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The Size of the Proteasomal Substrate Determines Whether Its Degradation Will Be Mediated by Mono- or Polyubiquitylation

Nitzan Shabek,[1,6] Yifat Herman-Bachinsky,[1,6] Samuel Buchsbaum,[1] Oded Lewinson,[2] Mahmood Haj-Yahya,[3] Mirva Hejjaoui,[4] Hilal A. Lashuel,[4] Thomas Sommer,[5] Ashraf Brik,[3] and Aaron Ciechanover[1,*]
[1]Cancer and Vascular Biology Research Center
[2]Department of Microbiology
The Rappaport Faculty of Medicine and Research Institute,Technion-Israel Institute of Technology, Haifa 31096, Israel
[3]Department of Chemistry, Ben-Gurion University of the Negev, Beer Sheva 84105, Israel
[4]Brain Mind Institute, École Polytechnique Fédérale de Lausanne, CH-1015 Lausanne, Switzerland
[5]Max Delbrück Center for Molecular Medicine, 13125 Berlin, Germany
[6]These authors contributed equally to this work
*Correspondence: c_tzachy@netvision.net.il
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SUMMARY

A polyubiquitin chain anchored to the substrate has been the hallmark of proteasomal recognition. However, the degradation signal appears to be more complex and to contain also a substrate's unstructured region. Recent reports have shown that the proteasome can degrade also monoubiquitylated proteins, which adds an additional layer of complexity to the signal. Here, we demonstrate that the size of the substrate is an important determinant in its extent of ubiquitylation: a single ubiquitin moiety fused to a tail of up to ~150 residues derived from either short artificial repeats or from naturally occurring proteins, is sufficient to target them for proteasomal degradation. Importantly, chemically synthesized adducts, where ubiquitin is attached to the substrate via a naturally occurring isopeptide bond, display similar characteristics. Taken together, these findings suggest that the ubiquitin proteasomal signal is adaptive, and is not always made of a long polyubiquitin chain.

INTRODUCTION

Degradation of intracellular proteins via the ubiquitin-proteasome system (UPS) is involved in the regulation of a broad array of essential cellular processes, such as cell cycle progression, differentiation, apoptosis, DNA repair, cellular quality control, autophagy and regulation of transcription. UPS substrates are modified initially by covalent attachment of ubiquitin (Ub), where its carboxyl-terminal Gly76 usually generates an isopeptide bond with an ε-amino group of an internal Lys of the substrate. This is followed by generation of a polyUb chain where additional moieties are typically conjugated to Lys48 of the previously added Ub (Chau et al., 1989; Hershko and Ciechanover, 1998). Ub chains are highly dynamic due to the opposite activities of the E3 ligases and deubiquitylating enzymes (DUBs). These enzymes, along with other proteins, such as E2s (Ub-carrier proteins; Ub-conjugating enzymes - UBCs), determine not only the nature of the Ub chain's linkage(s) (Ub has seven Lys residues, all can be conjugated), but also the length of the chain (from a single Ub modification to a long polymeric chain) (See for example: Hibbert et al. [2011]; Kim et al. [2007]; Kirkpatrick et al., [2006]; Saeki et al. [2009]).

The polyUb-tagged substrates are recognized and degraded by the 26S proteasome which is a large (~2MDa) cryptic proteolytic complex. It is comprised of two sub-complexes: the 20S catalytic particle (CP), and the 19S regulatory particle (RP) (Finley, 2009; Lander et al., 2012). The catalytic sites that are responsible for hydrolysis of the substrate are located in the interior of the 20S barrel. The 19S complex is further subdivided into a "lid" and a "base." The "base" consists of six ATPases involved in unfolding and translocation of the substrate into the 20S CP, and additional subunits, among them Rpn10 and Rpn13, involved in recognition of the polyUb chain (Finley, 2009; Husnjak et al., 2008; Lam et al., 2002; van Nocker et al., 1996). Despite all that is currently known on the proteasome, the mechanism(s) by which a protein substrate is recognized and delivered into the catalytic chamber is not fully understood. It is widely accepted that the proteasomal proteolytic signal is generally bipartite: the first element is Ub which serves as an *in trans* recognition signal for the adduct, whereas the second is an intrinsic unfolded fragment in the substrate that serves as a translocation initiation site and mediates entry of the substrate into the proteolytic chamber (Fishbain et al., 2011; Prakash et al., 2004; Takeuchi et al., 2007).

The paradigm in the Ub field has been that the minimal Ub oligomer required for recognition by the proteasome is a tetraUb chain (Thrower et al., 2000), and that proteins modified by monoUb regulate nonproteolytic processes such as membrane transport and regulation of transcription (Hicke, 2001). However, an increasing number of reports have demonstrated that monoubiquitylation or multiple monoubiquitylations can also serve as proteolytic signals for the proteasome. For example, it has been

shown that the paired box 3 (PAX3) protein, a regulator of muscle differentiation, is degraded following monoubiquitylation on a specific Lys residue (Boutet et al., 2007). Likewise, syndecan-4 (SDC4), a cell adhesion receptor that is required for cell migration, is monoubiquitylated on its cytoplasmic tail in a WNT/DSH-dependent manner, and is subsequently degraded by the proteasome (Carvallo et al., 2010). Studies on α-globin (Shaeffer and Kania, 1995), ferritin (De Domenico et al., 2006), and lysozyme (Hershko et al., 1984) also suggested proteasomal degradation of these substrates following their monoubiquitylation. Proteasomal processing of the NF-κB precursor p105 to the active subunit p50 requires its modification by several single Ub moieties (Kravtsova-Ivantsiv et al., 2009), and proteasomal degradation of cyclin B1 (Dimova et al., 2012) and phospholipase D (PLD) (Yin et al., 2010) also depend on multiple monoubiquitylations. Importantly, it was demonstrated that proteins made of Ub fused to C-terminal extensions longer than 20 residues, are efficiently degraded by the proteasome without further ubiquitylation (Shabek et al., 2009; Shabek et al., 2007; Verhoef et al., 2009). The extension is probably homologous to the unstructured segment necessary in order to initiate entry of the entire substrate into the proteasomal 20S CP. The minimal 20 residues-length of the extension may be necessary in order to bridge the distance between the 19S recognition site(s) to the translocation machinery and/or the catalytic sites (Inobe et al., 2011; Lander et al., 2012; Shabek and Ciechanover, 2010). Interestingly, the naturally occurring Ub variant to which an open reading frame of additional 19 residues is attached in frame (UBB+1; van Leeuwen et al. [1998]), is stable (Lindsten et al., 2002; Shabek et al., 2009). However, addition of a single amino acid to the 19-residue extension renders this pathological protein susceptible to proteasomal degradation without further ubiquitylation (Shabek et al., 2009). Collectively, these findings raise the possibility that proteins of up to a certain length (i.e., above a minimal length of 20 residues) can be degraded following monoubiquitylation. Thus, the observed ability of the proteasome to recognize a variety of signals raises the possibility that degradation is not mediated by a homogenous long polymeric chain, but rather by a signal that is variable and adapted to basic characteristics of the substrates.

In the present study we demonstrate that proteins shorter than ~150 residues can be degraded following monoubiquitylation. Longer proteins require polyubiquitylation to promote their efficient proteolysis.

