# Zebrafish (*Danio rerio*) Presenilin Promotes Aberrant Amyloid $\beta$-Peptide Production and Requires a Critical Aspartate Residue for Its Function in Amyloidogenesis[†]

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ABSTRACT: Alzheimer's disease (AD) is characterized by the invariable accumulation of senile plaques composed of amyloid $\beta$-peptide (A$\beta$). Mutations in three genes are known to cause familial Alzheimer's disease (FAD). The mutations occur in the genes encoding the $\beta$-amyloid precursor protein ($\beta$APP) and presenilin (PS1) and PS2 and cause the increased secretion of the pathologically relevant 42 amino acid A$\beta$42. We have now cloned the zebrafish (*Danio rerio*) PS1 homologue (zf-PS1) to study its function in amyloidogenesis and to prove the critical requirement of an unusual aspartate residue within the seventh putative transmembrane domain. In situ hybridization and reverse PCR reveal that zf-PS1 is maternally inherited and ubiquitously expressed during embryogenesis, suggesting an essential housekeeping function. zf-PS1 is proteolytically processed to produce a C-terminal fragment (CTF) of approximately 24 kDa similar to human PS proteins. Surprisingly, wt zf-PS1 promotes aberrant A$\beta$42 secretion like FAD associated human PS1 mutations. The unexpected pathologic activity of wt zf-PS1 may be due to several amino acid exchanges at positions where FAD-associated mutations have been observed. The amyloidogenic function of zf-PS1 depends on the conserved aspartate residue 374 within the seventh putative transmembrane domain. Mutagenizing this critical aspartate residue abolishes endoproteolysis of zf-PS1 and inhibits A$\beta$ secretion in human cells. Inhibition of A$\beta$ secretion is accompanied by the accumulation of C-terminal fragments of $\beta$APP, suggesting a defect in $\gamma$-secretase activity. These data provide further evidence that PS proteins are directly involved in the proteolytic cleavage of $\beta$APP and demonstrate that this function is evolutionarily conserved.

Aggregation and precipitation of peptides appears to play a major role in neurodegenerative diseases such as Alzheimer's disease (AD)[1] (*1*), Parkinson's disease (*2*), and Huntington's disease (*3*). In AD the aggregating amyloid $\beta$-peptide (A$\beta$) accumulates in highly insoluble senile plaques, which are the defining pathological lesions of the disease (*4*). A$\beta$ is derived by proteolytic processing from the $\beta$-amyloid precursor protein ($\beta$APP; *4*). Two secretases have been postulated, which either generate the N-terminus ($\beta$-secretase) or the C-terminus ($\gamma$-secretase) of A$\beta$ (*5*). A$\beta$ is generated under physiological conditions in cultured cells and is secreted into the medium (*5*). In vivo A$\beta$ is detected in human plasma and cerebrospinal fluid (*5*).

In the majority of cases AD occurs sporadically with an increasing risk during aging (*4, 6*). However, rare mutations have been found to cause autosomal dominant early onset familial AD (FAD; *4, 6*). Mutations were found within the genes encoding $\beta$APP and presenilin (PS1) and PS2 (summarized in refs *4* and *6*). The analysis of these mutations in primary fibroblasts derived from FAD patients, transfected cells, and transgenic animals revealed that they all alter the production of A$\beta$ (*4, 6*). Interestingly, these mutations affect a common pathological mechanism by increasing the production of the long (42 amino acid) version of A$\beta$ (A$\beta$42). This peptide is known to aggregate much faster as compared to the more abundant A$\beta$40 (*7*) and is therefore predominantly found within senile plaques (*1, 8, 9*).

Besides the pathological function of mutant PS in A$\beta$42 generation, wt PS proteins appear to have a fundamental function in physiological A$\beta$ production, since it was recently

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[1] Abbreviations: A$\beta$, amyloid $\beta$-peptide; AD, Alzheimer's disease; $\beta$APP, $\beta$-amyloid precursor protein; PS, presenilin, NTF, N-terminal fragment; CTF, C-terminal fragment; FAD, familial Alzheimer's disease; zf-PS1, zebrafish PS1; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction.

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found that PS1$^{-/-}$ mice produce significantly reduced amounts of A$\beta$40 and A$\beta$42 (*10*). Since C-terminal fragments of $\beta$APP accumulate in neurons derived from the brains of PS$^{-/-}$ mice, it has been proposed that PS proteins may either activate $\gamma$-secretase or may even exhibit a $\gamma$-secretase activity by themselves (*10, 11*). This is supported by the very recent finding that two unusual aspartate residues are critically required for the function of human PS1 in amyloidogenesis (*12*). Mutagenesis of these residues results in a phenotype similar to the PS1 deletion in mice, since A$\beta$ production is severely reduced and C-terminal fragments of $\beta$APP accumulate to high levels (*12*). Moreover, endoproteolysis of PS1 is inhibited under these conditions. These observations lead Wolfe et al. (*12*) to the proposal that PS1 is an unusual aspartate protease, which exhibits the $\gamma$-secretase activity. In such a scenario endoproteolysis of PS1 is believed to occur by autoproteolysis and appears to be required for functional activation of presenilins (*12*). However, other models implicate that PS molecules are involved in targeting of selected membrane proteins including $\beta$APP to the cell surface (*13*).

PS proteins facilitate Notch signaling. This is supported by the finding that a defect in the PS homologue of *Caenorhabditis elegans* results in a Notch phenotype, which can be fully rescued by transgenic expression of human PS1 and PS2 (*14, 15*). Moreover, genetic evidence in *Drosophila melanogaster* also links PS function to Notch signaling (*16, 17*). Finally, a deletion of the PS1 gene in mice results in abnormal embryonic development causing a fatal phenotype, which is similar to that caused by the deletion of the Notch gene (*18, 19*). This is now explained by the finding that PS1 is required for the proteolytic release of the cytoplasmic domain of Notch 1 (*20*).

PS proteins are polytopic proteins with eight potential transmembrane domains (*21, 22*). Human presenilins are proteolytically processed to a N-terminal ~30 kDa fragment (NTF) and a C-terminal ~20 kDa fragment (CTF; *23*). In vivo these fragments accumulate and almost no full-length PS can be observed (*23*). PS fragments are bound to each other (*24*−*26*) and associate with other proteins such as $\beta$-catenin (*26, 27*). Recent evidence indicates that a recombinant NTF containing a FAD-associated mutation exhibits no pathological activity (*28*−*30*). Furthermore, such recombinant NTFs are also inactive in facilitating Notch signaling (*14*) and are not incorporated into the PS complex (*28, 31*). This raises the possibility that the formation of the PS complex composed of the NTF and the CTF may be required for the pathological activity of PS proteins.

