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Research report

Characterization of mouse Ire1α: cloning, mRNA localization in the brain and functional analysis in a neural cell line

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Abstract

In yeast, an endoplasmic reticulum (ER)-associated protein, Ire1p, is believed to initiate the unfolded protein response (UPR), that is responsible for protein folding in the ER under stressed conditions. Two mammalian homologs of Ire1p have been identified, Ire1α and Ire1β. We have previously reported that familial Alzheimer's disease linked presenilin-1 variants downregulate the signaling pathway of the UPR by affecting the phosphorylation of Ire1α. In the present study, we cloned the mouse homolog of Ire1α for generating genetically modified mice. Ire1α was ubiquitously expressed in all mouse tissues examined, and was expressed preferentially in neuronal cells in mouse brain. This led us to investigate the effects of the downregulation of the UPR on the survival of neuronal cells under conditions of ER stress. Morphological and biochemical studies using a dominant-negative form of mouse Ire1α have revealed that cell death caused by ER stress can be attributed to apoptosis, and that the downregulation of the UPR enhances the apoptotic process in the mouse neuroblastoma cell line, Neuro2a. Our results indicate that genetically modified mice such as transgenic mice with a dominant-negative form of Ire1α might provide further understanding of the pathogenic mechanisms of Alzheimer's disease and other neurodegenerative disorders.

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1. Introduction

Various cell stresses such as perturbation of calcium homeostasis, deprivation of trophic factors and oxidative insults cause accumulation of unfolded proteins in the endoplasmic reticulum (ER). Cells respond to these ER stresses by increasing transcription of genes encoding ER-resident chaperones such as GRP78/BiP, GRP94, calreticulin and protein disulfide isomerase (PDI), which facilitate protein folding in the ER. This induction system is termed the unfolded protein response (UPR; reviewed in Ref. [14]). In yeast, the UPR signaling is initiated by Ire1p/Ern1p, an ER-associated type I transmembrane protein that contains both serine/threonine kinase and ribonuclease domains in its cytosolic portion [1,8,13,18]. The ER-luminal portion of Ire1p is thought to sense the perturbed environment in the ER, followed by oligomerization and trans-phosphorylation of the kinase domain [12,18], and downstream signaling through the activation of a sequence-specific ribonuclease activity [13]. Two mammalian homologs of yeast Ire1p have been identified, Ire1α [16] and Ire1β [17]. The expression of Ire1β is limited to the lung and the intestine, where its expression level is very low (our unpublished data). Recently we showed that familial Alzheimer’s disease (FAD) linked presenilin-1 variants downregulate the signaling pathway...
of the UPR by affecting the phosphorylation of Ire1α [4], suggesting that Ire1α might be one of the components that participate in pathogenic mechanisms in brains of FAD patients.

In mammals, little has been understood about in vivo expression of Ire1α and the mechanism by which Ire1α senses ER stress and activates the downstream pathway of the UPR. Here, we cloned the mouse homolog of Ire1α for generating genetically modified mice, and investigated the expression of Ire1α mRNA in mouse tissues. In situ hybridization analysis has revealed that in mouse brain Ire1α mRNA is expressed preferentially in neuronal cells, which led us to investigate the effects of the downregulation of the UPR in a mouse neuroblastoma cell line, Neuro2a, using a dominant-negative form of mouse Ire1α. Morphological and biochemical studies have shown that cell death caused by ER stress can be attributed to apoptosis, and that the downregulation of the UPR enhances the apoptotic process.

2. Materials and methods

2.1. Animals

Adult ddY mice (8 weeks old) weighing ~25 g were anesthetized with ether inhalation and decapitated. Several tissues were then promptly removed. Brains were also removed from embryonic 14- and 18-day, and postnatal 1-, 7- and 14-day ddY mice.

2.2. cDNA cloning

Approximately one million colonies from a unidirectional oligo (dT) primed mouse brain cDNA library (C57 Black/6, female, 19 weeks; Stratagene) were screened by hybridizing to the insert of a mouse expressed sequence tag (EST) clone J0729C04 (Genbank accession #AU016754). The library screening was performed by standard methods.

