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Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and γ-secretase activity

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Accumulation of the amyloid-β protein (Aβ) in the cerebral cortex is an early and invariant event in the pathogenesis of Alzheimer's disease. The final step in the generation of Aβ from the β-amyloid precursor protein is an apparently intramembranous proteolysis by the elusive γ-secretase(s)[1]. The most common cause of familial Alzheimer's disease is mutation of the genes encoding presenilins 1 and 2, which alters γ-secretase activity to increase the production of the highly amyloidogenic $Aβ_{42}$ isoform[2]. Moreover, deletion of presenilin-1 in mice greatly reduces γ-secretase activity[3], indicating that presenilin-1 mediates most of this proteolytic event. Here we report that mutation of either of two conserved transmembrane (TM) aspartate residues in presenilin-1, Asp 257 (in TM6) and Asp 385 (in TM7), substantially reduces Aβ production and increases the amounts of the carboxy-terminal fragments of β-amyloid precursor protein that are the substrates of γ-secretase. We observed these effects in three different cell lines as well as in cell-free microsomes. Either of the Asp → Ala mutations also prevented the normal endoproteolysis of presenilin-1 in the TM6 → TM7 cytoplasmic loop. In a functional presenilin-1 variant (carrying a deletion in exon 9) that is associated with familial Alzheimer's disease and which does not require this cleavage[4], the Asp 385 → Ala mutation still inhibited γ-secretase activity. Our results indicate that the two transmembrane aspartate residues are critical for both presenilin-1 endoproteolysis and γ-secretase activity, and suggest that presenilin 1 is either a unique diaspartyl cofactor for γ-secretase or is itself γ-secretase, an autoactivated intramembranous aspartyl protease.

The β-amyloid precursor protein (APP) is cleaved by α- and β-secretases (Fig. 1a), causing the release of soluble derivatives of the protein (α-APPs and β-APPs) and the retention of membrane-anchored 83- and 99-amino-acid C-terminal fragments (C83 and C99)[1]. These fragments serve as substrates for γ-secretase, generating the 4K Aβ fragment from C99 and a 3K peptide, p3, from C83 (ref. 5). A limited portion of presenilin-1 (PS1), which has eight transmembrane segments[6] (Fig. 1a), undergoes endoproteolysis within the exon-9 region of the cytosolic loop between TM6 and TM7 (more specifically, within the hydrophobic sequence Thr 291–Ala 299)[7,8], and the resultant N- and C-terminal fragments form 1:1 heterodimers that have a much longer half-life than the holoprotein and are thought to be the biologically active form of PS1 (ref. 9). The