RESULTS

Isopeptide Bond-Linked Peptides Require a Free Segment Longer than 20 Residues to Undergo Proteasomal Degradation

We have previously shown that noncleavable Ub (Ub^{VV}) linearly fused to the N-terminus of peptides longer than 20 residues can be degraded by the proteasome without further ubiquitylation. Shorter tailed fusions of Ub are stable (Shabek et al., 2009). To further corroborate these findings using natural conjugates, we synthesized peptides of different lengths to which Ub was conjugated in an isopeptide bond to an internal Lys residue. Initially, we studied the degradation of pure histone H2B-derived peptides of 8 and 31 residues where the Ub moiety was attached to Lys residues 4 and 27, respectively (denoted Ub-8-K4 and Ub-31-K27). The first peptide has free segments of 3 and 4 residues flanking the Ub-anchoring Lys, while the second contains free segments of 26 and 4 residues (Figure 1Ai; Kumar et al. [2011]). The longer peptide was rapidly degraded by purified 26S proteasome, whereas the shorter one was stable (Figure 1Aii). Proteasome inhibition resulted in stabilization of the degradable conjugate (Figure 1Aiii). Next, it was important to study whether for the proteasome to digest this adduct, the free segment (up or downstream to the Ub-anchoring site) has to be, as we hypothesized before, longer than 20 residues. To that end, we synthesized a peptide of 31 residues with amino acid sequence identical to the one described above, except that the Ub moiety was attached to Lys16 (Ub-31-K16; Figure 1Bi; its analysis is shown in Figure S1). This monoubiquitylated peptide has two free segments of 15 residues on each side of the Ub-anchoring site (Figure 1Bi). As can be seen in Figure 1Bii, this peptide was stable compared to its Ub-31-K27 counterpart. This finding strongly suggests that proteasomal degradation of peptides that are modified with a single Ub requires also a free segment longer than a minimal length.

A Single Ub Fused to a Polypeptide of ~150 Residues Can Target It for Proteasomal Degradation without Additional Ubiquitylation: The Case of HA Repeats

At that point it was important to determine the maximal length of such peptides, beyond which polyubiquitylation becomes necessary for their degradation. Thus, we sought to establish a set of substrates that will serve as a "length ruler." To that end, we first generated a noncleavable and nonpolymerizable Ub, denoted $LLUb^{VV}$ where the C-terminal -$G^{75}G^{76}$ residues of Ub were substituted with Val (Ub^{VV}) (Johnson et al., 1992), and all internal Lys residues were substituted with Arg (lysine-less Ub, LLUb). This Ub variant was fused linearly to polypeptides comprised of an increasing number (six to twelve) of HA repeats (Figure 2A; $LLUb^{+79aa}$ (6HA), $LLUb^{+114aa}$ (9HA) and $LLUb^{+150aa}$ (12HA)]. In all these proteins, the extensions are predicted to be unstructured/disordered (Figure S2A). We monitored the stability of these fusion proteins and found that $LLUb^{+79aa}$ and $LLUb^{+114aa}$ were rapidly degraded in a cell-free system, whereas $LLUb^{+150aa}$ was more stable (Figure 2B). The HA repeat itself, to which Ub was not fused, was stable (Figure S2B), strongly suggesting that the proteasomal degradation was dependent on the Ub moiety. Similar results were obtained in HEK293 cells (Figure 2C), where $LLUb^{+79aa}$ and $LLUb^{+114aa}$ were degraded efficiently by the proteasome, whereas $LLUb^{+150aa}$ was rather stable. To demonstrate that the fused peptide of 150 residues can be degraded following polyubiquitylation, we generated a similar protein to which polymerizable (yet noncleavable) WTUb was fused (Ub^{+150aa}). As shown in Figure 2D, this Ub fusion was rapidly degraded by the proteasome following its polyubiquitylation. Taken together these results and those that follow raise the hypothesis that proteins of up to 150 residues can be targeted to the proteasome by a single Ub moiety, whereas larger proteins require polyubiquitylation. Mechanistically, it is possible that small proteins bind to the proteasome efficiently following their monoubiquitylation, whereas for longer

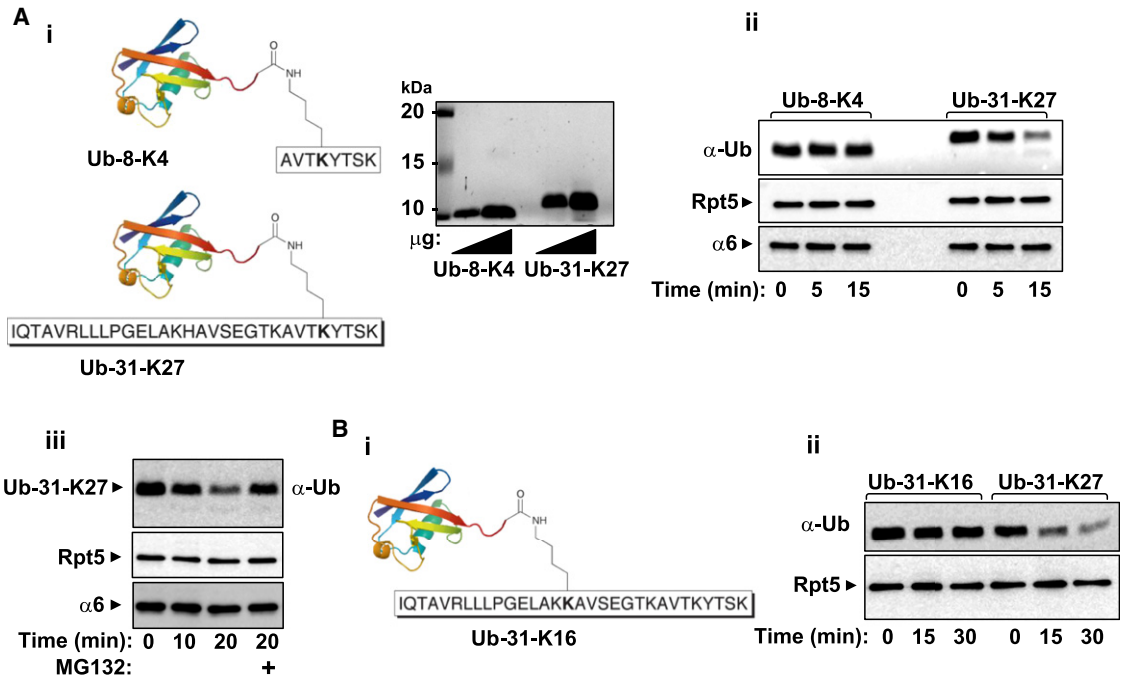

Figure 1. Isopeptide Bond-Linked Monoubiquitylated Peptides Require Free Segments Longer than a Minimal Length in Order To Be Degraded by the 26S Proteasome

(A) (i) Schematic presentation of monoubiquitylated peptides with different length segments flanking the Ub-anchoring site (left panel). Ub was chemically conjugated to Lys4 in an H2B-derived 8-mer peptide (Ub-8-K4), and to Lys27 in an H2B-derived 31-mer peptide (Ub-31-K27) as described under Experimental Procedures. 1 and 2 μg of the purified monoubiquitylated peptides were resolved by SDS-PAGE and detected using coomassie blue staining (right panel). (ii) Ub-8-K4 and Ub-31-K27 were subjected to ATP-dependent degradation in a cell-free system containing purified 26S proteasome. Reactions were incubated for the indicated times. (iii) Degradation of Ub-31-K27 by the proteasome is sensitive to MG132. Reactions were carried out as described under (ii) and MG132 was added as indicated.

