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review

Programmed Proteolysis by ATP-dependent Protease Complexes*

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Summary

Programmed intracellular degradation of proteins is essential for cell survival. In eukaryotic cells, there are two major pathways: (i) Proteolysis of endocytosed proteins such as membrane receptors or extracellular proteins is largely confined to the lysosomal/vacuolar system. (ii) Energy-dependent proteolysis by proteasomes is the major site for the degradation of cellular proteins and plays an important role in many aspects of cellular regulation. Proteasome activity is intimately coupled to the ubiquitin system. More simple proteasomes and proteasome-like complexes have been discovered also in archebacteria and eubacteria. In mitochondria and chloroplasts, a significant part of programmed proteolysis for 'quality control' is achieved by membrane-bound protease complexes. Remarkably, the ATPases in all of these complexes have been found to be members of the ATPase associated activities (AAA) family, which can be considered a specialized subfamily of the ATPase superfamily. This review focuses on the recent advancement of our understanding of the control mechanisms underlying the functions of proteasomes and proteasome-like complexes. Additional systems structurally and functionally related to these complexes are considered briefly.

Key words: proteasomes, energy-dependent proteolysis, ATPases associated activities (AAA) family, proteasome-like complexes

Introduction

Among cellular regulatory mechanisms, programmed proteolysis has received increasing attention. Degradation not only serves to remove unassembled or misfolded proteins but, more importantly, to regulate important cellular processes. For instance, it is needed for cells to adapt to environmental changes or in the control of time-dependent cellular programmes. Proteolysis is advantageous over other control mechanisms: it is fast and therefore enables the cell to efficiently reduce the levels of distinct components; it is irreversible, thus guaranteeing complete loss of function of proteins. However, to prevent unspecific degradation, proteolysis has to be highly selective and must be controlled by superior circuits. Two major types of remarkably sophisticated proteolytic systems have been shown to exist: *(i)* proteasomes that have been found in all eukaryotic cells and in more simple forms in archea and some eubacteria, *(ii)* several protease complexes that occur in prokaryotes and mitochondria and can be considered proteasome-like structures. In the following, I will focus on those proteolytic complexes that contain subunits belonging to a novel protein family, the AAA family (for family of ATPases Associated Activities).

The AAA Family

The AAA proteins are a fast growing family of ATPases, which are characterized by a highly conserved domain of about 230 amino acids, present in one or two copies *(1)*. Each of these domains comprise well-conserved, but specialized Walker A and B motifs for ATP binding. In the various members of the family, the ATPase modules are flanked by other domains, suggesting that in evolutionary terms they have been generated by lin-

* Dedicated to Professor Pavao Mildner for his 80th birdthday

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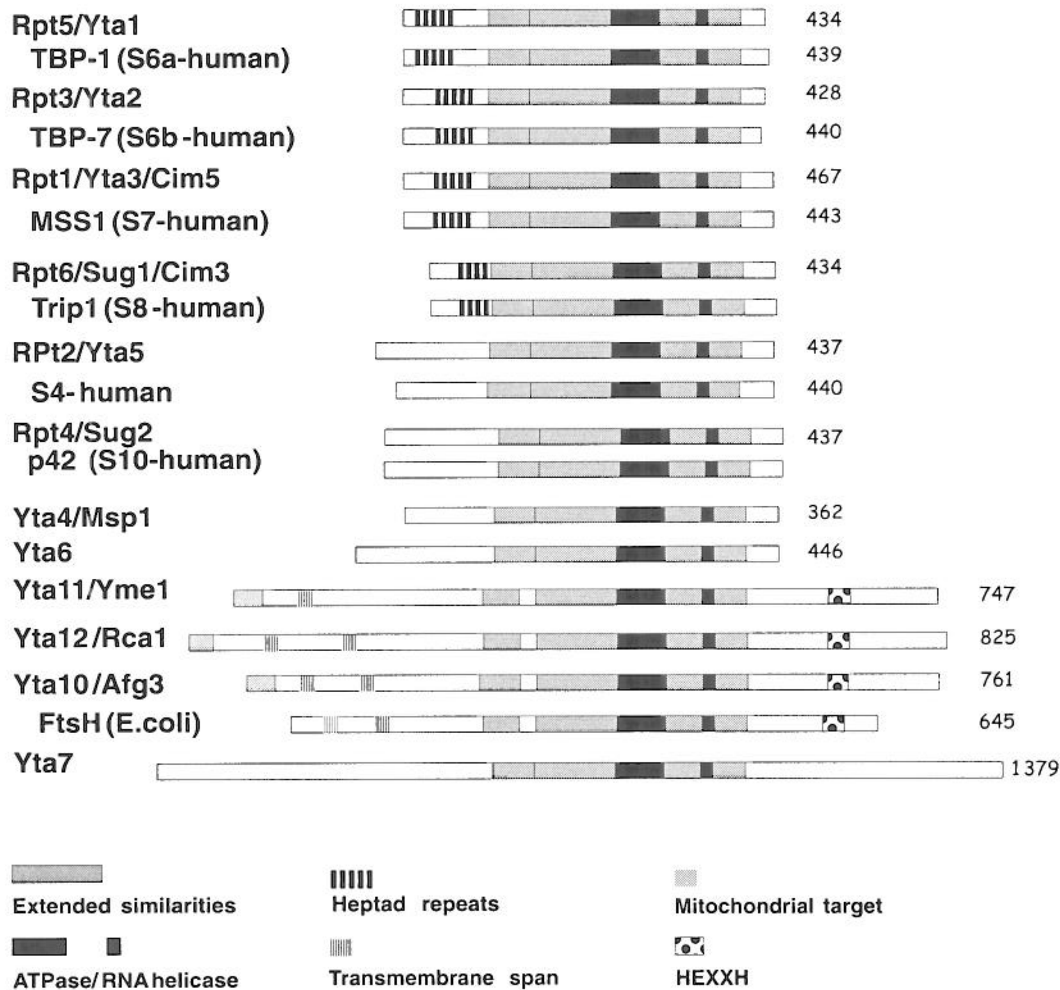

Fig. 1. Schematic representation of members of the yeast AAA family containing one ATPase module and some of their orthologues

king separate functional entities to form mosaic molecules. To date, more than 200 members of this family have been identified in eubacteria, archaebacteria and eukaryotes; a complete list can be found at web address http://yeamob.pci.chemie.uni-tuebingen.de/. Twenty four AAA proteins have been found in connection with the genome project of the yeast, *Saccharomyces cerevisiae* *(2,3)* (Fig. 1). Members of this family are involved in a variety of cellular processes, including membrane fusion, organellar assembly, cell cycle control and protein degradation. Despite the diversity of cellular activities, the structural conservation of the ATPase module points to a basic biochemical role of the AAA proteins, which includes a chaperone-like function in the dissociation and assembly of protein complexes.

