The Influence of Endoproteolytic Processing of Familial Alzheimer’s Disease Presenilin 2 on Aβ42 Amyloid Peptide Formation*

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Mutant presenilins (PS) contribute to the pathogenesis of familial Alzheimer’s disease (FAD) by enhancing the production of Aβ42 from β-amyloid precursor protein. Presenilins are endoproteolytically processed to N-terminal and C-terminal fragments, which together form a stable 1:1 complex. We have mapped the cleavage site in the PS2 protein by direct sequencing of its C-terminal fragment isolated from mouse liver. Three different N-terminal residues were identified starting at Val-299, Thr-301, and Leu-307 that correspond closely to the previously described N termini of the C-terminal fragment of human PS1. Mutational analysis of the PS2 cleavage site indicates that the principal endoproteolytic cleavage occurs at residues Met-298/Val-299 and that the N terminus is subsequently modified by secondary proteolytic cleavages. We have generated cleavage defective PS2 constructs, which accumulate exclusively as full-length polypeptides in transfected Neuro2a cells. Functional analysis of such cleavage defective PS2 carrying the FAD mutation Asn-141→Ile showed that its Aβ42 producing activity was strongly reduced compared with cleavage-competent FAD PS2. In contrast, cleavage defective PS2 was active in rescuing the egg-laying defect of a sel-12 mutant in Caenorhabditis elegans. We conclude that PS2 endoproteolytic cleavage is not an absolute requirement for its activities but may rather selectively enhance or stabilize its functions.

The presenilin 1 (PS1) and presenilin 2 (PS2) genes are genetically linked to early-onset familial Alzheimer’s disease (FAD) (1–3). Mutations in the PS1 gene account for over 25% of all cases of early FAD and numerous mutations (>40) are currently known. FAD-linked PS2 mutations are less often observed, and only two have been reported up to now (4). All FAD-linked PS mutations affect single residues of the polypeptide and often lead to rather conservative amino acid substitutions. This appears also to be true for the PS1 Δexon 9 mutant, where the FAD effect was shown to be due to the point mutation Ser-290→Cys and not to the deletion of exon 9 and the resulting absence of endoproteolytic cleavage (5). FAD mutations to both PS1 and PS2 affect the processing of the β-amyloid precursor protein (APP), leading to an increased production of the more fibrillogenic Aβ42 peptide in vivo and in vitro (6–9). It is presumed that this elevation of the Aβ42 peptide is crucial for the pathogenic activity of mutated PS. Deficiency in PS1 expression causes a profound decrease in both Aβ42 and Aβ40 peptide production (10).

Presenilins are integral membrane proteins predominantly of the endoplasmic reticulum and Golgi. Topological models predict a protein with 8 TM domains, with the N- and C termini and the large hydrophilic loop between TM6/7 facing the cytosol (11, 12). PS1/2 polypeptides share ~70% overall identity, with the most conserved parts being the TM regions and the C-terminal sequences. Close homologues have been found in various vertebrates and invertebrates, and human PS2 can functionally replace the Caenorhabditis elegans homologue sel-12 in vivo (13, 14).

Endogenous PS occur predominantly as endoproteolytically processed N- and C-terminal fragments of approximately 34 and 20 kDa, respectively, in a 1:1 complex. The pathway leading to proteolytic cleavage is easily saturated when overexpressing PS in transfection experiments, and under such experimental conditions the accumulation of full-length PS is observed (15–18). Recent experiments show that the NTF and CTF in a complex are the stable species of PS1 (t1/2 > 12 h), whereas the full-length protein is short-lived (t1/2 < 1 h). There may also be subtle differences in their respective intracellular distribution; while the full-length PS1 is primarily located in the endoplasmic reticulum, the complex of NTF/CTF is also found in the Golgi (16, 18–20). A notable exception to these observations is the PS1 Δexon 9 mutant, which occurs exclusively as uncleaved holoprotein due to the deletion of the cleavage site in exon 9. However, its subcellular distribution and its stability are similar to the endoproteolytically processed wild type PS1 and distinct from localization and stability of the PS1 holoprotein (19, 20). Radiolabeling of the human PS1 CTF isolated from transfected cells indicated the presence of three different species starting at position Met-292, Val-293, or Ala-299 (21). In addition to the constitutive proteolytic cleavage, an inducible, caspase-cleavage site in the large hydrophilic loop between TM6 and TM7 has been reported whose physiological significance and regulation remains to be established (22, 23).

