Title: Tight regulation of the unfolded protein sensor Ire1 by its intramolecularly antagonizing subdomain

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Abstract

Accumulation of unfolded proteins in the endoplasmic reticulum (ER) accompanies ER stress and causes the type-I transmembrane protein Ire1 to trigger the unfolded protein response (UPR). When dimerized, the core stress-sensing region (CSSR) of Ire1 directly captures unfolded proteins and forms a high-order oligomer, leading to clustering and activation of Ire1. The CSSR is N-terminally flanked by an intrinsically disordered subdomain, which we previously named Subregion I, in *Saccharomyces cerevisiae* Ire1. In this study, we describe tight repression of Ire1 activity by Subregion I under no or weak stress conditions. Weak hyperactivation of an Ire1 mutant lacking Subregion I slightly retarded growth of yeast cells cultured under unstressed conditions. Fungal Ire1 orthologs and the animal Ire1-family protein PERK carry N-terminal intrinsically disordered subdomains with a similar structure and function as Subregion I. Our observations presented here cumulatively indicate that Subregion I is captured by the CSSR as an unfolded-protein substrate. This intramolecular subdomain interaction is likely to compromise self-association of the CSSR, explaining why Subregion I can suppress Ire1 activity when ER-accumulated unfolded proteins are not abundant.
Introduction

The endoplasmic reticulum (ER) of eukaryotic cells is a cellular compartment where secretory and membrane proteins are folded. Impaired protein folding in the ER accompanies dysfunction of the ER, namely ER stress, and evokes the unfolded protein response (UPR). The UPR is a cellular protective event through which proteins in the ER are transcriptionally induced (Mori, 2009; Walter and Ron, 2011). Ire1 is an ER-located type-I transmembrane endoribonuclease conserved through eukaryotic organisms and functions as an ER-stress sensor that triggers the UPR.

It is widely believed that Ire1 functions as a receptor for unfolded proteins accumulated in the ER (Credle et al. 2005; Kimata et al., 2007; Gardner and Walter 2011). The luminal domain of Ire1 has a tightly folded region (Kimata et al., 2004; Credle et al., 2005; Oikawa et al., 2005) called the core stress-sensing region (CSSR; Fig. S1A). According to the X-ray crystallographic analysis reported by Credle et al. (2005), the dimeric form of CSSR has a deep groove which captures unfolded proteins. Gardner and Walter (2011) proposed that the CSSR is highly self-oligomerized when directly associated with unfolded proteins. This finding well explains the molecular mechanism by which Ire1 clusters during ER stress (Fig. S1A; Kimata et al., 2007; Argon et al., 2009; Li et al., 2010). According to X-ray crystallographic and biochemical analyses of the Ire1 cytosolic domain, clustered Ire1 molecules exhibit a potent RNA-cleaving activity (Korennykh et al., 2009).

In the case of Saccharomyces cerevisiae (hereafter called yeast), Ire1 performs splicing of the HAC1-gene transcript ($HAC1^0$) to yield the $HAC1^u$ form, which is translated into a transcription factor that induces the UPR-target genes (Cox and Walter, 1996). Cell growth is damaged when Ire1 is improperly activated or when the UPR is artificially induced (Mori et al., 2000; Chawia et al., 2011; Rubio et al., 2011), probably because the yeast cell transcriptome is drastically changed by the UPR (Travers et al., 2000; Kimata et al., 2006). This finding explains the reason for Ire1 being tightly regulated through additional mechanisms.

We and others have previously reported that Ire1 is negatively regulated by the ER-located molecular chaperone BiP (Bertolotti et al., 2000; Kimata et al., 2003; Kimata et al., 2004). While the association of BiP with Ire1 is likely to inhibit the self-association of Ire1 in unstressed cells (Bertolotti et al., 2000), ER stress causes
dissociation of BiP from Ire1 (Fig. S1A). The BiP-binding subdomain is located at the juxtamembrane position (Subregion V of yeast Ire1; Kimata et al., 2004) and is loosely folded (Oikawa et al., 2005). As BiP is induced by the UPR, the negative regulation of Ire1 by BiP is likely to be a feedback-control system (Pincus et al., 2010).

Metazoan cells also carry another Ire1-family ER-stress sensor called PERK that attenuates protein synthesis upon ER stress (Harding et al., 1999). Ire1 orthologs and PERK commonly possess juxtamembrane BiP-binding subdomains and sequentially similar regions corresponding to the CSSR (Fig. S1B; Liu et al., 2000; Kimata and Kohno, 2011). Thus, we believe that Ire1 orthologs and PERK are regulated and activated in a similar manner.

In addition, PERK and yeast Ire1, but not higher eukaryotic Ire1 orthologs, have sequentially unconserved subdomains at the N terminus (N-terminal unconserved region (NUCR); Fig. S1B). The NUCR of yeast Ire1 is called Subregion I (Figs S1A,B; Kimata et al., 2004) and appears to be intrinsically disordered, as it is susceptible to partial proteolysis of an Ire1 recombinant luminal-region fragment (Oikawa et al., 2005).

In the present study, we describe the physiological importance and molecular mechanism of tight negative regulation of Ire1 by the NUCR.
Results

Subregion I suppresses yeast Ire1 activity

At the beginning of this study, we checked some of the partial deletion mutants of yeast Ire1 (Fig. 1A) for their UPR-inducing ability using a UPRE-lacZ reporter gene that expresses β-galactosidase under control of the UPR-target promoter element (UPRE; Mori et al., 1992). In the experiment shown in Fig. 1B, cells were cultured under unstressed conditions before measuring cellular β-galactosidase activity. The UPR was slightly induced by the ΔV mutation, namely a full-length deletion of Subregion V (compare column 4 to 1), probably because this mutation abolishes the interaction between Ire1 and BiP.

