A Conserved Unfoldase Activity for the p97 AAA-ATPase in Proteasomal Degradation

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The multifunctional AAA-ATPase p97 is one of the most abundant and conserved proteins in eukaryotic cells. The p97/Npl4/Ufd1 complex dislocates proteins that fail the protein quality control in the endoplasmic reticulum to the cytosol where they are subject to degradation by the ubiquitin/proteasome system. Substrate dislocation depends on the unfoldase activity of p97. Interestingly, p97 is also involved in the degradation of specific soluble proteasome substrates but the exact mode of action of p97 in this process is unclear. Here, we show that both the central pore and ATPase activity of p97 are necessary for the degradation of cytosolic ubiquitin-fusion substrates. Addition of a flexible extended C-terminal peptide to the substrate relieves the requirement for p97. Deletion mapping reveals a conserved length dependency of 20 residues for the peptide, which allows p97-independent degradation to occur. Our results suggest that initiation of unfolding may be more complex than previously anticipated and that the 19S regulatory complex of the proteasome can require preprocessing of highly folded, ubiquitylated substrates by the p97/Npl4/Ufd1 complex. Our data provide an explanation for the observation that p97 is only essential for a subpopulation of soluble substrates and predict that a common characteristic of soluble p97-dependent substrates is the lack of an initiation site to facilitate unfolding by the 26S proteasome.

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Introduction

The ubiquitin/proteasome system (UPS) is required to process and degrade a wide range of intracellular proteins. Overall, UPS substrates have a diverse variety of structures and stabilities. The UPS itself is a pathway with a clear beginning, the marking of substrates by a conjugation system, and an irreversible end, the 26S proteasome.¹ Proteins destined for proteasomal degradation are modified by conjugation of ubiquitin moieties to internal lysine residues through the concerted action of E1, E2, and E3 enzymes. Repeated rounds of conjugation lead to the formation of a polyubiquitin chain attached to the target protein, making it a preferred substrate for the 26S proteasome. The 26S proteasome, which hydrolyzes the targeted proteins, is composed of a 20S proteolytic core flanked by one or two 19S regulatory particles.² The 19S regulatory particle has been proposed to contain a number of distinct activities. First, it acts as a ubiquitin receptor to acquire ubiquitylated substrates to the proteasome and removes ubiquitin chains by means of its deubiquitylation activity prior to degradation.³ In addition, the 19S regulatory particle opens the gated pore of the 20S proteolytic core to allow access to the proteolytic sites and directs substrates into the proteolytic chamber.⁴,⁵ Targeted proteins must be unfolded to accomplish this final step.⁶,⁷ The 19S regulatory particle contains six related ATPase subunits that are positioned within the base of the complex.⁸ These ATPase subunits are believed to
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unfold substrates to allow threading of the substrates into the 20S proteolytic core complex.

The p97 protein is a highly conserved ATPase belonging to the family of AAA (ATPase associated with various cellular activities) ATPases that is found at high abundance in the cytoplasm and nucleus. The p97 consists of six identical subunits, each possessing two highly conserved ATPase domains that are arranged in a homo-hexameric ring configuration. The "bottom" of the hexameric ring forms a central pore that appears to be closed and has been proposed to associate with substrates as shown for synaptotagmin 1. Overall, the hexameric p97 undergoes large dynamic conformational changes as it proceeds through the ATP hydrolysis cycle that may assist protein unfolding.

The p97 protein has been shown to play a role in a wide range of cellular mechanisms and pathways, such as apoptosis, reassembly of the nucleus, postmitotic homotypic membrane fusion of the Golgi apparatus and endoplasmic reticulum (ER), protein aggregate handling and clearance, dislocation of misfolded proteins from the ER, and ubiquitin-dependent proteasomal degradation. It has been proposed that all these diverse roles of p97 may be mechanistically reduced to a single function, namely, a segrease-like activity responsible for extracting polypeptides from protein complexes, lipid membranes, or chromatin.

The mode of action of p97 in cells is dictated by its various adaptor proteins. The p47 adaptor forms a well-studied complex with p97 that serves an essential function in membrane fusion. Another important complex encompasses p97 and the heterodimeric cofactor Ufd1/Npl4, which is involved in ER-associated degradation (ERAD), escorting substrates from the cytoplasmic surface of the ER to the proteasome. The Ufd1/Npl4 dimer competes with p47 for binding to p97 and has been proposed to serve separate pathways. In metazoans, both cofactors of the Ufd1/Npl4 complex can bind ubiquitin chains. Ufd1 binds ubiquitin through a UIM domain, and Npl4 ubiquitin binding is due to an NZF domain. Previous studies indicate that p97 complexes indirectly bind to the 26S proteasome itself, suggesting that p97 may facilitate interaction between ubiquitylated proteins, bound to Ufd1/Npl4, and the proteasome. A recent study by Besche et al. suggests that p97 complexes and 26S proteasomes exist largely as separate complexes but with a fraction transiently associated. These findings suggest that, in association with its cofactors, p97 escorts ubiquitylated substrates to the 26S proteasome.

A subset of natural soluble substrates (protein kinase Cdc5, GI-CDK inhibitor Far1p, chaperone UNC-45, HIF1α-lph) as well as well-defined N-end rule and UFD substrates require p97 for their degradation, although the function of p97 in this pathway remains elusive. It is not clear if the ATPase activity of p97, which is instrumental for the degradation of ERAD substrates, also plays a role in handling of soluble substrates. Notably, in vitro studies have shown that simple binding of polyubiquitylated substrates to the proteasome is not always sufficient for proteasomal degradation, suggesting that extraproteasomal factors may play a role in substrate handling.

