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

Rubwd Mathuranyanon, Tomoko Tsukamoto, Asumi Takeuchi, Yuki Ishiwata-Kimata, Yuichi Tsuchiya, Kenji Kohno and Yukio Kimata*

ABSTRACT

Accumulation of unfolded proteins in the endoplasmic reticulum (ER) accompanies ER stress and causes the type-I transmembrane protein Ire1 (also known as ERN1) to trigger the unfolded protein response (UPR). When dimerized, the core stress-sensing region (CSSR) of Ire1 directly captures unfolded proteins and forms a higher-order oligomer, leading to clustering and activation of Ire1. The CSSR is N-terminally flanked by an intrinsically disordered subdomain, which we previously named Subregion I, in Saccharomyces cerevisiae. In this study, we describe tight repression of Ire1 activity by Subregion I under conditions of no or weak stress. Weak hyperactivation of an Ire1 mutant lacking Subregion I slightly retarded growth of yeast cells cultured under unstressed conditions. Fungal Ire1 orthologs and the animal Ire1 family protein PERK (also known as EIF2AK3) carry N-terminal intrinsically disordered subdomains with a similar structure and function to that of Subregion I. Our observations presented here cumulatively indicate that Subregion I is captured by the CSSR as an unfolded protein substrate. This intramolecular subdomain interaction is likely to compromise self-association of the CSSR, explaining why Subregion I can suppress Ire1 activity when ER-accumulated unfolded proteins are not abundant.

KEY WORDS: Unfolded protein response, Stress response, Endoplasmic reticulum, Intrinsically disordered region, Molecular chaperone, Misfolded protein

INTRODUCTION

The endoplasmic reticulum (ER) of eukaryotic cells is a cellular compartment where secretory and membrane proteins are folded. Impaired protein folding in the ER accompanies dysfunction of the ER, namely ER stress, and evokes the unfolded protein response (UPR). The UPR is a cellular protective event through which proteins in the ER are transcriptionally induced (Mori, 2009; Walter and Ron, 2011). Ire1 (also known as ERN1) is an ER-located type-I transmembrane endoribonuclease conserved among eukaryotic organisms, and it functions as an ER-stress sensor that triggers the UPR.

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

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

And others have reported previously that Ire1 is negatively regulated by the ER-located molecular chaperone BiP (encoded by KAR2) (Bertolotti et al., 2000; Kimata et al., 2003; Kimata et al., 2004). The association of BiP with Ire1 is likely to inhibit the self-association of Ire1 in unstressed cells (Bertolotti et al., 2000), and ER stress causes dissociation of BiP from Ire1 (supplementary material Fig. S1A). The BiP-binding subdomain is located at the juxtamembrane position (Subregion V of yeast Ire1; Kimata et al., 2004) and is loosely folded (Oikawa et al., 2005). As BiP is induced by the UPR, the negative regulation of Ire1 by BiP is likely to be a feedback-control system (Pincus et al., 2010).

Metazoan cells also carry another Ire1-family ER-stress sensor called PERK (also known as EIF2AK3) that attenuates protein synthesis upon ER stress (Harding et al., 1999). Ire1 orthologs and PERK commonly possess juxtamembrane BiP-binding subdomains and highly conserved regions corresponding to the CSSR (supplementary material Fig. S1B; Liu et al., 2000; Kimata and Kohno, 2011). Thus, we believe that Ire1 orthologs and PERK are regulated and activated in a similar manner.

In addition, PERK and yeast Ire1, but not higher eukaryotic Ire1 orthologs, have unconserved subdomains at the N-terminus...
The NUCR of yeast Ire1 is called Subregion I (supplementary material Fig. S1A,B; Kimata et al., 2004), and it appears to be intrinsically disordered, as Subregion I is susceptible to partial proteolysis (Oikawa et al., 2005). In the present study, we describe the physiological importance and molecular mechanism of tight negative regulation of Ire1 by the NUCR.

RESULTS

Subregion I suppresses yeast Ire1 activity

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

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

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

Does the ΔI single mutation exhibit any apparent phenotype? The UPRE-lacZ reporter assay did not reveal a difference in activity between wild-type Ire1 and ΔI Ire1 (Fig. 1B, compare lane 2 to 1). We then employed another highly sensitive version of the UPR reporter assay in which expression of the lacZ gene was controlled under five tandem copies of the UPRE [(UPRE)5-LacZ;...
Promlek et al., 2011]. This method allowed us to monitor weak induction of the UPR at high resolution and to observe slightly higher ΔI-Ire1 activity than that of wild-type Ire1 in unstressed cells (Fig. 1D). As shown in supplementary material Fig. S2A, the ΔI or the ΔV mutations did not considerably affect cellular abundance of Ire1.

