Increased Production of β-Amyloid and Vulnerability to Endoplasmic Reticulum Stress by an Aberrant Spliced Form of Presenilin 2*

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An alternative spliced form of the presenilin 2 (PS2) gene (PS2V) lacking exon 5 has previously been reported to be expressed in human brains in sporadic Alzheimer's disease (AD). PS2 encodes the amino-terminal portion of PS2, which contains residues Met1-Leu119 and 5 additional amino acid residues (SSMAG) at its carboxyl terminus. Here we report that PS2V protein impaired the signalling pathway of the unfolded protein response, similarly to familial AD-linked PS1 mutants and caused significant increases in the production of both amyloid β40 and β42. Interestingly, PS2V-encoding protein was expressed in neuropathologically affected neurons of the hippocampal CA1 region and temporal cortex in AD patients. These findings suggest that the aberrant splicing of the PS2 gene may be implicated in the neuropathology of sporadic AD.

Alzheimer's disease (AD) is a neurodegenerative disorder clinically characterized by progressive loss of memory and other cognitive abilities. Pathologically, severe neuronal loss, glial proliferation, extracellular deposition of senile plaques composed of amyloid β protein (Aβ), and intraneuronal neurofibrillary tangles are found in the AD brain (1). Direct relationships, however, between these morphological changes and the molecular mechanisms of AD onset have not been established. Familial forms of AD (FAD) have been linked to mutations in three different genes; the amyloid precursor protein (APP) gene, which leads to generation of mRNA lacking exon 5 in chromosome 21 (2), the presenilin 1 (PS1) gene on chromosome 17 (11) and the presenilin 2 (PS2) gene on chromosome 1 (3), and the presenilin 2 (PS2) gene on chromosome 1 (4, 5). Because the pathological features of both FAD and sporadic AD brains are thought to be identical or quite similar, genes mutated in FAD are considered to be logical candidates for further investigation of the etiology of sporadic AD.

Alternative splicing represents a typical mechanism underlying regulation of gene expression in eukaryotic cells (6, 7). Exon selection results in the production of different protein isoforms from the same gene, isoforms that may share functions with the original form. Alternatively, variant protein isoforms may either lack function or confer novel characteristics on their cellular environment. In fact, two splicing defects lacking exon 4 (8, 9) and exon 9 (10) of the PS1 transcript have been identified in FAD, and tau splicing mutations that increase four-repeat isoforms containing exon 10 of tau were found in frontotemporal dementia and Parkinsonism linked to chromosome 17 (11). In addition, aberrant transcripts of the excitatory amino acid transporter-2 gene are commonly present in sporadic amyotrophic lateral sclerosis patients (12).

Recently, we found an alternatively spliced form of the PS2 gene, which leads to generation of mRNA lacking exon 5 in sporadic AD brains, and this product (PS2V) was preferentially expressed in AD brains compared with those of age-matched controls (13). From in vitro experiments, the aberrant splicing was demonstrated to be induced in cultured cells under hypoxia. The neuroblastoma lines that were stably transfected with PS2V were shown to be susceptible to various cell stresses. However, the mechanisms by which PS2V sensitizes cells to various stresses have been unknown, and it also has been unclear whether PS2V is implicated in the neuropathology of sporadic AD.

We report here that PS2V protein affected the unfolded protein response (UPR) and also caused an increase in production of both Aβ40 and Aβ42. Furthermore, PS2V-encoding protein was indeed translated from the aberrant spliced form of the PS2 gene in neuropathologically affected neurons of the hippocampus and the temporal cortex in sporadic AD patients.

EXPERIMENTAL PROCEDURES

Antibodies—An antibody to PS2V was obtained by serial immunization of rabbits with the synthetic peptide SSMAG. The anti-SSMAG antibody was then purified using a peptide affinity column. An antibody recognizing the PS2 amino terminus has been described before (13). The anti-Aβ42 monoclonal antibody was obtained by serial immunization of mice with the synthetic peptide MVGVVIA. The anti-Aβ42 antibody was then purified using a peptide affinity column. The anti-TAU2 monoclonal antibody, anti-β-actin monoclonal antibody, and anti-Bcl-xl antibody were purchased from Sigma, Stressgen, and MBL, respectively.

