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N-Myristoylation of the Rpt2 subunit of the yeast 26S proteasome is implicated in the subcellular compartment-specific protein quality control system

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ABSTRACT

Ubiquitination is the posttranslational modification of a protein by covalent attachment of ubiquitin. Controlled proteolysis via the ubiquitin–proteasome system (\UPS) alleviates cellular stress by clearing misfolded proteins. In budding yeast, UPS within the nucleus degrades the nuclear proteins as well as proteins imported from the cytoplasm. While the predominantly nuclear localization of the yeast proteasome is maintained by the importin-mediated transport, *N*-myristoylation of the proteasome subunit Rpt2 was indicated to cause dynamic nucleo-cytoplasmic localization of proteasomes. Here, we quantitatively analyzed the ubiquitinated peptides using anti-K-ε-GG antibody in yeast cell lines with or without a mutation in the N-myristoylation site of Rpt2 and detected upregulated ubiquitination of proteins with nucleo-cytoplasmic localizations in the mutant strains. Moreover, both the protein and ubiquitinated peptide levels of two Hsp70 family chaperones involved in the nuclear import of misfolded proteins, Ssa and Sse1, were elevated in the mutant strains, whereas levels of an Hsp70 family chaperone involved in the nuclear export, Ssb, were reduced. Taken together, our results indicate that *N*-myristoylation of Rpt2 is involved in controlled proteolysis via regulation of the nucleo-cytoplasmic localization of the yeast proteasome.

1. Introduction

More than 90% of proteins in eukaryotes are ubiquitinated and targeted for degradation by the ubiquitin–proteasome system (UPS) [1]. Ubiquitination is the post-translational modification of a protein by covalent attachment of ubiquitin, a 76-amino acid protein with a highly stable structure. During ubiquitination, an isopeptide bond is formed between the carboxyl group of the ubiquitin C-terminus and the ε-amino group of target Lys residue in a reaction catalyzed by the three enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3) [2]. Ubiquitin conjugated to a target protein can itself be ubiquitinated at all seven of its Lys residues as well as its *N*-terminal amino group, resulting in polyubiquitin chains of various topologies that differ in regard to the number and linkage types of ubiquitin conjugates [3]. The Lys48 linkage predominantly targets UPS substrates, whereas the other ubiquitination topologies trigger a broad range of biological processes (e.g., DNA repair, signal transduction, and endocytosis, which are triggered by the Lys63 linkage [2,4]). Recent work showed that polyubiquitin chains other than the K63 linkage also target proteins for proteasome-dependent degradation [5–7], although the extent of the contribution of each type of polyubiquitin chain needs to be further characterized. A recently developed antibody-based method for the enrichment of ubiquitin signature peptide (K-ε-GG peptide), which is produced by trypsin digestion of ubiquitinated proteins [8–10], enables high-throughput analysis of polyubiquitination-mediated protein homeostasis (proteostasis).

Proper folding is critical for protein functions in various cellular processes. Misfolded proteins continually arise during protein synthesis and are also induced by mutations, transcription errors, or various proteotoxic stresses. Because misfolding exposes hydrophobic amino-acid residues that are usually buried in the protein structure, such proteins are prone to accumulate within cells to form potentially harmful aggregates. To protect against proteotoxic stress, various stress-regulated chaperones constantly survey the cell for abnormally folded proteins. Upon encountering misfolded proteins, the chaperones attempt to refold or degrade them through a process called the protein quality control (PQC) system, which works in concert with the UPS. The PQC in the endoplasmic reticulum (ER), which is called ER-associated degradation (ERAD), has been most extensively studied for its role in clearing proteins that fold incompletely or improperly during protein synthesis. Degradation of misfolded proteins is a highly regulated

Abbreviations: UPS, ubiquitin proteasome system; PQC, protein quality control; ER, endoplasmic reticulum; ERAD, ER-associated degradation; CytoQC, cytoplasmic protein quality control; Ssa, stress seventy subfamily A; Ssb, stress seventy subfamily B; Sse, stress seventy subfamily E.

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process, not only in the ER, but also in the cytoplasm and other organelles such as the nucleus and mitochondrion [11–13]; however, little is known about the mechanisms operating in these compartments.

