β-secretase cleavage at the amino acid residue 34 in the Amyloid β peptide is dependent upon the γ-secretase activity

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Abstract

The amyloid β peptides (Aβ) are the major components of the senile plaques characteristic of Alzheimer’s disease (AD). Aβ peptides are generated from the cleavage of amyloid precursor protein (APP) by β- and γ-secretases. β-secretases (BACE), a type-I transmembrane aspartyl protease, cleaves APP first to generate a 99 amino acid membrane-associated fragment (CT99) containing the N-terminus of Aβ peptides. γ-secretase, a multi-protein complex, then cleaves within the transmembrane region of CT99 to generate the C-termini of Aβ peptides. The production of Aβ peptides is, therefore, dependent on the activities of both BACE and γ-secretase. The cleavage of APP by BACE is believed to be a prerequisite for γ-secretase-mediated processing. In the present study we provide evidence both in vitro and in cells that BACE mediated cleavage between amino acid residues 34 and 35 (Aβ-34-site) in the Aβ region is dependent on γ-secretase activity. In vitro the Aβ-34-site is processed specifically by BACE1 and BACE2, but not by cathepsin D, a closely related aspartyl protease. Moreover, the cleavage of the Aβ-34-site by BACE1 or BACE2 only occurs when Aβ 1-40 peptide, a γ-secretase cleavage product, was used as substrate and not the non-cleaved CT99. In cells, over-expression of BACE1 or BACE2 dramatically increased the production of the Aβ 1-34 species. More importantly, the cellular production of Aβ 1-34 species induced by over-expression of BACE1 or BACE2 was blocked by a number of
known γ-secretase inhibitors in a concentration dependent manner. These γ-secretase inhibitors had no effect on enzymatic activity of BACE1 or BACE2 in vitro. Our data thus suggest that γ-secretase cleavage of CT99 is a prerequisite for BACE mediated processing at Aβ-34-site. Therefore, BACE and γ-secretase activity can be mutually dependent.

Introduction

Aβ peptides are principal components of the neuritic plaques that represent one of the hallmarks of AD pathology. Production of Aβ peptides is initiated first by activities of BACE1 which cleaves APP at the β-site to yield a membrane-associated APP C-terminal fragment of 99 amino acid residues (CT99) (1-6). A subsequent cleavage within the transmembrane domain of CT99 by γ-secretase then release Aβ peptides of 39-42 amino acid residues (1-6). APP can also be cleaved within it’s Aβ region by α-secretase(s) to generate CT83 (5), which is also a substrate for γ-secretase (1, 7).

In cells the activity of BACE and γ-secretase to process APP has been observed in the membrane fractions, particularly in the trans-Golgi network (TGN) (1, 8-11). Production of Aβ have also been detected in both ER and TGN (12-15). Several lines of experimental evidence suggest that cellular APP processing is sequential and that cleavage of APP by α- or β-secretase is a prerequisite for γ-secretase-mediated processing. Firstly, only two species of APP N-terminal products cleaved by endogenous secretases were reportedly detected in cells, the BACE-cleaved product, sAPPβ, and the
α-secretase-cleaved product, sAPPα (1-7). A γ-secretase cleaved N-terminal APP, or so called sAPPγ without prior α- or β-secretase cleavage, was never detected or reported. Secondly, in an in vitro cell free assay system, the N-terminally truncated form of APP, CT99 (or CT100), has been demonstrated as an efficient substrate for γ-secretase (16, 17). In contrast, the full length APP is a very poor substrate for γ-secretase (Li, Y-M., et al., unpublished results). Therefore, removal of the N-terminal region of APP appears to be essential for γ-secretase mediated cleavage.

In the present study we found that BACE mediated cleavage at the Aβ-34-site is dependent on the γ-secretase activity. BACE only cleaves the Aβ-34-site in vitro within Aβ 1-40 peptide, a product from γ-secretase, not the site in the non-cleaved CT99 fragment. Moreover, the cellular production of Aβ 1-34 species induced by over-expression of BACE1 or BACE2 was blocked by known γ-secretase inhibitors in a concentration dependent manner, whereas the γ-secretase inhibitors had no effect on enzymatic activity of BACE1 or BACE2 in vitro. Our results suggest that the cleavage activity of BACE at Aβ-34-site, both in vitro and in cells, is dependent on γ-secretase activity. Therefore BACE and γ-secretase activity can be mutually dependent.

Materials and Methods

BACE1 and BACE2 expression and purification

Cloning of human BACE1 and BACE2 cDNA and purification of human BACE1 and BACE2 full length protein from transiently transfected human embryonic kidney 293T
cells were described previously (18). Expression and purification of truncated version of BACE1 from recombinant baculovirus expression system was performed as described previously (19).