(B) (i) Schematic presentation of Ub-31-K16. (ii) Ub-31-K16 and Ub-31-K27 were subjected to ATP-dependent proteasomal degradation as described under (A) (ii). Rpt5 and α6 served as loading controls. Ub structure shown in all schemes is based on PDB: 1UBQ. In all degradation experiments, peptides were detected after SDS-PAGE and Western blotting using anti-Ub antibody.

proteins a single modification may not be sufficient: their monoubiquitylated adducts may not bind at all or will bind weakly, which renders their degradation inefficient. The interaction of nonpolymerizable and WTUb fused to 150 residues with the proteasome was studied in HEK293 cells. It appears that $WTUb^{+150aa}$ binds slightly stronger (~1.5-fold) to the proteasome compared to $LLUb^{+150aa}$ (Figure S2C). To further corroborate these findings in a different system, we expressed $LLUb^{+79aa}$ and $LLUb^{+150aa}$ in yeast and followed their degradation. Similar to the findings in mammalian cells, $LLUb^{+79aa}$ was extremely short-lived, whereas $LLUb^{+150aa}$ was stable (Figure S2D). Importantly, similar to mammalian cells, the binding of the unstable $LLUb^{+79aa}$ to the yeast proteasome was significantly tighter compared to that of its stable counterpart $LLUb^{+150aa}$ (Figure S2Ei). We sought to rule out the remote possibility that Ub may play a new, unexpected noncanonical role in the degradation of short tailed Ub extensions, and show that binding to the proteasome plays a role in the degradation of these model substrates as well. Accordingly, we monitored the degradation of such a substrate in a yeast Rpn10-deleted strain (Δ*rpn10*). As can be seen in Figure S2Eii, Rpn10 is essential for efficient degradation of $LLUb^{+20aa}$ and $LLUb^{+79aa}$. As expected, in both WT and Rpn10-deleted strains, $LLUb^{+150aa}$ was stable. In that context, it was also important to study the potential role of Ub adaptors such as the UbL-UbA shuttle proteins Rad23 and Dsk2 in mediating the transfer of the Ub fusion proteins to the proteasome (Hartmann-Petersen and Gordon, 2004; Zhang et al., 2009). As can be seen in Figure S2F, $LLUb^{+79aa}$ was equally unstable in yeast strains lacking RAD23, DSK2 or both.

A Single Ub Fused to a Polypeptide of ~150 Residues Can Target It for Proteasomal Degradation without Additional Ubiquitylation: The Case of GFP

To further investigate the relationship between the length of the protein target and the requirement for its polyubiquitylation, we studied the degradation of Ub fused to intact and truncated derivatives of the Green Fluorescent Protein (GFP). Toward that end, we fused Ub^{VV} and $LLUb^{VV}$ to GFP (the complete GFP protein has 238 residues) (Figure 3A). It has been shown by others that Ub fused to the complete protein can serve as a degradation signal following its polyubiquitylation (Dantuma et al., 2000; Johnson et al., 1992). As expected, Ub^{VV}GFP was ubiquitylated both in a cell-free system and in cells, whereas the LL derivative was not (Figure S3A). In correlation with this finding, Ub^{VV}GFP was unstable in cells and its degradation was sensitive to MG132, whereas the nonpolymerizable Ub

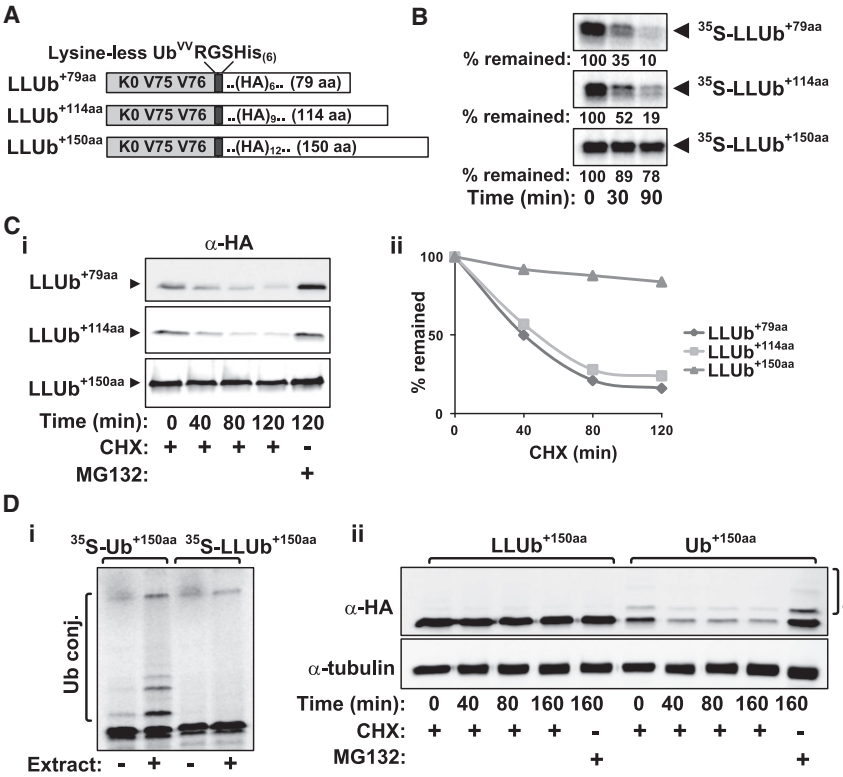

Figure 2. The Susceptibility to Proteasomal Degradation of a Single Ub-Modified HA Tag Is Dependent on the Number of the Repeats

(A) Schematic presentation of lysine-less Ub^{VV}His (denoted: LLUb) extended by an increasing number of HA tags - 6xHA (+79 amino acids; aa), 9xHA (+114aa) and 12xHA (+150aa).

(B) [^{35}S]-labeled LLUb^{VV} variants were subjected to cell-free ATP-dependent degradation in the presence of FrII. Degradation was assessed based on the radioactivity that had remained along time relative to time 0.

(C) (i) Stability of LLUb^{VV} variants was monitored in HEK293 cells after the addition of CHX. MG132 was added as indicated. (ii) Quantitative representation of the degradation of LLUb^{VV} variants shown in (i).

(D) (i) Cell-free ubiquitylation of [^{35}S]-labeled Ub^{+150} or LLUb^{+150} in the presence or absence of HeLa cell extract. (ii) The stability of Ub^{+150} or LLUb^{+150} was monitored in HEK293 cells as described under (C) (i). The expressed proteins were detected by Western blot using anti-HA antibody. Tubulin was used to assess equal protein loading. Ub always denotes WTUb that contains the complete cohort of its lysine residues.

derivative was stable (Figure 3B). Next, we generated a series of truncated derivatives of Ub^{VV}GFP where the Ub moiety was fused to the first 25, 40, 100, 120 and 150 residues of GFP (Figure 3Ci), and monitored their stability. As shown in Figure 3Cii–iv, and in consistency with the results obtained with Ub fused to the HA repeats (Figure 2), experiments carried out in both cells (Figure 3Cii) and cell-free systems (Figure 3Ciii–iv) showed that fusions containing up to 120 residues were unstable, whereas the chimera that contains 150 residues was stable. This chimera (WTUb^{VV}GFP$_{150}$) required exogenous Ub for its proteasomal cell-free degradation (Figure 3Ciii and Figure S3B). As expected, LLUb^{VV}GFP$_{150}$ was not degraded even following addition of free Ub (Figure S3B).

