Self-association and BiP dissociation are not sufficient for activation of the ER stress sensor Ire1

Daisuke Oikawa, Yukio Kimata* and Kenji Kohno*

Laboratory of Molecular and Cell Genetics, Graduate School of Biological Sciences, Nara Institute of Science and Technology (NAIST), 8916-5 Takayama, Ikoma, Nara 630-0192, Japan

*Authors for correspondence (e-mail: kimata@zero.ad.jp; kkouno@bs.naist.jp)

Accepted 8 March 2007
Journal of Cell Science 120, 1681-1688 Published by The Company of Biologists 2007
doi:10.1242/jcs.002808

Summary

Ire1 is a type I transmembrane protein located on the endoplasmic reticulum (ER). Upon ER stress, Ire1 releases the ER chaperone BiP and self-associates. This activates Ire1 and triggers the unfolded protein response in the yeast Saccharomyces cerevisiae. We isolated and characterized an Ire1 luminal domain mutant lacking both the N-terminal and the juxtamembrane loosely folded subregions. Although this ‘core’ mutant was able to self-associate and failed to bind BiP even under nonstressed conditions, its activation was still dependent on ER stress. Furthermore, although substitution of Pro for Ser103 (S103P) in the Ire1 luminal domain of full-length Ire1 caused neither BiP dissociation nor a change in self-association, the substitution in combination with the core mutation resulted in constitutive activation. This phenotype of the S103P mutation required a cluster of positively charged amino acid residues (Arg or Lys) located close to the mutation site in the Ire1 sequence. These observations indicate that in addition to BiP dissociation and self-association of Ire1, another unknown change on the luminal side is crucial for Ire1 activation.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/120/9/1681/DC1

Key words: Molecular chaperone, Misfolded proteins, Stress response, Endoplasmic reticulum, Unfolded protein response

Introduction

The endoplasmic reticulum (ER) is a membranous organelle where folding, disulfide bond formation, subunit assembly and glycosylation of secretory and membrane proteins are facilitated. A variety of conditions, collectively called ER stress, inhibit these events and lead to accumulation of unfolded proteins in the ER. To alleviate ER stress, eukaryotic cells activate a cytoprotective signaling cascade designated the unfolded protein response (UPR).

The UPR signaling pathway is best understood in Saccharomyces cerevisiae. Ire1, an ER-resident type I transmembrane protein, transmits an ER-stress signal to the cytosol (Cox et al., 1993; Mori et al., 1993). The cytosolic domain of Ire1 possesses kinase and RNase activities. ER stress causes autophosphorylation of Ire1, which leads to conformational change and activation as an RNase (Shamu and Walter, 1996; Papa et al., 2003). The target of this RNase is a precursor form of HAC1 mRNA (HAC1 u) (Cox and Walter, 1996). Ire1 α is involved in splicing the pre-mRNA of the XBP1 transcription factor (Yoshida et al., 2001; Calfon et al., 2002). Ire1 β has been shown to cleave ribosomal RNA (Iwawaki et al., 2001). PERK is an ER-resident type I transmembrane protein that carries a kinase domain. Upon ER stress, PERK phosphorylates eukaryotic translation initiation factor 2 (Harding et al., 1999). The attenuation of protein synthesis by PERK or Ire1 β is thought to reduce the protein loading into the ER. ATF6 is also recognized as an ER stress sensor (Yoshida et al., 1998), but it has no structural similarity to Ire1 or PERK.

Because Ire1 and PERK have similar luminal domains, the mechanism by which these two proteins sense ER stress is likely to be similar. The structure of the yeast Ire1 luminal domain has been elucidated (Kimata et al., 2004; Oikawa et al., 2005; Credle et al., 2005). Analysis of a 10-amino-acid-long deletion-scanning mutagenesis of Ire1 predicted that its luminal domain is composed of five subregions, among which subregions II and IV are crucial for activity (Kimata et al., 2004) (see Fig. 1A, WT). Ire1 orthologs of higher eukaryotes do not carry sequences corresponding to subregion I, and the functions of this subregion are unclear. High accessibility to limited proteolysis suggests that subregions I and V are loosely folded (Oikawa et al., 2005). The luminal domain excluding subregion I and V was therefore designated the core stress-sensing region. The crystal structure of this region reported by Credle et al. (Credle et al., 2005) indicates that it forms one tightly folded domain.

