Amyloidogenic Function of the Alzheimer's Disease-Associated Presenilin 1 in the Absence of Endoproteolysis†

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ABSTRACT: Alzheimer's disease (AD) is characterized by the invariant accumulation of senile plaques predominantly composed of the pathologically relevant 42-amino acid amyloid β-peptide (Aβ42). The presenilin (PS) proteins play a key role in Aβ generation. FAD-associated mutations in PS1 and PS2 enhance the production of Aβ42, and PS1 is required for physiological Aβ production, since a gene knockout of PS1 and dominant negative mutations of PS1 abolish Aβ generation. PS proteins undergo endoproteolytic processing, and current evidence indicates that fragment formation may be required for the amyloidogenic function of PS. We have now determined the sequence requirements for endoproteolysis of PS1. Mutagenizing amino acids at the previously determined major cleavage site (amino acid 298) had no effect on PS1 endoproteolysis. In contrast, mutations or deletions at the additional cleavage site around amino acid 292 blocked endoproteolysis. The uncleavable PS1 derivatives accumulated as full-length proteins and replaced the endogenous PS1 proteins. In contrast to the previously described aspartate mutations within transmembrane domains 6 and 7, the uncleaved PS1 variants do not act as dominant negative inhibitors of Aβ production. Moreover, when a FAD-associated mutation (M146L) was combined with a mutation blocking endoproteolysis, Aβ42 production still reached pathological levels. These data therefore demonstrate that endoproteolysis of presenilins is not an absolute prerequisite for the amyloidogenic function of PS1. These data also show that accumulation of the PS1 holoprotein is not associated with the pathological activity of PS1 mutations as suggested previously.

Presenilin (PS)[1] proteins play a fundamental role in the proteolytic generation of Amyloid β-peptide (Aβ). Alzheimer's disease (AD)-associated mutations in both PS genes result in the enhanced production of the highly amyloidogenic Aβ42, which preferentially accumulates in senile plaques (*1*, *2*), and a deletion of the PS1 gene in mice abolishes physiological Aβ generation (*3*). Very recently, dominant negative mutations were created by mutagenizing two highly conserved Asp residues within putative transmembrane domains 6 and 7 of PS1 (*4*). Interestingly, these mutations not only inhibited endoproteolysis (see below) of PS1 but also severely affected proteolytic processing of the β-amyloid precursor protein (βAPP). C′-Terminal fragments of βAPP generated by β- and α-secretase accumulate to high levels, and Aβ production is abolished, suggesting a defect in γ-secretase activity (*4*). The critical Asp residues of PS1 are functionally conserved. Mutagenesis of the corresponding Asp residues within human PS2 as well as zebrafish PS1 results in very similar effects on βAPP metabolism (*5*, *6*). On the basis of the finding of these functionally important Asp residues, Wolfe et al. (*4*) suggested that PS1 might be an unusual aspartyl protease, which directly acts as the γ-secretase. In such a scenario, autoactivation of PS by endoproteolysis appears to be required for its proteolytic activity in Aβ generation, since mutagenesis of the Asp residues inhibits both Aβ production and endoproteolysis of PS1. Interestingly, mutagenesis of the critical Asp residues in PS1 and PS2 not only affects Aβ production but also inhibits Notch signaling in *Caenorhabditis elegans* (*5*, *7*). Moreover, it has recently been demonstrated that a gene deletion of PS1 inhibits the proteolytic release of the cytoplasmic domain of Notch-1 (*8*). Therefore, the same γ-secretase activity of PS1 might be associated with Aβ production, Notch-1 cleavage, and autoproteolysis.

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[1] Abbreviations: Aβ, amyloid β-peptide; AD, Alzheimer's disease; βAPP, β-amyloid precursor protein; PS, presenilin; NTF, N-terminal fragment; CTF, C-terminal fragment; FAD, familial Alzheimer's disease.