According to Shaner et al. (Shaner et al., 2013), mNeonGreen is a bright green fluorescent protein that works as a superior substitute for the Aequeorea GFP derivatives. In the experiments shown in Fig. 1E,F, mNeonGreen-tagged Ire1 or its ΔIΔV mutant version was expressed from a single-copy plasmid under control of the authentic ire1 promoter. Both wild-type Ire1 and ΔIΔV Ire1 distributed diffusely, probably on the ER, under unstressed conditions, whereas they exhibited dot-like distribution in response to ER stress caused by dithiothreitol (DTT; a disulfide reducing agent). This finding suggests that neither Subregion I nor V is involved in the cluster-formation step of Ire1. It should be noted that our observation presented here is inconsistent with our previous report in which ΔIΔV Ire1 constitutively clustered even under unstressed conditions when artificially overexpressed from a multicopy plasmid (Kimata et al., 2007).

Next, we directly checked the splicing of HAC1 mRNA to compare activities of wild-type Ire1 and ΔI Ire1 in cells (Fig. 2A). Supporting the result shown in Fig. 1D, the ΔI single mutation weakly enhanced HAC1 mRNA splicing under unstressed conditions (Time 0). Moreover, whereas UPR activation profiles following treatment with 3 mM DTT were almost equal in cells expressing wild-type ire1 or the ΔI mutant at the early time-points (15 min to 1 h), attenuation of HAC1 mRNA splicing under long-term stress (2–5 h) appeared to be slightly retarded by the ΔI mutation.

We then performed the same experiment as that shown in Fig. 2A using not only wild-type ire1 and ΔI ire1 cells but also ΔV-ire1 and ΔIΔV-ire1 cells (Fig. 2B). As reported previously (Pincus et al., 2010; Ishiwata-Kimata et al., 2013), the ΔV mutation also caused an impaired attenuation of Ire1 activity upon prolonged ER stress. The HAC1 mRNA splicing assay, as well as the UPRE-lacZ reporter assay shown in Fig. 1, demonstrated an aggravated hyperactivation of Ire1 resulting from the combination of the ΔI and the ΔV mutations.

![Fig. 2. Timecourse profile of wild-type, ΔI, ΔV and ΔIΔV ire1 activity.](Image)

After transformation with a single-copy ire1 plasmid (pRS313-IRE1; wild type) or its mutants, KMY1516 ire1ΔΔΔ cells were stressed with 3 mM DTT for the indicated times. HAC1 mRNAs were then amplified by RT-PCR from total RNA samples to obtain the HAC1 mRNA splicing efficiency values, which are expressed as the mean±s.d. from three independent transformant clones.

Table 1. Growth retardation of unstressed yeast cells due to the ΔI, the ΔV or the ΔIΔV mutations

<table>
<thead>
<tr>
<th></th>
<th>Abundance relative to wild-type cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔI-IRE1</td>
<td>50.8±6.8</td>
</tr>
<tr>
<td>ΔV-IRE1</td>
<td>48.1±3.6</td>
</tr>
<tr>
<td>ΔIΔV-IRE1</td>
<td>47.4±5.6</td>
</tr>
</tbody>
</table>

The IRE1 plasmid (pRS313-IRE1; wild type) and its mutants (ΔI, ΔV or ΔIΔV) were used to transform KMY1516 ire1ΔΔΔ cells. The resulting cells expressing wild-type Ire1 or mutant Ire1 (2×10⁶ cells each) were mixed and cultured for a 1-day incubation, which was followed by continuous cycles of 1/32 dilutions and 1-day incubations of the culture. The abundance of cells expressing mutant Ire1 relative to that of cells expressing wild-type Ire1 was determined as described in Materials and Methods, and the values are presented as the mean±s.d. from three independent cultures in which independent transformant clones were inoculated.

In the experiment shown in Table 1, we checked whether the ΔI mutation affects cellular growth under unstressed conditions. Cells expressing wild-type ire1 and those expressing ΔI-ire1 were mixed and cultured for a long duration to observe subtle differences in the growth rate between the two cell lines. Then, their relative abundance was monitored, which indicated that the ΔI mutation retarded cellular growth. The ΔV and the ΔIΔV mutations also caused growth retardation.

Primary structural properties of Subregion I

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

As illustrated in Fig. 3A, the Subregion I 60-amino-acid portion was partitioned into six 10-amino-acid segments (Segments 1–6; see supplemental material Table S1), which were serially deleted.

![Image 95x91 to 517x274]
from ΔV Ire1 (refer to supplementary material Table S2 for the resulting amino acid sequences). We observed that ΔV Ire1 was not as highly activated as ΔΔV Ire1 in unstressed cells even when carrying any of the partial deletions (Fig. 3B). Nevertheless, Fig. 3B also shows that the Δ4–6, Δ3–4 and Δ4 mutations, but not the Δ1–3, Δ1–2, Δ5–6 or Δ3 mutations, partially activated ΔV Ire1. Therefore, no specific Subregion I sequence was absolutely required for the Ire1-suppressing ability, whereas Segment 4 appears to be relatively important. All Ire1 mutants responded well to ER stress induced by tunicamycin (Fig. 3C). Moreover, these partial deletion mutations did not considerably affect cellular abundance of ΔV Ire1 (supplementary material Fig. S2B).

