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Site-specific cleavage of CD59 mRNA by endoplasmic reticulum-localized ribonuclease, IRE1

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Abstract

IRE1, an ER-localized transmembrane-RNase, plays a central role in ER stress response. Upon ER stress, IRE1 induces various adaptive genes through the processing of mRNA encoding the transcription factor XBP1. Moreover, it was recently reported that in fly IRE1 attenuates the expression of several genes by cleaving mRNAs, but it has been unclear whether such a mechanism also exists in mammal. In this study, we searched for IRE1α-cleaved mRNAs in mammalian cells and identified human CD59 (complement defense 59) mRNA as a novel cleavage target. In addition, the expression of CD59 was significantly attenuated by overexpression of IRE1α or ER stress. These results suggest that IRE1α-mediated mRNA cleavage functions even in mammals as a common system to regulate gene expression.

Keywords: CD59; ER stress response; IRE1; RNase

The endoplasmic reticulum (ER) plays a central part in the synthesis and modification of secretory and membrane proteins in all eukaryotic cells. The exposure of cells to various stresses interfering with the functions of the ER leads to the accumulation of unfolded protein in the ER lumen. Under these conditions, known as ER stress, a signal transduction pathway called the unfolded protein response (UPR), is activated to increase the expression of ER stress response genes, such as ER chaperones [1,2].

IRE1, a highly conserved ER-localized type I transmembrane protein with kinase and ribonuclease domains in its cytoplasmic region plays a central role in the UPR [3]. The luminal domain of IRE1 senses the accumulation of unfolded proteins [4–6]. Upon ER stress, IRE1 activates its ribonuclease domain via oligomerization and autophosphorylation [7] and cleaves specific exonic–intronic sites in the mRNA encoding the transcription factor X-box-binding protein 1 (XBP1) [8,9]. This cleavage initiates an unconventional splicing reaction, leading to production of an active transcription factor and induction of various adaptive genes.

IRE1 regulates gene expression not only by the XBP1 pathway, but also by other mechanisms. There are two IRE1 paralogues in mammals, IRE1α [10], and IRE1β [11]. IRE1α auto-regulates its expression by cleaving its own mRNA [12]. On the other hand, IRE1β is thought to attenuate protein synthesis by cleaving ribosomal RNA under stressed conditions [13]. In addition, it was recently reported that IRE1 attenuates the expression of several genes by cleaving mRNAs in fly cells [14]. However, it has been unclear whether such a mechanism also exists in mammalian cells.

In this study, we searched for mammalian mRNAs cleaved by IRE1α using the recently reported *in vitro* RNA cleavage system [15]. As a result, we identified human CD59 (complement defense 59) mRNA as a novel cleavage target for IRE1α, and its expression was significantly attenuated by overexpression of IRE1α or ER stress. These results suggest that IRE1α-mediated mRNA cleavage functions even in mammalian cells as a common system to regulate gene expression.

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Materials and methods

Cell culture, transfection, and treatment. HeLa cells and HEK293T cells were cultured in DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum at 37 °C in 5% CO_2. The calcium phosphate-DNA precipitation method was used to introduce plasmid DNA into the cells. To induce ER stress, cells were treated with tunicamycin for the indicated times.

Plasmids. pCAX-F-h*IRE1α*(467–977) [15] was used to express the cytoplasmic domain of human IRE1α. pCAG-h*IRE1α*-HA was made by insertion of the h*IRE1α* full-ORF with three tandem HA-tags at its C-terminus into the EcoRI site of pCAGGS. To make its kinase mutant derivative, the K599A mutation was introduced by PCR techniques.

pBS-XBP1(266–602) contains the partial cDNA fragment of human XBP1 (266–602 of the coding region) [15].

To make the pBS-CD59 series, each human CD59 cDNA fragment was inserted into KpnI/NheI sites of pBlueScript II SK (−). The following primer sets were used to synthesize each CD59 cDNA fragment: for 143–1455 nt, primers A, B; for 1036–2559 nt, primers C, D; for 2132–3656 nt, primers E, F; for 3223–4753 nt, primers G, H; for 4336–5853 nt, primers I, J; for 5431–6907 nt, primers K, L; for 6529–7526 nt, primers M, N. Primers are shown in Supplementary Table. pBS-CD59 (64–646) was obtained from the screening mentioned below, which contains 64–646 nt of CD59 mRNA. The position of nt in CD59 mRNA is numbered according to data from the GenBank (NM_203331).

