Review

The ubiquitin-proteasome system

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The 2004 Nobel Prize in chemistry for the discovery of protein ubiquitination has led to the recognition of cellular proteolysis as a central area of research in biology. Eukaryotic proteins targeted for degradation by this pathway are first 'tagged' by multimers of a protein known as ubiquitin and are later proteolyzed by a giant enzyme known as the proteasome. This article recounts the key observations that led to the discovery of ubiquitin-proteasome system (UPS). In addition, different aspects of proteasome biology are highlighted. Finally, some key roles of the UPS in different areas of biology and the use of inhibitors of this pathway as possible drug targets are discussed.

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1. Introduction

In an incisive article, J Goldstein, the 1985 Nobel laureate for the regulation of cholesterol metabolism (together with M Brown) and Chair for the Jury for the Lasker awards, laments the fact that it is hard to pick out truly original discoveries among the plethora of scientific publications (Goldstein 2004). He suggests that there are two ways to analyse a scientific discovery which, for ease of understanding, can be categorized into two groups: "top to bottom" or "bottom to top." In the first category are papers that are published in highly ranked journals and often arrive with lots of media coverage but are soon forgotten, i.e. they start at the top but end up at the bottom. On the other hand, it is harder to spot a "bottom to top" discovery as it starts off at the bottom and its arrival is unheralded. However, with the passage of time, the significance of this discovery is increasingly appreciated, resulting in its emergence at the top with true impact! The discovery of protein ubiquitination is a fine example of a "bottom to top" discovery and its role in 'big' biological processes, e.g. transcription, cell cycle, antigen processing, cellular defense, signalling etc. is now well established (Ciechanover and Iwai 2004; Varshavsky 2005).

During the early days in the field of cytosolic protein degradation, cell biologists were intrigued by the requirement of ATP in this process as it is well known that peptide bond hydrolysis does not require metabolic energy. The strategy used by A Hershko and colleagues involved biochemical fractionation of reticulocytes (terminally differentiated red blood cells lacking lysosomes), purification, identification of the components involved and reconstitution of the ATP-dependent cytosolic protein degradation *in vitro*. These studies were supported by genetic studies and data from other groups on the *in vivo* roles of cytosolic protein degradation in different biological processes. The steps that led to the discovery and appreciation of the ubiquitin-proteasome system (UPS) are highlighted in table 1. For their pioneering studies on the role of ubiquitination of proteins during cytosolic protein degradation, Avram Hershko, Aaron Ciechanover and Irwin Rose were awarded the 2004 Nobel Prize in Chemistry.

Keywords. Cellular proteolysis; proteasomes; protein degradation; ubiquitination.

Abbreviations used: AAA-ATPase, ATPases associated with various cellular activities-ATPase; BrAAP, branched chain amino acids preferring; CSN, COP9 signalosome; ER, endoplasmic reticulum; HECT, homologous to E6-AP carboxyl terminus; IFN, interferon; MHC, major histocompatibility complex; NTN, N-terminal nucleophile; PA, proteasome activator; PAC, proteasome assembling chaperone; PAN, proteasome-activating nucleotidase; PI, proteasome inhibitor; POMP, proteasome maturing protein; RING, really interesting new gene; Rpn, regulatory particle non-ATPase; Rpt, regulatory particle tripleA-ATPase; SCF, Skp-Cullin-F box; SNAAP, small neutral amino acids preferring; UBH, ubiquitin hydrolase; UBP, ubiqutin processing; UMP1, ubiquitin mediated proteolysis-1; UPS, ubiquitin-proteasome system

Table 1. Key landmarks leading to the discovery of the ubiquitin-proteasome system.

Key landmarks	Contributors
Development of a rabbit reticulolysate system to study non-lysosomal and ATP-dependent protein degradation	Etlinger and Goldberg 1977
Fractionation of reticulolysate led to the identification of two fractions, active principle of fraction (APF)-I and APF-II. The combination of APF-I and APF-II reconstituted protein degradation	Ciechanover *et al* 1978
APF-II was subfractionated into two fractions: APF-IIa and APF-IIb. APF-IIb contained the E1-E3 ubiquitin conjugating enzymes	Hershko *et al* 1979
APF-IIa was later shown to contain proteasomes	Hough *et al* 1986
APF-I identified as ubiquitin	Wilkinson *et al* 1980
High molecular conjugates of ubiquitin and substrate proteins were formed in the presence of conjugating enzymes and ATP	Hershko *et al* 1980
Deubiquitinating enzyme activity identified that was capable of recycling ubiquitin bound to substrate proteins	Hershko *et al* 1980
The carboxyl terminal glycine of ubiquitin was found to be activated by the E1 enzyme	Hershko *et al* 1981
The amount of ubiquitin-protein conjugates increased in reticulocytes during the formation of abnormal proteins, demonstrating a link between ubiqutination and protein degradation	Hershko *et al* 1982
Purification and identification of all three ubiquitin conjugating enzymes, E1, E2 and E3	Hershko *et al* 1983
Genetic experiments revealed physiological roles of the UPS. E1 enzyme was responsible for the lack of growth of the mutant mammary cell line ts85 in non permissive temperature, suggesting the involvement of ubiquitination during cell cycle progression	Finley *et al* 1984
The degradation of short-lived proteins in ts85 cell line was inhibited at the non-permissive temperature	Ciechanover *et al* 1984
An E3 ligase was found to demonstrate substrate specificity	Hershko *et al* 1986
Several groups were involved in the characterization of a large molecular weight, neutral protease from different sources: bovine pituitary, rat liver and the archaebacterium, *Thermoplasma acidophilum*	Wilk and Orlowski 1983 Tanaka *et al* 1986 Dahlmann *et al* 1989
This protease was initially called as 'pro-some' as it was thought to be associated with RNA	Schmid *et al* 1984
Interestingly, a large molecular weight complex consisting of small subunits was detected using an allo-MHC serum. Several years later, genes encoding two IFN-γ-inducible proteasome subunits, β1i and β5i, were found to be in the MHC locus in mammals	Monaco and McDevitt 1984
Characterization of a high molecular protease that degraded ubiquitin adducts of protein but not untagged protein	Hough *et al* 1986
The term proteasome was coined to indicate its proteolytic and particulate nature	Arrigo *et al* 1988
Active 26S proteasomes were generated on mixing purified 20S proteasomes and 19S regulators in the presence of ATP	Hoffman *et al* 1992

Cellular protein degradation occurs in different cellular compartments: cytosol, membrane, lysosome and endoplasmic reticulum. In addition, regulatory intramembrane proteolysis is emerging as a major player in several critical processes, e.g. cellular differentiation, lipid metabolism, the unfolded protein response, Alzeimer's disease etc. (Brown *et al* 2000). Lysosomal degradation is, primarily, mediated by cathepsins, belonging to the cysteine or aspartate family of proteases. Patients lacking cathepsin K suffer from pycnodysostosis, a disorder in bone remodelling (Turk *et al* 2001). Calpains belong to the group of Ca^{2+} dependant cysteine proteases, which are involved in adhesion, locomotion, cytoskeletal rearrangements, inflammation and apoptosis. Typically, calpains consist of a large ~ 80 kDA subunit together with a small ~ 30 kDa subunit. Disruption of the small subunit that is common to all calpains, results in embryonic lethality (Arthur *et al* 2000) and deficiency in calpain 3 leads to muscular dystrophy (Richard *et al* 2000). The majority of cytosolic protein degradation in eukaryotes occurs via the UPS, the main focus of this review. In this process, cellular proteins targeted for degradation are tagged by multimers of an evolutionarily conserved protein known as ubiquitin and are degraded by the 26S proteasome, a giant cytosolic protease.

The steady state levels of cellular proteins depend on their rate of synthesis and degradation. How are cellular proteins targeted for cytosolic protein degradation? The initial events are not completely understood and are dependent on multiple factors: the presence of specific sequences (e.g. the destruction box in cyclins) or the amino-terminal residue may be important (also known as the N-end rule), for e.g. proteins with a basic amino acid at the amino-terminus are less stable compared to those containing small amino acids. In addition, aged proteins may display a

hydrophobic patch due to denaturation that are recognized and routed for degradation. More importantly, cellular signalling events target proteins by post translation modifications (e.g. phosphorylation or oxidation) that undergo degradation. It is this selective degradation of proteins that play crucial roles during cellular decision making and render this process important (Varshavsky 2005).