2.3. RT-PCR

Total RNA was isolated from mouse tissues by extraction with Rneasy Mini Kit (QIAGEN) according to the manufacturer’s instructions. Five micrograms of each total RNA were reverse-transcribed into first strand cDNA using Ready-To-Go You-Prime First-Strand Beads (Amersham Pharmacia) with the reaction volume of 33 μl according to the manufacturer’s instructions. One microlitre of the reaction mixture was then amplified by PCR using Taq DNA polymerase (TaKaRa) and primer pairs designed for the specific detection of mouse Ire1α and β-Actin (as an internal control) cDNA target sequences. PCR was performed using the following conditions: after an initial denaturation of 2 min at 95°C, the amplification profile consisted of 30 s at 95°C, 1 min annealing, 30 s at 72°C for 29 cycles (for the tissue distribution of Ire1α), 31 cycles (for the developmental changes of Ire1α in brain) or 18 cycles (for β-Actin). The annealing temperatures were 68°C for Ire1α and 60°C for β-Actin. The primers used in this experiment were 5′-CCCTGATAGTTGAATCCCTG-GCTATGTG-3′ (sense) and 5′-AATCTATGCCTAATTGCTGTGCTCTG-3′ (antisense) for Ire1α and 5′-AGCT-GAGAGCCTCGTGC-3′ (sense) and 5′-ACCAGAC-AGCCTGTGTTGG-3′ (antisense) for β-Actin. The PCR products were separated by 4% acrylamide gel electrophoresis and visualized by staining with ethidium bromide.

2.4. In situ hybridization

Frozen sections (15 μm thick) of adult mouse brain (8 weeks old) were cut in various planes and thaw-mounted onto poly-L-lysine-coated slides. Digoxigenin-labeled cRNA probes (antisense and sense) were generated by in vitro transcription using a cDNA fragment (2980–3426 nt) of mouse Ire1α as a template in the presence of digoxigenin-labeled dUTP (Boehringer Mannheim). Hybridization and posthybridization procedures were performed as described previously [3].

2.5. Expression plasmids

The coding region of mouse Ire1α cDNA was cloned into pcDNA3.1(+) at KpnI and ApaI sites. A truncated form of mouse Ire1α cDNA, ΔIre1α, that lacked the sequence of kinase and ribonuclease domains, was generated and tagged with a Flag-epitope sequence at its C-terminal by PCR using wild-type cDNA as a template, and also cloned into pcDNA3.1(+) at the same sites.

2.6. Cell culture and assessment of apoptosis by Hoechst 33258 staining

Neuro2a cells, a mouse neuroblastoma cell line, were stably transfected with mock (empty plasmid), wild-type mouse Ire1α cDNA or a truncated form, ΔIre1α. Two lines were selected from the stable lines transfected with mock (#3, #8), wild-type (#3, #10) and ΔIre1α (#2, #5), respectively. They were repleted on 10-cm dishes at a density of 2×10^5 cells/cm^2, maintained in alpha-MEM (Life Technologies) containing 10% fetal calf serum at 37°C for 48 h and then treated with 2 μg/ml tunicamycin (Sigma) or 3 μM A23187 (Sigma) in the same medium. At 10, 20, 30 or 40 h after treatment, cells were stained with 5 μg/ml Hoechst 33258 (Molecular Probes) in PBS for 15 min. The average percentages of cells with chromatin condensation in randomly selected fields were scored as an indicator of apoptosis under UV illumination.
Fig. 1. Amino acid sequence analysis and structure of mouse Ire1α. (A) Alignment of mouse and human Ire1α amino acid sequences. Black boxes and shaded boxes represent nonconserved and conserved amino acids, respectively. All other amino acids are identical. Mouse and human sequences share a 92% identity. GenBank accession number for the mouse cDNA sequence is AB031332. (B) Domain organization of mouse Ire1α. SP and TM refer to the potential signal peptide and the putative transmembrane domain, respectively.
The expression of mouse Ire1α mRNA was investigated by RT-PCR. Total RNA was isolated from different tissues of adult mice (A) and brains of mice at several developmental stages (B), and reverse-transcribed into cDNA, that was then amplified by PCR using a primer pair designed for the specific detection of mouse Ire1α. β-Actin was also examined as an internal control. E14, E18, P1, P7, P14 and 8W refer to embryonic 14 and 18 days, and postnatal 1, 7 and 14 days, and 8 weeks old, respectively.

2.7. Electron microscopy

Neuro2a cells expressing ΔIre1α were treated with 2 μg/ml tunicamycin for 40 h. PBS containing 2% glutaraldehyde and 4% paraformaldehyde was then employed in fixing the cells at 4°C for 45 min. They were post-fixed in 1% OsO₄ at 25°C for 30 min, dehydrated in the graded ethanol solutions and embedded in Quetol 812 (NISSHIN EM Co.). Areas containing cells with apoptotic morphology were block-mounted and cut into 80-nm sections. The sections were stained with uranyl acetate (10% in 50% ethanol) and lead citrate, and then examined with a Hitachi H-7100 electron microscope.

