Usa1 Protein Facilitates Substrate Ubiquitylation through Two Separate Domains

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Abstract

**Background:** Defects in protein folding are recognized as the root of many neurodegenerative disorders. In the endoplasmic reticulum (ER), secretory proteins are subjected to a stringent quality control process to eliminate misfolded proteins by the ER-associated degradation (ERAD) pathway. A novel ERAD component Usa1 was recently identified. However, the specific role of Usa1 in ERAD remains obscure.

**Methodology/Principal Findings:** Here, we demonstrate that Usa1 is important for substrate ubiquitylation. Furthermore, we defined key cis-elements of Usa1 essential for its degradation function. Interestingly, a putative proteasome-binding motif is dispensable for the functioning of Usa1 in ERAD. We identify two separate cytosolic domains critical for Usa1 activity in ERAD, one of which is involved in binding to the Ub-protein ligase Hrd1/Hrd3. Usa1 may have another novel role in substrate ubiquitylation that is separate from the Hrd1 association.

**Conclusions/Significance:** We conclude that Usa1 has two important roles in ERAD substrate ubiquitylation.

Introduction

The majority of selective proteolysis in eukaryotes is handled by the proteasome [1]. Substrates of the proteasome are often covalently modified by the ubiquitin (Ub) molecule, an abundant 76-residue protein [2,3]. Ub is activated and transferred to the substrate via several enzymes including a Ub-activating enzyme (E1), a Ub-conjugating enzyme (E2), and a Ub-protein ligase (E3). The rate-limiting step is likely the recognition and ubiquitylation of the substrate by the E3 enzyme. The ubiquitylated substrate is then degraded by proteasome. Defects in the Ub/proteasome system can lead to cancers and neurodegenerative diseases [4].

The Ub/proteasome pathway is a part of the protein quality control system responsible for the destruction of misfolded polypeptides [5,6]. Nearly one third of cellular proteins enter the endoplasmic reticulum (ER) on their way to various cellular destinations. The folding state of secretory proteins is actively monitored in the ER. Immature proteins are retained to fold properly by ER chaperones. To prevent toxicity by the accumulation of aberrant proteins, terminally misfolded proteins are disposed of via a process termed ER-associated protein degradation (ERAD) [5,7]. More specifically, these malfolded proteins are returned to the cytosol and recognized by a Ub-protein ligase (E3), which decorates misfolded proteins with Ub molecules that mark the substrate for proteasome-mediated proteolysis [4]. Failure of ERAD can lead to protein aggregation and cell death.

Multiple ERAD pathways are employed to eliminate aberrant proteins [6,8]. Recent findings suggest that at least two checkpoints are employed to sort ERAD substrates into different degradation pathways based on the location of the misfolded domain (e.g. membrane, lumen, or cytosol) and the topology of the protein [9,10,11]. ERAD substrates with lesions exposed in the cytosol, termed ERAD-C, are selected for degradation by the Doa10 (E3) pathway. ERAD substrates with lesions in either ER membrane (ERAD-M) or ER lumen (ERAD-L) are ubiquitylated by an E3 complex composed of Hrd1 (a RING finger containing protein) and Hrd3. Interestingly, ERAD-L requires two additional proteins residing in the ER membrane, Usa1 and Der1 [10,11]. While Der1 is proposed to be involved in the substrate retro-translocation since it has four transmembrane domains, the specific role of Usa1 in ERAD is unknown.

In the cytosol, the ATPase Cdc48 in complex with two Ub-binding proteins Ufd1 and Npl4 recognizes Ub chains and uses its chaperone activity to extract ubiquitylated proteins out of the ER [6,8]. How the ubiquitylated ERAD substrates are transferred to the proteasome is not clear. Some, but not all ERAD substrates require Ub receptors Rad23 and/or Rpn10 [6,8]. Since Usa1 contains a putative proteasome-binding Ub-like motif (UBL) [10,12,13], we considered the possibility that Usa1 may have a role in bringing the proteasome close to the ER membrane and thereby shuttling substrates to the proteasome. We show herein that the UBL motif is largely dispensable for the functioning of Usa1 in ERAD-L substrate degradation. We demonstrate that Usa1 is specifically involved in the ERAD substrate ubiquitylation step. Our deletion analysis uncovers two domains essential for Usa1 function, one of which binds the Hrd1-Hrd3 E3 complex.

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Our data reveal that the function of Usa1 requires its association with the Hrd1-Hrd3 E3, and further suggest that Usa1 may have another undefined role in substrate ubiquitylation.