We have mapped and characterized the endoproteolytic cleavage site in PS2 by direct sequencing of CTFs from mouse liver and by mutational analysis of human PS2. As for PS1, we found three CTF species of PS2 differing at their N-termini. Mutational analysis, however, suggests that endoproteolysis occurs only at the most upstream position (Met-298). Furthermore, we demonstrate that inhibition of proteolytic cleavage greatly diminishes the Aβ42 production in FAD PS2-express-
ing cells, which indicates that the proteolytically derived presenilin fragments are the physiologically relevant species for the FAD pathology.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Amino acids 1–80 of human PS2 (N terminus) and 270–387 of murine PS2 (large hydrophobic loop between transmembrane domains 6 and 7) were expressed in *Escherichia coli* as (NANP)n fusion proteins and used for immunization. PST20 is a monoclonal antibody specific for the hydrophobic N-terminal region of human and mouse PS2, while monoclonal antibody PST18 reacts with the PS2 hydrophobic loop region, having preference for murine PS2. The rabbit antisemur 53217 is directed against the hydrophobic loop of PS2 (23).

**Expression and Mutagenesis of Human Presenilin 2**—A cDNA encoding the human PS2 was isolated by PCR amplification and inserted in the expression vector pcDNA3.1 (Invitrogen) as described previously (23). For fast mutagenesis of the endoproteolytic cleavage site, a PS2 cassette construct was used; the sequence encoding amino acids Ser-296 to Gln-312 was deleted, and codons for Ser-295/Gly-313 were changed to (23). For fast mutagenesis of the endoproteolytic cleavage site, a PS2 specific for the hydrophilic N-terminal region of human and mouse PS2, the regions around the mapped cleavage sites indicates a similar fragmentation pattern of the two proteins, implying that the processing of both PS1 and PS2 is mediated by a similar or identical protease (Fig. 1B). However, the regions around the cleavage sites do not reveal any obvious sequence motifs, which would be indicative for a particular proteolytic class.

**Characterization of the PS2 Cleavage Sites**—To confirm and characterize the endoproteolytic cleavage sites of mouse PS2, we performed a mutational analysis of the corresponding sequence in the human PS2 homologue. The peptide sequence of the cleavage domain is completely conserved, and the PS2 of both species is efficiently processed in transfected cells of either mouse or human origin. In a first experiment, we constructed

**RESULTS**

**Determination of the PS2 Endoproteolytic Cleavage Sites**—In order to study the endoproteolytic cleavage of PS2, we analyzed a number of cell lines and tissues for high PS2 expression to facilitate purification and protein chemical characterization of the cleavage site(s). Substantial amounts of PS2 were found in mouse liver, as determined by Western blotting, with a major C-terminal fragment migrating as a doublet at around 20/22 kDa and a minor fragment at around 18 kDa (Fig. 1A). Samples derived from mouse brain or mouse kidney showed a single 20-kDa C-terminal fragment (Fig. 1A). The PS2 CTFs were subsequently isolated from a pool of 26 mouse livers by immunoaffinity chromatography using monoclonal antibody PST18, which recognizes an epitope on the large hydrophilic loop of murine PS2. The eluate from the PST18 column was further separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. After staining with Amido Black, the bands corresponding to the 20/22-kDa fragments were sequenced by Edman degradation in a ABI 490cLC sequencer.

**Isolation of the C-terminal Fragment of PS2 and Sequencing**—For isolation of the C-terminal fragment of PS2 from mouse liver were similar, i.e. 1.8, 2.2, and 2.3 pmol, respectively. The larger 22-kDa CTF presumably carries a secondary modification, which is most pronounced in liver tissue. Multiple phosphorylation sites in PS2 have been reported previously (25, 26). The 18-kDa band corresponds by its size to the caspase-cleavage derived fragment starting at Ser-330, which we have described previously (23). Alignment of PS1 and PS2 sequences around the mapped cleavage sites indicates a similar fragmentation pattern of the two proteins, implying that the processing of both PS1 and PS2 is mediated by a similar or identical protease (Fig. 1B). However, the regions around the cleavage sites do not reveal any obvious sequence motifs, which would be indicative for a particular proteolytic class.

