Presenilins mutated at Asp257 or Asp385 restore Pen-2 expression and Nicastrin glycosylation but remain catalytically inactive in the absence of wild type Presenilin.

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Abbreviations: PS: Presenilin; APP: Amyloid Precursor Protein, MEF: mouse embryonic fibroblasts; BN: Blue native.

Abstract

The Presenilins are part of the γ-secretase complex that is involved in the regulated intramembrane proteolysis of Amyloid Precursor Protein and other type I integral membrane proteins. Nicastrin, Pen2 and Aph1 are the other proteins of this complex. The Presenilins contribute likely the catalytic activity to the protease complex. However, several investigators reported normal Aβ-peptide generation in cells expressing Presenilins mutated at the putative catalytic site residue Asp257, contradicting this hypothesis. Because endogenously expressed wild type Presenilin could contribute to residual γ-secretase activity in these experiments, we have reinvestigated the problem by expressing mutated Presenilins in a Presenilin negative cell line. We confirm that Presenilins with mutated Asp residues are catalytically inactive. Unexpectedly, these mutated Presenilins are still partially processed into amino- and carboxyterminal fragments by a “Presenilinase” like activity. They are also able to rescue Pen-2 expression and Nicastrin glycosylation in Presenilin negative cells and become incorporated into large ~440 kDa complexes as assessed by blue native gel electrophoresis. Our study demonstrates that the catalytic activity of Presenilin and its other functions in the generation, stabilization and transport of the γ-secretase complex can be separated, and extend the concept that Presenilins are multifunctional proteins.
Introduction

The γ-secretase cleavage of the Amyloid Precursor Protein (APP) is the final step in the release of the amyloid peptide Aβ, the main constituent of the amyloid plaques in the brain of patients suffering from Alzheimer’s Disease (1). This proteolytic event, thought to occur in the hydrophobic environment of the cell membrane, is mediated by a multimolecular protein complex consisting of Presenilin (PS), Aph-1, Pen-2 and Nicastrin reviewed in (8). How these different proteins precisely interact with each other, how this complex can cleave substrates in their transmembrane domain, and whether additional proteins or lipids are needed for its full activity are questions that remain to be fully addressed.

The Presenilins have been identified some years ago by positional cloning(9). Missense mutations of Presenilin 1 (PS-1) and its homologue Presenilin 2 (PS-2) are the major cause of dominant inherited early onset Alzheimer’s Disease and have been shown invariably to affect γ-secretase processing of APP, increasing the production of the longer, more amyloidogenic Aβ42- species(10-13). Genetic inactivation of PS in mice(14-17), Caenorhabditis elegans(18) and Drosophila melanogaster(19,20) abrogates γ-secretase processing of APP(21,22) and of other substrates like Notch(19,20,23), Erb-B4(24), LRP(25), Nectin 1-alpha(26), CD44(27,28), E-Cadherin(29), Delta and Jagged(30) and others(31). The lethal phenotype of the PS knock out animals can almost entirely be explained by Notch deficiencies, indicating that the major in vivo task of PS during embryogenesis is the regulation of Notch signaling(14-17). Likewise, inactivation of the genes encoding the other members of the γ-secretase complex, at least in Drosophila and C. Elegans, also results in deficiencies in γ-secretase processing of APP, and a Notch like phenotype (2,3,32,33).

While the genetic evidence implying PS in γ-secretase processing is therefore strong, it remains an issue of debate whether PS contains the active, catalytic site of the γ-secretase complex(34) or whether PS have a more general role in trafficking, maturation and/or stabilization of the different components of the γ-secretase complex like Nicastrin (35-39) and Pen2(40), and some other proteins including APP(22), Telencephalin(41) and the nicotinic acetylcholine receptor(42). Cell biological and pharmacological evidence(43-45) is consistent with the hypothesis that two aspartate residues, Asp257 and Asp385, located in putative transmembrane domain 6 and 7, respectively, constitute the core of an aspartyl type catalytic site in PS1 and PS2 (34,46-48). In further support to this hypothesis, a minimal motif around the apartyl residues is conserved in all PS in evolution (49) and in a series of other bacterial(50) and mammalian candidate proteases(51) including the recently discovered signal peptide peptidase(52).