For PQC in the cytoplasm (CytoQC), abnormally folded proteins must be separated from native or newly synthesized polypeptides in order to avoid inappropriate interactions that would cause deactivation or aggregation of normal proteins [14]. Recent work in yeast showed that the nuclear UPS degrades not only misfolded nuclear proteins but also various abnormal proteins that are imported from the cytoplasm [14–17]. In a unicellular eukaryote such as yeast, which has a closed mitosis (i.e., mitosis without nuclear envelope breakdown), the nucleus is a major site of proteasome activity and serves as a segregated compartment for degradation of a subset of cytoplasmic proteins [14,15]. In mammalian cells, proteasomes are localized both in the cytoplasm and the nucleus [18,19]; however, similar mechanisms that enclose protein aggregates for the UPS-mediated degradation have been proposed to exist in the nucleus and juxtanuclear compartment of mammals [20–23].

In *Saccharomyces cerevisiae*, multiple mechanisms are involved in the dynamic nucleo-cytoplasmic transport of the proteasome and its substrate proteins. Many chaperones normally localized in the cytoplasm are imported into the nucleus over the course of cell-cycle progression or upon exposure to proteotoxic stress (e.g., heat shock) [13]. Together with the importins, the chaperones Ssa and Sse1 import the misfolded proteins into the nucleus for degradation by the nuclear PQC system [15,17,24]. By contrast, another Hsp70 chaperone, Ssb, exports misfolded proteins from the nucleus via nuclear export signals on its C-terminus [25]. Three pathways for nuclear import of the proteasome have been identified in budding yeast [21,26]: 1) separate import of the core and regulatory particle of the 26S proteasome by the canonical nuclear import system involving importin/karyopherin α and β, which recognize the nuclear localization signals on several proteasome subunits and the proteasome-binding protein Sts1 [27,28]; 2) import of the core particle from the proteasome storage granule within the cytoplasm by the karyopherin β-related protein Blm10 [29]; and 3) direct import of the assembled 26S proteasome by an unknown mechanism [30]. Although the molecular mechanism involved in nuclear export of the proteasome remains unknown, the Sis1-related *S. pombe* protein Cut8 was reported to anchor proteasomes on the inner nuclear membrane to prevent them from leaking out of the nucleus [31]. In addition, *N*-myristoylation of *S. cerevisiae* Rpt2, a highly conserved lipid modification found in eukaryotic proteasomes, reduces the nuclear export of proteasomes during progression of yeast growth phase [32]. Mutation of the *N*-myristoylation site not only changes the subcellular localization pattern of proteasomes (specifically, proteasome signals decrease in the nucleus and aggregate within the cytoplasm), but also increases the levels of polyubiquitinated proteins and exacerbates the cell's sensitivity to stress induced by misfolded proteins. A defect in UPS-mediated proteolysis is expected in these mutant strains, despite the fact that proteasome activity itself was not affected by the mutation [32]. These results led us to speculate that novel mechanisms involving *N*-myristoylation are responsible for compartmentalizing the PQC system by controlling the intracellular localization of the proteasome.

In this study, we performed a ubiquitin proteome analysis using the *S. cerevisiae* strains with or without a mutation in the *N*-myristoylation site of Rpt2, in order to obtain a comprehensive view of the UPS system controlled by this modification [32]. The K-ε-GG peptide levels of proteins with putative nucleo-cytoplasmic localization were significantly elevated in the mutant strains, whereas those of proteins exclusively localized in the nucleus or the cytoplasm exhibited a slight decrease. Furthermore, the protein and K-ε-GG peptide levels of Ssa and Sse1, the Hsps involved in nuclear import of CytoQC substrates, were significantly upregulated in the mutant strains, whereas those of the chaperone involved in the nuclear export, Ssb, was downregulated. These results indicated a possible role for proteasome *N*-myristoylation in the alternative CytoQC system within the nucleus.

