Biochemical Characterization of the γ-Secretase Activity That Produces β-Amyloid Peptides

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ABSTRACT: Recent studies of γ-secretase have pointed out that it may be comprised of a multisubunit complex with presenilin 1 and presenilin 2 as central components. Elucidation of the biochemical mechanism of this enzymatic activity will provide important information for developing γ-secretase inhibitors in Alzheimer's disease therapy. Here we describe the biochemical characterization of γ-secretase activities using a sensitive, membrane-based assay system. Membranes were isolated from 293 cells expressing C99, the substrate of γ-secretase. Upon incubation at 37 °C, C99 is cleaved by the endogenous γ-secretase, and Aβ peptides are liberated. Aβ40 and Aβ42 γ-secretase activities are very similar in terms of their kinetic profiles and pH dependence, supporting the notion that a single enzyme is involved in both Aβ40 and Aβ42 production. Pepstatin A inhibited Aβ40 and Aβ42 γ-secretase activities with similar potency. Peptide difluoroketone and peptide aldehyde inhibitors inhibited Aβ40 production in a dose-dependent fashion, enhanced Aβ42 production at low concentrations, and inhibited Aβ42 production at high concentrations. Although the selective increase of Aβ42 by low concentrations of peptide difluoroketone and peptide aldehyde inhibitors has been reported in intact cells, the finding that this phenomenon occurs in a membrane-based assay system suggests that these compounds increase Aβ42 by a direct effect on γ-secretase. The ability of these compounds to increase Aβ42 production may reflect allosteric modulation of the γ-secretase complex by a mechanism related to that responsible for the increase of Aβ42 production by mutations in presenilins.

Abnormal extracellular deposition of β-amyloid peptide (Aβ)[1] is one of the major pathological hallmarks of Alzheimer's disease (AD). Clinical mutations that are linked to familial Alzheimer's disease (FAD) have been identified on genes encoding the β-amyloid precursor protein (APP), presenilin 1 (PS1), and presenilin 2 (PS2) [reviewed by (*1*−*3*)]. It is now well established that all FAD mutations share a common pathological phenotype of altered APP processing that eventually leads to elevated production of either total Aβ or a longer, more fibrillogenic form of Aβ known as Aβ42. These findings point to an important role of Aβ in AD pathogenesis. Modulating Aβ production, therefore, becomes a prime target for drug development in AD therapy.

Aβ is the product of two sequential proteolytic cleavages of APP. The first step is catalyzed by a single transmembrane domain aspartyl protease termed β-secretase (or BACE, for β-amyloid cleavage enzyme) (*4*−*7*). Following BACE processing, the C-terminal fragment of APP (C99) undergoes a unique intramembrane cleavage by a second protease known as γ-secretase to yield two major variants of Aβ, Aβ40 and Aβ42 (*2*, *3*). Several lines of evidence suggest that γ-secretase activity is closely related to the function of PS1 and PS2. First, deletion of the PS1 and PS2 genes (*8*, *9*) or mutation of two conserved aspartate residues in the transmembrane domains of PS1 (*10*) abolishes the γ-secretase activity. Second, several structurally distinct γ-secretase inhibitors bind to PS1 and PS2 in cross-linking experiments (*11*−*13*). Finally, γ-secretase activity is eluted as a high molecular weight complex that includes PS1 during gel filtration chromatography (*14*). These findings suggest that PSs are essential components of a multisubunit protein complex responsible for γ-secretase activity or that PS1 and PS2 are themselves γ-secretases. Elucidating the molecular and biochemical mechanism of such a unique protease activity will provide important insight into AD pathogenesis and drug development strategies.