We have now isolated the PS1 homologue of zebrafish (*Danio rerio*) to study its function in amyloidogenesis and to provide further evidence for the critical requirement of an unusual aspartate residue within the seventh putative transmembrane domain.

## EXPERIMENTAL PROCEDURES

*cDNA Cloning.* Zebrafish were kept and raised under standard laboratory conditions. RNA was prepared by extraction of 100 24-h-old *Danio rerio* embryos with Trizol reagent (Gibco) according to the instructions. One microgram of total RNA was used for the 5′-RACE (Gibco) together with the degenerate gene-specific primer 5′-CIG CIA RIA TIA RCC AIG CIG TCC in the first strand cDNA synthesis. In the first PCR (35 cycles) the nested degenerated primer 5′-CCC GAA TTC GCC ATI ARI GCI SWI ATC ATI ATI ARR TA (D2) together with the abridged anchor primer was used to amplify a 458 bp fragment. After reamplification in another 25 cycles with the primer D2 and the universal amplification primer the fragment was cloned into pBluescript II KS(+). The identity of the cDNA fragment was verified by DNA sequencing. The digoxigenine-labeled (Boehringer Mannheim) fragment was then used to screen a zebrafish $\lambda$-ZAP II cDNA library ($2 \times 10^6$ phages) generated from 20−28-h-old fishes. The cDNA insert was isolated, and both strands were sequenced. For expression of the zf-PS1 cDNA in human cells, the cDNA was amplified by PCR under standard conditions with the primers 5′-GGG GAA TTC ATG GCT GAT TTA GTG and 5′-TAT CAA ATG CGG CCG CCT ATA TGT AGA ACT. The resulting DNA fragment was subcloned into pcDNA3.1 Zeo vector (*24*). Expression vectors were stably transfected into HEK 293 cells as described (*28, 32*).

*Antibodies.* The rat polyclonal antibody zf-PS$_{loop}$ was raised to the loop domain (amino acids 289−365). The respective coding region was amplified by PCR with the primers 5′-GGG GAA TTC GAC AGT GCT GAA and 5′-CCC AAG CTT TTA GTC ATC ATC TGC. The resulting fragment was subcloned into the *Eco*RI/*Hin*dIII restriction site of pMAL-C2 (New England Biolabs). The fusion protein was expressed in *Escherichia coli* DH 5$\alpha$, purified on amylose resin (New England Biolabs) according to the supplier's instructions, and inoculated in rats.

Antibody 3027 to the large loop of human PS1 was described previously (*32*).

The rabbit polyclonal antibody 5818 was raised to the C-terminus of human $\beta$APP$_{659}$ (amino acids 652−695). The respective coding region was amplified by PCR with the primers 5′-CGC GAA TTC CAG TAC ACA TCC ATT CAT C and 5′-TGC GGT CGA CCT AGT TCT GCA TCT GCT C and subcloned into pMal-C2. Bacterial expression and purification of the fusion protein was performed as described above. The purified recombinant protein was inoculated into rabbits.

Antibody 3927 used to detect A$\beta$ in conditioned medium was described previously (*33*).

*ELISA.* The ELISA used to specifically detect the relative amounts of A$\beta$40 and A$\beta$42 in conditioned media was described previously (*28*).

*In Situ Hybridization.* In situ hybridizations were carried out as described (*34*).

*Metabolic Labeling, Immunoprecipitation, Immunoblotting, and Electrophoresis.* Pulse−chase experiments, immunoprecipitations, and immunoblotting was carried out as described (*28*).

*Mutagenesis.* The cDNA encoding zf-PS1 Asp374Asn was constructed by a two-step PCR procedure. The following primers were used: for the first PCR, (1) 5′-GGG GAA TTC ATG GCT GAT TTA GTG and (2) 5′-GTA AAA GAT GAA ATT TCC CAA ACC C; and for the second PCR, (3) 5′-GGG TTT GGG AAA TTT CAT CTT TTA C and (4) 5′-TAT CAA ATG CGG CCG CCT ATA TGT AGA ACT. After gel purification, the PCR products were mixed and

subjected to a final PCR with primers 1 and 4. The resulting PCR product was digested with *Eco*RI/*Not*I and cloned into the pcDNA3.1/Zeo(+) expression vector (Invitrogen). The cDNA was sequenced to verify successful mutagenesis. Transfection was carried out as described above (*28*).

*RNA Isolation and Reverse PCR.* Total RNA was extracted from the described developmental stages using standard protocols. Five micrograms of total RNA was reverse-transcribed. zf-PS1 cDNA was then amplified by PCR with the forward primer GCATCCACTGGAAGGG and the reverse primer GCCGAGCGCTGGGCAT. After 25 cycles amplified cDNAs were analyzed on agarose gels. Negative controls were performed without reverse transcription of mRNA.

## RESULTS

*Sequence Analysis of zf-PS1.* The zebrafish homologue of human PS1 was isolated by standard procedures (see also Experimental Procedures). Sequence analysis reveals an open reading frame of 456 amino acids, which would correspond to a protein with a calculated molecular mass of 50.1 kDa. Sequence comparison to human and *Xenopus* presenilins (*35*−*38*) demonstrates that the protein encoded by the zebrafish cDNA is 73.9% identical to PS1 and 61.8% identical to PS2 (Figure 1). The higher homology to human PS1 together with the fact that the zebrafish PS protein can be phosphorylated by protein kinase C in vivo (data not shown), as can human PS1 (*32*, *39*), suggests that the isolated clone encodes a PS1 homologue of zebrafish (zf-PS1). Analysis of the hydrophobicity of zf-PS1 by the Kyte−Doolittle algorithm reveals a high degree of structural conservation. Similar to human PS (*21*, *22*), zf-PS1 probably contains eight transmembrane domains with a relatively large loop between TM6 and TM7 (Figure 1). Immediately C-terminal to the putative TM6, a very high degree of sequence homology is observed. This includes the potential cleavage sites for constitutive proteolytic processing (*40*, *41*; Figure 1). In contrast, downstream of these cleavage sites very little sequence conservation is observed until amino acid 365. Very little sequence conservation is also observed in the N-terminal region before the putative transmembrane domain 1, whereas the C-terminal domain after amino acid 434 is almost completely conserved. Interestingly, two aspartate residues at position 246 and 374 within the putative transmembrane domains 6 and 7 are completely conserved in all PS1 sequences (Figure 1 and data not shown). The unusual intramembraneous localization of these two charged residues together with their evolutionary conservation might indicate a crucial function (see also ref *12*).

