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Invited Review

The role of the proteasome in cellular protein degradation

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Summary. Eukaryotic cells contain a major intracellular proteolytic activity known as the proteasome. The proteasome is a strongly conserved cylindrical structure of high molecular weight (650 kDa, ~20 S) and desmonstrates multiple endopeptidase activities. The general structural, biochemical and genetic features of the proteasome are conserved from archaebacteria through yeast to humans. This structure fulfills an essential role by functioning as the proteolytic core of a 26 S multienzyme complex responsible for the energy-dependent degradation of ubiquitinated proteins. The bulk of intracellular proteolysis appears to be through the ubiquitin-dependent pathway. Incorporation of the proteasome into the 26 S multienzyme complex appears to confer both a specificity for ubiquitinated proteins as well as a means to tightly regulate proteolytic activity. Thus, one function of the proteasome is required for the degradation of either abnormal or certain regulatory proteins by the ubiquitin pathway. Proteasome subunits appear to be encoded by a related gene family as defined by extensive sequence similarities. The gene products are confined to either of two general classes: α-type which appear to be structural and ß-type which may be catalytic. Genes encoding at least two proteasome subunits map to the Major Histocompatibility Complex. Accumulating evidence points to the proteasome (or a specialized form) participating in the cytosolic degradation of these viral proteins upon cellular infection. Through a previously unforseen mechanism, it appears that the products from the digestion of the viral proteins may be rescued from further digestion to amino acids and shuttled from the cytoplasm through the endoplasmic reticulum to the cell surface where they serve as antigenic peptides for recognition by the immune system. The proteasome may have been recruited by the immune system to serve as the cytosolic activity responsible for generating these antigenic peptides. The proteasome may function in the ubiquitin-dependent degradation of not only certain self-proteins but may fulfill a second essential role in the degradation of proteins originating from viral infection.

Key words: Proteasome, Ubiquitin, Protein degradation, Antigen processing

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Introduction

Although it has been over half a century since the dynamic turnover of cellular constituents was recognized, until only recently little progress was made in understanding this process (Schoenheimer, 1942). Cellular proteins levels are in a constant state of turnover being controlled by the rates of synthesis and degradation. The overall process of protein degradation is highly selective with the half-lives of individual proteins varying from seconds to being virtually stable for days (Hershko and Tomkins, 1971; Goldberg and St. John, 1976; Hershko and Ciechanover, 1992). The widely different rates at which individual proteins are degraded reflect the highly selective nature of protein degradation. The degradation of cytosolic proteins has an important function in selectively eliminating damaged or abnormal proteins that are produced during normal, or more frequently, under stress conditions. Furthermore, cytosolic proteolysis also modulates the levels of specific proteins controlling the levels of certain transcriptional factors, cell-cycle regulators and certain key enzymes in metabolic pathways (Finley and Chau, 1991; Hershko and Ciechanover, 1992; Varshavski, 1992).

The ubiquitin-dependent proteolytic system appears to be the major selective proteolytic system operating in eukaryotic cells. Cellular proteins are marked for their degradation by their ligation to the small polypeptide ubiquitin. The covalent attachment of ubiquitin to

protein substrates is a multistep process. Briefly, ubiquitin-specific conjugating enzymes, in concert with accessory factors, recognize specific signals on proteins and attach ubiquitin to defined lysine residues on the substrate. It is the attachment of the ubiquitin moiety (or moieties) that commits that protein for degradation. These ubiquitin-conjugated proteins are degraded in an energy-dependent process by a specific proteolytic complex. The nature of the signals that trigger the recognition and ubiquitination of a proteolytic substrate are only beginning to be understood. A further understanding of the rules governing the ubiquitination of certain cellular proteins should provide a better understanding of selective protein turnover.

