2 Introduction to the Proteasome and its Inhibitors

Biochemistry and Cell Biology

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CONTENTS

ABSTRACT

The development of proteasome inhibitors for treatment of multiple myeloma and probably other cancers has followed an unusual course but is clearly linked to recent basic advances in our understanding of intracellular protein breakdown. After the discovery of the ATP-dependent pathway for protein degradation in the 1970s, ATP was shown necessary for the conjugation of ubiquitin to cell proteins, which marks them for degradation by the 26S proteasome. Its 19S regulatory complex uses ATP to unfold proteins and to inject them into the 20S core proteasome where proteins are digested to small peptides. The active sites in the 20S proteasome function by a novel threonine-based mechanism which allows their selective inhibition (e.g., by the boronate, Velcade). Surprisingly, we initially organized a biotechnology company to develop inhibitors of the ubiquitin–proteasome pathway not for cancer therapy, but with the goal of reducing the excessive proteolysis seen in atrophying muscle or cachexia, as well as inhibition of MHC class I antigen presentation, both of which depend on proteasome function. The availability of proteasome inhibitors has greatly advanced our understanding of the many functions of the proteasome, such as its key role in the activation of the transcription factor NF-κB, which led to a recognition that proteasome inhibitors might have anti-inflammatory and antineoplastic actions. The unexpected discovery that these inhibitors cause apoptosis selectively in neoplastic cells led to systematic studies and clinical trials against cancer. Amongst their multiple actions, proteasome inhibitors (1) cause the accumulation of

From: *Cancer Drug Discovery and Development: Proteasome Inhibitors in Cancer Therapy*
Edited by: J. Adams © Humana Press Inc., Totowa, NJ

abnormal proteins, which can trigger apoptosis; (2) stabilize tumor suppressors (p53, p27); (3) inhibit production of NF-κB, which is antiapoptic and generates important growth factors and cell adhesion molecules. However, the actual importance of these mechanisms in vivo in combating cancer remains uncertain.

Key Words

Proteasome inhibitors; proteasome mechanisms; proteasome functions; NF-κB; Velcade (PS-341); cancer.

1. INTRODUCTION

Although there can be many frustrations in a life devoted to biomedical research, there can also be unique rewards, especially the satisfaction that comes from seeing one's work lead to a greater understanding of living organisms and even, on occasion, to improvements in medical care. Consequently, those of us whose research has focused on the mechanisms of intracellular protein breakdown and the functions of the proteasome view with particular satisfaction and some parental pride the exciting emergence of proteasome inhibitors in the treatment of various cancers. Also highly gratifying has been the enormous utility that proteasome inhibitors have had as research tools that have greatly expanded our knowledge of the importance of the ubiquitin–proteasome pathway in cell regulation, immune surveillance, and human disease.

The research that led up to the synthesis of bortezomib (Velcade™; formerly known as PS-341) and its introduction for the treatment of cancer has had a curious, unpredictable history that is very much linked to the multiple strands of my own research career. In this chapter, I review the scientific findings that led to the development of bortezomib, which is now showing such exciting promise in clinical trials against cancer. Interestingly, this research, and even the organization of a biotechnology company whose primary program was to generate inhibitors of the ubiquitin–proteasome pathway, were not undertaken to find new cancer therapies. However, advances in biochemistry and drug development often do not follow predictable paths, and new insights in this area, as in others, have often come about by unexpected routes. This chapter, in addition to explaining the scientific background and rationale for the preclinical development of proteasome inhibitors, attempts to summarize our present understanding of the proteasome's function that has emerged from 25 yr of biochemical studies and studies using proteasome inhibitors.

1.1. Why Did We Want to Generate Inhibitors of the Proteasome?

The research paths leading to bortezomib and our decision in 1992 to undertake an effort to synthesize proteasome inhibitors for possible therapeutic applications derived not from the field of cancer biology, but from more arcane studies I had initiated almost 40 yr ago as a medical-graduate student to clarify the mechanisms of muscle atrophy, such as that occurring with denervation, disuse, and many major systemic diseases, including cancer cachexia, sepsis, renal failure, and AIDS *(1,2)*. That early work demonstrated that this loss of cell protein following denervation was caused not by a simple reduction in muscle protein synthesis (as we had expected), but rather by an acceleration of the rate of protein breakdown *(3)*. This finding was the first evidence that overall rates of protein breakdown in mammalian cells are regulated and can be of major importance in human disease.

At the time, virtually nothing was known about the pathways for protein catabolism in cells *(4,5)*. Therefore, in starting my own laboratory in 1969, I decided to focus my research on elucidating this degradative pathway through biochemical studies, not in muscle, but rather in simpler cells, initially *Escherichia coli* (because of the opportunity to use genetic approaches) and then mammalian reticulocytes. In the 1970s and 1980s, these studies led to the discovery of the soluble ATP-dependent proteolytic system and of the roles of the 26S proteasome and ubiquitin in this pathway. Although seemingly unrelated to muscle wasting or cancer therapy, these very basic studies have eventually influenced both areas. Ten years ago, these biochemical discoveries and parallel findings about muscle protein degradation suggested to us that selective pharmacologic inhibition of the ubiquitin–proteasome pathway might be feasible and could provide a rational approach toward developing agents to combat muscle wasting and cachexia. Although that therapeutic goal has not been achieved, in pursuing that objective, many important scientific insights have been obtained, and, most excitingly, bortezomib has emerged.

1.2. Discovery of the Ubiquitin–Proteasome Pathway

A major motivation for our undertaking to study protein degradation in bacteria and reticulocytes was that these cells lack lysosomes, which were then believed to be the site of protein breakdown in cells. However, I had become convinced that these organelles could not account for the extreme selectivity and regulation of intracellular proteolysis. Our present understanding of this process emerged from a series of developments, first through key in vivo findings and then in vitro studies of their biochemical basis.

Of particular importance was my finding that bacteria, which were long believed not to degrade their own proteins, could not only activate intracellular proteolysis when starved for amino acids or aminoacyl tRNA *(6)*, but that even during exponential growth, these cells rapidly degraded proteins with abnormal structures, such as those that may arise by mutation or postsynthetic damage *(7)*. Reticulocytes were also shown to carry out the degradation of abnormal proteins, which further suggested to us the existence of a nonlysosomal system for selective protein breakdown in eukaryotic cells *(5)*.

