FEBS Letters 584 (2010) 3203–3208

FEBS Letters
journal homepage: www.FEBSLetters.org

Domain compatibility in Ire1 kinase is critical for the unfolded protein response

Juthakorn Poothong [a], Pattarawut Sopha [a], Randal J. Kaufman [b], Witoon Tirasophon [a,*]

[a] The Institute of Molecular Biosciences, Mahidol University, Salaya, Nakhon Pathom 73170, Thailand
[b] Department of Biological Chemistry and Howard Hughes Medical Institute, University of Michigan, Ann Arbor, MI 48109, USA

ARTICLE INFO

Article history:
Received 16 March 2010
Revised 17 May 2010
Accepted 3 June 2010
Available online 10 June 2010

Edited by Robert Barouki

Keywords:
Unfolded protein response
Ire1
Domain swapping
Protein kinase
Endoribonuclease
HAC1 splicing

ABSTRACT

The unfolded protein response is a mechanism to cope with endoplasmic reticulum stress. In *Saccharomyces cerevisiae*, Ire1 senses the stress and mediates a signaling cascade to upregulate responsive genes through an unusual *HAC1* mRNA splicing. The splicing requires interconnected activity (kinase and endoribonuclease (RNase)) of Ire1 to cleave *HAC1* mRNA at the non-canonical splice sites before translation into Hac1 transcription factor. Analysis of the truncated kinase domain from Ire1 homologs revealed that this domain is highly conserved. Characterization by domain swapping indicated that a functional ATP/ADP binding domain is minimally required. However the overall domain compatibility is critical for eliciting its full RNase function.

1. Introduction

The endoplasmic reticulum (ER) is the site for the folding and assembly of secretory and membranous proteins. The environment inside the lumen of the ER is optimized to facilitate folding of the nascent polypeptide into a correct tertiary structure prior to trafficking to the Golgi. Perturbation of the homeostatic environment inside the ER results in accumulation of unfolded proteins that disrupts ER function leading to cell death [1]. An exquisite quality control system ensures that only properly folded proteins exit the ER, and those that are irreparably misfolded are directed to degradation through ER-associated proteasomal degradation and/or autophagy. The cell has evolved the ability to adjust the protein folding capacity to meet fluctuations in the protein folding demand [2]. The unfolded protein response (UPR) is a set of adaptive signaling pathways that evolved to limit accumulation of misfolded proteins by (1) reducing the load on the ER by inhibiting mRNA translation initiation, (2) inducing the transcription of genes encoding protein folding, processing, and trafficking functions, and (3) increasing the degradation rate of irreversibly misfolded proteins. Activation of the UPR is initiated through three ER-localized transmembrane protein sensors Ire1, Perk and ATF6 [3–5]. These proximal effectors of the UPR monitor the protein folding status in the ER through detecting the free pool of the molecular chaperone BiP that is available to facilitate protein folding [6].

Ire1 is the most ancient UPR sensor and is conserved in all eukaryotes. The Ire1 signaling has been well characterized in *Saccharomyces cerevisiae* [6–8]. Its amino terminus resides in lumen ER lumen to sense the protein folding status. The carboxyl terminus resides in the cytosol to initiate a unique signaling through its serine/threonine kinase and endoribonuclease (RNase) activities to induce an unconventional splicing reaction that removes a 252 nucleotide intron from *HAC1* mRNA, encoding a transcription factor that activates UPR-responsive genes [9,10]. The Ire1 kinase comprises 11 subdomains folded into two distinct lobes. A small lobe at the amino terminus is mainly involved in nucleotide binding domain whereas a large lobe at the carboxyl terminus participates in substrate binding [8,11]. Ire1 activation is mediated by oligomerization and trans-autophosphorylation leading to a conformational change that fully elicits the RNase activity [10,12,13].

Here, we describe an inducible Ire1 platform that is amendable for modulating the UPR response in *S. cerevisiae*. Through domain swapping we demonstrate that specific coordination within the kinase domain is critical role for activating the RNase function.