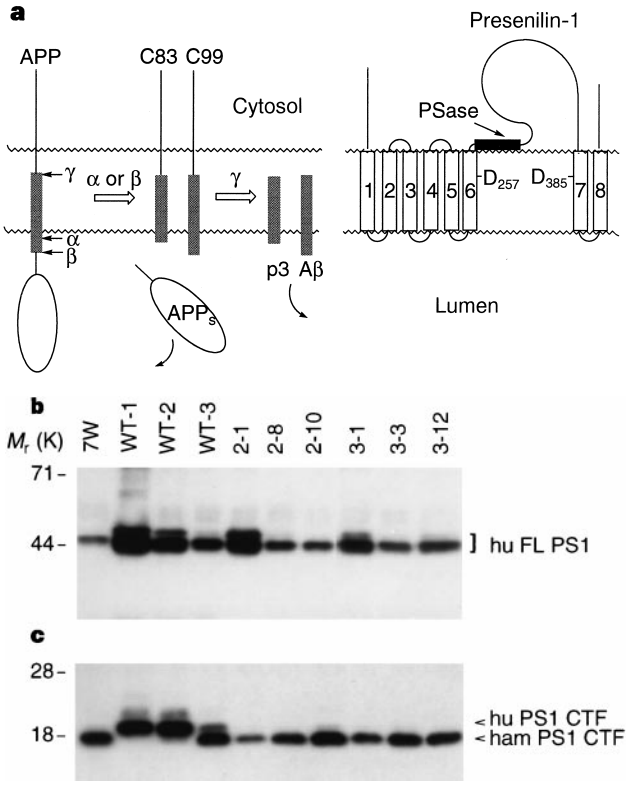

Figure 1 Expression of wild-type and TM Asp → Ala mutant PS1 holoproteins and CTFs in stable transfectants. **a**, Structures of APP (left) and PS1 (right), proteolytic processing sites of APP (cleaved by α, β and γ secretases) and PS1 (cleaved by PSase), and locations of Asp 257 in TM6 and Asp 385 in TM7 of PS1. **b**, PS1 holoproteins (precipitated by antibody 4627 and blotted with 13A11) from CHO cell line 7W stably expressing human APP751 (lane 1), and 7W cell lines stably co-expressing wild-type PS1 (clones WT-1, WT-2 and WT-3 in lanes 2–4), D257A PS1 (clones 2-1, 2-8 and 2-10 in lanes 5–7) or D385A PS1 (clones 3-1, 3-3 and 3-12 in lanes 8–10). Lines with high expression of PS1 characteristically have two holoproteins, both of which have been sequenced and are SDS-stable conformers of full-length (FL) PS1 (ref. 12). **c**, PS1 CTFs immunoprecipitated and blotted as in **b** from the same cell lines. Exogenous human (hu) and endogenous hamster (ham) CTFs are indicated. Replacement of hamster by human CTF is complete in the high-PS1-expressing WT-1 and WT-2 lines, partial[7] in WT-3, and does not occur in any of the six Asp-mutant lines. In **b**, **c**, the migration positions of relative molecular mass markers are indicated on the left.

specific function of the presenilins, including their role in Aβ generation, is unknown. The aspartate residues in TM6 and TM7 are the only two potentially charged residues clearly within the theoretical membrane boundaries of PS1 and PS2 (ref. 2). Where aspartates are found within TM regions of other proteins (for example, adrenergic receptors[10]), they can be critical for function. Thus, the conserved TM aspartates in presenilins may be required for their functions, including their ability to mediate γ-secretase activity.

We constructed plasmids containing wild-type, D257A or D385A human PS1 complementary DNA, and stably transfected Chinese hamster ovary (CHO) cells overexpressing the 751-residue wild-type human APP (APP751) with each PS1 plasmid. The processing of human APP and PS1 in CHO cells has been extensively characterized[11,12]. Using antibody 4627 for immunoprecipitation from cell lysates and antibody 13A11 for western blotting (both raised against the PS1 C-terminal fragment (CTF)), we found that expression of the ~44K wild-type and mutant PS1 holoproteins varied between nine independent stable cell lines (Fig. 1b). Pulse-chase experiments done as controls revealed no difference in the overall metabolic turnover of the mutant as compared to the wild-type holoproteins (both have short half-lives) (results not shown), and membrane fractionation revealed that the subcellular distribution of the holoproteins was normal (see below), indicating that the single Asp → Ala substitutions did not detectably alter the membrane insertion and transport of the holoproteins. Furthermore, the Asp → Ala substitutions did not appear to increase PS1 aggregation, as no high-molecular-weight SDS-stable bands were seen (results not shown).

Consistent with previous evidence that PS1 N- and C-terminal fragments are tightly regulated by competition for limiting cellular factors[13], overexpression of wild-type human PS1 led to the replacement of endogenous hamster CTFs by the human CTFs (Fig. 1c). The extent of replacement depended on the amount of wild-type human PS1 expressed (Fig. 1b, c; lanes 2–4), as reported[7]. In contrast, there was no endoproteolysis of D257A or D385A PS1 to human CTFs (lanes 5–10). Apparently, mutation of either of the two PS1 transmembrane aspartates prevented endoproteolysis in the TM6 → TM7 loop. Endogenous hamster CTFs were not replaced in most of the cell lines expressing mutant human PS1; an exception was the D257A cell line 2-1, the highest expressor of mutant holoprotein (Fig. 1b, lane 5), in which endogenous CTFs were markedly decreased (Fig. 1c, lane 5); the second-highest expressor, the mutant line 3-1, also showed a decrease in endogenous CTFs (Fig. 1c, lane 8).

Cells transfected with wild-type or Asp → Ala mutant PS1 showed comparable expression of the 110K–120K *N*-, and *N* and *O*-glycosylated APP holoproteins (Fig. 2a). Likewise, the amounts of the soluble ectodomain derivatives generated by the β- and α-secretases (β-APP$_s$ and α-APP$_s$) were essentially unchanged (Fig.