The observation that site directed mutagenesis of either Asp257 or Asp385 towards alanine (and also other amino acid) residues resulted in a dominant negative version of PS was pivotal for this hypothesis(34). These investigators found that strong overexpression of such “aspartate mutants” could suppress the expression of wild type PS, resulting in the complete inactivation of γ-secretase activity. This “replacement phenomenon” of endogenously expressed wild type PS by overexpressed mutant PS is not well understood. Probably competition of endogenously and exogenously expressed PS for “limiting cellular factors”, responsible for the stabilization of newly synthesized PS, could explain this phenomenon(53). It is possible that the recently discovered Aph1, Pen2 and Nicastrin are these limiting factors(5-8). Precisely
because of this “replacement phenomenon” experiments studying overexpressed PS containing Asp mutations (PSD/A) in a wild type background are difficult to interpret. Effects can indeed be due to a loss of catalytic function of the mutated PS itself, but it is as likely that these aspartyl mutations distort the three-dimensional structure of PS and therefore hamper the assembly of a “normal” active γ-secretase complex(4). Indeed, and in general, dominant negative effects of proteins are difficult to interpret mechanistically, opening opportunities for alternative interpretations(54). Using similar approaches for the generation of stable PSD/A overexpressing cell lines, some investigators found that all Aβ generation failed in their cell lines (indicating the total absence of γ-secretase processing activity) (34,47) while others saw no significant effects on Aβ production, in particular with the single Asp257 mutant(55-57). Both groups of investigators claimed the total replacement of endogenously synthesized PS by the mutant PS, making it difficult to explain this discrepancy. While the second series of observations are in essence not compatible with the hypothesis that Asp257 is part of the catalytic site, these results are not conclusive because it is extremely difficult to rule out the possibility that variable, small amounts of endogenously expressed wild type PS confuse the interpretation of the experiments(58). Therefore we decided to reevaluate the effects of the Asp mutations on PS function in a PS deficient cell line (39,59). We also analyzed the role of these aspartyl residues in the proteolytic cleavage of PS itself, the so-called “presenilinase” activity that previously was proposed to be autocatalytic(34) and the incorporation of these mutant PS into the γ-secretase complex.

Methods

Cell culture, generation of stable cell lines and transduction with adenovirus
PS1&2 deficient mouse embryonic fibroblast (MEF) cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Sigma) containing 10% foetal calf serum. At 50% confluency, MEFs were transduced using a replication-defective recombinant retroviral system (Clontech) harboring the respective inserts (human PS1, PS1D257A, PS1D385A and PS1D257/385A). Stable transfected cell lines were selected using 5µg/ml Puromycine (Sigma).

Confluent stable MEF cell lines were transduced with human APP-695 by infection at 50 MOI (multiplicity-of-infection) for 2 hours at 37°C with recombinant adenovirus Ad5/CMV-APP (see below), using a minimal volume of DMEM. After 2 hours, cells were transferred to DMEM containing 1% foetal bovine serum and maintained overnight at 37°C. Conditioned medium then was collected and immunoprecipitated with mab 4G8 (Senetek), recognizing Aβ, and 30µl protein-G-Sepharose (Pharmacia). The bound material was eluted from the beads using Nu-Page sample buffer, boiled for 10 min and proteins were separated on a 12% Nu-PAGE precasted gel (Invitrogen). After transfer, western blot analysis was performed using mab WO2 (1µg/ml)(60), followed by HRP-conjugated secondary antibodies and ECL-detection.
Recombinant adenovirus

Replication-deficient, recombinant adenovirus Ad5/CMV-APP that expresses wild type human APP-695 was prepared by direct ligation as described (61). Briefly, the APP-695 expression cassette, which includes a 5’ CMV immediate-early promoter and a 3’ polyadenylation sequence, was inserted into the E1a genomic region of human type 5 adenovirus (Ad5), and the resulting construct was propagated in permissive (HEK 293) cells. NotchΔE and NICD cDNA was cloned in the pIPspAdapt6 plasmid and transfected with the appropriate cosmid into PerC6/E2A cells, yielding the Ad5/dE1dE2A/CMV NotchΔE virus (62).

