γ-Secretase Processing and Effects of γ-Secretase Inhibitors and Modulators on Long Aβ Peptides in Cells*

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Background: Aβ peptides are generated by stepwise cleavage of the amyloid precursor protein.

Results: Aβs longer than Aβ1–48 are efficiently cleaved by γ-secretase in cells with different response to inhibitors (GSIs) and modulators (GSMs).

Conclusion: The initial γ-secretase cleavage does not precisely define subsequent product lines.

Significance: Understanding how different species of Aβ are generated and modulated by small molecules has broad therapeutic implications.

Although it has been modified and challenged, the amyloid cascade hypothesis remains the most accepted hypothesis regarding the etiology of Alzheimer disease (1). The core tenant of this hypothesis—that accumulation of the amyloid β (Aβ)3 peptide aggregates in the brain triggers a complex pathophysiological process leading to widespread brain organ failure—is strongly supported by genetic, pathological modeling, and more recently clinical studies in living humans (2–4). In most cases of Alzheimer disease, Aβx-42 and possibly other longer Aβ peptides (e.g., Aβx-43) have been implicated as key pathogenic species that are at least required for initiating Aβ aggregation and accumulation (5–10). Thus, understanding how different species of Aβ are generated remains a critically important issue in Alzheimer disease and has important implications for development of therapies designed to affect production of Aβ.

Three proteolytic activities are involved in the secretory processing of amyloid β precursor protein (APP) (11). In the periphery, APP is primarily processed by α-secretase (12). α-Secretase primarily cleaves APP after residue 16 of Aβ, which is considered a nonamyloidogenic pathway. ADAM10 and 17 have been identified as the major α-secretases (13–15), although other proteases may also cleave APP near this site (e.g., BACE2) (11, 16). Aβ1-x is the cleavage product of APP by two membrane-bound proteases: β- and γ-secretase. β-Secretase (BACE1) primarily cleaves APP at the N terminus of the Aβ peptide and releases the sAPPβ ectodomain (17–19). Cleavage of the resulting C-terminal fragment (CTF) by γ-secretase results in the release of Aβ (20, 21). As opposed to processing in peripheral cells and organs, a much greater amount of APP is processed in the amyloidogenic β-secretase pathway in the brain, especially in neurons (19).

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3The abbreviations used are: Aβ, amyloid β peptide; APP, amyloid precursor protein; PSEN1, presenilin 1; BRI2, integral membrane protein 28; IP/MS, immunoprecipitation MALDI-TOF mass spectrometry; CHAPSO, 3-[(3-Cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonate; GSI, γ-secretase inhibitor; GSM, γ-secretase modulator; iGSM, inverse GSM; CTF, C-terminal fragment.
γ-Secretase cleavage of APP CTFs has been proposed to occur in three distinct steps: (i) an initial e-cleavage near the cytoplasmic face of the transmembrane domain that liberates the cytoplasmic tail of the substrate (22, 23), (ii) stepwise tri- or tetra-peptide carboxyl-peptidase-like cleavages, and (iii) a final cleavage releasing the Aβ peptide (24). The most commonly observed final products are Aβ1–37, 1–38, 1–39, 1–40, and 1–42 (25). Aβ1–45 and Aβ1–46 that can be generated during the stepwise cleavage have typically only been detected in the presence of certain γ-secretase inhibitors (GSIs) or in in vitro assays (26–28), although some studies have provided limited evidence that these species may be present in human and APP transgenic mouse brain (27, 29). Aβ1–48 and Aβ1–49, the main Aβs produced from the initial e-cleavage are typically not detectable in physiological models, but their generation is inferred from the identification of the corresponding APP intracellular domains, C51 and C50 (22, 23). These e-sites are considered the main initial cleavage sites within the APP CTF. A third site generating Aβ1–51 and the APP intracellular domain C49 has also been identified in broken cell γ-secretase assays and in presenilin APP mutant cell lines (30). Shorter Aβ peptides such as Aβ1–17 through Aβ1–20 have also been reported, but whether they are produced by γ-secretase alone or the combined action of γ-secretase and other proteases has not been resolved (25, 31–33).