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‡ The abbreviations used are: AD, Alzheimer's disease; FAD, familial AD; APP, amyloid precursor protein; PS, presenilin; PS2V, an alternative spliced form of the PS2 gene; Aβ, amyloid β protein; ER, endoplasmic reticulum; UPR, unfolded protein response; GRP78/BiP, glucose-regulated protein/immunoglobulin-binding protein; Tm, tunicamycin; SFV, Semliki Forest virus.
Immunoprecipitation and Ire1 Phosphorylation Assay—Immunoprecipitation was performed on 20 μg of crude lysate from HEK293T cells transfected with flag-tagged wild PS2 or PS2V using anti-PS2N or anti-SSMAG polyclonal antibodies. Western blotting analysis was performed with anti-FLAG monoclonal antibody.

Human GRP78 Semliki Forest Virus (SFV) Expression System—Human GRP78 cDNA was subcloned into the pSFV1 expression plasmid (Life Technologies). The cDNA was transformed into Competent E. coli (Life Technologies). The transformants were selected on kanamycin plates. The cDNA was purified and subcloned into the expression plasmid pSFV1. The plasmid was transformed into E. coli. The cells were infected with the SFV recombinant viral particles. The SFV recombinant viral particles were harvested and concentrated by ultracentrifugation.

Enzyme-linked Immunosorbent Assay and Metabolic Labeling of AP—Neuro 2a cells were plated on six-well dishes, and the medium was changed the next day. Then, the culture media was collected 24 h after the medium change and subjected to sandwich enzyme-linked immunosorbent assays as developed by Takeda Chemical Industries, Ltd. (15). For metabolic labeling of AP, stable transformants of each PS2 construct were incubated for specified times with [35S]methionine. The cells were extracted by Nonidet P-40 lysis buffer and immunoprecipitated using anti-APP antibody (22C11; Roche Molecular Biochemicals).

RESULTS

In Vitro Expression of PS2V Protein—Recently, we found an alternatively spliced form of the PS2 gene, which leads to generation of mRNA lacking exon 5 in sporadic AD brains, and we found that this product was preferentially expressed in AD brains compared with those of age-matched controls (15). The lack of exon 5 causes a frameshift in exon 6. This product (PS2V) encodes the amino-terminal portion of PS2, which contains residues Met1-Leu119 and an additional 5 amino acid residues (SSMAG) at its carboxyl terminus (Fig. 1A). We generated a polyclonal antibody specific for this "SSMAG" (anti-SSMAG) and a polyclonal antibody specific for the PS2 amino-terminal portion (anti-PS2N). The specificities of these were checked as shown in Fig. 1B. Immunofluorescence assay showed that wild PS2 protein was localized to intracellular membrane compartments, especially the endoplasmic reticulum (ER) and Golgi apparatus (data not shown). These findings
were consistent with those of previous studies (16). PS2V immunoreactivities were also found in the ER and Golgi as well as in wild PS2 (data not shown).

We examined whether the PS2 splice variant could actually be translated to the PS2V protein under conditions of hypoxia, because the aberrant splicing of PS2 was induced by hypoxic stress in cultured cells (13). Human neuroblastoma SK-N-SH cells were exposed to hypoxia for 20 h, and immunoprecipitation experiments were performed using an anti-SSMAG antibody. PS2V protein, the molecular mass of which was ~15 kDa, was detected in extracts of hypoxic cells by Western blotting using an anti-PS2N antibody (Fig. 1C).

PS2V Affects the UPR Signaling and the Sensitivity to ER Stress—Previously, missense mutations in PS2 were shown to decrease the phosphorylation of endogenous presenilins (data not shown).

