

Proteasomes

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Keywords

AAA ATPases

A large subfamily of ATPases involved in separating and unfolding proteins. The six ATPases of the 19S regulatory complex are members of the AAA subfamily.

Aggresome

“Abnormal proteins” – pericentrosomal accumulations of abnormal proteins that recruit chaperones and proteasomes.

Base

A nine-protein subcomponent of the 19S regulatory complex, containing six ATPases and three proteins that recognize ubiquitin or ubiquitin-like proteins.

Chaperone

Chaperones are proteins that assist in the folding of nascent proteins or the refolding of denatured proteins. The heat shock proteins such as hsp90 or hsp70 are prime examples.

COP9 Signalosome (Csn)

A protein complex containing a metalloisopeptidase that removes the ubiquitin-like protein NEDD8 from ubiquitin ligases. Each of the eight Csn subunits is evolutionarily related to the eight lid subunits in the 19S regulatory complex.

Degron

Short peptide motifs (or even single N-terminal amino acids) that confer rapid proteolysis on the polypeptides bearing them.

Ecm29

A proteasome-associated protein proposed to function either as a clamp holding the 19S RC to the 20S proteasome or as an adaptor that localizes the 26S proteasome to specific membrane compartments within cells.

Epoxomicin

A bacterial compound that is a specific inhibitor of the proteasome.

Hybrid Proteasome

A 20S proteasome with a 19S regulatory complex at one end and either PA28 or PA200 at the other.

Immunoproteasome

A 20S proteasome where each of the three constitutive catalytic subunits is replaced by an active subunit with altered substrate specificity. Immunoproteasomes are induced by immune cytokines and play a role in specifying epitopes presented by the Class-I immune pathway.

Lactacystin

A fungal metabolite that is a reasonably specific inhibitor of the proteasome's active sites.

Lid

An eight-protein subcomponent of the 19S regulatory complex, containing a metalloisopeptidase that removes ubiquitin chains from the substrate protein.

Metabolic Regulation

Metabolic regulation refers to the control of biological processes by changes in the concentration of metabolites or proteins or by changes in the activities of enzymes. Phosphorylation and ubiquitination are widespread mechanisms for controlling enzyme activity.

PA28s (aka 11S REGs)

Donut shaped, heptameric protein complexes that bind the ends of the 20S proteasome and promote peptide entry to or efflux from its central proteolytic chamber.

PA200

A large nuclear protein that binds the ends of the 20S proteasome and is thought to play a role in DNA repair.

Proteasome Activators

Single polypeptide chains or small protein complexes that bind 20S proteasomes and stimulate peptide hydrolysis. To date, three have been discovered.

20S Proteasome

A cylindrical proteolytic particle composed of 28 subunits arranged as a stack of four heptameric rings. The enzyme's active sites face an internal chamber.

26S Proteasome

The only ATP-dependent protease discovered so far in the nuclear and cytosolic compartments of eukaryotic cells. The enzyme consists of one or two 19S regulatory complexes attached to the ends of the 20S proteasome.

19S Regulatory Complex (aka PA700)

A multisubunit particle containing six ATPases and eleven additional proteins that functions to bind, unfold, and translocate substrate proteins into the central proteolytic chamber of the 20S proteasome.

Ubiquitin

An exceptionally conserved 76-amino acid protein that is covalently attached to a wide variety of other eukaryotic proteins. Chains of ubiquitin attached to substrate proteins target them for destruction by the 26S proteasome.

VCP

A large hexameric ATPase that transfers some polyubiquitylated substrates to the 26S proteasome.

Velcade

A peptide boronic acid inhibitor of the proteasome used clinically to treat multiple myeloma.

■ The 20S proteasome was discovered in 1980 and the 26S proteasome six years later. Research over the past two decades has made it abundantly clear that the Ub-proteasome system is of central importance in eukaryotic cell physiology and medicine. At the cellular and biochemical levels, there are a number of unresolved problems. We need a crystal structure of the 19S RC, or better yet, the 26S proteasome, for they would surely provide an insight into the mechanism by which the 26S proteasome degrades its substrates. How the 26S proteasome itself is regulated and the extent to which proteasomal components vary among

tissues in higher eukaryotes are other important unresolved problems. As for the medical perspective, we need to know how many diseases will be found to arise because of defects in the ubiquitin–proteasome system. Hopefully, these problems will generate research on the UPS by some readers of this article.

1 Proteasomes – A Quick Summary

Proteasomes are multisubunit, cylindrical proteases found in eukaryotes, eubacteria, and archaeobacteria. The proteasome's active sites face a central chamber buried within the cylindrical particle. Thus, the proteasome is an ideal intracellular protease because cellular proteins can only be degraded if they are actively transferred to the enzyme's central chamber. Eukaryotic proteasomes come in two sizes, the 20S proteasome and the considerably larger ATP-dependent 26S proteasome. The latter is formed when the 20S proteasome binds one or two multisubunit ATPase-containing particles known as 19S *regulatory complexes*. The 26S proteasome is responsible for degrading ubiquitylated proteins and is therefore essential for a vast array of cellular processes including cell-cycle traverse, control of transcription, regulation of enzyme levels, and apoptosis. Being the key protease of the ubiquitin system, the 26S proteasome also impacts a number of human diseases, especially cancer, cachexia, and neurodegenerative diseases. Both 20S and 26S proteasomes can associate with other protein complexes. As their name implies, proteasome activators stimulate peptide hydrolysis and may serve to localize 20S and 26S proteasomes within cells. Hybrid proteasomes consist of the 20S proteasome with a 19S regulatory complex bound at one end and a proteasome activator at the other. Immunoproteasomes

are formed when the catalytic subunits found in constitutive proteasomes are replaced by interferon-inducible subunits with different substrate specificities. Immunoproteasomes are found mainly in immune tissues where they play a role in Class-I antigen presentation.

