the central pore of the Rpt ring with sufficient force to promote unfolding of substrate structure domains and translocate them to the CP channel for degradation. Among the six Rpt subunits, only Rpt2, Rpt3, and Rpt5 contain the C-terminal hydrophobic-tyrosine-X (HbYX) motif, and the Rpt2 and Rpt5 subunits specifically facilitate the gate opening (Kohler et al., 2001; Smith et al., 2007; Gillette et al., 2008; Rabl et al., 2008).

The eukaryotic 26S proteasome is thought to have evolved from a simpler complex, called proteasome-activating nucleotidase (PAN) protease complex of archaea. PAN is a homohexameric ATPase ring related to the eukaryotic base. Recent structural studies of the PAN and related actinobacterial ARC have revealed that each subunit has an ATPase domain and N-terminal oligosaccharide binding (OB) and coiled-coil (CC) domains (Djurunic et al., 2009; Zhang et al., 2009a, b). The ATPase domains associate with the CP by inserting their C-terminal tails and drives ATP-dependent functions, whereas the CC-OB domains serve as an entry port of the substrate translocation channel. Goldberg and coworkers investigated the role of ATP in proteasome-mediated proteolysis, using biochemical analysis of PAN, i.e., how nucleotides bind to PAN (Smith et al., 2005, 2011; Horwitz et al., 2007). Although PAN has six identical subunits, it binds ATPS in pairs, and its subunits exhibit three conformational states with high, low, or no affinity for ATP. When PAN binds two ATPS molecules or two ATPγS plus two ADP molecules, it is maximally active in binding protein substrates, associating with the CP, and promoting the opening of the α ring gate of the CP. In contrast, binding of four ATPγS molecules reduces the above functions. Based on these findings, the group proposed an ordered cyclical mechanism in which two ATPase subunits bind ATP simultaneously and dock into the 20S (Smith et al., 2011). This mechanism can explain how these hexameric ATPases interact with and ‘wobble’ on top of the heptameric 20S proteasome (Saeki and Tanaka, 2007; Smith et al., 2007). Finley and colleagues studied the contacts between RP and CP subunits of the 26S proteasome applying a protein cross-linking approach. Some Rpt subunits were found to be engaged with the CP in a dynamic fashion, which was regulated by nucleotide binding and hydrolysis (Tian et al., 2011). Interestingly, the base complex shows chaperone activity in vitro, which

![Figure 4](image-url) A model describing the function of the 19S RP.

The substrate protein (red) conjugated to the polyubiquitin chain (yellow) is recognized by the Ub receptor subunits Rpn10 and Rpn13 (cyan). The ATPase ring comprising the Rpt1-6 (orange) unfolds the substrate protein and translocates it into the channel of the CP (blue). Ubs are spared by three DUBs, Rpn11, Uch37/UCHL5, and Ubp6/USP14 (green). Finally, the substrate protein is hydrolyzed to oligopeptides in the CP (not shown). (For details, see Lander et al., 2012; Lasker et al., 2012; Sakata et al., 2012.)
can cause refolding of unfolded proteins in the presence of ATP, but neither in the presence of ADP nor the absence of ATP (Braun et al., 1999).

**PA28 and PA200**

The CP is also activated by proteasome activators other than the RP, such as PA28 family proteins and PA200. PA28, also known as the 11S regulator (REG), was identified as another protein activator of the latent CP (Rechsteiner et al., 2000). EM studies revealed that PA28 forms conical caps by associating with both ends of the central CP. PA28 complexes are composed of three structurally related members designated α, β, and γ; their primary structures display approximately 50% homology (Tanahashi et al., 1997). Whereas the PA28α and PA28β assemble into heteroheptameric complexes whose stoichiometric compositions remain uncharacterized due to lack of their atomic structures, the PA28γ appears to form homopolymeric complexes. Immunofluorescence analysis revealed that both PA28α and PA28β are located mainly in the cytoplasm, whereas PA28γ is located predominantly in the nucleocyttoplasm (Wojcik et al., 1998). The crystal structure of the PA26 (Trypanosoma brucei PA28)-CP complex shows that PA26 binds to 20S proteasomes by inserting three C-termini into the intersubunit pocket between adjacent α subunits. In addition, the ‘activation loop’ domain of PA26 stabilizes the open-gate conformation (Figure 1, middle right) (Whitby et al., 2000).

The PA28 protein stimulates all of the peptidase activities of the CP without affecting the destruction of large protein substrates, even if the proteins have already been polyubiquitylated except in the case of the hybrid proteasome that contains PA28 and RP simultaneously (see below). Thus, PA28 does not play a central role in the initial cleavage of protein substrates in cells. It presumably has a stimulating effect on the degradation of intermediate-size polypeptides that are generated by the 26S proteasome, implying that the 26S proteasome and the PA28-proteasome complex may function sequentially or cooperatively.

