BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

pubs.acs.org/biochemistry

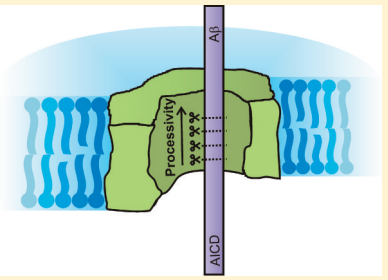
Dissociation between the Processivity and Total Activity of γ-Secretase: Implications for the Mechanism of Alzheimer's Disease-Causing Presenilin Mutations

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, United States

Supporting Information

ABSTRACT: The amyloid β-peptide (Aβ), 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 Aβ production and increase the ratio of the aggregation-prone 42-residue form (Aβ42) to the 40-residue form (Aβ40). The connection between these two effects is not understood. Besides Aβ40 and Aβ42, γ-secretase produces a range of Aβ peptides, the result of initial cutting at the ε site to form Aβ48 or Aβ49 and subsequent trimming every three or four 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 the overall activity of the wild-type enzyme did not consistently result in increased proportions of longer Aβ peptides. Low salt concentrations and acidic pH were notable exceptions that subtly alter the proportion of individual Aβ 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, Aβ42:Aβ40 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 Aβ peptides.



The 4-Da amyloid β-peptide (Aβ) 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 Aβ peptides varying at their C-termini. As the C-terminus is derived from the transmembrane domain, longer Aβ peptides contain more hydrophobic residues and are more prone to aggregation.[5] Soluble aggregates of Aβ 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 Aβ production, increasing either the total amount of Aβ or the ratio of the 42-residue Aβ species (Aβ42) to the otherwise predominant 40-residue form (Aβ40).[9] Thus, although Aβ is normally produced in the brains of all individuals, aggregation of slightly longer forms, particularly the 42-residue form (Aβ42), is thought to initiate AD pathogenesis. Therefore, a full understanding of how these longer Aβ 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 in which C99 is processively cleaved by γ-secretase. Initial proteolysis at the ε site primarily produces the 50-residue APP intracellular domain

Received: May 9, 2011
Revised: September 14, 2011
Published: September 15, 2011

(AICD)[27] and Aβ49;[28] however, γ-secretase can also cut at an alternative ε site nearby to produce a 51-residue AICD[29] and Aβ48.[28,30] After the ε cut, Aβ48 and Aβ49 are trimmed every three or four residues, creating the C-termini of the other Aβ peptides, ranging from 38 to 46 residues in length.[30] There is threefold evidence of γ-secretase trimming, or processivity. First, longer AICDs that would complement the shorter Aβ peptides from single cleavage events have not been detected. Second, the Aβ peptides created by ε cleavage, Aβ48 and Aβ49, can be processed by γ-secretase into shorter Aβ peptides, including Aβ40 and Aβ42.[31] Third, Takami et al. recently identified tri- and tetrapeptides produced by γ-secretase cleavage of APP that 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, respectively) result in an increase in the Aβ42:Aβ40 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 Aβ42:Aβ40 ratio, leading to both a reduction in the overall activity and a gain in the proportion of toxic Aβ species.[40,41] That is, a less active protease may lead to slower trimming, with more opportunity for longer forms of Aβ to be released from the enzyme before further trimming.

Here we report testing of 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 Aβ peptides produced. We conclude from these investigations that activity and processivity can be dissociated. A shift in the proportion of Aβ 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 Aβ peptides. That is, factors that affect overall activity differ from factors that affect trimming to alter the proportion of Aβ peptides.

EXPERIMENTAL PROCEDURES

Plasmids and Cell Lines. The pAph1HA plasmid carrying the wild-type Aph-1α2 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). The A246E mutant was a generous gift from the laboratory of D. Selkoe. Other FAD mutations have previously been described (L286V and ΔE9[35]). The coding sequences were then amplified via polymerase chain reaction 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 Aph-1α2 and nicastrin-V5/His, was 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 μg/mL hygromycin, 150 μg/mL G418, and 10 μg/mL blasticidin. Finally, cell lines overexpressing 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 μg/mL puromycin to the C-20 medium.

Substrate Purification. SUMO-C99-Flag protein was expressed in *Escherichia coli* BL21(DE3). Bacteria were grown to log phase at 37 °C and induced with 1 mM 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, and 1% Triton X-100, and lysed by being passed 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, and 1 mM DTT, and the SUMO tag was removed by overnight digestion at 30 °C with SUMO protease (Invitrogen). C99-Flag was eluted from M2 beads by boiling in reducing sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) sample buffer for 5 min and then loaded on preparative Criterion 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 with a French press in 50 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES) buffer (pH 6.0), 150 mM NaCl, 5 mM $MgCl_2$, and 5 mM $CaCl_2$ with complete protease inhibitors (F. Hoffmann-La Roche, Basel, Switzerland). Nuclei and unbroken cells were removed by centrifugation at 3000*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 being passed through a 27.5 gauge needle in 160 μL (WT or mutants) or 2 mL (S-20) of Hepes buffer A [50 mM Hepes (pH 7.0), 150 mM NaCl, 5 mM $MgCl_2$, and 5 mM $CaCl_2$] with 1% CHAPSO. Insoluble membranes were removed by centrifugation 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% CHAPSO (final

concentration). Lipids dissolved in chloroform for the assays were dried under nitrogen and rehydrated in 1% CHAPSO Hepes buffer A. All reactions were stopped when the mixtures were snap-frozen 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 at varying pH values, 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) was substituted for Hepes buffer A in the assay protocol. These buffers contained 150 mM NaCl. For reactions at varying salt concentrations, 50 mM Hepes (pH 7.0) with 0−300 mM NaCl or $NaSO_4$, at 50 mM increments, was substituted for Hepes buffer A. The temperature was varied in a gradient-capable thermal cycler (MyCycler, Bio-Rad) from 15 to 65 °C, with standard conditions of pH (7.0) and salt (150 mM NaCl) held constant. All pH, salt, and temperature reaction mixtures were prepared with a final CHAPSO concentration of 0.25%.

Gel Electrophoresis and Western Blotting. For analysis of AICD, samples were run on Criterion 12% Bis-Tris gels (Bio-Rad) using MES running buffer. To exclude the uncut substrate from further steps, the gel was cut on the basis of a BenchMark prestained protein ladder and Novex Sharp prestained protein ladder (Invitrogen) and transferred to a PVDF membrane at 400 mA for 2 h on ice. Membranes were blocked in a 6% milk/PBST mixture for 5 min followed by incubation for 1 h with the primary M2 anti-FLAG antibody (Sigma-Aldrich) (1:1000 in blocking buffer) and 45 min with secondary anti-mouse HRP (GE Healthcare Biosciences, Pittsburgh, PA) (1:10000 in PBST). Bands were illuminated using ECL Plus (GE Healthcare Biosciences) and captured on film.

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 as follows. 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 the separating gel and 11 cm for the spacing gel. Bicine/urea gels were run at 12 mA for 1 h and then at 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 being boiled in PBS for 5 min. The membrane was blocked in an 8% milk/PBST mixture for 1 h, washed in PBST overnight, and then incubated with primary antibody 6E10 (Sigma-Aldrich) 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 to 20% gels (Invitrogen) and transferred to a 0.2 μm PVDF membrane for 75 min at 180 mA. Membranes were blocked in a 6% milk/PBST mixture and probed with antibodies 1563 for PS1-NTF (Chemicon, Millipore, Billerica, MA) (1:1000), 13A11 for PS1-CTF (Donated by Elan Corp., Dublin, Ireland (1:1,000), anti-HA for Aph-1α2-HA (F. Hoffmann-La Roche) (1:2,000), anti-Flag M2 for Flag-Pen-2 (Sigma-Aldrich) (1:1000), and anti-GST for NCT-GST (Sigma-Aldrich) (1:3,000). Secondary HRP-conjugated antibodies were diluted 1:10,000.