RNA cleavage assay. In vitro cleavage reactions were performed as described previously [15]. Briefly, *in vitro* transcribed RNA was incubated with the cytoplasmic domain of human IRE1α at 37 °C, and the resulting fragments were resolved on a 2% denaturing agarose gel and stained with ethidium bromide. As a control, the XBP1 RNA fragment (266–602) was used.

Screening. cDNA was synthesized from total RNA of HeLa cells or human placenta RNA (from the SMART mRNA Amplification Kit (Clontech)) using the SMART PCR cDNA Synthesis Kit (Clontech). The cDNA fragments were PCR-amplified with XhoI-5′-PCR-primer-IIA (5′-CCGCTCGAGAAGCAGTGGTATCAACGCAGAGT-3′) and BamHI-5′-PCR-primer-IIA (5′-CGCGGATCCAAGCAGTGGTATCAACGCAGAGT-3′), and inserted into the XhoI/BamHI sites of pBlueScript II SK (−). Plasmids that had cDNA inserts more than 500 bp in length were selected and used as a template for *in vitro* transcription. From the selected plasmids, RNA fragments were synthesized using the Riboprobe *in vitro* Transcription System (Promega). All of the fragments were subjected to the RNA cleavage reaction described above. The plasmid producing the cleaved RNA was sequenced with T7 primer, and the inserted cDNA was identified.

Primer extension. A partial CD59 RNA fragment derived from pBS-CD59 (64–646) was subjected to the *in vitro* cleavage assay. For each primer extension reaction, 0.1 μg of cleaved or uncleaved RNAs were used as templates for reverse transcription. The oligonucleotide complementary to positions 364–387 in the CD59 mRNA (sequence: 5′-GTGACGTCGTTGAAATTGCAATGC-3′) was [^{32}P]-labeled with MEGALABEL (Takara) and used as a primer. Resulting products were resolved on a sequencing gel (7 M urea, 6% acrylamide with Long Ranger Gel Solution (Takara)). To map the products onto the CD59 mRNA, the same radiolabeled primer was used to sequence from pBS-CD59 (64–646). Sequencing was carried out using the BcaBEST Dideoxy Sequencing kit (Takara). The sequencing reactions were run in lanes adjacent to the primer extension reactions, and the resolved gel was dried for autoradiography.

Northern blot analysis. Total RNA was prepared using the Isogen reagent (Nippon Gene). Aliquots of total RNA (5 μg) were loaded into each lane of a 1% denaturing agarose gel and transferred onto Hybond-N membranes (Amersham-Pharmacia). Hybridization was performed at 65 °C in Church buffer [16]. After three washes in Church buffer, signals were detected using the BAS 2500 system (Fuji Film). To detect BiP, GAPDH and CD59, the following probes were used: BiP, nt 1−1959 of the coding region; GAPDH, nt 75−1019 of the coding region; CD59, nt 64–646 of the mRNA. These probes were radiolabeled using a Random Primer DNA Labeling kit ver. 2.0 (Takara).

Western blot analysis. The cells were lysed in lysis buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton-X, 1% glycerol), and heated at 98 °C for 5 min with sample buffer. SDS–PAGE was performed to resolve the proteins in the lysate. After electrophoresis, the proteins were electrotransferred onto a polyvinylidene fluoride microporous membrane and immunodetection was performed with an anti-CD59 antibody (Serotec) or anti-GAPDH antibody (Abcam) using standard procedures.

Results

Screening for IRE1α-cleaved RNA

To search for novel mRNAs cleaved by IRE1α, we performed the screening as shown in Fig. 1A. First, a cDNA library was made from total RNA of HeLa cells or human placenta, and RNA fragments were synthesized from the cDNA library by *in vitro* transcription. Next, each synthesized fragments were subjected to the *in vitro* cleavage assay with IRE1α [15]. As a control experiment in the cleavage assay, we confirmed that the partial XBP1 RNA fragment that is a known target for IRE1α [15] was surely cleaved in this assay (Fig. 1B).

