Ubiquitin degradation with its substrate, or as a monomer in a ubiquitination-independent mode, provides clues to proteasome regulation

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Contributed by Aaron Ciechanover, May 27, 2009 (sent for review April 21, 2009)

The mechanisms that regulate the ubiquitin (Ub)-proteasome system's own components, although critically important, are largely unknown. Ub, a principal component of the system, must be maintained at adequate levels to support cellular homeostasis under basal and stressed conditions. It was suggested that Ub is degraded as part of the polyubiquitin chain along with its substrate. Here, we demonstrate in a direct manner that Ub is indeed degraded in a "piggyback" mechanism. Also, it has been shown that monomeric Ub can be rapidly degraded when a C-terminal tail of a minimal length is fused to it. The tail, which may represent the substrate or part of it, or a naturally occurring extended form of Ub, probably allows entry of the protein into the 20S catalytic chamber, while Ub serves as an anchor to the 19S complex. Here, we show that shorter-tailed Ubs, such as UBB^{+1}, bind to the proteasome but because they cannot be efficiently degraded, they inhibit the degradation of other Ub system's substrates such as Myc, p21, Mdm2, and MyoD. The inhibition depends on the ability of the tailed Ubs to be ubiquitinated: their mere binding to the proteasome is not sufficient. Interestingly, the inhibition affects only substrates that must undergo ubiquitination for their degradation: ornithine decarboxylase that is targeted by the proteasome in a Ub-independent manner, is not affected by the short-tailed ubiquitinated Ubs, suggesting it binds to the 19S complex in a site different from that to which ubiquitinated substrates bind.

UBB^{+1} | neurodegeneration | extended ubiquitin | proteasomal recognition

Modification by ubiquitin (Ub) affects the fate of countless proteins and plays important roles in numerous basic cellular processes in health and disease. Therefore, regulation of the system's own components is vital.

Ub exists as a free monomer or in a conjugated form, and the ratio between these two forms is determined by the balanced activities of conjugation and deubiquitination. The maintenance of Ub homeostasis is essential for cell viability, and an adequate supply of Ub is crucial for the ability of the cell to withstand stress (1–3). Under such conditions, aberrant proteins are formed in large amounts, leading to an increase in the activity of the Ub-proteasome system (UPS), which partially results from accelerated rate of synthesis of Ub and other components of the UPS (4–6). Interestingly, the total amount and ratio between free and bound Ub vary among different tissues under basal conditions (7), and significant changes occur in Ub homeostasis during different pathologies (4, 8). The mechanisms that underlie Ub regulation under different pathophysiological conditions are largely unknown.

Early reports showed that under basal conditions the degradation of Ub is nonlysosomal and ATP-dependent, and the protein has a half-life of ≈10 h (9, 10) that varies between different organisms and cells (1, 2, 11). Importantly, Ub degradation was significantly accelerated in yeast harboring mutated Doa4 and Ubp6, the proteasome-associated deubiquitinating enzymes (DUBs) (2, 3). Moreover, a considerable decrease in Ub level was observed in mice carrying mutations in Usp14 (12), suggesting that malfunction of DUBs results in the degradation of unreleased Ub along with its target substrate. Depletion of Ub was also observed under stress conditions such as elevated temperature or after treatment with translation inhibitors (1, 2, 13), suggesting that general elevated activity of the UPS leads to accelerated Ub degradation. It was proposed that under stress elevated Ub conjugation may lead to accumulation of aggregation-prone ubiquitinated substrates that are not degraded (14).

In an attempt to find out whether Ub is degraded as a free monomer or as part of the targeted substrate, we showed that monomeric Ub is rapidly degraded by the proteasome when fused to a long (>20 residues) C-terminal extension (13). Similar findings were reported later by Verhoef et al. (15). An unsolved problem relates to the physiological relevance of such an extension. It can represent a proteolytic intermediate where the tail that is derived from the substrate is bound to Ub. This intermediate rebinds to the proteasome via its Ub moiety and via the tail reaches the catalytic chamber of the 20S complex, pulling the entire molecule behind it: Ub itself or Ub with a tail that is shorter than a certain size cannot reach the 20S-buried proteolytic sites. It can also represent the entire conjugated substrate that is degraded after monoubiquitination (16, 17). An extended Ub can also represent naturally occurring derivatives. One such protein is coded by the Ub B gene and was designated UBB^{+1}. This molecule is generated from molecular misreading of the mRNA resulting in translation of an additional C-terminal segment of 19 residues (18). UBB^{+1} was implicated in the pathogenesis of an early-onset form of Alzheimer's disease and other forms of neurodegeneration (18). The toxicity of the protein is probably related to its stability and ability to inhibit the proteasome in an as yet unknown mechanism (19). An interesting finding was that a slightly longer tail renders the protein unstable (15).