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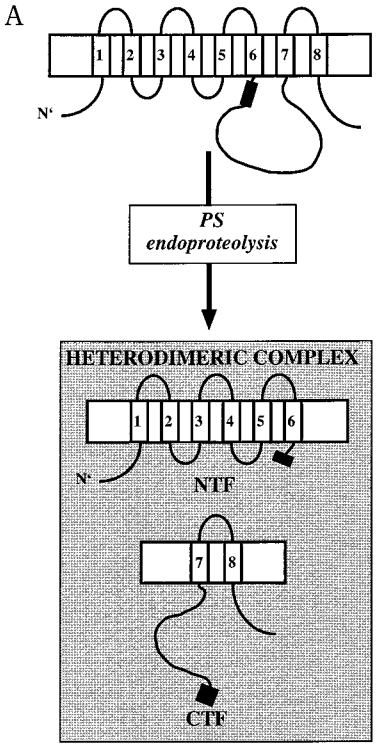

B

cDNA construct	amino acid sequence (290 S T M V W L W N M A E 300; 291, 292, 298)
PS1	S T M V W L W N M A E
Δ291-298	S - - - - - - - - - E
M292D/V293A	S T **D A** W L W N M A E
M298E/A299P	S T M V W L W N **E P** E
M292D/V293A/M298E/A299P	S T **D A** W L W N **E P** E
M292D	S T **D** V W L W N M A E

FIGURE 1: PS endoproteolysis and cDNA constructs. **(**A) PS is endoproteolytically processed within the large cytoplasmic loop between TM6 and TM7. The resulting NTFs and CTFs form stable heterodimeric complexes (*11*−*14*). The black box represents the cleavage site domain. (B) Amino acid sequence of the cleavage site domain (from amino acid position 290 to amino acid position 300). The various cleavable and noncleavable PS1 proteins used in this study are shown. Arrows denote the sites of endoproteolytic cleavage determined by Podlisny et al. (*18*).

PS proteins are endoproteolytically processed (*9*) and form a complex composed of the NTF and CTF (Figure 1) and probably other proteins as well (*10*−*14*), and evidence that both fragments together are required for PS function accumulates. Recombinant N-terminal fragments (NTFs) of PS1 and PS2 containing an AD-associated point mutation are not pathologically active (*15*−*17*). Moreover, coexpression of such recombinant mutant NTFs with C-terminal fragments (CTFs) of PS2 was not sufficient to allow overproduction of Aβ42 (*16*), which is due to the failure of complex formation (*14*, *17*). PS fragments associated with the complex are remarkably stable (*17*−*19*). In contrast to the highly stable fragments, the holoprotein is unstable and rapidly turned over (*17*−*20*). Expression of the PS complex is regulated, and PS fragments not incorporated into the complex as well as the holoprotein itself are rapidly removed by multiple proteolytic activities, including the proteasome (*17*, *21*, *22*) and caspases (*17*, *23*−*26*), as well as a cysteine protease activity (*17*).

Taken together, current evidence therefore indicates that endoproteolysis (or autoactivation) of presenilins may be a prerequisite for their amyloidogenic function. Endoproteolysis may even be affected by FAD-associated PS mutations, although conflicting data have been published claiming either a hyperaccumulation of fragments (*27*) or inhibition of PS endoproteolysis (*28*−*30*).

We wanted to prove if fragment formation is required for PS function by inhibition of endoproteolytic processing of PS1 without affecting the functionally important Asp residues. We therefore mutagenized the cleavage sites to inhibit endoproteolysis. The resultant uncleavable PS variants were then analyzed for their activity in Aβ generation. Surprisingly, we found normal Aβ production in the absence of endoproteolysis.

MATERIALS AND METHODS

Cell Culture and Cell Lines. K293 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycine, 200 μg/mL G418 (to select for βAPP expression), and 200 μg/mL zeocin (to select for presenilin expression). K293 cells stably expressing mutant PS1 variants were generated by transfection of K293 cells stably expressing βAPP containing the Swedish mutation (*31*) with the respective cDNAs. K293 cells stably coexpressing Swedish βAPP695 and wild-type PS1 were described previously (*32*).

Construction of cDNAs Encoding Mutant PS1 Derivatives. All cDNAs encoding PS1 mutant derivatives were constructed by PCR-mediated mutagenesis using appropriate primers. The sequences of the oligonucleotides used are available upon request. The PCR products were cloned into expression vector pcDNA3.1 containing a zeocin-resistance gene (Invitrogen). The cDNAs were sequenced to verify successful mutagenesis.