**BACE Activity in vitro assay**

Aβ 1-40 was purchased from Enzyme Systems Product (Livermore, CA). Human Cathepsin D was purchased from Cal Biochem (La Jolla, CA). The production of recombinant APP-CT100-Flag was described previously (16). Treatment of CT100-Flag with aminopeptidase (#A8200, Sigma, St. Louis, MO) resulted in a CT99-flag with amino acid residue “D” at its N-terminus. CT99-flag was confirmed by mass spectrometric analysis and by its reactivity with a neo-epitope specific antibody, FCA-18, purchased from Dr. Checler (20). Assays were performed for the indicated time in the presence of 50 mM ammonium acetate, 0.15 M NaCl, pH 4.5 or 5.0 and 0.1 mg/ml BSA at 37°C. The reaction was terminated by heating samples at 75°C for 5 min. Samples were analyzed by mass spectrometry or/and by reverse-phase HPLC.

BACE1 or BACE2 activity in vitro was also assessed by cleavage of a FRET substrate encompassing P8-P4' of the APPsw β-site, TAMRA-5-CO-EEISEVNLDAEF-NH-QSY7, similar to the one described by Ermolieff et al., (21). The reaction condition is the same as described above and the cleavage product was measured by a LJL Analyst AD instrument (LJL BioSystems, Sunnyvale, CA) with excitation of 530 nm and emission of 580 nm.
Cell culture, transfection and analysis of APP processing products

HEK293T cells stably expressing human APP695 were seeded in 100 mm dishes and transfected with either BACE1 or BACE2 DNA described above. Transfection was performed using Lipofectamin reagent (Gibco, Rockville, MD) according to the instructions by the manufacture. Media were changed next day and either DMSO control or compound-1 was added to the new media. Following an additional 24 hours incubation at 37 °C, the media and cells were harvested. Aβ level in media was assayed by Origen system as described previously using biotinylated 6E10 antibody (Senetek, Missouri) and ruthenylated G2-10 antibody (16) licensed from University of Heidelberg.

Mass spectrometric analysis of BACE cleaved products

MALDI mass analysis was performed with a SELDI-TOF mass spectrometer from Ciphergen Biosystems (Fremont, CA) and a Voyager DE/RP MALDI-TOF from Applied Biosystems (Framingham, MA). Typically 1 microliter of reaction mixture was mixed with 1 microliter of a saturated MALDI matrix solution (α-cyanohydroxycinnamic acid in 1:1 water:acetonitrile with 0.1% TFA) and was then spotted onto Ciphergen NP2 MALDI chips. After drying and crystal formation, sample spots were typically analyzed with 100 laser shots.

In all figures containing MALDI mass spectrometric data, the generic notation Aβ X-X refers to the length of the Aβ fragment, such as Aβ 1-40 or Aβ1-34. This notation is used to denote the Aβ fragment corresponding to the mass of the singly charged peptide ion.
(M+H)^+. Additionally, in cases where the doubly charged ion, (M+2H)^2+ is visible, as in Figures 2, the generic notation \((A\beta X-X + 2H)^2+\), denotes the A\beta species corresponding to the mass of the doubly charged peptide ion.

**Mass Spectrometric analysis of APP processing products in media**

For analysis of APP processing species secreted into media, the collected media samples were immunoprecipitated (IP) with 6E10 antibody and protein G beads (Protein G ultralink, Pierce Chemical Co). Following incubation at room temperature for one hour, the supernatants were removed and the IP beads were first washed three times with a buffer containing 150 mM NaCl, 10 mM Tris, 2 mM EDTA, 0.1% triton X-100, 0.1% IGEPAL CA630 (pH 7.8), then washed twice with a buffer consisting of 500 mM NaCl, 10 mM Tris, 2 mM EDTA, 0.1% triton X-100, 0.1% IGEPAL CA630 (pH 7.8) and finally washed three times with 100mM NH₄HCO₃ (pH 7.8). Following the final wash, the captured products were eluted from the beads with a minimal volume of a saturated solution of \(\alpha\)-Cyano-4-Hydroxycinnamic acid dissolved in 50% Acetonitrile : 0.1% TFA. One \(\mu\)L of the bead eluate was then spotted on to the an NP-2 Surface Enhanced Laser Desorption/Ionization (SELDI) sample target (Ciphergen Biosystems) or a stainless steel target for an ABI Voyager and allowed to air dry. The dried targets were placed into the SELDI-Time-of-Flight (TOF) and Voyager mass spectrometers and matrix/analyte coprecipitates were desorbed with a 337 nm N₂ laser. The laser power was attenuated and the detector sensitivity manipulated such that spectra, which were the average of 100 laser shots, provided a qualitative assessment of the IP captured cleavage products.
Results

