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Proteasomes: Structure and Biology[1]

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The proteasome is a multisubunit protease complex with an apparent sedimentation coefficient of 20S. Two types of regulatory complexes, named PA700 and PA28, bind to both ends of the cylindrical 20S proteasome to form the dumbbell-like and football-like proteasomes, respectively. The former complex, named the 26S proteasome, is a eukaryotic ATP-dependent protease and appears to be well organized as a large complex of 2 MDa, consisting of approximately 40 polypeptides, to facilitate rapid proteolysis. It is assumed to be a protein "death machine", destroying a variety of cellular proteins that have acquired a specific degradation signal(s) such as a multiubiquitin chain. Recently data on *in vivo* substrates for the ubiquitin-proteasome pathway have been accumulating rapidly, implying its involvement in many biologically important processes, such as cell-cycle regulation, signal transduction, protein quality control, and the immune response. The newly-identified PA28 family proteins are inducible by interferons, and may cooperate with the 26S proteasome or play additional roles. Since the proteasome is capable of catalyzing breakdown of proteins not only irreversibly, but also rapidly and timely, it is thought to be a new regulatory system for biological reactions in eukaryotes.

Key words: ATP-dependent proteolysis, 20S and 26S proteasomes, PA700, PA28, ubiquitin.

Forty-five years ago it was found that metabolic energy was required for protein degradation in living cells (for this history, see a recent review, Ref. *1*), but the molecular mechanism has not been clarified until recently. In 1978 Hershko and his coworkers discovered ubiquitin (Ub) as a protein factor supporting ATP-dependent proteolysis. Ub was found to be covalently attached to target proteins in an ATP-dependent fashion to form the degradation signal, suggesting that energy for proteolysis in mammalian cells is needed for covalent modification of the substrate. Moreover, in 1983 we proposed a new model for the energy-dependency of eukaryotic protein degradation in which ATP is utilized at two distinct steps, one requiring and the other independent of Ub (*2*). However, instead a great deal of progress was made on the study of the Ub system thereafter, the mechanism of the latter Ub-independent process remained to be uncovered for a long time.

On the other hand, many investigators had noticed that eukaryotic cells contain a novel protease with an unusually large size and multiple catalytic functions, which enzyme is distributed widely in all eukaryotes. A variety of terminology covering over 10 names was proposed for the enzyme, for example, high molecular weight protease or multicatalytic proteinase complex; and finally, in 1988, the term "proteasome" was proposed based on its structural features (reviewed in Ref. *3*). Before long, Rechsteiners' group indicated that two large proteases were present in cells, one being the 20S proteasome and the other, an ATP-activated protease with an apparent sedimentation coefficient of 26S (*4*). The larger 26S protease was shown to contain the 20S proteasome together with multiple components containing potential ATPase, and this protease was called the 26S proteasome to distinguish it from the original 20S proteasome (*3*). The 26S proteasome has been established as a eukaryotic ATP-dependent protease that appears to be responsible for the process we first pointed out, in which a second step of ATP-requirement is involved in addition to ubiquitination. Thus the eukaryotic ATP-dependent system for proteolysis consists of two distinct processes: first, recognition of target proteins by Ub and then degradation of Ub-ligated proteins by the 26S proteasome, with metabolic energy being required for both steps.

During the past decade the 20S and 26S proteasomes have been studied as a central protease in eukaryotic cells, providing much information concerning not only their protein and gene structures, but also their significance (*3*). In this review, I summarize current information on structural aspects of proteasomes, and discuss their biological roles identified recently. The proteasome study appears to be coupled to the tremendous progress in research on the Ub system (*5*), and thus I wish to address it briefly prior to describing proteasome structure (I cite recent papers; and for others, see reviews Refs. *3-7*).

The ubiquitin system

Ubiquitin is an 8.6-kDa highly conserved polypeptide,

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which is covalently ligated to target proteins by a multi-enzymatic system consisting of E1 (Ub-activating), E2 (Ub-conjugating), and E3 (Ub-ligating) enzymes, through an isopeptide linkage (Fig. 1). Multiple Ub moieties become attached repeatedly to target proteins to form branched multi-Ub chain. This post-translational modification system was discovered by Hershko's laboratory using biochemical methods (*1*) and confirmed by Varshavsky's group who employed yeast genetics and showed the pathway to operate by the so-called N-end rule (*6*). In these studies, E3 was shown to serve as a recognition protein, indicating that it is capable of associating with both E2-Ub and target protein for promoting an efficient ligating reaction between them. However, E3 species recently identified have an active Cys residue accepting Ub from E2-Ub to form a thioester-bond, indicating that it functions as an actual ligase to transfer Ub to the target protein; and thus ubiquitination seems to be a cascade reaction of E1-E2-E3 (see below). To date, accumulating evidence indicates multiple species of E3 with apparently distinct properties in terms of their sizes and subunit organizations, but it is unknown so far how many E3s are present in cells. Characterization of novel E3 species in various eukaryotes may provide new insight for clarifying the broad roles of the Ub system.

