

## Possible Involvement of Ubiquitin Ligase HRD1 Insolubilization in Amyloid $\beta$ Generation

Masayuki Kaneko,<sup>a,#</sup> Ryo Saito,<sup>a,#</sup> Yasunobu Okuma,<sup>a</sup> and Yasuyuki Nomura<sup>\*,b</sup>

<sup>a</sup>Department of Pharmacology, Faculty of Pharmaceutical Sciences, Chiba Institute of Science, 15–8 Shiomi-cho, Choshi, Chiba 288–0025, Japan; and <sup>b</sup>Laboratory of Pharmacotherapeutics, Yokohama College of Pharmacy, 601 Matano-cho, Totsuka-ku, Yokohama 245–0066, Japan.

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**Endoplasmic reticulum (ER)-associated degradation (ERAD) selectively retro-transportes and degrades unfolded proteins accumulated in the ER. We have demonstrated that the ubiquitin ligase HRD1 involved in ERAD was significantly decreased in the cerebral cortex of Alzheimer's disease patients. Furthermore, the HRD1 level was negatively correlated with amyloid  $\beta$  ( $A\beta$ ) production levels. Here we found that the HRD1 protein level decrease was due to its insolubilization. Moreover, these protein levels extracted from detergent insoluble fraction were positively correlated with those of SEL1L and  $A\beta$ s ( $A\beta$ 40 and  $A\beta$ 42). Thus, the insolubilization-induced decrease in the HRD1 and SEL1L levels might involve in  $A\beta$  generation.**

**Key words** Alzheimer's disease; amyloid  $\beta$ ; HRD1; endoplasmic reticulum stress; SEL1L; insolubilization

Alzheimer's disease (AD) is a progressive neurodegenerative disease of unknown etiology. Accumulation of amyloid  $\beta$  ( $A\beta$ ) plaques and neurofibrillary tangles are the well-known neuropathological hallmarks of AD.<sup>1)</sup> The toxic  $A\beta$  peptides,  $A\beta$ 40 and  $A\beta$ 42, are generated from amyloid precursor protein (APP) via sequential proteolytic cleavages by  $\beta$ -secretase (BACE1) and  $\gamma$ -secretase complexes.<sup>2)</sup> APP is a type-I transmembrane glycoprotein, which is folded and *N*-glycosylated in the endoplasmic reticulum (ER) and is subsequently transported to the Golgi complex for further maturation.

The ER is involved in the following several cellular functions: (i) membrane and secretory protein folding, post-translational modification, and transport to the Golgi complex; (ii) maintenance of intracellular calcium homeostasis; (iii) synthesis of lipids and sterols; and (iv) regulation of cellular survival through the unfolded protein response (UPR) transducers, such as IRE1, ATF6, and PERK. Impairment of these processes causes an accumulation of unfolded proteins in the ER lumen, which is termed ER stress. ER stress activates the UPR pathways such as translational arrest, induction of ER chaperone, and ER-associated degradation (ERAD).<sup>3)</sup> ERAD pathways act as a defense mechanism against ER stress. In this pathway, ERAD targets are selected by a protein quality control mechanism within the ER lumen, subsequently removed by retrograde transport from the ER to the cytosol, and finally degraded by the ubiquitin–proteasome system.<sup>4,5)</sup> Recent studies have shown an involvement of ER stress and dysfunction of ERAD pathway in AD.<sup>6,7)</sup>

In previous studies, we identified and characterized a human homolog of yeast Hrd1p/Der3p (HRD1) and Hrd3p (SEL1L). HRD1 has an E3 ubiquitin ligase activity and it colocalizes and forms a complex with its stabilizing factor SEL1L in the cerebral nerve. Furthermore, HRD1 is expressed as a result of ER stress and protects against ER stress-induced apoptosis.<sup>5,8–12)</sup> We recently found that HRD1 promoted the ubiquitination and degradation of APP in ERAD pathway, resulting in decreased  $A\beta$  production. On the other hand, suppression of HRD1 expression caused an accumulation of APP and an increase in  $A\beta$  levels, accompanied by ER stress and apoptosis. Interestingly, we found that HRD1 protein levels were significantly low in the NP-40 detergent-soluble fraction

of the cerebral cortex of AD patients, and that low level of HRD1 protein in AD patients did not result from a decrease in mRNA expression but from a decrease in its protein levels.<sup>13)</sup> Moreover, the decrease in the protein levels of HRD1 highly correlated with  $A\beta$  production.<sup>14)</sup> However, it was not clear why HRD1 protein levels were reduced in the cerebral cortex. In this study, we investigated the protein levels of HRD1 and SEL1L in the NP-40 detergent-insoluble fraction of the controls and AD brains. Furthermore, we calculated Pearson product-moment correlation coefficient to determine whether HRD1 insolubilization is correlated to  $A\beta$  production.