Quantitation of Films. All films were preflashed with diffuse white light before being exposed to the ECL signal. To quantitate Western signals from film, correction for the error introduced by flatbed scanning of the films must be made. 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 versus exposure time. From these data, dose−response curves followed the exponential equation:

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

where y is the pixel intensity on a scale of 0−255, x is the exposure time, and y_{max} is the calculated maximum value of y (243.8 under our conditions). A curve fit was generated using Sigmaplot (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

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

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

$$y_{corr}' = ay_{corr} = a \times \log\left(\frac{y_{max}}{y_{max} - y}\right) \quad (3)$$

Factor a equaled 88.7 under our experimental conditions. ImageJ's mathematical macro function (National Institutes of Health, Bethesda, MD) was used to process each scanned image according to eq 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 (Bio-Rad).

RESULTS

We first explored whether a change in overall activity correlates with a change in processivity by measuring AICD and the various forms of Aβ produced by wild-type γ-secretase complexes under different conditions. In particular, do conditions that lower the level of AICD production change in parallel with increased proportions of longer forms of Aβ at the expense of shorter forms? 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 Aβs 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 are 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 Aβ peptides identical to those produced in cells, and standard synthetic Aβ 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 Aβ peptides[30,44] to allow for the simultaneous analysis of Aβ38, -40, -42, -43, -45, and -46+ (unresolved Aβ46, -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 Aβ antibodies and can comigrate with and obscure shorter Aβ 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 Aβ peptides, and as a result, we were able to reproducibly resolve Aβ38 from excess substrate. Note that under these conditions, longer Aβ 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 Aβ species failed to provide reliable linear production with time for the longer Aβ forms (larger than Aβ42). Therefore, we chose to monitor Aβ production in a steady-state end point fashion. Under these in vitro conditions, time course experiments demonstrated that the formation of products becomes nonlinear after incubation for 6 h at 37 °C for 1 μM 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 Aβ peptides. Thus, the substrate concentration was in constant excess during the time frame of our analysis.

For our first attempt to identify 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 pH values ranging from 5.5 to 9.0. To achieve these pH values, different buffers were needed: MES for pH 5.5−6.5, HEPES for pH 7.0−8.0, and bicine for pH 8.5−9.0. 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 high pH values. 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 Aβ peptides (Figure S1 of the Supporting Information and data not shown, respectively). Generated Aβ peptides were separated and detected using our modified procedure; each band was carefully measured by densitometry (see Experimental Procedures), and the proportions of the individual Aβ peptides were graphed (Figure 1B and Figure S2A of the Supporting Information). No statistically significant differences were seen for any of the specific Aβ peptides at the various pH values with the exception of Aβ38 and Aβ46+. The proportion of Aβ38 was decreased at pH 5.5 and 6.0 as compared to that at higher pH values, and the proportion of Aβ46+ was increased at pH 8.5 compared to that at pH 7.0 (regraphed for the sake of clarity and with error bars in Figure 1C). Comparison of these changes in Aβ to the changes in total activity shows discordance; at high pH values where there is a substantial reduction in activity, there appears to be both an increase and a decrease in processivity (more Aβ38 and more Aβ46+, respectively). A small reduction in processivity (less Aβ38) is seen at mildly acidic pH values, 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 at different concentrations and in different types of salts. Under otherwise standard conditions [0.25% CHAPSO in HEPES buffer (pH 7.0) at 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 Aβ peptides of ≥42 residues precipitated (i.e., were salted out), even with a mixture of standard Aβ 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 NaCl (Figure 2A, shown only up to 150 mM), while activity was dramatically reduced in the absence of NaCl. In examining the range of Aβ peptides produced, again we found no significant differences in the amounts or proportion of the individual Aβ 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 Aβ peptides with ≥46 residues was observed to more than double compared to that seen in the presence of 150 mM NaCl (Figure 2C and Figure S2B of the Supporting Information). These same experiments were also conducted using Na_2SO_4. Similar to what was seen with NaCl, AICD production was statistically equivalent over the higher Na_2SO_4 concentrations ranging from 150 to 300 mM (data not shown). Again, concentrations of >300 mM led to precipitation of longer Aβ peptides, even from a standard mixture of the peptides alone, and could not be tested for effects on

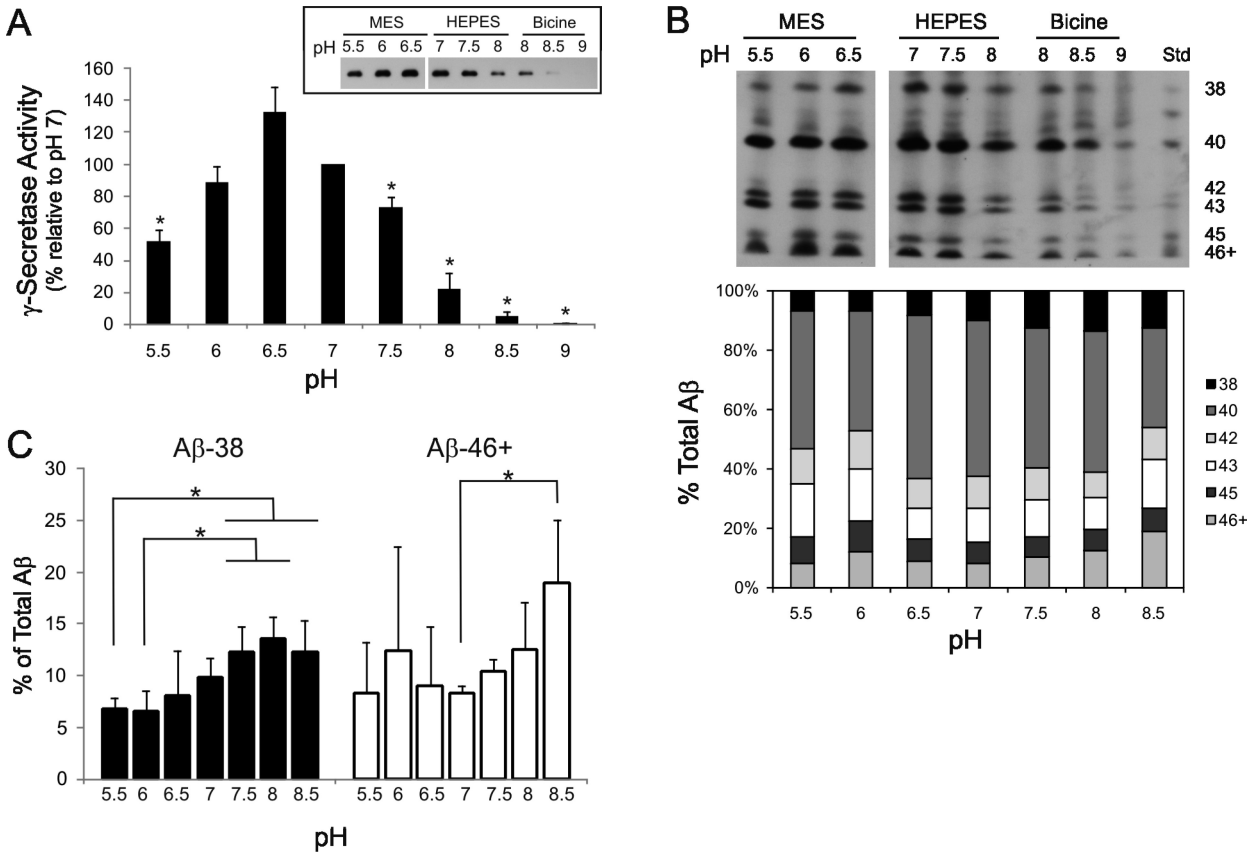


Figure 1. Effects of pH on γ-secretase activity and processivity. γ-Secretase assays were performed from pH 5.5 to 9.0. To span this range, we used three buffering agents: MES at pH 5.5, 6.0, and 6.5; HEPES at pH 7.0, 7.5, and 8.0; and bicine at pH 8.0, 8.5, and 9.0. (A) The total activity of γ-secretase 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 for HEPES buffer are displayed, although bicine at pH 8.0 gave the same result (Figure S1 of the Supporting Information). Asterisks denote values that are significantly different from the pH 7.0 value. (B) The Aβ fragments produced in these assays were analyzed on bicine/urea gels (top) and identified using known Aβ standards (Std). Quantitation of each band allowed for determination of the relative contribution of each of the Aβ species (bottom). The data at pH 8.0 are from the HEPES buffer assay. The bands for pH 9.0 were too faint to be reliably quantified. (C) Aβ38 and Aβ46+ show significant changes in their contribution to total Aβ with a change in pH. Statistical significance was determined by the unpaired two-tailed Student's *t* test ($P < 0.05$). The data are averages from three independent experiments.