It is of outstanding note that ubiquitination of target proteins appears to be a reversible reaction, because a set of de-ubiquitinating enzymes are present in cells; for example, there are 17 genes encoding proteins with the conserved catalytic sites for de-ubiquitinase in budding yeast (*5*). Their action has been implicated in the generation of Ub from poly-Ub and Ub-fused gene products, "proofreading" of incorrectly ubiquitinated proteins, or "trimming" of abnormal multi-Ub structures; but the reason why so many enzymes are present in the cells is still unknown. Irrespective of clarification of their exact roles, the reversibility of the ubiquitinating reaction implies that the proteasome is no doubt a most important enzyme responsible for determining the final fate/stability of proteins in cells (see Fig. 1).

The protein death machinery

The 20S proteasome was found initially as a multifunctional protease complex with diverse peptidase activities, and later it was demonstrated as the catalytic portion of the 26S proteasome. The molecular weight of the 20S proteasome has been determined by various physical techniques to be 700–750 kDa. The 20S proteasome is composed of a set of small subunits of 21–32 kDa, having strikingly different isoelectric points of 3–9 and being arranged in a stack of four rings that comprise a cylindrical particle. These proteasome subunits can be divided into two distinct subfamilies, α and β (*3, 5*). Seven homologous α- and β-polypeptides are assembled into each α- and β-ring, respectively, as observed by electron microscopy; and these rings are associated in the order $\alpha\beta\beta\alpha$ to form the cylindrical particle (7, and Fig. 2, middle part). Recently, high-ordered structural analysis of yeast proteasomes by

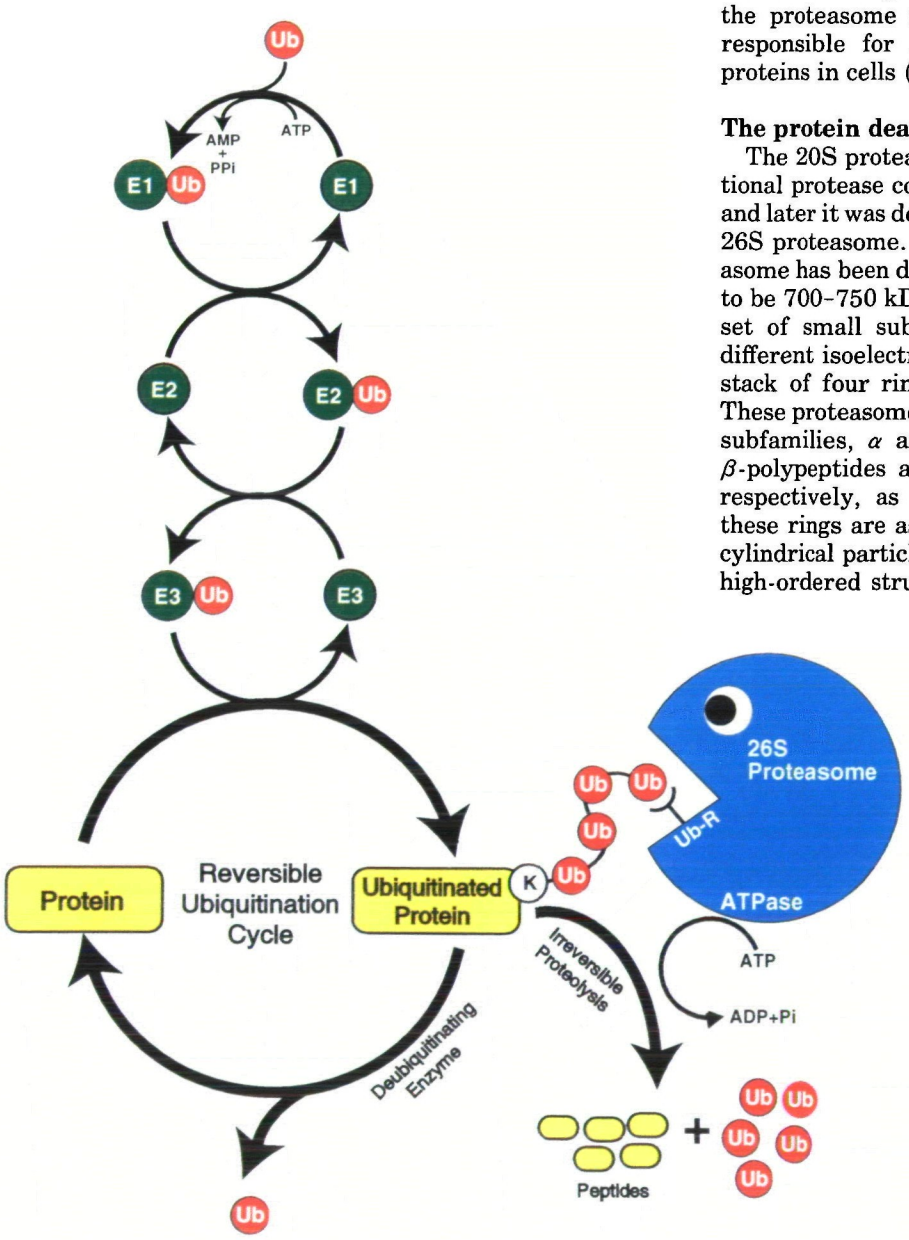

Fig. 1. **The ubiquitin-proteasome system.** For details, see text. Ub, ubiquitin, E1 (Ub-activating), E2 (Ub-conjugating), and E3 (Ub-ligating) enzymes. Ub-R, multi-ubiquitin receptor.