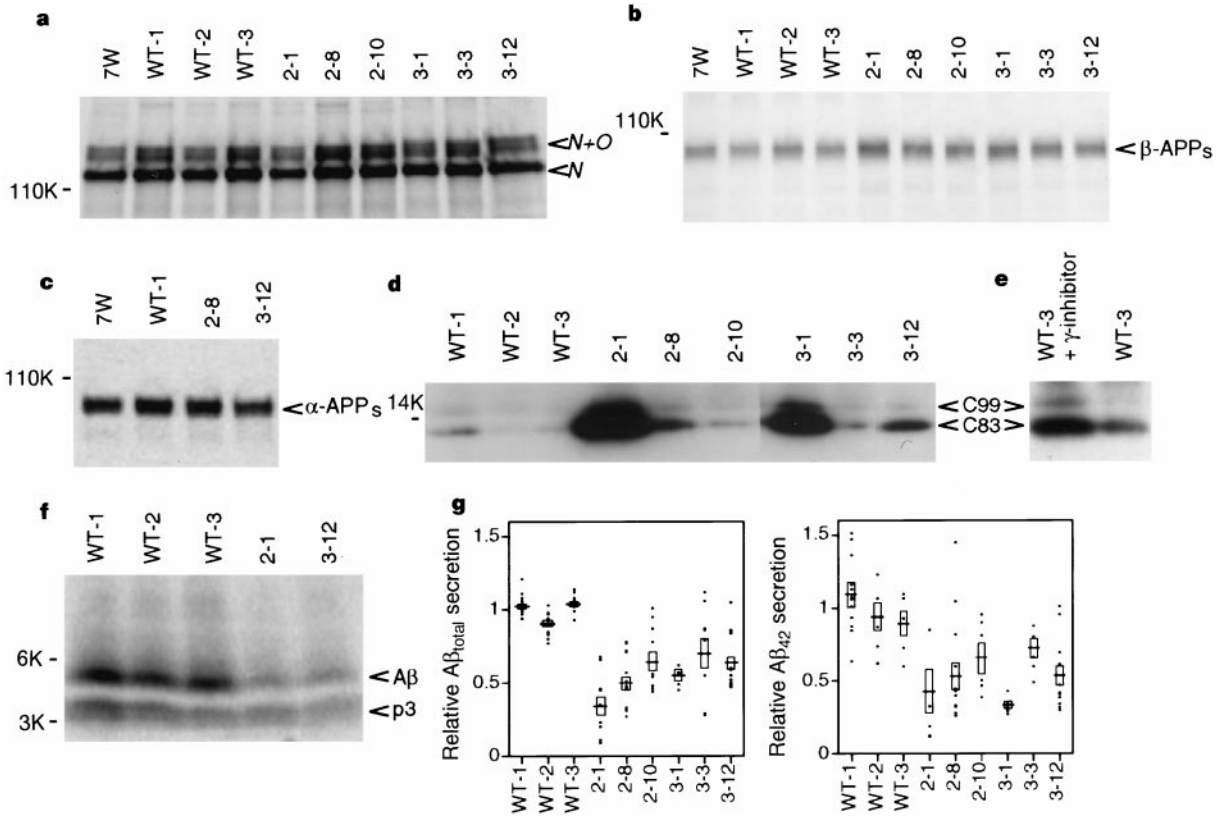

Figure 2 Effect of wild-type and transmembrane Asp → Ala mutant PS1 on APP processing in stable CHO transfectants. **a**, *N*- and *N* + *O*-glycosylated holoAPP metabolically labelled with [^{35}S]Met and precipitated from lysates with antibody C7. Cell lines were as described for Fig. 1b. **b**, β-APP$_s$ precipitated from medium with antibody 192. **c**, α-APP$_s$ precipitated from medium with antibody 1736. The other six lines gave similar results. **d**, C83 and C99 precipitated with antibody C7 were blotted with 13G8; all lines have C83 and C99, although C99 is barely visible in WT-2. C83 and C99 levels from 7W cells (not shown) were comparable to those for WT cell lines. **e**, Line WT-3 was left untreated (right) or treated with a published γ-secretase inhibitor[14] for 4 h at 25 μM (left), and the lysates were precipitated and blotted as for **d**. **f**, Aβ and p3 precipitated from medium of metabolically labelled cells by antibody 1280 against Aβ. The decreases in Aβ in lines 2-1 and 3-12 as shown by phosphorimaging were similar to those quantified by ELISA (**g**); the amount of p3 in these lines was reduced by ~40–50%. **g**, ELISAs for total Aβ and Aβ$_{42}$. Relative amounts of Aβ (dots) were determined by calculating the ratio of the amount of Aβ secreted by each mutant cell line to the mean amount of Aβ secreted by the 3 WT lines in each experiment (normalized to 1). Boxes, s.e.m.; horizontal bars, mean.

