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

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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 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.

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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; Sidrauski and Walter, 1997). After a splicing reaction facilitated by Ire1, the mature mRNA (*HAC1*^{i}) is translated into a transcription factor that induces various genes involved in alleviating ER stress (Travers et al., 2000; Kimata et al., 2006). One of these genes encodes an ER-resident molecular chaperone BiP. The BiP gene carries a promoter element named the UPR element (UPRE), where the Hac1 protein binds (Kohno et al., 1993; Mori et al., 1992; Mori et al., 1996).

Unlike yeast, mammalian cells have been found to carry multiple ER-stress sensors. There are two Ire1 paralogs, Ire1α and Ire1β (Tirasophon et al., 1998; Wang et al., 1998; Iwawaki et al., 2001). Ire1α is involved in splicing the pre-mRNA of the XBP1 transcription factor (Yoshida et al., 2001; Calfon et al., 2002). IRE1β 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 IRE1β 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

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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 yeast 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 UPRE-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

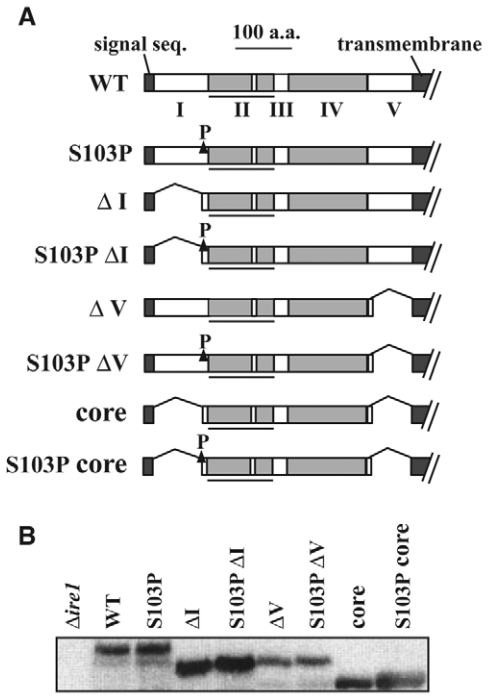

Fig. 1. Ire1 mutants and their cellular expression. (A) The luminal domain of wild-type (WT) Ire1 and luminal domain mutants is shown schematically. Ire1 is inactivated by internal 10-a.a. deletions in the shaded region, and subregions I to V were designated based on this observation (Kimata et al., 2004) (subregion I corresponds to a.a. positions 32-111; subregion II, 112-242; subregion III, 243-272; subregion IV, 273-454; and subregion V, 455-524). The ΔI and the ΔV mutations are deletions of T32 to R91 and T463 to N523, respectively. The core mutation is the combination of the ΔI and ΔV mutations. The position of the S103P point mutation is also indicated. (B) Lysates were prepared from KMY1015 (*ire1* null mutant) cells carrying pRS315-Ire1-HA (centromeric plasmid for expression of Ire1-HA) or mutant alleles under denaturing conditions, and subjected to anti-HA western blotting with 15 μg total protein per lane. The empty vector control (*Δire1*) carried pRS315 (Sikorski and Hieter, 1989) instead of pRS315-Ire1-HA.

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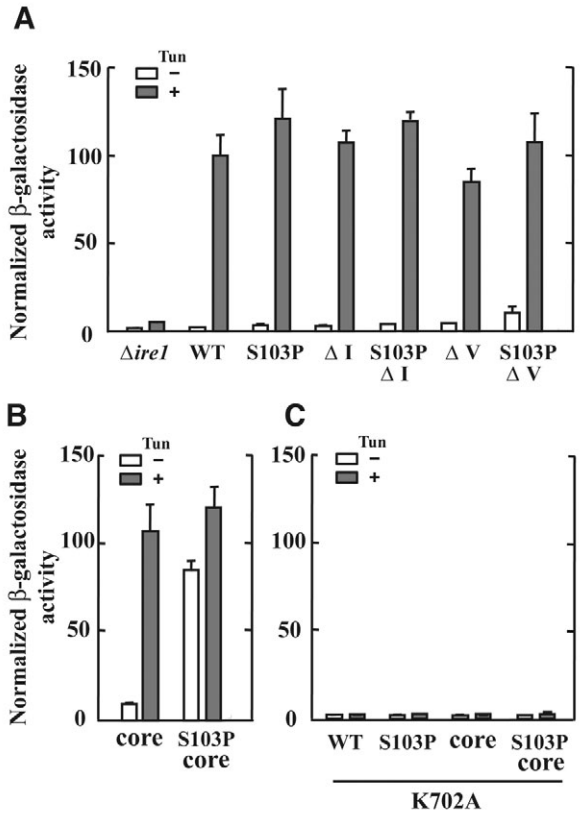