The release of amino acids from an intact protein can be distinguished into two parts, based on the utilization of metabolic energy: the proximal ATP-dependent steps are followed by ATP-independent events (figure 1). This basic model of protein unfolding followed by degradation is conserved and involves several steps (Chandu and Nandi 2002, 2004). Proteins targeted for degradation are often polyubiquitinated in eukaryotes and are bound to regulators of ATP-dependent proteases. Second, these proteins are unfolded by regulators via conformation changes driven by ATP hydrolysis. Third, unfolded proteins are actively translocated into the proteolytic chambers present in proteases. Finally, polypeptides are degraded in an ATP-independent manner by endopeptidases, aminopeptidases and carboxypeptidases. Interestingly, enzymes involved in the proximal ATP-dependent steps of this process are selective, i.e. ClpP and Lon are the major enzymes in prokaryotes, whereas 26S proteasomes are responsible for cytosolic protein degradation in eukaryotes (Chandu and Nandi 2004). However, key peptidases involved in the latter ATP-independent steps are conserved in all kingdoms, from *Escherichia coli* to humans (Chandu and Nandi 2002).

2. The ubiquitination system

Cellular proteins targeted for degradation by ATP-dependent 26S proteasomes are tagged to ubiquitin, a protein composed of 76 amino acids. It is covalently bound to the target protein by an isopeptide linkage between the carboxy terminal glycine of ubiquitin and, usually, the ε-amino group of lysine in the target protein. Similar isopeptide linkage is formed between the carboxy terminus of ubiquitin with the ε-amino group of lysine of another ubiquitin molecule to form polyubiquitin chains (Pickart 2001; Weissman 2001; Ciechanover and Iwai 2004). In some proteins (for e.g. in proteins lacking lysine), poly-ubiquitination may occur at the amino terminal residue (Ciechanover and Ben-Saadon 2004). Also, the lysine residue on which polybiquitination occurs is important. For e.g. formation of polyubiquitin chains by linkage at Lys-48 (well studied) and Lys-29 (less studied) of ubiquitin can act as a signal for proteasome mediated degradation, whereas ubiquitination at other lysine residues (e.g. Lys-63) may act as signals for DNA repair, activation of transcription factors etc. (Weissman 2001). On the other hand, mono-ubiquitination of proteins has other functions, e.g. endocytosis, histone regulation, virus budding etc. (Hicke 2001).

The process of ubiquitin mediated substrate delivery to 26S proteasomes can be summarized in this simplistic description (figure 2). An activating enzyme, E1, transfers ubiquitin to a carrier E2 enzyme, which in turn tags ubiquitin to the doomed substrate with the help of E3 enzymes.

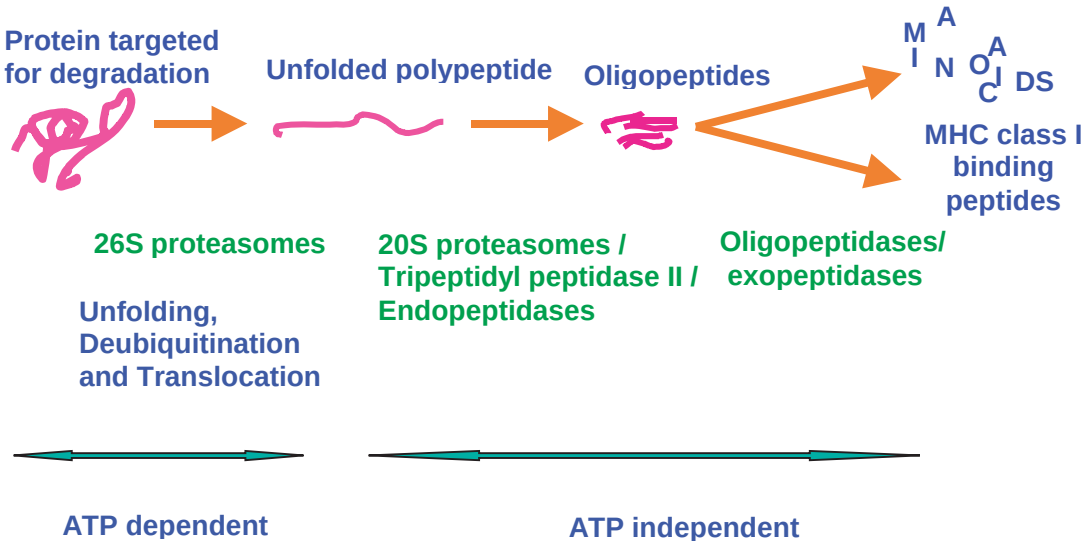

Figure 1. Overall view of the cytosolic protein degradation pathway in mammals. The cytosolic protein degradation pathway leading to free amino acids is represented in this schematic diagram. The proximal steps in this pathway are ATP-dependent and performed by 26S proteasomes whereas the latter steps are executed by ATP- independent proteases and peptidases. In addition, this pathway also generates 8–15 amino acid long peptides that are transported into the endoplasmic reticulum and bind to MHC class I molecules for perusal by $CD8^+$ T cells. Binding of these peptides to MHC class I protects them from further degradation by exopeptidases.

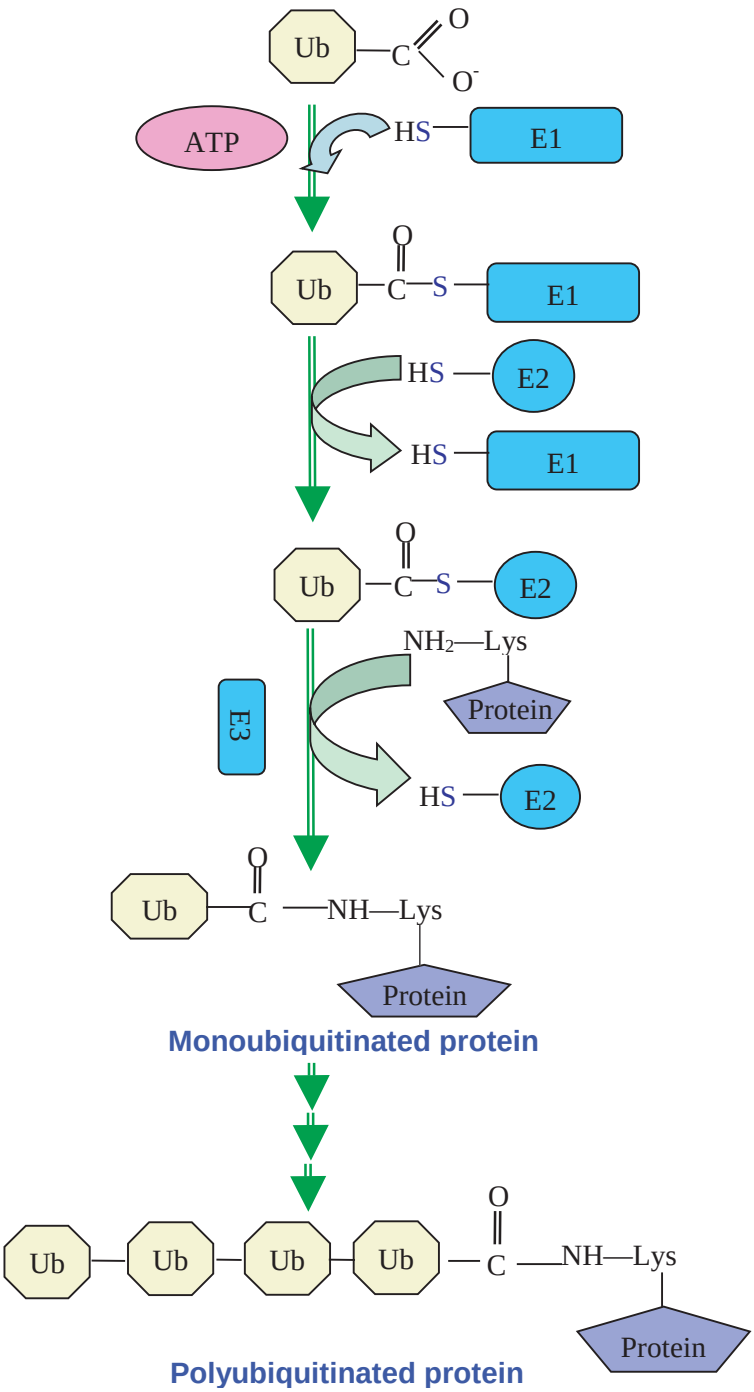

Figure 2. The protein ubiquitination pathway. Ubiquitin (Ub) is activated by E1 and transferred to the E2 enzyme and is, finally, conjugated to substrate proteins with a specific E3 ligase. Further polyubiquitination is required to target proteins for degradation.

Although 26S proteasomes recognize poly-ubiquitin as the main tag, diverse E3 ligases recognize substrates harbouring various degradation signals and contribute to selectivity and specificity of the UPS (Voges *et al* 1999; Pickart 2001; Weissman 2001; Ciechanover and Iwai 2004; Pickart and Cohen 2004). Comparative genome analysis reveals few genes encoding E1, tens of E2 encoding genes and hundreds of E3 encoding ligases (Semple *et al* 2003). By using E2 and E3 mediated specificity, the UPS regulates and eliminates specific proteins while leaving the others intact. This specific

regulation of protein degradation enables cells to regulate cellular 'decisions' in a dynamic fashion (Varshavsky 2005).