*zf-PS1 Is Maternally Inherited and Ubiquitously Expressed.* We have employed whole-mount in situ hybridization with digoxigenin-labeled sense and antisense RNA probes as well as reverse PCR to determine the spatial and temporal expression of zf-PS1 during embryogenesis of zebrafish. zf-PS1 mRNA was detected in blastomeres before the onset of zygotic transcription (Figure 2A,D), demonstrating maternal inheritance. At all other stages we have examined, zf-PS1 RNA shows a ubiquitous distribution (Figure 2A−C). Maternal inheritance of zf-PS1 together with its ubiquitous distribution in all stages analyzed suggests an essential function during embryonic development.

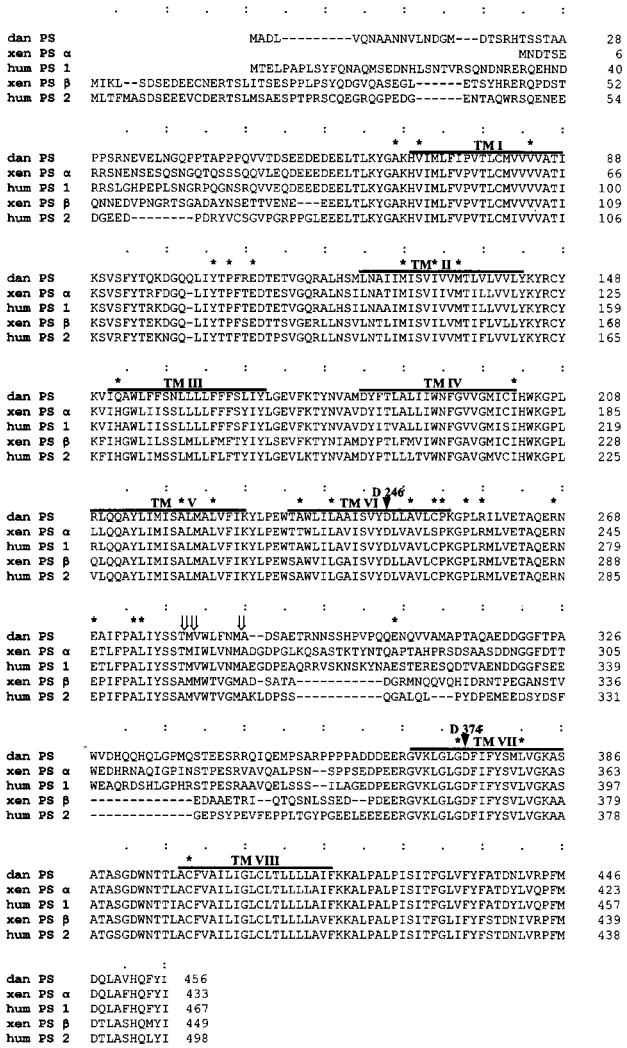


FIGURE 1: Deduced amino acid sequence of zf-PS1 (dan PS) and comparison to human (hum) and *Xenopus* (xen) PS1 and PS2 (*35*−*38*). Sites of AD-associated mutations in human PS1 and PS2 are indicated by asterisks. Putative transmembrane domains are indicated by black bars. Arrows indicate the cleavage sites of human PS1 by the constitutive proteolytic activity (*40*, *41*). Accession number of the zebrafish PS1 cDNA is AJ132931.

*Proteolytic Processing of zf-PS1.* Human full-length PS is rapidly turned over by the proteasome (*28*, *42*, *43*) and only very small amounts are processed to the remarkably stable CTFs and NTFs (*23*, *25*, *28*). In order to study proteolytic processing and degradation of zf-PS1, the corresponding cDNA was stably transfected in human kidney 293 cells (HEK 293). This cell line is frequently used to study the proteolytic turnover and the amyloidogenic function of human presenilins (*24*, *28*, *29*, *40*, *44*−*46*). Cell lines stably expressing zf-PS1 were pulse-labeled with [$^{35}$S]methionine for 15 min and chased for the indicated time points in the presence of excess amounts of unlabeled methionine. Cell lysates were immunoprecipitated with antibody zf-PS1$_{loop}$. As shown in Figure 3A, full-length zf-PS1 is rapidly turned over. Consistent with human PS1 (*23*, *25*, *28*, *40*) the half-life of zf-PS1 is approximately 30−40 min. As observed for human PS1 (*23*), very little zf-PS1 is turned over into the stable CTFs (data not shown). This confirms and extends previous findings (*23*, *25*, *28*) suggesting that most of the newly synthesized PS proteins are rapidly degraded and only very minor

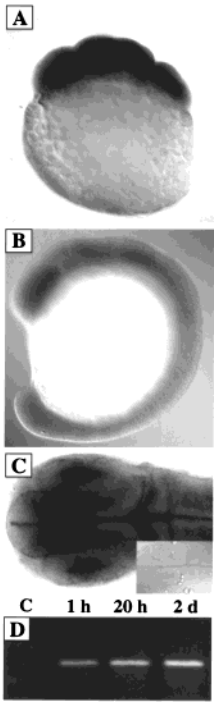


FIGURE 2: Developmental expression of zf-PS1. (A) Maternal zf-PS1 message is detected in blastomeres at the eight-cell stage, prior to onset of zygotic transcription. (B) Uniform expression during the segmentation stage, at 16 h; lateral view. (C) Dorsal view of a zebrafish head at 24 h of development. Uniform expression is seen in the CNS and other tissues; variations of intensity are due to different densities of cells or nuclei. Inset: control sense probe detects no signal. (D) Detection of zf-PS1 mRNA by reverse PCR. zf-PS1 mRNA was amplified by reverse PCR from the indicated stages.