In order to elucidate the role of p97 in the degradation of soluble proteins, we have used a dominant-negative set of ATPase mutants for p97 that have been previously applied in functional analyses of the p97 ATPase domains in ERAD. We have made corresponding mutations in the Drosophila p97 homologue TER94 to determine if the unfoldase activity of p97 is essential for specific cytosolic UPS substrates. We demonstrate that substrates require preprocessing by the p97 ATPase prior to degradation by the 26S proteasome and further show that the addition of unassociated peptide sequences removes the requirement for the p97 complex. Our findings suggest a significantly greater general role for the p97 ATPase in processing ubiquitylated protein substrates for proteasome proteolysis.

Results

The requirement for the p97Ufd1/Npl4 complex in the degradation of UFD substrates is conserved in metazoans

It has been previously reported that depletion of p97 by RNA interference (RNAi) strongly stabilizes ubiquitin conjugates. Targeting various regions of the p97 transcript with different double-stranded RNAs (dsRNAs) also led to a readily detectable accumulation of ubiquitin conjugates. Ubiquitin conjugates accumulated in response to dsRNAs targeting the region of the p97 encoding for the large ATPase domains or the 3′ untranslated region (UTR) that is not shared by other ATPases. Finally, the ability to successfully knock down p97 using dsRNA directed to the 3′ UTR allows complementation studies to be carried out as shown below.

The p97 hexamer interacts with a wide range of cofactor proteins, and we identified nine Drosophila genes that contain the conserved p97-interacting UBX domain as annotated on the SMART domain server. (Supplementary Fig. 1). In order to determine which potential Drosophila p97 cofactors are required for cytoplasmic degradation of soluble proteins, we performed an RNAi screen using specific dsRNAs (Fig. 1). This screen included orthologs of Ufd1 and Npl4, which are involved in the degradation of the soluble UFD proteins in yeast, and pairs of related genes that share a high level of similarity to Ufd2 proteins. The RNAi screen was carried out in Drosophila Schneider-2 (S2) cells expressing the green fluorescent protein (GFP) fluorescent UFD reporter substrate UbG76VGFP (Fig. 1b). To confirm that RNAi treatments were functioning and specific for reducing the targeted mRNA on samples that showed no significant stabilization, we carried out semiquantitative PCR reactions (Supplementary Fig. 2).
In addition to the knockdown of p97 or the proteasome subunits, knockdowns of two cofactors showed significant stabilization of the reporter, namely, the Drosophila Ufd1 and Npl4 orthologs (Fig. 1c). Double knockdowns of p97+Npl4, p97+Ufd1, or Npl4+Ufd1 were also carried out and reached similar levels of UbG76VGFP reporter accumulation to single knockdown treatments (Fig. 1c). These results underline that Npl4 and Ufd1 share the same functional pathway and show that the p97/Ufd1/Npl4 complex is required for the degradation of UFD substrates in Drosophila cells. Western blots confirmed stabilization of the UFD reporter UbG76VGFP by RNAi of the p97/Ufd1/Npl4 complex (data not shown). Together, our

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**Fig. 1.** RNAi screen for additional cofactor proteins required for cytoplasmic degradation of Ub{sup G76V}GFP. (a) p97 RNAi knockdown in Drosophila cells. RNAi depletion of Drosophila p97 in S2 cells using long dsRNA targeted to either coding or the 3' UTR. Cellular levels of p97 and ubiquitin conjugate proteins in RNAi p97-depleted cells, as determined by immunoblotting. Top panel, p97 levels; middle panel, ubiquitin conjugates; lower panel, α-tubulin levels that serve as a loading control. (b) Triplicate RNAi knockdowns on proposed Drosophila cofactors of p97. Percentage of Ub{sup G76V}GFP stabilization is ranked from highest to lowest. As positive controls, proteasome subunits were also knocked down with double-stranded RNAi. For all proteasome subunits targeted, p97 and cofactors Ufd1 and Npl4, Student's t test \( p < 5 \times 10^{-4} \) compared to mock RNAi treatment. (c) Comparison of individual versus double RNAi knockdowns of Drosophila Npl4 and Ufd1 and resulting stabilization of Ub{sup G76V}GFP. Average results of triplicate knockdowns presented as percentage of cells gated as positive for GFP fluorescence.
data show that the role of the p97<sup>Ued1<sub>1</sub>/Npl4</sup> in the UFD pathway is conserved in insect cells and no additional cofactors were observed to be essential.

**Mutations in the ATPase domains of p97 block degradation of a soluble UFD substrate**

While a number of recent studies indicate that p97 is implicated in degradation of cytoplasmic UPS substrates<sup>25,28,31</sup> it is not clear whether p97 acts by functioning solely as a scaffold for ubiquitin-binding cofactors to interact with the substrates, or if the enzymatic activity of p97 itself plays an essential role in substrate handling. To address this question, a series of *Drosophila* p97 ATPase mutants were made. The use of specific ATPase mutants has been used successfully in a number of studies to decipher the requirements for either ATP binding and/or hydrolysis in the

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**Fig. 2**

*Fig. 2.* p97 ATPase mutants block degradation of UbG76 VGFP. (a) The p97 mutants located with R7 H141, E426, and K439 and E548 are shown in red. WA mutants ATP binding defect WB mutants ATP hydrolysis defect. (b) Left images, transient transfection of ATPase mutants and UbG76 VGFP in *Drosophila* S2 cells. All images contain equal amounts of total cells; white images not shown. Right panel, ATPase and p97 mutants. Stable lines induced expression of p97 variants that lack the native 3′UTR, allowing expression of mutants during RNAi knockdown directed to the native 3′UTR of endogenous p97. RNA treatment concurrent with complementation is termed RNAi+c. Student’s t-test p-value is 2.2 × 10<sup>−5</sup> for p97 wild-type complementation compared to maximum stabilization seen in proteasome deficient in D1 ATPase, ATPase mutants showed partial rescue of UbG76 VGFP degradation; p = 3.3 × 10<sup>−5</sup>.