2b, c). In contrast, expression of D257A or D385A PS1 resulted in consistently increased amounts of C99 and C83 compared to wild-type PS1 (Fig. 2d). The increase correlated roughly with the level of expression of mutant holoprotein (Fig. 1b); for example, the PS1 D257A-containing cell line 2-1, which expressed the most mutant holoprotein and markedly reduced levels of endogenous PS1 C-terminal fragments (Fig. 1b and c, lane 5), always produced the most C99/C83 (Fig. 2d, lane 4), and cell line 3-1 produced the next highest levels of mutant PS1 and C99/C83 (Fig. 2d, lane 7). The effects of the PS1 mutations on the amounts of C99/C83 produced were similar to those caused by a peptidomimetic γ-secretase inhibitor (Fig. 2e)[14]. Thus, accumulation of C99 and C83 caused by the Asp → Ala mutations results from interference with γ-secretase activity and not from increased α- and β-secretase cleavage of holoAPP, consistent with the results of deleting the PS1 gene[3].

All of the cell lines stably expressing Asp → Ala mutant PS1 secreted substantially less total Aβ (mean 57 ± 3%) and $Aβ_{42}$ (53 ± 4%) than cell lines expressing wild-type PS1 ($P < 0.001$ in each mutant line), as assessed by sandwich ELISA (Fig. 2g). This decrease in Aβ secretion in the mutant PS1-containing cell lines was confirmed by [^{35}S]-methionine labelling and immunoprecipitation of medium with Aβ antibody 1280 (Fig. 2f). In general, the decrease in Aβ secretion among the six stable mutant lines correlated roughly with the amount of expression of the mutant holoprotein, with cell line 2-1 showing the greatest inhibition of total Aβ production (36 ± 9% ($n = 10$) of the mean Aβ production of the three wild-type PS1 cell lines), a reduction approaching that obtained by deleting the *PS1* gene[3]. We assume that the residual Aβ production is due to PS2 (ref. 3). Moreover, the levels of total Aβ (principally $Aβ_{40}$) and $Aβ_{42}$ both fell synchronously as the expression of the Asp → Ala mutant holoproteins rose (compare Figs 2g and 1b), again mimicking the PS1-knockout effect. Thus, these single aspartate point mutations have the same effect on APP processing (inhibiting γ-secretase activity) as deleting the entire *PS1* gene and appear to act as dominant-negative effectors with respect to endogenous PS1. No other PS1 mutation reported to date has these effects, underscoring the critical importance of each of the two TM aspartates for PS1 function.

We assessed the effect of the PS1 Asp → Ala mutations directly on C99 processing by transiently transfecting monkey COS-1 cells with plasmid pCI-C99, encoding C99 fused to the APP signal sequence. Cells transfected with C99 alone contained large amounts of this protein compared with COS-1 cells transfected with APP alone, in which C99 was not detectable (not shown). Co-transfection of C99 with D257A or D385A PS1 substantially increased C99 compared with wild-type PS1 (Fig. 3a), again indicating that elimination of either aspartate protects C99 against catabolism by γ-secretase. Interestingly, blocking γ-secretase activity resulted in accumulation of not only C99 but also of the endogenous C83 substrate of γ-secretase (Fig. 3a). Transfection of either Asp → Ala-mutant PS1 alone (without C99) also gave increased amounts of C83 compared with wild-type PS1 (not shown), indicating that the PS1 mutants block the normal catabolism of endogenous monkey kidney C83. To examine a third cell type, we also transiently transfected the PS1 Asp → Ala mutants into a human kidney 293 cell line stably expressing the K595N/M596L ('Swedish') double mutation of human APP[1]. Again, both D257A and D385A PS1 caused markedly increased C99 and C83 compared with wild-type PS1 (Fig. 3b, lanes 1–4). Thus, the PS1 Asp → Ala mutants interfered with γ-secretase processing of C99 and C83 in cell lines from three different species (including human), and the effect was observed for endogenous and exogenous γ-secretase substrates and in transient as well as multiple stable transfectants. Aβ production in these transiently transfected cells was too variable and too close to the limit of ELISA detection for quantification.

