Mutational Analysis of Intrinsic Regions of Presenilin 2 That Determine Its Endoproteolytic Cleavage and Pathological Function*

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Keiro Shirotani§, Keikichi Takahashi, Wataru Araki, Kei Maruyama, and Takeshi Tabira‡

From the §Division of Demyelinating Disease and Aging, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawamizuchi, Kodaira, Tokyo 187-8502 and the ‡Department of Molecular Biology, Tokyo Institute for Psychiatry, Tokyo 156-8585, Japan

To investigate the significance of endoproteolytic processing of presenilin 2 (PS2) on its pathological function, we constructed PS2 cDNAs causing amino acid substitutions or deletions around the cleavage site. We found that a PS2 mutant (Del3) with a 20-amino acid deletion was not endoproteolytically processed, while other PS2s with amino acid substitutions and short deletions were cleaved. Overproduction of all the mutant proteins led to a compensatory decrease of endogenous PS1 fragments, but did not affect the amyloid β peptide X-42/Aβ X-40 ratio without the familial Alzheimer’s disease mutation. The Del3 mutant did not exhibit significant deficits in γ-secretase activity. The turnover rate of the Del3 holoprotein was the same as that of full-length PS2. These data suggest that the determinants of the PS2 cleavage site reside within a large region and that the pathological function of PS2 is exerted by familial Alzheimer’s disease mutations not related to the cleavage of holoproteins. We also found that PS2 with an 18-amino acid deletion at the C-terminal end was not processed. Its overexpression led neither to diminished accumulation of endogenous PS1 fragments nor to increased production of amyloid β peptide X-42. The C-terminal end of PS2 seems to possess the signal for entry into the processing pathway.

Mutations in homologous presenilin 1 and 2 (PS1 and PS2) genes are associated with early-onset autosomal dominant familial Alzheimer’s disease (FAD) (1–3). To date, more than 50 different pathogenic missense mutations and one splice site mutation (loss of exon 10, designated as PS1ΔE10) have been found in PS1, and two missense mutations have been identified in PS2. The gene products of PS1 and PS2 are thought to contain six or eight transmembrane (TM) domains, and the N and C termini and a large hydrophilic loop region following the sixth TM domain are located within the cytoplasm (4–6). PS1 proteins are involved in cell fate decisions by facilitating Notch signaling pathway (7–9). Recently, PS1 proteins have been shown to have a role in the endoproteolytic processing of β-amyloid precursor protein (APP) (10, 11) and Notch (12–14), and trafficking of proteins including APP (15) and Notch (12, 13), while the physiological function of PS2 proteins is poorly understood. The amount of highly amyloidogenic Aβ42 (amyloid β peptide 42) increases in cells and transgenic mice with the PS1 and PS2 mutant genes (16–20), supporting a hypothesis that mutations in PS lead to Alzheimer’s disease by increasing the extracellular levels of Aβ42.

The PS proteins are proteolytically cleaved at the hydrophilic loop region and detected predominantly as N-terminal fragments (NTF) and C-terminal fragments (CTF) in culture cells and tissues (19, 21–23). The formation of PS fragments is highly regulated in a saturable and stoichiometric manner, since overproduction of full-length PS in transfected cells and transgenic mice does not yield a linear increase of fragments (21) and causes a compensatory decrease of the endogenous PS fragments (21, 24, 25). However, the enzyme involved in the normal PS cleavage remains to be determined. The processed fragments form stable heterodimers and occur as a high molecular weight complex, while the remaining full-length proteins are rapidly degraded (20, 23, 25–30). Therefore, it is proposed that these heterodimers consisting of NTF and CTF are functional units of PSs. Interestingly, PS1ΔE10 has a longer half-life than that of full-length PS1, although it does not undergo endoproteolytic processing because of the lack of the cleavage region. In addition, the overexpression of PS1ΔE10 causes a compensatory decrease of endogenous presenilins and it is incorporated into the high molecular weight complex (17, 21, 25, 27, 28). In contrast, exogenous expression of NTF or CTF alone leads to neither diminished accumulation of endogenous presenilin fragments nor increased production of Aβ42, even though they have FAD mutations (31–35). Moreover, the overexpressed NTF is not incorporated into the heterodimer (33, 35). These data indicate that the processing step of full-length PSs, including the stabilization, endoproteolytic cleavage and the formation of the heterodimer, plays a key role in producing pathological forms, although the possibility cannot be excluded that the cleavage of holoprotein is not essential to form functional PSs.

