Published in final edited form as:

Biochemistry. 2011 October 25; 50(42): 9023–9035. doi:10.1021/bi2007146.

Dissociation between Processivity and Total Activity of γ -Secretase: Implications for the Mechanism of Alzheimer-Causing Presenilin Mutations†

Omar Quintero-Monzon[‡], Morgan M. Martin[‡], Marty A. Fernandez, Christina A. Cappello, Amanda J. Krzysiak, Pamela Osenkowski, and Michael S. Wolfe^{*}

Center for Neurologic Diseases, Harvard Medical School and Brigham and Women's Hospital, 77 Avenue Louis Pasteur, Boston, Massachusetts, 02115, USA

Abstract

The amyloid β -peptide ($A\beta$), strongly implicated in the pathogenesis of Alzheimer's disease (AD), is produced from the amyloid β -protein precursor (APP) through consecutive proteolysis by β - and γ -secretases. The latter protease contains presenilin as the catalytic component of a membrane-embedded aspartyl protease complex. Missense mutations in presenilin are associated with early-onset familial AD, and these mutations generally both decrease $A\beta$ production and increase the proportion of the aggregation-prone 42-residue form ($A\beta_{42}$) over the 40-residue form ($A\beta_{40}$). The connection between these two effects is not understood. Besides $A\beta_{40}$ and $A\beta_{42}$, γ -secretase produces a range of $A\beta$ peptides, the result of initial cutting at the ϵ site to form $A\beta_{48}$ or $A\beta_{49}$ and subsequent trimming every 3–4 residues. Thus, γ -secretase displays both overall proteolytic activity (ϵ cutting) and processivity (trimming) toward its substrate APP. Here we tested whether a decrease in total activity correlates with decreased processivity using wild type and AD-mutant presenilin-containing protease complexes. Changes in pH, temperature and salt concentration that reduced overall activity of the wild type enzyme did not consistently result in increased proportions of longer $A\beta$ peptides. Low salt concentrations and acidic pH were notable exceptions that subtly alter the proportion of individual $A\beta$ peptides, suggesting that the charged state of certain residues may influence processivity. Five different AD-mutant complexes, representing a broad range of effects on overall activity, $A\beta_{42}$ -to- $A\beta_{40}$ ratios, and ages of disease onset were also tested, revealing again that changes in total activity and processivity can be dissociated. Factors that control initial proteolysis of APP at the ϵ site apparently differ significantly from factors affecting subsequent trimming and the distribution of $A\beta$ peptides.

The 4 kDa amyloid β -peptide ($A\beta$) is the principle protein component of the cerebral plaques found in Alzheimer's disease (AD) (1, 2). This peptide is produced by successive proteolysis of the type I integral membrane amyloid β -protein precursor (APP), first by β -secretase (3), which releases the luminal/extracellular ectodomain, and then by γ -secretase (4), which cleaves within the transmembrane region of the membrane-bound remnant (C99). Heterogeneous proteolysis of C99 by γ -secretase results in a range of $A\beta$ peptides varying at

†This work was supported by a grant to M.S.W. from the National Institutes of Health (R01 AG17575 and a Minority Supplement to this grant) and a fellowship to M.A.F. from the Howard Hughes Medical Institute.

*To whom correspondence should be addressed: Center for Neurologic Diseases, Brigham and Women's Hospital and Harvard Medical School, 77 Avenue Louis Pasteur, H.I.M. 754, Boston MA 02115; Tel: (617)525-5511; Fax: (617)525-5252; mwolfe@rics.bwh.harvard.edu.

‡These authors contributed equally to this work

SUPPORTING INFORMATION

Supplemental figures S1–S3 show pH overlap results as well as full $A\beta$ profiles under various conditions or with different PS1 mutants including error bars. This material is available free of charge via the internet at <http://pubs.acs.org>.

their C-termini. As the C-terminus is derived from the transmembrane domain, longer A β peptides contain more hydrophobic residues and are more prone to aggregation (5). Soluble aggregates of A β are neurotoxic and impair synapse function, long-term potentiation and memory in rodent brains (6–8). Dominant missense mutations in APP and presenilin, the catalytic component of γ -secretase, cause familial AD (FAD) in midlife and alter A β production, increasing either total A β or the proportion of 42-residue A β species (A β 42) over the otherwise predominant 40-residue form (A β 40) (9). Thus, although A β is normally produced in the brains of all individuals, aggregation of slightly longer forms, particularly the 42-residue form (A β 42), are thought to initiate AD pathogenesis. Therefore, a full understanding of how these longer A β peptides are produced by γ -secretase is critical.

γ -Secretase is a complex of four integral membrane proteins (10–13): presenilin (PS), nicastrin (NCT), anterior pharynx-defective 1 (Aph-1) and presenilin enhancer 2 (Pen-2), with presenilin containing a membrane-embedded aspartyl protease active site (14–16). Assembly of the complex in the endoplasmic reticulum is followed by trafficking to the Golgi (17), endoproteolysis of presenilin into N-terminal fragment (NTF) and C-terminal fragment (CTF) subunits (18–20), and complex N- and O-glycosylation of nicastrin (21–25). The NTF and CTF subunits of presenilin each possess one of the catalytic aspartates (14); to gain access to the active site, the APP-derived substrate C99 apparently first docks on the outer surface of presenilin, followed by entry into the internal active site (26).

Experimental evidence supports a model whereby C99 is processively cleaved by γ -secretase. Initial proteolysis at the ϵ site primarily produces the 50-residue APP intracellular domain (AICD) (27) and A β 49 (28); however, γ -secretase can also cut at an alternative ϵ site nearby to produce a 51-residue AICD (29) and A β 48 (28, 30). After the ϵ cut, A β 48 and A β 49 are trimmed every 3–4 residues, creating the C-termini of the other A β peptides, ranging from 46 to 38 residues long (30). The evidence for γ -secretase trimming, or processivity, is threefold. First, longer AICDs that would complement the shorter A β peptides from single cleavage events have not been detected. Second, the A β peptides created by ϵ cleavage, A β 48 and A β 49, can be processed by γ -secretase into shorter A β peptides, including A β 40 and A β 42 (31). Third, Takami *et al.* recently identified tri- and tetrapeptides produced by γ -secretase cleavage of APP which correspond to the predicted products of processive trimming (32).

The ϵ cut and subsequent trimming are accomplished by a single active site: one of each component of the complex is sufficient for γ -secretase activity (i.e., each complex contains one presenilin and therefore one active site) (33). Otherwise, the relationship between ϵ cleavage and trimming is unclear. For instance, do conditions and mutations that affect one type of activity impact the other? FAD mutations in presenilin-1 and -2 (PS1 and PS2) result in an increase in the A β 42:A β 40 ratio, which can be considered a “gain-of-function” (34–37). Conversely, these same mutations also tend to lower total γ -secretase activity (38), leading others to conclude that presenilin FAD mutations cause a “loss-of-function” (39). Wolfe and De Strooper have previously proposed independently that perhaps these two ideas can be reconciled; slowing of the protease may be mechanistically linked to a shift in the A β 42:40 ratio, leading to both a reduction of overall activity as well as a gain in the proportion of toxic A β species (40, 41). That is, a less active protease may lead to slower trimming, with more opportunity for longer forms of A β to be released from the enzyme before further trimming.

Here we report testing this hypothesis by altering the *in vitro* conditions (pH, salt concentration, and temperature) of the wild-type protease and testing the effects of a variety of FAD mutants on overall activity and the proportion of the various A β peptides produced. We conclude from these investigations that activity and processivity can be dissociated. A

shift in the proportion of A β peptides is not necessarily the result of reduced γ -secretase activity, and reduced activity is not necessarily accompanied by a change in the distribution of A β peptides. That is, factors that affect overall activity differ from factors that affect trimming to alter the proportion of A β peptides.

EXPERIMENTAL PROCEDURES

Plasmids and Cell Lines

The pAph1HA plasmid carrying the wild-type Aph-1 α 2 coding sequence with a C-terminal hemagglutinin (HA) tag was previously described (12). FAD mutations were introduced into wild-type human PS1 using QuikChange mutagenesis (L166P and G384A, Stratagene, Agilent Technologies, Wilmington, DE), were a generous gift from the laboratory of Dr. Dennis Selkoe (A246E), or have previously been described (L286V and Δ E9 (35)). The coding sequences were then PCR amplified and subcloned into pIRESpuro3 (Clontech, Mountain View, CA) with EcoRV and EcoRI. The gene for the FLAG-tagged APP C-terminal fragment C99, was subcloned from pET2-21b-C100Flag (42) into the pET-SUMO expression vector (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions to generate an N-terminal SUMO fusion protein. All plasmid open reading frames were verified by DNA sequencing.

S-20, a Chinese hamster ovary (CHO) cell line stably expressing human PS1, N-terminally FLAG-tagged Pen-2, and C-terminally HA-tagged Aph1 α 2 and nicastrin-V5/His, were grown as previously described (43). A separate CHO cell line, designated C-20, was made by stably integrating human FLAG-Pen-2, Aph-1-HA and GST-NCT and was cultured in DMEM supplemented with 10% FBS, 250 μ g/mL hygromycin, 150 μ g/mL G418 and 10 μ g/mL blasticidin. Finally, cell lines over-expressing all four human γ -secretase components were generated by stably transfecting this cell line with wild type or mutant human PS1 plasmids and adding 2.5 μ g/mL puromycin to the C-20 media.