The above results indicate that the two PS1 transmembrane aspartates are essential for PS1 endoproteolysis and γ-secretase activity. However, it was unclear whether the reduced γ-secretase activity resulted solely from eliminating endoproteolysis of the mutant PS1 holoproteins. We therefore introduced the D385A mutation into PS1ΔE9, a functional PS1 variant that lacks exon 9 and does not undergo conventional endoproteolysis[4,7], and transfected the 293 cells expressing APP695 K595N/M596L with this PS1 variant. As expected, the mutant holoproteins were expressed, but PS1 C-terminal fragments were not generated from either PS1ΔE9 or its D385A counterpart (not shown). Expression of PS1ΔE9, a mutation that causes familial Alzheimer's disease, did not alter either C83 or C99 levels (Fig. 3b, lane 5) compared to vector or wild-type PS1 (lanes 1 and 2). However, cells transfected with ΔE9-D385A PS1 accumulated substantially more C83 and C99 than those transfected with PS1ΔE9 (lanes 5 and 6). Thus, this transmembrane aspartate is independently critical for γ-secretase activity even in a PS1 variant that does not require endoproteolysis to function in APP processing.

Because a critical negative charge was lost in each of the Asp → Ala mutations, it was possible that the protein was misfolded (that is, the aspartates serve an important structural, rather than functional, role). We therefore introduced a glutamate mutation into PS1 to conserve the charge (D257E) and transfected this construct into CHO 7W cells expressing APP751 and into HEK 293 cells expressing APP695 K595N/M596L. In each cell line, expression of D257E-mutant PS1 increased C83 and C99 compared with wild-type PS1 (Fig. 3c; only shown for 7W cells), demonstrating that aspartate is specifically required in this position.

We examined the ability of microsomes isolated from selected cell lines to generate Aβ from C99 *in vitro*. Microsomes were prepared from the CHO cells stably expressing wild-type or Asp → Ala mutant PS1. Immunoprecipitation and western blotting with anti-

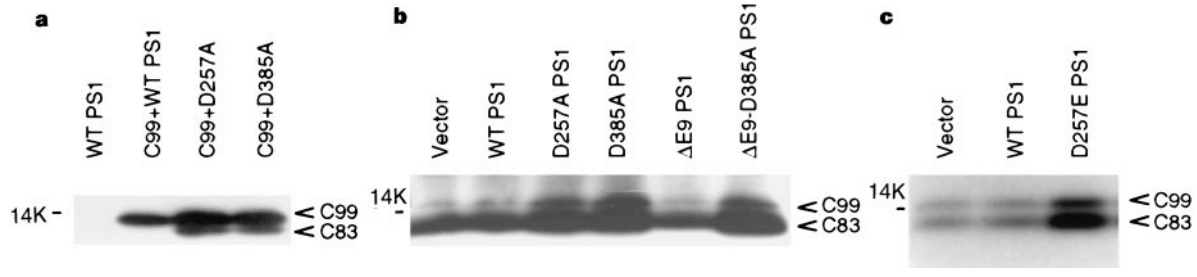

Figure 3 Effect of wild-type and transmembrane Asp → Ala mutant PS1 on APP processing in transient transfectants. **a**, C83 and C99 (precipitated and blotted as for Fig. 2d) from monkey COS-1 cells co-transfected with C99 and either WT or mutant PS1. Note the strong C83 band when Asp-mutant PS1 is expressed. **b**, C99 and C83 from human 293 cells stably transfected with human APP695 K595N/M596L and transiently transfected with vector alone, WT PS1, D257A PS1, D385A PS1, ΔE9 PS1 or ΔE9 D385A PS1. **c**, C99 and C83 from CHO cell line 7W stably transfected with human APP751 and transiently transfected with vector alone, WT PS1, or Asp → Glu (D257E) PS1.