There is a monomeric activator of the CP that was named PA200 in mammals (Blm10 in yeast), which is a single large protein of approximately 200 kDa with HEAT repeat domains, broadly expressed in the nucleus (Ustrell et al., 2002; Schmidt et al., 2005). It can open the gate of the CP partially, thereby facilitating substrate access to the proteolytic chamber (Sadre-Bazzaz et al., 2010; Stadtmueller and Hill, 2011). Interestingly, the CP is conserved in all eukaryotes, but plants and yeast only contain PA200/Blm10 and lack both isoforms of the PA28 activator, PA28αβ and PA28γ. Only sparse information is available on biochemical analysis of PA200, and its function remains controversial. Nevertheless, it was reported recently that Blm10 stimulates the degradation of specific substrates such as Sfp1, a transcription factor of ribosome biogenesis, and tau (Dange et al., 2011; Lopez et al., 2011). The three-dimensional molecular structure determined by X-ray crystallography of Blm10-CP provides a model for activation (Figure 1, right) (Sadre-Bazzaz et al., 2010). The carboxy terminus of Blm10 inserts into a dedicated pocket in the outer ring of the CP surface, whereas multiple HEAT-like repeats fold into an asymmetric solenoid wrapping around the central pore to stabilize a partially open conformation. Furthermore, the crystal structure of the Blm10-CP complex indicates that Blm10 surrounds the proteasome entry pore in the 1.2-MDa complex to form a largely closed dome that is expected to restrict access of potential substrates. The Blm10 C-terminus binds in the same manner as seen for a PA26 activator and inferred for 19S/PAN activators and indicates a unified model for gate opening, displaying essentially the same mechanisms for activation of the latent CP by various factors. In addition to the proteolytic function, Blm10 is thought to regulate the RP-CP assembly and CP maturation (Marques et al., 2007; Lehmann et al., 2008).

### Emerging roles in physiology and pathology in mammals

One widely held view is the involvement of the proteasome and its partner ‘Ub’ in multiple arrays of physiologically important cellular processes, such as cell cycle progression, gene regulation, immune responses, and developmental programs. Consequently, it is anticipated that their impairments can cause intractable diseases especially in today’s aging society. Several studies addressed the importance of dysfunctional abnormalities of the target molecules controlled by the proteasome; however, little is known about proteasomal defects that can induce pathological abnormalities. In this context, proteasomal mutations may be incompatible with life and lead to early embryonic death. Accumulating data point to the pathophysiological roles of the proteasome, mainly based on mouse genetic analyses and to a lesser extent on human diseases. In this section, we discuss the physiology and pathology related to proteasomal malfunctions, citing mainly murine genetic analysis, but we will not include cellular and organ-related dysfunctions associated with abnormalities of the Ub system, which have been already discussed by others (Rubinsztejn, 2006; Schwartz and Ciechanover, 2009; Matsuda and Tanaka, 2010; Lipkowitz and Weissman, 2011; Weissman et al., 2011).

### Role of standard proteasome

As described in a previous section, the 20S CP exists in a latent form in the cell, and accordingly, there is no biological role for the free CP itself. However, some researchers reported the existence of various approaches to open the gate of the α ring in addition to the RP over the last two decades. In the late 1980s, the 20S proteasome was discovered and isolated as a multicytolytic or multifunctional protease that can hydrolyze various substrates with broad specificity in *in vitro* degradations assays that included synthetic fluorogenic peptides. Conceptually, it is believed that the proteasome can degrade unfolded/denatured proteins (e.g., proteins damaged by oxidation) generated in response to environmental stressors in cells (Grune et al., 1997). Although the mechanisms...
that control the gate opening of the closed α ring are poorly understood, the binding of denatured proteins, which contain hydrophobic surface patches formed by partial unfolding to the α ring, can lead to the opening of the gate (Jung and Grune, 2008). While this process has been examined in vitro, it is still unclear whether the CP itself is responsible for proteolysis in vivo without facilitation by other activator protein(s).

Similar scenarios are also thought for proteasome-mediated degradation of natively unfolded proteins, which lack well-defined secondary and tertiary structures under physiological conditions (Prakash et al., 2004). Indeed, computer-assisted genome-wide research of protein sequences indicates that considerably large populations of cellular proteins belong to this type of intrinsically unstructured proteins in higher organisms, and those proteins are directly degraded by the CP without ubiquitylation in vitro experimental settings (Baugh et al., 2009). Moreover, in vivo evidence suggests that latent (closed) and activated (open) proteasomes degrade certain natively disordered substrates at internal peptide bonds even when they lacked accessible termini, suggesting that these substrates themselves promoted gating of the CP pore and that endoproteolysis by the 20S proteasome that accesses internal unfolding multidomain proteins and misfolded proteins is physiologically important, even if this degradation is less efficient (Liu et al., 2003).

As the majority of CP subunits are essential for cellular biology, genetic factors involved in the regulation of the proteasome level itself are not practical experimentally. Currently, experimental techniques have been developed to define the molecular mechanisms underlying the assembly of the CP by identifying a set of proteasome-dedicated assembling chaperones. We identified four proteasome-dedicated chaperones that assist α ring formation named proteasome assembly chaperone (PAC) 1–4 in mammals and found that these chaperones form a pair of functional heterodimers, PAC1-PAC2 and PAC3-PAC4 (Hirano et al., 2005, 2006; Yashiroda et al., 2008). While the PAC1-PAC2 and PAC3-PAC4 complexes have different roles at different steps, they cooperate with each other in the assembly of α rings. Intriguingly, knockdown of PAC1 or PAC2 expression in mammals decreased the number of α rings and resulted in accumulation of dead end products, causing presumably unusual α ring dimers. This finding suggests that PAC1/PAC2 prevents the off-pathway assembly. On the other hand, the PAC3-PAC4 complex effectively promotes the formation of α ring. After completion of α ring assembly, the β subunits are incorporated onto the α ring at their specific positions in a defined order, forming the 20S proteasome [reviewed in (Kusmierekzyk and Hochstrasser, 2008; Ramos and Dohmen, 2008; Murata et al., 2009), for details].

