REVIEW

Substrate selection by the proteasome through initiation regions

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Received 1 April 2019; Accepted 6 May 2019
DOI: 10.1002/pro.3642
Published online 23 May 2019 proteinscience.org

Abstract: Proteins in the cell have to be eliminated once their function is no longer desired or they become damaged. Most regulated protein degradation is achieved by a large enzymatic complex called the proteasome. Many proteasome substrates are targeted for degradation by the covalent attachment of ubiquitin molecules. Ubiquitinated proteins can be bound by the proteasome, but for proteolysis to occur the proteasome needs to find a disordered tail somewhere in the target at which it initiates degradation. The initiation step contributes to the specificity of proteasomal degradation. Here, we review how the proteasome selects initiation sites within its substrates and discuss how the initiation step affects physiological processes.

Keywords: proteasome; ubiquitin; protein degradation; initiation region; protein unfolding

Introduction

To maintain cellular homeostasis, protein abundance is regulated by adjusting the rates of synthesis and degradation. A large fraction of regulated intracellular protein degradation is performed by the ubiquitin proteasome system (UPS), which plays pivotal roles in a variety of cellular processes including cell cycle regulation, membrane trafficking, and DNA repair.[1–3] Proteins are targeted to proteasomal degradation through conjugation of the small globular protein ubiquitin to Lys residues in the substrate.[4–6] The specificity and processivity of the UPS ensure proper cellular function and thus the UPS is indispensable for cells to survive.

The proteasome is a large protein complex with a molecular weight of ~2.5 MDa and consists of a 20S core particle (CP) and 19S regulatory particles (RP, also known as PA700) (Fig. 1) (Unverdorben P et al, PubMed ID: 24706844). In cells, the CP is predominantly capped at either or both ends by a specific RP, forming the holoenzyme known as the 26S proteasome.[7] The CP possesses the proteolytic sites at the surface of an internal chamber.[8] The RP contains three well-characterized ubiquitin receptors (Rpn1, Rpn10, and Rpn13) and thus is responsible for

Significance statement: The ubiquitin proteasome system surveils the proteome. Proteasome substrates are tagged with the small protein ubiquitin, but ubiquitination also signals other cellular fates. Proteolysis requires a disordered region in the substrate at which the proteasome initiates degradation, and this step contributes to target selection. Proteasome failure is associated with neurodegenerative diseases, and methods to induce protein degradation artificially could be a powerful therapeutic strategy.

Abbreviations: CDK, cyclin-dependent kinases; CKI, CDK inhibitor; CP, core particle; CTD, C-terminal domain; DHFR, dihydrofolate reductase; ODC, ornithine decarboxylase; PROTAC, proteolysis targeting chimera; RP, regulatory particles; UPS, the ubiquitin proteasome system; Vif, viral infectivity factor.

Grant sponsor: National Cancer Institute R21CA196456; Grant sponsor: National Institute of General Medical Sciences R01GM124501; Grant sponsor: Welch Foundation F-1817.

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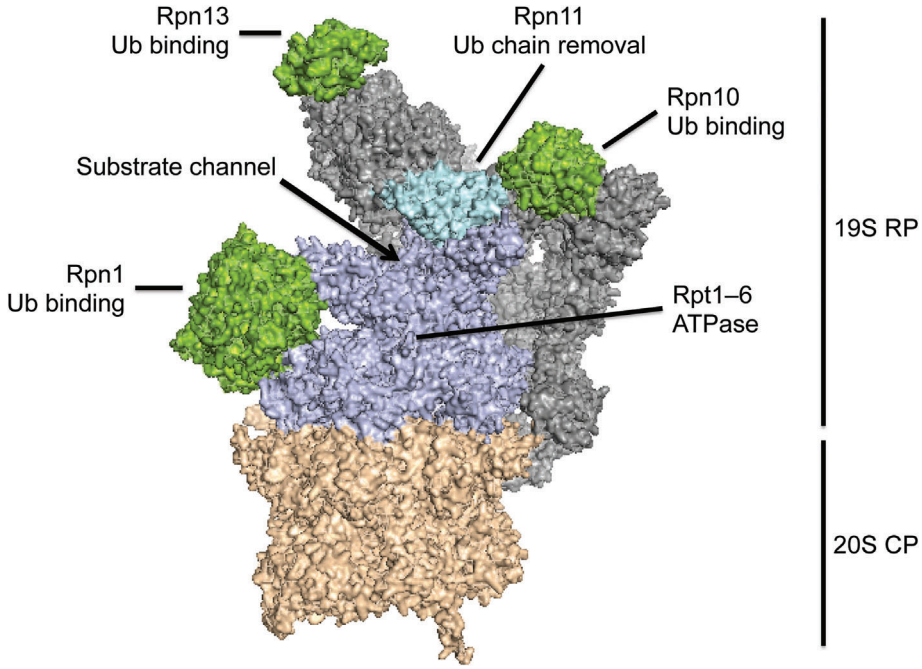

