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A highly sensitive assay of IRE1 activity using the small luciferase NanoLuc: Evaluation of ALS-related genetic and pathological factors

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ABSTRACT

Activation of inositol-requiring enzyme 1 (IRE1) due to abnormal conditions of the endoplasmic reticulum (ER) is responsible for the cleavage of an unspliced form of X-box binding protein 1 (uXBP1), producing its spliced form (sXBP1). To estimate IRE1 activation, several analytical procedures using green fluorescence protein and firefly luciferase have been developed and applied to clarify the roles of IRE1-XBP1 signaling pathways during development and disease progression. In this study, we established a highly sensitive assay of IRE1 activity using a small luciferase, NanoLuc, which has approximately 100-fold higher activity than firefly luciferase. The NanoLuc reporter, which contained a portion of the spliced region of XBP1 upstream of NanoLuc, was highly sensitive and compatible with several types of cell lines. We found that NanoLuc was secreted into the extracellular space independent of the ER-Golgi pathway. The NanoLuc activity of an aliquot of culture medium from the neuroblastoma-spinal neuron hybrid cell line NSC-34 reflected the toxic stimuli-induced elevation of intracellular activity well. Using this technique, we evaluated the effects of several genetic and pathological factors associated with the onset and progression of amyotrophic lateral sclerosis (ALS) on NanoLuc reporter activity. Under our experimental conditions, inhibition of ER-Golgi transport by the overexpression of mutant Sar1 activated luciferase activity, whereas the co-expression of mutant SOD1 or the C-terminal fragment of TDP-43 (TDP-25) did not. The addition of homocysteine elevated the reporter activity; however, we did not observe any synergistic effect due to the overexpression of the mutant genes described above. Taken together, these data show that our analytical procedure is highly sensitive and convenient for screening useful compounds that modulate IRE1-XBP1 signaling pathways as well as for estimating IRE1 activation in several pathophysiological diseases.

1. Introduction

The endoplasmic reticulum (ER) is responsible for folding and modifying newly synthesized transmembrane and secretory proteins [1,2]. A number of pathophysiological conditions disrupt ER function and cause the accumulation of unfolded and/or misfolded proteins in the ER. These phenomena, referred to as ER stress, activate various stress responses that are mediated by three major ER-resident stress sensors: PERK [3], IRE1 [4] and ATF6 [5,6]. A variety of genes have been identified as downstream targets of these three sensors, some of which, including those related to ER-resident chaperones and ER-associated degradation, control the quality of newly synthesized proteins in the ER and alleviate the damage caused by ER stress [7]; others, such as GADD153, have been demonstrated to promote cell death in various types of cells [8]. Among the three sensors, IRE1 is the most conserved signal transducer and possesses both Ser/Thr protein kinase and endoribonuclease activity, which enables it to splice unspliced XBP1

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(uXBP1) mRNA, converting it into spliced XBP1 (sXBP1), a potent transcription factor. To date, many types of genes that regulate protein quality control, protein glycosylation, and ER and Golgi biogenesis have been reported to be downstream targets of sXBP1 [9,10]. sXBP1 has been demonstrated to play crucial roles in the regulation of cellular functions in cells with well-developed ER, such as plasma B cells [11], the exocrine cells of the pancreas and salivary glands [12] and hepatocytes for liver lipogenesis [13]. Moreover, aberrant activation of the IRE-XBP1 pathway has been suggested to participate in the onset and progression of various types of diseases. Therefore, it is necessary to develop a procedure that enables more precise estimation of the activation of the IRE1-XBP1 pathway. Iwawaki et al. have reported unique reporter constructs that consist of a region around the splice site of XBP1, followed by firefly luciferase or the fluorescence protein Venus [14,15]. Very recently, we established a highly sensitive assay for the post-translational regulation of an ER stress-inducible trophic factor, mesencephalic astrocyte-derived neurotrophic factor (MANF), based on the application of a small luciferase, NanoLuc [16,17]. In the current study, we utilized NanoLuc to establish a highly sensitive assay for IRE1 activity. ER stress responses have been reported to be associated with the onset and progression of several neurodegenerative diseases [18]. Amyotrophic lateral sclerosis (ALS) is a common adult-onset motor neuron disease [19] and is characterized by the selective loss of motor neurons. Several humoral and genetic factors related to ALS [20–22] have been reported to be associated with ER stress responses [21,23]; however, the precise mechanisms underlying ALS are not fully understood. In the present study, we evaluated the effects of several ALS-related genetic and pathological factors on IRE1 activity using our NanoLuc-based assay system.

2. Materials and methods

2.1. Materials

Thapsigargin (Tg), tunicamycin (Tm), homocysteine (Hcy), N-acetylcysteine (NAC), and buthionine sulphoximine (BSO) were obtained from Sigma–Aldrich. Antibodies against Flag-, Myc-epitope, GADD153, actin and EGFP were purchased from Sigma–Aldrich, Santa Cruz Biotech, Calbiochem and Roche Life Sci, respectively.