We examined whether PS2V protein binds to Ire1 as well as PS1 (17). HEK293T cells were transiently cotransfected with expression plasmids for PS2V and Ire1-flag. Lysates were immunoprecipitated with anti-PS2N antibody and then blotted with anti-flag antibody (Fig. 1C). PS2V protein was coprecipitated with Ire1 (Fig. 3B). These results indicate that PS2V directly binds to Ire1 on the membrane of the ER. However, we could not ascertain the cause of the decrease in phosphorylated Ire1 by PS2V in the present study.

To confirm that vulnerability to ER stress by the repression of GRP78 mRNA induction in PS2V transfectants, SK-N-SH cells stably transfected with PS2V were infected with recombinant SFV-GRP78, and sensitivity to various ER stresses was examined. Increased sensitivity to ER stressors in

FIG. 2. Sensitivity to ER stress and effects on the UPR in SK-N-SH cells expressing PS2V. A, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Stable transfectants of wild PS2 (w14, w17, w20, and w23) and PS2V (dEX5-1, -3, -4, -5, -6) were treated with 0.5 μg/ml tunicamycin (left) or 0.5 μM A23187 (right). MTT reduction was quantified as a percentage of the cells at 0 h. *p < 0.01 compared with corresponding values for wild PS2-transfected cells (n = 4; mean ± S.D. is shown). B, after treatment of SK-N-SH cells stably transfected with wild PS2 or PS2V with Tm (0.5 or 1 μg/ml) or A23187 (0.5 or 1 μM). A human GRP78 cDNA fragment was used as a probe (upper panel). A β-actin cDNA fragment was used as a control probe (lower panel). N, samples before treatment with Tm or A23187.

When misfolded proteins accumulate in the endoplasmic reticulum (ER) of cultured cells, gene expression of molecular chaperones such as GRP78/BiP and GRP94 is known to be immediately induced to refold the unfolded proteins (18, 19). Before and after treatment with Tm or A23187, total RNAs were isolated from each stable transfectant. Under nonstress conditions, the levels of GRP78 mRNA in cells expressing PS2V were reduced to ~50% compared with those of the transfectants of mock or wild PS2 (Fig. 2B). When cells were treated with 0.5 μg/ml Tm for 6 h, GRP78 mRNA was induced ~20-fold in mock or wild PS2 transfectants. In contrast, it was markedly inhibited in PS2V transfectants to ~10% of that in the controls (Fig. 2B). Treatment of each cell line with 0.5 μM A23187 for 6 h led to the same results (Fig. 2B). Decreased induction of GRP78 mRNA in cells expressing PS2V was caused by the impaired phosphorylation of Ire1, an ER stress sensor, which is known to oligomerize and be autophosphorylated by its own kinase domain on accumulation of unfolded protein in the ER (Ref. 20 and Fig. 3A). In PS2V transfectants, the levels of autophosphorylation of Ire1 were decreased to ~50% compared with the mock-transfected cells. Phosphorylation of tau or GSK-3β was not changed in any of these cell lines (data not shown).

We examined whether PS2V protein binds to Ire1 as well as PS1 (17). HEK293T cells were transiently cotransfected with expression plasmids for PS2V and Ire1-flag. Lysates were immunoprecipitated with anti-PS2N antibody and then blotted with anti-flag antibody. PS2V was coprecipitated with 140-kDa Ire1-flag (Fig. 3B). As the reverse experiments, we prepared immunoprecipitates using the anti-flag antibody and blotted with anti-PS2N antibody. The PS2V protein was coprecipitated with Ire1 (Fig. 3B). Bcl-xl, which is a transmembrane protein of the ER and mitochondria, did not coimmunoprecipitate with Ire1 (Fig. 3C). These results indicate that PS2V directly binds to Ire1 on the membrane of the ER. However, we could not ascertain the cause of the decrease in phosphorylated Ire1 by PS2V in the present study.