X-ray crystallography showed that 14 (7α- and 7β-) independent subunits were found to be assembled in a single complex (*8*). Interestingly, the center of the α-ring is closed, preventing penetration of proteins into the inner surface of the β-ring on which the catalytic threonine center of three β-subunits resides, suggesting that potential regulatory complexes such as PA700 or PA28 (see below) attached to the α-ring must open the gate of the β-ring for entry of the protein substrate.

The 26S proteasome is a eukaryotic ATP-dependent protease that was found as a large multisubunit complex with a molecular mass of approximately 2 MDa. It consists of a central cylindrical 20S proteasome, functioning as a catalytic machine, and two large V-shaped terminal modules, named PA700, having possible regulatory roles, attached to the central 20S proteasome in opposite orientations ATP dependently. The 26S proteasome thus, appears as a dumbbell-shaped particle (7, and Fig. 2, lower panel). The PA700 rectangular complex consists of a characteristic set of heterogeneous subunits of 25–110 kDa which are classified into two subgroups, *i.e.*, a subgroup of at least 6 ATPases that constitute a unique multi-gene family encoding homologous polypeptides conserved during evolution and a subgroup of over 15 non-ATPase subunits, most of which are structurally unrelated to each other (*3, 5*). One role of the ATPase is to supply energy continuously for the selective degradation of target proteins by the active 26S proteasome. Presumably the energy may be utilized for unfolding of proteins to allow them to penetrate the channel of the α- and β-rings of the 20S proteasome. However, it is unknown why multiple ATPases with high homologies are associated with PA700, although they are suggested to have distinct functions. These 6 ATPases are members of a large protein family termed AAA proteins (ATPases associated with a variety of cellular activities), characterized by a conserved 200-amino acid domain containing a consensus sequence for an ATP binding module. Some of the proteasomal ATPases have unique motifs related to the leucine-zipper or helicase and were discovered as transcriptional factors independent of the proteasome story, suggesting that they may have dual functions, but their exact biochemical roles are unknown.

The primary structures of approximately 15 subunits of the human PA700 that are structurally unrelated to members of the ATPase family have been determined, but their functions are unknown except for one subunit, a multi-Ub receptor. This obligatory subunit, which was identified as S5a/MBP1, is needed to trap ubiquitinated proteins for their degradation by the 26S proteasome (*9*). Surprisingly the yeast homologue Mcb1 (equivalent to Sun1) is present in free form without binding to PA700 or the 26S proteasome, being present as a free pool; and thus play dual roles freely recruiting ubiquitinated target proteins to the 26S proteasome for trapping and serving some other functions independent of the 26S proteasome (*10*). Moreover, and remarkably, disruption of the Mcb1/Sun1 gene had no effect on cell proliferation, suggesting another recognition system for trapping ubiquitinated proteins (*10, 11*). However, no proteins with similarity to Sun1 are encoded by the yeast genome, indicating that a presumptive 2nd Ub-receptor differs from Mcb1/Sun1 in the recognition mechanism. Another functional subunit associated with PA700 is a de-ubiquitinating enzyme that generates the Ub moiety from the multi-Ub chain for its reutilization. Recently, one protein of the PA700 complex, corresponding to the size of approximately 30 kDa, was identified as a Ub isopeptidase by affinity labeling using an inhibitor of the de-ubiquitinase (*12*). At present the functions of the many other non-ATPase subunits comprising PA700 are unknown, although most of them have been conserved during evolution from yeast to human.

Recently, another activator protein of the 20S proteasome was found and named PA28 (or 11S regulator) (*13*). The purified PA28 protein greatly stimulated multiple peptidase activities of the 20S proteasome without affecting destruction of large protein substrates, even though the proteins had already been ubiquitinated, suggesting that it may cooperate with the 26S proteasome in a sequential proteolytic pathway. Electron microscopic investigations indicate that PA28, free of the proteasome, is a ring-shaped particle, and that it associates with the 20S proteasome ATP independently by forming a football-like structure on both ends of the complex (*14*), indicating that PA28 occupies the same site on the 20S core particle as the regulator complex does in the case of the 26S proteasome. An average projection image of the PA28 associated with the 20S proteasome is shown in Fig. 2 (upper panel).

PA28 is composed of two homologous proteins, which are named PA28α and PA28β (*15*). Surprisingly, they are homologous to a previously described protein, Ki antigen, which was identified as a nuclear protein detected with autoantibodies found in sera of patients with systemic