2.8. Measurement of caspase-3-like activity

Stable Neuro2a lines treated with 2 μg/ml tunicamycin for 40 h were washed with PBS, harvested and lysed by ICE buffer (50 mM Tris–HCl (pH 7.4), 1 mM EDTA and 10 mM EGTA) containing 10 μM digitonin. The lysates were then incubated at 37°C for 10 min and centrifuged at 15,000×g for 10 min. Each sample (50 μg protein) was incubated with 50 μM of Ac-DEVD-MCA (Peptide Institute, Inc.), the substrate for caspase-3, in ICE buffer with the reaction volume of 1 ml at 37°C for 30 min. Levels of released 7-amino-4-methylcoumarin (AMC) were measured using a spectrofluorometer with excitation at 380 nm and emission at 460 nm.

2.9. Western blot analysis

The expression levels of wild-type Ire1α and ΔIre1α in stable Neuro2a lines were demonstrated by Western blot analysis. Cells were washed with PBS, harvested and lysed in NP-40 lysis buffer (1% NP-40, 10 mM Tris–HCl (pH 7.8), 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF) and 10 μg/ml aprotinin). The lysates were then incubated on ice for 50 min, centrifuged at 15,000×g for 20 min and boiled for 5 min with SDS sample buffer. Equal amounts of protein (30 μg) were subjected to 10% SDS–PAGE, transferred to PVDF membrane and blotted with rabbit polyclonal anti-Ire1α antibody, which was made by injecting rabbits with the cytosolic portion of Ire1α-GST fusion protein. The membrane was then incubated with alkaline phosphatase-conjugated sheep anti-rabbit IgG antibody (Boehringer Mannheim). For Western blot analysis of caspase-3, Neuro2a lines which were treated with 2 μg/ml tunicamycin for 40 h were lysed in NP-40 lysis buffer. The samples were prepared by the same procedures as the analysis of Ire1α. Equal amounts of protein (30 μg) were subjected to 12% SDS–PAGE, and Western blot analysis was performed using rabbit polyclonal anti-caspase-3 antibody (Santa Cruz), which recognizes pro-caspase-3 and active subunits, as the primary antibody, and anti-rabbit IgG antibody as the secondary antibody. For Western blot analysis of cytochrome c, Neuro2a cells expressing ΔIre1α which were treated with 2 μg/ml tunicamycin for 12, 24 or 40 h were harvested by centrifugation, and the cell pellets then washed with PBS and resuspended with buffer A (50 mM Tris–HCl pH 8.0, 1 mM EDTA, 0.32 M sucrose and 0.1 mM PMSF). The cells were homogenized with 10 strokes of a Teflon homogenizer, and the homogenates then separated as cytosol and membrane fractions by ultracentrifugation at 105,000×g for 60 min. Equal amounts of protein (6 μg) from the cytosol fraction were subjected to 12% SDS–PAGE, and Western blot analysis was performed using mouse monoclonal anti-cytochrome c antibody (PharMingen) as the primary antibody, and goat anti-mouse IgG antibody (Sigma) as the secondary antibody.

