Review

The proteasome: Overview of structure and functions

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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

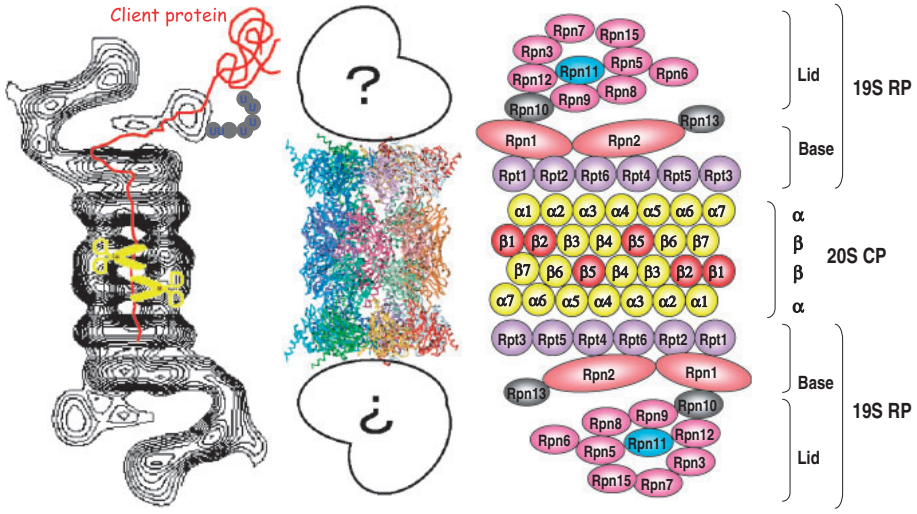

Fig. 1. Schematic diagram of the 26S proteasome. Left panel: Averaged image of the rat 26S proteasome complex based on electron micrographs. Photograph kindly provided by W. Baumeister. U, ubiquitin. Middle panel: The overall tertiary structure of the bovine 20S proteasome (central portion); the structures 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	α1	PSMA6	iota	SCL1, YC7	(252) 246	NLS	+	
	subunits	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	β1	PSMB6	Y, delta	PRE3	(19+196) 34+205	Ntn	+	Caspase-like
	subunits	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	ATPase	Rpt1	PSMC2	S7, Mss1	YTA3, CIM5	(467) 433	AAA	+	ATPase
(19S RP)	subunits	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	Rpn1	PSMD2	S2, p97	HRD2, NAS1/mts4	(993) 908	PC	+	PIPs scaffold
	subunits	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			PSME1	PA28α, REGα	—	249		(−)	PSM activator
(11S REG)			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:

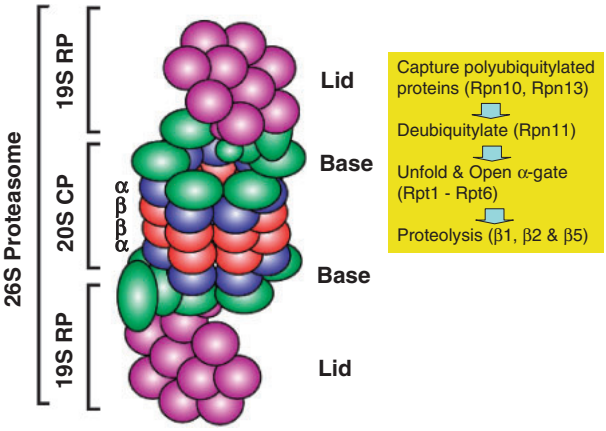

Fig. 2. Schematic diagram of proteolysis by the 26S proteasome. Positions of subunits indicated (i.e., Rpn10, Rpn13, Rpn11, Rpt1-Rpt6, β1, β2 and β5 are represented in Fig. 1. The β1, β2 and β5 subunits are associated with caspase-like, trypsin-like and chymotrypsin-like activities, respectively (for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

roles: capturing client proteins via ubiquitin recognition, promoting substrate unfolding and opening the channel in the α-ring (Fig. 2). Recently, a novel functional unit within the lid complex, comprising two subunits, Rpn1 and Rpn2 was proposed; i.e., Rpn2 interfaces with the 20S, whereas Rpn1 sits atop Rpn2, serving as a docking site for a substrate-recruitment factor.[25] The lid ATPases encircle the Rpn1-Rpn2 stack, covering the remainder of the 20S surface. Both Rpn1-Rpn2 and the ATPases are required for substrate translocation and gating of the proteolytic channel.

Rpn10 and Rpn13 function as integral ubiquitin receptors and efficiently trap polyubiquitylated substrates. Rpn10 achieves this function via a C-terminal ubiquitin-interacting motif (UIM).[26] More recently, Rpn13 was identified as a second ubiquitin receptor.[27–29] The N-terminal domain of Rpn13 shows no similarity to known ubiquitin-binding motifs, but instead contains the novel 'pleckstrin-like receptor for ubiquitin' (Pru) domain. The Pru domain in human Rpn13 shows a high affinity for diubiquitin. It is interesting that the C-terminal domain of Rpn13 produces the DUB activity, which may collaborate with the N-terminal ubiquitin receptor within the same molecule to facilitate proteolysis. It is possible that Rpn13 is not an intrinsic proteasome subunit, because the 26S proteasome, which does not contain Rpn13, exists to a lesser extent in mammalian cells.[22] In addition, although Rpt5 has been reported to bind polyubiquitylated proteins *in vitro*,[30] there is no direct evidence that it functions as a ubiquitin receptor in the cell.

In addition to the intrinsic ubiquitin receptors, there are several extrinsic UBL (ubiquitin-like)–UBA (ubiquitin-associated) ubiquitin receptors, such as Rad23, Dsk2 and Ddi1 (Table 2).[31,32] In higher eukaryotes, these UBL–UBA proteins interact with the 26S proteasome via their UBL domains. Indeed, the UBL domain can bind directly the Rpn10, which also functions as an acceptor site of polyubiquitylated proteins due to the UBA domain. Thus, UBL–UBA proteins also function cooperatively with several intrinsic receptors to recognize polyubiquitylated proteins.[33] In addition, the p62/SQSTM1, which contains N-terminal PB1 whose tertial structure resembles UBL, C-terminal UBA domains and VCP (also known as CDC48 or p97 ATPase), also binds both the proteasome and polymerized ubiquitin chains, thereby localizing client proteins to the 26S proteasome.[34,35] It was reported recently, however, that p62 functions as a key factor to direct ubiquitylated proteins to autophagy (i.e., self-destruction through the lysosomal machinery).[36,37] It is worth noting that Rpn1 (and Rpn2) is also responsible for acceptance of various UBL proteins,[38] similar to Rpn10.