We showed that a key feature of this process was that it required ATP *(5,7)*, which clearly distinguished this intracellular process from known proteolytic enzymes and implied novel biochemical mechanisms. This energy requirement, in fact, was the critical clue to our finding and elucidation of the responsible degradative system and was, at the time, most puzzling, because there was clearly no thermodynamic necessity for energy to support peptide bond hydrolysis and no ATP requirement for the function of known proteases. Although studies in mammalian cells in the 1960s and early 1970s, especially by Schimke, Tomkins, and co-workers, had indicated that many tightly regulated enzymes were rapidly degraded in vivo *(4)*, this process ceased when cells were broken open, in large part because of this energy requirement for intracellular proteolysis *(8)*. It had been speculated that energy might be required for lysosomal function; however, our finding of a similar ATP requirement in bacteria and reticulocytes indicated that another explanation must apply.

On this basis, Joseph Etlinger and I searched for such a system in cell-free extracts of reticulocytes and were able to demonstrate a soluble, nonlysosomal (neutral pH optimum) proteolytic system that carried out the selective degradation of abnormal proteins when provided with ATP in a similar fashion to intact cells *(9)*. The biochemical dissec-

tion of this degradative system focused on an attempt to understand the molecular basis for this mysterious ATP requirement.

A fundamental advance was the discovery by Avram Hershko and Aaron Ciechanover of the involvement in this process of a small, heat-stable protein, subsequently identified by others to be the polypeptide ubiquitin, which was known to be linked in vivo to certain histones *(10,11)*. Their seminal work, together with that of Ernie Rose (1979–1982), established that ATP was necessary for covalent linkage chains of ubiquitin to protein substrates, which marks them for rapid degradation by the 26S proteasome. These workers also identified the three types of enzymes that are involved in the activation (E1), transfer (E2), and ligation (E3) of the ubiquitin. Mammalian cells appear to contain at least 20–30 different E2s and hundreds of distinct ubiquitination ligases, which provide the selectivity to this degradative pathway and are already emerging as very attractive drug targets *(10,11)*.

In parallel studies of protein breakdown in *E. coli* and mitochondria, we discovered a very different explanation of the ATP requirement for proteolysis. The bacteria and these organelles were shown to contain a soluble ATP-dependent proteolytic system for degradation of abnormal proteins. However, in exploring the basis for this requirement, we discovered a new type of proteolytic enzyme, very large proteolytic complexes (20–100 times the size of typical proteases) that are both proteases and ATPases (Figs. 1 and 2) *(12,13)*. Bacteria and mitochondria lack ubiquitin or a similar substrate-marking system. Instead, selective protein breakdown involves several ATP-dependent proteolytic complexes (in *E. coli* named Lon or La, ClpAP or Ti, ClpXP, HslUV, or FtsH) that hydrolyze ATP and proteins in a linked process and are selective for different kinds of substrates.

These investigations thus initially suggested two very different explanations for the ATP requirement for proteolysis, ubiquitin ligation in eukaryotes and ATP-dependent proteases in prokaryotes. However, subsequent work indicated that both mechanisms must also function in mammalian cells and that the degradation of ubiquitinated proteins and certain non-ubiquitinated substrates must be by an ATP-dependent protease *(12,14)*. In 1987, in fact, we had found such an ATP-stimulated proteolytic activity very early, but its isolation, characterization, and precise role took many years to clarify *(15)*. Finally, Rechsteiner's *(16)* and our group *(17)* were able to isolate a very large complex that degraded ubiquitin-conjugated proteins in an ATP-dependent process, the complex we subsequently named the 26S proteasome.These ATP-dependent proteases appear to function through similar ATP-dependent mechanisms as the 26S proteasome. In fact, one of these ATP-dependent proteases, HslUV, in eubacteria and the homologous proteasomes of archaebacteria and actinomyces appear to be the evolutionary ancestors of the eukaryotic 26S proteasome. Thus, proteasomes evolved quite early, before protein breakdown became linked to ubiquitin conjugation, which clearly provided opportunities for greater selectivity and regulation.

Initially, this proteolytic complex appeared to be very different from the 600-kDa proteolytic complex, which we now call the 20S proteasome (20S and 26S refer to their sedimentation rates). This structure, containing multiple peptidases, had been discovered in the early 1980s in several contexts—as a peptidase complex that hydrolyzes neuropeptides by Wilk and Orlowski *(18)*, as a nonproteolytic "ribonucleoprotein" particle by Scherrer, and as a major cytosolic endoprotease by DeMartino, our lab, and others. In the literature, there were actually at least 17 different names and multiple functions proposed

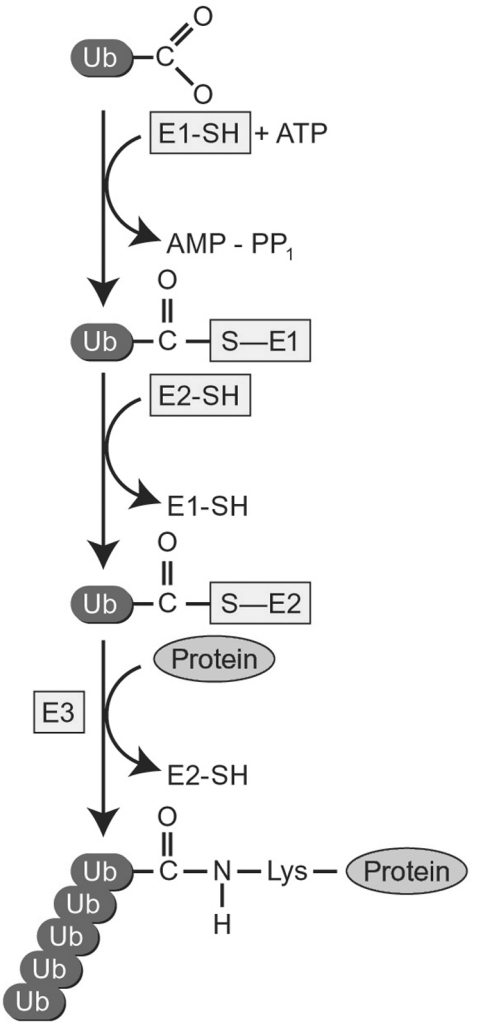

Fig. 1. Ubiquitin (Ub) conjugation to protein substrates.

for this complex. Eventually, we were able to show that these different structures corresponded to the same particle, which we named the 20S proteasome to indicate that it was a particle with protease function *(19)*. The next critical step was the demonstration by immunoprecipitation that the 20S proteasome was essential in the ubiquitin-dependent degradation of proteins in reticulocytes *(20)*. Finally, we *(21)* and Hershko's lab *(22)* were able to show that in the presence of ATP, the 20S particle was incorporated into the larger 26S (2.2-kDa) complex that degrades ubiquitin conjugates, which we then named the 26S proteasome (Fig. 3).