Results

Usa1 regulates ERAD-L substrate ubiquitylation

To determine the execution point of Usa1, we compared the ubiquitylation pattern of misfolded CPY*, a well-defined ERAD-L substrate, in wild-type and usa1Δ cells [9,10,11]. To this end, we co-transformed the plasmid expressing Flag-tagged CPY* and the plasmid bearing HA-tagged Ub to these cells. Ubiquitylated CPY* species were seen in wild-type cells expressing both CPY* and HA-Ub, but not in control cells lacking either CPY* or HA-Ub (Figure 1A). Ubiquitylated CPY* bands were significantly reduced in usa1Δ cells (Figure 1A), suggesting that Usa1 facilitates substrate ubiquitylation.

Usa1 was shown to bind the Hrd1-Hrd3 E3 complex [10]. Although both Hrd1 and Hrd3 are inserted into the ER membrane, their topology and functional assignments are different [6,7]. Whereas most of Hrd3 sequences are in the ER lumen, majority of Hrd1 sequences are on the cytosolic side. In combination with two luminal proteins Yos9 and Kar2, Hrd3 recognizes misfolded substrates and targets them for ubiquitylation [11]. Hrd1, a RING finger domain protein, assembles Ub-chains onto the substrates in the cytosol. To further delineate the interaction between Usa1 and Hrd1/Hrd3, we examined these interactions in the presence or absence of one of the subunits. Usa1 binds Hrd3 (Figure 1B). In the absence of Hrd1, the interaction of Usa1-Hrd3 remained unaltered (Figure 1B). The Usa1-Hrd1 interaction was also maintained in the absence of Hrd3 albeit at slightly reduced level (Figure 1C). Note that Hrd1 level is known to be reduced in hrd3Δ cells [14,15]. Combined, these results suggest that Usa1 interacts with Hrd1 and Hrd3 separately in vitro.

The UBL motif is not essential for Usa1 function

The majority of Usa1 sequences is projected on the cytosolic side [10]. Besides its membrane-localization domains, the only recognizable element in Usa1 is the UBL motif, which can bind various components of the Ub/proteasome pathway including the proteasome and a Ub chain elongation factor Ufd2 [13,16]. Unlike Rad23, another UBL-containing protein, Usa1 was not found in association with the proteasome or Ufd2 (Figure 2A, B).

To determine the importance of this putative UBL element, we deleted the region encompassing the UBL motif (amino acids 250–350; UBLΔ also termed as d2 in Figures 3 and 4) at the genomic USA1 locus. To evaluate the effect of the UBL deletion on Usa1 function, we examined the stability of two soluble ERAD-L substrates, misfolded CPY* and ricin A chain (RTA), in usa1Δ mutant cells. Consistent with previous reports, CPY* is markedly stabilized in usa1Δ null mutant (Figure 2C, D) [10]. Surprisingly, deletion of the UBL domain does not significantly alter the degradation of CPY* or RTA (Figure 2C, 3C), suggesting that the UBL motif is not crucial for the functioning of Usa1 in proteolysis.

Two N-terminal domains are essential for Usa1 function in ERAD-L

To understand how Usa1 facilitates substrate ubiquitylation, we sought to identify critical sequence elements in Usa1 through mutational analysis. We deleted the C-terminal cytosolic segment (amino acids 595–838), the other two non-UBL regions of the N-terminal Usa1 (amino acids 31–240, 351–525) separately (Figure 3A) at the genomic locus of USA1. We found that CPY* degradation was markedly impaired in mutants lacking one of the N-terminal domains (Figure 3B), indicating that these two segments are essential for Usa1 function. In contrast, the C-terminal cytosolic tail, which accounts for nearly one third of the protein, was not required for CPY* degradation (Figure 3B) and was not further analyzed. Similarly, the turn-over of another ERAD-L substrate RTA [17] is compromised by deletions of the N-terminal, non-UBL domains (Figure 3C).

Mutations in Usa1 do not affect its membrane localization and protein stability

Next, we examined whether the loss of function caused by these deletions was due to the change of Usa1 localization and/or stability. To facilitate the detection of Usa1 protein, Usa1 and its
mutant alleles were separately fused to Flag-epitope. Yeast extracts expressing USA1 derivatives were divided into total, soluble, and membrane fractions. Whereas the soluble Rad23 protein partitioned into supernatant portion, Usa1 mainly resides in the membrane fraction (Figure 3D). None of the N-terminal deletions affected the localization of Usa1 to the membrane (Figure 3D). Small amount of Usa1 (less than 5%) was also detected in the soluble fractions. Whether this is due to insufficient fractionation or related to its role in pre-mRNA splicing remains further investigation [18]. Then, we also carried out pulse-chase assays to measure the stabilities of Usa1 and its derivatives. Wild-type and mutant Usa1 are stable proteins (Figure 3E and data not shown).