Here, we demonstrate in a direct manner that Ub is degraded via the proteasome along with its conjugated target substrate in a "piggyback" mechanism. Further studies on the mechanism of degradation of tailed Ub showed that it does not require further ubiquitination, highlighting in an as yet additional case the ability of the proteasome to recognize and degrade monoubiquitinated substrates (16, 17). This was true also for UBB^{+1} that was further extended to have a tail >19 residues. We also show that short-tailed Ubs such as UBB^{+1}

Author contributions: N.S., Y.H.-B., and A.C. designed research; N.S. and Y.H.-B. performed research; N.S., Y.H.-B., and A.C. contributed new reagents/analytic tools; N.S., Y.H.-B., and A.C. analyzed data; and N.S., Y.H.-B., and A.C. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0905746106/DCSupplemental.

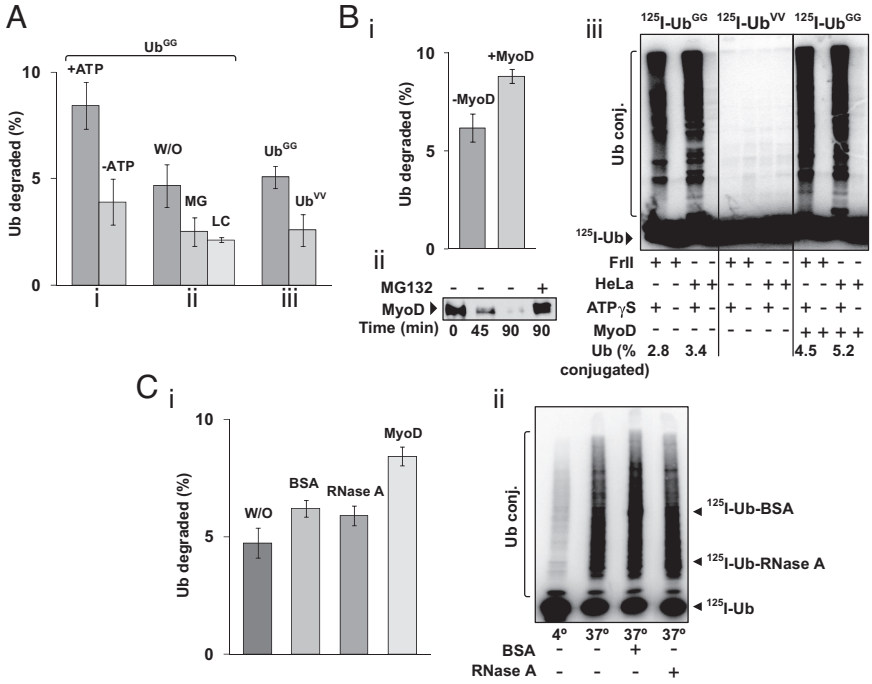

Fig. 1. Ub degradation is enhanced in a cell-free system when it is conjugated to its substrate. (*A*) ATP- and proteasome-dependent degradation of WT (Ub^{GG}) and inert (Ub^{VV}) Ubs. (*i* and *ii*) ^{125}I- Ub^{GG} was subjected to in vitro degradation in reticulocyte fraction II (FrII) in the presence or absence of ATP (*i*) and in the presence or absence of the proteasome inhibitors MG132 (MG) or lactacystin (LC) (*ii*). (*iii*) Degradation of ^{125}I-Ub^{GG} was compared with that of ^{125}I-Ub^{VV}. Bars in *ii* and *iii* represent net ATP-dependent values. (*B*) Ubiquitination and degradation of ^{125}I- Ub^{GG} in the presence and absence of added MyoD. (*i*) ^{125}I- Ub^{GG} was subjected to in vitro degradation in the presence of HeLa cell extract with or without MyoD (5 μg). (*ii*) Purified MyoD was subjected to degradation in vitro as described in *Materials and Methods*. (*iii*) ^{125}I-Ub^{GG} and ^{125}I-Ub^{VV} were subjected to in vitro ubiquitination in the presence of HeLa cell extract or reticulocyte FrII, ATP, and purified MyoD (5 μg) as indicated. (*C*) ATP-dependent degradation (*i*) and ubiquitination (*ii*) of ^{125}I-Ub^{GG} in the presence of FrII. Purified MyoD and unlabeled iodine-modified BSA and RNase A were added as indicated (5 μg each). Degradation of labeled Ub was monitored by using release of TCA-soluble radioactivity, and degradation of MyoD was monitored after SDS/PAGE and Western blot analysis as described in *Materials and Methods*. Conjugation of labeled Ub was carried out as described in *Materials and Methods*. All degradation values of Ub represent the average of 5 independent experiments (SD is shown). Ub conj. denotes Ub conjugates.