Blue native gel electrophoresis:

Blue-Native (BN) PAGE was performed in a mini-gel apparatus (Protean II, Bio-Rad). The 5% to 13.5% polyacrylamide gradient, as well as all buffers used for electrophoresis, were prepared as described by Schägger et al.(63). The cathode buffer consisted of 50mM Tricine, 15mM Bis-Tris/HCl, pH 7.0 and Coomassie Blue G-250(0.01%). The gel buffer contained 150mM Bis-tris/HCl, 1.5M aminocaproic acid, pH 7.0 and the anode Buffer finally contained 50mM Bis-Tris/HCl, pH 7.0 (4°C). Microsomal membranes were solubilized in buffer containing 0.5% dodecyl-maltoside, 20% glycerol and 25mM Bis-Tris-HCl, (pH 7.0) for 30 min on ice. After ultracentrifugation (100,000 g, 30 min), the protein concentration of the supernatant was measured (protein assay of BioRad). Finally, extracts were aliquoted and stored at −80°C until use. For BN-Page, 30µl extract was mixed with 3µl of the following buffer: 5% Coomassie brilliant blue G250, 200mM Bis-Tris-HCl, 1M 6-amino caproic-acid, pH 7.0 and 30% sucrose. 70µg of total protein extract was loaded in each lane. BN-Page was performed at 4-6°C. After an initial run at 80V to allow the protein sample to run into the stacking gel, electrophoresis was continued at 200V for 1 hour. Next, the Coomassie brilliant blue G250 containing cathode buffer was replaced by a buffer without the dye and run for another 1.5 hrs. The gel was incubated in 0.1% SDS, 25mM Tris, 192mM glycine,20% methanol for 10 min at room temperature and transferred on polyvinylidifluoride membranes. The blot was de-stained for 1 hour in distilled water/methanol/acetic acid (60/30/10) and reacted with the appropriate antibodies.

Antibodies:

Polyclonal antibodies directed against human PS1 (B14.5 and SB129) and Nicastrin (B59.4) have been described (39,64),(65). For the detection of the additional γ-secretase components, polyclonal antibodies were generated in New Zealand white rabbits. MNLERVSNEEKLNLC in the C-terminus of Pen-2 and CSRVMVYSAALRIPPED in the C-terminus of the long splice variant of Aph1a were coupled to keyhole limpet hemocyanin (Pierce Imject). All polyclonal antibodies were affinity purified against the peptide antigen immobilized on a NHS-sepharose matrix according to the manufacturer’s instructions (Amersham Pharmacia). Antibody SZ 909 was raised against PALIYST in PS1 and kindly provided by Dr. Helmut Jacobsen (Hoffmann La Roche, Basel, Switzerland). Polyclonal 4627 recognizing the amino acids 457-467 of PS1 was kindly provided by Dr. Dennis Selkoe (Harvard, USA). “Cleaved Notch1 (Val 1744) antibody”, specific for the γ-secretase cleaved Notch intracellular domain (NICD) fragment was purchased from Cell Signaling Technology (USA).
Cell free assay:
For the cell free assay, MEFs were harvested and centrifuged. The cell pellet was resuspended in 250mM sucrose, 5 mM Tris-HCl (pH 7.4) and 1 mM EGTA supplemented with protease inhibitors and homogenized using a ball-bearing cell cracker (10 passages, clearance 10µm). After low-speed centrifugation (800g, 10 minutes), the post nuclear supernatant was ultracentrifuged (100,000 g, 1 hour). The resulting microsomal pellet was washed twice in 0.02% saponin, resuspended in 5mM Tris-1mM EDTA (pH 7) containing 0.5% CHAPS, and incubated for 1hr at 4°C. Next, cleared extracts (100,000 g, 1 hr) were incubated overnight (37°C) with recombinant flag-tagged APP C100 (66). Finally, de novo formed Aβ was analyzed by SDS-PAGE on 10% Bis-Tris NuPAGE gels (Invitrogen) in MES running buffer followed by Western blotting and ECL-detection.

