F-actin and a Type-II Myosin Are Required for Efficient Clustering of the ER Stress Sensor Ire1

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ABSTRACT. Endoplasmic reticulum (ER) stress causes the ER-resident transmembrane protein Ire1 to self-associate, leading to the formation of large oligomeric clusters. In yeast cells, this induces strong unfolded protein response (UPR) through splicing of HAC1 mRNA. Here, we demonstrate that highly ER-stressed yeast cells exhibited poor Ire1 clustering in the presence of the actin-disrupting agent latrunculin-A. Under these conditions, Ire1 may form smaller oligomers because latrunculin-A only partially diminished the Ire1-mediated splicing of HAC1 mRNA. Ire1 cluster formation was also impaired by deletion of the type-II myosin gene MYO1 or SAC6, which encodes the actin-bundling protein fimbrin. Finally, we demonstrated that Ire1 clusters are predominantly located on or near actin filaments. Therefore, we propose that actin filaments play an important role in ER stress-induced clustering of Ire1.

Key words: UPR, molecular chaperone, stress response, cytoskeleton, organelle

Introduction

The endoplasmic reticulum (ER) consists of an extensive membranous network, which includes the nuclear envelope (perinuclear ER) and emanates through the cytoplasm. In Saccharomyces cerevisiae (hereafter called yeast), the cytoplasmic ER is mainly juxtaposed with the plasma membrane (peripheral ER). Accumulation of unfolded proteins in the ER often accompanies disturbances in ER function, namely ER stress, and evokes the unfolded protein response (UPR).

Ire1 is an ER-localized type-I transmembrane protein, which is conserved through eukaryotic evolution and triggers the UPR (Ron and Walter, 2007; Mori, 2009). Upon ER stress, Ire1 molecules cluster to form high-order homooligomers (Fig. 1), which can be visualized as punctate spots using GFP-tagging or fluorescence immunostaining techniques (Kimata et al., 2007; Aragon et al., 2009; Li et al., 2010). The cytosolic domain of Ire1 has an endoribonuclease motif. In yeast cells, the clustered Ire1 molecules exhibit strong splicing activity toward HAC1 mRNA (Korennykh et al., 2009), leading to subsequent translation of the Hac1 transcription factor that functions to alleviate ER stress.

The luminal domain of Ire1 has a tightly folded segment called the core stress-sensing region (CSSR), which self-associates through two different interfaces, known as Interfaces I and II (Credle et al., 2005). Importantly, this self-association of CSSR is likely to be the structural basis for the Ire1 cluster formation (Fig. 1; Kimata et al., 2007). In addition, Ire1 clustering can be negatively regulated by its loosely folded segments, Subregions I and V (Fig. 1) because an Ire1 mutant in which both of these segments are deleted (the ΔIΔV mutation) has been shown to self-associate even in the absence of ER stress (Oikawa et al., 2007). Moreover, the CSSR has the ability to capture unfolded proteins (Promlek et al., 2011; Gardner and Walter, 2011). According to our observations proposed previously (Kimata and Kohno, 2011), the association of unfolded proteins with CSSR results in complete activation of clustered Ire1 molecules.

It is obscure whether cells have additional machineries that promote or accelerate the Ire1 cluster formation. Therefore, we examined if the cytoskeletal system contributes to Ire1 clustering in yeast cells because it is often involved in intracellular movement of cellular compartments and macromolecular complexes. In this report, we propose that actin filaments, but not microtubules, are involved in Ire1 cluster formation during ER stress.
Materials and Methods

Chemicals

Chemical stock solutions used in this study were 20 mM latrunculin-A (Wako Pure Chemical, Japan) in DMSO, 20 mg/ml nocodazole (Nakarai Tesque) in DMSO, and 1 M dithiothreitol (Nakarai Tesque, Japan) in water. The chemical stock solutions were added into culturing media at 1:100 dilution for latrunculin-A (200 μM final), 1:1000 dilution for nocodazole (20 μg/ml final), and 1:100 dilution for dithiothreitol (10 mM final).