Emerging evidence has provided significant insights into the roles of the ATPases in the base subcomplex. Substrates access the catalytic sites through the central pore in the α-rings. Free CP exists in an autoinhibited form in which the N termini of the α subunits create a gate that blocks substrate entry. The base contains six ATPase subunits (Rpt1-6) organized into a hexameric ring that facilitates the opening of the gate and allows the substrate to reach the catalytic sites. How proteasomal ATPases promote the gate opening in proteasomes remains a long-standing question. 20S proteasomes are activated following opening of the gate by the proteasome activators PA700 (i.e., the base of 19S RP) and PA28 (Ref. 39 see below for details). The PA28-mediated mechanism involved in gate control is well established. PA26 from *Trypanosoma brucei*, which is similar to mammalian PA28, is a homoheptameric complex. The crystal structure of the PA26–20S proteasome complex shows that PA26 binds to 20S proteasomes by inserting its C terminus into the intersubunit pocket between adjacent α subunits.[40] In addition, the "activation loop" domain of PA26 stabilizes the open-gate conformation. Interestingly, activation of 20S proteasomes by PAN, an archaeal homohexameric ATPase complex that is related to eukaryotic 19S ATPases, also requires the C-terminal residues of PAN. This suggests that the proteasomal ATPases and PA26 employ similar mechanisms to open the proteasome gate.

The archaeal PAN ATPase complex and the three 19S ATPase subunits each contain a conserved C-terminal hydrophobic-tyrosine-X (HbYX) motif required for gate opening. The C termini of the PAN molecules are inserted into the 20S pockets to induce gate opening through a mechanism that resembles a key and a lock.[41,42] Among the six 19S ATPases, only Rpt2, Rpt3 and Rpt5 contain the HbYX motif, and it is clear that multiple HbYX motifs from the three subunits

specifically facilitate the gate opening. One unresolved question is how the 6-fold symmetric ATPase ring associates with the 7-fold symmetric 20S α-ring to stabilize the open-gate conformation. Because only 2–4 subunits of the hexameric ATPase structure are thought to simultaneously bind ATP, not all of proteasomal ATPase subunits synchronously work to open the gate. Thus, only a subset of the C-terminal ends of the ATPase subunits inserts into the 20S pockets, which may occur sequentially to stabilize the open gate through an apparent "wobbling" of the proteasomal ATPases. Alternatively, the subunits may show different ATPase rates; e.g., Rpt2 and Rpt5 may be always bound to ATP to stabilize the open-gate conformation, whereas the remaining ATPase subunits may move dynamically through the ATPase cycle to promote protein unfolding. In this model, the 26S proteasome is rather a stable complex and two or three ATPase subunits simultaneously bind the 20S pockets to open the gate. It is noteworthy that similar to the PA28-mediated mechanism, ATP binding is sufficient for gate opening without requiring ATP hydrolysis.[43)]

The base subcomplex ATPases are required for not only α-ring channel opening but also substrate unfolding. Because the protein-degrading sites lie inside the 20S CP and are accessible only through the narrow channel, substrate proteins must be unfolded before they can reach the active sites in the β-ring chamber. Although details of the underlying ATP-dependent mechanism are still largely unknown, it is clear that the base subcomplex ATPases play a central role in substrate unfolding, through a process that requires ATP hydrolysis.[44)] Intriguingly, the base complex shows a chaperone activity *in vitro* that can cause refolding of unfolded proteins in the presence of ATP, but neither in the presence of ADP nor the absence of ATP.[45)]

4. PA28 and Hybrid Proteasome

PA28 or the 11S regulator (REG) was identified as another protein activator of the latent 20S proteasome (Table 1).[39)] Electron microscopic examination revealed that PA28 forms conical caps by associating with both ends of the central 20S CP.[7)] PA28 complexes are composed of three structurally-related members designated α, β and γ; their primary structures display approximately 50% homology.[46)] Whereas the PA28α and PA28β assemble into hetero-oligomeric complexes with alternating α and β subunits, 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 nucleus outside of the nucleolus.[47)] X-ray crystallographic analysis of recombinant REGα (PA28α) revealed a heptameric complex.[48)] Whether PA28α and PA28β form heteroheptameric (i.e., $\alpha3\beta4$ or $\alpha4\beta3$) complexes in cells, however, requires further investigation.

4.1 The PA28$\alpha\beta$ complex. The PA28 protein stimulates all of the peptidase activities of the 20S proteasome without affecting the destruction of large protein substrates, even if the proteins have already been polyubiquitylated. 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.

To assess the precise role of the PA28α/β complex *in vivo*, we generated mice lacking both the PA28α and PA28β genes.[49)] No obvious gross abnormalities were observed in the mutant mice. Available evidence indicates that the proteasome functions as a processing enzyme responsible for the generation of major histocompatibility complex (MHC) class I ligands, which are essential for the initiation of cell-mediated immunity in vertebrates.[50)–52)] Intriguingly, the immunomodulatory cytokine interferon (IFN)-γ induces overexpression of PA28α and PA28β.[46)] In addition, IFN-γ also overexpresses the majority of proteins related to the MHC class I ligand presentation pathway, such as MHCs and transporter associated with antigen processing (TAP); thus, it is plausible that PA28α/β contributes to efficient production of cytotoxic T lymphocyte (CTL) epitopes. Although splenocytes from PA28$\alpha^{-/-}$PA28$\beta^{-/-}$ mice displayed no apparent defects in the processing of ovalbumin and normal immune responses against infection with influenza A virus, they almost completely lacked the ability to process a 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.[49]

4.2 The PA28γ complex. The function of PA28γ, which is not involved in the processing of intracellular antigens, remains largely unknown. To investigate the roles of PA28γ *in vivo*, we generated mice lacking the PA28γ gene.[53] PA28γ-deficient mice were born without appreciable abnormalities in any of the examined tissues, but their growth after birth was retarded compared with that of 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 subcellular localization of PA28α/β was affected in the PA28$\gamma^{-/-}$ cells. These results indicate that PA28γ functions as a regulator of cell proliferation and body growth in mice and demonstrates that neither PA28α nor PA28β compensates for the PA28γ deficiency.

Recently, numerous reports have addressed the importance of PA28γ in nuclear proteolysis. This protein contributes to the turnover of p53 via MDM2-mediated proteasomal degradation. The polymer form of PA28γ facilitates the physical interaction between MDM2 and p53, promoting MDM2-dependent ubiquitylation and subsequent proteasomal degradation of p53; this process limits p53 accumulation, and thereby inhibits apoptosis after DNA damage.[54] These findings indicate the involvement of PA28γ in apoptosis and cell proliferation. In addition, PA28γ promotes proteasome-mediated degradation of steroid receptor coactivator-3 (SRC-3), which is encoded by an oncogene that is frequently amplified and overexpressed in breast cancers, highlighting an alternative proteasome-directed degradation mechanism, independent of 19S RP.[55] Moreover, PA28γ also enhances the degradation of the cell-cycle regulator $p21^{Cip1}$, independent of ubiquitylation.[56] The role of PA28γ in cell-cycle regulation may extend beyond its effect on p21, because $p16^{INK4A}$ and $p19^{Arf}$ also bind to PA28γ and are stabilized in PA28γ-deficient cells.[57] In addition, 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.[58] These findings underscore a previously uncharacterized function of PA28γ in centrosomes and chromosomal stability. The discovery that PA28γ controls cell-cycle regulators is consistent with a previous study that described body growth retardation of PA28γ-deficient mice.[59] Finally, PA28γ plays a crucial role in the development of liver pathology induced by hepatitis C virus (HCV) infection, because knocking out of PA28γ gene induced HCV core protein accumulation in hepatocyte nuclei of HCV core gene transgenic mice and disrupted the development of both hepatic steatosis and hepatocellular carcinoma.[60] Thus, PA28γ appears to play a diverse set of functions in mammals.