Abbreviations: UPR, unfolded protein response; RNase, endoribonuclease; RT-PCR, reverse transcribed-polymerase chain reaction; *Sca*, *Saccharomyces carlsbergensis*; *Sl*, *Saccharomycodes ludwigii*; *Pt*, *Pachysolen tannophilus*; *Td*, *Torulasopora delbrueckii*; *Wf*, *Wickerhamia fluorescens*

* Corresponding author. Fax: +1 662 800 2732.
E-mail address: mbwtr@mahidol.ac.th (W. Tirasophon).

0014-5793/$36.00
doi:10.1016/j.febslet.2010.06.003

2. Materials and methods

2.1. Strains and manipulations

S. cerevisiae AWY14 (W303-1A, UPRE-CYC1-LacZ, UPRE-CYC1-LEU 2), *S. cerevisiae* AWY 19 (same as AWY14 except Δire1::kanamycin^r) [14], *Saccharomyces carlsbergensis* (*Sca*), *Saccharomycodes ludwigii* (*Sl*), *Pachysolen tannophilus* (*Pt*), *Torulasopora delbrueckii* (*Td*), *Wickerhamia fluorescens* (*Wf*) were maintained in YEPD. Transformation of recombinant plasmids into the AWY19 strain was performed by the standard lithium acetate method. Strains carrying the plasmid were grown on uracil dropout medium. *Escherichia coli* DH5α was used for propagation and construction of all plasmids.

2.2. Construction of Ire1 expression plasmid

The entire open reading frame of the *IRE1* gene was PCR amplified from *S. cerevisiae* genomic DNA into 3 fragments by Vent DNA polymerase (New England Biolab) using primers shown in Fig. 1 and Table 1. Silent mutations were introduced to the gene to create two unique restriction recognition sites, *Bam*H I *and Xho* I, at positions 2047 and 2421 (relative to the translation initiation site). The fragments were sequentially assembled into pYES-2 plasmid (Invitrogen) downstream of the GAL1 promoter resulting in pYES-*IRE1*. The nucleotide sequence was verified by automated DNA sequencing.