2 The 20S Proteasome

2.1 Structure

We know the molecular anatomy of archaeobacterial, yeast, and bovine proteasomes in great detail since high-resolution crystal structures have been determined for all three enzymes. The archaeobacterial proteasome is composed of two kinds of subunits, called α and β . Each subunit forms heptameric rings that assemble into the 20S proteasome by stacking four deep on top of one another to form a “hollow” cylinder. Catalytically inactive α -rings form the ends of the cylinder, while proteolytic β -subunits occupy the two central rings. The quaternary structure of the 20S proteasome can therefore be described as $\alpha_7\beta_7\beta_7\alpha_7$. The active sites of the β -subunits face a large central chamber about the size of serum albumin (see Fig. 1). The α -rings seal off the central proteolytic chamber and two smaller antechambers from the external solvent. Archaeobacterial proteasomes, with

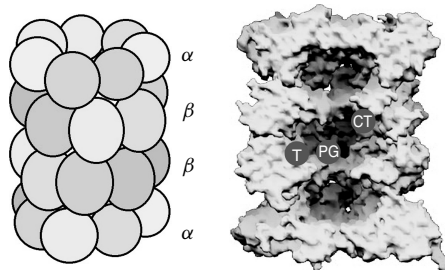


Fig. 1 20S Proteasomes. At the left is a schematic representation of a eukaryotic 20S proteasome. Different colors serve to emphasize the diversity of α and β subunits. At the right is a cutaway view of the yeast proteasome, showing the three internal cavities and the three protease activities: T (trypsin-like), PG (post glutamyl cleaving) and CT (chymotrypsin-like).

their fourteen identical β -subunits, preferentially hydrolyze the peptide bonds following hydrophobic amino acids and are therefore said to have chymotrypsin-like activity. Eukaryotic proteasomes maintain the overall structure of the archaeobacterial enzyme, but they exhibit a more complicated subunit composition. There are seven different α -subunits and at least seven distinct β -subunits arranged in a precise order within their respective rings (see Fig. 1). Although current evidence indicates that only three of its seven β -subunits are catalytically active, the eukaryotic proteasome cleaves a wider range of peptide bonds containing, as it does, two copies each of trypsin-like, chymotrypsin-like, and post-glutamyl-hydrolyzing subunits. For this reason, it is capable of cleaving almost any peptide bond, having difficulty only with proline-X, glycine-X, and, to a lesser extent, with glutamine-X bonds.

2.2

Enzyme Mechanism and Proteasome Inhibitors

Whereas standard proteases use serine, cysteine, aspartate, or metals to cleave peptide bonds, the proteasome employs an unusual catalytic mechanism. N-terminal threonine residues are generated by self-removal of short peptide extensions from the active β -subunits, and they act as

nucleophiles during peptide-bond hydrolysis. Given its unusual catalytic mechanism, it is not surprising that there are highly specific inhibitors of the proteasome. The fungal metabolite lactacystin and the bacterial product epoxomicin covalently modify the active-site threonines and inhibit the enzyme. Both compounds are commercially available; other inhibitors include vinylsulfones and various peptide aldehydes that are generally less specific. Recently, Velcade, a peptide boronate inhibitor of the proteasome, has been approved for the treatment of multiple myeloma.

2.3

Immunoproteasomes

Interferon γ is an immune cytokine that increases expression of a number of cellular components involved in Class-I antigen presentation. Among the IFN γ inducible components are three catalytically active β -subunits of the proteasome, called LMP2, LMP7, and MECL1. Each replaces its corresponding constitutive subunit, resulting in altered peptide-bond cleavage preferences of 20S immunoproteasomes. For example, immunoproteasomes exhibit much reduced cleavage after acidic residues and enhanced hydrolysis of peptide bonds following branch-chain amino acids such as isoleucine or valine. Class-I molecules preferentially bind peptides with hydrophobic or positive C-termini,

and proteasomes generate the vast majority of Class-I peptides. Hence, the observed β -subunit exchanges are well suited for producing peptides able to bind Class-I molecules.

3

The 26S Proteasome

3.1

The Ubiquitin–proteasome System

Bacteria can express as many as five ATP-dependent proteases. By contrast, the 26S proteasome is the only ATP-dependent protease discovered so far in the nuclear and cytosolic compartments of eukaryotic cells. Because the 20S proteasome's internal cavities are inaccessible to intact proteins, openings must be generated in the enzyme's outer surface for proteolysis

to occur. A number of protein complexes have been found to bind the proteasome and stimulate peptide hydrolysis. (see Fig. 2). The most important of the proteasome-associated components is the 19S regulatory complex (RC), for it is a major part of the 26S ATP-dependent enzyme that degrades ubiquitin-tagged proteins in eukaryotic cells. Ubiquitin (Ub) is treated in a separate chapter of this Encyclopedia. Still, this important protein must be briefly covered since it plays a central role in substrate recognition by the 26S proteasome.

Ubiquitin is a small, evolutionarily conserved eukaryotic protein that can be attached to a wide variety of intracellular proteins, including itself. Although Ub serves nonproteolytic roles, such as histone modification or viral budding, the protein's major function is targeting proteins for destruction. To do so,

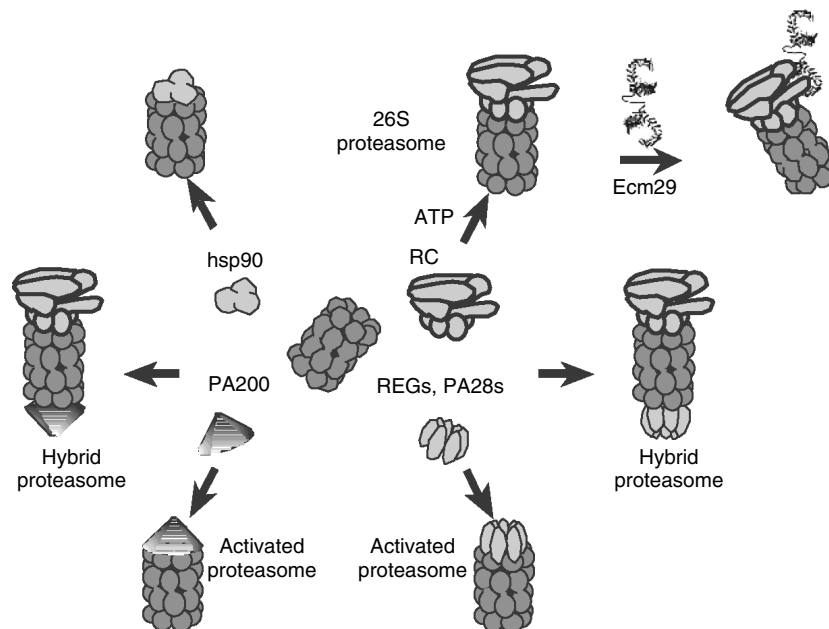


Fig. 2 Schematic representation of the 20S proteasome assembling with various activator proteins (RC, REGs, PA200) or with the chaperone hsp90, a protein that inhibits the enzyme.

the carboxyl terminus of ubiquitin is activated by an ATP-consuming enzyme (E1) and is transferred to one of several small carrier proteins (E2s) in the form of a reactive thiolester. The carboxyl terminus of an activated Ub then forms an isopeptide bond with lysine amino groups on proteolytic substrates (S) that have been selected by members of several large families of Ub ligases or E3s. Chains of Ub are formed, and the Ub-conjugated substrate is recognized by the 26S proteasome and degraded; Ub is recycled for use in further rounds of proteolysis (see Fig 3). It is important to note that ubiquitin contains seven lysine residues and polyUb chains formed via Lys6, Lys27, Lys29, Lys48, and Lys63 exist in nature. Lys29 and

Lys48 chains form directly on proteolytic substrates and target them for destruction. Lys27 chains have been found on the cochaperone BAG1, and they target degradation of misfolded proteins bound by the Hsp70 chaperone to the 26S proteasome. Ub monomers linked to each other through Lys63 are involved in endocytosis and DNA repair, but not, apparently, in targeting proteins to the 26S proteasome.