Fig. 2. Activity of the Ire1 mutants as measured by a UPRE-lacZ reporter. KMY1015 (*ire1* null mutant) cells carrying both pCZY1 (UPRE-lacZ reporter plasmid) and pRS315-Ire1-HA or mutant alleles were cultured with (+) or without (–) 2 μg/ml tunicamycin (Tun) for 4 hours and subjected to β-galactosidase assay. For the empty vector control (*Δire1*), pRS315 was used instead of pRS315-Ire1-HA. In all three panels, each value is the mean ± s.d. for three independent clones and was normalized to that of the Tun+ wild-type Ire1-HA control, which was set at 100.

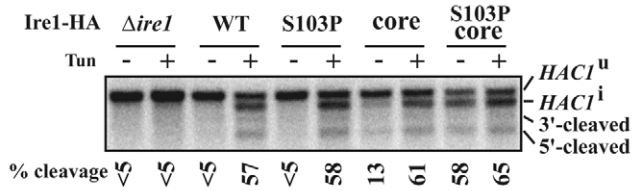

Fig. 3. *HAC1*^{u} cleavage by the Ire1 luminal domain mutants. KMY1516 (*ire1* null mutant) cells carrying pRS313-Ire1-HA (centromeric plasmid for expression of Ire1-HA) or mutant alleles were cultured with (+) or without (–) 2 μg/ml tunicamycin (Tun) for 60 minutes and total RNA was analyzed by northern blotting using the *HAC1* probe. For the empty vector control (*Δire1*), pRS313 was used instead of pRS313-Ire1-HA. The positions of the uncleaved (*HAC1*^{u}), the cleaved and ligated (*HAC1*^{i}) and the cleaved but unligated (5′-cleaved and 3′-cleaved) versions of *HAC1* mRNA are indicated (Kawahara et al., 1998). The percentage of *HAC1* mRNA cleavage was calculated as described in the Materials and Methods.

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 UPRE-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 Ire1 variants was not changed by the S103P mutation (Fig. 1B). According to the results from a UPRE-lacZ reporter assay (Fig. 2A and B), the core mutant, but not the wild-type, ΔI or ΔV Ire1, was constitutively activated by the S103P mutation. These findings are not artifacts caused by addition of HA epitope to Ire1, because similar results were obtained when untagged Ire1 and its mutants were used (supplementary material Fig. S2).

We next evaluated the activity of the Ire1 mutant directly by checking the cleavage of *HAC1*^{u}, and obtained results consistent with those of the UPRE-lacZ reporter assay. Wild-type or mutant Ire1 cells were treated with tunicamycin or cultured under nonstressed conditions, and total RNA was analyzed by northern blotting using the *HAC1* probe (Fig. 3). No *HAC1*^{u} cleavage (<5% cleavage) was detected in empty vector control cells lacking *IRE1*. In wild-type and S103P Ire1 cells, *HAC1*^{u} cleavage, which was not obvious under nonstressed condition (<5% cleavage), was clearly induced by tunicamycin treatment (wild type, 57%; S103P, 58%). In core mutant Ire1 cells, *HAC1*^{u} was cleaved slightly, even under nonstressed conditions (13% cleavage). Tunicamycin treatment increased this cleavage efficiency to 61%, which is almost the same value as for wild-type and S103P Ire1 cells. Constitutive activation by the S103P core double mutation was also observed by this *HAC1*^{u} cleavage analysis. This doubly mutated Ire1 cleaved *HAC1*^{u} to a significant extent, even in nonstressed cells (58% cleavage). This cleavage efficiency was only slightly increased by tunicamycin treatment (65% cleavage). 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 UPRE-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.