4.3 The hybrid proteasome. Immunoprecipitation analysis revealed that the PA28 and PA700 activators simultaneously bind to the 20S proteasome; PA28 and PA700 rings bind at opposite ends of the 20S particle, forming the PA700–20S–PA28 complex (Table 1). This complex has been named the "hybrid proteasome".[61] Because electron microscopic analysis revealed PA28 and PA700 occupy the same site on the 20S CP and the 26S proteasome, respectively, it is surprising that both activators can associate with the same 20S proteasome in opposite orientations.[62),63]

The hybrid proteasome seems to contribute to efficient proteolysis; intact substrate proteins may be first recognized by PA700 and then fed into the cavity of the 20S proteasome, which shows markedly enhanced cleavage activity in the presence of the PA28αβ complex. Indeed, this complex catalyzes ATP-dependent degradation of ornithine decarboxylase (ODC) without ubiquitylation, although it does require antizyme, an ODC inhibitory protein, as does the 26S proteasome.[64] Intriguingly, IFN-γ appreciably enhances the expression of PA28αβ and consequently promotes the formation of hybrid proteasomes, implying that this complex could be also responsible for the immunological processing of intracellular antigens. Moreover, the hybrid proteasome enhances the hydrolysis of small peptides and generates a pattern of peptides different from those generated by the 26S proteasome, without altering the mean product length.[62] Presumably, this change in the peptide profile accounts for the capacity of PA28 to enhance antigen presentation. The existence of hybrid

proteasomes may explain the physiological importance of PA28$\alpha\beta$ and/or PA28γ as described in previous sections. Therefore, it is plausible that the 26S and hybrid ATP-dependent proteasomes both contribute to the proteolytic pathway in mammalian cells.

5. PA200 or Blm10

Yeast Blm10 (formally Blm3 and equivalent to mammalian PA200) is reported to regulate proteasome assembly and/or proteolytic activity, although there are discrepant reports about its precise roles.[65),66)] Blm10 was identified in proteasome precursors purified with Ump1 in yeast. Blm10-decicient cells grew apparently normal under normal conditions but the turnover of Ump1 as well as the processing of β5 were accelerated, suggesting a role in preventing premature formation of 20S proteasomes.[67)] On the other hand, the combination of Blm10 deletion and β7 C-terminal truncation resulted in severe impairment of proteasome activity and β2 processing, indicating that Blm10 promotes proteasome maturation, presumably by stabilizing nascent 20S proteasomes.[68)] The discrepancy in the two paradoxical roles of Blm10 has not yet been explained.

Interestingly, the Blm10-CP-RP complex (i.e., PA200-20S-PA700 in Table 1) is found predominantly. Electron microscopy (EM) studies have shown that Blm10 has a highly elongated, curved structure, and adapts to the end of the CP cylinder, where it is properly positioned to activate the autoinhibited CP (i.e., closed-gate conformation) by opening the axial channel into its proteolytic chamber.[66)] In contrast, cryo-electron micrographs of the singly bound complex, PA200, shows an asymmetric dome-like structure with major and minor lobes.[69),70)] PA200 makes contact with all α-subunits except α7, and this interaction induces the opening of the axial channel through the α-ring, indicating that the activation mechanism of PA200 is expressed via its allosteric effects on the CP, perhaps facilitating release of digestion products or the entrance of substrates. It was proposed recently that whereas the single-capped Blm10-CP shows peptide hydrolysis activity, the peptide hydrolysis activity is repressed in double-capped Blm10-CP-Blm10, suggesting that that Blm10 distinguishes between gate conformations and regulates the activation of CP.[71)]

On the other hand, PA200 was also identified as a broadly expressed nuclear protein that activates proteasomal hydrolysis of peptides, but not proteins.[65)] In addition, in response to ionizing radiation, PA200 forms alternative hybrid proteasomes with PA200 and PA700 (i.e., PA200-CP-PA700) that accumulate on chromatin, leading to an increase in proteolytic activity, preferentially cleavage after acidic residues *in vitro*. Importantly, cells with PA200-knockdown by small interfering RNA (siRNA) show genomic instability and reduced survival after exposure to ionizing radiation, suggesting a unique role for PA200 in genomic stability that is likely mediated through its ability to enhance postglutamyl cleavage by proteasomes.[72)] However, PA200-deficient embryonic stem cells do not exhibit increased sensitivity to either ionizing radiation or bleomycin, implying that it is not essential for the repair of DNA DSBs generated in these experimental settings.[73)] Intriguingly, PA200 knockout mice are viable and exhibit no gross developmental abnormalities, but loss of PA200 led to a marked reduction in male, but not female, fertility, suggesting an important nonredundant function during spermatogenesis.

6. PI31

PI31, a previously described inhibitor of 20S proteasomes, prevents the activation of the proteasome by each of two proteasome regulatory proteins, PA700 and PA28, suggesting that it plays an important role in control of proteasome function.[74)] PI31 is a proline-rich protein, particularly within its carboxyl-terminal half where 26% of the amino acids are proline, which appears to have an extended secondary structure. Proteasome inhibition is conferred by the proline-rich domain of PI31. However, it also is reported that PI31 represents 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.[75)] Surprisingly, recent studies reported that PI31 is structurally related to Fbxo7, the substrate-recognition component of the SCF^{Fbxo7} E3 ligase.[76)] 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 PI31 FP domain mediates heterodimerization of SCF^{Fbxo7} and PI31.

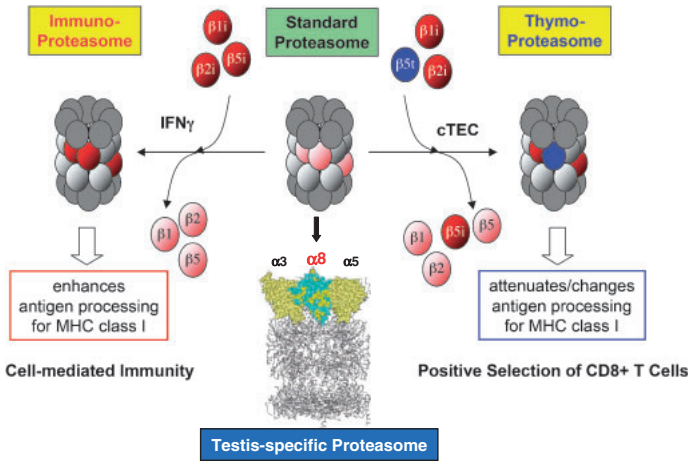

Fig. 3. Schematic representation of diverged proteasomes. 20S proteasomes are responsible for the proteolytic activity of the proteasomes and are composed of 28 subunits arranged as a cylinder containing four heteroheptameric rings with an $\alpha_{1\text{-}7}\beta_{1\text{-}7}\beta_{1\text{-}7}\alpha_{1\text{-}7}$ arrangement (middle-upper drawing, constitutive or standard proteasomes). In vertebrates, the β1i, β2i and β5i subunits are expressed in response to IFN-γ and are preferentially incorporated into proteasomes, resulting in the immunoproteasomes (left). The newly identified β5t catalytic subunit of 20S proteasomes is incorporated in place of β5 or β5i, together with β1i and β2i to form thymoproteasomes, which are specifically found in cTECs (right). The middle-lower drawing represents the putative testis-specific proteasome, in which α4 of mammalian standard proteasomes is replaced by a novel subunit, α8.