BACE1 or BACE2, but not cathepsin D, cleaves the Aβ-34-site in vitro

The activity of purified human full length BACE1, BACE2 or cathepsin D, a closely related aspartyl protease, was tested for cleavage of a synthetic Aβ peptide 1-40 in vitro. When BACE1 or BACE2 was incubated with a synthetic Aβ peptide 1–40 in vitro, a cleavage site located between Aβ residues 34 and 35 was clearly identified through mass spectrometric analysis shown in Fig 1 (B1, B3 and B4). The theoretical mass value for Aβ 1-34 is 3786.9 and the observed mass values for the cleaved 1-34 products were 3785.6 and 3786.6 for BACE1 and BACE2, respectively. However, when Aβ 1-40 peptide was incubated with cathepsin D, Aβ species cleaved at residues between 19 and 20 were observed by mass spectrometric analysis, but not the Aβ 1-34 product (Fig. 1, B1 and B2). The absence of Aβ 1-34 species in cathepsin D-treated sample is not due to a secondary cleavage of Aβ 1-34, since a shorter incubation time and with less enzyme showed similar results (data not shown). In contrast, both purified full length human BACE1 (Fig. 1, B3) and BACE2 (Fig. 1, B4) processed the Aβ-34 site. Thus, the cleavage of Aβ-34-site is specific for BACE1 and BACE2, not for cathepsin D.

BACE1 lacking the transmembrane and cytoplasmic regions cleave the Aβ-34-site in vitro

One of the major differences between BACE and cathepsin D is that cathepsin D does not contain a transmembrane domain and cytoplasmic tail. To investigate whether the
transmembrane and cytoplasmic regions of BACE contribute to its specific activity of processing the Aβ-34 site, we tested the activities of truncated forms of BACE1 (460) lacking both the transmembrane and cytoplasmic domains using the Aβ 1-40 substrate. Results from mass spectrometric analysis showed that truncated BACE1, both the pro-(amino acid 22-460) and the mature (amino acid 46-460) forms (19), cleaves the Aβ-34-site in vitro (Fig. 2). Therefore, the differential cleavage of Aβ-34-site between BACE and cathepsin D is likely due to their difference in enzyme active site. It is interesting to note that the truncated forms of BACE1 did not cleave the Aβ-20-site as seen with the full length enzyme.

**BACE1 or BACE2 does not cleave Aβ-34-site using CT99 as substrate**

To further investigate BACE in vitro cleavage specificity, we tested BACE activity using a recombinant CT99 protein substrate. CT99 or CT100 has been described previously as an efficient in vitro substrate for γ-secretase (16, 17). Control mass spectrometric analysis of CT99 alone (Fig. 3A) at ms range 1000-5000 did not show any notable species (Fig. 3B). To our surprise, when CT99 was incubated with BACE1, cleavage products of CT99 at either Aβ-20-site or Aβ-34-site was not observed (Fig. 3C). This was true for both full length and truncated forms of BACE1 (data not shown). The absence of BACE1 mediated cleavage at Aβ-20 or Aβ-34 sites in CT99 was not caused by substrate depletion, since the substrate peak was still observed by mass spectrometry at the end of the reaction (data not shown); nor was it due to the enzyme inactivation, because that addition of a small peptide substrate encompassing the β-scissile bond at the end of the
CT99 reaction yielded appropriate cleavage product (data not shown). Likewise, BACE2 also did not cleave CT99 at the Aβ-34-site, although it cleaved at the Aβ-19 and -20 sites (Fig. 3D). Since the CT99 used here is in solution and not embedded in the membrane, the hydrophobic domain of the CT99 might be "abnormally" folded in the buffer. To test whether addition of a limited amount of detergent could expose the Aβ-34 site in CT99 for processing, the in vitro reaction with CT99 was also performed in the presence of 0.05% Triton X-100. Both BACE1 and BACE2 were fully functional in the presence of 0.05% Triton X-100 to cleave the β-site of APP (data not shown) (3). However, neither of them showed any cleavage of Aβ-34-site in CT99 (data not shown). Therefore, unlike Aβ 1-40 peptide, CT99 is not a substrate for Aβ-34 site cleavage by either BACE1 or BACE2 in vitro.

*Over-expression of BACE1 or BACE2 in cells increase Aβ 1-34 production*

To evaluate the effect of BACE1 or BACE2 on cellular Aβ 1-34 production, HEK293T cells stably expressing APP695 (HEK293T/APP695) were either mock transfected, or transfected with BACE1 or BACE2 cDNA. Aβ1-40 level secreted in the media by these cells was determined by a modified ELISA assay using antibodies (6E10 and G2-10) (16) against Aβ 1-40 (Fig. 4A). Consistent with previous reports, over-expression of BACE1 moderately increased the Aβ 1-40 production (Fig. 4A, 2) (4), and over-expression of BACE2 abolished the generation of Aβ 1-40 (Fig. 4A, 3) (22). When the conditioned media were subjected to immunoprecipitation and mass spectrometric analysis (IP/MS)(Materials and Methods), results showed that HEK293T/APP695 cells produced
little Aβ 1-34 species (Fig. 4, B1); Transfection of BACE1 increased the relative amount of Aβ 1-34 species, in addition to Aβ 1-20 and Aβ 1-40 (Fig. 4, B2); Transfection of BACE2 similarly increased Aβ 1-34 level, despite abolishing Aβ 1-40 (Fig. 4, B3). This suggests that in cells BACE2 prefers Aβ-19, -20 or -34 sites over the Aβ-1-site. This result not only agrees well with BACE2 activity data in vitro (18, 23) but also provides a plausible explanation for the negative effect of BACE2 on cellular Aβ 1-40 production.