The activation of ubiquitin is carried out by the E1 enzyme in the presence of ATP, resulting in the formation of ubiquitin-AMP as intermediate and ubiquitin-E1 thiol ester as the final product. The E1-ubiquitin thiol ester is recognized by multiple E2s, to which ubiquitin is transferred by another thiol ester linkage. E2 enzymes aid in carrying the activated ubiquitin from E1 to the substrate and hence, are often called as ubiquitin-conjugating or ubiquitin-carrier proteins. All E2 enzymes harbour a conserved core that is utilized, along with support from the termini, in E2-E3 binding. E2 enzymes associate with E3 enzymes in a specific manner although each E2 can bind more than one E3. Importantly, E3 enzymes are responsible for the final target selection and specificity. E3 enzymes belong to two distinct families: (i) E3s that harbour the ~ 350 aa homologous to E6-AP carboxyl terminus (HECT) domain with the conserved catalytic cysteine residue. The E6-AP protein is encoded by the human papilloma virus and is responsible for targeting certain proteins (e.g. p53) for ubiquitination. HECT domain proteins form a covalent (thiolester) bond with the ubiquitin before transferring it to the substrate. (ii) On the other hand, E3 enzymes that harbour the really interesting new gene (RING) domain contain cysteines and histidines amino acids that co-ordinate two metal atoms. Many observations facilitated identification of this group including the fact that all E3s lacking HECT domain harbour a RING finger domain. Ubiquitination via RING finger E3s involves binding to E2-ubiquitin complex and facilitating direct transfer of ubiquitin to the targeted protein without the additional thiol ester formation as observed in the HECT family of E3 ligases (Pickart 2001; Weissman 2001; Ciechanover and Iwai 2004). A subset of E3 ligases are the Skp-Cullin-F box (SCF) proteins that are characterized by: Skp–1 or Skp–1-like protein that is involved in substrate recognition, Cullin subunit e.g. Cul–1, an F-box containing protein and the catalytic RING finger containing protein, Rbx–1. Modification of Cullins by an ubiquitin-like protein Nedd/Eub–1, results in neddylation and activation of SCF-ligases due to increased recruitment of E2 enzymes (Cardozo and Pagano 2004). The conventional ubiquitination enzymes E1, E2, and E3 add only limited number of ubiquitin moieties to protein substrates and polyubiquitination is required to act as a degradation signal. E4 enzymes (e.g. Ufd2) support the formation of multi-ubiquitin conjugates (Hoppe 2005) and *Saccharomyces cerevisiae* lacking *ufd2* are defective in proteolysis of ubiquitin-substrates (Koegl *et al* 1999). Ufd2a is involved in the degradation of pathological forms of ataxin type 3 that are responsible for a neurodegenerative disease known as spinocerebellar ataxia type 3 (Matsumoto *et al* 2004).

Ubiquitin needs to be removed from tagged proteins before they enter the proteolytic core of proteasomes. The classical deubiquitinaing enzymes belong to ubiquitin processing (UBP) and ubiquitin carboxy-terminal hydrolases (UBH) families. In general, UBPs remove ubiquitin from poly-ubiquitinated proteins whereas UBHs remove small adducts from ubquitin and regenerate free monomeric ubiquitin. Also, ubiquitin genes are transcribed and translated as a polyubiquitin chain, which then needs to be acted upon by UBHs to release single ubiquitin moieties (Weissman 2001; Kim *et al* 2003). As ubiquitin is bound to enzymes by thio ester linkage, deubiquitinating enzymes are thiol proteases. Genome analysis has revealed that genes encoding UBPs are higher in number compared to UBHs in most organisms. Also, the crystal structures of two UBH enzymes reveal structural similarities with papain-like cysteine proteases, especially in the active site (Kim *et al* 2003). Other novel deubiquitinating enzymes may also exist. For e.g. the deubiquitinating enzyme identified to be a part of the PA700 or 19S regulator complex is an ATP-dependent metalloprotease with a JAMM motif (Rpn11; see table 2) (Berndt *et al* 2002) and is essential for 26S proteasomal degradation (Verma *et al* 2002; Yao and Cohen 2002). The functional role of the Rpn11 subunit demonstrates the close relationship between deubiquitination and proteasome mediated protein degradation.

3. PA700, the 19S regulator

The process of tagging with multi-ubiquitin marks proteins for degradation by 26S proteasome, a huge cytosolic protease complex. This structure consists of the 20S proteasome, harbouring the proteolytic core, bound to a 19S cap also known as PA700 (figure 3). 20S proteasomes degrade unfolded or loosely folded proteins and peptides in an ATP-independent manner; however, they cannot degrade ubiquitin-protein conjugates. The proteolytic active sites of 20S proteasome are sequestered within the lumen of this cylindrical complex, to avoid non-specific degradation of cellular proteins. PA700 binds to either or both ends of 20S proteasomes, in the presence of ATP, to channelize ubiquitinated proteins into the central active site chamber for degradation (Glickman *et al* 1998; Voges *et al* 1999; Pickart and Cohen 2004).

PA700 is a large complex comprising several subunits, which impart the complex with diverse activities: ATPase, ubiquitin-binding, deubiquitinating, reverse chaperone etc. PA700 from *S. cerevisiae* harbours at least 17 subunits, regulatory particle non-ATPase (Rpn)1–12 and regulatory particle tripleA-ATPase (Rpt)1–6. PA700 can be dissociated into two sub-complexes, base consisting of nine subunits and lid consisting of eight subunits. The base harbours six essential ATPases and three nonATPase subunits, including the polyubiquitin-interacting protein S5a (table 2). These ATPases belong to the ATPases associated with various cellular activities (AAA)-ATPase family. By utilizing its ATPase activity, the base complex acts as a reverse chaperone to unfold target proteins and also facilitates opening up the narrow pore of 20S

Table 2. Nomenclature of 19S regulator (PA700) subunits.

	S. cerevisiae	Humans	Functions	
Base subunits				
Rpt1	Cim5/Yta3	S7/Mss1	ATPase	
Rpt2	Yta5	Rpn1	ATPase	Controls substrate entry and product egress from proteasome
Rpt3	Yta2/Ynt1	S6/Tbp7	ATPase	
Rpt4	Crl13/Sug2	S10b	ATPase	
Rpt5	Yta1	S6'/Tbp1	ATPase	Binds polyubiquitinated substrates
Rpt6	Sug1/Cim3	S8/Trip1	ATPase	
Rpn1	Hrd2/Nas1	S2/Trap2	Non ATPase	Interacts with deubiquitinating enzyme, Ubp6
Rpn2	Sen3	S1	Non ATPase	NLS important for nuclear proteasome localization
Rpn10	Mcb1/Sun1	S5a	Non ATPase	Binds polyubiquitinated substrates
Lid subunits				
Rpn3	Sun2	S3	Non ATPase	
Rpn4	Son1/Ufd5		Non ATPase	
Rpn5	Nas5		Non ATPase	Important for proper assembly of 19S regulator
Rpn6	Nas4	S9	Non ATPase	Important for proper assembly of 19S regulator
Rpn7		S10	Non ATPase	Important for proper assembly of 19S regulator
Rpn8		S12	Non ATPase	
Rpn9		S11	Non ATPase	Important for proper assembly of 19S regulator
Rpn11	Mpr1	Poh1	Non ATPase	Deubiquitinating activity, Zn^{2+} dependent metallo-protease
Rpn12	Nin1	S14	Non ATPase	

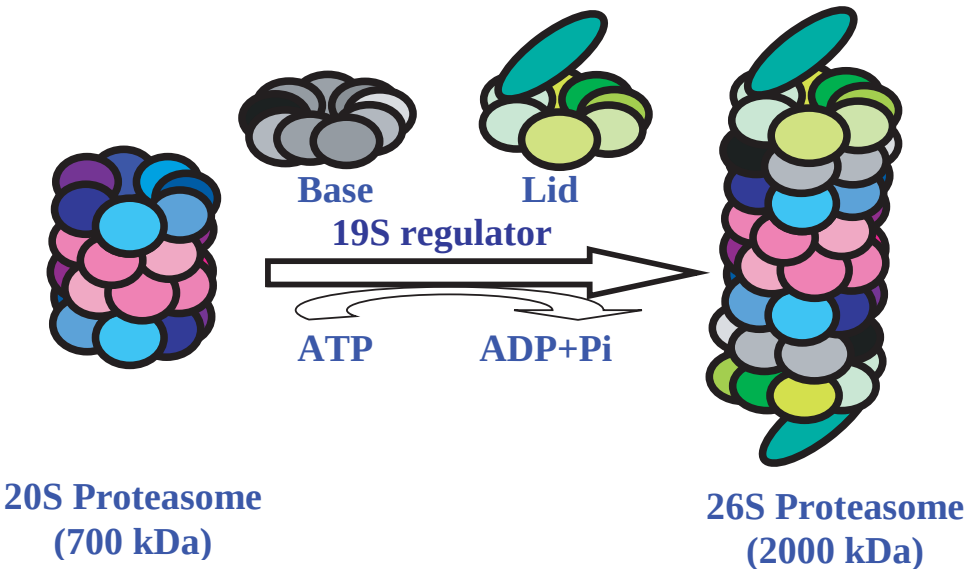

Figure 3. Formation of 26S proteasome. These are formed by the combination of catalytic 20S proteasome with PA700, also known as 19S regulators, in an ATP-dependent manner.

