Endoplasmic Reticulum Stress-inducible Protein, Herp, Enhances Presenilin-mediated Generation of Amyloid β-Protein

Received for publication, December 26, 2001, and in revised form, January 16, 2002. Published, JBC Papers in Press, January 17, 2002, DOI 10.1074/jbc.M112372200

Xiaorei Sai,a Yuuki Kawamura,a,c Koichi Kokame,a Hiroyasu Yamaguchi,a Hirohisa Shiraiishi,a Ryu Suzuki,a Toshiharu Suzuki,b Masashi Kawaichi,a Toshiyuki Miyata,db Toshio Kitamura,d Bart De Strooper,e Katsuhiro Yanagisawa,a and Hiroto Komanoa,j

From the aDepartment of Dementia Research, National Institute for Longevity Sciences, 36-3 Gengoro, Morioka, Ohi, Aichi 474–8522, Japan, bJapan Science and Technology Corporation (JST), Kawaguchi, Saitama 332–0012, Japan, cNational Cardiovascular Center Research Institute, Osaka 565-8565, Japan, dGunma University School of Health Sciences, Maebashi Gunma 371-8511, Japan, eNeuroscience, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan, fDivision of Gene Function in Animals, Nara Institute of Science and Technology, Ikoma, Nara 630-01, Japan, gDivision of Cellular Therapy, Advanced Clinical Research Center, Institute of Medical Sciences, University of Tokyo, Tokyo 108-8639, Japan, and the iNeuronal Cell Biology Laboratory, K.U. Leuvan and Flanders Interuniversitary Institute for Biotechnology, Herestraat 49, B-3000 Leuven, Belgium

Presenilin (PS) is essential for the γ-cleavage required for the generation of the C terminus of amyloid β-protein (Aβ). However, the mechanism underlying PS-mediated γ-cleavage remains unclear. We have identified Herp cDNA by our newly developed screening method for the isolation of cDNAs that increase the degree of γ-cleavage. Herp was originally identified as a homocysteine-responsive protein, and its expression is up-regulated by endoplasmic reticulum stress. Herp is an endoplasmic reticulum-localized membrane protein that has a ubiquitin-like domain. Here, we report that a high expression of Herp in cells increases the level of Aβ generation, although not in PS-deficient cells. We found that Herp interacts with both PS1 and PS2. Thus, Herp regulates PS-mediated Aβ generation, possibly through its binding to PS. Immunohistochemical analysis of a normal human brain section with an anti-Herp antibody revealed the exclusive staining of neurons and vascular smooth muscle cells. Moreover, the antibody strongly stained activated microglia in senile plaques in the brain of patients with Alzheimer disease. Taken together, Herp could be involved in Aβ accumulation, including the formation of senile plaques and vascular Aβ deposits.

Mutations in the presenilin genes, PS1 and PS2, cause early onset familial Alzheimer disease (AD) (1–3) and lead to an increase in the ratio of Aβ42/Aβ40 generation from β-amyloid precursor protein (APP) (reviewed in Ref. 4). PS is required for intramembranous cleavage of APP (termed γ-cleavage) and Notch (5–8). Recently, ErbB4 has been found to be another natural substrate of the PS-mediated intramembranous proteolysis (9). Interestingly, although the γ-cleavage of APP is a critical step toward the production of Aβ, the major intramembranous cleavage site of APP was found to be distinct from the γ-cleavage site (10, 11). The mechanism underlying PS-mediated intramembranous proteolysis including γ-cleavage remains to be clarified. To date, a number of PS-interacting proteins have been identified, but no natural interactors with PS have been found to modulate Aβ generation, although some mutant forms of nicastrin, which was identified as a component of the PS complex, increase Aβ production (12). To elucidate the mechanism underlying the PS-mediated γ-cleavage, we have recently developed a new functional screening method for identifying cDNAs that increase the degree of γ-cleavage using a combination of γ-cleavage-dependent purumycin-resistant assay and Aβ quantitation (see “Experimental Procedures”; the details of this screening method will be described elsewhere). Using this method, we have identified Herp, which was originally identified as a homocysteine-induced protein (13). Interestingly, elevated levels of homocysteine are correlated with multiple neurological disorders (14–16). Plasma homocysteine levels have been reported to be elevated in some cases of AD (16) and could be an early marker of cognitive impairment in the elderly (17). Herp mRNA is constitutively expressed in various tissues, but its expression is up-regulated by homocysteine or the inducers of ER stress such as 2-mercaptoethanol, A23187, and thapsigargin (13, 18). Herp is a membrane protein localized in the ER, and it has an N-terminal ubiquitin-like domain; the function of this protein, however, is not known (13). In contrast to ER stress-induced molecular chaperones including BiP and GRP94 in the ER lumen, a major portion of Herp molecules are present in the cytoplasmic side of the ER membrane (13). Herp may have a role distinct from that of molecular chaperones under ER stress. Here, we show that Herp enhances PS-mediated Aβ generation and that it interacts with PS.