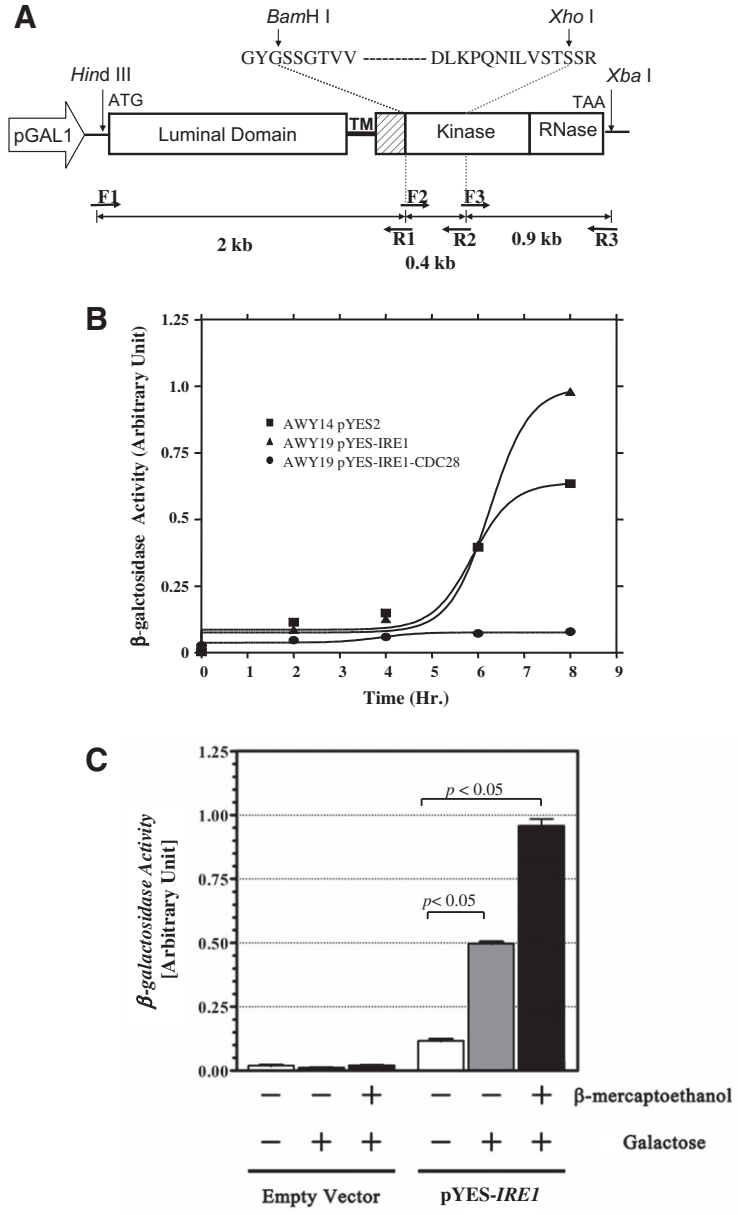

Fig. 1. UPR restoration by inducible Ire1. (A) Construction of the inducible Ire1 expression plasmid. The *S. cerevisiae IRE1* gene was PCR amplified as three consecutive fragments then cloned under GAL 1 promoter in pYES-2 plasmid. *BamH I* and *Xho I* restriction sites (indicate by arrows) were introduced into the kinase subdomains I and VI, respectively. Positions of primers and the approximate size of the amplified fragments are indicated. GYGSSGTVV and DLKPQNILVSTSSR are conserved amino acid sequences in subdomain I and VI used to design degenerate primers. (B) Measure of UPR reporter gene activity. Wild type (*AWY14*) or *ire1* null (*AWY19*) strains with indicated plasmids were cultured in uracil dropout containing 2% raffinose, 1% galactose and 10 mM β-mercaptoethanol. β-Galactosidase activity was measured at indicated time. (C) The UPR activity of the recombinant Ire1 in the presence of absence of ER stress was determined (mean ± S.E.M.) ($n = 3$). ** Represent significant difference of the reporter ($P < 0.05$, $n = 3$) by one-way ANOVA test.

Table 1
Oligonucleotide primers used in this study.

Primer	Sequence
F1	5′ ATT TTA AAG CTT TGA AAA ATG CGT CTA CT 3′ *Hind* III
R1	5′ TCC TGA GGA TCC GTA ACC TAA AAT TTT 3′ *Bam* HI
F2	5′ GGT TAC GGA TCC TCA GGA ACA GTA GTT T 3′ *Bam* HI
R2	5′ AAA CCT ACT CGA GGT AGA AAC GAG AAT AT 3′ *Xho* I
F3	5′ TT TCT ACC TCG AGT AGG TTT ACT GCC 3′ *Xho* I
R3	5′ AGG GTC TAG AAC ATG TTA TGT ATA C 3′ *Xba* I
Degenerate F1	5′ GGN TAY GGA TCC TCN GGN ACN GTB GT 3′ *Bam* HI
Degenerate R1	5′ CCT ACT CGA GGT AGA AAC GAG RAT RTT YTG NGG YTT NAG RTC 3′ *Xho* I
Degenerate CDC28 F	5′ GGN GAR GGA TCC TAY GGN GTB GTB TAY AA 3′ *Bam* HI
Degenerate CDC28 R	5′ CCT ACT CGA GGT AGA AAC GAG RAT RTT YTG NGG YTT NAG RTC 3′ *Xho* I
HAC1F	5′ GCC CAA GAG TAT GCC GAT TCC G 3′
HAC1R	5′ ACC CTC GAG CGA TTG TCT TCA TG 3′
ACT1F	5′ GCC GGA TCC GCC GGT GAC GAC GCT CC 3′
ACT1R	5′ TCG TCG AAT TCT TGT TTT GAG ATC C 3′

2.3. Cloning of IRE1 homologs

Partial fragments of the *IRE1* homologs were PCR amplified from genomic DNA of various yeast species using Vent DNA polymerase (New England Biolabs) and Degenerate F1 and Degenerate R1 primers designed from the amino acid sequence of the *S. cerevisiae* Ire1 kinase in subdomain I and VI (Table 1). The homologous region from CDC28 from *S. cerevisiae* DNA was amplified by Degenerate CDC28 F and Degenerate CDC28 R primers.