3.2

Ultrastructure of the 26S Proteasome and Regulatory Complex

Electron micrographs (EMs) of purified 26S proteasomes reveal a dumbbell-shaped particle, approximately 40 nm in

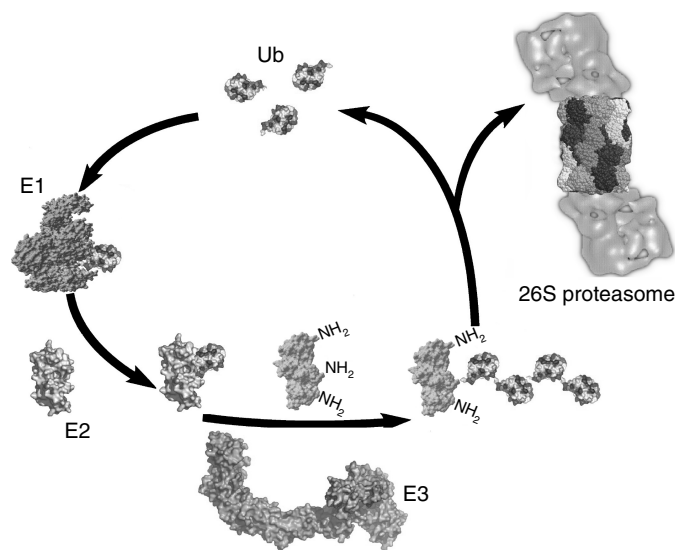


Fig. 3 Schematic representation of the ubiquitin-proteasome pathway. Ubiquitin molecules are activated by an E1 enzyme (shown in green at one-third scale) in an ATP-dependent reaction, transferred to a cysteine residue (yellow) on an E2 or Ub carrier protein and subsequently attached to amino groups (NH₂) on a substrate protein (lysozyme shown in purple) by an E3 or ubiquitin ligase, (the multicolored SCF complex). Note that chains of Ub are generated on the substrate, and these are recognized by the 26S proteasome depicted in the upper right at one-twentieth scale. (See color plate p. xxiv).

length, in which the central 20S proteasome cylinder is capped at one or both ends by asymmetric regulatory complexes looking much like Chinese dragonheads (see Fig 4). In doubly capped 26S proteasomes, the regulatory complexes face in opposite directions, indicating that contact between the proteasome's α -rings and the RC is highly specific. However, the contacts may not be especially tight since image analysis of *Drosophila* 26S proteasomes suggests movement of the RCs relative to the 20S proteasome. EM images of the 26S proteasome appear the same in all organisms, indicating that the overall architecture of the enzyme has been conserved in evolution. This conclusion is also supported by sequence conservation among RC subunits (see Sect. 3.4). A yeast mutant lacking the RC subunit Rpn10 contains a salt-labile RC that dissociates into two subcomplexes called the *lid* and the *base* (see Fig 4). The base contains 9 RC subunits, which include six ATPases described below in Sect. 3.4, the two largest RC subunits S1 and S2, as well as S5a; the lid contains the remaining RC subunits. Thus, the RC is composed of two subcomplexes separated on one side by a cavity, that is, the dragon's mouth. Ultrastructural studies have also been performed on the lid and on a related protein complex called the *COP9 signalosome*. Both particles

lack obvious symmetry. Some particles exhibit a negative-stain-filled central groove; other classes of particles exhibit seven or eight lobes in a disc-like arrangement. Since both particles are composed of eight subunits, the lobes may represent individual subunits.

3.3

The 19S Regulatory Complex (RC)

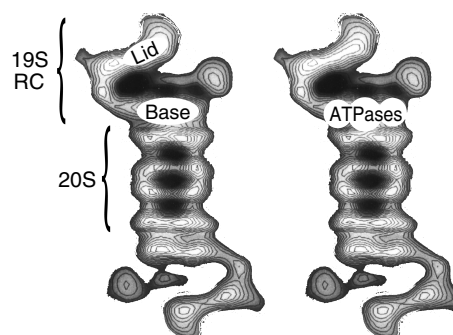
The regulatory complex is also called the 19S cap, PA700, and the μ -particle. As its most common name suggests, the 19S regulatory complex is roughly the same size as the 20S proteasome. In fact, it is a more complicated protein assembly containing 17 or 18 different subunits, ranging in size from 25 to about 110 kDa. In animal cells, the subunits are designated S1 through S15. Homologs for each of these subunits are present in budding yeast where an alternate nomenclature has been adopted (see Table 1). Sequences for the 18 RC subunits permit their classification into a group of 6 ATPases and another group containing the 12 nonATPases.

3.4

ATPases of the RC

The six ATPases belong to the rather large family of AAA ATPases (for ATPases

Fig. 4 Electron microscopic reconstructions of the 26S proteasome. Two images of negatively stained, doubly capped 26S proteasomes are presented to illustrate the positions of the 19S RC lid and base subcomplexes and to identify the most probable location of the RC ATPases.



Tab. 1 Subunits of the 19S regulatory complex.