Yeast plasmids, strains, and culturing conditions

A yeast centromeric plasmid carrying \textit{IRE1} (pRS313-IRE1) was described previously (Kimata et al., 2004) and called the wild-type \textit{IRE1} plasmid in our study. The W426A mutation was introduced into pRS313-IRE1 through \textit{in vivo} homologous recombination (Kimata et al., 2007). The \textit{IRE1} promoter sequence of pRS313-IRE1 was replaced with a stronger promoter sequence (the \textit{TEF1} promoter, ChrXVI position 700180-700560), and the resulting plasmid was named as pRS313-TRF1p-IRE1. In-frame insertion of the GFP-tagging sequence into the Ire1-coding region \textit{Ire1-GFP} (Aragon et al., 2009) of pRS313-TRF1p-IRE1 was achieved through \textit{in vivo} homologous recombination. To express hemagglutinin epitope-tagged Ire1 (Ire1-HA) and its ΔIΔV mutant version, we used a yeast 2μ plasmid (pRS423-IRE1-HA) and its ΔIΔV mutant derivative (Kimata et al., 2003; Oikawa et al., 2007). Plasmid pPM28 was used for cellular expression of an ER-lumen localized GFP variant (Merksamer et al., 2008).

Haploid strains carrying the \textit{ire1Δ} mutation, KMY1516, and KMY1015, were described previously (Kimata et al., 2004) and used according to their nutrient requirements. KMY1005 is a congenic \textit{IRE1+} strain (Mori et al., 1996). KMY1516 and KMY1015 were transformed with a KanMX4-based EUROSCARF (http://www.web.uni-frankfurt.de/fb15/mikro/euroscarf/) gene-disruption module for knocking-out \textit{SAC6} to generate YSD001 and YSD002, respectively. The \textit{myo1Δ} strain (BY23665) and the \textit{myo2-66ts} strain (BY24093), both of which are congenic to YPH499, were provided by the National Bio-Resource Project (NBRP) of the MEXT, Japan. BY23665 was transformed with \textit{ADE2} in order to eliminate auto-fluorescence caused by its \textit{ade2} mutation. The \textit{myo3Δ} strain (Y04979), the \textit{myo4Δ} strain (Y00379), the \textit{myo5Δ} strain (Y06549), and the congenic wild type strain (BY4741) were provided by EUROSCARF. These strains were transformed with the aforementioned plasmids.

Except for the \textit{myo2-66ts} cells, which were incubated at a semi-permissive temperature of 23°C, yeast cells were cultured in synthetic dextrose (SD) medium at 30°C.

Staining and microscopic observation of cells

Anti-HA immunofluorescent staining of formalin-fixed cells was performed as described previously (Kimata et al., 2007). For double staining of actin filaments and the HA epitope, we modified this method as follows. Spheroplast cells were permeabilized through suspension in PBS containing 1% Triton-X100 for 5 min. The permeabilized cells were then washed twice through brief spin-down and re-suspension in PBS in standard 1.5 ml microtubes and suspended in PBS containing 1% bovine serum albumin (blocking buffer) for 30 min. Subsequently, cells were suspended in blocking buffer containing 12CA5 anti-HA antibody for 3 h, washed 4 times with blocking buffer, suspended in blocking buffer containing an FITC-labeled secondary antibody, and washed again with blocking buffer (4 times) and PBS (twice). See Kimata et al. (2007) for information on antibodies and concentrations. Cells were then suspended in PBS containing Alexa Fluor 594-phalloidin.
Fig. 2. Latrunculin-A but not nocodazole inhibits Ire1-HA cluster formation. A, Experimental procedures used for treatment of ire1Δ cells (KMY1516) carrying the wild-type (WT) Ire1-HA-expression plasmid with dithiothreitol (DTT; 10 mM) and/or latrunculin-A (Lat-A, 200 μM). B-I, The WT Ire1-HA-expressing cells were treated as indicated in A and subjected to anti-HA immunofluorescent staining. Punctate spots in G and H are denoted with pink arrowheads. J and K, Latrunculin-A-treated and untreated ire1Δ cells (KMY1516) carrying the ΔIAV Ire1-HA-expression plasmid. L-N, Nocodazole (Noc; 20 μg/ml) treatment of the WT Ire1-HA-expressing cells. L, Nocodazole-treated and untreated cells were stained with DAPI, and their morphology and nuclear position were analyzed. N, Cells were treated with nocodazole for 2 h, and then dithiothreitol was added to the culture, which was further incubated for 30 min. O, Negative staining control using ire1Δ cells (KMY1516) carrying the non-tagged wild-type IRE1 plasmid.
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(30 U/ml; Molecular probes) for 2 h and washed twice with PBS. The doubly stained cells were observed using an LSM710 confocal fluorescent microscope (Carl Zeiss), which was also used to capture the fluorescent signal from eroGFP, and an Apotome deconvolution microscope (Carl Zeiss). In other cases, cells were observed under a conventional fluorescence microscope (Kimata et al., 2007).