inhibit the UPS, but the inhibitory effect is not direct: it rather results from their ability to be ubiquitinated. This inhibitory effect is restricted to ubiquitination-dependent substrates of the proteasome: ornithine decarboxylase (ODC), a ubiquitination-independent substrate, was not affected. Thus, it appears that the minimal requirements for degradation of a substrate by the 26S proteasome is a binding Ub moiety and a long enough extension that will allow it to reach the proteolytic sites or be ratcheted into the proteolytic chamber. The polyubiquitin chains are probably required to increase the affinity of the target substrate to the proteasome, but they become inhibitory if the substrate does not have one feature that renders it susceptible for proteasomal degradation, e.g., a long enough free tail.

Results

Ub Is Degraded by the UPS in a Piggyback Mechanism Along with Its Conjugated Substrate. To demonstrate directly that Ub is degraded along with its conjugated substrate, we used a cell-free reconstituted proteolytic system that is comprised of fraction II (which is depleted from endogenous Ub but contains most of the remaining enzymes of the UPS) and radiolabeled ^{125}I-Ub. The stability of Ub was monitored by following the release of trichloroacetic acid (TCA)-soluble radioactivity. As can be seen in Fig. 1*A*, Ub degradation is both ATP- and proteasome dependent (*i* and *ii*). The finding that Ub^{GG} (WT Ub) was a significantly more efficient substrate than Ub^{VV} that cannot be conjugated (Fig. 1 *Aiii* and *Biii*) further suggests that it is degraded along with its conjugated substrates.

To address this assumption directly, we monitored Ub degradation in the presence of exogenously added substrates of the UPS. As can be seen in Fig. 1*Bi*, addition of MyoD (ref. 20 and Fig. 1*Bii*) stimulated Ub degradation. As expected, the addition of MyoD was accompanied by enhanced conjugation (Fig. 1*Biii*), most probably of MyoD itself. This enhancement could be observed when both FrII and HeLa extracts were used. Similar results were observed by using BSA, and RNase A (21) (Fig. 1*C i* and *ii*). It should be noted that both FrII and HeLa extracts are rich with endogenous substrates, and the added substrates are riding over this "background." Taken together, these findings clearly show that Ub is degraded as part of its conjugated substrate.

26S Proteasome Can Bind and Degrade Extended Monomeric Ub in a Ubiquitination-Independent Manner. We and others previously reported that C-terminal tails longer than ≈20 residues destabilize Ub (13, 15). For example, $Ub^{VV}RGS_6His$ (denoted: $Ub^{VV}His$) was significantly more stable than $Ub^{VV}RGS_6HisHA$ (denoted $Ub^{VV}HisHA$) (13). To study whether ubiquitination is required for degradation, we examined the stability of similar Ub derivatives that are lysineless (LL) and blocked at their N-terminal residue (by a Myc tag), so they cannot be ubiquitinated (13, 20). As can be seen in Fig. 2*A*, in cells, LL or N-terminally-blocked long-extended Ubs were rapidly degraded similar to the nonmodified Ub species (compare Fig. 2*A v* and *vi* with *iii*). As expected, tailless Ub or short-tailed Ub are stable (Fig. 2 *A i* and *ii* and *B i* and *ii*). The combined LL and N-terminally blocked long-tailed extended Ub was also degraded, although somewhat

