Trimming of Ubiquitin Chains by Proteasome-associated Deubiquitinating Enzymes*

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The proteasome generally recognizes substrate via its multiubiquitin chain followed by ATP-dependent unfolding and translocation of the substrate from the regulatory particle into the proteolytic core particle to be degraded. Substrate-bound ubiquitin groups are for the most part not delivered to the core particle and broken down together with substrate but instead recovered as intact free ubiquitin and ubiquitin chains. Substrate deubiquitination on the proteasome is mediated by three distinct deubiquitinating enzymes associated with the regulatory particle: RPN11, UCH37, and USP14. RPN11 cleaves at the base of the ubiquitin chain where it is linked to the substrate, whereas UCH37 and apparently USP14 mediate a stepwise removal of ubiquitin from the substrate by disassembling the chain from its distal tip. In contrast to UCH37 and USP14, RPN11 shows degradation-coupled activity; RPN11-mediated deubiquitination is apparently delayed until the proteasome is committed to degrade the substrate. Accordingly, RPN11-mediated deubiquitination promotes substrate degradation. In contrast, removal of ubiquitin prior to commitment could antagonize substrate degradation by promoting substrate dissociation from the proteasome. Emerging evidence suggests that USP14 and UCH37 can both suppress substrate degradation in this way. One line of study has shown that small molecule USP14 inhibitors can enhance proteasome function in cells, which is consistent with this model. Enhancing protein degradation could potentially have therapeutic applications for diseases involving toxic proteins that are proteasome substrates. However, the responsiveness of substrates to inhibition of proteasomal deubiquitinating enzymes may vary substantially. This substrate specificity and its mechanistic basis should be addressed in future studies. ***Molecular & Cellular Proteomics 10: 10.1074/mcp.R110.003871, 1–5, 2011.***

The eukaryotic proteasome is dedicated primarily to the degradation of proteins tagged by ubiquitin (1). Proteasomes strongly prefer multiubiquitinated protein substrates. The successive addition of ubiquitin groups to the substrate by ubiquitin ligases is usually accomplished through the formation of ubiquitin chains. The proteasome has much in common with the simple ATP-dependent proteases of prokaryotes and mitochondria (2, 3), although only the proteasome recognizes the ubiquitin modification. In all cases, the ATPases form a hexameric ring complex. These rings are homomeric in the case of the prokaryotic and mitochondrial proteases, whereas in eukaryotic proteasomes, the ATPase ring is heteromeric. Proteasomes and the simple ATP-dependent proteases are fundamentally similar in that they all have an ATPase ring (found within the regulatory particle [RP][1] in proteasomes, also known as the 19S particle and PA700) abutting a proteolytic complex (the core particle [CP] in proteasomes, also known as the 20S particle), although in some cases, the ATPase and protease domains are present on the same polypeptide chain (Fig. 1). Furthermore, this ancient organization of ATP-dependent proteases involves stacked ring complexes. Substrates are translocated from one ring to the next via the central pore within each ring. For most substrates, movement from ring to ring is driven by ATP hydrolysis. Thus, the substrate is captured by the ATPase ring of the RP and then translocated into the central cavity of the CP where it is hydrolyzed.

The pathway of translocation contains a series of narrow constrictions through which folded proteins cannot pass. The inability of a typical folded protein to pass through these "filters" defines in part the selectivity of such proteases. However, the ATPases can exert a pulling force on the substrate that is strong enough to unfold the protein, which allows for passage through the series of constrictions. This force is exerted within the central channel of the ATPase complex. Thus, translocation and unfolding of the substrate are generally coupled events (1–3).

Although not departing from this paradigm, the eukaryotic proteasome interacts with substrate in a more complex manner as a result of interactions involving the ubiquitin tag. Thus, many of the 13 subunits that were added to the evolutionarily ancient ATPase complex to form the RP in the eukaryotic lineage participate in recognition and processing of the ubiquitin tag (1). For example, the yeast proteasome has five and probably more distinct ubiquitin receptors, two that are inte-

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[1] The abbreviations used are: RP, regulatory particle; CP, core particle; DUB, deubiquitinating enzyme; USP, ubiquitin-specific protease; UCH, ubiquitin C-terminal hydrolase; Ub, ubiquitin; AMC, 7-amino-4-methylcoumarin; JAMM, JAB1/MPN/Mov34 metalloenzyme.

