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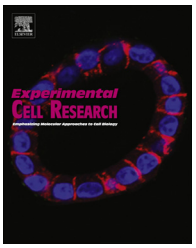

Research Article

Ubiquitin-specific protease 19 regulates the stability of the E3 ubiquitin ligase MARCH6

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ABSTRACT

Ubiquitin-specific protease (USP)19 is a recently identified deubiquitinating enzyme (DUB) having multiple splice variants and cellular functions. One variant encodes an endoplasmic reticulum (ER)-anchored DUB that rescues misfolded transmembrane proteins from ER-associated degradation (ERAD), but the underlying mechanism remains to be elucidated. Here, we show that USP19 interacts with the ERAD-associated E3 ubiquitin ligase MARCH6. Overexpression of USP19 delayed the degradation of MARCH6, leading to an increase in its protein level. In contrast, USP19 depletion resulted in decreased expression of MARCH6. We also show that USP19 overexpression reduced ubiquitination of MARCH6, while its knockdown had the opposite effect. In particular, USP19 was found to protect MARCH6 by deubiquitination from the p97-dependent proteasomal degradation. In addition, USP19 knockdown leads to increased expression of mutant ABCB11, an ERAD substrate of MARCH6. Moreover, USP19 is itself subjected to endoproteolytic processing by DUB activity, and the processing cleaves off an N-terminal cytoplasmic region of unknown function. However, elimination of this processing had no evident effect on MARCH6 stabilization. These results suggest that USP19 is involved in the regulation of ERAD by controlling the stability of MARCH6 via deubiquitination.

Introduction

In eukaryotic cells, secretory and membrane proteins are synthesized in the endoplasmic reticulum (ER) and nascent polypeptides fold into their mature conformation with the assistance of molecular chaperones and folding enzymes. Both unfolded and misfolded proteins are recognized and translocated back to the cytosol for proteasomal degradation by ER quality control processes and ER-associated degradation (ERAD) [1]. In addition to aberrant ER proteins, degradation via ERAD is also carried out on normal, short-lived ER proteins, such as hydroxymethylglutaryl-CoA reductase (HMGR), in order to regulate their enzymatic activities [2]. Polyubiquitination is required for dislocation (or retrotranslocation) of most of the ERAD substrates from the ER into the cytosol. The cytosolic ATPases associated with diverse cellular activities (AAA), cdc48 in yeast and p97 (also termed VCP) in mammals, recognize polyubiquitinated ERAD substrates and

Abbreviations: USP, ubiquitin-specific protease; DUB, deubiquitinating enzyme; ER, endoplasmic reticulum; ERAD, ER-associated degradation; E3, E3 ubiquitin ligase; NPT II, neomycin phosphotransferase II; FBS, fetal bovine serum; siRNA, small interference RNA; NEM, *N*-ethylmaleimide; K48-Ub, K48-linked ubiquitin; UBL, ubiquitin-like

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drive their extraction from the ER membrane for delivery to the 26S proteasome [3–7]. Ubiquitination is catalyzed by the sequential activities of three enzymes, an E1 ubiquitin activating enzyme, an E2 ubiquitin activating enzyme and an E3 ubiquitin ligase that confers substrate specificity [8]. The E3 ubiquitin ligases (hereafter, "E3s") involved in ERAD have been identified and are currently being studied [9]. Yeast contains the two E3 ubiquitin ligases Hrd1 and Doa10, which have been shown to degrade all of the identified ERAD substrates [10–12]. Mammals contain many more types of E3s that participate in ERAD, including HRD1 (synoviolin), GP78 (AMFR), MARCH6 (TEB4), RMA1, RFP2 and TRC8, which diversity apparently reflects an expansion of the ERAD substrates [13–19]. Ubiquitination can be reversed by deubiquitination via deubiquitinating enzymes (DUBs) in a variety of cellular processes. Recent studies have reported that DUBs have a role in ERAD. For example, Rpn11, UCH37 and USP14 are proteasome-associated DUBs involved in removal of polyubiquitin from proteasomal substrates prior to proteolysis for the recycling of free ubiquitin [20]. Ataxin-3 and YOD1 promote the deubiquitination of p97-associated ERAD substrates, and facilitate delivery to the proteasome [21–24]. Recently, USP19 and USP25 were shown to rescue transmembrane ERAD substrates from proteasomal degradation [25,26].