**RNA and protein analyses**

Extraction of total RNA samples and RT-PCR amplification of the HAC1 products were performed as described previously (Kimata et al., 2003; Promlek et al., 2011). Cell lysis and subsequent centrifugal fractionation were performed as described previously (Promlek et al., 2011), and the resultant supernatant and pellet fractions were analyzed by Western blotting as described previously (Kimata et al., 2003).

**Results**

Yeast cells were treated with dithiothreitol, a potent ER-stressing reagent, and latrunculin-A, an actin-disrupting agent, following the scheme shown in Fig. 2A, and checked for the cellular localization of HA-tagged Ire1 (Ire1-HA). In non-stressed cells, Ire1-HA exhibited a double-ring-like typical ER distribution pattern (Fig. 2B; perinuclear and peripheral ER), indicating that Ire1-HA was diffusively localized over the ER. As reported previously (Kimata et al., 2007), the localization pattern of Ire1-HA became punctate following dithiothreitol treatment (Fig. 2C and D), indicating Ire1 cluster formation upon ER stress. Latrunculin-A treatment did not significantly affect the diffuse ER localization pattern of Ire1-HA in cells not stressed with dithiothreitol (Fig. 2E and F). However, pretreatment of cells with latrunculin-A for 40 min drastically impaired the cluster formation of Ire1-HA upon induction of ER stress (Fig. 2G and H).

As aforementioned, the ΔΔV mutant version of Ire1-HA constitutively clusters even in the absence of ER stress (Fig. 2J). This pattern appeared to remain unaffected by latrunculin-A treatment (Fig. 2K). Moreover, cells treated with latrunculin-A after the dithiothreitol treatment showed considerable clustering of wild-type Ire1-HA (Fig. 2I). Therefore, we considered that latrunculin-A inhibits the formation of Ire1 clusters without promoting their dissociation.

Two-hour treatment with the microtubule-disrupting compound nocodazole considerably increased the portion of single-nuclear budded cells (Fig. 2L) because the microtubule-dependent nuclear migration was blocked. As shown in Fig. 2M and N, under this condition, Ire1-HA clustered efficiently upon induction of ER stress. Therefore, we consider that Ire1 clustering occurs in a microtubule-independent manner.

These observations were further confirmed using another Ire1 construct, Ire1-GFP, in which the GFP tag was inserted at a different position (juxtamembrane; Aragon et al., 2009) than the HA tag (C-terminus). As seen with Ire1-HA, clustering of Ire1-GFP was inhibited by latrunculin-A but unaffected by nocodazole (Fig. 3).

In addition, we quantified the data from our fluorescence microscopy studies (Fig. 2 and Fig. 3) by counting the number of cells in which Ire1-HA or Ire1-GFP were clustered. As shown in Fig. S1, this quantitation confirmed that latrunculin-A inhibition of dithiothreitol-induced cluster formation was statistically significant.

In Fig. S2, we washed out latrunculin-A from a culture

![Fig. 3. Inhibition of Ire1-GFP clustering by latrunculin-A. The *ire1Δ* cells (KMY1015) carrying the wild-type (WT) Ire1-GFP-expression plasmid were observed using fluorescence microscopy after treatment with dithiothreitol (DTT; 10 mM), nocodazole (Noc; 20 μg/ml), and/or latrunculin-A (Lat-A; 200 μM) as indicated. Punctate spots in F are denoted with pink arrowheads.](image-url)
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Fig. 4. Latrunculin-A poorly alleviates ER stress and only partially diminishes the activity of Ire1 to splice HAC1 mRNA. A, Experimental procedures used for treatment with DTT (10 mM) and/or latrunculin-A (Lat-A; 200 μM) of ire1Δ cells (KMY1516) carrying the wild-type (WT) IRE1 plasmid or its W426A variant. B, The wild-type (WT) Ire1-expressing cells were lysed in the presence of non-denaturing detergent (Triton X-100) and then centrifuged at 8,000×g for 20 min. The resulting supernatant fractions (equivalent to 0.1 OD600 cells) and pellet fractions (equivalent to 1.0 OD600 cells) were then analyzed by anti-BiP Western blotting. C, Total RNA samples from the wild-type Ire1-expressing or the W426A Ire1-expressing cells were analyzed by RT-PCR for HAC1 species, and the amplified fragments were used to estimate “HAC1-mRNA splicing %” as described in Promlek et al. (2011). Error bars refer to standard deviation of triplicate experiments.

Fig. 5. Deletion of SAC6 impairs the cluster formation of Ire1. The sac6Δire1Δ cells producing wild-type (WT) Ire1-HA (YSD001; A and B) or Ire1-GFP (YSD002; C and D) were left untreated or ER-stressed with dithiothreitol (DTT; 10 mM) and cellular localization was analyzed as mentioned in Fig. 2 and Fig. 3.

containing both latrunculin-A and dithiothreitol, and the cells were further stressed using dithiothreitol. In this case, Ire1-GFP clustered after the removal of latrunculin-A.

