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#### Citation
FEBS Letters, 581(28): 5355-5360

#### Issue Date
2007-11-27

#### Doc URL
http://hdl.handle.net/2115/32746

#### Type
article (author version)

#### File Information
kaneko.pdf
A different pathway in the endoplasmic reticulum stress-induced expression of human HRD1 and SEL1 genes

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Abbreviations: 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
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
INTRODUCTION

A number of environmental changes that affect the function of the 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-N9-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, 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 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.

MATERIALS and METHODS

Real-time PCR

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

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 293 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).
RESULTS

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).

Inhibitory effects of IRE1 and ATF6 on the expression of HRD1 and SEL1 induction 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 5.1- to 2.3-fold in 293 cells stably expressing a dominant-negative IRE1 mutant (Supplemental Fig. 1); in addition, the expression of ER degradation-enhancing alpha-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 significantly reduced by the IRE1 mutant (Fig. 2A). In contrast, the increased expression of SEL1 was significantly attenuated by ATF6 siRNA, while that of HRD1 was not significantly affected by siRNA. 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.

**Promoter region of HRD1 and SEL1**

Transcriptional activation of ER stress-responsive genes, including GRP78, CHOP, SERCA2, 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 (ERSE1 and 2) 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).
**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 to the overexpression of ATF6 (4.7-fold) (Fig. 3B). These results are consistent with the increased mRNA expression induced by ATF6 and XBP1 overexpression (Fig. 1C).

**Deletion and mutation analyses of HRD1 promoter**

We investigated the cis-acting elements responsible for the induction of HRD1 by ER stress and UPR signal transducers. Unexpectedly, a region from −599 to +17 in the HRD1 promoter with ERSE effectively reduced the transcriptional activity in response to Tg and Tm by approximately half as compared with the full length promoter (Fig. 4A). Moreover, a region from −175 to +17 in the HRD1 promoter lacking ERSE2 completely abolished the responsiveness to Tg and Tm to the basal levels (Fig. 4A).

To identify cis-acting elements responsible for ER stress-induced transcriptional
activation in the HRD1 promoter, we next investigated mutation effect of the ERSE motifs on
the activation under ER stress. In deletion promoter analysis, the region from –175 to +17 in
HRD1 lacking the ERSE motif was most effective, but another ERSE sequence (ERSE1)
existed downstream of ERSE2. We therefore tested the effect of ERSE2 disruption (mutant),
which is the replacement of the ATF6 binding site, on the transcriptional activation of the HRD1
promoter by ER stress. ERSE2 mutant almost completely blocked the HRD1 promoter activity
in response to treatment with Tm and Tg (Fig. 4B).

We next analyzed the deletion and mutation promoters using ATF6 and XBP1.
Deletion of the region from –599 to –175, including ERSE2, completely abolished the
transcriptional activation of HRD1 by each transducer as well as the ER stress condition (Fig.
4C).

We also examined the mutation effect of the ERSE on the HRD1 promoters in
transcriptional activation induced by ER stress transducers. In the HRD1 promoters, ERSE2
mutation reduced the transcriptional activation induced by ATF6 and XBP1 (Fig. 4D). These
results suggest that the induction of HRD1 expression is dependent on the ERSE (ERSE2).

DISCUSSION

In this study, we demonstrated that the expression of human HRD1 was induced by
the overexpression of both ATF6 and XBP1, and that human SEL1 was induced by only ATF6.
Additionally, the Tm-induced HRD1 expression was significantly suppressed by the
dominant-negative IRE1 mutant, while the SEL1 expression was significantly suppressed by
siRNA-mediated ATF6 silencing. HRD1 has a complete ERSE responsive to ER stress and
overexpressed ATF6 and XBP1 in the promoter region, whereas SEL1 does not have a complete
ERSE but responds to ER stress and ATF6. These results suggest that the induction signaling of
these ERAD genes is dependent on the different transcription factors due to variations of 
cis-elements in the promoter regions.

Ire1p (IRE1) mediates the induction of yeast ERAD gene expression, including 
HRD1 and HRD3, under ER stress [6]. The responsiveness of the ERAD gene expression, 
including human HRD1 and SEL1, to ER stress, appears to be conserved from yeast to 
mammals. Although several signal transducers, in addition to IRE1, participate in mammalian 
UPR, their different roles in the induction of mammalian ERAD gene are unknown. Yoshida et 
al. has reported that EDEM, which is involved in ERAD, is induced by ER stress via the 
IRE1-dependent pathway, and concluded that the genes constituting ERAD are induced by the 
IRE1-XBP1 pathway, while the induction of ER-resident chaperones is mediated by the ATF6 
pathway [12]. Our finding that the ER stress-induced HRD1 expression was dependent on IRE1 
is consistent with this hypothesis. In contrast, the induction of SEL1 appears to be an exception 
to that rule because of its dependence on ATF6. If SEL1 acts as an ERAD component with 
HRD1, these genes are likely to be induced concomitantly via a common signaling pathway 
under ER stress or, alternatively, the expression of SEL1 may precede that of HRD1 in waiting 
for HRD1 protein production, since yeast Hrd1p is unstable by itself without Her3p. Therefore, 
it seems reasonable that SEL1 expression prior to HRD1 is induced via ATF6 pathway 
expression in response to ER stress. However, protein upregulation of SEL1 did not precede that 
of HRD1 (Fig. 1B). Thus, it remains unclear why SEL1 is induced via ATF6 pathways under ER 
stress, and whether all ERAD genes use IRE1-XBP1 pathways in an ER stress-induced 
expression.