In yeast, similar PAC-like chaperones named proteasome biogenesis-associated protein (Pba) 1–4 involved in proteasome assembly have also been identified, but deletions of Pba1-Pba2 in yeast resulted in only subtle phenotypes (Li et al., 2007a; Kusmierekzyk et al., 2008, 2011). Indeed, none of these chaperones are essential for yeast viability, suggesting differences in their roles between the two organisms, but the reason why PAC chaperones display species-specific distinct functions in CP biogenesis remains to be elucidated.

Knockdown of PAC1 does not cause complete loss of the CP, suggesting the existence of both chaperone-dependent and -independent assembly pathways, but the contribution of the chaperone-dependent pathway remains unclear. To elucidate the biological significance of PAC1-mediated proteasome assembly in vivo, we generated PAC1-conditional knockout mice (Sasaki et al., 2010). PAC1-null mice exhibited early embryonic lethality, i.e., mice that systemically lacked PAC1 died around E6.0. However, this phenotype is still milder than that of mice deficient in proteasome base ATPases, Rpt3 and Rpt5, which died before development into blastocysts (E3.5) (Sakao et al., 2000) (as described below). This finding supports the notion that proteasome biogenesis is not entirely dependent on the assembly chaperones, i.e., the existence of a PAC1-independent assembly pathway. Nonetheless, efficient PAC1-based proteasome biogenesis is needed to develop beyond the blastocysts and thus is essential for mouse development. Analysis of brain-specific PAC1-deficient mice after birth showed a large decrease in the levels of both 20S and 26S proteasomes, which resulted in severe impairment in the development of the central nervous system (CNS). Taken together, the study clearly demonstrated that PAC1 is essential for mammalian development, especially for explosive cell proliferation.

In contrast, in quiescent adult hepatocytes, PAC1 is responsible for the production of the majority of the CP. Surprisingly, the free latent CP decreased almost completely, but the levels of the 26S proteasome remained unchanged at least for 6–12 months after PAC1 ablation, indicating that the normal levels of the 26S proteasome were maintained by efficient production in a PAC1-independent assembly pathway. Mice lacking PAC1 in the liver exhibit premature senescence (i.e., aging) phenotypes, suggesting that chaperone-assisted biogenesis of proteasomes is essential for liver homeostasis. Intriguingly, accumulation of polyubiquitin-conjugated proteins was noted in the PAC1-deficient liver, suggesting that the free CP apparently participates in the degradation of ubiquitylated protein. As there is no proof that CP directly recognizes and degrades polyubiquitylated proteins, these findings imply that some proteins that are usually degraded directly by the CP in an Ub-independent manner accumulate in the cell and are subsequently polyubiquitylated. These results demonstrate the physiological importance of the PAC1-dependent assembly pathway and of the CP itself in the maintenance of cell integrity (Sasaki et al., 2010).

Role of immunoproteasome

MHC class I molecules bind peptides produced through proteolysis of cytosolic proteins and consequently display them on the cell surface. This mechanism enables CTLs to detect and destroy abnormal cells that contain tumor antigens or other foreign proteins invading the cell, such as viral proteins. In the early 1990s and soon after the discovery of the transporter associated with antigen processing (TAP), a heterodimeric peptide pump, localized in the endoplasmic reticulum (ER)
membranes, the proteasome was identified as a candidate for the enzyme that processes endogenous antigens (Rock et al., 1994). Identification of the immunoproteasome, which contributes to efficient production of peptide epitopes for CTLs, has been highlighted in the MHC class I-restricted antigen-processing pathway in cell-mediated immunity. Indeed, acquisition of the immunoproteasomes during evolution has enabled organisms to produce MHC class I ligands and combat pathogens more efficiently.

Mice lacking genes that encode immunoproteasome subunits (i.e., β1i, β2i, or β5i) display defective antigen processing and, consequently, compromised immune responses [reviewed in detail in (Tanaka and Kasahara, 1998; Kloetzel, 2001; Rock et al., 2002; Kloetzel and Ossendorp, 2004; Groettrup et al., 2010)]. These initial studies emphasized the immunological importance of this enzyme in mice. Further studies addressed more convincingly the pivotal roles of these immunosubunits in the production of MHC class I ligands peptides in double and/or triple KO mice for β1i, β2i, or β5i, i.e., the immunoproteasome plays a much more important role in antigen presentation than previously thought (Fehling et al., 1994; Van Kaer et al., 1994; Sijs and Kloetzel, 2011; Kincaid et al., 2012).

Other unexpected roles of the immunoproteasome, independent of antigen presentation, were recently identified, e.g., the immunoproteasome contributes to prevention of cell death by IFN-induced oxidative stress through the prevention of accumulation of harmful protein aggregates (Seifert et al., 2010). Efficient clearance of these aggregates by the enhanced proteolytic activity of the immunoproteasome is important for the preservation of cell viability under IFN-induced oxidative stress, suggesting that the immunoproteasome contributes to fundamental functions other than a specific role in the production of class I MHC ligands. Other evidence also suggests the roles of the immunoproteasome beyond the antigen-processing pathway. In one landmark study, a specific inhibitor of β5i, named PR-957, blocked β5i-mediated presentation of MHC-I-restricted antigens (Muchamuel et al., 2009). Interestingly, the same inhibitor resulted in profound reductions in cellular infiltration, cytokine production, and autoantibody body levels, hence, attenuating the progression of experimental arthritis. These studies highlight a unique role for β5i in the control of pathogenic immune responses and provide a therapeutic rationale for targeting β5i in autoimmune disorders.

