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Proteasome Inhibitors and Antigen Presentation

Abstract: *Protein degradation plays an important role in the control and regulation of many crucial biological functions, ranging from cell cycle progression to presentation of viral antigens for scrutiny by cells of the immune system. At the heart of many of these catabolic events is the multicatalytic proteinase complex known as the proteasome. This large barrel-shaped protein complex executes a remarkable set of functions ranging from the complete destruction of abnormal and misfolded proteins to the specific proteolytic activation of crucial signaling molecules. Inhibitors of this proteolytic complex have thus been extremely useful for perturbing its function and deciphering its role in these diverse biological processes. Inhibitors of the proteasome consist mainly of peptides that are modified at the predicted site of hydrolysis with a reactive functional group capable of modifying the attacking nucleophile, either reversibly or irreversibly. Many of these inhibitors can be used in living cells and have proved to be invaluable tools for the study of proteasome function.* © *1997 John Wiley & Sons, Inc. Biopoly* ***43:*** *269–280, 1997*

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INTRODUCTION

The proteasome is a multisubunit complex responsible for the degradation of many if not all cytosolic proteins.[1] It plays a crucial role in a variety of biological processes including degradation of key regulatory proteins such as the cyclins and the activation of transcription factors by removal of inhibitory factors.[2–6] The proteasome is also required for the generation of antigens for presentation to cytotoxic T-cells via the major histocompatibility complex (MHC) class I pathway.[7,8] Its role in the presentation of viral antigens has been uncovered largely through the use of inhibitors and genetic mutants that allow for the controlled blocking of proteolytic function.

The topic of antigen presentation has been the subject of many review articles[9–11] and therefore the details of these pathways will not be discussed at length in this review. Figure 1 shows a general scheme for presentation of peptides via the two main routes. Presentation of peptides via major histocompatibility complex (MHC) class II molecules begins with the endocytosis of foreign material. Once inside the endocytic pathway, proteins are broken down into peptide fragments by lysosomal proteases known as the cathepsins.[11] These proteases have optimal activity at the acidic pH of the lysosomal compartment. Consequently, neutralization of the endosome leads to a block in antigen presentation via this pathway.[12,13] At the same time, MHC class II molecules are transported from the endoplasmic reticulum through the golgi apparatus and are targeted to vesicles containing the foreign pep-

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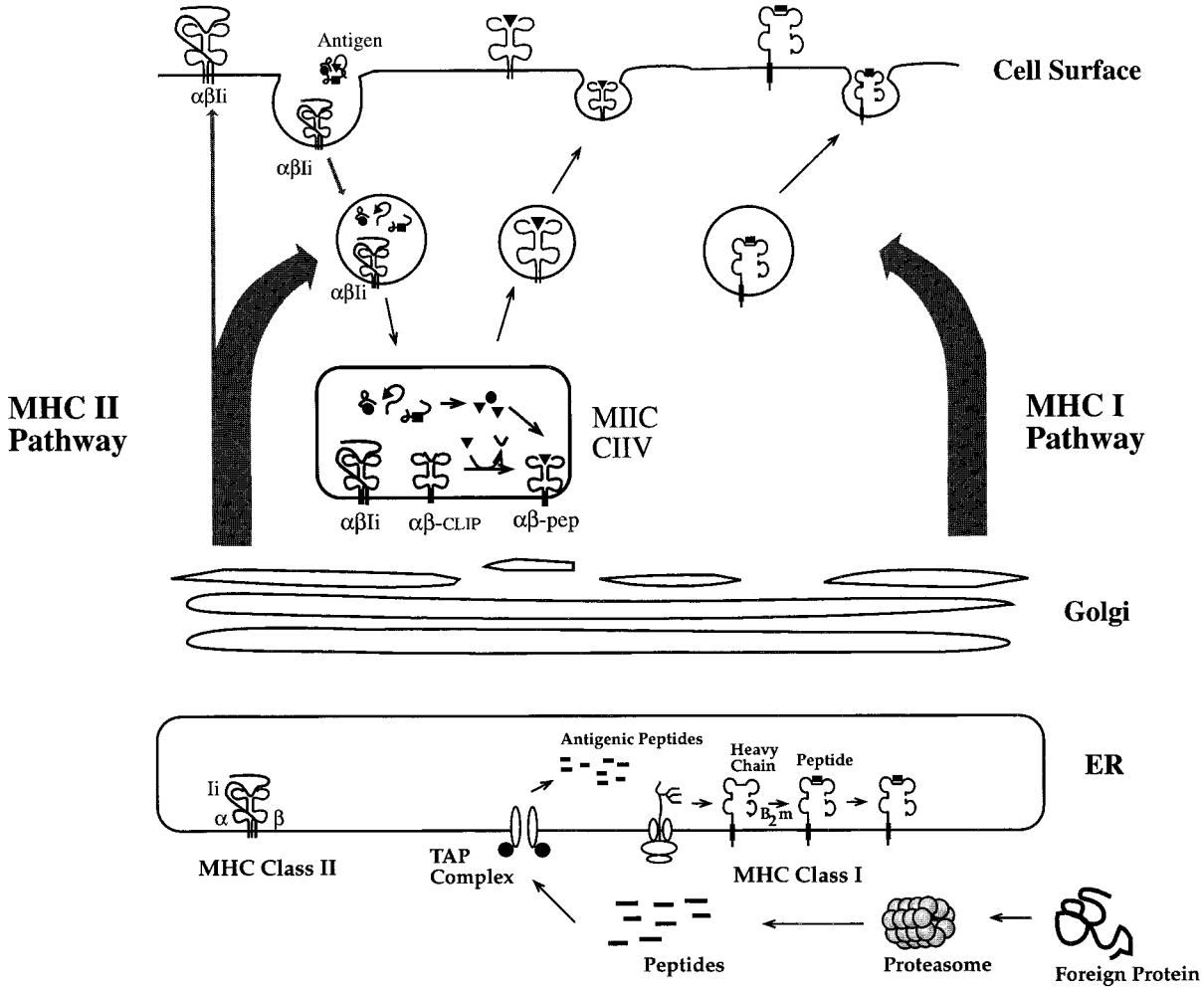


FIGURE 1 Presentation of antigenic peptides via MHC class I and class II molecules.

tides. These peptides may then bind in the groove of a class II molecule and move on as a complex to the cell surface for display to $CD4^+$ helper *T* cells. Engagement of the proper class II–peptide complex with the *T* cell receptor of a $CD4^+$ *T* cell can lead to an inflammatory response and to the production of antibodies.