What events occur in the Ire1 luminal domain to activate Ire1
upon ER stress? BiP binds to Ire1 and dissociates in response to ER stress (Bertolotti et al., 2000; Okamura et al., 2000). Cells carrying certain mutant alleles of the BiP gene, including kar2-113, exhibit attenuated Ire1 activity together with impaired dissociation of the mutant BiP protein from Ire1 (Kimata et al., 2003). Abolishment of BiP binding by deletion of subregion V (designated ΔV, see Results for details of the deletion position) strongly suggests that the BiP-binding site is located in this subregion (Kimata et al., 2004). Although the ΔV mutant version of Ire1 was not constitutively active, it is activated in response to ER stress and the following observations indicate that BiP binding to subregion V is a biologically meaningful event that negatively regulates Ire1 (Kimata et al., 2004). First, depression of Ire1 activity by the kar2-113 mutation was rescued by the ΔV mutation. Second, high temperature and ethanol, neither of which is considered as ER stress, failed to activate the wild type but did activate the ΔV Ire1 mutant. Taken together, we conclude that BiP dissociation is required but not sufficient for the activation of Ire1. It is likely that binding and dissociation of BiP contributes to the precise regulation of Ire1.

Moreover, ER stress facilitates self-association of Ire1. According to density gradient fractionation analysis of mammalian cell lysates (Bertolotti et al., 2000), Ire1 dimerizes upon ER stress. Recombinant Ire1 fragments with a full-length luminal domain or core stress-sensing region exist as dimers in solution (Liu et al., 2002; Oikawa et al., 2005). In vivo self-association of native Ire1 protein probably depends on this dimer-forming ability of the core stress-sensing region, because mutations that abolished the latter also impaired the former (Oikawa et al., 2005; Zhou et al., 2006). Ire1 auto-phosphorylation that occurs upon ER stress takes place in trans, i.e. one Ire1 molecule phosphorylates another in a complex (Shamu and Walter, 1996).

In this report, we show that a yeast Ire1 mutant lacking most of subregions I and V (the ‘core’ mutant) was still regulated by ER stress, in spite of constitutive self-association and loss of BiP-binding ability. A luminal-domain point mutation of Ire1, which did not facilitate BiP dissociation or self-association, rendered the core mutant constitutively active. We conclude that the Ire1 luminal domain is involved in an unknown activation step in addition to playing roles in BiP dissociation and self-association.

Results

Constitutive activation of Ire1 by the core and S103P double mutations

Some of the Ire1 mutants used in this study are illustrated in Fig. 1A. Deletion of amino acids 32-91, consisting of nearly all subregion I, was designated ΔI. Deletion of amino acids 463-523, consisting of nearly all subregion V, was designated ΔV. The positions of amino acid residues were renumbered according to data from the Saccharomyces Genome Database (SGD; http://www.yeastgenome.org/), in which the initiation ATG is assigned 21 bases upstream from that assigned in GenBank data accession number AAB68894. Because we employed the latter data in our previous reports (Kimata et al., 2004; Oikawa et al., 2005), amino acid numbering differs from that in the present report by seven amino acids.

Because we designated the tightly folded region formed by the Ire1 luminal domain excluding subregions I and V the core stress-sensing region, we called the ΔI ΔV double mutant the core mutant. These alleles were expressed as C-terminal hemagglutinin (HA)-tagged versions from low copy (centromeric) plasmids under the control of the native promoter in a Δire1 strain, unless noted otherwise. As shown in Fig. 1B, none of the mutations significantly changed Ire1 expression levels. After treatment with or without the ER stressor tunicamycin, cells were assayed for UPR-LacZ reporter activity (Fig. 2A,B). Although no activity was detected in cells carrying an empty vector control, the reporter was clearly activated by tunicamycin treatment in the wild-type Ire1 cells. The ΔI and the ΔV single mutants exhibited almost the same result as wild-type Ire1 cells. In the case of the core mutant carrying both the ΔI and the ΔV mutations, reporter activity was slightly higher under nonstressed conditions and was upregulated by tunicamycin.