Defective Usa1-E3 binding affects substrate ubiquitylation

To examine the specific defects caused by these deletions, we examined the ubiquitylation pattern in usal mutants. As expected, CPY* ubiquitylation was compromised in cells lacking USA1 or HRDL (Figure 4A). We found reduced ubiquitylation of CPY* in two non-UBL deletion mutants (Figure 4A), suggesting that these two domains are required for substrate ubiquitylation. Interestingly, CPY* ubiquitylation is consistently more severely reduced in d3Δ mutant than in usal null. Although the underlying mechanism is not known, one possible explanation is that the deletion may exert a dominant negative effect since, unlike usal null mutant, it still has other functional domains such as the first N-terminal region and membrane anchoring domain. We suspect that these two mutations may affect the association between Usa1 and other ERAD-L ubiquitylation components. First, we determined the interaction between Usa1 derivatives and Hrd1 by co-immunoprecipitations. Interestingly, deletion of the middle portion (i.e. d3Δ) abolished the binding between Usa1 and Hrd1 (Figure 4B) and also reduced the Usa1-Hrd3 interaction (Figure 4D), suggesting that the interaction between Usa1 and the Hrd1-Hrd3 E3 complex is important for ERAD. The N-terminal fragment (amino acids 1-525) binds efficiently to Hrd1 in the absence of transmembrane domain (Figure 4C and data not shown). We also examined the binding between Usa1 and Der1. To this end, we employed Der1-TAP, which does not support ERAD but, nevertheless, associates with the other components of the Hrd1-complex [10]. None of the mutations affects the Usa1-Der1 interaction (Figure 4E). Usa1 is also known to interact with the chaperone complex Cdc48-Ufd1-Npl4 [10], which recognize and ubiquitylated proteins out of the ER membrane [19,20,21]. Since CPY* ubiquitylation is unaltered in cdc48 mutant [19,20], the Usa1-Cdc48 association is likely indirect and not relevant for the functioning of Usa1 in CPY* ubiquitylation. Since the d1Δ mutant retained the bindings to Hrd1, Hrd3 and Der1, the results also suggest that the extreme N-terminal domain of Usa1 plays a different, but undefined essential function in ERAD-L ubiquitylation.

Discussion

Since the discovery of ERAD over a decade ago, the physiological significance of ERAD has been increasingly appreciated owing to its emerging, prominent role in human diseases [4,6]. However, many important questions remain: For example, how are substrates selected for degradation? How are substrates retro-translocated across the ER membrane? How are ubiquitylated substrates transferred to the proteasome? Studies in yeast have led the way in uncovering critical mechanistic attributes and the physiologic functions of ERAD. Many key players in ERAD were first identified in yeast, and nearly all of them have human counterparts [8,11]. Assigning each ERAD factor to the specific events mentioned above presents the first step to unravel this highly coordinated choreography of ERAD.
Compromised CPY* ubiquitylation in usa1 mutants suggests the involvement of Usa1 in ERAD-L ubiquitylation. Currently, it is unknown whether Usa1 regulates substrate ubiquitylation directly or indirectly. Given the membrane barrier, ERAD-L ubiquitylation is likely much more complicated than typical ubiquitylation carried out by a normal E2–E3 pair. Besides Ubc6 E2 and Hrd1 E3, other proteins (e.g. Cue1, Hsp70 chaperones) have also been implicated in ERAD substrate ubiquitylation [22,23]. Identification of these ubiquitylation regulators would facilitate in vitro reconstitution of ERAD ubiquitylation.

Given its ER membrane localization and mainly cytosolic topology [10], Usa1 has the potential to play multiple key roles in the ERAD pathway. Interestingly, the putative UBL motif and the C-terminal tail are not essential for ERAD-L substrate degradation (Figure 2, 3). Usa1 bridges Der1 and Hrd1 [10], which in turn could ensure the coupling of retrotranslocation and ubiquitylation. Our results also suggest that likely Usa1 has another function in ERAD in addition to its associations with Der1, Hrd1 and Hrd3 since the d1Δ mutant with largely intact bindings to Der1 and Hrd1/Hrd3 is defective for CPY* ubiquitylation (Figure 4).
specific function of the first 250 amino acids of Usa1 is unknown, but may bring other cytosolic factors required for substrate ubiquitylation close to Hrd1 [6,7]. Our results suggest that Usa1 also works with the Hrd1–Hrd3 E3 complex to facilitate substrate ubiquitylation (Figure 4). How might Usa1 assist Hrd1 in attaching ubiquitins onto misfolded proteins? In the presence of E1 and E2, Hrd1 alone is sufficient to catalyze self-ubiquitylation in vitro, indicating that Hrd1 has the Ub-protein ligase activity [24]. Usa1 is likely not essential for turning on the enzymatic activity of Hrd1, but may still stimulate Hrd1 activity. It remains a possibility that Usa1 could be involved in substrate retro-translocation. Our data provides new insights regarding the mechanism governing ERAD substrate ubiquitylation and lays the foundation for uncovering the more detailed events in the ERAD pathway.