Here we report the biochemical characterization of γ-secretase activity in a cell-free assay system in which γ-secretase cleaves the C99 substrate directly in the membrane. Such a membrane-based reaction most closely reflects the native enzymatic activity under physiologic conditions. Our study reveals that Aβ40 and Aβ42 γ-secretase activities are very similar in terms of kinetic profiles and pH-dependence. We also demonstrate that Aβ42 γ-secretase activity in membranes can be potentiated by low concentrations of peptide

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[1] Abbreviations: Aβ, β-amyloid peptide; AD, Alzheimer's disease; ALLN, *N*-acetyl-leucyl-leucyl-norleucinal; APP, β-amyloid precursor protein; BFA, brefeldin A; C99, C-terminal fragment of APP after β-secretase cleavage; ECL, electrochemiluminescence; ER, endoplasmic reticulum; FAD, familial Alzheimer's disease; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PS1, presenilin 1; PS2, presenilin 2; TGN, trans-Golgi network.

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aldehyde and difluroketone inhibitors. This finding suggests that these compounds allosterically modulate the γ-secretase complex, perhaps by a mechanism similar to that responsible for the increase in Aβ42 γ-secretase activity produced by mutations in PS1 and PS2.

EXPERIMENTAL PROCEDURES

Reagents. Antibodies W02, G2-10, and G2-11 were obtained from Dr. Konrad Beyreuther (University of Heidelberg, Heidelberg, Germany). W02 recognizes residues 5−8 of Aβ peptide, while G2-10 and G2-11 recognize the specific C-terminal structure of Aβ40 and Aβ42, respectively (*15*). Biotin-4G8 was purchased from Senetec (St. Louis, MO). All tissue culture reagents used in this work were from Life Technologies, Inc., unless otherwise specified. Pepstatin A was purchased from Roche Molecular Biochemicals; DFK167 was from Enzyme Systems Products (Livermore, CA).

cDNA Constructs, Tissue Culture, and Cell Line Construction. The construct SPC99-Lon, which contains the first 18 residues and the C-terminal 99 amino acids of APP carrying the London mutation, has been described previously (*17*). Upon insertion into the membrane, the 17 amino acid signal peptide is processed, leaving an additional leucine at the N-terminus of Aβ. SPC99-lon was cloned into the pcDNA4/TO vector (Invitrogen) and transfected into 293 cells stably transfected with pcDNA6/TR, which is provided in the T-REx system (Invitrogen). The transfected cells were selected in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, 250 μg/mL zeocin, and 5 μg/mL blasticidin (Invitrogen). Colonies were screened for Aβ production by inducing C99 expression with 0.1 μg/mL tetracycline for 16−20 h and analyzing conditioned media with a sandwich immunoassay (see below). One of the clones, designated as pTRE.15, was used in the studies described in this report.

Membrane Preparation. C99 expression in cells was induced with 0.1 μg/mL tetracycline for 20 h. The cells were pretreated with 1 μM phorbol 12-myristate 13-acetate (PMA) and 1 μM brefeldin A (BFA) for 5−6 h at 37 °C before harvesting. The cells were washed 3 times with cold phosphate-buffered saline (PBS) and harvested in buffer A containing 20 mM Hepes (pH 7.5), 250 mM sucrose, 50 mM KCl, 2 mM EDTA, 2 mM EGTA, and Complete protease inhibitor tablets (Roche Molecular Biochemicals). The cell pellets were flash-frozen in liquid nitrogen and stored at −70 °C before use.

To make membranes, the cells were resuspended in buffer A and lysed in a nitrogen bomb at 600 psi. The cell lysate was centrifuged at 1500*g* for 10 min to remove nuclei and large cell debris. The supernatant was centrifuged at 100000*g* for 1 h. The membrane pellet was resuspended in buffer A plus 0.5 M NaCl, and the membranes were collected by centrifugation at 200000*g* for 1 h. The salt-washed membrane pellet was washed again in buffer A and centrifuged at 100000*g* for 1 h. The final membrane pellet was resuspended in a small volume of buffer A using a Teflon−glass homogenizer. The protein concentration was determined, and membrane aliquots were flash-frozen in liquid nitrogen and stored at −70 °C.

