The ubiquitin-like domain of Herp is involved in Herp degradation, but not necessary for its enhancement of amyloid β-protein generation

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Abstract Herp is an endoplasmic reticulum (ER)-stress-inducible membrane protein, which has a ubiquitin-like domain (ULD). However, its biological function is as yet unknown. Previously, we reported that a high expression level of Herp in cells increases the generation of amyloid β-protein (Aβ) and that Herp interacts with presenilin (PS). Here, we addressed the role of the ULD of Herp in Aβ generation and intracellular Herp stability. We found that the ULD is not essential for the enhancement of Aβ generation by Herp expression and the interaction of Herp with PS, but is involved in the rapid degradation of Herp, most likely via the ubiquitin/proteasome pathway. Thus, the ULD of Herp most likely plays a role in the regulation of the intracellular level of Herp under ER stress.

Key words: Presenilin; Herp; Amyloid β-protein; Endoplasmic reticulum stress; Ubiquitin-like domain; Proteasome

1. Introduction

Amyloid β-protein (Aβ), which is the major component of senile plaques in the brains of patients with Alzheimer's disease (AD), is generated from the amyloid precursor protein (APP) through its sequential proteolytic cleavage catalyzed by β- and γ-secretases (reviewed in [1]). β-Secretase was identified as a membrane-tethered aspartyl protease [2]; γ-secretase, however, remains to be clarified, although presenilin (PS) was found to be essential for inducing γ-secretase activity [3–6]. Mutations in the PS genes, *PS1* and *PS2*, cause early-onset familial AD (reviewed in [1]). PS is a multiple transmembrane protein and forms a high-molecular-weight complex with several other proteins (reviewed in [6]). Recent studies have shown that the PS complex, including nicastrin, PEN-2 and APH-1, is responsible for inducing γ-secretase activity [7–10]. However, it still remains unknown how the PS-mediated γ-cleavage is regulated. In order to elucidate how the γ-cleavage is regulated, we have recently developed a new functional screening method for identifying cDNAs that enhance γ-cleavage using the combination of puromycin resistance assay and Aβ quantitation [11]. Previously, we have identified *Herp* using this screening method [12]. *Herp* was originally identified as a homocysteine-inducible gene and was found to be also inducible by endoplasmic reticulum (ER) stress [13,14]. Homocysteine or the inducers of ER stress, including thapsigargin, induce the expression of Herp mRNA approximately 50-fold over levels observed in untreated cells [13]. We have demonstrated that a high expression level of Herp, which is comparable to the level induced by homocysteine or ER stress, increases PS-mediated Aβ generation, possibly through Herp binding to PS [12]. Interestingly, elevated levels of homocysteine in plasma are likely to be a risk factor for AD [15]. Therefore, Herp induced by a high level of homocysteine may play an important role in Aβ accumulation, including the formation of senile plaques or vascular Aβ deposits in AD. Herp is a membrane protein localized in the ER and it has an N-terminal ubiquitin-like domain (ULD) [13]; however, the precise function of this protein and the role of the ULD of Herp have not been established.

Ubiquitin is a highly conserved small protein widely present in all eukaryotic cells. The modification of cellular proteins with ubiquitin targets them for degradation by proteasome. Accumulating evidence has shown that ubiquitin-like proteins (ULPs), which have structural similarity to ubiquitin, are also present in cells. ULPs are divided into two subclasses (reviewed in [16]): small, type-1 ULPs, such as SUMO-1 and NEDD8, which are ligated to target proteins in a manner analogous to the ubiquitination pathway (reviewed in [17]), and type-2 ULPs that contain a ubiquitin-like structure in large proteins, such as elongin B [18], Rad23 [19], parkin [20] and Herp [13]. Although type-1 ULPs constitute a new type of post-translational protein-modifying system, the biological significance of type-2 ULPs remains to be clarified. The ULD of elongin B appears to have a chaperone-like function [18], whereas a recent report showed that the intracellular level of parkin is regulated by the ULD [21]. In this

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Abbreviations: Aβ, amyloid β-protein; PS, presenilin; AD, Alzheimer's disease; APP, β-amyloid precursor protein; ER, endoplasmic reticulum; ELISA, enzyme-linked immunosorbent assay; ULD, ubiquitin-like domain; ULP, ubiquitin-like protein; ERAD, ER-associated degradation

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study, we determined the role of the ULD of Herp. For this purpose, we first investigated whether the ULD of Herp is involved in the pathway leading to the enhancement of Aβ generation caused by a high expression level of Herp, since the precise mechanism underlying this action is not known. Second, we determined whether the cellular level of Herp is regulated by this domain via the ubiquitin/proteasome pathway similar to the function of ubiquitin.