**Characterization of the PS2 Cleavage Sites**—To confirm and characterize the endoproteolytic cleavage sites of mouse PS2, we performed a mutational analysis of the corresponding sequence in the human PS2 homologue. The peptide sequence of the cleavage domain is completely conserved, and the PS2 of both species is efficiently processed in transfected cells of either mouse or human origin. In a first experiment, we constructed
two mutants; we deleted sequences encompassing the mapped N termini Val-299/Thr-301 and introduced a point mutation at position Lys-306 (mutant Δ296–301 plus Lys-306 → Glu) or deleted the residues surrounding the downstream N terminus (mutant Δ306–308). We studied the processing of these and all other constructs in stably transfected Neuro2a cells (Fig. 2). We used stably transfected cells since transient high level expression of PS2 resulted preferentially in caspase processing of the full-length precursor (23). While the Δ306–308 mutation did not change the processing pattern compared with wild type PS2, the Δ296–301/Glu-306 mutant accumulated exclusively as full-length protein in transfected clones. This observation suggested that the preferred cleavage sites are located between positions 299 and 301 or, at least, that residues in this domain were essential for proteolytic cleavage, whereas amino acids Lys-306/Leu-307/Asp-308 were not strictly required.

In the next series of experiments, we attempted to prevent any proteolytic cleavage of PS2 by combinations of point mutations. For this purpose, we employed a cassette mutagenesis approach (see “Experimental Procedures”). The goal of our mutations was (i) to find the minimal alteration of the PS2 sequence that prevented cleavage and (ii) to locate the exact cleavage site(s) within the investigated amino acid stretch. We assumed that a protein needs to be in an extended conformation to be cleaved by a protease and, therefore, the predicted helical structure for the investigated sequence must unfold. In all mutants Lys-306 was exchanged by a negatively charged or hydrophilic residue in order to prevent cleavage between Lys and Leu by a protease that recognizes a lysine in the S1 pocket. Changes in the region of Met-298–Thr-301 were designed such that the respective new side chains would not fit tightly in the recognition pockets of a protease optimized to cleave the wild type sequence, due to steric, charge, or polar incompatibilities. Other mutations were introduced to probe the roles of Gly-303 and Pro-309, which could be essential in stabilizing or destabilizing secondary structure and therefore could affect the efficiency of proteolytic processing. The PS2 mutations, named DB1–DB10, are listed in Fig. 3A. Proteolytic cleavage of the mutants was evaluated by immunoblot analysis in stably transfected clone populations of Neuro2a cells. A control construct, into which an oligonucleotide encoding the wild type PS2 sequence (“PS2”) of the cleavage site was inserted, was processed to an NTF of the expected size, proving the validity of this experimental approach.

For all mutants the amount of proteolytically processed NTF was strongly reduced whereas full-length protein accumulated to significant amounts, proving that the cleavage domain was very sensitive to the different sets of mutations that had been introduced (Fig. 3B). Interestingly, insertion of an oligonucleotide encoding the corresponding sequence of human PS1 also resulted in a processed protein (lane PS1), which again suggests that both presenilins are cleaved by the same protease or, at least, by proteases of very similar specificity.

In a subsequent experiment, we set out to obtain a more precise understanding of the PS2 cleavage site by a more restricted mutagenesis of residues Met-298, Val-299, Lys-306, and Leu-307 (Fig. 4A). Processing was again assayed in clone populations of Neuro2a cells selected for G-418 resistance (Fig. 4). Of the 6 mutants, only DB24/2 was processed to a significant extent; the other 5 constructs appeared to be completely resistant to endoproteolytic processing. DB24/2 was the only mutant where residues 298/299 remained unchanged. In contrast, mutant DB23/2 with residues 298/299 replaced by Asp/Ala but positions 306 and 307 left unchanged, accumulated exclusively as full-length protein. These results suggest that the primary cleavage occurs at or close to positions 298/299 and that the generation of the fragment starting at Leu-307 is a secondary event, possibly through a trypsin-like or a aminopeptidase activity. Our data further indicate that both residues Met-298 and Val-299 are important for endoproteolysis since substitutions of Asp or Ala at either position led to its complete suppression. A role for both residues in the processing step is also supported by the results from the previous cassette mutagenesis. Although the mutants DB2/DB3/DB10 and DB4/DB5/DB6 were wild type at one of the residues at positions Met-298 or
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Val-299, they were all completely resistant to endoproteolytic cleavage. Even DB31/2m with Ala in positions 298 and 299 was not processed, although neither charge nor polarity of the side chains were changed and steric hindrances with the protease can be excluded.