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**Fig. 2 (legend on next page)**
dislocation of ERAD substrates. The two ATPase domains of p97, D1 and D2, contain Walker A and B motifs, which are extremely conserved among different species and show almost no variance between Drosophila and human sequences.

Site-directed mutagenesis was carried out on the Drosophila homologue of p97 mutating the Walker A and Walker B regions (Fig. 2a). K248A or K521A mutations in the Walker A regions prevent ATP binding to p97, while E302Q or E575Q mutations in the Walker B region allow binding of ATP molecules but prevent hydrolysis, potentially by impairing the dramatic conformational changes within the hexameric ring structure. Expression of the mutant p97 proteins, but not wild-type p97, had a dominant-negative effect and blocked the ability of Drosophila S2 cells to degrade the fluorescent UFD substrate despite the presence of endogenous wild-type p97 (Fig. 2b).

Mutations residing internally within the pore have recently been characterized for murine p97 and, notably, these pore residues are important for p97’s dislocation activity in the degradation of ER substrates. The neighboring Trp and Phe residues, which face the upper regions of the D2 C-terminal pore, have been proposed to be required for the interaction of the p97 hexamer with protein substrates (Fig. 2a). We found that expression of a corresponding Drosophila p97 W548A/F549A pore mutant also displayed a dominant-negative effect on the degradation of the soluble UFD substrate similar as observed with the ATPase mutants (Fig. 2b).

Stable cell lines were generated for each of the ATPase and pore p97 mutants to test if any p97 mutants could complement for the loss of wild-type p97. These stable cell lines express p97 variants that lack the native p97 3′ UTR, allowing expression of the mutants during RNAi knockdown directed to the native 3′ UTR of endogenous p97. In addition to wild-type p97, which gave a near to complete rescue of degradation of the UFD substrate in p97 RNAi-treated cells, only the E302Q displayed a partial rescue, whereas the other mutants were unable to restore p97-dependent degradation of the UFD substrate (Fig. 2c). These data show that both the ATP binding to the D1 domain and the ATPase activity of the D2 domain of p97 are essential for the degradation of UFD substrates. The requirement for W548 and F549 residues in the pore also suggests that the unfoldase activity of p97 may be implicated in degradation of soluble substrates.

**An extended peptide sequence allows UFD substrates to bypass p97**

The requirement for p97 ATPase activity in the degradation of the soluble fluorescent substrates could indicate that UFD substrates need to be partially unfolded before the substrates interact with the proteasome. However, it remained possible that the ATPase requirement instead reflected ATP-dependent rearrangement of the p97 with its cofactors consistent with its role as a scaffold for ubiquitylation.

It has generally been thought that simple localization of substrates to the proteasome was the critical step needed before degradation could be initiated. However, the process of substrate recognition may, in fact, not be that simple. Proteasomes require an unstructured initiation site to start unraveling the polypeptide chain. The above findings led us to ask the following question regarding the role of p97 in the degradation of soluble substrates: Does p97 act by extracting or unfolding a region away from the main folded body of the substrate? To test this, we constructed a hybrid reporter consisting of the UFD substrate with an additional 35-residue sequence fused to its C-terminus. For this purpose, we initially used the commonly used V5-His epitope tag. We used structural prediction algorithms to predict the secondary structure of the V5-His extension. This hidden Markov modeling predicted the V5-His extension to be mostly random coil. In support of this, proteins whose structures have been solved containing V5-His C-terminal extensions do not show secondary structural elements or were unsolvable for the extension (Refs. 42 and 43 and personal communication).

Consistent with a role for p97 in preprocessing of proteasome substrates, we observed that addition of a V5-His epitope tag to a UFD substrate was sufficient to abrogate the strict requirement for p97 for the degradation of the UFD substrate. Side-by-side comparison of UbC56V-RFP with and without C-terminal peptide extensions by flow cytometry showed that substrates with the C-terminal extension were able to be degraded in cells with strongly
reduced levels of p97 (Fig. 3). To make a side-by-side comparison of substrates with and without the unstructured extension, we co-expressed two fluorescent UFD substrate reporters based on a GFP (Ub<sup>C76V</sup>GFP) and a red fluorescent protein (RFP) (Ub<sup>C76V</sup>RFP) in Drosophila cell lines. Cells were