Largely distinct from this pathway is presentation of peptides by MHC class I molecules. In this pathway, class I heavy chains are synthesized and assembled with light chains (termed β_2m) in the endoplasmic reticulum (ER). A peptide of 7–9 residues in length is the third and final component of this complex and is required for proper assembly and release of the class I complex from the ER. Peptides presented by class I molecules are in most cases derived from cytosolic proteins by a process described below. Thus, the host cell must have a mechanism that allows for the transport of these peptides across the ER membrane. This translocation process is carried out by a dedicated transporter called TAP (for Transporter associated with Antigen Presentation). TAP is a heterodimeric complex consisting of two transmembrane proteins (TAP1 and TAP2), which utilize energy from ATP to translocate peptides from the cytosol to the lumen of the ER. Once a peptide binds to a class I molecule in the ER, the complex is allowed to leave the ER and is transported through the secretory pathway. Eventually this peptide MHC complex is displayed to cytotoxic *T* cells at the cell surface. Upon engagement of a *T* cell receptor with an MHC class I molecule containing an appropriate peptide, the *T* cell becomes activated and may proceed to kill the infected cell.

In order for a cell to advertise the presence of an intruding organism to cytotoxic *T* cells, it must first be able to generate peptide fragments from proteins synthesized by the presenting cell. Crucial to

presentation of antigens by this pathway is the generation of peptides suitable for transport by TAP and also capable of binding to class I molecules. The proteasome is a key player in the generation of these antigenic peptides and its role in this pathway will be the focus of further discussion throughout this review.

STRUCTURE AND MECHANISM OF THE PROTEASOME

The proteasome is a large, barrel-like structure made up of four stacked rings of 7 subunits each.[1,14,15] These rings form a tunnel with openings at either end and an inner core where controlled proteolysis takes place. There are two types of subunits termed α and β, with the α subunits playing mainly a structural role while the β subunits are endowed with catalytic activity. The simplest type of proteasomes are found in bacteria such as *Rhodococcus*[16] and in the archaebacterium *Thermoplasma acidophilum.*[15] In these organisms the proteasome is made up of only a single type of α subunit and a single type of β subunit. The eukaryotic proteasome, although structurally similar to the eubacterial enzyme, is much more complex, consisting of 7 unique but related α and 7 unique but related β subunits.[1,15,17,18] The eukaryotic proteasome exists as a 20S complex made up of two rings of catalytic β subunits (7 subunits per ring) and two rings of α subunits (also seven subunits per ring), or as a larger 26S complex in which regulatory subunits (termed the 19S complex) are added to the core 20S complex. These regulatory subunits include ATPase, isopeptidases (discussed below) and several proteins thought to be responsible for the unfolding of a protein substrate prior to insertion into the proteolytic core of the 20S proteasome. The structure of the 20 and 26S proteasomes is shown in Figure 2.

The 26S complex, unlike the 20S complex, binds ATP and is responsible for the degradation of proteins that have been targeted for degradation by conjugation with a 72 amino acid polypeptide known as ubiquitin.[19] Ubiquitin is attached to a target protein by an isopeptide bond formed between the ϵ-amino group of lysine on the target and the C-terminal glycine reside of ubiquitin. This conjugation is performed by a series of enzymes called E1, E2, and E3.[19] The mechanism by which ubiquitin is conjugated to a protein for degradation is shown in Figure 3. Ubiquitin conjugating enzymes act in series by transferring a ubiquitin chain from

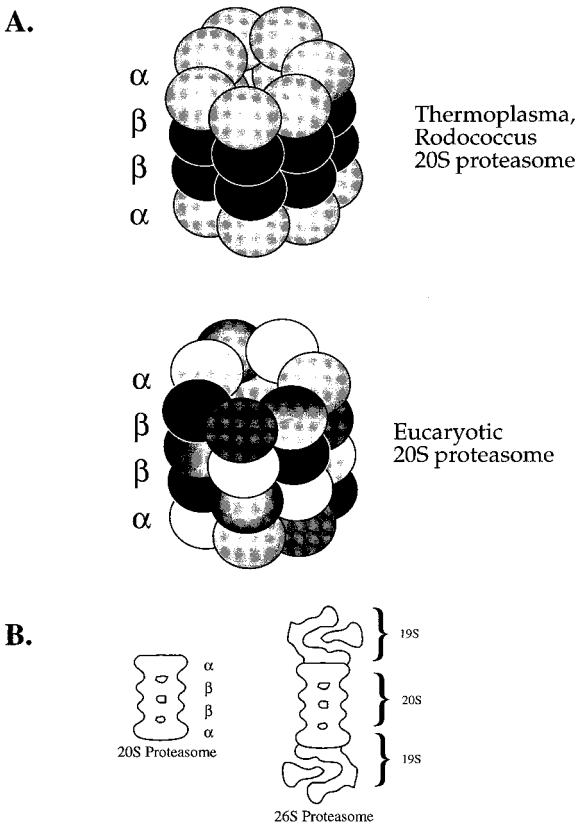


FIGURE 2 Structure of the eukaryotic and prokaryotic 20S and 26S proteasome. (A) cartoon representation and (B) sketch based on the electron micrograph.