Although a constitutively active yeast Ire1 mutant carrying multiple amino acid substitutions in the luminal domain has been reported (Papa et al., 2003), it is unclear which point
mutation(s) is actually responsible for the constitutively active phenotype. Here, we introduced five of these point mutations into the core mutant. As shown in supplementary material Fig. S1, the resulting core 5m mutant cells exhibited significant activation of the DRE- lacZ reporter even without extrinsic ER stress. Reverse mutation analysis shown in supplementary material Fig. S1 indicated that the S103P mutation alone contributes significantly to this phenotype, because the S103P core mutant was activated to nearly the same extent as the core 5m mutant. Anti-HA western blot analysis of cell lysates showed that the expression level of the core mutant was not changed by the S103P mutation alone (see supplementary material Fig. S1). Therefore, we speculate that the doubly mutated Ire1 is almost fully activated even under nonstressed conditions.

Activation of Ire1 by the luminal-domain mutations does not obviate the need for phosphorylation

As described earlier, autophosphorylation of the cytosolic domain is required for Ire1 activation upon ER stress. Is the phosphorylation also required for Ire1 activation by the luminal domain mutations? The kinase domain mutation K702A is known to abolish phosphorylation and activation of Ire1 in response to ER stress (Shamu and Walter, 1996). Here we introduced the K702A mutation into the luminal domain mutants used in this study. The DRE-lacZ reporter assay shown in Fig. 2C indicates that the K702A mutation completely abolished activation of Ire1 both by tunicamycin treatment and the S103P core double mutations. The K702A mutation did not significantly reduce cellular expression of the Ire1 variants (data not shown). These results strongly suggest that kinase activity is required for Ire1 activation both by ER stress and by the luminal-domain mutations. In other words, activation of Ire1 by the luminal domain mutations does not abolish the requirement for phosphorylation of the cytosolic domain.
Bip binding and dissociation from mutated Ire1

The UPR-lacZ reporter and the HAC1<sup>a</sup> cleavage assays indicated that combining the S103P and the core mutations activated Ire1. As noted earlier, Bip dissociation from Ire1 is a prerequisite for Ire1 activation upon ER stress. Are Bip binding or dissociation from Ire1 altered by these mutations? In Fig. 4, cells expressing the wild-type or the mutant versions of Ire1-HA were lysed under non-denaturing conditions and subjected to anti-HA immunoprecipitation. In this experiment, and in that shown in Fig. 5A, C-terminal epitope-tagged versions of Ire1 were expressed on high copy (2-μm) plasmids, which alone, does not cause constitutive activation of the UPR signaling pathway (Okamura et al., 2000).

The uppermost and the third panels of Fig. 4, respectively, indicate adequate expression and anti-HA immunoprecipitation of Ire1-HA variants. The Ire1-HA variants not carrying the ΔI mutation (wild type, S103P, ΔV and ΔV S103P) run as double bands in SDS gels owing to partial degradation in subregion I. As shown in the second panel, the expression level of Bip protein was not altered significantly in this experiment. In the fourth panel, Bip bound to Ire1-HA was detected by anti-Bip western blot analysis of anti-HA immunoprecipitates. The Bip signal was not detected in the anti-HA immunoprecipitates prepared from empty vector control cells not expressing Ire1-HA (lanes 1 and 2). Wild-type, S103P, ΔI and ΔI S103P Ire1-HA showed similar levels of Bip binding (lanes 3, 5, 7 and 9), which was almost equally reduced by tunicamycin treatment (lanes 4, 6, 8 and 10). By contrast, consistent with our previous report (Kimata et al., 2004), Bip did not bind to the Ire1-HA variants lacking subregion V (ΔV, ΔV S103P, core, core S103P; lanes 11 to 18).

Core mutation results in constitutive self-association of Ire1

How is the self-association status of Ire1 affected by these luminal-domain mutations? In Fig. 5A, cells co-expressing Ire1-HA and Ire1-Flag were subjected to anti-HA immunoprecipitation, which was followed by anti-Flag western blotting to detect Ire1-Flag bound to Ire1-HA. To promote formation of an Ire1-HA/Ire1-Flag homo-complex, Ire1-HA and Ire1-Flag were expressed from low copy (centromeric) and high copy (2-μm) plasmids, respectively. Anti-HA and anti-Flag western blot analyses of cell lysates indicated adequate expression of both Ire1-HA and Ire1-Flag (Fig. 5A). Anti-HA immunoprecipitation was also adequate. A control experiment showed that Ire1-Flag is not detected in the anti-HA immunoprecipitate from cells not expressing Ire1-HA (supplementary material Fig. S3). This indicates that Ire1-Flag bound to Ire1-HA was actually detected as shown in Fig. 5A. To quantify self-association efficiency, the signal intensity of co-immunoprecipitated Ire1-Flag was corrected against that of immunoprecipitated Ire1-HA as described in the Fig. 5A legend.