Based on the initial functional characterization of AAA proteins, at least four subtypes can be distinguished: *(i)* Various AAA proteins, typically characterized by the presence of two AAA modules, have been demonstrated to be substantial factors for membrane fusion processes such as Sec18p/NSF *(4)* and Cdc48p/p97 *(5)*, or the PEX proteins *(6)* involved in peroxisome biogenesis. Cdc48p even exhibits multiple functions, it is involved in stress response connected to the ubiquitin pathway *(7)* and it mediates cell cycle apoptosis *(8)*. *(ii)* As detailed below, six closely related AAA proteins are subunits of the regulatory complex of the 26S proteasome *(9)*, there fullfilling key functions in the assembly of the subunits and the unfolding and transport of substrate polypeptides into the cavity of the 20S moiety of the proteasome. Typically, in most of these proteins the ATPase module is preceded by a coiled-coil domain. *(iii)* Membrane-bound AAA proteins (mAAA proteins) with metal-dependent peptidase activity (metallo-proteases) have been identified in several prokaryotes, in mitochondria (reviews: *10,11*), and in chloroplasts *(12)*. *(iv)* Homotypic AAA proteins whose cellular activities are presently only ill-defined comprise factors involved in endosomal traficking such as Vps4p *(13)* and a large variety of other factors.

The Ubiquitin-proteasome System

General features

The ubiquitin-proteasome system (Fig. 2) plays key regulatory roles in numerous aspects of cellular regulation, including metabolic or environmental adaptation, cell differentiation, cell-cycle progression, signal trans-

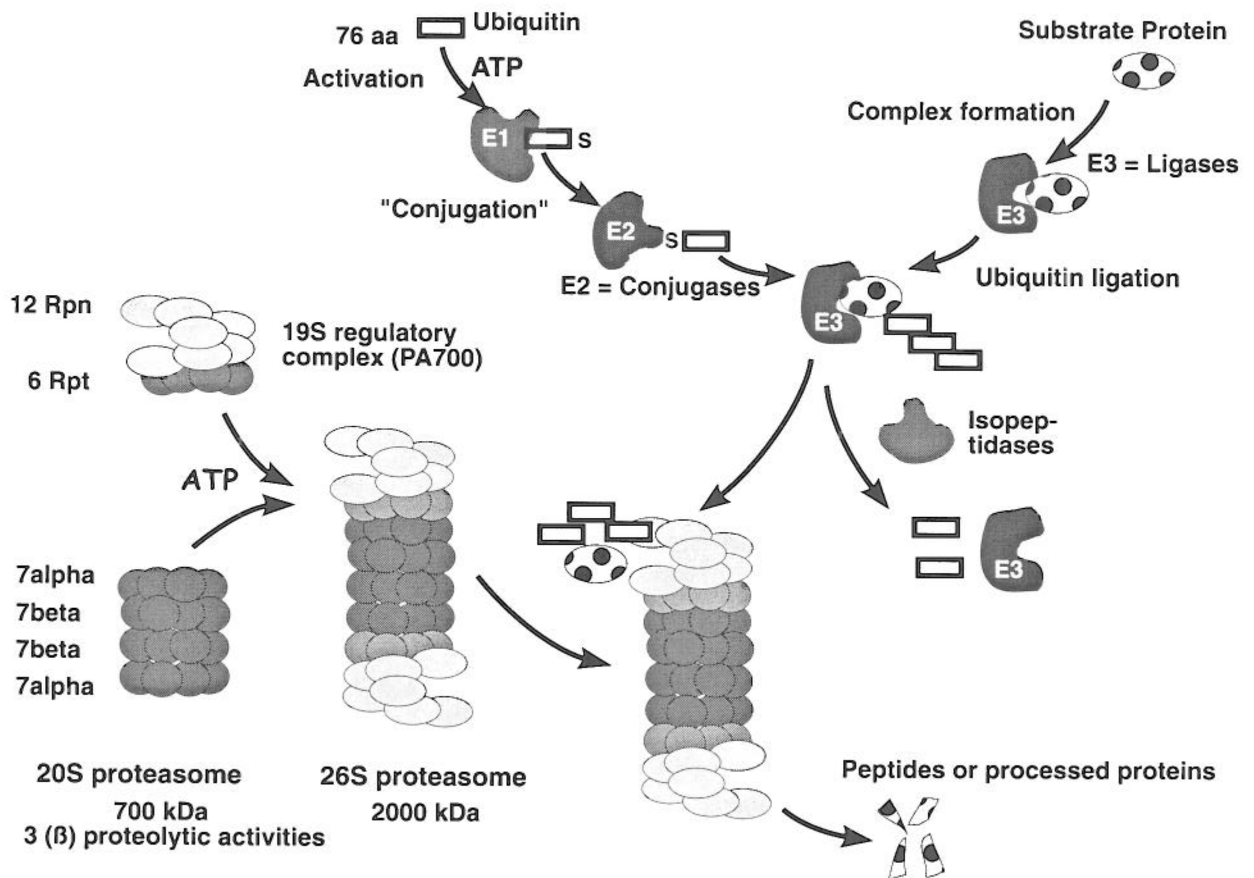

Fig. 2. The ubiquitin-proteasome pathway

duction, transcriptional regulation, receptor-down regulation, and endocytosis; it has also been implicated in the immune response, developmental processes, and apoptosis (reviews: *14–18*). Proteolysis has more complexity than was thought initially. There is growing evidence that although most of the proteins removed or processed by the 26S proteasome are tagged by ubiquitination, alternate pathways exist which on the one hand allow flexibility and on the other hand ensure selectivity at various levels of proteolysis. Additionally, isoforms of proteasomes and regulatory complexes that modulate proteasome activity for specialized functions have been discovered.