Intriguingly, PR39, a 39-amino acid cell-permeable antibiotic peptide composed of 49% proline and 24% arginine is a noncompetitive and reversible inhibitor of 20S proteasomes.[77] PR39 changes the conformational dynamics of the proteasome following their interaction, inducing a unique allosteric process that allows specific inhibition of degradation of selected proteins.[78] The suppressive effect of PR39 is universal for proteasomes from yeast to human, although its physiological role remains unknown.

7. Diversity of Proteasomes

The proteasome complex has been highly conserved during evolution due to its fundamental roles in cells. Budding yeast has seven α- and β-type subunits, consistent with the seven-subunit α- and β-ring observed in the 20S proteasome. In contrast, vertebrates have remarkably more than seven β-type subunits. Accordingly, the proteasome system in vertebrates has acquired considerable diversity among the catalytic subunits, which have evolved during the acquisition of adaptive immunity. In this section, I describe the diverse functions of the proteasome with particular emphasis on its immunological roles. Moreover, flies and plants contain multiple proteasome genes (e.g., duplicated or triplicated), though their biological roles are unknown.[79),80] For example, the *Arabidopsis thaliana* encodes 13 α-subunits and 10 β-subunits of 20S proteasomes.[81] In this section, I summarize advances related to this issue.

7.1 Immunoproteasome. Which are structurally related to β1, β2 and β5, respectively (Table 2). The expression of these three subunits with highly similar amino acid sequences to other β subunits indicates that IFN-γ may cause the β1, β2 and β5 subunits to be replaced with β1i, β2i and β5i, respectively. Accordingly, we have proposed that IFN-γ–inducible proteasomes should be called 'immunoproteasomes' (Fig. 3) to emphasize their specialized functions in immune responses and to distinguish them from complexes containing only constitutively expressed subunits.[50] On the other hand, the 20S proteasomes that include the constitutively expressed β1, β2 and β5 catalytic subunits are often called standard or constitutive proteasomes. MHC class I molecules bind peptides produced through proteolysis of cytosolic proteins and display them on the cell surface. This mechanism enables CTLs to detect and destroy abnormal cells that contain viral or other foreign proteins or tumor antigens. More than 15 years ago, the proteasome was identified as a candidate for the

enzyme that processes intracellular (or endogenous) antigens. To date, the roles of the immunoproteasome, which contributes to the efficient production of peptide epitopes for CTLs, have been highlighted in the MHC class I-restricted antigen-processing pathway and cell-mediated immunity.[50)–52)]

The three IFN-γ–regulated immunosubunits contain active threonine residues, indicating that the exchange of subunits induced by IFN-γ is likely to confer different functions to the proteasome. In fact, IFN-γ alters the proteolytic specificities of proteasomes, increasing their chymotrypsin- and trypsin-like activities to cleave peptide bonds on the carboxyl side of hydrophobic and basic amino-acid residues, respectively, and decreasing their caspase-like activities for peptides containing acidic amino acid residues.[50),52)] Comparison of the tertiary structures of the standard proteasome and the immunoproteasome using computer-assisted modeling suggested that the caspase-like activity is reduced and the chymotryptic activity is enhanced in the immunoproteasome.[13)] The altered peptidase activities suggest that immunoproteasomes in IFN-γ–treated cells should generate more peptides with hydrophobic or basic carboxyl termini and fewer peptides with acidic carboxyl termini. The peptides generated by the immunoproteasome are more likely to bind in the peptide-binding pocket of MHC class I molecules, because hydrophobic or basic (to a lesser extent) carboxyl terminal peptide residues normally serve as anchors for binding in these class I complexes. Thus, IFN-γ promotes the production of immunoproteasomes with an altered proteolytic specificity that may be more appropriate for the immunological processing of antigens.[50)–52)] It is likely that the acquisition of the immunoproteasomes during evolution enabled organisms to produce MHC class I ligands and combat pathogens more efficiently. Indeed, mice lacking β1i, β2i or β5i display defective antigen processing and consequently compromised immune responses.[82)–84)]

Sequence comparison of β-type subunits of standard and immunoproteasome genes indicates that each subunit pair that is exchanged in response to IFN-γ is encoded by genes derived from a common ancestor gene.[50)] We previously proposed a chromosomal duplication model to explain the emergence of the IFN-γ–regulated β-type subunits.[85)] The basic assumption of this model is that all of the IFN-γ–regulated β-type immunosubunits emerged simultaneously as a result of duplication of the MHC region during a genome-wide duplication.[50)]

7.2 Thymoproteasome. Recently, we identified a novel catalytic subunit designated β5t that is expressed exclusively in cortical thymic epithelial cells (cTECs); these cells are responsible for positive selection of developing thymocytes. The mechanism underlying the thymus-specific expression of β5t is not known. β5t is structurally related to β5 and β5i and is encoded by an intronless gene, in contrast to the three-exon β5 and β5i genes. Whereas the chymotrypsin-like activity of proteasomes is thought to be important for production of peptides with high affinities for MHC class I clefts, incorporation of β5t into proteasomes instead of β5 or β5i selectively reduces this activity. Although this characteristic differs from the imunoproteasome, the high overall sequence similarity suggests these subunits belong to the same family. β5t and the immunosubunits β1i and β2i, but not their standard counterparts, are incorporated into a vertebrate-specific alternative 20S proteasome referred to as the 'thymoproteasome' (Fig. 3).[86)] We found that β5t-deficient mice displayed major (but not total) defects in the thymic development of $CD8^+$ T cells, although no obvious abnormality was observed in the thymic architecture, suggesting that β5t is not essential for the differentiation and proliferation of cTECs. Importantly, no obvious alterations in the $CD4^+CD8^+$ (co-receptors of TCR) double-positive thymocytes and $CD4^+$ T cell populations were observed in $\beta5t^{-/-}$ mice. These results suggest a key role for β5t in the development of the MHC class I-restricted $CD8^+$ T cell repertoire during thymic selection.

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 with high avidity with self-peptide–MHC complexes are eliminated through apoptosis, a process referred to as negative selection.[87)–89)] In addition, thymocytes that lack functional T cell receptors also undergo apoptosis, a process referred to as null selection. To

date, however, the mechanism by which cTECs provide the specialized signals for positive selection has not been elucidated. Considering that proteasomes are essential for the production of MHC class I ligands and that β5t specifically attenuates the proteasomal chymotryptic activity without changing the caspase- and trypsin-like activities, it is possible that thymoproteasomes in cTECs predominantly produce moderate avidity MHC class I ligands rather than high-affinity ligands, which would support positive selection. The discovery of the thymoproteasome may contribute to our understanding of how positive selection occurs in the thymus. The types of antigenic peptides generated by the thymoproteasome, the underlying mechanism, and the roles in positive selection require further examination.