Consistent with our previous report (Kimata et al., 2004), the level of Ire1 self-association was increased by tunicamycin treatment of wild-type Ire1 cells (lanes 3, 4, 13 and 14). When cells carried the ΔI or the ΔV single mutations, the level of self-association still increased upon ER stress, although it was modest but higher than that of wild-type Ire1 cells even under nonstressed conditions (lanes 5, 6, 7 and 8). By contrast, the core mutant exhibited significant self-association signals even under nonstressed conditions (lanes 9 and 17), and those signals were not enhanced by tunicamycin treatment (lanes 10 and 18). The S103P mutation did not change the degree of self-association in the wild type or in the core mutant Ire1 (lanes 15, 16, 19 and 20). Thus, it is likely that the core mutation causes constitutive self-association of Ire1, independently on the S103P mutation.

To further investigate the self-association status of Ire1, cell lysates were fractionated by density gradient centrifugation in the presence of the nonionic detergent Triton X-100 (Fig. 5B). The same cells used in Fig. 1B, Fig. 2A and Fig. 2B, which expressed Ire1-HA variants from low copy (centromeric) plasmids, were analyzed in this experiment. Ire1-HA in density gradient fractions was concentrated by anti-HA immunoprecipitation and detected by anti-HA western blotting. The core mutant was detected in fractions 4-10 when a lysate from nonstressed cells was analyzed (Fig. 5B, uppermost panel). As deduced from the peak positions of the
Early steps in Ire1 activation

1685

of these positively charged amino acid residues in the constitutively active phenotype of the S103P core mutant was tested. As shown in Fig. 6B, partial replacement of the Lys or Arg residues with Ser (RR1-SS, KK-SS, RR2-SS or RR1/2-SS) partially lowered the activity of the S103P core mutant, whereas complete replacement (RR1/2KK-SS) abolished the activating phenotype of S103P. When six Arg residues were clustered in this position (6R), the mutant exhibited the highest activity. The S103 residue was then replaced with six different amino acids other than Pro in the core mutant (Fig. 6C). Although substitution of Ala, Trp or Thr for S103 did not significantly change activity, substitution with Arg activated the core mutant, although more moderately than the S103P mutation. On the other hand, substitution with Asp reduced activity. These observations suggest that highly positive local charge at this position contributes to constitutive activation of the core mutant. All mutants tested in this experiment, including those that exhibited low activity, were highly active when they were incubated with tunicamycin. As for the mutants that exhibited high activity even in the absence of tunicamycin, the results from the UPRE-lacZ reporter assay were confirmed by checking cleavage of \( HAC1 \)u, which gave consistent results (Fig. 6D).

Discussion

To elucidate the early molecular steps in the activation of Ire1 upon ER stress, we analyzed the phenotypes of yeast Ire1 luminal-domain mutants. Consistent with our previous observation that the BiP-binding site is located in subregion V, BiP did not bind to the core mutant Ire1 (Fig. 4). Although the level of self-association of wild-type Ire1 clearly increased upon ER stress, the Ire1 core mutant exhibited significant constitutive self-association, which was not enhanced by ER stress (Fig. 5A). As for the \( \Delta I \) and \( \Delta V \) single mutants, the level of self-association was slightly higher than that of wild-type Ire1 under nonstressed conditions, and increased upon ER stress, similar to that of wild-type Ire1 (Fig. 5A). Considering that the core mutation is a combination of the \( \Delta I \) and the \( \Delta V \) mutations, it is likely that both subregions I and V repress self-association of Ire1 under nonstressed conditions. Because the only known role of subregion V is BiP binding, we now speculate that BiP binding represses self-association of Ire1.

molecular mass markers that were similarly fractionated, the Ire1 core mutant was fractionated as a monomer and dimer, whose peak fractions overlapped. Neither the tunicamycin treatment nor the S103P mutation (second and third panels) shifted this fractionation pattern significantly. We were unable to fractionate wild-type Ire1 clearly, as it appeared as a broad band in many of the fractions (data not shown).