A Single Ub Fused to a Polypeptide of ~150 Residues Can Target It for Proteasomal Degradation without Additional Ubiquitylation: The Case of DHFR

Another protein we used in order to study the relationship between the length of the UPS target substrate and the requirement for polyubiquitylation for its proteasomal degradation is Dihydrofolate Reductase (DHFR). This protein (186 residues) is unfolded and assumes a tight folded conformation in the presence of methotrexate (MTX)(Bolin et al., 1982; Johnston et al., 1995). We studied the stability of DHFR to the N-terminus of which we fused either WTUb^{VV} or LLUb^{VV} (Figure 4Ai). In cells, Ub^{VV}DHFR was ubiquitylated, whereas the LL derivative was not (Figure 4Aii). This ubiquitylation destabilized WTUb-DHFR (Figure 4Aiii), whereas DHFR to which LLUb fused remained stable (Figure 4Aiv). As expected, nonubiquitylated DHFR was also stable (Figure 4Aiii). Addition of MTX inhibited the degradation of Ub^{VV}DHFR, most probably because the drug-bound enzyme is tightly folded (Figure 4Aiv). To further test the "length hypothesis," we generated two chimeras where LLUb was fused to the first 125 and 153 residues of DHFR (Figure 4Bi), and monitored their degradation in cells. Both LLUb^{VV}DHFR$_{125}$ and LLUb^{VV}DHFR$_{153}$ were degraded by the proteasome compared to the intact LLUb^{VV}DHFR that remained stable (Figure 4Bii). Taken together, these findings strongly suggest that the size of the protein plays an important role in determining the extent of ubiquitylation required for its proteasomal degradation. The stabilization of WTUb-DHFR by MTX corroborates previously published data (Johnston et al., 1995; Prakash et al., 2004) that an unstructured segment is necessary to initiate the degradation, probably regardless and independently of the size of the protein.

Monoubiquitylation Is Sufficient to Target Small Naturally Occurring Proteins for Proteasomal Degradation

Our next aim was to identify physiological substrates that are targeted following monoubiquitylation. We initially searched the database for low molecular mass proteins, and in particular those that may be regulated by proteolysis such as cell cycle control and stress response proteins. Two such proteins are the human cyclin-dependent kinases regulatory subunit 2 (Cks2) and the yeast Hug1. Cks2 is a 79 residues protein which has an essential function in cell cycle progression. It binds to the catalytic subunit of cyclin-dependent kinases and probably targets them to their substrates (Pines, 1996). High levels of Cks2 were observed in a broad range of human malignancies (Liberal et al., 2012). Interestingly, a recent report has shown

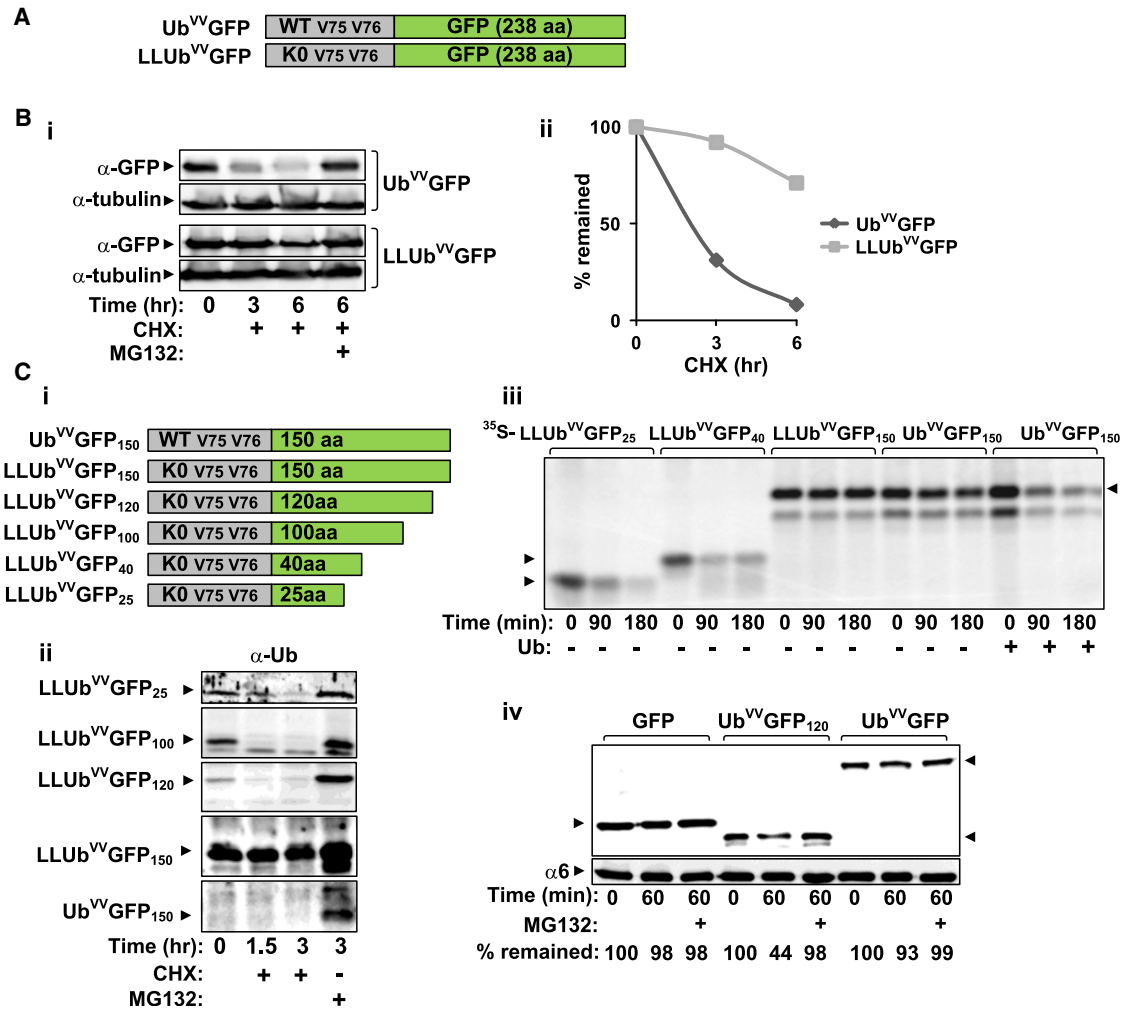

Figure 3. The Susceptibility to Proteasomal Degradation of a Single Ub Fused to GFP and Its Truncated Forms Is Dependent on the Length of Fused Segment

(A) Schematic presentation of Ub^{VV} and $LLUb^{VV}$ fused to GFP.

(B) (i) Stability of Ub^{VV}GFP and $LLUb^{VV}$GFP in HEK293 cells was monitored following the addition of CHX and MG132 as indicated. (ii) Quantitative representation of the degradation of the proteins shown in (i).