Results and discussion
We stably transduced PS1&2 deficient MEFs (PS-/-)(39) with wild type human PS1 (HuPS1), and with HuPS1 containing Asp to Ala mutations at position Asp257 (PS1/D257A), at position Asp385 (PS1/D385) or at both positions together (PS1/D257/385A). Transduced wild type HuPS1 was expressed and processed into amino-terminal (NTF) to the same extent as endogenously expressed mouse PS1 (MoPS1) in wild type fibroblasts (fig 1, upper row). Antibody SB129 recognizes human and mouse PS1 full length and PS1-NTF fragments equally well. Also Nicastrin glycosylation (fig 1) and γ-cleavage of APP (see below, fig 4 and 5) were restored in the PS-/- cells, indicating that the expression levels of HuPS1 in the reconstituted fibroblasts were physiological relevant.

Similar levels of expression were obtained with HuPS1 containing the different Asp mutations (PS1/D/A, fig 1, three last lanes). All three Asp mutants displayed also partial proteolytic processing into NTF and CTF fragments, albeit less efficiently and to a much more variable extent than wild type HuPS1 (Fig 2B and quantitated in C). The fact however that they can be cleaved in a PS negative background is surprising since it is believed that Presenilins are cleaved by an autocatalytic process depending on these aspartate residues (7,34). One possibility is that the PS1D/A proteins are cleaved by caspases (67,68). However the caspase cleaved CTF fragments of both HuPS1 and PS1D/A are much smaller than the fragments observed here (result not shown). This was further ruled out since antibody Pep35.2 against amino acids 310-331 that is aminoterminal to the caspase cleavage site in the PS1 loop, reacts with the PS1-CTFs (fig 2, panel Bc). This experiment also confirmed that the observed cleavage occurs closely to the region where “Presenilinase” cleaves PS1 (69). The “Presenilinase” like activity therefore identified in the current experiments is apparently not autocatalytic since cleaving the PS1D/A proteins. It should be noticed that the “Presenilinase” cleavage site displays heterogeneity and that it is not proven that all cleavages are performed by one and the same protease (69). One of the major cleavage sites identified occurs carboxyterminal to the P284ALYYSST291 sequence. We obtained antibody SZ909 that reacts with full length PS1 and PS1-NTF that are
specifically cleaved at this cleavage site (Fig 2Bb). In our cell culture conditions only a marginal fraction of the total pool of PS1-NTF is reacting with this antibody, suggesting that the major “Presenilinase-like” processing occurs at the other identified positions (M298 and A299,(69)).

PS deficiency also causes impaired glycosylation of Nicastrin (fig 1, see also (35,36,38,39), and destabilizes Pen-2 expression (fig 1)(70). The expression of another γ-secretase component, Aph1a, is not affected by the absence of PS expression (Fig1). Interestingly, HuPS1/D257A, HuPS1/D385A and HuPS1/D257/385A, were all able to reconstitute Nicastrin maturation and Pen-2 expression to a similar extent as wild type HuPS1, indicating that these Asp mutations do not interfere with the roles of PS in the transport of Nicastrin through the Endoplasmic Reticulum and the Golgi complex, and the stabilization of Pen-2.

As discussed above, some authors have suggested that the Asp mutations affect the three-dimensional structure of PS1, thereby interfering with the normal incorporation of PS1D/A into the γ-secretase complex. We investigated this problem using blue-native gel electrophoresis. In these non-denaturing gels PS1 migrates at ~440 kDa because it is non-covalently bound to other proteins like Nicastrin and Pen-2 (fig 3, lane 2). Interestingly, similar complexes are observed in PS-/- fibroblasts expressing PS1D385A or PS1D257/385A, indicating that the PS1D/A mutants become incorporated into a complex together with Nicastrin and Pen-2 (Fig 3). In PS-/- fibroblasts, no ~440 kDa complex is observed, and Nicastrin and Pen-2 migrate at a lower apparent Mr. Thus, the incorporation of both proteins into the ~440 kDa complex depends on PS. Interestingly Pen-2 (that is expressed at significantly lower levels in PS-/- cells, see also fig 1) is distributed over two Mr fractions (fig 3).