Substrate purification

SUMO-C99-Flag protein was expressed in *E. coli* BL21 (DE3). Bacteria were grown to log phase at 37 °C and induced with 1mM IPTG for 16 h at 25 °C. Each 1 L of cells was pelleted, resuspended in 10 mL of 10 mM Tris (pH 7.0), 200 mM NaCl, 1% Triton X-100 and lysed by passing twice through a French press. Following centrifugation at 3000 g for 15 min, the supernatant was absorbed on M2-agarose beads (Sigma-Aldrich, St. Louis, MO). After binding, beads were washed with 20 mM Tris-HCl (pH 8.0), 125 mM NaCl, 1 mM DTT, and the SUMO tag was removed by overnight digestion at 30 °C with SUMO protease (Invitrogen, Carlsbad, CA). C99-Flag was eluted from M2 beads by boiling in reducing SDS-PAGE sample buffer for 5 min and then loaded on preparative CriterionTM 12 % Bis-Tris gels (Bio-Rad, Hercules, CA). To maximize yield, the isolated band was not stained; to identify the migration of C99-Flag, the gel edges were cut and stained with Instant Blue (Fisher Scientific, Pittsburgh, PA) and placed to flank the unstained gel. The C99-Flag band was excised, washed for 10 min with water, crushed through metal mesh fitted at the bottom of a 1 mL syringe and eluted by incubation for 20 min in 50 mM ammonium bicarbonate buffer. Gel pieces were pelleted at 3000 g, and pure C99-Flag in the supernatant was dried by speed vacuum, resuspended in water and aliquoted.

Detergent-Solubilized γ -Secretase Preparations

Approximately 4×10^9 CHO cells (20 confluent 15-cm dishes) overexpressing wild-type or mutant γ -secretase or 8×10^9 S-20 cells were used for preparation of solubilized γ -secretase. Cells were lysed by French press in 50 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer, pH 6.0, 150 mM NaCl, 5 mM MgCl₂, 5 mM CaCl₂ with complete protease inhibitors

(F. Hoffmann-La Roche, Basel, CH). Nuclei and unbroken cells were removed by spinning at 3,000 g for 10 min. Membranes were pelleted by centrifugation at 100,000 g for 1 h at 4 °C. Membranes were washed with ice-cold 100 mM sodium bicarbonate (pH 11.3) and centrifuged again at 100,000 g for 1 h. The membrane pellet was solubilized by passage through a 27½ gauge needle in 160 µL (WT or mutants) or 2 mL (S-20) Hepes buffer A (50 mM Hepes [pH 7.0], 150 mM NaCl, 5 mM MgCl₂, 5 mM CaCl₂) with 1 % CHAPSO. Insoluble membranes were removed by spinning at 100,000 g for 1 h at 4 °C. The resulting solubilized membranes were aliquoted and snap frozen with liquid nitrogen.

γ-Secretase Assays

Solubilized membranes from cells expressing WT and FAD mutant γ-secretase complexes were incubated with 1 µM C99 substrate for 4 h at 37 °C in Hepes buffer A, 0.1 % phosphatidylcholine, 0.025 % phosphatidylethanolamine, 0.00625 % cholesterol, and 0.45 % final concentration of CHAPSO. Chloroform dissolved lipids for the assays were dried under nitrogen and rehydrated in 1 % CHAPSO Hepes buffer A. All reactions were stopped by snap freezing on dry ice and stored for analysis at −80 °C. The N-terminal and C-terminal cleavage products (Aβ peptides and AICD, respectively) were detected using Western blotting.

For reactions with varying pH, 50 mM MES (pH 5.5, 6.0, and 6.5), 50 mM PIPES (pH 6.5, 7.0, and 7.5), 50 mM Hepes (pH 7.0, 7.5, and 8.0) or 50 mM bicine (pH 8.0, 8.5, and 9.0) were substituted for Hepes buffer A in the assay protocol. These buffers contained 150 mM NaCl. For reactions varying salt, 50 mM Hepes (pH 7.0) with 0 to 300 mM NaCl or NaSO₄, at 50 mM increments, was substituted for Hepes buffer A. Temperature was varied in a gradient-capable thermal cycler (MyCycler™, Bio-Rad, Hercules, CA) from 15 °C to 65 °C, with standard conditions of pH (7.0) and salt (150 mM NaCl) held constant. All pH, salt and temperature reactions were prepared with a final concentration of 0.25 % CHAPSO.

Gel Electrophoresis and Western Blotting

For analysis of AICD, samples were run on Criterion™ 12 % Bis-Tris gels (Bio-Rad, Hercules, CA) using MES running buffer. To exclude uncut substrate from further steps, the gel was cut based on a BenchMark pre-stained protein ladder and Novex™ Sharp pre-stained protein ladder (Invitrogen, Carlsbad, CA) and transferred to a PVDF membrane at 400 mA for 2 h on ice. Membranes were blocked in 6 % milk/PBST for 5 min followed by 1 h incubation with primary M2 anti-FLAG antibody (Sigma-Aldrich, St. Louis, MO 1:1,000 in blocking buffer), and 45 min secondary anti-mouse HRP (GE Healthcare Biosciences, Pittsburgh, PA 1:10,000 in PBST). Bands were illuminated using ECL Plus (GE Healthcare Biosciences, Pittsburgh, PA) and captured on film.

In order to analyze the Aβ products of γ-secretase, Bicine/Tris gels containing 8 M urea described by Klafki *et al.* (44) were used. The system was modified in the following ways: the acrylamide concentration of the separating gel was reduced to 11% T/2.6 % C as previously reported by Qi-Takhara *et al.* (30), the spacer gel was identical to the separation gel but contained 4 M urea, the comb gel acrylamide concentration was reduced to 4% T/3.3 % C, and the gel length was increased to 13.5 cm for separating and 11 cm for spacing gel. Bicine/urea gels were run at 12 mA for 1 h, then 34 W for 3.8 h. The region of the gel immediately below the 12 kDa C99 substrate was cut and transferred to a 0.2 µm PVDF membrane. Following transfer, Aβ peptides were fixed to the membrane by boiling in PBS for 5 min. The membrane was blocked in 8 % milk/PBST for 1 h, washed in PBST overnight and then incubated with primary antibody 6E10 (Sigma-Aldrich, St. Louis, MO) in PBST with 1.5 µg/mL Congo Red for 1 h. Incubation with 1:10,000 secondary antibody

anti-mouse HRP in PBST was followed by 3×5 min washes and ECL Plus/film capture of the signal.

Human γ -secretase components (PS1-NTF, PS1-CTF, Aph-1, Pen-2, and NCT) in preparations were analyzed with specific antibodies for each protein by western blotting. Proteins were separated on Novex Tris-glycine 4–20% gels (Invitrogen, Carlsbad, CA) and transferred to 0.2 μ m PVDF membrane for 75 min at 180 mA. Membranes were blocked in 6% milk/PBST and probed with antibodies 1563 for PS1-NTF (Chemicon, Millipore, Billerica, MA 1:1,000), 13A11 for PS1-CTF (Donated by Elan Corporation, Dublin, Ireland, 1:1,000), anti-HA for Aph-1 α 2-HA (F. Hoffmann-La Roche, Basel, CH 1:2,000), anti-Flag M2 for Flag-Pen-2 (Sigma-Aldrich, St. Louis, MO 1:1,000), and anti-GST for NCT-GST (Sigma-Aldrich, St. Louis, MO 1:3,000). Secondary HRP-conjugated antibodies were diluted 1 to 10,000.

Quantitation of Films

All films were pre-flashed with diffuse white light before exposure to the ECL signal. To quantitate western signals from film, correction for the error introduced by flatbed scanning of the films must be done. Control films were used to produce a mathematical correction equation. Briefly, films were exposed to white light for defined times to produce a gradient of exposures and scanned using a flatbed scanner. A previously described logarithmic correction method was used to compensate for light-scattering interference during film scanning (45). The correction parameter (y_{max}) was empirically determined by plotting intensity vs. exposure time. From these data, dose-response curves followed the exponential equation:

$$y = y_{max}(1 - e^{-bx}) \quad \text{equation 1}$$

where y is the pixel intensity in a scale of 0 to 255, x is the exposure time, and y_{max} is the calculated maximum value of y (243.8 in our conditions). A curve fit was generated using Sigma plot (Molecular Devices, Sunnyvale, CA). Equation 1 was logarithmically linearized to $y_{corr} = bx$, where y_{corr} (corrected signal in the scanned image) can be calculated by:

$$y_{corr} = \log \left\{ \frac{y_{max}}{y_{max} - y} \right\} \quad \text{equation 2}$$

Equation 2 was modified with linear factor a , to render y'_{corr} values in image format (pixel values in the 0 to 255 scale):

$$y'_{corr} = a * y_{corr} = a * \log \left\{ \frac{y_{max}}{y_{max} - y} \right\} \quad \text{equation 3}$$

Factor a corresponded to 88.7 in our experimental conditions. ImageJ software's mathematical macro function (U. S. National Institutes of Health, Bethesda, MD) was used to process each scanned image according to equation 3 generating a new corrected image where each pixel is now directly proportional to the corresponding film intensity. After this correction, protein bands were quantitated using Quantity-one software (Bio-Rad, Hercules, CA).