Two of our previous reports (Kimata et al., 2004; Oikawa et al., 2005) and a web-based computer prediction (supplementary material Fig. S3A; Ishida and Kinoshita, 2007) strongly suggest that Subregion I is intrinsically disordered. We next replaced the Subregion I 60-amino-acid portion of ΔV Ire1 with other 60-amino-acid sequences to determine whether intrinsically disordered

![Graphs showing partial deletion and substitution mutations of Subregion I](https://example.com/graph.png)

**Fig. 3.** Partial deletion and substitution mutations of Subregion I. (A) The Subregion I 60-amino-acid (a.a.) portion (amino acids 32–91) was partitioned into six 10-amino-acid segments (Segments 1–6), which were serially deleted from ΔV Ire1 as illustrated. (B,C) A similar experiment and data presentation to that shown in Fig. 1B,C was performed using the Ire1 mutants shown in panel A. (D,E) A similar experiment and data presentation to that shown in Fig. 1B,C was performed using the ΔV ire1 variants on which the Subregion I 60-amino-acid portion was replaced with San1 IDR, Top2 IDR, a six tandem repeat of the 5-amino-acid peptide GGGSS or the three tandem repeat of Segment 4. Data in B–E show the mean ± s.d.
unrelated peptides could work as Subregion I (refer to supplementary material Table S3 for amino acid sequences of the peptides). As shown in Fig. 3D, the intrinsically disordered region (IDR) of the unrelated protein San1 (Rosenbaum et al., 2011) suppressed Ire1 activity in unstressed cells when substituted for Subregion I (compare column 3 to columns 1 and 2). Moreover, the IDR of Top2 (Berger et al., 1996) and a six tandem repeat of a 5-amino-acid peptide GGGSS also exhibited Ire1-suppressing ability (columns 4 and 5), although less potently. Notably, a three tandem repeat of Segment 4 had stronger Ire1-suppressing ability than that of the authentic Subregion I (compare columns 6 and 1). (Segment 4)3 ΔV Ire1, as well as the other Ire1 mutants employed here, responded well to tunicamycin-induced ER stress (Fig. 3E). Moreover, the cellular abundance of (Segment 4)3 ΔV Ire1 did not differ from that of the other Ire1 mutants (supplementary material Fig. S2C).

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

We then asked whether the NUCRs of the fungal Ire1 ortholog and mammalian PERK, which are predicted to be intrinsically disordered (supplementary material Fig. S3B), also have the Subregion-I-like Ire1-suppressing ability. Thus, we constructed yeast ΔV Ire1 chimeric mutants in which the Subregion I 60-amino-acid portion was replaced with similar-length (52-amino-acid) NUCR sequences derived from mammalian PERK or Aspergillus oryzae Ire1 (refer to supplementary material Table S3 for the amino acid sequences). According to the UPRE-lacZ reporter assay results shown in Fig. 4A, the chimeric constructs were less active than ΔV Ire1, which carried the authentic Subregion I, in unstressed cells, whereas they responded well to ER stress (Fig. 4B). These chimeric mutations did not considerably affect cellular abundance of ΔV Ire1 (supplementary material Fig. S2C,D). This observation strongly suggests a potent function of the NUCRs in suppressing Ire1 activity.

Next, the 52-amino-acid NUCR sequences were partitioned into two half-length segments, which were substituted for the Subregion I 60-amino-acid portion of ΔV Ire1 (Fig. 4C; see supplemental material Table S3). We then observed that, in unstressed cells, the posterior segments commonly suppressed Ire1 function to a greater extent than the anterior segments did.

![Fig. 4. Replacement of yeast Ire1 Subregion I with the NUCRs of other IRE1 family proteins.](image)

(A,B) A similar experiment and data presentation to that shown in Fig. 1B,C was performed using the ΔV Ire1 variants on which the Subregion I 60-amino-acid (a.a.) portion was chimerically replaced with the NUCRs of mouse PERK (mPERK), human PERK (hPERK) or Aspergillus oryzae Ire1 (AIre1). (C) As illustrated, the NUCRs of the other IRE1 family proteins (orange colored) were divided into anterior and posterior portions and substituted for the Subregion I 60-amino-acid portion of ΔV Ire1.

(D) A similar experiment to that shown in Fig. 1D was performed using the Ire1 mutants shown in panel C. Data are normalized to that of the ΔV-IRE1 samples (set to 1.0). Data in A,B,D show the mean ± s.d.
(Fig. 4D, compare column 3 to 2, 5 to 4 and 7 to 6). Notably, the posterior 30-amino-acid segment had an Ire1-suppressing ability that was greater than that of the anterior 30-amino-acid segment also in the case of authentic yeast Ire1 Subregion I (Fig. 3B, compare column 3 to 4). Thus, we think that the NUCRs have a common structural feature that cannot be predicted from a simple sequence comparison.

