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A different pathway in the endoplasmic reticulum stress-induced expression of human HRD1 and SEL1 genes

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Abstract Human HRD1 and SEL1 are components of endoplasmic reticulum-associated degradation (ERAD), which is a retrograde transport mechanism from the ER to the cytosol for removing unfolded proteins. The expression of HRD1 and SEL1 was induced by ER stress-inducing agents and overexpression of both ER stress-responsive transcription factors, ATF6 and XBP1. Inhibition of IRE1 and ATF6 revealed that ER stress-induced HRD1 and SEL1 expressions are mediated by IRE1-XBP1- and ATF6-dependent pathways, respectively. These results suggest that the ER stress-induced ERAD gene expressions are mediated by different pathways, which are attributed to the differences in the promoter regions.

Keywords: HRD1; SEL1; ER stress; ATF6; XBP1; ERAD

1. Introduction

A number of environmental changes that affect the function of the endoplasmic reticulum (ER) lead to an accumulation of unfolded proteins in the ER lumen. Under such conditions, a form of signaling called the unfolded protein response (UPR) is initiated, and the crisis is transduced from the ER to the nucleus across the ER membrane [1].

Mammalian cells induce a variety of genes in response to ER stress [2]. The UPR transducer ATF6 is processed and liberated from the membrane during ER stress, and subsequently translocated to the nucleus as a transcription factor [1,3]. The processed ATF6 binds to two motifs, CCAAT-N_9-CCACG/A and ATTGG-N-CCACG, termed as the ER stress response element (ERSE)-I and -II, respectively, resulting in the transcriptional induction of ER stress response genes [1,3,4]. IRE1, a protein kinase and ribonuclease in the ER membrane, activates itself via *trans*-autophosphorylation [1]. IRE1 activated by ER stress initiates spliceosome-independent splicing of XBP1 mRNA and the spliced XBP1 then encodes an additional open reading frame and activates the transcription of its target genes through the mammalian unfolded protein response element (UPRE; TGACGTCC/A) [1,5].

A series of gene expressions including *HRD1*/*DER3*, *HRD3* is induced by the UPR through the Ire1p pathway in yeast [6]. Proteins encoded by these genes serve to remove unfolded proteins by retrograde transport from the ER back to the cytosol with subsequent degradation by the ubiquitin–proteasome system designated as the ER-associated degradation (ERAD) [7]. On the other hand, in other species, including humans, these homologs remain largely uncharacterized. We have identified HRD1 as a human homolog of yeast Hrd1p/Der3p, a ubiquitin ligase located in the ER membrane, and shown that this gene expression was induced by ER stress [8]. SEL1L (SEL1) was identified as a human homolog of yeast Hrd3p that interacts with Hrd1p for stabilization [9].

It is unclear in mammals how ERAD genes are induced under ER stress and which transducer, IRE1 or ATF6, mediates UPR signaling to induce the ERAD gene expression. In this study, we show that: (1) the induction pathway of HRD1 expression by ER stress depends on IRE1-XBP1, whereas that of SEL1 depends on ATF6, (2) a *cis*-element ERSE is responsible for the transcriptional induction of HRD1.

2. Materials and methods

2.1. Real-time PCR

The expression of mRNA was measured by real-time PCR assay with Assays-on-Demand™ primer and probe sets (Applied Biosystems).

2.2. Construction of reporter plasmids and firefly dual-luciferase assay

Based on the NCBI gene database (HRD1; Chromosome: 11; Location: 11q13; Gene ID: 84447) or the published sequences of the human synoviolin (identical to HRD1), GRP78, and SEL-1L genes [3,10,11], a 1041-bp fragment of the HRD1 promoter (GenBank accession number AB162192; −1022 to +19 region; numbers indicate the nucleotide position relative to the transcription start site), a 1188-bp fragment of the SEL1 promoter (−1017 to +171 region) and a 549-bp fragment of the GRP78 promoter (−542 to +7 region) were generated by PCR from HEK293 genomic DNA and cloned into the pGL3-Basic vector (Promega). Mutants of ERSE were changed at the ATF6 binding site from 5′-CCACG-3′ to 5′-AACAT-3′. Firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega).