<i>Mammalian nomenclature</i>	<i>Yeast nomenclature</i>	<i>Function</i>	<i>Motifs</i>
S1	Rpn2	Ub/Ub/ binding	Leucine-rich repeats, KEKE
S2	Rpn1	Ub/Ub/ binding	Leucine-rich repeats, KEKE
S3	Rpn3	?	PCI
p55	Rpn5	?	PCI
S4	Rpt2	ATPase	AAA nucleotidase
S5a	Rpn10	polyUb binding	UIM, KEKE
S5b	none	?	
S6	Rpt3	ATPase	AAA nucleotidase
S6'	Rpt5	ATPase	AAA nucleotidase
S7	Rpt1	ATPase	AAA nucleotidase
S8	Rpt6	ATPase	AAA nucleotidase
S9	Rpn6	?	PCI
S10a	Rpn7	?	PCI, KEKE
S10b	Rpt4	ATPase	AAA nucleotidase
S11	Rpn9	?	PCI
S12	Rpn8	?	MPN, KEKE
S13	Rpn11	Isopeptidase	MPN
S14	Rpn12	?	PCI

Associated with a variety of cellular Activities) whose members include the motor protein dynein, the membrane fusion factor NSF, and the chaperone VCP/Cdc48. The six ATPases, denoted S4, S6, S6', S7, S8, and S10b in mammals, are about 400 amino acids in length and homologous to one another. On the basis of their sequences, one can distinguish three major regions: (1) a central nucleotide binding domain of about 200 amino acids, which is roughly 60% identical among members of the RC subfamily; (2) the C-terminal region, approximately 100 amino acids in length and with a lesser, though significant, degree of conservation (~40%); and (3) a highly divergent N-terminal region (<20% identity) around 120 amino acids in length; this region contains heptad repeats characteristic of coiled-coil proteins. Despite sequence differences among RC ATPases within an organism, each ATPase has been conserved

during evolution with specific subunits being almost 75% identical between yeast and humans. The high degree of conservation encompasses the entire sequence, making it likely that even the divergent N-terminal regions play an important role in RC function. Conceivably, they are used to select substrates for degradation by the 26S proteasome. The "helix-shuffle" hypothesis proposes that the N-terminal coiled-coils of S4 subfamily ATPases bind unassembled substrate proteins such as fos or jun through the latter's unpaired leucine zippers. Alternatively, the variable N-terminal regions in the RC ATPases may be involved in the assembly of the RC by promoting the specific placement of the ATPase subunits within the complex. In this regard, the six ATPases associate with one another in highly specific pairs: S4 binds S7, S6 binds S8, and S6' binds to S10b. Moreover, the N-terminal regions of RC ATPases are required for

partner-specific binding. Staining patterns of two-dimensional gels show the six RC ATPases to be present at comparable levels and the affinity capture of yeast 26S proteasomes indicate the presence one copy of each in the regulatory complex. Mutational analysis in yeast demonstrates that the ATPases are not functionally redundant since mutation of yeast S4 has a particularly profound effect on peptide hydrolysis. It is probable that the ATPases form a hexameric ring like other members of the AAA family of ATPases such as NSF or VCP/Cdc48. But this assumption has not been experimentally verified. Finally, it is quite likely that the ATPases directly bind the α -ring of the 20S proteasome. Evidence favoring this arrangement comes from chemical cross-linking experiments and the presence of the ATPases in the base subcomplex of the yeast 26S proteasome.

3.5

The non-ATPase Subunits

Whereas the six RC ATPases are homologous and relatively uniform in size, the nonATPases are heterogeneous in size and sequence. Nonetheless, they can be grouped on the basis of their location, for example, lid versus base, on the presence or absence of certain sequence motifs, for example, PCI and MPN domains, and on their affinity for Ub chains or Ub-like proteins. Eight RC subunits are found in the lid subcomplex. One of these subunits (S13) is a metalloisopeptidase that removes Ub chains from the tagged substrate prior to its translocation into the proteasome for degradation. Each of the eight lid subunits is homologous to a subunit in a separate protein complex called the COP9 signalosome. Six of the eight lid subunits contain PCI domains, stretches of about 200 residues so named from

their occurrence in Proteasome, COP9 signalosome, and the eukaryotic Initiation factor 3 subunits. The PCI domains are thought to mediate subunit-subunit interactions. Two lid subunits contain 140 amino acid long MPN domains (Mpr1p and Pad1p N-terminal regions), with one of these subunits being the S13 isopeptidase. Although several models have been proposed from 2-hybrid screens of lid and COP9-signalosome subunits, the arrangement of the eight subunits within the lid subcomplex is not known with certainty. S13 stands out because it exhibits isopeptidase activity. Functions of the remaining seven lid subunits have not been discovered, although two lid subunits, S3 and S9, are critical for the degradation of specific substrates. The presence of the S13 isopeptidase in the lid explains why the lid is necessary for degradation of ubiquitylated proteins, even though the RC-base complex supports the ATP-dependent degradation of some small nonubiquitylated proteins. Interestingly, the COP9-signalosome also exhibits isopeptidase activity that removes the ubiquitin-like protein, NEDD8, from certain ubiquitin ligases.

In addition to the six ATPases, the base subcomplex contains the two largest RC subunits (S1, S2) and a smaller subunit called S5a. Besides their common location, these three subunits share the property of binding polyUb chains or Ub-like domains. S5a binds polyubiquitin chains even after it has been transferred from SDS-PAGE gels, and it displays many features that match polyubiquitin recognition by the 26S proteasome. However, S5a cannot be the only ubiquitin recognition component in the 26S proteasome because deletion of the gene-encoding yeast S5a has only a modest impact on proteolysis. This strongly suggests that there

are other ubiquitin recognition components in the RC, with S1 and S2 being prime candidates. S1 and S2 display significant homology to each other, and both can be modeled as α -helical toroids. They have been shown to bind the ubiquitin-like domains of RAD23 and Dsk2, adapter proteins that target ubiquitylated proteins to the 26S proteasome. It has also been found that the S6' ATPase can be cross-linked to Ub. Currently, then it appears that the RC contains three, or possibly four, subunits able to recognize Ub or Ub-like proteins. As discussed below, there are other ways in which the RC can select proteins for destruction.

3.6

Biochemical Properties of the Regulatory Complex

3.6.1 Nucleotide Hydrolysis

Both the 26S proteasome and the regulatory complex hydrolyze all four nucleotide triphosphates, with ATP and CTP preferred over GTP and UTP. K_m s for hydrolysis by the 26S proteasome are two- to fivefold lower for each nucleotide and virtually identical to the K_m s required for Ub-conjugate degradation. Although ATP hydrolysis is required for conjugate degradation, the two processes are not strictly coupled. Complete inhibition of the peptidase activity of the 26S proteasome by calpain inhibitor I has little effect on the ATPase activity of the enzyme. The nucleotidase activities of the RC and the 26S proteasome closely resemble those of *Escherichia coli* Lon protease, which is composed of identical subunits that possess both proteolytic and nucleotidase activities in the same polypeptide chain. Like the regulatory complex and 26S proteasome, Lon hydrolyzes all four ribonucleotide triphosphates, but not ADP or AMP.