The NUCR of yeast Ire1, namely Subregion I, is composed of 80 amino-acid (a.a.) residues. In our previous studies (Oikawa et al., 2007; Kimata et al., 2007), we deleted a 60-a.a.-length portion of Subregion I (hereafter called the Subregion-I 60-a.a. portion) to obtain the ΔI mutant. Reproducing our previous findings (Oikawa et al., 2007; Kimata et al., 2007), we observed that the ΔI mutation clearly activated Ire1 in unstressed cells when combined with the ΔV mutation (Fig. 1B, compare column 5 to 1, 2, and 4).

The ΔI_{full} mutation is a full-length deletion of Subregion I (Fig. 1A). The activity of ΔI_{full}-ΔV Ire1 was higher than that of ΔV Ire1, but lower than that of ΔIΔV Ire1 in unstressed cells (Fig. 1B, compare column 6 to 4 and 5). Thus, we employed the ΔI mutation, but not the ΔI_{full} mutation, in the subsequent experiments to determine the Ire1-supressing ability of Subregion I. As shown in Fig. 1C, all Ire1 mutants used were considerably activated by treating cells with tunicamycin, which inhibits N-glycosylation and induces ER stress (note that the y-axis scale value of Fig. 1C is 10-fold larger than that of Fig. 1B).

Does the ΔI single mutation exhibit any apparent phenotype? The UPRE-lacZ reporter assay did not produce a difference in activity between wild-type Ire1 and ΔI Ire1 (Fig. 1B, compare lane 2 to 1). We then employed another highly sensitive version of UPR reporter in which expression of the lacZ gene was controlled under five tandem copies of the UPRE ((UPRE)_5-LacZ; Promlek et al., 2011). This method allowed us to monitor weak evocation of UPR at high resolution and to observe slightly higher
ΔI-Ire1 activity than that of wild-type Ire1 in unstressed cells (Fig. 1D). As shown in
Fig. S2A, the ΔI or the ΔV mutation did not considerably affect cellular abundance of
Ire1.

According to Shaner et al. (2013), mNeonGreen is a bright green fluorescent
protein which works as a superior substitute of the Aequorea GFP derivatives. In the
experiments shown in Figs 1E,F, mNeonGreen-tagged Ire1 or its ΔΔV mutant version
was expressed from a single-copy plasmid under control of the authentic IRE1 promoter.

Both wild-type Ire1 and ΔΔV Ire1 diffusively distributed probably on the ER under
unstressed conditions, while they exhibited dot-like distribution in response to ER stress
caused by dithiothreitol (DTT; a disulfide reducing agent). This finding suggests that
neither Subregion I nor V is involved in the cluster-formation step of Ire1. It should be
noted that our observation presented here is inconsistent with our previous report in
which ΔΔV Ire1 constitutively clustered even under unstressed conditions when
artificially overexpressed from a multicopy plasmid (Kimata et al., 2007).

Next, we directly checked splicing of HAC1 mRNA to compare activities of
wild-type Ire1 and ΔI Ire1 in cells (Fig. 2A). Reproducing the result shown in Fig. 1D,
the ΔI single mutation weakly enhanced HAC1-mRNA splicing under unstressed
conditions (Time 0). Moreover, while UPR activation profiles by 3 mM DTT were
almost equal in wild-type-IRE1 cells and ΔI-mutant cells at the early time points (15
min to 1 hr), attenuation of HAC1-mRNA splicing under long-term stress (2–5 hr)
appeared to be slightly retarded by the ΔI mutation.

We then performed the same experiment as Fig. 2A using not only wild-type-IRE1
and ΔI-IRE1 cells but also ΔV-IRE1 and ΔΔV-IRE1 cells (Fig. 2B). As reported
previously (Pincus et al., 2010; Ishiwata-Kimata et al., 2013), the ΔV mutation also
exhibited an impaired attenuation of Ire1 activity upon the prolonged ER stress. The
HAC1-mRNA splicing assay, as well as the UPRE-lacZ reporter assay shown in Fig. 1,
demonstrated an aggravated hyperactivation of Ire1 by the combination of the ΔI and
the ΔV mutations.

In the experiment shown in Table 1, we checked if the ΔI mutation affects cellular
growth under unstressed conditions. Wild-type-IRE1 cells and ΔI-IRE1 cells were
mixed and cultured for long duration to observe subtle differences in the growth rate
between the two cell lines. Then, their relative abundance was monitored, which indicated that the ΔI mutation retarded cellular growth. The ΔV and the ΔIΔV mutations also caused growth retardation.

Primary-structural properties of Subregion I

The UPRE-lacZ reporter values of ΔV-IRE1 and ΔIΔV-IRE1 cells under unstressed conditions were considerably different (Fig. 1B), allowing us to perform quick and high-resolution monitoring of the Ire1-suppressing ability of Subregion I and its mutants. Thus, we modified ΔV Ire1 by introducing various mutations into its Subregion I and tested for its activity to induce UPRE-lacZ reporter in unstressed cells to address the primary structural requirements of Subregion I for the Ire1-suppressing ability.