proteasome. The lid complex is essential for degradation of ubiquitinated proteins and comprises eight subunits that lack ATPase activity. Also, the PA700 associated deubiquitinating enzymes reside in the lid complex. The different components of the PA700 and their functions have been extensively discussed (Glickman *et al* 1998; Voges *et al* 1999; Pickart and Cohen 2004). Interestingly, the components of the PA700 lid are homologous to subunits of the COP9 signalosome (CSN), which was first discovered to be involved in signal transduction in plants and is, subsequently, shown to be involved in different biological processes (Schwechheimer 2004). In fact, deficiency of the Csn2 subunit results in disruption of the CSN

complex, increased levels of Cyclin E and p53, resulting in arrest of embryo development in mice (Lykke-Andersen *et al* 2003). The CSN directly interacts with 26S proteasomes and competes with the lid for assembly. In addition, the CSN modifies cullin subunits by cleaving off the Nedd8 (i.e. deneddylation) and modulating the assembly and activity of SCF-E3 ligases. Additionally, CSN regulates proteolysis by associating with protein kinases and deubiquitinating enzymes (Schwechheimer 2004).

Eubacterial enzymes involved in cytosolic protein degradation also associate with ATPase regulators, for e.g. ClpP binds to ClpA or ClpX (Chandu and Nandi 2004). Although there is no evidence of protein-ubiquitination in archaea and eubacteria, 20S proteasomes present in some bacteria in these kingdoms may bind to ATPase regulators. 20S proteasomes from some archeaebacteria, e.g. *Methanococcus jannaschii*, are known to interact with the proteasome-activating nucleotidase (PAN) complex which is homologous to PA700 present in 26S proteasomes complex (Zwickl *et al* 1999). PAN (~ 650 kDa) is an oligomeric complex with a subunit size of 50 kDa and increases the protein degradation (but not peptide degradation) ability of proteasomes. Substrate binding to PAN activates nucleotidase activity, which in turn is utilized for substrate unfolding, 20S proteasome terminal pore opening and protein translocation (Benaroudj *et al* 2003). Although ATP is most efficient in hydrolyzing proteins, other nucleotides could also significantly support hydrolysis and hence the name, nucleotidase (Zwickl *et al* 1999). Further light scattering and fluorescence experiments demonstrated that PAN inhibits protein aggregation and facilitates protein unfolding in an ATP-dependent manner. PAN, similar to PA700 ATPases, acts as a reverse-chaperone that can facilitate degradation by unfolding targeted proteins (Benaroudj and Goldberg 2000). Also, AAA-ATPase in eubacteria form homo-hexameric ring shaped complexes (ARC). In fact, the proteasomal ATPase in *Mycobacterium tuberculosis* is important in resisting host-encoded nitrosative stress (Darwin *et al* 2003, 2005).

4. Proteasomes

20S proteasomes are composed of four heptameric stacked rings ($\alpha_7\beta_7\beta_7\alpha_7$) and the outer rings are made up of α-type subunits whereas the inner two rings are made up of β-type subunits. The quarternary structure of 20S proteasomes is conserved from bacteria, including archaea, to mammals and the active sites are present inside a central "chamber for degradation" (Voges *et al* 1999; Pickart and Cohen 2004). Archaebacterial proteasomes act as prototype of all proteasomal sequences and all other proteasomal subunits probably arose from these by gene duplications and lateral transfer. In fact, the definition of α and β subunits is also based on the similarity to α and β subunit of *Thermoplasma acidophilum* proteasomes. Often the *T. acidophilum* proteasome is called as the '*Urproteasome*' or the ancestral proteasome; however, with evolutionary complexity, the subunit composition has changed. All the subunits in a ring are identical in the 20S proteasome from *T. acidophilum*. In eukaryotes, each ring is composed of as many as 7 different α-type or 7 different β-type subunits (figure 4; table 3).

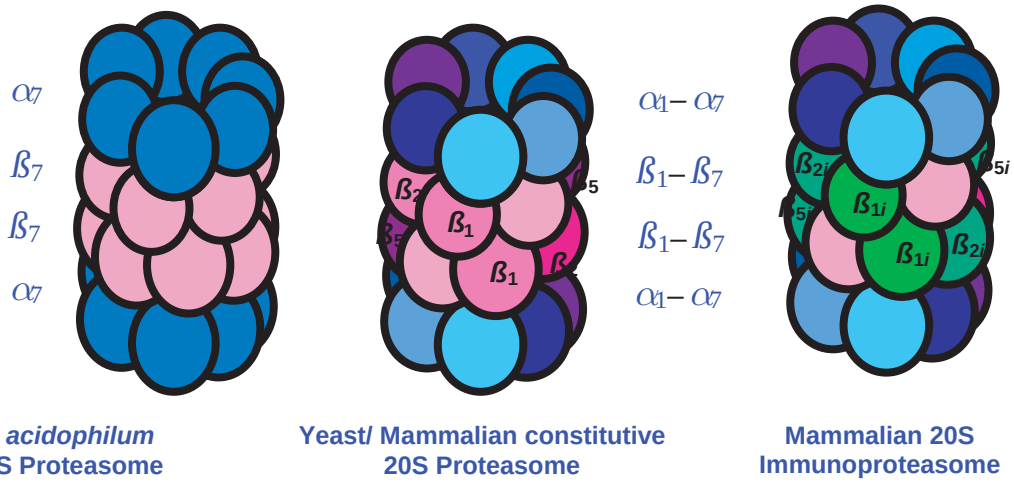

Figure 4. Comparison of the subunit composition of 20S proteasomes from different organisms. The proteasome of archaebacterium *T. acidophilum* contains single α and β proteasome subunits and the outer rings are composed of identical α subunits whereas the inner rings are composed of the identical β subunit. On the other hand, yeast proteasomes are composed of seven different α and β subunits. In mammals, three constitutive proteasomal β subunits, β1, β2 and β5, are replaced by βli, β2i, and β5i, which are induced in response to inflammatory signals, e.g. IFNγ.

Table 3. Nomenclature of 20S proteasome subunits.

Subunit	*T. acidophilum*	*S. cerevisiae*	Mammals	
			Constitutive	Immunoproteasomes
α1	α	Prs2/C7	PSMA6/ Iota/ LMP11	PSMA6/ Iota/ LMP11
α2	α	Y7	PSMA2/ C3/ LMP8	PSMA2/ C3/ LMP8
α3	α	Y13	PSMA4/ C9/ LMP14	PSMA4/ C9/ LMP14
α4	α	Pre6	PSMA7/ C6/ XAPC7/ LMP16	PSMA7/ C6/ XAPC7/ LMP16
α5	α	Pup2	PSMA5/ Zeta/ LMP1	PSMA5/ Zeta/ LMP1
α6	α	Pre5	PSMA1/ C2/ LMP13	PSMA1/ C2/ LMP13
α7	α	Prs1/C1	PSMA3/ C8/ LMP18	PSMA3/ C8/ LMP18
β1	β	Pre3	PSMB6/ Y/ delta/ LMP19	**PSMB9/ β1i/ LMP2**
β2	β	Pup1	PSMB7/ Z/ LMP9/	**PSMB10/ β2i/ LMP10/ MECL1**
β3	β	Pup3	PSMB3/ C10/ LMP5	PSMB3/ C10/ LMP5
β4	β	Pre1/C11	PSMB2/ C7/ LMP6	PSMB2/ C7/ LMP6
β5	β	Pre2/Doa3	PSMB5/ X/ MB1/ LMP17	**PSMB8/ β5i/ LMP7**
β6	β	Prs3/C5	PSMB1/ C5/ LMP15	PSMB1/ C5/ LMP15
β7	β	Pre4	PSMB4/ N3/ beta/ LMP3	PSMB4/ N3/ beta/ LMP3

IFNγ-inducible β subunits present in immunoproteasomes are indicated in bold.

Functionally, proteasomes from *T. acidophilum* are essential only during heat shock (Ruepp *et al* 1998). However, out of 14 proteasome subunits in *S. cerevisiae*, 13 are essential for viability and an extra copy of α4/Pre6 subunit can substitute for the α3/Pre9 subunit (Velichutina *et al* 2004). 20S proteasomes are absent in *E. coli* and the complete sequences of various eubacterial genomes revealed the presence of single α and β proteasome subunit encoding genes in the order actinomycetales of eubacteria (Lupas *et al* 1994). Interestingly, 20S proteasomes in *Mycobacterium smegmatis* are dispensable (Knipfer and Shrader 1997) but are essential in the pathogen *Mycobacterium tuberculosis* (Sassetti *et al* 2003).