3.6.2 Chaperone-like Activity

AAA nucleotidases share the common property of altering the conformation or association state of proteins. So, it is not surprising that the RC has been shown to prevent aggregation of several denatured proteins including citrate synthase and ribonuclease A. The chaperone activity of the RC may explain why the RC plays a role in DNA repair even in the absence of an attached 20S proteasome.

3.6.3 Proteasome Activation

The 20S proteasome is a latent protease because of the barrier imposed by the α -subunit rings on peptide entry. Consequently, a readily measured activity of the RC is activation of fluorogenic peptide hydrolysis by the 20S proteasome. The extent of activation is generally found to be in the range 3- to 20-fold. Activation is relatively uniform for all three proteasome catalytic subunits and presumably reflects opening by the attached RC of a channel, leading to the proteasome's central chamber.

3.6.4 Ubiquitin Isopeptide Hydrolysis

The channel through the proteasome α -ring into the central chamber measures 1.3 nm in diameter, a size too small to permit passage of a folded protein even as small as ubiquitin. This consideration coupled with the fact that Ub is recycled intact upon substrate degradation requires an enzyme to remove the polyUb chain prior to or concomitant with proteolysis. Several isopeptidases that remove Ub from substrates has been found associated with the 26S proteasome. Of these, S13 is an integral component of the enzyme. S13 is a metalloisopeptidase stimulated by nucleotides and is active only in the fully assembled 26S proteasome. Thus, it is not

strictly correct to list isopeptidase activity as a property of the RC.

3.6.5 Substrate Recognition

It is clear that the RC plays a predominant role in selecting proteins for degradation. This important topic is covered in Sect. 5 in the context of substrate recognition by both 20S and 26S proteasomes.

4

Proteasome Biogenesis

4.1

Subunit Synthesis

The synthesis of proteasome subunits is markedly affected by proteasome function. For example, inhibition of proteasome activity by lactacystin induces coordinate expression of both RC and 20S proteasome subunits. Similarly, impaired synthesis of a given RC subunit results in overexpression of all RC subunits. Proteasome subunit synthesis in yeast is controlled by Rpn4p, a short-lived positive transcription factor that binds PACE elements upstream of proteasome genes. Rpn4p is a substrate of the 26S proteasome, suggesting that the transcription factor functions in a feedback loop in which proteasome activity limits its concentration, thereby regulating proteasome levels. To date, an Rpn4-like factor has not been identified in higher eukaryotes; however, the presence of such a factor could explain why proteasome inhibition results in higher expression of proteasome subunits in mammalian and *Drosophila* cells.

4.2

Biogenesis of the 20S Proteasome

Proteasome β -subunits are synthesized with N-terminal extensions, and they are

inactive because a free N-terminal threonine is required for peptide-bond hydrolysis. The precursor β -subunits assemble with α -subunits to form half proteasomes composed of one α - and one β -ring. These two ring intermediates dimerize to form the 20S particle, and the N-terminal extensions are removed, thereby generating a new unblocked N-terminal threonine in the catalytically active β -subunits. A small accessory protein called Ump1 in yeast and POMP or proteasemblin in mammalian cells assists in the final assembly of the 20S proteasome. Interestingly, Ump1/POMP is apparently trapped in the proteasome's central chamber and degraded upon maturation of the enzyme.

4.3

Biogenesis of the RC

Assembly pathways for the RC are virtually unknown. As mentioned above, the ATPases interact with one another and complexes containing all six S4 subfamily members have been observed following *in vitro* synthesis. Impaired synthesis of a lid subunit can result in the absence of the entire lid, so presumably, lid and base subcomplexes assemble independently and associate in the final stages of RC formation cells. *In vivo*, 26S proteasomes assemble from preformed regulatory complexes and 20S proteasomes.

4.4

Posttranslational Modification of Proteasome Subunits

Proteasome and RC subunits are subjected to a variety of posttranslational modifications including phosphorylation, acetylation, and even myristoylation in the case of the RC ATPase S4. In yeast, all seven α -subunits as well as two

β -subunits are acetylated. Since acetylation of the N-terminal threonine in an active β -subunit would poison catalysis, it has been suggested that the propeptide extensions function to prevent acetylation. Three members of the S4 ATPase subfamily (S4, S6, and S10b) and two 20S α -subunits (C8 and C9) are known to be phosphorylated. Phosphorylation appears to be particularly important for 26S proteasome assembly since the kinase inhibitor staurosporine reduces 26S proteasome levels in mouse lymphoma cells.

4.5

Biogenesis of the 26S Proteasome

The RC and 20S proteasome associate to form the 26S proteasome in the presence of ATP. Comparison of the cross-linking patterns of RC and assembled 26S proteasomes indicates that this association is accompanied by subunit rearrangement. In yeast, two proteins play a special role in 26S proteasome assembly or stability. Nob1p is a nuclear protein required for biogenesis of the 26S proteasome. It is degraded following assembly of the 26S enzyme, suffering the same fate as Ump1 does following 20S maturation. The molecular chaperone Hsp90 also plays a role in the assembly and maintenance of yeast 26S proteasomes since functional loss of Hsp90 results in 26S proteasome dissociation.

5

Substrate Recognition by Proteasomes

5.1

Degradation Signals (Degrons)

One of the major insights of twentieth century cell biology was the recognition that

proteins possess built-in signals, targeting them to specific locations within cells. Selective proteolysis can be considered to be targeting out of existence, and a number of short peptide motifs have been discovered to confer rapid proteolysis on their bearers. These include PEST sequences, destruction boxes, KEN boxes, and even the N-terminal amino acid. These motifs are recognized by one or more ubiquitin ligases that mark the substrate protein by addition of a polyUb chain. However, some proteins are degraded by the 26S proteasome without prior marking by Ub. Denatured proteins are also selectively degraded by both 26S and 20S proteasomes. It is not clear what features of denatured proteins are recognized by proteasomes or by components of the Ub proteolytic system.

5.2

Ubiquitin-dependent Recognition of Substrates

Most well-characterized substrates of the 26S proteasome are ubiquitylated proteins, so our discussion starts with them. Efficient proteolysis of ubiquitylated proteins by the 26S proteasome requires a chain containing at least four Ub monomers. This matches well with the Ub-binding characteristics of the RC subunit S5a. It also selectively binds ubiquitin polymers composed of four or more ubiquitin moieties and exhibits increased affinity for longer chains. In addition, binding to S5a is impaired by mutations in ubiquitin that allow chain formation but reduce the targeting competence of the chains. These properties and the fact that denatured S5a can readily regain affinity for polyUb suggest that multiple, short sequences within S5a form “loops” that are able to bind “grooves” in the polyubiquitin

chain. S5a molecules from a number of higher eukaryotes contain two repeated motifs that are independent polyubiquitin binding sites. Each motif is approximately 30-residues long and is characterized by five hydrophobic residues that consist of alternating large and small hydrophobic residues, for example, leu-ala-leu-ala-leu. Similar motifs have been found in other proteins of the ubiquitin system and are now called UIMs, for Ubiquitin Interacting Motifs. Because a hydrophobic patch on the surface of ubiquitin is critical for substrate targeting, current models envision direct hydrophobic interaction between the UIMs in S5a and the hydrophobic patches on ubiquitin molecules in polyUb chains.

