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Targeting proteins for degradation

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Abstract

Protein degradation plays a central role in many cellular functions. Misfolded and damaged proteins are removed from the cell to avoid toxicity. The concentrations of regulatory proteins are adjusted by degradation at the appropriate time. Both foreign and native proteins are digested into small peptides as part of the adaptive immune response. In eukaryotic cells, an ATP-dependent protease called the proteasome is responsible for much of this proteolysis. Proteins are targeted for proteasomal degradation by a two-part degron, which consists of a proteasome binding signal and a degradation initiation site. Here we describe how both components contribute to the specificity of degradation.

Introduction

The proteasome faces a challenge when selecting proteins to degrade. In the classical examples of substrate specificity, enzymes recognize a defined three-dimensional surface. For example, restriction enzymes and conventional proteases recognize specific nucleotide or amino acid sequences in the substrates they cleave. The proteasome, however, has to be able to digest hundreds of unrelated proteins and even foreign proteins the cell has not seen before. Therefore, it cannot have strong sequence preferences for its substrates. On the other hand, the proteasome is located in the cytosol and the nucleus, where it is exposed to the entire complement of cellular proteins. Therefore, it must be exquisitely specific and discriminatory to avoid reckless protein destruction.

The solution to this problem lies in the structure of the proteasome. The proteasome contains six proteolytic sites, which individually show weak sequence preferences in their substrates but together are able to degrade almost all proteins¹. The cleavage sites are sequestered inside the proteasome, where they are inaccessible to most folded proteins. Only substrates that contain specific degradation signals, or degrons, are recognized by the proteasome, processively unfolded², threaded into the degradation chamber, and digested. Thus, the proteasome controls access to the proteolytic sites. An overwhelming number of factors

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affect how proteins are targeted to the proteasome. In this article, we will try to put these into a simple mechanistic framework.

The proteasome

The proteasome is a large cylindrical particle consisting of at least 33 subunits, with a total molecular weight of approximately 2.5 MDa^{3,4}. There are several variants of the proteasome that perform slightly different functions. For example, cells of the immune system contain a particular form of the proteasome that produces peptides for display at the cell surface⁵. We will focus on the version of the proteasome that is found in all cells and is responsible for the specific degradation of regulatory proteins and the removal of damaged proteins. This form, called the 26S proteasome, is composed of a 20S core particle capped by a 19S regulatory particle^{3,4} at one or both ends (Fig. 1a). In bacteria, a series of ATP-dependent proteases share a common structural design with the proteasome and fulfill equivalent functions (Fig. 1b). The proteasome and these bacterial proteases are distantly related and belong to the group of AAA+ proteins (ATPases Associated with diverse cellular Activities).

The 20S core particle is a stack of four heptameric rings, which are assembled to form a cylindrical structure³. The outer two rings are made of α subunits, and the inner two rings are composed of β subunits, which contain the proteolytic active sites in a central cavity (Fig. 1a). The degradation chamber can be reached through a channel that runs along the long axis of the core particle. The entrance to the channel is narrow, about 13 Å^{6,7}, such that folded proteins must be at least partially unfolded before they can be translocated into the 20S core particle and degraded⁸.

The 19S regulatory particle is composed of at least 19 subunits arranged into two subcomplexes – the lid and the base. The regulatory particle contains ATPase subunits, gates the entrance to the degradation channel, and plays a role in substrate recognition, unfolding, and translocation into the 20S core particle^{4,5} (Fig. 1a).

The degron

The degron that leads to the degradation of a folded protein has two parts. Most proteins appear to be targeted for degradation by the covalent attachment of a tag that consists of several copies of the small protein ubiquitin^{9,10}. However, ubiquitinated proteins are stable, at least *in vitro*, unless they also contain an unstructured region¹¹, which is then the second component of the degron. The ubiquitin modification delivers the substrate to the proteasome and the unstructured region is the site at which degradation initiates¹¹. Typically both parts of the degron are present in the same protein. However, the ubiquitin tag and initiation site also work when they are located on two distinct polypeptide chains that bind to each other to form a complex¹².