Because there is evidence that the initial cleavage of APP at Aβ1–51 or Aβ1–48 generates a higher ratio of Aβ1–42, whereas cleavage at Aβ1–49 generates higher levels of Aβ1–40, Aβ1–40 and Aβ1–42 have been proposed to be generated from two different "product lines" in a stepwise cleavage model. Funamoto et al. (34) provided initial evidence for this model using truncated Aβ minigenes in which Aβ was expressed in mammalian cells as APP signal peptide: Aβ fusion proteins. Cells expressing Aβ1–49 generated predominantly Aβ1–40 and cells expressing Aβ1–48 generated much more Aβ1–42, although the absolute amount of Aβ generated was very low (~0.2 pm compared with >200 pm from Aβ1–49). Takami et al. (24) subsequently provided direct evidence for stepwise cleavage using a very elegant strategy, which combined in vitro γ-secretase cleavage and LC-MS/MS to quantify the specific peptides that were postulated to be released during stepwise processing. They showed that Aβ1–40 can be preferentially derived from Aβ1–49 through sequential removal of ITL, IVV, and IAT peptides, whereas Aβ1–42 is preferentially derived from Aβ1–48 through sequential cleavage of VIT and TVI peptides. More recently, additional support for this stepwise cleavage model has come from several studies showing that FAD-linked mutations in APP and PSEN can not only shift the initial e-cleavage site, but also can alter subsequent processivity, thus increasing the relative production of long Aβ peptides (35, 36). One of these studies also suggested that Aβ1–38 arises from an additional stepwise cleavage of Aβ1–42 (36), a finding directly supported by a recent study showing that Aβ1–42 could be further processed into Aβ1–38 in vitro, whereas Aβ1–43 is cleaved into both Aβ1–40 and Aβ1–38 (37).

In the present study, we further explored the stepwise cleavage model initially proposed by Ibara and co-workers (24, 34) using BRI2-Aβ1-x fusion proteins that produced Aβ peptides ranging from 1–38 to 1–55. We have previously employed this strategy to efficiently express individual Aβ proteins in cells and in the brains of transgenic mice (38–40). Because many of the studies supporting a stepwise cleavage model for γ-secretase processing of APP have been performed in in vitro assays or in cells utilizing signal peptide Aβ peptide expression constructs that produce Aβ peptides that are inefficiently secreted, we hypothesized that the BRI2 fusion protein strategy, which results in efficient processing and secretion of Aβ from the fusion protein, might provide a more physiologic model to assess γ-secretase processivity. Although our studies further support the sequential γ-secretase cleavage model, they show that in cells the initial γ-secretase cleavage site does not absolutely define subsequent product lines. Unexpectedly, we also find (i) that under some circumstances long Aβ peptides generated in cells following processing of the BRI2 fusion protein are efficiently secreted as intact soluble peptides; (ii) that the long Aβ peptides dramatically decrease the relative potency of multiple GSIs compared with the potency of the same GSIs when APP or C99 is used as a substrate; and (iii) that two PSEN1 mutants selectively alter processing for Aβ1–51, but not Aβ1–49.

**Experimental Procedures**

**DNA and Cell Culture**—Fusion constructs encoding the first 243 amino acids of BRI2 protein followed by Aβ peptides encompassing various Aβ species from Aβ38 to Aβ55 or C99 (99 amino acids on the C-terminal of APP) were generated as previously described (38). The fragments were ligated into the expression vector pAG3. Sequences were verified by DNA sequencing. The overexpression was performed by transiently transflecting human embryonic kidney (HEK 293T) and CHO cells. Cells were grown in either DMEM (HEK) or Ham’s F-12 (CHO) media supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin/streptomycin (Invitrogen). Briefly, 2.7 μg of DNA was applied to a 75% confluent 6-well plate (Corning) using the polycation polyethyleneimine transfection method (41). When BRI2-Aβ constructs were co-transfected with either wild-type PSEN1 or the mutants M139V or Δexon9 into CHO cells, 1.35 μg of each DNA were used. In some experiments, 10-cm dishes were used with appropriately adjusted amounts of DNA. Cells were incubated with transfection reagent for 12–16 h, after which the growth medium was replaced with fresh medium. DMSO (Sigma), GSIs, or GSMs were added to the appropriate concentration. The GSIs L685,458 and MRK680 were purchased from Tocris; the GSIs sulindac sulfide and fenofibrate were purchased from Sigma. (Z-LL)2 ketone was from Calbiochem. LY411,575, GSM1, and compound 2 were synthesized by A. Faqu at the Mayo Clinic Clinical Core. 24 h later, the medium was collected for assay by ELISA and MS. Stable CHO cell lines were selected with 0.8 μg/ml hygromycin B. To evaluate any potential non-γ-secretase-mediated proteolytic cleavage of the long secreted Aβs, 1 μM synthetic Aβ1–49 was incubated with CHO cells overnight, and then Aβ in the media was analyzed by immunoprecipitation MALDI-TOF mass spectrometry (IP/MS), as described
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below. A sample with 1 μM synthetic Aβ1–49 in fresh media was used as control.