In summary, our results indicate that over-expression of BACE1 or BACE2 in cells increase the production of Aβ 1-34.

**γ-secretase inhibitors block the production of Aβ 1-34 in cells**

To evaluate the effect of γ-secretase inhibitors on the cellular production of Aβ 1-34 species, HEK293T/APP695 cells, either mock-transfected, or transfected with BACE1 or BACE2, were treated with either DMSO, or with 10 μM of each of the two known γ-secretase inhibitors, compound-1 and compound-2 (24, 25). As expected, both compounds inhibited Aβ 1-40 production in HEK293TAPP695 cells (Fig. 5A, 1-3) and the cells transfected with BACE1 (Fig. 5A, 4-6). The effect of compounds on the cellular production of Aβ 1-40 was further confirmed by mass spectrometric analysis of the Aβ species in the conditioned media of these cells (Fig.5B). Notably, in BACE1 over-expressing cells these γ-secretase inhibitors not only blocked the production of Aβ 1-40, but also that of Aβ 1-34 species (Fig. 5B, 4-6). Likewise, in BACE2 over-expressing cells, the increased generation of Aβ 1-34 species was also inhibited by these compounds.
In contrast, the generation of other Aβ species such as 1-16, 1-19 or 1-20 were largely unblocked (Fig. 5B).

Inhibition of Aβ 1-34 production by γ-secretase inhibitor is concentration-dependent and coincides with the blockade of Aβ 1-40 production

To further evaluate the effect of γ-secretase inhibitors on the cellular production of Aβ 1-34 species, another known γ-secretase inhibitor with a different structural class, compound-3 (17, 26), was tested in cells at various concentrations (1 µM and 10 µM). Results from the modified Aβ ELISA assay revealed a concentration dependent inhibition of Aβ 1-40 production in both HEK293T/APP695 cells (Fig. 6A, 1-3) and cells transfected with BACE1 (Fig. 6A, 4-6). Furthermore, the mass spectrometric analysis of Aβ species in conditioned media of these cells showed that both the production of Aβ 1-40 and Aβ 1-34 species were blocked by compound-3 in a dose-dependent manner (Fig. 6B). Notably, in BACE1 over-expressing cells, the extent of Aβ 1-34 inhibition coincided with the blockade of Aβ 1-40 production (Fig. 6B, 4-6). In BACE2 over-expressing cells, the generation of Aβ 1-34 was similarly inhibited by compound-3 in a dose-dependent manner (Fig. 6B, 7-9).

Because both BACE and γ-secretase are aspartyl proteases (2, 3, 16, 17), we performed experiments to determine whether any of the γ-secretase inhibitors used in our cellular study blocked the activities of BACE1 or BACE2 in vitro. When BACE1 or BACE2
were incubated in vitro with a peptide substrate containing the APPsw β-site sequence, a steady formation of the cleavage products was observed with time (Fig. 7). The known BACE inhibitor, StatV (3) inhibited the cleavage activities of both BACE1 and BACE2. However, no inhibitory effect was observed with any of the γ-secretase inhibitors (Fig. 7), whereas at the same concentration (10 µM) all the 3 compounds completely blocked γ-secretase activity in cells (Fig. 5 and 6).

Discussion

Aberrant Aβ production is believed to be one of the major causes for AD (1). BACE and γ-secretase are two critical enzymes responsible for the generation of Aβ peptides and, therefore, have been considered as potential therapeutic targets for AD (1-7). Studies of the relationship between BACE, γ-secretase and APP substrate could shed light on APP metabolism and facilitate our understanding of Aβ generation process. Several lines of experimental evidence suggest that BACE cleavage in APP is a prerequisite for γ-secretase mediated processing. In this current investigation we present evidence that, both in vitro and in cells, BACE and γ-secretase activities can be mutually dependent. An example described here is the generation of Aβ 1-34 species.

First, we show that the Aβ-34 site is cleaved by BACE both in vitro and in cells; and second, we demonstrate that γ-secretase activity is required for BACE cleavage at the Aβ-34 site both in vitro and in cells.
In vitro, human BACE, both the full length and the truncated form lacking transmembrane and cytoplasmic domains, cleaved the Aβ-34 site within Aβ 1-40 peptide substrate. However, cathepsin D, a closely related aspartyl protease, did not cleave the Aβ-34 site, although it shares many of the cleavage sites in APP with BACE, including the β-scissile bond (our unpublished results) and the Aβ-19 or -20 sites (Fig.1). Thus, the Aβ-34 site appears to be rather specific for BACE. Notably, the sequence around the Aβ 34-site, IIGL-MVGG, shows a high homology to a reported in vitro “optimized sequence” for BACE1, EIDL-MVLD (27).