Figure 1. Structure of the 26S proteasome (based on PDB: 4CR2). The 26S proteasome consists of a 20S CP (orange) and a 19S RP (gray or indicated colors). The CP is responsible for proteolysis. The RP contains the ATPase subunits Rpt1–6 (purple), the deubiquitinase Rpn11 (cyan), and the ubiquitin receptors Rpn1, Rpn10, and Rpn13 (green). Substrates enter the proteasome through a channel at the center of a ring formed by the ATPase subunits.

substrate recognition.[9] Once bound to the proteasome, substrate is unfolded by the action of six ATPase subunits (Rpt1–6) located in the RP and translocated from the RP into the CP, where it is cleaved into peptides of 3–8 amino acids.[10] The ubiquitin chain is removed by the deubiquitinating activity of subunit Rpn11 in the RP as the substrate is pulled into the proteasome particle.[11–14] In addition to the stoichiometric proteasome subunits, several accessory proteins, such as the deubiquitinases Ubp6 (USP14 in mammals) and UCH37 (not present in yeast) and the shuttle factors Rad23 (HR23A/B), Dsk2 (UBQLNs) and Ddi1 (DDI1/2), bind transiently to the proteasome to play auxiliary roles in degradation.[15] The proteasome also cooperates with some upstream factors including the ubiquitin-selective chaperone Cdc48 (p97/VCP).[16,17]

The mechanism of proteasomal degradation can be divided into three steps, substrate recognition, unfolding, and proteolysis. Most proteins are targeted to the proteasome by the covalent attachment of multiple ubiquitin molecules through a cascade of three classes of enzymes acting sequentially: ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3). The E1 activates ubiquitin by catalyzing the formation of a ubiquitin adenylate. The activated ubiquitin is transferred to the E2. Finally, the E3 recognizes a short sequence or degron in the target protein and mediates the transfer of ubiquitin from the E2 to the target. The ubiquitin moieties are attached through isopeptide bonds between their C-termini and amino groups in the substrate, usually the ε-amino groups of Lys residues. Ubiquitin itself contains seven lysine residues (Lys 6, Lys 11, Lys 27, Lys 29, Lys 33, Lys 48, and Lys 63) as well as an α-amino group at the N-terminus (Met 1), and the ubiquitination process typically forms poly-ubiquitin chains on proteins, as the first ubiquitin tag becomes itself modified and so on.[18] Chains formed by ubiquitin molecules linked through Lys 48 are the most abundant in cells and represent the canonical degradation signal that targets substrates to the proteasome.[19] The next most common linkage is through Lys 63, and chains of this type are associated with membrane trafficking, as are tags consisting of a single ubiquitin molecule.[6,20] Chains in which ubiquitin moieties are linked C- to N-terminus through peptide bonds form protein complexes in signaling cascades. Branched chains in which ubiquitin moieties are tagged at two Lys residues are also observed. For example, chains with Lys 11 and Lys 48 linkages are made during cell cycle regulation.[21] However, there is not a strict one-to-one correspondence between ubiquitin modification and cellular process and, for example, multiple monoubiquitin and Lys 63 chains can also target proteins for proteasomal degradation in some circumstances.[6,22]

On the proteasome, ubiquitin chains are recognized by the three stoichiometric proteasome subunits mentioned above, through one (yeast) or two (human) ubiquitin-interacting motif domains in Rpn10, the pleckstrin-like receptor for ubiquitin domain of Rpn13, and the T1 site of Rpn1. Cells encode additional proteins that seem to function as supplementary substrate

receptors. These shuttle receptors bind to ubiquitin chains through ubiquitin-associated domains and are recruited to the proteasome via their N-terminal ubiquitin-like domains.[23–31] The receptors perform largely overlapping functions though mutation of individual receptors affects degradation of subsets of proteasome substrates.[32–34]