We further analyzed yeast cells that were treated with dithiothreitol and/or latrunculin-A, as summarized in Fig. 4A. In Fig. 4B, cell lysates were fractionated by centrifugation. Similar to our previous observation (Promlek et al., 2011), anti-BiP Western blotting revealed that dithiothreitol treatment of cells considerably increased the level of BiP in the pellet fractions (compare lanes 3 and 1 in Fig. 4B), suggesting that dithiothreitol denatures proteins, which aggregates with BiP in the ER. Since latrunculin-A did not change the level of BiP in the pellet fractions (compare lane 2 to 1, and lane 4 to 3 in Fig. 4B), we believe that latrunculin-A does not alleviate ER stress per se.

Next, we examined if latrunculin-A diminish the ability of Ire1 to splice HAC1 mRNA (Fig. 4C or Fig. S3 for the raw data). In cells carrying wild-type Ire1, dithiothreitol treatment strongly induced splicing of the HAC1 mRNA, which was diminished only partially by pretreatment of cells with latrunculin-A. This may reflect the partial dot-like
distribution of Ire1-HA and Ire1-GFP under these conditions (Fig. 2G, H, and Fig. 3F, pink arrow heads), indicating that latrunculin-A does not completely abolish Ire1 clustering. It is also possible that in cells treated with both latrunculin-A and dithiothreitol, a considerable portion of the Ire1 molecules may form smaller but active oligomers that cannot be visualized by fluorescence microscopy. We could not demonstrate the existence of such small oligomers through biochemical analyses, as self-association through Interface II is weak and dissociates upon cell lysis (Kimata et al., 2007). As expected, latrunculin-A did not affect the weak activity of the W426A mutant version of Ire1 (Fig. 4C or Fig. S3 for the raw data), which cannot self-associate through Interface II (Credle et al., 2005), and functions not as an oligomer but as a dimer (Kimata et al., 2007).

We next visualized cells expressing eroGFP, which is a GFP variant that localizes to the ER lumen (Merksamer et al., 2008). Neither dithiothreitol nor latrunculin-A disrupted the double-ring-like ER morphology (Fig. S4).

To test whether Ire1 cluster formation was inhibited by genetic damage to the actin cytoskeleton, we examined the Ire1 clustering in SAC6- (a non-essential gene encoding the actin-bundling protein fimbrin (Adams et al., 1991)) deleted strain. We found that SAC6 deletion impaired the cluster formation of Ire1. The myo1Δ and the myo2-66ts strains (and their congenic wild-type strain YPH499) and the myo3Δ, myo4Δ, and myo5Δ strains (and their congenic wild-type strain BY4741) carrying the wild-type Ire1-GFP-expression plasmid were left untreated or ER-stressed with dithiothreitol (DTT; 10 mM).

**Fig. 6.** Deletion of MYO1 impairs the cluster formation of Ire1. The myo1Δ and the myo2-66ts strains (and their congenic wild-type strain YPH499) and the myo3Δ, myo4Δ, and myo5Δ strains (and their congenic wild-type strain BY4741) carrying the wild-type Ire1-GFP-expression plasmid were left untreated or ER-stressed with dithiothreitol (DTT; 10 mM).
formation of both Ire1-HA and Ire1-GFP in dithiothreitol-treated cells (compare Fig. 5B with Fig. 2D and Fig. 5D with Fig. 3B), albeit not completely (localization patterns shown in Fig. 5B and D are somewhat less smooth than those in Fig. 5A and C). This inhibition of Ire1 clustering by the sac6Δ mutation was statistically significant (Fig. S1).

In order to address involvement of myosin proteins in the cluster formation of Ire1, we employed strains in which one of the myosin genes, MYO1, MYO3, MYO4 or MYO5, was deleted. Depending on the strain background, myo1Δ mutation can be viable. Because MYO2 is essential, we used cells carrying a temperature-sensitive allele of MYO2, myo2-66ts, and cultured them at a semi-permissive temperature of 23°C. Ire1-GFP clustered efficiently in the myo2-66ts, myo3Δ, myo4Δ, and myo5Δ cells as well as in wild-type cells upon dithiothreitol stress (Fig. 6 and Fig. S1). However, the myo1Δ mutation caused considerable impairment of the Ire1-GFP cluster formation (Fig. 6 and Fig. S1).