The ubiquitin tag

Ubiquitin is attached to proteins by a series of three enzymatic activities. Individual ubiquitin molecules are activated by ubiquitin activating enzymes (E1s), transferred to

ubiquitin conjugating enzymes (E2s) and from there to the substrate, which is recognized by ubiquitin protein-ligase enzymes (E3s), reviewed in¹⁰. Depending on the type of E3, the ubiquitin is either transferred from the E2 to the E3 and then to the substrate, or directly from the E2 to the substrate. Ubiquitin is almost always attached to the substrate through an isopeptide bond between the α -carboxyl group of the ubiquitin backbone and the ϵ -amino group of a lysine in the substrate. In a few rare cases ubiquitin has been found to be conjugated to N-terminal residues¹³ or cysteines¹⁴. Oddly enough, the modification reaction does not usually stop after the first ubiquitin is attached to a protein but continues so that an additional ubiquitin moiety is attached to a lysine of the first ubiquitin, another ubiquitin is attached to this second one, and so on. At times, an additional enzyme, called an E4, is involved in this reaction, reviewed in¹⁵. As a result, long ubiquitin chains form on substrates.

There are seven lysines in ubiquitin so polyubiquitin chains can form through different linkages, reviewed in¹⁶. In the classical view, the minimal signal necessary for proteasome targeting is a chain of four ubiquitin molecules linked through lysine 48 (K48)⁹. However, ubiquitin tags where the ubiquitin moieties are linked through other lysine residues such as K11¹⁷, K63^{18,19}, and probably others²⁰ can also target proteins for proteasomal degradation. In a few cases, it appears that monoubiquitination is sufficient to target a protein for proteasome-mediated degradation²¹⁻²³ but it is not clear if this targeting mode is widespread. Polyubiquitin chains linked through every lysine in ubiquitin are present *in vivo* in yeast^{20,24}. Some of these polyubiquitin modifications, and the attachment of a single ubiquitin, have functions outside of degradation, such as in membrane trafficking²⁵. Indeed, ubiquitination is emerging as a general and transferable protein-protein interaction signal, not unlike phosphorylation²⁶.

Delivery of ubiquitinated substrates to the proteasome

Ubiquitinated proteins are delivered to the proteasome by various routes and the complete picture of how these pathways fit together has yet to emerge, reviewed in²⁷. Some substrates bind directly to the proteasome by interacting with the 19S regulatory particle subunits Rpn10²⁸ or Rpn13²⁹, and probably Rpt5³⁰ (Fig. 2a). Alternatively, ubiquitinated substrates can be brought to the proteasome by adaptors that bind both the proteasome and the ubiquitin chain on the substrate to deliver it for degradation³¹⁻³³ (Fig. 2b). Interestingly, a large proportion of Rpn10 is not associated with the proteasome and this population of Rpn10 may act much like the adaptor proteins discussed next. Lastly, some proteins are degraded by the proteasome but are not ubiquitinated (Fig. 2c); these proteins will be discussed later.

Three proteasome adaptor proteins, which appear to function in very similar ways, have been identified: Rad23³⁴⁻³⁶, Dsk2^{35,36}, and Ddi1³⁶. All three of these contain a ubiquitin-like (UBL) domain that interacts with the proteasome and one or two ubiquitin-associated (UBA) domains that bind polyubiquitin chains³⁶⁻³⁸. The adaptors can bind the ubiquitinated substrate and proteasome simultaneously, and either guide the substrate directly to degradation by allowing the proteasome to engage the substrate at its initiation site, or by first handing the substrate off to ubiquitin-binding proteasome subunits⁵. It is not clear

which substrates reach the proteasome directly and which flow through adaptors, although it has been suggested that proteins with long ubiquitin chains are more likely to take the direct path³⁹. Genetic studies show that ubiquitin receptors can have overlapping functions and probably cooperate to degrade proteins^{33,40}.

There may be yet other ways in which the proteasome can recognize ubiquitinated substrates. Proteasomal degradation is required for yeast to live, yet yeast strains in which all of the known ubiquitin receptors are inactivated are viable²⁹, indicating that there may be other unidentified ubiquitin receptors. Some E3 ubiquitin-protein ligases bind to the proteasome²⁷, in certain cases through UBL domains⁴¹. Of course, E3s also interact with substrates, so they may not only ubiquitinate their substrates but also deliver them to the proteasome, much like the previously mentioned adaptors²⁷. E3 ligases can also mediate the binding of substrates to adaptor proteins²⁷. For example, the adaptor Ddi1 interacts with the E3 ligase Ufo1, and Ddi1-Ufo1 binding is required for degradation of Ufo1's substrate⁴². The adaptor proteins and many associated proteins involved in protein targeting to the proteasome are present in both mammals and yeast²⁷. Therefore, the overall targeting mechanism appears to be widely conserved.