Protein binding to the proteasome is not sufficient for degradation. A substrate is degraded only when the proteasome is able to engage it at a disordered region in the protein to initiate degradation. The length, location, and amino acid sequence of the disordered region determine how well it is recognized by the proteasome and thus how rapidly a protein is degraded. In this review, we highlight the mechanism by which the proteasome initiates degradation. We review the advances of the field from early conceptualization to recent progress, including application to disease treatment.

The Initiation Region of Proteasomal Degradation

Identification of the initiation region: Ubiquitination is not sufficient

Ubiquitination serves as the proteasome targeting signal, but it does not always lead to rapid degradation.[19,35] Substrates have to reach the proteolytic sites inside the proteasome through a channel, and the pore at its entrance has a diameter of approximately 13 Å.[36,37] Thus, folded proteins must be unraveled and threaded through the substrate channel to the proteolytic sites to be degraded. Early studies exploring how the proteasome unfolds proteins and initiates proteolysis analyzed the degradation of artificial substrates constructed from well-defined building blocks. At their center were tightly folded domains derived from the ribonuclease barnase or dihydrofolate reductase (DHFR). The domains can be stabilized against unfolding by tightly binding ligands, barstar for barnase, and methotrexate for DHFR.[38–41] The proteins were then targeted to the proteasome by artificial degradation tags at their N-termini composed of four ubiquitin domains fused to each other in frame through short linkers. Thus, the resulting hybrid proteins consisted entirely of compact domains. The proteasome was only able to degrade these targets if an unstructured region was also attached to their C-termini.[42] These and other experiments showed that the proteasome degrades proteins by engaging them at an unstructured region and then pulling them from there into the substrate channel and on to the proteolytic sites, unraveling any folded domains in the process.[43] Thus, the proteasome degrades proteins sequentially from an initiation site that does not have to coincide with the ubiquitin tag.[42,43]

Experiments investigating the regulation of ornithine decarboxylase (ODC) came to similar conclusions. ODC is degraded without ubiquitination but requires the cofactor antizyme.[44] The proteasome recognizes ODC at an unstructured region at its C-terminus and initiates its degradation there.[45,46] This C-terminal region can induce the degradation of other proteins when attached to their C-termini.[47] Degradation of ODC itself requires binding of antizyme, to induce a conformational change that exposes the C-terminal tail and to provide an additional interaction surface for proteasome binding.[48] These observations led to the identification of initiation regions or initiation sites of proteasomal degradation: a disordered region at which the proteasome engages its substrates and initiates unfolding and degradation [Fig. 2(a,b)]. This initiation step can explain the behavior of physiological proteins. For example, Ubp6, mentioned above as an accessory factor in degradation, binds to the proteasome near the entrance to the substrate channel but escapes degradation of itself because it lacks efficient initiation regions.[31]

Length: Be long enough to reach the proteasome

The proteasome is able to initiate degradation not only at N- or C-terminal tails but also at internal disordered regions within a protein.[49,50] Interestingly, the disordered regions have to be much longer to allow efficient proteasomal degradation when they are located internally