### MATERIALS AND METHODS

**Antibodies** Antibodies were purchased as follows: Anti-HRD1/SYVN1 (1:2000 dilution; C-term; Sigma-Aldrich, St. Louis, MO, U.S.A.), Sel1L (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.),  $\beta$ -actin (1:2000 dilution; C4; Santa Cruz Biotechnology), anti-rabbit immunoglobulin G (IgG) and anti-mouse IgG horseradish peroxidase (1:2500 dilution; GE Healthcare, U.K., Ltd., Buckinghamshire, U.K.), anti-goat IgG horseradish peroxidase (1:5000 dilution; Promega, Madison, WI, U.S.A.).

**Analysis of Protein in Human Brains** Human brain samples were purchased from Analytical Biological Services (Wilmington, DE, U.S.A.) through KAC (Kyoto, Japan). These specimens showed no significant differences in age, postmortem interval, or gender between AD patients and controls. Gray matter was dissected from the cerebral cortex of deceased AD patients ( $n=5$ ) and controls ( $n=5$ ), which were selected based on the  $A\beta$  accumulation levels to provide biochemical evidence.<sup>14)</sup>

The tissues were extracted as described previously.<sup>13)</sup> NP-40 detergent-insoluble fractions were removed by centrifugation at 30000 $\times g$  for 30 min, and then the insoluble fractions were extracted by supersonic treatment in a buffer consisting of 10 mM Tris–HCl (pH 8.0), 420 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 0.5% sodium deoxycolate, 0.1% sodium dodecyl sulfate, 10  $\mu$ g/mL aprotinin, 10  $\mu$ g/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride, 100  $\mu$ M sodium orthovanadate, 10 mM sodium fluoride, and

\* To whom correspondence should be addressed. e-mail: y.nomura@hamayaku.ac.jp

# These authors contributed equally to this work.

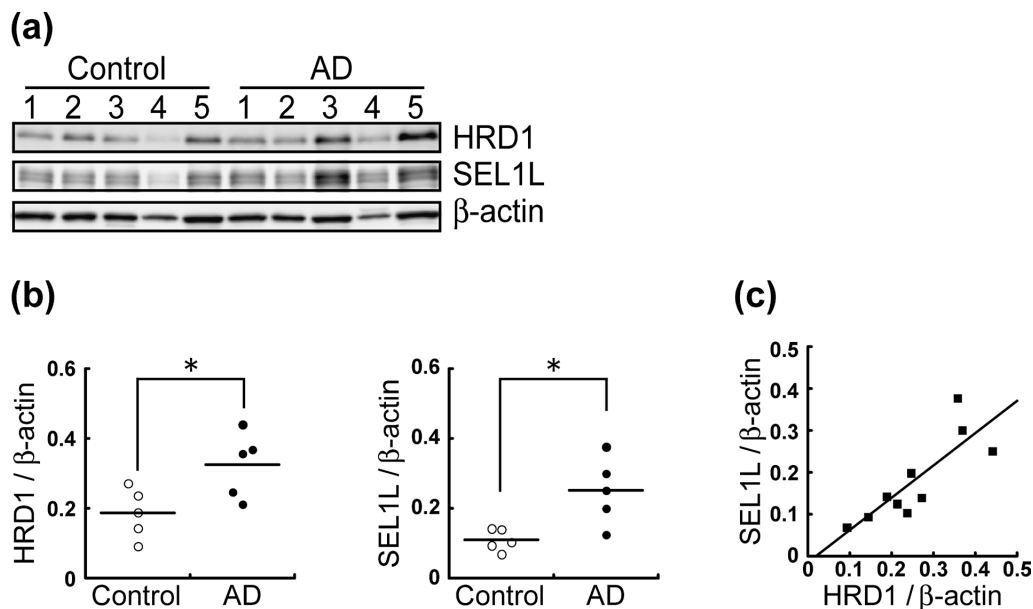


Fig. 1. Protein Levels of HRD1 and SEL1L in the NP-40 Detergent-Insoluble Fraction

(a) Typical images of immunoblot analyses for HRD1, SEL1L, and  $\beta$ -actin in the detergent-insoluble fraction of control and AD brains. The cerebral cortex of control and AD were subjected to Western blotting. (b) Statistical analysis of (a). These data are normalized to the amount of  $\beta$ -actin; results are expressed as a dot plot (control,  $n=5$ ; AD,  $n=5$ ). Asterisk represents a significant difference (Student's  $t$ -test,  $*p<0.05$ ). (c) Correlation of HRD1 and SEL1L protein levels in the detergent-insoluble fractions (Pearson correlation coefficient,  $r=0.81$ ).