γ-secretase activity. In this case, lower concentrations of Na_2SO_4 (50 mM) did show substantially reduced overall proteolytic activity, although it was still higher than that seen without added salt (Figure 2A). The reasons behind the subtle differences in concentration-dependent effects of NaCl and Na_2SO_4 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 Na_2SO_4, the proportion of the various Aβ peptides was not significantly different between the enzyme reactions conducted in 150 mM Na_2SO_4 (Figure 2B,C and Figure S2B of the Supporting Information). The only difference observed was again found with Aβ peptides with ≥46 residues when comparing no added salt to added Na_2SO_4 (Figure 2C). Thus, no added 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 conducted under the standard conditions of pH (7.0) and salt concentration (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 surprisingly, 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, the activity seems to decrease quite rapidly above 50 °C, with little activity seen at 55 °C. A more gradual decrease in activity was observed for temperatures of <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 Aβs produced at these different temperatures, we noted changes in the distribution of many Aβ peptides (Figure 3B and Figure S2C of the Supporting Information). Interestingly, although the longest forms of Aβ (≥46 residues) were found in greater proportions at the temperature extremes (i.e., with the lowest overall activities), the short forms (Aβ38 and Aβ40) were found in smaller proportions as the temperature was increased over 35 °C but did not change when the activity was impaired by lowering the temperature (Figure 3C and Figure S2C of the Supporting Information). Strikingly, the proportions of Aβ42 and Aβ43 increased with temperature throughout almost the entire range (Figure 3C and Figure S2 of the Supporting Information), showing no correlation with the total activity profile generated

dx.doi.org/10.1021/bi2007146 | *Biochemistry* 2011, 50, 9023–9035

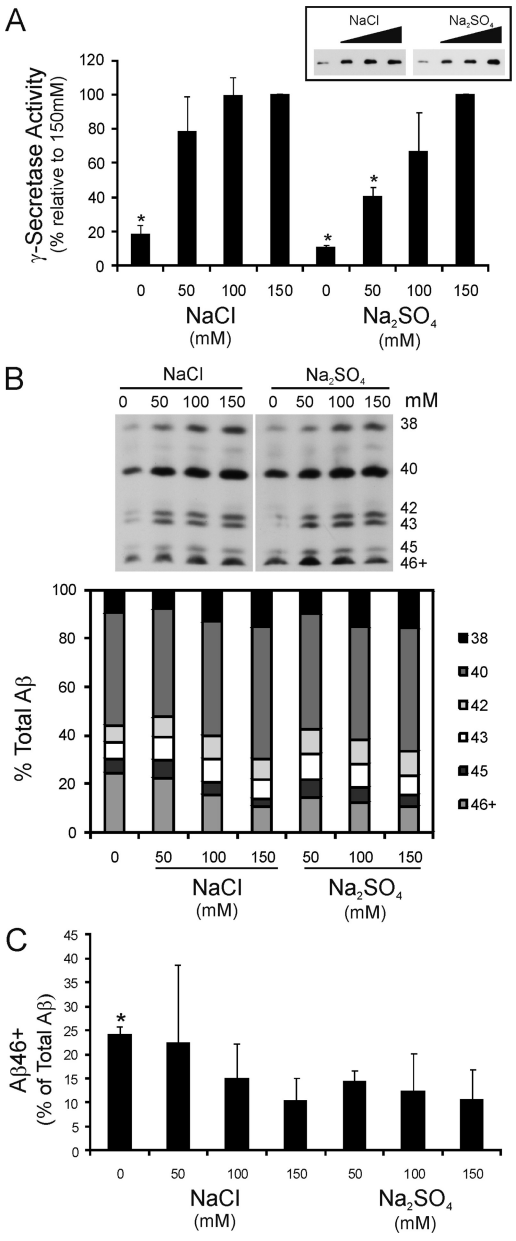


Figure 2. Effects of salinity on γ-secretase activity and processivity. Shown are γ-secretase assays performed with 0−150 mM NaCl or Na_2SO_4. (A) The total activity of γ-secretase was measured using anti-FLAG Western blotting to detect AICD-FLAG (inset). Bands were quantified and normalized to 150 mM salt. Asterisks denote columns that are significantly different from the 150 mM salt column. (B) The Aβ fragments produced in these assays were analyzed on bicine/urea gels (top). Quantitation of each band allowed for determination of the relative contribution of each of the Aβ species (bottom). (C) Aβ46+ shows significant changes in contribution to total Aβ. The asterisk denotes a value that is significantly different from the 150 mM NaCl value. Statistical significance was determined by the unpaired two-tailed Student's t test ($P < 0.05$). The data are averages from two independent experiments.

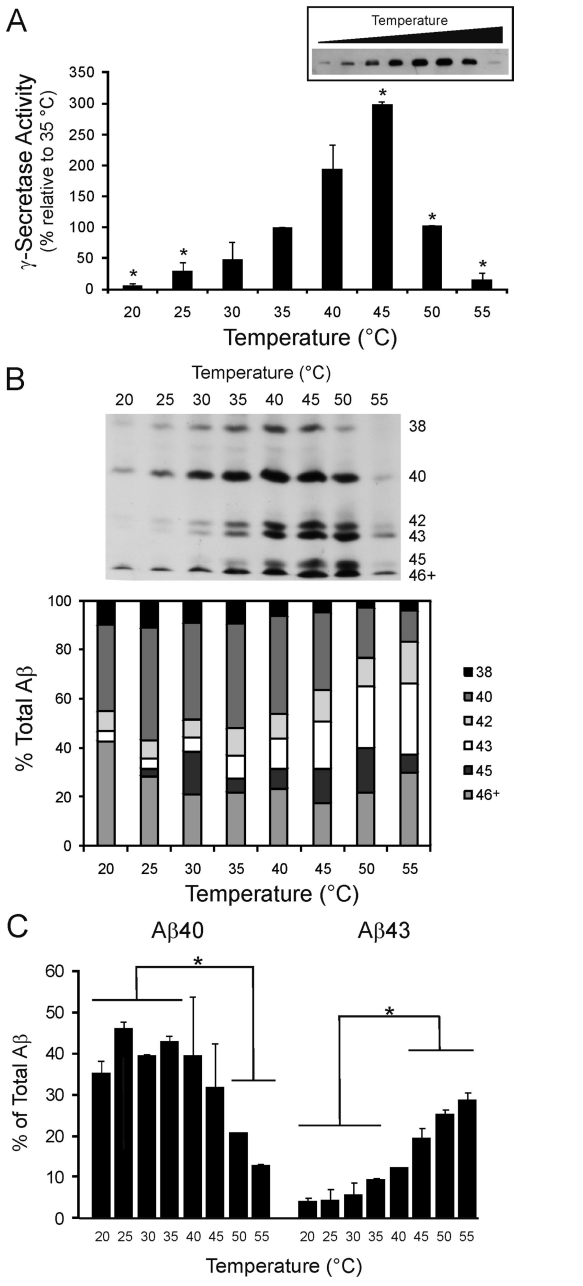


Figure 3. Effects of temperature on γ-secretase activity and processivity. Shown are results from γ-secretase assays performed between 20 and 55 °C. (A) The total activity of γ-secretase was measured using anti-FLAG Western blotting to detect AICD-FLAG (inset). Bands were quantified and normalized to 35 °C. Asterisks denote values that are significantly different from the 35 °C value. (B) The Aβ fragments produced in these assays were analyzed on bicine/urea gels (top). Quantitation of each band allowed for determination of the relative contribution of each of the Aβ species (bottom). (C) Aβ40 and Aβ43 show significant changes in their contribution to total Aβ. Statistical significance was determined by the unpaired two-tailed Student's t test ($P < 0.05$). The data are averages of two independent experiments.