The ubiquitin modification is dynamic

Although ubiquitin tags are an effective proteasome targeting signal, the conjugation of ubiquitin to proteins does not always lead to their degradation. One reason for this is that the ubiquitin modification can be very dynamic. Cells contain a large number of enzymes that remove ubiquitin chains from proteins and a few of these deubiquitinating enzymes are associated with or part of the proteasome, reviewed in⁴³. One of them, a 19S subunit called Rpn11 in yeast, cleaves the entire ubiquitin chain off of proteins by hydrolyzing the isopeptide bond between the lysine in the substrate and the first ubiquitin moiety in the tag^{44,45}. This cleavage occurs when the substrate is fully committed for degradation⁴⁴ and allows the ubiquitin to escape from the proteasome and be recycled. In fact, protein degradation is impaired upon Rpn11 inactivation^{44,45} suggesting that polyubiquitin tags hinder substrate unfolding and/or translocation into the proteasome⁵.

Two other deubiquitinating enzymes associated with the proteasome, named Ubp6 and Uch37 in yeast, trim ubiquitin chains sequentially from the distal end^{46,47}. This activity probably acts like a timer. When the ubiquitinated substrate is first bound, the proteasome tries to degrade the substrate but at the same time the ubiquitin tag begins to shrink. Eventually, the tag will be removed and if degradation has not yet initiated, the substrate will escape (Fig. 3a). Given that a chain of four K48-linked ubiquitin molecules binds to the proteasome with high affinity and longer ubiquitin chains have only slightly higher affinities⁹, it is likely that the purpose of longer ubiquitin chains is to enhance a substrate's resistance to deubiquitinating enzymes and thus increase the time a protein spends at the proteasome^{9,47}. The activity of Ubp6 and Uch37 likely prevents degradation of lightly or improperly ubiquitinated protein substrates^{47,48}. The deubiquitinating enzymes also prevent "clogging" of the proteasome. If a substrate spends too much time at the proteasome, potentially blocking other proteins from degradation, then the deubiquitinating enzymes will

remove that protein's ubiquitin chain and promote the release of the indigestible substrate, thus preventing a backup.

However, the entire story is more complicated. A ubiquitin ligase with E4 activity, Hul5, is also associated with the proteasome and acts in opposition to the deubiquitinating enzymes^{48,49}. That is, the previously mentioned “timer” is fine tuned by Hul5, which extends the ubiquitin chains on substrates, thereby increasing the time substrates spend at the proteasome and the likelihood of their degradation (Fig. 3b). In this way, the activities of both a ubiquitin ligase and deubiquitinating enzymes at the proteasome play a role in selecting substrates for proteasome-mediated degradation.

One example of how this ubiquitin “timer” could work comes from the mechanism by which degradation of cell cycle regulators is ordered in time. The E3 anaphase-promoting complex (APC) regulates cell cycle progression in mitosis and G1, reviewed in⁵⁰. At the end of mitosis, the APC ubiquitinates a series of substrates to target them for degradation in a specific temporal order, which is known as substrate ordering. This substrate ordering depends on the processivity of ubiquitination by the APC⁵¹. The substrates that obtain their polyubiquitin chains in a single binding event to the APC are degraded first. The substrates that are degraded later require multiple APC binding events to become polyubiquitinated. Because these substrates bind to and dissociate from the APC repeatedly, they are more vulnerable to deubiquitinating enzymes. Thus, it seems that the proteins that receive their polyubiquitin chains first are also degraded earlier.

The Cdc48 / p97 targeting machine

Most ubiquitination and deubiquitination probably occurs away from the proteasome. Many E3 ubiquitin protein-ligases are soluble proteins by themselves or are bound to a small number of partners. However, enzymes that alter ubiquitin modifications can assemble into a large complex, which can function as a protein-targeting machine. A broad range of proteins, including E3 and E4 ligases, deubiquitinating enzymes, and ubiquitinated substrates assemble on Cdc48⁵². Cdc48 (p97 in mammals) is an ATPase ring complex which is distantly related to the ATPases in the 19S regulatory particle²⁷. Cdc48 may determine the fate of a substrate by balancing or regulating its various associated enzymatic activities, reviewed in⁵³. In one scenario, the Cdc48-associated ubiquitin ligases can lengthen the ubiquitin chain on the substrate. The ubiquitinated protein is then shuttled to the proteasome by the adaptor proteins Rad23 and Dsk2 and degraded³⁹ (Fig. 4a). Alternatively, deubiquitinating enzymes recruited by Cdc48 can trim the ubiquitin chain and rescue the protein from proteolysis^{39,53} (Fig. 4b). The subunits of Cdc48 themselves are ATPases and it is possible that Cdc48 can unfold ubiquitinated proteins and facilitate proteasomal degradation by pre-denaturing substrates²⁷.