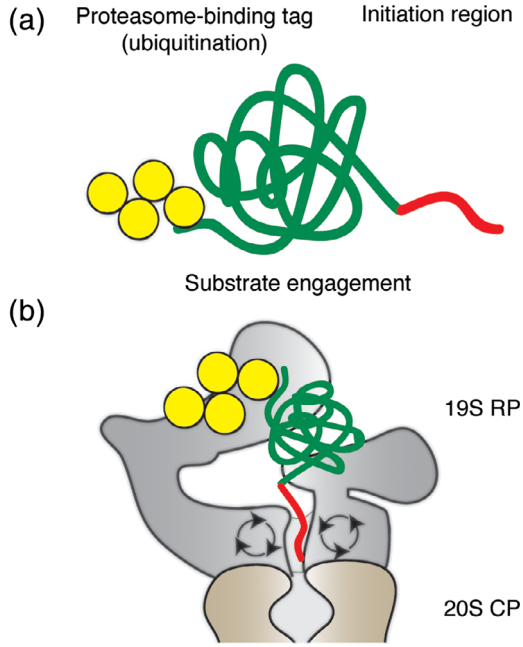

Figure 2. The proteasomal degradation signal has two parts. (a) Schematic representation of a proteasome substrate. A substrate protein (green) has to contain a proteasome-binding tag (typically poly-ubiquitin chains, yellow) and an unstructured region (red) for efficient degradation. (b) Substrate engagement by the proteasome. The proteasome recognizes a substrate via the ubiquitin tag and engages it at the disordered region for unfolding and translocation from the 19S RP to 20S CP.

than as tails.[50] This observation is compatible with a mechanism in which the disordered region has to engage a receptor in a channel before translocation and unfolding can occur. Recent high-resolution structures of the proteasome and elegant biochemical experiments show that this is indeed how proteasomal protein degradation takes place.[51,52] The proteasome engages its substrates with loops (pore-1 loops) in the proteasome's ATPase subunits. The loops contain Tyr residues that face the substrate channel and change their orientation during the ATPase cycle, apparently moving the substrate through channel. These Tyr loops are located some 35 Å from the entrance to the channel.[9] This arrangement is compatible with the experimental observation that initiation regions at the C-terminus of a protein have to be some 30 amino acids long and much longer when flanked by folded domains to allow the proteasome to engage the substrate effectively [Fig. 3(a)].[31,42,47,53–56] After the proteasome grabs the initiation region, the ubiquitin chain is removed from substrate by the deubiquitinase Rpn11, which is located above the central pore of the proteasome and repositions upon substrate engagement.[9,11–14]

Composition: The proteasome likes sequences with diverse amino acid compositions

The proteasome is able to process almost any protein presented for destruction. At the same time, the surface of the initiation regions to be recognized by the proteasome will vary with the amino acids sequence and the unique chemical features of their side chains. Therefore, it is possible that the proteasome will recognize dissimilar initiation sites with different efficiencies.

An effect of the amino acid sequence of a protein on its degradation by the proteasome was originally proposed based on the observation that the Epstein–Barr virus protein EBNA1 escapes proteolysis and the generation of peptides to be displayed by MHC complexes and thus allows host cells that harbor the provirus in their genome to avoid detection by the immune system.[57,58] It appeared that the stability of EBNA1 was due to the presence of a long stretch of Gly-Ala repeats within the protein that protected it from proteasomal degradation.[59–62]

The proteasome's preference for the amino acid sequence of initiation regions was examined systematically by measuring the degradation of model proteins. An early small-scale screen revealed that proteins in which the initiation regions have biased amino acid compositions show longer half-lives than proteins with unbiased sequences in the regions.[53] Analysis of ~100 different initiation regions indicated that in addition to compositional complexity, hydrophobicity, charge, and flexibility of the sequence also affect proteasomal recognition; the proteasome prefers hydrophobic and nonpolar amino acid residues and stiffer polypeptide chains, whereas polar, acidic, and structurally flexible sequences are avoided.[54]

These sequence preferences seem to matter in the cell [Fig. 3(b)]. The ubiquitin-conjugating enzyme