To confirm that vulnerability to ER stress by the repression of GRP78 mRNA induction in PS2V transfectants, SK-N-SH cells stably transfected with PS2V were infected with recombinant SFV-GRP78, and sensitivity to various ER stresses was examined. Increased sensitivity to ER stressors in
SK-N-SH cells expressing PS2V was reversed by the overexpression of GRP78 (Fig. 4).

Effects of Production of Aβ—One of the most common pathological features in AD brain is the deposition of Aβ (21, 22). Therefore, it is of interest to determine whether PS2V enhances secretion of endogenous Aβ into the cultured media. Twenty-four hours after changes of conditioned media of Neuro 2a cell lines, which stably expressed mock, wild PS2, PS2V, or FAD-linked PS2 mutant (Volga German type, N141I), we collected those and measured the amounts of released Aβ40 and Aβ42 by enzyme-linked immunosorbent assay (15). Secreted Aβ40 and Aβ42 were both significantly increased by 1.3-fold in stable transfectants of PS2V compared with the cells expressing the mock or wild PS2 (Fig. 5A). However, the ratios of Aβ42/Aβ40 plus Aβ42 were nearly equal to those of the control cells. FAD-linked PS2 mutant specifically led to elevation of Aβ42 secreted into the media but did not affect secretion of Aβ40.

Having demonstrated that PS2V affects the function of Ire1, we examined whether dominant negative Ire1 causes an increase in Aβ production, as well as PS2V. This Ire1 derivative DIRE1 has a truncated cytoplasmic region and therefore lacks the kinase and RNase L domains, and we have confirmed that cells expressing DIRE1 showed down-regulation of GRP78 induction and significantly increased vulnerability to ER stress (17). Stable Neuro 2a transformants of DIRE1 showed that secreted Aβ40 and Aβ42 were significantly increased by ~1.3-fold compared with the cells expressing the mock (Fig. 5B). The ratio of Aβ42/Aβ40 plus Aβ42 was not changed in these cells.

We speculated that PS2V affects the correct folding and maturation of APP, because PS2V down-regulates the UPR signaling mediated by disturbed Ire1 and also decreases the expression levels of GRP78 in a steady-state condition. Therefore, we examined the process of biosynthesis of APP in stable transformants of wild PS2 and PS2V. The cells were labeled for 10 min with [35S]methionine and chased for 0–50 min. As
shown in Fig. 5C, immunoprecipitates at 0 min of chase showed a single band of 95 kDa that apparently corresponds to N-glycosylated APP in both cells. In wild PS2-expressing cells, two new N- and O-glycosylated forms at 105 and 130 kDa were shown within a 20-min chase period. At the 50-min chase time, the bands of the matured forms of APP reached maximum intensity. In contrast, N- and O-glycosylated forms of APP were not detected within the 20–40-min period, and small amounts of those were observed at 50 min in PS2V-expressing cells (Fig. 5, C and D). These results indicate that PS2V caused inhibited intracellular maturation of APP, involving the retention of immature forms of APP in the ER.

**Expression of PS2V Protein in Sporadic AD Brains**—To confirm that PS2V was translated in sporadic AD brains, we carried out immunoprecipitation followed by Western blotting. PS2V protein was only detected in extracts of AD brain (AD 9; Table I) by Western blotting (Fig. 1D). On immunohistochemical analysis using the anti-SSMAG and anti-PS2N antibodies, PS2V-immunoreactive cells were observed in the CA1 region of the hippocampus (Fig. 5, B–F) and the temporal cortex (data not shown). Immunoreactivity was completely abolished by the preabsorption of the antibodies with an excess of synthetic peptide antigen (10 μM; Fig. 6C). In the hippocampus, the cells expressing PS2V protein were pyramidal neurons and were scattered throughout the CA1 region. Judging from the morphology, PS2V-immunoreactive cells could be divided into two cell types. The first type had neurons showing moderate immunoreactivity for PS2V and were localized to perinuclear regions (Fig. 6, B and D). This type of cell was diffusely distributed throughout CA1 of the hippocampus, and some of these cells exhibited degenerative changes such as shrinkage and loss of neurites. The other type of cell had apoptotic neurons containing PS2V-immunoreactive inclusion bodies in the cytoplasm (Fig. 6, E, F, H, and I) and occasionally also contained neurofibrillary tangles (Fig. 6H). These neurons were sparsely distributed in the CA1 regions. Double labeling of PS2V and Aβ showed that some of the PS2V-immunoreactive neurons lay closely adjacent to extracellular amyloid deposits (Fig. 6D).