Cdc48 clearly also has activities other than functioning as a binding platform for the ubiquitination machinery. It is one of the most abundant proteins in cells⁵² and is involved in many pathways, including endoplasmic reticulum (ER)-associated degradation and membrane fusion, reviewed in^{27,52,53}. For example, Cdc48 removes ubiquitinated proteins from protein complexes, and extracts ubiquitinated substrates from membranes such as the

ER membrane and then directs the extracted proteins to the proteasome^{54,55} as described above (Fig. 4a). During the last few years it has become clear that the Cdc48-proteasome pathway plays a much larger role in regulating protein turnover than previously thought⁵².

Initiation of degradation

Ubiquitin modification of a folded protein alone is not sufficient for its rapid degradation^{9,56,57}. In addition to a proteasome targeting signal, a protein must contain a region for degradation initiation^{11,58}. This initiation site is an unstructured region where the proteasome can engage the substrate and begin degradation. It can be at the end of a polypeptide chain¹¹ or internal^{11,59,60} and flanked by folded domains on both sides¹¹. In the simplest mechanism, the ubiquitin modification tethers a substrate to the proteasome and orients it so that the initiation site can be engaged by the proteasome's translocation motor. Based on studies of the bacterial analogues of the proteasome⁶¹⁻⁶⁵, the motor is probably located in the ATPase ring of the 19S regulatory particle⁶⁶. Loops that protrude into the central pore of the ATPase ring most likely grab the unstructured initiation region and then pull on the substrate and cause unfolding and translocation^{62,66} (Fig. 1 and 3).

Proteins that lack an unstructured region can also be degraded because folded domains are in equilibrium with unfolded states. However, if unfolding fluctuations are rare or short-lived the ubiquitin modification may be removed before degradation initiates, and the substrate will dissociate from the proteasome before it is degraded.

Requirements for an effective initiation site

Unstructured regions are common in eukaryotic proteins. Therefore, the requirement for an initiation site is not likely to limit degradation of most proteasome substrates. However, there are situations where the initiation site seems to play a critical role in selecting substrates for degradation. Some proteins can bind to the proteasome or be heavily ubiquitinated, yet escape degradation. For example, the adaptor Rad23 binds to the proteasome³⁸ and delivers substrates for proteolysis, but itself remains stable^{38,67}. Perhaps Rad23 escapes destruction because the proteasome is unable to initiate its degradation. Interestingly, some of the proteins that avoid degradation contain disordered segments, suggesting that not all unstructured regions can promote degradation. There may be specific physical and chemical requirements for initiation sites. For example, they may have to be of a minimum length^{11,68} or be located in the substrate within a certain distance from the ubiquitin tag¹¹. The amino acid composition and thus chemical properties of the unstructured region could also be important^{56,69}.

Trans initiation

The two parts of the degron, the ubiquitin modification and the unstructured initiation site, can function together even when they are separated onto two polypeptide chains¹². This observation is surprising, because the proteasome is able to select, extract, and degrade specific subunits from larger complexes while leaving the other subunits intact. This subunit-specificity of degradation was first described using model proteins^{70,71} and plays a critical role in many biological processes. For instance, as a cell moves through the cell

cycle, cyclin-dependent kinases (cdks) are activated by cyclins at particular steps. For the cell to continue to progress through the cell cycle, the cdks have to be switched off again and this happens through the destruction of the cyclins. The cyclins become ubiquitinated⁷² and are degraded while complexed with the cdk, which itself remains intact^{72,73}.

When the ubiquitin tag and the initiation site are separated onto different polypeptide chains¹², it appears that the proteasome may degrade first the subunit with the most effective initiation site. This idea fits with some examples of subunit specificity. For instance, in the Cdk2-cyclin A complex, Cdk2 has a compact tertiary structure with no unfolded or disordered regions⁷⁴. In contrast, the N-terminus of cyclin A is mostly unstructured, contains the sites of ubiquitination, and is degraded^{72,75,76}. Cdk2 may escape degradation simply because it lacks an effective initiation site for the proteasome.