with AICD. Furthermore, examination of the Aβ spectra at the temperature that gave maximal activity (45 °C) reveals a shift toward aggregation-prone Aβ43 in direct opposition to the hypothesis that it is the loss of γ-secretase activity that is responsible for transition to the longer Aβ forms. Thus, again we noted dissociation between activity and processivity; lower activities did not correlate with a greater proportion of longer forms of Aβ. It is important to point out that we did not observe precipitation or loss of Aβ peptides from a standard mixture at extremes of pH or temperature or without added NaCl or Na_2SO_4, nor did APP substrate precipitate or display an increased level of aggregation (data not shown). That is, the

dx.doi.org/10.1021/bi2007146 | Biochemistry 2011, 50, 9023–9035

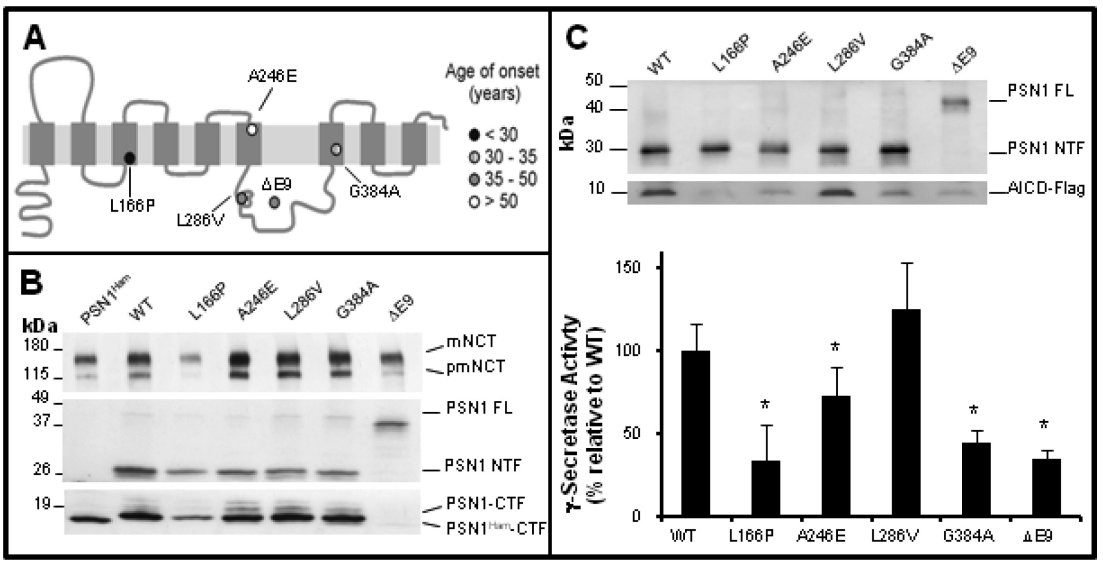


Figure 4. PS1 FAD mutants differ in their rates of AICD formation. (A) Schematic of the location and age of onset for PS1 FAD mutants used in this study. (B) Solubilized membranes containing PS1 FAD mutants were normalized for levels of NTF and analyzed by Western blotting for other γ-secretase components and presenilin endoproteolysis. From top to bottom: anti-GST for detecting GST-nicastrin, anti-presenilin N-terminal fragment (NTF) for detecting full-length presenilin and NTF, and anti-presenilin C-terminal fragment (CTF) for detecting 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 (bottom blot). Bands were quantified and normalized to a wild-type PS1 control (top blot). All PS1 FAD mutants, except L286V, show significant statistical difference relative to the wild type (graph, columns with asterisks). Statistical significance was determined by the unpaired two-tailed Student's *t* test ($P < 0.05$). The data are averages of three independent experiments.

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 Aβ42 to Aβ40 and also decrease overall proteolytic activity. We asked whether γ-secretase complexes containing PS1 mutations associated with familial, early-onset AD (FAD) indeed decrease the overall level of 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 ΔE9 (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 Aβ42:Aβ40 ratios and ages of disease onset (Figure 4A). For instance, the L166P mutation, which resulted in disease onset at 24 years of age, and the G384A mutation, which has an average age of onset of 34 years, have been reported to cause large increases in the Aβ42:Aβ40 ratio.[49−53] In contrast, the A246E and L286V mutations are associated with more subtle effects on the Aβ42:Aβ40 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 the active form.[18−20] Indeed, this endoproteolytic event occurs only 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 ΔE9, 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 ΔE9 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 ΔE9 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 mixture via adjustment for amounts of PS1 NTF (or full-length protein in the case of the ΔE9 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 that of the 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 ΔE9, nor could we distinguish

dx.doi.org/10.1021/bi2007146 | *Biochemistry* 2011, 50, 9023−9035

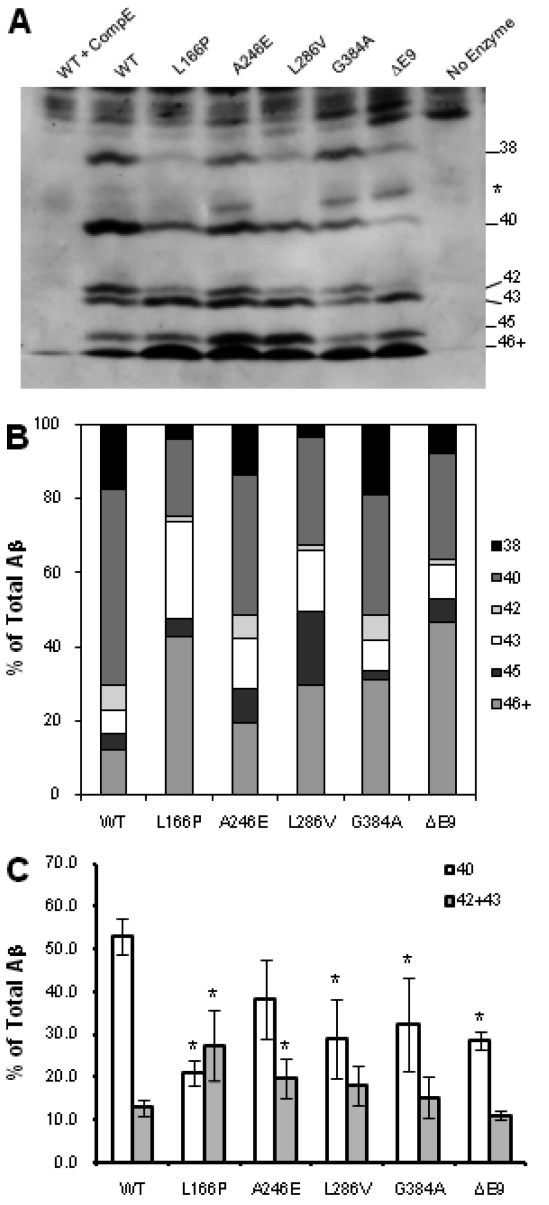


Figure 5. FAD mutations result in large differences in Aβ distribution. The Aβ peptides produced by each FAD mutant and wild-type γ-secretase were separated via bicine/urea SDS–PAGE and visualized by anti-Aβ 6E10 Western blotting. Asterisks denote band artifacts as previously reported.[30] (B) Quantitation 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 the level of Aβ40, increase the level of Aβ42 + Aβ43, or both. Asterisks denote values that are significantly different from the wild-type value. Statistical significance was determined by the unpaired two-tailed Student's t test ($P < 0.05$). The data are averages of four independent experiments.

differences between the wild-type enzyme and PS1 L286V mutation-containing enzyme.

The production of the spectrum of Aβ peptides by these various γ-secretase complexes was then examined by bicine/urea PAGE and Western blotting (Figure 5A). To compensate for the reduced overall activity of L166P, A246E, G384A, and ΔE9 and allow quantitation of all Aβ peptides (i.e., to obtain sufficiently strong bands), the loaded samples were normalized for total Aβ. This allowed facile comparison of changes in the proportions of the different Aβ species rather than confounding these changes with differences in total Aβ production, which mirror the differences in AICD production.[28] All of the mutant-containing complexes appear to increase the proportion of longer forms of Aβ ($\geq$42 residues). The PS1 L166P mutant protease showed the most dramatic effect (Figure 5B and Figure S3A of the Supporting Information). Complexes with PS1 A246E and G384A appear to have the smallest effects on processivity; the A246E mutant shows an increase in the level of Aβ43, and the G384A mutant shows a decrease in the levels of Aβ40 and Aβ45 and a concomitant increase in the level of $\geq$Aβ46. Complexes with PS1 ΔE9 and L286V displayed intermediate effects with an overall decrease in the level of Aβ peptides with $\leq$42 residues and an increase in the level of Aβ peptides with >42 residues.