To construct chimeric *IRE1s*, the 0.4 kb of *Bam*H I *and Xho* I fragment encoding kinase subdomain I–VI in pYES-*IRE1* was substituted with the homologous region from CDC28 or *IRE1* homologs. DNA sequencing was performed to determine their sequences and ensure appropriate translational frame.

2.4. β-Galactosidase assay

Yeast cells were grown in uracil dropout medium using 2% raffinose as carbon source. Activation of Ire1 expression was performed by addition of galactose (1% final) for 4 h. β-Mercaptoethanol (10 mM) (or 2 μg/ml tunicamycin) was added to induce ER stress for 4 h (or 6 h) before the β-galactosidase assay [15]. Total protein in the cell extract was measured by Bradford (BioRad) for normalization.

2.5. Reverse transcription-PCR

Total RNA was extracted from yeast strains using the phenol–chloroform method [19]. First strand cDNA synthesis was performed using oligo-dT primer and Improm pt II reverse transcriptase (Promega). PCR amplifications were carried out using specific primers (Table 1). *HAC1* mRNA splicing was measured using HAC1F and HAC1R primers flanking the 252 base intron region. The level of *IRE1* transcript was determined by multiplex PCR using F3 and R3 primer. The amplicon of Actin with ACT1F and ACT1R primers served as an internal control.