However, in some instances the mechanism is not quite as obvious, such as in the case of the cell cycle regulator Sic1. Sic1 binds to a cdk-cyclin complex and inhibits its kinase activity. To activate the kinase, Sic1 has to be degraded and cdk-cyclin left intact, but both Sic1 and cyclin contain unstructured regions⁷⁷ (Fig. 5a). The disordered region in Sic1 might be the more effective initiation site because it is closer to the ubiquitin tag. Once the ubiquitinated Sic1 is degraded, the rest of the complex would be safe from proteolysis until the cyclin becomes ubiquitinated in turn. There are some hints as to what physical and chemical factors affect initiation but clearly much work remains to be done to define the rules of initiation site selection^{11,56,68,69}.

Degradation without ubiquitin

The observation of *trans* initiation suggests another interesting targeting mechanism: a ubiquitinated adaptor protein can shuttle many binding partners with initiation sites to the proteasome for degradation and itself remain stable^{27,33,35,38}. This is the biochemical basis of the mechanism proposed for the Rad23, Dsk2, and Ddi1 adaptor proteins described above. This mechanism may also explain the degradation of some other proteins. For example, the antiretroviral cytidine deaminase APOBEC3G (A3G) is targeted for degradation by a viral infectivity factor (Vif). Vif has been shown to be polyubiquitinated, but a lysine-less A3G that cannot be ubiquitinated is still degraded⁷⁸. This could be a case of *trans* targeting, in which the ubiquitinated Vif acts as an adaptor protein to bring A3G to the proteasome, where A3G would then be engaged through its initiation site¹² (Fig. 5b). Another example that may follow a similar mechanism is the human papillomavirus protein E7 and its binding partner, the retinoblastoma tumor suppressor protein (Rb). It is known that E7 binds to and targets Rb for degradation⁷⁹. E7 is ubiquitinated and binds to the proteasome⁸⁰. Ubiquitinated E7 might act as an adaptor that targets Rb for degradation by *trans* initiation¹².

An unstructured region in a protein might under some circumstances function as a proteasome initiation site by itself in the absence of ubiquitin (Fig. 2c). This could occur if the initiation site sequence has a particularly high affinity for the site on the proteasome that engages substrates, or when the proteasome and substrate are present at high concentrations so that even a weak interaction leads to substrate binding.

Indeed, ubiquitin is not always required to target proteins for proteasomal degradation, reviewed in⁸¹⁻⁸⁴. The original and best-understood example of ubiquitin-independent proteasomal degradation is ornithine decarboxylase (ODC)⁸⁵. ODC is a folded protein yet it can be degraded efficiently in the absence of ubiquitin by the full proteasome particle in the canonical ATP-dependent process⁸⁵. This degradation requires the protein antizyme 1 (AZ1), which binds to ODC but itself escapes proteolysis⁸⁶ (Fig. 2c). AZ1 increases the association of ODC with the proteasome⁸⁶. AZ1 binding exposes a 37 amino acid region in the C-terminus of ODC⁸⁶ from which degradation begins⁸⁷. Thus, ODC may be degraded because its initiation site has a particularly high affinity for the proteasome, or because AZ1 functions like an adaptor protein.

There are several additional proteins that may be degraded in the absence of ubiquitin (*e.g.*, p21^{CIP1}, p53, c-Jun, I κ B α , thymidylate synthase, T cell antigen receptor chain α , Fra-1, and Hif-1 α , reviewed in⁸¹⁻⁸⁴). In many cases, the evidence for ubiquitin-independent degradation was obtained *in vitro*, so that the physiological relevance of this degradation is unclear. In fact, the physiological degradation of some of these proteins depends strongly on ubiquitin. Nevertheless, unstructured proteins can be degraded by the 20S core particle in a ubiquitin- and ATP-independent manner, reviewed in⁸³. Although it is not known what forms of the proteasome exist and in what proportion *in vivo*, some singly-capped or uncapped 20S core particles are likely present. At the same time, purified 26S proteasomes are able to cleave some proteins that are not ubiquitinated at internal disordered regions, leaving the structured domains intact⁸⁸.