RESULTS

We first explored whether a change in overall activity correlates with a change in processivity by measuring AICD and the various forms of A β produced by wild type γ -secretase complexes under different conditions. In particular, do conditions that lower the level of AICD production parallel with increased proportions of longer forms of A β at the expense of shorter forms? In order to answer this question, we chose to perturb γ -secretase activity in an *in vitro* system; this is particularly important because we aimed to analyze all γ -secretase products. In a cellular system, normal differences in product half-lives and retention of longer A β s in the cell membrane would preclude this type of in-depth analysis.

Membranes containing γ -secretase complexes were isolated from S-20 cells, Chinese hamster ovary (CHO) cells stably overexpressing all four components of the human protease complex (43). Overexpression of human PS1 in CHO cells results in replacement of endogenous hamster presenilins in γ -secretase complexes (14, 20). Combined with the stable overexpression of V5-His-Nicastrin, HA-Aph-1, and Flag-Pen-2, the S-20 cell line has 17-fold more activity than the parental CHO cell line (43). Thus, γ -secretase preparations from this cell line is composed of the overexpressed human components, and CHAPSO-solubilized membranes from the S-20 cells display high γ -secretase activity that has been well characterized (43). In addition, the S-20 cell line has previously been used as a source of purified protease complexes for structural studies by cryoelectron microscopy (46) and to determine the effects of various lipids on γ -secretase activity (47). For γ -secretase substrate, we utilized recombinant C99-Flag, which is identical to the 99-residue membrane-bound fragment of APP produced by β -secretase but with a FLAG epitope tag on the C-terminus to allow facile purification and ready detection of the AICD-Flag proteolytic product. γ -Secretase cleavage of this substrate also produces A β peptides identical to those produced in cells, and standard synthetic A β peptides can be used to identify those formed in our enzyme assays. C99-Flag was expressed in *E. coli* as a SUMO-tagged fusion protein. We took advantage of the ability of SUMO protease to leave no additional prime-side residues after cleavage. Digestion of SUMO-C99-Flag with SUMO protease released C99, and a two-step purification (immunoaffinity chromatography followed by preparative electrophoresis) provided a highly pure substrate.

To quantitatively study the γ -secretase reaction *in vitro*, we also needed to improve the reproducibility of an electrophoretic technique for separating various A β peptides (30, 44) to allow for the simultaneous analysis of A β 38, 40, 42, 43, 45 and 46+ (unresolved A β 46, 48, and 49) in the presence of a large excess of C99-Flag substrate. A challenge for electrophoretic analysis of γ -secretase activity is that unreacted C99-Flag is recognized in Western blots by general A β antibodies and can comigrate with and obscure shorter A β products, thereby interfering with quantification. We modified the composition and length of the spacer gel in the bicine/urea gel system to improve the separation of unreactive C99 substrate from A β peptides, and as a result we were able to reproducibly resolve A β 38 from excess substrate. Note that under these conditions, longer A β peptides migrate more quickly than the shorter ones, possibly because the longer peptides retain some folded structure (and are therefore more compact), even in the presence of high concentrations of urea.

Attempts to develop methods for kinetic analysis of all A β species failed to provide reliable linear production with time for the longer A β forms (>A β 42). Therefore, we chose to monitor A β production in a steady-state endpoint fashion. Under these *in vitro* conditions, time-course experiments demonstrated that the formation of products becomes nonlinear after six hours of incubation at 37°C for 1 μ M of C99-Flag (data not shown). For the following experiments, we utilized an end-point (4 h) that was well within the linear range

of product formation and that gave reproducible results for all A β peptides. Thus, the substrate concentration was in constant excess during the time-frame of our analysis.

For our first attempt at finding conditions in which γ -secretase activity is reduced, CHAPSO-solubilized S-20 cell membranes were incubated with C99-Flag at 37 °C in buffers at pHs ranging from 5.5 to 9.0. To achieve these pH values, different buffers needed to be employed: MES for pH 5.5 to 6.5, HEPES for pH 7.0 to 8.0, and bicine for pH 8.5 to 9.0. Measurement of AICD production showed a bell-shaped relationship between pH and overall γ -secretase activity (Figure 1A). The pH optimum was 6.5, in general concordance with previous reports (42, 48). We note, however, that in these prior studies buffers had been used outside their pH buffering ranges, giving the impression that γ -secretase is substantially active even at certain pHs. Thus, the results shown in Figure 1A should be a more accurate measure of the pH dependence of CHAPSO-solubilized γ -secretase. For instance, both prior reports indicated that γ -secretase is still quite active up to the highest pH tested (9.0), which is surprising for an aspartyl protease, while here we found that with an appropriate buffering system (bicine) there is very little activity at this highly basic pH. Running these enzyme reactions in different buffers with overlapping pH values demonstrated that changing the buffer type *per se* did not alter either AICD production or the proportions of A β peptides (Figure S1 and data not shown, respectively). A β peptides generated were separated and detected using our modified procedure; each band was carefully measured by densitometry (see Experimental Procedures), and the proportions of the individual A β peptides were graphed (Figure 1B and S2A). No statistically significant differences were seen for any of the specific A β peptides at the various pHs with the exception of A β 38 and A β 46+. The proportion of A β 38 was decreased at pH 5.5 and 6.0 as compared to higher pHs, and the proportion of A β 46+ was increased at pH 8.5 as compared to pH 7.0 (regraphed for clarity and with error bars in Figure 1C). Comparison of these changes in A β to the changes in total activity shows discordance; at high pHs where there is a substantial reduction in activity, there appears to be both an increase and a decrease in processivity (more A β 38 and more A β 46+, respectively). A small reduction in processivity (less A β 38) is seen at mildly acidic pH in concert with a moderate reduction in activity, but in general, processivity did not change in concert with changes in overall activity.

Next, we tested the effect of changes in total activity on processivity of γ -secretase in different concentrations and types of salt. Under otherwise standard conditions (0.25 % CHAPSO in HEPES buffer [pH 7.0], 37 °C), the concentration of NaCl, which is typically held at 150 mM, was varied from 0 to 300 mM. Above 300 mM, we found that the longer A β peptides ≥ 42 residues precipitated (i.e., were salted out), even with a mixture of standard A β peptides alone (data not shown), consistent with the tendency of these highly hydrophobic peptides to aggregate. Thus, NaCl concentrations higher than 300 mM could not be reliably tested for effects on γ -secretase activity. No statistical difference was observed in AICD production from 50 to 300 mM of NaCl (Figure 2A, shown only up to 150 mM), while activity was dramatically reduced in the absence of NaCl. In examining the range of A β peptides produced, again we found no significant differences in the amounts or proportion of the individual A β peptides under conditions ranging from 100 to 300 mM NaCl (Figure 2B, shown only up to 150 mM). However, in the absence of NaCl, the proportion of A β peptides ≥ 46 residues was observed to more than double compared to that seen in the presence of 150 mM of NaCl (Figure 2C and S2B). These same experiments were also carried out using Na₂SO₄. Similar to what was seen with NaCl, AICD production was statistically equivalent over the higher Na₂SO₄ concentrations ranging from 150 to 300 mM (data not shown). Again, concentrations over 300 mM led to precipitation of longer A β peptides, even from a standard mixture of the peptides alone, and could not be tested for effects on γ -secretase activity. In this case, lower concentrations of Na₂SO₄ (50 mM) did show substantially reduced overall proteolytic activity, although still higher than that seen in

the absence of salt (Figure 2A). The reasons behind the subtle differences in concentration-dependent effects of NaCl and Na₂SO₄ on total activity are unclear but may be due to the nature of the anion, as chloride is a “hard” anion (e.g., dense charge) while sulfate is a “softer” anion (e.g., diffused charge). Nevertheless, despite the lower activity at 50 mM Na₂SO₄, the proportion of the various A β peptides was not significantly different between the enzyme reactions run in 150 mM Na₂SO₄ (Figure 2B,C and S2B). The only difference observed was again found with A β peptides $\geq$ 46 residues when comparing the absence of salt to added Na₂SO₄ (Figure 2C). Thus, the absence of salt can lead to some reduction in processivity, but in general processivity does not decrease in parallel with decreases in total activity under different salt conditions.