The α subunits are more conserved than the β subunits and form a selective barrier between the catalytic chamber and the cytoplasm. They are the sites for the binding of various regulatory particles, entry and exit of substrates. On the other hand, β subunits harbour the catalytic site. Most β subunits have a prosequence that is cleaved off to expose a threonine residue at the N terminus. This Thr-1 is considered as a critical part of the active site of the proteasome, there by making it a distinct member of a new class of proteases known as N-terminal nucleophile (NTN) hydrolase family. This N-terminal threonine is physiologically important as replacement with serine results in reduced cleavage of peptide bonds (Kisselev *et al* 2000). All the β subunits of *T. acidophilum* possess this N-terminal threonine. In eukaryotes, only three of the seven β subunits (β1, β2, and β5) in each ring are processed to expose the N terminal threonine (Seemuller *et al* 1995). However, the crystal structure determination of 20S proteasomes from bovine liver has revealed processing of another β subunit (β7) with an exposed N-terminal Thr-1 (Unno *et al* 2002).

Originally described as a complex of multiple peptidase activities, studies with inhibitors and specific substrates have defined important activities of this enzyme. Eukaryotic proteasomes display three major peptidase activities, based on cleavage of fluorogenic peptides that can be easily assayed: chymotrypsin-like activity (cleavage after hydrophobic amino acids), trypsin-like activity (cleavage after the basic amino acids) and caspase-like activity (cleavage after acidic amino acids). In addition, two activities, e.g. cleavage after branched chain amino acids (BrAAP) and small neutral amino acids (SNAAP) are also known. The three major activities of yeast 20S proteasomes, caspase-like, trypsin-like and chymotrypsin-like activities can be correlated directly to the three subunits β1 (Pre3), β2 (Pup1) and β5 (Pre2), respectively, as demonstrated by the mutation studies (Heinemeyer *et al* 1997; Groll *et al* 1999). The other four subunits either have unprocessed (β3 or Pup3, β4 or C11) or partially processed (β6 or Pre7, β7 or Pre4) propeptides (Groll *et al* 1999). Studies of human 20S proteasomes on peptide libraries demonstrated that proteasomes can cleave peptide bonds at the P1 position (the amino acid immediately proximal to the peptide bond that is cleaved) of most amino acids, with a preference for leucine and alanine. Also, amino acids proximal to the P1 position, i.e. at P3 and P4 positions, glutamine, valine, isoleucine, leucine and asparagine influence peptide cleavage by 20S proteasomes (Harris *et al* 2001). Interestingly, no major difference in the size of peptides generated after cleavage of proteins was observed from proteasomes from *T. acidophilum*, which contain 14 active sites, and humans, which contain 6 well defined active sites. Peptides generated vary in length from 3–25 amino acids and decreasing amounts of peptides are produced with increase in size (Kisselev *et al* 1998, 1999). The data suggests that

proteasomes process peptides until they are small enough to diffuse out of the proteolytic chamber.

In addition to the 'constitutive' proteasome described so far, there also exists the 'immunoproteasome', which is primarily induced by microbial infections and the immunomodulatory cytokine, IFNγ (figure 4). This cytokine induces the expression of three mammalian proteasome subunits, β1i (LMP2), β5i (LMP7) and β2i (MECL-1), which are incorporated in the place of the constitutive β subunits (Y, X and Z respectively) to form immunoproteasomes (Frentzel *et al* 1994; Nandi *et al* 1997; Griffin *et al* 1998). Interestingly, β1i and β1 are mutually exclusive and belong to the same subgroup as is the case with β2i and β2 or β5i and β5 (table 3), implying that the IFNγ-inducible β subunits in mammals probably arose by gene duplication. The presence of genes encoding *β1i* and *β5i* in the MHC locus of mammals implies that the immune system has utilized the services of already existing set of genes by imparting specialized functions. This is further corroborated by the observation that yeast proteasomes, that lack the three IFNγ-inducible β proteasome subunits, hydrolyze proteins and generate peptides that can be loaded on to MHC I molecules (Niedermann *et al* 1997). Immunoproteasomes are present in hematopoietic cells in mammals, unlike the house keeping proteasomes, which are found in all cells. Apart from IFNγ, other cytokines, for e.g. IFNβ and tumour necrosis factor-α also induce these subunits. In fact, it has been recently demonstrated that the constitutive levels of immnoproteasome subunits are IFNγ independent and IFNγ is essential only for the induction of these subunits and incorporation into proteasomes *in vivo* (Barton *et al* 2002). Interestingly, microbial infections modulate proteasomal subunit expression, resulting in significant replacement of constitutive proteasomes by immunoproteasomes (Khan *et al* 2001; Barton *et al* 2002). The importance of the immunoproteasome subunits is reflected in mice lacking these subunits. Mice lacking *β1i* or *β5i* display differences in peptidase activities and are defective in some immune responses. *β1i*$^{-/-}$ and *β5i*$^{-/-}$ mice display diminished CD8^{+} T cells and MHC class I expression, respectively. These mice also display defects in processing some, but not all, antigens e.g. influenza virus by *β1i*$^{-/-}$ mice and H-Y antigen by *β5i*$^{-/-}$ mice (Fehling *et al* 1994; Van Kaer *et al* 1994). *β1i* and *β5i* are polymorphic and are associated with some diseases, e.g. acute anterior uveitis, juvenile rheumatoid arthritis etc. (Nandi *et al* 1998).

5. Structure and mechanism of action of proteasomes

The X-ray crystallographic analysis of 20S proteasomes from *T. acidophilum* (Lowe *et al* 1995), *S. cerevisiae* (Groll *et al* 1997) and bovine liver (Unno *et al* 2002) revealed similarity in size, shape, active sites, catalytic mechanism, binding to inhibitors etc., despite the complex subunit composition in eukaryotes. *T. acidophilum* 20S proteasomes are 148 Å × 113 Å, where as bovine 20S proteasomes are 150 Å × 115 Å in size. These crystal structures confirmed previous electron micrographic findings that the four rings of proteasome form a cylindrical structure with a narrow channel harbouring a catalytic chamber with a maximum diameter of ~ 53 Å. Although the three active site harbouring β subunits (β1, β2 and β5) are conserved in bovine 20S proteasomes, another subunit, β7 displayed propeptide processing at Thr1 including the presence of residues important for catalysis in correct positions and may display SNAAP activity. However, Thr1 of bovine β7 is placed in a completely different orientation and close to the α ring-β ring junction, unlike Thr1 of other active β subunits, which stay in the chamber formed by two β rings. The comparison of predicted structure of immunoproteasomes with that of the crystal structure of bovine constitutive proteasomes revealed that the active center of immunoproteasomes is apolar, where as it is positively charged in the constitutive proteasomes (Unno *et al* 2002).

20S proteasomes from *T. acidophilum* allow entry of the substrates to the active sites through the central channel as the outer rings made of α subunits harbour an entry port of 13 Å suggesting that translocation may require prior unfolding of the substrate. However, the hydrolytic chamber in *S. cerevisiae* and bovine liver proteasomes does not have easy access from the axial pore as it is surrounded by the N-termini of the α subunits. Thus, eukaryotic proteasomes display low levels of *in vitro* activity, also termed as latent; however, activity increases in the presence of low amounts of SDS and poly-lysine (Tanaka *et al* 1989) or addition of natively unstable protein substrates, e.g. the CDK inhibitor p21^{cip1} and α-synuclein (Liu *et al* 2003). The most likely explanation is that low amounts of SDS, poly-lysine etc. enhance pore opening leading to increased proteasomal activity. A series of structures of 20S proteasomes bound to activators revealed that the proteasome activators PA700, PA28, PA200 and PAN, induce opening of the axial gates of α rings by outward displacement of a set of turns surrounding the gate that allows for better access of substrates into the catalytic chamber of proteasomes (Whitby *et al* 2000; Forster *et al* 2003, 2005). Together, these structures demonstrate that opening the gate pore by activators is critical for the 20S proteasome ability to perform proteolysis.

The structures of 20S proteasomes from *T. acidophilum* and *S. cerevisiae* bound to an inhibitor indicated the presence of Thr1, Glu/Asp17, Lys33, Ser129, Asp166 and Ser169 to be in the vicinity of active sites. Further, the key players are: the amino group of Thr1 which acts as a proton donor, the Thr1Oγ which acts as a nucleophile, Glu17 is important in proper orientation of Lys33 and the positive charge of Lys33 lowers the pK_a of Thr1 to enhance its nucleophilicity. The mechanism of cleaving of peptide bond

by the N-terminal threonine of proteasome involves the initial formation of an acyl-enzyme intermediate by the hydroxyl group of the active site Thr1 forming a bond with the carbonyl of the substrate. Further, activated water molecules act as the general base and acid and hydrolyzes the acyl-enzyme intermediate to free enzyme and product (Lowe *et al* 1995; Seemuller *et al* 1995; Groll *et al* 1997).