Targeting of proteins by covalent ligation to ubiquitin, a 76-amino-acid-residue protein, requires the sequential action of three enzymes: *(i)* the C-terminus of ubiquitin provides high selectivity for proteolysis *(16)*. Ubiquitin-protein ligation is activated in an ATP-dependent step by a specific activating enzyme (E1) to yield an ubiquitin-E1-thiolester. *(ii)* Activated ubiquitin is transferred to an ubiquitin-carrier protein or 'conjugase', E2. *(iii)* By a ubiquitin-protein ligase or E3 enzyme, ubiquitin is linked by its C-terminus in an isopeptide bond to a lysine residue of the substrate. After linkage of ubiquitin to the substrate protein, a polyubiquitin chain is usually formed, and these conjugates are finally recognized and processed by the 26S proteasome, which also contains isopeptidase activity (Fig. 2). Importantly, many E2s and multiple families of E3s or E3 multiprotein complexes exist, and mainly specific E3s appear to be responsible for the selectivity of proteolysis.

The 26S proteasome, a multisubunit enzyme complex (relative molecular mass ~2000 kDa) forming a dumbbell-shaped structure, appears to be present in all eukaryotic cells. Its subunit composition has been found to be highly conserved among species *(14,15,17)*. cDNAs or genes encoding almost all subunits of human and the budding yeast proteasomes have been isolated and characterized by molecular-biological techniques. Orthologues have been detected in all those organisms from which appropriate sequence data became available. 26S proteasomes occur in the cytosol as well as in the nucleus; they have been isolated from and functionally studied in a multitude of eukaryotes.

The subunits of the 26 proteasome are distributed between two subassemblies, the core particle (20S particle or CP) and the regulatory particle (19S particle, PA700, or RP). The core particle houses the proteolytic activities of the proteasome, while the regulatory particle confers ATP-dependence and specificity for ubiquitin-protein conjugates. The postulated mechanism of the proteasome is novel in that, after an initial encounter with the regulatory particle, the proteolytic substrate is thought to be unfolded and threaded into an internal compartment of the core particle for hydrolysis *(19)*.

The 20S complex is a barrell-like structure composed of two times two ring-shaped layers, in eukaryotes each comprising seven different but structurally related α-type and seven β-type subunits housing the proteolytic activities of the proteasome. These subunits are synthesized as precursors and have to be processed during assembly of the 20S particle. It should be noted that the occurrence of 20S complexes is not restricted to eukaryotes. In archebacteria (e.g. *Methanococcus jannaschii*), the 20S proteasome is complexed with a close relative of the 19S cap ATPases, which activates the 20s protease for programmed proteolysis, and in several actinomycetes this function appears to be exerted by a more distantly related ATPase of the AAA family *(20)*. Further, in prokaryotes there are only two different but related subunits, α and β. However, X-ray structures of the 20S proteasomes from *Thermoplasma acidophilum (21)* and yeast *(22)* have confirmed the high structural similarity of the particles from different organisms and permitted insights into the mechanism of proteasomal proteolysis.

The six Rpt proteins are believed to form a ring at the interface between the 19S cap complex and the 20S core. Structural details on the architecture of the 19S complex have still to be worked out. Though many of the 12 or so 'non-ATPase' subunits have been shown to represent homologues in different organisms, there might be slight variations in subunit composition.

In mammals, a further cap complex, 11S or PA28, has been characterized (e.g.,*14*). It consists of α and β (and probably γ) subunits that form a ring-shaped particle which can associate with the 20S core particle at both ends. The formation of the 11S-20S complex is induced by γ-interferon, triggers the exchange of three particular subunits in the 20S core particle, and dramatically enhances its proteolytic capacity. The modified 20S core then associates with 19S caps to constitute the so--called 'immunoproteasomes' which are responsible for antigen processing (see below).

Controlled degradation of regulatory proteins through the ubiquitin-proteasome system

As indicated above, proteasomes are involved in regulating a multitude of key cellular processes. Even basal cellular activities have been shown to be controlled by programmed proteolysis. For example, some histones become ubiquitinated and subject to degradation. The activity of topoisomerase type I is controlled by ubiquitination as is the activity of RNA polymerase II. If the CTD tail of the largest subunit of RNA pol II remains unphosphorylated, this subunit is degraded thus preventing transcription. In the following, I will briefly outline some aspects of controlled degradation of regulatory proteins. Details can be found in recent review articles cited below.

Cell cycle control factors. The eukaryotic cell cycle is driven by oscillations in the activities of cyclin-dependent kinases (Cdks), which are controlled by periodic synthesis and degradation of positive regulatory factors, the cyclins, as well as by fluctuations of the levels of negative factors, the Cdk inhibitors (Ckis) and by reversible phosphorylation *(23)*. Cdks are activated at distinct intervals during the cell cycle by association with cyclins specific for the G1, S, or M-phases and inactivated through selective proteolysis of these cyclins by the proteasome. Notably, the single cyclins are recognized for ubiquitination by specific E2-E3 systems. Certain cyclin/Cdk complexes can only act when the inhibitor is removed by selective proteolysis. For example, Sic1p from yeast inhibits the activity of Cdk1 associated with mitotic B-type cyclins but not that of Cdk1/G1-cyclin. Rapid degradation of Sic1p at the G1 to S-phase transition thus permits the activation of S-phase promoting B-type cyclins to trigger DNA replication. The degradation of Sic1p is mediated by phosphorylation and a number of regulatory genes. Similar Cki systems have been described in *Sacch. pombe* and mammalian cells.

The machinery that degrades mitotic cyclins is also involved in programmed proteolysis of other cell cycle regulators, such as the anaphase inhibitors, which may act as 'molecular clamps' that glue sister chromatids together and are degraded to allow disassembly of the mitotic spindle at the end of the anaphase.