We then examined the temperature dependence of γ -secretase catalysis. These assays were otherwise carried out under the standard conditions of pH and salt concentration (pH 7.0 and 150 mM NaCl). Unexpectedly, we found that this human protease complex was maximally active at 45 °C (Figure 3A). Apparently, the heteropentameric enzyme complex is stable even at this elevated temperature. More surprising, the γ -secretase complex retained considerable ability to generate AICD even at 50 °C, producing slightly more than what is seen at 35 °C. However, activity seems to drop quite rapidly above 50 °C, with little activity seen at 55 °C. A more gradual decrease in activity was observed for temperatures lower than 35 °C, down to 20 °C which displayed very little ability to generate AICD. These temperatures, from 20 to 55 °C, provided a good range of proteolytic activities to further address the connection between overall activity and processivity. In examining the A β s produced at these different temperatures, we noted changes in the distribution of many A β peptides (Figure 3B and S2C). Interestingly, although the longest forms of A β ($\geq$ 46 residues) were increased in proportion at the temperature extremes (i.e., with the lowest overall activities), the short forms (A β 38 and A β 40) decreased in proportion as the temperature was increased over 35 °C but did not change when the activity was impaired by lowering the temperature (Figure 3C and S2C). Strikingly, the proportions of A β 42 and A β 43 increased with temperature throughout almost the entire range (Figure 3C and S2), showing no correlation with the total activity profile generated with AICD. Furthermore, examination of the A β spectra at the temperature that gave maximal activity (45 °C) reveals a shift towards aggregation prone A β 43 in direct opposition to the hypothesis that it is the loss of γ -secretase activity that is responsible for transition to the longer A β forms. Thus, again we noted dissociation between activity and processivity; lower activities did not correlate with a greater proportion of longer forms of A β . It is important to point out that we did not observe precipitation or loss of A β peptides from a standard mixture at extremes of pH or temperature or with no added NaCl or Na₂SO₄, nor did APP substrate precipitate or display increased aggregation (data not shown). That is, the effects we noted above upon altered conditions can be attributed to changes in γ -secretase activity.

We then turned our attention to AD-associated mutations in PS1. In general, these mutations have been found to increase the proportion of A β 42 to A β 40 and also decrease overall proteolytic activity. We asked whether γ -secretase complexes containing PS1 mutations associated with familial, early-onset AD (FAD) indeed decrease overall proteolysis of APP in a cell-free biochemical assay and whether decreased activity occurs in parallel with decreased processivity. To that end, we first established stable cell lines overexpressing human γ -secretase complexes with different FAD-associated PS1 mutations. A CHO cell line was generated that stably overexpresses the other three components of γ -secretase: Aph-1, NCT and Pen-2. From this common parental cell line, six different variants of PS1 were stably introduced: wild type (WT), L166P, A246E, L286V, G384A and Δ E9 (a deletion of PS1 exon 9). The five FAD-associated PS1 mutants chosen are located in diverse regions of the PS1 sequence and represent a broad range of reported effects on A β 42 to A β 40 ratios and ages of disease onset (Figure 4A). For instance, the L166P mutation, which

resulted in disease onset at 24 years, and the G384A mutation, which has an average age of onset of 34 years, have been reported to cause large increases in the A β 42:40 ratio (49–53). In contrast, the A246E and L286V mutations are associated with more subtle effects on the A β 42:40 ratio and much later ages of onset (53 and 48 years, respectively) (50, 54).

Expression of mature γ -secretase complexes and complete replacement of endogenous hamster PS1 by the exogenous human PS1 variants were verified for each stable cell line. All showed the presence of a higher molecular weight, more highly glycosylated form of NCT that is found in mature, active γ -secretase complexes (21–25) (Figure 4B). Processing of PS1 into NTF and CTF is also part of the maturation of protease complexes to active form (18–20). Indeed, this endoproteolytic event only occurs upon assembly of all four components (10–13). Using antibodies specific for the N-terminus of human PS1, we found that each of the exogenous PS1 variants was well expressed, and all but one were processed to NTF; the exception was PS1 Δ E9, which lacks the exon 9 region encoding the endoproteolytic cleavage site but nevertheless enters into mature, active protease complexes (18, 19, 55–57). The PS1 CTF was also examined, as the human and hamster forms display slightly different mobilities in SDS-PAGE, and therefore, replacement of the endogenous hamster PS1 with the exogenous human form can be confirmed (18, 20). The human PS1 CTF is observed in each case, with PS1 Δ E9 again being the expected exception. For all stable cell lines overexpressing human PS1 variants, the hamster PS1 CTF is absent, with only a very faint band detected in Δ E9 and G384A, ensuring that the γ -secretase activities we observe are overwhelmingly due to the human γ -secretase complexes of interest.

After membrane preparation and solubilization in CHAPSO, each human γ -secretase complex variant was tested for its ability to produce AICD (Figure 4C). Approximately equal amounts of γ -secretase were added to each reaction by adjusting for amounts of PS1 NTF (or full-length protein in the case of the Δ E9 variant). Also, relative specific AICD values were obtained by measuring NTF and AICD simultaneously in these reactions, eliminating important sources of variability. We found that most of the studied mutants had substantially reduced overall proteolytic activity relative to wild type, with the exception of complexes containing the PS1 L286V mutation. We could not distinguish statistical differences between the relative activities of protease complexes with PS1 L166P, A246E, G384A or Δ E9, nor could we distinguish differences between wild type enzyme and PS1 L286V-containing enzyme.

The production of the spectrum of A β peptides by these various γ -secretase complexes was then examined by bicine-urea PAGE and western blotting (Figure 5A). In order to compensate for the reduced overall activity of L166P, A246E, G384A and Δ E9 and allow quantitation of all A β peptides (i.e., to obtain strong enough bands), the samples loaded were normalized for total A β . This allowed facile comparison of changes in the proportions of the different A β species rather than confounding these changes with differences in total A β production, which mirror the differences in AICD production (28). All of the mutant-containing complexes appear to increase the proportion of longer forms of A β ($\geq$ 42 residues). The PS1 L166P mutant protease showed the most dramatic effect (Figure 5B and S3A). Complexes with PS1 A246E and G384A appear to have the smallest effects on processivity; the A246E mutant shows an increase in A β 43, and the G384A mutant shows a decrease in A β 40 and A β 45 and a concomitant increase in $\geq$ A β 46. Complexes with PS1 Δ E9 and L286V displayed intermediate effects with an overall decrease in A β peptides $\leq$ 42 residues and an increase in A β peptides longer than 42 residues.

PS1 FAD mutant γ -secretase complexes are known to increase the A β 42:A β 40 ratio compare to the wild-type enzyme (34–37), and this shift to longer, more aggregation-prone amyloid peptides is thought to contribute to AD pathogenesis (58). Most investigators use

enzyme-linked immunosorbent assays (ELISAs) or short bicine/urea gels to quantitate A β 40 and A β 42. In our hands (data not shown) and as recognized previously (59–62), these methods do not completely distinguish or separate A β 43 from A β 42. Moreover, a recent study has demonstrated that the frequently overlooked A β 43 peptide can be primarily produced by at least one FAD PS1 mutant, and transgenic mice expressing this FAD PS1 mutant produce amyloid pathology in the brain (63). Therefore, for each of our γ -secretase mutants we calculated the (A β 42+A β 43):A β 40 ratio which was significantly increased compared to that produced by the wild type enzyme (Figure S3B). As previously reported, the ratio can be increased by a decrease in A β 40, an increase in A β 42/43, or a decrease in both A β 40 and A β 42/43 if A β 40 decreases proportionately more (50, 64, 65). Dissecting the cause for this increase in ratio for our mutants shows three groupings: L286V, G384A, and Δ E9 have significantly decreased A β 40, A246E has significantly increased A β 42/43, and L166P has both decreased A β 40 and increased A β 42/43 (Figure 5C). These results are concordant with previous studies (49, 50, 65–72) which have shown G384A and L286V to have reduced A β 40 levels, A246E to have increased A β 42 levels, and L166P to have both decreased A β 40 and increased A β 42. For Δ E9 our results deviate from previous studies (49, 52, 65, 68, 71) in that we did not detect a significant increase in A β 42. However, consistent with these previous studies, we found that upon transfection of APP, the relative level of secreted A β 42 compared to total A β , as determined by specific ELISAs, is increased in the cell line stably expressing the PS1 Δ E9 mutant compared to the wild type PS1-expressing cell line (data not shown). It is important to bear in mind that the A β secreted from cells is not all the A β produced and that longer forms are not secreted well, and the longest forms not at all.

As with the wild-type enzyme under different conditions, we observed dissociation between the effects of these PS1 mutations on the overall activity of γ -secretase and their effects on the processivity of the enzyme. The L286V PS1-containing complex had indistinguishable total activity as compared to the wild type enzyme, but nevertheless displayed a clear decrease in processivity. In contrast, the G384A PS1 mutation caused a substantial decrease in total activity but led to subtler effects on processivity. As one means of deciphering the relationship between activity and processivity, we determined the ratio of long to short A β peptides, long being defined as ≥ 42 residues and short being the sum of A β 38 and A β 40, a ratio defined here as the “processivity index”, with higher values indicating reduced processivity. This value was plotted as a function of activity (Figure 6A). Clearly, no obvious correlation exists between the level of overall γ -secretase activity and processivity for γ -secretase mutants. In addition, the same analysis was done for the temperature data from Figure 3, and again no relationship between activity and processivity could be distinguished (Figure 6B).