More recently, several groups independently reported that a homozygous missense mutation of the β5i gene (PSMB8) causes the autoimmune disorder, Nakajo-Nishimura syndrome (NNS), a disorder inherited in an autosomal recessive fashion characterized clinically by autoimmunization and lipodystrophy (Arima et al., 2011; Kitamura et al., 2011). It is likely that the G201V and G197V mutations disrupt the β-sheet structure, resulting in its protrusion from the loop that interfaces with the β4 subunit and locates in close proximity to the catalytic threonine residue (Unno et al., 2002). These β5i mutants are not efficiently incorporated during immunoproteasome biogenesis, resulting in reduced activities of all proteasomal peptidases within cells expressing immunoproteasomes, i.e., not only chymotrypsin-like but also, to a lesser extent, caspase-like and trypsin-like activities, and accumulation of ubiquitylated and oxidized proteins due to defective proteasome assembly, which leads to stress-dependent activation of the p38 MAP kinase pathway. Consequently, high expression of IL-6 is detected in the skin and B cells of the affected patients, together with reduced expression of β5i. These changes may account for the exaggerated inflammatory response and periodic fever observed in these patients. Moreover, downregulation of β5i inhibited the differentiation of murine and human adipocytes in vitro. These findings identify β5i as an essential component and regulator not only of inflammation, but also of adipocyte differentiation, indicating that immunoproteasomes have pleiotropic functions in maintaining the homeostasis of a variety of cell types.

Furthermore, it has been reported that the T75M mutation of β5i also causes two autoinflammatory syndromes, joint contractures, muscle atrophy, and panniculitis-induced lipodystrophy (JMP) syndrome and chronic typical neutrophilic dermatosis with lipodystrophy and elevated temperature (CANDLE) syndrome (Agarwal et al., 2010; Liu et al., 2011). The mutation is predicted to disrupt the tertiary structure. Compared to normal lymphoblasts, the inherited mutant immunoproteasome of an affected patient showed significantly reduced chymotrypsin-like proteolytic activity without affecting the other two peptidase activities, indicating that mutation in the β5i gene causes JMP syndrome, most probably by affecting MHC class I antigen processing. Apparently, the proteasomal defect due to T75M mutation profoundly differs from those of G201V and G197V mutations, but the discrepancy in proteasomal impairment remains unknown at present.

Role of thymoproteasome

Diverse sets of proteasomes have been identified with a special reference to the immune response. Another landmark study was the discovery of the thymoproteasome (Murata et al., 2007). To define the in vivo role, we generated β5t-ablated mice, which displayed major defects in thymic development of CD8+ T cells. Because no obvious abnormalities were observed in the thymic architecture including the size and histology, β5t does not seem essential for the differentiation and proliferation of cTECs; specifically, no obvious changes were observed in the numbers of cTECs, the CD4+CD8− (co-receptors of TCR) double-positive thymocytes, CD4+ T cell populations, and all other cells including Aire-expressing medullary thymic epithelial cells (mTEC). In addition, the β5t-deficient mice displayed no change in negative selection and no impairment in T cell responses. In contrast, the immunoproteasomes were increased, instead of the thymoproteasome (i.e., β5t changed to β5i in the thymus cortex), which could be responsible for the production of different MHC ligands that could not fulfill positive selection. This finding is compatible with the observation of lack of changes in the expression levels of MHC class I in β5t-deficient cTEC, indicating that most MHC class I molecules on the cTEC surface are associated with the peptides irrespective of β5t deficiency. These results emphasize the role of β5i in the development of
MHC class I-restricted CD8+ T cell repertoire during thymic selection, indicating that the peptides generated by the thymoproteasomes are responsible for the positive selection of developing thymocytes (Murata et al., 2007).

During positive selection, double-positive cells that interact with self-peptide-MHC complexes expressed on cTECs with a sufficiently modest avidity [i.e., affinity (MHC-TCR interaction) X density (surface MHC levels)] are rescued from intrathymic death and induced to differentiate into CD4+ or CD8+ single-positive thymocytes. In contrast, double-positive cells that interact at high affinity or avidity with self-peptide-MHC complexes are eliminated through apoptosis, a process referred to as negative selection (Klein et al., 2009). In addition, thymocytes that lack functional T cell receptors also undergo apoptosis, a process referred to as null selection.

The selection mechanisms are known as the thymic education of the immune system in the textbook. To date, however, the mechanism by which cTECs provide specialized signals for positive selection remains poorly understood. Considering that the proteasome is essential for the production of MHC class I ligands and that β5t specifically attenuates the proteasomal chymotryptic activity without changing caspase- and trypsin-like activities, it is possible that the thymoproteasome in cTECs predominantly produces moderately avid MHC class I ligands rather than high-avidity ligands, which would support the positive selection hypothesis. The discovery of the thymoproteasome can enhance our understanding of the mechanism of positive selection in the thymus (Murata et al., 2008; Takahama et al., 2012).