In cells, over-expression of BACE1 or BACE2 increased the generation of Aβ 1-34 species (Fig. 3), suggesting that cellular production of Aβ 1-34 is dependent on BACE levels. The Aβ 1-34 species has also been observed in cells stably over-expressing BACE1 (10, 28). However, in all these cases it is difficult to completely rule out the possibility that over-expressed BACE1 increases the cleavage of Aβ-1 site and other enzymes such as γ-secretase could be responsible for the cleavage at the Aβ-34 site in cells. In this aspect, our data from BACE2 over-expression studies is particularly noteworthy. The over-expression of BACE2 similarly increased Aβ 1-34 level, but abolished Aβ 1-40 production [Fig. 4B-(3)]. This indicates that two different enzymes must be responsible for the cleavage of Aβ-34 and Aβ-40 sites. In fact, we and others have observed that BACE1 prefers the Aβ-1 site while BACE2 prefers the internal cleavage sites within Aβ 1-40, such as the Aβ-19 or -20 sites and Aβ-34 site (18). These "internal" cleavage activities by BACE2 lead to a decrease in Aβ 1-40 level and also
expectedly in Aβ 1-34 level if the Aβ-34 site were indeed processed by γ-secretase. Thus, taken together, our data indicate that in cells Aβ 34-site is cleaved by BACE1 or BACE2, not the γ-secretase.

Several lines of evidence indicate that BACE cleavage of the Aβ-34-site is dependent on γ-secretase activity. In vitro, BACE, both BACE1 or BACE2, only cleaves Aβ-34 site within the Aβ 1-40 peptide, the product of γ-secretase cleavage; BACE did not process the Aβ-34 site in CT99, the substrate for the γ-secretase. In contrast, BACE2 cleavage of Aβ-19 and -20 sites occurred with both Aβ 1-40 and CT99 substrates. We were unable to observe any BACE1 cleavage product using CT99 as substrate, although the enzyme was active toward the β-scissile bond cleavage. One of the possible explanations is that other BACE1-mediated cleavages within CT99 could also be dependent on γ-secretase activity. More experiments are needed to test this hypothesis. In cells, cleavage of the Aβ-34-site induced by BACE1 or BACE2 is blocked by several different classes of γ-secretase inhibitors. Moreover, the inhibition of Aβ-34 site processing is dose-dependent and coincides with the blockade of Aβ-40 site cleavage. None of the γ-secretase inhibitors tested here showed any inhibitory effect on enzymatic activity of BACE1 or BACE2 in vitro. Therefore, the most likely explanation would be that γ-secretase cleavage of CT99 in cells is a prerequisite for BACE mediated processing of Aβ–34-site. Additionally, two recent studies also reported that γ-secretase inhibitors blocked the production of the Aβ 1-34 species in cells (28, 29). During the revision of our manuscript, Fluhrer et al., has
published a study with similar findings using presenilin dominant negative mutants to inhibit cellular γ-secretase activity (30).

A question that remains is how the activity of γ-secretase affects the BACE cleavage of the Aβ–34-site. Results from the in vitro experiments using either Aβ1-40 or CT99 substrates suggest that it is the feature in CT99 fragment that interferes with BACE cleavage of Aβ-34 site. One possibility is that the hydrophobic domain of the CT99, normally embedded in the membrane, might be mis-folded and prevented the access of BACE. However, addition of a limited amount of detergent, 0.05% Triton X-100, did not promote the processing. Alternatively, CT99 fragment could still possess an "unfit" conformation or contain an inhibitory element downstream from the γ-secretase processing sites. In any case, CT99 needs to be truncated at the γ-secretase cleavage sites in order for BACE to cleave the Aβ-34 site. In vitro such requirement is fulfilled by directly using the γ-secretase cleaved product, a synthetic Aβ 1-40 peptide, as substrate, and in cells this is accomplished through the cellular γ-secretase activities. A γ-secretase inhibitor(s) blocked the "truncation" of CT99 and, therefore, inhibited BACE cleavage at the Aβ–34 site in cells.

In summary, we show that BACE, both BACE1 or BACE2, cleaves Aβ-34 site in vitro and in cells, but only following γ-secretase mediated processing of APP. It is interesting to note that N-terminal truncation of APP by BACE or α-secretase is a prerequisite for γ-secretase cleavage around Aβ-40 sites. And it is, in turn, as shown in the present study, that γ-secretase cleavage at the C-terminus of Aβ peptide is a prerequisite for BACE
mediated processing at the Aβ-34 site. Our data indicate a sequential and mutual
dependence of BACE and γ-secretase activities in APP processing. We and others have
observed and reported that BACE cleaves at least 4 sites within a stretch of 50 amino acid
residues of APP, around and within the Aβ region with approximately every 10 amino
acid residues apart (18). Results described in this study further support such a complex
picture in APP metabolism: product from one enzyme can become substrate for the other
and verse visa.

The biological role of Aβ 1-34 species is still unclear. Although Aβ 1-34 species, either
in soluble or insoluble forms, has not been reported in AD brain, its presence in human
cerebrospinal fluid (CSF) has been observed (31). The contribution of Aβ 1-34 species to
AD pathogenesis remains to be determined. The discovery that BACE cleavage at Aβ-34
site is dependent on γ-secretase activity could facilitate our understanding of APP
processing by these two critical enzymes.