USP19 was first characterized as being upregulated in the atrophying skeletal muscle of rats, a process in which USP19 controls the expression levels of myofibrillar proteins [27,28]. USP19 has multiple functions, in part due to the presence of its variant forms. For example, a soluble isoform of USP19 has been shown to control fibroblast cell proliferation [29,30]. Its DUB activity stabilizes the KPC1 E3 ubiquitin ligase, resulting in the downregulation of the cyclin inhibitor $p27^{Kip1}$ and hence cell cycle progression [29,30]. On the other hand, Hassink et al. [26] have identified another USP19 isoform which is a transmembrane protein anchored to the ER membrane. The transmembrane USP19 has been shown to stabilize transmembrane ERAD substrates, such as the mutant CFTR channel and T-cell receptor α-chain, possibly by preventing their proteasomal degradation [26]. Thus, ER-anchored USP19 is thought to contribute to ER quality control and/or ERAD, but the precise mechanism involved remains to be elucidated.

However, little is known about how the protein expression and activity of ERAD E3s are regulated. A number of studies have reported that various DUBs interact with certain E3s so as control their stability and functions [31]. Indeed, USP19 has been shown to associate with several of the soluble E3s, such as KPC1, c-IAPs and SIAHs, in order to regulate the cell cycle [29,30], apoptosis [32] and stability of USP19 [33]. Thus, the ER pattern of localization led us to hypothesize that USP19 affects the stability of the ERAD E3(s). In this study, we show that USP19 deubiquitinates and stabilizes MARCH6. In addition, USP19 knockdown resulted in an increased expression of mutant ABCB11, an ERAD substrate of MARCH6. These findings provide insight into the detailed regulatory mechanisms underlying the stability and activity of the ERAD E3s.

Materials and methods

Construction of mammalian expression plasmids

Mouse *Usp19* cDNA (IMAGE clone 6400986) was obtained from OpenBiosystems (Huntsville, AL). A cDNA fragment encoding full-length USP19 was amplified by PCR and was then subcloned into pcDNA3 (Invitrogen, Carlsbad, CA), yielding a mammalian expression vector for USP19. FLAG–USP19 and Myc-USP19 were constructed by cloning the open reading frame of USP19 into p3 × FLAGCMV-10 (Sigma-Aldrich, St. Louis, MO) and pcDNA3 encoding a 6 × Myc epitope tag, respectively. USP19–FLAG was generated by cloning cDNA fragments encoding residues 1–1360 of USP19 into p3 × FLAGCMV-14 (Sigma-Aldrich). Point mutations were introduced by oligonucleotide-directed mutagenesis. FLAG–MARCH6 was constructed by cloning a cDNA fragment encoding human MARCH6 into the EcoRV site of p3 × FLAGCMV-10. His-p97 was constructed by a cDNA fragment encoding human p97 into the EcoRI and XbaI sites of pcDNA3 encoding a 6 × His epitope tag. His-p97 QQ was generated by introducing E305Q and E578Q mutations by oligonucleotide-directed mutagenesis into the pcDNA3-His-p97 plasmid. FLAG-$ABCB11^{G238V}$ was constructed by cloning cDNA fragments encoding human ABCB11 containing a G238V mutation into the KpnI/XbaI sites of p3 × FLAGCMV-10. The sequences of all of the plasmids were verified by DNA sequencing.

Antibodies

The rabbit anti-USP19 polyclonal antibody (A301-587A) was purchased from Bethyl Laboratories (Montgomery, TX), which had been raised against residues 900–950 of human USP19, and was used for detecting endogenous human USP19 by Western blotting. The anti-USP19 polyclonal antibody (#799) was raised in a rabbit against GST-fusion proteins of residues 409–1222 of mouse USP19 (GST-DUB) and was antigen-affinity purified with HiTrap NHS-activated HP (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). The anti-MARCH6 polyclonal antibody (#917) was raised in a rabbit against GST-His_6-fusion proteins of residues 2–78, 164–276 and 866–910 of human MARCH6 and was antigen-affinity purified. The animal protocols and procedures were approved by the Institutional Animal Care and Use Committee of Tokyo Institute of Technology. The following mouse monoclonal antibodies were purchased: anti-α-tubulin and anti-FLAG M2 antibodies (Sigma-Aldrich); anti-c-Myc antibody (Roche, Indianapolis, IN); anti-neomycin phosphotransferase II (NPT II; clone AC113) and anti-K48-linked ubiquitin antibodies (clone Apu2; Merck Millipore, Billerica, MA).

Cell culture and plasmid transfection

293T cells were cultured in Dulbecco modified Eagle medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Plasmid transfection was performed with TransFectin reagent (Bio-Rad, Hercules, CA) according to the manufacturer's instruction.

RNA interference

The human *USP19*-specific Stealth small interference RNA (siRNA) duplex oligonucleotides (5′-ggaggcaugauugguggccacuaca-3′) were purchased from Invitrogen. Stealth RNAi Negative Control Medium GC Duplex #3 (Invitrogen) was used as a negative control. One day before siRNA transfection, 293T cells (0.5×10^5 cells) were seeded onto a 24-well plate. Cells were incubated in 500 μl