EXPERIMENTAL PROCEDURES

Isolation of Herp cDNA—We designed a functional screening system for isolating cDNAs that increase the degree of γ-cleavage as follows.
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Fig. 1. Effects of Herp expression on Aβ generation from full-length APP. a, HEK293 cells stably expressing APP695 retrovirally infected with the pMX-puro vector (14) harboring Herp cDNA or the vector alone. Cells stably expressing Herp were selected in the presence of puromycin. Aβ40 secreted from cells (4 × 10⁵) during 12 h was immunoprecipitated with 6E10 and detected with B27. Immunoprecipitated Aβ from cells grown on one culture dish was loaded in one lane. The lysates (10 μg) were immunoblotted with anti-Herb antibody. WB, Western blots. b, ELISA for Aβ secreted from the cells stably expressing Herb after a 12-h culture (for Aβ40) or a 48-h culture (for Aβ42). HEK293 cells stably expressing APP were retrovirally infected with the pMX-puro vector harboring Herb cDNA or vector alone. Cells stably expressing Herb were selected in the presence of puromycin. The relative amounts of Aβ were determined by calculating the ratio of the amount of Aβ secreted by cells expressing Herb to the mean amount of Aβ secreted by a mock transfectant (normalized to 1). Values are the means ± S.D. of two independent experiments of two independent Herb or mock-stable transfectants (n = 4). *, p < 0.02; **, p > 0.05 (Mann-Whitney's U test). c, PS1/PS2 double-deficient fibroblasts or wild-type fibroblasts (5 × 10⁵) plated on a 100-mm tissue culture dish and retrovirally infected with retroviral vector, pMX (mock), PMX-Herb, or pMX-PS1, together with pMX-APP695. Aβ40 secreted during a 24-h incubation was detected. The level of intracellular APP (holo-APP) was determined as reported previously (23). HEK293 cells stably expressing APP695 were retrovirally infected with the pMX-puro vector harboring cDNAs generated from A5 cells treated with a retrovirus-mediated infection was carried out as reported previously (20). The retrovirus-mediated infection was carried out as reported previously (20). The retrovirus-mediated infection was carried out as reported previously (20). PS-deficient Fibroblasts—Tissue from PS-deficient embryo (24) was digested with collagenase and cultured in Dulbecco's modified Eagle's medium-F12 containing 10% fetal calf serum. Outgrowing cells were immobilized with large T antigen and split twice a week.

Antibodies, Immunoprecipitation, Immunoblotting, and Sandwich ELISA—An affinity-purified rabbit anti-Herb antibody was prepared as described previously (13). A rat anti-PS1 antibody (for the N-terminal fragment of PS1) and a rabbit anti-PS2 antibody (for the N-terminal fragment) were purchased from Chemicon International, Inc. (Temecula, CA) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), respectively. A monoclonal antibody 6E10 specific to human Aβ1–17 was purchased from Senetek (St. Louis, MO). BA27 specific for the Aβ40 terminal site, BC05 specific for the Aβ42 terminal site, BAN50 raised against Aβ1–6, and BNT77 raised against human Aβ1–28 have all been characterized previously (25). Affinity-purified rabbit antibody 369 was raised against the C-terminal residues of APP695 (26). Affinity-purified rabbit antibody B12/4 was raised against 20 C-terminal amino acid residues of APP695 (27). Cultured HEK293 cells were lysed in RIPA buffer (150 mM NaCl, 10 mM Tris/HCl pH 7.5, 1% Nonidet P-40, 0.1% SDS, and 0.2% sodium deoxycholate) containing a protease inhibitor mixture. The solubilized proteins were subjected to immunoprecipitation as described previously (28). The precipitated proteins were resolved by SDS-PAGE on 4–20% gel for the detection of PS and Herb and on 7.5% gel for the detection of intracellular APP. Immunoblotting was performed as reported previously (28). The secreted Aβ was immunoprecipitated and detected using a highly sensitive immunoblotting technique.

