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Oligomerization of the transmembrane domain of IRE1α in SDS micelles

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ABSTRACT

IRE1α (Inositol-requiring enzyme 1 α), an endoplasmic reticulum (ER)-resident sensor for mammalian unfolded protein response, is a type I transmembrane protein which has a bifunctional enzyme containing kinase and RNase domains. Although the luminal domain and cytosolic domain of IRE1α are thought to play crucial roles in regulating the protein activity, no functional and structural studies of the transmembrane domain exist thus far. Herein, using CD spectroscopy, we report that the transmembrane domain of the IRE1α is alpha-helical in a membrane-like environment. In addition, SDS–PAGE and FRET analyses support that the transmembrane domain forms oligomers in SDS micelles. Thus, the study would provide insights into how the transmembrane domain plays a role in regulating the IRE1α protein activity.

1. Introduction

The endoplasmic reticulum (ER) is a major compartment within the eukaryotic cell responsible for folding of secretory and transmembrane proteins. Perturbation of the protein folding environment in the ER leads to the accumulation of unfolded proteins, eventually causing ER stress. This ER stress is sensed by the cells through three ER transmembrane proteins, IRE1α (Inositol-requiring enzyme 1 α), PERK (protein kinase R (PKR)-like ER kinase), and ATF6α (activating transcription factor 6 α) [1]. They activate signaling processes to restore ER homeostasis, and are collectively termed the unfolded protein response (UPR). UPR signaling coordinates the cellular response by down-regulating protein translation, enhancing expression of ER chaperone proteins that promote protein refolding, and activating proteases involved in the degradation of misfolded proteins. When these corrective actions are insufficient to attenuate ER stress, the UPR switches to a pro-apoptotic mode [2].

IRE1α (Inositol-requiring enzyme 1 α) is a type I transmembrane protein with a N-terminal luminal domain as an ER stress sensor and a C-terminal cytosolic domain carrying protein kinase and endoribonuclease activities. The luminal domain associates with the BiP protein in the absence of ER stress [3]. Upon accumulation of unfolded or misfolded proteins, the BiP dissociates allowing the dimerization of the luminal domain [4,5]. This promotes the face-to-face dimerization of the kinase domain, facilitating trans-autophosphorylation and subsequently activating the RNase domain [6]. The activated IRE1α cleaves a 26-nucleotide intron from the XBP1 (X-box binding protein 1) mRNA which generates an active transcription factor (XBP1s) [7]. The sliced transcription factor XBP1s activates genes that enhance ER protein-folding capacity and degrade unfolded or misfolded ER proteins [7,8].

The dimerization of the luminal and of the cytosolic domains was found to upregulate IRE1α activity. The interface of the luminal domain dimerization is stabilized by hydrogen bonds and hydrophobic interactions and disruption of these interactions reduces both the autophosphorylation and the RNase activities of IRE1α in cells [5,9]. The structure of the IRE1α cytosolic domain was recently resolved and the dimerization interface was identified [6]. Mutation of the residues implicated in the face-to-face dimerization interface significantly diminished autophosphorylation of IRE1α, thereby lowering the RNase activity as compared with the wild-type protein [6]. Thus the dimerization of either the luminal or the cytosolic domains is crucial for regulating IRE1α enzymatic activities. Although conformational and functional studies on the dimerization of the luminal and the cytosolic domains of IRE1α have been performed [3,5,6,10,11], to the best of our knowledge, no information exists on the transmembrane (TM) domain of the IRE1α protein. Information on the TM domain of IRE1α could provide important insight into possible connections between the dimerization of the luminal and the cytosolic domains.

IRE1α is thought to have similar mechanisms to receptor tyrosine kinases (RTKs), where the dimerization of the N-terminal extracellular domain is a universal activation mechanism for the regulation of cytosolic domain activities. RTKs, the largest group of type I transmembrane proteins, transmit signaling from the

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extracellular domain to the cytosolic domain through lateral movement (dimerization) of the TM domain [12,13]. Dimerization of the transmembrane domain plays a key role in bringing the C-terminal cytosolic domains into juxtaposition and stabilizing the dimerization conformation. Thus this evokes whether the TM domain of IRE1α has a similar role. In other words, can the IRE1α TM domain dimerize and thus potentially facilitate the stabilization of the dimer conformation of the IRE1α protein?