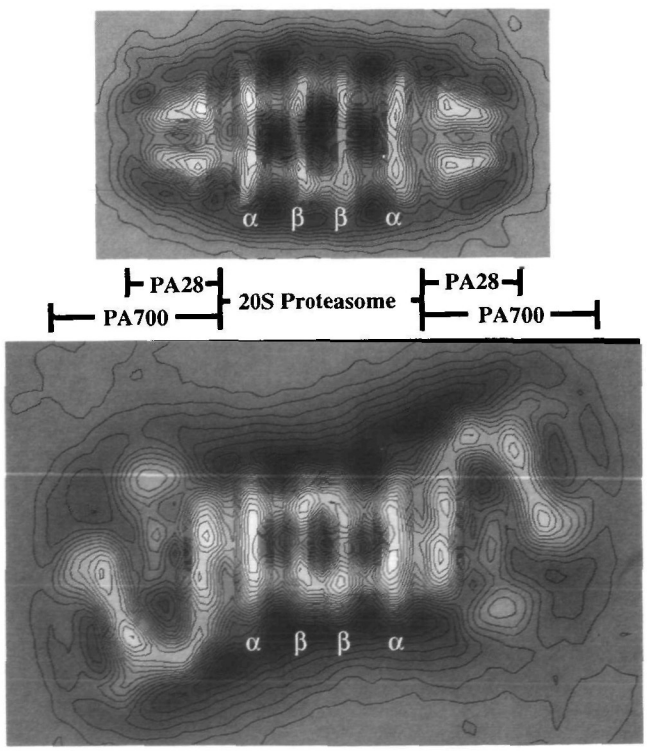

Fig. 2. **Averaged image, based on electron micrographs, of the complex of the football-like PA28-20S proteasome (upper panel) and dumbbell-like PA700-20S proteasome (lower panel) from rat liver.** The α and β rings of the 20S proteasome are indicated. The photos were taken in collaboration with W. Baumeister.

lupus erythematosis. Ki antigen was co-immunoprecipitated and associated reversibly with the 20S proteasome, as observed for PA28α and PA28β, indicating that Ki antigen is a genuine component of the PA28 family proteins; and, therefore, Ki antigen was renamed PA28γ (*16*). Thus, PA28s constitute a new protein family generated by duplication of a common ancestral gene, although their counterpart genes are not present in budding yeast. PA28α and PA28β were shown to be assembled into a hetero-hexamer complex with alternating α and β subunits, *i.e.*, PA28$(\alpha\beta)_3$. In contrast, the complex of PA28γ is distinct from that of PA28α and PA28β, suggesting that PA28γ forms perhaps a single homopolymer complex, presumably PA28$(\gamma)_6$ (*16*). Very recently we found that an antibody against p45 ATPase, a subunit of the PA700 complex, immunoprecipitated not only a set of 26S proteasome components, but also PA28α and PA28β, indicating simultaneous binding of PA28 and PA700 activators to the 20S proteasome (Hendil, Khan, and Tanaka, submitted). This "hybrid-type proteasome" may contribute to more efficient proteolysis; *i.e.*, perhaps intact substrate proteins are recognized first by PA700 and fed into the cavity of the proteasome whose cleavage ability is greatly stimulated by PA28.

These PA28 family proteins are unrelated to known proteins, but PA28α and PA28β, but not PA28γ, contains a "KEKE" motif, a hydrophilic region rich in alternating positively charged (lysine) and negatively charged (glutamate) amino acids (*17*). The KEKE motif is predicted to be responsible for a protein-protein interaction including assembly between PA28α and PA28β, but its exact role remains to be determined. Moreover, PA28α and PA28γ, but not PA28β, have possible nuclear location signals. Indeed, immunofluorescence analysis revealed that PA28α and PA28β are mainly found in the cytoplasm and diffusely present in the nucleus; whereas PA28γ is predominantly located in the nucleus, without appreciable localization in the cytoplasm (*18*, Wojcik, Paweletz, Tanaka, and Wilk, submitted). Thus, PA28γ may be involved in the functions *via* nuclear proteolysis by proteasomes, although the exact function of PA28γ is still unknown at the present time. It is interesting that the 20S proteasome apparently is present in considerable molar excess to PA28 and PA700 complexes. Nevertheless, considerable amounts of PA28 complexes are present in their free forms without associating with the 20S proteasome (*16*), suggesting that PA28 may have distinct role(s) unrelated to the proteasomal function. Alternatively there may be present unknown factor(s) capable of regulating association of PA28 with the 20S proteasome in cells. The physiological relevance of the PA28-proteasome complex has not yet been established, but PA28 plays an essential role in immunology (see below).

Biological consequences of protein death

In general, it seems difficult to determine the biological roles of intracellular proteases. Recently, yeast proteasomal mutants and membrane-permeable inhibitors have been used to determine the *in vivo* functions of proteasomes. The budding yeast mutants lacking some peptidase activities have contributed greatly to our understanding of the involvement of proteasomes in the degradation of many unstable key proteins (*19*), but their application to multicellular organisms appears to be difficult. Various substrate-related peptidyl aldehydes have been devised as potent inhibitors of proteasomes, such as Z-LLnV-H, Z-LLL-H (equivalent to MG115 and MG-132, respectively), and PSI, because they are likely to across plasma membranes in living mammalian cells (for abbreviations and Refs. *22-28*, see Table I). 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. Therefore, a negative control using other compounds, such as Z-LLM-H and Z-LL-H, which do not affect proteasomal activity at low concentrations, is required in determining the involvement of the proteasome (*28, 30*). Recently, the non-aldehyde peptidyl inhibitor Z-L_3VS was synthesized, but its specificity is unknown, although it strongly bound to cathepsins B and S (*24*). In contrast to these compounds, a new microbial metabolite, lactacystin (LS), was found to be a selective proteasome inhibitor that does not affect other proteases examined so far (*29, 21*). Recently, β-lactone converted from LS spontaneously by lactonization seems to be the actual form inhibiting the proteasome, and is considerably more effective than LC (*22*). Both LS and β-lactone are very effective in living cells, inducing almost complete loss of proteasome-mediated proteolysis.