(The details of the screening system will be described elsewhere.) We established a cell line (designated as A5–9), Ba/F3 cells (19) stably transfected with pcXN-C53NICD (see below) and pHES1-pac (see below) in which an increase in the degree of γ-secretase cleavage concerns on the cells an increase in puromycin resistance. Ba/F3 cells do not express endogenous APP. To initiate screening, a human hippocampus-derived cDNA library in a retroviral vector, pMX, was infected into A5–9 cells and designated as pHES-pac. We ensured that the cells expressing (CLONTECH) was inserted into PGV-B-HES-1. The final plasmid was designated as PGV-B-HES-1. (ii) A reporter system (13) into pcDNA3.1 or pMX. The effect of the Herp cDNA (13) reported previously (23). HEK293 cells stably transfected with APP695 and human PS1 were generated from HEK293 cells stably expressing APP695 by the transfection of the cells with the pcDNA 3.1-Hygro vector (Invitrogen) carrying human PS1. PS-deficient Fibroblasts—Tissue from PS-deficient embryonic (24) was digested with collagenase and cultured in Dulbecco’s modified Eagle’s medium-P12 containing 10% fetal calf serum. Outgrowing cells were immortalized with large T antigen and split twice a week.

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[2] H. Komano, Y. Kawamura, H. Shiraiishi, X. Sai, R. Suzuki, M. Kawaichi, T. Kitamura, and K. Yanagisawa, unpublished data.

[3] H. Komano and K. Yanagisawa, unpublished data.
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Fig. 2. Interaction of presenilin and Herp. a, HEK293 cells stably expressing APP and PS1 transiently transfected with pcDNA-Herp. RIPA-solubilized lysates (100 μg) were immunoprecipitated with anti-PS1 N-terminal fragment (NTF) antibody and immunoblotted with anti-Herp antibody. The lysates (10 μg) were also immunoblotted with the anti-HERP antibody (right two lanes). WB, Western blots. b, HEK293 cells stably expressing APP and PS1 were transiently transfected with the recombinant pcDNA-Herp. RIPA-solubilized lysates (100 μg) were immunoprecipitated with anti-Herp and immunoblotted with anti-PS1 N-terminal fragment antibody. The lysates (10 μg) were also immunoblotted with anti-PS1 N-terminal fragment antibody (right two lanes). The same result was also obtained when the lysate was prepared using a different detergent such as digitonin, which extracts the complex of nicastrin and the endoproteolytic product of PS (12). FL, full-length. c, HEK293 cells transiently transfected with C-terminal FLAG-tagged (and also N-terminal Myc-tagged) Herp (Herp-F) and PS2. RIPA-solubilized lysates (100 μg) were immunoprecipitated with the anti-PS2 N-terminal fragment antibody and immunoblotted with anti-FLAG antibody. The lysates (10 μg) were also immunoblotted with the anti-FLAG antibody (right two lanes). d, RIPA-solubilized lysates (100 μg) of HEK293 cells transiently transfected with PS2 and FLAG-tagged Herp were immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-PS2 N-terminal fragment antibody. The lysates (10 μg) were also immunoblotted with the anti-PS2 antibody (right two lanes).