Like the immunoproteasome genes, the β5t and PA28α/β genes appear to result from modification and duplication of existing nonimmune genes, such as β5 and PA28γ, respectively, and may have been instrumental in the emergence of the adaptive immune system.

7.3 Other subtypes of proteasomes. Surprisingly, in *Drosophila*, about a third of the 32 proteasome subunits have testis-specific isoforms, encoded by paralogous genes.[90] Analysis of GFP-tagged transgenes showed that whereas the *Drosophila* α6 (Prosα6) subunit is expressed in early stages of spermatogenesis, gradually fading away following meiosis, the expression of testis-specific Prosα6T becomes prominent in spermatid nuclei and cytoplasm after meiosis, and persists in mature sperms. Moreover, a loss-of-function mutant of Prosα6T reveals that homozygous males are sterile and show spermatogenic defects in sperm individualization and nuclear maturation, consistent with the expression pattern of Prosα6T, indicating a functional role for testis-specific proteasomes during *Drosophila* spermatogenesis.[90]

Interestingly, although examination of the various α-rings shows that most organisms, except flies and plants have seven α subunits, an eighth α subunit (PAMA8: 20q13.33) that resembles the ubiquitously expressed α4 subunit is present in humans (PAMA7: 11q11.2; our unpublished results). The limited expression of the α8 subunit in cells and tissues, mainly in the testis, implies that it is involved in spatially and temporally restricted regulatory programs. Here I propose the term 'mammalian testis-specific proteasome' for the proteasome complex containing the α8 subunit instead of α4 (see Fig. 3).

As described in a previous section, Rpn10 functions as a receptor to trap polyubiquitylated client proteins for ultimate breakdown by the 26S proteasome. Intriguingly, mouse Rpn10 mRNA occurs in at least five distinct forms, Rpn10a–e, due to developmentally regulated alternative splicing.[91] These isoforms, with the exception of the universally expressed Rpn10a, are expressed in tissue-specific and/or developmental stage-specific manners. For example, Rpn10e is specifically expressed in the embryonic brain in mice, implying the existence of 'the brain-specific proteasome'. Knocking out the mouse Rpn10 gene was embryonically lethal,[92] although the specific reason was not determined. It is interesting that Rpn10a knock-in mice lacking the Rpn10 gene are born without any apparent abnormalities, suggesting that Rpn10a is a particularly important Rpn10 family protein.

Intriguingly, protein misfolding caused by exposure to arsenite induces the expression of arsenite-inducible proteasomal 19S regulatory-associated protein (AIRAP), which binds to Rpn1. Cells lacking AIRAP contain more polyubiquitylated proteins and exhibit higher levels of stress when exposed to arsenite, suggesting that AIRAP adapts the core protein degradation machinery to counteract the proteotoxicity of this environmental toxin.[93] In mammals, a second constitutively expressed AIRAP-like gene (AIRAPL) encodes a proteasome-interacting protein.[94] Whereas most AIRAP is associated with the 26S proteasome, AIRAPL is detected as a free form; exposure to arsenite, however, shifts some of this protein to heavier fractions that also contain the AIRAP peak colocalized with the 26S proteasome. Therefore, proteasomes containing these newly described subunits may be referred to as 'stress-specific proteasome'.

8. Proteasome Assembly

How the complex structures of the 20S and 26S proteasomes are organized remains largely unknown. For example, the mechanism responsible for the correct positioning of the various sets of different, but structurally-related subunits in the 20S proteasome is unclear. Recent studies have

examined this issue, particularly the biogenesis of eukaryotic 20S proteasomes from 28 subunits, each of which occupies a defined position within the 20S proteasome particle.

8.1 Assembly of 20S proteasome. The prokaryotic 20S proteasome consists of homo-oligomers of the same α and β subunits; these subunits can assemble autonomously into functionally mature proteasomes without the help of any chaperone proteins.[11] On the other hand, the assembly of the eukaryotic 20S proteasome is more complex; it requires a set of extrinsic [i.e., proteasome assembling chaperones (PAC) 1-4 and Ump1] and intrinsic [i.e., propeptides and C-tail of β subunits] chaperones (Table 2).[95)–99)] Among these potential regulatory processes, current studies aim to clarify the assembly mechanism of 20S proteasomes in yeast and human cells, which share common assembly and intramolecular chaperones, although some of their roles differ considerably.[98)]

8.1.1 *α-ring formation assisted by extrinsic chaperones.* The first assembly step for the eukaryotic 20S proteasome is α-ring formation, which was thought to occur autonomously before the identification of multiple chaperone molecules specialized for proteasome assembly. Remarkably, recent studies identified two heterodimeric complexes dedicated to proteasome assembly in human cells (PAC1–PAC2 and PAC3–PAC4) and in yeast (Pba1/Poc1, Pba2/Poc2, Pba3/Poc3/Dmp2, and Pba4/Poc4/Dmp1; orthologs of human PAC1, PAC2, PAC3 and PAC4, respectively) (Fig. 4).[98)]

Knockdown of PAC1 expression using siRNA resulted in the loss of PAC2, and *vice versa*, indicating that PAC1 and PAC2 are stable only when they form a heterodimer.[100)] Coexpression of PAC1 and PAC2 in *Escherichia coli* supported this hypothesis. The human PAC1–PAC2 heterodimer promotes α-ring formation *in vitro* and is mainly associated with an α-subunit proteasome assembly intermediate *in vivo*. Intriguingly, knockdown of PAC1 or PAC2 expression decreased the number of α-rings and resulted in accumulation of off-pathway products, presumably unusual α-ring dimers. These findings indicate that PAC1–PAC2 does not only promote α-ring formation but also prevents aberrant dimerization of the α-rings, which seems to be an intrinsic characteristic of α-rings. PAC1–PAC2 binds to proteasome precursors until the complete formation of the 20S proteasome; the complex is

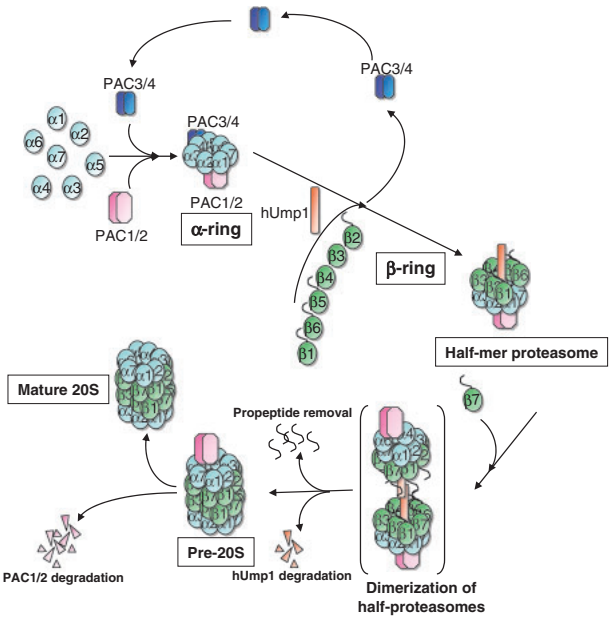

Fig. 4. A schematic model of mammalian 20S proteasome assembly. The PAC1–PAC2 and PAC3–PAC4 heterodimeric complexes are involved in the formation of the α-ring. Then, sequential incorporation of the β subunits begins with the binding of β2 and hUmp1 on the α-ring. hUmp1 is required for the association of β2 in early assembly intermediates. PAC3–PAC4, which is released at the time of β3 association, maintains the structural integrity of the intermediates until β3 is incorporated on the α-ring. The subsequent ordered incorporation of other β subunits is assisted by intramolecular chaperones, such as the propeptides of β2 and β5 and the C-terminal tail of β2. Dimerization of half-mers (i.e., half-proteasomes lacking β7) is assisted by the C-terminal tail of β7. This is followed by removal of the β subunit propeptides (β1, β2, β5, β6 and β7) as well as hUmp1 and PAC1–PAC2 degradation. Note that the role of Ump1 for dimerization of half-proteasomes or checkpoint of half-mers is emphasized in yeast, but its exact role is somewhat difference in mammals (for details, see text).