one enzyme to the next by an activated thioester linkage to a cysteine residue. The final step is a transfer of the activated ubiquitin chain from the E2 enzyme to the target protein. The monoubiquitinated protein is then acted on again and the same ubiquitin conjugating enzymes attach an additional ubiquitin to the previous one at either of two possible lysine residues. Ubiquitin conjugation continues and results in a high molecular weight polyubiquitin protein complex. This heterogeneous population of ubiquitin tagged molecules is then the target for rapid degradation by the 26S proteasome.[1]

Once a ubiquitin-protein conjugate begins to be destroyed by the 26S proteasome, ubiquitin is recycled by removal of the large, branched poly-ubiquitin chain.[20] The resulting polymer is subsequently cleaved to individual monomer units by enzymes called isopeptidases, which perform the cleavage of the ubiquitin isopeptide bond. In some cases an isopeptidase may remove a polyubiquitin chain from a protein, thus saving the potential substrate from destruction by the proteasome.[21] There are several known isopeptidases in this family of enzymes

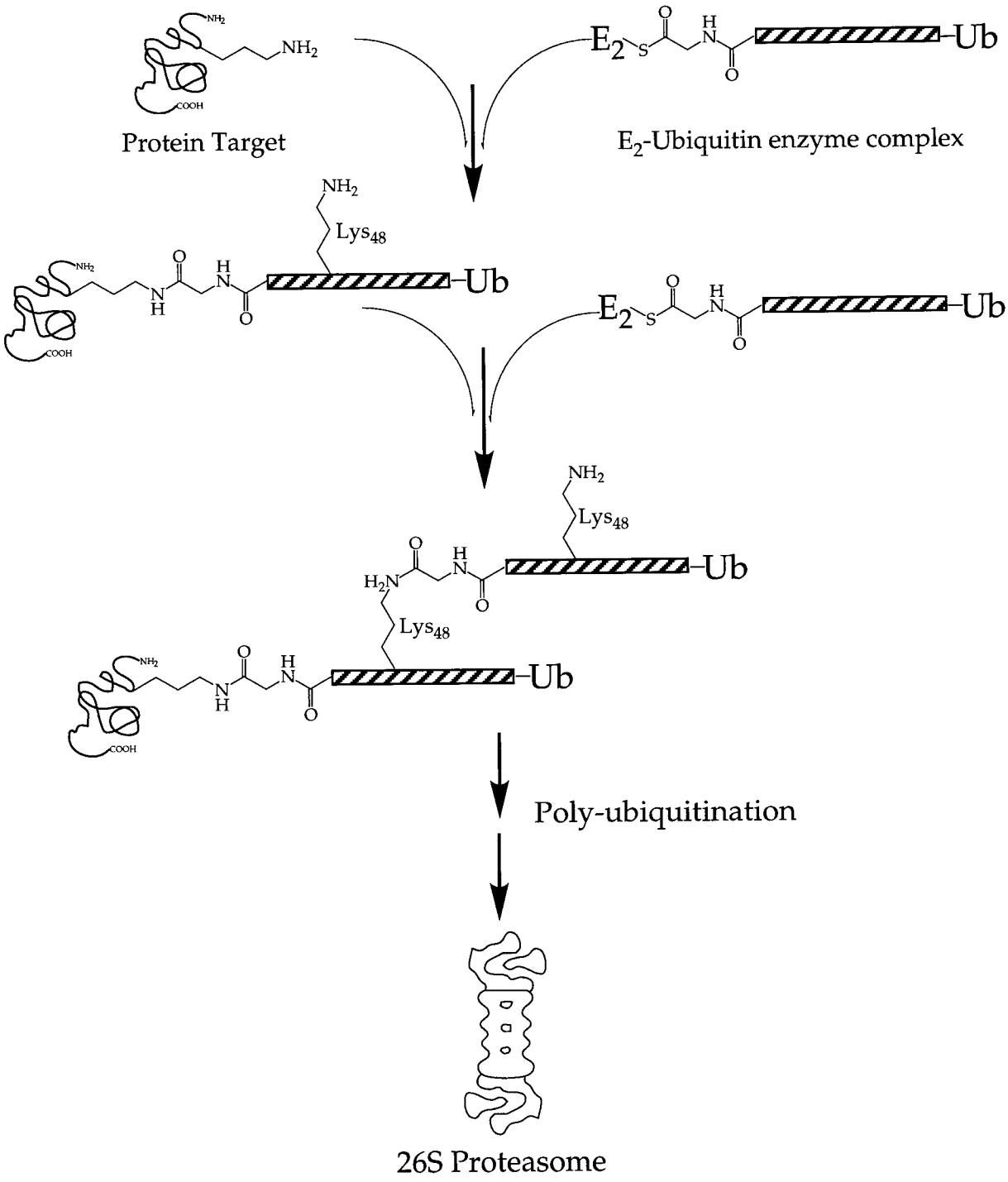


FIGURE 3 Conjugation of ubiquitin to a target protein for destruction by the 26S proteasome.

but still little is known about the mechanism by which these enzymes act.[20–23] Cell-permeable inhibitors of these peptidases could be of great importance in deciphering the role of ubiquitin conjugation in protein degradation.