RESULTS

We first investigated whether transfection of Herp cDNA increases the extent of Aβ generation from full-length APP using HEK293 cells stably expressing APP. We found that a stably high expression level of Herp increased the extent of Aβ40 generation (Fig. 1a). Statistical analysis of ELISA results using the cells stably expressing Herp showed that the increase in the Aβ40 level caused by the high expression of Herp was about 1.8-fold (Fig. 1b). In contrast, the effect of Herp on the Aβ42 level was not significant (Fig. 1b). We next tested whether an increase in the level of Aβ generation by Herp expression can occur in PS-deficient cells since it is not conclusive yet whether PS itself is a γ-secretase. As shown in Fig. 1c, no Aβ was detected in PS-deficient fibroblasts expressing Herp, whereas Herp expression in wild-type fibroblasts increased the Aβ level. We also confirmed that the intracellular APP level was not changed by the high expression level of Herp (Fig. 1c). These results indicate that Herp enhances PS-mediated Aβ40 generation. In addition, the level of intracellular β-secretase-cleaved C-terminal APP fragment (C99) was significantly reduced by Herp transfection, whereas the level of soluble α-secretase-cleaved N-terminal APP fragment (α-APPs) was not changed (Fig. 1d). These results strongly suggest that Herp preferentially increases a degree of γ-secretase cleavage.

Since both PS and Herp reside in the ER, it is likely that Herp interacts with PS, resulting in an increase in the extent of Aβ generation. Therefore, we next investigated the interaction of PS with Herp by coimmunoprecipitation studies using HEK293 cells stably expressing PS1 plus APP. An anti-PS1 antibody coimmunoprecipitated with Herp (Fig. 2a). The level of the coimmunoprecipitated Herp was approximately equivalent to that of Herp contained in the 10% starting lysate used for immunoprecipitation (Fig. 2a). Considering that the efficiency of immunoprecipitation by the anti-PS1 antibody is about 10% (data not shown), it is likely that almost the entire amount of Herp interacts with PS1. PS undergoes endoproteolysis, forming N- and C-terminal fragments (29). Interestingly, an anti-Herp antibody immunoprecipitated the full-length PS1, but it only immunoprecipitated a small amount of the N-terminal fragment of PS1 (Fig. 2b). These results indicate that Herp mainly interacts with the full-length PS1. The study of PS2 and Herp interaction was also performed using

**Histochemical Analysis**—Acetone-fixed cryostat sections or Kryofix-paraffin sections were prepared from five nondemented and five AD brains. Cryostat sections were used only for the double immunofluorescent study. Acetone-fixed cryostat sections or Kryofix-paraffin sections were reacted with a primary antibody mixture (anti-Herp, 500 μg/ml) followed by biotinylated secondary antibodies. Positive signals were visualized by incubating the sections in a diaminobenzidine-H2O2 solution. For the double immunofluorescent study, after pretreatment with 0.1% Sudan black B in 70% ethanol for 7 min to mask autofluorescence, Kryofix paraffin sections were reacted with a primary antibody mixture (anti-Herp, 5 μg/ml and anti-Aβ40, 4G8, monoclonal, 1:1,000) and then reacted with the anti-Herp antibody (5 μg/ml) or the anti-HLA-DR, DP, and DR antibodies (major histocompatibility complex II antigen, 3 μg/ml; DAKO, CR3/43) antibody followed by biotinylated secondary antibodies. Positive signals were visualized by incubating the sections in a diaminobenzidine-H2O2 solution.
HEK293 cells that transiently expressed PS2 and FLAG-epitope-tagged Herp. As shown in Fig. 2c, full-length PS2 coprecipitated with FLAG-epitope-tagged Herp. Thus, Herp interacts with both PS1 and PS2, and the major PS molecule that interacts with Herp was the full-length PS. The expression of Herp did not alter the steady-state levels of full-length PS and N-terminal fragment (Fig. 2, b and d), suggesting that Herp does not enhance the endoproteolysis of PS. APP and Herp interaction was also studied. Herp only coprecipitated APP at less than 1% of the total APP in the cells (data not shown). Therefore, in all probability, APP does not specifically interact with Herp. Thus, it is likely that the direct interaction of the full-length PS with Herp causes an increase in the degree of PS-mediated γ-cleavage of APP. Alternatively, at present, we cannot exclude the possibility that Herp indirectly affects the Aβ level, for example, by regulating the intracellular calcium level, since it was suggested that the intracellular calcium level modifies the Aβ level (30). Herp expression is up-regulated by calcium ionophores (13), and Herp may potentially regulate the intracellular calcium level.