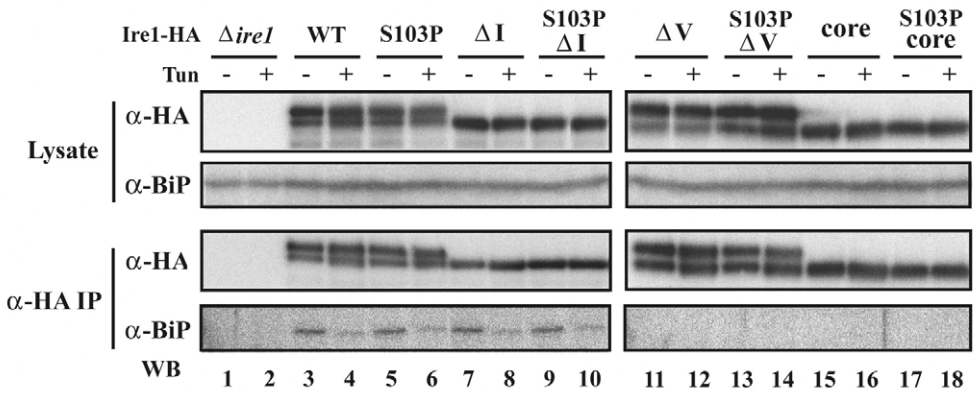

Fig. 4. Binding of BiP to luminal domain mutated Ire1. KMY1516 (*ire1* null mutant) cells carrying pRS423-Ire1-HA (2-μm plasmid for expression of Ire1-HA) or mutant alleles were cultured with (+) or without (–) 2 μg/ml tunicamycin (Tun) for 60 minutes and their lysates were then used for anti-HA immunoprecipitation. The cell lysates (equivalent to 3×10^6 cells) and the anti-HA immunoprecipitates (equivalent to 1×10^7 cells; α-HA IP) were analyzed by anti-HA (α-HA) and anti-BiP (α-BiP) western blotting (WB). For the empty vector control (*Δire1*), pRS423 was used instead of pRS423-Ire1-HA.

BiP binding and dissociation from mutated Ire1

The UPRE-lacZ reporter and the *HAC1*^{u} 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 nondenaturing 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 complex over an Ire1-HA 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

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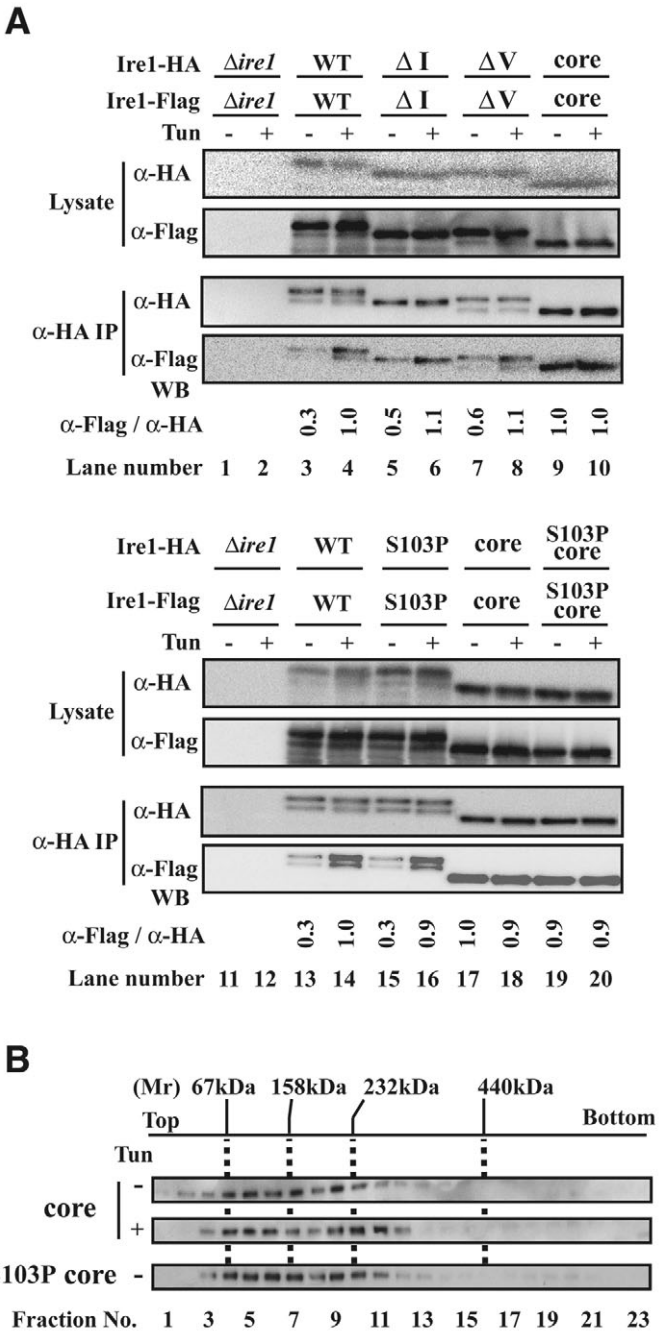