Intriguingly, Z-LLL-H and LS were first found as novel factors having strong activity for initiating neurite outgrowth from immature neural cells, but later they were also found not only to block cell-cycle progression but also to induce apoptotic cell death (*31*). Moreover, apoptosis induced by LS is blocked partly by inhibitors of caspase-3 (CPP32) and LS induced CPP32-like activity, suggesting that the proteasome plays an essential role in the upstream of the CPP-32-mediated apoptotic pathway (S. Ohmi, personal communication). On the other hand, the proteasome has been recently shown to play an essential role in thymocyte apoptosis and in the programmed cell death of NGF-derived sympathetic neurons, because treatment with proteasome inhibitors resulted in almost complete blockage of these processes. It is unknown why these same inhibitors would also induce apoptosis as mentioned above, but it seems likely that proteasomal inhibitors induce appreciably apoptosis of rapidly-growing cells, whereas they inhibit greatly apoptosis of non-dividing cells induced by extracellular signals (*31*).

There is accumulating evidence that the proteasome is involved in the destruction of bulk proteins with rapid and slow turnover rates in a wide variety of biological processes such as cell-cycle progression, transcriptional control, signal transduction, and metabolic regulation. Here I focus on several topics to indicate the importance of the Ub-proteasome pathway. It should be noted how the proteasomal death machinery traps target substrates for accurate and timely destruction. The phosphorylation of proteins may serve to trigger ubiquitination and subsequent proteasomal breakdown. Indeed, many proteins, such as Cln2 (*32*), Cln3 (*33*), cyclin E (*34*), cyclin D1 (*35*), Cut 2 (*36*), p27 (*37*), Sic1 (*38*), Rum1 (*39*), I-κBα (*40*), STAT1 (*41*), IRAK (*42*), and β-catenin (*43*), are known to be phosphorylated prior to ubiquitination, implying that proteolysis is located downstream of signal transduction. This fact would be able to explain highly-regulated destruction of proteins, but it is not able to explain logically the mechanism whereby

phosphorylation leads to ubiquitination. One could postulate that there is a specific Ub-ligase capable of recognizing phosphorylated proteins, but so far there is no evidence to support this idea. Alternatively, the "PEST" polypeptide sequence, enriched in Pro, Glu, Ser, and Thr, and which serves as a proteolytic signal (*44*), may contribute to provide a site for phosphorylation related to ubiquitination. In contrast, however, there are some proteins, such as c-Mos (*45*), c-Fos (*46*), and c-Jun (*47*), whose phosphorylation blocks their rapid proteolysis, implying that the unphosphorylated forms are metabolically very labile in cells; but the mechanism as to how phosphorylation prevents ubiquitination of these proteins remains to be determined.

A finding of great impact was the discovery that B-type cyclin is degraded by the Ub-proteasome pathway (*48*), because it a key regulator of Cdc2 kinase, which acts as an engine for M-phase progression of the cell cycle. By further study, a novel Ub-ligase for cyclin B was found, which is a very large protein complex named the 20S APC (anaphase-promoting complex) or cyclosome, consisting of at least 8 distinct proteins (for details, see recent review Ref. *49* and references therein). The ligase may play a key role in M-phase progression by participating in the destruction of Pds1 (*50*), Cut2 (*36*), and Ase1 (*51*) in addition to cyclin B. Interestingly, APC itself appears to be regulated by phosphorylation in an M-phase-specific manner, suggesting that the phosphorylation-dephosphorylation system perhaps plays a critical role for maintaining normal cell-cycle progression. Moreover, the Ub-proteasome pathway plays a pivotal role at the G1/S transition of the cell cycle by causing destruction of CDK (cyclin-dependent protein kinase) inhibitors (Sic1, p21, and p27) and G1/S-phase cyclins (Cln 1-3, Clbs 5-6, and cyclins E, D1, and A). The Ub-ligase responsible for these degradations may be composed of a large complex named PULC (phosphoprotein-Ub-ligase complex, see Ref. *49*) containing Cdc53, Cdc4, and Skp1 (*38*).

The first indication of the direct involvement of the proteasome in cell proliferation was obtained by gene disruption analysis of budding yeast. Disruption of chromosomal genes encoding most of the 20S proteasomal subunits by homologous recombination had a lethal effect, indicating that the proteasome is essential for proliferation of yeast (*5, 19*). These findings suggest that each subunit may play a distinct and indispensable role in cell growth, or more probably, that all these subunits are required for the proper functional assembly of the proteasome complex. In addition, mutations of some ATPases of the 26S proteasome induced abnormalities in cell-cycle progression, *e.g.*, arrest in the M-phase of the cell cycle (*52, 53*). These findings are apparently consistent with our report that Nin1p, a non-ATPase regulatory subunit of the 26S proteasome of yeast, is required for activation of Cdc28 kinase at the G1/S and G2/M boundaries (*54*). The main reason for the important role of proteasomes may be that proteolysis can catalyze irreversible biological reactions, and that such definite regulations appear to be essential for cell-cycle traverse.