Immunoprecipitation and Mass Spectrometry—50 μl of magnetic sheep anti-mouse IgG beads (Invitrogen) were incubated with 4.5 μg of Ab5 antibody for 30 min at room temperature with constant shaking. The beads were then washed with PBS and incubated with 1–10 ml of conditioned medium, to which 0.1% Triton X-100 was added for 1 h. For cell lysate IP, cells from a 10-cm dish were washed twice with PBS and then incubated with 1 ml of PBS with 1% Triton X-100 on ice for 20 min. Lysates were centrifuged at 1500 × g for 10 min at 4 °C with the resulting supernatants diluted with 9 ml PBS and incubated with Ab5-pretreated beads. Bound beads were washed sequentially with 0.1% and 0.05% octyl glucoside (Sigma) followed by water. Samples were eluted with 10 μl of 0.1% trifluoroacetic acid (Thermo Scientific, Rockford, IL) in water. 2 μl of elute was mixed with an equal volume of saturated α-cyano-4-hydroxycinnamic acid (Sigma) solution in 60% acetonitrile, 40% methanol. 1 μl of sample mixture was loaded on to α-cyano-4-hydroxycinnamic acid pretreated MSP 96 target plates (Bruker Daltonics Inc., Billerica, MA). The samples were analyzed with a Bruker Microflex (Bruker Daltonics Inc.) mass spectrometer.

Aβ ELISA—Sandwich ELISAs used for Aβ detection were performed as described previously (42). Aβ40 and Aβ42 were captured with mAb 13.1.1 (human Aβ35–40 specific) and mAb 2.1.3 (human Aβ35–42 specific), respectively (43, 44). Both were detected with HRP-conjugated mAb Ab5 (human Aβ1–16 specific) (45). Total Aβ was captured with mAb Ab5 and detected by HRP-conjugated mAb 4G8 (Covance, Princeton, NJ). All ELISAs were developed with TMB substrate (KPL, Gaithersburg, MD).

In Vitro Assay—Aβ1–48, Aβ1–49, and C99 (CTF99 plus a methionine at the N-terminal and FLAG tag at the C-terminal) were used for in vitro assay. Aβ48 and Aβ49 were purchased from AAPPTEC (Louisville, KY). The peptides were pretreated with 1,1,3,3,3-hexafluoroisopropanol. After drying, the peptides were dissolved in 0.1% ammonia hydroxide. C99 was purified as described with modifications (46). Briefly, inclusion bodies were dissolved in Tris-HCl-urea buffer (25 mM Tris, 8 M urea, pH 8.0). After addition to a Hitrap Q column (GE Healthcare), the column was washed with 10 × column volume of Tris-HCl-urea buffer and then Tris-HCl-CHAPSO buffer (25 mM Tris, 1% CHAPSO, pH 8.0). C99 was eluted using Tris-HCl-CHAPSO buffer with NaCl gradient. Gel filtration and anti-FLAG M2 affinity column can be used if further purification is necessary. CHAPSO-solubilized γ-secretase was prepared from CHO S-1 cells as previously described (47). Briefly, S-1 cells were washed once with cold PBS and then resuspended in PBS with Complete protease inhibitors (Roche Applied Science). After nitrogen cavitation at 750 p.s.i. on ice for 1 h, cell debris and nuclei were removed by centrifugation at 1000 × g for 10 min. The supernatant was centrifuged at 100,000 × g for 1 h to pellet the total membranes. The membrane pellet was resuspended and homogenized in cold sodium bicarbonate buffer (100 mM NaHCO3, pH 11.0). After centrifugation at 100,000 × g for 1 h, the pellet was solubilized with CHAPSO buffer (150 mM sodium citrate, 1% CHAPSO, 5 mM EDTA) by slightly vortex and incubated on ice for 1 h. The insoluble debris was removed by centrifugation at 100,000 × g for 1 h. For in vitro assay, substrates (25 μM for MS, 1 μM for ELISA) were incubated with CHAPSO solubilized γ-secretase (100 μg/ml protein concentration) in sodium citrate buffer (150 mM, 1X complete protease inhibitor, pH 6.8) for 3 h at 37 °C in the presence or absence of γ-secretase inhibitors.