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Abbrevations: EDEM, ER degradation-enhancing α-mannosidase-like protein; ER, endoplasmic reticulum; ERAD, ER-associated degradation; ERSE, endoplasmic reticulum stress response element; Tg, thapsigargin; Tm, tunicamycin; UPR, unfolded protein response; UPRE, unfolded protein response element

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3. Results

3.1. Induction of HRD1 and SEL1 expression

We initially examined the expression of HRD1 and SEL1 induced by ER stress-inducing agents, such as thapsigargin (Tg; sarco/endoplasmic reticulum Ca^{2+}-ATPase inhibitor) and tunicamycin (Tm; *N*-glycosylation inhibitor). Increased HRD1 and SEL1 mRNA expression was observed and it peaked 6 h after the addition of Tm (Fig. 1A). On the other hand, the expression of GRP78 and CHOP, well-known ER stress-inducible genes, and mRNA was concomitantly elevated (Fig. 1A). Furthermore, we examined the expression of HRD1 and SEL1 proteins induced by ER stress in HEK293 cells. The expression of HRD1 and SEL1 was upregulated by treatment with Tm and Tg, and peaked 12–24 h following the peak of mRNA expression (Fig. 1B).

We determined the signaling pathways, IRE1-XBP1 or ATF6, used for the induction of HRD1 and SEL1 expression. The expression of HRD1 mRNA was increased by overexpression of ATF6 and XBP1 up to 4-fold (Fig. 1C, *upper left*). On the other hand, the expression of SEL1 mRNA was induced by only ATF6 up to 25-fold, while XBP1 did not affect the expression levels of SEL1 (Fig. 1C, *upper right*). The expression of GRP78 mRNA was increased by both ATF6 and XBP1, although the induction levels of XBP1 were lower than ATF6 (Fig. 1C, *lower left*). The induction form of CHOP, responsive to only ATF6, was similar to that of SEL1 (Fig. 1C, *lower right*). The protein levels of HRD1 and SEL1 were also increased by overexpression of ATF6 and XBP1 (Fig. 1D).

3.2. Inhibitory effects of IRE1 and ATF6 on the expression of HRD1 and SEL1 induced by ER stress

We examined the effects of inhibition of ATF6 and IRE1 on the expression of HRD1 and SEL1 induced by ER stress. The Tm-induced expression of HRD1 was significantly reduced from 7.2- to 3.8-fold in 293 cells stably expressing a dominant-negative IRE1 mutant (Supplemental Fig. 1); in addition, the expression of ER degradation-enhancing α-mannosidase-like protein (EDEM), an already known IRE1-XBP1-dependent gene [12], also decreased (Fig. 2A). On the other hand, the increased expression of SEL1 was not reduced by the IRE1 mutant (Fig. 2A). In contrast, the increased expression of SEL1 was significantly attenuated from 4.6 to 1.7 by ATF6 siRNA, while that of HRD1 was not significantly affected by siRNA (Fig. 2B). In addition, expression of GRP78 that is partially dependent on ATF6 also decreased (Fig. 2B). These results suggest that HRD1 is predominantly dependent on the IRE1-XBP1 pathway, while SEL1 is dependent on the ATF6 pathway.

3.3. Promoter region of HRD1 and SEL1

Transcriptional activation of ER stress-responsive genes, including GRP78, CHOP, and Herp, is induced via the ERSE [3,4]. To investigate the induction mechanism of the ERAD gene, we cloned promoter regions of HRD1 and SEL1 (Supplemental Fig. 2A, B, C). ERSE has so far been characterized to be of two types: ERSE-I (CCAAT-N_9-CCACG/A) and ERSE-II (ATTGG-N-CCACG) [3,4]. A complete ERSE-I (ERSE2) and an ERSE-I-like sequence (ERSE1) exist within the cloned HRD1 promoter region, while SEL1 has two ERSE-I-like motifs but not complete ERSE (Supplemental Fig. 2C, D). It has been reported that the activated form of XBP1 spliced by IRE1 induces the transcription of ER stress-responsive genes through binding to the mammalian UPRE (TGACGTGG/A) [5]. However, no UPRE motif was found in either cloned promoter, and we therefore decided to investigate the ERSE-I (ERSE2) of HRD1 (SEL1 promoter analyses: Supplemental Fig. 3).