One of the qualities that distinguishes the proteasome from many other proteolytic enzymes is its multiple peptidase activities. Initial studies of the proteasome using fluorogenic substrates consisting of a variety of sequences indicated that the proteolytic activity of the proteasome could be categorized into three main activities: cleavage after hydropho-

bic side chains (chymotrypsin-like), cleavage after acidic side chains (postglutamyl peptidase), and cleavage after basic side chains (trypsin-like).[24–26] These activities were found to be the result of distinct active sites and could be modulated by mutation of β subunits or by changing the subunit composition of the proteasomal complexes.[8,27] In addition to the three major proteolytic activities of the proteasome, two other activities have been discovered as the result of their resistance to inhibition by the serine protease inhibitor 3,4-dichloroisocoumarin.[28] These two proteolytic activities result in cleavage after branched-chain amino acids (BrAAP activity) and cleavage after small neutral amino acids (SNAAP activity).[28]

Initial studies of the proteasome were unable to classify it into a category with other known proteases mainly due to lack of homology and unusual reactivity with protease inhibitors.[29,30] It was initially thought to utilize a cysteine or serine as the catalytic nucleophile, but exhaustive mutational analysis found neither of these residues to be required for catalytic activity.[31] Recently mutational analysis of the β subunits of the thermoplasma proteasome showed the N-terminal threonine residue to be required for peptide bond hydrolysis.[32] This, combined with the x-ray crystal structure of the 20S proteasome from thermoplasma with an inhibitor bound in the active site, provided strong evidence that the sidechain hydroxyl of Thr1 was the catalytic nucleophile.[14,32] The proposed mechanism of peptide hydrolysis by a β subunit of the proteasome is shown in Figure 4. Activation of the side chain hydroxyl of threonine is thought be catalyzed by either the free amino terminus or the amine of a nearby lysine. This lysine residue, in conjunction with a glutamic acid residue, may act as a charge relay system analogous to that found in most serine proteases. Thus the proteasome is an example of a N-terminal hydrolase, of which there are only a few known other examples.[33–35]

Like many proteolytic enzymes, activation of the N-terminal nucleophile found on the catalytically active subunits of the proteasome is a process involving a proteolytic processing step. The proteasome synthesizes all of its catalytic β subunits as inactive precursor proteins that must first be activated by removal of an N-terminal pro-sequence. This pro-sequence is remarkably diverse from subunit to subunit and there is also little homology within the pro-sequences of β subunits from different species.[36] Mutational analysis has shown that the catalytic Thr1 as well as a conserved glycine residue on the N-terminal side of Thr1 (termed Gly -1) are both required for efficient processing of the β subunits.[37,38] It has been reported by multiple laboratories working with yeast and mammalian systems that removal of the pro-sequence is not required for assembly of subunits into the large 20S and 26S complexes, and therefore it is believed that the pro-sequences prevent catalytic activation of β subunits before they become part of the larger proteolytic complex.[37,38] The mechanism of pro-sequence hydrolysis, however, still remains unclear. Several reports have proposed an intermolecular mechanism in which another β subunit is required for processing,[36,38] while a purely autocatalytic, intramolecular process remains possible. The proposed mechanism for autocatalytic processing of a β subunit is shown in Figure 5. The details of the processing events are difficult to address and the process may in fact be a combination of the two proposed mechanisms.

INHIBITORS OF THE PROTEASOME

Crucial to understanding proteasome function is the ability to modulate its activity in vivo. This goal has been accomplished with the advent of several classes of proteasome inhibitors that are able to penetrate living cells and block proteasome function without affecting normal biological processes such as ATP metabolism and protein synthesis.[39–42] The structures of several classes of proteasome inhibitors are shown in Figure 6. The first class of compounds to be studied as inhibitors of the proteasome were the C-terminal peptide aldehydes.[39,42–44] The C-terminal aldehyde group is capable of forming a covalent hemi-acetal with a threonine hydroxyl and may also form a stable oxizolidine ring by simultaneous reaction with the N-terminal amine and the side chain hydroxyl. The mechanism of inhibition of the proteasome by the peptide aldehydes as well as several other classes of inhibitors is shown in Figure 7. These types of covalent adducts, although energetically favored, are reversible and therefore peptide aldehyde inhibitors may be removed, resulting in the return of proteolytic function.[42]

Initial studies using peptide aldehydes were carried out with compounds previously described to inhibit other proteolytic enzymes. The tripeptide aldehyde acetyl-leu-leu-norleucinal (calpain I inhibitor) was found to reversibly inhibit the chymotrypsin-like activity of the proteasome while the tripeptide aldehyde leupeptin was found to be a weak inhibitor of the trypsin-like activity.[28,44] Because of the therapeutic possibilities of proteasome inhibitors