100 nM okadaic acid. Unbroken fractions were removed by centrifugation at  $20400\times g$  for 20 min. The protein concentrations were determined using the Bio-Rad Protein assay kit (Bio-Rad Laboratories, Richmond, CA, U.S.A.). Protein equivalent samples were subjected to Western blotting using a LAS-3000 luminescent image analyzer (Fujifilm, Tokyo, Japan). Quantitative analysis was performed using Multi Gauge software (Fujifilm).

**A $\beta$  Enzyme-Linked Immunosorbent Assay (ELISA)** For A $\beta$  determination, it was extracted as described previously.<sup>15</sup> Approximately 150 mg of tissue was dounce-homogenized (6 strokes) in 1 mL of 70% formic acid. Homogenates were centrifuged at  $100000\times g$  for 1 h to remove particulate material. The supernatant was recovered and neutralized with a 20-fold dilution in 1 M Tris base. After neutralization, the samples were measured by standard sandwich ELISA using a human amyloid  $\beta$  (1–40) and (1–42) assay kit (IBL, Takasaki, Japan).

**Statistical Methods** Statistical comparisons were done using two-tailed Student's  $t$ -test with significance placed at  $p<0.05$ . All data are expressed as mean  $\pm$  S.E. Correlation coefficient was calculated using Pearson product-moment correlation coefficient.

## RESULTS

We have previously reported that HRD1 protein levels in the NP-40 detergent-soluble fraction significantly decreased, whereas HRD1 mRNA showed a tendency to increase in the cerebral cortex of AD patients compared with controls.<sup>13</sup> To determine the cause of the decrease in HRD1 protein level in the NP-40 detergent-soluble fraction, we investigated protein levels in the detergent-insoluble fraction of the cerebral cortex of control and AD patients. In contrast to the observation of the detergent-soluble fraction, HRD1 protein levels

significantly increased in the insoluble fraction (Figs. 1a,b). Interestingly, SEL1L, which localizes in the ER lumen and interacts with HRD1 to stabilize, also significantly increased in the insoluble fraction (Figs. 1a,b). These results suggest that the decrease in HRD1 protein levels in the detergent-soluble fraction was due to its protein insolubilization, raising the possibility that HRD1 coisolubilized with SEL1L. To investigate this possibility, we calculated the correlation coefficient between HRD1 and SEL1L in both fractions. The calculation revealed that HRD1 protein levels highly correlated with SEL1L in the detergent-insoluble fractions (Fig. 1c,  $r=0.81$ ), suggesting coisolubilization of HRD1 and SEL1L. However, there was no correlation between HRD1 and SEL1L in the detergent-soluble fractions (data not shown).

In the previous study, we found a negative correlation between HRD1 protein levels in the detergent-soluble fraction and both A $\beta$ 40 and A $\beta$ 42 production.<sup>14</sup> To determine whether HRD1 insolubilization is associated with both A $\beta$ 40 and A $\beta$ 42 generation, we calculated the correlation coefficient. In contrast to the previous observation of the detergent-soluble fraction, HRD1 protein levels in the detergent-insoluble fractions were positively correlated with both A $\beta$ 40 (Figs. 2a,b;  $r=0.70$ ) and A $\beta$ 42 (Figs. 2a,c;  $r=0.70$ ). This result is consistent with the previous observation of the detergent-soluble fraction, supporting our hypothesis that insolubilization of HRD1 protein causes dysfunctional HRD1, and this induces A $\beta$  production.