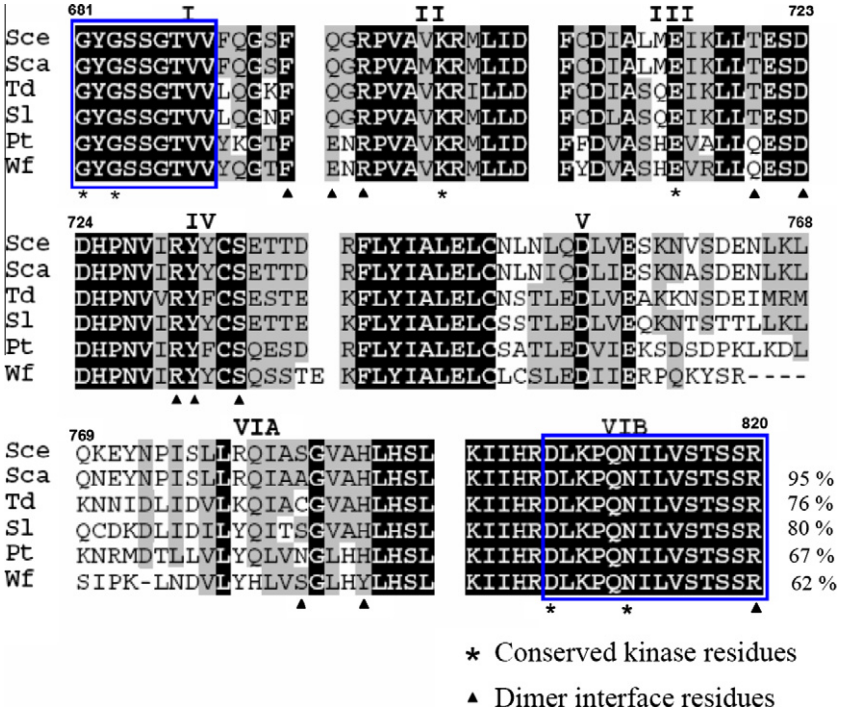

Fig. 2. Amino acid alignment of Ire1 homologs. Partial deduced amino acid sequences of Ire1 homologs from *Saccharomyces carlsbergensis* (accession number FN677757), *Saccharomycodes ludwigii* (accession number FN677758), *Pachysolen tannophilus* (accession number FN677759), *Torulasopora delbrueckii Wf* (accession number FN677760) and *Wickerhamia fluorescens* (accession number FN677761) were aligned to *Saccharomyces cerevisiae* Ire1. Roman numerals indicate kinase subdomains. Blocks represent the position of degenerate primers. Dark shading indicates identical residues. Light shading indicates conserved residues. Numbers indicate amino acid residues according to *S. cerevisiae* Ire1(Sce). Percent sequence identities to *S. cerevisiae* Ire1 are shown in the last line.

3. Results

3.1. UPR activation can be conferred by inducible Ire1 expression

The *IRE1* gene was modified to harbor a *Bam*H I site in the consensus kinase subdomain I motifs (GYG↓SSGTVV) and a *Xho*I site in subdomainVI (DLKPQNILVSTS↓SR) under an inducible GAL 1 promoter (pYES-*IRE1*) (Fig. 1A). This plasmid restored UPR signaling in an *ire1* null (AWY19) strain only when cultured in medium with raffinose and galactose but not glucose (Fig. 1C). The recombinant gene displayed comparable activity to the endogenous gene in AWY14 within the first 6 h of induction (Fig. 1B). The recombinant Ire1 exhibited greater activity (~40%) when the induction was prolonged to 8 h. Notably modulation of the recombinant Ire1 activity is constitutive however the magnitude was further increased under the stress condition (Fig. 1C). In contrast the chimeric Ire1 harboring the kinase subdomain from an unrelated CDC28 kinase (Ire1-CDC28), serving as a negative control, failed to restore the UPR. Together this evidence shows that the inducible Ire1 system is a functional platform.

3.2. The nucleotide binding domain is highly conserved among Ire1 homologs

The cytoplasmic half of Ire1 consists of dual catalytic modes, a protein ser/thr kinase and an RNase [16–18]. Activation of the