Examination with the 221 synthesized RNA fragments indicated three cleavage candidates (Fig. 1C). Direct sequencing of the plasmids producing these cleaved RNA fragments identified its containing genes, CSH1 (chorionic somatomammotropin hormone 1), CD59 (complement defense 59), and HBB (hemoglobin beta). Among them, CSH1 was cloned in the reverse direction on the library plasmid, and the expression of HBB was not detected by the Northern blot analysis with total RNA from HeLa cells (data not shown). Therefore, we removed these two candidates from consideration, continued to research about the RNA fragment of CD59.

Identification of the IRE1α-cleavage site on CD59 mRNA

Full-length, human CD59 has been reported as a 7678-nt long mRNA. As the cDNA obtained in the screening described above contains only a very short region (64–646 nt) of this mRNA, it remains possible that CD59 mRNA could be cleaved at a position other than this region. To investigate whether CD59 mRNA contains other cleavage sites, we performed a further experiment as described below (Fig. 2).

CD59 mRNA was divided into eight fragments, including the fragment obtained by the above screening, and subjected to the cleavage assay (Fig. 2A). Although the 64–646 fragment was also cleaved similar to that shown in Fig. 1, none of the remaining fragments were cleaved by IRE1α (Fig. 2B). Therefore, it is clear that the CD59 mRNA is cleaved only within the region of 64–646 nt.

Next, RT-driven primer extension was performed to map the cleavage site on CD59 mRNA (Fig. 3A). Mapping with the RNAs from the cleavage assay revealed that the CD59 mRNA was cleaved at the position of −6/−7, which is located just before the start codon (Fig. 3B).

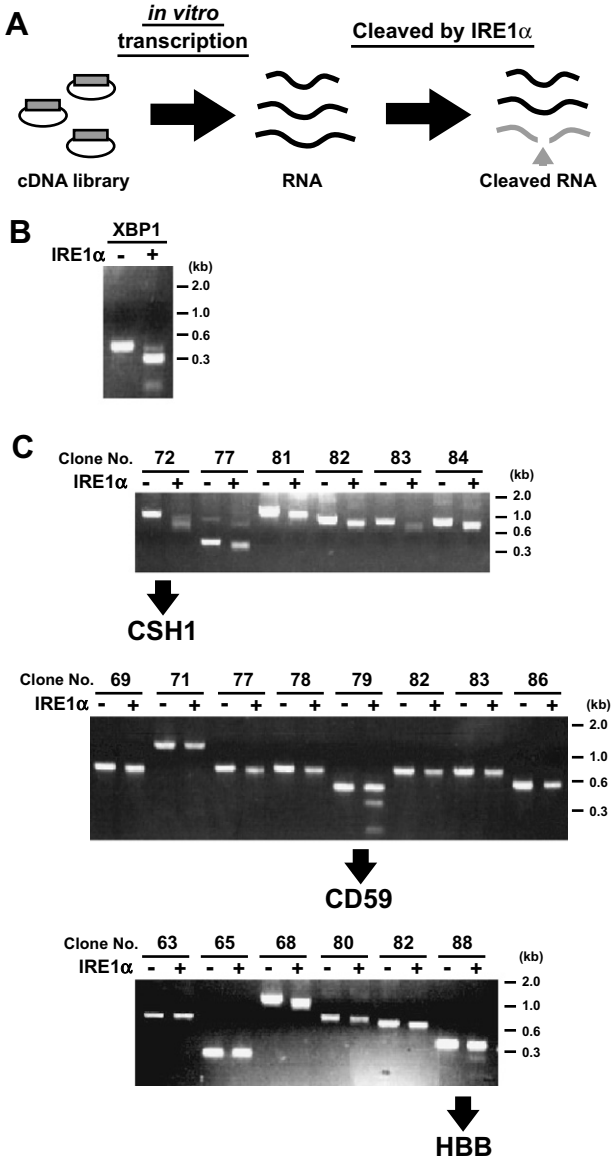

Fig. 1. Screening for IRE1α-cleaved RNA. (A) Schematic representation of the screening procedure. (B) *In vitro* cleavage assay with the partial XBP1 RNA fragment. The partial XBP1 RNA (266–602) synthesized by *in vitro* transcription was incubated with or without immunoprecipitated IRE1α cytoplasmic domain. Resulting fragments were resolved on a 2% denaturing agarose gel and stained with ethidium bromide. (C) A subset of the screening. RNA fragments incubated with or without IRE1α were resolved on a 2% denaturing agarose gel and stained with ethidium bromide. Direct sequencing of the library plasmids revealed that No. 72 was a partial RNA fragment of CSH1 (antisense), No. 79 was a partial fragment of CD59 (sense), and No. 88 was a partial fragment of HBB (sense).