1.3. The Path to Proteasome Inhibitors and Bortezomib

These major biochemical advances did not by themselves provide the therapeutic rationale for an effort to synthesize proteasome inhibitors. In addition to these biochemi-

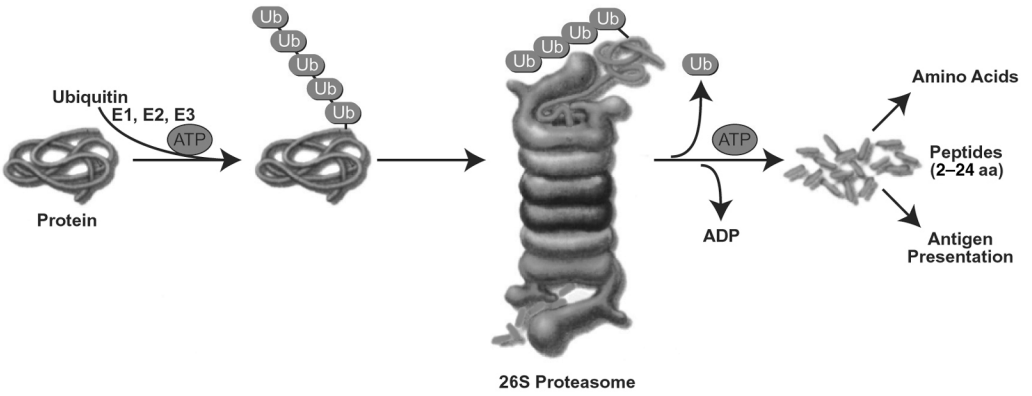

Fig. 2. The ubiquitin (Ub)–proteasome pathway.

cal studies, my lab continued to pursue physiologic studies of the control of protein breakdown in muscle in normal and disease states. A major advance was the development of simple in vitro techniques for precise measurement of rates of protein breakdown in incubated rodent muscles, which enabled us in the 1970s and 1980s to demonstrate that protein breakdown in muscle was accelerated in various diseases (e.g., cancer cachexia, renal failure, sepsis) in which muscle wasting is prominent (for reviews, see refs. **1** and **2**). Initially, it was assumed that this general acceleration of proteolysis was due to activation of the lysosomal (autophagic) pathway for proteolysis or the Ca^{2+}-activated proteases (calpains). However, blocking their function in isolated muscles had no effect on the excessive proteolysis, and, with time, we were able to demonstrate that the increased protein degradation was primarily due to an activation of the ubiquitin–proteasome pathway. This finding was surprising, since it had been generally assumed that this system primarily degraded the short-lived proteins in cells (e.g., abnormally folded polypeptides or regulatory molecules) *(10,11)* and not the long-lived proteins (e.g., contractile proteins), which comprise the bulk of cell proteins. In fact, it is now clearly established that atrophying muscles (whether due to fasting, denervation, cancer, sepsis, or diabetes) undergo a common series of transcriptional adaptations that enhance its capacity for proteolysis *(1,2)*, including increased expression of ubiquitin, proteasomes, and key new ubiquitination enzymes (E3s) *(23,24)*.

These insights led me to propose that it might be of major benefit to an enormous number of patients to be able to retard pharmacologically this degradative pathway in muscle, especially since only a relatively small increase in overall proteolysis (two- to threefold) appeared to be responsible for the rapid muscle atrophy. Therefore, our initial goal in undertaking to synthesize proteasome inhibitors was to partially inhibit the proteasome and thus to reduce muscle proteolysis to its normal rate in these catabolic states. On that basis, I convinced a group of Harvard colleagues to form a Scientific Advisory Board and to help found a biotechnology company whose primary goal would be to try to control the debilitating loss of muscle in these diseases by retarding the ubiquitin–proteasome pathway. Founding a company was attractive, because in a university setting, in which research programs are restricted by individuals' grants, it is really impossible to bring together chemists, biologists, and pharmacologists to work as a team toward a common goal. Venture-capital backing was obtained, and a biotechnology

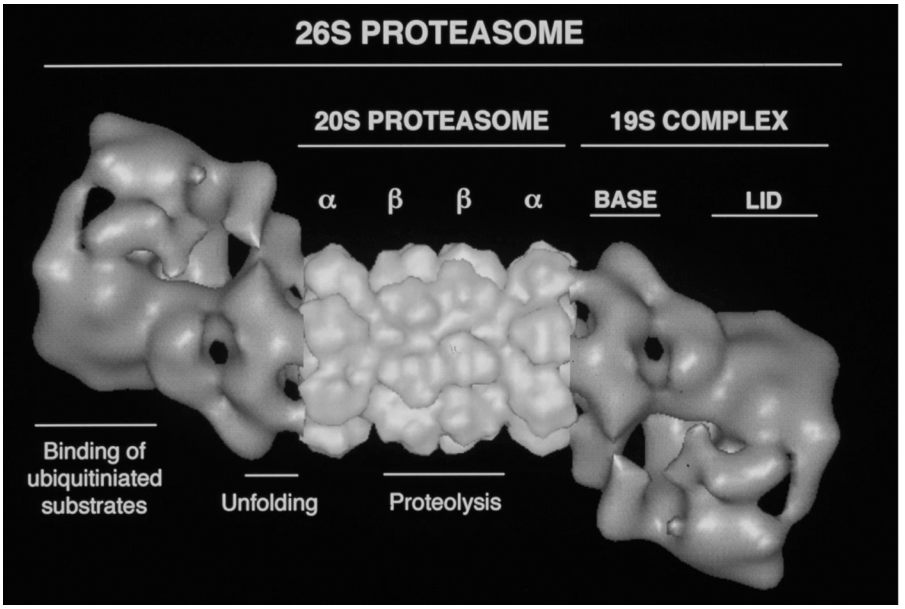

Fig. 3. 26S proteasome.

company was founded in 1993 and named MyoGenics to indicate our goal of preventing debilitating loss of muscle. Its first target was to develop inhibitors of the proteasome. With financial backing, we eventually assembled a talented team of enzymologists (led by Ross Stein), chemists (led by Julian Adams), and cell biologists (led by Vito Palombella), whose efforts led eventually to the development of the proteasome inhibitors bortezomib and PS-519, now in clinical trials.

This company was unusual in its close collaboration with academia; in fact, its scientific board met almost monthly, and the early scientific knowledge developed rapidly through fruitful collaborations between several of us Harvard-based founder-scientists and the talented enzymologists and biochemists in the company. For example , as soon as the first inhibitors were available, their effects on cells were analyzed in my lab and Kenneth Rock's (25,26), and subsequent studies on nuclear factor-κB (NF-κB) were pursued in collaboration between company scientists and Tom Maniatis's laboratory at Harvard *(27)*.