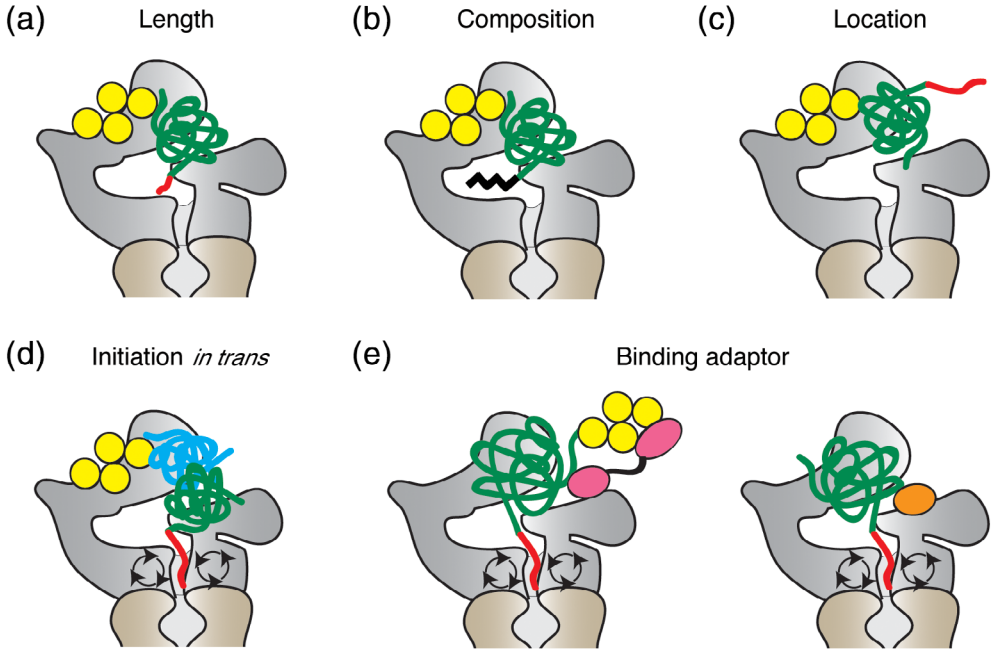

Figure 3. Initiation site selection by the proteasome. (a–c) Requirements for effective initiation regions. The initiation region has to be long enough (a), with a suitable amino acid composition (b), and be located in a position accessible to the proteasome (c). (d) Initiation of proteasomal degradation *in trans.* The proteasome can recognize a protein complex through ubiquitinated subunits (yellow and blue) but degrades only subunits with accessible initiation regions (green and red). (e) Speculative models of substrate recognition via degradation adaptors. The proteasome may recognize substrates through adaptor proteins (pink and orange) that recognize the ubiquitin chain attached to a substrate or the substrate directly without ubiquitination.

Cdc34 is ubiquitinated on a long disordered region at its C-terminus, but escapes degradation. The C-terminal tail is acidic and has biased composition, with 50 out of 130 amino acids being Asp or Glu residues preventing the proteasome form initiating degradation. Similarly, the shuttle receptor Rad23 contains three disordered linker regions of 60–68 amino acids but these regions do not serve as efficient initiation regions because of their biased amino acid composition. Cdc34 and Rad23 are easily degraded when short initiation regions are fused to their C-termini.[50,53]

The sequence preferences are also reflected in the behavior of proteins observed at the system-level when analyzing protein stability in large-scale proteomic studies. Proteins containing intrinsically disordered segments have significantly shorter half-lives than proteins without these features across species.[55] Protein stability furthermore correlates with the amino acid composition of the disordered region, as proteins in which the disordered region has strongly biased compositions are as stable as proteins without any disordered regions.[53,55]

The proteasome's sequence preferences are most likely due to the effect of these sequences on their recognition in the ATPase subunits during translocation.[63,64] For example, hydrophobic initiation sequences may be preferred because of their interaction with the pore-1 loops that drive substrate translocation.[54,65] Negatively charged surfaces of the substrate channel that flank the pore-1 loops may repel acidic sequences.[54,65] Stiffer polypeptide chains may access to the pore-1 loops more efficiently than more flexible sequences.[54] At the same time, the proteasome is able to recognize and degrade a vast number of proteins in cells that do not share any obvious consensus sequence.[53] Thus, the proteasome seems to be able to interact with a wide range of features within a stretch of amino acids, and only if pronounced sequence bias dilutes these features does recognition fail.[53,66]

Location: Fitting substrate's geometrical arrangement

The proteasome degrades proteins efficiently when it binds to a ubiquitin tag on the substrate and engages it at an initiation region. The arrangement of the proteasome-binding tag and the initiation region on the substrate presumably has to match the arrangement of their receptors on the proteasome [Fig. 3(c)].[56] This relationship can be demonstrated experimentally on model substrates by inserting spacer domains between ubiquitin tag and initiation region to increase the distance between the two. When the initiation site is located too close or too far from the proteasome-binding tag, the proteasome is unable to initiate degradation of the substrate.[56]