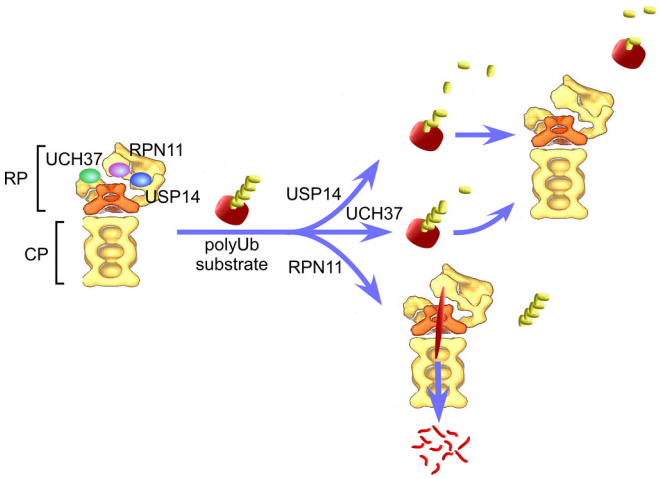

FIG. 1. **Deubiquitinating enzymes of proteasome.** In metazoans, three DUBs associate with the proteasome as shown. Each is associated with the 19-subunit RP. The detailed positioning of these enzymes on the RP is not known and is represented here schematically. RPN11 cuts at the base of the chain to release the chain en bloc. As shown, this is coupled (by an unknown mechanism) to translocation of the substrate from the RP to the CP to be degraded. In contrast, the action of USP14 and UCH37 is thought to promote substrate release from the proteasome rather than degradation. However, it should be noted that the attack of these enzymes on a substrate does not guarantee release, especially as their action on the chain is gradual, proceeding stepwise over time from the distal tip of the ubiquitin chain. Some substrates may carry more than one ubiquitin chain and thus be processed in a more complex manner. Moreover, more than one DUB might act on a given chain. The proteasome icon, adapted from Ref. 30 with permission, is based on cryo-EM imaging.

gral subunits and three that are reversibly proteasome-associated (4). In addition, proteasomes of mammals have three distinct deubiquitinating enzymes (DUBs). The multiplicity of DUBs points to a surprisingly complex role of deubiquitination in proteasome function.

PROTEASOMAL DEUBIQUITINATING ENZYMES

Why do proteasomes deubiquitinate substrate? The native state of ubiquitin is exceptionally stable: its single globular domain remains properly folded under extremes of temperature and pH (5). This tight structure may antagonize ubiquitin unfolding by the proteasome, thus obstructing translocation of the attached substrate; the extent to which attached ubiquitin impedes substrate translocation may vary from substrate to substrate. A second problem is that, if not cleaved from the substrate, some ubiquitin can be translocated into the CP along with substrate and degraded (6, 7). In the absence of wild-type DUB activity on the proteasome, particularly that of Ubp6 (the *Saccharomyces cerevisiae* ortholog of USP14), cells can become pleiotropically stress-sensitive due to ubiquitin deficiency (6). Thus, DUBs can minimize this collateral degradation of ubiquitin (6).

Because of these imperatives to remove ubiquitin from substrate, one might expect that proteasomes remove ubiquitin aggressively. However, unregulated deubiquitination has the potential to prevent degradation of not only ubiquitin but substrate as well because substrate is docked at the proteasome primarily through attached ubiquitin groups. Indeed, modulation by DUBs could potentially provide sensitive control over degradation rates of both ubiquitin and substrate.