In the present study, we investigated the nature and specificity of the endoproteolytic processing of PS2 and its relation to the pathological function using mutants with amino acid substitutions and deletions around the cleavage site. Our data indicate that the proteolytic cleavage of PS2 at Lys409Leu407 does not depend on amino acid sequence and that overexpression of any mutant PS2s has no effect on Aβ X-42/Aβ X-40 ratio without the N141I FAD mutation. We also found that the integrity of the C-terminal end of PS2 is a prerequisite for its entry into the processing pathway.
**EXPERIMENTAL PROCEDURES**

**Preparation of Mutant PS2 cDNAs—**The PS2-pCEP4 was constructed by ligation of full-length human PS2 cDNA into the PvuII site of an expression vector pCEP4 (Invitrogen, San Diego, CA), as reported previously (36). Mutations at the cleavage site of PS2 (K306A, K306E, KLA/P, and MAK3A) were introduced using a site-directed mutagenesis kit (Stratagene, La Jolla, CA). The DNA fragments with these mutations were excised with NotI from the PvuII product and ligationed into the NotI/HindIII-digested PS2-pCEP4. For constructing Del1, 5' and 3' regions encompassing amino acid residues 46–303 and 310–384 were separately amplified from PS2 cDNAs by PCR using primer pairs 5'-CAGTTGAGACCAGGAGAACGAG-3'/5'-CGGTAGCATCCGAG-3'. Primers for the 5' region were designed to have an additional SmaI site at their 3' end and the PCR product was subcloned into the SmaI site of pUC18 and sequenced. The DNA fragments corresponding to the 5' and 3' regions were excised with NotI/BamHI and HindIII, respectively, and were ligated into the NotI/HindIII-digested PS2-pCEP4, resulting in Del1 cDNA lacking residues 304–309. Del2 and Del3 were constructed by similar procedures using the primer pairs 5'-CAGTTGAGAACGAGA-3'/5'-CGGTAGCATCCGAG-3' for the 3' region and 5'-CGGATCCATCCGAG-3'/5'-GTCGACCATCCGAG-3' for the 5' region, respectively. The PCR product was subcloned into the SmaI site of pUC18 and sequenced. The DNA fragments corresponding to the 5' and 3' regions were excised with NotI/BamHI and HindIII, respectively, and were ligated into the NotI/HindIII-digested PS2-pCEP4, resulting in Del2 and Del3 lacking residues 300–312 and 297–316, respectively. PS2HA cDNA was prepared as described above and precleared with protein G-agarose over night at 4 °C. Immunoprecipitates were washed three times in a solution A containing 0.1% Triton X-100, and heated at 60 °C for 10 min in a sample buffer and subjected to SDS-PAGE. The gel was dried and analyzed with a BioImaging BAS5000 analyzer (Fuji, Tokyo, Japan).

**Results**

**Endoproteolytic Processing of PS2 with Cleavage Site Mutations—**To determine the nature and specificity of endoproteolytic cleavage, we constructed two series of PS2 cDNAs with mutations around the cleavage site (Fig. 1). The first series (K306A, K306E, KLA/P, and MAK3A) had substitutions with either an alanine, a glutamic acid or a proline residue for a lysine residue and its neighbors, since the lysine is suggestive of a possible trypsin-like specificity. The second mutants had deletions (6, 13, and 20 amino acid residues) around the native cleavage site. Expression and cleavage of mutant PS2 proteins were determined in stable cell lines transfected with these cDNAs using antibodies specific for PS2NTF and PS2CTF (PS2N53 and Ab333, respectively). We also monitored levels of NTF and CTF of endogenous PS2 using antibodies of PS1N62 and Ab111 to evaluate the ability of mutant proteins for entry into the processing pathway.