γ-Secretase Reaction and Aβ Analysis. To measure γ-secretase activity, membranes of the specified amount were incubated at 37 °C for 1 h in 50 μL of buffer containing 20 mM Hepes (pH 7.0) and 2 mM EDTA. At the end of the incubation, Aβ40 and Aβ42 were measured using an electrochemiluminescence (ECL)-based immunoassay. Aβ40 was identified with antibody pairs TAG−G2-10 and biotin−W02, while Aβ42 was identified with TAG−G2-11 and biotin−4G8. The ECL signal was measured using an ECL-M8 instrument (IGEN International, Inc.) according to the manufacturer's instructions. The data presented in this study were the means of the duplicate or triplicate measurements in each experiment, and error bars represented the standard deviations. The characteristics of γ-secretase activity described here were confirmed using more than five independent membrane preparations.

Immunoprecipitation and Mass Spectrometry. To isolate γ-secretase cleavage products, the γ-secretase reaction was carried out as described above with 1 mg of membrane protein for 1 h. At the end of the reaction, the membranes were solubilized at 4 °C for 1 h in 1× RIPA buffer that contained 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 23 μg/mL phenylmethylsulfonyl fluoride (PMSF), 11 μg/mL tosyl-L-phenylalanine chloromethyl ketone (TPCK), and 0.7 μg/mL pepstatin. The insoluble components were removed by centrifugation at 100000*g* for 30 min. The supernatants were preincubated with protein A plus G agarose (Oncogene) for 2 h. After removing the agarose, the solubilized membranes were incubated at 4 °C overnight with antibody W02. Protein A plus G agarose was then added to each reaction, and the mixture was incubated for another 2 h. At the end of this incubation, the agarose beads were collected by centrifugation and washed 3 times with 1× RIPA buffer and twice with 20 mM Tris (pH 7.4). The immunoprecipitated peptides were eluted from the beads with 10 μL of 10% acetonitrile/0.1% trifluoroacetic acid (TFA). To isolate Aβ products from conditioned media, cells expressing C99 were grown to 90% confluence and re-fed with fresh media. The conditioned media, harvested after 5−6 h of incubation, were mixed with 5× RIPA buffer and processed as described above.

Matrix-assisted laser desorption/ionization mass spectrometric (MALDI MS) analysis of Aβ was performed on a Voyager-DE STR mass spectrometer (PerSeptive Biosystems, Inc., Framingham, MA). The instrument is equipped with a pulsed nitrogen laser (337 nm). Mass spectra were acquired in the linear mode with an acceleration voltage of 20 kV. Each spectrum presented in this work represents an average of 256 laser shots. To prepare the sample−matrix solution, 1 μL of immunoprecipitated Aβ sample was mixed with 1 μL of saturated α-cyano-4-hydroxycinnamic acid solution in 0.1% TFA/acetonitrile. The sample−matrix solution was then applied to the sample plate and dried at ambient temperature prior to mass spectrometric analysis. All the spectra were externally calibrated with a mixture of bovine insulin and ACTH (18−39 clip).

General Methods. Protein concentration was determined with the Bio-Rad Protein Assay dye according to the manufacturer's instructions. Western blots were developed using the Enhanced Chemiluminescence system (Amersham-Pharmacia) according to the manufacturer's instructions.