Fig. 5. Self-association of the Ire1 luminal domain mutants. (A) KMY1015 (*ire1* null mutant) cells carrying both pRS426-Ire1-Flag (2-μm plasmid for expression of Ire1-Flag; wild type (WT) or mutant) and pRS315-Ire1-HA (WT or mutant) were cultured with (+) or without (–) 2 μg/ml tunicamycin (Tun) for 60 minutes, and lysates were then used for anti-HA immunoprecipitation. The cell lysates (equivalent to 3×10^6 cells) and the anti-HA immunoprecipitates (equivalent to 1×10^7 cells; α-HA IP) were analyzed by anti-HA (α-HA) and anti-Flag (α-Flag) western blotting (WB). The ratios of Ire1-Flag signal to Ire1-HA signal in α-HA IP were normalized to the Tun+ WT Ire1 control, which was set at 1.0, and are indicated. (B) KMY1015 cells carrying pRS315-Ire1-HA (core or S103P core mutant) were cultured with (+) or without (–) 2 μg/ml tunicamycin (Tun) for 60 minutes, and their lysates were fractionated by 5-25% glycerol gradient centrifugation. Ire1-HA in each fraction was detected by anti-HA immunoprecipitation followed by anti-HA Western blotting. The positions of protein M_r markers, fractionated in parallel on an identical gradient, are indicated.

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 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 ΔI and ΔV 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 ΔI and the ΔV 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. In