In summary, ubiquitin-independent degradation, in the context of the two-part degron model, could be explained by the fact that the unstructured regions within the proteins could function as particularly effective initiation sites. Alternatively, the proteins could be degraded because they are shuttled to the proteasome by adaptors or ubiquitinated binding partners.

Parallels with bacterial degrons

In bacteria, regulated protein degradation is carried out by ATP-dependent proteases such as ClpAP, ClpXP, Lon, HslUV, and FtsH, reviewed in⁸⁹⁻⁹¹. These bacterial proteases share a common structural design with the eukaryotic proteasome (Fig. 1b). Some bacteria even use a ubiquitin-like modifier to tag proteins for degradation^{92,93}. This modifier is a 64 amino acid protein found in *Mycobacterium tuberculosis* called prokaryotic ubiquitin-like protein or Pup⁹². Like ubiquitin, Pup targets proteins to a proteasome-like particle^{92,93}. A broad range of proteins appear to be modified by Pup^{92,93} and Pup is present in all major actinobacteria and a few other bacterial lineages⁹⁴. However, most bacteria do not use a ubiquitin-like degradation tag and at first glance, the mechanisms by which proteins are targeted for degradation in eukaryotes, bacteria, and archaea appear to be quite different. But like in eukaryotes, bacterial degrons have two functions: they target proteins to the protease and serve as the initiation site for degradation⁹⁰.

Proteins are targeted for degradation in bacteria by signals in their primary amino acid sequence, often near the N- or C-terminus⁹⁵, reviewed in⁹⁰. Degradation begins at these

degrons and they are therefore in some ways equivalent to the unstructured regions that serve as degradation initiation sites in proteasome substrates. In one particularly intriguing case, the degron is not encoded in the same gene as the substrate protein but is appended during translation by a switch of the template RNA. The switch leads to the synthesis of an 11 amino acid long tail at the C-terminus of the nascent protein and release from the ribosome⁹⁶. The *ssrA* tag targets proteins for degradation by several proteases⁹⁰. Most other bacterial degrons are not well characterized⁸⁹.

In eukaryotes, the substrate is in most cases tethered to the proteasome by the ubiquitin tag. In bacteria, this function can often be performed by the linear degradation signal itself. There are perhaps a few cases where two distinct sequences in a substrate could perform the protease binding and initiation functions^{97,98}. However, parallels with the eukaryotic targeting mechanism are clearest in the use of bacterial adaptor proteins in degradation, reviewed in^{89,90}.

For three bacterial adaptors, it is quite well understood how they regulate protein degradation and deliver proteins to proteases, reviewed in^{89,90}. One example is SspB, which targets the majority of *ssrA*-tagged proteins for degradation⁹⁹ by linking the protein directly to the protease¹⁰⁰. Like eukaryotic adaptor proteins, SspB functions as a shuttle²⁷. The substrate is degraded and SspB remains stable⁹⁰, allowing it to recapture additional proteins for destruction. Sometimes adaptor proteins themselves are regulated. For example, the activity of the adaptor RssB, which controls the σ^S subunit of RNA polymerase, is mediated by several anti-adaptors¹⁰¹⁻¹⁰³.

The adaptor protein ClpS is responsible for N-end rule^{104,105} degradation in bacteria¹⁰⁶. The N-end rule relates the stability of a protein with the identity of its N-terminal amino acid, called the N-degron^{104,105}. ClpS recognizes the N-degron¹⁰⁶ as well as ClpA¹⁰⁷ and shuttles the protein for degradation¹⁰⁶. The N-end rule also functions in eukaryotes, where proteins with N-degrons are recognized and ubiquitinated by E3 ligases called N-recognins, reviewed in¹⁰⁸. The domains in ClpS and the N-recognins that recognize the N-degrons are homologous¹⁰⁹⁻¹¹¹ showing that the N-end rule pathway is conserved between eukaryotes and bacteria even if the mechanistic details by which it functions are very different¹⁰⁸.

The unfolding ability of proteases

The fact that bacteria contain different ATP-dependent proteases allows a variation in the mechanism of substrate selection that is absent in eukaryotes. It appears that bacterial ATP-dependent proteases differ greatly in their ability to unfold substrate proteins¹¹². Some proteins might contain degrons that are recognized by a protease, but if the protease lacks the ability to unfold the substrates, they escape. Thus, information is found in both the targeting signal and in the stability of the folded domains of the substrates¹¹². The unfolding ability of the eukaryotic proteasome is greater than that of the bacterial proteases so under most circumstances proteins are unable to resist degradation. However, it is possible to stabilize some proteins by ligand binding such that they resist even the eukaryotic proteasome⁸. In addition, some protein aggregates that form as part of disease processes may be too stable to be denatured and degraded by the proteasome^{113,114}.