We then asked whether mammalian PERK is hyperactivated by the deletion mutation of its NUCR. In the experiment shown in Fig. 5, murine PERK-expression plasmids were transfected into a murine cultured cell line, NIH3T3. The pcDNAmPERK-Myc (Harding et al., 1999) was used for overexpression of Myc epitope-tagged murine PERK (PERK–Myc) under control of the strong CMV promoter. We also employed a truncated version of this plasmid for expression of a mutant form of PERK–Myc not carrying NUCR (the ΔNUCR mutation). We then monitored phosphorylation of eIF2α (also known as EIF2S1), which is the direct phosphorylation target of PERK. As shown in Fig. 5A,C, the phosphorylation level of eIF2α seemed to be equally induced by transfection of either the PERK–Myc plasmid or its ΔNUCR version (compare lanes 1–6 to 7–9). Probably because of the endogenous PERK protein, DTT treatment induced eIF2α phosphorylation even in the case of the empty-vector-transfected cells (Fig. 5A–C, compare lanes 16–18 and 25–27 to 7–9). Importantly, as compared with the wild-type PERK–Myc-transfected or the empty-vector-transfected cells, cells expressing the ΔNUCR mutant version of PERK–Myc exhibited a higher-level phosphorylation of eIF2α upon weak ER stress induced by 0.2 mM DTT (Fig. 5A,C, compare lanes 13–15 to 10–12 and 16–18). A similar tendency, although less pronounced, was observed when cells were treated with 0.5 mM DTT (Fig. 5B,C, compare lanes 22–24 to 19–21 and 25–27). The anti-Myc western blot analysis of cell lysates shown in Fig. 5D indicates that wild-type PERK–Myc and its ΔNUCR version were expressed at similar levels in this transfection experiment.

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

Because no specific primary structure of Subregion I appeared to be absolutely required for the Ire1-suppressing ability, we hypothesized that the CSSR, which captures substrate peptides rather nonspecifically, was involved (Gardner and Walter, 2011). Thus, we determined whether Subregion I could be a CSSR substrate. In the experiment shown in Fig. 6A, the Gal4 DNA-binding domain was fused to the Subregion I 60-amino-acid portion and used as bait in a yeast two-hybrid assay (see supplementary material Table S4 for the amino acid sequence). To yield prey, the Gal4 activation domain was fused to a CSSR peptide or that carrying the ΔIII or M229A/F285A/Y301A (MFY) mutation, which impairs the ability of the CSSR to capture substrate peptides (supplementary material Table S5; Kimata et al., 2007; Gardner and Walter, 2011; Promlek et al., 2011). Because the tester cells carry the AUR1-C gene controlled under a Gal4-inducible promoter, they acquire resistance to aureobasidin A when the two-hybrid system works. Subregion I and the CSSR exhibited a weak two-hybrid interaction, which, as expected, was impaired by the CSSR mutations.

As demonstrated in Fig. 6B, the CSSR also captured various peptides that appeared in the experiments shown in Figs 3, 4 (see supplementary material Table S4 for the amino acid sequences). In the two-hybrid analysis shown in Fig. 6B, the length of all the bait peptides was approximately 60 amino acids because the short peptides were tandemly repeated. Considering colony appearance and size on the aureobasidin A plate, the two-hybrid interaction of the GGGSS repeat, the Top2 IDR and the Segment 1–3 peptides with the CSSR was weaker than that of the other peptides. This
observation correlates well with our aforementioned finding that the Ire1-suppressing ability of the Top2 IDR, the (GGGSS)₆ and the Segment 1–3 (D₄–₆) peptides was weaker than that of the San1 IDR, the (Segment 4)₃, the Segment 4–6 (D₁–₃), the mPERK NUCR and the hPERK peptides (Figs 3, 4).

Next, we performed an in vitro competition assay to monitor the affinity of the Subregion I segments for the CSSR. A recombinant CSSR protein tagged with the maltose-binding protein (MBP–CSSR) was expressed in Escherichia coli and purified with amylose resin (Kimata et al., 2007). As originally described by Gardner and Walter (Gardner and Walter, 2011), a fluorescently labeled CSSR substrate, ΔEspP–FAM, exhibited increased fluorescence anisotropy when it was mixed with MBP–CSSR (supplementary material Fig. S4). Then, fluorescence anisotropy of ΔEspP–FAM was measured in the presence of MBP–CSSR and a chemically synthesized peptide carrying one of the Subregion I segment sequences (see supplementary material Table S6 for the amino acid sequences), indicating that Segment 4 compromises fluorescence anisotropy (by reducing the association between ΔEspP–FAM and MBP–CSSR), more effectively than the other segments did (Fig. 6C). This finding strongly suggests high-affinity capture of Segment 4 by the CSSR.

In the experiment shown in Fig. 7A, we used the yeast two-hybrid assay to monitor self-association of the CSSR (supplementary material Tables S4, S5). A two-hybrid interaction was observed when the CSSR was employed both as bait and prey. However, adding Subregion I to the bait CSSR (Subregion I-CSSR) abolished this two-hybrid interaction, which was restored by the ΔIII or the MFY mutation of Subregion