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References

1. Selkoe, D. J. (1999) Nature 399, A23-A31
2. Vassar, R., Bennett, B. D., Babu-Khan, S., Kahn, S., Mendiaz, E. A., Denis, P., Teplow, D. B., Ross, S., Amarante, P., Loeloff, R., Luo, Y., Fisher, S., Fuller, J., Edenson, S., Lile, J., Jarosinski, M. A., Biere, A. L., Curran, E., Burgess, T., Louis, J.-C., Collins, F., Treanor, J., Rogers, G., and Citron, M. (1999) *Science* **286**, 735-741

3. Sinha, S., Anderson, J. P., Barbour, R., Basi, G. S., Caccavello, R., Davis, D., Doan, M., Dovey, H. F., Frigon, N., Hong, J., Jacobson-Croak, K., Jewett, N., Keim, P., Knops, J., Lieberburg, I., Power, M., Tan, H., Tatsuno, G., Tung, J., Schenk, D., Seubert, P., Suomensaari, S. M., Wang, S., Walker, D., Zhao, J., McConlogue, L., and John, V. (1999) *Nature* **402**, 537-540

4. Yan, R., Bienkowski, M. J., Shuck, M. E., Miao, H., Tory, M. C., Pauley, A. M., Brashler, J. R., Stratman, N. C., Mathews, W. R., Buhl, A. E., Carter, D. B., Tomasselli, A. G., Parodi, L. A., Heinrikson, R. L., and Gurney, M. E. (1999) *Nature* **402**, 533-537

5. Cai, H., Wang, Y., McCarthey, D., Wen, H., Borchelt, D., Price, D., and Wong, P. (2001) *Nature Neuroscience* **4** (3), 233-234

6. Luo, Y., Bolon, B., Kahn, S., Bennett, B., Babu-Khan, S., Denis, P., Fan, W., Kha, H., Zhang, J., Gong, Y., Matin, L., Louis, J.-C., Yan Q., Richards, W., Citron, M., Vassar, R. (2001) *Nature Neuroscience* **4** (3), 231-232

7. Sisodia, S. S. (1992) *Proc. Natl. Acad. Sci. USA* **89** (13), 6075-6079

8. Kamal, A., Almenar-Queralt, A., LeBlanc, J. F., Roberts, E. A., and Goldstein, L. S. B. (2001) *Nature* **414**, 643-648

9. Citron, M., Teplow, D. B., and Selkoe, D. J. (1995) *Neuron* **14**, 661-670

10. Huse, J. T., Liu, K., Pijak, D. S., Carlin, D., Lee, V. M. Y., and Doms, R. W., (2002) *J. Biol. Chem.* **277**, 16278-16284

11. Benjannet, S., Elagoz, A., Wickham, L., Mamarbachi, M., Munzer J. S., Basak, A., Lazure, C., Cromlish, J. A., Sisodia, S., Checler, F., Chretien, M., and Nabil, G. S. (2001) *J. Biol. Chem.* **276**, 10879-10887

12. Yan R., Han, P., Miao, H., Greengard, P., and Xu, H. (2001) *J. Biol. Chem.* **276**, 36788-36796

13. Esler, W. P., Kimberly, W. T., Ostaszewski, B. L., Ye, W., Diehl, T. S., Selkoe, D. J., and Wolfe, M. S. (2002) *Proc. Natl. Acad. Sci. USA* **99** (5), 2720-2725

14. Cook, D. G., Forman, M. S., Sung, J. C., Leight, S., Kolson, D. L., Iwatsubo, T., Lee, V. M., and Doms, R. W. (1997) *Nature Medicine* **3** (9) 1021-1023
15. Greenfield, J. P., Tsai, J., Gouras, G. K., Hai, B., Thinakaran, G., Checler, F., Sisodia, S. S., Greengard, P., Xu, H. (1999) *Proc. Natl. Acad. Sci USA* **96**(2), 742-747

16. Li, Y-M., Lai, M-T., Xu, M., Huang, Q., DiMuzio-Mower, J., Sardana, M., Shi, X-P., Yin, K-C., Shafer, J. A., and Gardell, S. J. (2000) *Proc. Natl. Acad. Sci USA* **97**(11), 6138-6143

17. Li, Y-M., Xu, M., Lai, M-T., Huang, Q., Castro, J., DiMuzio-Mower, J., Harrison, T., Lellis, C., Nadin, A., Neduvilil, J. G., Register, R. B., Sardana, M., Shearman, M. K., Smith, A. L., Shi, X-P., Yin, K-C., Shafer, J.A., and Gardell, S. J. (2000) *Nature*, **405**, 689-694

18. Shi, X-P., Wu, G-X., Tugusheva, K., Register, R. B., Chen-Dodson, E., Price, E., Sardana, M., Lucka, A., Bruce, J. E., and Hazuda, D. J. (2002) *Neurobiology of aging* **23**, s180