Until the discovery of the thymoproteasome, little was known about how self-peptides displayed in the thymus contribute to the development of immunocompetent and self-protective T cells. In contrast, the role of thymic self-peptides in eliminating self-reactive T cells and thereby preventing autoimmunity has been well established. To date, we know that cTECs displayed thymoproteasome-specific peptide-MHC class I complexes essential for the positive selection of major and diverse repertoire of MHC class I-restricted T cells. CD8+ T cells generated in the absence of thymoproteasomes displayed a markedly altered T cell receptor repertoire that was defective in both allogeneic and antiviral responses (Nitta et al., 2010). Thus, the thymoproteasome is required for the generation of optimal cellularity of CD8+ T cells and displayed an essential role in the development of MHC class I-restricted CD8+ T cell repertoire during thymic positive selection.

Role of regulatory particles

The RP recognizes polyubiquitylated substrate proteins, removes the polyubiquitin chain and entraps the protein moiety, unfolds the substrate proteins, opens the α ring, and transfers the unfolded substrates into the CP for destruction. However, little is known about the molecular/physiological functions of individual subunits of the RP in mammals.

Ubiquitin receptor Proteasomal receptors that recognize Ub chains attached to substrates are key mediators of selective protein degradation in eukaryotes. Currently it is well known that the 26S proteasome has two integral Ub receptors, Rpn10 and Rpn13, which recognize and trap polyubiquitylated proteins directly (Deveraux et al., 1994; Husnjak et al., 2008; Schreiner et al., 2008). In addition to the intrinsic Ub receptors, there are many shuttling factors, functioning as extrinsic Ub receptors, such as the UBL-Ub-associated (UBA) domain-containing proteins (e.g., Rad23, Dsk2, and Ddi1) and CDC48/VCP/p97, which can recruit indirectly many substrate proteins to the 26S proteasome for destruction (Finley, 2009).

Rpn10 and Rpn13 are dispensable in the budding yeast because individual deletion has no effect on growth, but they function collaboratively under stress conditions, indicating functional linkage between these two Ub receptors (Husnjak et al., 2008). However, there is confusion about their exact functions and locations. Initial studies indicated that Rpn10 physically connects the lid to the base, as its deletion resulted in easy dissociation of these two subcomplexes in yeast. Rpn10 is reported to bind with multiple RP subunits, Rpn1, Rpn2, Rpn9, and Rpn12, whereas Rpn13 is associated only with the Rpn2 subunit (Takeuchi et al., 1999; Xie and Varshavsky, 2000; Seeger et al., 2003; Hamazaki et al., 2006; Qi et al., 2006; Yao et al., 2006; Chen et al., 2010; Riedinger et al., 2010). Although previous studies suggested that Rpn10 and Rpn13 are located close to the ATPase pore (Zhang et al., 2009c), a recent structural study suggested that these two Ub receptors are located in a distant site on the apical part of the RP complex near the periphery, where the sites have been thought to be the lid (Lander et al., 2012; Lasker et al., 2012; Sakata et al., 2012), thus explaining the rationale for efficient trapping of the polyubiquitylated substrates.

In mammals, the Rpn10 gene generates multiple forms of the 26S proteasome by developmentally regulated alternative splicing; e.g., Rpn10 mRNAs occur in at least five distinct forms, named Rpn10a to Rpn10e in mouse (Kawahara et al., 2000). Rpn10a is ubiquitously expressed, whereas Rpn10e is expressed only in embryos, with the highest levels of expression in the brain. Thus, the 26S proteasome occurs in at least two functionally distinct forms: one containing a ubiquitously expressed Rpn10a and the other an embryo-specific Rpn10e. While the former is thought to perform proteolysis constitutively in a wide variety of cells, the latter may play a specialized role in early embryonic development, although the biological significance of the two remains to be elucidated. Considering the circumstances, exploring the functions of Rpn10a is not simple in vivo. In particular, the importance of Rpn10 in Ub-mediated proteolysis is debatable, as Rpn10 deficiency results in different phenotypes in different organisms. Surprisingly, Rpn10 knockout mice showed early embryonic lethality, demonstrating the essential role of Rpn10 in mouse development (Hamazaki et al., 2007). Rpn10a knock-in mice exclusively expressed the constitutive type of Rpn10, but did not express other vertebrate-specific variants and grew normally, indicating that Rpn10 diversity is not essential for conventional development.
Rpn13 is another proteasomal receptor that interacts with the largest subunit Rpn2 of the base complex via its aminoterminal. The majority of 26S proteasomes contain Rpn13, although some do not, and therefore, it remains debatable whether Rpn13 is or is not an integral subunit of the 26S proteasome. The majority of Rpn13-deficient mice survived to adulthood, although they were smaller at birth and fewer in number than wild-type littermates (Al-Shami et al., 2010). Absence of Rpn13 produced tissue-specific effects on proteasomal function: increased proteasome activity in adrenal glands and lymphoid organs and decreased activity in the testes and brain. Adult Rpn13-ablated mice reached normal body weight but had increased body fat content and were infertile due to defective gametogenesis. In addition, Rpn13−/− mice showed increased T-cell numbers, resembling growth hormone-mediated effects. Indeed, serum growth hormone and follicular stimulating hormone levels were significantly increased in Rpn13-laking mice, while growth hormone receptor expression was reduced in the testes.

Deubiquitylating enzymes  The main function of the lid is to deubiquitylate the captured substrates, a process in which the metalloisopeptidase Rpn11 functions to cleave the polyubiquitin chain at a proximal site (Verma et al., 2002; Yao and Cohen, 2002). So far, there is no genetic evidence for the Rpn11 gene in mice. In *Drosophila*, however, the loss of function of Rpn11 caused early onset of reduced 26S proteasome activity and premature age-dependent accumulation of ubiquitylated proteins (Tonoki et al., 2009). It was also associated with a shorter life span and enhanced neurodegenerative phenotype. Conversely, overexpression of Rpn11 suppressed age-related reduction of the 26S proteasome activity and extended the fly life span.