As illustrated in Fig. 3A, the Subregion-I 60-a.a. portion was partitioned into six 10-a.a.-long segments (Segments 1–6), which were serially deleted from ΔV Ire1 (refer to Table S1B for the resulting amino-acid sequences). We observed that ΔV Ire1 was not as highly activated as ΔIΔV Ire1 in unstressed cells even when carrying any of the partial deletions (Fig. 3B). Nevertheless, Fig. 3B also shows that the Δ4–6, Δ3–4, and Δ4 mutations, but not the Δ1–3, Δ1–2, Δ5–6, or Δ3 mutations, partially activated ΔV Ire1. Therefore, no specific Subregion-I sequence was absolutely required for the Ire1-suppressing ability, whereas Segment 4 appears to be relatively important. All Ire1 mutants responded well to ER stress induced by tunicamycin (Fig. 3C). Moreover, these partial deletion mutations did not considerably affect cellular abundance of ΔV Ire1 (Fig. S2B).

Two of our previous reports (Kimata et al., 2004; Oikawa et al., 2005) and a web-based computer prediction (Fig. S3A; Ishida and Kinoshita, 2007) strongly suggest that Subregion I is intrinsically disordered. We next replaced the Subregion-I 60-a.a. portion of ΔV Ire1 with other 60 a.a.-long sequences to determine if intrinsically disordered unrelated peptides could work as Subregion I (refer to Table S1C for amino-acid sequences of the peptides). As shown in Fig. 3D, the intrinsically disordered region (IDR) of the unrelated protein San1 (Rosenbaum et al., 2011) suppressed Ire1 activity in unstressed cells when substituted for Subregion I (compare column 3 to 1 and
2). Moreover, the IDR of Top2 (Berger et al., 1996) and a six tandem repeat of a five a.a.-long peptide GGGSS also exhibited Ire1-suppressing ability (columns 4 and 5), although less potently. Notably, a three tandem repeat of Segment 4 had stronger Ire1-suppressing ability than that of the authentic Subregion I (compare column 6 to 1). (Segment 4)₃ΔV Ire1, as well as the other Ire1 mutants employed here, responded well to tunicamycin-induced ER stress (Fig. 3E). Moreover, the cellular abundance of (Segment 4)₃ΔV Ire1 did not differ from that of the other Ire1 mutants (Fig. S2C).

Subregion I is replaceable by the NUCRs of fungal Ire1 and mammalian PERK

We then asked if the NUCRs of the fungal Ire1 ortholog and mammalian PERK, which are predicted to be intrinsically disordered (Fig. S3B), also have the Subregion I-like Ire1-suppressing ability. Thus, we constructed yeast ΔV Ire1 chimeric mutants in which the Subregion-I 60-a.a. portion was replaced with similar-length (52 a.a.) NUCR sequences derived from mammalian PERK or Aspergillus oryzae Ire1 (refer to Table S1C for the amino-acid sequences). According to the UPRE-lacZ reporter assay results shown in Fig. 4A, the chimeric constructs were less active than ΔV Ire1, which carried the authentic Subregion I, in unstressed cells, whereas they responded well to ER stress (Fig. 4B). These chimeric mutations did not considerably affect cellular abundance of ΔV Ire1 (Fig. S2C,D). This observation strongly suggests a potent function of the NUCRs to suppress Ire1 activity.

Next, the 52-a.a.-long NUCR sequences were partitioned into two half-length segments, which were substituted for the Subregion-I 60-a.a. portion of ΔV Ire1 (Fig. 4C and Table S1C). We then observed that in unstressed cells, the posterior segments commonly suppressed Ire1 function stronger than the anterior segments did (Fig. 4D, compare column 3 to 2, 5 to 4 and 7 to 6). Notably, the posterior 30-a.a.-long segment had an Ire1-suppressing ability stronger than that of the anterior 30-a.a.-long segment also in the case of authentic yeast Ire1 Subregion I (Fig. 3B, compare column 3 to 4). Thus, we think that the NUCRs have a common structural feature that cannot be predicted from a simple sequence comparison.

We then asked if mammalian PERK is hyperactivated by the deletion mutation of its NUCR. In the experiment shown in Fig. 5, murine PERK-expression plasmids were
transfected into a murine cultured cell line NIH3T3. The pcDNAmPERK-Myc (Harding et al., 1999) was used for overexpression of Myc epitope-tagged murine PERK (PERK-Myc) under control of the strong CMV promoter. We also employed a truncated version of this plasmid for expression of a mutant form of PERK-Myc not carrying NUCR (the ΔNUCR mutation). We then monitored phosphorylation of eIF2α, which is the direct phosphorylation target of PERK. As shown in Fig. 5A,C, the phosphorylation level of eIF2α seemed to be equally induced by transfection of either the PERK-Myc plasmid or its ΔNUCR version (compare lanes 1-6 to 7-9). Probably because of the endogenous PERK protein, DTT treatment induced the eIF2α phosphorylation even in the case of the empty vector-transfected cells (Figs 5A,B,C, compare lanes 16-18 and 25-27 to 7-9). Importantly, as compared with the wild-type PERK-Myc plasmid-transfected or the empty vector-transfected cells, cells expressing the ΔNUCR mutant version of PERK-Myc exhibited a higher-level phosphorylation of eIF2α upon weak ER stress induced by 0.2 mM DTT (Figs 5A,C, compare lanes 13-15 to 10-12 and 16-18). A similar tendency, although less pronounced, was observed when cells were treated with 0.5 mM DTT (Figs 5B,C, compare lanes 22-24 to 19-21 and 25-27). The anti-Myc Western-blot analysis of cell lysates shown in Fig. 5D indicates that wild-type PERK-Myc and its ΔNUCR version were expressed at almost similar levels in this transfection experiment.