2. Materials and methods

2.1. Antibodies, reagents and cell lines

An affinity-purified rabbit anti-Herp antibody was prepared as previously described [13]. A rat anti-PS1 antibody (for the N-terminal fragment (NTF) of PS1) and a rabbit anti-PS2 antibody (for the NTF) were purchased from Chemicon International (Temecula, CA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. A monoclonal antibody 6E10 specific to human Aβ1–17 was purchased from Senetek (St. Louis, MO, USA). BA27 specific to the Aβ40 terminal site, BC05 specific to the Aβ42 terminal site and BNT77 raised against human Aβ11–28 have all been characterized previously [22]. The 369 antibody was raised against the C-terminal residues of APP695 [23]. A monoclonal anti-FLAG antibody and a rabbit polyclonal anti-FLAG antibody were purchased from Sigma. Anti-multi-ubiquitin antibody was purchased from MBL. Cycloheximide was purchased from Wako. Lactacystin was purchased from Kyowa. Monoclonal antibody against β-tubulin was purchased from Babco (Richmond, CA, USA). HEK293 cells stably transfected with APP695 and PS1, which were used for the study of the interaction between PS1 and Herp, were generated as previously reported [12].

2.2. Plasmids and retrovirus-mediated infection

pMX-Herp was generated as previously described. The ΔUb-Herp cDNA encoding Herp lacking Val^{10} to Cys^{86} of Herp was inserted at the *Eco*RI and *Sal*I sites of pMX (designated pMX-ΔUb-Herp). We generated C-terminally FLAG-tagged ΔUb-Herp using the polymerase chain reaction method (sense primer: GGAATTCGCCACCATGGAGTCCGAGACCGA; antisense primer: GCGTCGACTCACTTATCGTCATCGTCCTTGTAGTCGTTTGCGATGGCTGGGGGGC) from Herp cDNA. This cDNA was inserted into pCI (Promega) at *Eco*RI and *Sal*I sites (designated pCI-ΔUb-Herp-F). The *Eco*RI-*Sal*I fragment of pCI-ΔUb-Herp-F was also inserted into pcDNA 3.1 (Invitrogen) at *Eco*RI and *Sal*I sites (designated pcDNA-ΔUb-Herp-