We found a complete ERSE-I (ERSE2; CCAAT-Nקורס-CCACG) and an ERSE-I-like 
sequence (ERSE1; CCATT-NCors-CCATG). ERSE2 was necessary for the induction of HRD1 
transcriptional activation in response to ER stress, since the transcriptional induction of HRD1
was decreased to basal levels by the mutation and deletion of ERSE2. Although the promoter region (−1022 to +17) does not include UPRE, the transcriptional induction of HRD1 seems to be sufficient compared with that of SEL1 and GRP78. In this study, we demonstrated that HRD1 induction by ER stress was dependent on IRE1 and ERSE-I (ERSE2). Yoshida et al. have reported that XBP1 acts on UPRE rather than ERSE, and that UPRE is involved in IRE1-mediated transcriptional induction [5, 12]. Our promoter-reporter analysis indicated that ERSE2 was responsive to XBP1 as well as ATF6; on the other hand, in electrophoretic mobility shift assay (EMSA), ATF6 but not XBP1 was able to bind to the ERSE, which is in agreement with the previous study (Supplemental Fig. 4A and B). These inconsistencies may be due to the nonspecific effects caused by overexpression.

Interestingly, ERSE-II (ATTGG-N-CCACG), which has been found in the Herp gene [4], has been demonstrated to act as a cis-acting element for XBP1 [13], although it was not found in the cloned HRD1 promoter. On the other hand, the existence of UPRE in the genes that depend on the IRE1-XBP1 pathway has so far not been demonstrated, since the effects of IRE1 and XBP1 on UPRE were investigated using only reporter vectors containing a tandem repeat of UPRE. Therefore, further physiological experiments are needed to determine which of ERSE-I, ERSE-II, or UPRE are actually involved in the induction of other IRE1-XBP1 dependent genes in addition to HRD1.

ACKNOWLEDGEMENTS

We particularly thank Otsuka GEN Research Institute for generously donating the TaqMan probes and primers. This study was supported by Grant-in-Aid for Young Scientists (B), 19790070, 2007 and Grants-in-Aid for Scientific Research (B), 19300135, 2007 from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.
REFERENCES


**LEGENDS**

**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. (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. (n=3). D. Western blotting was performed using anti-HRD1 (top), SEL1 (middle), and anti-α-tubulin (bottom) antibodies.

Fig. 2. Inhibitory effects of IRE1 and ATF6 on HRD1 and SEL1 induction by ER stress. A. Normal HEK293 cells and those stably expressing IRE1α-K599A were treated with 5 µg/ml Tm for 6 h. Results were expressed as means ± SEM (n = 3; Student’s t-test; *, P < 0.05; **, P < 0.01; versus Normal). B. HEK293 cells were transiently transfected with siRNA of non-targeted control (NC) or ATF6α, and incubated for 48 h. The cells were then treated with 5 µg/ml Tm for 6 h. Results were expressed as means ± SEM (n = 3; Student’s t-test; *, P < 0.05; **, P < 0.01; versus NC).

Fig. 3. HRD1 and SEL1 promoter activities. A. Each promoter ligated to the pGL-Basic vectors was transiently introduced into HEK293 cells together with the pRL-SV40 reference plasmid. Thirty hours after transfection, the cells were treated with and without 1 µM Tg or 5 µg/ml Tm and incubated for 18 h, and the lysates were subjected to luciferase assay. The value represents fold induction, which is the ratio of induced to basal levels of relative reporter activity (means ± SEM, n=3). B. Each HRD1 and SEL1 promoter was transiently transfected into HEK293 cells together with the pRL-SV40 plasmid and an empty vector (pCR3.1; mock), ATF6 (1–373), and XBP1 (spliced) expression vectors and incubated for 48 h. The value
represents fold induction, which is the ratio of induced to mock transfection of relative reporter activity (means ± SEM, n=3).