The Ire1-suppressing ability of Subregion I likely results from its intramolecular interaction with the CSSR.

Because no specific primary structure of Subregion I appeared to be absolutely required for the Ire1-suppressing ability, we hypothesized involvement of the CSSR, which captures substrate peptides rather nonspecifically (Gardner and Walter, 2011). Thus, we determined if Subregion I could be a CSSR substrate. In the experiment shown in Fig. 6A, the Gal4 DNA-binding domain was fused to the Subregion-I 60-a.a. portion and used as bait in a yeast two-hybrid assay (refer to Table S1D for the amino-acid sequence). To yield prey, the Gal4 activation domain was fused to a CSSR peptide or that carrying the ΔIII or M229A/F285A/Y301A (MFY) mutation, which impairs the ability of the CSSR to capture substrate peptides (refer to Table S1E; Kimata et al., 2007; Gardner and Walter, 2011; Promlek et al., 2011). Because the tester
cells carry the AURI-C gene controlled under a Gal4-inducible promoter, they acquire resistance to aureobasidin A when the two-hybrid system works. As a result, Subregion I and the CSSR exhibited a weak two-hybrid interaction, which, as expected, was impaired by the CSSR mutations.

As demonstrated in Fig. 6B, the CSSR also captured various peptides that appeared in the experiments shown in Figs 3, 4 (refer to Table S1D for the amino-acid sequences). In the two-hybrid analysis shown in Fig. 6B, the length of the bait peptides was uniform (approximately 60 a.a.-long) by tandem repeats. Considering colony appearance and size on the aureobasidin-A plate, the two-hybrid interaction of the GGGSS repeat, the Top2-IDR and the Segment 1–3 peptides to the CSSR was weaker than that of the other peptides. This observation is correlated well with our aforementioned finding that the Ire1-suppressing ability of the Top2-IDR, the (GGGSS)$_6$, and the Segment 1–3 (namely Δ4–6) peptides was weaker than that of the San1-IDR, the (Segment 4)$_3$, the Segment 4–6 (namely Δ1–3), the mPERK NUCR and the hPERK peptides (Figs 3, 4).

Next, we performed an *in vitro* competition assay to monitor the affinity of the Subregion-I segments to the CSSR. A recombinant CSSR protein tagged with the maltose-binding protein (MBP-CSSR) was expressed in *Escherichia coli* and purified with amylose resin (Kimata et al., 2007). As originally described by Gardner and Walter (2011), a fluorescently labeled CSSR substrate, ΔEspP-FAM, exhibited increased fluorescence anisotropy when it was mixed with MBP-CSSR (Fig. S4). Then, fluorescence anisotropy of ΔEspP-FAM was measured in the presence of MBP-CSSR and a chemically synthesized peptide carrying one of the Subregion I-segment sequences (refer to Table S1F for the amino-acid sequences), indicating that Segment 4 compromises fluorescence anisotropy, namely the association between ΔEspP-FAM and MBP-CSSR, more effectively than the other Segments did (Fig. 6C). This finding strongly suggests high-affinity capture of Segment 4 by the CSSR.

In the experiment shown in Fig. 7A, we used the yeast two-hybrid assay to monitor self-association of the CSSR (refer to Table S1D,E). A two-hybrid interaction was observed when the CSSR was employed both as bait and prey. However, adding Subregion I to the bait CSSR (Subregion I-CSSR) abolished this two-hybrid interaction,
which was restored by the ΔIII or the MFY mutation of Subregion I-CSSR. This finding strongly suggests that Subregion I leads to dissociation of the self-associated CSSR molecules when intramolecularly captured by the CSSR as a substrate.
Discussion

Growth of yeast cells is severely retarded by deregulated expression of the spliced form of HAC1 mRNA (Mori et al., 2000). Moreover, according to Chawla et al. (2011) and Rubio et al. (2011), cells are damaged when Ire1 activity fails to be attenuated during long-term ER stress. Based on activation profiles of wild-type Ire1 and ΔI Ire1 (Figs 1D, 2A), it is likely that Subregion I contributes to suppression of Ire1 activity under unstressed conditions and long-term ER stress. Although the difference in activity between wild-type Ire1 and ΔI Ire1 was not drastic, we think that Subregion I is beneficial for long-term proliferation of cells over many generations. Indeed, wild-type IRE1 cells grew more predominantly than ΔI-IRE1 cells in mixed cultures (Table 1). As shown in Figs. 5A,C, it is likely that the deletion of the NUCR also causes hyperactivation of murine PERK especially under the weak ER stress condition.

Activation of Ire1 by the ΔI mutation was more obvious when it was combined with the ΔV mutation (Fig. 1B; Oikawa et al., 2007). Thus, we think that Subregion I and Subregion V suppress Ire1 activity in complementary fashions. Unlike the ΔV mutation, the ΔI mutation does not compromise the interaction between Ire1 and BiP (Oikawa et al., 2007). We thus think that Subregion I functions via a different mechanism in which BiP is not involved, whereas Subregion V serves as the BiP-binding site (Kimata et al., 2004).