RESULTS

BRI2-Aβ1-x Fusion Proteins Efficiently Produce Aβ Peptides Ranging from 1–38 to 1–55—We had previously employed the BRI2-Aβ1-x fusion protein strategy to efficiently generate Aβ1–40 and Aβ1–42 in the late secretory pathway; additionally we had also shown that a BRI2-C99 (CTFβ) fusion protein efficiently generated C99 that could be subsequently processed by γ-secretase to Aβ (38, 40, 48, 49). Here we evaluated whether the BRI2 fusion protein strategy could be used to generate Aβ peptides spanning from 1–38 to 1–55 to examine their subsequent proteolytic processing. Expression plasmids encoding the various BRI2-Aβ1-x proteins were transiently transfected into HEK-293 cells with Aβ assessed in the cells and in the media by both Aβ ELISAs and IP/MS. Each transfected BRI2 fusion construct resulted in overexpression and secretion of the full-length Aβ1-x peptides as assessed by IP/MS (Fig. 1A). The calculated molecular mass for each peptide and the average observed molecular mass of each peptide are listed in Table 1. Overall, mass accuracy was within 0.05%. To determine whether the secreted Aβ1-x peptides were present in exosomes or present as soluble secreted proteins, conditioned media were centrifuged at 100,000 × g for 1 h. Aβ1-x peptides remained in the supernatants with no depletion, indicating that they are produced as soluble proteins. Full-length Aβ1-x peptides were also detectable in the cell lysate (data shown for a subset of the fusion proteins, Fig. 1B). Western blotting of the cell lysate with BRI2 protein antibody and an antibody recognizing the N terminus of Aβ (6E10) demonstrated that the constructs were efficiently overexpressed (Fig. 1C). In the HEK-293 cell studies, we were not able to detect shorter Aβ cleavage products in the media by IP/MS. However using Aβ1–40 and Aβ1–42 ELISAs coupled with GSI treatment, we could establish that both Aβ1–40 and Aβ1–42 were being produced by γ-secretase processing from constructs longer than Aβ1–47 (Fig. 2A). Compared with other constructs, BRI2-Aβ1–51 showed a different production profile, which generated significant amount of Aβx-42. Aβx-42 was detected by ELISA from Aβ1–43, 44, 45, 46, and 47, but this likely represents detection of these species by mm2.1.3, the anti-Aβx-42 antibody used for capture. This antibody, like most anti-Aβ antibodies, shows cross-reactivity with longer Aβ peptides. Indeed, GSI treatment did not significantly lower levels of Aβx-42 detected by ELISA from these Aβ species (Fig. 2B).

Aβ1-x Peptides Longer than Aβ1–48 Are Processed by γ-Secretase in CHO Cells—In parallel studies using select constructs transfected into CHO cells, we were able to detect processing of the secreted Aβ peptides produced from BRI2-Aβ fusion proteins longer than Aβ1–48 by both IP/MS and Aβ ELISA. Aβ ELISA results from CHO cells (both transiently and stably transfected) were similar to those from HEK-293 cells (Fig. 3, A and B). All constructs longer than Aβ1–48 appeared
to be efficiently processed by γ-secretase to produce Aβx-40 and x-42 because 1 μM of the GSI LY-411,575 almost completely inhibited production of these Aβ peptides (Fig. 3C). Although small amounts of Aβ1–40 produced by Aβ1–47 or Aβ1–48 was also completely inhibited by the GSI, Aβ1–42 was not. The lack of complete inhibition of Aβ1–42 likely represents some detection of the full-length peptide by the ELISA. By IP/MS from 1 ml of media, intact Aβ1–47 from BRI2-Aβ1–47 cells and Aβ1–48 BRI2-Aβ1–48 could be detected. Although BRI2-Aβ1–48 was not efficiently processed by γ-secretase, it appeared to be processed preferentially to Aβ1–42, but not other processed Aβ species, and could be detected by IP/MS from 10 ml of conditioned media from BRI2-Aβ1–48 cells (Fig. 3D). Although small amounts of full-length Aβ1–49 could also be detected from cells expressing the BRI2-Aβ1–49 fusion protein, full-length species could not be detected from fusion proteins expressing longer Aβs (Aβ1–50 to 1–55). Consistent with the concept of a preferential product line for Aβ1–40, Aβ1–49 was cleaved predominately to Aβ1–40 and a small amount of Aβ1–37. Aβ1–50 showed a similar profile to Aβ1–49, although by ELISA a small amount of Aβ1–42 could be detected. In contrast, BRI2-Aβ1–51 showed a distinct profile producing a larger proportion of Aβ1–42 as assessed by both IP/MS and ELISA. Moreover, minor amounts of Aβ1–37, 38,
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TABLE 1

| Molecular mass of Aβ peptide detected in the conditioned medium | Construct | Molecular mass |
|---------------------------------------------------------------|-----------|----------------|
|                                                               | Calculated | Observed       |
| BR12-Aβ1–36                                                   | 4017.48    | 4018.04        |
| BR12-Aβ1–37                                                   | 4074.53    | 4074.13        |
| BR12-Aβ1–38                                                   | 4131.58    | 4130.99        |
| BR12-Aβ1–39                                                   | 4230.72    | 4230.10        |
| BR12-Aβ1–40                                                   | 4329.85    | 4329.12        |
| BR12-Aβ1–41                                                   | 4443.01    | 4442.80        |
| BR12-Aβ1–42                                                   | 4514.09    | 4513.74        |
| BR12-Aβ1–43                                                   | 4615.19    | 4615.05        |
| BR12-Aβ1–44                                                   | 4714.32    | 4713.41        |
| BR12-Aβ1–45                                                   | 4827.48    | 4826.27        |
| BR12-Aβ1–46                                                   | 4926.62    | 4924.26        |
| BR12-Aβ1–47                                                   | 5039.78    | 5038.99        |
| BR12-Aβ1–48                                                   | 5140.88    | 5139.85        |
| BR12-Aβ1–49                                                   | 5254.04    | 5254.18        |
| BR12-Aβ1–50                                                   | 5353.17    | 5353.56        |
| BR12-Aβ1–51                                                   | 5484.37    | 5482.94        |
| BR12-Aβ1–52                                                   | 5597.53    | 5596.55        |
| BR12-Aβ1–53                                                   | 5725.70    | 5725.36        |
| BR12-Aβ1–54                                                   | 5853.87    | 5852.34        |
| BR12-Aβ1–55                                                   | 5982.05    | 5983.31        |