DISCUSSION

Up to now, an analysis of all A β peptides produced from a variety of isolated AD-mutant γ -secretase complexes and purified substrate has not been carried out. Almost all of the previous studies examining the effects of presenilin mutations on γ -secretase cleavage of APP involved detection of A β peptides and AICD generated in cells, raising concerns about cleavage events by other proteases, degradation of γ -secretase products, or retention of long A β s in the cell membrane. Understanding the mechanism of action of the human γ -secretase complex and how FAD-causing PS mutants alter this proteolytic activity requires a fuller appreciation of the products that are formed and the relationship of these products to one another under more reductionistic conditions.

Here we considered the hypothesis (40, 41) that slowing of the proteolytic rate, whether of the wild-type enzyme under different reaction conditions or of complexes containing FAD-

mutant presenilin, might lead to reduced trimming of A β peptides (i.e., reduced processivity). In this way, a reduction of function (overall activity) could lead to a gain of function (an increase in the proportion of longer, more aggregation-prone forms of the peptide). A unification of these two seemingly opposed concepts would resolve the long-standing controversy over whether presenilin mutations cause AD through a loss or a gain of function. Even if our studies did not support this unifying hypothesis, we hoped to glean clues regarding the nature of processivity by γ -secretase complexes.

In analyzing the activity and processivity of γ -secretase under various conditions of pH, salt concentration and temperature, we did not observe a connection between overall proteolytic activity and processivity. Using buffering systems within their appropriate pH ranges, we found that optimal AICD generation occurred at pH 6.5. As the pH was increased, activity decreased; however, we observed an increase in the proportions of both A β 38 and A β 46+. As the pH was decreased from 6.5, again overall activity decreased, and for the most part the proportion of individual A β peptides did not change, the sole exception being a clear decrease in A β 38. Thus, processivity is slightly compromised at mildly acidic pH.

Using different salts and salt concentrations, we found that overall activity varied at lower concentrations of sodium sulfate. However, no change in the proportion of the various A β peptides was observed. Only in the absence of salt did we see a clear increase in the proportion of A β peptides ≥ 46 residues (that is, a decrease in processivity). That mildly acidic pH and the absence of salt both result in reduced processivity would be consistent with a role of one or more charged residues of γ -secretase in the trimming of long A β peptides to short ones. Residues with pKa values ~ 6 (e.g., histidines) would be reasonable candidates.

With respect to temperature, we observed that 45 °C led to optimal total activity and that the enzyme was active even at 50 °C. Given that the complex contains five components held together by noncovalent forces, this was quite surprising. However, we noted that changes in processivity did not track with changes in total activity at different temperatures. Changes in pH, salt identity, salt concentration, and temperature can alter the charged state, conformation, conformational dynamics, and reactivity of proteins and their functional groups. However, it appears that the factors that affect the initial ϵ cleavage of APP and those that affect the trimming of the initially formed A β peptides are generally different.

To explore the properties of FAD-mutant PS1-containing γ -secretase complexes on overall activity and processivity, we generated a stable cell line overexpressing human forms of all three of the other components of the protease (Aph-1, NCT and Pen-2) as a parental line in which to introduce different human PS1 variants. In analyzing a set of such mutant protease complexes, we found that the mutations generally decreased overall proteolytic activity, with the notable exception of the L286V mutant. Although PS1 L286V-containing complexes showed total activity comparable to that of the wild type enzyme, the L286V mutation nevertheless led to a clear change in processivity, with a greater proportion of longer A β peptides. Indeed, all of the AD-mutant PS1-containing protease complexes tested here displayed a decrease in processivity; that is, an increase in longer, more aggregation-prone A β peptides, consistent with the connection between the formation of A β assemblies and the pathogenesis of AD. However, overall activity and processivity were again dissociated, not only with the L286V mutation, but also with the G384A mutation, which shows a substantial decrease in activity but relatively mild effects on processivity. Nevertheless, it may be possible to correlate total activity and trimming with a subset of presenilin FAD mutations.

These findings with PS1 FAD mutations are consistent with several earlier reports examining the effects of such mutations on A β and AICD production in cell culture. For instance, Moehlmann and colleagues found a dissociation between AICD production and A β 42/40 ratios with a variety of mutations in PS1 L166, including the pathogenic L166P (49). Moreover, Bentahir *et al.* used rescue experiments with presenilin-deficient cells to show that while several PS1 and PS2 FAD mutants all reduced AICD production, these mutants had variable effects on the spectrum of secreted A β peptides, some decreasing A β 40 and others increasing A β 42 (50). Others have shown a lack of correlation between A β 38 and A β 42 levels, both with γ -secretase modulators (e.g., sulindac sulfide) and FAD PS1 mutations, arguing against a precursor-production relationship (62, 68). This particular conclusion, however, disregards the fact that A β 42 levels are determined not only by its trimming to A β 38 but also its production from A β 45, and the Ihara laboratory has detected the corresponding tetrapeptide of residues 39–42 generated by γ -secretase (32), clear proof that A β 38 can be directly derived from A β 42. Nevertheless, the apparent dissociation between A β 38 and A β 42 with PS1 FAD mutations is a clear illustration that the relationship between total activity and processivity is not a simple one.

It is remarkable that a protease with a single catalytic site (33) can be responsible for so many different proteolytic events: presenilin endoproteolysis into NTF and CTF (14, 73), initial ϵ cleavage to release the intracellular domains of a variety of type I integral membrane substrates (74), and processive proteolysis every 3–4 residues (32) in producing a spectrum of peptides (A β from APP, but also various counterpart peptides from the Notch receptor (75); other substrates of γ -secretase are likely to be similarly processed). The misregulation of processivity in particular is apparently critical to the pathogenesis of AD, resulting in the generation of longer A β species that are more prone to aggregation into neurotoxic assemblies. Understanding the mechanism of the trimming process of A β peptides will likely require a reductionist approach using biochemical assays and facile analytical methods for measuring all of the products formed: the intracellular domain, the full spectrum of A β peptides (not only those secreted from cells), as well as the tri- and tetrapeptide fragments. Such methods could then be coupled with targeted mutagenesis of γ -secretase components to test specific mechanistic hypotheses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Hanna Laudon for generating PS1 mutant constructs L166P and G384A, the laboratory of Dennis Selkoe for providing the PS1 mutant A246E construct, and Amy Emerman for help in setting up the salinity experiments.

ABBREVIATIONS

AD	Alzheimer's disease
Aβ	amyloid β -peptide
APP	amyloid β -protein precursor
AICD	amyloid β -protein precursor intracellular domain
Aph1	anterior pharynx-defective 1
CHO	Chinese hamster ovary
ELISA	enzyme-linked immunosorbent assay

FAD	familial Alzheimer's disease
HA	hemagglutinin
NCT	nicastatin
PS1	presenilin-1
PS2	presenilin-2
CTF	presenilin C-terminal fragment
Pen-2	presenilin enhancer 2
NTF	presenilin N-terminal fragment
WT	wild type

References

1. Glenner GG, Wong CW. Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem Biophys Res Comm*. 1984; 120:885–890. [PubMed: 6375662]
2. Masters CL, Simms G, Weinman NA, Multhaup G, McDonald BL, Beyreuther K. Amyloid plaque core protein in Alzheimer disease and Down syndrome. *Proc Natl Acad Sci U S A*. 1985; 82:4245–4249. [PubMed: 3159021]
3. Cole SL, Vassar R. The role of APP processing by BACE1, the beta-secretase, in Alzheimer's disease pathophysiology. *J Biol Chem*. 2008; 283:29621–29625. [PubMed: 18650431]
4. Wolfe MS. The gamma-secretase complex: membrane-embedded proteolytic ensemble. *Biochemistry*. 2006; 45:7931–7939. [PubMed: 16800619]
5. Jarrett JT, Berger EP, Lansbury PT Jr. The carboxy terminus of the beta amyloid protein is critical for the seeding of amyloid formation: implications for the pathogenesis of Alzheimer's disease. *Biochemistry*. 1993; 32:4693–4697. [PubMed: 8490014]
6. Walsh DM, Klyubin I, Fadeeva JV, Cullen WK, Anwyl R, Wolfe MS, Rowan MJ, Selkoe DJ. Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo. *Nature*. 2002; 416:535–539. [PubMed: 11932745]
7. Lesne S, Koh MT, Kotilinek L, Kaye R, Glabe CG, Yang A, Gallagher M, Ashe KH. A specific amyloid-beta protein assembly in the brain impairs memory. *Nature*. 2006; 440:352–357. [PubMed: 16541076]
8. Shankar GM, Li S, Mehta TH, Garcia-Munoz A, Shepardson NE, Smith I, Brett FM, Farrell MA, Rowan MJ, Lemere CA, Regan CM, Walsh DM, Sabatini BL, Selkoe DJ. Amyloid-beta protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory. *Nat Med*. 2008; 14:837–842. [PubMed: 18568035]
9. Tanzi RE, Bertram L. Twenty years of the Alzheimer's disease amyloid hypothesis: a genetic perspective. *Cell*. 2005; 120:545–555. [PubMed: 15734686]
10. Francis R, McGrath G, Zhang J, Ruddy DA, Sym M, Apfeld J, Nicoll M, Maxwell M, Hai B, Ellis MC, Parks AL, Xu W, Li J, Gurney M, Myers RL, Himes CS, Hiebsch R, Ruble C, Nye JS, Curtis D. aph-1 and pen-2 are required for Notch pathway signaling, gamma-secretase cleavage of betaAPP, and presenilin protein accumulation. *Dev Cell*. 2002; 3:85–97. [PubMed: 12110170]
11. Takasugi N, Tomita T, Hayashi I, Tsuruoka M, Niimura M, Takahashi Y, Thinakaran G, Iwatsubo T. The role of presenilin cofactors in the gamma-secretase complex. *Nature*. 2003; 422:438–441. [PubMed: 12660785]
12. Kimberly WT, LaVoie MJ, Ostaszewski BL, Ye W, Wolfe MS, Selkoe DJ. γ -Secretase is a membrane protein complex comprised of presenilin, nicastrin, aph-1, and pen-2. *Proc Natl Acad Sci U S A*. 2003; 100:6382–6387. [PubMed: 12740439]
13. Edbauer D, Winkler E, Regula JT, Pesold B, Steiner H, Haass C. Reconstitution of gamma-secretase activity. *Nat Cell Biol*. 2003; 5:486–488. [PubMed: 12679784]