In the present study, we report the TM domain of IRE1α forms oligomers in SDS micelles. Furthermore, we found that the secondary structure of the TM domain is alpha-helical, maintaining native-like structure.

2. Materials and methods

2.1. Materials

TFE (2,2,2-trifluoroethanol) and SDS were purchased from Sigma–Aldrich. All other reagents were of the highest grade commercially available.

2.2. Peptide synthesis and purification

Unlabeled peptide and N-terminal labeled-peptides with FITC (Fluorescein isothiocyanate) or Rhodamine B were synthesized from Peptide 2.0 Inc (Chantilly, VA, USA). The peptides were purified using reverse-phase HPLC and confirmed by MALDI-TOF mass spectrometry.

2.3. Sample preparation

To prepare TM peptides in aqueous solutions of SDS, the peptides were initially dissolved in TFE and dried under a stream of nitrogen. The dried peptides were then mixed with a freshly made 20 mM SDS solution containing 10 mM Tris–HCl (pH 7.0) and 50 mM NaCl. The samples were sonicated for 15 min and incubated for 1 h at room temperature before further experiments.

2.4. Circular dichroism (CD) spectroscopy

CD spectra were recorded with Chirascan™ CD Spectrometer (Applied Photophysics) in the 190–260 nm regions (0.5 nm step, 10 nm/min, 1 nm slit width). The 0.1 cm path-length quartz cell was used for the measurements. Baseline was measured for TFE and SDS micelles without the peptide and subtracted from the corresponding sample spectrum. Peptide secondary structure was analyzed with CDNN program [14].

2.5. SDS–PAGE

Different amounts of the TM peptide were dissolved in 20 mM SDS solution containing 10 mM Tris–HCl (pH 7.0) and 50 mM NaCl and mixed with a SDS–PAGE loading buffer (Cell signaling). SDS–PAGE analysis was performed using precast 12% NuPAGE Bis-Tris mini gels in MES-SDS running buffer (Invitrogen). Spectra Multicolor Low Range Protein Ladder (Thermo Fisher Scientific Inc.) was used as a molecular weight marker. The peptides were visualized with Silver staining (Thermo Fisher Scientific Inc.).

2.6. Förster resonance energy transfer (FRET) measurements

FRET measurements were performed at room temperature using a FluoroMax-4 (Horiba). For FITC/Rhodamine B labeled peptide samples, the excitation wavelength was set at 439 nm and emission spectra were collected from 480 to 640 nm with both the excitation and emission slit width set at 5 nm. FRET efficiency was calculated from measurements of donor intensity at 515 nm in the absence and presence of the acceptor: $FRET\ efficiency = (ID - IDA)/(ID)$, where ID and IDA are the donor intensities of samples containing only donor-labeled peptides and samples with both donor- and acceptor-labeled peptides, respectively. The contribution to the emission at 515 nm from the direct excitation of the acceptor was removed by subtracting the spectra of samples containing only acceptor-labeled peptides.

3. Results and discussion

The sequence of the IRE1α TM domain has not been experimentally determined but its potential sequence (444–464 aa) was obtained from UniProt (http://www.uniprot.org/uniprot/O75460) (Table 1). We additionally simulated the potential TM domain with several web-based programs, i.e. DAS [15], HMMTOP [16], OCTOPUS [17], Phobius [18], SOSUI [19], TMHMM [20]. The N-terminus of the TM domain was predicted to begin at M444 expect with DAS which predicted A445, but the C-terminal residue varied from I462 to L466, depending on the simulation program (Table 1). Residues 444–466 were chosen as the transmembrane domain, thus an extended TM model peptide was designed (A435–H469) that included several N-terminal and C-terminal flanking residues in IRE1α.

SDS is a well-characterized system applied to mimic transmembrane proteins, and is commonly used to uncover helix–helix

Table 1
Prediction of IRE1α Transmembrane (TM) Segment from various prediction programs.