The CSSR is likely to capture Subregion I, which is intrinsically disordered, as an unfolded protein substrate, because their two-hybrid interaction was abolished by the ΔIII or the MFY mutation (Fig. 6A). Our findings shown in Figs 3, 4 indicate that various intrinsically disordered peptides exhibited potent or weak Ire1-suppressing ability when substituted for Subregion I. Peptides showing potent Ire1-suppressing ability commonly exhibited two-hybrid interactions with the CSSR stronger than those of peptides only weakly suppressing Ire1 (Fig. 6B). According to the in vitro competition analysis shown in Fig. 6C, the CSSR captured Segment 4, which is relatively important for the Ire1-suppressing ability of Subregion I (Fig. 3B), more preferentially than other segments. These findings strongly suggest an intramolecular interaction between the CSSR and Subregion I (or peptides substituted on Subregion I).
that suppresses Ire1 activity in unstressed cells. This interaction is likely to compromise
self-association of the CSSR (Fig. 7A).

As shown in Fig. 7B, we thus propose a role of Subregion I as an intramolecular
antagonist of the CSSR. We speculate that under unstressed conditions, Subregion I is
captured by the CSSR as an unfolded-protein substrate and covers the dimerization
interface of the CSSR, homo-association of which is then inhibited. It may be also
possible that the dimeric form of the CSSR is dissociated through its intramolecular
interaction to the CSSR. According to our model presented here, Subregion I and
unfolded proteins compete for association with the CSSR. Therefore, instead of the
intramolecular interaction of the CSSR to Subregion I, unfolded proteins are
intermolecularly captured by the CSSR, which then forms the high-order oligomer
Gardner and Walter, 2011), under ER-stress conditions (Fig. 7C). This mechanism is
highly unique, as a receptor protein is carrying an antagonizing sequence.

The BiP-Ire1 association is also likely to contribute to maintaining Ire1 as a
non-self-associated form (Bertolotti et al., 2000). Therefore, we think that Subregions I
and V function as “double locks” that compromise self-association of Ire1 under
unstressed conditions. In the absence of this “double lock”, Ire1 remains a dimer, as
\( \Delta \text{I} \Delta \text{V} \) Ire1 was self-associated constitutively but clustered in response to ER stress (Figs
1E,F; Oikawa et al., 2007). According to Gardner and Walter (2011), the intermolecular
interaction between unfolded proteins and dimeric CSSR molecules leads to formation
of the Ire1 cluster.

In the present paper, we propose that the NUCR portions of the Ire1 family
proteins, which commonly show high disorder probability values in the PrDOS analysis
(Fig. S3), are captured by the CSSR as disordered peptides to suppress Ire1 activity. On
the other hand, it also should be noted that the disorder probability values of a peptide
does not always correlate to its ability to be captured by the CSSR and to suppress Ire1
activity. For instance, the disorder probability of Segment 4 is not particularly higher
than those of the other segments (Fig. S3A). According to our data shown in Fig. 4D,
the posterior-half segments of the NUCR portions of the Ire1 family proteins commonly
suppressed Ire1 function stronger than the anterior-half segments did. In agreement with
this observation, the posterior-half segments of the NUCR portions of mouse and
human PERK show the disorder probability values which are higher than those of the
anterior-half segments, while, *A. oryzae* Ire1, however, does not show such a tendency (Fig. S3B).

PERK, but not mammalian Ire1 orthologs (hereafter called IRE1), carries the NUCR (Fig. S1B), which exhibited Ire1- or PERK-suppressing ability (Fig. 4, 5). We thus speculate that in mammalian cells, PERK may be inactivated more tightly than IRE1 under no or weak ER-stress conditions. Since PERK inhibits global protein synthesis, it sounds reasonable that there exist a molecular machinery which suppress PERK activity in healthy cells. On the other hand, IRE1 works under various physiological situations of development and homeostasis maintenance in mammals without external stress stimuli (Iwawaki et al., 2009; Iwawaki et al., 2010; Tsuru et al., 2013). As internal physiological stress stimuli sensed by IRE1 may not be potent, we speculate that too-tight suppression of IRE1 may be unfavorable for mammals. Indeed, according to Ma et al. (2010), IRE1 but not PERK is activated upon differentiation of mature B cells to plasma cells, which secrete a large amount of antibody. We think that this idea may explain why the higher eukaryotic IRE1 does not carry the NUCR.
Materials and Methods

Yeast cultures and strains:

Unless otherwise noted, yeast cells were exponentially grown at 30°C under liquid-shaking culture in synthetic dextrose medium (2% glucose, 0.66% Difco yeast nitrogen base without amino acids, appropriate auxotrophic requirements). See Mori et al. (1996), Kimata et al. (2004), and Promlek et al. (2011) for congenic haploid strains KMY1015 (MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ 901 lys2-801 ire1Δ::TRP1) and KMY1516 (MATα LEU2::UPER-GFP::leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 LYS2::(UPRE)-lacZ::lys2-801 ire1Δ::TRP1). The MATα haploid strain Y2HGGold and the MATα haploid strain Y187 were obtained from a commercial yeast two-hybrid system kit (Matchmaker Gold; Clontech).