The DUBs are a diverse family of enzymes, which fall into five distinct classes (8): the USP family (over 50 members in humans), the JAMM family (at least four members, one working on the ubiquitin-like protein Nedd8), the ovarian tumor (OTU) family (14 members), the Josephin family (four members), and the UCH family (four members). DUBs are thiol proteases with the exception of the metalloenzyme JAMM family. DUBs vary in their preference for cleaving ubiquitin chains of different linkages types, their preference for substrate-bound ubiquitin chains as opposed to unanchored (free) ubiquitin chains, and their cellular localization (8). Some DUBs appear to function with high specificity toward one or a few substrates, although only a few examples of this are known as yet. The functional specialization of DUBs often reflects their residence in specific protein complexes, and a remarkable example of this is the proteasomal DUBs.

In humans, the three proteasomal DUBs are RPN11 (also known as POH1), USP14, and UCH37 (also known as UCH-L5). This set of proteasomal DUBs is well conserved evolutionarily with the exception of the lack of a recognizable UCH37 ortholog in *S. cerevisiae*. The proteasomal DUBs each belong to a different DUB family and are thus anciently diverged in evolution: RPN11, USP14, and UCH37 belong to the JAMM, USP, and UCH families, respectively.

Among the most interesting features of proteasomal DUBs are their effects on degradation of the substrate. Whereas RPN11 appears to promote substrate degradation (9, 10), USP14 and UCH37 appear to antagonize degradation (11–14) (Fig. 1). It is generally thought that the distinction between degradation-promoting proteasomal DUBs and degradation-antagonizing proteasomal DUBs reflects ATP coupling on the part of RPN11 and the lack of it for USP14 and UCH37 (1). RPN11 is not itself an ATPase and is part of a larger proteasome subassembly, the lid, which contains nine subunits, none of which is thought to be an ATPase. Presumably RPN11-mediated deubiquitination events are ATP-dependent because they are contingent on ATP hydrolysis by the ATPase ring complex, which, as described above, supports substrate translocation and unfolding. For example, one may speculate that substrate translocation by the ATPases brings the substrate into the proximity of the active site of RPN11. In this model, RPN11-mediated deubiquitination events may occur somewhat "late" in the reaction pathway after the ATPases have productively engaged the substrate within the central translocation channel. In contrast, the removal of ubiquitin from substrate by UCH37 and USP14 is expected to commence upon docking of the substrate to the proteasome. Although the exact timing of these events has not been di-

rectly probed, the functional distinctions among these enzymes are obvious and quite striking.

A second distinction between RPN11 and the other proteasomal DUBs is that RPN11 appears to cut at the base of the chain, whereas UCH37 and most likely USP14 cut at the distal tip of the chain (12, 15) (Fig. 1). The longer a chain is, the stronger its interaction with the proteasome will be (16). Thus, attrition of the chain at its distal tip has the potential to finely regulate the lifetime of the proteasome-substrate interaction. In one scenario, the longer the substrate remains docked at the proteasome, the shorter its chain may become and the more likely it would be to dissociate.

SMALL MOLECULE INHIBITOR OF USP14

In a recent study of USP14, we have assessed several of these ideas about how deubiquitination may regulate proteasome function (11). A high throughput assay for the inhibition of USP14 catalytic activity was developed using ubiquitin-AMC (Ub-AMC), a fluorogenic substrate of many DUBs. USP14 is activated ~800-fold by proteasomes, so the screen was done in the presence of proteasomes. Of 63,000 compounds screened, we found 215 true USP14 inhibitors, but of the strong inhibitors, only three proved specific for USP14 in comparison with a panel of other DUBs. One of these, IU1, was characterized in detail. When tested against purified proteasomes, IU1 produced a sharp reduction in the rate of trimming of ubiquitin chains bound to cyclin B and of other ubiquitin chains. This effect of IU1 was observed only with proteasomes containing USP14. Thus, USP14 is a major chain-trimming DUB of the mammalian proteasome (11).

With these findings in hand, IU1 was used to test whether chain trimming can provide for regulation of substrate proteolytic rates. Two ubiquitinated substrates of the proteasome, cyclin B (17) and a tagged form of Sic1 ($Sic1^{PY}$ [18]), were both degraded by purified proteasomes more rapidly in the presence of IU1 (11), indicating that the catalytic activity of USP14 can suppress substrate degradation. In support of this view, a catalytically inactive form of USP14 (an alanine substitution of the active site cysteine of this thiol protease) was largely or completely defective in suppressing degradation. Moreover, the inactive form of USP14 does not confer sensitivity to IU1.