---

**Fig. 6. Two-hybrid and in vitro interaction of the CSSR with various peptides.** (A,B) A yeast two-hybrid analysis was performed using the CSSR and its mutants as prey. The Subregion I 60-amino-acid portion (Sub-I), Segment 4 (Seg4; six tandem repeat), GGGSS (12 tandem repeat), Segments 1–3 (Segs1–3; amino acids 32–61 of yeast Ire1; two tandem repeat), Segments 4–6 (Segs4–6; amino acids 62–91 of yeast Ire1; two tandem repeat), the NUCR of human or mouse PERK (hPERK or mPERK), the Top2 IDR (Top2; two tandem repeat) and the San1 IDR (San1) were used as bait. Tester cell cultures were serially diluted tenfold, spotted on agar plates, incubated for 2–4 days and photographed. WT, wild type. (C) A fluorescently labeled peptide ΔEspP–FAM (10 μM final concentration) was mixed with MBP–CSSR (5 μM final concentration) and a competitor peptide [Segment (Seg) 2–6] and measured for fluorescence anisotropy. The resulting values from triplicate assays (mean ± s.d.) are normalized to that for the no competitor peptide condition.
I-CSSR. This finding strongly suggests that Subregion I leads to dissociation of the self-associated CSSR molecules when intramolecularly captured by the CSSR as a substrate.

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

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

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

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

The BiP–Ire1 association is also likely to contribute to maintaining Ire1 as a non-self-associated form (Bertolotti et al., 2000). Therefore, we think that Subregions I and V function as ‘double locks’ that compromise self-association of Ire1 under unstressed conditions. In the absence of this double lock, Ire1 remains as a dimer, as ΔIAV Ire1 was self-associated constitutively but clustered in response to ER stress (Fig. 1E,F; Oikawa et al., 2007). According to Gardner and Walter (Gardner and Walter, 2011), the intermolecular interaction between unfolded proteins and dimeric CSSR molecules leads to the formation of the Ire1 cluster.
In the present paper, we propose that the NUCR portions of the Ire1 family proteins, which commonly show high disorder probability values in the PfDOS analysis (supplementary material Fig. S3), are captured by the CSSR as disordered peptides to suppress Ire1 activity. By contrast, it also should be noted that the disorder probability values of a peptide do not always correlate with its ability to be captured by the CSSR and to suppress Ire1 activity. For instance, the disorder probability of Segment 4 is not particularly high compared with those of the other segments (supplementary material Fig. S3A). According to our data shown in Fig. 4D, the posterior-half segments of the NUCR portions of the Ire1 family proteins commonly suppressed Ire1 function to a greater extent than the anterior-half segments did. In agreement with this observation, the posterior-half segments of the NUCR portions of mouse and human PERK show disorder probability values that are higher than those of the anterior-half segments; A. oryzae Ire1, however, does not show such a tendency (supplementary material Fig. S3B).

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

MATERIALS AND METHODS

Yeast cultures and strains

Unless otherwise noted, yeast cells were grown exponentially at 30°C under liquid-shaking culture in synthetic dextrose medium (2% glucose, 0.66% Difco yeast nitrogen base without amino acids, appropriate auxotrophic requirements). See Mori et al. (Mori et al., 1996), Kimata et al. (Kimata et al., 2004) and Promlek et al. (Promlek et al., 2011) for congeneric haploid strains KMY1015 (MATα leu2-3,112 ura3-52 his3-120 trp1-A 901 lys2-801 ire1Δ::TRP1) and KMY1516 (MATα LEU2::UPER-GFP:::leu2-3,112 ura3-52 his3-120 trp1-A901 lys2::(UPER)3-lacZ::lys2-801 ire1Δ::TRP1). KMY1015 is a generous gift from Dr Kazutoshi Mori (Kyoto University, Japan). The MATα haploid strain Y2HGold and the yeast strain Y2HGold and the pRS313-IRE1, and then DNA fragments corresponding to the peptides shown in Fig. 6A,B. See supplementary material Tables S4 and S5 for the genotypic analysis of yeast cells in mixed culture

A mixed culture of cells expressing wild-type IRE1 (KMY1516 transformed with pRS313-IRE1) and cells expressing ΔI-IRE1 (KMY1516 transformed with the ΔI-mutant version of pRS313-IRE1) was diluted and plated on agar plates to obtain isolated colonies. We then checked 100 colonies for their IRE1 genotypes by PCR with the primer set 5′-CCATTACCTTCTCCATAC-3′ and 5′-GCAATTCTAAATTCGGTT-3′ to check the ΔI-mutant genotype to obtain the relative abundance of ΔI-IRE1 cells (%) from mixed cultures of cells expressing wild-type IRE1 cells or ΔI-IRE1 cells. We used the PCR primer set 5′-CAAGCGCTAGGAT-TTTAATGGT-3′ and 5′-AATCTTCCAGCTTATAATTCG-3′ to check the ΔI-mutant genotype to obtain the relative abundance of ΔI-IRE1 cells (%) from mixed cultures of cells expressing wild-type IRE1 cells or ΔI-IRE1 cells.

RNA analysis

After extraction from cells as described by Kimata et al. (Kimata et al., 2003), total RNA samples were used as templates for reverse transcription (RT)-PCR amplification of the HAC1 mRNA species, which was then separated by electrophoresis (Promlek et al., 2011; Ishiwata-Kimata et al., 2013). DNA fluorescence images of the resulting gels were captured and quantitatively analyzed using the LAS-4000 cooled CCD camera system. The resulting data were used to calculate the HAC1 mRNA splicing efficiency (%) from the formula 100×[(HAC1I band signal)/(HAC1I band signal+HAC1a band signal)].