3. Results

3.1. Isolation of complementary DNA encoding mouse Ire1α

A mouse expressed sequence tag (EST) clone J0729C04 was found to have significant sequence homology to a portion of human Ire1α cDNA. The clone was used as the probe to screen a mouse brain cDNA library (C57 Black/
The preferential expression of Ire1α in neuronal cells in mouse brain led us to perform function analysis of Ire1α in a mouse neural cell line. In yeast, overexpression of a truncated form of Ire1p that lacks an effector domain can downregulate the activation of the pathway that leads to the induction of KAR2/GRP78 [8,12]. In mammalian cells, derivatives of Ire1α and β that lack the cytosolic portion have been shown to demonstrate similar dominant-negative effects on the UPR [8,10]. In the present study, a truncated form of mouse Ire1α, ΔIre1α, that lacked kinase and ribonuclease domains (Fig. 4A) was used to interfere with the activity of the endogenous inducers of the UPR in Neuro2a cells, a mouse neuroblastoma cell line. We confirmed that the levels of GRP78 mRNA induction in Neuro2a cells expressing ΔIre1α were lower than those in cells expressing wild-type Ire1α or mock (empty plasmid) after treatment with tunicamycin or A23187 (data not shown), indicating dominant-negative effects of ΔIre1α on the UPR; tunicamycin and A23187 are known to provoke ER stress by inhibition of protein glycosylation or perturbation of calcium homeostasis, respectively. To analyze the effects of the downregulation of the UPR, we examined the time-course of cell viability under conditions of ER stress in Neuro2a cells by investigating nuclear morphology with Hoechst 33258 staining (Fig. 4C and D). Neuro2a cells expressing a truncated form of Ire1α, ΔIre1α, displayed chromatin condensation, that is the morphological feature of apoptosis, at a significantly higher rate than cells expressing wild-type or mock at 20 h or later after treatment with 2 μg/ml tunicamycin (Fig. 4C). A significantly increased rate of apoptotic morphology of cells expressing ΔIre1α was also observed after treatment with 3 μM A23187 (Fig. 4D), suggesting that the downregulation of the UPR enhances the apoptotic process under conditions of ER stress in Neuro2a cells. The morphological change described above that was revealed by Hoechst 33258 staining is shown in Fig. 5A. Neuro2a cells expressing ΔIre1α treated with tunicamycin were also examined under electron microscopy (Fig. 5B). They exhibited changes consistent with apoptosis, such as loss of cytoplasm and nuclear fragmentation. Enlarged ER lumens were also observed, indicating that the cells were under conditions of ER stress.

In mammalian cells, a family of cysteine proteases termed caspases are known to be activated during the apoptotic process (reviewed in Ref. [15]). To examine the biochemical features of cell death induced by the downregulation of the UPR, we measured caspase-3-like protease activity in ER-stress exposed Neuro2a cells (Fig. 6A). At 40 h after treatment with tunicamycin, caspase-3-like activity in Neuro2a cells expressing ΔIre1α was significantly higher than that in cells expressing wild-type
Fig. 3. In situ hybridization analysis of Ire1α mRNA in mouse brain. Signals were detected in neuronal cells of the cortex and hippocampus (A), and in the olfactory bulb (D). High magnification views are shown for cortical neurons (B), hippocampal CA1 pyramidal cells (C), and olfactory mitral and granule cells (E). DG, dentate gyrus; py, pyramidal cell layer; gr, granule cell layer; mi, mitral cell layer. Scale bars: 100 μm for (A), (D); 10 μm for (B), (C); 20 μm for (E).

or mock. Caspase-3 is synthesized as a 32-kDa precursor (pro-caspase-3), that is activated proteolytically into 20, 19 or 17 kDa subunits when cells are undergoing the apoptotic process [2]. To confirm the involvement of caspase-3 in cell death induced by the downregulation of the UPR, we examined the cleavage of caspase-3 in ER-stress exposed Neuro2a cells by Western blot analysis using an anti-caspase-3 antibody that can detect the proform and active subunits (Fig. 6B). The processing of caspase-3 was observed in Neuro2a cells treated with tunicamycin for 40 h. Active fragments of caspase-3 were more abundant in cells expressing ΔIre1α than in cells expressing wild-type or mock, suggesting that caspases contribute to cell death enhanced by the downregulation of the UPR in Neuro2a cells.

When cells are exposed to stimuli that trigger apoptosis, cytochrome c is rapidly released from mitochondria into the cytosol where it activates caspases [5–7,19]. We therefore examined cytosolic cytochrome c in ER-stress exposed Neuro2a cells expressing ΔIre1α by Western blot analysis (Fig. 6C). Cytochrome c was detected in the cytosol fraction at 12 h after treatment with tunicamycin, and became more abundant at 24 and 40 h. This cytochrome c release into the cytosol from mitochondria preceded the morphological changes of cells demonstrated in Fig. 4, suggesting that cytochrome c might play a key role in the course of cell death enhanced by the downregulation of the UPR in Neuro2a cells.

These morphological and biochemical findings suggest that cell death caused by ER stress in Neuro2a cells can be attributed to apoptosis and that the downregulation of the UPR enhances the apoptotic process.
Fig. 4. The time-course of cell viability under conditions of ER stress was examined by investigating nuclear morphology. A truncated form of mouse Ire1α, ΔIre1α, which lacks kinase and ribonuclease domains, was used for its dominant-negative effects on the UPR. The structures of ΔIre1α and wild-type Ire1α are shown in (A). SP and TM refer to the potential signal peptide and the putative transmembrane domain, respectively. Neuro2a cells, a mouse neuroblastoma cell line, were stably transfected with mock (empty plasmid), wild-type Ire1α or ΔIre1α. Two lines were selected from the stable lines transfected with mock (#3, #8), wild-type (#3, #10) and ΔIre1α (#2, #5), respectively. The expression levels of the transgenes in these lines were demonstrated by Western blot analysis (B). Arrows indicate wild-type Ire1α and ΔIre1α. Size markers (in kilodaltons) are on the left. At indicated time points after treatment with 2 μg/ml tunicamycin (C) or 3 μM A23187 (D), the stable lines were stained with Hoechst 33258, and the average percentages of cells with chromatin condensation in randomly selected fields were scored as an indicator of apoptosis. Data are means±S.D. of four independent experiments.