2. Materials and methods

2.1. Yeast culture, lysate preparation, and protein digestion

S. cerevisiae strains with a mutation in the *N*-myristoylation site of Rpt2 and a GFP tag attached to proteasome subunit Rpn11 (Rpt2-normal/rpn11-GFP, rpt2-G2A/rpn11-GFP, rpt2-G2Δ/rpn11-GFP) [32] were grown for 3 days at 30 °C in synthetic complete medium lacking leucine (SC-Leu). Localization of rpn11-GFP was monitored by observing an aliquot of unfixed cells under a fluorescence microscope (BZ-9000, Keyence) equipped with a fluorescence filter for GFP and a Plan Apo 100× 1.40 oil objective lens (Nikon). Cells were harvested at 3000 ×*g* for 10 min, washed twice in sterile water, and resuspended in lysis buffer [9 M Urea/20 mM HEPES (pH 8.0)] supplemented with protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) and 50 μM PR-619 (Life Technologies, Carlsbad, CA, USA). The cell suspension was vortexed with glass beads and centrifuged to obtain cell lysates. Proteins were precipitated with a 3× volume of cold acetone and resuspended in lysis buffer. A total of 3.3 mg protein extracted from total cell lysate from triplicate cultures of each cell line was reduced with 10 mM dithiothreitol at 37 °C for 1 h, alkylated with 50 mM 2-chloroacetamide [33] (Sigma, Madison, WI, USA) at room temperature for 30 min, diluted with 3× volume of 20 mM HEPES (pH 8.0) and digested at 37 °C for 16 h with trypsin (Sigma) at a protease:protein ratio of 1:10.

2.2. Enrichment and MS analysis of K-ε-GG peptides

Peptide mixtures were acidified with 1/20th volume of 20% trifluoroacetic acid (TFA), cleared by centrifugation, and desalted using SepPak Light C18 columns (Waters, Milford, MA, USA). The desalted peptide mixtures were frozen overnight at −80 °C, lyophilized using a vacuum freeze-dryer (Techcorp FD-3-85-MP), and reconstituted in 462 μl of IAP buffer (Cell Signaling Technology, Danvers, MA, USA). Peptide mixtures were added to 26.4 μl of a 50% slurry of K-ε-GG antibody beads (Cell Signaling Technology) and rotated overnight in cold room. Beads were washed three times with IAP buffer and twice with distilled water. Bound peptides were eluted twice with 100 μl of 0.15% TFA and desalted using C18 Stage Tips [34]. Eluted peptides were evaporated and reconstituted in 0.1% formic acid. Mixed sample of all the samples was also analyzed as a reference run for the following label-free quantitation. K-ε-GG peptides prepared from 1.65 mg of lysate protein were separated on a Dionex Ultimate 3000 nano HPLC system (Dionex Softron, Germering, Germany) equipped with a nanoscale C18 capillary LC column (Acclain PepMap 100 C18 column, 75 μm id, 150 mm length, 3 μm particle size, and 100 Å pore size; Dionex Benelux, Amsterdam, The Netherlands). The mobile phase consisted of 2:98 (v/v) acetonitrile/water (mobile phase A) and 95:5 (v/v) acetonitrile/water (mobile phase B), both of which were supplemented with 0.1% formic acid. The peptides were concentrated on a C18 Trap Column (Acclain PepMap 100, 100 μm id, 20 mm length, 5 μm particle size, and 100 Å pore size; Dionex Benelux) equilibrated with mobile phase A and eluted at a flow rate of 0.3 μl/min with a linear gradient of mobile phase B from 2 to 33% for 120 min and 95% B for 10 min. The liquid was directly electrosprayed with electrospray ionization (ESI) source in a positive ion mode using a SilicaTip PicoTip nanospray emitter (10 μm id, top non-coated; New Objective Inc., Woburn, MA, USA) at a spray voltage of 2.1 kV and capillary temperature of 250 °C, and analyzed on an LTQ Orbitrap Velos (Thermo Fisher Scientific, Bremen, Germany). MS/MS analysis was performed in a Data-Dependent Scanning mode with a full-range scan (m/z of 350 to 1200) followed by product ion scans for the 15 most intense ions selected from the MS-scan spectrum. For quantitation of total lysate protein, aliquots of yeast cell lysate containing 10 μg of protein were trypsin-digested and desalted. A total of 1 μg of digested peptides were analyzed as described above.