Plasmids:

The yeast 2µ plasmid pCZY1 carries a UPRE-lacZ reporter construct (Mori et al., 1992). The yeast IRE1 gene carrying the authentic 5‘- and 3‘-untranslated regions was previously cloned into the yeast single-copy vector pRS313 (Sikorski and Hieter, 1989) to obtain plasmid pRS313-IRE1 (Kimata et al., 2004). Partial deletion mutations were introduced into the IRE1 gene on pRS313-IRE1, as described by Kimata et al. (2004), using the overlap PCR and in vivo homologous recombination techniques. We inserted the SpeI-restriction sequence (ACTAGT) and the MluI-restriction sequence (ACGCGT), respectively, after nucleotide position 93 and before nucleotide position 274 on the IRE1 gene to introduce Subregion-I substitution mutations into pRS313-IRE1, and then, DNA fragments corresponding to the peptides shown in Figs 3, 4 were placed in-frame between these two restriction sites. The San1 IDR (a.a. 339–394) and Top2IRD (a.a. 634–659) were modified to carry the SpeI-restriction or MluI-restriction sequence (2-a.a.-long each) on each end using the PCR technique. The NUCR fragments correspond to a.a. 29–80 of mouse PERK, a.a. 31–82 of human PERK, and a.a. 28–79 of Aspergillus oryzae Ire1 and also carry the artificially attached SpeI-restriction or MluI-restriction sequence on each end. See Table S1C for amino-acid sequences of the Subregion-I substitution mutants.
We used the pGBKT7 bait vector and the pGADT7 prey vector, both of which were obtained from the Matchmaker Gold kit (Clontech), for the two-hybrid analysis. The \textit{IRE1}-gene fragment corresponding to the CSSR (nucleotide positions 334–1362) or its mutants were cloned into the \textit{BamHI}/\textit{XhoI} sites of pGADT7. Gene fragments of \textit{IRE1} and its mutants were cloned into the \textit{Ncol}/\textit{BamHI} sites of pGBKT7 for the experiment shown in Fig. 7A. DNA fragments corresponding to the bait peptides were cloned into the \textit{EcoRI}/\textit{BamHI} sites of pGBKT7 for the experiments shown in Figs 6A,B. See Table S1D,E for amino-acid sequences of the bait and the prey peptides.

For overexpression of PERK-Myc, we used a mammalian expression plasmid pcDNA mPERK-Myc (Harding et al., 1999). As the empty-vector control, pCDNA3.1(+) (Life Technologies) was employed. In order to generate the \textit{\DeltaNUCR} mutant (deletion of a.a. 29-99) version of pcDNA mPERK-Myc, we performed PCR amplification of this plasmid with the PCR primer set

\begin{align*}
5'-TCCTTGGTATCATCAGCACTTTAGAGG-3' \quad \text{and} \\
5'-CGCAGAGATCCCGCGCCAGCCCAGCAG-3',
\end{align*}

the product of which was phosphorylated by T4 polynucleotide kinase and self-ligated.

Genotypic analysis of yeast cells in mixed culture:

A mixed culture of wild-type-\textit{IRE1} cells (KMY1516 transformed with pRS313-\textit{IRE1}) and \textit{\DeltaI-IRE1} cells (KMY1516 transformed with the \textit{\DeltaI}-mutant version of pRS313-\textit{IRE1}) was diluted and plated on agar plates to obtain isolated colonies. One hundred colonies were then checked for their \textit{IRE1} genotypes by PCR with the primer set

\begin{align*}
5'-CCATTATCAGTGCTTCCATCA-3' \quad \text{and} \\
5'-GCAATTCTAAATCTAAATGC-3'.
\end{align*}

The “relative abundance of \textit{\DeltaI-IRE1} cells (%)” was calculated from the formula $100 \times \frac{\text{[the number of \textit{\DeltaI-IRE1} colonies]}}{\text{[the number of \textit{\DeltaI-IRE1} colonies] + [the number of wild-type-\textit{IRE1} colonies]}}$.

The same method was used to obtain the “relative abundance of \textit{\DeltaAV-IRE1} cells (%)” from mixed cultures of wild-type-\textit{IRE1} cells and \textit{\DeltaAV-IRE1} cells. We used a PCR primer set

\begin{align*}
5'-CAAGCGATTTAGATTTAGAATTG-3' \quad \text{and} \\
5'-AATACTCCAGTCTCTATATAATTGAATTTG-3',
\end{align*}

to check the \textit{\DeltaAV}-mutant genotype to obtain
the “relative abundance of ΔV-IRE1 cells (%)” from mixed cultures of wild-type-IRE1 cells and ΔV-IRE1 cells.

RNA analysis:

After extracted from cells as described by Kimata et al. (2003), total RNA samples were used as templates for reverse transcription (RT)-PCR amplification of the HAC1-mRNA species, which was then fractionated by electrophoresis (Promlek et al., 2011; Ishiwata-Kimata et al., 2013). DNA fluorescence images of the resulting gels were captured and quantitatively analyzed using the LAS-4000 cooled CCD camera system. The resulting data were used to calculate the “HAC1-mRNA splicing efficiency (%)
from the formula 100 × \[\text{HAC1}^i \text{ band signal}] / \[(\text{HAC1}^i \text{ band signal}) + (\text{HAC1}^u \text{ band signal})\].

Fluorescent-protein tagging and localization of Ire1

An mNeonGreen-coding sequence (Shaner et al., 2013) with a codon usage optimized for yeast was inserted into the Ire1-coding region of pRS313-IRE1 and its ΔIAV mutant. See Aragon et al. (2009) for the insertion position on the IRE1 gene. After transformation of the KMY1015 strain with the resulting plasmid, mNeonGreen fluorescent images of cells were captured using the Delta Vision Elite microscopy system (Applied Precision) with the GFP excitation/emission filter set.

UPRE-lacZ reporter assay:

The KMY1015 strain carrying pCZY1 or the KMY1516 strain were transformed with IRE1-gene plasmids and checked for cellular β-galactosidase, as described previously (Kimata et al., 2003). Data from multiple (>3) independent transformant clones were used to calculate the means and standard deviations.

