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Activation mechanism of ER stress in yeast and mammalian cells

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Research aims

Eukaryotic cells activate the unfolded protein response (UPR) upon endoplasmic reticulum (ER) stress, where the stress is assumed to be accumulation of unfolded proteins in the ER[1]. Of the ER-membrane proteins that mediate the intracellular signal for the UPR, only Ire1 is known to be evolutionarily conserved throughout eukaryotes[1,2]. Ire1 is a very unique type-I transmembrane protein carrying both kinase and endoribonuclease activities in its cytosolic region (Fig. 1)[2]. The luminal portion of yeast Ire1 has been precisely analyzed and core stress-sensing region (CSSR) in Ire1 luminal domain plays a central role in the recognition of the accumulation of misfolded proteins in the ER (Fig. 1)[3]. Recent our *in vitro* study showed that unfolded model proteins directly bound the Ire1 CSSR domain[4]. However there has been no evidence that unfolded protein could directly bind Ire1 luminal region *in vivo*. To address this question, we tried to detect *in vivo* physical interaction between Ire1 and a misfolded model protein CPY*, which is a R255G missense mutant of carboxypeptidase Y.

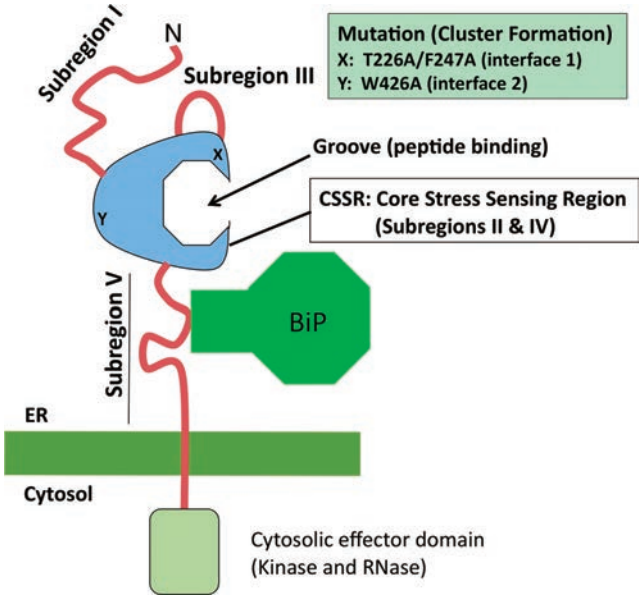

Fig. 1. Structure of the luminal domain of *S. cerevisiae* Ire1. The luminal domain of yeast Ire1 can be divided into five subregions. Subregions I (aa 32–111), III (aa 243–272), and V (aa 455–524) are loosely folded, while subregions II (aa 112–242) and IV (aa 273–454) form the tightly folded CSSR[3,6]. The CSSR dimer associated via interface I forms a groove, by which unfolded proteins may be captured[6]. Mutation of Interface 1 (X: T226A/F247A) and another mutation of Interface II (Y: W426A) disrupt the groove structure and the cluster formation, respectively, resulting in the great reduction of full activation of UPR under ER stress[3,6].

Methods[5]

Plasmids: *S. cerevisiae IRE1* plasmid pRS313-IRE1 and C-terminally HA-tagged *IRE1* (Ire1-HA) plasmids pRS315-IRE1-HA and pRS423-IRE1-HA were respectively produced from centromeric vectors pRS313, pRS315 and 2μ vector pRS423. Plasmid pRS426-IRE1-HA is the *URA3*-marker variant of pRS423-IRE1-HA. To introduce point or partial-deletion mutations into the *IRE1* gene on these plasmids, overlap PCR and *in vivo* homologous recombination (gap repair) techniques were employed[3,4].

Results

In order to support the idea of a physical interaction between unfolded proteins and Ire1, we investigated whether such complexes are formed in yeast cells. The R225G mutant of Prc1 carboxypeptidase Y (CPY) known as CPY* fails to be correctly folded and transported to the vacuole. In Figure 2, GFP-tagged wild-type CPY (CPY-GFP) or CPY* (CPY*-GFP) was constitutively produced from the strong *TEF1* promoter. Activation of the UPR by these proteins was checked by induction of a lacZ reporter controlled by the UPR promoter element (UPRE), which the Hac1 protein directly activates. As expected, the reporter was induced by expression of CPY*-GFP and less strongly by CPY-GFP (Fig. 2A). This observation was reproduced by an assay for Ire1-dependent *HAC1*[i] mRNA production, in which cellular RNA samples were used for reverse transcriptase (RT)-PCR amplification of the *HAC1* mRNAs. We thus think that CPY-GFP may be somewhat unfolded, while CPY*-GFP acts as a more obviously unfolded protein model. In Figure 2B, cells were treated with the chemical crosslinker dithiobis(succinimydylpropionate) (DSP) before cell lysis and anti-GFP immunoprecipitation (IP). In agreement with the ER retention of CPY*-GFP, this protein appeared as a single protein band in an anti-GFP Western blot of the lysate and the anti-GFP IP samples. Also consistent with the above result, CPY-GFP partially converted to the fast-mobility vacuolar form. Importantly, co-expressed HA epitope-tagged Ire1 (Ire1-HA) was co-immunoprecipitated with CPY*-GFP but less abundantly with CPY-GFP (Fig. 2B). Next we performed a reverse immunoprecipitation ex-