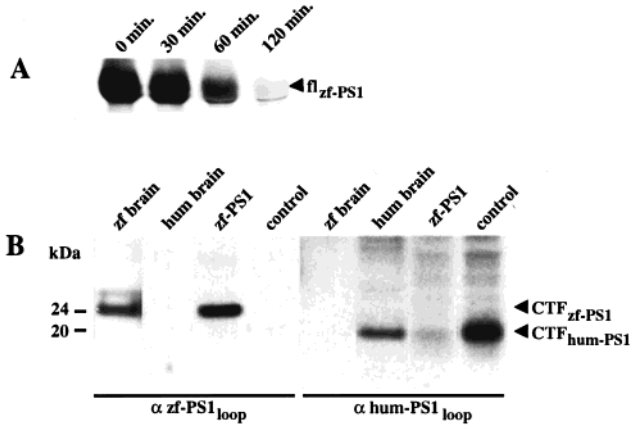


FIGURE 3: Proteolytic processing and degradation of zf-PS1. (A) Full-length zf-PS1 is rapidly degraded. HEK 293 cells stably expressing zf-PS1 were pulse-labeled for 15 min with [$^{35}$S]-methionine and chased for the indicated time points. Cell lysates were immunoprecipitated with zf-PS1$_{loop}$. (B) zf-PS1 is proteolytically processed. Lysates from zebrafish/human brain, untransfected HEK 293 cells (control), and HEK 293 cells stably transfected with zf-PS1 were immunoblotted with antibodies zf-PS1$_{loop}$ (to detect the zebrafish CTF) or antibody 3027 (α hum-PS1$_{loop}$; to detect the human PS1 CTF). Comigrating CTFs of zf-PS1 are detected in fish brain as well as in lysates prepared from human cells overexpressing zf-PS1. Zebrafish CTFs migrate to a slightly higher molecular weight as compared to human CTFs. Overexpressed zf-PS1 displaces endogenous human PS1 CTFs.

amounts are processed to the stable CTFs and NTFs.

As described above, human presenilins predominantly occur as proteolytically processed NTFs and CTFs in vivo (*23*). Since these fragments are highly stable, they are very difficult to detect in pulse-labeling experiments (*23*, *25*, *28*, *29*, *40*). We therefore performed immunoblots on brain extracts of adult zebrafish using antibody zf-PS$_{loop}$. Robust amounts of a ~23 kDa CTF were detected (Figure 3B). This CTF migrated above the human CTF detected in human brain and HEK 293 cells with antibody 3027 (*32*; Figure 3B). Due to the nonconserved amino acid sequence of the antigen used to generate antibody zf-PS1$_{loop}$ (see Figure 1 and Experimental Procedures), this antibody did not cross-react with human PS fragments derived from brain or cell extracts (Figure 3B). However, upon stable expression of the zf-PS1 cDNA in HEK 293 cells, a CTF comigrating with that observed in zebrafish brain extracts was also generated in human cell lines (Figure 3B). This demonstrates that human cells are able to correctly process zebrafish PS1.

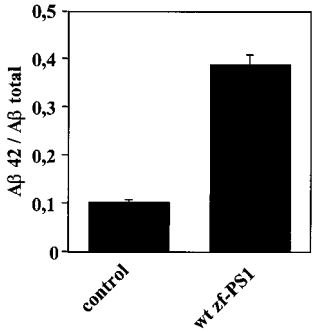


FIGURE 4: Amyloidogenic activity of zf-PS1. Conditioned media from HEK 293 cells overexpressing Swedish mutant βAPP (control) or HEK 293 cells overexpressing Swedish mutant βAPP and zf-PS1 were collected and the ratio of Aβ42/Aβ total was determined by a previously described ELISA (*28*, *46*). Bars represent the mean ± SE of six independent experiments.

Overexpression of exogenous presenilins results in the displacement of their endogenous counterparts (*23*, *25*, *47*, *48*). To determine whether zf-PS1 also affects expression of endogenous PS in human cells, immunoblots were performed on extracts from untransfected cells as well as independent cell lines overexpressing zf-PS1. Membrane preparations were immunoblotted with zf-PS1$_{loop}$ to detect the zebrafish CTF and with antibody 3027 (*32*) to detect the human PS1 CTF. In untransfected HEK 293 cells, antibody 3027 detected endogenous human PS1 CTF as expected (Figure 3B). In contrast, only very minor amounts of the human fragments could be detected in cell lines overexpressing zf-PS1 (Figure 3B). Therefore, overexpression of zf-PS1 results in an efficient displacement of endogenous PS even within a heterologous system.

*Amyloidogenic Activity of zf-PS1.* In order to study the amyloidogenic activity of zebrafish PS1, HEK 293 cells expressing Swedish mutant βAPP were stably transfected with the zf-PS1 cDNA. Coexpression of PS cDNA constructs together with the Swedish mutant βAPP facilitates the detection of Aβ (*28*, *29*, *44*−*46*, *49*). The relative concentrations of Aβ40 and Aβ42 were estimated in conditioned media from independent cell clones by a previously described ELISA (*28*, *46*). Surprisingly, we observed that expression of wt zf-PS1 results in an approximately 3−4-fold increase of Aβ42 secretion as compared to the Aβ42 levels secreted from cells expressing endogenous human PS1 (Figure 4). Such a 3−4-fold increase of Aβ42 secretion is usually observed upon the expression of presenilins containing FAD-associated point mutations (*6*). We therefore analyzed the

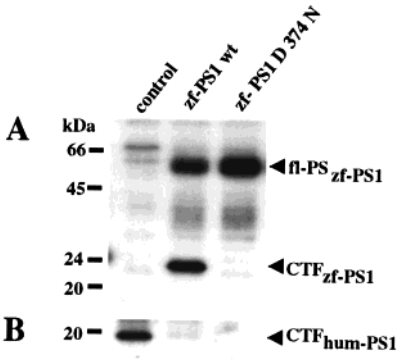


FIGURE 5: Mutation of a critical aspartate residue of zf-PS1 at codon 374 inhibits endoproteolytic processing. (A) Membrane preparations from untransfected HEK 293 cells (control), HEK 293 cells stably transfected with wt zf-PS1 or zf-PS1 Asp374Asn were immunoblotted with antibody zf-PS1$_{loop}$. The mutation of aspartate 374 inhibits the formation of the CTF. (B) Similar to wt zf-PS1, overexpression of zf-PS1 Asp374Asn results in the displacement of the endogenous human PS1 CTFs. Membrane preparations used for the experiments shown in panel A were immunoblotted with antibody 3027 to detect human PS1 CTFs.

deduced amino acid sequence of zf-PS1 for variations at positions with known point mutations causing FAD in humans. This revealed a sequence variation at codon 152 (Figure 1). At the corresponding codon in human PS1, His163Tyr, and His163Arg mutations were found previously (Figure 1). Moreover, several amino acid exchanges in the highly conserved transmembrane domains were observed as well (Figure 1). Identical amino acid exchanges were obtained from several independent cDNA clones, therefore excluding the possibility that such sequence variations are caused by PCR errors (data not shown). Therefore, naturally occurring sequence variations in wt zf-PS1 may cause a high level of A$\beta$42 secretion.