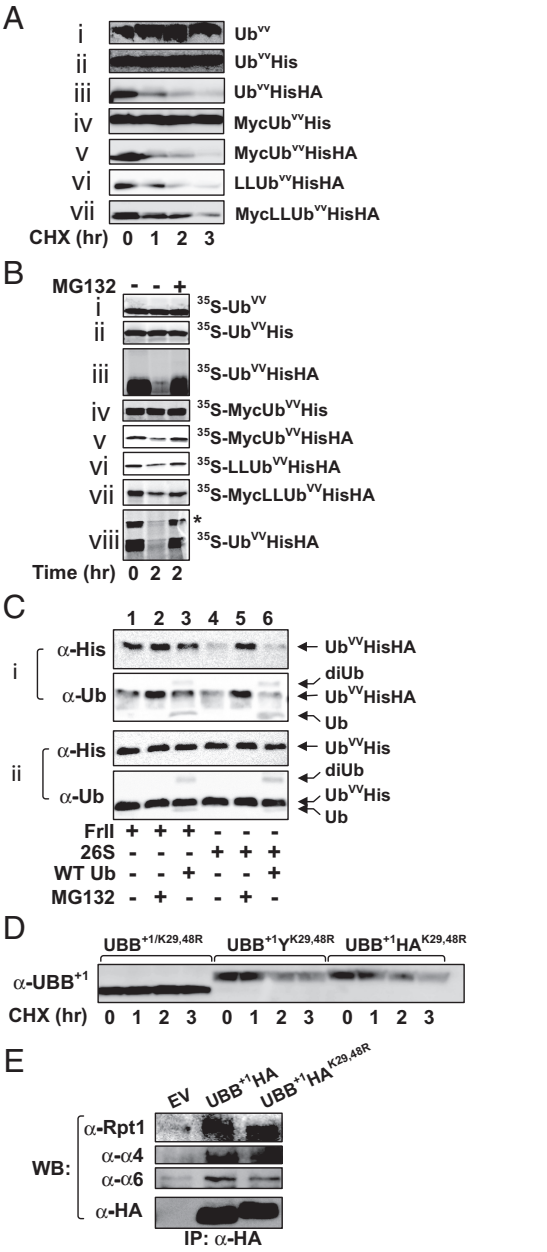

Fig. 2. Ubiquitination-independent degradation of extended Ubs by the 26S proteasome. (*A*) cDNAs coding for the indicated Ub^{VV} variants was expressed in HEK-293 cells, and the stability of the proteins was monitored after the addition of CHX. Proteins were visualized by using anti-Ub (*i*) or anti-RGS·His (*ii–vii*). (*B*) ^{35}S-labeled Ub^{VV} variants were translated in S30 bacterial extract (except for ^{35}S-Ub^{VV}HisHA shown in *viii* that was translated in wheat germ extract) and subjected to in vitro ATP-dependent degradation in FrII without Ub. MG132 was added as indicated. The asterisk in *viii* marks monoubiquitinated Ub^{VV}HisHA that was generated during translation. (*C*) His-tagged bacterially expressed and purified Ub^{VV}HisHA (*i*) and Ub^{VV}His (*ii*) were subjected to in vitro ATP-dependent degradation in the presence of FrII (lanes 1–3) or purified 26S proteasome (lanes 4–6). MG132 and Ub were added as indicated. Reaction mixtures were incubated for 2 h. Proteins were detected by using anti-RGS·His (*Upper*) or anti-Ub (*Lower*; membranes were reblotted). (*D*) HEK-293 cells were transfected with cDNAs (in pCS2) coding for $UBB^{+1/K29,48R}$, $UBB^{+1}Y^{K29,48R}$, and $UBB^{+1}HA^{K29,48R}$, and their stability was monitored after the addition of CHX. (*E*) HEK-293 cells were transfected with cDNAs coding for UBB^{+1}HA, $UBB^{+1}HA^{K29,48R}$, and empty vector (EV). HA-tagged proteins were immunoprecipitated as described in *Materials and Methods*, and the association with the proteasome was detected with the indicated antibodies. In all experiments, proteins were resolved via SDS/PAGE.

less rapidly (Fig. 2*Avii*). Similar degradation patterns of these substrates were observed in a reconstituted cell-free system (Fig. 2*B*). Of note is that the substrates in this experiment were translated in bacterial extract (Fig. 2*Bi–vii*) that does not contain Ub, and their degradation was monitored in a Ub-depleted FrII.

To further substantiate these findings, we examined the degradation of short-tailed and long-tailed Ub derivatives generated in bacteria, using purified 26S proteasome. As can be seen in Fig. 2C *i* and *ii* (lanes 4–6), the purified proteasome degraded the long- but not the short-tailed Ub. Notably, addition of WT Ub did not affect the degradation of the unstable species (Fig. 2*Ci*, lane 6).

Taken together, these findings strongly indicate that long-enough extended Ub can be rapidly degraded without further ubiquitination.

It was previously demonstrated that ubiquitination of UBB^{+1} was abrogated when Lys-29 and Lys-48 were substituted with Arg [($UBB^{+1/K29,48R}$ (22)]. Therefore, we sought to use this substrate to further corroborate our findings that monomeric extended Ub is degraded in a ubiquitination-independent manner. To this end, we further extended the C-terminal segment of $UBB^{+1/K29,48R}$ by adding either a single residue (Tyr) or a HA tag, thus having a tail >20 residues that destabilizes the protein. As can be seen in Fig. 2*D*, both $UBB^{+1}Y^{K29,48R}$ and $UBB^{+1}HA^{K29,48R}$ were unstable in cells compared with the nonextended $UBB^{+1/K29,48R}$, demonstrating that these derivatives were degraded in a ubiquitination-independent manner. Interestingly, both $UBB^{+1}HA^{K29,48R}$ and UBB^{+1}HA were able to bind the 19S regulatory subunit Rpt1 and the 20S subunits α4 and α6 (Fig. 2*E*), suggesting that the binding to the proteasome does not require ubiquitination.