Antibodies. The polyclonal and monoclonal antibodies against amino acids 263−407 of PS1 (3027; BI.3D7) were described previously (*33*, *34*). Antibody 3926 to synthetic Aβ (*35*) and antibodies C7 (to the last 20 C-terminal amino acids of βAPP; *36*) were described previously.

Analysis of βAPP Metabolites. Stably transfected K293 cell lines were grown to confluence. For the analysis of Aβ in conditioned media, cells were metabolically labeled with 450 μCi of [^{35}S]methionine (Promix, Amersham) for 2 h,

and chased for 2 h in medium containing excess amounts of unlabeled methionine. Conditioned media were immunoprecipitated with antibody 3926 and separated on 10 to 20% Tris-Tricine gels (Novex). To analyze βAPP−CTFs, cell lysates were subjected to immunoprecipitation with antibody C7 (*36*) and separated on 10 to 20% Tris-Tricine gels (Novex). Quantitations were carried out as described in ref *5*.

Combined Immunoprecipitation and Western Blotting of PS. Stably transfected K293 cell lines were grown to confluence. Cell extracts were prepared and subjected to immunoprecipitation using the polyclonal antibody 3027 to PS1 (*33*). Following gel electrophoresis, immunoprecipitated PS were identified by immunoblotting using the monoclonal antibody BI.3D7 (*34*). Bound antibodies were detected by enhanced chemiluminescence (ECL, Amersham).

Analysis of Aβ40 and Aβ42. Conditioned media (2 mL) were collected from confluent K293 cells grown in six-well dishes for 18−24 h. The media were assayed for Aβ40 and Aβ42 using a previously described ELISA (*17*).

RESULTS

The endoproteolytic cleavage site of PS1 was previously analyzed by amino acid sequencing. Abundant cleavage products starting at or close to amino acid 299 of PS1 (*18*, *37*, *38*) were observed. In addition, cleavage activity was detected after amino acids 291 and 292 (*18*; Figure 1). To generate uncleavable PS1 derivatives, we mutagenized the known cleavage sites. We first deleted the domain between amino acid 291 and amino acid 298 (Figure 1). The deletion was introduced in a PS1 cDNA containing the M146L mutation to follow pathological Aβ42 generation. The resultant cDNA construct (PS1 M146L/Δ291−298) was stably transfected into human kidney 293 cells (K293 cells). K293 cells are suitable for the analysis of PS function and endoproteolysis as demonstrated by numerous previous publications (*9*, *11*, *13*, *15*, *17*, *18*, *32*, *34*). Cell lysates of control cells (expressing PS1 M146L) and cells expressing PS1 M146L/Δ291−298 were immunoprecipitated with a polyclonal antibody to the large loop of PS1 (*33*), and precipitated PS1 derivatives were identified by immunoblotting using the monoclonal antibody BI.3D7 (*34*). As shown in Figure 2A, deleting the domain encoding the cleavage site of PS1 completely abolished its endoproteolysis and resulted in the accumulation of the holoprotein. As observed previously, ectopic expression of presenilins results in the replacement of endogenous PS molecules; therefore, no fragments are observed in the absence of endoproteolysis (*9*). To introduce the minimal required mutation, we scanned the cleavage domain by introducing point mutations. Stable expression of PS1 M146L/M292V/D293A also inhibited endoproteolysis (Figure 2B), indicating that residues which are required for endoproteolysis are located several amino acids N-terminal to the major cleavage site observed by amino acid sequencing (*18*, *37*, *38*). To further prove this prediction, we mutagenized amino acids 298 and 299 (PS1 M146L/M298E/A299P). Stable expression of PS1 M146L/M298E/A299P had no detectable effect on endoproteolysis (Figure 2B). Combining the point mutations at residues 292 and 293 and 298 and 299 (PS1 M146L/M292V/D293A/M298E/A299P) also blocked endoproteolysis (Figure 2B).