Sequence comparison revealed two highly conserved aspartate residues at amino acids 246 and 374 (Figure 1). Interestingly, these charged amino acids are located within the putative transmembrane domains 6 and 7 (Figure 1) and have been demonstrated to be essential for the function of human PS1 in A$\beta$ production (*12*). Moreover, our previous work indicated that the aspartate located within the putative TM7 is required for Notch signaling in *C. elegans* (*49*). We therefore mutagenized aspartate 374 of zf-PS1 to asparagine and analyzed the consequences for endoproteolytic cleavage and $\beta$APP processing. The cDNA construct zf-PS1Asp374Asn was stably transfected in the above-mentioned HEK 293 cells expressing Swedish mutant $\beta$APP. Membrane preparations of these cells were then immunoblotted with antibody zf-PS1$_{loop}$. This revealed high levels of the full-length protein, whereas no CTFs could be observed (Figure 5A). In contrast, cells expressing wt zf-PS1 showed lower levels of the full-length protein and robust levels of the CTF (Figure 5A). Therefore, the mutation of aspartate 374 inhibits endoproteolysis of zf-PS1, resulting in the accumulation of the uncleaved full-length protein. To determine whether the mutant zf-PS1 Asp374Asn is still capable of inhibiting endogenous (human) PS fragment accumulation, aliquots of the same membrane preparation shown in Figure 5A were immunoblotted with antibody 3027 to detect human PS1 CTFs. As shown in Figure 5B, overexpression of zf-PS1 Asp374Asn inhibits accumulation of endogenous (human) PS fragments, similar to wt zf-PS1 (Figures 5B and 3B).

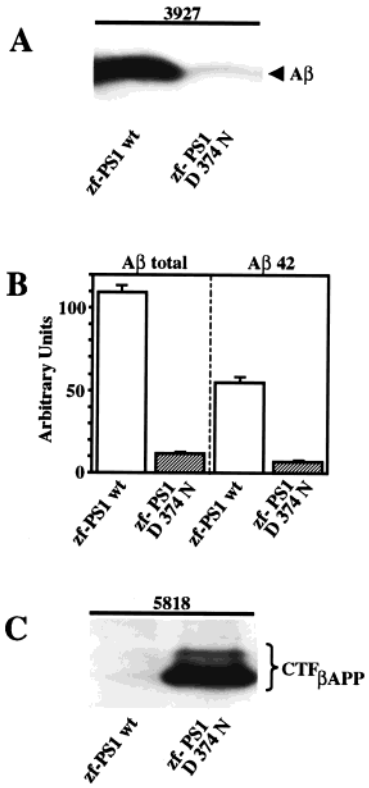


FIGURE 6: Expression of the Asp374Asn mutation affects $\beta$APP processing. (A) Overexpression of zf-PS1 Asp374Asn inhibits A$\beta$ secretion. HEK293 cells overexpressing Swedish mutant $\beta$APP and zf-PS1 Asp374Asn or HEK 293 cells overexpressing Swedish mutant $\beta$APP and wt zf-PS1 were metabolically labeled with [$^{35}$S]-methionine. Conditioned media were immunoprecipitated with antibody 3927 (*33*) to detect A$\beta$. Expression of zf-PS1 Asp374Asn results in a significant reduction of A$\beta$. (B) Quantitation of total A$\beta$ (A$\beta$40 and A$\beta$42; left panels) and A$\beta$42 secretion (right panels) by a previously described ELISA (*28*, *46*). Bars represent the mean $\pm$ SE of six independent experiments. (C) Overexpression of zf-PS1 Asp374Asn causes the accumulation of C-terminal fragments of $\beta$APP. Cell lysates from HEK293 cells overexpressing Swedish mutant $\beta$APP and zf-PS1 Asp374Asn or HEK 293 cells overexpressing Swedish mutant $\beta$APP and wt zf-PS1 were immunoblotted with antibody 5818 to detect C-terminal fragments of $\beta$APP. In contrast to wt zf-PS1, overexpression of zf-PS1 Asp374Asn results in a significant accumulation of C-terminal $\beta$APP fragments.

In order to study the effect of the aspartate 374 mutation on the amyloidogenic activity of zf-PS1, total A$\beta$ was analyzed in conditioned media from these cell lines. HEK 293 cells expressing wt zf-PS1 or zf-PS1 Asp374Asn were metabolically labeled with [$^{35}$S]methionine. After a 2 h chase, conditioned media were immunoprecipitated with antibody 3927, which detects all species of A$\beta$ (*33*). Interestingly, expression of zf-PS1 Asp374Asn significantly inhibited secretion of A$\beta$ (Figure 6A). Quantitative analysis by our previously described ELISA (*28*, *46*) revealed an approximately 90% inhibition of total A$\beta$ secretion as compared to cell lines expressing wt zf-PS1 (Figure 6B). The aspartate mutation at codon 374 also inhibits A$\beta$42 secretion to a similar extent (Figure 6B). Inhibition of A$\beta$ secretion was accompanied by the accumulation of C-terminal proteolytic fragments of $\beta$APP (Figure 6C), which may suggest a defect in $\gamma$-secretase cleavage of $\beta$APP or a deficiency in transport of $\beta$APP and/or its processing enzymes to the cellular compartments required for proteolytic processing. We point out that wt zf-PS1 and the aspartate mutation displace

endogenous human presenilins (Figures 3 and 5A,B). Since wt zf-PS1 but not zf-PS1 Asp374Asn allows A$\beta$ generation, the effects of the aspartate mutation are not due to the reduction of the human presenilins but rather reflect zf-PS1 activity.