6. Proteasome biogenesis

The general model of proteasome assembly involves the formation of a ring containing α subunits followed by incorporation of β subunits into another ring. These 'half' proteasomes undergo dimerization followed by cleavage of the prosequences in the β subunits, most likely by autocatalysis (Kruger *et al* 2001). *T. acidophilum* proteasomes over-expressed in *E. coli* assemble into complexes indistinguishable from native 20S proteasomes. Over-expressed α subunits form a ring by themselves; however, β subunits cannot form a ring by themselves and require preformed α rings for their assembly. The processing of the β subunit to expose the N-terminal active site threonine is autocatalytic and is dependent on the presence of the catalytic Thr-1 and Lys-33. In addition, this processing occurs after the assembly of complete proteasome complex to prevent non-specific hydrolysis of cellular proteins (Zwickl *et al* 1994).

Although the broad pathway is similar, there are some differences between the assembly and processing of *T. acidophilum* and eukaryotic proteasomes. The formation of a α ring is followed by binding of β subunits and formation of the tetrameric ring complex followed by the processing of propeptides of β subunits harbouring active sites. As mentioned before, the *S. cerevisiae* proteasome harbours 7 different α and 7 different β subunits. However, only three β subunits undergo processing of propeptides after assembly and hence, *S. cerevisiae* proteasome harbours only three active sites per β ring, unlike the *T. acidophilum* proteasome which harbours seven active sites per ring. Also, propeptides of β subunits in *T. acidophilum* are dispensable, and fully assembled proteasomes can be dissociated and reassembled (Grziwa *et al* 1994; Zwickl *et al* 1994). Remarkably, propeptides of yeast β subunits are specific to each subunit; for e.g. the β5 (Doa3) propeptide is indispensable, and the subunit lacking propeptide cannot be incorporated into the proteasome complex. However, the β5 (Doa3) propeptide expressed separately (i.e. in trans) can facilitate its incorporation into the proteasome. Also, the β5 (Doa3) subunit responsible for the "chymotrypsin" like-activity in yeast 20S proteasome cannot undergo processing if a mutation disrupts its contact with the complementary inactive subunit β4 (Pre1) from other ring (Chen and Hochstrasser 1996). Also, β2 (Pup1) in the opposite ring is responsible for the processing of β6 (Pre7) and β7 (Pre4) into their final forms. The β5 propeptide plays a greater role during proteasome assembly and activity, which is essential in *S. cerevisiae,* compared to β2 and β1 propeptides (Arendt and Hochstrasser 1999; Groll *et al* 1999; Jager *et al* 1999).

The assembly pathway of mammalian proteasomes has been delineated using antibodies specific for precursor and processed subunits. Interestingly, intermediate forms of proteasome (~ 300 kDa) have been observed, which harbours the precursor subunits and probably correspond to half proteasomes. It has been proposed that following the formation of a ring consisting of α subunits, the first β subunits that become a part of these precursor proteasomes are unprocessed β subunits lacking the catalytic threonine, β4 (C7) and β3 (C10). Interestingly, the other precursor β subunits are incorporated at different times and undergo processing of propeptides at different rates. Perhaps, the incorporation of one β facilitates the incorporation of another β as the localization of different subunits within the proteasome ring is fixed. Two half proteasomes containing a ring of α subunits and another ring of precusor β subunits dimerize followed by the autocatalytic processing of β subunits (Frentzel *et al* 1994; Nandi *et al* 1997). Mammalian proteasomes display displacement of the active β subunits, β1 (Y/delta), β2 (Z) and β5 (X) by interferon (IFN)-γ inducible subunits β1i (LMP2), β2i (MECL) and β5i (LMP7) respectively. A model has been proposed to explain the preferential incorporation of these IFNγ-inducible subunits into immunoproteasomes. Here β1i (LMP2) and β2i (LMP10) are incorporated in 'early' proteasomes and the incorporation of β5i (LMP7) results in formation of active immunoproteasomes (Nandi *et al* 1997, Griffin *et al* 1998). Propeptides play key roles in this process, for e.g. the β5i (LMP7) propeptide is more efficient in incorporation into proteasomes compared to its counterpart, i.e. the β5 (X) propeptide (Kingsbury *et al* 2000). Similarly, β2i (MECL) containing the propeptide of β2 (Z) is incorporated in precursor proteasomes containing constitutive subunits, β5 (X) and β1 (Y) (De *et al* 2003). These experiments clearly demonstrate the key roles of propeptides during proteasome assembly in eukaryotes. Propeptides play multiple roles and support the proper folding and assembly of β subunits; in addition, they protect the N-terminal threonine from acetylation mediated inhibition (Arendt and Hochstrasser 1999; Groll *et al* 1999; Jager *et al* 1999).

Studies have also demonstrated a role of ubiquitin mediated proteolysis-1 (UMP1), encoding an ~ 17 kDa protein, for proper proteasome maturation in *S. cerevisiae*. Remarkably, Ump1p binds to precursor proteasomes and gets degraded by the same proteasome after maturation (Ramos *et al* 1998). Homologues of UMP1 known as protassemblin have been identified in mouse (Griffin *et al* 2000), and in humans known as hUMP1 (Burri *et al* 2000), or the proteasome maturation protein (POMP) (Witt *et al* 2000). POMP has been shown to be up-regulated in cells treated with IFNγ (Burri *et al* 2000) or proteasome

inhibitors. In fact, it has been observed that proteasomal inhibition leads to increased expression of proteasomal genes, facilitating formation of new functional proteasomes to compensate for the loss (Meiners *et al* 2003). Although the function of *S. cerevisiae* and mammalian UMP1 appears to be similar, mammalian UMP1 cannot complement the loss of yeast UMP1 perhaps due to differences in the primary sequence. Mutation studies performed with human UMP1 identified the proteasome-interacting domain to residues 68–72 (RNIQG), as deletion of these five amino acids severely diminishes its precursor proteasome-binding ability (Burri *et al* 2000). POMP is induced by IFNγ and binds to β5i (LMP7) more efficiently than β5 (X), resulting in accelerated formation of immunoproteasomes. In fact, reduced POMP expression results in lower formation of proteasomes, reduced MHC class I and induction of apoptosis (Heink *et al* 2005). Recent studies have identified a proteasome assembling complex (PAC) consisting of two chaperones, PAC1 and PAC2, that binds to α subunits and ensures proper formation and stability of the ring of α subunits (Hirano *et al* 2005). Therefore, UMP1 and the PAC complex play distinct roles during mammalian proteasome assembly.

7. Cellular localization of proteasomes

In mammalian cells, proteasomes are primarily localized in cytosol but also display significant association with cytoskeletal elements, ER, nucleus and plasma membrane; however the ratios of proteasomes associated with different organelles varies in different cells (Wojcik and DeMartino 2003). Studies performed with GFP-tagged β1i (LMP2) in a human cell line revealed that proteasomes are distributed in both nucleus and cytoplasm, though they are excluded from nucleolus and ER lumen. These experiments demonstrated that preformed proteasomes move from cytosol to nucleus in a slow and unidirectional manner. It is also possible that proteasomes from nucleus and cytosol mix after the breakdown of the nuclear envelope after mitosis (Reits *et al* 1997). In *S. cerevisiae*, proteasomes are mainly localized in the nuclear membrane-ER network (Enenkel *et al* 1998). However, localization of proteasomes at different times is dependent on the physiological state of the cell. In fission yeast *S. pombe*, proteasomes are localized at the nuclear periphery through out mitosis and is dispersed in the nucleus during the first meiotic division. However, proteasomes are observed at the interface between the two nuclei during the second meiotic division (Wilkinson *et al* 1998). Proteasomes are responsible for protein degradation in the nucleus. Some of the α subunits harbour nuclear localization signals (NLS), which probably aid in nuclear targeting of proteasomes. In fact, *T. acidophilum* proteasomes when expressed in HeLa and 3T3 cells can translocate into nucleus (Wang *et al* 1997).

Although proteasomes are distributed throughout the cells, it is possible that they play important roles in selected proteolytic centers within cells, e.g. the centrosome, a perinuclear structure that plays important roles in organizing the mitotic spindle to separate chromosomes. Recent evidences demonstrate that cellular proteins targeted for degradation are delivered to the centrosome via the nocadozole sensitive microtubule mediated transport system. On treatment of cells with proteasomal inhibitors, an accumulation of protein aggregates, known as aggresomes, consisting of chaperones, ubiquitinated proteins, together with components of the 26S proteasomes occurs. The assembly of aggresomes serves as a site for recruitment and concentration of the unfolded protein response (Johnston *et al* 1998; Wigley *et al* 1999; Fabunmi *et al* 2000). The identification of the roles of centrosomes in cell division and protein degradation reinforces the close interrelationship between these two processes.