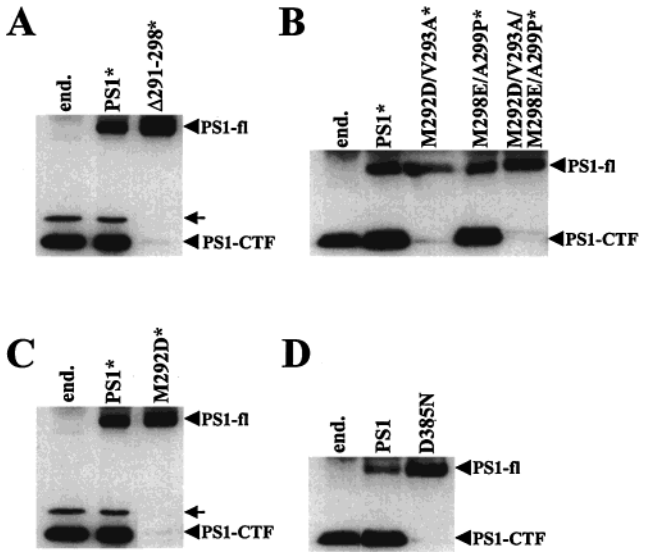

FIGURE 2: Expression of cleavable and noncleavable PS1 variants. (A) Lack of endoproteolytic cleavage in cells overexpressing PS1 containing a deletion of the cleavage site. Cell lysates from K293 cells expressing endogenous presenilins and from cell lines overexpressing PS1 M146L or PS1 M146L/Δ291−298 were immunoprecipitated with antibody 3027 specific to the large loop of PS1 (*33*) and analyzed by immunoblotting using the monoclonal antibody BI.3D7 (*34*). Uncleaved full-length PS1 accumulates in the cell line expressing PS1 M146L/Δ291−298. An arrow denotes the phosphorylated form of the PS1−CTF (*10*, *33*). (B) Mutations at the minor but not at the major cleavage site inhibit endoproteolysis. Cell lysates from K293 cells expressing endogenous presenilins and from cell lines overexpressing PS1 M146L or the PS1 variants M146L/M292D/V293A, M146L/M298E/A299P, and M146L/M292D/V293A/M298E/A299P were analyzed as described for panel A. Uncleaved full-length PS1 accumulates in the cell lines expressing PS1 M146L/M292D/V293A or PS1 M146L/M292D/V293A/M298E/A299P. Endoproteolysis occurs in cells expressing PS1 M146L/M298E/A299P. (C) A single point mutation at amino acid position 292 blocks endoproteolytic cleavage of PS1. Cell lysates from K293 cells expressing endogenous presenilins and from cell lines overexpressing PS1 M146L and PS1 M146L/M292D were analyzed as described for panel A. An arrow denotes the phosphorylated form of the PS1−CTF (*10*, *33*). (D) Lack of endoproteolytic cleavage in cells overexpressing PS1 D385N. Cell lysates from K293 cells expressing endogenous presenilins and from cell lines overexpressing wild-type PS1 and PS1 D385N were analyzed as described for panel A. As observed previously (*4*), endoproteolytic cleavage is inhibited in the cell line overexpressing PS1 D385N. All constructs containing the M146L mutation are marked with an asterisk.

To generate the smallest possible mutation, which blocks endoproteolysis, we next mutagenized the Met residue at codon 292 to an Asp residue. The cDNA (PS1 M146L/M292D) was stably transfected into HEK293 cells, and endoproteolysis of PS1 was monitored by the combined immunoprecipitation and immunoblotting protocol. As shown in Figure 2C, this revealed that indeed a single amino acid exchange at amino acid 292 is sufficient to block endoproteolysis of PS1.

Abolishing endoproteolysis of PS1 by mutagenizing amino acid 292 produces a PS1 molecule with biochemical properties, which are highly similar to the previously described mutations of the critical Asp residues in putative TM6 and TM7 (*4*). Like the above-described mutations at amino acid 292, expression of PS1 D385N in HEK293 cells results in the accumulation of the PS1 holoprotein and inhibits endoproteolysis (*4*; Figure 2D). Since the latter mutation has