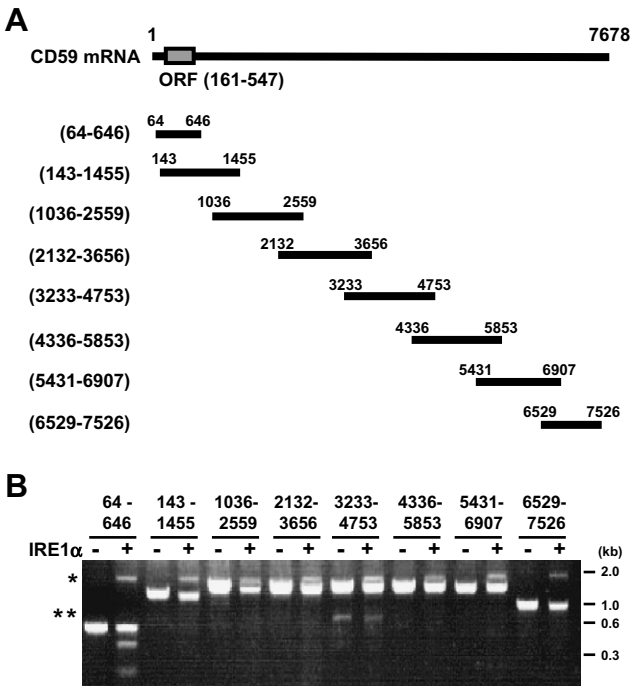

Fig. 2. Search for the IRE1α-cleavage site on CD59 mRNA. (A) Schematic representation of the partial CD59 RNA fragments used in the cleavage assay. (B) *In vitro* cleavage assay for each CD59 RNA fragment. Each CD59 RNA fragment was subjected to the cleavage assay similarly to the method described in Fig. 1. An unidentified fragment immunoprecipitated with IRE1α was marked with an asterisk. An artificial fragment was marked with a double-asterisk.

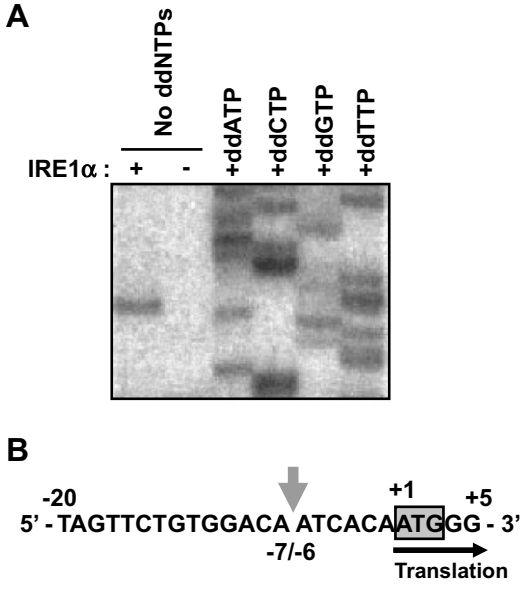

Fig. 3. Mapping of the IRE1α-cleavage site on CD59 mRNA. (A) Primer extension analysis for mapping of CD59 mRNA cleavage sites. RNA fragments prepared from the *in vitro* cleavage assay were used as templates for the primer extension analysis. Direct sequencing of CD59 mRNA is shown to the right side of the panel. (B) Partial sequence of CD59 mRNA including the cleavage site. The cleavage site is represented by a gray arrow. +1 above the "A" indicates the translational initiation site.