Perhaps the most fundamental finding from our own initial studies at Harvard in collaboration with Ken Rock was that reducing or blocking proteasome function in vivo did not immediately kill cells or interfere with their normal functions, both of which were theoretical possibilities. In fact, my real fear in initiating this company was that inhibition of the proteasome would rapidly lead to an accumulation of short-lived cell proteins in inactive ubiquitin-conjugated forms and thus be highly toxic. However, the presence in cells of a large number of enzymes that remove ubiquitin from proteins (i.e., isopeptidases) means that only a small fraction of cell proteins accumulate in the ubiquitinated form, even after marked inhibition of proteasomes. In other words, cells could function quite well for hours or even days with significantly reduced proteasomal capacity, as became clearly evident from subsequent animal studies and recent clinical trials.

An important motivation (at least for me, personally) for generating such inhibitors was purely scientific—the recognition that the availability of inhibitors that could enter cells and block proteasome function would be of tremendous value in clarifying its various physiologic roles. However, venture capitalists, stockholders, and company managers are not solely motivated by their interest in advancing biological science, for understandable reasons. Thus, this motivation was a hidden agenda of mine, although it has proved to be a major legacy of MyoGenics/ProScript, because their introduction of proteasome inhibitors greatly advanced our understanding of this area of biology *(28–30)*. In fact, very few biotechnology or pharmaceutical companies have had such a marked effect on an area of science. Moreover, this company did distribute some inhibitors freely to academic investigators, whose efforts rapidly advanced our knowledge of their effects. For example, early academic studies with these inhibitors remarkably altered our knowledge of the proteasome's importance in cancer, apoptosis, inflammation, and antigen presentation. At the same time, these studies ruled out certain potential therapeutic applications, such as the possibility of using them to suppress immune responses.

The first proteasome inhibitors synthesized by the company were simply peptide aldehydes *(29)*, which were analogs of the preferred substrates of the proteasome's chymotrypsin-like active site. Genetic studies in yeast had suggested that this site was the most important one in protein breakdown *(14,31)*, and we knew that hydrophobic peptides would be likely to penetrate cell membranes readily. The first inhibitors synthesized at the company were called MG compounds (for MyoGenics), such as MG132, which is still the proteasome inhibitor most widely used in basic research in cell biology because it is inexpensive, and its actions are readily reversible *(28,30)*. Most other inhibitors, including bortezomib, were derived from these initial molecules by peptide chemistry, in which a peptide with high affinity for the proteasome's chymotrypsin site was linked to different inhibitory pharmacophores. Julian Adams, who introduced the highly potent boronate as the inhibitory warhead, led these synthetic efforts. This pharmacophore had originally been developed to inhibit serine-proteases by scientists at DuPont and led to much more potent and selective inhibitors of the proteasome. Further medicinal chemistry efforts to modify the peptidic portion yielded bortezomib, a dipeptide-boronate, which was initially named MG341, but has since undergone multiple name changes as the company underwent various transitions. A major development leading to a change in the company's focus came from the discovery by Palombella et al. *(32)* (in a collaboration between Maniatis's lab and my own) of the involvement of the proteasome in activation of the key transcription factor, NF-κB, which plays a fundamental role in inflammation and cancer. Eventually, the company's role in muscle atrophy research became secondary, and its focus changed to the anti-inflammatory and antineoplastic actions of these inhibitors. The company therefore changed its name to ProScript, for Proteasomes and Transcription (hence, PS-341) and was taken over by a larger biotech company, Leukosite (hence, LDP-341) and then by Millennium Pharmaceuticals (MLN-341), who recently rechristened the drug bortezomib for commercial purposes.

It is noteworthy that these promising inhibitors were initially synthesized based on simple biochemical knowledge of the specificity of the proteasome's active sites, through directed medicinal chemistry, and by the use of classical enzyme and intracellular assays. They were not developed through a purely random screening of huge chemical libraries, as is the practice in most drug development efforts. Moreover, at the time, the nature of

Table 1
Distinctive Properties of the 26S Proteasome

Very large complex (2.5 MDa) composed of 44 polypeptides
Activity linked to ATP hydrolysis
Six proteolytic sites: two "chymotrypsin-like," and two "trypsin-like," and two caspase-like
Degrades proteins processively
Unfolds globular proteins
Degrades primarily proteins with polyubiquitin chain
Has isopeptidases that disassemble ubiquitin chains

the proteasome's architecture was unknown *(31)*; therefore, no structural information was available from X-ray crystallographic analysis to facilitate drug optimization. Nevertheless, exciting drug candidates emerged within 1 yr of the start of this company with less than a dozen talented scientists.

2. THE 26S AND 20S PROTEASOME

2.1. The 20S Core Particle

The degradation of most cellular proteins is catalyzed by the 26S proteasome, an extremely large (2.4-MDa), ATP-dependent proteolytic complex that differs in many respects from typical proteolytic enzymes *(14,31)* (Fig. 1 and Table 1). The great majority of proteases (which typically are 20–40-kDa enzymes) cleave the substrate once and then release the two fragments. By contrast, proteasomes (and other ATP-dependent proteases) are highly processive *(33)*, i.e., they cut polypeptides at multiple sites without the release of polypeptide intermediates. They degrade the proteins all the way down to small peptides ranging from 2 to 24 residues in length, with a median size of six to seven residues *(34)*. The 26S proteasome consists of a cylindrical proteolytic particle, the 20S (720-kDa) proteasome, in association with one or two 19S (890-kDa) regulatory complexes *(35)*, also termed PA700. These complexes associate with each other in an ATP-dependent process *(14,31)*. It remains unclear whether the symmetric (19S-20S-19S) complexes or the asymmetric, singly capped (19S-20S) complexes have different functional properties and are of equal importance in protein breakdown in vivo.

Free 20S proteasomes also exist in mammalian cells *(36,37)*, but when isolated by gentle approaches, they are inactive against peptide or protein substrates *(14,31)*. In the absence of the 19S particle, the 20S proteasomes are not ATP-dependent and are unable to degrade ubiquitin-conjugated proteins. Thus, they are unlikely to play a major role in intracellular proteolysis, which in vivo is largely an ATP-dependent process *(5,15,38)* and generally requires substrate modification by ubiquitination. Additional forms of the proteasome exist in vivo; for example, the cytokine interferon-γ induces a heptameric ring complex, PA28 (also termed Reg), which enhances peptide entry and exit in vivo *(39)*. Also, single-capped 26S particles may associate with the PA28 (11S) complex *(36)* to form hybrid 19S-20S-PA28 complexes *(40)*. These structures enhance the production of MHC class I-presented antigenic peptides by proteasomes *(41)* in most cells during inflammation and constitutively in immune tissues *(42,43)*.