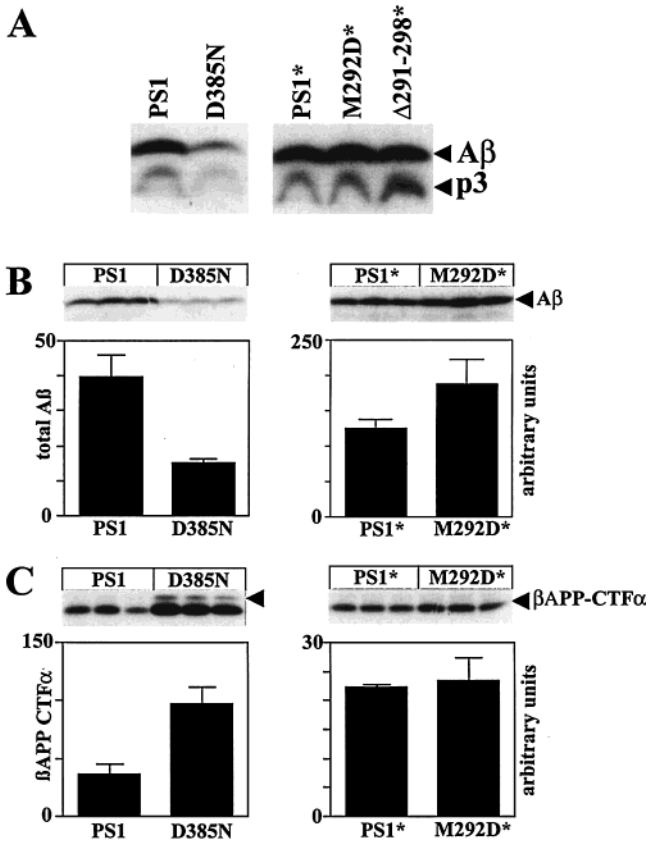

FIGURE 3: Proteolytic processing of βAPP in cells expressing cleavable and noncleavable PS1 variants. (A) Aβ production is not affected in cells expressing the noncleavable PS1 M146L/M292D or M146L/Δ291−298 mutants. K293 cells stably expressing the indicated PS1 proteins were metabolically labeled with [^{35}S]-methionine for 2 h followed by a cold chase for an additional 2 h. Conditioned media were immunoprecipitated with antibody 3926 specific to synthetic Aβ (*35*). In contrast to decreased Aβ and p3 levels in cell lines overexpressing PS1 D385N, cell lines overexpressing the noncleavable PS1 mutant M146L/M292D produce normal levels of Aβ and p3. (B) Quantitation (lower panels) of independent immunoprecipitations (upper panels) confirms that the M292D mutation allows physiological Aβ production, whereas the D385N mutation significantly reduces the level of Aβ generation as described previously (*4*). K293 cells stably expressing the indicated PS1 proteins were metabolically labeled with [^{35}S]-methionine for 2 h followed by a cold chase for an additional 2 h. Conditioned media were immunoprecipitated with antibody3926. For constructs marked with an asterisk, the cDNA contains the M146L mutation. Bars represent the mean ± standard error of three independent experiments. (C) Expression of noncleavable PS1 M146L/M292D does not result in increased levels of C-terminal fragments of βAPP generated by α- and β-secretase. Cell lysates of the samples shown in panel B were immunoprecipitated with antibody C7 (*36*) to detect the C-terminal fragments of βAPP. In contrast to that of PS1 D385N, overexpression of PS1 M146L/M292D does not lead to increased levels of βAPP−CTFs. For constructs marked with an asterisk, the cDNA contains the M146L mutation. Bars represent the mean ± standard error of three independent experiments; the arrowhead indicates β-secretase-generated βAPP-CTFs, which accumulate only in cells expressing the D385N mutation.

been shown to alter βAPP processing due to a defect in γ-secretase cleavage (*4*), we now analyzed Aβ production in cell lines expressing either the point mutation M292D or the Δ291−298 deletion (Figure 3A). Cells were pulse labeled with [^{35}S]methionine and chased for 2 h in the presence of excess amounts of unlabeled methionine. Conditioned media were immunoprecipitated with antibody 3926 (*35*), and Aβ production was analyzed. In contrast to the PS1 D385N mutation, which significantly inhibited Aβ production as shown previously (*4*; Figure 3A,B), the uncleavable PS variants M292D and Δ291−298 did not block Aβ generation (Figure 3A,B). Quantitation of independent immunoprecipitations confirmed that Aβ production is not inhibited by the uncleavable PS1 M292D derivative (Figure 3B). Therefore, the mutations at amino acid 292 inhibit endoproteolysis without affecting PS activity in Aβ generation. To prove this further, we also investigated the production of βAPP-CTFs. Cell lysates from the experiments described above (Figure 3B) were immunoprecipitated with antibody C7 (*36*) to detect βAPP−CTFs. Accumulation of βAPP−CTFs in cells stably expressing PS1 D385N could be fully confirmed (*4*; Figure 3C). However, the mutation at amino acid 292 did not cause an increase in the levels of these βAPP metabolites, suggesting that inhibition of PS1 endoproteolysis does not necessarily affect βAPP processing (Figure 3C).