Positively charged amino acid residues contribute to the activating phenotype of the S103P mutation

Fig. 6A shows the amino acid sequence around the S103P mutation site in Ire1, where a cluster of positively charged amino acid (Lys or Arg) residues exists. Potential involvement
In contrast to subregion V, it is unclear how subregion I contributes to regulation of Ire1 self-association. Because Ire1 orthologs of higher eukaryotes do not contain sequences corresponding to subregion I, their self-association may be regulated only by binding and dissociation of BiP.

To monitor BiP binding and self-association of Ire1, epitope-tagged Ire1 was expressed from high copy (2-μm) plasmids, which we believe does not lead to artifactual results, i.e. misfolding of Ire1 that can cause non-native BiP binding. This is because the binding of BiP that we observed is highly likely to be a biologically meaningful event (see Introduction) and because Ire1 expressed from 2-μm plasmids was observed to function as well as endogenous single copy Ire1 to control the UPR pathway in response to ER stress (Okamura et al., 2000). However, development of a method that does not require high copy expression of Ire1 would have the advantage of allowing direct determination of potential artifacts caused by Ire1 overexpression.

Consistent with a recent report on a similar Ire1 mutant (Credle et al., 2005), the core mutant was slightly more activated than wild-type Ire1 under nonstressed conditions, whereas ER stress activated both the wild type and the core mutant approximately equal levels (Figs 2 and 3). This observation indicates that both BiP dissociation and self-association are insufficient to activate Ire1. Thus, an additional unknown event is required for activation of Ire1 upon ER stress. We previously reported (Kimata et al., 2004) that an Ire1 mutant carrying both the S103P mutation and the 10-a.a. deletion in the core stress-sensing region completely lost activity, although it self-associated upon ER stress. Although this observation implied that activation requires an as yet unknown event, it was not possible to rule out the idea that the 10-a.a. deletion caused a global perturbation of the structure of the core stress-sensing region that artificially inactivated the mutant Ire1. The present study provides the first plausible evidence for an unknown additional requirement for Ire1 activation.

Substitution of Pro for S103, juxtapositioned to the core-stress sensing region, in the core mutant caused constitutive activation of Ire1 (Figs 2 and 3). Because the S103P mutation itself did not stimulate BiP dissociation or self-association of Ire1 (Fig. 4 and Fig. 5A), it is highly likely that this luminal domain point mutation eliminates the need for the unknown activating event. Significantly, this scenario further supports the possibility that the unknown activating event actually occurs on the luminal side and is required for activation of Ire1. The present study provides the first plausible evidence for an unknown additional requirement for Ire1 activation.

Substitution of Pro for S103, juxtapositioned to the core-stress sensing region, in the core mutant caused constitutive activation of Ire1 (Figs 2 and 3). Because the S103P mutation itself did not stimulate BiP dissociation or self-association of Ire1 (Fig. 4 and Fig. 5A), it is highly likely that this luminal domain point mutation eliminates the need for the unknown activating event. Significantly, this scenario further supports the possibility that the unknown activating event actually occurs on the luminal side and is required for activation of Ire1. The present study provides the first plausible evidence for an unknown additional requirement for Ire1 activation.

Substitution of Pro for S103, juxtapositioned to the core-stress sensing region, in the core mutant caused constitutive activation of Ire1 (Figs 2 and 3). Because the S103P mutation itself did not stimulate BiP dissociation or self-association of Ire1 (Fig. 4 and Fig. 5A), it is highly likely that this luminal domain point mutation eliminates the need for the unknown activating event. Significantly, this scenario further supports the possibility that the unknown activating event actually occurs on the luminal side and is required for activation of Ire1. The present study provides the first plausible evidence for an unknown additional requirement for Ire1 activation.
activated by ER stress or the S103P mutation. A similar observation was reported for mammalian IRE1α (Bertolotti et al., 2000). Nonetheless, Credele et al. (Credele et al., 2005) proposed that Ire1 is activated as a high-order oligomer. They reported that a recombinant fragment of the yeast Ire1 core stress-sensing region formed a multimer in a crystal used for X-ray structural analysis, and that point mutations predicted by the crystal structure that abolish multimer formation were found to inactivate Ire1. We suspect that Ire1 may dissociate artifically to a dimer in cell lysates in our experiments, even if it forms a higher multimer in vivo. If so, it is possible that the unknown event may be conversion of dimeric Ire1 to a higher multimer.