(C) (i) Schematic presentation of $LLUb^{VV}$ extended by sequential truncations of GFP ($Ub^{VV}GFP_{25}$ through $Ub^{VV}GFP_{150}$). (ii) Degradation of Ub^{VV} and $LLUb^{VV}$ GFP fusion variants in HEK293 cells after the addition of CHX or MG132. (iii) Degradation of [^{35}S]-labeled Ub^{VV}- and $LLUb^{VV}$-GFP fusion variants in a cell-free system in the presence of FrII and in the presence or absence of Ub as indicated. (iv) The degradation of bacterially expressed and purified GFP (purified proteins are shown in Figure S3C), $Ub^{VV}GFP_{120}$ and Ub^{VV}GFP was monitored in the presence of purified 26S proteasome. MG132 was added as indicated. Ub^{VV}GFP variants were detected using anti-Ub antibody. The 20S subunit α6 was used to assess equal protein loading.

that Cks2 is a short-lived protein (Eden et al., 2011), but its mechanism of degradation had not been identified. To examine whether Cks2 is targeted by the UPS, we first demonstrated that it can be ubiquitylated in a cell-free system (Figure 5A). Importantly, methylated Ub (MeUb), in which all the amino groups (the N-terminal and those of the internal lysines) were chemically modified - resulting in its inability to polymerize (Hershko and Heller, 1985), was also conjugated to the protein and generated mostly a single adduct. In contrast, WTUb generated several adducts, most of them of higher molecular mass. Next, we demonstrated that MeUb stimulates degradation of Cks2 in a cell-free system as efficiently as its WT counterpart (Figure 5B), suggesting that polyUb chain synthesis is not necessary in order to target the protein for degradation. As a control, we used Ring1B, another bona fide substrate of the UPS (Ben-Saadon et al., 2006) and demonstrated that its degradation in the cell-free system is strongly inhibited by MeUb (Figure S4A). The degradation of Cks2 in both cell-free system and in cells was proteasome-dependent, as it was sensitive to the inhibitor MG132 (Figure 5C). Not surprisingly, it required also internal lysines in the substrate as lysine-less Cks2 was stable (Figure 5C). The degradation of Cks2 in both cell-free system and in cells is accompanied by formation of Ub adducts which, as expected, is less efficient for LLCks2 (Figure S4B). It should

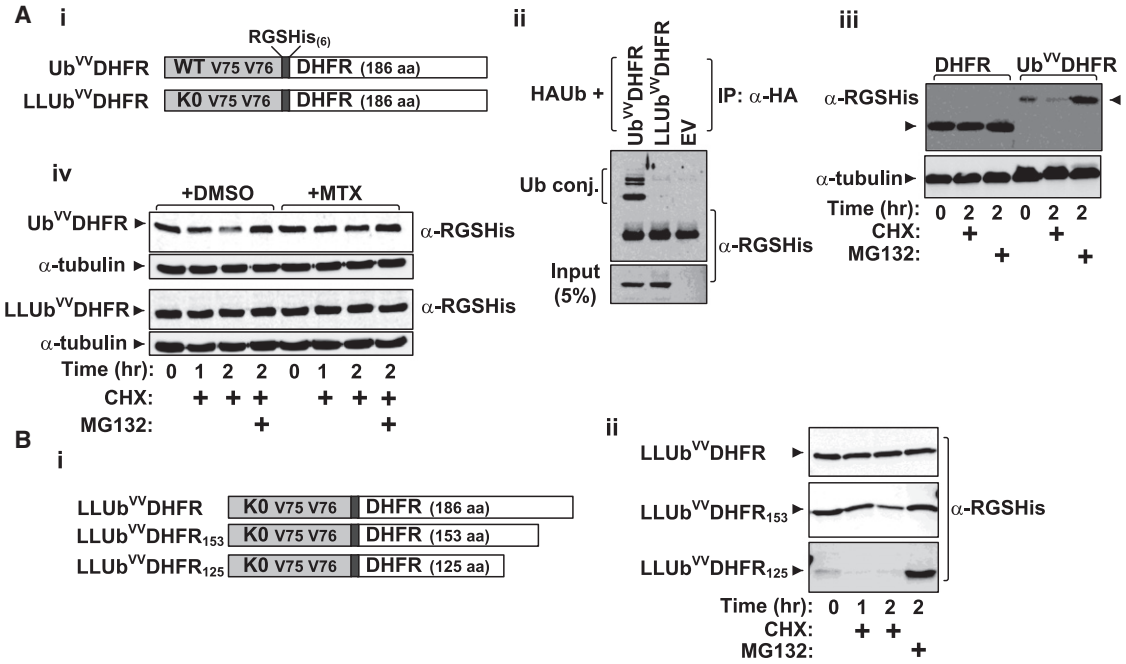

Figure 4. The Susceptibility to Proteasomal Degradation of a Single Ub Fused to DHFR and Its Truncated Forms Is Dependent on the Length of Fused Segment

(A) (i) Schematic presentation of Ub^{VV}His and LLUb^{VV}His fused to DHFR (denoted: Ub^{VV}DHFR, LLUb^{VV}DHFR). (ii) Ubiquitylation of Ub^{VV}DHFR and LLUb^{VV}DHFR in HEK293 cells. All cells were co-transfected also by a cDNA coding for HA-Ub. Empty vector (EV) was used as a control. Cell lysates were immunoprecipitated using anti-HA antibody and conjugates were visualized using anti-RGSHis (upper panel). 5% of the total cell lysates were resolved and proteins detected using anti-RGSHis (lower panel). (iii) Time-dependent degradation of DHFR and Ub^{VV}DHFR in HEK293 cells after the addition of CHX or MG132. The proteins were visualized using anti-RGSHis antibody, and tubulin was used to assess equal protein loading. (iv) The stability of Ub^{VV}DHFR and LLUb^{VV}DHFR was monitored in HEK293 cells as described under (iii). Methotrexate (MTX) or DMSO were added as indicated.

(B) (i) Schematic presentation of LLUb^{VV} extended by different lengths fragments of DHFR (LLUb^{VV}DHFR$_{125}$ and LLUb^{VV}DHFR$_{153}$). (ii) Degradation of the LLUb^{VV}DHFR variants in HEK293 cells was carried out as described under A.

be noted that endogenous Cks2, like the expressed protein, was also short-lived, and its degradation was dependent on the proteasome (Figure 5D). Similarly, in cells, the degradation of Cks2 proceeded also in the presence of nonpolymerizable K0Ub (Figure 5E). In contrast, the degradation of Mdm2, a larger protein substrate of the UPS, was significantly inhibited in cells expressing K0Ub (Figure 5E).

The yeast Hug1 is a 68 residues protein involved in the Mec1p-mediated checkpoint pathway that responds to DNA damage or replication arrest (Basrai et al., 1999). First we established that Hug1 is a UPS substrate that can be degraded following monoubiquitylation. Similar to Cks2, Hug1 was ubiquitylated in a cell-free system supplemented with FrII and Ub (Figure 6A) with lower MW conjugates formed in the presence of the nonpolymerizable Ub species K0Ub and MeUb. Also similar to Cks2, Hug1 was degraded in a cell-free system in a WT-, K0Ub- and MeUb- and proteasome-dependent modes (Figure 6B). In cells, the protein was short-lived, and its degradation was sensitive to proteasomal inhibition (Figure 6C). Last, we showed that in cells, expression of K0Ub has no effect on the short life of Hug1, yet it inhibits significantly the degradation of Mdm2 (Figure 6D). It should be noted that unlike the fused Ub constructs (to HA repeats, truncated GFP and truncated DHFR) that are unstructured, folding prediction of both Cks2 and Hug1 (Figure S5) show that both proteins are largely folded. Taken together, these results strongly suggest that the proteasomal degradation of Cks2 and Hug1 does not require the formation of polyUb chains. However, one cannot say with certainty whether these proteins require multiple monoubiquitylations for their degradation, or a modification by a single Ub moiety is sufficient. Mutational analysis of the lysine anchors in both proteins will provide better insight to this yet unsolved problem.

