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Review

The complexity of recognition of ubiquitinated substrates by the 26S proteasome☆

Aaron Ciechanover [a], Ariel Stanhill [b,*]

[a] The David and Janet Polak Cancer and Vascular Biology Research Center, The Rappaport Faculty of Medicine and Research Institute, Technion-Israel Institute of Technology, Haifa 31096, Israel
[b] Department of Biochemistry, The Rappaport Faculty of Medicine and Research Institute, Technion-Israel Institute of Technology, Haifa 31096, Israel

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ABSTRACT

The Ubiquitin Proteasome System (UPS) was discovered in two steps. Initially, APF-1 (ATP-dependent proteolytic Factor 1) later identified as ubiquitin (Ub), a hitherto known protein of unknown function, was found to covalently modify proteins. This modification led to degradation of the tagged protein by – at that time – an unknown protease. This was followed later by the identification of the 26S proteasome complex which is composed of a previously identified Multi Catalytic Protease (MCP) and an additional regulatory complex, as the protease that degrades Ub-tagged proteins. While Ub conjugation and proteasomal degradation are viewed as a continued process responsible for most of the regulated proteolysis in the cell, the two processes have also independent roles. In parallel and in the years that followed, the hallmark signal that links the substrate to the proteasome was identified as an internal Lys48-based polyUb chain. However, since these initial findings were described, our understanding of both ends of the process (i.e. Ub-conjugation to proteins, and their recognition and degradation), have advanced significantly. This enabled us to start bridging the ends of this continuous process which suffered until lately from limited structural data regarding the 26S proteasomal architecture and the structure and diversity of the Ub chains. These missing pieces are of great importance because the link between ubiquitination and proteasomal processing is subject to numerous regulatory steps and are found to function improperly in several pathologies. Recently, the molecular architecture of the 26S proteasome was resolved in great detail, enabling us to address mechanistic questions regarding the various molecular events that polyubiquitinated (polyUb) substrates undergo during binding and processing by the 26S proteasome. In addition, advancement in analytical and synthetic methods enables us to better understand the structure and diversity of the degradation signal. The review summarizes these recent findings and addresses the extrapolated meanings in light of previous reports. Finally, it addresses some of the still remaining questions to be solved in order to obtain a continuous mechanistic view of the events that a substrate undergoes from its initial ubiquitination to proteasomal degradation. This article is part of a Special Issue entitled: Ubiquitin-Proteasome System.

The diversity and complexity of the ubiquitin proteasomal degradation signal

Ub, similar to many post-translational modifiers, mediates protein–protein interactions. However, in contrast to many other types of modifications it enables a complex and diverse array of biological processes. As for proteasomal degradation, the prototypic 'canonical' signal is a polyUb chain where the Ub moieties are linked to one another via an isopeptide bond between the C-terminal Gly76 of the distal moiety and internal Lys48 in the proximal one. The most proximal Ub moiety is linked to an ε-NH_2 group of an internal lysine (Lys) residue in the target substrate [1–3]. Furthermore, it was suggested that the shortest chain recognized by the proteasome has to contain at least four Ub moieties [4]. Recent investigations show however that the proteasomal proteolytic signal is far more complex and diverse: chains based on different internal linkages, linear — head-to-tail chains, mixed chains made of Ub-like (UbL) proteins and Ub, and surprisingly also a single Ub moiety, can be recognized by the proteasome. Also, chains conjugated to internal residues other than Lys as well as to the N-terminal residue were described, all challenging the current 'canon'. All these different modifications are depicted in Fig. 1.

1. Single and multiple Ub moieties and structural characteristics of the substrate can be parts of the proteolytic signal

Several recent investigations demonstrate that the proteasome can recognize and degrade protein targets that were conjugated by a single or multiple single Ubs. One of the first findings described was that a single ubiquitination on a specific Lys residue on paired box 3 protein (PAX3) – a regulator of muscle differentiation – targets the protein for degradation [5]. The ubiquitination reaction is catalyzed by the TAF1 ligase [6]. Similarly, the conjugation of a single Ub in response to Wnt

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* Corresponding author at: Department of Biochemistry, Faculty of Medicine, Technion-Israel Institute of technology, POB 9649, Haifa 31096, Israel. Tel.: +972 4 829 5306; fax: +972 4 853 5245.
E-mail address: stanhill@tx.technion.ac.il (A. Stanhill).

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signaling results in proteasomal degradation of the cell adhesion membrane receptor Syndecan 4 (SCD4) that is involved in regulating cell migration during embryonic development [7].

Detailed mechanistic studies of monoubiquitination as a degradation signal revealed that Ub fused linearly to a peptide longer than 20 residues can efficiently target itself for degradation with no need for further chain extension [8]. The hypothesis raised was that peptides shorter than 20 residues that are fused to Ub cannot bridge the distance between the bound Ub and the proteolytic chamber in the 20S subcomplex – where the proteolytically active β-subunits are located – and therefore cannot be degraded. Hence, only peptides longer than 20 residues can target the N-terminally fused Ub for degradation. Since there is a minimal length of a peptide the degradation of which can be driven by a single Ub moiety, the question raised was whether there is a maximal length of a protein the degradation of which can be driven by a single Ub moiety, and whether the length of a protein plays a role in the extent of ubiquitination that leads to its degradation. It was shown that a single Ub moiety can support the degradation of extensions of up to ~150 residues [9]. Of note is that most of the extensions used in these studies appeared to be artificial and raised the concern that they are misfolded/denatured, and do not represent naturally occurring proteins. However, a random and preliminary search for cellular targets demonstrated that Cks2, a 79 residues cell cycle regulator, and Hug1, a yeast protein involved in Mec1p-mediated checkpoint pathway that responds to DNA damage or replication arrest, are degraded both in a cell free system and in cells following monoubiquitination [9]. Similarly, properly folded monoubiquitinated α-Synuclein, a 140 residue protein that was generated by a combination of chemical and biological methods, was degraded by a purified proteasome in a manner that was completely dependent on the presence of the conjugated single Ub moiety [9]. Furthermore, it was shown that that α-globin (142 residues) can be degraded following monoubiquitination [10,11].