The human homologue of Usa1 is thought to be Herp since Herp partially restores the ERAD-L pathway in usa1D yeast cells [10]. Herp is induced upon ER stress and important for cell survival under stress [25,26]. The precise function of Herp remains elusive. Like Usa1, Herp also interacts with the mammalian Hrd1–Hrd3 complex and is required for the degradation of ERAD substrates (e.g., CD3-delta, connexin 43) [26,27,28]. Interestingly, the only sequence similarity between Usa1 and Herp is the UBL motif [10,25], which is essential for Herp-mediated connexin 43 degradation but not for Usa1-mediated CPY* or RTA turnover (Figure 2C, 3C). Whereas Herp is an unstable protein subjected to proteasome-mediated proteolysis [26], Usa1 is quite stable (Figure 3E and data not shown). Given our findings here, it remains to be seen whether Herp and Usa1 perform a similar role in ERAD.

In summary, both two N-terminal, non-UBL domains are important for the function of Usa1 in substrate ubiquitylation. Our results lay the foundation to further uncover the detailed events involved in ERAD ubiquitylation.

Materials and Methods

Yeast Strains and Plasmids

Cultures were grown in rich (YPD) or synthetic media containing standard ingredients and 2% glucose (SD medium), or 2% raffinose (SR medium), or 2% galactose (SG medium), or 2% raffinose + 2% galactose (SRG medium). Wild-type strain BY4741 and isogenic mutant strains usa1Δ, hrd1Δ, hrd3Δ, der1Δ and Der1-TAP were obtained from Open Biosystems (Huntsville, AL). Yeast strains YHR161 (USA1Δ31–240) in BY4741 background;
in the SRG medium to an OD$_{600}$ of $\sim1$, followed by preparation of extracts, immunoprecipitation with the beads coated with indicated antibody, SDS-8% PAGE, and immunoblotting, separately, with various antibodies.

Detection of ubiquitylated CPY*

Analysis of CPY* ubiquitylation was carried out as described previously [32]. Briefly, CPY* was immunoprecipitated from yeast extracts treated with MG132 and the immunoprecipitates were resolved by SDS-PAGE, and immunoblotted with the antibody recognizing ubiquitin (Biomol).

Membrane localization of Usa1 and its derivatives

Soluble and membrane fractions of yeast cells expressing either wild-type or mutant Usa1 were prepared by centrifugation [33]. Briefly, cells expressing Flag tagged Usa1 or Rad23 were labeled for 1 hour with 0.1 mCi of $^{35}$S-Express (Perkin Elmer), and lysed by glass beads. Cell debris was removed by centrifugation at 4,000 rpm for 5 minutes. Soluble proteins were separated from membrane fraction by centrifugation at 14,000 rpm for 10 minutes. Total extract, soluble and membrane fractions were subjected to immunoprecipitation with Flag beads, followed by SDS-8% PAGE, and autoradiography.

Acknowledgments

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Author Contributions

Conceived and designed the experiments: IK, HR. Performed the experiments: IK, YL, PM. Analyzed the data: IK, HR. Contributed reagents/materials/analysis tools: IK. Wrote the paper: IK, HR.

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Detection of protein-protein interaction by immunoprecipitation was done as described [16,17]. Briefly, yeast cells were grown on galactose-containing medium. Cells were labeled for 10 min with 0.16 mCi of $^{35}$S-Express (Perkin Elmer), followed by the chase with 4 mM methionine and 2 mM cysteine. Samples were taken at the indicated time points and processed for immunoprecipitation with Flag beads (Sigma-Aldrich), followed by SDS-PAGE and autoradiography.

Detection of protein-protein interaction by immunoprecipitation was done as previously described [31]. To construct the plasmid containing Flag-tagged full-length Usa1 or various deletions, USA1 gene was amplified by PCR from wild type or USA1 deletion strains and incorporated into pRS416Gal1 vector containing Flag epitope at C-terminus. Flag-tagged RTA was previously described [17].

Pulse-chase analysis

Pulse-chase analysis was done as described previously [17]. Yeast cells carrying plasmids that express Flag-tagged CPY*, or RTA, or Usa1 derivatives from the P$_{GAL}$ promoter were grown at 30°C to an OD$_{600}$ of $\sim1$ in galactose-containing medium. Cells were labeled for 10 min with 0.16 mCi of $^{35}$S-Express (Perkin Elmer), followed by the chase with 4 mM methionine and 2 mM cysteine. Samples were taken at the indicated time points and processed for immunoprecipitation with Flag beads (Sigma-Aldrich), followed by SDS-PAGE and autoradiography.

Co-immunoprecipitation/immunoblotting assay

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Author Contributions Conceived and designed the experiments: IK, HR. Performed the experiments: IK, YL, PM. Analyzed the data: IK, HR. Contributed reagents/materials/analysis tools: IK. Wrote the paper: IK, HR.
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