We specifically introduced the cleavage site mutations into a PS1 molecule carrying an AD-associated mutation (M146L), since that would allow us to monitor pathological Aβ42 production of the FAD-associated PS1 M146L mutation in the absence of endoproteolysis. Conditioned media from control cells and cells expressing either PS1 M146L/M292D or PS1 M146L/Δ291−298 were collected, and the ratio of Aβ42 to $Aβ_{total}$ was determined using a previously described ELISA (*17*). Analysis of the conditioned media revealed that the uncleavable PS1 derivatives containing the FAD-associated M146L mutation still caused an elevated level of Aβ42 generation compared to the wild-type control (Figure 4A). However, we found a slight reduction in the Aβ42/$Aβ_{total}$ ratios as compared to those in cells expressing only the M146L mutation (Figure 4A). Nevertheless, the observed Aβ42/$Aβ_{total}$ ratios are still within a pathological range (*1*, *2*). This result demonstrates that the FAD-associated PS1 M146L mutation can promote pathological Aβ42 generation in the absence of endoproteolysis. To exclude the possibility that the elevated Aβ42/$Aβ_{total}$ ratios were due to the artificially introduced mutation of position 292 rather than due to the FAD mutation M146L, we also analyzed a PS1 variant containing only the M292D mutation. As shown in Figure 4B, expression of PS1 M292D did not lead to elevated Aβ42/$Aβ_{total}$ ratios, thus demonstrating that the artificially introduced mutation at amino acid position 292 is not responsible for the abnormal Aβ42 generation observed in cell lines expressing PS1 M146L/M292D. Taken together, these data demonstrate that endoproteolytic cleavage of PS1 is not an absolute prerequisite for its role in Aβ40 or Aβ42 generation.

DISCUSSION

We have demonstrated here that a primary determinator for endoproteolytic cleavage of PS1 is not located at the previously characterized major cleavage site(s) at amino acids 298 or 299 (*18*, *37*, *38*) but is surprisingly located around amino acid 292. However, data derived by amino acid sequencing also provided evidence for further endoproteolytic cleavage products of PS1 starting at amino acids 292 and 293 (*18*). When these data are taken together with our mutagenesis analysis, it appears likely that PS1 is first cleaved at or close to amino acid 292. After the first cleavage, which is dependent on the primary amino acid sequence,

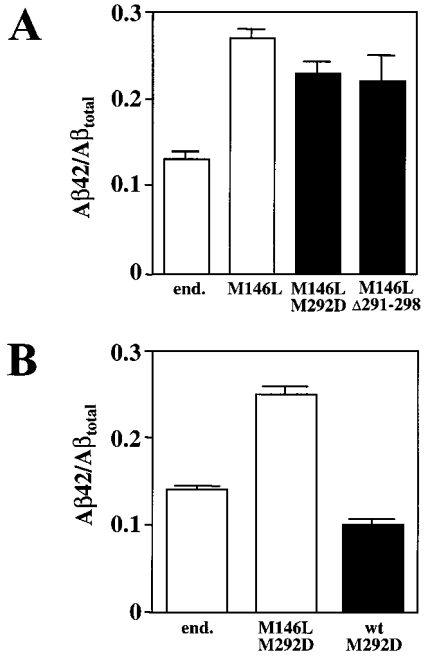

FIGURE 4: The lack of endoproteolytic cleavage still allows production of pathogenic Aβ42 levels. (A) Conditioned media of K293 cells expressing the indicated PS1 proteins were collected and analyzed using a previously described highly specific ELISA (*17*). Pathological levels of Aβ42 are detected in cells expressing the noncleavable PS1 variants M146L/M292D and M146L/Δ291−298. Bars represent the mean ± standard error of three independent experiments. (B) Mutagenesis of Met 292 to Asp does not create an artificial point mutation leading to pathogenic levels of Aβ42. Conditioned media of K293 cells expressing either PS1 M146L/M292D or PS1 M292D were collected and analyzed using a previously described highly specific ELISA (*17*). Elevated levels of Aβ42 are only detected in cells expressing the noncleavable PS1 M292D together with the M146L mutation. Bars represent the mean ± standard error of three independent experiments.