Uch37, a proteasome-associating DUB, is also involved in the editing of polyubiquitin chains. Intriguingly, deletion of Uch37 resulted in prenatal lethality in mice associated with severe defect in embryonic brain development, suggesting that Uch37 and Rpn13 play distinct roles in mouse development (Al-Shami et al., 2010), though Uch37 is associated with Rpn13.

USP14/Ubp6, another proteasome-interacting DUB, regulates the degradation of Ub-protein conjugates both in *vitro* and in cells (Hu et al., 2005; Koulbach et al., 2008; Lee et al., 2010). Curiously, the catalytically inactive variant of USP14 has reduced inhibitory activity, indicating that inhibition is not mediated by trimming of the Ub chain on the substrate (Hanna et al., 2006; Lee et al., 2010). Interestingly, mutation in USP14 was identified as a recessive neurological *axl* mutation, which resulted in ataxia phenotype in mice. The *axl*/*axl* mice had significantly low levels of the USP14 protein, which were associated with defective synaptic transmission in the central and peripheral nervous systems, though this was not associated with any detectable neuronal cell loss (Wilson et al., 2002). By a quantitative proteomics approach, we found near stoichiometric binding of Ubp6 to the base precursors of the RP as well as the mature 26S proteasome (Sakata et al., 2011). Ubp6 facilitates proteasomal assembly by clearing ubiquitylated substrates from assembly precursors through its deubiquitylating activity.

It is noteworthy that a selective small-molecule inhibitor of the deubiquitylating activity of USP14, identified by a high-throughput screening and referred to as IU1, enhanced the degradation of several proteasome substrates that have been implicated in neurodegenerative diseases (Lee et al., 2010). Inhibition of USP14 accelerated the degradation of oxidized proteins and enhanced resistance to oxidative stress. Thus, enhancement of proteasome activity through inhibition of USP14 may offer a strategy to reduce the levels of aberrant proteins in cells under proteotoxic stress. More recently, a small compound b-AP15 that induces the lysosomal apoptosis pathway was reported as an inhibitor of the proteasomal deubiquitylating activity, specifically USP14 and Uch37 activities (D’Arcy et al., 2011). Treatment by b-AP15 results in accumulation of the polyubiquitylated proteins and induces tumor cell apoptosis. Thus, the DUBs of the 19S RP are attractive drug targets as well as the CP.

ATPases  Rpt3 and Rpt5, components of the base complex of the RP, show a significant degree of amino acid similarity, especially in the conserved ATPase domain. Ablation of the Rpt3 (*PSMC4*) and Rpt5 (*PSMC3*) genes resulted in death before uterine implantation in mice, representing defective blastocyst development. In other words, Rpt3 and Rpt5 have similar and essential roles in early embryogenesis, and further, both ATPases have noncompensatory functions *in vivo* (Sakao et al., 2000). The conditional knockout mouse (cKO) of the Rpt2 gene (*PSMC1*) using the Cre/loxP system was produced to spatially restrict inactivation. The generated mice displayed depletion of the 26S proteasome in targeted neurons, in which the 20S proteasome was not affected (Bedford et al., 2008). Intriguingly, specific impairment of 26S proteasome-mediated protein degradation caused intraneuronal Lewy body-like inclusions, called pale body, and extensive neurodegeneration in the nigrostriatal pathway and forebrain regions. This report showed that 26S dysfunction in neurons is sufficient to trigger neurodegenerative disease and is involved in the pathology of neurodegenerative disease.

Like the CP assembly pathway, the base assembly is also a highly organized process assisted by four base-dedicated chaperone proteins, including Nas2/p27, Nas6/gankyrin =p28, Rpn14/PAAF1, and Hsm3/S5b (yeast/human: miscellaneous nomenclature) (Funakoshi et al., 2009; Kaneko et al., 2009; Le Tallec et al., 2009; Park et al., 2009; Roelofs et al., 2009; Saeki et al., 2009). Recent studies proposed renaming of Rpn14, Nas6, Nas2, and Hsm3, to RP assembling chaperones (RACs), i.e., RAC1, 2, 3, and 4, respectively (Tomko and Hochstrasser, 2011). Curiously, several reports addressed the importance of gankyrin, which is an ankyrin-repeat protein with structural resemblance to the CDK inhibitor p21Cip1 and is a new oncoprotein overexpressed early in human hepatocarcinogenesis (HCC) and in hepatocellular carcinomas and regulator of pRb and p53 (Higashitsuiji et al., 2000; Dawson et al., 2002, 2006). Based on the role of gankyrin in malignancy, it could also have a unique function solely independent on the chaperone function.
Role of PA28 and PA200

PA28α/β  To assess the in vivo role of the PA28α/β complex, we generated mice lacking both the PA28α and PA28β genes (Murata et al., 2001). No obvious growth abnormalities were observed in the mutant mice. Available evidence indicates that the proteasome functions as a processing enzyme responsible for the generation of MHC class I ligands, which are critical for the initiation of cell-mediated immunity in vertebrates. As mentioned, various immunomodulatory cytokines, mainly IFN-γ, induce overexpression of PA28α and PA28β in most cells. In addition, IFN-γ also overexpresses the majority of proteins related to the MHC class I ligand presentation pathway, such as MHCs, transporter associated with antigen processing (TAP); thus, it is plausible that PA28α/β contributes to efficient production of CTL epitopes. Although splenocytes from PA28α−/PA28β− double knock-out mice displayed no apparent defects in the processing of ovalbumin and normal immune responses against infection with influenza A virus, they lacked almost completely the ability to process the melanoma antigen TRP2-derived peptide. These findings indicate that PA28α/β is not required for antigen presentation, in general, but instead plays an essential role in the processing of certain antigens. These results are in marked contrast to the previous finding of impaired immunoproteasome assembly and immune responses in PA28β−/− mice (Preckel et al., 1999), but the reason for this discrepancy remains entirely unknown.