ultimately degraded by the newly formed 20S proteasome and therefore has a half-life of approximately 30 minutes, which agrees with the maturation period of the 20S proteasome. In budding yeast, Pba1–Pba2 appears to be the counterpart of human PAC1–PAC2 because Pba1 and Pba2 have similar sequences to those of PAC1 and PAC2, respectively, though the degree of sequence identity is marginal. Although the yeast and human proteins share several characteristics, such as heterodimer formation, association with the α-ring assembly intermediate, and short half-life, yeast strains lacking Pba1–Pba2 displayed only subtle de-

fects.[101] This contrasts with mammals, because PAC1 deficiency in mice caused early embryonic lethality (see below).

PAC3 and PAC4 were copurified with α-rings and PAC1–PAC2 in human cells (Ref. 102 and unpublished results). Knockdown of PAC3 and PAC1 expression produced additive effects on proteasome assembly; simultaneous depletion of PAC1 and PAC3 caused severe reduction in cellular levels of α-rings and 20S proteasomes compared with depletion of either PAC1 or PAC3 alone. These findings suggest that PAC1–PAC2 and PAC3–PAC4 function differently but cooperate with each other in the assembly of α-rings and half-proteasomes.

Pba3, the ortholog of PAC3 in budding yeast, and its binding partner Pba4, were identified by several groups independently.[103–106] Pba3 and Pba4 form a heterodimer complex, similar to PAC3 and PAC4; this complex specifically binds to proteasome precursors containing all seven α subunits and the unprocessed β2 subunit *in vivo*, whereas Pba3–Pba4 was found to interact directly and specifically with α5 *in vitro*.[104] Deletion of Pba3 or Pba4 in cells markedly decreased the level of 20S proteasomes and caused accumulation of assembly intermediates.[105] Taken together, these results suggest that Pba3–Pba4 catalyzes correct subunit orientation in the α-ring, presumably by collaborating with PAC1–PAC2 and facilitating the recruitment of α subunits.

X-ray crystallography revealed that the quaternary structure of the Pba3–Pba4 heterodimer displays profound similarity to that of the PAC3 homodimer, which may not exist *in vivo*, despite the divergence of the primary structures of these subunits.[104] The tertiary structures of all of the α and β subunits closely resemble each other, which, together with the obvious homology in their sequences, imply that they were derived from a common ancestral gene. Interestingly, the overall structure of the Pba3–Pba4 complex also resembles those of proteasomal α- and β-subunits. Structural analysis of the Pba3–Pba4–α5 complex revealed that Pba3–Pba4 binds on the surface of the α-ring at a position where the β subunits are assembled. The binding mode of Pba3–Pba4, however, is different from that of the β subunits; Pba3–Pba4 is located near the inner surface of the α-ring, which enables this complex to interact with three different α subunits, α4, α5 and α6, whereas the β subunits interact with two neighboring α subunits. This feature of Pba3–Pba4 might be helpful for initiating α-ring assembly. The location of Pba3–Pba4 is consistent with the results of biochemical analyses, which indicate that Pba3–Pba4 and PAC3–PAC4 detach from the α-rings during β-ring formation. Unfortunately, the molecular roles of PAC1–PAC2 in the assembly process are unclear, because structural information about PAC1–PAC2 and Pba1–Pba2 are not yet available. However, one can predict that PAC1-PAC2 makes contact with the surface of the α-ring, because this chaperone complex prevents the spontaneous dimerization of α-rings (i.e., the formation of off-pathway assemblies). On the other hand, Ump1 may posit inside in the newly formed proteasome, because it can associate with α-ring and is protected by tryptic digestion *in vitro*. Based on the findings that none of these dedicated chaperones is strictly dispensable in the budding yeast, and together with the observations that the α- and β-subunits can self-assemble *in vitro*, forming off-pathway products, unassisted assembly may be error prone.[97] However, genetic ablation of mouse orthologs of Ump1 or PAC1 caused early embryonic lethality (our unpublished observations), emphasizing the importance of the chaperone-mediated proteasome assembly pathway in embryonic development.

8.1.2 *β-ring formation assisted by intramolecular chaperones.* No β-ring assembly intermediates have been detected in cells, implying that the half-proteasome is not formed through the association of an α-ring with a β-ring. Rather the α-ring serves as a scaffold for the assembly of the β subunits; i.e., each β subunit is progressively added to the complex on the α-ring, resulting in half-proteasomes that consist of one copy each of the α- and β-rings. siRNA-mediated silencing of the expression of each β subunit in mammalian cells caused the accumulation of an "assembly-arrested" intermediate, representing the structure just before incorporation of the knocked-down β subunit. This technique allowed the order of β-subunit assembly on the α-ring to be defined: β2, followed by β3, β4, β5, β6, β1 and β7 (Fig. 4). Although β1 has been experimentally incorporated at various steps, its incorporation most likely follows that of β6. A recent report using yeast showed that the addition of other β subunits, excluding β7, form another

intermediate referred to as the "half-mer" precursor complex.[101] During β-ring assembly in human cells, release of PAC3 (and perhaps with PAC4) is coupled to β3 incorporation, which is consistent with the observation that Pba3–Pba4 was selectively copurified with β2 but not with other β subunits in yeast, indicating a conserved mechanism in the roles of PAC3–PAC4 and Pba3–Pba4 during proteasome assembly.

Propeptides and the tails of 20S proteasome β subunits facilitate proteasome assembly; these types of domains are called 'intramolecular chaperones'.[107] The N-terminal propeptides and C-terminal tails of β subunits play pivotal roles in proteasome assembly through specific interactions with *cis* and *trans* β-rings in yeast and humans. For example, the propeptide from β2 influences cooperative proteasome assembly.[108] The β5 propeptide facilitates the incorporation of this subunit and is essential for yeast viability.[109] On the other hand, the β5 propeptide does not appear to be required for incorporation of β5 but rather it is used for β6 recruitment in human cells.[110] The propeptides of β1 and β2 are dispensable for cell viability in yeast, although mutants lacking these two propeptides displayed subtle defects in proteasome biogenesis. Thus, the role(s) of these propeptides remains obscure. In human cells, loss of the β2 propeptide eliminated β3 recruitment and was thus fatal to the cells. Of note, the C-terminal tail of β2, which wraps around β3 within the same β-ring, is also essential for proteasome biogenesis both in yeast and human cells.