body 4627 showed that the PS1 holoproteins were present in each microsomal preparation but, as observed in whole-cell lysates, microsomes isolated from the cells expressing Asp → Ala mutant PS1 did not have the corresponding CTFs (not shown). The microsomes were added to a cell-free rabbit reticulocyte transcription/translation system containing [^{35}S]Met and pCI-C99, a DNA construct with a T7 promoter suitable for C99 expression in this system. After sufficient time at 30 °C for C99 expression, samples were incubated at 37 °C for 1 h under either neutral or mildly acidic (pH 6.4) conditions. The latter condition was chosen because γ-secretase appears to have some properties of an aspartyl protease[15]. Reactions were quenched, precleared, and immunoprecipitated with Aβ antibody 1280. When the incubation was performed at neutral pH, C99 was well expressed, but virtually no Aβ was detected (Fig. 4a). However, under mildly acidic conditions, a new 4K band which co-migrated with ^{125}I-labelled Aβ appeared only with the microsomes purified from cell lines expressing wild-type PS1 (Fig. 4a). Preabsorption of antibody 1280 with Aβ peptide eliminated the 4K band (not shown).

We also looked for *de novo* Aβ generation from endogenous, full-length APP in microsomes purified from the same cells stably expressing wild-type PS1 (line WT-1) or Asp → Ala mutant PS1 (lines 2-1 and 3-1). The microsomes were incubated for 4 h at 37 °C (or at −80 °C to determine basal Aβ levels), and their contents were then assayed by Aβ ELISA. Microsomes expressing wild-type PS1 produced a 2–3 fold increase (mean: 160 pg ml^{-1}; $n = 3$, $P < 0.05$) in total Aβ over basal levels (mean: 60 pg ml^{-1}; $n = 3$), whereas microsomes expressing Asp → Ala mutant PS1 had undetectable basal Aβ and produced no new Aβ after incubation at 37 °C. Quantitatively similar results were obtained using an Aβ ELISA that specifically detects only Aβ species ending at residue 40, confirming the occurrence of bona fide γ(40)-secretase cleavage of holoAPP in the PS1 wild-type microsomes. These results in isolated microsomes, both with *in vitro*-generated C99 and intrinsic APP, mirror the inhibition of Aβ production observed in our Asp → Ala mutant cells (Fig. 2f, g) and in neurons of PS1-knockout mice[3], and suggest that PS1 plays a direct role in γ-secretase cleavage, rather than influencing the transport of the component proteins of this reaction. In support of this conclusion, the subcellular distribution of holoAPP, C83 and C99 after gradient fractionation of total microsomes was the same in the presence (+/+) as in the absence (−/−) of PS1 in mouse fibroblasts[16], and in the presence of wild-type or Asp → Ala mutant PS1 in CHO cells (not shown). Furthermore, aspartate-mutant PS1 showed the same subcellular distribution as wild-type PS1 in CHO cells stably expressing these proteins (Fig. 4b), providing further evidence against a principal role in protein transport for PS1. There were also no changes in the subcellular distribution of endogenous PS1 C-terminal fragments in cells expressing Asp → Ala mutant PS1 compared with wild-type PS1 (not shown), although the amounts of these endogenous fragments in cells expressing Asp → Ala mutant PS1 were reduced compared to wild-type PS1, as expected (Fig. 1c).