Recent studies reported that PA28α/β deficiency reduced the production of MHC class I-binding peptides both in cells with and without immunosubunits, in the latter cells, further decreasing an already diminished production of MHC ligands in the absence of immunoproteasomes (de Graaf et al., 2011). These findings demonstrate that PA28α/β and the proteasome immunosubunits use fundamentally different mechanisms to enhance the supply of MHC class I-binding peptides; however, only the immunosubunit-imposed effects on proteolytic epitope processing appear to have substantial influence on the specificity of pathogen-specific CD8+ T-cell responses.

PA28γ  To investigate the roles of PA28γ in vivo, we generated mice lacking the PA28γ gene (Murata et al., 1999). PA28γ-deficient mice were born without appreciable abnormalities in any of the examined tissues, but they showed growth retardation after birth compared with wild-type mice. We also investigated the effects of PA28γ deficiency in vitro using cultured embryonic fibroblasts; cells lacking PA28γ were larger and displayed a lower saturation density than their wild-type counterparts. Neither the expression of PA28α/β nor the cytosolic localization of PA28α/β was affected in the PA28γ−/− cells. These results indicate that PA28γ functions as a regulator of cell proliferation and body growth in mice independent of PA28α or PA28β.

The PA28γ is localized in the nucleus and is not involved in the processing of intracellular antigens, but accumulating evidence suggests the importance of PA28γ in nuclear proteolysis (Zhang and Zhang, 2008). This protein contributes to the turnover of p53 via MDM2-mediated proteasomal degradation. The PA28γ facilitates the physical interaction between MDM2 and p53, promoting MDM2-dependent ubiquitylation and subsequent proteasomal degradation of p53, implying that PA28γ-mediated inactivation of p53 is one of the mechanisms involved in cancer progression. In addition, PA28γ promotes proteasome-mediated degradation of steroid receptor coactivator-3 (SRC-3) and the cell cycle regulator p21Cip1 independent of ubiquitylation (Li et al., 2006, 2007b). The role of PA28γ in cell cycle regulation may extend beyond its effect on p21 because p16INK4A and p19ARF also bind to PA28γ and are stabilized in PA28γ-deficient cells (Chen et al., 2007). It is worth noting that PA28γ is localized in the nucleus in interphase cells and on chromosomes in telophase cells, suggesting a role in mitotic progression. This conclusion is supported by the marked aneuploidy (chromosomal gain and loss), supernumerary centrosomes, and multipolar spindles observed in the fibroblasts of PA28γ-deficient mice (Zammini et al., 2008). These findings underscore a previously uncharacterized function of PA28γ in centrosomes and chromosomal stability.

The ability of PA28γ to enhance viral protein degradation suggests its involvement in viral pathogenesis (Mao et al., 2008). Indeed, PA28γ plays a crucial role in the development of liver pathology induced by hepatitis C virus (HCV) infection; PA28γ gene knockout induced accumulation of HCV core protein in hepatocyte nuclei of HCV core gene transgenic mice and disrupted the development of both hepatic steatosis and hepatocellular carcinoma (Morishii et al., 2007). Thus, PA28γ does not only play a role in the pathogenesis but also in the propagation of HCV by regulating the degradation of the core protein. Taken together, PA28γ appears to have a diverse set of functions in mammals, presumably in the ATP-independent proteolytic pathway in the nucleus.

Intriguingly, it has become clear that the RP and PA28 activators simultaneously bind to the CP; PA28 and RP bind at opposite ends of the CP, forming the RP-CP-P28 complex (Tanahashi et al., 2000). This complex has been named the ‘hybrid proteasome’. EM analysis demonstrated that PA28 and RP can associate with the same CP in opposite orientations (Cascio et al., 2002). The hybrid proteasome seems to contribute to efficient proteolysis; intact substrate proteins may be first recognized by RP and then fed into the cavity of the CP, which shows markedly enhanced cleavage activity in the presence of the PA28αβ complex. Indeed, this complex catalyzes ATP-dependent degradation of substrate proteins, as does the 26S proteasome. The existence of hybrid proteasomes may explain the physiological importance of PA28αβ and/or PA28γ as described above. Therefore, it is plausible that both the 26S and hybrid ATP-dependent proteasomes contribute to the proteolytic pathway in mammalian cells.