The 20S proteasome is a hollow, cylindrical particle consisting of four stacked rings. Each outer ring contains seven distinct but homologous α-subunits; each inner ring contains seven distinct but homologous β-subunits *(5,14,31,44)*. Three of these β-subunits contain proteolytic active sites, which were first identified by X-ray diffraction as the sites of binding of a peptide aldehyde inhibitor *(45)*. These active sites face the inner chamber of the cylinder. Because the outer walls of the proteasome are very tightly packed, the only way for substrates to reach this central degradative chamber or for products to exit is by passage through the narrow gate channels in the α-rings *(45,46)*. This channel is too narrow to be traversed by tightly folded globular proteins; therefore, the breakdown of most proteins requires their unfolding prior to translocation into the core particle *(47)*.

This entry channel in the α-ring is tightly regulated. It has long been recognized that 20S proteasomes can be isolated in an active or an inactive (latent) form, which can be activated by various treatments (e.g., low concentrations of detergents, heat, dialysis against low ionic strengths). In the inactive 20S proteasomes, these entry channels are closed, as demonstrated by X-ray diffraction *(45)*, whereas the active forms all have open gates allowing substrate entry. Spontaneous gate opening and activation are inhibited by intracellular concentrations of potassium (*48*; K.M. Woo and A.L. Goldberg, in preparation), and one key function of the 19S (PA700 complex) is to facilitate substrate entry. Binding of ATP by this particle can trigger gate opening as part of the ATP-dependent translocation of substrates into the 20S particle (*48*; I. Bize and A.L. Goldberg, in preparation). The diameter of this gate in the α-ring also influences the sizes of products generated during proteolysis *(48)*. In fact, the interferon-γ-induced complex, PA28, increases the yield of peptides appropriate for antigen presentation in part by promoting gate opening *(39)* and allowing larger products to exit *(49)*. In addition, small hydrophobic peptides allosterically trigger gate opening, which may represent a mechanism by which peptide products exit *(50)*.

Because the particle's active sites are localized on its inner cavity, this architecture must have evolved to prevent the uncontrolled destruction of cellular proteins. Similarly, the 19S complexes, as well as substrate ubiquitination, may be viewed as mechanisms that ensure the entry only of substrates into the 20S proteolytic core particle in a highly selective, carefully regulated manner.

2.2. The 19S Regulatory Particle and the Role of ATP

Dramatic progress has been made recently in defining the composition of the 19S (PA700) regulatory particle and the functions of individual subunits *(11,31)*. The 19S particle can be separated into a base and a lid *(11,51)*. The lid contains at least nine polypeptides, including multiple isopeptidases that disassemble the poly-ubiquitin chain, allowing free ubiquitin to be reutilized in further rounds of proteolysis. The removal of the ubiquitin chain is an ATP-dependent process catalyzed by a specific subunit (rpn11), and this step is essential for ATP-dependent degradation of the substrate *(52,53)* by the 20S particle. The base, which associates with the 20S particle, contains eight polypeptides, including six homologous ATPases, which serve multiple functions. They interact directly with the α-rings of the 20S, allowing ATP-dependent opening of the channel, which is essential for polypeptide entry into the proteolytic chamber. In fact, one ATPase (Rpt2) subunit appears to be especially important in regulating this gating process *(48)*.

Table 2
Essential Roles of ATP in Protein Degradation by 26S Proteasome[a]

Association of 19S regulatory complex with 20S proteasome
Unfolding of globular protein
Translocation of protein substrates into the 20S particle
Gate-opening into 20S particle (especially rpn2)
Binding of ubiquitin chains
Action of isopeptidase (rpn11)

[a]Based on studies with 26S complex or PAN-20S complex.

The ATPases also have chaperone-like functions that enable them to bind a polypeptide substrate, trigger gate opening, unfold a globular protein, and catalyze protein translocation into the 20S proteasome *(54)* (Table 2).

Much has been learned about the role of ATP hydrolysis by studying the analogous complexes from archaebacteria, which lack ubiquitin but contain simpler forms of the 20S proteasomes *(55)*. These particles function in protein breakdown together with the hexameric ring ATPase complex termed PAN, whose subunit is highly homologous to the ATPases in the base of the 19S complex *(56,57)*. PAN shares more than 40% identity with the six ATPases in the 19S complex and thus appears to be the evolutionary precursor to the 19S base, as well as regulated proteasome function before proteolysis became linked to ubiquitination in eukaryotes.

These ATPases are all members of the AAA family of multimeric ATPases *(58)*, which includes the ATP-dependent protease Lon and the regulatory components of the bacterial ATP-dependent proteases ClpAP, ClpXP, and HsIUV, which catalyze protein breakdown in bacteria and mitochondria *(59)*. Like these enzymes, PAN increases its rate of ATP consumption several fold when it binds an appropriate substrate *(54)*. This complex of ATPases has been shown to catalyze ATP-dependent unfolding of the globular model substrate GFPssrA *(60)*. This process occurs somehow on the surface of the ATPase ring *(61)*, but its mechanism is completely unclear. In addition, the ATPase complex is necessary for the ATP-dependent entrance of substrates, even denatured proteins, into the core proteasome. These substrates appear to be translocated through a central opening in the ATPase ring and then through the gate in the α-ring *(61)*. Some proteins enter exclusively in a C to N direction, whereas others are translocated in an N to C direction (Navon and Goldberg, in preparation). Recent studies have determined the actual amounts of ATP utilized during degradation of model proteins by this complex. Surprisingly, for the model unfolded substrate casein and the tightly folded protein GFPssrA, the same amount of ATP is hydrolyzed, about 350 ATP molecules/molecule of the protein, which is perhaps a third of what is consumed by the ribosome in synthesis of a polypeptide of this size *(54)*. The inhibitors of the proteasome available now all inhibit the active sites of the 20S particle, primarily the chymotrypsin-like site. However, the 26S particle contains many other subunits and enzymatic activities in its 19S component. Therefore, it seems very likely that this particle contains many other possible targets for selective inhibition and perhaps drug development in the future. At least in theory, agents affecting the 19S

particle might even be anticipated to affect degradation of different substrates of the proteasome differentially.

2.3. The Proteasome's Unusual Proteolytic Mechanism

Proteasomes comprise a new class of proteolytic enzymes called threonine proteases, whose catalytic mechanism differs from that of other types of proteases *(29,31)*. The active sites in proteasomes utilize the N-terminal threonines of certain β-subunits as the nucleophile that attacks peptide bonds. The proteasome thus is an N-terminal hydrolase, a family of enzymes that have similar three-dimensional structures and utilize the side chains of their N-terminal serine, threonine, or cysteine residues to cleave various amide bonds *(62)*. Much of our understanding of this unique proteolytic mechanism has come through studies using proteasome inhibitors *(29)* and site-directed mutagenesis *(8,63)*. The first strong evidence that the threonine hydroxyl is the catalytic nucleophile was the finding by X-ray diffraction that a peptide aldehyde inhibitor (ALLN) forms a hemiacetal bond with the hydroxyl group of the N-terminal threonines of the proteasome's β-subunits *(45,64)*. Also, mutation of this threonine to alanine completely abolished the activity of the proteasome, whereas mutation to a serine retained significant activity against small peptides *(8,65)*. This catalytic threonine residue is covalently modified by different irreversible proteasome inhibitors, lactacystin *(66)*, vinyl sulfones *(67)*, and epoxyketones *(68)*.