In cells transfected with PS2WT and PS2N141I (referred to as PS2WT and PS2N141I cells), PS2 polypeptides were detected as a 55-kDa band for the full-length protein, a 35-kDa band for PS2NTF, and a 23-kDa band for PS2CTF (Fig. 2A, top and bottom panels, lanes 2–4). Amounts of these bands increased in PS2WT and PS2N141I cells, as compared with cells transfected with the pCEP4 vector alone (referred to as pCEP4 cells; Fig. 2A, lane 1), while overproduction of exogenous PS2 diminished the levels of endogenous PS1NTF and CTF (Fig. 2B, lanes 2–4), as described previously (34). Similarly, stable cells transfected with the first series of mutant cDNAs (K306A, K306E, KLA/P, and MAK3A) showed increased amounts of the full-length polypeptides, NTF and CTF (Fig. 2A, top and bottom panels, lanes 5–12), and decreased levels of endogenous PS1NTF and CTF (Fig. 2B, lanes 5–12). The sizes of the PS2 fragments were indistinguishable from native fragments, indicating that these mutant PS2s were endoproteolytically cleaved at or near the original cleavage site. Thus, the MAKL sequence around the cleavage site is not a critical determinant for the proteolytic processing.

In stable cells expressing the second mutants (Del1-Del3 cells, Fig. 2C, top panel, lanes 3–16), increased amounts of full-length proteins, which migrated slightly faster than full-length PS2 (Fig. 2C, top panel, lane 2), and decreased levels of endogenous PS1 fragments (Fig. 2D) were also observed. The level of endogenous PS2CTF (23 kDa) was decreased in Del2 and Del3 cells (Fig. 2C, bottom panel, lanes 7–16), indicating
that processing of endogenous PS2 was diminished. However, amounts and sizes of processed NTFs and CTFs varied among different mutant cells (Fig. 2C, top and bottom panels, lanes 3–16). In the cases of Del3 cell lines, no band corresponding to PS2CTF was found. In contrast, several faint bands with sizes ranging from 35 to 38 kDa were detected by an antibody specific for PS2NTF in three cell lines that expressed full-length proteins more abundantly (Fig. 2C, lanes 12–14). The 38-kDa band may represent a cleaved product by the caspase family, because its level was induced after treatment with staurosporine (not shown), an activator of caspases (41, 42). Other bands are likely to be degrading intermediates by proteasome, since amounts of those bands greatly increased by treatment with lactacystin (data not shown), which is a potent inhibitor of proteasome. Thus, Del3 protein, similar to PS1E10, does not undergo endoproteolytic processing despite its ability to compete with endogenous PS1 and PS2 for the cellular factor(s), which is predicted to be involved in the stabilization and the endoproteolytic cleavage of presenilin holoprotein (24). On the other hand, PS2CTFs in Del1 were detected mainly as doublet bands at 22.5 and 23 kDa, and PS2CTF in Del2 cells was observed as a band with 21.5 kDa, while the sizes of NTFs were not apparently changed. The decreases of molecular weight for Del1 and Del2 CTFs (loss of 0.5 and 1.5 kDa) were roughly consistent with the size of the deletions (6 and 13 amino acids, respectively). It is also noted that similar results were obtained for mutant PS2s with the N141I FAD mutation, indicating that the introduction of the FAD mutation did not affect levels or sizes of NTF and CTF.

Aβ42-promoting Activity of Mutant PS2 Proteins—We investigated whether amino acid substitutions or deletions would affect the pathological generation of Aβ42. Fig. 3 (A and B) shows the ratio of amyloid β species (Aβ42/Aβ40) in media from stable cell lines. As described previously (34), the ratio in PS2N141I cells was 8–9 times higher than those in pCEP4 or PS2WT cells (Fig. 3A). This Aβ42-promoting activity was not observed for all mutants with amino acid substitutions or deletions around the cleavage site, unless they harbored the N141I mutation. In contrast, in cases of PS2s with the N141I mutation, levels of Aβ42 promotion varied significantly. K306A, K306E, KL/AP, and MAK/3A mutants with the N141I mutation could increase the Aβ42/Aβ40 ratio as effectively as PS2N141I (Fig. 3A), while Del1, Del2, and Del3 with the FAD mutation showed decreased Aβ42-promoting activity with increasing sizes of deletion (Fig. 3B). As compared with PS2N141I cells, the average of Aβ42/Aβ40 ratio was 72% for Del1N141I, 46% for Del2N141I, and 36% for Del3N141I. The different Aβ42-promoting activity among PS2 mutants was not due to the expression level of the PS2 holoprotein, since the highest amount of full-length form was detected in Del3N141I cells (Fig. 2C, top panel). These results indicate that the pathological function of PS2 is affected by deletions, but not by amino acid substitutions around the cleavage site.

