Endoplasmic reticulum stress-regulated degradation of a translocon-associated protein is independent of integrated stress response transcription factor Gcn4p

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Abstract

Figure 1: Effect of ER stress on Deg1*-Sec62 degradation in wild type and gcn4Δ yeast. (A) Schematic depiction of Deg1*-Sec62 following aberrant translocon engagement. Deg1*-Sec62 consists of Deg1* (a modified version of the amino-terminal 67 amino acids from the yeast transcriptional repressor MATα2p (Rubenstein et al. 2012)), a Flag (F) epitope, the two-transmembrane protein Sec62p, and two copies of the S. aureus Protein A (PrA). Following translocon engagement, Deg1*.Sec62 is modified by N-linked glycosylation and is targeted for degradation by the Hrd1p ubiquitin ligase. Degradation of Deg1*.Sec62 is impaired by ER stress (Buchanan et al. 2019). The primary glycosylated asparagine amino acid is portrayed as a blue circle. Ub, ubiquitin. (B) Cycloheximide chase of wild type and gcn4Δ yeast expressing Deg1*-Sec62 cultured in the absence or presence of 6 mM DTT. Cultures were treated with DTT (or water for “No Treatment” controls) for 60 min prior to cycloheximide addition; DTT was maintained at the same concentration during incubation with cycloheximide. Deg1*-Sec62 signal intensity was normalized to Pgk1, and the percentage of Deg1*-Sec62 remaining at each time point is presented below the image. This experiment was performed three times. GCN4 deletion was confirmed by PCR. Vec, empty vector.

Description

Most eukaryotic proteins that reside in the secretory pathway or that are released to the extracellular space cross the endoplasmic reticulum (ER) membrane by passing through the Sec61p translocon channel (Lang et al. 2017). Arrested translocation prevents the movement of other proteins and impairs cellular fitness (Izawa et al. 2012; Ast et al. 2016). Eukaryotes have evolved multiple quality control mechanisms to degrade proteins that aberrantly and persistently engage the translocon. Proteins that stall in the Saccharomyces cerevisiae translocon are targeted for proteasomal degradation by the Hrd1p (Rubenstein et al. 2012) or Ltn1p/Rkr1p (Crowder et al. 2015) ubiquitin ligases or cleaved by the Ste24p protease (Ast et al. 2016). These mechanisms have been intensively investigated in yeast and are likely conserved in mammalian systems (Fisher et al. 2011; von der Malsburg et al. 2015; Ast et al. 2016).

When misfolded or unfolded proteins accumulate in the ER, cells experience ER stress. ER stress is characteristic of a number of human diseases, and prolonged ER stress leads to cell death (Chadwick and Lajoie 2019). Further, some human cells (e.g. professional secretory cells) exist at elevated basal levels of stress (van Anken and Braakman 2005).

During ER stress in yeast, degradation of model translocon-associated substrates of Hrd1p and Ste24p is impaired (Buchanan et al. 2019). How ER stress impairs degradation of such proteins is not known. Impaired degradation of the Hrd1p substrate Deg1*-Sec62 (Figure 1A) during stress is not due to broadly inhibited Hrd1p function (Buchanan et al. 2019). Further, stress-dependent Deg1*-Sec62 stabilization does not require three established ER stress response mechanisms (unfolded protein response, ER surveillance, or stress-induced homeostatically regulated protein degradation) or one predicted ER stress response pathway (rapid ER stress-induced export), at least non-redundantly (Babour et
The plasmids used in this study were pVJ27/pRS316 (Reagents using an Odyssey CLx Infrared Imaging System and Image Studio Software (Li-Cor). AlexaFluor-680-conjugated rabbit anti-mouse secondary antibody (1:40,000). Membranes were imaged and analyzed.