Cleavage Site Mutations Decrease the Amyloidogenic Activity of FAD PS2—We asked whether inactivation of the cleavage site would affect the enhanced Aβ42 production of human FAD PS2 (PS2.1, Asn-141 Ile). Clones of Neuro2a cells stably transfected with PS2 cleavage site mutants were assayed for Aβ42 versus Aβ40 when compared with the cleavage-deficient mutants. The Aβ peptide measurements were obtained from 3 to 6 independent clones for each construct, and similar observations have been obtained from the G-418-resistant clone populations of constructs DB1–DB10. In Fig. 5B, the absolute concentrations of secreted Aβ40 and Aβ42 are shown for the various constructs and clones that are summarized in Fig. 5A. While the clonal variations in the absolute amounts of secreted Aβ peptides are quite high as has been noted previously (24), they are in agreement with our interpretation that changes in the Aβ42/Aβ total ratio for the different constructs are primarily due to changes in the amounts of secreted Aβ42.

These results show that the full-manifestation of the FAD phenotype of PS2 requires its prior proteolytic processing and, thus, indicate that the N- and C-terminal fragments form the active and physiologically relevant species for enhanced Aβ42 production.

Cleavage Site Mutations Do Not Abolish the Activity of PS2 in the Notch Signaling Pathway—Presenilins have recently been shown to function in the Notch pathway of intercellular signaling in C. elegans, Drosophila melanogaster, and mouse. They facilitate Notch activation by contributing in a yet unidentified way to the proteolytic release of the intracellular domain (NotchIC) that is a prerequisite for its signal transduction (27–29). Overexpression of presenilin mutants resulted in reduced proteolytic cleavage of the Notch intracellular signaling domain. In order to test whether our cleavage site mutations reduce the activity of PS2 in vivo, we tested their function in C. elegans. For this purpose, we expressed PS2 with wild...
DISCUSSION

Our data demonstrate that the CTF of PS2 has a heterogeneous N terminus and comprises three species starting at positions Val-299, Thr-301, and Leu-307. These positions correspond closely but not exactly to the previously reported N termini of the PS1 CTFs, which were derived by radiosequencing of a transfected cDNA (21). Shirotani et al. (32, 33) reported that human PS2 transfected into SH-SY5Y cells yielded one CTF starting at position Leu-307 and PS1 CTF isolated from human brain had Glu-300 as unique N-terminal residue. While we started initially on the assumption of independent endoproteolytic cleavage events resulting in the different CTF species, our mutagenesis studies showed that endoproteolysis occurs at one position, which is at or close to Met-298 and that subsequently the newly formed N terminus is modified, probably by exoproteolytic cleavages. This conclusion is supported by the phenotypes of the deletion mutant del.306–308 and by the point mutant DB24/2 (Lys-306 → Glu/Leu-307 → Pro), which both allowed significant endoproteolysis comparable to wild type PS2. Furthermore, the heterogeneity of the N termini of the PS2 CTFs with respect to the predicted residues in the P1 position of the cleavage site would almost certainly require endoproteolysis by at least two different proteases, which favor either a hydrophobic (Met-298, Tyr-300) or a basic residue endoproteolysis (31). Since endoproteolysis occurs in vivo as consequence of reduced Notch signaling (30). Since overexpression of PS2 mutants with an inactivated presenilin cleavage site substantially decreased the Aβ42 levels (Fig. 5), we first asked whether these cleavage site mutants could exhibit a dominant-negative effect on Notch signaling. For this purpose, mutant presenilins were transformed into wild type animals and the resulting transgenic lines were tested for an impairment of their egg-laying behavior. We did not observe any influence of either wild type or mutant presenilin protein expression on the egg-laying behavior of C. elegans. Next, we crossed presenilin-expressing strains with sel-12(ar171) mutant animals and screened for the presence of both the sel-12 mutant and the transgene. sel-12(ar171) animals never lay eggs due to a stop mutation of sel-12 in codon 225 in the sixth TM domain. As shown previously, expression of wild type PS2 efficiently rescues this egg-laying defect (Table 1). sel-12 transgenic animals with either DB22/2 or DB27/2 mutants also showed profound egg-laying, indicating that the respective PS2 variants are functional in vivo. In contrast, the mutation of a preserved Asp-385 that has been suggested to be part of the active core of the PS1, and which inhibits its endoproteolytic cleavage, completely abolished its activity in the sel-12 rescue assay.

Thus, we conclude that inactivation of the constitutive endoproteolytic cleavage site does not abolish the in vivo function of PS2 in the Notch signaling pathway.

A similar or identical proteolytic activity involved in the processing of both PS1 and PS2 is further supported by the con-
struct which carries the PS1 cleavage site in a PS2 cDNA (PS1 in Fig. 3B). This construct is obviously processed to yield an NTF of the expected size. While all the data reported here were obtained from transfected mouse Neuro2a cells, most of the PS2 mutations were also studied in stable transfections of human HEK293 cells and showed the same inhibition of proteolytic cleavage (data not shown).