Fig. 3. Recovery of degradation in p97-depleted cells by the addition of a C-terminal peptide extension tag. (a) Upper left panel, flow cytometry analysis of cells expressing and degrading both Ub<sup>C76V</sup>RFP and Ub<sup>C76V</sup>GFP. Upper right panel, RNAi p97-depleted cells cotransfected with Ub<sup>C76V</sup>GFP and Ub<sup>C76V</sup>RFP. Each panel is a single representative from a set of experiments. Cells that contain both Ub<sup>C76V</sup>GFP and Ub<sup>C76V</sup>RFP are in the upper right quadrant, and the average stabilization of triplicate experiments is shown below. Control flow cytometry runs with cells only expressing GFP or RFP confirmed the specificity of each fluorescent signal with little or no channel bleeding (data not shown). (b) Drosophila S2 cells cotransfected with Ub<sup>C76V</sup>GFP and a Ub<sup>C76V</sup>RFP containing a C-terminal V5-His extended peptide sequence (left panel). Middle panel, identical cotransfection with RNAi p97 depletion resulting in positive Ub<sup>C76V</sup>GFP cells but few Ub<sup>C76V</sup>RFP V5-His tag positive cells; graph of average stabilization from triplicate experiments. Lower right panel, restoration of dual positive cells, after cells were treated with proteasome inhibitor. Green bar graph represents cells that were only GFP positive, and striped bar represents GFP- and RFP-positive cells.
cotransfected with Ub\(^{G76V}\)GFP and Ub\(^{G76V}\)RFP with or without a C-terminal V5-His peptide. The coexpressed extension-less reporters were rapidly degraded, while p97 knockdown by RNAi treatment stabilized both substrates (Fig. 3a). However, degradation of the Ub\(^{G76V}\)RFP reporter containing an extended C-terminal peptide, V5-His, was still degraded after p97 knockdown, whereas the coexpressed Ub\(^{G76V}\)GFP was stabilized (Fig. 3b, bottom middle panel).

Stabilization was apparent for the Ub\(^{G76V}\)RFP with the C-terminal V5-His tags after proteasome inhibition treatment and demonstrates that both fluorescent proteins were expressed. The stability of RFP lacking a fused ubiquitin was not affected by the introduction of the tag excluding that the V5-His tag functions as a degradation signal and underscoring our model that the tag assists in the degradation of the substrates but does not play a role in the actual targeting (Supplementary Fig. 3). Treatment with the calpain inhibitor leupeptin or the autophagosome formation inhibitor 3-methyladenine does not stabilize the reporters (Supplementary Fig. 4). We therefore conclude that the introduction of an unstructured peptide enables soluble reporter substrates to be degraded by the proteasome in a p97-independent fashion.

Length requirement of C-terminal peptide extensions that allow p97-independent degradation

The addition of a C-terminal 35-amino-acid residue sequence allowed the degradation of Ub\(^{G76V}\)RFP by the proteasome to occur independent of the p97\(^{Ufd1/Npl4}\) complex. Two recent studies demonstrate that proteasomal substrates, both ubiquitin-independent\(^{44}\) and ubiquitin-dependent\(^{45}\), require short, extended peptides of around 20 to 30 aa in order to be efficiently degraded by the 26S proteasome. To map the length requirement of the extended flexible sequence, we carried out a series of deletions in the V5-His tag sequence to shorten the extension in a stepwise manner (Fig. 4a). Whereas C-terminal tags of 20 or more amino acids were functional in bypassing the requirement for p97, substrates carrying a 15-amino-acid extension relied again on p97 for efficient degradation (Fig. 4b).

Treatment of the cells with proteasome inhibitor caused accumulation of the substrates regardless of the length of the extension, confirming that all fusions are expressed and subject to proteasomal degradation. As a control, half the cells for each transfection were treated with the proteasome inhibitor epoxomicin, and as expected, all deletion constructs were expressed and stabilized after proteasome inhibition (Fig. 4c). Two of the Ub\(^{G76V}\)RFP deletions along with the tagged and non-tagged forms were also confirmed to be UFD substrates. Mutating the ubiquitin Lys residues 29 and 48, which are known to be targeted for ubiquitylation in UFD substrates\(^{46}\), to Arg residues resulted in stabilization of Ub\(^{G76V}\)RFP fusions (Supplementary Fig. 3d). The mapped length restriction corresponds closely to the minimal length requirement for ubiquitin-dependent proteasomal degradation observed previously.\(^{45}\) The p97-cofactors Ufd1 and Npl4 are required for degradation of Ub\(^{G76V}\)GFP that lacks a C-terminal peptide extension (Fig. 1b). Double knockdowns of Ufd1 and Npl4 showed, as expected, that reporters containing
long peptide extensions did not require the cofactors for degradation either in control cells or p97-depleted cells (data not shown). Our data suggest that provision of the substrate with a sufficiently long, extended flexible sequence to allow direct degradation by the proteasome abrogates the need for p97 for efficient degradation. These observations suggest that the p97 dependency may reflect the need to generate an initiation site consistent with a role for p97’s unfoldase activity in the degradation of soluble substrates.

**Constraints of amino acid compositions of extended peptides**

A series of short, extended peptide sequences were appended to Ub\(^{G76V}\)RFP and tested for degradation in control and p97 RNAi-treated cells to investigate the constraints of the extended peptide sequence in enabling p97-independent degradation of the reporter substrate (Fig. 5). The glycine-rich sequence located in the NFKB precursor functions as a stop signal that terminates proteasomal degradation, which has been previously claimed to be due to the unfoldase of the proteasome being unable to translocate the polypeptide.\(^{47,48}\) We found that the introduction of a glycine repeat, unlike the V5-His tag, was unable to facilitate p97-independent degradation. Another problematic sequence for the proteasome is the expanded polyglutamine repeats that are responsible for a number of neurodegenerative diseases.\(^{49,50}\) It has been reported that the metazoan proteasome is unable to degrade polyglutamine peptides.\(^{51,52}\) Our analysis also showed that a C-terminal extension consisting of glutamine residues was unable to allow p97-independent proteasomal degradation (Fig. 5a). Notably, we confirmed that the UFD substrates with glycine and glutamine extensions were still degraded by the UPS albeit in a p97-dependent way.