P53, the 'guardian of the cell cycle' *(24)*, is a relatively short-lived protein. Upon stress-induced DNA damage, p53 is transiently stabilized to act as a transcriptional activator for appropriate target genes, to induce cell cycle arrest, and to exert its apoptotic functions (Fig. 3). During this interval, the level of p53 is elevated. Concomitantly, the transcription of the Mdm2 oncoprotein is increased in an autoregulatory feedback loop. It has recently been proved that Mdm2 is the p53 specific ubiquitin ligase that targets p53 for degradation by the proteasome. Thus the interval between p53 stabilization and proteolysis induced by elevated levels of Mdm2 provides a time window for p53 to fulfil its repair activities and allowing subsequent cell cycle progression. Remarkably, p53 degradation is accelerated by the human papilloma virus (HPV) E6 protein, which interacts with a cognate E3 enzyme, E6-AP, to achieve rapid ubiquitination of p53. Thus, infection of cells with HPV allows cell proliferation and prevents apoptosis.

The transcription factors of the E2F and E2A families play an important role in regulating cell-cycle progression at the G1/S-transition, E2F-1 being the best studied example *(25)*. Among multiple control mechanisms, E2F-1 is degraded by the ubiquitin-proteasome system but ubiquitination is prevented if E2F-1 is bound to the retinoblastom tumor suppressor protein (Rb). As only the dephosphorylated form of Rb can bind E2F-1 and multiple phosphorylated forms of Rb exist, phosphorylation of Rb by Cdks/cyclins is used as a superior control mechanism. Interestingly, the degradation of E2F-1 is enhanced by the HPV oncoprotein E7.

Transcription regulators. Transcription factors that are rapidly degraded in mammalian cells by the ubiquitin-proteasome system include c-Jun and c-Fos, but not their transforming counterparts, v-Jun or v-Fos. The degradation of mammalian c-Jun is induced by mitogen-activated kinases. Long-standing examples in yeast are the mating type regulator MATalpha2 and Gcn4p, an orthologue of c-Jun. If hypoglycosylated, SP1, a transcription factor necessary for the expression of TATA--less genes is degraded by the proteasome. HIF-1, the hypoxia-inducible factor complex that stimulates ery-

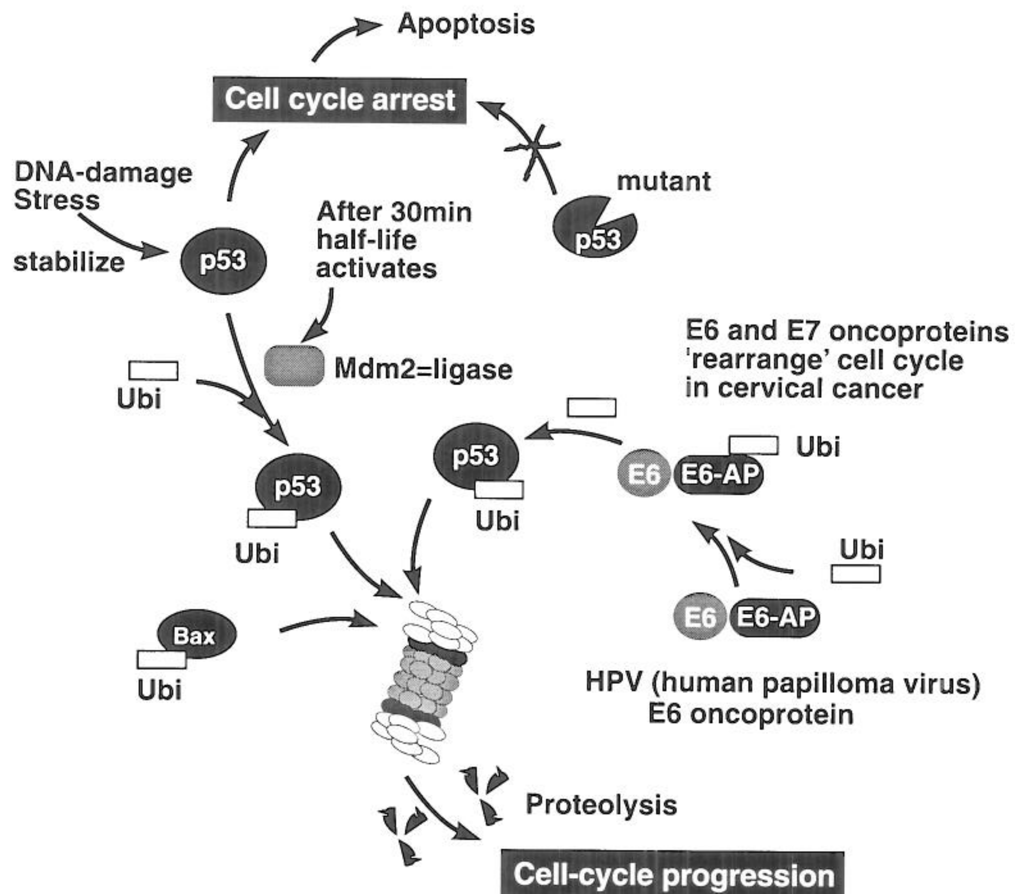

Fig. 3. Programmed proteolysis of p53 is involved in cell cycle regulation

thropoietin synthesis is composed of two subunits (HIFα and HIFβ). Under normoxic conditions, this complex is inactivated by proteolysis of HIFα.

Particularly challenging cases are the various members of the NF-κB family, inducible transcription factors involved in central immune, inflammatory, stress and developmental processes. They are composed of two subunits (e.g., p50 and p65 in NF-κB1; p52 and p65 in NF-κB2), the smaller of which are the processed products of larger precursor proteins (p105 and p100, respectively). Both proteolytic cleavage at a particular site and degradation of the C-terminal part are carried out by the proteasome *(e.g., 26)*. The processed factor is masked by the association with inhibitors (IκBs) and retained in the cytosol of non-stimulated cells. Only upon extracellular stimuli through cytokines, viral or bacterial components, or stress, the inhibitors are degraded by the proteasome, and active NF-κB heterodimers are imported into the nucleus.