PS1 FAD mutant γ-secretase complexes are known to increase the Aβ42:Aβ40 ratio compared to that of 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 Aβ40 and Aβ42. In our hands (data not shown) and as recognized previously,[59−62] these methods do not completely distinguish or separate Aβ43 from Aβ42. Moreover, a recent study has demonstrated that the frequently overlooked Aβ43 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 (Aβ42+Aβ43):Aβ40 ratio, which was significantly increased compared to that produced by the wild-type enzyme (Figure S3B of the Supporting Information). As previously reported, the ratio can be increased by a decrease in the level of Aβ40, an increase in the level of Aβ42 and Aβ43, or a decrease in both the level of Aβ40 and the level of Aβ42 and Aβ43 if Aβ40 decreases proportionately more.[50,64,65] Dissecting the cause for this increase in the ratio for our mutants shows three groupings: L286V, G384A, and ΔE9 have significantly decreased levels of Aβ40, A246E has a significantly increased level of Aβ42 and Aβ43, and L166P has both a decreased level of Aβ40 and an increased level of Aβ42 and Aβ43 (Figure 5C). These results are concordant with previous studies[49,50,65−72] that have shown G384A and L286V have reduced Aβ40 levels, A246E has increased Aβ42 levels, and L166P has both a decreased Aβ40 level and an increased Aβ42 level. For ΔE9, our results deviate from previous studies[49,52,65,68,71] in that we did not detect a significant increase in the level of Aβ42. However, consistent with these previous studies, we found that upon transfection of APP, the relative level of secreted Aβ42 compared to the total level of Aβ, as determined by specific ELISAs, is increased in the cell line stably expressing the PS1 ΔE9 mutant compared to the wild-type PS1-expressing cell line (data not shown). It is important to bear in mind that the Aβ secreted from cells is not all the Aβ 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 that of the wild-type enzyme but nevertheless displayed a clear decrease in processivity. In contrast, the PS1 G384A 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 short to long Aβ peptides, long being defined as ≥42 residues and short being the sum of Aβ38 and Aβ40, a ratio defined here as the "processivity index", with higher values indicating increased processivity. This value was plotted as a function of activity (Figure 6A). Clearly, no obvious correlation

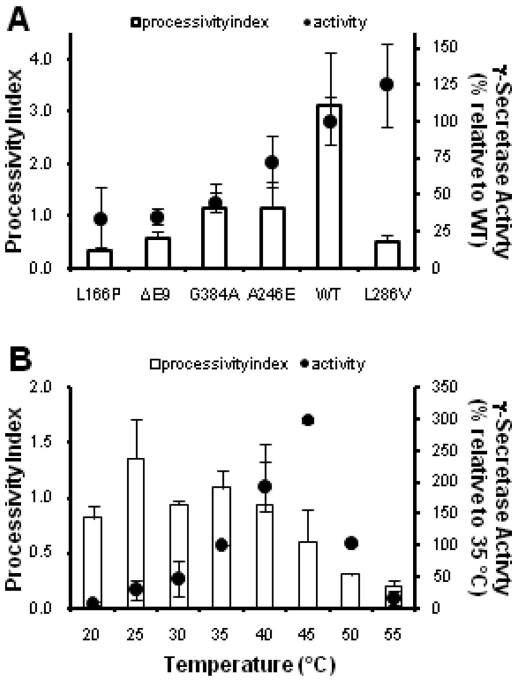


Figure 6. Lack of correlation between total γ-secretase activity and the general distribution of Aβs. (A) The processivity index (sum of short Aβ38 and Aβ40, divided by the sum of long Aβs, Aβ42 to Aβ46+) was plotted in order of total activity for each FAD mutant. (B) The processivity index and total activity were plotted for the γ-secretase assays conducted at different temperatures.

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

Until now, an analysis of all Aβ peptides produced from a variety of isolated AD mutant γ-secretase complexes and purified substrate had not been conducted. Almost all of the previous studies examining the effects of presenilin mutations on γ-secretase cleavage of APP involved detection of Aβ peptides and AICD generated in cells, raising concerns about cleavage events by other proteases, degradation of γ-secretase products, or retention of long Aβs 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 reducing 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 a reduced level of trimming of Aβ peptides (i.e., reduced processivity). In this way, a decrease in 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 about 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 pH, salt concentration, and temperature conditions, 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 Aβ38 and Aβ46+. As the pH was decreased from 6.5, again overall activity decreased, and for the most part, the proportion of individual Aβ peptides did not change, the sole exception being a clear decrease in the level of Aβ38. 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 Aβ peptides was observed. Only without added salt did we see a clear increase in the proportion of Aβ peptides with ≥46 residues (that is, a decrease in processivity). That mildly acidic pH and no added 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 Aβ peptides to short ones. Residues with pK_a values of ∼6 (e.g., histidines) would be reasonable candidates.

With respect to temperature, we observed that a temperature of 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 Aβ peptides are generally different.

To explore the properties of FAD mutant PS1-containing γ-secretase complexes in terms of 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 into 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 mutation. Although PS1 L286V mutation-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 Aβ 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 Aβ peptides, consistent with the connection between the formation of Aβ assemblies and the pathogenesis of AD. However, overall activity and processivity were again dissociated, not only with the L286V mutation but also with the

dx.doi.org/10.1021/bi2007146 | Biochemistry 2011, 50, 9023–9035

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 Aβ and AICD production in cell culture. For instance, Moehlmann and colleagues found a dissociation between AICD production and Aβ42:Aβ40 ratios with a variety of mutations of PS1 L166, including the pathogenic L166P mutation.[49] Moreover, Bentahir et al. used rescue experiments with presenilin-deficient cells to show that while several PS1 and PS2 FAD mutants all reduced the level of AICD production, these mutants had variable effects on the spectrum of secreted Aβ peptides, some decreasing the level of Aβ40 and others increasing the level of Aβ42.[50] Others have shown a lack of correlation between Aβ38 and Aβ42 levels, both with γ-secretase modulators (e.g., sulindac sulfide) and with FAD PS1 mutations, arguing against a precursor−production relationship.[62,68] This particular conclusion, however, disregards the fact that Aβ42 levels are determined by not only it being trimmed to Aβ38 but also its production from Aβ45, and the Ihara laboratory has detected the corresponding tetrapeptide of residues 39−42 generated by γ-secretase,[32] clear proof that Aβ38 can be directly derived from Aβ42. Nevertheless, the apparent dissociation between Aβ38 and Aβ42 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: endoproteolysis of presenilin 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 three or four residues[32] in producing a spectrum of peptides (Aβ 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 Aβ species that are more prone to aggregation into neurotoxic assemblies. Understanding the mechanism of the trimming process of Aβ 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 Aβ peptides (not only those secreted from cells), and the tri- and tetrapeptide fragments. Such methods could then be coupled with targeted mutagenesis of γ-secretase components to test specific mechanistic hypotheses.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplemental Figures S1−S3 (including error bars) show pH overlap results as well as full Aβ profiles under various conditions or with different PS1 mutants. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Center for Neurologic Diseases, Brigham and Women's Hospital and Harvard Medical School, 77 Avenue Louis Pasteur, HIM 754, Boston, MA 02115. Telephone: (617) 525-5511. Fax: (617) 525-5252. E-mail: mwolfe@rics.bwh.harvard.edu.

Author Contributions

O.Q.-M. and M.M.M. contributed equally to this work.

Funding

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.

■ 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, nicastrin; 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, G. G., and Wong, C. W. (1984) Alzheimer's disease: Initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem. Biophys. Res. Commun. 120*, 885−890.