To quantify the number of neurons bearing the PS2V protein in sporadic AD (10 cases), age-matched controls (nonneurological diseases, 6 cases), and control diseased brain (7 cases; Table I), immunohistochemical analysis was performed on three sections of the hippocampal CA1 region in each case. PS2V immunoreactivity was observed in all specimens from AD brains, and the number of positive neurons in AD brains was almost more than approximately ~100 per field (Table I). In contrast, only one specimen from the age-matched control group (specimen 3; Table II) showed a small number of PS2V-immunoreactive neurons. The other age-matched control and diseased control specimens had no positive neurons in any of the sections. The number of neurons containing PS2V protein in this control case (specimen 3) was extremely low, and the neurons were located only in the CA1 region. This case showed no clinical symptoms of dementia, but a small number of amyloid plaques were pathologically observed in the CA1 region and cortex.
FIG. 6. PS2V immunoreactivity in sporadic AD brain. A, staining with cresyl violet in hippocampal sections of the human AD brain. B, staining with anti-SSMAG antibody in the boxed area in A. The arrowhead shows the PS2V-immunoreactive structures. C, absorption of the antibody by excessive amounts of synthetic peptide (SSMAG, 10 μM). Morphologically intact neurons (B) and degenerative neurons (D) show PS2V immunoreactivities stained by anti-SSMAG antibody. E, PS2V-immunoreactive inclusion bodies stained by anti-SSMAG antibody (arrowhead). F, PS2V-immunoreactive inclusion body stained by anti-PS2N antibody. G, double staining of PS2V (anti-SSMG) and tau. Colocalization of PS2V and a neurofibrillary tangle in single neurons is shown (brown, PS2V; blue, neurofibrillary tangle). H, electron microscopy of a PS2V-immunoreactive neuron in sporadic AD brain (anti-SSMAG). Deposition of DAB reaction product within a single neuron (arrowhead) is shown. Scale bar, 4 μm.

Table I

| Group                        | No. of patients | M/F | Age at death | Postmortem delay |
|------------------------------|-----------------|-----|--------------|------------------|
| Sporadic AD                  | 10              | 3/7 | 76 ± 5.3     | 2.4 ± 0.7        |
| Nonneurological disease      | 6               | 3/3 | 80 ± 5.8     | 2.8 ± 0.9        |
| Vascular dementia            | 2               | 1/1 | 76.5 ± 6.5   | 1.65 ± 0.25      |
| Hypoxia                      | 3               | 1/2 | 64 ± 7.0     | 2.1 ± 1.3        |
| Huntington’s disease         | 2               | 1/1 | 46.5 ± 10.5  | 7.4 ± 4.9        |

Table II

| Patient | Diagnosis | Gender | Age | PS2V |
|---------|-----------|--------|-----|------|
| 1       | C         | M      | 83  | –    |
| 2       | C         | F      | 81  | –    |
| 3       | C         | F      | 90  | +    |
| 4       | C         | F      | 74  | –    |
| 5       | C         | M      | 79  | –    |
| 7       | AD        | M      | 81  | +++  |
| 8       | AD        | F      | 78  | +    |
| 9       | AD        | F      | 82  | +++  |
| 10      | AD        | M      | 82  | +++  |
| 11      | AD        | M      | 64  | +++  |
| 12      | AD        | M      | 75  | +    |
| 13      | AD        | F      | 78  | +    |
| 14      | AD        | F      | 74  | +    |
| 15      | AD        | F      | 76  | +++  |
| 16      | AD        | F      | 65  | +++  |