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**Fig. 5.** Sequence requirements for extended peptides to degrade Ub\(^{G76V}\)RFP in p97-depleted cells. (a) Western blot analysis of Drosophila S2 cells expressing Ub\(^{G76V}\)RFP plasmids containing various extended peptides: the V5-His C-terminal peptide, 25-Gln residues, 25-Ala residues, 25-Gly residues, no extended peptide; all extended peptide tags are linked to the C-terminus of RFP by an additional 8-aa linker region SCRPLESS. Western blots from p97-depleted cells expressing Ub\(^{G76V}\)RFP with different peptide extension tags. A nonspecific Drosophila protein detected by the anti-RFP antibodies is marked (*) and also serves as a loading control. For each transfection, the RNAi p97-depleted cells were split into two samples and either directly Western immunoblotted with anti-RFP antibody (top blot) or additionally treated with proteasome inhibitor for 12 h and then immunoblotted (lower blot). A 25-Ala peptide extension promotes degradation of Ub\(^{G76V}\)RFP in p97-depleted cells while 25-Gln and 25-Gly extensions fail to promote degradation. (b) Representative fluorescence micrographs of single cells after stabilization of Ub\(^{G76V}\)RFP reporters with epoxomicin proteasome inhibition, 12 h. All reporters are able to fold and be fluorescent with varying levels of aggregation: Ub\(^{G76V}\)RFP (upper left panel), Ub\(^{G76V}\)RFP-V5 (upper right panel Ub\(^{G76V}\)RFP-Gln25), (lower left panel), Ub\(^{G76V}\)RFP-Ala25 (lower middle panel), Ub\(^{G76V}\)RFP-Gly25 (lower right panel). The scale bars represent 10 μm.
We noted that fusions with glycine and glutamine extensions were prone to aggregation, as reported previously, and since protein aggregation can hinder proteasomal degradation, we wondered whether their inability to bypass p97 could be attributed to protein aggregation. Therefore, we also tested the effect of an extension solely consisting of alanine residues, a strongly aggregation-prone sequence also implicated in human genetic diseases. We found that an Ala extension was able to circumvent the need for p97 similar to what we observed for the V5-His sequence despite the fact that it caused the reporter to aggregate (Fig. 5a). Cells expressing UbG76VRFP-Ala25 and treated with the proteasome inhibitor epoxomicin were aggregated but highly fluorescent, suggesting proper folding of the RFP (Fig. 5b). This suggests that protein aggregation cannot be the sole factor accountable for the inability of glycine and glutamine extensions to facilitate p97-independent degradation. Together, these data show that the nature of the C-terminal extension is an important determinant and underscores the problematic nature of glycine and glutamine repeats.

Requirement for short extensions to bypass p97 is conserved in human cells

An earlier study showed that human cells also require p97 for the degradation of UFD substrates. We therefore wondered whether the p97 dependency of UFD substrates could be bypassed by the introduction of short extensions also in human cells. We analyzed four different small interfering RNAs (siRNAs) directed against human p97 and found that each of the siRNAs caused a reduction of the basal p97 levels in a human cervix carcinoma HeLa cell line stably expressing UbG76VGFP (data not shown). Western blot and fluorescence microscope analyses showed that RNAi-mediated reduction in p97 levels was accompanied by an increase in the levels of the UbG76VGFP reporter substrate (Fig. 6a and b). Quantitative analysis of the micrographs showed that whereas the levels of UbG76VGFP remained unaltered in cells with normal p97 levels, cells with reduced levels of p97 consistently displayed elevated levels of the reporter (Fig. 6c). The increase in fluorescence in UbG76VGFP-expressing cells with successfully reduced p97 levels roughly corresponded to the levels obtained by treating the cells with the proteasome inhibitor MG132 (Fig. 6d).

Next, we established two HeLa cell lines that stably express UFD substrates with C-terminal extensions of 15 and 20 aa of the V5 epitope and are identical with the fusions used in the Drosophila cell lines. p97 siRNA treatment of HeLa cells expressing the UFD substrate with the 15-amino-acid extension gave an increase comparable to the effect obtained by incubating the cell with proteasome inhibitor, similar to the effect observed with UbG76VGFP minus the tag (Fig. 6e). On the contrary, an extension of 20 aa allowed efficient degradation of this fusion in the absence of p97, suggesting that a difference of only 5 aa converts this substrate from a p97-dependent to a p97-independent substrate (Fig. 6f).

Taken together, our data show that the requirement for p97 can be bypassed by providing the substrate with a C-terminal extension of at least 20 aa. The function of p97 in the degradation of soluble proteasome substrates demonstrated in the present study implicates an unfoldase activity for p97, which is conserved between insect and mammalian cells.

Discussion

The AAA-ATPase p97 (VCP/Cdc48) is one of the most abundant proteins in eukaryotic cells. The p97 homo-hexamer changes its functional role depending upon the specific adaptor proteins that associate with it. This work indicates that the role for p97/Ufd1/Npl4 in ERAD and soluble protein degradation share many functional similarities. Both degradation pathways are dependent on the ATPase activity and require the C-terminal pore region of the p97 complex. A number of studies have proposed that the diverse roles of p97 are mediated through a segregase activity, which disintegrates higher-order structures by unfolding endogenous substrate, which could explain its involvement in processes as diverse as dislocation of ER substrates, homotypic membrane fusion, and inhibition of protein aggregation. The present study suggests an additional role for p97’s unfoldase activity in the degradation of tightly folded proteins (Fig. 7).