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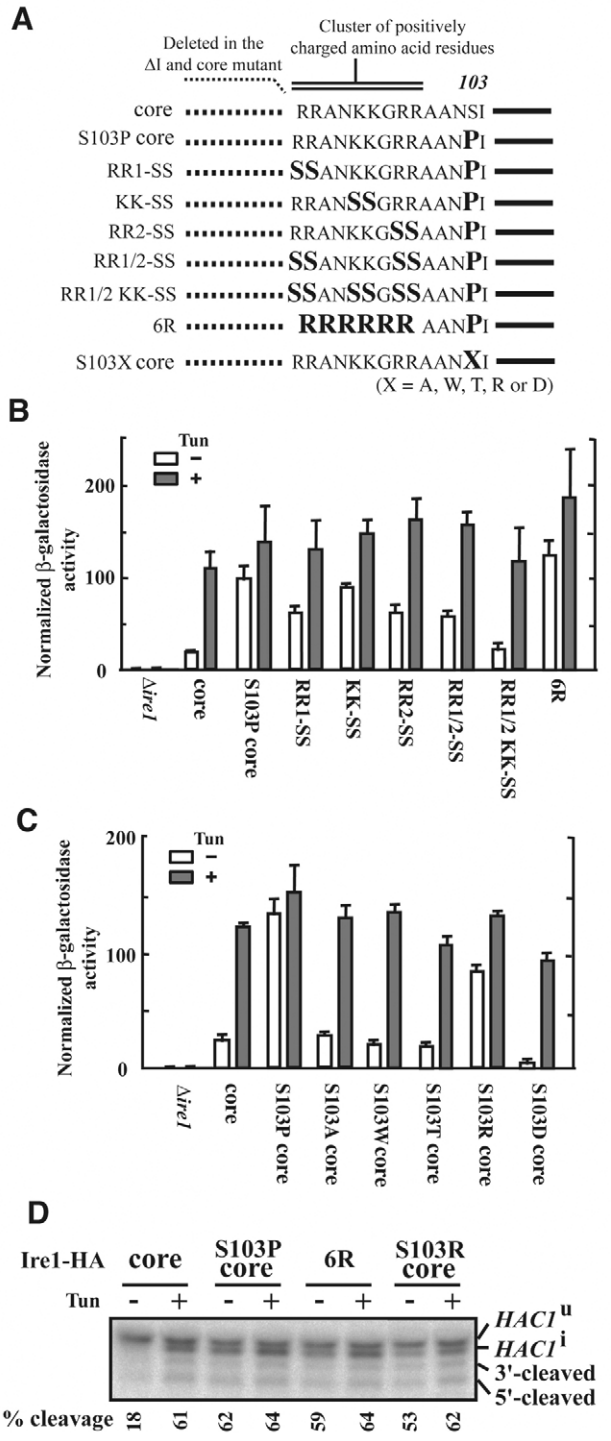

Fig. 6. Analysis of additional amino acid substitutions at and around S103 in Ire1. (A) Amino acid sequences of the mutant alleles used in this assay. The mutated residues are in bold. Note that all alleles carry the core mutation. (B,C) KMY1015 (*ire1* null mutant) cells carrying both the UPRE-lacZ reporter pCZY1 and a mutant allele of pRS315-Ire1-HA were cultured with (+) or without (–) 2 μg/ml tunicamycin (Tun) for 4 hours, and subjected to β-galactosidase assay. Each value is the mean ± s.d. for three independent clones and was normalized to that of the Tun+ wild-type Ire1-HA control, which was set at 100. (D) KMY1015 (*ire1* null mutant) cells carrying the indicated mutant alleles of pRS315-Ire1-HA were cultured with (+) or without (–) 2 μg/ml tunicamycin (Tun) for 60 minutes, and total RNA was analyzed by northern blotting using the *HAC1* probe. See Fig. 3 legend for further details.

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 ΔV 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. We speculate that both ER stress and the luminal domain mutations induce shared downstream events occurring on the cytosolic side of Ire1. Consistent with this idea, the kinase activity of the cytosolic domain was required for activation of Ire1 both by ER stress and the luminal domain mutations (Fig. 2C).

What is the unknown event? According to the density gradient fractionation analysis shown in Fig. 5B, yeast Ire1, at least the core mutant, does not form high-order oligomers, even if it is

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activated by ER stress or the S103P mutation. A similar observation was reported for mammalian IRE1α (Bertolotti et al., 2000). Nonetheless, Credle et al. (Credle 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 artifactually 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 Credle et al. (Credle 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 trp1-Δ901 lys2-801 Δire1::TRP1*) and KMY1516 (*MATα ura3-52 his3-Δ200 trp1-Δ901 LYS2::(UPRE)$_5$-CYC1 core promoter-lacZ::lys2-801 LEU2::UPRE-CYC1 core promoter-GFP::leu2-3,112 Δire1::TRP1*) as described (Kimata et al., 2004). Because these two strains were phenotypically identical with respect to the UPR, one or the other was chosen for experiments as a function of their auxotrophic markers.