Source	Sequence[a]
UniProt	^{435}APVDSMLKD<u>MATIILSTFLLIGWVAFIITY</u>PLSMH^{469}
DAS	^{435}APVDSMLKD<u>MATIILSTFLLIGWVAFIITYP</u>LSMH^{469}
HMMTOP	^{435}APVDSMLKD<u>MATIILSTFLLIGWVAFIITYPL</u>SMH^{469}
OCTOPUS	^{435}APVDSMLKD<u>MATIILSTFLLIGWVAFIITY</u>PLSMH^{469}
Phobius	^{435}APVDSMLKD<u>MATIILSTFLLIGWVAFIITYPL</u>SMH^{469}
SOSUI	^{435}APVDSMLKD<u>MATIILSTFLLIGWVAFIITYPL</u>SMH^{469}
TMHMM	^{435}APVDSMLKD<u>MATIILSTFLLIGWVAFII</u>TYPLSMH^{469}

[a] Underlined sequences represent residues predicted by each prediction source as occurring in the protein TM segment.

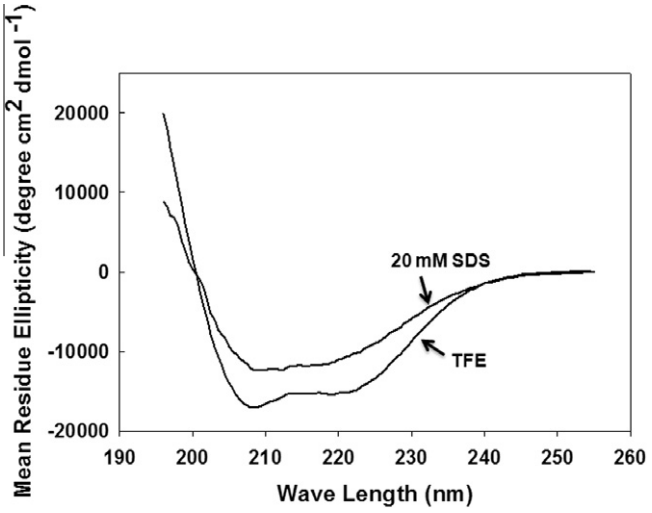

Fig. 1. Secondary structure of IRE1α TM model peptide determined by circular dichroism spectroscopy. CD spectra are shown for the peptide in TFE and in detergent buffer containing 20 mM SDS, 10 mM Tris–HCl (pH 7.0), 50 mM NaCl. The peptide concentration was 40 μM in each case.

interactions of TM domains given its ability to maintain the native secondary and tertiary structures of the TM segments [21–24]. However, long hydrophobic peptides frequently result in (non-native) β-sheet characteristics, which can lead to insolubility and unstable peptide solutions. Using CD spectroscopy, we checked whether the model peptides are helical in the SDS micelles. The CMC (critical micelle concentration) of SDS at 50 mM NaCl is reported to be 2.25 mM [25]. Therefore we used 20 mM SDS, which is well above the CMC, in a buffer solution (10 mM Tris (pH 7.0), 50 mM NaCl). In addition, TFE (2,2,2-trifluoroethanol), known to promote α-helix formation, was used as a positive control environment for the TM peptide. The CD spectra of the TM peptide in TFE showed a characteristic α-helical profile with negative maxima at 208 and 222 nm (Fig. 1). Likewise, the CD spectrum of the TM peptide in 20 mM SDS also showed α-helical conformation. CDNN (the CD spectrum deconvolution software) analysis [14] of the spectra revealed a high helical content of the TM peptide in SDS and TFE, 43.97 ± 0.47% and 54.80 ± 3.63%, respectively.

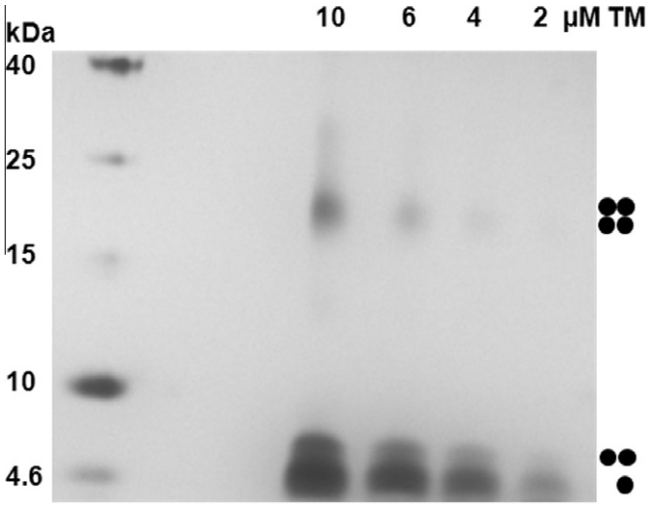

Fig. 2. Oligomer formation of IRE1α TM model peptide by SDS–PAGE. IRE1α TM model peptide dissolved in 20 mM SDS-containing sample buffer were loaded onto 12% NuPage gel at different concentrations (2, 4, 6, 10 μM). Bands were visualized using silver staining.