Practical applications

The principles that govern protein targeting to the proteasome can be exploited to control protein degradation artificially and several methods have been developed to manipulate protein stability, reviewed in¹¹⁵. For example, fusion of a target protein to ODC can destabilize the protein and its binding partners¹¹⁶. ODC degradation is ubiquitin-independent so this method does not require ubiquitination for target protein degradation.

Other artificial degradation systems use inducible dimerization domains derived from FKBP and FRB. Both of these domains bind the naturally-occurring small molecule rapamycin such that the addition of rapamycin will cause any two fusion proteins containing these domains to dimerize^{117,118}. Perhaps the most direct implementation of this system has been developed in yeast¹¹⁹, where a proteasome subunit is tagged with the FKBP domain and the FRB domain is fused to the target protein. On addition of rapamycin, the FRB and FKBP domains dimerize and the target protein becomes bound to the proteasome, where it is then degraded¹¹⁹. Like the ODC-fusion method, this system causes degradation of the target protein without ubiquitination.

In a method used to create conditional alleles, a target protein is destabilized by fusing it to a modified FRB or FKBP domain. The altered FRB or FKBP domains act as degrons, most likely because they contain mutations that cause partial unfolding^{120,121}. The target protein can then be stabilized by rapamycin-induced binding of endogenous FKBP to the FRB degron, or small molecule ligand binding to the FKBP degron. In another method, called Split Ubiquitin for Rescue of Function or SURF¹²², the target protein is released completely unmodified following stabilization. Here several FRB domains and half of the ubiquitin coding sequence are fused to the N-terminus of the target protein, which leads to its rapid degradation. The other half of ubiquitin is fused to FKBP. When rapamycin addition causes the dimerization of FRB and FKBP, the two halves of ubiquitin are brought together and form a complete ubiquitin molecule. The reconstituted ubiquitin is then recognized by cellular enzymes that cleave the ubiquitin and FRB domains off the target protein, which is released in its native form.

Another method manipulates the ubiquitin-conjugation machinery itself to artificially target specific proteins for degradation. A protein-binding domain can be fused to an E2 so that the E2 ubiquitinates the protein that is brought into its vicinity¹²³. Similarly, some E3s can be redirected to new targets when their substrate recognition subunits are modified to recognize a specific target^{124,125}. Another possibility is to bring the E3 to a substrate with a bifunctional ligand that recognizes both the E3 and the target protein¹²⁶. These ligands have been called Proteolysis-targeting chimeric molecules or Protacs, and several cell-permeable ligands for different proteins are effective in cell culture¹²⁷.

Lastly, the N-end rule can be used to manipulate the stability of target proteins in yeast. Fusion with a short peptide that leads to the display of a destabilizing N-terminal residue can greatly decrease the half-life of a target protein¹²⁸. Further, if a destabilizing N-terminal residue is masked in a heat labile protein domain, N-end rule degradation can be made

temperature-dependent. Degradation of a protein fused to this domain can then be induced by increasing the temperature¹²⁹.

Conclusion

In summary, we propose that the great complexity of mechanisms by which proteins are targeted for degradation by ATP-dependent proteases can be explained by a simple chemical mechanism. The degron contains two parts: a tether that allows the protease to grab the substrate, and an initiation site where degradation begins. In eukaryotes the tether is usually formed by a reversibly-attached ubiquitin tag, and degradation initiates at an unstructured region in the substrate. However, there are many variations on this theme. For example, sometimes the substrate is recognized through adaptor proteins that bind the substrate and proteasome at the same time. It is possible that the initiation site by itself may be sufficient to target proteins for degradation in some cases. In bacteria, it is common for substrates to be recognized by their initiation sites alone but adaptors that recognize the substrate and protease also play an important role. Covalently attached tags seem to be rarer in bacteria but at least two examples exist^{92,93,96}. Thus, eukaryotes and bacteria appear to have similarly solved the problem of how to utilize a proteolytic process that can hydrolyze nearly all proteins in a controlled and specific manner. Protein targeting and degradation apparently follow the same overall principle in bacteria and in eukaryotes⁹⁰.

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Figure 1.