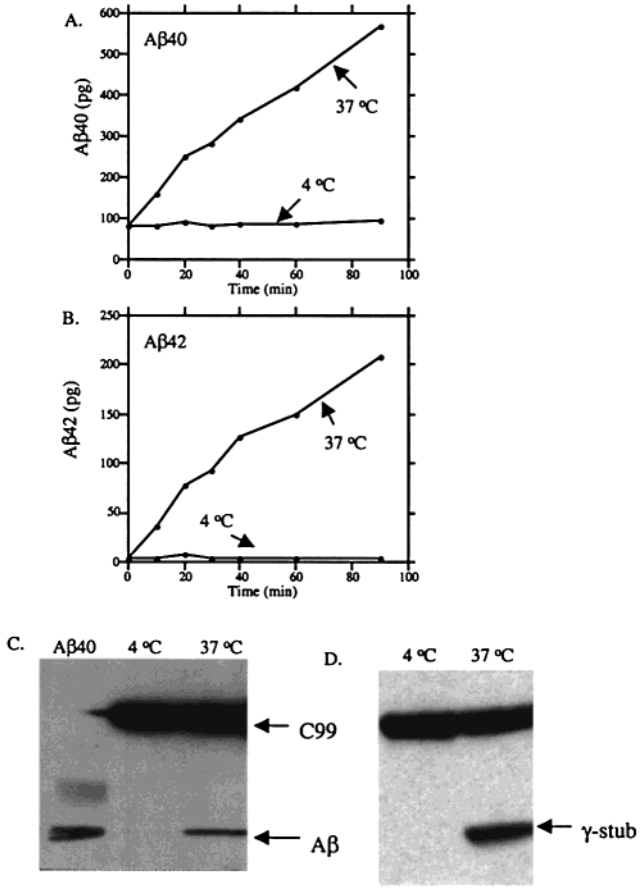

FIGURE 1: Time-dependent Aβ production in the membrane-based γ-secretase reaction. (A) Time course of Aβ40 production. Two micrograms of membrane protein was used in each assay, and the reactions were stopped at the specified time points. (B) Time course of Aβ42 production. Five micrograms of membrane protein was used in each assay. (C) C99 and the Aβ products were separated on a 16% gel and detected at the end of a 1 h reaction at either 4 °C or 37 °C by immunoblot with antibody W02. The left lane shows the mobility of Aβ40 standard. (D) C-terminal product of γ-secretase cleavage detected with antibody 369.

RESULTS

To characterize the biochemical properties of γ-secretase activity, we developed a cell-free assay using membranes isolated from cells expressing C99, the C-terminal fragment of APP that is the substrate of γ-secretase. Several strategies were used to increase the amount of Aβ generated by the cell-free system. Since our previous study suggests that C99 substrate concentration is a limiting factor for cellular Aβ production (*17*) and since the toxic effect of C99 accumulation in cells may limit its expression level (*18*), an inducible system was utilized to maximize C99 expression. A FAD London mutation was also included in the substrate construct to facilitate the production of Aβ42. In addition, based on the observation that activation of protein kinase C (PKC) by the phorbol ester PMA can stabilize C99 in cells (L. Song and L. Zhang, unpublished data), cells were pretreated with PMA before harvesting them for membrane preparation.

Upon incubation at 37 °C, C99 was cleaved by the endogenous γ-secretase in this intact membrane system, producing Aβ40 and Aβ42 in a time-dependent fashion (Figure 1A,B). Consistent with the observation that Aβ40 is the dominant form of Aβ secreted from cells, Aβ40 γ-secretase activity measured in the membrane assay is much higher