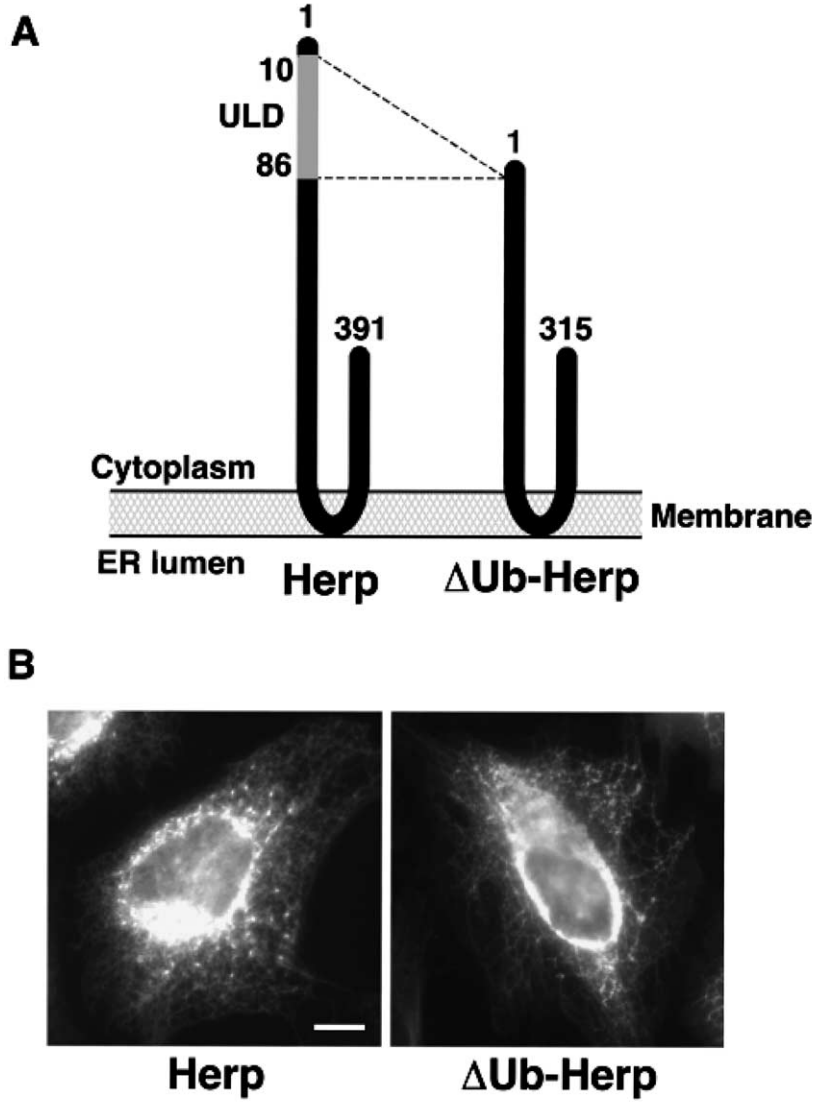

Fig. 1. Schematic representation of Herp lacking the ULD (ΔUb-Herp) (A) and the cellular localization of ΔUb-Herp (B). A: ΔUb-Herp encodes Herp that lacks the residues Val^{10}–Cys^{86}. The gray box illustrates the ULD. B: HeLa cells were transiently transfected with pCI-Herp-F or pCI-ΔUb-Herp-F. The cells were immunostained with anti-FLAG antibody. Scale bar, 10 μm.