Attenuation of CD59 in cells

To investigate the *in vivo* expression level of CD59 mRNA that is cleaved by IRE1α, Northern blot analysis was performed (Fig. 4). The full-length CD59 mRNA is registered as 7678-nt long. However, this gene is reported to contain multiple polyadenylation sites in its 3′-UTR,

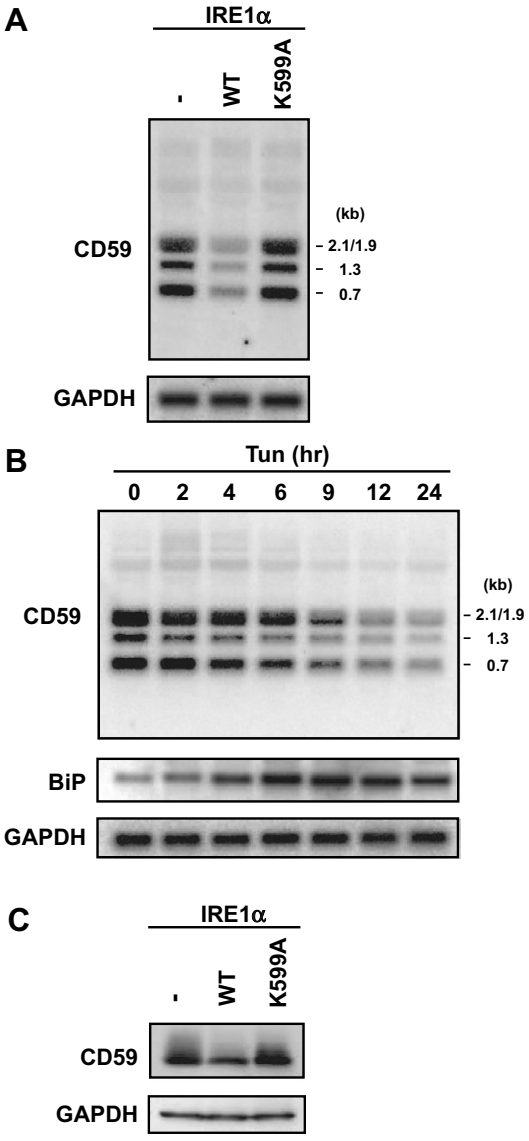

Fig. 4. Attenuation of CD59 in HeLa cells. (A) Northern blot analysis of CD59 mRNA with overexpression of IRE1α. HeLa cells were transiently transfected with human IRE1α -HA expressing vectors (wild-type or mutant). As a control, empty vector (pCAGGS) was used. Total RNAs (5 μg per lane) from these cells were used in Northern blot analysis. GAPDH was used as an internal standard. (B) Northern blot analysis of CD59 mRNA under stressed condition. Total RNAs (5 μg per lane) derived from HeLa cells treated with 2.5 μg/ml tunicamycin (Tun) for the indicated times were used in Northern blot analysis. BiP was used as a marker for induction of ER stress. GAPDH was used as an internal standard. (C) Western blot analysis of CD59 protein with overexpression of IRE1α. Total cell lysates (40 μg per lane) deprived from HeLa cells transiently transfected with empty vector (pCAGGS) or human IRE1α-HA expressing vectors (wild-type or mutant) were used in Western blot analysis. GAPDH was used as an internal standard.

and to be mainly expressed as 0.7-, 1.3-, 1.9-, and 2.1-kb transcripts by the alternative polyadenylation in HeLa cells [17].

In Fig. 4A, the effect of IRE1α overexpression was examined. Upon overexpression of wild-type IRE1α, major variants of the CD59 mRNAs were attenuated, though the level of GAPDH mRNA was not changed. On the other hand, expression of the K599A mutant, which loses autophosphorylation and RNase activity, had no effect on the levels of CD59 mRNAs. Treatment with tunicamycin to induce ER stress also reduced the CD59 mRNA (Fig. 4B). In this case, we confirmed that tunicamycin-induced BiP mRNA, which is a known ER stress-responsive gene, but did not change the level of GAPDH mRNA.

Western blot analysis confirmed that the expression of CD59 protein was also attenuated (Fig. 4C). The overexpression of wild-type IRE1α, not K599A mutant, reduced the CD59 protein, while the level of GAPDH protein did not change. From these results, we conclude that CD59 is attenuated via IRE1α under stressed conditions.

Discussion

In this study, we identified the human CD59 mRNA as a novel target for IRE1α in mammalian cells (Fig. 1). Moreover, it was revealed that the CD59 mRNA, which is cleaved by IRE1α (Figs. 2 and 3), was attenuated by the overexpression of IRE1α or by ER stress in cells (Fig. 4). These results suggest that IRE1α-mediated mRNA cleavage, which had been found in fly cells [14], also functions in mammalian cells to regulate gene expression.