Although considerable research has focused on the mechanism and enzymes responsible for the conjugation of ubiquitin to proteins, until recently very little was known about the mechanism used to achieve the degradation of such ubiquitinated proteins. The focus of this review is the proteasome which comprises the proteolytic core of the multienzyme complex responsible for the degradation of proteins ligated to ubiquitin. Although proteins are selected for degradation at the stage of ubiquitination, considerable specificity and regulation are necessary and achieved at the proteolytic step as well. Selectivity of this complex ensures that non-ubiquitinated proteins are not recognized and damaged and that (for the most part) only proteins conjugated to ubiquitin are destroyed. To understand how the cell achieves such selectivity and regulation during intracellular protein degradation, it was essential to identify and biochemically characterize the cytosolic factors responsible for this activity. Reconstitution experiments indicate that the proteasome functions within this process as a component of a 26 S multienzyme complex responsible for the degradation of ubiquitinated proteins. Thus, a critical understanding of the biochemical and catalytic features of the proteasome, its assembly into the multienzyme complex, its regulation (by both nucleotides and other protein factors) and biologic roles are essential to the understanding of the ubiquitin pathway and hence cellular protein turnover.

The proteasome: general, structural and biochemical properties

Proteasomes are non-lysosomal proteolytic complexes of unusually high molecular mass (~650 kDa) found in all eukaryotic cells examined to date (Rivett, 1989; Orlowski, 1990; Goldberg, 1992). The proteasome is found in both the cytosol and nucleus and can constitute up to 1% of total protein in tissue homogenates. Proteasomes have been isolated from numerous tissues of a broad range of organisms. These proteolytic complexes are composed of several small nonidentical but related polypeptide subunits with molecular weights that range from 20 to 33 kDa and isoelectric points that range from 3 to 10. These subunits are noncovalently associated and are arranged in a characteristic cylindrical structure. The cylinder appears to be hollow and arise by the stacking of four ring-like arrangements. Such structures have been observed from archaebacteria, yeast, various plants, *Xenopus*, mice, rat, rabbit and humans.

Upon the fractionation of eukaryotic cells, the proteasome appears to provide the major proteolytic activity detected at neutral pH. The proteasome possesses (endo)proteolytic activity as evidenced by the capacity to cleave a variety of natural or fluorogenic peptides as well as certain small unstructured polypeptides. This proteolytic activity is demonstrated against a variety of peptides each bearing different residues at the P1 position, i.e., the amino acid residue preceding the scissile bond. Hence the proteasome has been come to be known also as the multicatalytic protease.

The proteasome isolated from archaebacteria, yeast and higher mammals all demonstrate a general ring-like structure. A detailed microscopic investigation of the *Thermoplasma* proteasome using image analysis of negatively stained particles indicates 7-fold symmetry for the particle (Dahlmann, 1989). It would appear that each of the four ring consists of 7 subunits. The stoichiometry of the archaebacterial complex would be $\alpha_{14}\beta_{14}$. Analysis suggests that the α subunits form the two outer rings framing the two inner rings which appear to be exclusively ß subunits. Electron microscopy of the rat proteasome also suggests a similar cylindrical structure and a two-fold rotational symmetry. The dimensions of the complex are in the range of ~11-12 nm in diameter with a height of 16-20 nm.

At present it is uncertain how many distinct proteolytic activities reside within the proteasome. Early studies provided evidence that the proteasome exhibits at least three catalytic activities.The activities were designated as tryptic, chymotryptic or peptidylglutamyl-like based on the ability to cleave on the carboxyl side of basic, hydrophobic or acidic residues. The observed substrate specificity of the proteasome could be interpreted as representing a single proteolytic active site existing within the complex that possessed very broad specificity and therefore accepted multiple substrates. Alternatively, these observations could also indicate the presence of multiple proteolytic sites each with a distinct substrate specificity residing within the same complex. (Further possibilities, expanding on these two options are also possible, e.g., individual proteasome complexes containing a subset of all available active sites. Such an arrangement would thereby offer proteasome populations biased to cleave only after certain residues but coexisting in the cell with other distinct proteasome populations). Recently reported evidence has suggested at least five different catalytic activities within the mammalian proteasome. It is uncertain whether distinct active sites are responsible for these activities. In addition, it is not certain whether there exists overlap in capacity for substrates between these multiple active

sites. Some proteasome activities may be specific for the hydrolysis of protein substrates and not participate in the hydrolysis of peptides. Further studies will be necessary to determine conclusively the number of activities residing within the proteasome as well as address the specificity of these catalytic activities.