Developmental factors. In *Drosophila*, several factors involved in signal transduction and differentiation have been reported to be subject to programmed degradation, such as *notch*, determining the body axes, or *tramtrack*, mediating the development of photoreceptor cells. Homologous systems have been described in mammals. A most interesting case is MyoD, a basic helix-loop-helix transcription factor involved in the activation of genes encoding skeletal muscle-specific proteins. Independent of its ability to transactivate muscle-specific genes, MyoD can also act as a cell cycle inhibitor. Recent studies have demonstrated that hyperphosphorylated MyoD is targeted for rapid degradation by the ubiquitin-proteasome pathway. Additionally, the 26S proteasome has been implicated in the bulk turn-over of cellular proteins during apoptosis, particularly in muscular dystrophies. A most interesting aspect is the participation of the ubiquitin-proteasome system in the cleavage of components involved in Alzheimer's disease: the amyloid β-protein (AP2) and the presenilin-2 membrane protein (PS2). Further, the switch from short to long-term facilitation in synapses is mediated by proteasomal degradation of inhibitors of the 'long term' pathway. Even in plant development, the proteasome has an important role in that its activity is controlled by light thereby regulating the circadian rhythm *(27)*.

Membrane receptors and hormonal control. While most cell surface receptors appear to be transferred to the lysosome/vacuole for degradation, several cases have been reported in which ubiquitinylated membrane proteins are targeted to the proteasome. These include the platelet-derived growth factor receptor (PDGF-R), the growth hormone receptor (GHR), and at least one subunit of the T-cell receptor (TCR-ξ), and the hepatocyte growth factor/scatter factor (HGF/SF). The insulin-degrading enzyme (IDE) has been found to be directly

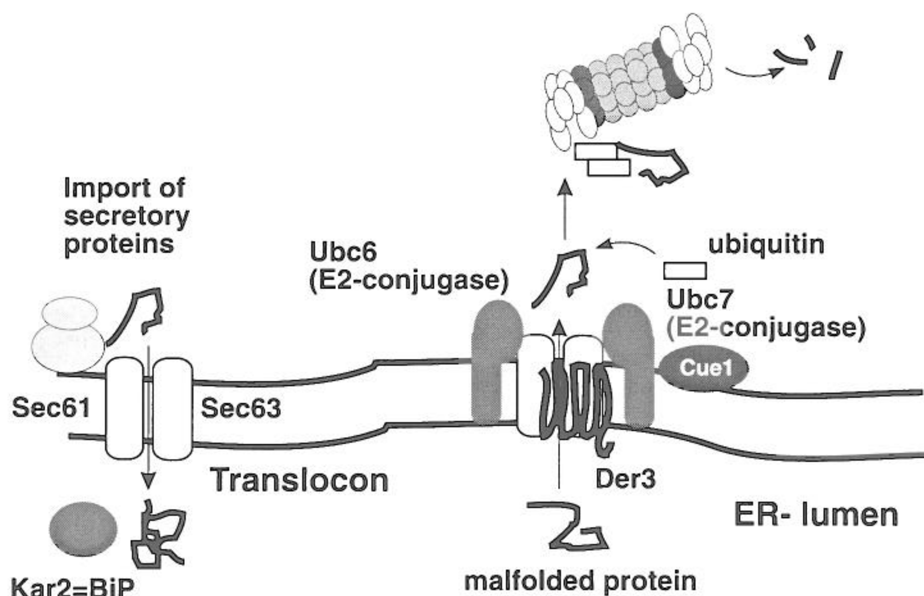

Fig. 4. Retrograde protein transport in the endoplasmic reticulum is coupled to proteolysis for quality control. The yeast system is presented as an example

coupled to the proteasome. Unclear cases are the IgE receptor, the prolactin receptor, and the EGF receptor. Recently, it has been demonstrated that angiotensin II down-regulates inositol trisphosphate receptors *via* degradation by the proteasome.

Programmed proteolysis coupled to retrograde protein transport. The existence of protein degradation associated with the endoplasmic reticulum (ER) has been apparent for some time. Recently, however, detailed facets of this process have been clarified from studies in yeast and mammalian systems. The vast majority of secreted proteins are synthesized by cytosolic ribosomes and translocated into the lumen of the ER, where they undergo maturation, such as posttranslational modifications and correct folding. Only active or properly assembled proteins are released. This is achieved by coupling retrograde transport to a 'quality control' system (including, for example, a membrane-bound ubiquitin-conjugating enzyme in yeast) which targets immature, misfolded or abnormal products for proteolysis by cytosolic proteasomes (Fig. 4). Importantly, this process has been shown to be highly selective for specific proteins. To date, at least 25 eukaryotic proteins can be considered to be substrates in this type of control *(28–30)*.

Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) that facilitates chloride transport in epithelial cells cause the human genetic disease cystic fibrosis. The resulting abnormal or immature forms of CFTR are rapidly degraded by the proteasome *(31)*. Similar observations hold for immature products of at least one (TCR-α) of the integral membrane subunits of the T-cell receptor that assemble in the ER before delivery. Ste6p, the yeast α-pheromone receptor, and GHR, the growth hormone receptor bound to its ligand, are two further examples where assembly is controlled by this pathway.

One ER-resident enzyme whose activity has been shown to be controlled indirectly by specific proteolysis is HMG-CoA reductase *(32)*. Upon cholesterol depletion, the sterol receptor element binding protein (SREBP), which itself is an ER-integral protein and acts as a 'sensor' for the intracellular level of cholesterol, is proteolytically cleaved to yield a transcription factor that binds to the serum response element (SRE) of two important target genes, those for HMG-CoA reductase and the LDL receptor. This ensures transient endogenous synthesis of sufficient amounts of cholesterol and augments its import from LDL. The process is reversed by proteasomal degradation of the transcription factor. Interestingly, also the production of LDL is controlled indirectly by proteolysis: *De novo* synthesis and subsequent secretion of this lipoprotein affords import of apoB100 into the ER-lumen and the availability of sufficient amounts of triglycerides and cholesterol in this compartment. Insufficient amounts of triglycerides prevent translocation of apoB100 which is then degraded by the proteasome *(33)*.