Taken together, these data demonstrate that zf-PS1 exhibits an amyloidogenic function, which promotes $\gamma$-secretase cleavage of $\beta$APP and depends on the presence of the critical aspartate 374 within TM7.

## DISCUSSION

The PS gene family appears to be highly conserved during evolution. PS genes have now been isolated from human, mouse, rat, zebrafish, *Xenopus*, *Drosophila*, and *C. elegans* (*35*−*38*, *50*−*56*). According to the highly conserved hydrophobic domains, which most likely represent the transmembrane regions, all known PS proteins adopt a similar conformation. On the basis of the current model (*6*) PS proteins appear to contain eight transmembrane domains with the N- and C-termini as well as the large loop located within the cytoplasm. The positions of the AD-associated mutations also appear to be largely conserved in all known PS species, which strongly indicates that these pathogenic mutations occur at critical positions. However, we also found sequence variants at the corresponding positions where FAD-related mutations have been identified earlier. These substitutions may be responsible for the very surprising observation that the wt zf-PS1 gene promotes aberrant A$\beta$42 secretion. Exploring the amyloidogenic activity of zf-PS1 may now shed light on the fact that although FAD-associated mutations are scattered along the PS primary amino acid sequence, they all cause a selective increase of A$\beta$42 secretion. Such investigations will contribute to new insights or concepts on PS structure and function relationship. The aberrant amyloidogenic activity of wt zf-PS1 may be caused by changes in folding of the native PS molecule. In that regard it is important to note that we have expressed the zf-PS1 gene in human cells. Due to the different temperature optima of mammals and fishes, it may be possible that aberrant folding of zf-PS1 occurs at 37 °C. This may indicate that rather subtle amino acid changes could cause the enhanced secretion of A$\beta$42 in FAD patients due to structural rearrangements of the PS complex. Our results will help to narrow down such "target amino acids".

Presenilins appear to play an important role in cell fate decisions via Notch signaling during early embryonic development (*10*, *14*−*19*, *57*). Such an essential function of presenilins for embryogenesis is supported by our finding that zf-PS1 is ubiquitously distributed in development and maternally inherited.

Interestingly, proteolytic processing of endogenous PS proteins appears to occur not only in mammals but also in fish. Here, we demonstrate that CTFs derived from zf-PS1 could be detected in fish brain. Moreover, very similar if not identical fragments are generated upon expression of zf-PS1 in human cells. The fish CTFs migrate to a slightly higher molecular weight as compared to the human fragments. However, on the basis of the highly conserved cleavage site (Figure 1) we predict that identical proteolytic fragments are generated in fish tissues. This is also supported by results obtained in mouse tissue, since Thinakaran et al. (*23*) found differences in gel migration of human and mouse PS fragments. Therefore, proteolytic processing of presenilins appears to be conserved during evolution, which might indicate that the cleavage of PS1 is essential for their biological and pathological activity. Such a hypothesis is supported by the findings that PS fragments are the predominant species found in vivo whereas the full-length protein is hardly detectable (*23*, *40*). Moreover, expression of PS proteins is highly controlled by multiple proteolytic systems (*28*). Furthermore, N- and C-terminal fragments of presenilins are known to form a very stable complex (*24*−*28*, *39*), which appears to be required for their biological function in Notch signaling (*14*) as well as for its amyloidogenic function (*28*−*31*). Since the full-length protein is very unstable (refs *25*, *28*, and *40*; Figure 3A) and most of it is rapidly degraded, it seems likely that the biological function of endoproteolysis of PS is required for its conversion into a stable complex (*48*, *28*).

Exchanging aspartate 374 with either asparagine (Figure 5 and 6) or alanine (data not shown) residues blocked endoproteolytic processing of zf-PS1 and severely reduced A$\beta$ secretion, very similar to the ablation of the human PS1 gene (*10*). Therefore, our data indicate that the aspartate 374 mutation is functionally inactive. A loss of function of this mutation is also supported by our previous results showing that transgenic expression of the corresponding human PS mutant (PS1 Asp385Asn) in *C. elegans* failed to rescue the phenotype caused by a defective endogenous PS homologue (*48*). Moreover, these results are consistent with data demonstrating that the corresponding aspartate residues in human PS1 (*12*) and PS2 (*58*) are also required for endoproteolytic processing of presenilins as well as their function in A$\beta$ generation.

Further investigations are required to determine the function of the critical aspartate residue. Mutagenesis of this residue might interfere with the structural integrity of zf-PS1. The mutation might then affect the PS complex and inhibit its potential function in protein transport. Such a scenario is supported by recent data demonstrating that the trafficking of selected proteins, including $\beta$APP, is affected in cells lacking the PS1 gene (*13*). However, it may also be possible that the mutant PS molecules directly affect an essential and evolutionary conserved function of presenilins in $\gamma$-secretase activity as suggested by Wolfe et al. (*12*).

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## REFERENCES

1. Teplow, D. (1998) *Amyloid 5*, 121−142.
2. Mezey, E., Dehejia, A., Harta, G., Papp, M. I., Polymeropoulos, P., and Brownstein, M. J. (1998) *Nat. Med. 4*, 755−757.
3. Georgalis, Y., Starikov, E. B., Lurz, R., Scherzinger, E., Saenger, W., Lehrach, H., and Wanker, E. E. (1998) *Proc. Acad. Natl. Sci. U.S.A. 95*, 6118−6121.
4. Selkoe, D.J. (1996) *J. Biol. Chem. 271*, 18295−18298.
5. Haass, C., and Selkoe, D. J. (1993) *Cell 75*, 1039−1042.