19. Shi, X-P., Chen, E., Yin, K-C., Na, S., Garsky, V. M., Lai, M-T., Li, Y-M., Platchek, M., Register, R. B., Sradana, M., Tang, M-J., Thiebeau, J., Wood, T., Shafer, J. A., and Gardell, S. J. (2001) *J. Biol. Chem.* **276**, 10366-10373

20. Barelli, H., Lebeau, A., Vizzavona, J., Delaere, P., Chevallier, N., Drouot, C., Marambaud, P., Ancolio, K., Buxbaum, J. D., Khorkova, O., Heroux, J., Sahasrabudhe, S., Martinez, J., Warter, J-M., Mohr, M., and Checler, F. (1997) *Molecular Medicine*, **3**, 695-707

21. Ermolieff, J., Loy, J.A., Koelsch G., and Tang, J. (2000) *Biochemistry*, **39**(40), 12450-12456

22. Yan, R., Munzner, J. B., Shuck, M. E., and Bienkowski, M. J. (2001) *J. Biol. Chem.* **276**, 34019-34027

23. Farzan, M., Schnitzler, C., Vasilieva, N., Leung, D., and Choe, H. (2000) *Proc. Natl. Acad. Sci USA* **97**, 9712-9717

24. Seiffert, D., Bradley, J. D., Rominger, C. M., Rominger, D. H., Yang, F., Meredith, J. E., Jr., Wang, Q., Roach, A. H., Thompson, L. A., Spitz, S. M., Higaki, J. N., Prakash, S. R., Combs, A. P., Copeland, R. A., Arneric, S. P., Hartig, P. R., Robertson, D. W., Cordell, B., Stern, A. M., Olsen, R. E., and Zaczek, R. (2000) *J. Biol. Chem.* **275**, 34086-34091

25. Beher, D., Wrigley, J. D. J., Nadin, A., Evin, G., Masters, C. L., Harrison, T., Castro, J. L., and Shearman, M. S. (2001) *J. Biol. Chem.* **276**, 45394-45402
26. Shearman, M. S., Beher, D., Clarke, E. E., Lewis, H. D., Harrison, T., Hunt, P., Nadin, A., Smith, A. L., Stevenson, G., and Castro, J. L. (2000) *Biochemistry* **39**, 8698-8704

27. Turner III, R. T., Koelsch, G., Hong L., Castenheira, P., Ghosh, A., and Tang, J., (2001) *Biochemistry* **40** (34), 10001-10006

28. Vandermeeren, M., Geraerts, M., Pype, S., Dillen, L., Van Hove, C., Mercken, M. (2001) *Neurosci. Lett.* **315**, 145-148

29. Beher, D., Wrigley, J. D. J., Owens, A. P., and Shearman, M. (2002) *J. Neurochem* **82**, 563-575

30. Fluhrer, R., Multhaup, G., Schlicksupp, A., Okochi, M., Takeda, M., Lammich, S., Willem, M., Westmeyer, G., Bode, W., Walter, J., and Haass, C. (2003) *J. Biol. Chem.* **278**, 5531-5538

31. Vigo-Pelfrey, C., Lee, D., Keim, P., Lieberburg, I., and Schenk, D.B. (1993) *J. Neurochem* **61** (5), 1965-1968

**Figure Legends**

**Figure 1:**
A. Schematic representation of APP derived substrates used in this study.

Full length APP with the Aβ region and the transmembrane domain is indicated in the top (the distance is not proportional). The C-terminal product of BACE cleavage at the β-site of APP, CT99, is depicted in the middle of the Fig. 1. The amino acid sequence corresponding to the region around Aβ peptides is indicated in the lower panel. Arrows above the amino acid sequence indicate the major cleavage sites by BACE (β), α-secretase (α) and γ-secretase (γ). The arrows below the amino acid sequence indicate the major Aβ species with the cleavage sites identified by mass spectrometric analysis from our studies both in vitro and in cells.

B. BACE1 and BACE2, but not Cathepsin D, cleave the Aβ-34-site in vitro.
Aβ 1-40 substrate (50 µM) was incubated either alone (B1), or in the presence of 0.1 µM of human Cathepsin D (B2), or 0.1 µM of BACE1 (B3) or 0.1 µM of BACE2 (B4) for 20 hrs at 37°C under the conditions described in Materials and Methods. Samples were analyzed by mass spectrometric analysis using a surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF). Results are representative of three independent experiments.

Figure 2:
BACE1 lacking the transmembrane and cytoplasmic regions, both the pro- and mature forms, cleave the Aβ-34-site in vitro.

Aβ 1-40 substrate (50 µM) was incubated either alone (A), or in the presence of 0.2 µM of pro forms of BACE1(22-460) (B) or 0.2 µM of mature form of BACE1(46-460) (C) for 20 hrs at 37°C under the conditions described in Materials and Methods. Samples were analyzed by mass spectrometric analysis using a surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF). (Aβ X-X+2H)2+ denotes the Aβ species corresponding to the mass of the doubly charged peptide ion. Results are representative of three independent experiments.