Since SDS–PAGE is commonly used to qualitatively examine the oligomeric states of the TM domain, we performed SDS–PAGE to determine whether the IRE1α TM domain oligomerizes in SDS. The results at different peptide concentrations (2, 4, 6, 10 μM) are presented in Fig. 2. The TM peptides migrated as three distinct forms, corresponding to a monomer (3.91 kDa), dimer (7.82 kDa) and tetramer (15.64 kDa) fractions on 12% NuPAGE gel. In addition, the dimer and tetramer fractions increased with peptide concentration, suggestive of the existence of dimeric and tetrameric TM peptide species. These results support that IRE1α TM domain oligomerizes in SDS in the absence of the luminal and cytosolic domains.

To confirm the oligomerization status, we further employed FRET analysis. FRET is used to evaluate the self-association of the TM peptides in SDS micelles [24,26,27]. The selected fluorophores (FITC (Fluorescein isothiocyanate) and Rhodamine B) are a well-known donor–acceptor pair with a R_0 (Förster radius that is a distance where FRET efficiency is 50%) of ~55 Å. SDS–PAGE results indicated similar migration of the unlabeled-TM peptide and labeled-TM peptides (FITC-TM and Rhodamine B-TM) (data not shown), demonstrating that the attachment of the fluorophores did not influence the oligomerization ability of the peptide. Fig. 3A shows typical emission spectra of the donor-only sample (FITC-labeled TM peptide) (dashed line) and the acceptor-only sample Rhodamine B-labeled TM peptide) (dotted line) in SDS micelles. However, when both the donor and acceptor are present in the sample, FRET occurs as indicated by the decrease in donor fluorescence and the appearance of sensitized acceptor fluorescence (Fig. 3A). Thus, the results suggest that the FITC- and Rhodamine B-labeled TM peptides could self-associate in SDS micelles.