39, 40, and 41 were produced from the BR12-Aβ1–51 construct. IP/MS profiles and ELISA data from BR12-Aβ1–52, Aβ1–55, and C99 were similar, with each producing a typical spectrum of Aβ peptides with Aβ1–40 as the major species and minor amounts of Aβ1–37, 38, 39, and 42 detected. To evaluate the potential non-γ-secretase-mediated proteolytic cleavage of the long secreted Aβs, synthetic Aβ1–49 was incubated with CHO cells overnight, and then Aβ1–49 in the media was analyzed by IP/MS. These data revealed that Aβ1–49 is quite stable in the media and not cleaved to smaller Aβ species (Fig. 3E).

To determine whether FAD PSEN1 variants change the production of long Aβ substrates, PSEN1 wt, M139V and Δexon9 expression plasmds were transiently co-transfected into CHO cells along with expression plasmds for BR12-C99, BR12-Aβ1–49, or BR12-Aβ1–51. When Aβ levels were assessed in the conditioned media using Aβ40 and Aβ42 ELISAs, we found that both M139V and Δexon9 significantly increased the Aβ42/Aβ40 ratio from BR12-C99 and BR12-Aβ1–51 but not from BR12-Aβ1–49 transfected cells (Fig. 4).

**Truncated Substrates Alter IC_{50} values of Several GSIs but Do Not Alter Effect of GSs**—The effects of two GSIs (1 μM of L65,458 and 100 nM MRK560 (50, 51)) on production of secreted Aβ1–40 from cells transfected with BR12-Aβ49 to BR12-Aβ55, BR12-C99, and APP was assayed by ELISA (Fig. 5, A and B). At these concentrations both GSIs showed much less ability to inhibit Aβ1–40 production from the BR12-Aβ constructs compared with GSIs on full-length APP and for L65,458; the BR12-Aβ constructs also were less responsive to the GSI than BR12-C99. To more fully explore this phenomena, we conducted dose-response studies comparing the effects of L65,458 on total Aβ and Aβ1–40 production from transiently transfected HEK 293 cells expressing BR12-Aβ1–49 and APP (Fig. 5C). In these studies, the IC_{50} of L65,458 for inhibition of Aβ1–40 shifted from 6.9 nM for APP to 362 nM for BR12-Aβ49, and for total Aβ shifted from 4.8 nM for APP to 14 μM for BR12-Aβ49. Tests of L65,458 with stably transfected CHO cells show similar effects (Fig. 5D), with the IC_{50} for inhibition of Aβ1–40 shifted from 65.6 nM for APP to 771.3 nM for BR12-Aβ49. Additional dose-response studies using the GSIs MRK560 and LY-411,575 (52) also show that the IC_{50} for inhibition of Aβ1–40 production from BR12-Aβ1–49 is greatly increased as compared with APP (Table 2). We also assessed whether similar effects could be observed with in vitro γ-secretase assays using recombinant C99 and synthetic Aβ1–49 as substrate. As shown in Fig. 5E and Table 2, inhibition of Aβ1–40 production from Aβ1–49 (IC_{50} 637 nm) was markedly less sensitive to inhibition by MRK560 than inhibition of C99 cleavage (0.3 nm).

In contrast to the loss in potency for multiple GSIs, we found no evidence for differential effects of two potent GSIs (GSM1 and compound 2) and two inverse GSIs (fenofibrate and (Z,L)-ketone) on modulation of Aβ1–42 generation from cells expressing BR12-Aβ1–49, 51, and 55 (Fig. 6A). Even though the yield of Aβx-42 from BR12-Aβ49 was very low (5–10 pM, which is close to the detection limit of the ELISAs 1–2 pM), we were still be able to evaluate the modulation. At the concentrations used, all modulatory compounds showed a similar effect on Aβx-42 production as we had previously observed for APP or BR1-C99. The lack of differential modulation was further established by directly comparing the effect of varying concentrations of GSM1 (Fig. 6B) and compound 2 (Fig. 6C) on Aβx-42 production in CHO stable lines expressing BR12-Aβ1–49, Aβ1–51, and Aβ1–55 and C99. No significant difference in dose response to these GSIs was observed.