Interestingly, the amino-acid sequences of the human β-subunit propeptides are considerably different from those of their yeast counterparts, unlike the mature β subunits, which are well conserved between yeast and humans. Such differences are also found in the extrinsic proteasome assembly chaperones, such as PAC1-4 and Ump1 (i.e., 5–20% identity), as discussed previously.[98] Why the chaperones have diverged during evolution is unknown; nonetheless, their basic functions and tertiary structures are highly conserved.

Unexpectedly, intermediates resulting from siRNA-mediated knockdown of each β subunit accumulated as two major and minor bands, in which the composition of each major and minor band in terms of α and β subunits was identical.[110] PA28 was associated with the slow-migrating minor bands, different from PAC1 and Hsp90α, which were detected only in the major bands. Hsc70 was observed in both the major and minor bands. Neither Hsp90α nor Hsc70 was detected in the α-ring. At present, it is unknown whether these conventional chaperones really have any roles in proteasome biogenesis or whether they are merely associated with the intermediates as experimental artifacts.

8.1.3 *Role of another chaperone Ump*1 *and dimerization of half-proteasomes.* Ump1 was identified in mutant yeast defective for *u*biquitin–*m*ediated *p*roteolysis and is the first identified extrinsic assembly factor for 20S proteasomes.[111] Ump1 specifically associates with the assembly intermediates of 20S proteasomes and appears to enter the assembly pathway after association of β2, β3 and β4 in yeast. Upon dimerization of the half-proteasomes, Ump1 is encapsulated and degraded within the newly formed 20S proteasome like PAC1 and PAC2. Loss of Ump1 caused accumulation of assembly intermediates as well as half-proteasomes with unprocessed β subunits, indicating that Ump1 coordinates the processing of β subunits and dimerization of half-proteasomes in yeast.[111] On the other hand, Ump1 is also thought to function as an assembly checkpoint factor that inhibits dimerization of half-proteasomes until a full set of β subunits have been recruited to the α-ring.[101]

The human ortholog of Ump1 (hUmp1, Proteassemblin, or POMP) was identified using homology searches.[112,113] hUmp1 is included in precursor proteasomes with unprocessed β subunits and is degraded upon completion of proteasome assembly with a similar half-life to that of PAC1–PAC2.[110] Interestingly, knockdown of hUmp1 expression inhibited β5 recruitment, and resulted in the accumulation of α-rings with no β subunits. Moreover, hUmp1 can bind to the α-ring in the absence of β subunits and incorporation of hUmp1 is coupled with β2 binding, suggesting that hUmp1 is incorporated into proteasome precursors earlier than yeast Ump1. Therefore, hUmp1 is required for the initiation of β-ring formation, differing from the reported role of yeast Ump1. In the final step of β-ring assembly, the C-terminal tail of β7 is inserted into a groove between β1 and β2 in the opposite half-mer precursor, which triggers dimerization of the half-proteasomes in both yeast and humans.[68,101] Correct dimerization of half-protea-

somes is followed by removal of the β propeptides and degradation of Ump1 and PAC1–PAC2 (for details, see Ref. 98.) (Fig. 4).

8.2 Assembly of immune response proteasomes. Vertebrates encode four additional catalytic β-subunits: IFN-γ–inducible β1i, β2i and β5i and thymus-specific β5t (Fig. 3). These alternative proteasomes play key roles in acquired/adaptive immunity by altering antigen processing as mentioned above. Accumulating evidence has clarified the molecular mechanism of immunoproteasome assembly.[98] Despite the coexistence of both immunoproteasome and standard subunits in some cells, immunoproteasomes are preferentially assembled.[114] The propeptides of the immunosubunits and hUmp1 play key roles in this cooperative assembly.[115] Interestingly, β1i enters the assembly pathway of immunoproteasomes earlier than in the standard proteasome assembly process, resulting in an assembly intermediate containing the α-ring, β1i, β2i, β3 and β4. In this intermediate, incorporation of β2i depends on β1i, and incorporation of β1i is facilitated by β2i. β5i is incorporated preferentially over β5 into the intermediates containing β1i and β2i.[114] This interdependency supports the homogenous formation of immunoproteasomes containing all three inducible subunits. Indeed, β2i processing and incorporation is severely impaired in β1i-deficient cells, and β1i incorporation is partially inhibited in β2i-deficient cells, whereas β5i incorporation, which is dependent on the β5i propeptide but not β5i catalytic activity, is not affected in either of these mutant cell lines.[110] β5i-deficient cells exhibited significantly retarded proteasome assembly and accumulation of proteasome precursors containing unprocessed β1i and β2i. Intriguingly, IFN-γ stimulation increased transcription of hUmp1 and immunosubunit mRNA, but decreased hUmp1 protein levels due to $\sim$4-fold augmentation of hUmp1 protein turnover.[116] This rapid turnover was coupled with the maturation of active immunoproteasomes, indicating that the rate of immunoproteasome generation is four times faster than that of standard proteasomes. The higher affinity of hUmp1 for β5i than for β5 is likely to contribute to the rapid maturation of immunoproteasomes.[116]

How the thymoproteasome, another vertebrate-specific 20S proteasome, is assembled is currently unknown. When β5t was ectopically expressed in a human cell line that does not express immunosubunits, the protein was readily processed and incorporated into the proteasome, suggesting that β5t is preferentially incorporated compared with β5 and that β1i and β2i (i.e., partners of thymoproteasomes) are not required for β5t incorporation.[87] Because the majority of proteasomes in cTECs are thymoproteasomes, it is thought that β5t is preferentially incorporated before β5i in the thymus, suggesting that thymoproteasomes employ a specific assembly mechanism. Indeed, considering the high expressions of β1i and β2i, β5i whose gene and β2i gene are located at the same MHC class II region must be expressed in cTECs. According to the scenario for the immunoproteasome assembly, it is plausible that the propeptide or the extended C-terminal tail of β5t contributes to the assembly of the thymoproteasome as an intramolecular chaperone, but there is no available information at present in support of this assumption.[98]

8.3 Assembly of 19S RP and 26S proteasome. Currently, the assembly mechanism for the 19S RP is poorly understood. The yeast lid complex seems to be subdivided into two clusters: one is made up of Rpn5, Rpn6, Rpn8, Rpn9 and Rpn11, and the other contains Rpn3, Rpn7, Rpn12 and Rpn15. The interaction between Rpn3 and Rpn5 connects these two clusters, implying a hierarchy in the incorporation of Rpn subunits into the lid complex.[117] Recently, it was proposed that the 20S proteasome functions as an assembly factor for the RP due to aberrant RP formation in the presence of defective 20S proteasomes in yeast.[105] It was also proposed that the base and the lid are assembled independently, and then joined together.[118] The base is composed of six related AAA-ATPase subunits and four non-ATPase subunits. Putative chaperones may discriminate and arrange the six homologous ATPase subunits in a defined order, as is observed in the assembly of 20S α-ring. Whether assembly chaperones are required for the assembly of the ATPase ring, the lid, the base, and/or the 19S RP complex requires further studies.