Figure 3:
Mass spectrometric analysis of the in vitro cleavage of CT99 substrate by BACE1 and BACE2.

Recombinant CT99 substrate (34 µM) was incubated either alone (A, for mass range 7000-14000 and B, for mass range 1000-5000), or in the presence of 0.1 µM of BACE1 (C) or 0.1 µM of BACE2 (D) for 20 hrs at 37°C under the conditions described in Materials and Methods. Samples were analyzed by mass spectrometric analysis using a surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF). Results are representative of four independent experiments.
Figure 4:
Over-expression of BACE increase cellular Aβ 1-34 production

HEK293T cells stably expressing APP695 (HEK293TAPP695) were transiently transfected either with BACE1 or BACE2 cDNA as described in the Materials and Methods. Forty eight hours post-transfection, media were harvested and Aβ species secreted in the media were analyzed by a modified ELISA and mass spectrometry. (A) Aβ level secreted in media determined by a modified ELISA using 6E10 and G2-10 antibody. (B) Mass spectrometric analysis of Aβ species in the media captured by 6E10 antibody. Mock-transfected HEK293T/APP695 cells (A and B, 1); HEK293T/APP695 cells transfected with BACE1 (A and B, 2); HEK293T/APP695 cells transfected with BACE2 (A and B, 3).

Figure 5:
γ-secretase inhibitors blocked the production of Aβ 1-34 in HEK293T/APP695 cells transfected with BACE.

HEK293T/APP695 cells were either mock-transfected, or transfected with BACE1 or BACE2 cDNA. Twenty-four hours-post transfection, media were changed. Either DMSO control, compound-1 (10 μM), or compound-2 (10 μM) was added to the cells. Media were harvested following additional 24 hours incubation. Panel A, Aβ level in the media determined by a modified ELISA using 6E10 and G2-10 antibodies. Panel B, mass spectrometric analysis of Aβ species in the same media captured by 6E10 antibody.
Mock-transfected cells (A and B, 1-3); cells transiently transfected with BACE1 (A and B, 4-6), or with BACE2 (A and B, 7-9); DMSO control (A and B, 1, 4 and 7); Compound-1 (10 μM) (A and B, 2, 5 and 8); Compound-2 (10 μM) (A and B, 3, 6 and 9). Results are representative of three independent experiments.

Figure 6:
Blockade of cellular Aβ 1-34 production by γ-secretase inhibitor is concentration-dependent.

Compound-3 (1 μM and 10 μM), was tested for its ability to block Aβ 1-34 production in HEK293TAPP695 cells. Panel A, Aβ level in the media determined by a modified ELISA using 6E10 and G2-10 antibodies; Panel B, Mass spectrometric analysis of Aβ species in the same media captured by 6E10 antibody. Mock-transfected cells (A and B, 1-3); cells transiently transfected with BACE1 (A and B, 4-6), or with BACE2 (A and B, 7-9); DMSO control (A and B, 1, 4 and 7); Compound-3 (1 μM) (A and B, 2, 5 and 8); Compound-3 (10 μM) (A and B, 3, 6 and 9). Results are representative of two independent experiments.

Figure 7
γ-secretase inhibitors have no effect on the activities of BACE1 or BACE2 in vitro

BACE1, 6 nM (Panel A) or BACE2, 60 nM (Panel B) was incubated with 2.5 μM of a FRET peptide substrate and one of the following agents: DMSO, 2% (○); BACE inhibitor, StatV, 10 μM (●); γ-secretase inhibitors: compound-1, 10 μM (▲); compound-2, 10 μM (◇) and compound-3, 10 μM (△). Product formation was determined at various times using a LJL Analyst AD instrument with excitation of 530 nm and emission of 580 nm. Results are representative of three independent experiments.
Fig. 1

A

....EVKMDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVATVI....

Aβ-sites: 1 16 19/20 34 40

B

1 3

2 4

Fig. 1
Fig. 3
Fig. 4

Aβ Level (E10/G2-10, ECL X10e3)

| Group | 1  | 2  | 3  |
|-------|----|----|----|
| ctrl | 0  | 0  | 0  |
| 1    | 100| 200| 300|
| 2    | 400| 500| 600|
| 3    | 700| 800| 900|

m/z

Fig. 4
B (continued)

Fig. 5
Fig. 6

A

Compound-3

B

![Mass Spectra](mass_spectra.png)

Aβ Levels (ECL X 10^4)

| Sample | Aβ Peptide |
|--------|------------|
| 1      | Aβ 1-19    |
| 2      | Aβ 1-20    |
| 3      | Aβ 1-40    |

m/z Range:

- Peptide 1: 2000 - 5000
- Peptide 2: 3000 - 6000
- Peptide 3: 4000 - 7000
Fig. 6

B (continued)
Fig. 7

A.

B.
Beta-secretase cleavage at the amino acid residue 34 in the amyloid beta peptide is dependent upon the gamma-secretase activity

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