Fluorescent-protein tagging and localization of Ire1

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

**UPRE-lacZ reporter assay**

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

**Yeast two-hybrid assay**

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

**Handbook, Clontech) for selecting mated cells (the growth control plates) and those containing 125 ng/ml aureobasidin A for checking the two-hybrid interaction. In the experiment shown in Fig. 7A, agar plates for checking the two-hybrid interaction did not contain histidine, because the two-hybrid interaction provides the tester diploid cells with histidine prototrophy as the two-hybrid interaction did not contain histidine.**

**In vitro competition assay for peptide binding to the CSSR**

The His-tagged MBP–CSSR protein was expressed in *E. coli* and purified using a nickel affinity column and elution buffer A [50 mM HEPES pH 8.0, 100 mM KCl, 5 mM MgCl2, 200 mM imidazole and 10% (v/v) glycerol] as reported previously (Kimata et al., 2007). A 5-carboxyfluorescein (5-FAM)-tagged peptide (ΔEspP–FAM; Gardner and Walter, 2011) was chemically synthesized by GL Biochem (supplementary material Table S6; Shanghai, China). The untagged competitor peptides NH2–KKA-[10 amino acids of Segment 2, 3, 4, 5 or 6]–AAKKK–COOH were chemically synthesized by Sigma-Aldrich (supplementary material Table S6; the peptide screening value pack). The lysine clusters on the competitor peptides were expected to serve as solubilization tags. Segment 1 was not used in this assay because of its low chemical synthesis yield. After incubating the mixture in elution buffer A for 30 min, fluorescence anisotropy was measured using the BEACONTM2000 fluorescence polarization reader (Invitrogen) at 25°C.

**Mammalian cell culturing and manipulation**

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

**Antibodies**

For western blot analysis, we used 12CA5 anti-HA mouse monoclonal antibody (Roche), 9E10 anti-e-Myc mouse monoclonal antibody (Boehringer Mannheim) and anti-εIF2α[pS45] rabbit polyclonal antibody (BioSource). As the secondary antibodies, we used goat anti-mouse IgG horse radish peroxidase (HRP)-linked antibody (Jackson ImmunoResearch) and goat anti-rabbit IgG HRP-linked antibody (Dako).

**Acknowledgements**

We thank Dr Kazutoshi Mori (Kyoto University) for materials.

**Funding**

This work was supported by the Ministry of Education, Culture, Sports, Science and Technology (MEXT)/Japan Society for the Promotion of Science (JSPS) KAKENHI (grant numbers 22657030 and 24370081 to Y.K., and 20380062 and 24228002 to K.K.). Deposited in PMC for immediate release.

**References**


Figure S1 Structure and regulation of the Ire1 family proteins

A, Regulation of yeast Ire1 by BiP association/dissociation and by the direct interaction of unfolded proteins is schematically represented. See Kimata et al. [J. Cell Biol., Vol. 167, 445–456 (2004)] for partitioning of the Ire1 luminal domain into Subregions I–V. Subregions II–IV are tightly folded and correspond to the CSSR (a.a. 112–454). Upon ER stress, Ire1 is clustered and activated by dissociation of BiP from Subregion V (a.a. 455–524) and interaction of unfolded proteins with the CSSR [Kimata and Kohno, Curr. Opin. Cell. Biol., Vol. 23, 135–142 (2011)]. Meanwhile, the regulatory function of Subregion I (a.a. 32–111) has been obscure.

B, Structural comparison of the luminal domains of Ire1-family proteins is schematically represented. The CSSR show low but significant homology, whereas the amino-acid sequences of the NUCRs and the BiP-binding sites are less conserved. Highly conserved motifs 1–4 observed on the CSSRs [Liu et al., J. Biol. Chem., Vol. 275, 24881–24885 (2000)] are marked with red lines. Modified from Kimata and Kohno [Curr. Opin. Cell. Biol., Vol. 23, 135–142 (2011)].
**Figure S2**  Cellular expression levels of Ire1 and its mutants

KMY1015 *ire1Δ* cells were cultured under unstressed conditions after transformation with pRS315-IRE1-HA [a yeast single-copy plasmid carrying the wild-type (WT) *IRE1* gene modified to harbor the C-terminal hemagglutinin (HA) epitope; Kimata et al., J. Cell Biol., Vol. 167, 445–456 (2004)] or its mutants. Cell lysates equivalent to 10 µg total protein were analyzed by anti-HA Western blotting as described in Kimata et al. [J. Cell Biol., Vol. 167, 445–456 (2004)]. The two panels in B are from the same gel. The two panels in C are also from the same gel.
Figure S3

Figure S3 Protein structure prediction of the luminal regions of Ire1 family proteins carrying the NUCRs Amino acid sequences of the luminal regions of yeast Ire1 (A) and others (B) were inputted into PrDOS (http://prdos.hgc.jp/cgi-bin/top.cgi).
**Figure S4**

In vitro association of the CSSR with a fluorescently labeled peptide

The ΔEspP-FAM fluorescently labeled peptide (10 µM final) was mixed with MBP-CSSR, and fluorescence anisotropy was measured. The resulting values from triplicate assays (means plus standard deviations) are normalized against that for the maximum value.
### Supplementary tables

Amino-acid sequences of the Subregion-I mutations, the two-hybrid preys and baits and the synthetic peptides employed in this study.