14. Wolfe MS, Xia W, Ostaszewski BL, Diehl TS, Kimberly WT, Selkoe DJ. Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and γ -secretase activity. *Nature*. 1999; 398:513–517. [PubMed: 10206644]
15. Li YM, Xu M, Lai MT, Huang Q, Castro JL, DiMuzio-Mower J, Harrison T, Lellis C, Nadin A, Neduvellil JG, Register RB, Sardana MK, Shearman MS, Smith AL, Shi XP, Yin KC, Shafer JA, Gardell SJ. Photoactivated gamma-secretase inhibitors directed to the active site covalently label presenilin 1. *Nature*. 2000; 405:689–694. [PubMed: 10864326]
16. Esler WP, Kimberly WT, Ostaszewski BL, Diehl TS, Moore CL, Tsai JY, Rahmati T, Xia W, Selkoe DJ, Wolfe MS. Transition-state analogue inhibitors of γ -secretase bind directly to presenilin-1. *Nature Cell Biology*. 2000; 2:428–434.
17. Kaether C, Capell A, Edbauer D, Winkler E, Novak B, Steiner H, Haass C. The presenilin C-terminus is required for ER-retention, nicastrin-binding and gamma-secretase activity. *Embo J*. 2004; 23:4738–4748. [PubMed: 15549135]
18. Thinakaran G, Borchelt DR, Lee MK, Slunt HH, Spitzer L, Kim G, Ratovitsky T, Davenport F, Nordstedt C, Seeger M, Hardy J, Levey AI, Gandy SE, Jenkins NA, Copeland NG, Price DL, Sisodia SS. Endoproteolysis of presenilin 1 and accumulation of processed derivatives in vivo. *Neuron*. 1996; 17:181–190. [PubMed: 8755489]
19. Ratovitski T, Slunt HH, Thinakaran G, Price DL, Sisodia SS, Borchelt DR. Endoproteolytic processing and stabilization of wild-type and mutant presenilin. *J Biol Chem*. 1997; 272:24536–24541. [PubMed: 9305918]
20. Thinakaran G, Harris CL, Ratovitski T, Davenport F, Slunt HH, Price DL, Borchelt DR, Sisodia SS. Evidence that levels of presenilins (PS1 and PS2) are coordinately regulated by competition for limiting cellular factors. *J Biol Chem*. 1997; 272:28415–28422. [PubMed: 9353300]
21. Edbauer D, Winkler E, Haass C, Steiner H. Presenilin and nicastrin regulate each other and determine amyloid beta-peptide production via complex formation. *Proc Natl Acad Sci U S A*. 2002; 99:8666–8671. [PubMed: 12048259]
22. Arawaka S, Hasegawa H, Tandon A, Janus C, Chen F, Yu G, Kikuchi K, Koyama S, Kato T, Fraser PE, St George-Hyslop P. The levels of mature glycosylated nicastrin are regulated and correlate with gamma-secretase processing of amyloid beta-precursor protein. *J Neurochem*. 2002; 83:1065–1071. [PubMed: 12437577]
23. Kimberly WT, LaVoie MJ, Ostaszewski BL, Ye W, Wolfe MS, Selkoe DJ. Complex N-linked glycosylated nicastrin associates with active gamma-secretase and undergoes tight cellular regulation. *J Biol Chem*. 2002; 277:35113–35117. [PubMed: 12130643]
24. Leem JY, Vijayan S, Han P, Cai D, Machura M, Lopes KO, Veselits ML, Xu H, Thinakaran G. Presenilin 1 is required for maturation and cell surface accumulation of nicastrin. *J Biol Chem*. 2002; 277:19236–19240. [PubMed: 11943765]
25. Tomita T, Katayama R, Takikawa R, Iwatsubo T. Complex N-glycosylated form of nicastrin is stabilized and selectively bound to presenilin fragments. *FEBS Lett*. 2002; 520:117–121. [PubMed: 12044882]
26. Kornilova AY, Bihel F, Das C, Wolfe MS. The initial substrate-binding site of gamma-secretase is located on presenilin near the active site. *Proc Natl Acad Sci U S A*. 2005; 102:3230–3235. [PubMed: 15722417]
27. Weidemann A, Eggert S, Reinhard FB, Vogel M, Paliga K, Baier G, Masters CL, Beyreuther K, Evin G. A Novel var epsilon-Cleavage within the Transmembrane Domain of the Alzheimer Amyloid Precursor Protein Demonstrates Homology with Notch Processing. *Biochemistry*. 2002; 41:2825–2835. [PubMed: 11851430]
28. Kakuda N, Funamoto S, Yagishita S, Takami M, Osawa S, Dohmae N, Ihara Y. Equimolar production of amyloid beta-protein and amyloid precursor protein intracellular domain from beta-carboxyl-terminal fragment by gamma-secretase. *J Biol Chem*. 2006; 281:14776–14786. [PubMed: 16595682]
29. Sato T, Dohmae N, Qi Y, Kakuda N, Misonou H, Mitsumori R, Maruyama H, Koo EH, Haass C, Takio K, Morishima-Kawashima M, Ishiura S, Ihara Y. Potential link between amyloid beta-protein 42 and C-terminal fragment gamma 49–99 of beta-amyloid precursor protein. *J Biol Chem*. 2003; 278:24294–24301. [PubMed: 12707272]