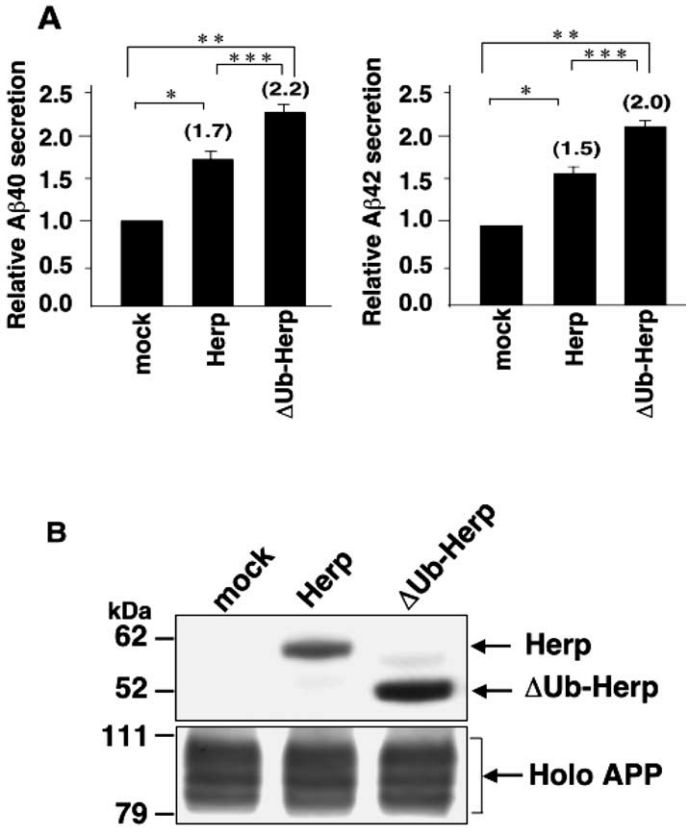

Fig. 2. Effect of the deletion of the ULD of Herp on Aβ generation. A: Murine fibroblasts (2×10^5) expressing APP695 were retrovirally transfected with pMX (mock), pMX-Herp or pMX-ΔUb-Herp. Aβ secreted during a 72-h culture was detected by ELISA. Values are means ± S.D. of four independent dishes ($n=4$). Similar results were obtained from four independent experiments. Relative amounts of Aβ (figures in parentheses) were determined by calculating the ratio of the amounts of Aβ secreted from the cells expressing Herp or ΔUb-Herp to the mean amount of Aβ secreted by a mock transfectant (normalized to 1). For Aβ40, $^*P<0.01$; $^{**}P<0.01$; $^{***}P<0.01$ (Mann–Whitney *U*-test). For Aβ42, $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.05$. B: Lysates (10 μg) were immunoblotted with the anti-Herp antibody (upper panel). Intracellular APP level (holo APP) was detected with the anti-C-terminal APP antibody, 369 (lower panel). WB, Western blots.

F). The *Bsu*36I-*Sal*I fragment of ΔUb-Herp-F cDNA was replaced with that of wild-type Herp and inserted at the *Eco*RI and *Sal*I sites of pMX, producing pMX-ΔUb-Herp. The retrovirus-mediated infection was carried out as previously reported [11].

2.3. Fluorescent immunocytochemistry

HeLa cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum on 35-mm dishes. The cells were transfected with pCI-Herp-F or pCI-ΔUb-Herp-F using FuGENE6 (Roche). At 24 h post transfection, the cells were immunostained with anti-FLAG rabbit polyclonal antibody (Sigma) followed by Alexa Fluor 488-labeled secondary antibody (Molecular Probes) as previously described [13]. Fluorescence was visualized with the Axiovert 200 microscope equipped with AxioCam (Carl Zeiss).

2.4. Immunoprecipitation, immunoblotting and sandwich ELISA

Cultured cells were lysed in RIPA buffer (150 mM NaCl, 10 mM Tris–HCl pH 7.5, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS) and 0.2% sodium deoxycholate) containing a protease inhibitor cocktail. The solubilized proteins were subjected to immunoprecipitation as previously described [24]. The precipitated proteins were resolved by SDS–polyacrylamide gel electrophoresis on 4–20% gel for the detection of PS and Herp, and on 7.5% gel for the detection of intracellular APP. Immunoblotting was performed as previously reported [24]. Enzyme-linked immunosorbent assay (ELISA) for Aβ was performed as previously described [22]. The capture antibody used was BNT77. Detector antibodies were horseradish peroxidase (HRP)-conjugated BA27 (for Aβ40) and HRP-conjugated BC05 (for Aβ42). ELISA data were statistically analyzed by ANOVA using StatView-J.4.11.

2.5. Cycloheximide and lactacystin treatment

Cells (5×10^5) plated on 60-mm tissue culture dishes were grown for 24 h, then cycloheximide and/or lactacystin were added to final concentrations of 30 μg/ml and 100 μM, respectively. At various times after the addition of cycloheximide and/or lactacystin, the cells were harvested and then lysed in RIPA buffer.

3. Results and discussion

As previously reported, Herp has the N-terminal region, Val^{14}–Val^{85}, which includes the stretch sharing 32% identity with the Val^5–Val^{70} stretch of ubiquitin [13]. In order to elucidate the role for this ULD of Herp, we generated Herp lacking the residues Val^{10}–Cys^{86} (designated ΔUb-Herp) (Fig. 1A). We confirmed with the immunostaining study that there is no significant difference in intracellular localization between Herp and ΔUb-Herp (Fig. 1B). Previously we demonstrated that a high expression level of Herp enhances PS-mediated Aβ generation. Therefore, in this study, we first investigated whether the expression of ΔUb-Herp also increases Aβ generation to determine whether the ULD is necessary for the increase in Aβ generation caused by a high expression level of Herp. As shown in Fig. 2A, ΔUb-Herp expression also increased the level of Aβ. Thus, we concluded that the ULD of Herp is not necessary for the enhancement of Aβ generation. It was noted in our previous study that Herp