Overall, it is clear from these experiments that the D/A mutations do not affect the incorporation of PS1 into the fully mature complex. Previously, Yu et al investigated the incorporation of PS1D/A mutants in high Mr complexes using glycerol gradients (4). They found that PS1D/A is not incorporated into such complexes in contrast to their wild type counterpart. However, it is very well possible that under the overexpression conditions used in those experiments, the other protein partners of the γ-secretase complex became rate limiting. This increases the chance that only partial complexes are generated, providing an explanation for the discrepancy between these previous findings and those presented here. In any event it is clear from our data that the Asp mutations do not necessarily affect the interaction of PS1 with the other members of the γ-secretase complex, in line also with the results displayed in figure 1.

Since under our experimental conditions the PS1D/A mutants are incorporated into the fully mature γ-secretase complex, we found it important to reinvestigate the effects on γ-secretase activity and thus cleavage of APP, especially given the existing controversy in the literature concerning the catalytic activity of these mutants. As shown in fig 4, APP-CTF fragments generated from endogenously expressed mouse APP clearly accumulate in PS-/- cells, while expression of HuPS1 restores the level of APP-CTF to the ones observed in wild type fibroblasts. None of the PS1D/A mutants were active in this assay and APP-CTF fragments accumulated to a similar extent (between 2.3 and 4.0 fold compared to wild type cells) as in the PS-/- cells (3.3 fold compared to wild type cells). It should be noticed that the levels of endogenously produced murine Aβ are at the detection limits of our assays (59). We therefore also analyzed processing of human APP transduced in the cells using adenoviral vectors.
A clear accumulation of (human) APP-CTF was again observed in the PS-/− fibroblasts, which was reversed in the wild type HuPS1, but not in the HuPS1D/A expressing cells (fig 5A). As shown in the same panel, wild type fibroblasts generated substantial amounts of Aβ under those conditions while PS-/− cells did not produce any detectable Aβ (59). Expression of HuPS1, but not HuPS1D/A, in these cells restored completely Aβ generation. We also investigated the activity of the γ-secretase complexes under cell free conditions. This assay allows to measure not only newly generated Aβ but also the cytoplasmic fragment of APP (APP intracellular domain or AICD). As demonstrated in fig 5B, none of the PS1D/A mutants was able to cleave the APP substrate, providing further evidence that the D/A mutations in PS1 inactivate the catalytic site in the γ-secretase complex.

We finally analyzed processing of another γ-secretase substrate, NotchΔE (23). Again, wild type human PS1 was able to restore processing in PS deficient cells, while the aspartate mutants were not active (Fig 5C).

Since the Ala mutations at positions Asp257 and Asp385 do not affect the incorporation of PS1 into the mature γ-secretase complex and since Pen-2 protein expression and Nicastrin glycosylation can be completely reconstituted with these mutants, we conclude that the Asp mutations do not alter significantly the overall structure of the PS protein. Therefore these mutations affect very specifically the catalytic activity of the complex, strongly agreeing with the interpretation that these two aspartate residues constitute indeed the catalytic site of the γ-secretase complex. The ongoing controversy in the field is based on the observation that the Asp257 mutation in PS1 did not significantly affect Aβ generation in certain cell lines (see introduction). In contrast to these previous experiments however, potential incomplete “replacement” of endogenous PS does not complicate the interpretation of the current results. It is possible that in previous experiments using transfected PS1D/A, small amounts of endogenously expressed wild type PS1 and/or PS2 have escaped detection in the western blot techniques used to confirm replacement. A low level of residual γ-secretase activity in these cells could indeed lead to an accumulation of APP-CTF substrate as observed by these investigators. Aβ will however still be generated at a lower rate, and it is possible that in the time course of the experiment a steady state level is reached in the medium that is comparable with the one reached in the wild type cells. While Notch processing was invariably affected in these experiments, the time course of the experiments analyzing Notch processing and APP processing are quite different. It is therefore plausible that clear deficits in Notch processing are observed while the deficits in APP processing are much less obvious.