Do these restrictions on the geometry of degradation signals affect the behavior of physiological proteins? Ubiquitin tag and initiation region have to be close in space, but they can be separated onto different proteins [Fig. 3(d)].[67] In protein complexes targeted for destruction, the proteasome degrades the subunits that contain accessible initiation regions while leaving the others intact. An example of this principle may be the remodeling by the proteasome of the protein complexes formed by cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors (CKIs). Both cyclins and CKIs are highly reactive proteasome substrates, yet the proteasome is able to extract them one by one from trimeric complex. One explanation for the selective destruction would be that the initiation region on the first subunit to be degraded is placed such that it is recognized more easily than that of the other units, thus competing for degradation more effectively. Once the subunit with the ubiquitin tag is degraded, the remaining subunits are stable until they in turn become ubiquitinated and targeted for destruction.[68]

At the same time, degradation signal structure may also explain the needs for shuttle receptors. It is possible that one function of these accessory factors is to enhance the degradation of some substrates by presenting them to the proteasome more favorably for initiation than the intrinsic ubiquitin receptors are able to [Fig. 3(e)].

Accessory factor Cdc48: The proteasome occasionally needs help

The degradation of some proteins by the proteasome requires the action of the ATPase Cdc48 (p97/VCP) as an accessory factor.[69,70] Cdc48 cooperates with cofactors such as Ufd1 (UFD1L) and Npl4 (NPLOC4), which serve as ubiquitin receptors and regulate Cdc48-dependent degradation pathways.[71–74] Most of these proteins are associated with membranes or are subunits of protein complexes.[69,70] For example, Cdc48 is a component of endoplasmic-associated protein degradation where it mediates the transfer of proteins from the retrotranslocation machinery in the endoplasmic reticulum membrane to the proteasome.[71,72] Other examples are the transcription factors Spt23 and Mga2, which are synthesized as membrane-anchored precursors and become activated when they are liberated by partial degradation by the proteasome in a Cdc48-dependent manner.[75]

Biochemically, the role of Cdc48 may be to unfold proteins that do not contain regions that would allow the proteasome to initiate degradation.[17,76,77] This function could explain the role of Cdc48 in the ubiquitin-fusion degradation pathway.[78–81] For example, a chimeric protein consisting of ubiquitin domain attached to the N-terminus of green fluorescent protein is not degraded by the proteasome *in vitro* unless a disordered region is fused to the protein, but can be unfolded by Cdc48.[70,74,82] Similarly, Rpb1 (RPB1), a subunit of RNA polymerase II requires Cdc48 for

degradation by the proteasome.[83] Rpb1 has a long disordered region at its C-terminus (the C-terminal domain or CTD), which consists of many copies of a heptad repeat motif and serves as the binding site for cofactors and regulators of transcription. Rpb1 becomes ubiquitinated at the CTD when the polymerase stalls at sites of DNA damage but degradation of Rpb1 requires Cdc48.[80,84–87] Cdc48 may be needed to unfold ubiquitinated Rpb1 and present it to the proteasome, because initiation at the CTD is prevented by the strong bias of its amino acid sequence.

Ubiquitin-independent degradation: Another targeting mechanism?

If a protein has a particularly effective initiation region, it might be possible that it is degraded by the proteasome without ubiquitination, just as bacterial ATP-dependent proteases recognize some substrates at C- or N-terminal targeting signals.[88] Indeed, some proteins are degraded in a ubiquitin-independent manner either because they are recognized directly by the proteasome or because they interact with the proteasome via an adaptor protein [Fig. 3(e)]. The best-established example is the ubiquitin-independent degradation of ODC mediated by antizyme as described above.[44,89] Another example may be the degradation of the cytidine deaminase APOBEC3G, which is targeted to the proteasome by ubiquitinated viral infectivity factor (Vif).[90] APOBEC3G can be degraded even when all Lys residues in the protein are mutated to prevent ubiquitination, presumably by ubiquitinated Vif acting as a proteasome adaptor.[67]

Several other proteins have been reported to be degraded in a ubiquitin-independent manner, including NAD(P)H:quinone-oxidoreductase-1, steroid receptor coactivator 3, the CDK inhibitors $p21^{Cip1}$, $p16^{Ink4a}$, and $p19^{Arf}$, p53, IκBα, a regulator of the transcription factor NFκB, and the transcription factor Rpn4, although some of these are also degraded through ubiquitin-dependent pathways.[91–102] A basal level of ubiquitin-independent degradation of regulatory proteins may be common in signaling networks to allow them to respond rapidly to signals and revert back to steady state after the signal is withdrawn.[103,104] We expect that there are still a large number of substrates of ubiquitin-independent degradation to be identified.