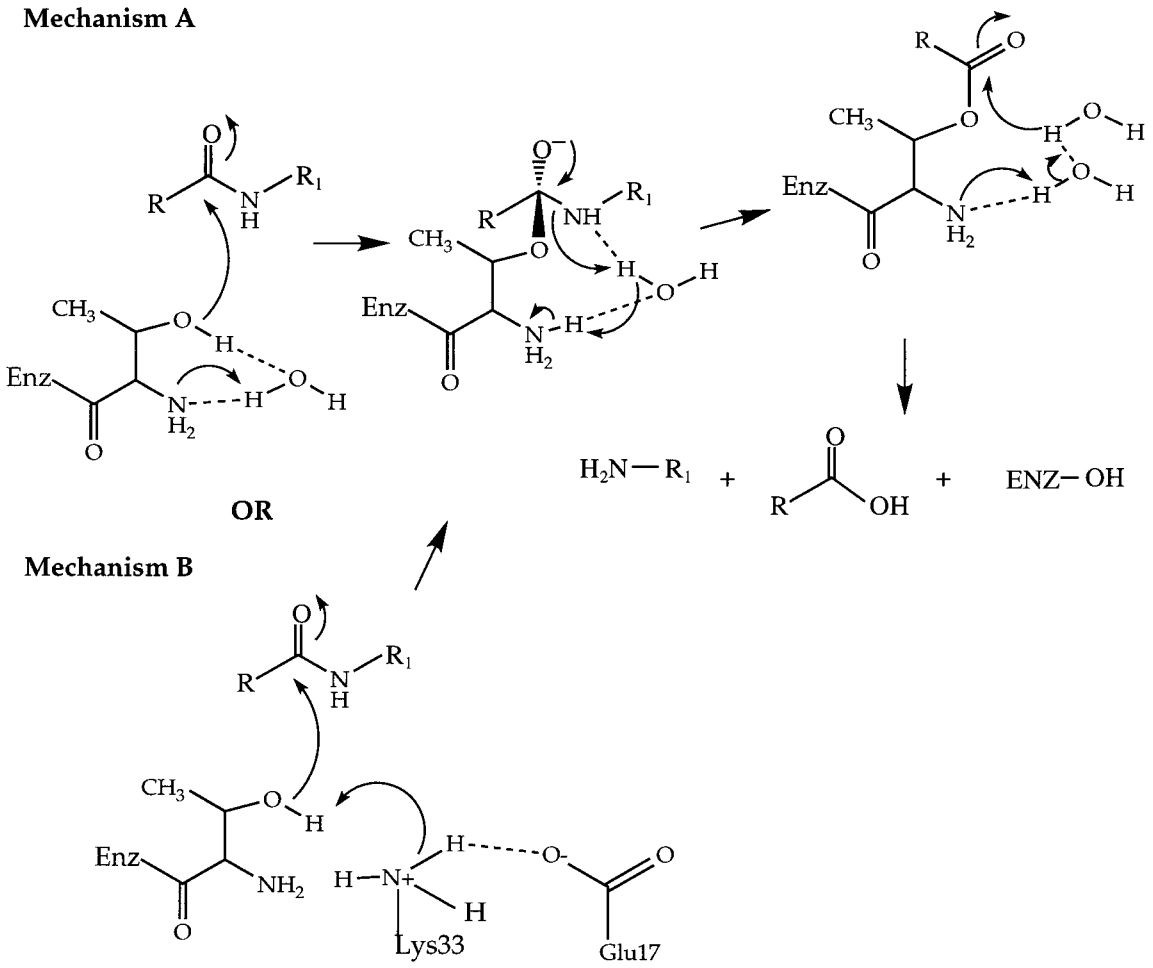


FIGURE 4 Peptide bond hydrolysis by the proteasome by two possible mechanisms. The side chain hydroxyl of threonine is activated by either the N-terminal amino group (mechanism A) or by a lysine amino group (mechanism B).

and driven by the need for tools to dissect proteasome function, other more potent inhibitors of the proteasome including the tri peptide aldehyde, Cbz-leu-leu-leucinal have been reported.[42–47] This tripeptide aldehyde has been subsequently used to block proteasome function in living cells and was shown to be a reversible inhibitor of the post glutamyl and trypsin-like as well as the chymotrypsin-like activities of the proteasome. Although these types of inhibitors have found widespread use, caution must be taken when interpreting effects of the peptide aldehydes due to their known inhibitory effect on other proteases such as the lysosomal cathepsins and calpains.[1,48] In addition, the highly reactive aldehyde functional group allows for possible side effects as the result of Schiff 's base formation with circulating free amines.

More recently peptides modified at the C-terminus by a vinyl sulfone moiety have proven to be another class of compounds capable of inhibiting proteasome function.[48] This type of C-terminally modified peptides are Michael acceptors and were therefore initially designed to be reactive toward soft nucleophiles such as thiols in the active site of a lysosomal cysteine proteases.[49–51] However, this class of inhibitors, when equipped with the proper tripeptide sequence, is capable of covalent modification of the N-terminal threonine of the proteasome. In addition, since the vinyl sulfones act as ''suicide substrates'' for the active site nucleophile, attachment of a radioisotope to these peptides results in an active site label that can covalently tag the proteasome in living cells.[48] Figure 8 shows the sodium dodecyl sulfate–polyacrylamide gel electrophoresis profile resulting from the labeling of intact cells with a trileucine peptide vinyl sulfone

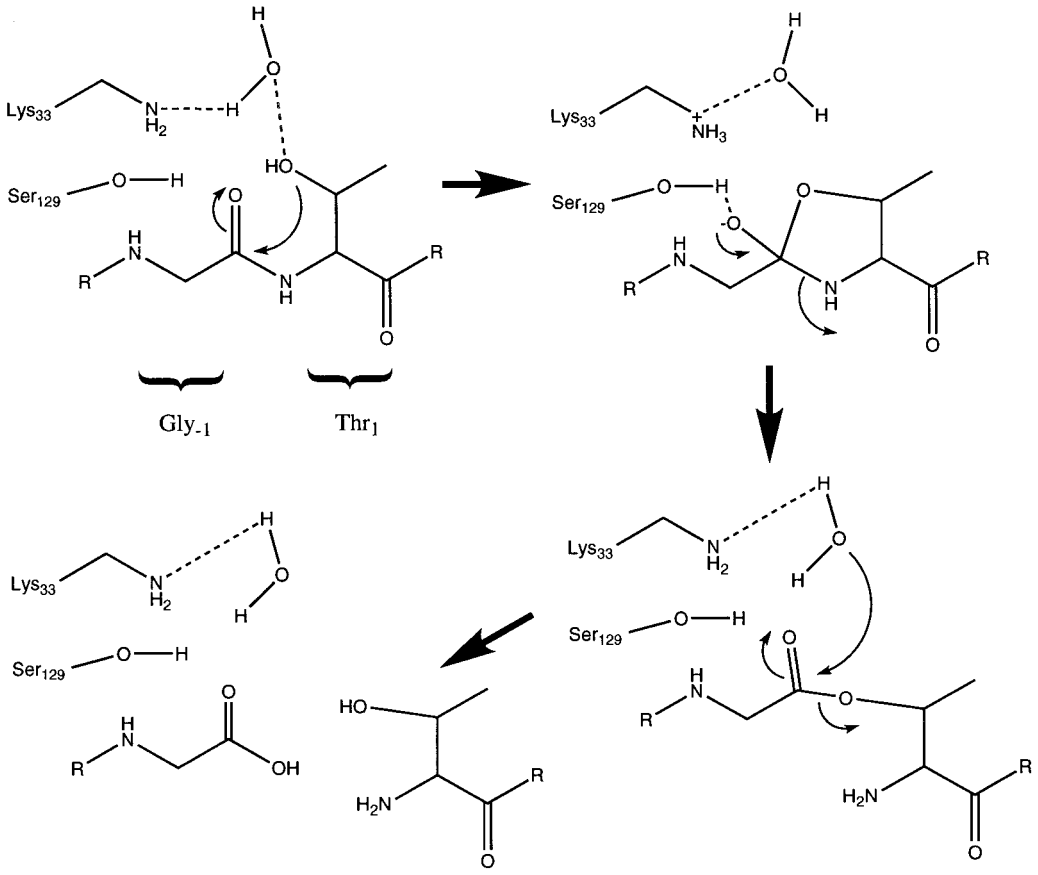
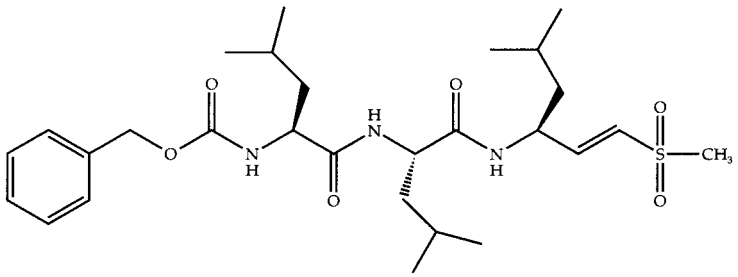