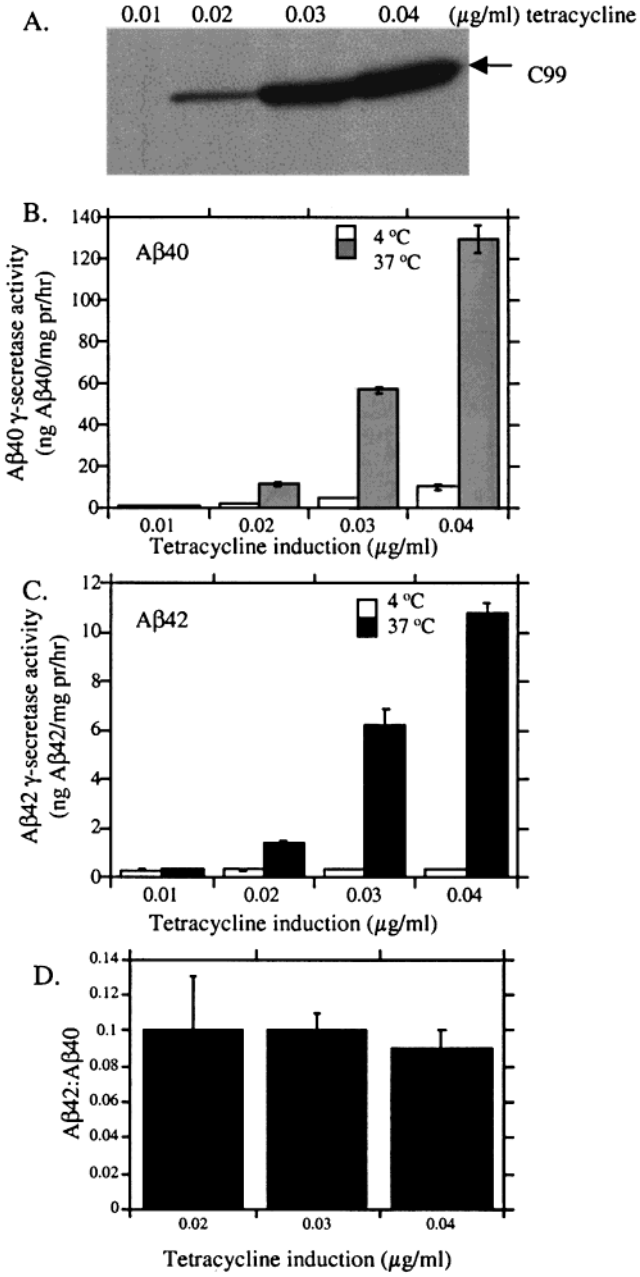

FIGURE 2: Substrate dependence of Aβ production in membranes. Membranes expressing different levels of C99 substrate were isolated from cells induced with 0.01, 0.02 0.03, and 0.04 μg/mL tetracycline. (A) Expression of C99 at different induction conditions. (B) Dependence of Aβ40 production on substrate concentration. (C) Dependence of Aβ42 production on substrate concentration. (D) Aβ42 to Aβ40 ratio at different substrate concentrations.

than that of Aβ42. Western blot analysis revealed both Aβ (Figure 1C) and the C-terminal cleavage product (Figure 1D). Only a small percentage of the substrate was converted to Aβ during the reaction (Figure 1C,D), which was consistent with the linear reaction rates of Aβ40 and Aβ42 production (Figure 1A,B). Membranes with different substrate levels were isolated from cells induced with different concentrations of tetracycline (Figure 2A). When γ-secretase activity was analyzed in these membranes, both Aβ40 and Aβ42 exhibited a substrate-dependent increase of production (Figure 2B,C), while the Aβ42:Aβ40 ratio remained unchanged (Figure 2D), indicating that the two activities responded similarly to the changes of the substrate concentration. Aβ40 and Aβ42