3.4. Induction of HRD1 and SEL1 promoter activity

To examine whether the promoter regions of HRD1 and SEL1 are responsive to ER stress, we performed luciferase assay with each promoter ligated into the reporter gene (firefly luciferase). As expected, in the HRD1 promoter, luciferase expression was induced by treatment with Tg and Tm up to 2-fold, compared to the basal level (Fig. 3A). Moreover, the SEL1 promoter was responsive to these stressors at the same levels (2-fold) compared with HRD1 and GRP78, although there is no complete ERSE in the SEL1 promoter (Fig. 3A).

Next, we examined the effects of ATF6 and XBP1 on each promoter. The overexpression of ATF6 was most effective in the expression of the HRD1 reporter gene (3.2-fold) followed by XBP1 (2.4-fold), compared with mock transfection (Fig. 3B). Similarly, the GRP78 reporter gene was induced by both inducers, ATF6 (3.6-fold) and XBP1 (1.4-fold). On the other hand, the induction of SEL1 was only responsive

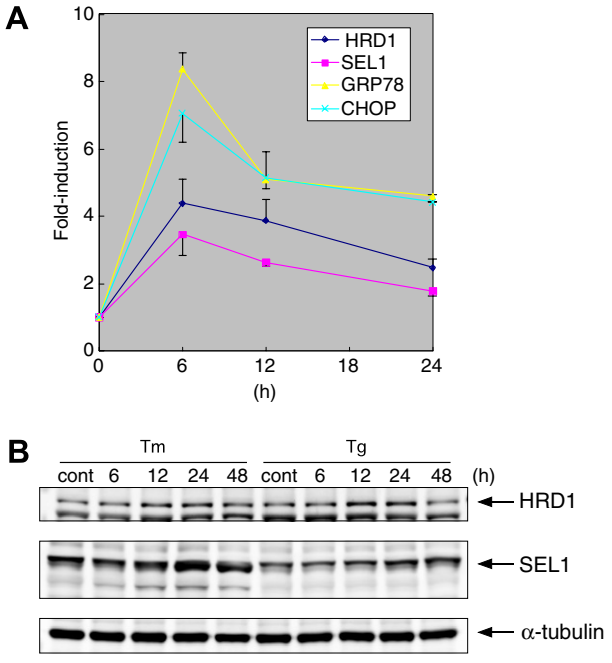

Fig. 1. Induction of HRD1 and SEL1 expression. (A) HEK293 cells were treated with 5 μg/ml of tunicamycin (Tm) for the periods indicated. The expression levels of mRNA were normalized for those of GAPDH and expressed as a fold increase compared with untreated cells and as means ± S.E.M. ($n = 3$). (B) HEK293 cells were exposed to 5 μg/ml of Tm or 1 μM of thapsigargin (Tg) for the periods indicated. Western blotting was performed using anti-HRD1 (*top*; C-term, ABGENT), SEL1 (*middle*; MSel1, ALEXIS), and anti-α-tubulin (loading control, *bottom*; tu-01, ZYMED) antibodies. (C) HEK293 cells were overexpressed with ATF6α (1–373 amino acid residues) or XBP1 (spliced form) using the T-REx™ system. The cells were treated with 1 μg/ml tetracycline for the periods indicated. Results were expressed as means ± S.E.M. ($n = 3$). (D) Western blotting was performed using anti-HRD1 (*upper*) and SEL1 (*lower*) antibodies.