Yeast two-hybrid assay:

Y2HGold cells transformed with a bait plasmid and Y871 cells transformed with a prey plasmid were mated through mixed culturing, as described in the manufacturer’s instructions (Clontech). The resulting cultures were serially 10-fold diluted and spotted...
onto agar plates, incubated for 2–4 days at 30°C, and photographed. The agar plates were synthetic dextrose supplemented with the –Leu–Trp Dropout supplement (Yeast Protocol Handbook, Clontech) for selecting mated cells (the growth control plates) and those containing 125 ng/ml aureobasidin A for checking the two-hybrid interaction. In the experiment shown in Fig. 7A, agar plates for checking the two-hybrid interaction did not contain histidine, because the two-hybrid interaction provides the tester diploid cells with histidine prototrophy as well as with aureobasidin-A resistance.

In vitro competition assay for peptide binding to the CSSR

The His-tagged MBP-CSSR protein was expressed in E. coli and purified using a nickel affinity column and elution buffer A [50 mM Hepes (pH 8.0), 100 mM KCl, 5 mM MgCl₂, 200 mM imidazole, and 10% (v/v) glycerol], as previously reported (Kimata et al., 2007). A 5-carboxyfluorescein (5-FAM)-tagged peptide (ΔEspP-FAM; Gardner and Walter, 2011) was chemically synthesized by GL Biochem (Table S1F; Shanghai, China). The untagged competitor peptides NH₂-KKKA-[10 a.a. of Segment 2, 3, 4, 5, or 6]-AAKKKK-COOH were chemically synthesized by Sigma-Aldrich (Table S1F; the peptide screening value pack). The lysine clusters on the competitor peptides were expected to serve as solubilization tags. Segment 1 was not used in this assay because of its low chemical synthesis yield. After incubating the mixture in elution buffer A for 30 min, fluorescence anisotropy was measured using the BEACON™2000 fluorescence polarization reader (Invitrogen, USA) at 25°C.

Mammalian cell culturing and manipulation

NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (37 °C, 10% CO₂). Plasmids were transfected into NIH3T3 cells by Lipofectamine LTX with PLUS Reagent (Invitrogen) according to manufacturer protocol. Standard RIPA buffer was used for preparation of cell lysates.

Antibodies

For Western blot analysis, we used 12CA5 anti-HA mouse monoclonal antibody (Roche), 9E10 anti-c-Myc mouse monoclonal antibody (Boehringer Mannheim) and anti-eIF2α[pS52] rabbit polyclonal antibody (BioSourse).
Acknowledgement

We thank Dr. Kazutoshi Mori (Kyoto University) for materials. This work is supported by MEXT/JSPS KAKENHI grants 22657030 and 24370081 to Y.K. and 20380062 and 24228002 to K.K.
References


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573
575 A 22 bp cis-acting element is necessary and sufficient for the induction of the yeast KAR2
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Figure legends

Figure 1 Yeast Ire1 mutants carrying full-length or almost full-length deletions of Subregion I and/or Subregion V

A, The Ire1 mutations used in the experiments shown in panels B–D are illustrated. The ΔI, ΔI\text{full}, and ΔV mutations correspond to deletion of a.a. 32–91, a.a. 32–111, and a.a. 463–524 of yeast Ire1, respectively. The N-terminal ER translocation signal is purple-colored. B and C, After transformation with a single-copy IRE1 plasmid (pRS313-IRE1; wild-type (WT)) or its mutants (or the empty vector pRS313 (ire1Δ)), KMY1015 ire1Δ cells carrying the UPRE-lacZ reporter plasmid (pCZY1) remained unstressed (B) or were stressed with 2 µg/ml tunicamycin for 4 h (C), and cellular β-galactosidase activity was measured and normalized to that of the unstressed ΔV-IRE1 samples (set to 1.0). D, A similar experiment to that shown in panel B was performed using ire1Δ KMY1516 ((UPRE)\text{5}-lacZ) cells. Data are normalized to that of the wild-type (WT) IRE1 samples (set to 1.0). E and F, Yeast cells producing mNeonGreen-tagged Ire1 (wild-type (WT) Ire1-mNeonGreen) or its ΔIΔV mutant were subjected to fluorescence-microscopic imaging.

Figure 2 Time-course profile of wild-type, ΔI, ΔV and ΔIΔV Ire1 activity

After transformation with a single-copy IRE1 plasmid (pRS313-IRE1; wild-type (WT)) or its mutants, KMY1516 ire1Δ cells were stressed with 3 mM DTT for the indicated times. HAC1-mRNAs were then amplified by RT-PCR from total RNA samples to obtain the HAC1-mRNA splicing efficiency values, which are expressed as means plus standard deviations from three independent transformant clones.

Figure 3 Partial deletion and substitution mutations of Subregion I

A, The Subregion-I 60-a.a. portion (a.a. 32–91) was partitioned into six 10-a.a.-long segments (Segments 1–6), which were serially deleted from ΔV Ire1 as illustrated. B and C, A similar experiment and data presentation as shown in Figs 1B,C were performed using the Ire1 mutants shown in panel A. D and E, A similar experiment and data presentation as shown in Figs 1B,C were performed using the ΔV Ire1 variants on which the Subregion-I 60-a.a. portion was replaced with San1 IDR, Top2 IDR, a six tandem repeat of the 5-a.a. peptide GGGSS or the three tandem repeat of Segment 4.
**Figure 4** Replacement of yeast Ire1 Subregion I with the NUCRs of other IRE1-family proteins

**A and B,** A similar experiment and data presentation as shown in Figs 1B,C were performed using the ΔV Ire1 variants on which the Subregion-I 60-a.a. portion was chimerically replaced with the NUCRs of mouse PERK (mPERK), human PERK (hPERK), or *Aspergillus oryzae* Ire1 (Alre1). **C,** As illustrated, the NUCRs of the other IRE1-family proteins (orange colored) were two-partitioned (anterior and posterior) and substituted on the Subregion-I 60-a.a. portion of ΔV Ire1. **D,** A similar experiment as shown in Fig. 1D was performed using the Ire1 mutants shown in panel C. Data are normalized to that of the ΔV-IRE1 samples (set to 1.0).