The overall structure of the eukaryotic proteasome and the bacterial ClpAP protease.

The two proteases have a common architecture. The protease sites are buried in an internal chamber of the core particle, which is capped by regulatory particles that control access to the proteolytic chamber and contain an ATPase hexameric ring.

A: Side-on cross-section of the eukaryotic 26S proteasome. The 20S core particle is flanked by 19S regulatory particles, and the proteolytic sites are located in the β -rings of the 20S core particle. The scaffold proteins Rpn1 and Rpn2, the ubiquitin receptors Rpn10 and Rpn13, and the loops lining the ATPase ring are depicted. Only one set of loops is shown for clarity.

B: Side-on cross-section of the ClpAP protease from *Escherichia coli*. ClpP contains the proteolytic sites and is capped on both ends by the ClpA ATPase ring.

Figure 2.

Mechanisms of targeting ubiquitinated proteins to the proteasome.

A: Some substrates bind directly to the 19S regulatory particle subunits Rpn10 or Rpn13.

B: Ubiquitinated substrates can also be carried to the proteasome by adaptor proteins, which bind both to the ubiquitin modification of the substrate and to the proteasome.

C: Several proteins are targeted to the proteasome independent of ubiquitination. Two cases are shown. On the left, a protein is engaged by the proteasome in the absence of any separate proteasome-binding motif. This might occur if the initiation site has a particularly high affinity for the site on the proteasome that engages substrates. On the right, ornithine decarboxylase (ODC) is shown as it is degraded by the proteasome when bound to antizyme 1 (AZ1). AZ1 binding to ODC exposes a 37 amino acid proteasome recognition signal in the C-terminus of ODC, where degradation then begins. The 37 amino acid signal may function as a high affinity proteasome binding site by itself, or AZ1 may act like an adaptor protein.

Figure 3.

An initiation site is required for protein degradation.

A: A protein lacking an initiation site escapes degradation.

The proteasome-targeted protein does not have an effective initiation site. The deubiquitinating enzymes trim the ubiquitin chain before the proteasome engages the substrate fully and initiates degradation. Thus, the intact substrate is released from the proteasome.

B: A protein containing an effective initiation site is degraded by the proteasome.

A ubiquitinated protein is recognized by the proteasome, and the ubiquitin ligase Huf5 and the deubiquitinating enzymes Ubp6 and Uch37 extend or trim the ubiquitin chain, respectively. The proteasome engages the protein at its unstructured region, which leads to unfolding and translocation of the polypeptide into the degradation chamber. In this case, the proteasome engages the substrate so rapidly that the deubiquitinating enzymes are not able to remove enough ubiquitin moieties to cause substrate release.

Figure 4.

The Cdc48 / p97 targeting machine.

Cdc48 / p97 is an ATPase ring complex involved in a range of cellular processes. It can serve as a scaffold for the assembly of E3 and E4 ligases, deubiquitinating enzymes, and ubiquitinated substrates. The fate of a substrate may be determined by the various enzymatic activities associated with Cdc48.

A: The Ufd1-Npl4 heterodimer recruits a polyubiquitinated substrate to Cdc48. The polyubiquitin chain on the substrate is lengthened by Ufd2, which has E4 activity. The substrate is then passed from Ufd2 to the adaptor protein Rad23 (or Dsk2), which shuttles the protein to the proteasome for degradation. Figure adapted from³⁹.

B: Cdc48-associated deubiquitinating enzymes (DUBs), such as Otu1, can trim the polyubiquitin chain on a substrate and prevent its degradation.

Figure 5.

Degradation of specific subunits from larger complexes.

A: The cell cycle regulator Sic1 binds to a cyclin dependent kinase (cdk)-cyclin complex and inhibits its activity. During the cell cycle, Sic1 is degraded in a process that leaves cdk-cyclin intact despite the fact that both Sic1 and cyclin contain unstructured regions that can serve as initiation sites. Sic1 could be degraded first because its disordered region is a more effective initiation site in this complex.

B: Viral infectivity factor (Vif) targets the antiretroviral cytidine deaminase APOBEC3G (A3G) for degradation. Polyubiquitinated Vif can mediate the degradation of lysine-less A3G that cannot be ubiquitinated. This could occur by *trans* targeting, where the ubiquitinated Vif acts as an adaptor protein to bring A3G to the proteasome. Degradation begins when A3G is engaged by the proteasome at its initiation site.