the resulting fragment is either trimmed by an exopeptidase or cleaved a second time at or close to amino acid 298. This is supported by the recent finding that PS2 is also cleaved at the methionine corresponding to amino acid 292 of PS1 (H. Jacobsen, personal communication) and then further processed to produce the in vivo-observed CTF starting at amino acid 307 (*39*; H. Jacobsen, personal communication). This strongly indicates that both presenilins are processed by a very similar proteolytic activity. Our data do not formally exclude the possibility that PS cleavage occurs by autoproteolysis due to the proposed intrinsic aspartyl protease activity, which may be identical with γ-secretase (*4*). However, on the basis of our results, such a possibility appears to be rather unlikely. γ-Secretase activity is known to be quite independent of the primary amino acid sequence at the recognition site, since deletions and amino acid exchanges at the γ-secretase site did not significantly abolish Aβ generation (*40*−*42*). In contrast, endoproteolysis of PS1 is abolished by the mutagenesis of a single amino acid (Met 292). Therefore, both proteolytic activities appear to be quite different in regard to their substrate specificity. Moreover, aspartyl protease inhibitors known to block Aβ generation do not affect PS endoproteolysis (T. Golde in ref *43*; C. Haass, K. Fechteler, and A. Capell, unpublished observation).

Our data now raise the question of why PS endoproteolysis is required at all. As described above, there is plenty of evidence that PS fragments are in vivo the biologically active units. However, here we show that the holoprotein is active in amyloidogenesis; therefore, the activity of presenilins in amyloidogenesis is not directly affected by endoproteolytic processing. We want to point out that in vivo very little holoprotein is detected since it is very unstable (*9*, *17*−*20*) and rapidly degraded by the proteasome in a ubiquitin-dependent pathway (*17*, *21*, *22*), whereas the PS complex composed of the two fragments is highly stable (*17*−*19*). It may therefore be possible that uncleaved and cleaved PS molecules are both active in amyloidogenesis, but only differ in their stability. In such a model, fragment formation would be required to convert PS into a stable molecule, which is resistant to degradation. As discussed previously (*11*), structural rearrangements may be responsible for the substantially increased stability of PS fragments. Such structural changes are expected to take place within the only known uncleavable PS variant that does exist in vivo (PS1 Δexon9; *9*, *11*). The exceptional PS1 Δexon9 mutation accumulates as an uncleaved holoprotein in vivo due to the lack of the exon encoding the cleavage site (*9*). This again demonstrates that uncleavable PS1 holoproteins are fully functional in amyloidogenesis and do not necessarily require endoproteolysis for their activation.

In this study, we have demonstrated that the FAD-associated PS1 M146L mutation is pathologically active in the absence of endoproteolysis, indicating that endoproteolysis may not be required for the pathological activity of PS1 mutations. This is supported by our previous finding that the pathological activity of the PS1 Δexon9 protein is independent of its inability to undergo endoproteolysis but is due to a single point mutation occurring at the splice junction (*34*). Additional work is now required to prove if PS1 mutations other than the M146L mutation used in this work can act independently of PS1 endoproteolysis. However, our data demonstrate that the lack of endoproteolysis is not directly associated with elevated levels of Aβ42 generation as suggested previously (*28*−*30*). Therefore, reduced endoproteolysis levels may not be responsible for the increased Aβ42 levels observed in patients with PS1 mutations.

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NOTE ADDED IN PROOF

Uncleavable PS2 allows normal Aβ production and functionally rescues mutant sel-12 in *C elegans* (Jacobsen, H., Reinhardt, D., Brockhaus, M., Bur, D., Kocyba, C., Kurt, D., Grim, M. G., Baumeister, R., and Loetscher, H. The influence of endoproteolytic processing of FAD presenilin 2 on Aβ42 amyloid peptide formation. *J. Biol. Chem.*, in press).

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