(2) Masters, C. L., Simms, G., Weinman, N. A., Multhaup, G., McDonald, B. L., and Beyreuther, K. (1985) Amyloid plaque core protein in Alzheimer disease and Down syndrome. *Proc. Natl. Acad. Sci. U.S.A. 82*, 4245−4249.

(3) Cole, S. L., and Vassar, R. (2008) The role of APP processing by BACE1, the β-secretase, in Alzheimer's disease pathophysiology. *J. Biol. Chem. 283*, 29621−29625.

(4) Wolfe, M. S. (2006) The γ-secretase complex: Membrane-embedded proteolytic ensemble. *Biochemistry 45*, 7931−7939.

(5) Jarrett, J. T., Berger, E. P., and Lansbury, P. T. Jr. (1993) The carboxy terminus of the β amyloid protein is critical for the seeding of amyloid formation: Implications for the pathogenesis of Alzheimer's disease. *Biochemistry 32*, 4693−4697.

(6) Walsh, D. M., Klyubin, I., Fadeeva, J. V., Cullen, W. K., Anwyl, R., Wolfe, M. S., Rowan, M. J., and Selkoe, D. J. (2002) Naturally secreted oligomers of amyloid β protein potently inhibit hippocampal long-term potentiation in vivo. *Nature 416*, 535−539.

(7) Lesne, S., Koh, M. T., Kotilinek, L., Kayed, R., Glabe, C. G., Yang, A., Gallagher, M., and Ashe, K. H. (2006) A specific amyloid-β protein assembly in the brain impairs memory. *Nature 440*, 352−357.

(8) Shankar, G. M., Li, S., Mehta, T. H., Garcia-Munoz, A., Shepardson, N. E., Smith, I., Brett, F. M., Farrell, M. A., Rowan, M. J., Lemere, C. A., Regan, C. M., Walsh, D. M., Sabatini, B. L., and Selkoe, D. J. (2008) Amyloid-β protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory. *Nat. Med. 14*, 837−842.

(9) Tanzi, R. E., and Bertram, L. (2005) Twenty years of the Alzheimer's disease amyloid hypothesis: A genetic perspective. *Cell 120*, 545−555.

(10) Francis, R., McGrath, G., Zhang, J., Ruddy, D. A., Sym, M., Apfeld, J., Nicoll, M., Maxwell, M., Hai, B., Ellis, M. C., Parks, A. L., Xu, W., Li, J., Gurney, M., Myers, R. L., Himes, C. S., Hiebsch, R., Ruble, C., Nye, J. S., and Curtis, D. (2002) aph-1 and pen-2 are required for Notch pathway signaling, γ-secretase cleavage of βAPP, and presenilin protein accumulation. *Dev. Cell 3*, 85−97.

(11) Takasugi, N., Tomita, T., Hayashi, I., Tsuruoka, M., Niimura, M., Takahashi, Y., Thinakaran, G., and Iwatsubo, T. (2003) The role of presenilin cofactors in the γ-secretase complex. *Nature 422*, 438−441.
(12) Kimberly, W. T., LaVoie, M. J., Ostaszewski, B. L., Ye, W., Wolfe, M. S., and Selkoe, D. J. (2003) γ-Secretase is a membrane protein complex comprised of presenilin, nicastrin, aph-1, and pen-2. *Proc. Natl. Acad. Sci. U.S.A. 100*, 6382−6387.
(13) Edbauer, D., Winkler, E., Regula, J. T., Pesold, B., Steiner, H., and Haass, C. (2003) Reconstitution of γ-secretase activity. *Nat. Cell Biol. 5*, 486−488.
(14) Wolfe, M. S., Xia, W., Ostaszewski, B. L., Diehl, T. S., Kimberly, W. T., and Selkoe, D. J. (1999) Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and γ-secretase activity. *Nature 398*, 513−517.
(15) Li, Y. M., Xu, M., Lai, M. T., Huang, Q., Castro, J. L., DiMuzio-Mower, J., Harrison, T., Lellis, C., Nadin, A., Neduvelil, J. G., Register, R. B., Sardana, M. K., Shearman, M. S., Smith, A. L., Shi, X. P., Yin, K. C., Shafer, J. A., and Gardell, S. J. (2000) Photoactivated γ-secretase inhibitors directed to the active site covalently label presenilin 1. *Nature 405*, 689−694.
(16) Esler, W. P., Kimberly, W. T., Ostaszewski, B. L., Diehl, T. S., Moore, C. L., Tsai, J.-Y., Rahmati, T., Xia, W., Selkoe, D. J., and Wolfe, M. S. (2000) Transition-state analogue inhibitors of γ-secretase bind directly to presenilin-1. *Nat. Cell Biol. 2*, 428−434.
(17) Kaether, C., Capell, A., Edbauer, D., Winkler, E., Novak, B., Steiner, H., and Haass, C. (2004) The presenilin C-terminus is required for ER-retention, nicastrin-binding and γ-secretase activity. *EMBO J. 23*, 4738−4748.
(18) Thinakaran, G., Borchelt, D. R., Lee, M. K., Slunt, H. H., Spitzer, L., Kim, G., Ratovitsky, T., Davenport, F., Nordstedt, C., Seeger, M., Hardy, J., Levey, A. I., Gandy, S. E., Jenkins, N. A., Copeland, N. G., Price, D. L., and Sisodia, S. S. (1996) Endoproteolysis of presenilin 1 and accumulation of processed derivatives in vivo. *Neuron 17*, 181−190.
(19) Ratovitski, T., Slunt, H. H., Thinakaran, G., Price, D. L., Sisodia, S. S., and Borchelt, D. R. (1997) Endoproteolytic processing and stabilization of wild-type and mutant presenilin. *J. Biol. Chem. 272*, 24536−24541.
(20) Thinakaran, G., Harris, C. L., Ratovitski, T., Davenport, F., Slunt, H. H., Price, D. L., Borchelt, D. R., and Sisodia, S. S. (1997) Evidence that levels of presenilins (PS1 and PS2) are coordinately regulated by competition for limiting cellular factors. *J. Biol. Chem. 272*, 28415−28422.
(21) Edbauer, D., Winkler, E., Haass, C., and Steiner, H. (2002) Presenilin and nicastrin regulate each other and determine amyloid β-peptide production via complex formation. *Proc. Natl. Acad. Sci. U.S.A. 99*, 8666−8671.
(22) Arawaka, S., Hasegawa, H., Tandon, A., Janus, C., Chen, F., Yu, G., Kikuchi, K., Koyama, S., Kato, T., Fraser, P. E., and St George-Hyslop, P. (2002) The levels of mature glycosylated nicastrin are regulated and correlate with γ-secretase processing of amyloid β-precursor protein. *J. Neurochem. 83*, 1065−1071.
(23) Kimberly, W. T., LaVoie, M. J., Ostaszewski, B. L., Ye, W., Wolfe, M. S., and Selkoe, D. J. (2002) Complex N-linked glycosylated nicastrin associates with active γ-secretase and undergoes tight cellular regulation. *J. Biol. Chem. 277*, 35113−35117.
(24) Leem, J. Y., Vijayan, S., Han, P., Cai, D., Machura, M., Lopes, K. O., Veselits, M. L., Xu, H., and Thinakaran, G. (2002) Presenilin 1 is required for maturation and cell surface accumulation of nicastrin. *J. Biol. Chem. 277*, 19236−19240.
(25) Tomita, T., Katayama, R., Takikawa, R., and Iwatsubo, T. (2002) Complex N-glycosylated form of nicastrin is stabilized and selectively bound to presenilin fragments. *FEBS Lett. 520*, 117−121.
(26) Kornilova, A. Y., Bihel, F., Das, C., and Wolfe, M. S. (2005) The initial substrate-binding site of γ-secretase is located on presenilin near the active site. *Proc. Natl. Acad. Sci. U.S.A. 102*, 3230−3235.
(27) Weidemann, A., Eggert, S., Reinhard, F. B., Vogel, M., Paliga, K., Baier, G., Masters, C. L., Beyreuther, K., and Evin, G. (2002) A Novel ε-Cleavage within the Transmembrane Domain of the Alzheimer Amyloid Precursor Protein Demonstrates Homology with Notch Processing. *Biochemistry 41*, 2825−2835.
(28) Kakuda, N., Funamoto, S., Yagishita, S., Takami, M., Osawa, S., Dohmae, N., and Ihara, Y. (2006) Equimolar production of amyloid β-protein and amyloid precursor protein intracellular domain from β-carboxyl-terminal fragment by γ-secretase. *J. Biol. Chem. 281*, 14776−14786.
(29) Sato, T., Dohmae, N., Qi, Y., Kakuda, N., Misonou, H., Mitsumori, R., Maruyama, H., Koo, E. H., Haass, C., Takio, K., Morishima-Kawashima, M., Ishiura, S., and Ihara, Y. (2003) Potential link between amyloid β-protein 42 and C-terminal fragment γ 49−99 of β-amyloid precursor protein. *J. Biol. Chem. 278*, 24294−24301.
(30) Qi-Takahara, Y., Morishima-Kawashima, M., Tanimura, Y., Dolios, G., Hirotani, N., Horikoshi, Y., Kametani, F., Maeda, M., Saido, T. C., Wang, R., and Ihara, Y. (2005) Longer forms of amyloid β protein: Implications for the mechanism of intramembrane cleavage by γ-secretase. *J. Neurosci. 25*, 436−445.
(31) Funamoto, S., Morishima-Kawashima, M., Tanimura, Y., Hirotani, N., Saido, T. C., and Ihara, Y. (2004) Truncated carboxyl-terminal fragments of β-amyloid precursor protein are processed to amyloid β-proteins 40 and 42. *Biochemistry 43*, 13532−13540.
(32) Takami, M., Nagashima, Y., Sano, Y., Ishihara, S., Morishima-Kawashima, M., Funamoto, S., and Ihara, Y. (2009) γ-Secretase: Successive tripeptide and tetrapeptide release from the transmembrane domain of β-carboxyl terminal fragment. *J. Neurosci. 29*, 13042−13052.
(33) Sato, T., Diehl, T. S., Narayanan, S., Funamoto, S., Ihara, Y., De Strooper, B., Steiner, H., Haass, C., and Wolfe, M. S. (2007) Active γ-secretase complexes contain only one of each component. *J. Biol. Chem. 282*, 33985−33993.
(34) Duff, K., Eckman, C., Zehr, C., Yu, X., Prada, C. M., Perez-tur, J., Hutton, M., Buee, L., Harigaya, Y., Yager, D., Morgan, D., Gordon, M. N., Holcomb, L., Refolo, L., Zenk, B., Hardy, J., and Younkin, S. (1996) Increased amyloid-β42(43) in brains of mice expressing mutant presenilin 1. *Nature 383*, 710−713.
(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., and Selkoe, D. J. (1997) Mutant presenilins of Alzheimer's disease increase production of 42-residue amyloid β-protein in both transfected cells and transgenic mice. *Nat. Med. 3*, 67−72.
(36) Scheuner, D., Eckman, C., Jensen, M., Song, X., Citron, M., Suzuki, N., Bird, T. D., 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., and Younkin, S. (1996) Secreted amyloid β-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. 2*, 864−870.
(37) Lemere, C. A., Lopera, F., Kosik, K. S., Lendon, C. L., Ossa, J., Saido, T. C., Yamaguchi, H., Ruiz, A., Martinez, A., Madrigal, L., Hincapie, L., Arango, J. C., Anthony, D. C., Koo, E. H., Goate, A. M., Selkoe, D. J., and Arango, J. C. (1996) The E280A presenilin 1 Alzheimer mutation produces increased Aβ42 deposition and severe cerebellar pathology. *Nat. Med. 2*, 1146−1150.
(38) Song, W., Nadeau, P., Yuan, M., Yang, X., Shen, J., and Yankner, B. A. (1999) 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. 96*, 6959−6963.
(39) Shen, J., and Kelleher, R. J. III (2007) The presenilin hypothesis of Alzheimer's disease: Evidence for a loss-of-function pathogenic mechanism. *Proc. Natl. Acad. Sci. U.S.A. 104*, 403−409.
(40) Wolfe, M. S. (2007) When loss is gain: Reduced presenilin proteolytic function leads to increased Aβ42/Aβ40. Talking Point on the role of presenilin mutations in Alzheimer disease. *EMBO Rep. 8*, 136−140.