The proteasome thus lacks the catalytic triad characteristic of serine and cysteine proteases *(36)*. Instead, the free N-terminal amino group of catalytic threonine is likely to accept the proton from the side chain hydroxyl. To summarize the proteasome's catalytic mechanism: First, the hydroxyl group of the catalytic threonine directly attacks the scissile bond, resulting in the formation of the tetrahedral intermediate, which then collapses into an acyl enzyme with the release of the first reaction product. Deacylation of the catalytic threonine residue by water leads to the release of the second peptide product and the regeneration of the free N-terminal threonine on the proteasome's active site.

3. PHYSIOLOGIC FUNCTIONS OF THE PROTEASOME

Prior to the development of proteasome inhibitors, the functions of the ubiquitin-proteasome pathway and its different cellular roles were studied primarily by biochemical methods or by genetic analysis of yeast mutants defective in this process. The degradation of a model protein was typically studied using cell-free extracts (especially from mammalian reticulocytes or, more recently, frog oocytes). These approaches, unfortunately, are often technically difficult, and genetic analysis can be quite time-consuming. Also, many complex cellular processes involving the proteasome have to this day never been reconstituted in cell extracts and cannot be studied in yeast (e.g., antigen presentation or muscle atrophy). The availability since 1994 of specific inhibitors of the proteasome that enter intact cells and block or reduce its function *(28)* has allowed much more rapid analysis of the role of the proteasome in the breakdown of specific proteins and in complex cellular responses *(28–30)*. Thus, if such inhibitors prevent a decrease in activity of an enzyme or cause an increase in the cellular content of a protein, then proteasome-mediated degradation is very likely to play a key role, especially if these inhibitors cause the protein to accumulate in a ubiquitin-conjugated, high-molecular-weight form. However, further biochemical analysis of the process is still necessary to

identify the responsible ubiquitination enzymes and the critical regulatory factors (e.g., kinases that may trigger ubiquitination and proteasomal degradation).

3.1. Proteasomes Degrade Short-Lived Regulatory Proteins

The nonlysosomal ATP-dependent proteolytic system, which we now call the ubiquitin–proteasome pathway, was first discovered as the system responsible for the selective degradation in mammalian reticulocytes of proteins with highly abnormal conformations *(9)*. Such abnormal proteins may result from nonsense or missense mutations, intracellular denaturation, damage by oxygen radicals, or failure of polypeptides to fold correctly. Both prokaryotic and eukaryotic cells have evolved mechanisms to degrade such proteins selectively, whose accumulation could be highly toxic. This process is particularly important in various human inherited diseases, for example, in the various "unstable hemoglobinopathies" and cystic fibrosis, in which the mutant protein fails to accumulate because it is degraded very rapidly. It is also likely that a failure of this degradative process somehow plays a major role in the accumulation of abnormal polypeptides that form toxic aggregates in various neurodegenerative diseases *(69)*. Interestingly, treatment with proteasome inhibitors prevents the rapid degradation of abnormal proteins and causes the accumulation of aggregates of the abnormal proteins resembling the inclusions seen in such diseases *(69)*.

Another critical role of the ubiquitin–proteasome pathways is in the degradation of various short-lived regulatory proteins, including many transcription factors, oncogene products, tumor suppressors, cell-cycle regulatory proteins (e.g., the various cyclins and cyclin-dependent kinase-inhibitors), and rate-limiting enzymes (Table 3) *(10,11)*. These proteins have evolved short half-lives, because their rapid degradation is important for regulation of cell growth and metabolism. Such proteins turn over within minutes after synthesis or, at most, with half-lives of a few hours. Thus, their levels can rise or fall rapidly with changes in physiologic conditions *(5)*, and they can serve as timing devices (e.g., in the cell cycle). Many of these important functions of the ubiquitin–proteasome pathway were uncovered initially by biochemical or genetic approaches but have been firmly established in cultured mammalian cells by treatment with proteasome inhibitors (e.g., MG132 or lactacystin). These inhibitors block up to 90% of the degradation of abnormal and short-lived proteins *(26)*. Moreover, studies with these inhibitors have uncovered many additional such substrates of the proteasome and have tremendously advanced our understanding of the role of protein degradation in normal and disease states, as discussed below *(10,11)*. The ability of bortezomib to promote apoptosis is probably due to its stabilization of certain key short-lived regulatory proteins (e.g., p53 or p27) and perhaps also to the stabilization of abnormal polypeptides whose accumulation can be toxic.

For a detailed discussion of the proteasome's regulation of NF-κB, *see* Chapter 6.

3.2. Proteasomes Degrade the Bulk of Cell Proteins

Although significant numbers (perhaps 20%) of newly synthesized proteins are short lived, with half lives of less than 3 h, most cell proteins are much more stable, with half-lives of many hours or days. The degradation of such long-lived proteins had long been believed to occur within lysosomes, an incorrect conclusion still stated in many textbooks. However, the long-lived and short-lived cell proteins show a similar ATP depen-

Table 3
Important Regulatory Proteins Rapidly Degraded by the 26S Proteasome

Oncogenic products and tumor suppressors

p53 and MDM2
c-fos
c-jun
c-Mos
E2A proteins

Cell cycle regulatory proteins

CDK inhibitors (p27, p21, and others)
Cyclins (mitotic cyclins, G1 cyclins, and others)

Transcriptional regulators

β-catenins
IκB and NK-κB (p105)
HIF1 (hypoxia-inducible factor 1)
ATF2 (activating transcription factor 2)
STAT proteins

Enzymes

DNA topoisomerase
Ornithine decarboxylase
Receptor-associated protein kinases
RNA polymerase II large subunit
IRF2 (iron regulatory protein 2)

dence and a similar sensitivity to proteasome inhibitors *(26,70)*. Thus, 80–90% of long-lived proteins in cultured mammalian cells, under optimal nutritional conditions, are also degraded by the proteasome pathway. By contrast, inhibitors of the lysosome block only a small fraction (10–20%) of the total protein degradation, perhaps only the breakdown of endocytosed membrane-associated components *(26)*. (In fully differentiated cells, such as liver cells, however, the lysosomes, through autophagic vacuole formation, may account for a larger fraction of degradation, especially upon cell starvation or glucagon treatment.) Although proteasomal involvement in the breakdown of these long-lived components is clear, it still remains uncertain whether their degradation also requires ubiquitination of the substrate molecules, as is required for degradation of most short-lived cell proteins.