2.2. Plasmid construction

The NanoLuc gene, which contained a Myc/His-epitope (NanoLuc-MH) at its C-terminus, or the Firefly luciferase gene were inserted into the pcDNA3.1 vector. A portion of the mouse XBP1 splice region (118 aa – 185 aa in mouse XBP1) from the unspliced XBP1 [14,15] was fused with NanoLuc-Myc/His, and the gene was inserted into the pFlag CMV vector (dXBP1-NL) (Fig. 1A). To prepare the human SOD1 constructs, the wild-type (wt) SOD1 and mutant SOD1 genes [G85R and G93A] were also amplified by PCR and inserted into the pFlag CMV vector. For the human TDP-25, the C-terminal region was amplified from the wild-type (wt) and mutant [M337V] full-length TDP-43 gene (from Dr. Leonard Petrucelli) by PCR and inserted into the pFlag CMV vector. An HA-tagged mutant Sar1 construct (Sar1[H79G]) was kindly provided by Dr. Wei Liu and Dr. Jennifer Lippincott-Schwartz [24]. A mutant Rab1α[S25N] construct generated by Dr. Terry Hébert was obtained from Addgene (#46777) [25].

2.3. Cell culture and treatments

NSC-34 [26], Neuro2a and HT-29 cells were maintained in Dulbecco's modified Eagle's minimum essential medium (DMEM) containing 8% fetal bovine serum. NIH3T3 cells were cultured in DMEM containing 10% bovine serum. Transfection of the indicated constructs was performed using Lipofectamine-Plus (Life Technologies) and PEI-Max reagents (Polysciences) as previously described, with a slight modification [27]. To establish NSC-34 cells that stably expressed dXBP1-NL, cells transfected with the dXBP1-NL reporter construct were selected with the appropriate amount of G418. To detect both dXBP1-NL protein and luciferase activities, cells were seeded into 6- or 12-well plates. For the analysis of luciferase activity, the indicated cells or NSC-34 cells that stably expressed an empty vector (mock) or dXBP1-NL were seeded into 48- or 96-well plates, grown to semi-confluence and used for subsequent experiments. The treatments used in this study were as follows: BSO, hydrogen peroxide (H_2O_2), homocysteine (Hcy), NAC, Tg and Tm, at the indicated concentrations.

2.4. Measurement of luciferase activity

After cells that transiently or stably expressed each of the indicated luciferase constructs were treated with the indicated reagents, the culture medium and cell lysates prepared with Passive Lysis buffer (Promega) were collected. After a brief centrifugation, the luciferase activity in each culture medium and lysate was measured using the Single Luciferase and NanoLuc assay systems (Promega), respectively [17]. By calculating the total luciferase activity in both the culture medium and the cell lysates, the percentage of secreted luciferase in each well was estimated. In some cases, Passive Lysis buffer was directly added to each culture well, and the total lysates were collected to measure the total luciferase activity, including both the culture medium and the cells.

2.5. Reverse-transcription polymerase chain reaction

To estimate the expression level of each gene by RT-PCR, total RNA was extracted from cells lysed with TRIzol reagent (Life Technologies) and converted to cDNA by reverse transcription using random nine-mers as primers for Superscript III RNase$^-$ reverse transcriptase (RT) (Life Technologies) [17,27]. Specific cDNAs were mixed and amplified with a PCR reaction mixture (Taq PCR Kit, Takara). The RT-PCR primers used in this study were as follows: *XBP1* sense primer, 5′-ACGCTTGGGAATGGACACG-3′; *XBP1* antisense primer, 5′-ACTTGTCCAGAATGCCCAAAAG-3′; *GADD153* sense primer, 5′-GAATAACAGCCGGAACCTGA-3′; *GADD153* antisense primer, 5′-GGACGCAGGGTCAAGAGTAG-3′; *GRP78* sense primer, 5′-ACCAATGACCAAAACCGCCT-3′; *GRP78* antisense primer, 5′- GAGTTTGCTGATAATTGGCTGAAC-3′; *GADD45α* sense primer, 5′-AGAGCAGAAGATCGAAAGGA-3′; *GADD45α* antisense primer, 5′-GATGTTGATGTCGTTCTCGC-3′; *p21* sense primer, 5′-GAGAACGGTGGAACTTTGAC-3′; *p21* antisense primer, 5′-GTGCAAGACAGCGACAAGG-3′; *GAPDH* sense primer, 5′-ACCACAGTCCATGCCATCAC-3′; and *GAPDH* antisense primer, 5′-TCCACCACCCTGTTGCTGTA-3′. The typical reaction cycling conditions were 30 s at 96 °C, 30 s at 58 °C and 30 s at 72 °C. The results represent 20–29 cycles of amplification. The cDNAs were separated by electrophoresis through 2.0% agarose gels and visualized using ethidium bromide.

2.6. Western blotting analysis

We detected the amount of spliced dXBP1-NL protein in the cell lysates as previously described, with a slight modification [17,27]. The cells were lysed with homogenate buffer [20 mM Tris–HCl (pH 8.0) containing 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1% TritonX-100, 1 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml pepstatin A]. After the protein concentration was determined, each cell lysate was dissolved with an equal amount of sodium dodecyl sulfate