Short-Tailed Ubs Inhibit the Degradation of UPS Substrates via Their Ability To Be Ubiquitinated. The results shown in Fig. 2 suggest that extended Ubs bind to the 26S proteasome via their Ub domain and are degraded after protrusion of their tails/extensions into the 20S catalytic chamber. A possible implication of this mechanism is that short-tailed Ubs, where the tail can enter the proteasome only partially not being able to be ratcheted efficiently or to reach the 20S catalytic sites, can occupy the proteasome and impair its function, as was suggested for UBB^{+1} (15, 22). Indeed, our findings (Fig. 3*A*) show that Ub^{VV}His, unlike Ub^{VV}HisHA, inhibits the degradation of four substrates of the UPS, MyoD, Mdm2, Myc, and p21. Ub^{VV} had an intermediate effect. We assume that not having even a short tail makes the binding of Ub to the proteasome weaker compared with that of Ub^{VV}His. Importantly, $LLUb^{VV}$His did not have any inhibitory effect (Fig. 3*B*), suggesting that it is the ubiquitination of these short-tailed Ubs that make them inhibitory. To further consider this hypothesis, we tested the effect of UBB^{+1} and $UBB^{+1/K29,48R}$ on the degradation of transfected Myc and endogenous p21. As can be seen in Fig. 3*Ci*, only the nonmutated UBB^{+1} that can be ubiquitinated was inhibitory. It should be noted that it was previously reported that in cells $UBB^{+1/K29,48R}$ does not exhibit the inhibitory effect seen with UBB^{+1} toward a UFD artificial substrate (22). As further evidence that short-tailed extended Ubs that can be ubiquitinated inhibit the proteasome, we monitored the level of Ub conjugates in cells expressing UBB^{+1} and $UBB^{+1/K29,48R}$. As can be seen in Fig. 3*Cii*, the level of total Ub conjugates in cells expressing UBB^{+1} is significantly higher than in cells expressing its counterpart that cannot be modified.

The Inhibitory Effect of Short-Extended Ub Derivatives Affects Ubiquitination-Dependent Substrates. We have shown that UBB^{+1} and its double lysine mutant interact with the 26S proteasome in cells (Fig. 2*E*). To further dissect the mode of proteasomal inhibition, we sought to determine in a cell-free reconstituted system whether shorter-tailed extended Ub can also bind to the proteasome. As can

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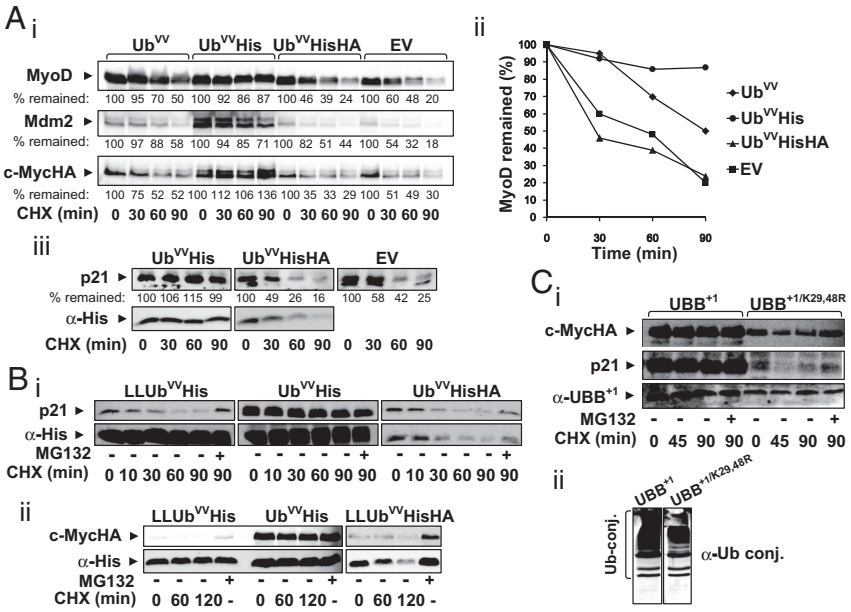