An alternative attractive hypothesis is that the unknown event is a change in the ternary structure of the Ire1 dimer on the luminal side, which arises by analogy to other membrane-bound sensor kinases. In the case of the erythropoietin receptor, the extracellular domain orientation between two receptor molecules is tightly coupled to the cytosolic signaling event (Livnah et al., 1998). Moreover, the conformational change after dimerization triggered by ligand binding is required for signal transmission (Remy et al., 1999; Livnah et al., 1999).

PhoQ, a membrane-bound sensor kinase found in gram-negative bacteria, also requires a conformational change triggered by interaction with a ligand and metal ions (Bader et al., 2005). In addition, as shown in Fig. 6, a cluster of positively charged amino acid residues in a site flanking the core stress-sensing region contributes to Ire1 activation by bypassing the unknown event. This observation supports our proposal that the unknown event is a conformational change on the luminal side that facilitates downstream events on the cytosolic side of Ire1.

In conclusion, the classical model whereby BiP dissociation and self-association simply lead to Ire1 activation, which received recent experimental support (Zhou et al., 2006), must be modified. According to the crystal structure reported by Credele et al. (Credele et al., 2005), the core stress-sensing region forms a major histocompatibility complex (MHC)-like groove. By analogy with the MHC, peptide fragments, and more speculatively, unfolded proteins, may bind to this groove. An attractive model is that direct recognition of unfolded proteins by the core stress-sensing region triggers the activating event. Further studies will be required to verify this scenario, including a demonstration that unfolded proteins bind Ire1 directly.

**Materials and Methods**

**Yeast strains and culture conditions**

Yeast S. cerevisiae strains were grown and genetic manipulations were performed by standard techniques (Kaiser et al., 1994). Basically, cells were cultured in minimal SD medium supplemented with appropriate nutrients at 30°C. Strains used in this study were KMY1015 (MATα ura3-52 leu2-3,112 his3-Δ200 trpl-Δ01 lys2-801 Δire1::TRP1) and KMY1516 (MATα ura3-52 his3-Δ200 trpl-Δ01 lys2-801 Δire1::TRP1) and KMY1516 (MATα ura3-52 his3-Δ200 trpl-Δ01 lys2-801 Δire1::TRP1). Yeast cells were lysed under non-denaturing or denaturing conditions as described (Kimata et al., 2004). Anti-HA immunoprecipitation of the non-denaturing lysates was also performed as described (Kimata et al., 2004) using protein-A-conjugated Sepharose beads (protein-A-Sepharose 4 FF; Amersham Biosciences).

The lysates and immunoprecipitates were denatured in SDS/DDT-sampling buffer and analyzed by anti-HA, anti-BiP and anti-Flag western blotting, using HRP-conjugated secondary antibodies and the ECL system (Amersham Biosciences) as described (Kimata et al., 2004). ECL signals were detected by a cooled CCD camera system LAS-1000plus (Fujifilm: Fig. 1B, Fig. 4, Fig. 5A and B) or X-ray film (Fig. 5C). Exposure times were 30-60 seconds for BiP, 1-10 seconds for Ire1-Flag, 1-2 minutes for Ire1-HA expressed from centromeric plasmids, and 1-10 seconds for Ire1-HA expressed from 2-μm plasmids. ImageJ, a software product from http://rsb.info.nih.gov/ij/imagej/, was used to quantify the signal intensity of the LAS image.

**Density gradient fractionation**

Nondenaturing lysates in gradient buffer (50 mM Tris-HCl pH 7.9, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 4% glycerol) from cells producing Ire1-HA and high molecular weight markers (Pharmacia) were separately centrifuged through a Nondenaturing lysates in gradient buffer (50 mM Tris-HCl pH 7.9, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 4% glycerol) from cells producing Ire1-HA and high molecular weight markers (Pharmacia) were separately centrifuged through a 5-25% glycerol gradient at 200,000 g for 12 hours. The resulting gradient was divided into 25 fractions (500 μl each). Each fraction was subjected to anti-HA immunoprecipitation with 15 μl of protein A-conjugated Sepharose beads to trap anti-HA antibody. The beads were collected by centrifugation (600 g for 10 seconds), washed three times with gradient buffer without glycerol, and heated in SDS/DDT-sampling buffer for western blot analysis.