**Fig. 4. Deletion and mutation effects of HRD1 promoter.** A. Deletion effects of HRD1 promoter on ER stress-induced transcriptional activation. Thirty hours after transfection, the cells were treated with and without 1 µM Tg or 5 µg/ml Tm and incubated for 18 h. The value represents fold induction, which is the ratio of induced to basal levels of relative reporter activity (means ± SEM, n=3). B. Mutation effects of HRD1 promoter on ER stress-induced transcriptional activation. C. Deletion effects of HRD1 promoter on ATF6 and XBP1-induced transcriptional activation. Forty-eight hours after transfection, the cells were harvested and lysates were subjected to luciferase assay. The value represents fold induction, which is the ratio of induced to mock transfection levels of relative reporter activity (means ± SEM, n=3). D. Mutation effects of HRD1 promoter on ATF6 and XBP1-induced transcriptional activation.
Fig. 1

A

![Graph showing fold-induction over time for HRD1, SEL1, GRP78, and CHOP.](image)

B

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- **HRD1**
- **SEL1**
- **α-tubulin**
Fig. 1

C

**HRD1**

- ATF6
- XBP1

**SEL1**

- ATF6
- XBP1

**GRP78**

- ATF6
- XBP1

**CHOP**

- ATF6
- XBP1

(h)
Fig. 1

D

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(h)

- HRD1
- SEL1
Fig. 2

A

![Bar chart A: Fold-increase for HRD1, SEL1, and EDEM.](chartA.png)

- **Normal**
- **KA-IRE1**

B

![Bar chart B: Fold-increase for HRD1, SEL1, and GRP78.](chartB.png)

- **NC**
- **ATF6**

* **Fold-increase**

** Fold-increase
Fig. 3

A

Fold Increase

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B

Fold Increase

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Fig. 4

A

HRD1 Promoter

--- ERSE2 ERSE1 Luc -1022
--- ERSE2 ERSE1 Luc -599
--- ERSE1 Luc -175
--- Luc -126

Fold increase

0 1 2 3

B

HRD1 Promoter

--- ERSE2 ERSE1 Luc WT
--- ERSE2 ERSE1 Luc mutant

Fold increase

0 1 2 3

none □ Tg □ Tm
Fig. 4

**C**

**HRD1 Promoter**

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Legend: □ MOCK □ ATF6 □ XBP1

**D**

**HRD1 Promoter**

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Legend: □ MOCK □ ATF6 □ XBP1
Supplemental Fig. 2

C

-231  GCGCATTCTG  GGAATTTGAG  TCTCGGATGA  GCCTCTTTCT  CCCAGCTTTG
-181  GACTCACGCCG  CTGATTCGGG  TTGAACCCAG  GGGCTACCAA  TTAGCTGGGG
-131  *CTCATGCTCCGT  GAGTCTCCCG*  CCAAACCAGA  AAGCTGGTGT  GGGAGCTTGC
-81    TACAATGATT  GCCAAATGAA  TGACCCCCGG  GCGGAGCAA  TGGGGGTGGG
-31    CCGGGAGACC  ATGGTGATTG  GACCCAGAAC  CCGCAGCGGC  GGGCGGGAT
  +20   TGGCTGCGCG  CTGGGTCAGG  GAAGGCTGGG  AAGGGGCAGA  GGAAGGAGAC
  +70    TAGAGCAGGA  AGACGACGGG  CGAGGCCGGG  GTGGTGCCGTG  AGTCCGCTGT
+120    GGCAGAGGCG  AAGGCAGACG  CTCTAGGGGT  TGGCACCAGCC  CCCGAGGGA
+170    GGA*GCGGT  CCGATAGGG  CTGACGCTGC  TGCTGTCGTC  GGTGCTGCTG
+220   AGCTTGGCCT  CGGGCTCCCT  GGgtcagt

D

HRD1 Promoter

-1022  ERSE2  ERSE1  Luc

SEL1 Promoter

-1017  ERSE2  ERSE1  Luc

GRP78 Promoter

-542  ERSE3  ERSE2  ERSE1  Luc

NF-Y binding site (CCAAT)  sense  antisense

ATF6 binding site (CCACG)  sense  antisense
**Supplemental Fig. 3**

**A**

SEL1 Promoter

- **ERSE2**
- **ERSE1**
- Luc

- **ERSE2**
- **ERSE1**
- Luc

- **ERSE1**
- Luc

- **ERSE1**
- Luc

**Fold increase**

- 0
- 1
- 2
- 3

**B**

SEL1 Promoter

- **ERSE2**
- **ERSE1**
- Luc

- **ERSE2**
- **ERSE1**
- Luc

- **ERSE1**
- Luc

**WT**

- **ERSE2**
- **ERSE1**
- Luc

- **ERSE1**
- Luc

**mutant**

- **ERSE2**
- **ERSE1**
- Luc

- **ERSE1**
- Luc

**Fold increase**

- 0
- 1
- 2
- 3

**Legend**

- □ none
- □ Tg
- □ Tm
Supplemental Fig. 3

C  SEL1 Promoter

- ERSE2 ERSE1 Luc
- ERSE2 ERSE1 Luc
- ERSE1 Luc
- ERSE1 Luc

Fold increase

D  SEL1 Promoter

- ERSE2 ERSE1 Luc
- ERSE2 ERSE1 Luc

WT

mutant

Fold increase
Supplemental Fig. 4

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<td>HRD1</td>
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1 2 3 4 5 6

ATF6+ERSE complex
ERSE complex

B

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Shifted ATF6+ERSE complex
ATF6+ERSE complex
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