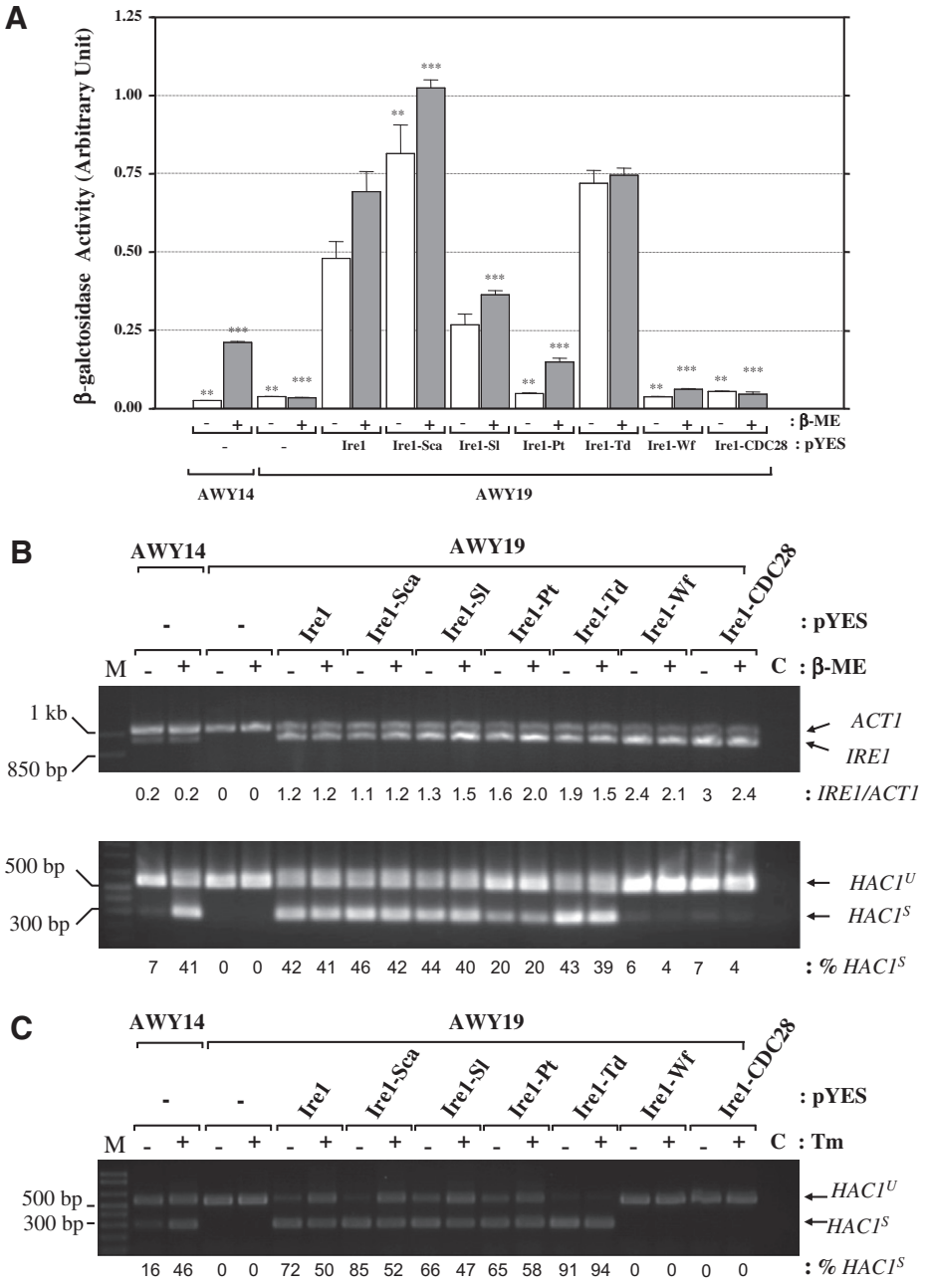

Fig. 3. UPR complementation by chimeric Ire1. (A) UPR reporter gene assay. *AWY14* or *AWY19* strains harboring the indicated plasmids were cultured in medium containing galactose for 4 h followed by addition of β-ME for 4 h. The activities were reported as mean ± S.E.M. ($n = 3$). ** and *** represent significant difference ($P < 0.05$) compared to recombinant Ire1 activity under the same conditions by one-way ANOVA. (B) Relative expression of chimeric *IRE1* compared to *ACT1* mRNA and efficiency of *HAC1* mRNA splicing was analyzed by Scion Image program. *HAC1* mRNA splicing upon 2 μg/ml Tunicamycin (Tm) was shown in (C). C: RT-PCR without reverse transcriptase. $HAC1^u$ and $HAC1^s$ represent unspliced and spliced *HAC1*. M: 1 kb plus DNA ladder.

intrinsic kinase is prerequisite for activating its adjacent RNase, implicating a close communication between the two domains [16,17]. Using degenerate primers designed from sequences in subdomain I and VIB of Ire1, covering the entire small lobe and part of the big lobe designated as the ATP/ADP binding domain, we cloned the 0.4 kb homologous fragments from several yeast strains: *S. carlsbergensis, S. ludwigii, P. tannophilus, T. delbrueckii* and *W. fluorescens*. The deduced amino acids of these fragments showed highest homology (62–95%) to *S. cerevisiae* Ire1 (Fig. 2). All (except *W. fluorescens*) sequences have subdomain organization and identical length as found in *S. cerevisiae* Ire1. The sequences in subdomains I, II, III and IV are highly conserved. In contrast, a high degree of variation was found clustered in subdomain V and VIA [8,11]. The homolog from *S. carlsbergensis* is almost identical to *S. cerevisiae* Ire1. Only 6 amino acid substitutions (V701M, L752I, V756I, V761A, K770N and S783A) of a total 130 residues were found. All substitutions (except V701A) are localized in subdomain V and VIA.