(41) De Strooper, B. (2007) Loss-of-function presenilin mutations in Alzheimer disease. Talking Point on the role of presenilin mutations in Alzheimer disease. *EMBO Rep. 8*, 141−146.
(42) Li, Y. M., Lai, M. T., Xu, M., Huang, Q., DiMuzio-Mower, J., Sardana, M. K., Shi, X. P., Yin, K. C., Shafer, J. A., and Gardell, S. J. (2000) Presenilin 1 is linked with γ-secretase activity in the detergent solubilized state. *Proc. Natl. Acad. Sci. U.S.A. 97*, 6138−6143.
(43) Cacquevel, M., Aeschbach, L., Osenkowski, P., Li, D., Ye, W., Wolfe, M. S., Li, H., Selkoe, D. J., and Fraering, P. C. (2008) Rapid purification of active γ-secretase, an intramembrane protease implicated in Alzheimer's disease. *J. Neurochem. 104*, 210−220.
(44) Klafki, H., Abramowski, D., Swoboda, R., Paganetti, P. A., and Staufenbiel, M. (1996) The carboxyl termini of β-amyloid peptides 1−40 and 1−42 are generated by distinct γ-secretase activities. *J. Biol. Chem. 271*, 28655−28659.
(45) Tarlton, J. F., and Knight, P. J. (1996) Comparison of reflectance and transmission densitometry, using document and laser scanners, for quantitation of stained Western blots. *Anal. Biochem. 237*, 123−128.
(46) Osenkowski, P., Li, H., Ye, W., Li, D., Aeschbach, L., Fraering, P. C., Wolfe, M. S., Selkoe, D. J., and Li, H. (2009) Cryoelectron microscopy structure of purified γ-secretase at 12 Å resolution. *J. Mol. Biol. 385*, 642−652.
(47) Osenkowski, P., Ye, W., Wang, R., Wolfe, M. S., and Selkoe, D. J. (2008) Direct and potent regulation of γ-secretase by its lipid microenvironment. *J. Biol. Chem. 283*, 22529−22540.
(48) Fraering, P. C., Ye, W., Strub, J. M., Dolios, G., LaVoie, M. J., Ostaszewski, B. L., Van Dorsselaer, A., Wang, R., Selkoe, D. J., and Wolfe, M. S. (2004) Purification and Characterization of the Human γ-Secretase Complex. *Biochemistry 43*, 9774−9789.
(49) Moehlmann, T., Winkler, E., Xia, X., Edbauer, D., Murrell, J., Capell, A., Kaether, C., Zheng, H., Ghetti, B., Haass, C., and Steiner, H. (2002) Presenilin-1 mutations of leucine 166 equally affect the generation of the Notch and APP intracellular domains independent of their effect on Aβ42 production. *Proc. Natl. Acad. Sci. U.S.A. 99*, 8025−8030.
(50) Bentahir, M., Nyabi, O., Verhamme, J., Tolia, A., Horre, K., Wiltfang, J., Esselmann, H., and De Strooper, B. (2006) Presenilin clinical mutations can affect γ-secretase activity by different mechanisms. *J. Neurochem. 96*, 732−742.
(51) Steiner, H., Kostka, M., Romig, H., Basset, G., Pesold, B., Hardy, J. A., Capell, A., Meyn, L., Grim, M. G., Baumeister, R., Fechteler, K., and Haass, C. (2000) Glycine 384 is required for presenilin-1 function and is conserved in bacterial polytopic aspartyl proteases. *Nat. Cell Biol. 2*, 848−851.
(52) De Jonghe, C., Cras, P., Vanderstichele, H., Cruts, M., Vanderhoeven, I., Smouts, I., Vanmechelen, E., Martin, J. J., Hendriks, L., and Van Broeckhoven, C. (1999) Evidence that Aβ42 plasma levels in presenilin-1 mutation carriers do not allow for prediction of their clinical phenotype. *Neurobiol. Dis. 6*, 280−287.
(53) Cruts, M., Backhovens, H., Wang, S. Y., Van Gassen, G., Theuns, J., De Jonghe, C. D., Wehnert, A., De Voecht, J., De Winter, G., Cras, P., et al. (1995) Molecular genetic analysis of familial early-onset Alzheimer's disease linked to chromosome 14q24.3. *Hum. Mol. Genet. 4*, 2363−2371.
(54) Sherrington, R., Rogaev, E. I., Liang, Y., Rogaeva, E. A., Levesque, G., Ikeda, M., Chi, H., Lin, C., Li, G., Holman, K., et al. (1995) Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature 375*, 754−760.
(55) Capell, A., Grunberg, J., Pesold, B., Diehlmann, A., Citron, M., Nixon, R., Beyreuther, K., Selkoe, D. J., and Haass, C. (1998) 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. 273*, 3205−3211.
(56) Zhang, J., Kang, D. E., Xia, W., Okochi, M., Mori, H., Selkoe, D. J., and Koo, E. H. (1998) Subcellular distribution and turnover of presenilins in transfected cells. *J. Biol. Chem. 273*, 12436−12442.
(57) Baumeister, R., Leimer, U., Zweckbronner, I., Jakubek, C., Grünberg, J., and Haass, C. (1997) Human presenilin-1, but not familial Alzheimer's disease (FAD) mutants, facilitate *Caenorhabditis elegans* Notch signalling independently of proteolytic processing. *Genes Funct. 1*, 149−159.
(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, I. C., Rousseau, F., Schymkowitz, J., and De Strooper, B. (2010) Neurotoxicity of Alzheimer's disease Aβ peptides is induced by small changes in the Aβ42 to Aβ40 ratio. *EMBO J. 29*, 3408−3420.
(59) Maruyama, K., Tomita, T., Shinozaki, K., Kume, H., Asada, H., Saido, T. C., Ishiura, S., Iwatsubo, T., and Obata, K. (1996) Familial Alzheimer's disease-linked mutations at Val717 of amyloid precursor protein are specific for the increased secretion of Aβ42(43). *Biochem. Biophys. Res. Commun. 227*, 730−735.
(60) Kounnas, M. Z., Danks, A. M., Cheng, S., Tyree, C., Ackerman, E., Zhang, X., Ahn, K., Nguyen, P., Comer, D., Mao, L., Yu, C., Pleynet, D., Digregorio, P. J., Velicelebi, G., Stauderman, K. A., Comer, W. T., Mobley, W. C., Li, Y. M., Sisodia, S. S., Tanzi, R. E., and Wagner, S. L. (2010) Modulation of γ-secretase reduces β-amyloid deposition in a transgenic mouse model of Alzheimer's disease. *Neuron 67*, 769−780.
(61) Tokuda, T., Fukushima, T., Ikeda, S., Sekijima, Y., Shoji, S., Yanagisawa, N., and Tamaoka, A. (1997) Plasma levels of amyloid β proteins Aβ1−40 and Aβ1−42(43) are elevated in Down's syndrome. *Ann. Neurol. 41*, 271−273.
(62) Czirr, E., Cottrell, B. A., Leuchtenberger, S., Kukar, T., Ladd, T. B., Esselmann, H., Paul, S., Schubenel, R., Torpey, J. W., Pietrzik, C. U., Golde, T. E., Wiltfang, J., Baumann, K., Koo, E. H., and Weggen, S. (2008) Independent generation of Aβ42 and Aβ38 peptide species by γ-secretase. *J. Biol. Chem. 283*, 17049−17054.
(63) Saito, T., Suemoto, T., Brouwers, N., Sleegers, K., Funamoto, S., Mihira, N., Matsuba, Y., Yamada, K., Nilsson, P., Takano, J., Nishimura, M., Iwata, N., Van Broeckhoven, C., Ihara, Y., and Saido, T. C. (2011) Potent amyloidogenicity and pathogenicity of Aβ43. *Nat. Neurosci. 14*, 1023−1032.
(64) Walker, E. S., Martinez, M., Brunkan, A. L., and Goate, A. (2005) Presenilin 2 familial Alzheimer's disease mutations result in partial loss of function and dramatic changes in Aβ42/40 ratios. *J. Neurochem. 92*, 294−301.
(65) Kumar-Singh, S., Theuns, J., Van Broeck, B., Pirici, D., Vennekens, K., Corsmit, E., Cruts, M., Dermaut, B., Wang, R., and Van Broeckhoven, C. (2006) Mean age-of-onset of familial alzheimer disease caused by presenilin mutations correlates with both increased Aβ42 and decreased Aβ40. *Hum. Mutat. 27*, 686−695.
(66) Shimojo, M., Sahara, N., Murayama, M., Ichinose, H., and Takashima, A. (2007) Decreased Aβ secretion by cells expressing familial Alzheimer's disease-linked mutant presenilin 1. *Neurosci. Res. 57*, 446−453.
(67) Fluhrer, R., Fukumori, A., Martin, L., Grammer, G., Haug-Kroper, M., Klier, B., Winkler, E., Kremmer, E., Condron, M. M., Teplow, D. B., Steiner, H., and Haass, C. (2008) Intramembrane proteolysis of GXGD-type aspartyl proteases is slowed by a familial Alzheimer disease-like mutation. *J. Biol. Chem. 283*, 30121−30128.
(68) Page, R. M., Baumann, K., Tomioka, M., Perez-Revuelta, B. I., Fukumori, A., Jacobsen, H., Flohr, A., Luebbers, T., Ozmen, L., Steiner, H., and Haass, C. (2008) Generation of Aβ38 and Aβ42 is independently and differentially affected by familial Alzheimer disease-associated presenilin mutations and γ-secretase modulation. *J. Biol. Chem. 283*, 677−683.
(69) Kretner, B., Fukumori, A., Gutsmiedl, A., Page, R. M., Luebbers, T., Galley, G., Baumann, K., Haass, C., and Steiner, H. (2011) Attenuated Aβ42 Responses to Low Potency γ-Secretase Modulators Can Be Overcome for Many Pathogenic Presenilin Mutants by Second-generation Compounds. *J. Biol. Chem. 286*, 15240−15251.
(70) Qi, Y., Morishima-Kawashima, M., Sato, T., Mitsumori, R., and Ihara, Y. (2003) Distinct mechanisms by mutant presenilin 1 and 2 leading to increased intracellular levels of amyloid β-protein 42 in Chinese hamster ovary cells. *Biochemistry 42*, 1042−1052.