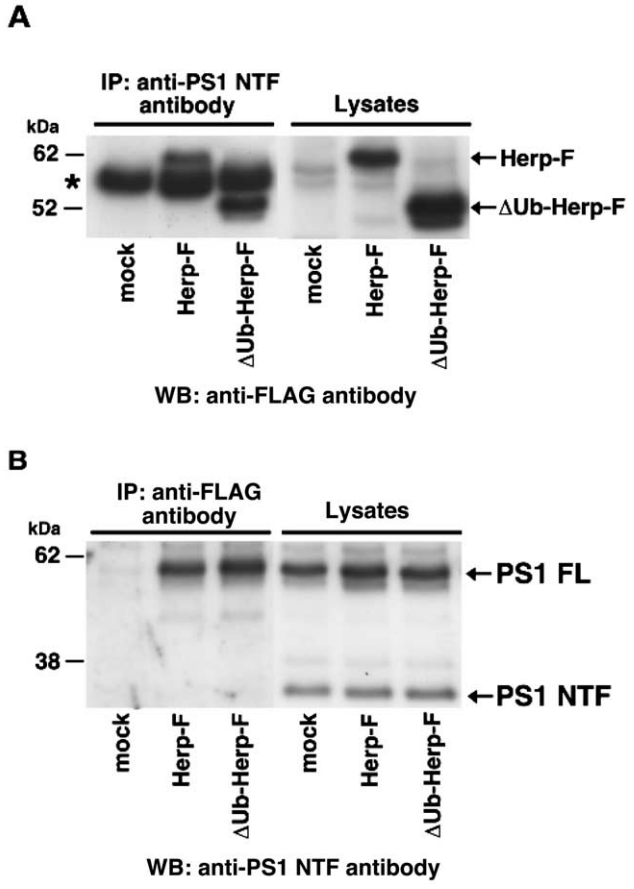

Fig. 3. Interaction between PS and Herp lacking the ubiquitin-like domain. A: HEK293 cells stably expressing APP and PS1 transiently transfected with pcDNA (mock), pcDNA-Herp-F or pcDNA-ΔUb-Herp-F. RIPA-solubilized lysates (100 μg) were immunoprecipitated with the anti-PS1 NTF antibody and immunoblotted with the anti-FLAG antibody (left, three lanes). The lysates (10 μg) were also immunoblotted with the anti-FLAG antibody (right, three lanes). IP, Immunoprecipitation; WB, Western blots. Herp-F, C-terminally FLAG-tagged Herp; ΔUb-Herp-F, C-terminally FLAG-tagged ΔUb-Herp. The asterisk indicates a non-specific band. B: The same RIPA-solubilized lysates (100 μg) were immunoprecipitated with the anti-FLAG antibody and immunoblotted with the anti-PS1 NTF antibody (left, three lanes). The lysates (10 μg) were also immunoblotted with the anti-NTF antibody (right, three lanes). Note: The anti-FLAG antibody was used for the immunoprecipitation of Herp-F and ΔUb-Herp-F since the anti-Herp antibody used in this study cannot immunoprecipitate ΔUb-Herp.

induced a greater increase in Aβ40 level than Aβ42 level in HEK293 cells [12], but in fibroblasts the extents of increase in both Aβ40 and Aβ42 levels were almost the same (Fig. 2A). The effect of Herp on Aβ42 generation appears to be different between the two cell types. It was also noted that, as shown in Fig. 2A, the extent of increase induced by the expression of ΔUb-Herp was slightly higher than that induced by the expression of wild-type Herp, probably because the protein level of ΔUb-Herp was higher than that of Herp (Fig. 2B; see below: the ULD is involved in Herp degradation). We next determined whether ΔUb-Herp can also bind to PS, since Herp interacts with the full-length PS as previously reported [12]. As shown in Fig. 3, ΔUb-Herp bound to the full-length PS1, but failed to bind to PS1 NTF. We also noted that ΔUb-Herp bound to the full-length PS2 (data not shown). Taken together, these data show that the ULD of Herp is not involved in the pathway leading to the enhancement of Aβ generation and in the interaction with PS.