Dithiothreitol treatment seems to somehow cause actin cytoskeleton depolarization. As shown in Fig. S5, dithiothreitol-treated and non-stressed cultures were subjected to cellular staining with fluorophore-labeled phalloidin. Most of the small-budded cells in non-stressed cultures carried normally polarized actin filaments. Namely, actin patches were enriched in the buds, and actin cables extended from the mother cells to the buds. However, dithiothreitol treatment considerably increased the number of small-budded cells in which such distribution of actin cytoskeletons was disturbed. We speculate that the depolarized distribution of actin filaments may contribute to efficient cluster formation, both in the mother cells and buds.

Finally, we examined the spatial relationship between the actin cytoskeleton and Ire1 clusters. As shown in Fig. 7 and Fig. S6, Ire1-HA-expressing cells were stressed by dithiothreitol treatment and doubly stained with the anti-HA antibody and Alexa Fluor 594-phalloidin, and then observed by deconvolution fluorescence microscopy. Punctate spots of Ire1-HA are denoted with pink arrowheads.

Fig. 7. Juxtaposition of Ire1 clusters along actin filaments. KMY1516 ire1Δ cells carrying the wild-type (WT) Ire1-HA-expression plasmid were stressed with 10 mM dithiothreitol for 30 min, doubly stained with mouse anti-HA antibody 12CA5/FITC-conjugated goat anti-mouse antibody and Alexa Fluor 594-phalloidin, and then observed by deconvolution fluorescence microscopy. Punctate spots of Ire1-HA are denoted with pink arrowheads.
**Discussion**

Here we demonstrated that ER-stress-induced cluster formation of Ire1 is inhibited by latrunculin-A and the sac6Δ mutation (Fig. 2, Fig. 3, Fig. 5, and Fig. S1). Thus, we propose that although may not be absolutely required, actin filaments are involved in Ire1 cluster formation. Because latrunculin-A diminished the HAC1-mRNA splicing only slightly (Fig. 4), we speculate that Ire1 forms small but active oligomers even when the actin cytoskeleton is destabilized. Considering these observations from another angle, it is possible that Ire1 requires the actin cytoskeleton for exerting its highest activity.

How do actin filaments contribute to the cluster formation of Ire1? In higher eukaryotic cells, myosin proteins are physically linked to the ER and promote its intracellular movement (Ueda et al., 2010; Wagner et al., 2011). Moreover, actin filaments and the class-V myosin Myo4 are reported to be important for mother-to-daughter-cell transport of the peripheral ER in yeast cells (Estrada et al., 2003). Moreover, fine morphology and dynamics of the peripheral ER are likely to be affected by actin-cytoskeleton aberrancy (Prinz et al., 2000; Fehrenbacher et al., 2002), although the broad outline of ER morphology (the double ring) was not changed by latrunculin-A treatment (Fig. S4; Prinz et al., 2000). Therefore, it is possible that the impaired cluster formation of Ire1 due to actin aberrancy is an indirect phenomenon because some properties of the ER are likely to depend on actin filaments.

However, it should be noted that latrunculin-A treatment and the sac6Δ mutation diminished the Ire1 cluster formation not only on the peripheral ER but also on the nuclear ER (Fig. 2, Fig. 3, and Fig. 5), structure and movement of which do not seem to be tightly linked to actin filaments (Fehrenbacher et al., 2002). Moreover, the Ire1 clusters were predominantly located along the actin filaments (Fig. 7). Therefore, we speculate an unidentified link between the Ire1 clusters and actin cytoskeletons.

**MYO1** encodes the sole class-II myosin heavy chain, the deletion of which impaired Ire1 cluster formation, unlike deletion or point mutation of the other myosin genes, **MYO2, MYO3, MYO4**, or **MYO5** (Fig. 6). We believe that this finding also implies an undisclosed role of the actin cytoskeleton in Ire1 clustering because to our knowledge, Myo1 is not reported to be closely related to the ER. He et al. (2012) recently reported a physical association between IRE1α, the major mammalian Ire1 parologue, and non-muscle myosin IIB in mammalian cells. Knocking-out of this myosin protein abolished the cluster formation of IRE1α (He et al., 2012), although it has not been reported what happens when actin filaments per se are damaged in mammalian cells. It also should be noted that according to our unpublished observation, the IRE1α clusters are located on or near actin filaments in mammalian cells, similar to yeast Ire1 clusters. Taken together, we speculate that similar machineries promote the Ire1 cluster formation both in yeast and mammalian cells. Actin filaments may function as a scaffold for Ire1 cluster formation, in which class-II myosin proteins may participate.

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**References**


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