3.3. Swapping of nucleotide binding domain in chimeric Ire1 kinase alters UPR activity

The high conservation among the Ire1 homologs prompted us to investigate its significance for Ire1 function. These homologous 0.4 kb fragments were swapped into pYES-*IRE1* for functional assessment. The chimeric Ire1 proteins derived from plasmid carrying the fragments from *S. carlsbergensis, S. ludwigii, P. tannophilus, T. delbrueckii or W. fluorescens* were designated as Ire1-Sca, Ire1-Sl, Ire1-Pt, Ire1-Td and Ire1-Wf, respectively.

The UPR activity of the chimeric Ire1s could be divided into 3 groups (Fig. 3A). First, the highly active chimeras (Ire1-Sca, Ire1-Sl or Ire1-Td as well as recombinant Ire1) exhibiting high and constitutive UPR activity regardless of ER stress. Intriguingly Ire1-Sca and Ire1-Td showed reproducibly stronger UPR activation than recombinant Ire1. Second, the moderately active chimera (Ire1-Pt) had dramatically reduced UPR activity but retained ER stress responsiveness. Finally the inactive group (Ire1-Wf and Ire1-CDC28) had almost undetectable activity.

3.4. Chimeric Ire1s with an intact nucleotide binding domain trigger HAC1 mRNA splicing

Lacking a sensitive Ire1 antibody for detection, reverse transcribed-polymerase chain reaction (RT-PCR) was used to measure chimeric Ire1 expression at mRNA level. All chimeric *IRE1* mRNAs were expressed at least threefold higher than the endogenous *IRE1* in AWY14 (Fig. 3B). Their expression was not altered by ER stress. The activities of the Ire1 chimeras were analyzed by their ability to restore *HAC1* mRNA splicing in the AWY19. The highly- and moderately active Ire1 chimeras efficiently spliced *HAC1* mRNA independent to β-mercaptoethanol stress (Fig. 3B). Interestingly, these chimeras behave differently in modulating *HAC1* splicing upon activating ER stress by blocking the N-linked glycosylation process by Tunicamycin (Fig. 3C). While the *HAC1* splicing meditated by the recombinant Ire1, and several chimeras (Ire1-Sca, Ire1-Sl and Ire1-Pt) were slightly compromised by this stressor, Ire1-Td mediated *HAC1* splicing was not affected. Surprisingly, a very low reproducible level of *HAC1s* was detected in AWY19 expressing the Ire1-Wf chimera or the Ire1-CDC28 chimera comparable to the non-stressed AWY14. Unlike the endogenous Ire1, the Ire1-Wf and Ire1-CDC28 chimeras failed to respond to ER stress. Lastly, we tested if these chimeras modulate *HAC1* mRNA splicing with different kinetic. Two Ire1 chimeras exhibited distinct kinetic from Ire1. Ire1-Sca swiftly activated the splicing at early time point (30 min) under the stress (Fig. 4). Ire1-Td, on the other hand, exhibited more splicing efficiency at late time point (6 h). Together these results indicate that all chimeric proteins were produced, however they exhibited distinct catalytic activities.

4. Discussion

We established an inducible Ire1 expression platform to enable analysis of structure-function relationships. The transcriptional induction by the GAL 1 promoter generated high-level expression of the recombinant Ire1, thus causing constitutive UPR activation. This data correspond to previous findings of ER stress-independent activation of Ire1 upon over expression [12,16].