30. Qi-Takahara Y, Morishima-Kawashima M, Tanimura Y, Dolios G, Hirotsu N, Horikoshi Y, Kametani F, Maeda M, Saido TC, Wang R, Ihara Y. Longer forms of amyloid beta protein: implications for the mechanism of intramembrane cleavage by gamma-secretase. *J Neurosci*. 2005; 25:436–445. [PubMed: 15647487]
31. Funamoto S, Morishima-Kawashima M, Tanimura Y, Hirotsu N, Saido TC, Ihara Y. Truncated carboxyl-terminal fragments of beta-amyloid precursor protein are processed to amyloid beta-proteins 40 and 42. *Biochemistry*. 2004; 43:13532–13540. [PubMed: 15491160]
32. Takami M, Nagashima Y, Sano Y, Ishihara S, Morishima-Kawashima M, Funamoto S, Ihara Y. gamma-Secretase: successive tripeptide and tetrapeptide release from the transmembrane domain of beta-carboxyl terminal fragment. *J Neurosci*. 2009; 29:13042–13052. [PubMed: 19828817]
33. Sato T, Diehl TS, Narayanan S, Funamoto S, Ihara Y, De Strooper B, Steiner H, Haass C, Wolfe MS. Active gamma-secretase complexes contain only one of each component. *J Biol Chem*. 2007; 282:33985–33993. [PubMed: 17911105]
34. Duff K, Eckman C, Zehr C, Yu X, Prada CM, Perez-tur J, Hutton M, Buee L, Harigaya Y, Yager D, Morgan D, Gordon MN, Holcomb L, Refolo L, Zenk B, Hardy J, Younkin S. Increased amyloid-beta42(43) in brains of mice expressing mutant presenilin 1. *Nature*. 1996; 383:710–713. [PubMed: 8878479]
35. Citron M, Westaway D, Xia W, Carlson G, Diehl T, 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, Selkoe DJ. Mutant presenilins of Alzheimer's disease increase production of 42-residue amyloid beta-protein in both transfected cells and transgenic mice. *Nat Med*. 1997; 3:67–72. [PubMed: 8986743]
36. Scheuner D, Eckman C, Jensen M, Song X, Citron M, Suzuki N, Bird TD, Hardy J, Hutton M, Kukull W, Larson E, Levy-Lahad E, Viitanen M, Peskind E, Poorkaj P, Schellenberg G, Tanzi R, Wasco W, Lannfelt L, Selkoe D, Younkin S. Secreted amyloid beta-protein similar to that in the senile plaques of Alzheimer's disease is increased in vivo by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease. *Nat Med*. 1996; 2:864–870. [PubMed: 8705854]
37. Lemere CA, Lopera F, Kosik KS, Lendon CL, Ossa J, Saido TC, Yamaguchi H, Ruiz A, Martinez A, Madrigal L, Hincapié L, Arango JC, Anthony DC, Koo EH, Goate AM, Selkoe DJ, Arango JC. The E280A presenilin 1 Alzheimer mutation produces increased A beta 42 deposition and severe cerebellar pathology. *Nat Med*. 1996; 2:1146–1150. [PubMed: 8837617]
38. Song W, Nadeau P, Yuan M, Yang X, Shen J, Yankner BA. Proteolytic release and nuclear translocation of Notch-1 are induced by presenilin-1 and impaired by pathogenic presenilin-1 mutations. *Proc Natl Acad Sci U S A*. 1999; 96:6959–6963. [PubMed: 10359821]
39. Shen J, Kelleher RJ 3rd. The presenilin hypothesis of Alzheimer's disease: evidence for a loss-of-function pathogenic mechanism. *Proc Natl Acad Sci U S A*. 2007; 104:403–409. [PubMed: 17197420]
40. Wolfe MS. When loss is gain: reduced presenilin proteolytic function leads to increased Abeta42/Abeta40. Talking Point on the role of presenilin mutations in Alzheimer disease. *EMBO Rep*. 2007; 8:136–140. [PubMed: 17268504]
41. De Strooper B. Loss-of-function presenilin mutations in Alzheimer disease. Talking Point on the role of presenilin mutations in Alzheimer disease. *EMBO Rep*. 2007; 8:141–146. [PubMed: 17268505]
42. Li YM, Lai MT, Xu M, Huang Q, DiMuzio-Mower J, Sardana MK, Shi XP, Yin KC, Shafer JA, Gardell SJ. Presenilin 1 is linked with gamma-secretase activity in the detergent solubilized state. *Proc Natl Acad Sci U S A*. 2000; 97:6138–6143. [PubMed: 10801983]
43. Cacquevel M, Aeschbach L, Osenkowski P, Li D, Ye W, Wolfe MS, Li H, Selkoe DJ, Fraering PC. Rapid purification of active gamma-secretase, an intramembrane protease implicated in Alzheimer's disease. *J Neurochem*. 2008; 104:210–220. [PubMed: 17986218]
44. Klafki H, Abramowski D, Swoboda R, Paganetti PA, Staufenbiel M. The carboxyl termini of beta-amyloid peptides 1–40 and 1–42 are generated by distinct gamma-secretase activities. *J Biol Chem*. 1996; 271:28655–28659. [PubMed: 8910499]
45. Tarlton JF, Knight PJ. Comparison of reflectance and transmission densitometry, using document and laser scanners, for quantitation of stained Western blots. *Anal Biochem*. 1996; 237:123–128. [PubMed: 8660546]

46. Osenkowski P, Li H, Ye W, Li D, Aeschbach L, Fraering PC, Wolfe MS, Selkoe DJ, Li H. Cryoelectron microscopy structure of purified gamma-secretase at 12 Å resolution. *J Mol Biol.* 2009; 385:642–652. [PubMed: 19013469]
47. Osenkowski P, Ye W, Wang R, Wolfe MS, Selkoe DJ. Direct and potent regulation of gamma-secretase by its lipid microenvironment. *J Biol Chem.* 2008; 283:22529–22540. [PubMed: 18539594]
48. Fraering PC, Ye W, Strub JM, Dolios G, LaVoie MJ, Ostaszewski BL, Van Dorsselaer A, Wang R, Selkoe DJ, Wolfe MS. Purification and Characterization of the Human gamma-Secretase Complex. *Biochemistry.* 2004; 43:9774–9789. [PubMed: 15274632]
49. Moehlmann T, Winkler E, Xia X, Edbauer D, Murrell J, Capell A, Kaether C, Zheng H, Ghetti B, Haass C, Steiner H. Presenilin-1 mutations of leucine 166 equally affect the generation of the Notch and APP intracellular domains independent of their effect on Abeta 42 production. *Proc Natl Acad Sci U S A.* 2002; 99:8025–8030. [PubMed: 12048239]
50. Bentahir M, Nyabi O, Verhamme J, Tolia A, Horre K, Wiltfang J, Esselmann H, De Strooper B. Presenilin clinical mutations can affect gamma-secretase activity by different mechanisms. *J Neurochem.* 2006; 96:732–742. [PubMed: 16405513]
51. Steiner H, Kostka M, Romig H, Basset G, Pesold B, Hardy JA, Capell A, Meyn L, Grim MG, Baumeister R, Fichteler K, Haass C. Glycine 384 is required for presenilin-1 function and is conserved in bacterial polytopic aspartyl proteases. *Nature Cell Biol.* 2000; 2:848–851. [PubMed: 11056541]
52. De Jonghe C, Cras P, Vanderstichele H, Cruts M, Vanderhoeven I, Smouts I, Vanmechelen E, Martin JJ, Hendriks L, Van Broeckhoven C. Evidence that Abeta42 plasma levels in presenilin-1 mutation carriers do not allow for prediction of their clinical phenotype. *Neurobiol Dis.* 1999; 6:280–287. [PubMed: 10448055]
53. Cruts M, Backhovens H, Wang SY, Van Gassen G, Theuns J, De Jonghe CD, Wehnert A, De Voecht J, De Winter G, Cras P, et al. Molecular genetic analysis of familial early-onset Alzheimer's disease linked to chromosome 14q24.3. *Hum Mol Genet.* 1995; 4:2363–2371. [PubMed: 8634711]
54. Sherrington R, Rogaev EI, Liang Y, Rogaeva EA, Levesque G, Ikeda M, Chi H, Lin C, Li G, Holman K, et al. Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature.* 1995; 375:754–760. [PubMed: 7596406]
55. Capell A, Grunberg J, Pesold B, Diehlmann A, Citron M, Nixon R, Beyreuther K, Selkoe DJ, Haass C. The proteolytic fragments of the Alzheimer's disease-associated presenilin-1 form heterodimers and occur as a 100–150-kDa molecular mass complex. *J Biol Chem.* 1998; 273:3205–3211. [PubMed: 9452432]
56. Zhang J, Kang DE, Xia W, Okochi M, Mori H, Selkoe DJ, Koo EH. Subcellular distribution and turnover of presenilins in transfected cells. *J Biol Chem.* 1998; 273:12436–12442. [PubMed: 9575200]
57. Baumeister R, Leimer U, Zweckbronner I, Jakubek C, Grünberg J, Haass C. Human presenilin-1, but not familial Alzheimer's disease (FAD) mutants, facilitate *Caenorhabditis elegans* Notch signalling independently of proteolytic processing. *Genes and Function.* 1997; 1:149–159. [PubMed: 9680315]
58. Kuperstein I, Broersen K, Benilova I, Rozenski J, Jonckheere W, Debulpaep M, Vandersteen A, Segers-Nolten I, Van Der Werf K, Subramaniam V, Braeken D, Callewaert G, Bartic C, D'Hooge R, Martins IC, Rousseau F, Schymkowitz J, De Strooper B. Neurotoxicity of Alzheimer's disease Abeta peptides is induced by small changes in the Abeta42 to Abeta40 ratio. *Embo J.* 2010; 29:3408–3420. [PubMed: 20818335]
59. Maruyama K, Tomita T, Shinozaki K, Kume H, Asada H, Saido TC, Ishiura S, Iwatsubo T, Obata K. Familial Alzheimer's disease-linked mutations at Val717 of amyloid precursor protein are specific for the increased secretion of A beta 42(43). *Biochem Biophys Res Commun.* 1996; 227:730–735. [PubMed: 8886002]
60. Kounnas MZ, Danks AM, Cheng S, Tyree C, Ackerman E, Zhang X, Ahn K, Nguyen P, Comer D, Mao L, Yu C, Pleynt D, Digregorio PJ, Velicelebi G, Stauderman KA, Comer WT, Mobley WC, Li YM, Sisodia SS, Tanzi RE, Wagner SL. Modulation of gamma-secretase reduces beta-amyloid

deposition in a transgenic mouse model of Alzheimer's disease. *Neuron*. 2010; 67:769–780. [PubMed: 20826309]