4. Discussion

In the present study, we cloned the mouse homolog of Ire1α for generating genetically modified mice. From the 92% identity of the amino acid sequence between mouse and human Ire1α, and the ubiquitous expression of Ire1α mRNA in mouse tissues, it is likely that Ire1α protein is a main stress sensor that plays an essential role in cell survival against ER stress in mammalian organs. Relatively high expression levels of Ire1α were observed in thymus, liver and lung, suggesting the possibility that in these organs Ire1α might participate in organ-specific stress response systems as well as the UPR. The low oxygen environment in embryonic circulation might be connected with the lower expression levels of Ire1α mRNA in mouse brain at embryonic stages compared to postnatal and adult stages.

Like many types of nonneuronal cells, neurons respond to extracellular stimuli that provoke stressed conditions in the ER. For example, transient cerebral ischemia is known to induce a stress response which is in many respects identical to that triggered by the depletion of ER calcium pools, implying the possibility that disturbance of the ER calcium homeostasis may play a major role in ischemia-induced cell injury of neurons [9–11]. In the present study, in situ hybridization analysis has revealed that in mouse brain Ire1α mRNA is preferentially expressed in cortical, hippocampal and olfactory neurons and in other neuronal cells containing Purkinje cells and brainstem neurons, suggesting that Ire1α is the essential proximal sensor of the UPR pathway in neuronal cells.

We have previously reported that familial Alzheimer’s disease (FAD) linked presenilin-1 mutations downregulate the signaling pathway of the UPR by affecting the phosphorylation of Ire1α [4]. However, the character of the resulting vulnerability to ER stress has been unclear. Our present study, using a dominant-negative form of Ire1α, has revealed that the downregulation of the UPR enhances the apoptotic process under conditions of ER stress in a mouse neural cell line, by investigating the morphological changes of cells, activation of caspase-3 and cytochrome c release from mitochondria into the cytosol. Together with
Fig. 5. Morphological study of Neuro2a cells expressing ΔIre1α treated with 2 μg/ml tunicamycin for 40 h. (A) Hoechst 33258 staining of tunicamycin-treated cells (right panel) and untreated Neuro2a cells (left panel). Arrows indicate the cells with chromatin condensation, which is the morphological feature of apoptosis. (B) Examination under electron microscopy of treated cells (right panel) and untreated cells (left panel). Enlarged ER lumens are indicated by arrows. Scale bars: 2 μm.

the preferential expression of Ire1α in neuronal cells in mouse brain, this finding raises the possibility that the perturbed ER-stress response might be involved in the neuron-specific apoptosis observed in brains of Alzheimer’s disease patients containing FAD ones. Enhanced apoptosis by the downregulation of the UPR in a mouse neural cell line suggests that transgenic mice with a dominant-negative form of Ire1α targeted to neurons would be a good animal model for evaluating the effects of the disturbed stress response on the mammalian nervous system, and that generating genetically modified mice on Ire1α including dominant-negative form transgenic mice might contribute to further understanding of the pathogenic mechanisms of Alzheimer’s disease and other neurodegenerative disorders.

In our analysis by in situ hybridization for Ire1α mRNA, signals were undetectable in oligodendrocytes and astrocytes, implying the possibility that the major ER-stress sensing molecule in these types of cells might not be Ire1α, or that they might mediate ER stress by a mechanism different to the UPR.

In the present study, we have shown that the downregulation of the UPR enhances the apoptotic process in a neural cell line. This apoptotic process is thought to be triggered by the accumulation of unfolded proteins in the ER lumen, which cannot undergo folding and maturation due to the inhibition of the ER-resident chaperones’ induction. However, the signaling pathway by which the stress in the ER lumen activates caspases which reside in the cytosol is unclear, even though our results suggest the involvement of cytochrome c release from mitochondria into the cytosol. Further studies should be performed to...
investigate the mechanisms by which the accumulation of unfolded proteins in the ER provokes downstream apoptotic signaling in neuronal cells.

References