We conclude that the Asp257 and Asp385 are indeed part of the catalytic site in PS1 as initially suggested by Wolfe et al. (34). However, and in disagreement with these authors, our experiments suggest that the proteolytic maturation of PS is not always dependent on the γ-secretase activity of PS1. Indeed, the catalytically dead PS1D/A mutants, when expressed at physiological relevant levels in a PS-/− background, are still processed by an elusive “Presenilinase” (fig 1 and 2). While several authors found that γ-secretase inhibitors inhibit “Presenilinase” activity (71,72), their inhibitory profiles are not entirely overlapping, suggesting that “Presenilinase” and “γ-secretase” are pharmacologically different activities. For instance pepstatin A is a good inhibitor of “Presenilinase”, while it is only in very high concentrations also active as a γ-secretase inhibitor (72). Moreover, the cleavages at the Presenilinase site
in PS1 are heterogeneous (69), implying that further work is needed to understand fully the exact molecular processes underlying the proteolytic maturation of Presenilin.

The most important implication from our work is probably that the assembly, activity and biology of the Presenilins (and the other components of the γ-secretase complex) have to be investigated at physiological relevant expression levels. Not only the subcellular localization (73), but also the interaction with the different γ-secretase components (this manuscript), the processing by Presenilinase (this manuscript), and the glycosylation of Nicastrin(39), are all heavily affected by the overexpression of Presenilin or one of the other proteins of the complex. Therefore if one wants to make reliable conclusions concerning the physiology of γ-secretase, it is needed to remain as close as possible to physiological relevant levels of expression.

Finally, our experiments confirm that deficiency of PS has important repercussions for the stability of other components of the γ-secretase complex. This suggests strongly an important role for PS in stabilization and trafficking of these proteins. Apparently this function of PS1 can be dissociated from its role in the catalytic cleavage of integral membrane proteins, indicating that PS are multifunctional proteins.

Figure legends

Figure 1: PS with Asp mutants are proteolytically processed and can rescue Nicastrin glycosylation and Pen 2 expression in PS deficient fibroblasts

Mouse embryonic fibroblasts (MEF) deficient in Presenilin expression (PS-/-) were stably transduced with wild type human PS1 (HuPS1) or with HuPS1 in which Asp257, Asp 385 or both were replaced with an alanine (PS1D257A, PS1D385A and PS1D257/385A respectively). 40µg membrane protein extracts were applied on 10% Nu-Page and analyzed by western blotting for the presence of the indicated proteins. Following antibodies were used: SB129 (Full length human and mouse PS (FL PS1) and their aminoterminal fragments (NTF PS1)); B59.4 (Mature and immature Nicastrin); B80.2 (Aph1a); B95.2 (Pen-2); Monoclonal anti β-actin as a loading control.

Figure 2: PS with Asp mutants are cleaved by a “Presenilinase” like protease at a similar position as wild type PS1.

Panel A: Schematic drawing of PS1 indicating the epitopes recognized by the different antibodies used in this study. The approximate position of the Presenilinase cleavage sites (indicated by an arrow) in the hydrophilic loop close to transmembrane domain 6 is indicated. The approximate position of the caspase cleavage site is indicated by an arrowhead.