These *in vitro* data were largely reproduced in cultured cells using different substrates. Proteins such as tau, TDP-43, and ataxin-3, which have been linked to various neurodegenerative diseases, showed reduced levels upon IU1 treatment (11). Mouse embryonic fibroblast cells were used for these studies because of the availability of *Usp14*-null mouse embryonic fibroblast cells, a critical specificity control. However, because this is not a neuronal cell type and because these transfection-based studies do not reproduce physiological expression levels, it remains uncertain whether USP14 controls the degradation rates of these particular proteins in a physiological setting. Nonetheless, these data indicate that USP14 can control proteasome activity in a cell-based assay. Another open question is whether accelerated degradation of such proteins such as tau and TDP-43 can ameliorate their associated toxicities. However, one indication that accelerated proteasome-mediated degradation can mitigate toxicity related to misfolded proteins has been seen under conditions of oxidative damage. Damage to the genome has traditionally been viewed as the most harmful aspect of oxidative damage, although it has become increasingly clear that oxidized proteins play a significant role in toxicity (19). The ability of cells to survive exposure to oxidizing agents was strongly increased by concurrent treatment with IU1 (11), and furthermore, this increased survival correlated with increased clearance of oxidatively damaged proteins. Thus, under conditions of general proteotoxic stress, the enhancement of proteasome activity can have a beneficial effect on cell viability.

It appears that a specific subset of substrates is responsive to catalytic inactivation of USP14 (11). RPN11 and UCH37 have been found to discriminate strongly among different types of chain linkages (14, 20), and this may be true of USP14 as well. Experiments to resolve this are in progress. Chain length may be equally critical (see below). A third substrate feature that may determine USP14 susceptibility is the amenability of the substrate to proteasome-directed unfolding. Substrates that cannot be readily unfolded and translocated into the CP may bind to the proteasome for longer periods, thus allowing more time for trimming to proceed. The persistence of the proteasome-substrate interaction may also be controlled by USP14 itself in a noncatalytic function of the protein. Studies carried out originally in yeast (15) and recently extended to mammalian cells (11) have shown that USP14 has a second mode of proteasome inhibition that does not involve chain trimming. However, if slowing substrate degradation allows a longer exposure of the substrate to the trimming activity, the two mechanisms may be related. Finally, USP14 can regulate the opening of the substrate translocation channel in the CP, providing yet another avenue by which this intricate DUB influences the interaction between proteasomes and ubiquitin conjugates (21). We have as yet essentially no understanding of how these three modes of proteasome regulation by USP14 are related; this is a major challenge for future work.

USP14 AND UCH37: HOW DO THEY COMPARE?

An important early study (in 1997) on chain trimming by the proteasome focused on an activity that was unidentified at the time (12) but now understood to be UCH37 (22). Inhibition by ubiquitin aldehyde resulted in enhanced degradation of ubiquitinated globin polypeptides. Interestingly, this enhancement was strongly dependent on chain length: a strong, nearly 4-fold rate enhancement was observed for monoubiquitinated globin, but this effect was quite weak for species bearing two or more ubiquitins (12). Thus, the effect observed by Lam *et al.* (12) seems to differ markedly from that seen with USP14 in

that USP14 can suppress the degradation of substrates bearing long chains or many ubiquitin groups. A second difference is that USP14 is able to suppress degradation over a very brief time scale. The globin degradation assays were 1-h assays (12), whereas in the USP14 assays using $Sic1^{PY}$, for example, the suppressive effect of USP14 was clear after only 2.5 min (11). It will be interesting to test whether these differences would be sustained if the behavior of these enzymes were examined on a wider variety of substrates.