The nature of the “presenilinase” is currently unknown, and inhibitor experiments that could hint at the protease class have not been reported. It was recently suggested that presenilins themselves are unusual aspartyl proteases with an autoproteolytic activity and a γ-secretase activity (31). Mutation of the predicted critical Asp residues at positions 263 and 366 in PS2 indeed abolished its endoproteolysis and led to the exclusive production of full-length precursor. Thus, mutations of the cleavage site or replacement of one of the critical Asp residues cause the same phenotype on the PS2 protein, i.e., the inhibition of endoproteolysis.

We also evaluated the functional consequences of mutations that prevent PS2 processing by measuring the levels of secreted Aβ40 and Aβ42. In these studies we used clones of Neuro2a cells stably transfected with the various PS2 constructs but expressing solely the endogenous APP. Our data show that cleavage site mutations which abolish or significantly reduce endoproteolysis also reduce the increase in Aβ42 caused by the FAD mutation Asn-141 → Ile. These results support previous suggestions that the complex of NTF and CTF, plus additional proteins, is the physiologically active and relevant species for the enhanced Aβ42 production (16, 17, 19).

However, in contrast to the above mentioned Asp mutants which can have a dominant-negative effect on the overall amyloid peptide production, i.e., Aβ42 and Aβ40 are reduced below the levels seen with wild type PS, an inactivation of the cleavage site reverses the increase in Aβ42 caused by the PS2 FAD transgene but does not reduce the overall amount of Aβ secretion, indicating that the activities of the endogenous presenilins are not affected. Functional differences between the PS2 cleavage site mutants and mutations of the putative catalytic Asp residues were also observed in the rescue of the egg-laying defect of sel-12 mutant C. elegans. While the endoproteolytically inactive PS2 mutants DB22/2m and DB27/2 efficiently restore the egg-laying behavior to levels comparable to wild type PS2, the mutant PS1 Asp-385 → Asn was devoid of any rescuing activity as was the PS2 mutant Asp-366 → Asn (24, 34). In this in vivo system, full-length PS2 obviously retains enough activity to allow signaling through the Notch pathway, as proven by its rescue of the egg-laying behavior. This differential effect of cleavage site inactivation versus mutation of the critical Asp residues in the rescue of the sel-12 egl phenotype could be readily explained within the framework of the recent hypothesis that presenilins themselves are aspartyl proteases. While any substitution for a catalytic aspartic acid is expected to completely abolish the enzymatic activity, inhibition of endoproteolytic cleavage could still allow for some proteolytic activity of the full-length protein.

There are several conceivable ways in which endoproteolytic processing (or lack thereof) could change the amyloidogenic activity of presenilin. Their function in APP processing, e.g., either as a trafficking factor or as a direct γ-secretase activity, could require proteolytic processing to obtain the fully active conformation. The assumption that PS holoprotein and the processed NTF:CTF complex differ functionally is indirectly supported by their occurrence in different sized protein complexes and by differences in their subcellular distribution. Endoproteolytic cleavage would thus be equivalent to a functional maturation step. Alternatively, endoproteolysis could primarily affect protein stability. It has been shown that overexpressed PS accumulated as full-length protein with limited stability (t1/2 < 1 h), which can be degraded via the proteasome pathway while the NTF:CTF complex is remarkably stable (t1/2 > 12 h) (19, 21, 35). Therefore, cleavage could be a means of ensuring the stability of PS and would, thus, only indirectly modulate the biological function of the protein. In either case, endoproteolysis appears to be an essential step for the activity of FAD presenilin in Aβ42 formation.

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### TABLE I

| Presenilin Variant | Strain genotype | No. of strains tested | Egg-laying behaviora |
|-------------------|-----------------|-----------------------|----------------------|
| PS2 wild type     | sel-12(ar171)   | 3                     | +++                  |
| Vector            | sel-12(ar171)   | 5                     | ++                   |
| PS2 DB22/2m       | sel-12(ar171)   | 2                     | ++                   |
| PS2 DB27/2        | sel-12(ar171)   | 2                     | ++                   |
| PS2 D385N         | sel-12(ar171)   | 3                     | ++                   |

a Strain carried an additional unc-1(e538) mutation that does not affect egg laying (30).

b ++, over 30 progeny laid; ++, 15–30 progeny laid; +, 5–15 progeny laid; –, no progeny laid.

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