Because these BR12-Aβ constructs were still sensitive to modulation, we used IP/MS to explore the changes in secreted Aβ profiles induced by GSM1, compound 2, fenofibrate, and (Z,L)-ketone from cells expressing BR12-Aβ1–49, 1–51, and 1–55. Representative spectra from these studies are depicted in Fig. 7A, and the data are summarized in Fig. 7B. Studies of modulatory effects on BR12-Aβ1–49 showed that GSM1 slightly increased relative levels Aβ1–37 and enabled the detection of Aβ1–38. Compound 2 dramatically shifted the spectra with large increases in Aβ1–36 and 1–37 noted and also a decrease in 1–40. In contrast, both iGSMS appeared to decrease the detection of shorter Aβ peptides but did not increase Aβ1–42. The effects of the compounds on the spectra from BR12-Aβ1–51 were more dramatic; both GSM1 and compound 2 decreased Aβ1–39, 40, and 42 and increased Aβ1–37 and 38, although the effects of compound 2 were more dramatic in terms of reduction of Aβ1–39, 40, and 42. iGSMS decreased shorter Aβ levels and increased Aβ1–42. For BR12-Aβ1–55, the results were similar to BR12-Aβ1–51, but less dramatic for most compounds. GSM1 lowered Aβ1–42 and increased Aβ1–37 in 1–38. Compound 2 increased Aβ1–36, 37, and 38 and lowered Aβ1–39, 40, and 42. Both iGSMS increased the level of Aβ1–42.

**DISCUSSION**

Understanding the precise mechanism by which γ-secretase generates different Aβ peptide species has both pathologic and therapeutic relevance. Our data, which use both ELISA and IP/MS methods to evaluate γ-secretase processing of Aβ1-x peptides ranging in length from 38 to 55 amino acids in cells, support and extend a number of recent studies demonstrating that stepwise cleavage by γ-secretase results in the generation of Aβ49.
Although our data in general are consistent with the stepwise model of \( \gamma \)-secretase cleavage of APP along different product lines that preferentially produce A\( \beta \)1–40 or A\( \beta \)1–42 (34), they demonstrate a number of novel observations regarding the solubility of these long A\( \beta \) peptides and their processing by \( \gamma \)-secretase. First, we find that following transient transfection in HEK cells, all of the A\( \beta \)1-x peptides are efficiently secreted as intact proteins that are soluble and easily detectable by IP/MS. Notably, both the long peptides derived from the BRI constructs and synthetic A\( \beta \)1–49 are quite stable in the media. This result is unexpected given that A\( \beta \)s longer than A\( \beta \)1–42 have been reported to be difficult to detect by mass spectrometry and might be expected to be membrane-associated because they contain a portion of or the entire

![FIGURE 2. Processing of BRI2-A\( \beta \)1-x in transiently transfected HEK 293T cells is detectable by A\( \beta \) ELISA. (A) A\( \beta \)x-40 and A\( \beta \)x-42 ELISA of conditioned media from transiently transfected HEK 293T cells expressing the various BRI2-A\( \beta \)1-x vectors. (B) Percentage of inhibition of A\( \beta \)x-42 determined by ELISA production for each construct in the presence of 1 \( \mu \)M LY411,575 compared with DMSO control. The numbers below each bar indicate the length of the corresponding BRI2-A\( \beta \)1-x constructs.](image)

![FIGURE 3. Constructs longer than A\( \beta \)1–48 are efficiently processed by \( \gamma \)-secretase in stable CHO cells. (A) A\( \beta \)x-40 and A\( \beta \)x-42 ELISA of conditioned media from stable cell line transfected with various BRI2-A\( \beta \)1-x constructs. (B) Percentage of inhibition of A\( \beta \)x-40 to A\( \beta \)x-40 production of each BRI2-A\( \beta \)1-x construct is shown. (C) Percentage of inhibition of A\( \beta \)x-42 determined by ELISA for each construct in the presence of 1 \( \mu \)M LY411,575 compared with DMSO control. (D) IP/MS spectra of select BRI2-A\( \beta \)1-x constructs. 10 ml of conditioned media was used for the BRI2-A\( \beta \)1–47 and BRI2-A\( \beta \)1–48 constructs, with 1 ml used for the remaining constructs. Labels in the spectra indicate the corresponding A\( \beta \) peptide, e.g., 37 for A\( \beta \)1–37. Intact A\( \beta \)1–47, 48, and 49 are labeled with asterisks. Peaks without labeling are nonspecific. (E) IP/MS spectra of fresh Ham’s F-12 medium with 1 \( \mu \)M synthetic A\( \beta \)1–49 (top panel) and media from CHO cells that was incubated with 1 \( \mu \)M A\( \beta \)1–49 overnight (bottom panel).](image)
transmembrane domain of APP. Although we do detect full-length Aβ1–47 and Aβ1–48 secretion from the stable CHO cell lines, we do not detect longer full-length Aβ peptides. The reasons for this difference between the two different experimental paradigms is not clear at this time but perhaps might reflect subtle differences in membrane lipid composition influencing both γ-secretase processing and membrane binding; it has been shown that lipid composition can influence γ-secretase processivity (53). Because these long Aβ peptides are challenging to produce synthetically, the ability to produce soluble long Aβ peptides in HEK cells could be exploited as a source of standards for other biological studies. Second, although shorter Aβ peptides can be shown to be processed in vitro by detergent-solubilized γ-secretase, we find that in cells, peptides shorter than Aβ1–49 are not efficiently processed by γ-secretase; presumably they do not efficiently insert into intact non-detergent-solubilized membranes. In stable CHO cell lines, Aβ1–47 and 1–48 undergo very limited γ-secretase processing, most of the secreted peptides detected in these cells are the full-length peptides and not shorter Aβs. However, based on GSI inhibition studies, it does appear that the small amount of Aβ1–40 and 1–42 produced is generated by γ-secretase cleavage. In contrast, Aβ1–49 is efficiently cleaved in the stable CHO line to Aβ1–40 and 1–37. Third, although our data support the concept of product lines with the initial ε-cleavage generating Aβ1–49 cleavage primarily leading to Aβ1–40 and initial ε-cleavages at Aβ1–48 or 1–51 generating primarily Aβ1–42, it is clear that these product lines are not completely distinct. Aβ1–49 generates small amounts of Aβ1–42, detectable by ELISA; Aβ1–48 generates Aβ1–37 and small amounts of