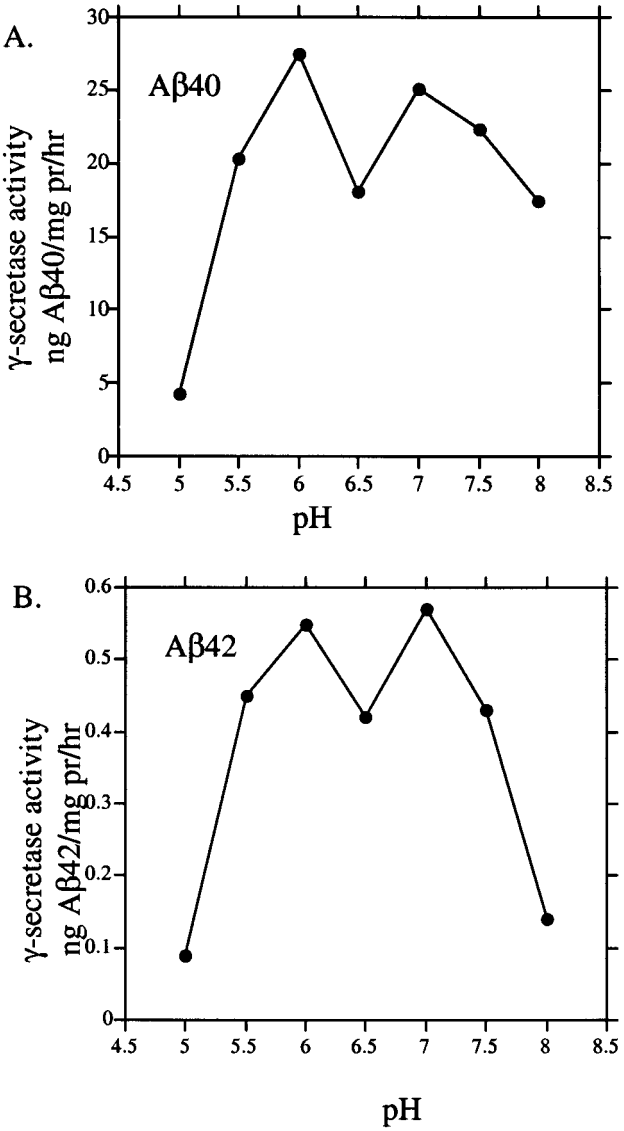

FIGURE 3: pH profile of γ-secretase activity in membranes. Aβ40 and Aβ42 γ-secretase activities were measured in the membranes at the specified pH buffer containing 2 mM EDTA. For pH 5.0−6.0, 20 mM acetate buffer was used, while 20 mM Hepes buffer was used for pH 6.5−7.5 and 20 mM Tris-HCl was used for pH 8.0. (A) pH dependence of Aβ40 γ-secretase activity. (B) pH dependence of Aβ42 γ-secretase activity.

γ-secretase activities were also active under similarly broad pH conditions (Figure 3A,B). Altogether, the data demonstrate that Aβ40 and Aβ42 γ-secretase activities in the membranes are remarkably similar in terms of their time-dependent, substrate-dependent, and pH-dependent profiles.

Aβ products generated by γ-secretase in the cell-free assay were purified using antibody W02 and subjected to MALDI-MS analysis. Several Aβ products were identified in the MS spectrum, including a major Aβ product with a molecular weight equivalent to Aβ40 and a minor product with a molecular weight equivalent to Aβ42 (Figure 4A). In addition, we also identified peptides with molecular weights identical to Aβ38 and Aβ33 (Figure 4A), although it is not clear whether they were generated from γ-secretase cleavage directly or derived from the degradation of Aβ40 and Aβ42. The Aβ products produced in the cell-free reaction were similar to those secreted from intact cells (Figure 4B), providing strong evidence that the γ-secretase activity in the

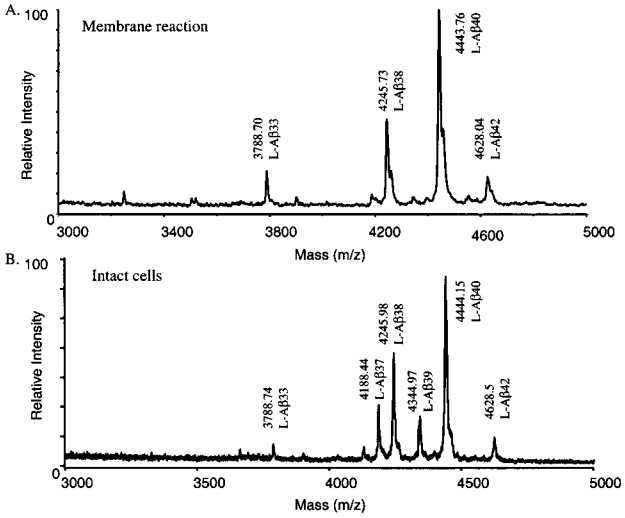

FIGURE 4: Mass spectrometric analysis of products generated by γ-secretase. The γ-secretase cleavage products were purified from the membrane reaction or from conditioned medium by immunoprecipitation using antibody W02 as described under Experimental Procedures. The purified peptides were subjected to MALDI-MS analysis using a Voyager-DE STR mass spectrometer. The N-terminus of Aβ peptides starts with an additional leucine residue due to the nature of the C99 construct (see Experimental Procedures). The calculated masses for Aβ peptides are as follows: Aβ42, 4426.25; Aβ40, 4442.01; Aβ1−39, 4342.88; Aβ38, 4243.74; Aβ37, 4186.69; Aβ36, 4129.64; Aβ33, 3786.15. (A) Aβ products generated in the cell-free γ-secretase reaction. (B) Aβ products secreted from intact cells.

cell-free assay is similar to that in intact cells under physiologic conditions. In conditioned medium, additional Aβ peptides were also identified that were equivalent to the molecular weights of Aβ39 and Aβ37. The heterogeneity at the C-terminus of Aβ is consistent with a previous report (*19*) and may reflect the degradation of Aβ in conditioned media.