The proteasome demonstrates unusual kinetic properties when compared to other proteases. The different activities that exist within the proteasome can cleave on the carboxyl side of hydrophobic, basic and acidic residues. Notably, these activities display distinct responses to various activators and inhibitors. The catalytic activity of the proteasome can be enhanced *in vitro* upon the addition of mild denaturants such as sodium dodecyl sulphate, highly basic reagents such as poly-L-lysine, dialysis against water and heat treatment. These observations would suggest that such treatments either expose cryptic catalytic sites or alternatively generate a more catalytically active conformational state amplifying hydrolysis by the existing active sites. It would be reasonable to assume (as noted below) that the artificial activation demonstrated *in vitro* would tend to be mimicking a biologic or protein factor that fulfills these functions *in vivo*. The effects of protease inhibitors are, likewise, dependent on the specific proteasome activity being assayed. Serine protease inhibitors, such as 3,4-dichloroisocoumarin, are potent and specific inhibitors of proteasome activities. However, the relative inhibition of this agent as well as most others inhibitors appears to be selective and substrate specific. A conservative judgement based upon cumulative work would support calling the proteasome an unusual type of serine protease.

An essential role in the ubiquitin-dependent pathway

In 1980, it was proposed that proteins ligated to ubiquitin are degraded by a specific proteolytic «system» that recognized such ubiquitin-protein conjugates (Hershko et al., 1980). More than half a decade later, a large ATP-dependent proteolytic activity from rabbit reticulocytes was partially characterized which demonstrate the ability to degrade ubiquitin-conjugates (Hough et al., 1987). A similar activity was reported by Goldberg and colleagues (Waxman et al., 1987). The sedimentation coefficient of this activity was ~26 and the molecular mass ranged from 1,000 to 1,500 kDa. These values were significantly larger than those for the proteasome. Thus, the system responsible for the degradation of ubiquitinated proteins appeared to be larger than the proteasome alone.

Based upon the relative molecular mass of the ubiquitin-conjugate degrading activity it was reasonably assumed that the activity was in fact a multienzyme complex (Ganoth et al., 1988). Using a insightful fractionation procedure, Ganoth et al. (1988) demonstrated that this structure was composed of multiple components. Studies showed that three different factors were required for reconstitution of ubiquitin-conjugate degradation. These three components were termed CF-1, CF-2 and CF-3 (for Conjugate-degrading Factor) and displayed molecular weights of 600, 250 and 650 kDa, respectively. All three factors were required for the ATP-dependent assembly of the multienzyme complex and further all three were incorporated into the complex.

By using a reductionist approach, it was possible to first identify individual components of this complex and then to attribute specific functions to these individual components. Using such an approach, two groups independently demonstrated that the proteasome corresponded to CF-3 of the 26 S multienzyme complex. Eytan et al. (1989) noted that preparations of CF-3 contained low levels of ATP-independent proteolytic activity. As cited above, proteasome preparations demonstrate a stimulation of proteolytic activity following treatment with low levels of mild detergents, such as SDS. Detergent treatment activated not only proteolytic activity of the proteasome but also of the purified 26 S complex. Further, following preincubation of CF-1, CF-2 and CF-3 with MgATP, the SDS-activated proteolytic activity shifted from the region of CF-3 to the 26 S region. Driscoll et al. (1990) incubated a crude reticulocyte fraction with or without MgATP and then precipitated with 38% ammonium sulfate (Driscoll and Goldberg, 1990). The procedure precipitates the 26 S complex, while the 20 S proteasome remains in the soluble fraction. Following preincubation with MgATP, it was found that an ATP-dependent activity that cleaved both fluorogenic peptides, certain proteins and (most importantly) ubiquitin-conjugates was precipitated at 38% ammonium sulfate. This newly appearing activity reacted with a monoclonal antibody generated against a subunit of the 20 S proteasome. The results indicated the incorporation of the multicatalytic protease into the 26 S complex in an ATP-dependent assembly process. The conclusions of both groups independently supported a role for the proteasome as a component of the 26 S complex and lead to the identification of CF-3 as being identical to the proteasome.