FIGURE 5 Possible mechanism for autocatalytic processing of a proteasomal β subunit.

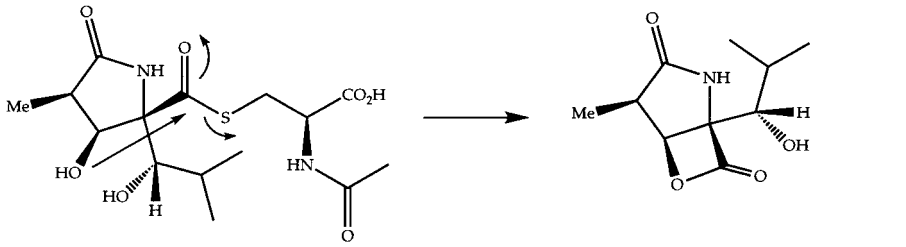
containing a radioiodinated nitro phenol moiety at the N-terminus. This labeled inhibitor was used to show that the trileucine peptide vinyl sulfone covalently inactivates all of the six known catalytically active β subunits of the proteasome. These compounds have also been found to be less reactive toward lysosomal proteases than peptide aldehydes and have no adverse effects on normal metabolic functions such as protein synthesis at concentrations that inhibit the proteasome.[48]

An inhibitor of the proteasome that is structurally quite different from the peptide-based inhibitors is the natural antibiotic lactacystin (Figures 6 and 7). This compound was initially identified by virtue of its ability to promote neurite outgrowth in cultured neurons.[52] Initially, a radiolabeled form of lactacystin was used to identify the target of the drug as a single catalytic β subunit (named *X*) of the eukaryotic proteasome.[40] Subsequently, several laboratories have shown that lactacystin in fact modifies all active β subunits.[48,53] Lactacystin is distinct from the peptide aldehydes and peptide vinyl sulfones in its specificity for the proteasome, having little or no effect on lysosomal proteolysis.[1,54] Lactacystin is a covalent inhibitor of the chymotrypsin-like and the trypsin-like activities of the proteasome and a weak reversible inhibitor of the postglutamyl peptidase activity.[40] Kinetic and biochemical studies of proteasomal inhibition by lactacystin and related derivatives have uncovered the unusual mechanism by which these compounds covalently modify the N-terminal threonine of the proteasome. Upon prolonged incubation in aqueous media, a hydroxyl group reacts with the thioester functional group resulting in the formation of a β-lactone (Figures 6 and 7).[55] It is this highly reactive β-lactone that is thought to be the species responsible for the covalent modification of the N-terminal threonine of proteasomal β subunits.

Still other classes of compounds have been described that are capable of either reversible or irreversible inhibition of the proteasome. These reagents include several di- and tripeptide aldehydes with sequences different from that of Cbz-leu-leu-leucinal,[39] peptide boron esters,[56,57] peptide α-ketocarbonyls,[56] and tripeptide α,β-epoxyketones.[58]



Cbz-leucyl-leucyl-leucine vinyl sulfone



lactacystin

clasto -lactacystin
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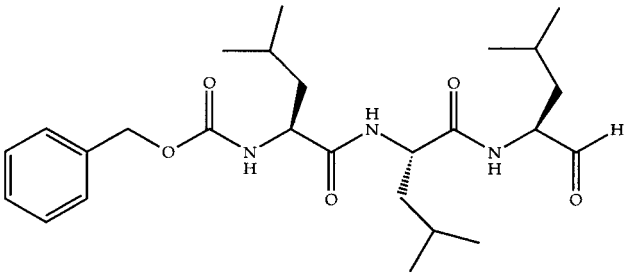


Cbz-Leucyl-Leucyl-Leucinal

FIGURE 6 Structure of several classes of inhibitors of proteasomal proteolysis.