#### Table S1. The Subregion-I 60-a.a. portion of wild-type yeast Ire1

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSRQRQIVEDEVASTKKNFNYGVDNINSPIPAPRTTEGLPNMKLSSYPTPNLLNTADNR</td>
<td>Segments 1, 3 and 5 are blue-colored. Segment 4 is orange-colored. Segments 2 and 6 are brown-colored.</td>
</tr>
</tbody>
</table>

#### Table S2. The partial-deletion mutations of the Subregion-I 60-a.a. portion

| Δ1-3 | IPAPRTTEGLPNMKLSSYPTPNLLNTADNR |
| Δ4-6 | TSRQRQIVEDEVASTKKNFNYGVDNISP |
| Δ1-2 | YGVKKNINSPIPAPRTTEGLPNMKLSSYPTPNLLNTADNR |
| Δ3-4 | TSRQRQIVEDEVASTKKNFNPNNMKLSSYPTPNLLNTADNR |
| Δ5-6 | TSRQRQIVEDEVASTKKNFNYGVDNINSPIPAPRTTEGL |
| Δ3 | TSRQRQIVEDEVASTKKNFINPAPRTTEGLPNMKLSSYPTPNLLNTADNR |
| Δ4 | TSRQRQIVEDEVASTKKNFINPAPRTTEGLPNMKLSSYPTPNLLNTADNR |

Segments 2, 4 and 6 are blue-colored. Segment 4 is orange-colored. Segments 2 and 6 are brown-colored.

#### Table S3. The peptides substituted on the Subregion-I 60-a.a. portion of ΔV Ire1

| San1-IDR | TSVPTIGNASSGQMLSRTGGLFPQNGQPLHNPVRLPPNDSRNGVNGPSSTTQNPTR |
| Top2-IDR | TSDKDYIDLAFSSKKADDKKEWLRQYEPTR |
| (GGGSS)$_n$ | TSGGSSGSGGGSSGGSSGGGGSSGGGGSSGT |
| (Segment 4)$_n$ | TSIPAPRTTEGLIPAPRTTEGLIPAPRTTEGL |
| mPERK-NUCR | TSVAPARSLAPSETVGGLAAAGPTSAARVPVATAEVTEDAEALPAAAGETR |
| hPERK-NUCR | TSGRARGLPAPTAAAFGLGAAAGPTSAATRVPAAGAVAAAEVTVEDAEALPAAATR |
| Alre1-NUCR | TSQLQPEHHDLPSTLSVPLGSTGHAVGKLYTPLNVSSTDASALT MALAGPGRTR |
| mPERK-NUCR anterior 26 a.a. | TSVAPARSLAPSETVGGLAAAGPTSTR |
| mPERK-NUCR posterior 26 a.a. | TSAARVPVATAEVTVEDAEALPAAAGETR |
| hPERK-NUCR anterior 26 a.a. | TSGRARGLPAPTAAAFGLGAAAGPTSAATR |
| hPERK-NUCR posterior 26 a.a. | TSTRVPAAGAVAAAEVTVEDAEALPAAATR |
| Alre1-NUCR anterior 26 a.a. | TSQLQPEHHDLPSTLSVPLGSTGHAVGKTR |
The amino-acid residues corresponding to the artificially added restriction sites (SpeI (TS) and MluI (TR)) are green-colored. Segment 4 is orange-colored.