Regulators of proteasome activity

The association with CF-1 and CF-2 seems to alter the catalytic and regulatory properties of the proteasome (Goldberg, 1992; Rechsteiner et al., 1993). Whereas the 20 S proteasome can, albeit weakly, hydrolyze certain polypeptides and demonstrates almost immeasurable activity upon ubiquitin-conjugates, the 26 S complex strongly prefers and very readily hydrolyzes ubiquitin-protein conjugates. In addition, the 26 S displays a requisite MgATP-dependence, in contrast to the 20 S proteasome. These alterations in specificity and regulation must be attributed, at least in part, to the physical and functional association of the proteasome with CF-1 and CF-2. Complete biochemical resolution of CF-1 and CF-2 at present has not been achieved. Hence, the contribution of these factors to 26 S activity

is not yet fully understood. Future studies will include experiments to understand how these factors confer ATP and ubiquitin dependence on the proteasome.

Recent studies have identified CF-2 as a 250 kDa, ATP-binding factor that upon incubation with the proteasome decreases its relative peptidase activity (Driscoll et al., 1992). Because of this factors effect on the isolated 20 S proteasome, the operational term «inhibitor» was applied. The inhibitor is essential for the reconstitution of ubiquitin-conjugate degradation in the presence of the proteasome (CF-3) and CF-1 and is incorporated into the 26 S protease complex in an ATP-dependent reaction. The 250 kDa factor is thus an essential component of the 26 S complex, most likely corresponding to CF-2. The inhibitor (CF-2) appears to be an ATP-binding component that regulates proteolysis within the 26 S complex. Quite possibly, during the multistep process of ubiquitin-conjugate degradation, the effect of this factor on proteasome activity reflects one of many steps integrated into the process of ubiquitin-conjugate degradation. Hence, either ATP hydrolysis or recognition of an appropriate substrate (bearing a ubiquitin chain) may temporarily release the inhibition of the proteasome and allow proteolysis.

The assembly and function of the 26 S complex both require ATP hydrolysis. The assembly is accompanied by the formation of the 26 S and accompanied by the formation of an NTPase (Armon et al., 1990). Putative subunits of the 26 S complex were cloned and revealed a homology to previously sequenced genes that comprise a family of ATPase members (Dubiel et al., 1992a,b). Thus, the genetic evidence supports the biochemical results. How these factors regulate the proteasome and there requirement during ubiquitin conjugate breakdown is not known. In addition, to generating an ATPase activity, formation of the 26 S protease complex also generates a ubiquitin-hydrolase. Each of the three components of the 26 S complex do not appear to display such an activity alone. The identification of the isopeptidase activity with an individual component (or components) should provide an understanding of how ubiquitin recognition and removal from a substrate is coupled to proteolytic hydrolysis of the substrate. A second isopeptidase activity, named isopeptidase T (or iso T), has been uncovered which functionally increases the relative rate of multiubiquitinated protein hydrolysis, possibly without physically interacting with the 26 S protease (Hadari et al., 1992). This hydrolase appears to act on free multiubiquitin chains that are cut from the target substrate. Clearly, analysis of the physical and functional role of the factors responsible for the ATPase and isopeptidase activities will bear on a complete understanding of the 26 S complex and on the regulation of the proteasome.