Fig. 3. Inhibition of degradation of UPS substrates depends on ubiquitination of short, C-terminally extended nondegradable Ub variants. (*A*) (i) HEK-293 cells were cotransfected with cDNAs coding for the indicated Ub^{VV} variants and UPS substrates (MyoD, Mdm2, or c-Myc), and the stability of the substrates was monitored after the addition of CHX for the indicated times. (*ii*) Quantitative representation of MyoD degradation as shown in *i*. (*iii*) Effect of the different Ub^{VV} variants on the degradation of endogenous p21. The experiment was carried out in a similar manner as described in *i*. (*B*) HEK-293 cells were transfected with cDNAs coding for Ub^{VV}His or Ub^{VV}HisHA, or their LL species. The stability of endogenous p21 (*i*) or cotransfected c-MycHA (*ii*) was monitored as described in *A*. (*C*) (*i*) cDNAs (in pcDNA3) coding for UBB^{+1} or $UBB^{+1/K29,48R}$ were cotransfected along with a cDNA coding for c-MycHA, and the stabilities of endogenous p21 and the expressed Myc were monitored as described in *A*. (*ii*) HEK-293 cells were transfected with cDNAs coding for UBB^{+1} and $UBB^{+1/K29,48R}$. Thirty hours after transfection, the total cell proteins were resolved via SDS/PAGE, and Ub conjugates were visualized after Western blot analysis using an anti-Ub conjugates antibody. Gels described in *Ai* and *Cii* were 10%. MG132 was added as indicated

be seen in Fig. 4*A*, both Ub^{VV}His and $LLUb^{VV}$His could be pulled down along with the 26S proteasome.

Next, we sought to determine whether the inhibitory effect is caused by specific occupation of Ub-binding sites in the 19S particle. To resolve this problem, we studied the effect of the inhibitory Ub derivatives Ub^{VV}His and UBB^{+1} on the degradation of ODC, a protein that is degraded in a ubiquitination-independent manner (23). Strikingly, not only that the degradation of ODC was not affected, but we observed further destabilization of this already short-lived protein (Fig. 4*B*). In an attempt to explain this finding, we studied the stability of the upstream key regulator of ODC, Antizyme (AZ), that targets ODC for degradation, and is degraded by the proteasome in a Ub-dependent manner (24). As can be seen in Fig. 4*C*, Ub^{VV}His stabilizes AZ in cells, suggesting that AZ accumulation enhances ODC degradation. As expected, $LLUb^{VV}$His was inert. Similar results were obtained in cells coexpressing UBB^{+1}. As can be seen in Fig. 4*D*, UBB^{+1} inhibits the degradation of AZ but not of ODC, whereas $UBB^{+1/K29,48R}$, similar to $LLUb^{VV}$His, is inert. Taken together, it appears that short-tailed Ub or UBB^{+1} inhibit the degradation of ubiquitination-dependent substrates by association with proteasomal Ub-binding sites, an effect that is strongly augmented by their own ubiquitination. In contrast, ubiquitination-independent substrates probably bind to a different proteasomal subunit.

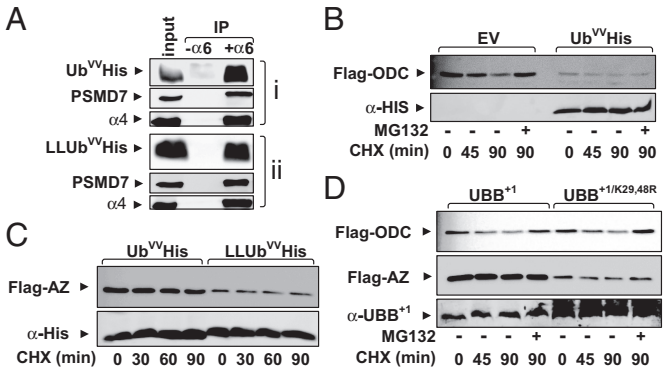

Fig. 4. Proteasome inhibition by extended Ub variants affects only ubiquitination-dependent substrates. (*A*) Ub^{VV}His and $LLUb^{VV}$His interact with the 26S proteasome. Bacterially expressed and purified Ub^{VV}His (*i*) and $LLUb^{VV}$His (*ii*) were incubated in the presence of HeLa cell extract. 26S proteasomes were precipitated by using anti-α6 (20S subunit) as described in *Materials and Methods*, and the Ub derivatives and proteasome subunits were detected after SDS/PAGE and immunoblotting. The input represents 10% of the lysate used for immunoprecipitation (IP). (*B*) HEK-293 cells were cotransfected with cDNAs coding for Ub^{VV}His and Flag-ODC as indicated. The level of ODC was monitored after the addition of CHX and MG132 at the indicated times. (*C*) HEK-293 cells were cotransfected with cDNAs coding for Flag-AZ and Ub^{VV}His or $LLUb^{VV}$His as indicated, and the stability of AZ was monitored after the addition of CHX for the indicated times. (*D*) Stabilities of transfected Flag-ODC and Flag-AZ were monitored in cells expressing UBB^{+1} or $UBB^{+1/K29,48R}$ (from pcDNA3) as described in *B* and *C*.