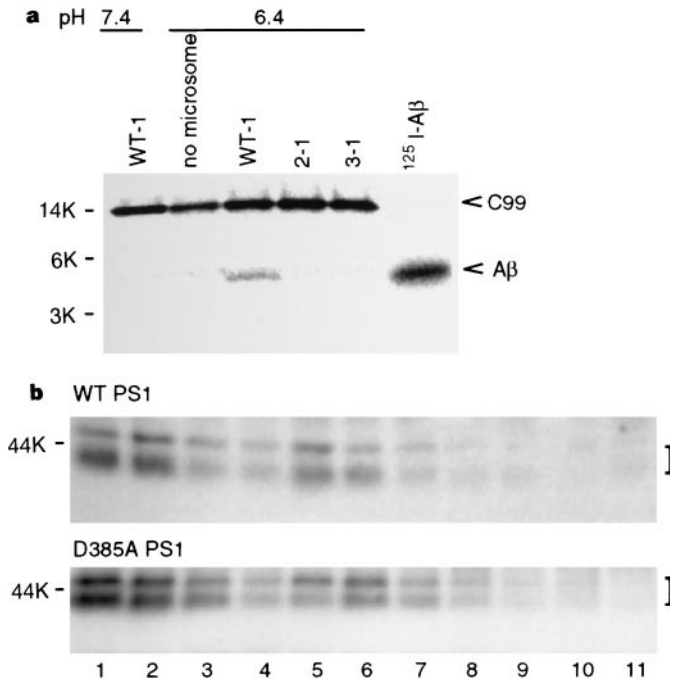

Figure 4 Cell-free generation of Aβ from isolated microsomes, and subcellular distribution of PS1. **a**, Aβ precipitated with antibody 1280 from a rabbit reticulocyte *in vitro* transcription/translation system containing [^{35}S]Met, plasmid pCI-C99 and microsomes isolated from the indicated CHO lines stably expressing WT or Asp → Ala mutant PS1. After protein synthesis, microsome reactions were incubated at 37 °C for 1 h under neutral (pH 7.4) or mildly acidic (pH 6.4) conditions (see Methods). **b**, Subcellular fractionation of microsomes isolated from stable cell lines WT-1 and 3-1 through an iodixanol step gradient[30]. The distribution of the PS1 holoproteins across the 12 fractions is shown by immunoprecipitation and western blotting; no retention or redistribution of the Asp-mutant PS1 was seen.

It is remarkable that two aspartates located in the middle of two adjacent transmembrane domains are independently critical for two separate proteolytic events: the cleavage of PS1 by the unidentified presenilinase and the cleavage of APP C-terminal fragments C99 and C83 by the unidentified γ-secretase. These aspartates are conserved even in *Caenorhabditis elegans* homologues SEL-12, SPE-4 and HOP-1[17,18], indicating that they are functionally important. In support of this idea, the mutation of Asp 385 to Asn eliminates the ability of human PS1 to rescue a lethal egg-laying defect in a *SEL-12* mutant of *C. elegans*[19]. Although most of the more than 50 PS mutations associated with familial Alzheimer's disease lie within transmembrane domains[2], and some of these sites are close to the two aspartate residues, natural mutation of the aspartates themselves has not been found. Such mutations would probably be lethal during embryonic development, as occurs in PS1-knockout mice[20,21] and should not lead to Alzheimer's disease as they cause a marked decrease in production of both $A\beta_{40}$ and $A\beta_{42}$.

What is the role of presenilins, and of these two transmembrane aspartates in particular, in γ-secretase function? Although the presenilins are mostly found in the endoplasmic reticulum and early Golgi[16,22], they are unlikely to be important for protein transport or chaperoning, considering our results with isolated cell-free microsomes and our subcellular fractionation studies. The presenilin aspartates are probably not critical for the translocation of nascent proteins (including APP) as α- and β-secretase processing are normal both in PS1 knock-out mice[3] and in cells expressing Asp → Ala mutant PS1 (Fig. 2). The presenilins may be cofactors for γ-secretase, analogous to the sterol-cleavage activating protein, also a multiple-spanning transmembrane protein found in the endoplasmic reticulum, which regulates the site-1 protease that cleaves the sterol regulatory-element-binding protein[23]. Although there is no precedence for two transmembrane aspartates being essential for a protease regulator, such a role for the presenilins in γ-secretase activity cannot be excluded.

Alternatively, presenilins themselves could be γ-secretases, which have some of the properties of aspartyl proteases[15]. The two transmembrane aspartates are predicted to align with each other (that is, they could associate) and also with the site in the transmembrane domain of APP that is cleaved by γ-secretase (so they could catalyse an intramembrane proteolysis). Our results are consistent with PS1 being an autoactivated aspartyl protease with γ-secretase activity: mutation of either transmembrane aspartate prevents both activities. As substitution of one of these aspartates with glutamate also disrupts γ-secretase activity, the presence of aspartate (not simply its charge) is critical, strongly favouring the

hypothesis that the presenilins are aspartyl proteases themselves and not regulators. Furthermore, Aβ is generated in cell-free microsomes containing wild-type PS1 under mildly acidic conditions; aspartyl proteases require two (and only two) activate-site aspartates and function optimally at acidic pH. The fact that overexpression of wild-type presenilins does not increase Aβ is not inconsistent with the idea that presenilins are γ-secretases, because even overexpressed presenilin is processed to tightly limited amounts of stable amino- and carboxy-terminal fragments, which are probably the active forms of the protein[7–9,13]. On the other hand, presenilin has no amino-acid sequence homology with known (cytosolic) proteases.