Immunoproteasomes. It is now clearly established that in higher vertebrates the proteasome is also involved in antigen processing generating peptides which are presented by MHC class I molecules to establish distinction between self and non-self. This process is controlled by cytokines (β- and γ-IFN) and TNFα which mediate the alteration of proteasome subunit composition *via* the 11S complex *(34)*. Three β-subunits of the 20S core are replaced by LMP2, LMP7, and MECL1, respectively, which most likely alter peptidase activity. The genes for these subunits reside within the MHC locus.

Selective inhibitors of proteasome activity. Inhibitors that can efficiently block proteasome function in intact cells have significantly contributed to an understanding of its physiological roles *(35,14)*. For example, chymostatin and acetyl-leu-leu-norleucinal reversibly inhibit the chymotryptic-like activity, while leupeptin inhibits the tryptic-like activity of the 20S core. Various compounds of this type have been synthesized. A more potent inhibitor is lactacystin, a natural compound pro-

duced by actinomycetes, which blocks both the chymotryptic- and tryptic-like peptidase moieties. The availability of specific proteasome inhibitors that do not affect other known proteases has raised great interest in developing drugs that could be of use for medical or industrial applications (see next section).

Cellular distribution of proteasomes and regulation of proteasome activity. Proteolysis in specific compartments of the cell, such as the nucleus or the nuclear envelope-ER network, may be regulated by changes in cellular distribution of the proteasomes, accompanied by changes in subunit composition *(36–38)*. Dramatic changes in proteasome content have been encountered during development, stimulated by mitogens, or during various pathological states owing to accelerated proteolysis. In mammals, skeletal muscles undergo rapid weight-loss as a consequence of various pathological constraints, including fasting, denervation atrophy, acidosis, cancer cachexia, severe infections or sepsis. Probably, increased levels of proteasomes (and components of the ubiquitin pathway) are mediated by changes in the levels of the corresponding mRNAs *(35,39)*. However, in mammals the mechanisms coordinating their expression are unclear, and our understanding as to the regulation of cellular content and intracellular distribution of the proteasomes is limited. None the less, proteasome inhibitors may turn out of importance for medical applications, for example, in protecting against ischemic injury, in organ transplantation, or in stimulating anti-inflammatory effects.

The yeast 26S proteasome as a model system

The yeast, *Saccharomyces cerevisiae*, has proved a powerful model system to study programmed proteolysis *in vivo (40)*. To date, 32 proteasomal subunits have been identified in this organism. Seventeen RP subunits have recently been characterized from purified yeast proteasomes *(9, 41)*, of which the six Rpt proteins belong to the AAA family. Together with their counterparts in other eukaryotes, the yeast Rpt proteins constitute a well-defined AAA subfamily: the primary structures of these subunits show high inter-subunit homology and high evolutional conservation among species. This is underlined by the possibility that particular Rpt proteins in yeast can be functionally replaced, for example, by their human counterparts *(42)*. However, each of the individual Rpt subunits is essential for proteasome function and cannot be replaced by another subunit of this set *(43)*. The remainder of the RP subunits (Rpn proteins) form a heterogeneous group. The important role of the 26S proteolysis system is underlined by the fact that in yeast most of the genes for its constituents have been shown to be essential genes. With one exception, individual chromosomal deletions of each of the 14 known yeast 20S proteasome genes are lethal (e.g., *15*), as is chromosomal deletion of most of the 19S cap genes.

Structural and functional aspects of the 20S core particle in yeast have been thouroughly studied *(18)*. Conceptions as to the structural and functional organization of the 19S particle, the molecular interplay between its constituting subunits, and the molecular interactions underlying the recognition of ubiquitinylated substrates to be attacked by the 26S proteasome are evolving rapidly *(43,44)*. Assembly of 26S proteasomes as well as their distribution between different cellular compartments appear to be dynamic processes thus resulting in distinct proteolytic complexes that are designed for specific functions. For example, the subcellular distribution of proteasomes implicates a major location of protein degradation in the nuclear envelope-ER network in yeast *(28–30,38)*. Alternate pathways involving ubiquitin-like proteins also seem to be connected to the ubiquitin-proteasome system *(45)*. Similarly, intrinsic ubiquitin-like moieties, as for example present in Rad23p, can serve in linking DNA repair to the ubiquitin-proteasome pathway *(46)*. Particular components of the 26S protease, such as Rpt6p, may have multiple functions. Rpt6p acts as a DNA-helicase *(47)* and the mammalian Rpt6p orthologue has been found to be a mediator of transcription activated by nuclear receptors *(48)*.

Given the high similarity among the six Rpt subunits and their essentiality in the assembly of the RP particle, one can wonder, whether the expression of the corresponding genes occurs independently or might be subject to coordinate control. A first indication for the latter possibility was our recent finding (unpublished) that the promoter regions of the *RPT* genes share a unique nonamer sequence motif (5'-GGTGGCAAA-3' or its complement) suggesting that this might function as a common *cis*-regulatory signal through interaction with specific DNA-binding factor(s). To address the functional significance of this sequence motif, we carried out promoter analyses to show that, in fact, it serves as a control element in the expression of selected *RPT* genes. We were able to demonstrate that Rpn4p, a protein previously found to be associated with the 26S proteasome *(49)*, binds to the nonamer sequence *in vitro* and *in vivo* and acts as a transcriptional activator. Interestingly, the element is found in the promoters of 26 of the 32 proteasomal genes implying that these subunits are subject to coordinate regulation at the transcriptional level. Therefore, we named the sequence element PACE (for proteasome associated control element).