61. Tokuda T, Fukushima T, Ikeda S, Sekijima Y, Shoji S, Yanagisawa N, Tamaoka A. Plasma levels of amyloid beta proteins Abeta1–40 and Abeta1–42(43) are elevated in Down's syndrome. *Ann Neurol*. 1997; 41:271–273. [PubMed: 9029078]
62. Czirri E, Cottrell BA, Leuchtenberger S, Kukar T, Ladd TB, Esselmann H, Paul S, Schubnel R, Torpey JW, Pietrzik CU, Golde TE, Wiltfang J, Baumann K, Koo EH, Weggen S. Independent generation of Abeta42 and Abeta38 peptide species by gamma-secretase. *J Biol Chem*. 2008; 283:17049–17054. [PubMed: 18426795]
63. Saito T, Suemoto T, Brouwers N, Slegers K, Funamoto S, Mihira N, Matsuba Y, Yamada K, Nilsson P, Takano J, Nishimura M, Iwata N, Van Broeckhoven C, Ihara Y, Saido TC. Potent amyloidogenicity and pathogenicity of Abeta43. *Nat Neurosci*. 2011 in press.
64. Walker ES, Martinez M, Brunkan AL, Goate A. Presenilin 2 familial Alzheimer's disease mutations result in partial loss of function and dramatic changes in Abeta 42/40 ratios. *J Neurochem*. 2005; 92:294–301. [PubMed: 15663477]
65. Kumar-Singh S, Theuns J, Van Broeck B, Pirici D, Vennekens K, Corsmit E, Cruts M, Dermaut B, Wang R, Van Broeckhoven C. Mean age-of-onset of familial Alzheimer disease caused by presenilin mutations correlates with both increased Abeta42 and decreased Abeta40. *Hum Mutat*. 2006; 27:686–695. [PubMed: 16752394]
66. Shimojo M, Sahara N, Murayama M, Ichinose H, Takashima A. Decreased Abeta secretion by cells expressing familial Alzheimer's disease-linked mutant presenilin 1. *Neurosci Res*. 2007; 57:446–453. [PubMed: 17210196]
67. Fluhner R, Fukumori A, Martin L, Grammer G, Haug-Kroper M, Klier B, Winkler E, Kremmer E, Condron MM, Teplow DB, Steiner H, Haass C. Intramembrane proteolysis of GXGD-type aspartyl proteases is slowed by a familial Alzheimer disease-like mutation. *J Biol Chem*. 2008; 283:30121–30128. [PubMed: 18768471]
68. Page RM, Baumann K, Tomioka M, Perez-Revuelta BI, Fukumori A, Jacobsen H, Flohr A, Luebbbers T, Ozmen L, Steiner H, Haass C. Generation of Abeta38 and Abeta42 is independently and differentially affected by familial Alzheimer disease-associated presenilin mutations and gamma-secretase modulation. *J Biol Chem*. 2008; 283:677–683. [PubMed: 17962197]
69. Kretner B, Fukumori A, Gutsmedl A, Page RM, Luebbbers T, Galley G, Baumann K, Haass C, Steiner H. Attenuated A{beta}42 Responses to Low Potency {gamma}-Secretase Modulators Can Be Overcome for Many Pathogenic Presenilin Mutants by Second-generation Compounds. *J Biol Chem*. 2011; 286:15240–15251. [PubMed: 21357415]
70. Qi Y, Morishima-Kawashima M, Sato T, Mitsumori R, Ihara Y. Distinct mechanisms by mutant presenilin 1 and 2 leading to increased intracellular levels of amyloid beta-protein 42 in Chinese hamster ovary cells. *Biochemistry*. 2003; 42:1042–1052. [PubMed: 12549925]
71. Jankowsky JL, Fadale DJ, Anderson J, Xu GM, Gonzales V, Jenkins NA, Copeland NG, Lee MK, Younkin LH, Wagner SL, Younkin SG, Borchelt DR. Mutant presenilins specifically elevate the levels of the 42 residue beta-amyloid peptide in vivo: evidence for augmentation of a 42-specific gamma secretase. *Hum Mol Genet*. 2004; 13:159–170. [PubMed: 14645205]
72. Winkler E, Hobson S, Fukumori A, Dumpelfeld B, Luebbbers T, Baumann K, Haass C, Hopf C, Steiner H. Purification, pharmacological modulation, and biochemical characterization of interactors of endogenous human gamma-secretase. *Biochemistry*. 2009; 48:1183–1197. [PubMed: 19159235]
73. Fukumori A, Fluhner R, Steiner H, Haass C. Three-amino acid spacing of presenilin endoproteolysis suggests a general stepwise cleavage of gamma-secretase-mediated intramembrane proteolysis. *J Neurosci*. 2010; 30:7853–7862. [PubMed: 20534834]
74. Haapasalo A, Kovacs DM. The Many Substrates of Presenilin/gamma-Secretase. *J Alzheimers Dis*. 2011 E-pub ahead of print.
75. Okochi M, Fukumori A, Jiang J, Itoh N, Kimura R, Steiner H, Haass C, Tagami S, Takeda M. Secretion of the Notch-1 Abeta-like peptide during Notch signaling. *J Biol Chem*. 2006; 281:7890–7898. [PubMed: 16434391]

Figure 1. Effects of pH on γ -secretase activity and processivity

γ -Secretase assays were performed from pH 5.5 to 9.0. In order to span this range, three buffering agents were used: MES (pH 5.5, 6.0, and 6.5), HEPES (pH 7.0, 7.5, and 8.0), and bicine (pH 8.0, 8.5, and 9.0). A) The total activity of γ -secretase was measured using anti-FLAG Western blotting to detect AICD-FLAG (inset). Bands were quantified and normalized to HEPES buffer pH 7.0. At pH 8.0 the data from HEPES buffer is displayed, although bicine at pH 8.0 gave the same result (Fig S1). Starred columns are significantly different from pH 7.0. B) The A β fragments produced in these assays were analyzed on bicine/urea gels (upper) and identified using known A β standards (Std). Quantitation of each band allowed for determination of the relative contribution of each of the A β species

(lower). The data at pH 8.0 is from the HEPES buffered assay. The bands for pH 9.0 were too faint to be reliably quantified. C) A β 38 and A β 46+ show significant changes in contribution to total A β with changing pH. Statistical significance was determined by the unpaired two-tailed student t-test. $P < 0.05$. The data is the average from three independent experiments.

Figure 2. Effects of salinity on γ -secretase activity and processivity

Shown are γ -secretase assays performed with 0 to 150 mM NaCl or Na₂SO₄. A) The total activity of γ -secretase was measured using anti-FLAG Western blotting to detect AICD-FLAG (inset). Bands were quantified and normalized to 150 mM salt. Starred columns are significantly different from 150 mM salt. B) The A β fragments produced in these assays were analyzed on bicine/urea gels (upper). Quantitation of each band allowed for determination of the relative contribution of each of the A β species (lower). C) A β 46+ shows significant changes in contribution to total A β . Starred column is significantly different from 150 mM NaCl. Statistical significance was determined by the unpaired two-tailed student t-test. $P < 0.05$. The data is the average from two independent experiments.

Figure 3. Effects of temperature on γ -secretase activity and processivity

Shown are results from γ -secretase assays performed between 20 °C and 55 °C. A) The total activity of γ -secretase was measured using anti-FLAG Western blotting to detect AICD-FLAG (inset). Bands were quantified and normalized to 35 °C. Starred columns are significantly different from 35 °C. B) The A β fragments produced in these assays were analyzed on bicine/urea gels (upper). Quantitation of each band allowed for determination of the relative contribution of each of the A β species (lower). C) A β 40 and A β 43 show significant changes in contribution to total A β . Statistical significance was determined by the unpaired two-tailed student t-test. $P < 0.05$. The data is the average of two independent experiments.

Figure 4. PS1 FAD mutants differ in their rate of AICD formation

A) Schematic of location and age of onset for PS1 FAD mutants used in this study. B) Solubilized membranes containing PS1 FAD mutants were normalized for levels of NTF and analyzed by Western blot for other γ -secretase components and presenilin endoproteolysis. From top to bottom: anti-GST to detect GST-nicastrin, anti-presenilin N-terminal fragment (NTF) to detect full-length presenilin and NTF, and anti-presenilin C-terminal fragment (CTF) to detect both exogenous human and endogenous hamster CTF. C) Equal amounts of wild-type and FAD mutant γ -secretase were included in C99 activity assays, and total activity was assessed using anti-FLAG Western blotting to detect AICD-FLAG (lower blot). Bands were quantified and normalized to a wild-type PS1 control (upper blot). All PS1 FAD mutants, except L286V, show significant statistical difference relative to wild type (graph, starred columns). Statistical significance was determined by the unpaired two-tailed student t-test. $P < 0.05$. The data is the average of three independent experiments.

Figure 5. FAD mutations result in large differences in Aβ distribution

The Aβ peptides produced by each FAD-mutant and wild type γ-secretase were separated on bicine/urea SDS-PAGE and visualized by anti-Aβ 6E10 Western blotting. * Denotes band artifacts as previously reported (ref). B) Quantitation of each band allowed for determination of the relative contribution of each of the Aβ species. C) Comparison of changes in Aβ40 and Aβ42+Aβ43 reveals PS1 FAD mutants that decrease Aβ40, increase Aβ42+Aβ43, or both. Starred columns are significantly different from wild-type. Statistical significance was determined by the unpaired two-tailed student t-test. $P < 0.05$. The data is the average of four independent experiments.

Figure 6. Lack of correlation between total γ -secretase activity and the general distribution of A β s

A) The processivity index (sum of short A β 38 and A β 40, divided by the sum of long A β s, A β 42 to A β 46+) was plotted in order of total activity for each FAD mutant. B) The processivity index and total activity was plotted for the γ -secretase assays run at different temperatures.