We next examined the effect of the deletion of the ULD of Herp on intracellular Herp stability, because it is well established that ubiquitin is involved in the protein degradation via the ubiquitin/proteasome pathway. Western blot analysis of intracellular degradation of Herp and ΔUb-Herp in fibroblasts following cycloheximide treatment revealed that Herp is rapidly degraded within 6 h, while ΔUb-Herp remains stable (Fig. 4A). The quantification of band intensity showed that the half-life of Herp is approximately 2.5 h, while that of ΔUb-Herp is more than 24 h (Fig. 4B). Furthermore, (i) Herp degradation was completely inhibited after treating the cells with the proteasome inhibitor lactacystin (Fig. 5A); (ii) the immunoprecipitated Herp and ΔUb-Herp contained smear bands on the gel, which were immunoreactive with the anti-polyubiquitin antibody (Fig. 5B), strongly suggesting that Herp and

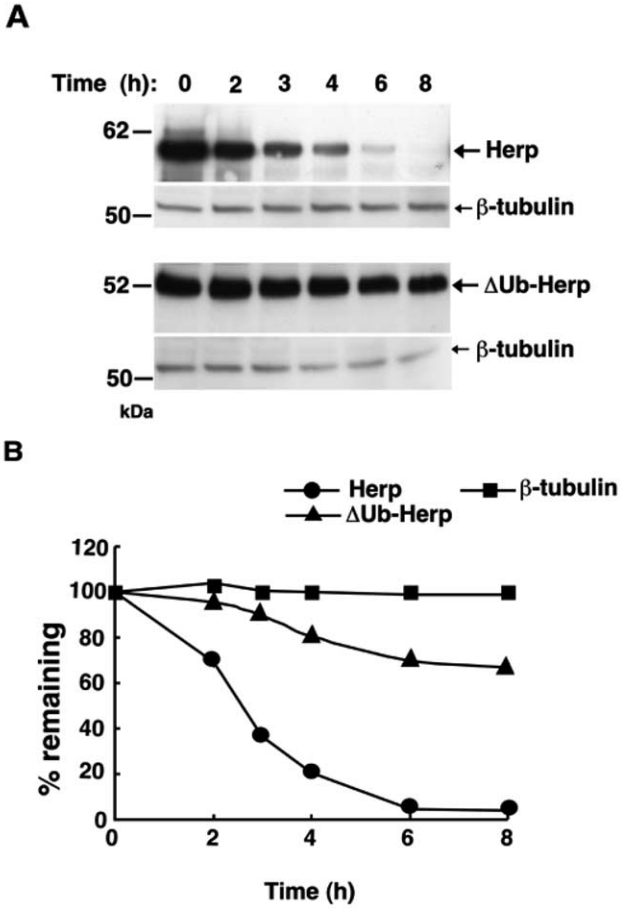

Fig. 4. Stabilization of Herp by deletion of the ULD. A: Wild-type fibroblasts retrovirally expressing Herp and ΔUb-Herp were treated with 30 μg/ml cycloheximide and then harvested at the times indicated. Herp or ΔUb-Herp in the RIPA-solubilized lysates (10 μg) was detected by immunoblotting with the anti-Herp antibody. As a control for the stable protein in the lysates, β-tubulin was immunodetected with anti-β-tubulin antibody. Upper panel: lysates from cells expressing Herp; lower panel: lysates from cells expressing ΔUb-Herp. The blots are representative of four independent experiments. B: The intensities of the bands corresponding to Herp, ΔUb-Herp and β-tubulin in A were quantified densitometrically using NIH Image software (PDI). Relative intensity was calculated as percentage of the intensity at time 0. Time, the time period of cycloheximide treatment.