Whereas S5a provides for direct recognition of polyubiquitylated substrates, a second mechanism involves adaptor proteins possessing both a UbL (a Ubiquitin-like domain) and one or more UbA domains (Ubiquitin-associated domains are polyUb binding domains found in several proteins of the Ub system). The adaptor proteins include RAD23 and Dsk2 in yeast and recruit substrates to the 26S proteasome through UbL binding to 26S proteasome subunits, while the UbA domain binds substrate-tethered Ub chains. In yeast, the RC subunits S1 and S2 serve as UbL binding components; in mammals, S5a also serves this purpose.

The cochaperone BAG1 illustrates a third way in which polyUb can target substrate proteins to the 26S proteasome. In this case, the substrate is not polyubiquitylated; rather, it is bound to the chaperone Hsp70. A polyUb chain, linked through Lys27, is attached to the Hsp70-associated cochaperone BAG1. Apparently, the Lys27 chain promotes association of the chaperone-substrate complex with the 26S proteasome, after which the substrate is

degraded while BAG1, Hsp70, and ubiquitin are recycled. Direct interaction between E3 ubiquitin ligases and RC subunits can also deliver ubiquitylated substrates to the protease. The yeast E3 UFD4 binds RC ATPases, and UFD4-mediated delivery of substrates bypasses the requirement for S5a. In what appears to be a similar delivery system, the mammalian E3 Parkin uses a UbL to bind the 26S proteasome. Mutational analyses in yeast have shown that whereas deletion of either S5a or Rad23 has mild impact on proteolysis, loss of both proteins produces a severe phenotype. Yeast lacking S5a, RAD23, and Dsk2 are not viable, indicating that direct delivery by E3s cannot compensate for the absence of all three proteins.

5.3

Substrate Selection Independent of Ubiquitin

The 26S proteasome also degrades nonubiquitylated proteins. The short-lived enzyme ornithine decarboxylase (ODC) and the cell-cycle regulator p21Cip provide well-documented examples of Ub-independent proteolysis by the 26S enzyme. ODC degradation is stimulated by a protein called *antizyme* that binds to both ODC and the 26S proteasome. Antizyme functions as an adapter much like RAD23 and Dsk2 except that polyUb chains are not involved, although interestingly, free Ub chains compete with antizyme-ODC for degradation. p21Cip is also degraded in a nonUb-dependent reaction. This was clearly demonstrated by substitution of arginines for all the lysine residues in p21Cip, thereby preventing ubiquitylation of p21Cip. The lysine-less protein was still degraded in human fibroblasts by the proteasome. The C-terminal region of p21Cip binds to the proteasome α -subunit C8,

and *in vitro* p21Cip is degraded by the 20S proteasome alone. Direct binding of p21Cip to the 20S proteasome may open a channel through the α -ring, allowing the loosely structured protein to enter the central chamber. c-Jun, “aged” calmodulin, troponin C, and p53 are other proteins that can be degraded by the 26S proteasome absent marking by Ub. The destruction of p53 and p21Cip can proceed by both Ub-dependent and Ub-independent pathways. Other 20S proteasome substrates, *in vitro* at least, include oxidized proteins, small denatured proteins, and loosely folded proteins such as casein. Whether the 20S proteasome degrades proteins within cells is an unresolved problem.

6

Proteolysis by the 26S Proteasome – Mechanism

6.1

Contribution of Chaperones to Proteasome-mediated Degradation

Chaperones are connected to proteasomes in at least four ways. First, chaperones can deliver substrates to the proteasome as described above for the cochaperone BAG1. In a similar fashion, the chaperone VCP/Cdc48 is required for the degradation of several Ub pathway substrates. VCP is a member of the AAA family of ATPases. The large hexameric ATPase appears to function as a protein separase able to remove ubiquitylated monomers from multisubunit complexes. In some cases, the liberated proteins are degraded by the 26S proteasome; in other cases, the separated proteins may change their intracellular location. The proteasome also degrades endoplasmic-reticulum membrane proteins. If these ER membrane proteins

possess a large cytoplasmic domain, their proteasomal degradation can require Hsps 40, 70, and 90 as well as VCP. Hsp90 is required to assemble and stabilize the yeast 26S proteasome, providing a third connection between chaperones and proteasomes. Hsp90 is also able to bind and suppress peptide hydrolysis by the 20S proteasome. Finally, both chaperones and proteasomes are induced by the accumulation of denatured proteins within eukaryotic cells.

6.2

Presumed Mechanism

Proteolysis of ubiquitylated proteins by the 26S proteasome can be thought to be consisting of seven steps: (1) chaperone-mediated substrate presentation; (2) substrate association with RC subunits; (3) substrate unfolding; (4) detachment of polyUb from the substrate; (5) translocation of the substrate into the 20S proteasome central chamber, (6) peptide-bond cleavage; and (7) release of peptide products as well as polyUb (see Fig 5). Step 1 is optional, depending on the substrate, and in principle, steps 3 and 4 could occur in either order. Step 4 is unnecessary for substrates like ODC that are not ubiquitylated. The other steps almost have to occur as presented. Although it is easy to conceptualize the reaction sequence, few experimental findings bear directly on any of the proposed subreactions, and virtually nothing is known about molecular movements within the 26S proteasome. It has been shown that steps 6 and 7 are not required for sequestration of ODC by the 26S proteasome. But which RC subunits actually recognize ODC–antizyme complexes has not been discovered. For ubiquitylated substrates, it is very likely