6. Price, D., and Sisodia, S. (1998) *Annu. Rev. Neurosci. 21*, 479−505.
7. Jarret, J. T., and Lansbury, P. T., Jr. (1993) *Cell 73*, 1055−1058.
8. Lemere, C. A., Lopera, F., Kosik, K. S., Lendon, C. L., Ossa, J., Saido, T. C., Yamaguchi, H., Ruiz, A., Martinez, A., Madrigal, L., Hincapie, L., Arango, J. C., Koo, E. H., Goate, A. M., Selkoe, D. J., and Arango, J. C. (1996) *Nat. Med. 2*, 1146−1150.
9. Mann, D. M., Iwatsubo, T., Cairns, N. J., Lantos, P. L., Nochlin, D., Sumi, S. M., Bird, T. D., Poorkaj, P., Hardy, J., Hutton, M., Prihar, G., Crook, R., Rossor, M. N., and Haltia, M. (1996) *Ann. Neurol. 40*, 149−156.
10. De Strooper, B., Saftig, P., Craessaerts, K., Vanderstichele, H., Guhde, G., Annaert, W., Von Figura, K., and Van Leuven, F. (1998) *Nature 391*, 387−390.
11. Haass, C., Selkoe, D. J. (1998) *Nature 391*, 339−340.
12. Wolfe, M. S., Xia, W., Ostaszewski, B. L., Diehl, T. S., Kimberly, W. T., and Selkoe, D. J. (1999) *Nature 398*, 513−517.
13. Naruse, S., Thinakaran, G., Luo, J.−J., Kusiak, J. W., Tomita, T., Iwatsubo, T., Qian, X., Ginty, D. D., Price, D. L., Borchelt, D. R., Wong, P. C., and Sisodia, S. S. (1998) *Neuron 21*, 1213−1221.
14. Baumeister, R., Leimer, U., Zweckbronner, I., Jakubek, C., Gruinberg, J., and Haass, C. (1997) *Genes Funct. 1*, 149−159.
15. Levitan, D., Doyle, T., Brousseau, D., Lee, M., Thinakaran, G., Slunt, H., Sisodia, S., and Greenwald, I. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 14940−14944.
16. Ye, Y., Lukinova, N., and Fortini, M. (1999) *Nature 398*, 525−529.
17. Struhl, G., and Greenwald, I. (1999) *Nature 398*, 522−525.
18. Shen, J., Bronson, R. T., Chen, D. F., Xia, W., Selkoe, D. J., and Tonegawa, S. (1997) *Cell 89*, 629−639.
19. Wong, P. C., Zheng, H., Chen, H., Becher, M. W., Sirinathsinghji, D. J. S., Trumbauer, M. E., Chen, H. Y., Price, D. L., Van der Ploeg, L. H. T., and Sisodia, S. S. (1997) *Nature 387*, 288−292.
20. De Strooper, B., Annaert, W., Cupers, P., Saftig, P., Craessaerts, K., Mumm, J. S., Schroeter, E. H., Schrijvers, V., Wolfe, M. S., Ray, W. J., Goate, A., and Kopan, R. (1999) *Nature 398*, 518−522.
21. Doan, A., Thinakaran, G., Borchelt, D. R., Slunt, H. H., Ratovitsky, T., Podlisny, M., Selkoe, D. J., Seeger, M., Gandy, S. E., Price, D. L., and Sisodia, S.S. (1996) *Neuron 17*, 1023−1030.
22. De Strooper, B., Beullens, M., Contreras, B., Levesque, L., Craessaerts, K., Cordell, B., Moechars, D., Bollen, M., Fraser, P., St. George-Hyslop, P., and van Leuven, F. (1997) *J. Biol. Chem. 272*, 3590−3598.
23. Thinakaran, G., Borchelt, D. R., Lee, M. K., Slunt, H. H., Spitzer, L., Kim, G., Ratovitsky, T., Davenport, F., Nordstedt, C., Seeger, M., Hardy, J., Levey, A. I., Gandy, S. E., Jenkins, N. A., Copeland, N. G., Price, D. L., and Sisodia, S. S. (1996) *Neuron 17*, 181−190.
24. Capell, A., Grünberg, J., Pesold, B., Diehlmann, A., Citron, M., Nixon, R., Beyreuther, K., Selkoe, D. J., and Haass, C. (1998) *J. Biol. Chem. 273*, 3205−3211.
25. Thinakaran, G., Regard, J. B., Bouton, C. M., Harris, C. L., Price, D. L., Borchelt, D. R., and Sisodia, S. S. (1998) *Neurobiol. Dis. 4*, 438−453.
26. Yu, G., Chen, F., Levesque, G., Nishimura, M., Zhang, D. M., Levesque, L., Rogaeva, E. A., Xu, M., Liang, Y., Duthie, M., St. George-Hyslop, P. H., and Fraser, P. E. (1998) *J. Biol. Chem. 273*, 16740−16475.
27. Zhang, Z., Hartmann, H., Do, V. M., Abramowski, D., Strchler-Pierrat, C., Staufenbiel, M., Sommer, B., van de Wetering, M., Clevers, H., Saftig, P., De Strooper, B., and Yankner, B. A. (1998) *Nature 395*, 698−702.
28. Steiner, H., Capell, A., Pesold, B., Citron, M., Kloetzel, P.-M., Selkoe, D. J., Romig, H., Mendla, K., and Haass, C. (1998) *J. Biol. Chem. 273*, 32322−32331.
29. Citron, M., Eckman, C. B., Diehl, T.S., Corcoran, C., Ostazewski, B. L., Xia, W., St. George-Hyslop, P., Younkin, S. G. and Selkoe, D. J. (1998) *Neurobiol. Dis. 5*, 107−116.
30. Tomita, T., Tokuhiro, S., Hashimoto, T., Aiba, K., Saido, T., Maruyama, K., and Iwatsubo, T. (1998) *J. Biol. Chem. 273*, 21153−21160.
31. Saura, C. A., Tomita, T., Davenport, F., Harris, C. L., Iwatsubo, T., and Thinakaran, G. (1999) *J. Biol. Chem. 274*, 13818−13823.
32. Walter, J., Grünberg, J., Capell, A., Pesold, B., Schindzielorz, A., Citron, M., Mendla, K., St. George-Hyslop, P., Multhaup, G., Selkoe, D. J., and Haass, C. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 5349−5354.
33. Wild-Bode, C., Yamazaki, T., Capell, A., Leimer, U., Steiner, H., Ihara, Y., and Haass, C. (1997) *J. Biol. Chem. 272*, 16085−16088.
34. Reifers, F., Böhli, H., Walsh, E. C., Crossley, P. H., Stainier, D. Y. R., and Brand, M. (1998) *Development 125*, 2381−2395.
35. Sherrington, R., Rogaev, E. I., Liang, Y., Rogaeva, E. A., Levesque, G., Ikeda, M., Chi, H., Lin, C., Li, G., Holman, K., Tsuda, T., Mar, L., Foncin, J.-F., Bruni, A. C., Montesi, M. P., Sorbi, S., Rainero, I., Pinessi, L., Nee, L., Chumakov, I., Pollen, D., Brookes, A., Sanseau, P., Polinsky, R. J., Wasco, W., da Silva, H. A. R., Haines, J. L., Pericak-Vance, M. A., Tanzi, R. E., Roses, A. D., Fraser, P. E., Rommens, J. M., and St. George-Hyslop, P. H. (1995) *Nature 375*, 754−760.
36. Levy-Lahad, E., Wasco, W., Poorkaj, P., Romano, D. M., Oshima, J., Pettingell, W. H., Yu, C., Jondro, P. D., Schmidt, S. D., Wang, K., Crowley, A. C., Fu, Y.-H., Guenette, S. Y., Galas, D., Nemens, E., Wijsman, E. M., Bird, T. D., Schellenberg, G. D., and Tanzi, R. E. (1995) *Science* 269, 973−977.
37. Rogaev, E. I., Sherrington, R., Rogaeva, E. A., Levesque, G., Ikeda, M., Liang, Y., Chi, H., Lin, C., Holamn, K., Tsuda, T., Mar, L., Sorbi, S., Nacmias, B., Piacentini, S., Amaducci, L., Chumakkov, I., Cohen, D., Lannfelt, L., Fraser, P. E., Rommens, J. M., and St. George-Hyslop, P. H. (1995) *Nature* 376, 775−778.
38. Tsujimura, A., Yasojima, K., and Hashimoto-Gotoh, T. (1997) *Biochem. Biophys. Res. Commun. 231*, 392−396.
39. Seeger, M., Nordstedt, C., Petanceska, S., Kovacs, D. M., Gouras, G. K., Hahne, S., Fraser, P., Levesque, L., Czernik, A. J., St. George-Hyslop, P., Sisodia, S. S., Thinakaran, G., Tanzi, R., Greengard, P., and Gandy, S. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 5090−5094.
40. Podlisny, M., Citron, M., Amarante, P., Sherrington, R., Xia, W., Zhang, J., Diehl, T., Levesque, G., Fraser, P., Haass, C., Koo, E., Seubert, P., St. George-Hyslop, P., Teplow, D., and Selkoe, D. J. (1997) *Neurobiol. Dis. 3*, 325−337.
41. Shirotani, K., Takahashi, K., Ozawa, K., Kunishita, T., Tabira, T. (1997) *Biochem. Biophys. Res. Commun. 240*, 728−731.
42. Kim, T.-W., Pettingell, W., Hallmark, O., Moir, R., Wasco, W., and Tanzi, R. (1997) *J. Biol. Chem. 272*, 11006−11010.
43. Marambaud, P., Ancolio, K., Lopez-Perez, E., and Checler, F. (1998) *Mol. Med. 4*, 147−157.
44. Citron, M., Westaway, D., Xia, W., Carlson, G., Diehl, T. S., Levesque, G., Johnson-Wood, K., Lee, M., Seubert, P., Davis, A., Kholodenko, D., Motter, R., Sherrington, R., Perry, B., Yao, H., Strome, R., Lieberburg, I., Rommens, J., Kim, S., Schenk, D., Fraser, P., St. George-Hyslop, P., and Selkoe, D. J. (1997) *Nat. Med. 3*, 67−72.
45. Xia, W., Zhang, J., Kholodenko, D., Citron, M., Teplow, D., Haass, C., Seubert, P., Koo, E. H., and Selkoe, D. J. (1997) *J. Biol. Chem. 272*, 7977−7982.
46. Steiner, H., Romig, H., Grim, M. G., Philipp, U., Pesold, B., Citron, M., Baumeister, R., and Haass, C. (1999) *J. Biol. Chem. 274*, 7615−7618.
47. Tomita, T., Maruyama, K., Saido, T. C., Kume, H., Shinozaki, K., Tokuhiro, S., Capell, A., Walter, J., Grünberg, J., Haass, C., Iwatsubo, T., and Obata, K. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 2025−2030.