**Assay for β-galactosidase activity**

Yeast cells (0.5 OD600 equivalent) were suspended in 800 μl of Z buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCL, 1 mM MgSO4, 0.27% 2-mercaptoethanol, pH 7.0). After addition of 20 μl of 0.1% SDS and 50 μl of chloroform, the mixture was vortexed vigorously for 20 seconds and equilibrated at 28°C for 5 minutes. β-Nitrophenyl-D-galactoside (4 mg/ml in Z buffer) was then added as substrate to a final concentration of 0.8 mg/ml. The reaction was terminated by addition of 0.5 ml of 1 M NaCO3, and the concentration of the product, o-nitrophenol (ONP), was measured at A405. One unit of β-galactosidase activity is defined as 1 nmol of ONP per minute of reaction per ml of yeast culture at OD600=1.

**RNA analysis**

A DNA probe corresponding to nucleotides –11 to 654 of the HAC1 mRNA was prepared by PCR using yeast genomic DNA as template. Total RNA was prepared using the hot phenol method (Collart and Olivierio, 1993). For northern blot analysis, 3 μg of total RNA was separated on a 1% agarose gel, 1.8% formaldehyde gel and transferred to a nylon membrane (Hybond-N; Amersham Biosciences). The membrane was subjected to prehybridization in 500 mM sodium phosphate pH 7.0, 1 mM EDTA, and 7% SDS. The membrane was then incubated with random-primed 32P-labeled probe. After washing, the membrane was exposed to an imaging screen (BAS- MS2040, Fuji), and radiation signal was detected and quantified using a Fuji BAS2500 image analyzer. The percentage of HAC1 mRNA cleavage was calculated using the equation (P-P'/P) × 100%, where P is the intensity of total HAC1 mRNA species and P' is the intensity of HAC1 mRNA.
and members of the Kohno laboratory for valuable discussion. This work was supported by Grants-in-Aids for Scientific Research on Priority Areas (14037240 to K.K., 18050024 to Y.K.) and for 21st Century COE Research from MEXT and JSPS.KAKENHI (18570179 to Y.K.).

References

Bader, M. W., Sanowar, S., Daley, M. E., Schneider, A. R., Cho, U., Xu, W., Kleif, R. E., Le Moul, H. and Miller, S. I. (2005). Recognition of antimicrobial peptides by a bacterial sensor kinase. Cell 122, 461-472.

Bertolotti, A., Zhang, Y., Hendershot, L. M., Harding, H. P. and Ron, D. (2000). Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. Nat. Cell Biol. 2, 326-332.

Calfon, M., Zeng, H., Urano, E., Till, J. H., Hubbard, S. R., Harding, H. P., Clark, S. G. and Ron, D. (2002). Ire1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. Nature 415, 92-96.

Christianson, T. W., Sikorski, R. S., Dante, M., Shero, J. H. and Hieter, P. (1992). Multifunctional yeast high-copy-number shuttle vectors. Gene 110, 119-122.

Collart, M. A. and Olivier, S. (1993). In Current Protocols in Molecular Biology (ed. F. M. Ausubel, R. Brent, R. E. Kingston, D. Moore, J. G. Seidman, J. A. Smit and K. Struhl), pp. 13.12.1-13.12.2. New York: Greene Publishing Associates.

Cox, J. S. and Walter, P. (1996). A novel mechanism for regulating activity of a transcription factor that controls the unfolded protein response. Cell 87, 391-404.

Cox, J. S., Shamu, C. E. and Walter, P. (1993). Transcriptional induction of genes encoding endoplasmic reticulum resident proteins requires a transmembrane protein kinase. Cell 73, 1197-1206.

Credle, J. J., Finer-Moore, J. S., Papa, F. R., Stroud, R. M. and Walter, P. (2005). On the mechanism of sensing unfolded protein in the endoplasmic reticulum. Proc. Natl. Acad. Sci. USA 102, 18773-18784.

Harding, H. P., Zhang, Y. and Ron, D. (1999). Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. Nature 397, 271-274.

Iwawaki, T., Hosoda, A., Okuda, T., Kamigori, Y., Nomura-Furuwatari, C., Kunita, Y., Tsuru, A. and Kohno, K. (2001). Translational control by the ER transmembrane protein response pathway regulates expression of genes for anti-oxidative stress and the unfolded protein response. Mol. Cell. Biol. 11, 5708-5717.

Kimata, Y., Yama, T., Yura, T. and Kuri, M. (1998). Unconventional splicing of the yeast HAC1/ERN4 mRNA required for the unfolded protein response. EMBO J. 17, 3028-3039.