8. Additional modulators of proteasomes

In addition to binding to PA700, 20S proteasomes can associate in an ATP-independent manner with another activator, PA28 or the 11S regulator, to form complexes that enhance proteasomal activity. In fact, PA28 and PA700 can simultaneously bind a 20S proteasome molecule (Hendil *et al* 1998). There are two forms of PA28: it can exist as a heteroheptameric complex of 180–200 kDa, comprising of two IFNγ inducible subunits, α and β, or a homohepatmeric complex of PA28γ subunits, also known as Ki antigen (Rechsteiner and Hill 2005). Incubation of human proteasomes with PA28$\alpha\beta$ greatly enhances activity against all amino acids whereas modest increase in proteasomal activity against basic amino acids is observed on incubation with PA28γ (Harris *et al* 2001). Functional studies performed on PA28$\alpha\beta$ revealed the importance of C-terminus in the proteasome activation. PA28 undergoes carboxypeptidase B mediated inactivation (Ping *et al* 1993) and site directed mutagenesis studies revealed the importance of the C-terminal 14 amino acids of each PA28 subunit in activating the proteasome (Zhang *et al* 1998a). Furthermore, chimeric PA28α containing the distal C-terminal residues of PA28β is more efficient at activating proteasomes compared to PA28α containing the distal C terminal residues of PA28γ (Li *et al* 2000). The crystal structure of PA28 revealed a narrow pore in the middle of the heptameric ring and suggested that it induces changes in the conformation of proteasomes facilitating access for substrates into the active site channel (Knowlton *et al* 1997; Forster *et al* 2005). Further, structure based mutations revealed the importance of the loop at the base of the PA28 in activating proteasome. A single mutation of Asn146 in the PA28α loop interferes in activation of proteasomes although binding to proteasomes is not affected. Mutation in the corresponding aspargines of PA28β

(N135Y) and PA28γ (N151Y) also display similar inactivation (Zhang *et al* 1998b). PA28 modulates the proteasomal cleavage of proteins, e.g. cytomegalovirus pp89, to release peptides that can bind MHC class I molecules (Dick *et al* 1996). Both the subunits of PA28 are distributed only in organisms with adaptive immune response and are uniformly localized in the cytosol and nucleus of the cell. In fact, mice lacking both subunits of PA28 display 15–25% reduction in ATP-dependent protein degradation suggesting the importance of complexes harbouring PA28 in proteolytic function. It is possible that PA28 is important in processing of some antigens; processing of an epitope from tyrosinase related protein, an antigen of B16 melanoma tumour, but not ovalbumin was severely defective in the *Pa28*$^{-/-}$ mice (Murata *et al* 2001). PA28$\alpha\beta$ is present mainly in the cytosol whereas PA28γ is predominantly localized in the nucleus (Soza *et al* 1997). PA28γ is a regulator of cell proliferation and mice lacking this subunit are smaller in size (Murata *et al* 1999). In addition, *Pa28*$\gamma^{-/-}$ mice display reduced numbers of $CD8^+$ T cells and are inefficient in processing some antigens (Barton *et al* 2004). Together, PA28 is involved in improving the antigen processing ability of proteasomes, by modulating the quantity and quality of peptides released.

Recently, another proteasome activator, PA200 was identified from bovine testis. PA200 adopts a solenoid structure, usually implicated in protein-protein interaction, suggesting that PA200 may act as an adaptor complex. PA200 stimulated the peptidase activities of proteasome by 2–10-fold, with higher folds of stimulations for hydrolysis after acidic residues. However, PA200 did not display any significant stimulation in hydrolysis of protein substrates. PA200 is localized primarily to the nucleus in HeLa cells under normal conditions, although upon IR irradiation PA200 localizes to certain nuclear foci. A mutation in yeast PA200 resulted in sensitivity to bleomycin, suggesting a probable role in DNA repair (Ustrell *et al* 2002). PA200 is induced under conditions of muscle wasting, although functional implications in mammals have not been delineated (Rechsteiner and Hill 2005).

In a search to detect native proteins that can modulate proteasome function, an inhibitor of ~ 31 kDa, PI31 (for proteasome inhibitor of 31 kDa), was identified along with PA28 and PA700 from bovine red blood cells (Chu-Ping *et al* 1992). Over-expressed mouse PI31 inhibits chymotryptic and tryptic activities, but has only marginal influence on the caspase-like activity of proteasomes. Further kinetic experiments revealed that PI31 acts as a competitive inhibitor of PA28 and PA700 and efficiently inhibits the degradation of large peptides (Zaiss *et al* 1999; McCutchen-Maloney *et al* 2000). PI31 hinders maturation of immunoproteasomes and processing of an epitope. Interestingly, induction of two MHC class I alleles H2-K^b as well as H2-D^b is also hampered in PI31 over-expressing cells (Zaiss *et al* 2002), which probably is the result of proteasome inhibition. It would be interesting to understand the regulation of PI31 in future and the conditions in which it associates with proteasomes, considering the fact that proteasomes are essential for survival in eukaryotes. Other proteinaceous proteasome inhibitors from various sources have also been described: δ-Aminolevulinic acid dehydratase, an enzyme involved in heme biosynthesis, is identical to a 240 kDa proteasome inhibitor (subunit size ~ 40 kDa) isolated from human blood (Guo *et al* 1994). Certain virus encoded proteins, e.g. HIV encoded Tat (Apcher *et al* 2003) and Hepatitis B virus encoded X protein (Hu *et al* 1999), also inhibit proteasome activity. Interestingly, aggregated proteins that occur in cells due to aging, oxidative stress or during disease progression, especially neuronal disease, etc. may be physiologically important as the binding of large amounts of aggregated protein to proteasomes results in formation of protein aggregates that are difficult to unfold and prevents the degradation of normal ubiquitinated proteins. As a result, high levels of aggregated proteins may act as dominant negative regulators of proteasome activity that may lead to cell death (Grune *et al* 2004). Finally, an antibacterial peptide PR39, which is rich in proline and arginine amino acids, binds to the α7 subunit, causes structural alterations and acts as a non-competative inhibitor of proteasome activity (Gaczynska *et al* 2003). This peptide reduces pancreatitis and myocardial infarctions in disease models by reducing NF-kB activation (Gao *et al* 2000).

9. Chemical inhibitors of proteasomes

Most protease inhibitors are peptide based ligand mimetics or transition state analogs of enzyme catalyzed reactions. They inhibit enzymes reversibly or irreversibly by formation of a covalent adduct between the inhibitor and the catalytic site of the enzyme. Inhibitors targeting proteasomal function (table 4) are attractive drug targets due to the importance of the UPS in numerous biological processes (Adams 2003; Groll and Huber 2004; Rajkumar *et al* 2005). The first discovered proteasome inhibitors were peptide aldehydes, e.g. N-acetyl-Leu-Leu-Norleucinal, also called Calpain inhibitor I. These compounds reversibly inhibit 20S proteasomes by modifying the catalytic hydroxyl group of threonine by forming a hemiacetal bond. They can rapidly dissociate from proteasomes and are rapidly oxidized into inactive acids. Calpain inhibitor I was found to primarily inhibit the chymotryptic-like activity of proteasomes and greatly reduce the rate of cytosolic protein degradation (Rock *et al* 1994). Peptide boronates are much more potent inhibitors of the proteasome compared to aldehydes and dissociate more slowly from the proteasome. The boronates also share the mode of inhibition with peptide aldehydes, by forming a tetrahedral adduct with the active site N-terminal threonine. Peptide vinyl sulfones are synthetic irreversible

Table 4. Modulators of proteasomal activity

1. Activators
   - PA 700
   - PA 28
   - PA 200
2. Inhibitors

A. Chemical Inhibitors

- Peptide aldehydes
  - MG132, PSI
- Peptide boronates
  - MG 262, PS341
- Peptide vinyl sulfones
  - NLVS, YLVS
- Peptide epoxyketones
  - Dihydroeponemycin
  - Epoximicin
- Lactacystin and derivatives
  - Lactacystin
  - clastolactacystin-β-lactone

B. Protein Inhibitors

- PI31
- PR39
- δ- aminolevulinic acid dehydratase
- HIV encoded Tat
- Hepatitis B virus encoded X protein
- Protein aggregates

inhibitors of proteasome that covalently modify the catalytic Thr1 present in all active β proteasome subunits. Lactacystin, the first natural proteasomal inhibitor identified, inhibits the chymotryptic activity to a great extent by covalently binding to the N-terminal Thr1 of the β5 proteasome subunit via an ester bond (Fenteany *et al* 1995; Groll *et al* 1997). Epoxymycin is probably the most selective as it does not inhibit any other cellular enzymes. Epoxymycin reacts with the catalytic N-terminal threonine of the proteasome to form an irreversible morpholino derivative (Groll *et al* 2000) and exhibits anti-tumour activity and anti-inflammatory activity *in vivo* (Meng *et al* 1999).

