Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 360 (2007) 122–127
BBRC
www.elsevier.com/locate/ybbrc

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

Daisuke Oikawa, Mio Tokuda, Takao Iwawaki *

Iwawaki Initiative Research Unit, Frontier Research System, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan

Received 22 May 2007
Available online 14 June 2007

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.

* Corresponding author. Fax: +81 48 467 8503.
E-mail address: iwawaki@riken.jp (T. Iwawaki).

0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.06.020

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).