48. Thinakaran, G., Harris, C. L., Ratovitski, T., Davenport, F., Slunt, H. H., Price, D. L., Borchelt, D. R., and Sisodia, S. S. (1997) *J. Biol. Chem. 272*, 28415−28422.
49. Brockhaus, M., Grünberg, J., Röhrig, S., Loetscher, H., Wittenburg, N., Baumeister, R., Jacobsen, H., and Haass, C. (1998) *NeuroReport 9*, 1481−1486.
50. Taniguchi, T., Hashimoto, T., Taniguchi, R., Shimada, K., Kawamata, T., Yasuda, M., Nakai, M., Terashima, A., Koizumi, T., Maeda, K., and Tanaka, C. (1997) *Gene 186*, 73−75.
51. Takahashi, H., Murayama, M., Takashima, A., Mercken, M., Nakazato, Y., Noguchi, K., and Imahori, K. (1996) *Neurosci. Lett. 206*, 113−116.
52. Tanahashi, H., and Tabira, T. (1998) *Biochim. Biophys. Acta 1396*, 259−262.
53. Takahashi, H., Mercken, M., Nakazato, Y., Noguchi, K., Murayama, M., Imahori, K., and Takshima, A. (1997) *Gene 197*, 383−387.
54. Boulianne, G. L., Livne-Bar, I., Humphreys, J. M., Liang, Y., Lin, C., Rogaev, E., and St. George-Hyslop, P. (1997) *NeuroReport 8*, 1025−1029.
55. Hong, C. S., and Koo, E. H. (1997) *NeuroReport 8*, 665−668.
56. Marfany, G., Del-Favero, J., Valero, R., De Jonghe, C., Woodrow, S., Hendriks, L., van Broeckhoven, C., and Gonzales-Duarte, R. (1998) *J. Neurogenet. 12*, 41−54.
57. Levitan, D., and Greenwald, I. (1995) *Nature 377*, 351−354.
58. Steiner, H., Duff, K., Capell, A., Romig, H., Grim, G., Lincoln, S., Hardy, J., Yu, X., Picciano, M., Fechteler, K., Citron, M., Kopan, R., Pesold, B., Keck, S., Baader, M., Tomita, T., Iwatsubo, T., Baumeister, B., and Haass, C. (1999) *J. Biol. Chem.* (in press).

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