Numerous groups have reported protein factors that activate the relative endopeptidase activity (activities) of the 20 S proteasome (Dubiel et al., 1992a,b; Rechsteiner et al., 1992). It appears reasonable to assume that these factors reflect the biological means to stimulate proteasome activity predicted by the earlier studies with *in vitro* treatments using nonphysiologic agents. It was reasoned that if a factor existed that stimulated the peptidase activity of the 20 S proteasome, the factor would be of biological significance only if it also stimulated the activity of the 26 S complex. Following preincubation with the 26 S protease complex, a 200 kDa activator isolated from reticulocytes stimulated the hydrolysis of not only peptides but also ubiquitin-conjugated proteins (Driscoll et al., 1993). These studies thus directly implicate the 200 kDa activator as a new factor in the ubiquitin-dependent proteolytic pathway. Importantly, this factor does not alter the substrate specificity of the 26 S protease complex, but does appear to regulate its hydrolytic activity and thereby controls the flux through the ubiquitin pathway.

An important question for future study is the regulation of the proteasome when not present in the 26 S protease complex. The levels of CF-3 (the 20 S proteasome) significantly exceed the intracellular levels of CF-1 and CF-2 (J. Driscoll, unpublished observations). Thus, substantial levels of the proteasome exist intracellularly that are not assembled into the 26 S complex. Further, since considerable levels of the «free» 20 S proteasome exist it is important to determine how its activity is regulated to prevent the digestion of cellular proteins. Quite possibly, this could be achieved through physical association with specific cytosolic protein factors or through covalent modifications which render the proteasome a less active «repressed» state (J. Driscoll, unpublished observations).

The proteasome gene family

While the numerous biochemical analyses lead to many insights into the mechanism of proteasome function and regulation, further genetic studies were essential to generate a better structural understanding of the proteasome and its function. The cDNA or genes of over 30 proteasome subunits have been isolated and sequenced. All proteasome genes examined to date encode previously unidentified genes that are evolutionarily related. The proteasome genes appear to be encoded by a homologous gene family and may possible have evolved from a common ancestor.

The primary structures of archaebacterial, yeast, xenopus, *Drosophila*, mouse, rat and human subunits have been deduced from the nucleotide sequences of cDNA isolated by recombinant DNA techniques (Tanaka, 1992). No evidence for significant homology with other known proteases (or proteins) was obtained. This would suggest that proteasome genes encode novel polypeptides. Because proteasomes display multiple peptidase activities, it may possibly consist of different subunits with distinct active sites that catalyze proteolytic reactions. Active site similarities of the subunits to those of seryl, cysteinyl, carboxyl or metalloprotease activities have not been detected. Therefore, its is possible that proteasome genes encode a

novel class of proteolytic activity.

Sequence similarities show the highest similarities compared within a given species, however, certain proteasome genes from different species show a marked homology. The proteasome complex which has been isolated from the archaebacteria *Thermoplasma acidophilum* may be considered an archetype to all eukaryotic proteasome forms. The *Thermoplasma* form consists of only two polypeptides (α and ß). Sequencing of these two show homology to other eukaryotic proteasome genes (Zwickl et al., 1992). The α subunits are presumed to fulfill a structural role while the ß subunits may be catalytic in function. These subunit subclasses can be applied to all proteasome genes sequenced to date.

Mechanistic insights of proteasome function have come from the generation of mutants in the yeast equivalent (yscE) of the proteasome. These mutants show a specific reduction in the activity of the proteolytic complex against certain peptide substrates. Specifically, three mutants (which fall into two complementation groups called PRE1 and PRE2) were found to be deficient for a «chymotryptic-like» activity which prefers hydrophobic residues at the cleavage site. Interestingly, mutations in two different genes lead to the generation of the same mutant phenotype. Possibly, these subunits either encode specific active sites or are essential for the incorporation of other subunits into the complex that are responsible for catalytic activity. The mutants are sensitive to elevated temperature (at which they accumulate ubiquitin-conjugates), demonstrate enhanced sensitivity to stress and amino acid analogs and exhibit lower total protein degradation rates (Hilt et al., 1993). An independent set of mutants, defective in the PRE3 and PRE4 genes, exhibits a specific reduction in the peptidylglutamyl-hydrolyzing activity but maintain wildtype chymotryptic- and tryptic-like activities. In contrast to the mutants in the PRE1 and PRE2 genes, these strains do not appear to demonstrate a sensitivity to stress or compromised ability to degrade ubiquitinated-proteins (Heinemeyer et al., 1993).