It has been suggested that PS1 is itself γ-secretase and mediates the endoproteolytic processing of PS1 (11). To assess whether the uncleaved Del3 mutant had endoproteolytic activity, we analyzed the levels of total Aβ polypeptide and APP C-terminal fragment (C100). Although clonal variability was observed, the total amounts of Aβ secreted from Del3 cells were comparable to those from other cells (Fig. 3C). The accumulation of APPC100 was detected neither in Del3 cells nor in cells expressing processed PS2 including wild type, Del1, and Del2 mutants (Fig. 3D). These results indicate that the deletion in Del3 does not affect γ-secretase activity.

Turnover of PS2 and Del3—We compared the stability of full-length PS2 and Del3, since PS1E10 has been reported to show a longer half-life than full-length PS1 (25, 27). PS2N141I and Del3N141I cells were labeled for 1 h and chased for up to 20 h, followed by immunoprecipitation with the PS2N53 antibody. Full-length PS2 was detected as broad 55-kDa bands in PS2N141I cells and declined with a half-life of 2.11 ± 0.470 h (Fig. 4, left). In the case of Del3, a holoprotein with slightly
faster migration also decreased immediately with a half-life of 2.26 ± 0.0147 h (Fig. 4, right). Thus, there is no significant difference in the turnover rate of holoprotein between PS2N141I and Del3N141I. The 35-kDa PS2NTF was detected after a 2-h chase in PS2N141I cells and increased substantially after almost all holoprotein had been catabolized. This result indicates that only a small part of the full-length protein was metabolized into the fragments and the remainder was rapidly degraded. In contrast, processed fragments were not detected in Del3N141I cells during the chase time.

PS2 Processing Requires Its Integrity of the C-terminal End—In the course of our study on PS2 processing, we noticed that tagging of influenza virus hemagglutinin at the C terminus of PS2 cDNA caused complete abolishment of endoproteolytic cleavage (data not shown), suggesting that the significance of the C-terminal end on PS2 processing. Therefore, we constructed a mutant (PS2–430) with an 18 amino acid deletion from the C-terminal end. The complete abolishment of endoproteolytic processing was observed in stable cells expressing the mutant PS2 (PS2–430 cells). Although increased levels of full-length PS2–430 were detected as a band with slightly lower molecular weight than full-length PS2 (Fig. 5A, top panel), there was no difference in patterns and intensities of bands corresponding to PS2NTF and PS2CTF between the pCEP4 and PS2–430 cell lines (Fig. 5A, top and bottom panels). The PS2CTF derived from PS2–430 should be detected as a band with faster migration than endogenous PS2CTF. This result also suggested that endoproteolytic processing of endogenous PS2 was not altered in PS2–430 cells. Furthermore, expression of PS2–430 affected neither levels of endogenous PS1 (Fig. 5B) nor levels of the AβX-42/AβX-40 ratio, even if they harbored N141I (Fig. 5C). These results suggest that the integrity of the PS2 C-terminal end is a prerequisite for entry into the processing pathway.

DISCUSSION

In the present study, we have demonstrated that the endoproteolytic cleavage of PS2 is prevented by a large (20-amino acid) deletion, but not by amino acid substitutions or short (up to 13-amino acid) deletions around the native cleavage site. Furthermore, the integrity of the PS2 C-terminal end is critical for targeting the holoprotein into the processing step, which is thought to include stabilization, cleavage, and formation of the heterodimer of PS2NTF and CTF.

We have previously reported that PS2 is endoproteolytically processed between Lys306 and Leu307 (36). Although the lysine has a possible trypsin-like specificity, the cleavage is not af-
Endoproteolytic Cleavage and Pathological Function of PS2

Endoproteolytic cleavage of PS2 (A), accumulation of endogenous PS1 fragments (B), and Aβ42-promoting activity (C) in stable cell lines expressing PS2-430. Stable cell lines were prepared using PS2–430 cDNAs as described under “Experimental Procedures.” PS2–430 proteins had an 18-amino acid deletion at the C-terminal end of PS2. Species of cDNAs used for transfection are indicated below the bottom panel of A, and other nomenclatures are the same as those in Fig. 1. A, full-length and NTF of PS2 were determined by PS2N53 (top panel), and PS2CTF was determined by Ab333 (bottom panel). B, samples were also subjected to Western blot analysis using PS1N62 for PS1NTF (top panel) and Ab111 for PS1CTF (bottom panels). C, the Aβ X-42/Aβ X-40 ratio was quantified in media prepared from PS2–430 cells with or without the N141I mutation.