PA200  The mammalian PA200 (alias yeast Blm10) localized in the nucleus was proposed to play a role in DNA repair and genomic stability, but the early indication for the repair of DNA damage could not be confirmed lately (Ustrell et al., 2002; Schmidt et al., 2005; Savulescu and Glickman, 2011). Genetic deletion of PA200 resulted in defective spermatogenesis in mice, suggesting that efficient
generation of male gametes has distinct protein metabolic requirements (Khor et al., 2006). This is consistent with the finding that loss of PA200 led to marked reduction in male, but not female, fertility. Intriguingly, deletion of Blm10 resulted in loss of mitochondrial function in yeast cells at high frequency, suggesting a role in the maintenance of mitochondrial inheritance, although this has not been observed so far in mammals (Sadre-Bazazz et al., 2010). With regard to the roles of PA200/Blm10, the data reported so far are inconsistent in many aspects, and thus, future studies are required to define its biological functions. In addition, the RP-CP-PA200 typed hybrid proteasome, like the RP-CP-PA28, has been detected in the cells, although their biological roles are entirely unknown (Ustrell et al., 2002; Schmidt et al., 2005).

### Other proteasome-interacting proteins (PIPs)

There are other auxiliary factors that regulate proteasome functions via direct binding, which are called proteasome-interacting proteins (PIPs). One example is Ecm29, which is a 200-kDa HEAT repeat protein that can bind to both the RP and CP in yeast (Leggett et al., 2002). EM clearly identified the Ecm29-CP complex, suggesting that Ecm29 stabilizes the 26S proteasome by tethering the CP to the RP. Recent studies suggested that Ecm29 could act as a proteasome-dedicated chaperone that controls the quality of RP-CP assemblies (Lehmann et al., 2010; Panasenko and Collart, 2011). Ecm29 could function as a scaffold protein during the remodeling of incompletely mature RP-CP assemblies into regular enzymes. In addition, Ecm29 has been proposed to regulate proteasome localization and promote proteasome disassembly upon oxidative stress (Gorbea et al., 2010; Wang et al., 2010).

Another interesting auxiliary factor is PI31. PI31 is a proline-rich protein that prevents the activation of the proteasome by each of RP/PA700 and PA28 (Chu-Ping et al., 1992; McCutchen-Maloney et al., 2000). PI31 is also a cellular regulator of proteasome formation and of proteasome-mediated antigen processing, based on the observation that PI31 selectively interferes with the maturation of immunoproteasome precursor complexes (Zaiss et al., 2002). Surprisingly, recent studies suggested that PI31 is structurally related to Fbxo7, the substrate-recognition component of the SCF<sup>Fbxo7</sup>-E3 ligase (Kirk et al., 2008). PI31 was identified as an Fbxo7-Skp1 binding partner whose interaction requires an N-terminal domain present in both proteins referred to as the FP (Fbxo7/PI31) domain. The crystal structure of the PI31 FP domain reveals a novel αβ-fold. The PI31 FP domain mediates heterodimerization of SCF<sup>Fbxo7</sup> and PI31. Consistent with this finding, the fly homologue DmPI31 was found as a binding partner of the F box protein Nutcracker, a component of a SCF Ub ligase required for caspase activation during sperm differentiation in Drosophila (Bader et al., 2011). DmPI31 binds Nutcracker via a conserved mechanism that is also used by mammalian FBXO7 and PI31. Furthermore, loss of DmPI31 function is lethal or causes cell cycle abnormalities, and defects in protein degradation, demonstrating that DmPI31 is physiologically required for normal proteasome activity.

To date, high-sensitive proteomics analysis has identified many other PIPs, but the functions of most of these PIPs remain unknown at this stage; thus, further studies are necessary to determine their pathophysiological significance.

### Outlook

In this review, we discussed the structure and molecular/pathophysiological functions of the core proteasome machinery and the main regulators of the CP, such as the RP, PA28, and PA200. While the three-dimensional structures of the 20S CP, PA26/PA28-CP, and Blm10/PA200-CP complexes have been determined by X-ray crystallography, the crystal structures of the 19S RP and the 26S proteasome remain elusive to date. However, two independent groups recently identified the complete subunit architecture of the 26S holocomplex by an integrative approach based on data from cryoelectron microscopy, X-ray crystallography, residue-specific chemical cross-linking, and several proteomics techniques, enhancing our understanding of the sequence of events preceding the degradation of polyubiquitylated substrates (Lander et al., 2012; Lasker et al., 2012) (for details, see model in Figure 4). Unfortunately, all efforts to unveil the atomic structures of the RP and/or the 26S proteasome have failed so far, but the complete molecular organization should be analyzed to understand how the proteasome catalyzes the destruction of target proteins mechanistically at atomic resolution.

Over the past 25 years, the divergent biological roles of the proteasome and the Ub system have been elucidated comprehensively. Currently, the components of the Ub-proteasome system (UPS) are estimated to comprise over 5% of all genes in the human genome, implying their biological importance. On another front, the incidences of intractable diseases, such as cancers, infectious diseases, and neurodegenerative diseases, have been increasing lately especially in the elderly population. In this context, there is general agreement that dysfunction of the UPS-mediated proteolysis machinery is a common abnormality in those diseases, at least in part. Accordingly, a multipronged approach for the analysis of the UPS system should contribute to the emergence of new bio-scientific field and the development of new therapies for the aforementioned diseases. In fact, the proteasome has recently emerged as a promising drug target in cancer therapy. For example, Velcade (bortezomib), a proteasome inhibitor, was first approved for refractory multiple myeloma, and its use has since been extended to mantle cell lymphoma. In addition, the next generation proteasome inhibitors, such as carfilzomib and salinosporamide A, have been developed and are already used in clinical trials (de Bettignies and Coux, 2010). Thus, we expect a rapid growth in the new field of proteasome biology, including both basic and clinical science, in the near future.

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