UPS and neurodegenerative diseases: Lack of initiation regions

Protein aggregation and inclusion body formation underlie the pathology of several neurodegenerative disorders including Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD), Parkinson's disease, and Alzheimer's disease.[105–108] For example, in HD the intensity of the phenotype in mouse models correlates with the accumulation of a protein fragment corresponding to exon 1 of a mutated *HTT* gene (Htt) in nuclear inclusions, and a gain of toxic function in Htt mutants is implicated in the pathogenesis of HD.[109] Despite evidence for the colocalization of ubiquitin and Htt as well as direct ubiquitination of Htt, the protein is not degraded and accumulates.[110–114] Indeed, proteasome subunits are detected in the inclusion bodies formed in HD, suggesting that the proteasome may attempt to clear them.[115] Autophagy, which is another bulk degradation process in cells, can also contribute to removal of protein aggregates but recent studies suggest that UPS inhibition has a greater effect on Htt accumulation than autophagy inhibition.[116]

A possible explanation for the failure of the UPS to eliminate these aggregates is an impairment of the proteasome. Protein aggregates can inhibit the UPS in culture cells and they may do so by clogging up the proteasome.[117,118] However, investigations *in vivo* have not yet reached a consensus. Several studies in HD animal models did not detect general defects in proteasome activity, while a cryo electron tomography study using an ALS/FTD model found that proteasome particles at the aggregates are in a substrate-processing conformation, suggesting stalled degradation.[119–121] Another possible reason for the stability of Htt aggregates is that Htt lacks an effective initiation region because the amino acid composition of its sequence is strongly biased. It consists of a short N-terminal sequence of 18 amino acids, followed by a stretch of at least 23 Gln residues (polyQ) and then a Pro-rich region of 50 amino acids, and does not allow the proteasome to initiate degradation in *in vitro* experiments.[53,122] In turn, attaching an effective initiation region to Htt leads to its proteasomal degradation *in vitro* and in yeast.[53,123] Thus, the pathogenesis of neurodegenerative diseases may in part be linked to the lack of proteasome initiation regions in aggregate-prone proteins.

Inducible degradation: For a better design of protein knockdown tools

The targeted destruction of proteins in cells is a useful tool to investigate their functions and potentially a powerful therapeutic strategy. Various inducible degradation systems have been developed, including proteolysis-targeting chimeras (PROTACs), which show promise for clinical use.[124–134] Unlike DNA- or RNA-targeting methods, inducible protein degradation systems could be effective for long-lived proteins and may be able to distinguish between otherwise identical target proteins with different post-translational modifications. The most common strategy is to induce ubiquitination of target proteins, in the case of PROTACs through small molecules that serve as adaptors that induce the interaction of the target with ubiquitin ligases.[125,127] The design of successful degradation tools is hindered by the fact that we do not fully understand the signals that control ubiquitination or

how ubiquitin modifications are interpreted in the cell. In some cases PROTACs fail to induce degradation of a target despite binding efficiently.[135,136] One possible explanation is that once ubiquitinated the target proteins are not presented to the proteasome in a manner that allows effective initiation and thus degradation. Accordingly, taking into account the initiation step of proteasomal degradation may be helpful in designing of inducible degradation systems.

Conclusions

Ubiquitin tags target proteins to the proteasome but proteolysis requires that the proteasome engage its substrates at a disordered region to initiate degradation. The initiation step contributes to the specificity of proteasomal degradation. However, there are gaps in our understanding of how initiation regions function in cells. It is still hard to map initiation regions on a proteome-wide scale, and the interplay between ubiquitination and initiation region remains elusive. The concept of proteasomal initiation has been developed mostly using model substrates, and it is necessary to translate existing knowledge to the behavior of natural proteins. Future studies will reveal the contribution of initiation regions in physiological processes and lead to a better understanding of protein degradation mechanisms in the UPS.

Acknowledgments

This work was supported by the National Institutes of Health through Grants R01GM124501 and R21CA196456 as well as the Welch Foundation through Grant F-1817. A.M. is a paid consultant for Kymera Therapeutics.

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