An important question that is related to the activity of the Ub derivatives is whether they inhibit degradation directly by competing with ubiquitinated substrates on binding sites in the proteasome as we assume, or they exert their effect via indirect mechanisms such as by inhibiting DUBs or the catalytic activities of the 20S proteasome. These two indirect mechanisms were ruled out. As can be seen in Fig. S1 and described in *SI Text*, we could not observe any effect of Ub^{VV}His on the overall DUB activity in the crude extract toward three substrates of the UPS (Fig. S1*A*; Ring1B, XIAP-1, and MyoD), and only a mild effect on proteasome-associated DUB activity (Fig. S1*B*). There was no effect on the catalytic activity of the 20S complex (Fig. S1*C*).

Discussion

Previous data have suggested that Ub can be degraded via two distinct mechanisms, piggybacking on its conjugated substrate, or as a monomer.

Data supporting the first mechanism were indirect: Ub levels were significantly lower in stressed cells and cells lacking Ub recycling enzymes. Here, we demonstrate directly that degradation of a ubiquitination-dependent substrate increases the degradation of Ub (Fig. 1). We assume that the most proximal part of the polyubiquitin chain is pulled along with the substrate into the proteasome, whereas the more distal part is rescued via the activity of DUBs. It is still not known which portion of the chain is digested along with the substrate. Even if small, this process may contribute significantly to the overall catabolism of Ub in the cell, where numerous substrates are degraded incessantly.

As for monomeric Ub, our results using a cell-free system (Fig. 1) and studies in cells (9, 25) demonstrate that Ub that cannot be conjugated to a target substrate is degraded: this protein (Ub^{VV}) that does not have a tail must be degraded therefore by an as yet to be discovered mechanism. However, we and others have shown that C-terminally extended Ub is an efficient substrate for the proteasome, and it appears that it is the tail length (>20 residues) rather than the nature of the tail that confers instability to the chimeric protein (13, 15, 25). Interestingly, we found that the fused protein does not require further ubiquitination for its binding to the proteasome and its subsequent degradation (Fig. 2). These findings define probably the minimal requirements for recognition and degradation by the proteasome: a binding Ub moiety and a long enough tail that allows the molecule to reach the catalytic sites or be pulled into the 20S complex. The tail probably must be unstructured and flexible to allow its entry and passage through the 19S complex, reaching the 20S complex (26). The lack of requirement for generation of a polyubiquitin chain in this and other cases (16, 17) raises questions about the role of the chain that appears to be necessary for the targeting of most substrates. It is possible that the chain increases the affinity, and thus the efficiency of degradation of large substrates. Binding via a single or several single Ub moieties may be weak, which decreases processivity. Indeed, affinity of the chains to the proteasome increases with their length, and when used alone, they become inhibitory (27). We show that ubiquitination of nondegradable extended Ubs inhibits degradation of substrates of the UPS, because the generated chains probably increase their affinity to the proteasome. In contrast, fusion derivatives that cannot be ubiquitinated are not inhibitory (Fig. 3). Although Ub derivatives that can be ubiquitinated appear to bind to the proteasome similar to those that cannot be modified (Figs. 2*E* and 4*A*), it will be interesting to monitor the binding affinities once the tail is further elongated. Our hypothesis is that the elongation will decrease the affinity/perturb the binding to a point that the substrate will not be degraded and will require further ubiquitination to increase the affinity/overcome the binding perturbation. Last, it was interesting to find out whether ubiquitinated substrates compete on proteasomal degradation with substrates that do not require ubiquitination for their degradation. We show that the degradation of ODC by the proteasome is even accelerated in the presence of the inhibitory Ub^{VV}His and found that this effect is caused by the inhibition of degradation of AZ, a protein that is required for the degradation of ODC and that its degradation depends on prior ubiquitination (Fig. 4). Thus, it is clear that the inhibitory effect of the chains is restricted only to substrates that are modified by Ub and that bind to specific Ub-recognizing proteasomal subunits: ODC is probably targeted after binding to a different subunit.

Materials and Methods

Monitoring of Protein Stability in Cells. Thirty hours after transfection, cells were treated with cycloheximide (CHX; 100 μg/mL) and/or the proteasome inhibitor MG132 (20 μM) and were harvested at the indicated times after addition of the inhibitors. After SDS/PAGE (15% except where indicated) of the reaction mixtures, proteins were visualized via Western blot analysis using the appropriate antibodies. All membranes were reprobed with an antibody against tubulin that served as a loading control.

In Vitro Translation of Proteins. Proteins were translated in vitro in the presence of [^{35}S]methionine by using wheat germ or *Escherichia coli* T7 S30-based kits for coupled transcription–translation according to the manufacturer's instructions.