Kurokawa, K., Kohno, K., Sant, A., Kohno, K. and Sambrook, J. (2005). An essential dimer-forming subregion of the endoplasmic reticulum stress sensor Ire1. Biochem. J. 391, 135-142.

Livnah, O., Stura, E. A., Middleton, S. A., Johnson, D. L., Jollife, K. L. and Wilson, I. A. (1999). Crystallographic evidence for preformed dimers of erythropoietin receptor before ligand activation. Science 283, 987-990.

Mori, K., Sant, A., Kohno, K., Normington, K., Gething, M. J. and Sambrook, J. F. (1992). A 22 bp cis-acting element is necessary and sufficient for the induction of the yeast KAR2 (BiP) gene by unfolded proteins. EMBO J. 11, 2583-2593.

Mori, K., Ma, W., Gething, M. J. and Sambrook, J. (1993). A transmembrane protein with a cdc2+cdc28-related kinase activity is required for signaling from the ER to the nucleus. Cell 74, 743-756.

Mori, K., Kawahara, T., Yoshida, H., Yanagi, H. and Yura, T. (1996). Signalling from endoplasmic reticulum to nucleus: transcription factor with a basic-leucine zipper motif is required for the unfolded protein-response pathway. Genes Cells 1, 803-817.

Oikawa, D., Kimata, Y., Takeuchi, M. and Kohno, K. (2005). An essential dimer-forming subregion of the endoplasmic reticulum stress sensor Ire1. Biochem. J. 391, 135-142.

Okamura, K., Kimata, Y., Higashio, H., Tsuru, A. and Kohno, K. (2000). Dissociation of Kar2p/BiP from an ER sensory molecule, Ire1p, triggers the unfolded protein response in yeast. Biochem. Biophys. Res. Commun. 279, 445-450.

Papa, F. R., Zhang, C., Shokat, K. and Walter, P. (2003). Bypassing a kinase activity with an ATP-competitive drug. Science 302, 1533-1537.

Remy, I., Wilson, I. A. and Michnick, S. W. (1999). Erythropoietin receptor activation by a ligand-induced conformation change. Science 283, 990-993.

Shamu, C. E. and Walter, P. (1996). Oligomerization and phosphorylation of the Ire1p kinase during intracellular signaling from the endoplasmic reticulum to the nucleus. EMBO J. 15, 3028-3039.

Sidrauski, C. and Walter, P. (1997). The transmembrane kinase Ire1p is a site-specific endonuclease that initiates mRNA splicing in the unfolded protein response. Cell 90, 1031-1039.

Sikorski, R. S. and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics 122, 19-27.

Takeuchi, M., Kimata, Y., Hiraata, A., Oka, M. and Kohno, K. (2006). Saccharomyces cerevisiae Rot1p is an ER-localized membrane protein that may function with Bip/Kar2p in protein folding. J. Biochem. (Tokyo) 139, 597-605.

Tiraphphon, W., Wellinhand, A. A. and Kaufman, R. J. (1998). A stress response pathway from the endoplasmic reticulum to the nucleus requires a novel bifunctional protein kinase/endonuclease (Ire1p) in mammalian cells. Genes Dev. 12, 1812-1824.

Travers, K. J., Patil, C. K., Wodicka, L., Lockhart, D. J., Weissman, J. S. and Walter, P. (2000). Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. Cell 101, 249-258.

Wang, X. Z., Harding, H. P., Zhang, Y., Jolicouer, E. M., Kuroda, M. and Ron, D. (1998). Cloning of mammalian Ire1 reveals diversity in the ER stress responses. EMBO J. 17, 5708-5717.

Yoshida, H., Haze, K., Yanagi, H., Yura, T. and Kuri, M. (1998). Identification of the cis-acting endoplasmic reticulum stress response element responsible for transcriptional induction of mammalian glucose-regulated proteins. Involvement of basic leucine zipper transcription factors. J. Biol. Chem. 273, 33741-33749.

Yoshida, H., Matoti, T., Yamamoto, A., Okuda, T. and Morl, K. (2001). XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. Cell 107, 881-891.

Zhou, J., Liu, C. Y., Buck, S. H., Clark, R. L., Pesach, D., Xu, Z. and Kaufman, R. J. (2006). The crystal structure of human Ire1 luminal domain reveals a conserved dimerization interface required for activation of the unfolded protein response. Proc. Natl. Acad. Sci. USA 103, 14343-14348.