The UFD substrates used in this study are clearly different from the majority of natural proteasome substrates. Instead of the initial ubiquitin being attached to an internal Lys residue by an E3 ligase, an N-terminal ubiquitin fused to the detectable target substrate functions as the proximal ubiquitin and donor of the polyubiquitin chain. In general, UFD substrates do have clear advantages and have allowed the identification of key steps in the UPS pathway such as the nature of polyubiquitylation recognition by the 26S proteasome and the need for loosely folded initiation sites in proteasome substrates. In the current study, the usage of UFD substrates has a clear second advantage as the designed reporter composed only of ubiquitin and a fluorescent protein presumably lacks any sites for initiation of unfolding by the proteasome. Notably, the N-terminal part is blocked by the fused ubiquitin and the C-terminus terminates within the highly folded barrel structure of the fluorescent proteins that lacks regions of loosely structured domains. It has been proposed that mono-ubiquitylated substrates are degraded significantly by macro-autophagic degradation. For our current study, the UFD reporters were strongly stabilized by proteasome inhibitor treatment. For the specific cell lines tested, stabilization was not observed with the calpain inhibitor leupeptin or the autophagosome formation inhibitor 3-methyladenine. This is also consistent with a more recent report in which it was shown that...
there is no appreciable degradation of UFD substrates by macro-autophagy. Using UFD model substrates, we have studied the role of p97\textsuperscript{Ufd1/Npl4} in protein degradation in the absence of complex associations. The model fluorescent substrates used in this study have no expected interaction partners, and hence, p97\textsuperscript{Ufd1/Npl4} can only assist in the direct unfolding of the model targets. RNAi knockdown of any one of the three components in p97\textsuperscript{Ufd1/Npl4} complex results in the stabilization of the UFD substrates and demonstrates that in vivo degradation depends on the p97 complex. Moreover, simultaneous knockdown of Ufd1 and Npl4 suggests an epistatic role of these two cofactors in degradation of UFD substrates in line with the notion that the activity is confined to the p97\textsuperscript{Ufd1/Npl4} complex. Accordingly, we found that none of the other proposed cofactors of the p97 ATPase was required for degradation of the substrate.

A number of observations in the current study lend support that p97 is functioning as an unfoldase for the 26S proteasome and is responsible for the generation of a loosely folded structure from the substrate to allow degradation by the proteasome. First, degradation of the substrate depends on the ATPase activity of p97. This argues that the mere presence of the p97 is not sufficient for degradation to occur but instead that the p97 is required to carry out structural rearrangement within its complex. Second, conserved residues within the hydrophobic pore of p97 are also required for proteasomal degradation of the reporter substrate. The pore is present on the opposite side of the p97 ATPase relative to the majority of ubiquitin binding cofactors and ubiquitin chain modifying components. The requirement for inner pore residues indicates that the p97\textsuperscript{Ufd1/Npl4} is not simply using the energy of ATPase hydrolysis to modify the ubiquitin chain

![Fig. 6. Requirement for short unfolded extensions to bypass p97 is conserved in human cells. (a) Western blot analysis of Ub\textsuperscript{G76V-GFP} HeLa cells transiently transfected with mock and p97-specific siRNA. p97 and Ub\textsuperscript{G76V-GFP} levels were detected using specific antibodies. As a loading control, the blot was reprobed with antibodies against glyceraldehyde 3-phosphate dehydrogenase. (b) Representative fluorescence micrographs of p97 immunostaining (upper panels) and native GFP fluorescence (lower panels) of Ub\textsuperscript{G76V-GFP} HeLa cells transiently transfected with mock (left panels) and p97 siRNA (right panels). The scale bar represents 50 μm. (c) Quantitative analysis of p97 immunostaining and native GFP fluorescence intensities of Ub\textsuperscript{G76V-GFP} HeLa cells transfected with mock (open circles) and p97 siRNA (filled circles). Normalized p97 and GFP intensities of individual cells are shown (n = 50). The threshold for classifying cells as ‘low p97’ or ‘high p97’ levels was set at 75% of the average p97 intensity in cells transfected with the control siRNA (indicated by the broken line in the graph). (d) Quantitative analysis of Ub\textsuperscript{G76V-GFP} levels in cells after transfection with mock or p97 siRNA or treatment with the proteasome inhibitor MG132. Student’s t test value (p < 0.0001) is shown. (e) Quantitative analysis of Ub\textsuperscript{G76V-GFP-15aa} levels after transfection with mock or p97 siRNA or treatment with the proteasome inhibitor MG132. Student’s t test value (p < 0.0001) is shown. (f) Quantitative analysis of Ub\textsuperscript{G76V-GFP-20aa} levels after transfection with mock or p97 siRNA or treatment with the proteasome inhibitor MG132. Student’s t test value (p = 0.20) is shown.](image-url)
length of the substrate but, instead, likely modifies the substrate itself. Cross-linking results indicate that an unfoldase activity for p97 would likely involve the C-terminal pore region of the p97.11 Third, the addition of extended peptide sequences to the UFD substrates abrogates the need for p97. The initial tested extended peptide sequence was a 35-residue sequence containing a V5 epitope with a His tag, V5-His. Deletion mapping of the V5-His sequence showed that 20 residues of extension were required to promote degradation. We propose that the size of the peptide extension corresponds with the minimum peptide length that the proteasome needs to engage the ATPase subunits present within the 19S regulatory particle and complete unfolding of the substrate. Thus, our results suggest that a p97-dependent process is required to generate a loosely folded structure on structured substrates that would promote unfolding directly by the proteasome.