(71) Jankowsky, J. L., Fadale, D. J., Anderson, J., Xu, G. M., Gonzales, V., Jenkins, N. A., Copeland, N. G., Lee, M. K., Younkin, L. H., Wagner, S. L., Younkin, S. G., and Borchelt, D. R. (2004) Mutant presenilins specifically elevate the levels of the 42 residue β-amyloid peptide in vivo: Evidence for augmentation of a 42-specific γ-secretase. *Hum. Mol. Genet. 13*, 159−170.

(72) Winkler, E., Hobson, S., Fukumori, A., Dumpelfeld, B., Luebbers, T., Baumann, K., Haass, C., Hopf, C., and Steiner, H. (2009) Purification, pharmacological modulation, and biochemical characterization of interactors of endogenous human γ-secretase. *Biochemistry 48*, 1183−1197.

(73) Fukumori, A., Fluhrer, R., Steiner, H., and Haass, C. (2010) Three-amino acid spacing of presenilin endoproteolysis suggests a general stepwise cleavage of γ-secretase-mediated intramembrane proteolysis. *J. Neurosci. 30*, 7853−7862.

(74) Haapasalo, A., and Kovacs, D. M. (2011) The Many Substrates of Presenilin/γ-Secretase. *J. Alzheimer's Dis.*, in press.

(75) Okochi, M., Fukumori, A., Jiang, J., Itoh, N., Kimura, R., Steiner, H., Haass, C., Tagami, S., and Takeda, M. (2006) Secretion of the Notch-1 Aβ-like peptide during Notch signaling. *J. Biol. Chem. 281*, 7890−7898.