It is well known that the portion of XBP1 mRNA surrounding the cleavage sites forms the characteristic secondary structure with stem-loop [8,9]. However, in the region of CD59 mRNA surrounding its cleavage site, such a characteristic structure was not found by MFOLD program analysis [18]. Thus, it is highly possible that IRE1α can recognize various RNAs that do not contain the XBP1-like structure as cleavage targets.

In HeLa cells, the CD59 gene is mainly transcribed as 0.7, 1.3, 1.9, and 2.1 kb mRNAs by alternative polyadenylation [17]. In Fig. 4, major variants of CD59 mRNA were attenuated by the overexpression of IRE1α or by ER stress. This result corresponds to the finding that the IRE1α-cleavage site is located just before the start codon (Figs. 2 and 3), which is contained in all of these mRNA variants. Because the CD59 mRNA was cleaved by IRE1α in *vitro* (Figs. 2 and 3), it is strongly suggested that IRE1α acts directly on the target mRNA in cells. Any cleavage products of CD59 mRNA was not detected in Fig. 4A and B, unlikely to the *in vitro* experiment (Fig. 2). Thus, the cleavage products may be rapidly degraded in cells.

What is the physiological role of IRE1α-mediated mRNA attenuation [14]? Under ER-stressed conditions, the unfolded proteins accumulate in the ER and inhibit ER function. Therefore, the burden on the ER exceeds its protein-folding capacity during ER stress. The IRE1α-mediated mRNA attenuation could lead to the reduction of protein loaded in the ER, and immediately lighten the burden on the ER, together with the general translational inhibition mediated by PERK [19]. Therefore, IRE1α

may prefer the ER-targeted mRNAs as its cleavage substrate. The CD59 transcripts, which are newly identified as a target for IRE1α, are carried to the cytosolic face of the ER membrane, because CD59 is a membrane protein that is made through translocon. Besides, the ribonuclease domain of the IRE1α also exists on the cytosolic side of ER membrane. This topological correlation between IRE1α and its substrate RNAs would enable the effective cleavage in cells.

The identification of CD59 as a target for IRE1α leads us to consider the relationship between the UPR and diseases. The human complement regulatory protein CD59 is a 20-kDa glycoprotein anchored to the membrane via glycosylphosphatidylinositol [20], which restricts human complement lysis by inhibiting the assembly of the membrane attack complex (MAC) [21]. IRE1α-mediated attenuation of this protein seems to have a certain specificity, because the mRNAs of other complement regulatory proteins, such a DAF (decay accelerating, CD55) or MCP (membrane cofactor protein, CD46), were not attenuated by the ER stress (data not shown). Attenuation of CD59 expression is reported in several diseases, such as lung cancer [22] and Alzheimer's disease (AD) [23]. Especially in AD, the expression of CD59 mRNA is significantly reduced, although the mechanism is still unknown, and this attenuation is though to lead to the neurite loss characteristic of AD-patient brains [23]. Meanwhile, other studies have proposed a relationship between UPR and neurodegenerative diseases, reporting that the UPR pathway is certainly activated in AD [24]. Thus in AD, the activated UPR pathway, perhaps activated IRE1α, may cleave CD59 mRNA to attenuate its expression, leading to the characteristic neurite loss in AD patients.

In this screening, only one cleavage substrate was identified from 221 RNA fragments. Considering the diversity of mRNAs, there must be more IRE1α-substrates in mammalian cells. To perform a genome-wide screening, more effective methods are required. In this respect, analysis with DNA micro-array seems to be extremely useful, as performed by Hollien and Weissman [14]. In this study, only mRNAs having a polyA tail were used as templates to generate RNA fragments for the *in vitro* cleavage screening. However in cells, IRE1α might cleave other types of RNA molecules. Recently, it is becoming clear that noncoding RNAs (ncRNAs), including microRNAs (miRNAs), function in many aspects of gene regulation [25]. Thus, IRE1α might involve in the regulation of various gene expression by the specific processing of these types of RNA molecules. In this regard, the reactivity between IRE1α and various types of RNA molecules should be further investigated.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.06.020.

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