Finally, we sought to test the degradation of a larger substrate, and we selected α-synuclein. The function(s) of this protein is poorly understood, yet, point mutations and whole locus multiplications have been implicated in the pathogenesis of Parkinson disease and related neurodegenerative disorders (Corti et al., 2011). This well-studied protein has 140 residues which we hypothesize is at the upper limit of the size scale of proteins we suggest are targeted by monoubiquitylation. Multiple proteolytic pathways have been reported to be involved in the degradation of α-synuclein, including the proteasome (Bennett et al., 1999; Rott et al., 2011), the endosomal-lysosomal system and autophagy (Cuervo et al., 2004; Tofaris et al., 2011). To determine if monoubiquitylation is sufficient to target α-synuclein for degradation, we generated monoubiquitylated α-synuclein in which the Ub moiety is linked to Lys12 via an isopeptide bond (Figure 7A). The modified protein was degraded

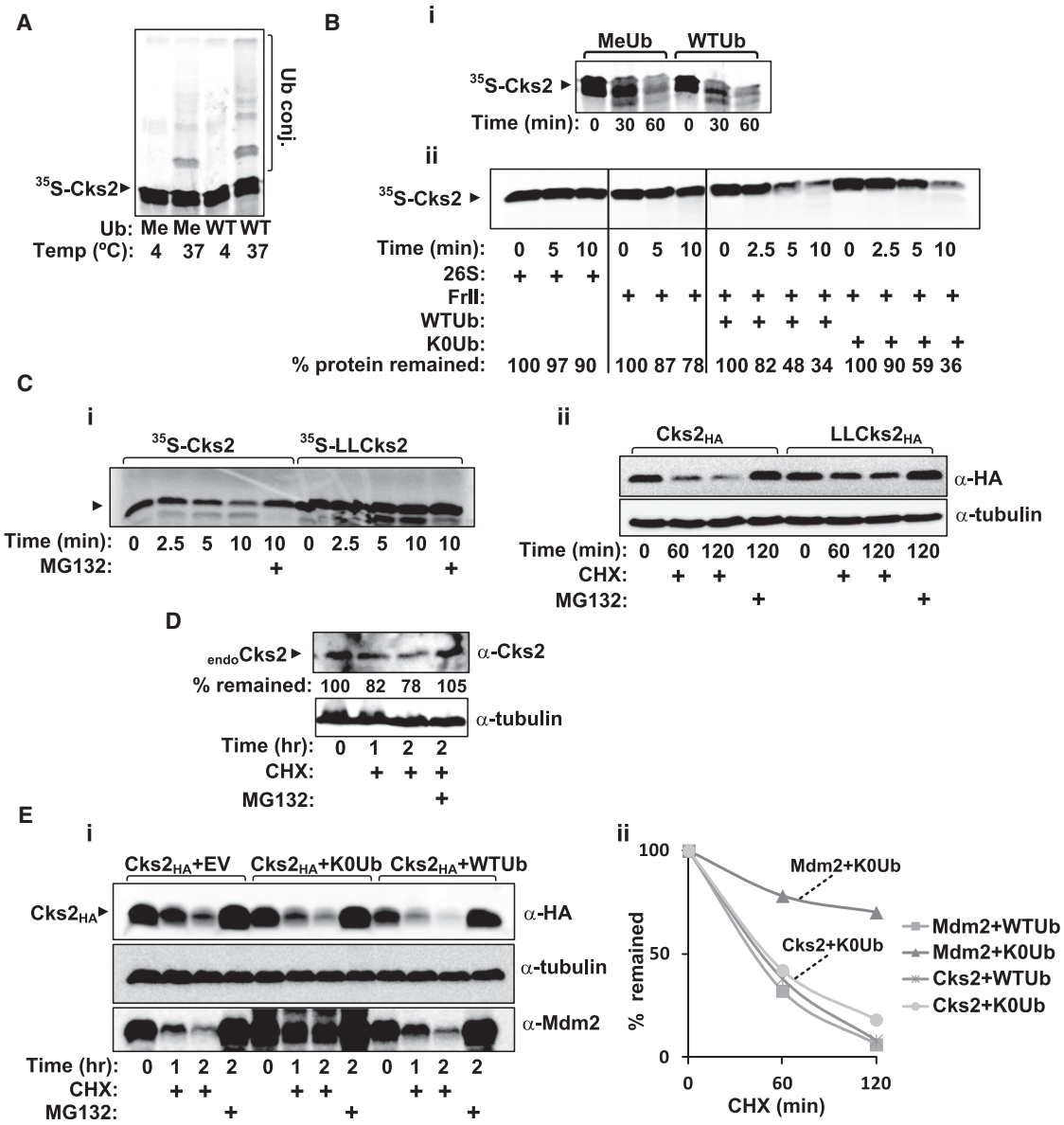

Figure 5. Cks2 Is Targeted for Proteasomal Degradation following Monoubiquitylation

(A) Cell-free conjugation of [^{35}S]-labeled Cks2 was monitored in the presence of FrII and in the presence of either WTUb or the nonpolymerizable MeUb as indicated.

(B) (i) Time-dependent degradation of [^{35}S]-labeled Cks2 in FrII-containing cell-free system in the presence of WTUb or MeUb as indicated. (ii) Nonpolymerizable Ub-dependent degradation of [^{35}S]-labeled Cks2 in FrII-containing cell-free systems. WTUb or K0Ub were added as indicated. A system supplemented only with 26S proteasome serves as a control for ubiquitylation dependence.

(C) (i) Cell-free degradation of [^{35}S]-labeled Cks2 or LLCks2 in the presence of HeLa cell extract. MG132 was added as indicated. (ii) Cellular stability of Cks2HA and LLCks2HA was monitored in a CHX chase experiment. MG132 was added as indicated. Proteins were visualized using anti-HA antibody and tubulin was used to assess equal protein loading.

(D) Degradation of endogenous Cks2 was monitored as described under (C) (ii) using anti-Cks2 antibody.

(E) (i) Effect of WT and nonpolymerizable Ub on cellular degradation of Cks2 and Mdm2. Cks2HA was co-expressed along with cDNAs coding for either WTUb or K0Ub. Its cellular stability was monitored along with that of endogenous Mdm2 as described under (C) (ii). Cks2 was detected using anti-HA antibody and endogenous Mdm2 was detected using anti-Mdm2 antibody. (ii) Quantitative presentation of the degradation of the proteins shown under (i).

efficiently in a cell-free system containing purified 26S proteasome. In contrast, unmodified α-synuclein was stable (Figure 7B). Thus, it appears that our hypothesis that modification by a single Ub moiety can target proteins of up to 150 residues for proteasomal degradation is true for naturally occurring proteins.