**Figure 5** Hyperactivation of murine PERK by its NUCR deletion mutation

**A, B and D,** After transfection of pcDNAmPERK-Myc (wild-type PERK-Myc overexpression), its ΔNUCR mutant or the empty vector pCDNA3.1(+), NIH3T3 cells were cultured for 24 h and stressed as indicated. Cell lysates were then analyzed by anti-phosphor-eIF2α (A,B) or anti-Myc (D) Western-blot analysis. Three independent samples prepared under identical condition were analyzed. **C,** Band densities of panels A and B were measured and are expressed.

**Figure 6** Two-hybrid and *in vitro* interaction of the CSSR with various peptides

**A and B,** A yeast two-hybrid analysis was performed using the CSSR and its mutants as prey. The Subregion-I 60-a.a. portion (Sub-I), Segment 4 (Seg4; six tandem repeat), GGGSS (12 tandem repeat), Segments 1–3 (Segs1–3; a.a. 32–61 of yeast Ire1; two tandem repeat), Segments 4–6 (Segs4–6; a.a. 62–91 of yeast Ire1; two tandem repeat), the NUCR of human or mouse PERK (hPERK or mPERK), the Top2 IDR (Top2; two tandem repeat) and the San1 IDR (San1) were used as bait. Tester cell cultures were serially diluted 10-fold, spotted on agar plates, incubated for 2–4 days, and photographed. **C,** A fluorescently labeled peptide DEspP-FAM (10 µM final) was mixed with MBP-CSSR (5 µM final) and a competitor peptide (Segment (Seg) 2–6) and measured for fluorescence anisotropy. The resulting values from triplicate assays (means plus standard deviations) are normalized against that for the no-competitor peptide.
Figure 7 Subregion I inhibits self-association of the CSSR

A, A yeast two-hybrid analysis was performed using the indicated peptide sequences as prey and bait. Subregion I-CSSR (Sub-I-CSSR) is a yeast Ire1 fragment carrying Subregion I and the CSSR (a.a. 33–454). **B and C**, Our proposing model for the function of Subregion I in Ire1 regulation is schematically presented. Under nonstress conditions, the groove-like structure (purple colored) of the CSSR (blue colored) captures Subregion I (orange colored), keeping the CSSR molecules monomeric (B). The CSSR forms a high-order oligomer when unfolded proteins are intermolecularly captured by the groove-like structure instead of Subregion I (C).
Table 1 Growth retardation of unstressed yeast cells due to the ΔI, the ΔV or the ΔIΔV mutation

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<th>Days of continuous culturing</th>
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<th>Relative abundance of ΔV-IRE1 cells</th>
<th>Relative abundance of ΔIΔV-IRE1 cells</th>
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The IRE1 plasmid (pRS313-IRE1; wild-type (WT)) and its mutants (ΔI, ΔV or ΔIΔV) were used to transform KMY1516 ire1Δ cells. The resulting WT-IRE1 and ΔI-IRE1 cells (2 × 10^5 cells each) were mix inoculated into 1 ml medium for a 1-day incubation, which was followed by continuous cycles of 1/32 dilutions and 1-day incubations of the culture. “Relative abundance of ΔI-IRE1 cells (or ΔV-IRE1 or ΔIΔV-IRE1 cells)” was determined as described in Materials and Methods, and the values are presented as means plus standard deviations from three independent cultures in which independent transformant clones were inoculated.
Figure 1

A. Schematic representation of NUCR and ER translocation.

B. Graph showing UPRE-lacZ reporter values under Nonstress conditions.

C. Graph showing UPRE-lacZ reporter values under Tunicamycin treatment.

D. Graph showing UPRE-Ire1-lacZ reporter values under Nonstress conditions.

E. Images of WT Ire1-mNeonGreen under Nonstress and DTT conditions.

F. Images of ΔΔV Ire1-mNeonGreen under Nonstress and DTT conditions.
Figure 2

A

HAC1-mRNA splicing efficiency (%)

Time (hr)

WT

ΔI

B

HAC1-mRNA splicing efficiency (%)

Time (hr)

WT

ΔI

ΔV

ΔIΔV
Figure 4

**A**
Nonstress

Chimeric substitution on the Subregion-I 60 a.a. portion of ΔV Ire1

**B**
Tunicamycin

Chimeric substitution on the Subregion-I 60 a.a. portion of ΔV Ire1

**C**

ΔV Ire1

NUCR (Subregion I)

ER translocation sig.

N-term

CSSR

ΔV

60 a.a.

 anterior 26 a.a.

posterior 26 a.a.

NUCR of other Ire1 family proteins

**D**
Nonstress

Chimeric substitution on the Subregion-I 60 a.a. portion of ΔV Ire1
Figure 5

A

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Figure 6

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C

Fluorescence anisotropy (normalized)

Conc. of competitors (µM)

- Seg 2
- Seg 3
- Seg 4
- Seg 5
- Seg 6
Figure 7

A

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B

Nonstress conditions

C

ER-stressed conditions