fected by substitutions of a lysine with a glutamic acid or an alanine residue with different charge and conformational structure. In addition, two other stable cell lines expressing mutated proteins with substitutions around this lysine residue (KL/AP and MAK/3A cells) showed a similar processing pattern to the lines expressing wild type PS2, indicating that the endoproteolytic cleavage of PS2 at Lys306/Leu307 is not mediated by protease(s) with strict sequence specificity, such as trypsin-type proteases. In cases of deletion mutants, the endoproteolytic cleavage was found for Del1 and Del2, but not for Del3, although they all lacked the amino acid sequences of the cleavage site. The 22.5- and 21.5-kDa CTF in Del1 and Del2 cells, respectively, corresponded approximately to the deleted sizes, while the sizes of NTF were not apparently changed. On the other hand, the fact that Del3 with 20 amino acid deletion was not processed indicates that the significant determinant of PS2 processing site resides between Ala297 and Val299. This region may be a binding site of the processing enzyme of PS2 or may contribute to the conformational feature around the native cleavage site, which is susceptible to the enzyme. It can be explained by another possibility that the primary cleavage of PS2 takes place upstream (between Ala297 and Val299 of PS2) and is followed by an additional endoproteolytic cleavage at Lys306/Leu307; since cleavage of PS1 occurs not only at Met298 but also at Thr291 and Met292 (26) and the amino acid sequence at the upstream site of PS1 is conserved in PS2. To investigate these two possibilities, an additional study will be needed to sequence the C-terminal amino acid of PS2NTF and/or introduce a small deletion and point mutations between Ala297 and Val299 of PS2. It is surprising that an amino acid substitution of either of two transmembrane aspartate residues in PS1 (Asp257 and Asp385 in the sixth and seventh TM domain, respectively) prevented endoproteolytic processing of PS1 (11). This may be due to a greatly altered structure between the sixth and seventh TM domains caused by the aspartate mutation, so that the processing enzyme of PS cannot recognize the cleavage site. Alternatively, the aspartate mutation may block autoproteolysis of PS1 by its intrinsic putative aspartyl protease function. However, the species of the putative presenilinase is still unknown, since any protease inhibitors tested did not block the formation of normally processed PS fragments (23, 33, 43). Recently, proteasome has been reported to be involved partially in the endoproteolytic cleavage of PS1 (44).

The intriguing finding in the present study is that Del3 without the N141I FAD mutation did not affect the production of Aβ42 species, which is in contrast to PS1ΔE10 (17), although neither proteins underwent endoproteolytic processing and their overexpression led to diminished accumulation of endogenous presenilin NTFs and CTFs. PS1ΔE10 identified in FAD patients (45, 46) is caused by a splice site mutation, which removes the whole sequence of exon 10 from PS1 (amino acid residue 291–319) and substitutes cysteine for serine at position 290. Therefore, the question arises whether the Aβ42-promoting activity of PS1ΔE10 is acquired by the 29-amino acid deletion or by the amino acid substitution. The fact that only Del3 with the N141I FAD mutation showed Aβ42-promoting activity suggests that the pathological function of PS1ΔE10 is ascribed to the Ser290 → Cys missense mutation, but not to deletion around the cleavage site. In fact, a recent report has shown that a PS1ΔE10E361K without the missense mutation has no Aβ42-promoting activity (47). Nevertheless, there are still two differences between Del3 with the N141I mutation and PS1ΔE10. First, the former (Del3N141I) was rapidly degraded with the same half-life as PS2N141I, while the latter has a longer half-life than the PS1 holoprotein (25, 27). Second, Del3N141I had the lowest Aβ42-promoting activity among the other PS2 mutants, in contrast to PS1ΔE10, which has similar or higher Aβ42-promoting activity among different FAD PS1 mutants (17, 48, 49). Possible explanations are that exon 10 of PS1 may have an inhibitory effect against the stabilization of a holoprotein or Del3 holoprotein is not stabilized so that its faster degradation gives rise to decreased Aβ42-promoting activity. It is also conceivable that the region around the cleavage site of PS2 is essential to the pathological function, since production of the Aβ42 species declined with increasing sizes of deletion.

Recent observations have suggested that PS1 is itself γ-secretase and involved in autoproteolysis (11). The fact that the uncleaved Del3 mutant in this study exhibited normal Aβ production indicates that PS2 endoproteolysis is not required for γ-secretase activity, even if the processing of PS2 is medi-
Entry 1

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