The finding of Ub-dependent degradation of p53, a tumor-suppressive gene product, has strongly affected subsequent study of the Ub pathway. The gene product E6 of high-risk human papillomavirus was found to stimulate the ubiquitination of p53; and subsequently an additional factor named E6-AP (E6-associated protein) was shown to be required for the E6-dependent ubiquitination of p53. Finally, E6-AP was proved to be a novel Ub-ligase, which has an acceptor Cys residue for the Ub moiety from E2-Ub (*55*). This finding was the first evidence for the cascade pathway consisting of E1-E2-E3 for ubiquitination. This finding provides a new concept that E3 acts as an actual Ub-ligase. More interestingly, the region containing the Ub acceptor residue is found to be common among various proteins and highly conserved during evolution, and this homologous region was named the "*hect* (homology to E6-AP carboxyl terminus)" domain (*56*). Thereafter, many proteins with the *hect* domain have been found to function as the catalytic domain of Ub-ligase, such as Nedd4, Rsp5, Tom1, Ufd4, Pub1, *etc.* (*5*). The protein containing the *hect* domain constitutes a family of new Ub-ligases, which may increase to an unusually large number. Interestingly, a potent inhibitor of p53, Mdm-2, prompts the rapid degradation of p53, indicating its involvement in the degradative system (*57*). Moreover, both Mdm-2 and p53-responsive protein Bax are degraded by the Ub-proteasome pathway (*58*).

TABLE I. **Inhibitors of proteasomes and calpains.**

Compound	Abbreviation	Proteasome[a]	Calpain[b]	Ref.
Lactacystin	LS	+++	−	(*20, 21*)
Clasto-lactacystin β-lactone	β-Lactone	++++	−	(*22*)
3,4-Dichloroisocoumarin	DCI	++	?	(*23*)
Z-Leu-Leu-Leu vinyl sulfone	Z-L_3VS	+++	?	(*24*)
Z-Gly-Pro-Phe-leucinal	Z-GPFL-H	+++	?	(*25*)
Z-Ile-Glu(*O-t*-butyl)-Ala-leucinal	Z-IE(OtBu)AL-H, PSI	+++	+++	(*26*)
Z-Leu-Leu-leucinal	Z-LLL-H, Z-L_3-H, MG132	++++	+++	(*27, 28*)
Z-Leu-Leu-norvalinal	Z-LLnV-H, MG115	+++	+++	(*29*)
Ac-Leu-Leu-norleucinal	Ac-LLnL-H, MG101, ALLN, calpain inhibitor-I	++	+++	(*29, 30*)
Ac-Leu-Leu-methional	Ac-LLM-H, calpain inhibitor-II	+	+++	(*29, 30*)
Z-Leu-norleucinal	Z-LnL-H, calpeptin	+	+++	(*30*)
Z-Leu-leucinal	Z-LL-H, Z-L_2-H	+	+++	(*26, 28*)

N-benzyloxycarbonyl = Cbz = Z, acetyl = Ac, peptidyl-aldehyde = -al = -CHO = -H. The inhibitory efficiency is presented as follows: none (−) and numbers of (+) show magnitude of the effect. ?, not reported. [a]The effect on the proteasome is shown for chymotrypsin-like activity with Suc-LLVY-MCA as a substrate, but the sensitivities differ from the effects for other activities. [b]Similar effects on m- and u-calpains. Various compounds affecting calpain activity appear to inhibit also strongly lysosomal cysteine proteases such as cathepsin B.

NF-κB, a heterodimeric complex of p50/p65, is one of the most important transcriptional factors, having various physiological roles in inflammation and immune responses. It is present in the cytoplasm of the unstimulated cell in an inactive form so by associating with a specific inhibitor protein, I-κB, which masks the nuclear targeting signal of NF-κB. Recent work demonstrated that a variety of extracellular signals, such as TNF-α, induced phosphorylation, ubiquitination, and subsequent proteasomal degradation of I-κB, which could promote the transfer of NF-κB into the nucleus, suggesting that proteolysis is a key regulatory process for signal transduction (*40*). Selective proteasome inhibitors such as Z-LLL-H, PSI, and LC almost completely inhibited rapid signal-dependent I-κB degradation, and induced accumulation of multiubiquitinated I-κB (*26*). Recently, I-κBα kinase was found to be a large complex of 700–900 kDa (*59*). This kinase is unusual in having many subunit compositions, which may contribute to explain the diversity of biological processes evoked by various extracellular signals for the NF-κB system, indicating that the Ub system acts as a positive regulator for this intelligent transcriptional pathway. However, what kinds of Ub-ligase are involved in the ubiquitination of I-κBα is unknown.

β-Catenin is a central component of the cadherin cell adhesion complex, and is a target of the Ub-proteasome pathway. Intriguingly, the adenomatous polyposis coli ("APC"), which was found to be associated with β-catenin, reduces the amount of cytoplasmic β-catenin (*43*). "APC" also binds to glycogen synthetase kinase 3β (GSK3β), which phosphorylates directly β-catenin, and induces ubiquitination-dependent destabilization of β-catenin. The phosphorylation consensus motif of β-catenin resembles a motif in I-κB that is required for phosphorylation-dependent degradation of I-κB *via* the Ub-proteasome pathway. Thus, degradation of β-catenin looks similar to that of I-κB. As for the role of "APC" in the degradation pathway of β-catenin, "APC" is thought to be a presumptive Ub-ligase.