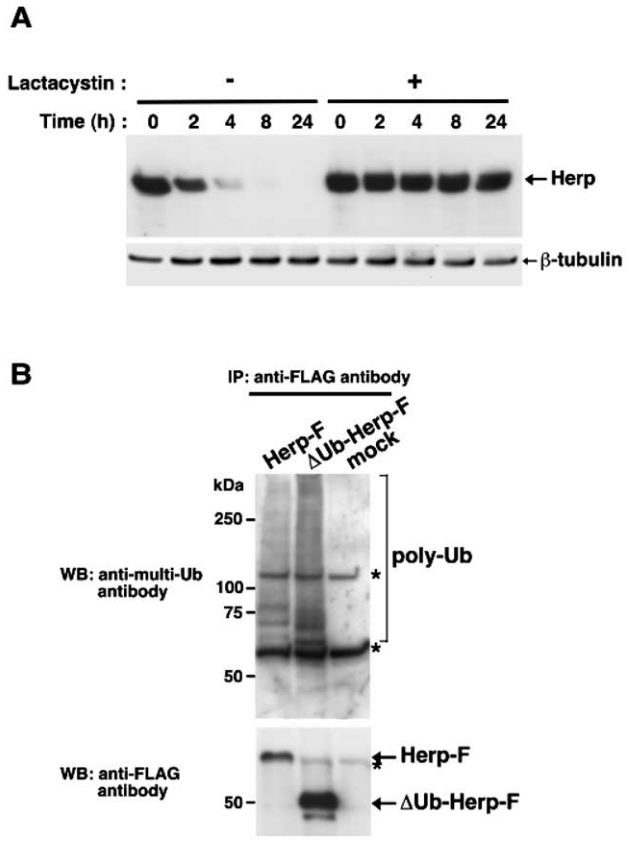

Fig. 5. Effect of lactacystin treatment on Herp degradation (A) and detection of Herp polyubiquitination (B). A: Wild-type fibroblasts retrovirally expressing Herp were treated with 30 μg/ml cycloheximide in the absence (−) or presence (+) of lactacystin and then harvested at the times indicated. Herp in the RIPA-solubilized lysates (10 μg) was detected by immunoblotting with the anti-Herp antibody. As a control for the stable protein in the lysates, β-tubulin was immunodetected with anti-β-tubulin antibody. B: Fibroblasts transiently transfected with pcDNA-Herp-F or pcDNA-ΔUb-Herp-F or pcDNA (mock) were treated with cycloheximide and lactacystin for 8 h, and then harvested. The cell lysates were immunoprecipitated with anti-FLAG mouse antibody and then immunodetected with anti-multi-ubiquitin antibody (upper panel) or anti-FLAG rabbit antibody (lower panel). Ub, ubiquitin, Herp-F, C-terminally FLAG-tagged Herp; ΔUb-Herp-F, C-terminally FLAG-tagged ΔUb-Herp. The asterisk indicates a non-specific band. IP, immunoprecipitation; WB, Western blots.

ΔUb-Herp undergo polyubiquitination. Taken together, Herp is degraded most likely via the ubiquitin/proteasome pathway, and the ULD is not involved in Herp polyubiquitination.

In this study, we noted that the ULD of Herp is not essential for the increase in Aβ generation mediated by Herp expression. We also demonstrated that the ULD of Herp is indeed involved in the degradation of Herp, most likely via the ubiquitin/proteasome pathway. Recently, it has been shown that the ULD of parkin regulates the intracellular parkin level and parkin polyubiquitination does not require the ULD [21]. In this regard, the ULD of Herp has a role very similar to that of parkin. However, it is not known how the ULD of Herp is involved in Herp degradation. Further study of the role of the ULD in Herp degradation is necessary. At present, the function of this protein is not known; Herp may, however, mediate the degradation of misfolded proteins induced by ER stress through the ULD, since Herp is the ER-stress-inducible ER-resident protein. The ER-associated degradation (ERAD) pathway is known to direct the ubiquitin-mediated degradation of various ER-associated misfolded proteins (reviewed in [25]). In response to ER stress, a series of ERAD-related genes are induced [26,27]. Parkin is also suggested to be involved in ERAD (reviewed in [28]). Therefore, *Herp* may also be an ERAD-related gene.

Since a strong ubiquitin immunoreactivity is associated with the pathogenesis of AD [29], the ubiquitin/proteasome protein degradation system appeared to be impaired in the brains of AD patients. We demonstrated that a high expression level of Herp enhanced Aβ generation, and Herp is degraded via the ubiquitin/proteasome pathway. Therefore, an impairment of the ubiquitin/proteasome system in AD may reduce Herp degradation, resulting in the enhancement of Aβ generation induced by a higher level of Herp.

Taking these data together, the ULD of Herp most likely plays a role in the regulation of the intracellular level of Herp under ER stress, and also possibly in AD pathogenesis. Further study of the mechanism underlying Herp degradation and the Herp-mediated increase in Aβ generation is required.

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