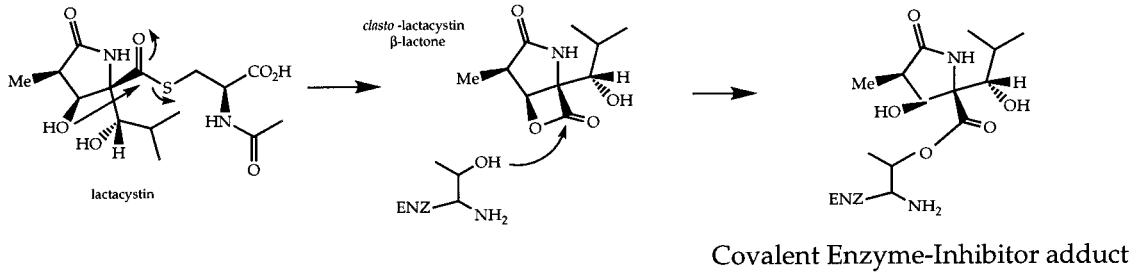
These compounds all have potential use in studying proteasome function but have not yet been exploited to their full potential.

THE PROTEASOME AND ANTIGEN PRESENTATION

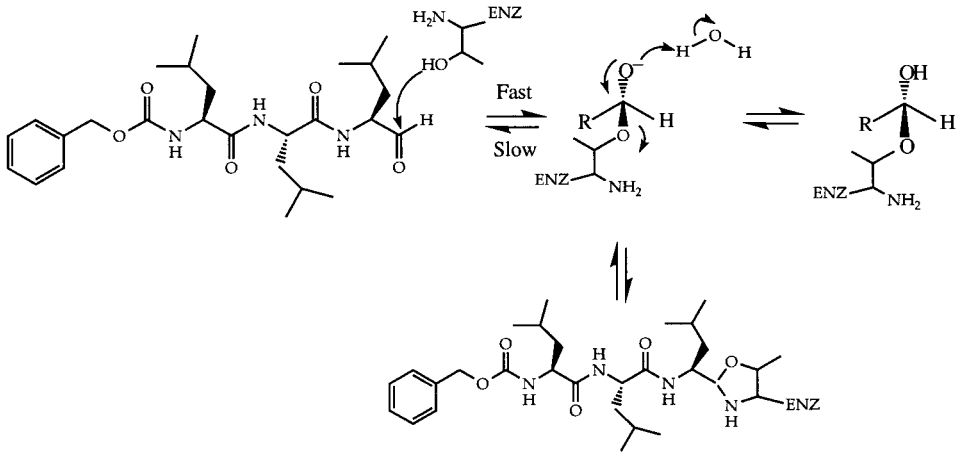
Cells of the immune system must be able to recognize when a cell is infected with a foreign pathogen and subsequently deal with the intruder by destruction of the infected cell. This communication between the *T* cell and a virus infected cell is mediated by molecules of the MHC. MHC proteins carry bits of foreign proteins, in the form of 8–10 residue peptides, to the cell surface to interact with antigen specific receptors on the surface of circulating *T* lymphocytes. These peptides must be generated from intact proteins by the action of proteolytic enzymes such as the proteasome.

Although the existence of a soluble ATP dependent proteolytic complex known as the proteasome was known since the late 1970s,[59] its role in the processing of class I antigens for presentation to cytotoxic T-lymphocytes has only recently been uncovered. Initial studies of genes encoded in the MHC region identified several low molecular weight proteins given the name the LMPs.[60] Since the expression of two of the LMP subunits was inducible by γ-interferon,[60] known to stimulate immune responses, it was proposed that these genes played a role in antigen presentation. Although the LMP subunits were similar in size to proteasomal subunits and formed complexes of high molecular weight similar to the proteasome, it was not until many years after their discovery that the two were found to be identical.[61–63]

Inhibition of the Proteasome by lactacystin



Inhibition of the Proteasome by C-Terminal Peptide Aldehydes



Inhibition of the proteasome by C-Terminal Peptide Vinyl Sulfones

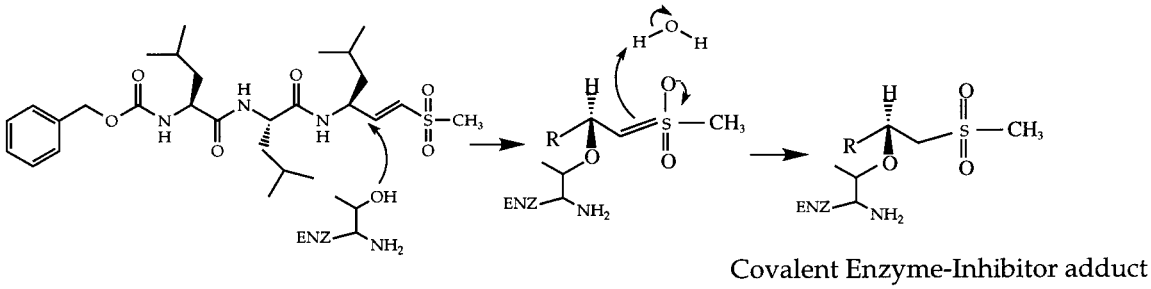


FIGURE 7 Mechanism of inactivation of the N-terminal threonine residue of catalytic β subunits by proteasomal inhibitors.

With this initial discovery, many laboratories began examining the details of the proteasome's involvement in antigen presentation. Initial studies focused on the two LMP subunits, LMP-2 and LMP-7, the genes for which were found adjacent to the TAP transporter genes in the MHC class II region and whose expression could be modulated with γ-interferon.[61–63] These two subunits were found to be homologous to the catalytically active β-type subunits of the proteasome.[18,64] The effect of these subunits on antigen presentation was studied using a mutant human cell line in which the MHC