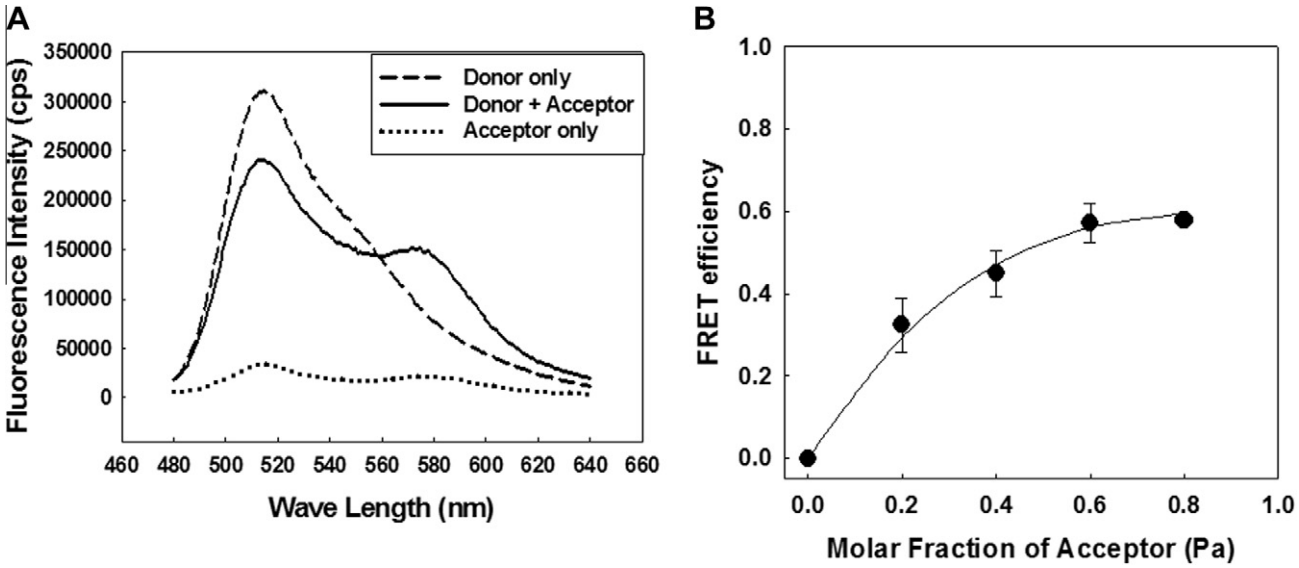

Fig. 3. Oligomer formation of IRE1α TM model peptide by FRET. (A) Fluorescence spectra of FITC/Rhodamine B-labeled TM peptides in SDS micelles. 0.75 μM of donor (FITC-TM) or/and acceptor (Rhodamine B-TM) peptides were dissolved in 20 mM SDS, 10 mM Tris–HCl (pH 7.0), and 50 mM NaCl. The excitation was fixed at 439 nm such that only the FITC was directly excited and the emission was scanned from 480 to 640 nm. The spectra were measured for the samples containing the donor and acceptor peptides (solid line), as well as control samples containing only the donor (dash line) and only the acceptor peptides (dotted line). (B) FRET analysis of IRE1α TM interactions in SDS micelles. FITC-labeled peptides (donor) at 0.75 μM were titrated with an increasing mole fraction of Rhodamine B-labeled peptides (acceptor) in 20 mM SDS, 10 mM Tris–HCl (pH 7.0), and 50 mM NaCl. The total peptide concentration was kept constant at 4 μM with the addition of unlabeled peptide. The FITC-labeled peptides were excited at 439 nm and the fluorescence intensity was measured from 480 to 640 nm. FRET efficiency was calculated and data fitted as described in the Section 2. Error bars correspond to the standard deviation of four experiments.

To further estimate the oligomer status, FRET efficiency was measured as a function of the molar fraction of the acceptor (Pa), while the concentrations of the donor and total peptide remained constant. According to the equation (FRET efficiency = $K(1-(1-Pa)^{n-1})$, where Pa is the molar fraction of the acceptor, n is the number of molecule, K is a constant), a linear dependence between the FRET efficiency and the molar fraction of the acceptor is indicative of dimer formation and deviation from linearity represents the formation of high-order oligomers [28]. As shown in Fig. 3B, the non-linear increase in FRET efficiency in the SDS micelles approximates a tetrameric state ($n = 4.33 \pm 0.187$ and $K = 0.58 \pm 0.01$ with $R = 0.997$), suggesting the IRE1α TM peptides is possibly forming tetramers. The FRET analysis (Fig. 3B) agrees with the SDS–PAGE results in Fig. 2, indicating the isolated TM domains have a propensity to oligomerize.

It has been shown that both the luminal and the cytosolic domains can oligomerize [5,6,9,11]. The oligomeric form of the luminal domain was identified by detecting exogeneously expressing proteins on SDS–PAGE gel [11]. In addition, structural and functional studies found that disruption of the dimer interface interaction on the luminal domain blocked the phosphorylation of the cytosolic domain, suggesting that dimerization of the luminal domain promotes dimerization and activation of the cytosolic domain [5]. In addition to dimers, the cytosolic domain also has been shown to form high-order oligomers (tetra-, hexa-, octomer as even-numbered oligomers) by velocity-analytical ultracentrifugation experiments, indicating the assembly of dimeric building blocks is important to the oligomerization process [9]. The enzymatic assay additionally suggested that the endoribonuclease activity of the human IRE1α is activated by self-association of four or more IRE1α monomers, presumably similar to that observed with yeast Ire1 [9,29]. Thus this higher-order oligomerization plays a crucial role in IRE1α activation. Similarly, our findings showed that the TM domain of IRE1α also is able to form dimers and tetramers, suggesting that the TM domain could participate in the oligomerization of the IRE1α protein. Therefore, as a next step in determining the role of the TM domain, in future studies one could evaluate whether the IRE1α TM domain is merely a passive membrane anchor in the oligomerization process or whether it contains critical structural information that positions the cytosolic domains in such a way that they can phosphorylate each other.

Using SDS–PAGE and FRET techniques, we provide experimental evidence that the TM domain of the IRE1α protein oligomerizes in a membrane-mimic environment, and maintains its helical structure. To best of our knowledge there is no report on the functional and structural roles of IRE1α TM domain. Thus this study opens the possibility that the IRE1α TM domain might play an important role in regulating the conformation of the cytosolic domain.

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