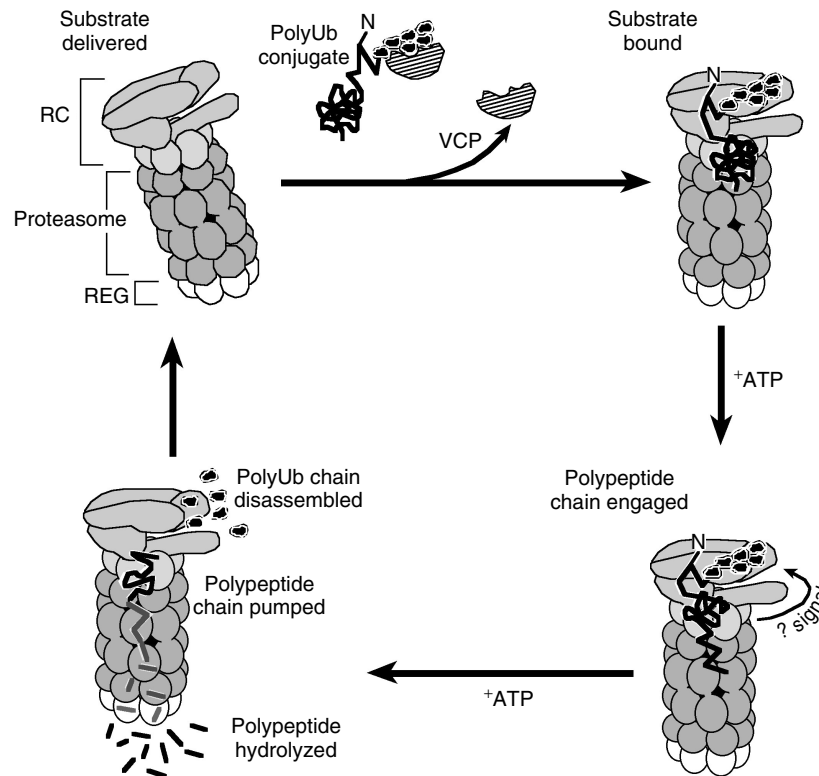


Fig. 5 Hypothetical reaction cycle for the 26S proteasome. A polyubiquitylated substrate is delivered to the 26S proteasome possibly by chaperones such as VCP (step 1). Substrate is bound by polyubiquitin-recognition components of the RC until the polypeptide chain is engaged by the ATPases (step 2). As the polypeptide chain is unfolded and pumped down the central pore of the proteasome, a signal is conveyed to the metalloisopeptidase to remove the polyUb chain (step 3). The unfolded polypeptide is eventually degraded within the inner chamber of the proteasome (step 4) and peptide fragments exit the enzyme.

that S1, S2, and S5a are recognition components, but this is not certain. Even if they are, we do not know whether they recognize just polyUb or polyUb and portions of the substrate. Translocation is thought to proceed by the six ATPases threading the polypeptide through a channel in the 20S proteasome's α -ring, but the possibility that convulsive movements transfer substrate has not been ruled out. It is also thought that the RC ATPases progressively unravel substrates from degradation signals within the polypeptide chain and

are able to "pump" the polypeptide chain in either N-terminal to C-terminal or the opposite direction. The ATPases may even be capable of transferring loops into the 20S enzyme. One thing that is certain is that the peptide fragments generated in the central chamber are generally 5 to 10 residues in length, but fragments as long as 35 amino acids can be present. How these fragments exit the central chamber is not known. Clearly, there is much to be discovered about the internal workings of the 26S proteasome.

6.3

Processing by the 26S Proteasome

In some cases, the 26S proteasome partially degrades the substrate protein releasing processed functional domains. The best-studied example of processing involves the transcriptional activator NF κ B. The C-terminal half of a 105-kDa precursor is degraded by the 26S proteasome to yield a 50-kDa N-terminal domain that is the active transcription component. A glycine-rich stretch of amino acids at the C-terminal boundary of p50 is an important factor in limiting proteolysis. It is possible that polypeptide translocation by the RC starts at the glycine-rich region and proceeds in only one direction because of the presence of the tightly folded N-terminal domain. Or the RC may start translocation at the C-terminus and stop when the ATPases encounter the glycine-rich region. Another example of partial processing involves SPT23, a yeast protein embedded in the endoplasmic-reticulum membrane. SPT23 controls unsaturated fatty acid levels and membrane fluidity regulates 26S proteasomal generation of a freely diffusible transcription factor from the SPT23 precursor. Partial processing may be a more widespread regulatory mechanism than is currently thought.

7

Proteasome Activators

In addition to the RC, there are two protein complexes, REG $\alpha\beta$ and REG γ , and a single polypeptide chain, PA200, that bind the 20S proteasome and stimulate peptide, but not protein, degradation (see Fig. 2). Like the RC, proteasome activators bind the ends of the 20S proteasome, and

importantly, they can form mixed or hybrid 26S proteasomes in which one end of the 20S proteasome is associated with a 19S RC and the other is bound to a proteasome activator. This latter property raises the possibility that proteasome activators serve to localize the 26S proteasome within eukaryotic cells.

7.1

REGs or PA28s

There are three distinct REG subunits called $\alpha\beta\gamma$. REG $\alpha\beta$ form donut-shaped hetero-heptamers found principally in the cytoplasm, whereas, REG γ forms a homo-heptamer located in the nucleus. REG $\alpha\beta$ are abundantly expressed in immune tissues while REG γ is highest in brain. The REGs also differ in their activation properties. REG $\alpha\beta$ activate all three proteasome active sites; REG γ only activates the trypsin-like subunit. There is reasonably solid evidence that REG $\alpha\beta$ play a role in Class-I antigen presentation, but we have no idea what REG γ does, especially since REG γ knockout mice have almost no phenotype. The crystal structure of REG α reveals that the seven subunits form a donut-shaped structure with a central aqueous channel, and the structure of a REG–proteasome complex provides important insight into the mechanism by which REG α activates the proteasome. The carboxyl tail on each REG subunit fits into a corresponding cavity on the α -ring of the proteasome and loops on the REG subunits cause N-terminal strands on several proteasome α -subunits to reorient upward into the aqueous channel of the REG heptamer. These movements open a continuous channel from the exterior solvent to the proteasome central chamber.

7.2

PA200

A new proteasome activator, called PA200, was recently purified from bovine testis. Human PA200 is a nuclear protein of 1843 amino acids that activates all three catalytic subunits, with preference for the PGPH active site. Homologs of PA200 are present in budding yeast, worms, and plants. A single chain of PA200 can bind each end of the proteasome, and when bound, PA200 molecules look like volcanoes in negatively stained EM images. Evidence from both yeast and mammals suggests that PA200 is involved in DNA repair.

7.3

Hybrid Proteasomes

As the α -rings at each end of the 20S proteasome are equivalent, the 20S proteasome is capable of binding two RCs, two PA28s, two PA200s, or combinations of these components. In fact, 20S proteasomes simultaneously bound to RC and PA28 or PA200 have been observed, and they are called *hybrid proteasomes*. In HeLa cells, hybrid proteasomes containing PA28 at one end and an RC at the other have been estimated to be twice as abundant as 26S proteasomes capped at both ends by 19S RCs. Hybrid 26S proteasomes containing PA200 appear to be much less abundant in HeLa cells.