So far the Ub-proteasome pathway for proteolysis is believed mainly to play physiological roles in the cytoplasm and nucleus. But recently this proteolysis machinery has also been shown to operate on various intrinsic membrane proteins. For example, degradation of various polypeptide receptors located on plasma membranes, such as platelet-derived growth factor receptor (*60*) and c-MET or hepatocyte growth factor receptor (*61*), is sensitive to proteasomal inhibitors, but not to lysosomotropic agents. Ligand-dependent ubiquitination and subsequent proteasomal degradation of these polypeptide receptors appear to play critical roles in signal termination by proteolysis. Moreover, cystic fibrosis transmembrane conductance regulator CFTR, the Cl^- channel protein (*27*), and an unstable yeast protein translocator, Sec61p, and its associated protein, Sss1p (*62*), all of which are located on membrane of the endoplasmic reticulum (ER), are degraded by the Ub-proteasome pathway. The ER membrane-associated HMG-CoA reductase (*63*) and apoprotein B100 (*64*) are indicated to be degraded by the proteasome, but multiple pathways may operate in their degradation, because proteasome inhibitors inhibited only partly. These observations suggest that the cytoplasmic domain of membrane-spanning proteins in both plasma and ER membranes is a site of ubiquitination, resulting in formation of targeting signals for the proteasome. However, ubiquitination of several intrinsic membranous proteins serves as a signal for endocytosis, in which process proteins are degraded by the lysosomal/vacuole system and not by the proteasome (*31*). The mechanism by which one group of ubiquitinated proteins is degraded by the proteasome and an other by the vacuole pathway is unknown.

Quality control of proteins

The proteasome plays a major role in the non-lysosomal pathway of not only ATP-dependent breakdown of naturally occurring unstable regulatory proteins but also the unregulated destruction of constitutively long-lived proteins. In addition, it appears to be indispensable for maintenance of cell viability to remove rapidly the proteins with aberrant structures that may be generated in cells by mutations or various environmental stresses, such as fever, oxidation, and exposure to heavy metals, suggesting that proteolysis may be a self-surveillance system to keep the cells from such stresses. The selective degradation of such harmful proteins is mediated by the Ub-proteasome pathway. Actually Ub is encoded by the poly-Ub gene, which belongs to the heat-shock gene family, indicating that cells must produce a large supply of Ub molecules under stress conditions to facilitate fast destruction of abnormal proteins induced by the stress (*5, 6*). Indeed, the Ub pathway was shown to be involved in the process, because inactivation of E1 in the cells caused by a defect in thermostability greatly blocked the degradation of abnormal proteins. In addition, proteasomal inhibitors were also effective in blocking the destruction of harmful proteins, indicating that the proteasome appears to be responsible for the selective degradation of such abnormal proteins. Moreover, the rapid degradation of some enzymes made abnormal by point mutation, which enzymes have been linked to certain severe diseases caused by metabolic abnormality owing to lack of the normal enzymes, has been found to be mediated by the proteasome. The selective breakdown of IRP-2 enhanced by oxidative-stresses is also mediated by the Ub-proteasome pathway (*65*, K. Iwai, personal communication). However, it is unknown how the Ub system is capable of recognizing the abnormal states of proteins. It would appear that specific E3s that can recognize abnormality of cellular proteins would be present in cells. Also, it is a generally accepted idea that molecular chaperones, such as hsp70 and hsp90, somehow support this process; because these chaperones are believed to be involved in not only the process of helping to fold denatured proteins to regain their proper structure, but also degradative removal of abnormal proteins that no longer serve for reutilization by refolding (*66*). Consistent with this observation, treatment with proteasome inhibitors results in induction of some chaperones located on the ER and in the cytoplasm, implying that cellular proteins that escape breakdown become a trigger for the stress response.

Thus protein quality control plays an essential for role in keeping the cell viable by protecting the cell from various environmental stresses that lead to protein abnormality. The initial concept of quality control proposed that unassembled, misfolded, or mutated proteins in the ER are degraded rapidly without further secretion or transport to target sites, and it has been accepted that the ER may have

a specific proteolytic system to serve this role. Surprisingly, however, unnecessary membrane and soluble proteins newly-synthesized in the ER were shown to be exported into the cytosol through the Sec61 translocon machinery and then degraded by the proteasome, suggesting involvement of the proteasome in the "quality control" of the ER (for details, see Refs. *67* and *68*).

Unusual selective proteolysis

As already mentioned, the proteasome is mainly responsible for the degradation of a variety of functional proteins with a multi-Ub chain as a degradation signal. However, the proteasome has been shown also to degrade non-ubiquitinated ornithine decarboxylase (ODC) when the latter is associated with antizyme, its specific inhibitor protein, which may expose the degradation signal of the ODC moiety (*69*). For several other proteins, such as c-Jun (*70*) and TCRα subunit (*71*), Ub-independent proteolysis has been suggested; but it seems to be difficult to determine whether these proteins are degraded by a Ub-independent pathway *in vitro* and/or *in vivo*, because of the difficulty in demonstrating ubiquitination, owing to a very high deubiquitinase activity in eukaryotic cells. Actually, for c-Jun degradation by the Ub-dependent route was demonstrated to occur *in vivo* (*47*). Thus, it now appears that ODC is the only protein that is degraded without ubiquitination. Nevertheless, the possibility that the proteasome may be able to attack diversed target proteins by recognizing various potential degradation signals cannot be ruled out.