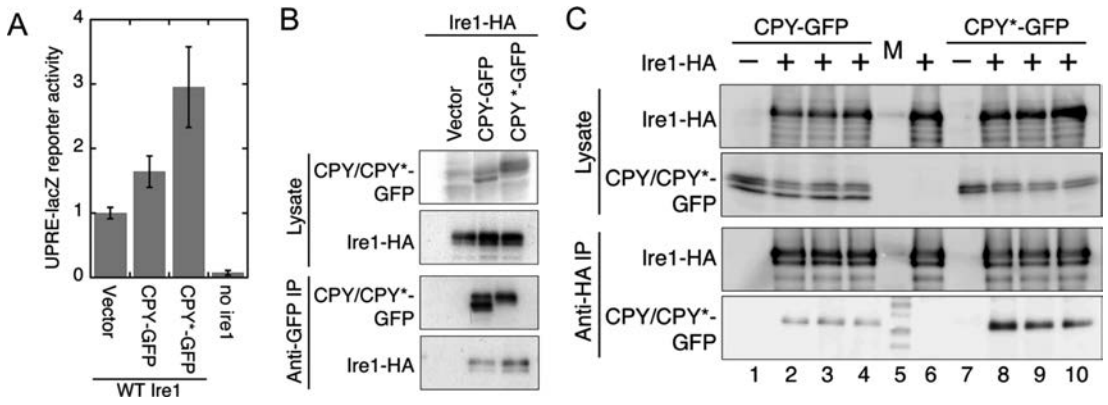

Fig. 2. *In vivo* association of CPY*-GFP with Ire1. (A) An ire1Δ strain KMY1015 carrying both the wild-type IRE1 (WT Ire1) plasmid pRS315-IRE1-HA and the UPRE-lacZ reporter plasmid pCZY1 was further transformed with the CPY-GFP or the CPY*-GFP expression plasmid (pRS313-TEF1pr-CPY-GFP or pRS313-TEF1pr-CPY*-GFP) or empty vector pRS313 (Vector). The transformant strains were then assayed for cellular β-galactosidase activity, the values of which are normalized against that of vector control cells (set at 1.00). In the "no ire1" sample, cells carried vector plasmids pRS315 and pRS313. Error bars represent the SDs from three independent transformants. According to Student's t test, all values are statistically different from each other ($p<0.05$). (B) The ire1Δ strain KMY1015 carrying both HA-tagged Ire1 plasmid pRS426-IRE1-HA and pRS313-TEF1pr-CPY-GFP or pRS313-TEF1pr-CPY*-GFP (or empty vector pRS313; Vector) was incubated with protein cross-linker DSP before cell lysis and anti-GFP IP. Subsequently, the lysate and the anti-GFP IP samples were analyzed by anti-HA or anti-GFP Western blotting. (C) The ire1Δ strain transformed with both pRS426-IRE1-HA (or empty vector pRS426 for lanes 1 and 7) and a GAL1 promoter-inducible CPY-GFP or CPY*-GFP plasmid, pRS313-GAL1pr-CPY-GFP or pRS313-GAL1pr-CPY*-GFP, was cultured in galactose-containing medium. After incubation with DSP, cells were lysed and analyzed by anti-HA IP, followed by anti-HA or anti-GFP Western blotting. In lanes 2, 3, and 4 and 8, 9, and 10, samples from three independent clones were analyzed. Cells for lane 6 carried an empty vector pRS313 instead of the CPY-GFP or CPY*-GFP plasmid. A molecular mass marker (M) was loaded in lane 5.

periment, in which CPY*-GFP was expressed from the inducible *GAL1* promoter, since we failed to transform *ire1* null strains with the CPY*-GFP constitutive expression plasmid. Consistent with the results in Figure 2B, Ire1-HA co-immunoprecipitates CPY*-GFP, and less abundantly the ER-retained form of CPY-GFP. The vacuolar form of CPY-GFP, which migrates faster on PAGE, was not co-immunoprecipitated with Ire1-HA (Fig. 2C, lanes 2–4).

Conclusion

Consistent with previous *in vitro* studies of the ER-luminal domain of the UPR initiator Ire1[4], the results of immnoprecipitation experiments indeed represent *in vivo* interaction between Ire1-HA and the ER-located model protein[5].

References

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