Numerous reports have demonstrated the anti-tumour ability of bortezomib (a boronic dipeptide; also known as velcade or PS–341), a reversible inhibitor of proteasomes. This compound has become the first proteasome inhibitor evaluated in human trials and has been approved to treat patients suffering from multiple myeloma (Rajkumar *et al* 2005). Proteasome inhibition can cause cellular apoptosis by modulating the levels of various short-lived proteins and inhibition of NF-kB activity. Consequently, one has to be cautious about the widespread use of proteasome inhibitors for therapeutic purposes. A smarter strategy may be to target different E3 ligases as they posses specialized functions. Each E3 ligase is specific for a small set of proteins; hence, inhibitors of E3 ligases can inhibit ubiquitination and 26S proteasome mediated degradation of specific cellular processes. Nutlin-3 is a *cis*-imidazoline analog, which binds to Mdm2, a Ring finger E3 ligase and modulator of tumour suppressor protein p53, and inhibits its association with p53. Consequently, high levels of p53 lead to apoptosis of cells in an experimental tumour model (Vassilev *et al* 2004).

10. Major roles of the UPS

The UPS play major roles in several biological processes and only key ones are listed below to reinforce its importance to the uninitiated reader.

(i) *Regulation of the cell cycle:* The levels of regulatory proteins (e.g. cyclin B, CDK inhibitor $p27^{kip1}$) are modulated at different phases of the cell cycle and the UPS is essential for cells to exit mitosis. The two major classes of E3 ligases that are involved in this process are the SCF complexes and the anaphase promoting complex/cyclosome. In general, SCF regulate entry into S phase and recognizes substrates post-phosphorylation. The anaphase promoting complex is important for sister chromatid separation, exit from mitosis and degrades cell cycle regulators containing a nine amino acid motif known as the destruction box (Murray 2004).
(ii) *Cancer and cell survival:* The tumour suppressor p53 plays a key role in regulating cell cycle arrest, DNA repair and apoptosis. Under normal conditions, p53 levels are low due to binding to Mdm2, an E3 ubiquitin ligase. After DNA damage, p53 is phosphorylated resulting in reduced interaction with Mdm2, and induction of cell death. Not surprisingly, mutations in p53 are often associated with different human cancers. Interestingly, the human papilloma virus encodes a E3 ligase (E6-AP) which degrades p53 and is involved in generation of cervical tumours (Ciechanover and Iwai 2004).
(iii) *Inflammatory responses:* NF-κB is a key transcription factor involved in the inflammatory response. NF-κB is bound to inhibitor-κB and is found in the cytosol. On appropriate stimulation, inhibitor-κB is phosphorylated and degraded by the UPS. Free NF-κB enters into the nucleus and induces the expression of several genes involved in the inflammatory response (Karin and Ben-Neriah 2000).
(iv) *Immune response:* MHC class I molecules present peptides to $CD8^+$ T cells. This process involves the digestion of self or microbial proteins into peptides by the UPS that are presented on MHC class I. Inhibition of UPS leads to the impairment of the biogenesis of MHC class I molecules (Kloetzel 2004).
(v) *Protein misfolding:* The UPS interacts with members of the heat shock family and cofactors to eliminate misfolded proteins. A direct relation between protein unfolding and degradation is via CHIP, an E3 ligase and a Hsc70 interacting protein (McDonough and Patterson 2003).
(vi) *ER associated degradation:* The UPS is also involved in the degradation of misfolded proteins in the ER which

involves retro-translocation of misfolded proteins from the ER to cytoplasm via the Sec61 translocon. Other components of this pathway also include a cytosolic N-glycanase, ATPases, e.g. Cdc48p/p97/valosin-containing protein, and associated cofactors (Kostova and Wolf 2003). A clinical manifestation of this is observed in patients suffering from cystic fibrosis where the mutant ΔF508 CFTR protein is retained exclusively in the ER and degraded by the UPS (Ward *et al* 1995). The role of chaperones and factors involved in ER-associated degradation is an active area of investigation.

(vii) *Disease progression:* Angelman syndrome is characterized by severe phenotypic defects including mental retardation, seizures and abnormal gait. Mutations in the E3 ligase, E6-AP cause Angelman syndrome, the first human disorder to be identified with a defect in the UPS (Kishino *et al* 1997). Another E3 ligase which contains the HECT domain, NEDD4, regulates the number of sodium channels on the cell surface. Mutations in *NEDD4* cause hypertension associated with hypokalemic metabolic alkalosis, low plasma renin activity, and suppressed aldosterone secretion, together termed as the Liddle syndrome (Staub *et al* 1997).

11. Summary and future directions

It has been a remarkable journey of the UPS from what was considered to be an esoteric post translation protein modification to appreciation of this pathway in different biological processes. Much has been learned in the past few decades about the genetics, biochemistry and structural aspects of components of the UPS; certainly more will be learned in the coming years. Not only is the UPS involved in the overall non-lysosomal degradation of cellular proteins but, more importantly, plays an important role in modulating cellular decision making. The UPS is able to perform this task, after receiving appropriate signals, by reducing levels of important proteins (e.g. cyclins) or partially degrading selective proteins, i.e. degrading of the C-terminal of a cohesion subunit which is important during chromosome segregation (Rao *et al* 2001). In fact, regulated protein degradation has challenged the predominant view that major cellular decisions are made at transcriptional and translational levels (Varshavsky 2005).

The roles of cellular enzymes involved in degradation of proteasomes needs to be understood. Inhibition of lysosomal enzymes revealed that proteasomes are present within the lumen of lysosomes and it is likely that they are degraded by lysosomal enzymes as a consequence of autophagy (Cuervo *et al* 1995). Interestingly, proteasomal activity is reduced in cells undergoing death because proteasome subunits are cleaved in a caspase-dependent manner (Adrain *et al* 2004; Sun *et al* 2004). This relationship between caspase activity and proteasomes is important because lowered proteasomal activity is often associated with cell death and further studies in this area will be important.

The UPS continues to remain attractive area of research with the human genome encoding multiple enzymes involved in ubiquitination and deubiquitination (Semple *et al* 2003). In addition, the 26S proteasome is composed of proteins encoded by at least 34 independent genes. With so many genes directly involved in the UPS, systematic inactivation of genes in mice may identify their physiological functions during different cellular processes; in addition, the challenge will be to decipher their cellular substrates. This is reinforced by the fact that enzymes involved in ubiquitination may be specific to different cellular processes; for e.g. E2–C is involved in cell cycle regulation (Hershko *et al* 1994), where as another E2 enzyme known as UbcM4 is involved in placental development (Harbers *et al* 1996).

In fact, there may be an underestimation of proteins involved in the UPS as some bind to PA700 in sub-stoichiometric amounts. Affinity-purified yeast proteasomes are associated with three proteins that are not observed in proteasomes purified by conventional purification methods, probably due to the high salt concentrations used in the latter procedures. It has been established that Ecm29, one of the associated proteins, is involved in the association of 20S proteasome and PA700. Hul5 and Ubp6 are the other two proteins associated with affinity-purified proteasomes. Ubp6 is responsible for hydrolysis of majority of the deubiquitinating activity associated with proteasomes (as detected by Ub-AMC hydrolysis) and is probably responsible for recycling of the proteasome-associated ubiquitin to the cellular pool (Leggett *et al* 2002). In fact, similar analysis of proteins associated with affinity purified proteasomes performed previously has also identified Ubp6, along with Hsp70 and Hsp82 and other regulatory complex proteins (Verma *et al* 2000). These proteasome associated proteins may play important and diverse roles. Yin6, a yeast homologue of Int6 (involved in breast cancer formation), has been identified to interact with proteasomal subunit Rpn5 and mediate the localization of proteasomes. *S. pombe* lacking yin6 has been demonstrated to harbour mislocalized proteasomes and thereby, improper degradation of proteins involved in mitotic regulation (Yen *et al* 2003). Recently, a proteasomal ATPase associated factor has been shown to interact with ATPases present in PA700 and inhibit proteasomal activity (Park *et al* 2005). Another interesting protein that interacts with proteasomes is Rad23, a component of nucleotide excision repair. Rad23 harbours a ubiquitin like domain, supports the formation of multiubiquitin chain and facilitates protein degradation. It has been demonstrated that Rad23 interacts with proteasomes via N-terminal domain and with DNA repair proteins via C-terminal domain (Schauber *et al* 1998). Also, the Cdc48/p97/valosin containing protein family of ATPases that unfold proteins are involved in docking ubiquitinated proteins to 26S proteasomes (Elsasser and Finley 2005). The identification of 26S proteasome-interacting

proteins will be of increasing importance in the future as they may shed light on how the UPS recruits additional proteins to modulate specific cellular responses. Further information on such types of protein interactions may lead to the generation of small molecule drugs that inhibit specific interactions that may ameliorate UPS-associated processes and diseases.

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