Panel B: Mouse embryonic fibroblasts (MEF) deficient in Presenilin expression (PS-/-) were stably transduced with wild type human PS1 (HuPS1) or with HuPS1 in which Asp257, Asp385 or both were replaced with an alanine (PS1D257A,
PS1D385A and PSAD257/385A respectively). 60µg membrane protein extracts were applied on 10% Nu-Page and analyzed by western blotting. Following antibodies were used: (a) B14.5 recognizing full length human PS1 (FL-HuPS1) and the aminoterminal fragments (NTF-HuPS1); (b) SZ909 directed against the P284ALIYSST291 sequence just aminoterminal to one of the major Presenilinase cleavage sites of PS1. This antibody reacts with full length PS1 and with stronger affinity with PS1-NTF containing a free PALIYSST sequence. The low amount of NTF fragments detected here indicates that “Presenilinase” processing in this experiment does not occur at this site; (c) pep3 5.2 monoclonal antibody against Human PS1 S310-D331); and (d) 4627 recognizing amino acids M457-I467 at the carboxyterminus of PS1 (CTF-HuPS1).

Panel C: Aminoterminal fragments (NTF-HuPS1) and full length PS1 (FL-HuPS1) were quantitated by densitometric scanning using a KODAK imager. The density of the NTF-bands is given relative to the total density of NTF and FL-HuPS1. The mean and standard error of 7 independent experiments is indicated.

Figure 3: PS with Asp mutants are incorporated in 440 kDa complexes.

70 µg membrane extracts of mouse embryonic fibroblasts were electrophoresed in a blue native gel (5-13.5%). Material was transferred to a pvdf membrane and probed with the indicated antibodies (see fig 1). No complex is generated in PS-/- cells. Molecular weight markers are indicated on the left, Ferritin (440kDa and 880kDa) and β-amylase (200 kDa).

Figure 4: Processing of endogenously expressed APP

Wild type mouse embryonic fibroblasts (MEFwt), or PS deficient fibroblasts (PS-/-), or PS deficient fibroblasts stably transfected with human PS1 (wt or containing the indicated Asp mutations), were analyzed by SDS Page (Nu page 10%) and western blotting. Mouse full length APP (FL-APP) and APP carboxyterminal fragments (APP-CTFs) were detected using antibody B10.4 recognizing the carboxyterminus of APP. Secreted APPs was detected in the medium using mAb22C11 recognizing the aminotermus of APP.
Figure 5: Absence of γ-secretase activity

Panel A: Fibroblasts as in fig 4 were transduced with adenovirus driving expression of human APP 695. Membrane extracts were analyzed as in fig 4. APP full length (FL-APP) and carboxyterminal fragments (APP-CTFs) were detected using antibody B10.4 recognizing the carboxyterminus of APP. Aβ-peptide from the conditioned media was immune precipitated using mAb 4G8 and detected in Western blotting with mAb WO-2. In the first and the third lane no adenovirus was added, and therefore only endogenously expressed mouse APP is detected.

Panel B: Membranes prepared from fibroblasts as indicated were solubilized in 0.5% CHAPS and APP substrate was added. After overnight incubation material was analyzed in a 12% Nu-Page and probed with Mab WO-2. Carboxyterminal fragment was analyzed using mAb M2 against the Flag tag incorporated in the synthetic APP substrate.

Panel C: Fibroblasts as indicated were transduced with adenovirus driving the expression of NotchDeltaE or Notch intracellular domain (NICD). Cell extracts were analyzed by Nu-PAGE(7% Tris Acetate) and NotchΔE or NICD was detected using respectively mAb recognizing the myc-tag (upper panel) and Cleaved Notch1(Val 1744) antibody (lower panel). This antibody only recognizes the NICD fragment after proteolytic cleavage by γ-secretase.

Acknowledgements

Research in the laboratory was supported by the Alzheimer’s Association (Pioneer award to BDS), the FWO, the VIB, the K.U.Leuven, the European union (DIADEM-QLK3-CT-2001-02362) and the federal office for scientific, technical and cultural affairs, Belgium ((IUAP P5/19). We sincerely thank Dr. D. Selkoe, Dr. R. Jacobsen, and Dr. B. Cordell for antibodies, Dr. R. Kopan for the Notch constructs and members of the lab at Galapagos NV for the generation of the NotchΔE adenovirus.
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Figure 1

Nyabi et al./ Fig1.
A. 

B. 

C. 

Nyabi et al./Fig.2
Figure 3

Figure 4

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J. Biol. Chem. published online July 28, 2003

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