Table S4. The two-hybrid bait peptides

<table>
<thead>
<tr>
<th>Sub-I (the Subregion-I 60-a.a. portion)</th>
<th>EFMSSRRQIVEDEVASTKCLNFNYGVDKNINSPIPAPRTEGLPNMKLSSYPTPNLLNTADNRGS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seg4 (six tandem repeats of Segment 4 (IPAPRTTEGL))</td>
<td>EFM1PAPRRTTEGLIPAPRRTTEGLIDIPARTTEGLIPAPRTTEGLIPAPRTTGS</td>
</tr>
<tr>
<td>GGGSS (tandem repeats of GGGSS)</td>
<td>EFMSSGGSSGGSSGGSSGGSSGGSSGGSSGGSSGGSSGGSSGGSSGGIDGGGGSSGS</td>
</tr>
<tr>
<td>Seg1-3 (two tandem repeats of Segment 1 to 3)</td>
<td>EFMSSRRQIVEDEVASTKCLNFNYGVDKNINSPIDTSRRQIVEDEVASTKCLNFNYGVDKNINSPGS</td>
</tr>
<tr>
<td>Seg4-6 (two tandem repeats of Segment 4 to 6)</td>
<td>EFM1PAPRRTTEGLPNMKLSSYPTPNLLNTADNRIDIPAPRTTEGLPNMKLSSYPTPNLLNTADNRGS</td>
</tr>
<tr>
<td>hPERK (the NUCR of human PERK)</td>
<td>EFMGRARGLPAATCAAGGIAAGAPTSRVPAAAGAVAAEVTEDAEALPAAGGQEPAGGS</td>
</tr>
<tr>
<td>mPERK (the NUCR of mouse PERK)</td>
<td>EFMVAPARSLAPASETVGLGAAGAAAPTSARVPAAVATAEVTEDAEALPAAGGQEPAGGS</td>
</tr>
<tr>
<td>Top2 (two tandem repeats of the Top2 IDR)</td>
<td>EFMKYDYMFLAFSSKMADDKREWQLYEPIDDDXYDILDAFSKKADDKREWQLYEPAGGS</td>
</tr>
<tr>
<td>San1 (the San1 IDR)</td>
<td>EFMVPTIGNASSGEGMLSRGFLVPQNGQPLHNPVRLPNDSDRUNGVPSSPTQNNSNGS</td>
</tr>
<tr>
<td>CSSR</td>
<td>PWSMVLNEISLSDLILDAADVGEGLHAVDNRNHIIWSTEMFNLQWLEIEQEPSRELTYETLTIIEPPGDMGINYFNAGQILQKLPSIRQLVSTPSPLHLKTNIVVNSKGIKVEDKertosonent_i_14294389129160</td>
</tr>
<tr>
<td>Sub-I-CSSR (Subregion 1 to 4)</td>
<td>PWSMSSRRQIVEDEVASTKCLNFNYGVDKNINSPIPAPRTEGLPNMKLSSYPTPNLLNTADNRANKKGRAANISVPYLENSRLNELSLSIDIAADVGEGLHAVDNRNHIIWSTEMFNLQWLEIEQEPSRELTYETLTIIEPPGDMGINYFNAGQILQKLPSIRQLVSTPSPLHLKTNIVVNSKGIKVEDKertosonent_i_14294389129160</td>
</tr>
<tr>
<td>The ΔIII mutant of Sub-I-CSSR</td>
<td>PWSMSSRRQIVEDEVASTKCLNFNYGVDKNINSPIPAPRTEGLPNMKLSSYPTPNLLNTADNRANKKGRAANISVPYLENSRLNELSLSIDIAADVGEGLHAVDNRNHIIWSTEMFNLQWLEIEQEPSRELTYETLTIIEPPGDMGINYFNAGQILQKLPSIRQLVSTPSPLHLKTNIVVNSKGIKVEDKertosonent_i_14294389129160</td>
</tr>
</tbody>
</table>
The amino-acid residues corresponding to the artificially added restriction sites (EcoRI (EF), BamHI (GS), Clal (ID) and NcoI (PW)) are green-colored. Segment 4 is orange-colored. Segments 2 and 6 are brown-colored. The CSSR is purple-colored. The mutation sites of the MFYT mutation are yellow-colored.

**Table S5. The two-hybrid prey peptides**

<table>
<thead>
<tr>
<th>CSSR</th>
<th>Segment 1</th>
<th>Segment 2</th>
<th>Segment 3</th>
<th>Segment 4</th>
<th>Segment 5</th>
<th>Segment 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSMRSLNELSLSDILIAADVEGGLHAVDRRNGHIWSTEPENFQPLIEIQ</td>
<td>EFSRLLETYELIIEPFQGDIYNYFAHQQKLPFLRSQVLSTPLHLKT</td>
<td>NIVVNDGKIVEDVGYTSMTMTYTIMLNGEIISAQPGSKNGYFGS</td>
<td>QSVDCSQPEEKIKLQECENIMIVIGKTIIFELG1H5YSGDASYNVYSTWQQNV</td>
<td>LDVPLALQNTSFKDGMCIAPFRDKSLLASDLDFRIARWSTPFTPIGVGL</td>
<td>FDVFNDRTNENILYPHEPSFPDCGHESISSNKNVLQDTNLSWFLASQSFNP</td>
<td>PSLVEASIRYASSDRWVSSIFEDTLFKNAIMGVHQIYNNEYDter</td>
</tr>
</tbody>
</table>

The amino-acid residues corresponding to the artificially added restriction site (BamHI (GS)) are green-colored. The CSSR is purple-colored. The mutation sites of the MFYT mutation are yellow-colored.

**Table S6. The chemically synthesized peptides**

<table>
<thead>
<tr>
<th>Fluorescently labeled peptide</th>
<th>M KKHKRLALCFLGLLQSSY SFAK-[5-FAM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Competitor peptides</td>
<td></td>
</tr>
<tr>
<td>Segment 2</td>
<td>KKKAVASTKKLNFAAKKK</td>
</tr>
<tr>
<td>Segment 3</td>
<td>KKA YGVDKNINS PAKKKK</td>
</tr>
<tr>
<td>Segment 4</td>
<td>KKA I PAPRT TEGLAAKKK</td>
</tr>
<tr>
<td>Segment 5</td>
<td>KKA PN MKLSS YTPA AAKKK</td>
</tr>
<tr>
<td>Segment 6</td>
<td>KKA PN LNT ADN RAAKKK</td>
</tr>
</tbody>
</table>

Segments 2 and 6 are brown-colored. Segments 3 and 5 are blue-colored. Segment 4 is orange-colored.