The finding that Ub (that is a stably folded protein except for a short C-terminal segment) is a long-lived protein, but a tail longer than 20 residues can target it for rapid degradation, strongly suggests that – besides Ub – the proteasomal degradation signal has to contain a second important characteristic, that of an unstructured tail or an initiation domain. This domain must be sufficiently long to cross the 19S subcomplex and to reach the catalytic sites/proteolytic chamber in the β rings, pulling the entire substrate behind it [12,13]. The question whether this domain is part of the natural structure of the protein, a result of misfolding, or generated by ubiquitination and/or 19S binding/processing, is still elusive.

For other proteins, a more extended modification by multiple single Ub moieties is necessary in order to promote their proteasomal degradation. For example, p105, the precursor of the NF-κB transcription factor p50, undergoes multiubiquitinations in the C-terminal domain of the precursor. These modifications result in processing of the precursor, releasing the N-terminal p50 active subunit of the transcription factor [14]. The cell cycle regulator cyclin B1 is also degraded by the proteasome following multiubiquitination catalyzed by the APC/C E3 Ub ligase [15]. Restricting the number of Lys residues that serve as Ub anchors in the case of p105 reduces the efficiency of processing whereas in the case of cyclin B1 it "forces" the generation of oligo- and/or polyUb chain(s).

The above findings suggest a new dynamic concept for the Ub signal. It seems that not all substrates require an equally long polyUb chains for targeting them for proteasomal degradation, and that the proteolytic signal can adapt itself to the substrate. Mechanistically, one can envision that in the cell the ubiquitination and degradation machineries are found in a loosely associated complex. As Ub moieties are added to the substrate and the chain is elongated, the avidity of the conjugate to the proteasome increases. Once the avidity reaches a certain threshold, and a stable binding of the adduct to the proteasome is secured, it is detached from the conjugating machinery, bound stably to the proteasome, and degraded processively and efficiently. With larger protein targets that may require a longer processing time, a longer polyUb chain may be necessary to generate the required avidity. Therefore, a single Ub moiety or a short chain is not sufficient to bind stably a long polypeptide for the proteasome to ascertain its processive digestion. For substrates that are multiubiquitinated, the spatial arrangement of a large enough number of single Ub moieties that bind to multiple points in the proteasomal Ub receptors ascertains the strong binding necessary for processive degradation (see Section 7). In the case of cyclin B1, restriction of the number of Ub anchors that "forces" the formation of oligo-/polyUb chains, can substitute for the multiple single moieties that were distributed among a higher number of anchors along the protein substrate.

2. PolyUb chains

2.1. Homogenous Ub chains based on a single internal link

Ub has 76 residues with seven lysines in positions 6, 11, 27, 29, 33, 48 and 63. As noted, the most common Ub polymer involved in targeting a substrate for degradation was thought to be a homogeneous chain where the Ub moieties are linked to one another via an isopeptide bond between the C-terminal Gly76 of the distal moiety and Lys48 of the previously conjugated one [1]. Mass spectrometry analysis has shown however that, in addition, chains based on Lys29, 11, 27 and 6 (in decreasing abundance) can also target proteins for proteasomal degradation, and their formation is dependent on different stress states [16]. Other studies have shown that homogeneous chains based on Lys11, generated by the APC/Cyclosome Ub ligase, can also target certain proteins for degradation during cell division ([17–19] and reviewed recently in [20]). These chains, like the 'canonical' Lys48-based chains, appear also to bind to the Rpn10/S5a subunit of 19S sub-complex [21], probably via a TEK box motif that is found on both the substrate and the Ub moiety [22]. Other studies have shown that Lys33- [23,24] and Lys63-based chains [25], are also recognized by the proteasome. It should be noted that some of these results were obtained in studies using cell free reconstituted systems. The validity of these systems was questioned recently in a study showing that while purified proteasome can degrade proteins conjugated with Lys63-based chains, this does not occur in cells [26]. The reason being that in cells factors such as ESCRT0 (Endosomal Sorting Complex Required for Transport) and its components, STAM and Hrs bind to the Lys63 chains and inhibit their association with the proteasome.

Thus, it appears that chains assembled via almost all Ub internal lysines can target proteins for degradation by the proteasome. It will be interesting to study the mechanistic and physiological conditions that lead to the assembly of such chains on different protein substrates. Some of the interesting questions are whether a single substrate can be modified by several types of chains?, is the synthesis of these chains catalyzed by a single or multiple ligases and under what conditions?, and why at all single or different substrates require different chains for their degradation?

2.2. Heterogeneous Ub chains based on different internal links

The fact that chains are synthesized enzymatically led naturally in the assumption that they must be homogenous, and all internal linkages are mediated via a single specific Lys residue in the Ub moiety. However, mass spectrometry analyses have shown that this is not the case, and heterogeneous/mixed chains – where the linkages involve different internal lysines – are also recognized by the proteasome. One example is cyclin B1 that, as noted, is targeted by multiubiquitinations [15], but was also shown to be targeted by short Ub chains containing K11, K48, and K63 internal linkages [27]. It is possible that in order to generate a proteasomal recognition signal of sufficient Ub avidity, different proteins use mixtures of chains of different lengths and internal linkages. The generation of such chains may be dependent on the

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