Next we examined the effect of a well-characterized vesicle-trafficking inhibitor, BFA, on γ-secretase activity. BFA blocks vesicle transport from ER to Golgi, therefore preventing secreted proteins and newly synthesized membrane proteins from moving downstream of the Golgi apparatus (*20*). Incubation of cells expressing C99 with BFA for 5 h resulted in a dramatic reduction of Aβ40 and Aβ42 secretion from intact cells (Figure 5A and data not shown), indicating that the secretory pathway was disrupted by BFA. Treatment of BFA, however, did not alter the steady-state C99 accumulation in cells (Figure 5B). Interestingly, analyzing γ-secretase activity in the membranes isolated from these BFA-treated cells revealed that γ-secretase activity was not impaired by BFA treatment. In fact, a 2−3-fold increase of both Aβ40 and Aβ42 production was observed in membranes isolated from cells pretreated with BFA (Figure 5C,D). BFA has no direct inhibitory effect on γ-secretase activity (Figure 5C,D, open bars). We have taken advantage of this observation by using BFA-pretreated cells to improve the signal-to-noise ratio of the cell-free γ-secretase assay.

To further understand the nature of γ-secretase activity in membranes, we tested a broad spectrum of commercial protease inhibitors for their effect on this activity. Most of them, including the common serine, cysteine, and metalloprotease inhibitors, did not affect either Aβ40 or Aβ42 γ-secretase activity (data not shown). Two types of protease