If γ-secretases effect intramembrane proteolysis, these enzymes should have several transmembrane regions, as do PS1 and 2, containing residues involved in catalysis and allowing hydrolytic water to enter through an opening formed by association of the TM domains. There is no clear precedent for a protease with a transmembrane active site, although there are several known multispanning transmembrane proteases in the endoplasmic reticulum, including the site-2 protease for the sterol regulatory element-binding protein[24] and the yeast Rce1 and Ste24 proteases[25] which process prenylated proteins with CAAX carboxy termini. Confirmation that presenilins are γ-secretases will require Aβ to be generated by artificial membranes expressing only PS and C99. However, this may be difficult if unknown limiting cellular factor(s) are required for the presenilins to function[13]. But even if the two aspartates function as part of a non-proteolytic co-factor in the presenilinase and γ-secretase cleavages, this will have implications for both the biology of proteases and the treatment of Alzheimer's disease and related disorders involving Aβ accumulation.

Our findings are also important for understanding the role of presenilins in Notch-directed cell-fate decisions during embryonic development. PS1 is part of the signalling pathway from Notch[21,26], a receptor that, upon ligand-induced activation, undergoes proteolytic processing which is strikingly similar to that of APP, including cleavage of a C-terminal fragment of Notch within its putative transmembrane region[27]. PS1 mediates this proteolysis of Notch, which is blocked by several γ-secretase inhibitors[28]. These findings, together with ours, make PS1 a prime candidate for the γ-secretase-like protease that processes Notch. □

Methods

Plasmids and transfections. Full-length wild-type (WT) or ΔE9 mutant PS1 was used as a template to introduce D257A, D385A or D257E by site-directed mutagenesis, and the genes were inserted into vector pCDNA3.1, which contains a zeocin-resistance gene. The identities of the mutant genes were confirmed by DNA sequencing. For transient expression, plasmids (10 μg) were transfected into the cell lines in 10-cm dishes using Lipofectamine. 72 h after transfection, cells were either first labelled with [^{35}S]Met for 1 h or collected and then lysed in buffer containing 50 mM Tris, pH 7.6, 1% NP-40, 2 mg ml^{-1} BSA, 150 mM NaCl, 2 mM EDTA and protease inhibitors. CHO cell lines stably co-expressing APP and mutant PS1 were generated from cell line 7W (ref. 11) (stably transfected with human APP751 and a neomycin-resistance gene) using zeocin and G418 for selection.

Immunoprecipitation, immunoblotting and sandwich ELISA. The PS1, APP, APP$_s$ and Aβ antibodies have all been characterized[5,8]. Cell lysates adjusted to equal protein concentration were precleared with protein A–Sepharose at 4 °C for 30 min and precipitated with primary antibody and protein A–Sepharose at 4 °C for 2 h. Beads were washed twice with 0.2% NP-40 buffer and heated for 5 min at 65 °C in reducing SDS-sample buffer. The precipitated proteins were resolved by SDS–PAGE on 14, 8–16 or 4–20% gels. Polyvinylidene difluoride immunoblots were developed with peroxidase-conjugated secondary antibody and enhanced chemiluminescence (ECL, Amersham). Immunoprecipitates of proteins labelled with [^{35}S]Met were analysed by phosphorimaging. ELISAs for total Aβ and Aβ$_{42}$ were performed as described[29]. Capture antibodies were 266 (to residues 13–28) for total Aβ and 21F12 (to residues 33–42) for Aβ$_{42}$. Reporter antibody was biotinylated 3D6 (to residues 1–5) in each assay.

***In vitro* Aβ generation and subcellular fractionation.** Cell homogenates prepared as described[16] were centrifuged at 3,000*g*, and the supernatant was spun at 100,000*g*. The microsomal pellet was taken up in a rabbit reticulocyte *in vitro* transcription/translation system suitable for transcription of plasmids containing the T7 promoter (Promega), together with [^{35}S]Met, and the volume was adjusted based on the protein content of the 3,000*g* supernatant. After addition of plasmid pCI-C99, samples were incubated at 30 °C for 1.5 h to allow transcription and translation to occur, then incubated at 37 °C for 1 h with or without an equal volume of 100 mM sodium citrate buffer, pH 5.6 (final pH, 6.4), quenched with Tris buffer containing 1% NP-40, precleared, and immunoprecipitated by Aβ antibody 1280. Precipitated proteins were separated by SDS–PAGE on 10–20% gels and analysed by phosphorimaging. Subcellular fractionation was achieved by passing resuspended microsomal pellets through an iodixanol step gradient as described[30].

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