FIGURE 4. PSEN1 mutants increase Aβ42:Aβ40 from CHO cells expressing BRI2-C99 and BRI2-Aβ1–51 but not from cells expressing BRI2-Aβ1–49. PSEN1 WT, variant M139V, or exon9 was transiently co-transfected with BRI2-C99 (A), BRI2-Aβ1–49 (B), or BRI2-Aβ1–51 (C), and then Aβx-40 and Aβx-42 were measured by ELISA. The data represent the results from four to seven independent experiments, with two or three replicates in each experiment per condition. Statistical analysis was performed by one-way analysis of variance. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

FIGURE 5. Effects of GSI on γ-secretase cleavage of long BRI2-Aβ1–x constructs. A and B, Aβx-40 production in the presence of 1 μM γ-secretase inhibitors L685,458 (A) or 0.1 μM MRK560 (B) using transiently transfected HEK cells. The ratio of Aβx-40 in the presence of inhibitors to Aβx-40 in the absence of inhibitors (DMSO) were used to evaluate the extent of inhibition. The data are expressed and graphed as means ± S.E. with statistical analysis performed by one-way analysis of variance followed by Dunnett’s post hoc testing for group differences. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001. C and D, dose response of L685,458 on Aβx-40 production in APP wild type and BRI2-Aβ1–49 from transiently transfected HEK-293 cells (C) or stable CHO cells (D), respectively. E, effect of MRK560 on Aβx-40 production in an in vitro γ-secretase assay using recombinant C99 and synthetic Aβ1–49.
TABLE 2
IC_{50} values of select γ-secretase inhibitors on APP constructs
Each assay was repeated at least two times. The values in the table are the means ± standard errors.

| GSI       | Cells* | APP | BR1-2Aβ1–49 nm | Aβ40 | APP | BR1-2Aβ1–49 nm | C99 | Aβ1–49
|-----------|--------|-----|----------------|------|-----|----------------|-----|------|
| L685,458  | HEK    | 4.8 ± 1.0 | 1400.0 ± 90.0 | 6.9 ± 1.5 | 362.4 ± 40.0 | 0.3 ± 0.1 | 637.0 ± 15.0 |
| L685,458  | CHO    | 12.8 ± 9.1 | 2794 ± 13.2 | 65.5 ± 2.1 | 771.3 ± 5.5 |
| MKR560    | HEK    | 6.9 ± 1.5 | >500 | 2.0 ± 1.0 | 53.3 ± 4.2 |
| LY411,575 | HEK    | 0.04 ± 0.01 | 4.30 ± 0.50 | 0.2 ± 0.3 | 5.5 ± 2.1 |
| LY411,575 | CHO    | 0.11 ± 0.05 | 3.26 ± 0.61 | 0.01 4.30 | 0.2 ± 0.3 |

* HEK 293 T cells were transient transfections; CHO cells are stable lines.
* Recombinant C99 (data generated from an in vitro assay).
* Synthetic Aβ1–49 (data generated from an in vitro assay).

Aβ1–40 and 1–42; and Aβ1–51 generates Aβ peptides 1–37 to 1–42. Notably, the diversity of species generated from Aβ1–51 would suggest that when produced in cells, the stepwise cleavage is more heterogeneous than what is observed for Aβ1–49.