## DISCUSSION

We have proposed that the loss of HRD1-mediated ERAD is possibly linked to the onset of AD. In the present report, we showed that insoluble HRD1 was significantly increased in AD patients compared with controls. Furthermore, an increase in the insoluble HRD1 protein level was positively correlated with A $\beta$  levels. These findings support our hypothesis that the

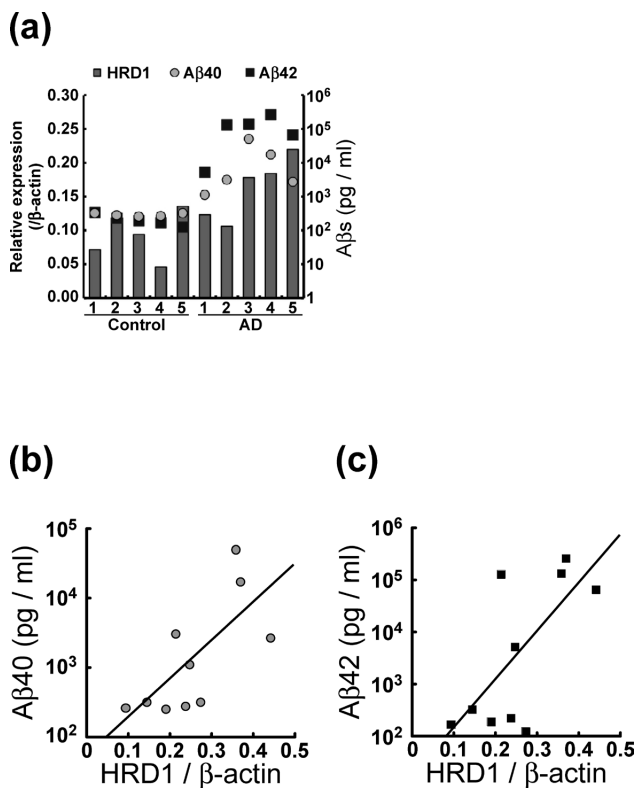


Fig. 2. Correlation between HRD1 Protein Level and A $\beta$  Production

(a) HRD1 protein level and A $\beta$ 40 and A $\beta$ 42 levels in the cerebral cortex of control and AD brains. HRD1 proteins in the insoluble fraction are normalized to the amount of  $\beta$ -actin. A $\beta$  levels were measured by a sandwich ELISA. (b,c) Correlation of HRD1 protein level with A $\beta$  production [Pearson correlation coefficient: (b) A $\beta$ 40,  $r=0.70$ ; (c) A $\beta$ 42,  $r=0.70$ ].

decrease in HRD1 protein levels in the cerebral cortex could be involved in A $\beta$  production during the pathogenesis of AD. However, it remains unclear how HRD1 protein is insolubilized. A recent report showed that Parkin, a ubiquitin ligase E3 that is involved in ERAD,<sup>16</sup> was induced to form aggregates by neurotoxins (1-methyl-4-phenyl pyridinium (MPP<sup>+</sup>), rotenone, 6-hydroxydopamine), nitric oxide, and dopamine.<sup>17</sup> Therefore, it is possible that a ubiquitin ligase HRD1 is also induced to form aggregates by such as agents that induce oxidative stress. On the other hand, it remains controversial whether the decrease in HRD1 protein promotes A $\beta$  generation or whether A $\beta$  neurotoxicity causes the decrease in HRD1 protein levels. It has been reported that high levels of A $\beta$  were also observed in non-AD patients containing mild cognitive impairment.<sup>18</sup> Thus, considering these cases, the decrease in HRD1 could mainly contribute to neurodegeneration in the pathogenesis of AD after A $\beta$  generation. Further investigation into the mechanism of HRD1 insolubilization may provide important clues about the underlying cause of AD.

There was a positive correlation between HRD1 and SEL1L levels in the insoluble fractions (Fig. 1c), whereas soluble HRD1 levels were not correlated with soluble SEL1L levels.<sup>13</sup> We speculate the reason for this inconsistency is a difference in protein content. In other words, HRD1 protein content might be much less than SEL1L. Although HRD1 and SEL1L necessarily form a complex, it is unknown whether other ubiquitin ligases (E3s) interact with SEL1L. If SEL1L insolubilization was caused by HRD1 insolubilization, the residual

SEL1L proteins might have interacted with other E3s that remained intact.

The present study suggests that a decrease in HRD1 levels as a result of its insolubilization may be involved in A $\beta$  generation, which may be associated with AD. In conclusion, our findings may propose a new model for AD pathogenesis associated with loss of ERAD.