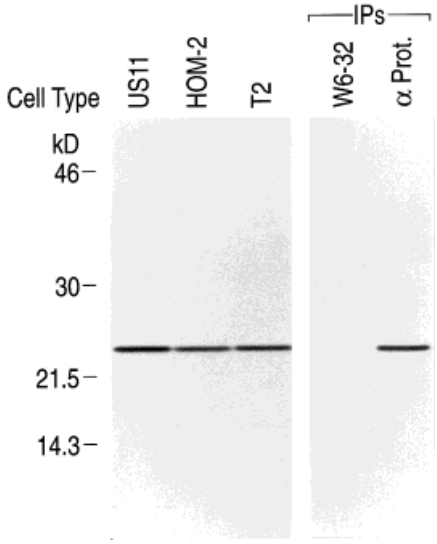


FIGURE 8 Covalent modification of proteasomal β subunits by the trileucine peptide vinyl sulfone 3-iodo (^{125}I)-4-hydroxy-nitrophenylacetly-leucinly-leucinyl-leucine vinyl sulfone (N^{125}IP-L$_3$-VS). The human astrocytoma cell line (US11), the human B-cell (HOM-2), and the human T2 cell line were labeled with N^{125}IP-L$_3$-VS. Immunoprecipitations using a proteasome specific antibody raised against an α subunit (α prot.) and an irrelevent antibody reactive against MHC class I molecules (W6–32) show that the labeling is specific for the proteasome. Reprinted with permission from Bogyo et al.[48]

region containing the LMP-2 and LMP-7 genes as well as the TAP-1 and TAP-2 genes had been deleted.[65,66] By replacing the TAP genes by transfection, it was possible to examine the effects of loss of LMP-2 and LMP-7 alone. Interestingly, several laboratories showed that the presentation of a variety of epitopes was unaffected by the absence of these subunits, indicating that LMP-2 and LMP-7 were not essential for the generation of epitopes.[66,67]

Initially, these findings seemed to dispute claims of the proteasome's involvement in antigen presentation. However, these experiments provided no information about more subtle effects of LMP-2 and LMP-7 on proteasome function. Several laboratories then examined the catalytic activities of the proteasome either containing or lacking the γ-interferon inducible subunits LMP-2 and LMP-7.[68,69] Activity of the proteasome against several different fluorogenic peptide substrates designed to act as substrates for the chymotrypsin-like, the trypsin-like, and the postglutamyl peptidase activities was compared for cells exposed to γ-interferon and their untreated controls. The results from these experiments showed that the activity of the proteasome changes quite dramatically upon inclusion of LMP2 and LMP7, favoring cleavage after hydrophobic and basic residues and strongly reducing cleavage after acidic residues. Furthermore, the activity of proteasomes isolated from mutant cells lacking the LMP-2 and LMP-7 subunits showed the exact opposite effects of γ-interferon treatment, favoring cleavage after acidic residues and reducing the propensity for cleavage after hydrophobic and basic residues.[70,71] These findings fit surprisingly well with the sequences of peptides known to bind with high affinity to MHC class I molecules. Several studies also showed the importance of hydrophobic or basic residues at the C-terminus of peptides for efficient transport by TAP.[72] These data restored the belief that the proteasome played an important role in antigen presentation.

More recently the requirement of the proteasome for generation of antigenic peptides was examined using inhibitors capable of blocking proteasome function.[41,42] Several peptide aldehydes comprised of different amino acid sequences were used to block proteasomal proteolysis. These inhibitors were able to cause a block in the rate of breakdown of both long- and short-lived proteins, as well as the degradation of abnormal proteins, all of which were believed to be the result of proteasomal inhibition. Further, when cells were pretreated with the peptide aldehyde inhibitors and subsequently assembly of class I molecules was examined by metabolic labeling and pulse chase analysis, it was found that inhibition of the proteasome led to a block in the formation of stable MHC class I heterodimers (heavy chain and β_2m). Since studies with cells lacking the TAP transporter showed a similar assembly defect due to a lack of peptides in the ER,[73] this result was presumed to be caused by a loss of peptides capable of binding to and stabilizing class I dimers. These data strongly suggested that proteasomal proteolysis was required for generation of peptides that bind to class I molecules in the ER.

Finally, proteasome inhibitors were found to have a dramatic effect on the ability of cells to present epitopes to cytotoxic T lymphocytes (CTLS).[42] When an antigenic protein was introduced into cells followed by preincubation with a peptide aldehyde known to inhibit proteasome function, there was a complete block in lysis by epitope specific T lymphocytes. However, when a mini gene encoding only the epitope required for transport, binding and presentation was introduced into the same cells, lysis by CTLs was resistant to treatment with proteasome inhibitors. Further, when a peptide aldehyde known to inhibit

lysosomal and soluble proteases other than the proteasome was used, there was no effect on antigen presentation.

FUTURE DIRECTIONS FOR PROTEASOME INHIBITORS

Although inhibitors of the proteasome have become widely used to dissect the role of the proteasome in many different cellular processes, there still remains much that can be done with these types of pharmacological agents. Compounds such as the peptide vinyl sulfones, which contain a peptide backbone similar to a protein substrate, can be equipped with an appropriate tag for labeling in living cells, and can covalently modify proteasomal active site nucleophiles, offer the possibility of designing inhibitors that target specific proteasomal β subunits. Such C-terminally modified peptides may be synthesized with a variety of different amino acid sequences using modern combinatorial methods. Subsequent labeling of proteasome subunits with mixtures of compounds could identify inhibitors that show selective binding properties. Subunit-specific compounds could then be used to correlate binding with inactivation of individual peptidase activities of the proteasome. Furthermore, such subunit-specific reagents would allow for the generation of pharmacological ''knock-outs'' in which the role of individual proteasomal peptidase activities in various metabolic processes such as antigen presentation could be explored.

While there is an emerging consensus as to the importance of the proteasome in the generation of antigenic peptides for presentation via MHC class I molecules, many of the details of the proteasome's role in this process remain a mystery. A wide range of inhibitors with differing specificity for proteasome active sites may be valuable in deciphering the details of how the proteasome is able to generate specific epitopes required for an infected cell to be recognized by a cytotoxic T lymphocyte.

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