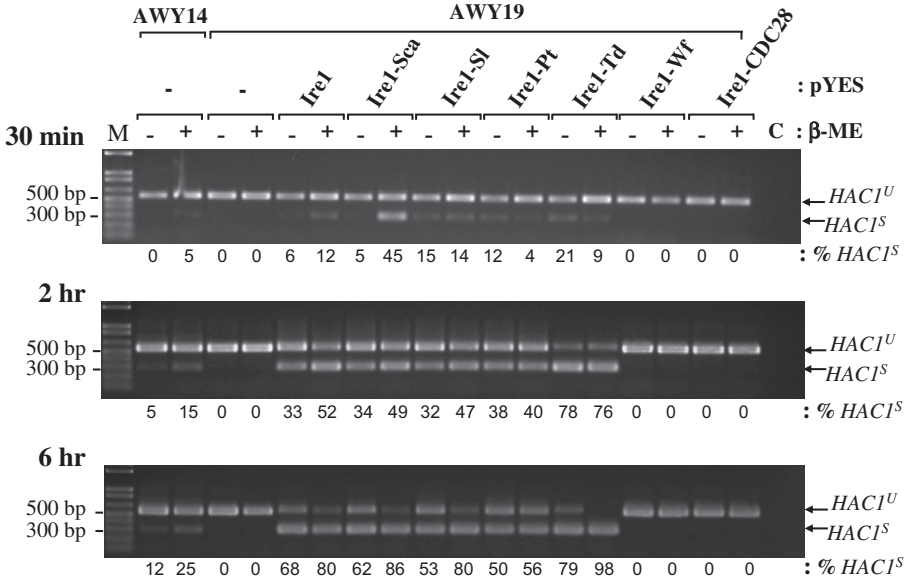

Fig. 4. Kinetic of *HAC1* mRNA splicing by chimeric Ire1. *AWY14* or *AWY19* strains harboring the indicated plasmids were cultured in medium containing galactose for 4 h. Status of *HAC1* mRNA splicing was monitored upon exposing to β-ME for indicated time. The efficiency of the splicing was analyzed by Scion Image program. M: 1 kb plus ladder.

The architecture of the Ire1 catalytic domain comprises kinase and RNase domains in close proximity [11,16,17,19]. The kinase activity is required to elicit the RNase during ER stress [4,12,13]. Based on the *S. cerevisiae* Ire1 crystal structure, subdomains I to VIB span the entire small domain, ATP/ADP binding cleft and the N-terminal region of the large domain. It is most likely that all of the Ire1 homologs we studied possess a functional ATP/ADP binding site. The high conservation of amino acid sequences, subdomain organization and dimer/oligomer interface among these homologs implicates that they possess similar structures and mechanisms for modulation [17]. This also emphasizes, partly if not entirely, the role of kinase domain as a molecular switch for UPR signaling.

The dramatically difference among chimeric Ire1s activity suggests that amino acid variation in certain regions may be crucial for activity. It is worth noting that sequence variations are concentrated in subdomains V and VIA (residues 749–783) which are equivalent to part of ATP/ADP binding cleft and hinge region. Of these residues, only Asn751 was shown to interact with the ribose ring of ADP whereas the functional significance of the other residues remains obscure [16]. Comparison between chimeric Ire1-Sca and Ire1, 5 of 6 substitution residues were in subdomains V and VIA, resulting in a significant increase in the UPR activity. Similarly, comparison between Ire1-Sl and Ire1-Td (91% homology), 17 of 23 residues amino acid substitutions were concentrated in the same region resulting in ~50% difference in activity. Lastly, our finding that the chimeras Ire1-CDC28 and Ire1-Wf were capable of triggering a basal level of *HAC1* mRNA splicing was unexpected. This suggests that a functional ATP/ADP binding domain per se is sufficient to elicit the RNase activity but their incompatibility within Ire1 backbone may prevent an overall conformational change required to fully activate Ire1.

In conclusion, this study provides the fist evidence showing that compatibility within Ire1 kinase subdomains is crucial for modulating its adjacent RNase activity. Unique amino acid sequences in subdomains V-VIA in the ADP/ATP binding domain may provide flexibility to fine tune the Ire1 kinase conformation that influences overall conformational plasticity of Ire1 during its transition into a fully active state.

Acknowledgements

We thank Dr. David Engelke, University of Michigan for providing yeast strains. Nucleotide sequences of these Ire1 homologs were submitted to EMBL. This work was partially supported by Thailand Research Fund to WT (BRG5080023). RJK is an Investigator of the Howard Hughes Medical Institute and supported by NIH Grants DK42394, HL52173, and PO1 HL057346.

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