The proteasome is an endoproteolytic protease catalyzing the exhaustive hydrolysis of proteins, and thus functions as a protein death machine. However, intriguingly it also has the capability of cleaving proteins in a limited proteolytic fashion. Indeed, the proteasome was found to have a new function as a real processing enzyme; namely, it was shown to activate transcriptional factor NF-κB through conversion of the p105 precursor to the mature p50 mature protein (*72*). Transcriptional factor NF-κB1 is synthesized as a p105 precursor, which is processed by degradation of its C-terminal domain containing ankylin repeats, and the resulting p50 N-terminal domain is associated with a partner p65 protein to form as functional transcriptional factor NF-κB. Interestingly, the p105 precursor is multiubiquitinated before the processing reaction, indicating that ubiquitination also is involved in this process. Why does the proteasome destroy only the C-terminal half of NF-κB? One possibility is that the Gly-rich motif in the middle part of the protein may confer resistance to denaturation to the N-terminal half prior to the proteasomal degradation, and thus the proteasome degrades only the C-terminal region (*73*). However, further study is required to clarify details of this mechanism.

Proteasomes and antigen processing

The Ub-proteasome system has been shown to be responsible for not only catalysis of exhaustive degradation of target proteins but also generation of antigenic peptides for presentation of MHC class I-restricted antigens, implying a distinct role of the proteasome for processing as well as for complete destruction of proteins (reviewed in Refs. *74* and *75*). But the former function might be an obligatory one to provide products for use by the immune system. Previously, a major immunomodulatory cytokine, γ-interferon (γ-IFN) was shown to play an important role in the MHC class I-restricted antigen processing pathway, because it induces replacement of three pairs of homologous proteasomal subunits; *i.e.*, X (MB1), Y (delta), and Z, by LMP7, LMP2, and MECL1, respectively, resulting in an alteration of the proteolytic specificity (reviewed in Ref. *76*). These γ-IFN-inducible proteasomal genes may have appeared by gene duplication from their constitutive counterparts during acquirement of the MHC class I-restricted immune system by ancient chromosomal duplication (*77*). Intriguingly, all of the active subunits with two N-terminal threonine residues, which have been conserved during evolution, seem to be regulated by γ-IFN, perhaps accounting for changes in the proteolytic functions of the proteasome. Thus, the altered molecular organization of the proteasome induced by γ-IFN may be responsible for acquisition of its functional changes in a variety of heterogeneous complexes. We have proposed that proteasomes containing these γ-IFN-inducible subunits should be named "immunoproteasomes" to emphasize their specialized functions in antigen presentation (*76*).

Recently, it was reported that PA28α and PA28β genes were coordinately regulated by γ-IFN (*15, 16*), which lead to greatly increase mRNA levels of both PA28s in cultured cells, implying their involvement in the immune response. The involvement of PA28 in antigen processing has been demonstrated further by experiments showing that it plays an important role in the induction of specific cytotoxic T cells *in vivo* (*78*) and in the production of a dominant MHC-ligand *in vitro* (*79, 80*). These findings suggest that PA28 could play an important role in the generation by the 20S proteasome of antigenic peptides suitable for presentation by the MHC class I molecules. I want to return again briefly to the roles of γ-IFN, which cytokine may induce not only the immunoproteasome but also the PA28 proteasome activator family proteins. Thus, these newly identified γ-IFN-regulated activator genes in combination with the three pairs of γ-IFN regulated proteasome genes perhaps act synergically to favor antigen presentation. Actually PA28 together with MHC-encoded LMP subunits increases the spectrum of peptides that can be generated in antigen-presenting cells. Although the PA28 protein greatly stimulates the multiple peptidase activities of the 20S proteasome, it fails to enhance hydrolysis of large protein substrates with native or denatured structures (*76*). Thus, PA28 does not play a central role in regulating the initial cleavage of cell proteins. It has perhaps a stimulating effect on the degradation of polypeptides of intermediate size that are generated by other endoproteinases, such as the 26S proteasome; functioning sequentially or cooperatively to catalyze the final cleavage reactions in the proteolytic pathway. Otherwise the "hybrid-type proteasome," namely the PA700-20S proteasome-PA28 complex (see above) may function as a processing enzyme of endogenous antigens for producing MHC class I-linked peptides.

Perspectives

In this review, I have emphasized the current progress in our understanding of the structure of and the biology mediated by proteasomes. A fundamental question arises as to why a simple peptide cleavage reaction must be catalyzed by a complex system consisting of Ub and proteasomes. Indeed, the 20S and 26S proteasomes, Ub-

ligases such as cyclosome/APC and PULC, and IκB kinase all are organized into unusually large complexes ranging from 700 to 2,000 kDa, which may serve to permit divergent functions, but the exact reason for this complexity is still unknown. In addition, members involved in the Ub- proteasome system are roughly estimated to account for over 1.5% of the entire open reading frames in the most primitive eukaryote, the budding yeast. Although the reason for such large numbers of proteins in this proteolysis system is unclear at the present time, such numbers may indicate the biological importance of proteolysis mediated by the Ub-proteasome system. Until recently, proteolysis seemed not to command much interest, biologically speaking, because proteases were thought simply to contribute to the destruction of unnecessary proteins only. However, the discovery of the Ub-proteasome system sheds new light on to a wide new field of biology as a previously unrecognized regulatory system for various biological reactions.

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