Immunohistochemical analysis of Herp in a human brain section was performed to identify the cell types expressing a high level of Herp in the brain; these cells are considered to generate a high level of Aβ. As shown in Fig. 3a, only neurons were positive for Herp in the parenchyma cerebral cortex of a nondemented human brain. Interestingly, microglia in senile plaques in the brain of AD patients were strongly stained (Fig. 3b). The staining of HLA-DP, DQ, and DR (markers of activated microglia (31)) and Herp in serial sections (Fig. 3c) indicates that some of the activated microglia in the center of senile plaques are highly immunoreactive with the anti-Herp antibody. Double immunostaining with anti-Herp and anti-HLA-DP, DQ, and DR antibodies also confirmed that activated microglia are strongly positive for Herp (see supplemental data).

In addition, smooth muscle cells in meningeal arteries and arterioles were strongly stained (Fig. 3d).

**DISCUSSION**

One hallmark of AD is the accumulation of Aβ, although the cause of AD may be multifactorial. Here, we showed that ER stress-inducible Herp enhances Aβ40 generation. Since a significant increase in the level of Aβ42 was not observed, the high expression level of Herp appears to mainly enhance 40-γ-secretase cleavage. A recent report showed that some nonsteroidal anti-inflammatory drugs preferentially decreased the generation of Aβ42 (32). Taken together with our result, it appears that the regulation of the generation of Aβ42 is distinct from...
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that of Aβ40, even though the generation of both Aβ forms requires PS. 

HSP was originally discovered as a homocysteine-induced protein. It is noteworthy that a high plasma homocysteine level was observed in some cases of AD (14). At present, we cannot determine whether HSP directly or indirectly activates γ-secretase. We have shown that HSP interacts with the full-length PS rather than with its endoproteolytic products. A transient overexpression of HSP did not enhance the endoproteolytic cleavage of PS nor change the PS level. Since endoproteolytic cleavage of PS is not essential for the biological function of PS or Aβ generation (34), one interpretation for the enhancement of Aβ generation caused by HSP expression is that HSP directly activates the full-length PS-mediated γ-secretase activity by binding to the full-length PS, possibly through a change in the conformation or the trafficking of PS. The physical interaction of HSP with PS also raises the possibility that the function of PS is modulated by ER stress through HSP expressed at high levels. It was reported that cells exposed to inducers of ER stress, such as calcium ionophore, A23187, or thapsigargin, have increased the extracellular Aβ level, suggesting that the intracellular calcium level regulates Aβ generation (30). Taken together with our results, it is most likely that these inducers cause a HSP-mediated increase in the Aβ level since these inducers strongly up-regulate HSP expression.

Our present study showed that, in the normal brain, HSP was expressed in neurons and highly expressed in vascular cells, suggesting that these cells are the major source of Aβ in the normal brain. Interestingly, HSP expression was up-regulated in activated microglia in senile plaques in the AD brain. Activated microglia are thought to play a role in the clearance of Aβ. However, some activated microglia, which strongly express HSP, may generate a high level of Aβ, thereby contributing to the formation of senile plaques or vascular Aβ deposits, in patients with AD.

In particular, it is interesting that HSP has an N-terminal ubiquitin-like domain. Several proteins were reported to have a ubiquitin-like domain in eukaryotic cells, but the biological significance of this domain still remains to be clarified. In some cases, such as ubiquitin itself, a ubiquitin-related domain is involved in targeting proteins for protein degradation (35), but other functions such as chaperon function or protein-protein interaction were also reported (36). Presenilin undergoes degradation via the ubiquitin/proteasome pathway (29). However, the steady-state level of PS in cells expressing HSP was not changed as compared with that in nontransfected cells, strongly suggesting that the high expression level of HSP is not involved in the degradation of PS. Mutations in the PARKIN gene were discovered in families with an autosomal recessive form of Parkinson disease (37). Parkin also has the ubiquitin-like domain in the N-terminal site, although the function of the domain is not known, and it is up-regulated by ER stress (38, 39). Recent reports indicate that parkin is involved in the ubiquitination of Pael receptor and α-synuclein (39, 40). Since a strong ubiquitin immunoreactivity is associated with the pathology of both AD and Parkinson disease, the complex of presenilin and HSP with a ubiquitin-like domain may also function in the elimination of misfolded proteins via the ubiquitin/proteasome pathway as parkin does. Further exploration of the biological significance of the interaction of HSP with PS will not only help determine the pathways by which γ-secretase activity is regulated by PS but may also further clarify the role of PS under ER stress or in AD pathogenesis.

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