Proteasome related genes have been found in the class II region of the Major Histocompatibility Complex (MHC). These genes were mapped adjacent to putative peptide transporters which are linked functionally to the class I antigen processing pathway. These two proteasome genes were cloned and shown to be homologous to the PRE2 gene from *S. cerevesiae*. Importantly, these MHC-linked gene products qualify as proteasome subunits and physically associate with cytoplasmic proteasome populations (Kelly et al., 1991; Martinez and Monaco, 1991).

The proteasome and the cytosolic generation of antigenic peptides

The mechanism by which endogenous proteins are cleaved to peptide fragments which then serve as antigenic peptides has remained a mystery. Certain peptides are passed through peptide transporters and into the lumen of the ER bound to the appropriate class I molecule from the ER to the cell surface to serve for recognition by the T-cell and subsequent destruction of the infected cell. Specific MHC-encoded gene products, which were mapped adjacent to the putative peptide transporters, were detected in a high molecular mass complex called the LMP (low molecular mass polypeptide) complex. Biochemical analysis indicated that the LMP bears extensive structural and serological similarity to the proteasome (Brown et al., 1991; Glynne et al., 1991). Further, co-immunoprecipitation experiments demonstrated that the LMP shares most, if not all of the proteasome subunits. These initial finding provided the first evidence supporting a role of the proteasome in the processing of class I antigenic peptides.

Sequencing of the two MHC-linked LMP genes revealed up to 30% homology to known proteasome genes. These genes map to a chromosomal region in which numerous components of the antigen presentation pathway cluster. Additionally, the LMP genes are gamma-interferon inducible and polymorphic: traits common to most components of the antigen presentation pathway. The cumulative evidence strongly supports a role for the proteasome (or a specialized form) in at least the initial cytosolic endoproteolytic events required for the generation of antigenic peptides.

Concluding remarks

Considerable progress has been made in understanding the biochemical, biologic and genetic features of the proteasome. The proteasome is still the only component of the intracellular machinery responsible for the hydrolysis of ubiquitinated proteins understood in some detail. Both the specificity for ubiquitinated proteins and the regulation of their degradation are achieved through incorporation of the proteasome into the 26 S multienzyme complex. The exact roles of the other components that comprise the 26 S complex remain to be clarified. Similarly, the regulated assembly of the 26 S and its regulation by nucleotides remain to be understood. Recent evidence supports a role for additional cytosolic factors in controlling flux through this complex and thus through the ubiquitin-pathway. The conjecture that the proteasome is altered by physiologically relevant changes and the result on the half-lives of intracellular proteins remains to be tested.

Tremendous interest was generated with reports that implicate the proteasome in the class I antigen processing pathway. Processing of endogenous proteins to peptides appears to be localized to the cytosol as is the majority of the proteasome. The cumulative evidence supports the proteasome as an attractive candidate as the generator of antigenic peptides. It must be pointed out that no direct evidence exists yet for this attribution. It remains to be seen if ubiquitin also is required at a prerequisite step during this process. Possibly, the

immune system has recruited an ancient structure and tailored it for a specialized proteolytic function. Future studies will surely provide more exciting and enlightening understanding of the proteasomes role in the class I antigen processing pathway.

The recent progress and interest in the proteasome has yielded a more comprehensive understanding of the proteasome and intracellular proteolysis in the regulation of eukaryotic cells. Similar to other multisubunit structures such as the ribosome and spliceosome, the proteasome (and analogously the 26 S complex) function to organize efficiently a defined sequence of reactions without allowing intermediates to escape. The combined use of molecular genetics with biochemical and cell biology approaches has added further unforseen dimensions to the proteasome and the general understanding of intracellular protein degradation.

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