Further, PACE is found in the promoter regions of 40 additional genes that can be supposed to be involved in the ubiquitin-proteasome pathway, thus forming a regulatory network. Most intriguing in this respect are the genes for the polyubiquitin precursor (Ubi4p), the ubiquitin activating enzyme Uba1p, the ubiquitin ligase binding protein Bul1p, and Cdc48p, a member of the AAA family which appears to have multiple functions in cell division, homotypic membrane fusion, ubiquitin-mediated proteolysis and apoptosis, as mentioned before. Another group of genes involved in programmed proteolysis, like *PIM1* (mitochondrial Lon protease) and *CCT6* (member of the TRiC complex), or *TFP1* (vacuolar homing endonuclease) possess the PACE box, but remarkably, none of the promoters of the mitochondrial AAA proteases (see below). It is tempting to speculate that also several genes for factors involved in cell wall synthesis, mRNA stability, protein folding, and several transcription factors may be subject to control by PACE. In this context, it has to be mentioned that we obtained preliminary results for two genes from the above list: both *RPN5* and *CDC48* appear to be transcriptionally controlled by PACE/Rpn4p. A problem that remains to

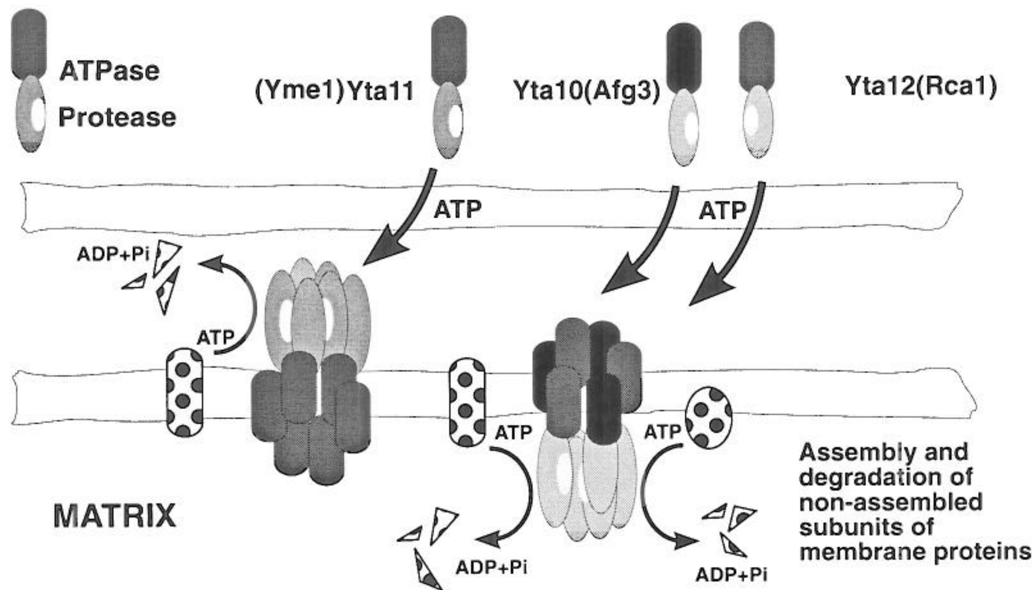

Fig. 5. Membrane-bound AAA protease complexes in yeast mitochondria. The intermembrane complex (i-AAA protease) is a hexamer composed of identical subunits, while the matrix-oriented complex (m-AAA protease) is made up of six heteromeric subunits

be solved is how and under which conditions Rpn4 activity itself may be regulated.

Taken together, the presence of PACE in most of the promoters for proteasomal and many other yeast genes together with the finding that Rpn4p is a PACE-binding factor suggests to us that we have uncovered a novel regulatory network involved in the coordinate control of programmed proteolysis and processes related to the ubiquitin-proteasome pathway in yeast.

Proteasome-like Particles

For a long time, proteasomes were believed to exist exclusively in eukaryotes, in certain archebacteria and actinomycetes. Recently, however, a proteasome-like particle, HslVU (ClpQY), has been discovered in *E. coli* *(35,50,51)*. HslVU is a two-component ATP-dependent protease composed of the proteasome-related peptidase HslV and the ATPase HslU. (The genes were found in a new heat shock operon why they were named **h**eat **s**hock **l**oci). The assembly of the active HslVU complex as well as the cleavage of small peptides and proteins require ATP. Though the overall architecture and the function of HslVU resemble those of the 26S proteasome, there are important structural differences. Both the HslV and the HslU subcomplexes, are ring-shaped particles composed of six homomeric subunits each, which form a cylindrical four-ring structure ($U_6V_6V_6U_6$). HslU reveals only moderate homology to the Rpt proteins but is 50 % homologous in primary structure to ATPases of the Hsp/Clp family such as ClpA or ClpX (see below). Most likely, HslU helps to unfold or disaggregate abnormal or heat-damaged proteins thereby assisting transfer of the substrate in an extended conformation into the proteolytic chamber.

Prokaryotic and Mitochondrial Metallo-proteases

The first member of this particular subgroup of the AAA family, FtsH (HflB), has been characterized in *E. coli (52,53)*, and meanwhile orthologues in many prokaryotes have been identified. FtsH is an integral membrane protein, with an essential Zn^{++}-binding region, constituted by the HEXXH motif. FtsH participates in several cellular processes including protein assembly into the membrane, protein export, programmed degradation of the heat shock factor σ32 and the transcriptional activator CII of phage lambda (λ CII). In *B. subtilis*, the FtsH homologue is involved in the regulation of sporulation. ATPase activity and Zn^{++}-binding as well as the membrane integrity of FtsH are essential for function *(54)*.

Eukaryotic organelles have a number of AAA metalloproteases, some of which are related to FtsH. Chloroplastic FtsH in plants is localized to the thylakoid membrane, its expression is dependent on light, and it is responsible for degrading unassembled proteins.

In yeast mitochondria, three members of the AAA family have been identified to constitute ATP-dependent metallopeptidases *(55–57)*, the subunits being organized in hexameric complexes. These structures can be viewed as kind of 'mini-proteasomes', in which the genetic entities for ATP-binding and protease activity have been fused. Both the matrix AAA protease (m-AAA) and the intermembrane AAA protease (i-AAA) are found integrated into the inner mitochondrial membrane but exposing their catalytic sites to opposite membrane surfaces (Fig. 5). Both complexes are essential for respiratory competence and co-operate in the programmed degradation of ('excess' or misfolded) inner membrane proteins. Additionally, the m-AAA protease has revealed a chaperone-like activity during the assembly of the mitochondrial respiratory and F_oF_1-ATP synthase complexes. These functions, which are coupled to ATPase and pro-

tease activities, represent a quality control system during membrane translocation of proteins and in the assembly of membrane-embedded protein complexes (review: *10*). A counterpart of Yta10p, named paraplegin, has recently been identified in human mitochondria *(58)*; mutations of this protease are the cause of the genetic disease paraplegia, leading to paralysis of both extremities and malfunctions in oxidative phosphorylation.