Iodination of Proteins. Ub was radiolabeled with ^{125}I as described (21). BSA and RNase A were modified in a similar manner by using unlabeled iodine.

Monitoring the Stability of Proteins in a Reconstituted Cell-Free System. ^{35}S-labeled proteins (≈20,000 cpm) or bacterially expressed and purified proteins (0.5 μg) were added to a reaction mixture that contained at a final volume of 12.5 μL: 5 μg of Ub, 0.25 μg of E1 [purified as described (28)], and crude HeLa cell extract or reticulocyte fraction II (50 μg) as a source of conjugating enzymes and proteasomes. Reactions were carried out in the presence of ATP (0.5 mM ATP and an ATP-regenerating system composed of 10 mM phosphocreatine and 0.5 μg of creatine phosphokinase) or in its absence (0.5 μg of hexokinase and 10 mM 2-deoxyglucose were added to deplete endogenous ATP). For proteasomal inhibition, MG132 (100 μM) or lactacystin (50 μM) were added. Reactions were incubated for the indicated times at 37 °C and terminated by the addition of 3-fold concentrated sample buffer. After boiling, reaction mixtures were resolved via SDS/PAGE, and proteins were visualized after Western blot analysis or PhosphorImaging.

Degradation of ^{125}I-Ub in a Reconstituted Cell-Free System. In vitro degradation assays were performed as described above, except that ^{125}I-Ub (50,000 cpm) was added at 100 ng per assay. Reaction mixtures were incubated for 3 h and terminated by the addition of BSA followed by TCA, and the released soluble radioactivity was determined as described (21). Degradation was expressed as: (T2 − T1)/total, where T2 is the soluble radioactivity at the reaction termination time, T1 is the soluble radioactivity at time 0, and total is the total radioactivity introduced into the reaction.

Conjugation of Proteins in a Reconstituted Cell-Free System. In vitro conjugation assays were carried essentially as described (13, 16). Briefly, in vitro-translated proteins (≈30,000 cpm) were incubated at 37 °C for 1 h (in a volume of 12.5 μL) in the presence of HeLa cell extract (50 μg), Ub (5 μg), E1 (0.25 μg), ATPγS (0.5 mM), and the isopeptidase inhibitor, Ub aldehyde (100 ng). In the case where ^{125}I-Ub was used to monitor conjugation, ^{125}I-Ub (50,000 cpm per assay) was added at 100 ng. Reactions were terminated by the addition of 3-fold concentrated sample buffer, and proteins were resolved via SDS/PAGE and visualized with a PhosphorImager.

Association of Ub^{VV} and UBB^{+1} Variants with the Proteasome. UBB^{+1}HA and $UBB^{+1}HA^{K29,48R}$-transfected cells were disrupted in TNH buffer [20 mM Hepes (pH 7.9), 100 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1.5 mM $MgCl_2$, 1 mM DTT, 5 mM ATP, and protease inhibitors mixture] as described (29), clarified at 15,000 × *g* for 10 min, and incubated with immobilized anti-HA for 1.5 h at 4 °C. After 5 washes with TNH buffer, proteins were resolved via SDS/PAGE and visualized after Western blot analysis. For monitoring association in a cell-free system, 5 μg of Ub^{VV}His or $LLUb^{VV}$His was incubated for 1 h at 37 °C in the presence of HeLa cell extract (200 μg) and TNH buffer in a final volume of 50 μL. Anti-α6 coupled to immobilized protein-G in TNH buffer (containing 0.5% Triton X-100) was added, and the reaction mixture was rocked gently for an additional 1.5 h at 4 °C. The beads were washed and proteins were resolved and visualized as described above.

The materials used and general methods are described in *SI Text*.

ACKNOWLEDGMENTS. We thank Dr. Fred van Leeuwen (University of Maastricht, Maastricht, The Netherlands) for the UBB^{+1} and $UBB^{+1/K29,48R}$ constructs and the antibody against the encoded proteins; Dr. Moshe Oren (Weizmann Institute, Rehovot Israel) for the anti-Mdm2 antibodies; Dr. Keiji Tanaka (Tokyo Metropolitan Institute of Medical Science, Tokyo) for the antibodies to the 20S proteasome subunits; Dr. Chaim Kahana (Weizmann Institute) for the expression vectors for ODC and AZ; and Dr. Ariel Stanhill (Technion) for thoughtful advice. Research in the laboratory of A.C. is supported by grants from the Dr. Miriam and Sheldon Adelson Foundation for Medical Research,

the Israel Science Foundation, the German–Israeli Foundation for Research and Scientific Development, the European Union Networks of Excellence NeuroNE and Rubicon, an Israel Cancer Research Fund USA Professorship, and a grant from the Foundation for Promotion of Research in the Technion.

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