DISCUSSION
Effects of PS2V Protein on UPR Signaling—SK-N-SH cells stably transfected with PS2V exhibited increases in susceptibility to various ER stresses. Overexpression of GRP78 almost completely restored resistance to some ER stresses up to the level of that of wild PS2 transfectants, suggesting that PS2V protein increases vulnerability to ER stress because of the inhibition of GRP78 mRNA induction. The decreased induction of GRP78 mRNA was attributable to the disturbance of UPR signaling caused by the reduction of Ire1 phosphorylation. Because PS2V directly binds to Ire1 on the membrane of the ER, the binding might affect phosphorylation of Ire1. On the basis of its structural features, it is unlikely that PS2V protein directly dephosphorylates Ire1 molecules. If PS2V protein directly interacts with Ire1, one possible mechanism responsible for the decrease in phosphorylated Ire1 is that PS2V may bind to the kinase domain of Ire1 or a particular region that is important for modulating its activity, and this causes inhibition of Ire1 phosphorylation. Alternatively, PS2V might inhibit the oligomerization of Ire1 and block transautophosphorylation by neighboring Ire1 (19).

Aβ Production in PS2V-expressing Cells—Secretion of both Aβx-40 and Aβx-42 was increased in stable transformants of PS2V compared with that in the controls. Recently, it was reported that secreted Aβ levels were reduced by transfection of GRP78, and it was suggested that GRP78 modulates Aβ secretion (23). Because basal levels of GRP78 expression were decreased in PS2V-expressing cells under the non-ER stress conditions, the increased secretion of Aβ could be associated with the down-regulation of GRP78. Stable transformants of dominant negative Ire1 also showed significantly increases of both Aβx-40 and Aβx-42 released into media under steady-state conditions. Therefore, it is possible that the cause of increased Aβ in PS2V expressing cells is based on the dysfunc-
tion of Ire1 under non-ER stress conditions. Furthermore, N- and O-glycosylated forms of APP in the transformants of PS2V at 20–50 min chase time after the pulse. Alternatively, N-glycosylated forms of APP were accumulated and could be considered to be retained in the ER, suggesting that correct folding of APP is probably impaired in cells expressing PS2V. Increase in total antibody could be associated with accumulation of unfolded APP in the ER or delay of sorting of APP from ER to Golgi. However, in this study, we could not measure the intracellular Aβ pool in PS2V- or ΔIre1-expressing cells. Therefore, to clarify the detailed mechanisms responsible for increased Aβ production in these cells, further analyses are needed.

Expression of PS2V Protein in Sporadic AD Brains—PS2V protein was detected in the cortex and hippocampus of all AD patients. In age-matched control and diseased control brains, PS2V was found in only one case. The number of neurons containing PS2V protein in this control case was extremely low, and the CA1 region was pathologically observed in the CA1 region and cortex. These findings suggest a close correlation between expression of PS2V protein and deposition of Aβ. At present, it is unknown whether aberrant splicing of the PS2 gene is a trigger for development of sporadic AD or whether it occurs as a result of neuronal damage in AD brains. However, it could be a factor that compromises neuronal viability in sporadic AD brains, because PS2V causes vulnerability to ER stress and increases in Aβ production. Indeed, immunohistochemically, cells showing intense immunoreactivities for PS2V were morphologically degenerative or apoptotic, suggesting that excessive production of PS2V protein may cause neuronal damage in sporadic AD brain.

In summary, our results indicate that PS2V, which is expressed in neuropathologically affected neurons of sporadic AD patients, affects both UPR signaling and Aβ production. Because both FAD-linked PS1 mutants and the PS2V protein caused down-regulation of the UPR pathway and resultant vulnerability to ER stress, regulation of components of the UPR pathway could provide an opportunity for the development of therapeutic strategies for AD.

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