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## REFERENCES

- Selkoe DJ. Alzheimer's disease: genes, proteins, and therapy. *Physiol. Rev.*, **81**, 741–766 (2001).
- Thinakaran G, Koo EH. Amyloid precursor protein trafficking, processing, and function. *J. Biol. Chem.*, **283**, 29615–29619 (2008).
- Schröder M, Kaufman RJ. The mammalian unfolded protein response. *Annu. Rev. Biochem.*, **74**, 739–789 (2005).
- Tsai B, Ye Y, Rapoport TA. Retro-translocation of proteins from the endoplasmic reticulum into the cytosol. *Nat. Rev. Mol. Cell Biol.*, **3**, 246–255 (2002).
- Kaneko M, Nomura Y. ER signaling in unfolded protein response. *Life Sci.*, **74**, 199–205 (2003).
- Katayama T, Imaizumi K, Manabe T, Hitomi J, Kudo T, Tohyama M. Induction of neuronal death by ER stress in Alzheimer's disease. *J. Chem. Neuroanat.*, **28**, 67–78 (2004).
- Huttunen HJ, Guénette SY, Peach C, Greco C, Xia W, Kim DY, Barren C, Tanzi RE, Kovacs DM. Htra2 regulates beta-amyloid precursor protein (APP) metabolism through endoplasmic reticulum-associated degradation. *J. Biol. Chem.*, **282**, 28285–28295 (2007).
- Omura T, Kaneko M, Tabei N, Okuma Y, Nomura Y. Immunohistochemical localization of a ubiquitin ligase HRD1 in murine brain. *J. Neurosci. Res.*, **86**, 1577–1587 (2008).
- Omura T, Kaneko M, Onoguchi M, Koizumi S, Itami M, Ueyama M, Okuma Y, Nomura Y. Novel functions of ubiquitin ligase HRD1 with transmembrane and proline-rich domains. *J. Pharmacol. Sci.*, **106**, 512–519 (2008).
- Kaneko M, Yasui S, Niinuma Y, Arai K, Omura T, Okuma Y, Nomura Y. A different pathway in the endoplasmic reticulum stress-induced expression of human HRD1 and SEL1 genes. *FEBS Lett.*, **581**, 5355–5360 (2007).
- Omura T, Kaneko M, Okuma Y, Orba Y, Nagashima K, Takahashi R, Fujitani N, Matsumura S, Hata A, Kubota K, Murahashi K, Uehara T, Nomura Y. A ubiquitin ligase HRD1 promotes the degradation of Pael receptor, a substrate of Parkin. *J. Neurochem.*, **99**, 1456–1469 (2006).
- Kaneko M, Ishiguro M, Niinuma Y, Uesugi M, Nomura Y. Human HRD1 protects against ER stress-induced apoptosis through ER-associated degradation. *FEBS Lett.*, **532**, 147–152 (2002).
- Kaneko M, Koike H, Saito R, Kitamura Y, Okuma Y, Nomura Y. Loss of HRD1-mediated protein degradation causes amyloid precursor protein accumulation and amyloid-beta generation. *J. Neurosci.*, **30**, 3924–3932 (2010).
- Saito R, Kaneko M, Okuma Y, Nomura Y. Correlation between decrease in protein levels of ubiquitin ligase HRD1 and amyloid-beta production. *J. Pharmacol. Sci.*, **113**, 285–288 (2010).
- Borchelt DR, Thinakaran G, Eckman CB, Lee MK, Davenport F, Ratovitsky T, Prada CM, Kim G, Seekins S, Yager D, Slunt HH, Wang R, Seeger M, Levey AI, Gandy SE, Copeland NG, Jenkins

- NA, Price DL, Younkin SG, Sisodia SS. Familial Alzheimer's disease-linked presenilin 1 variants elevate Abeta1-42/1-40 ratio *in vitro* and *in vivo*. *Neuron*, **17**, 1005—1013 (1996).
- 16) Imai Y, Soda M, Inoue H, Hattori N, Mizuno Y, Takahashi R. An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of Parkin. *Cell*, **105**, 891—902 (2001).
- 17) Wang C, Ko HS, Thomas B, Tsang F, Chew KC, Tay SP, Ho MW, Lim TM, Soong TW, Pletnikova O, Troncoso J, Dawson VL, Dawson TM, Lim KL. Stress-induced alterations in Parkin solubility promote Parkin aggregation and compromise Parkin's protective function. *Hum. Mol. Genet.*, **14**, 3885—3897 (2005).
- 18) Jack CR Jr, Knopman DS, Jagust WJ, Shaw LM, Aisen PS, Weiner MW, Petersen RC, Trojanowski JQ. Hypothetical model of dynamic biomarkers of the Alzheimer's pathological cascade. *Lancet Neurol.*, **9**, 119—128 (2010).