Other ATP-dependent Proteolytic Complexes

The ATPases of the AAA family can be viewed as a particular subfamily within the large superfamily of ATPases. In addition to proteasomes and proteasome-like particles, all cells enharbor various self-compartmentalizing proteolytic systems, which are related among each other and depend on regulatory ATPases (reviews: *11, 20, 59*). These systems include: *(i)* The Lon and Lon-like ATP-dependent proteases, which are functionally conserved from prokaryotes to eukaryotes and known to regulate gene expression and cell-cycle control; *(ii)* Ti proteases, which promote proteolysis through association of particular members of the Hsp100/Clp protein family of ATPases *(59)* with serine proteases of the ClpP type, were detected in any organism.

The Lon protease is a homo-oligomer of at least four subunits containing a central ATPase domain and a C-terminal protease domain. The coupling between the two domains appears to be as tight as seen in the mitochondrial AAA proteases. The ATPase domain belongs to the ATPase superfamily, and the protease is an unusual kind of serine protease, which is found in many bacterial proteins generally in association with an ATPase domain.

Energy-dependent Clp (Ti) proteases are composed of a proteolytic component, ClpP, and a regulatory ATPase, which can be either ClpA, ClpC or ClpX. Homologues of these proteins are found in most bacteria and organelles. Whereas in *E. coli* all three types of proteases occur, only two or one of the ATPase moieties occur in some other prokaryotes. The Cpl complexes assemble in an ATP-dependent manner, whereby the ATPase subunits activate ClpP for proteolytic activity and confer substrate specificity. Like the proteasome β-type subunits, ClpP is encoded as a precursor molecule that processes autocatalytically to the mature, active form. The crystal structure of ClpP from *E. coli* revealed a barrell-like complex consisting of two heptameric rings, which, for example associates with hexameric rings of ClpA at both ends, a structure which highly resembles that of proteasomes. The substrates of the Clp proteases include abnormal proteins and short-lived regulatory proteins. It has also been demonstrated that these complexes function in the assembly of proteins, a feature they share with the mitochondrial AAA proteases. This is not surprising in view of the fact that all members of the Hsp100/ Clp family are involved in various aspects of protein chaperoning and stress tolerance *(59)*.

Recently, two energy-dependent protease complexes have been characterized which form torodial structures with 32 symmetry, traversed by a channel along the threefold axis and enclosing a central cavity: Gal6/bleomycin hydrolase is capable of degrading bleomycin and probably other drugs. Tricorn protease has been detected only in some archebacteria. It can assemble into giant molecules similar in structure to isohedrical virus capsids. However, function and regulation of these complexes remain obscure thus far.

Concluding Remarks

The multitude of proteolytic systems present in cells and organelles underlines the importance of programmed protein degradation in the regulation of various cellular activities thus maintaining cellular integrity and productivity. In evolutionary terms, a number of strategies appear to have been employed to generate appropriate proteolytic machineries. Nevertheless, the resulting molecular complexes can be thaught variations of a common theme: association between specific protease moities and ATPase moities that participate in complex assembly and unfolding of the substrates. In the 26S proteasome, the ATPase subunits are part of the regulatory particle that is conferring substrate specificity. Likewise, in the Clp proteases, the ATPase subunits are associated with regulatory proteins *(11)*.

A feature common to all ATP-dependent proteases (unlike conventional proteases) is their highly processive fashion of substrate degradation, *i.e.* they seem to have mechanisms to bind tightly protein substrates and to make multiple cleavages in the polypeptide chain before releasing the peptide products and not to release partially digested polypeptides that are attacked at later rounds of degradation *(35)*. Processivity has been clearly demonstrated for Lon proteases and the Clp complexes, and can be extended to the proteasome *(35)*. As examplified by the release of a large polypeptide fragment (p50) during processing of the p105 precursor of NF-κB and the generation of peptides of defined length by immunoproteasomes, special mechanims seem to operate in these cases.

During the past years enormous progress has been made in understanding the biochemical mechanisms and intracellular functions of ATP-dependent proteases. This has already provided new insights into pathogenic states or genetic diseases that are caused by alterations in programmed proteolysis and, undoubtedly, will stimulate scientific exploration of still unsolved questions.

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Programirana proteoliza s ATP-ovisnim proteaznim kompleksima

Sažetak

Programirana razgradnja proteina unutar stanice bitna je za njezino preživljavanje. U eukariotskim stanicama postoje dva glavna puta razgradnje: (i) Proteoliza endocitoznih proteina, kao što su membranski receptori ili ekstracelularni proteini, ograničena je uglavnom na lizosomski / vakuoalarni sustav. (ii) O energiji ovisna proteoliza s proteasomima glavni je put razgradnje staničnih proteina, a ima važnu ulogu u mnogim procesima regulacije u stanici. Aktivnost proteasoma neposredno je povezana sa sustavom ubikitina. U Archebacteria i Eubacteria otkriveni su mnogo jednostavniji proteasomi i proteasomima slični kompleksi. U mitohondrijima i kloroplastima bitni dio programirane proteolize za »kontrolu kakvoće« postiže se s proteaznim kompleksima vezanim na membranu. Bitno je da su ATPaze u svim tim kompleksima dio obitelji novih proteina, tj. AAA obitelji (obitelj ATPazom povezanih aktivnosti), koja se može smatrati specijaliziranom podobitelji ATPazne nadobitelji. U ovom su prikazu izneseni najnoviji rezultati koji pridonose našem razumijevanju kontrolnih mehanizama koji čine osnovu djelovanja proteasoma i proteasomima sličnih kompleksa. Ostali sustavi strukturno i funkcionalno srodni tim kompleksima samo su ukratko navedeni.