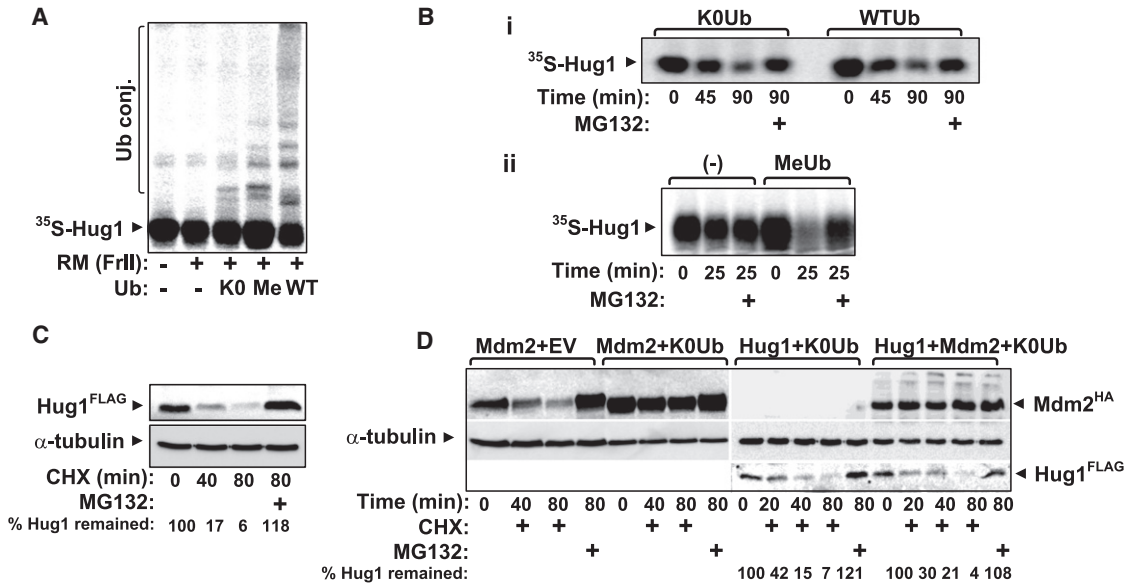

Figure 6. Hug1 Is Targeted for Proteasomal Degradation following Monoubiquitylation

(A) Cell-free conjugation of [^{35}S]-labeled Hug1 supplemented with FrII in the presence of either K0Ub (K0), MeUb (Me), or WTUb (WT) as indicated.

(B) Degradation of [^{35}S]-labeled Hug1 in FrII supplemented with either WTUb or K0Ub (i), or MeUb (ii). MG132 was added as indicated.

(C) Time-dependent degradation of Hug1-FLAG in HEK293 cells following the addition of CHX. MG132 was added as indicated.

(D) Effect of WT and nonpolymerizable Ub on the cellular degradation of Hug1 and Mdm2. cDNAs coding for the two proteins were co-transfected along with a cDNA coding for K0Ub or an EV as indicated. The stability of Hug1 and Mdm2 was monitored as described under (C), and the proteins were visualized using anti-FLAG and anti-HA antibodies. Tubulin was used to assess equal protein loading.

DISCUSSION

It was suggested that the proteasome can recognize monoubiquitylated targets longer than 20 residues (Shabek et al., 2009; Verhoef et al., 2009). This was demonstrated using linear Ub fusion proteins that though occur in nature, are not the common Ub adducts. In the current study we have further corroborated these initial findings, now using peptides to which Ub is conjugated by an isopeptide bond that is the hallmark of degradable natural UPS substrates (Figure 1). The main aim of this study was to identify possible common features that characterize substrates that are degraded following monoubiquitylation. As a single Ub is sufficient to promote the proteasomal degradation of peptides with a minimal length of 20 residues, it was important to determine the maximal size of peptides/proteins the degradation of which can still be supported by a single Ub modification. Synthesis of Ub molecules and assembly of polyUb chain is energetically costly. Furthermore, we have shown that part of the polyUb chain is degraded along with the substrate which is also costly (Shabek et al., 2009). Thus, it seems that not all substrates should require an equally long polyUb chain for their proteasomal degradation, and that the proteolytic signal is adaptive. Mechanistically, one can envision that in the cell the ubiquitylation and degradation machineries are found in a loosely associated complex. As Ub moieties are added to the substrate-bound elongating Ub chain, the affinity of the conjugate to the proteasome increases. Once the affinity is high enough to secure stable binding of the adduct to the proteasome, it is detached from the conjugating machinery, binds to the proteasome, and is degraded processively and efficiently. With larger substrates, a longer polyUb chain may become necessary. That because a single Ub moiety or a short chain are not sufficient to bind stably a long polypeptide to the proteasome to secure its processive digestion. To initially test this hypothesis, we monitored the proteasomal susceptibility of nonpolymerizable Ub fused to peptides of increasing length. Our constructs included Ub fused to peptides comprised of an increasing number of HA repeats (Figure 2), and to fragments of increasing length derived from GFP (Figure 3) and DHFR (Figure 4). In addition, we identified two small naturally occurring proteins, the cell cycle regulator Cks2, and the DNA damage response protein Hug1, that can also be targeted following monoubiquitylation (Figures 5 and 6, respectively). Furthermore, a single Ub conjugated via an isopeptide bond to α-synuclein, a protein of 140 residues, was sufficient to promote its proteasomal degradation (Figure 7). All these studies have shown that conjugation of a single Ub moiety can target for degradation proteins of up to ~150 residues. It should be noted that Rott and colleagues have recently brought evidence that α-synuclein is degraded in cells following monoubiquitylation, though their experiments do not rule out multiple monoubiquitylations of the molecule (Rott et al., 2011).

In this context it should be noted that recent structural studies of the 26S proteasome suggest that the distance between the proteasomal Ub receptors, Rpn10 and Rpn13, and the ATPase channel is ~70-80Å (Lander et al., 2012). We have shown that a substrate where Ub is fused to an unstructured tail of 20 residues (~70Å long), is degraded in an Rpn10-dependent mode, suggesting it acts as a "classical" substrate, the degradation

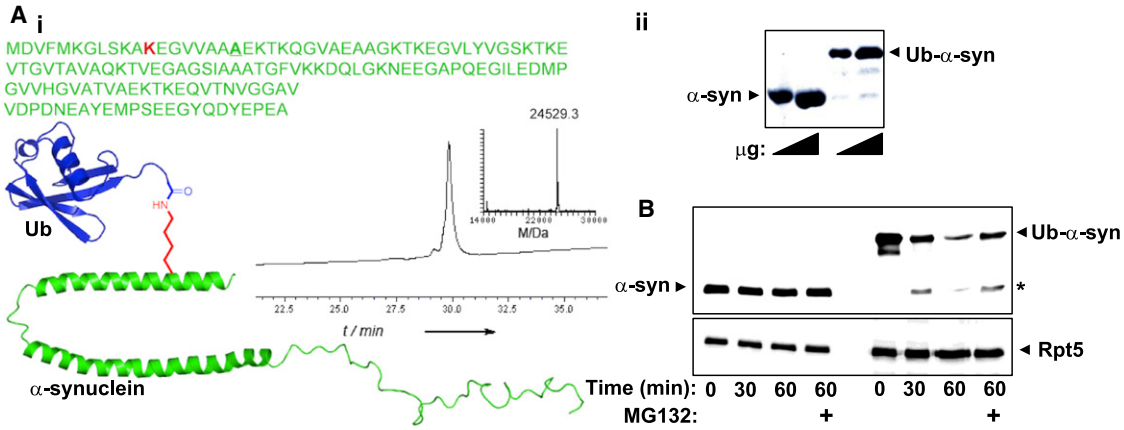

Figure 7. Isopeptide Bond-Linked Monoubiquitylated α-Synuclein Is Degraded by the Proteasome

(A) (i) Schematic presentation of monoubiquitylated α-synuclein (α-syn) that was generated by a semi-synthetic chemical approach. Ub was covalently linked to Lys12 and the adduct was analyzed by HPLC/ESMS. (ii) 1 and 2 μg of purified and monoubiquitylated α-syn (Ub-α-syn) were analyzed by coomassie blue staining following SDS-PAGE.