The assembly mechanism of the 26S proteasome is largely not understood. Hsp90 is thought to play a role in both the assembly and maintenance of the lid in yeast.[119] Inactivation of Hsp90 was found to cause disassembly of the lid complex, which was then partially reassembled into the 26S proteasome

following reactivation of Hsp90 *in vivo* or by adding Hsp90 and ATP *in vitro*. These findings suggest that the ATP-dependent chaperone activity of Hsp90 contributes to the assembly of the lid and 26S proteasomes. The function of Hsp90 in the assembly of 26S proteasomes, however, remains to be elucidated. Inhibition of proteasome active sites also stabilized 26S proteasomes, suggesting that the interface between the RP and the 20S proteasome changes depending on the activities of the 20S proteasome.[120] Related to this result, whether 26S proteasomes undergo obligatory disassembly and reassembly during protein degradation is currently a point of debate in this field. It was first reported that disassembly of the 26S proteasome and dissociation of the RP into subcomplexes or subunits are induced upon ATP-dependent degradation of a substrate protein in yeast.[121] In contrast, it was more recently reported that mammalian 26S proteasomes can degrade polyubiquitylated proteins without disassembling or the release of any subunits or subcomplexes.[122]

9. Proteasome Interacting Proteins (PIPs)

Recent proteomic analyses have identified auxiliary factors with known and unknown functions that are physically and/or transiently associated with the 26S proteasome.[123]–[125] These proteins, referred to as proteasome-interacting proteins (PIPs), can be categorized into two groups (Table 1). The first group contains protein factors that are related to the ubiquitylation system. In this article, I described the association of the deubiquitylating enzymes Usp14 and Uch37 with the base subunits Rpn1 and Rpn2 via Rpn13, respectively. The extrinsic UBL–UBA ubiquitin receptors may also belong to this group. In addition, emerging evidence indicates that many ubiquitin E3 ligases, such as Hul5/KIAA10, E6AP, and Parkin, are transiently associated with the 26S proteasome. Moreover, other E3s such as Ubr1, APC, Ufd4 and SCF^{CDC4} as well as some E2 enzymes are also reported to associate loosely with the 19S RP of 26S proteasomes.[9]

The second group contains auxiliary factors that regulate proteasome functions via direct binding. For example, Ecm29 is an approximately 200-kDa protein that can bind to both the RP and the 20S proteasome in yeast. Purified 26S proteasomes from *Δecm*29 cells tend to dissociate into RPs and 20S proteasomes. Together with the findings of electron micrographs of Ecm29–20S proteasome complexes, these results suggest that Ecm29 stabilizes the 26S proteasomes by tethering the 20S proteasome to the RP.[126],[127] The mechanism underlying this function, however, is unclear. As listed in Table 2, there are many other factors, such as p28/gankyrin, Rpn14, p27 and S5b that interact with proteasomes. Some of them are suggested to be responsible for the regulation of 26S proteasomes or the assembly of the lid and base complexes, the process is largely ambiguous to date, but the details of their functions are unknown and require further studies.

Perspectives

The UPS is essential for cells to proliferate, and consequently proteasome levels are tightly regulated. For example, the balance between 20S and 26S proteasomes fluctuates to respond to environmental conditions; e.g., while the 26S proteasome levels increase during growth and developmental stages the 26S proteasome attenuates with aging process in *Drosophila*.[128] In addition, proteasomes are predominantly distributed in the nuclei of rapidly proliferating mammalian cells and growing yeast, indicating that this localization may contribute to cell proliferation. Why the proteasome is predominantly located in this cellular compartment remains to be determined, although typical nuclear localization signals (NLSs) are found on several of the 20S proteasomal α subunits, but not the β subunits.[129] No clear NLSs have been identified in the 19S RP subunits, except Rpn2, but it is plausible that the lid and the base are transported into the nucleus independently (unpublished results); the mechanisms underlying this translocation are a complete mystery at present. In addition, the issue of nuclear export (i.e., nucleocytoplasmic transport) of proteasomes is totally open to investigation. Indeed, nuclear export signals (NESs) of 20S and 26S proteasomes remain undefined.

To date, various lines of evidence have supported the importance of proteasomes outside of their proteolytic functions, such as transcription, DNA repair, and chromatin modeling.[9] For example, the 19S RP may contribute to transcriptional control in cells, independent of the functions of the 20S proteasome.[130],[131] The non-proteolytic activities of the proteasome are important for co-

activator recruitment; i.e., the ATPase activity of PA700 drives a stable association of a transactivator with the SAGA histone acetyltransferase complex.[131] PA700 also acts nonproteolytically in nuclear excision repair (NER).[132),133] Chromatin remodeling is another nonproteolytic role of PA700, with implications for both transcription and DNA repair.[131] In addition, a proteasome-derived ATPase activity mediates relocalization of the substrates of endoplasmic reticulum-associated degradation (ERAD), a function that is primarily attributed to the AAA-ATPase p97/Cdc48.[134] ERAD eliminates aberrant proteins from the ER by localizing them to the cytoplasm where they are tagged by ubiquitin and degraded by the proteasome.

As described before, PI31 and PR39 are naturally occurring proteasome inhibitors, but their physiological functions are unclear. On the other hand, membrane-permeable synthetic inhibitors have been devised; e.g., various substrate-related peptidyl aldehydes have been designed as potent inhibitors of proteasomes, such as MG-132 (*N*-carbobenzoxy-Leu-Leu-leucinal) and PSI (*N*-carbobenzoxy-L-gamma-t-butyl-L-glutamyl-L-alanyl-L-leucinal), and the non-aldehyde peptidyl inhibitor Z-L_3VS (carboxybenzyl-leucyl-leucyl-leucine vinyl sulfone), which are often used in *in vitro* and *in vivo* experiments.[135),136] However, caution must be exercised in their use for inferring proteasome functions, because they inhibit not only proteasomes but also cysteine proteases such as calpains and lysosomal cathepsins.[135] In contrast to these compounds, microbial metabolites, lactacystin and epoxomicin, were found to be selective proteasome inhibitors that do not affect other proteases examined so far.[137),138] Of particular interest is bortezomib (also known as velcade or PS-341). Bortezomib as first-in-class proteasome inhibitor has proven to be highly effective in some hematological malignancies, and in fact it has been granted approval by the FDA for relapsed multiple myeloma and non-Hodgkin lymphoma (NHL) and has been used clinically in over 85 countries worldwide so far.[139] Moreover, preclinical studies demonstrate that proteasome inhibition potentiates the activity of other cancer therapeutics, and particularly, the combination of proteasome inhibition with novel targeted therapies is an emerging field in oncology.[140] Furthermore, Salinosporamide A (also called NPI-0052),[141] recently identified from the marine bacterium *Salinispora tropica*, is a potent inhibitor of 20S proteasome and exhibits therapeutic potential against a wide variety of tumors. In addition, many other proteasome inhibitors are being assessed clinically for therapeutic use.[142] Thus, proteasome inhibitors provide a powerful new tool as fashionable drugs against cancer and other diseases including inflammations.

Finally, it should be emphasized that studies of the proteasome continue to provide significant insights in the physiologic roles of these complexes. Many questions, however, remain to be uncovered.

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Profile

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