This involvement of the proteasome in the degradation of most cell proteins has had important implications for energy homeostasis in mammals and for evolution of the immune system. Cell proteins, especially proteins in skeletal muscle, also serve as a reservoir of metabolizable substrates, amino acids, that can be used for glucose production under poor nutritional states *(1,2)*. The activation of this degradative pathway in muscle during fasting or systemic disease helps mobilize essential amino acids from cell proteins for glucogenesis and is regulated by glucoregulatory hormones (e.g., insulin glucocorticoids). In muscles, the degradation of most cell proteins is normally quite slow unless it is activated by hormones (e.g., glucocorticoids) or cytokines (tumor necrosis

factor, interleukin-1). In fact, whether a muscle cell grows or atrophies is determined largely by the overall rate of proteolysis in the tissue. For example, it is now well established that the major cause of the muscle wasting seen in fasting, in cancer cachexia following denervation, and in patients with sepsis is the excessive proteolysis caused by a general activation of the ubiquitin–proteasome pathway *(2)*. Atrophying muscles show a series of adaptations indicating an activation of this pathway, including increased expression of ubiquitin and many proteasome genes, an accumulation of ubiquitinated proteins, the induction of specific E3s *(23,24)*, and more rapid ubiquitin conjugation in cell-free extracts *(2)*. Also, studies with proteasome inhibitors have proved that enhanced degradation of normally long-lived muscle proteins is the primary mechanism leading to the rapid loss of muscle mass in cancer and other disease states. In fact, as discussed above, these insights were the original justification for mounting an effort to synthesize proteasome inhibitors for therapeutic purposes.

3.3. Proteasomes and MHC Class I Antigen Presentation

Eukaryotic cells contain two primary systems for degrading proteins in the nucleus and cytosol—the ubiquitin–proteasome pathway and the lysosomes in animal cells (or the vacuole in plants and yeast). In higher vertebrate cells, these two major proteolytic systems also function in the generation of antigenic peptides presented to the two functional arms of the immune system. The breakdown of extracellular proteins by the lysosome–endosome pathway is the source of antigenic peptides that are presented on MHC class II molecules and elicit antibody production. Similarly, some of the peptides generated during breakdown of intracellular proteins by the proteasome are transported into the endoplasmic reticulum and are delivered to the cell surface bound to MHC class I molecules for presentation to cytotoxic T-lymphocytes. This process allows the immune system to monitor continually for non-native proteins within cells that may arise during viral infection or cancer. If non-native (e.g., viral and oncogenic proteins) epitopes are presented on the cell surface, then the presenting cells are quickly killed by cytotoxic T-cells.Another important finding that linked proteasome function to immune surveillance was the discovery by many laboratories that specialized forms of the 20S and 26S particles, often termed immunoproteasomes, are induced upon exposure of cells to interferon-γ and certain other cytokines (ror review, see ref. *71*). With time, these alternative forms replace the normal species in most tissues. These immunoproteasomes are found constitutively in spleen, thymus, and presumably other immune cells. They differ from normal 20S particles in containing three alternative interferon-γ-induced β-subunits, termed LMP2, LMP7, and MECL1, which are incorporated in newly synthesized proteasomes. They encode the active sites, and their incorporation in place of the normal subunits enhances the particle's ability to cleave proteins after hydrophobic and basic residues and reduce cleavages after acidic amino acids *(72)*. These alterations do not influence the rate at which proteins are degraded, but rather change the nature of the peptides generated *(40,49)*. As a consequence, more peptides are produced with C-termini that are appropriate for binding to MHC class I molecules (which require ligands with hydrophobic and basic C-termini). Surprisingly, these alternative subunits in immunoproteasomes also influence the length of antigenic peptides generated *(49)*.

The involvement of the proteasome in the generation of antigenic peptides had been proposed but remained controversial until the proteasome inhibitors were introduced

(26,71). Peptide aldehyde inhibitors (MG132) and lactacystin, at concentrations that block the ATP-dependent degradation of cell proteins, were shown to prevent MHC class I presentation of an antigenic peptide (SIINFEKL) derived from a microinjected protein, ovalbumin. These inhibitors prevented the generation of the antigenic peptide but did not affect its transport into the endoplasmic reticulum (ER) or delivery to the cell surface. Moreover, the presentation of most antigenic peptides was also blocked by proteasome inhibitors. Thus, the great majority of MHC class I-presented peptides are generated by 26S proteasomes during the course of protein breakdown, in accord with our previous demonstration that protein ubiquitination is important in class I antigen presentation *(73)*.

Nearly all MHC class I peptides are 8–9 (or occasionally 10) residues long. This length is necessary for a peptide to fit within the groove in the class I molecule. By contrast, 70% of proteasome products are too small to function in this process, whereas perhaps 15% are too large unless trimmed by cellular enzymes *(34)*. Recent studies with proteasome inhibitors have led to the surprising discovery that proteasomes, although essential for the generation of the C-termini of most antigenic peptides, are not required for the production of their N-termini *(43,71)*. In other words, the proteasome, while degrading polypeptides, generates longer precursors of the presented peptides with N-terminal extensions *(43,49,71)*. The proteolytic trimming of these N-extended precursors is then catalyzed by cellular aminopeptidases to generate the presented eight- or nine-residue peptide. In fact, in leukocytes normally and in all cells in inflammatory states, the specialized forms of proteasomes induced by interferon-γ (termed immunoproteasomes) and the PA28 proteasome–activator complex both favor the production of such N-extended antigenic precursors, which require subsequent trimming before association with MHC class I molecules. In addition, interferon-γ signals induction of two aminopeptidases, leucine aminopeptidases in the cytosol *(74)* and a novel aminopeptidase, which we named ERAP1 *(75)* in the ER, that trim the N-extended peptides to the presented epitopes *(75,76)*. Thus, multiple proteolytic enzymes are involved in the production of antigenic peptides, and each of these steps is altered in inflammatory states by interferon-γ so as to enhance the efficiency of production of antigenic peptides *(43)*. Although proteasome inhibitors in vivo can block the generation of most MHC class I-presented peptides in patients or animals receiving therapeutic doses of these inhibitors, no immune deficiencies have been observed, presumably because the inhibition of proteasome function is only partial and of limited duration.