Finally, the nature of the extended peptide sequence was also tested. Peptide sequences that have previously been shown to be problematic for the proteasome do not allow bypass of the p97 requirement. Peptide extensions solely containing Gln or Gly, which are known to interfere with proteasomal degradation, failed in promoting degradation in p97-depleted cells. It is currently unclear why certain extended sequences can promote degradation in p97-depleted cells while others fail to rescue and additional future work is required. As a beginning, the initial observation that a polyalanine extension can promote degradation in p97-depleted cells suggests that the results are not simply due to a basic difference between aggregated and non-aggregated reporters.

It is also currently unclear what fraction of soluble proteasome substrates requires preprocesing for efficient degradation to occur by the proteasome. The proteasome has been shown to be able to degrade proteins that lack accessible termini by endoproteolytic cleavage of substrates that contain internal natively disordered regions,59 and it has been clearly demonstrated in yeast that proteasomal degradation can occur from internal sites.60 Unfolding initiator sites could reside at the termini or internally as long as the unraveling domains are accessible to the 19S ATPase subunits of the proteasome. For proteins that naturally lack unraveling domains, a model can be proposed where the degradation of these substrates requires the assistance of the p97 complex that initiates unraveling and transfers directly to the 26S proteasome. This is the first evidence we are aware of that
indicates a role for p97 to modify the tertiary structure of a soluble proteasome target. Initiation of unfolding is likely a complex process where the steps and mechanisms of unraveling may vary depending upon the structural nature of the ubiquitylated protein.

Materials and Methods

Antibodies and reagents

Polyclonal antibodies against SDS-PAGE-purified Drosophila p97 protein were generated in rabbits by Agrisera. Rabbit polyclonal antibodies against TurboRFP (AB232) were purchased from Evrogen. Mouse monoclonal antibody against ubiquitin (U 0508) was purchased from Sigma-Aldrich. Hybridomycin (10667-010) was purchased from Invitrogen. Total lysates of HeLa cells were separated by SDS-PAGE and transferred to nitrocellulose membranes (Whatman). The membranes were incubated with a mouse monoclonal antibody specific to p97 in a 1:10,000 dilution or a rabbit polyclonal antibody against GFP (Molecular Probes) in a 1:5000 dilution. For loading controls, blots were reprobed with a mouse monoclonal glyceraldehyde 3-phosphate dehydrogenase (RDI Research Diagnostics) in a 1:10,000 dilution. After incubation with horseradish-peroxidase-conjugated secondary antibodies, the blots were developed by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Cell culture

S2 cells were cultured in Schneider’s Drosophila medium (Gibco) supplemented with 10% fetal calf serum. 2 mM l-glutamine, 50 U penicillin/ml, and 50 μg streptomycin/ml. The cells were grown at 24 °C and passed every 7 days at 1:5 dilution. To make cell lines, we plated 3 × 10⁶ cells in 3.5-cm wells in a total volume of 3 ml. The cells were transfected using calcium phosphate with 9.5 μg of either of the reporters (UbG76V-GFP, UbG76V-TurboRFP) and 9.5 μg of either the wild type or any of the p97 mutants. After 16–20 h of growth, the cells were carefully washed with Schneider’s Drosophila media (GIBCO) three times, and CuSO₄ was added for induction of the transfected DNA. The human HeLa cervix carcinoma cell lines were cultured in Iscove’s modified Dulbecco medium (Sigma-Aldrich) supplemented with 10% fetal calf serum (Sigma-Aldrich), 10 U/ml penicillin, and 10 μg/ml streptomycin (Sigma-Aldrich). The stable cell line expressing UbG76V-GFP has been described before. The cell lines expressing UbG76VYPF-15aa and UbG76V-GFP-20aa were created by transfection of HeLa cells with the corresponding plasmids. Clones were selected in the presence of 0.5 mg/ml G418 (Gibco) and screened for fluorescence upon inhibition of proteasome activity by MG132. 

Clones and plasmids

Constructs of EGFP (enhanced GFP) and TurboRFP N-terminally connected to ubiquitin (UbG76V-EGFP/ TurboRFP) were subcloned into the pAct vector (Invitrogen). Drosophila p97 wild type and ATPase mutants lacking the 3′ UTR were subcloned into the CuSO₄-inducible vector pMT/V5-hisB. Fusion of UbG76VYPF with the V5-His tag was achieved by homologous recombination of a UbG76VYPF PCR product with the pYES2-V5His plasmid in the yeast Saccharomyces cerevisiae, creating pYES2 UbG76VYPF-V5His. In order to generate a mammalian expression vector, the UbG76VYPF-V5His fragment was inserted in the backbone of EGFP-N1. Shortening of the V5-His extension to 15 and 20 aa was achieved by introducing stop codons at the relevant positions in the UbG76VYPF-V5His plasmid by site-directed mutagenesis using the QuikChange II Site-Directed Mutagenesis kit (Stratagene), resulting in the UbG76VYPF-V5His (15 aa) and UbG76V GPF-V5His (20 aa) plasmids. All construct were verified by DNA sequencing.

For simultaneous expression and detection of two substrate reporters in S2 cells, flow cytometry measuring both red and green fluorescence in Drosophila S2 cell populations was performed. Transfection of DNA constructs was performed by plating a total of 6 × 10⁴ Drosophila S2 cells onto 5-cm dishes in 5 ml of Schneider’s Drosophila medium (GIBCO). Per transfection, the cells with calcium phosphate and a total of 19 μg of expression plasmid per transfection after 24 h of growth. The cells were incubated with the DNA-calcium precipitate for another 24 h and then washed three times and allowed to grow in 3 ml of medium. For dual measurement of GFP and turbo-RFP, a FACS DiVa Sorter (Becton Dickinson) was used. Half of the cells were treated with MG132 added directly to the medium at a final concentration of 10 μM. Cells were harvested by centrifugation after 5 h of MG132 treatment.