7.4

ECM29

Another proteasome-associated protein, called ecm29, has been identified in several recent proteomic screens. Ecm29p is reported to stabilize the yeast 26S proteasome by clamping the RC to the 20S cylinder. However, in human

culture cells, ecm29p is found predominantly associated with the endoplasmic reticulum Golgi intermediate compartment, a location suggesting a role in secretion rather than stability of 26S proteasome. Ecm29p clearly associates with 26S proteasomes; whether it activates proteasomal peptide hydrolysis is unknown.

8

Protein Inhibitors of the Proteasome

A number of proteins have been found to suppress proteolysis by the proteasome. One of these is PI31, another is the abundant cytosolic chaperone, Hsp90, and a third is a proline/arginine-rich 39-residue peptide called PR39. Both PI31 and Hsp90 may affect how the proteasome functions in Class-I antigen presentation. PI31 is a 30-kDa proline-rich protein that inhibits peptide hydrolysis by the 20S proteasome and can block activation by both RC and REG $\alpha\beta$. Although surveys of various cell lines show PI31 to be considerably less abundant than RC or REG $\alpha\beta$, when overexpressed, PI31 is reported to inhibit Class-I antigen presentation by interfering with the assembly of immunoproteasomes. A number of studies have shown that Hsp90 can bind the 20S proteasome and inhibit its chymotrypsin-like and PGPH activities. Interestingly, inhibition by Hsp90 is observed with constitutive but not with immunoproteasomes, a finding consistent with proposals that Hsp90 shuttles immunoproteasome-generated peptides to the endoplasmic reticulum for Class-I presentation. PR39 was originally isolated from bone marrow as a factor able to induce angiogenesis and inhibit inflammation. Two hybrid screens showed that PR39 binds the 20S

proteasome. Apparently, PR39 affects angiogenesis and inflammation by inhibiting respectively the degradation of HIF1 or I κ B α , the latter being an inhibitor of NF κ B. Finally, HIV's Tat protein inhibits the 20S proteasome's peptidase activity. Tat also competes with REG $\alpha\beta$ for proteasome binding, and by doing so, Tat can inhibit Class-I presentation of certain epitopes.

9

Physiological Aspects of Proteasomes

9.1

Tissue and Subcellular Distribution of Proteasomes

Proteasomes are found in all organs of higher eukaryotes, but the degree to which the composition of proteasomes and its activators varies among tissues is largely unexplored territory. Proteasomes are very abundant in testis since the organ contains almost fivefold more 20S subunits than skeletal muscle. At the cellular level, there are about 800 000 proteasomes in a HeLa cell and roughly 20 000 proteasomes in a yeast cell. At the subcellular level, 26S proteasomes are present in cytosol and nucleus where they appear to be freely diffusible. They are not found in the nucleolus or within membrane-bound organelles other than the nucleus. When large amounts of misfolded proteins are synthesized by a cell, the aberrant polypeptides often accumulate around the centrosome in what are called "aggresomes." Under these conditions, 26S proteasomes, chaperones, and proteasome activators also redistribute to the aggresomes presumably to refold and/or degrade the misfolded polypeptides.

9.2

Physiological Importance

Deletion of yeast genes encoding 20S proteasome and 19S RC subunits is usually lethal, indicating that the 26S proteasome is required for eukaryotic cell viability. Known substrates of the 26S proteasome include transcription factors, cell-cycle regulators, protein kinases, and so on – essentially, most of the cell's important regulatory proteins. Surprisingly, even proteins secreted into the endoplasmic reticulum are returned to the cytosol for degradation by the 26S proteasome. Given the scope of its substrates, it is hardly surprising that the ubiquitin–proteasome system contributes to the regulation of a vast array of physiological processes, ranging in higher eukaryotes. Discussion of these fascinating regulatory mechanisms is beyond the scope of this article. A brief review of proteasomes and disease does, however, seem appropriate.

9.3

The Ubiquitin–proteasome System and Human Disease

As the central protease in the ubiquitin system, the 26S proteasome impacts a number of diseases, especially cancer, neurological diseases, and muscle wasting. Ubiquitin-mediated destruction of proteins driving cell division imparts directionality to the cell cycle. So it is not surprising that several cancers arise because of an impaired or overactive ubiquitin–proteasome system. VonHippel-Lindau (VHL) disease is a hereditary cancer syndrome, characterized by a wide range of malignant tumors. The protein defective in VHL is the substrate-recognition component of a Ub–ligase complex that targets

the transcription-component hypoxia-inducible factor 1 (HIF1) for destruction. HIF1 induces synthesis of VEGF, a growth factor important for angiogenesis, and many of the tumors in VHL involve the vasculature. The human papilloma virus oncoprotein E6 provides an example of an overactive Ub ligase causing cancer. E6 is a positive regulator of the Ub ligase that targets the tumor-suppressor protein p53 for degradation. Downregulation of p53 prevents apoptosis of HPV-transformed cells, thereby promoting malignancy. Angelman and Liddle's syndromes and juvenile-onset Parkinson's disease are three other human diseases involving E3 Ub ligases.

Impairment of the proteasome occurs in a number of neurodegenerative diseases, especially those characterized by formation of protein inclusions, such as Parkinson's and Huntington's diseases. In these diseases, proteasomes and ubiquitylated proteins accumulate at the inclusions. It is thought that sequestration and/or inhibition of proteasomes may impair normal protein turnover, eventually resulting in neuronal cell death.

The ubiquitin-proteasome system also plays a key role in cellular immunity. There is considerable evidence that proteasomes generate the majority of peptides presented on Class-I molecules to cytotoxic lymphocytes. Class-I antigen presentation is a major defense against viral infection, and viruses have devised a number of ways to prevent Class-I molecules from reaching the cell surface. In several cases, viruses actually hijack the Ub-proteasome system to degrade newly synthesized MHC Class-I molecules, thereby preventing surface presentation of viral peptides. Given its pervasive role in metabolic regulation, there is no doubt that many more human diseases

will be shown to result from malfunction of the Ub-proteasome system.

See also Electron Microscopy in Cell Biology; Electron Microscopy of Biomolecules; Membrane Traffic: Vesicle Budding and Fusion; Motor Proteins; Ubiquitin-Proteasome System for Controlling Cellular Protein Levels.

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**Protein Aggregation: see
Aggregation, Protein**