3.4. Proteasome Inhibitors and the Heat-Shock Response

It has long been known that in all cells an increase in the ambient temperature leads to the induction of a characteristic group of stress proteins, known as heat shock proteins. This adaptive response is induced not only by high temperatures, but also by a variety of stressful conditions that damage cell proteins, including exposure to heavy metals or oxygen radicals or incorporation of amino acid analogs *(69)*. The common feature of these various conditions is that they all cause the accumulation in cells of unfolded or denatured proteins. Accordingly, the expression or microinjection of an unfolded protein into intact cells causes the induction of heat shock proteins *(76,77)*. Therefore, the capacity of the ubiquitin–proteasome pathway to degrade such unfolded proteins rapidly is a major factor that indirectly suppresses the expression of heat shock proteins. However, if this degradative process is blocked with proteasome inhibitors, an accumulation of

unfolded proteins should occur, and as a result, induction of the heat shock response *(72,78,79)*. The resulting changes in expression of heat shock genes leads to production of various molecular chaperones, which promote protein folding, and of ubiquitin and other components of the degradative machinery *(76)*. Thus, cells are better able to cope with the onslaught of unfolded proteins. A similar protective response, termed *the unfolded protein response*, occurs when abnormal proteins arise in the ER *(73)* and enhances the level of chaperones in the ER. When cells are exposed to proteasome inhibitors, both responses occur *(80)*. As a consequence, the cells show an enhanced capacity to refold such damaged proteins, to prevent their aggregation, and to degrade the abnormal polypeptides.

The treatment of cells with proteasome inhibitors leads to a coordinate induction of many, if not all, cytosolic heat shock proteins, as well as various molecular chaperones in the ER *(72,79)*. This effect involves increased transcription of these genes and is seen within 1–3 h of exposure of mammalian cells to various proteasome inhibitors. In mammalian cells, this induction of heat shock proteins was shown to be mediated by the activation of the heat shock transcription factors (HSFs). Thus, the ubiquitin–proteasome pathway seems to degrade a short-lived transcription factor(s) (e.g., an HSF) that is stabilized by these inhibitors, leading to the induction of heat shock proteins. It is well established that the induction of heat shock proteins is a protective response that enhances cellular resistance to high temperatures and other highly toxic agents (e.g., oxygen radicals). Accordingly, exposure to the proteasome inhibitors dramatically increased the cell's resistance in mammalian cells and yeast to many lethal insults, such as exposure to heat, high concentration of ethanol, or oxygen radicals *(72,79)*. The magnitude of this protective effect depends on the duration and the extent of the inhibition of proteolysis. This effect may contribute to some of the therapeutic actions of the proteasome inhibitor. However, it is noteworthy that the continual accumulation of abnormal proteins in the cytosol or ER (e.g., as occurs in cells at higher temperatures and cells treated with proteasome inhibitors) eventually can exceed the protective capacity of these responses and can trigger apoptosis by activating JNK-kinase *(81)*. Such a mechanism may also be occurring and may be important in the anticancer actions of bortezomib, especially in myeloma cells, which generate large amounts of aberrant, rapidly degraded immunoglobulins.

3.5. Proteasomes Degrade Abnormal Secretory and Membrane Proteins

Another unexpected discovery resulting from the use of proteasome inhibitors has been that cytosolic proteasomes are also responsible for the rapid degradation of many membrane or secretory proteins during their passage through the ER. The presence of this degradative process and its importance in quality control in the secretory pathway had been recognized for some time, but it had been attributed to an unidentified degradative system within the ER. However, recent studies with the proteasome inhibitors and genetic analysis of yeast mutants have led to the recognition that many such proteins, if they are not folded properly or if they fail to bind cofactors or form correct oligomeric structures, are translocated from the ER to the cytosol for ubiquitin-dependent proteasome-mediated proteolysis *(82,83)*. For example, the human cystic fibrosis transmembrane conductance regulator (CFTR) *(84,85)*, mutant forms of human α_1-antitrypsin *(86)*, unlipidated apolipoprotein B *(87)*, and MHC class I molecules in cytomegalovirus-in-

fected cells *(88)* are rapidly degraded by the ubiquitin–proteasome pathway and stabilized by treatment with proteasome inhibitors. In addition to serving in quality control, this process is responsible for the tightly regulated degradation of ER-bound enzymes, such as 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, the key enzyme in cholesterol biosynthesis.

The selective destruction of these misfolded membrane or mutant secretory proteins by the ubiquitin–proteasome pathway requires that they be translocated back into the cytoplasm. In some cases, proteasome inhibition leads to an accumulation of the nondegraded proteins in the cytosol, whereas in others, their extraction from the ER is also blocked. The Sec61 complex, which functions in the translocation of polypeptides into the ER, is also a key component of this retrograde transport system. Other components in the ER, including the ER chaperones BiP, calnexin, and a novel ER membrane protein Cue1p, are also required for this process *(89)*. Apparently, these substrates can be ubiquitinated while in the membrane, but their extraction from the membrane seems to require the function of the ATP-dependent molecular chaperone p93 (cdc48) *(90)*. However, it remains to be elucidated how this translocation out of the ER takes place and which other components are required for this process. It is noteworthy that the cell's capacity to carry out the process is enhanced by exposure of cells to proteasome inhibitors, but if this adaptation (termed the *unfolded protein response*) fails to prevent the continued accumulation of abnormal proteins, the apoptotic cell program is activated *(91)*.

For a detailed discussion of proteasome inhibition and apoptosis, *see* Chapter 10.

4. CONCLUSIONS

This chapter has described the scientific origins of the development of bortezomib and reviewed our knowledge of how the proteasome functions both as an isolated molecular machine and in vivo as the key site for degradation of cell proteins. Aside from their inherent scientific interest and exciting medical promise, these stories nicely illustrate several truths about medical research, namely that:

1. Improved therapies (especially in cancer) are tightly linked to advances in our understanding of basic biochemistry and cell biology.
2. The teamwork possible between industrial applied scientists and academic investigators in biotechnology companies can achieve practical ends impossible in university settings.
3. The paths to scientific progress are often unpredictable. I certainly never anticipated that in studying the mechanisms of muscle wasting or the selective degradation of abnormal proteins in *E. coli* that this work might somehow lead to the discovery of the proteasomal apparatus, or that this finding would, in turn, lead to insights about immune surveillance or even indirectly to novel therapies for cancer. In fact, had we ever suggested in a grant proposal that this research program might have such benefits, every granting agency or study section would have rejected such statements as fantasy, nonsense, or pure hogwash. It would be good if the lessons clearly illustrated by the development of proteasome inhibitors were appreciated by the governmental, private, and industrial offices that decide on research policies.

ACKNOWLEDGMENTS

These studies have been supported by grants from the National Institutes of Health (NIGHS), the Muscular Dystrophy Association, the National Space Biology Research

Institute, and the Hereditary Disease Foundation. The author is very grateful for the expert assistance of Sarah Trombley in preparing this manuscript.

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