Plasmids

Plasmid pRS315-Ire1-HA (Kimata et al., 2004) is a yeast centromeric plasmid bearing the *LEU2* selectable marker and the yeast *IRE1* gene carrying three tandem C-terminal HA-epitope-tagging sequences (Ire1-HA). *IRE1* mutations were created by overlap PCR as described (Kimata et al., 2004). Mutagenic primers are listed in supplementary material Table S1. To create mutations in the luminal domain, partial fragments of the Ire1 gene were PCR amplified from pRS315-Ire1-HA using Pi1, Pi2 and internal mutagenic primers. Each fragment was digested with *Sal*I and *Xba*I, and fused with the *Sal*I-*Xba*I vector fragment of pRS315-Ire1-HA. The *IRE1* mutant plasmids used to generate the data shown in Fig. 6B were constructed not by standard recombinant DNA techniques, but rather, by in vivo gap repair in yeast as described (Kimata et al., 2004).

To create the K702A mutation, a 3.4-kb Ire1-HA gene fragment was amplified from pRS315-Ire1-HA using Pi1, T7 and internal mutagenic primers. This PCR product was digested with *Sal*I and *Not*I, and ligated to *Sal*I-*Not*I-digested pRS315-Ire1-HA.

To obtain pRS313-Ire1-HA or its mutant versions, Ire1-HA gene fragments were obtained by *Bam*HI-*Not*I digestion of pRS315-Ire1-HA or its mutant versions, and cloned into the *Bam*HI-*Not*I sites of the *HIS3* centromeric vector pRS313 (Sikorski and Hieter, 1989). Plasmid pRS423-Ire1-HA (Kimata et al., 2004) is a yeast 2-μm plasmid carrying the *HIS3* selectable marker and the Ire1-HA gene. To generate mutant alleles of pRS423-Ire1-HA, the *Bam*HI-*Not*I fragments carrying the mutant Ire1-HA gene were ligated to *Bam*HI-*Not*I-digested pRS423 (Christianson et al., 1992). Plasmid pRS426-Ire1-Flag is a yeast 2-μm plasmid carrying the *URA3* selectable marker and the yeast *IRE1* gene tagged with three tandem C-terminal Flag epitope-tagging sequences (Kimata et al., 2004). To generate its mutant alleles, Ire1 gene fragments were obtained by *Bam*HI-*Sph*I digestion of the mutant versions of pRS315-Ire1-HA, and ligated to *Bam*HI-*Sph*I-digested pRS426-Ire1-Flag. For untagged Ire1 derivatives, pRS313-Ire1 was employed, and mutations were introduced as described (Kimata et al., 2004).

Antibodies and protein analyses

Antibodies used were mouse anti-HA mAb 12CA5 (Roche Diagnostics), rabbit anti-yeast BiP antiserum (Takeuchi et al., 2006), anti-Flag mAb M2 (Sigma-Aldrich) and horseradish peroxidase (HRP)-conjugated commercial secondary antibodies.

Yeast cells were lysed under nondenaturing or denaturing conditions as described (Kimata et al., 2004). Anti-HA immunoprecipitation of the nondenaturing 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/DTT-sampling buffer and analyzed by anti-HA, anti-BiP and anti-Flag western blotting, using HRP-coupled 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 (Fuji; 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://rbs.info.nih.gov/nih-image/, 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 5-25% glycerol gradient at 200,000 ***g*** for 12 hours. The resulting gradient was divided into 23 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/DTT-sampling buffer for western blot analysis.

Assay for β-galactosidase activity

Yeast cells (0.5 OD_{600} equivalent) were suspended in 800 μl of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 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. *o*-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 Na_2CO_3, and the concentration of the product, *o*-nitrophenol (ONP), was measured at A_{420}. One unit of β-galactosidase activity is defined as 1 nmol of ONP per minute of reaction per ml of yeast culture at OD_{600}=1.

RNA analysis

A DNA probe corresponding to nucleotides –11 to 654 of *HAC1* was prepared by PCR using yeast genomic DNA as template. Total RNA was prepared using the hot phenol method (Collart and Oliviero, 1993). For northern blot analysis, 3 μg of total RNA was separated on a 1% agarose, 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 ^{32}P-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 $(I^t–I^u)/I^t \times 100\%$, where I^t is the intensity of total *HAC1* mRNA species and I^u is the intensity of *HAC1*^{u} mRNA.

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