When BR12-Aβ1–49 was co-transfected with either the M139V or Δexon9 PSEN1 mutants, neither altered the ratio of Aβ42:40 from BR12-Aβ1–49. In the present study, M139V only increased Aβ42 from BR12-C99 and BR12-Aβ1–51 but not from BR12-Aβ1–49. Although these data do not evaluate the effect on e-cleavage, our data suggest that M139V and Δexon9 mutants alter Aβ42 by specifically inhibiting processivity by one cycle along the Aβ1–48/51 but not the Aβ1–49 production line. We would note that previous studies on M139V have led to different conclusions; a cell-free assay had shown that M139V does not alter e-cleavage efficiency but does impair the fourth stepwise cleavage along both product lines when C99 was used as an in vitro substrate (36). In a separate report using a cell model, M139V increased Aβ42 but also reduced e-cleavage efficiency (54). However, our present results are consistent with a previous study from our group where we found that the M139V mutant selectively raised Aβ42 but did not alter levels of other peptides in CHO cells stably overexpressing this mutant (55). Discrepancies between these results might be explained by the variable methodology, especially in cell culture systems where there are admixtures of wild-type and mutant PSEN1.

Another unexpected result from our study was the finding that the long Aβ peptides that are efficiently processed by γ-secretase in cells (e.g., Aβ1–49 to 1–55) are much less sensitive to inhibition by various GSIs. This loss of sensitivity to GSIs was observed in cells and in vitro using recombinant substrates. The basis for the differential sensitivity of these long Aβ peptides to GSI inhibition is not clear. However, given the rather dramatic effects observed, in some cases as much as a 300-fold differential sensitivity between Aβ1–49 and C99 in vitro, these data suggest that IC_{50} measures established for a GSI with any truncated substrate for γ-secretase could be misleading. This could be of importance when investigating effects of GSIs on other substrates; if one does not establish an IC_{50} for cleavage using a full-length substrate, such data could prove to be misleading. Indeed, some GSIs that have been reported to be “Notch-sparing” appear to have no substrate selectivity when evaluated using identical assay conditions in vitro (36, 56, 57).

In contrast to the differential sensitivity to GSIs, long Aβ peptides appear to remain sensitive to modulation of cleavage by both GSMS and iGSMS. Previous findings have shown that acidic (e.g., GSM1) and nonacidic GSMS (e.g., compound 2) have different modulatory effects. For the most part, acidic GSMS selectively lower Aβ1–42 and increase Aβ1–38, whereas nonacidic GSMS lower both Aβ1–40 and 1–42 and raise both Aβ1–37 and 1–38 (44, 58). Our current data provide some insight into the differences between these two GSM classes that...
are consistent with previous reports showing that GSMs enhance processivity by favoring an additional stepwise cleavage without altering the initial cleavage event (49, 59, 60). Although both GSM1 and compound 2 reduce the small amount of A\beta\textsubscript{1-42} that can be detected by ELISA, IP/MS studies demonstrate that compound 2 has a much larger effect on A\beta\textsubscript{1-42} processivity, reducing A\beta\textsubscript{1-40} and increasing A\beta\textsubscript{1-36}, 1–37, and 1–38. In contrast, both GSMs dramatically alter processivity from BRI2-A\beta\textsubscript{1-51}, increasing A\beta\textsubscript{1-37} and 1–38 and lowering A\beta\textsubscript{1-42}. Although subtle, there does appear to be a stronger effect of compound 2 on reducing A\beta\textsubscript{1-40}. Although the effects of iGSMs on A\beta\textsubscript{1-42} derived from A\beta\textsubscript{1-49} were only detectable by ELISA, iGSMs showed a consistent effect on the three longer A\beta substrates decreasing the shorter A\beta peptides and increasing A\beta\textsubscript{1-42}. Overall these data suggest a model whereby the acidic GSMs preferentially enhance processivity along the A\beta\textsubscript{1-42} product line, whereas nonacidic GSMs promote processivity along both A\beta\textsubscript{1-40} and 1–42 product lines, with iGSMs decreasing processivity in both product lines.

Building on the pioneering work of Ihara and co-workers (24), multiple groups have now explored the mechanisms through which γ-secretase generates A\beta peptides with differing C termini. Using multiple different systems, ranging from homogenous purified reconstituted γ-secretase assays to cell culture studies, these studies provide detailed insights into how γ-secretase cleavage of APP generates A\beta peptides with differing C termini and how various factors such as mutations in
APP, PSEN1/2, small molecules, or other factors can alter the profile of Aβ produced (35–37, 61). Although the data generated from the different systems is not identical, the differences for the most part are quantitative in nature and not qualitative. Through both heterogeneous ε-cleavage and differential stepwise processing, there can suffice to quite dramatic effects on the final profile of Aβ peptides produced. For example, several studies now show that FAD-linked mutations in APP and PSEN1 and PSEN2 alter γ-secretase by altering ε-cleavage site utilization, processivity, or some combination of these two mechanisms (35, 36). In contrast, the action of GSMs and iGSMs seems to be restricted to effects on processivity, with GSMs promoting additional stepwisecleavages and iGSMs partially inhibiting the stepwise cleavage (36). Further study of γ-secretase cleavage of other substrates and the effect of GSMs and iGSMs using methods such as those established here should provide additional data regarding whether the cleavage mechanism observed for APP is utilized for other substrates.

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