We performed cycloheximide chase experiments to test the hypothesis that the ISR transcription factor Gcn4p is required for ER stress-dependent stabilization of the Hrd1p translocon-associated substrate Deg1*-Sec62 (Figure 1B). In wild type cells, Deg1*-Sec62 was rapidly degraded in non-stress conditions. As previously reported (Buchanan et al. 2019), Deg1*-Sec62 was profoundly stabilized during ER stress induced by dithiothreitol (DTT), which reduces disulfide bonds. ER stress also delayed N-linked glycosylation of Deg1*-Sec62, causing the protein to migrate as two species with distinct electrophoretic mobility. Deletion of GCN4 did not stabilize Deg1*-Sec62 in the absence of stress, nor did it restore protein degradation during stress.

Our results indicate that the ISR transcriptional regulator Gcn4p is not required for degradation of Deg1*-Sec62 or its stabilization by ER stress, unless the ISR functions redundantly with other mechanisms. The molecular details by which ER stress impedes protein degradation and the cellular consequences of reduced degradation remain active areas of investigation. We speculate that impaired destruction of translocon-associated proteins is an adaptive response to ER stress. Dampered turnover of proteins that persistently engage the translocon may slow the inward flux of new proteins into an already stressed ER.

Methods
Request a detailed protocol

Yeast and Plasmid Methods

Yeast were cultured at 30°C in standard plasmid-selective growth media (Guthrie and Fink 2004). An empty vector and a plasmid encoding Deg1*-Sec62 driven by the MET25 promoter were introduced to wild type yeast or congenic gcn4Δ yeast via lithium acetate transformation (Guthrie and Fink 2004). The genotype at the GCN4 locus in both yeast strains was confirmed by PCR.

Cycloheximide Chase Analysis, Cell Lysis, and Western Blotting

Cycloheximide chase analysis was performed as described previously (Buchanan et al. 2016). Briefly, yeast grown to mid-exponential phase at 30°C were concentrated to 2.5 OD₆₀₀ units/ml in fresh media. Cycloheximide was added to a final concentration of 250 μg/ml. Aliquots (2.4 OD₆₀₀ units) were harvested immediately, 30 min, and 60 min after cycloheximide addition.

Yeast were lysed as described previously (Kushnirov 2000; Buchanan et al. 2016). 2.4 OD₆₀₀ units of yeast were pelleted and suspended in 200 μl of 0.1 M NaOH. Suspended yeast were incubated for 5 min at room temperature. Yeast were pelleted, resuspended in 1X Laemmli sample buffer, and heated to 95°C for 5 min.

Lysates were subject to centrifugation to pellet insoluble material. Proteins in the soluble fraction were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes via wet transfer at 20 V for 60 min at 4°C. Membranes were blocked in 5% skim milk in Tris-buffered saline (TBS; 50 mM Tris-base, 150 mM NaCl) at 4°C overnight. Membranes were probed in a solution containing 1% skim milk in TBS with 1% Tween 20 (TBS/T) and the appropriate antibodies for 1 hour at room temperature, followed by three five-min washes in TBS/T.

Deg1*-Sec62 is C-terminally tagged with two copies of the Staphylococcus aureus protein A epitope (Figure 1A). Because S. aureus Protein A binds to mammalian immunoglobulins (Hjelm et al. 1972), AlexaFluor-680-conjugated rabbit anti-mouse antibody (Life Technologies, Inc; 1:40,000) was used to directly detect Deg1*-Sec62. Pgk1 was detected with mouse anti-phosphoglycerate kinase 1 (Pgk1; clone 22C5D8; Life Technologies, Inc; 1:20,000) followed by AlexaFluor-680-conjugated rabbit anti-mouse secondary antibody (1:40,000). Membranes were imaged and analyzed using an Odyssey CLx Infrared Imaging System and Image Studio Software (Li-Cor).

Reagents

The plasmids used in this study were pVJ27/pRS316 (URA3/CEN/ampR ( Sikorski and Hieter 1989)) and pVJ411 (LEU2/CEN/ampR/POMET25−Deg1*-Sec62 (Buchanan et al. 2019)). The yeast strains used in this study were VJY476 (BY4741 MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) and VJY779 (MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gcn4Δ::kanMX4) (Tong et al. 2001).
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References


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