Although direct comparisons of chain trimming by USP14 and UCH37 remain to be done, the present data raise the possibility that, at least for the few substrates examined to date, USP14 catalyzes chain trimming more rapidly to the extent that it may be dominant over UCH37. This would be surprising because several studies based on Ub-AMC assays have reported that UCH37 activity strongly predominates over that of USP14 (13, 14). However, the Ub-AMC assay may not be strongly predictive of the relative strengths of these activities against true ubiquitin-protein conjugates. For example, UCH-type DUBs may dominate over USP-type DUB for substrates with small leaving groups, such as Ub-AMC (23). It should be noted that, in the Lee *et al.* (11) study of 2010, whereas UCH37 was inhibited by Ub-vinyl sulfone (a derivative of ubiquitin in which the vinyl sulfone moiety is placed at the C terminus of ubiquitin; Ub-vinyl sulfone is an irreversible modifier of catalytic cysteine of thiol protease DUBs) to establish a high throughput assay of Ub-AMC hydrolysis for proteasome-bound USP14, UCH37 was not subjected to inhibition when the trimming of true ubiquitin chains or effects on protein degradation were examined (11). Thus, the observation of strong effects of USP14 on these processes was not achieved by silencing a competing deubiquitinating activity. Procedures used for purification in the Lam *et al.* (12) study of 1997 would have removed USP14 (13), the existence of which was not recognized at that time, raising the possibility that a stronger effect on the trimming of ubiquitin from globin might have been seen otherwise. Chain-trimming globin isopeptidase activity has been observed in reticulocyte extracts as well, and its inhibition by ubiquitin aldehyde also stimulates globin degradation (24). This is congruent with the studies using purified proteasomes, but whether the globin-directed chain trimming activity in the extracts is in fact UCH37, rather than USP14, which is also inhibited by ubiquitin aldehyde, is unclear.

We do not know why UCH37 and USP14 are functionally distinct, but one interesting scenario is that UCH37 acts preferentially on a subset of ubiquitin conjugates that dock at the proteasome via subunit RPN13. RPN13 has the remarkable property of being a receptor for both UCH37 and ubiquitin itself (4, 25). RPN13 is also an activator of UCH37 (22). If UCH37 acts quickly enough, chains bound to RPN13 might be released not only through simple dissociation but by UCH37-dependent chain disassembly as well. Alternatively, UCH37 might cleave RPN13-bound chains but not in such a way as to promote their release from RPN13. Several studies, performed both *in vivo* and *in vitro*, have suggested that UCH37 can suppress protein degradation (12–14). In contrast, a recent study has suggested that UCH37 may instead promote the degradation of specific proteasome substrates, thus working similarly to RPN11 (26). We know too little to resolve these seeming contradictions. It is possible that UCH37 suppresses the degradation of some substrates while promoting the degradation of others.

DIRECTIONS FOR FUTURE STUDIES

In summary, recent studies have highlighted the complexity and functional importance of proteasome-associated deubiquitination reactions. With growing interest in this problem, a much clearer picture should be available soon. Many basic issues that should be addressed are described above. In addition, it will be important to determine whether proteasomal DUBs are constitutive or regulated. One study in yeast has shown that Ubp6, the ortholog of USP14, is controlled by ubiquitin levels in a feedback regulatory pathway (27). The sensitive control over protein degradation rates that can be achieved via deubiquitination makes it an attractive means to modulate proteasome activity. In addition to endogenous mechanisms to regulate proteasome function, small molecules that selectively inhibit these enzymes might in principle have therapeutic applications in diseases involving toxic, mutationally defective, or otherwise harmful proteins that are also proteasome substrates. Reducing the levels of such harmful proteins through accelerated degradation might be beneficial. Proteasome substrates have been found among proteins involved in neurodegeneration and cancer (11). Enhancement of proteasome activity, by inhibition of chain trimming, could have detrimental effects as well. For example, *Usp14*-null mutations are lethal in mice (28). However, proteasome function is essential in many and possibly all mammalian cell types, yet partial proteasome inhibition has had a major impact in the treatment of multiple myeloma (29). It is possible that modest proteasome activation may similarly have beneficial effects in specific disease contexts.

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