(B) Degradation of α-syn and Ub-α-syn in a cell-free system. 1 μg of each substrate was subjected to ATP-dependent degradation in the presence of purified 26S proteasome. MG132 was added as indicated. Rpt5 is derived from the proteasome. Asterisk denotes free α-syn which was probably released by the proteasome-associated DUB.

of which is Ub-dependent. It should be noted that Ub itself may contribute to some extent to the final distance. Also, the proteasome's recognition/degradation machinery probably does not act in a binary - point-to-point - rigid mode, but rather as a dynamic and elastic apparatus allowing a variety of peptides and substrates to be efficiently processed following diverse ubiquitylation patterns.

It is interesting to point that a single Ub moiety can bind to the Rpn10 subunit of the proteasome in a similar affinity (as determined by surface plasmon resonance) to that of Ub fused to model proteins (intact and truncated GFP; Figures S3C and S3D). However, whereas the quantitative affinity data are in line with other published data (Zhang et al., 2009), they nevertheless do not appear to represent faithfully the cellular events. That because the association of Ub adducts with the intact proteasome and its multiple Ub-binding subunits is far more complex than the binding to a single immobilized proteasomal subunit used in our assay.

It was proposed that α-globin (142 residues) can be degraded following monoubiquitylation (Shaeffer, 1994; Shaeffer and Kania, 1995) which is in line with our hypothesis. However, PAX3 (479 residues) was also reported to be targeted following a similar modification (Boutet et al., 2007). Furthermore, recent studies reported that multiple-monoubiquitylations are required for the proteasomal processing of the p105 NF-κB precursor (Kravtsova-Ivantsiv et al., 2009) and for the degradation of phospholipase D (PLD) (Yin et al., 2010), and cyclin B1 (Dimova et al., 2012). It will be interesting to find out (though complicated structural studies are required to solve this problem), whether multiple monoubiquitylations - where the different Ub moieties bind to different proteasomal subunits or to different domains in the known Ub-anchoring subunits - can substitute for a single polyUb chain (the role of which is also not clear). Thus, it is clear that while the size of the protein substrate plays an important role in determining the length of the Ub chain required for targeting it for degradation, other factors may play a role as well.

One general argument can be that the folding state rather than the length of a protein may play a role in its ubiquitylation/degradation mode. In this context, one may claim correctly that the HA-repeats as well as the truncated GFP and DHFR are unfolded. Of note that for the HA repeats, the 114 residues extension is unstable, whereas the 150 residues extension is stable; for the GFP, the 120 is unstable, whereas the 150 (which is assumingly still unfolded) is stable; and for the DHFR, the 125 is unstable, the 153 (which does not bind methotrexate) is partially stable, whereas the full length protein (186 residues), even without methotrexate (the binding of which results in its folding) is stable (all obviously in the presence of nonpolymerizable Ub). Importantly, we show that naturally occurring small substrates that are properly folded (α-synuclein, Hug1 and Cks2, see Figures 7 and S5) are degraded following monoubiquitylation. Thus it appears that the size/length acts as a distance ruler that plays an important role in the ubiquitylation mode of the appropriate substrates that leads to their degradation regardless of their folding state. Nevertheless, it will be a challenge to distinguish between these two elements (folding versus length) and to be able to assess the role of each in the proteolytic process.

It should be noted that with currently available experimental tools, it is impossible to dissect the nature of Ub chains generated on proteins larger than 150 residues. However, recent emerging methodologies that enable chemical synthesis of well-defined Ub conjugates may allow further light to be shed on the adaptability of the proteolytic signal. Once we shall have the capacity to attach to proteins Ub chains of increasing size, we shall be able to test the contribution of the size of the target protein to that of the Ub chain necessary to efficiently target it for degradation.

EXPERIMENTAL PROCEDURES

Materials, antibodies, cDNA constructs, and standard methods are described under Supplemental Information.

Synthesis of Ubiquitylated Peptides and Monoubiquitylated α-Synuclein

The Ub-peptides were synthesized chemically using solid phase methodology coupled with native chemical ligation of Ub (NCL)/desulfurization] as described in Kumar et al. [2011]). Specifically, the synthesis of Ub- α-syn (K12) was accomplished following a similar strategy for the preparation Ub- α-syn (K6) (Hejjaoui et al., 2011). The α-syn(1-18)-SR peptide bearing δ-mercaptolysine at Lys12 was prepared using Fmoc-based solid phase peptide synthesis. Subsequently, this peptide was ligated with an expressed peptide fragment (19-140) bearing N-terminal Cys. After ligation of the two fragments to assemble the backbone of the α-syn polypeptide, an isopeptide ligation step with Ub-thioester - mediated by δ-mercaptolysine - was performed followed by a final desulfurization step to give the pure Ub-α-syn (K12). The purified conjugates were analyzed by HPLC/ESMS, and their folding state determined by circular dichroism (CD). They were dissolved and stored in 50 mM Tris pH 7.6, and their concentration determined using Pierce® BCA Protein Assay Kit.

Monitoring the Stability of Proteins in a Reconstituted Cell-Free System

Purified Ub-peptides (1 μg), [^{35}S]-labeled proteins (~20,000 cpm), or purified proteins (1 μg) were incubated at 37°C for the indicated times in a reaction mixture (RM) supplemented with ATP and ATP-regenerating system at a final volume of 12.5 μl as described previously (Shabek et al., 2009). The RM contained complete HeLa cell extract or Fraction II (FrII; Ub-depleted HeLa cell extract; was prepared as described under Supplemental Information) or purified 26S proteasome (0.3 μg, Enzo Life Sciences). The ratio of 26S proteasome to purified proteins was approximately 1:800. When indicated, the proteasome inhibitor MG132 was added in a concentration of 100 μM. Reactions were terminated by the addition of 3-fold concentrated sample buffer. Boiled samples were resolved via SDS-PAGE, and proteins were visualized using Western blot and chemiluminescence or PhosphorImaging. Band intensities were quantified using the Total Labs TL100 1D gel analysis software.

Monitoring Stability of Proteins in Cells

30 hr after transfection, cells were treated with cycloheximide (CHX; 100 μg/ml) and/or MG132 (20 μM), or methotrexate (MTX; 40 μM). Following addition of the inhibitors for the indicated times, cells were harvested in RIPA lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% NP-40, 0.1% SDS and protease inhibitors). Proteins were resolved via SDS-PAGE and detected following Western blotting as described above. All membranes were re-probed with anti-tubulin antibody that served as a loading control. Proteins were quantified as described above.

Conjugation of Proteins in a Reconstituted Cell-Free System

[^{35}S]-labeled proteins were incubated in reaction mixtures in a final volume of 12.5 μl in the presence of 30 μg HeLa cell extract or FrII (as a source of UPS conjugating enzymes) and other components as described previously (Shabek et al., 2009). 5 μg of WTUb, MeUb or K0Ub were added to the RM as indicated. The reactions were incubated at 37°C for 1 hr, and proteins were resolved by SDS-PAGE and visualized using PhosphorImaging.

Ub Conjugates in Cells

HEK293 cells were transiently transfected (as described under Supplemental Information) with cDNAs coding for the different Ub^{VV} fusions or Cks2 variants along with cDNAs coding for WT or K0 HA/FLAG-tagged Ub. After 24 hr, MG132 was added for 2 hr and the cells were lysed with RIPA buffer supplemented with freshly dissolved iodoacetamide and *N*-ethylmaleimide (5 mM each) to inhibit DUBs. Ubiquitylated substrates were immunoprecipitated with the indicated immobilized antibodies, washed three times with RIPA buffer, and resolved by SDS-PAGE. Free and conjugated substrates were visualized using the appropriate antibodies.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2012.07.011.

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