RNAi interference treatments and oligonucleotides used to generate dsRNA are described in Supplementary Methods.

Drosophila p97 mutants

Wild-type cDNA for Drosophila p97, TERA94 (GM02885), was purchased from Drosophila Genomics Resource Center. The single (K248A, E302Q, K521A, and E575Q) and double (K248/521A and E302/575Q) ATPase mutants, as well as the pore double mutant (W548A/F549A), were made with the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene) using oligos corresponding to the different mutants: K248A, 5′-CCCCGGGTACCGGCGCCACCCT-GATTGC-3′; E302Q, 5′-CGGCGCTATACATTTCTTATT-GATCAAAATCAGCCTATTTGCC-3′; K521A, 5′-CTCGGTTCGCCGGCCACACGTGTCTGGCC-3′; E575Q, 5′-CTCTTGTTGCTCTTCTTCTTCTTGACACGTGGACTC-GATCGCC-3′; and W548A/F549A, 5′-CTGACCATGGCGGCGCAGAGTCTG-3′. 

RFP deletions constructions and introduction of various extended tag sequences

The UbG76V-RFP pAc5.1/V5-His vector was mutated by site-directed mutagenesis to place a TGAATTC sequence at various positions in the V5-His region. In this way, a TGA stop codon was introduced within the V5-His region, as well as an EcoRI site to enable screening of the resulting colonies. Various different UbG76V-RFP plus extended peptides were generated at the C-terminal end of RFP by cutting at the 5′ end of the V5-His gene region, which contained an XbaI site. For each new extended peptide sequence tag, two long oligos were hybridized together and resulted in a double-stranded DNA with XbaI sticky ends but without the complete XbaI consensus sequence. After ligation, the plasmids were treated with XbaI to
select for plasmids that did not contain the original XbaI site. Minipreps were sequenced to find inserts that had ligated in the desired orientation. The following is the sequence of the forward oligos. Reverse complementary oligos are not shown.

Gln-25 tag, 5′-CTAGCCACAAACAAAGCACACAG-CACGAGGAGGAGGAGGAGGAGGGAGCCT-GCACGAGGAGGAGGAGGAGGAGGAGGAGGAG-3′

α-helix tag, a 23-residue α-helical sequence from the phage major coat protein Gp824, amino acid sequence: AFDSLQASATEMIGYAWAMVVIV, oligo sequence, 5′-GAATTAATACGACTCACTATAGGGAGAGGGCAGC-3′

RNAi knockdowns

For the Drosophila RNAi experiments, dsRNA was synthesized with the aid of the MEGAscript kit (Ambion) as previously described.32 Oligonucleotides were selected by searching the GadFly database. An approximately 700-bp fragment of exon sequence was amplified from genomic DNA extracted from S2 Drosophila S2 cells,63 and then imaged. attach to concanavalin-A-coated cover slides to spread the proteasome inhibitor epoxomicin and then allowed to incubation. The work in the Dantuma laboratory was supported by the Swedish Research Council, the Swedish Cancer Society. The work in the Dantuma laboratory was supported by the Swedish Research Council and the Swedish Cancer Society. The work in the Dantuma laboratory was supported by the Swedish Research Council and the Swedish Cancer Society.

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RNAi in human cells

HeLa cell lines were transfected with the commercially available and validated mock siRNA (AM4611, Ambion) or p97 siRNA (Flexitube siRNA SI03019730, Qiagen) using Lipofectamine 2000 reagent (Invitrogen). The cells were retransfected after 24 h and harvested after 3 days of incubation in total.

Fluorescence microscopy

HeLa cell lines were grown on coverslips, fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and stained with Hoechst 33258 (Molecular Probes). Immunostainings were performed using a mouse monoclonal antibody specific to p97 in a 1:1000 dilution and AlexaFluor 647 anti-mouse immunoglobulin G antibodies (Molecular Probes) in a 1:2000 dilution. Fixed cells were incubation in total.

RNAi treatment of Drosophila S2 cells was performed by introducing double-stranded RNAi diluting S2 cells to a concentration of 1×106 cells/ml in Express Five serum-free medium (Invitrogen). RNAi treatment of Drosophila S2 cells was performed as previously described. Drosophila S2 cell cultures were diluted to a concentration of 1×106 cells/ml in Express Five serum-free medium (Invitrogen). After cell attachment, the medium was replaced twice with 1 ml serum-free media, and the cells were grown in the serum-free medium for 1 to 2 h. To initiate RNAi, we directly added 20–60 μg of dsRNA to the medium under constant agitation. After 12 h, 2 ml Express Five serum-free medium was added, and the cells were cultured for 3 to 4 days prior to isolation.

For an initial RNAi screen to identify cofactor Drosophila proteins required for UbG76VGFP degradation, 600- to 700-bp-long PCR products containing T7 RNA polymerase promoter site sequences on both ends were created. dsRNA was synthesized using Ambion MEGAscript reactions as described above. The SMART genomic database37 was scanned to identify Drosophila genes that contain UBX domains, and oligos residing in exon regions were selected. The oligos used to PCR genomic Drosophila DNA are shown in Supplementary Fig. 5.

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Supplementary Data

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References