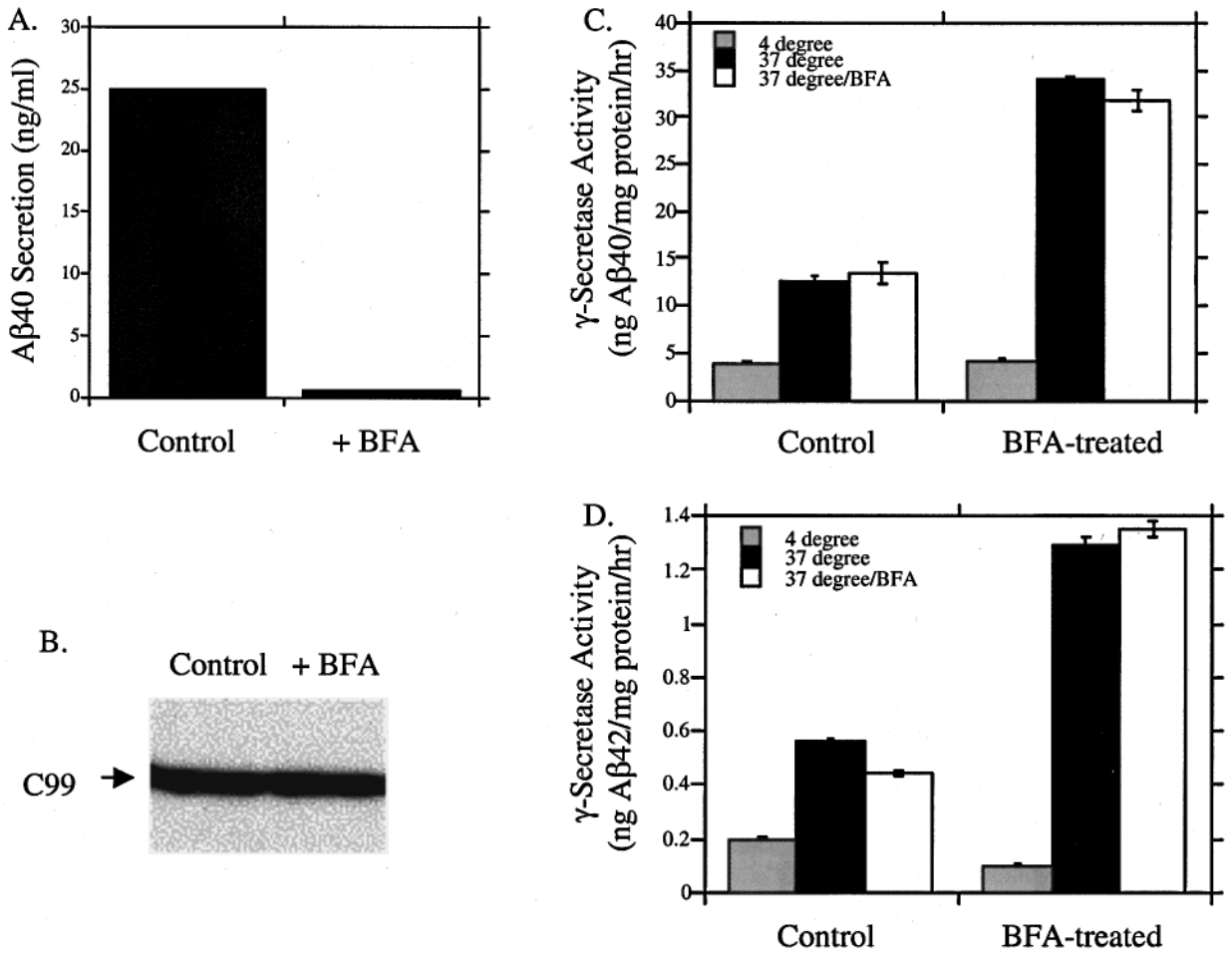

FIGURE 5: Treatment of cells with BFA blocks Aβ secretion but does not abolish γ-secretase activity in membrane. Cells expressing C99 were treated for 5 h with 1 μM PMA either in the presence or in the absence of 1 μM BFA before harvesting. Conditioned media were collected and analyzed for secreted Aβ40 and Aβ42. Membranes were isolated from cells, and Aβ40 and Aβ42 γ-secretase activity was measured as described in Figure 1 and under Experimental Procedures. C99 protein in the membranes was detected by Western blot using antibody W02. (A) Aβ40 secretion from intact cells treated with and without BFA. (B) C99 protein in membranes from cells treated with and without BFA. (C) Aβ40 γ-secretase activity in membranes isolated from cells treated with and without BFA. (D) Aβ42 γ-secretase activity in membranes isolated from cells treated with and without BFA.

inhibitors were found to display distinct dose-dependent effects on Aβ40 and Aβ42 production. The first type of γ-secretase inhibitor is exemplified by the aspartyl protease inhibitor pepstatin A, which inhibited Aβ40 and Aβ42 activities in a similar concentration-dependent fashion (Figure 6A). The second type of γ-secretase inhibitor includes the difluoroketone inhibitor DFK167 (*21*) and peptide aldehyde inhibitors such as MG132 (Figure 6B, MG132 data not shown). Although DFK167 and MG132 inhibited Aβ40 γ-secretase activity with similar potencies as pepstatin A, these two compounds potentiated Aβ42 production at low concentrations. At high concentrations, DFK167 and MG132 inhibited Aβ42 production (Figure 6B and data not shown). It should be noted that the effect of these protease inhibitors on γ-secretase activity in membranes is similar to their effect on Aβ production in intact cells (*17, 21*−*23*).

DISCUSSION

In this report, we studied the biochemical properties of γ-secretase activity using a cell-free assay in which Aβ40 and Aβ42 are produced by membranes containing endogenous γ-secretase and recombinant C99 substrate. Several observations argue that γ-secretase activity in membranes possesses the characteristics of the enzyme responsible for Aβ production in intact cells. First, Aβ40 is the predominant form of Aβ produced by membranes and intact cells while Aβ42 comprises only a small fraction of Aβ production (Figures 1 and 2). Second, mass spectrometric analysis confirms that Aβ products generated from γ-secretase cleavage in membranes are similar to those secreted from intact cells (Figure 4). Finally, γ-secretase inhibitors have a similar effect on Aβ40 and Aβ42 production in membranes and in intact cells (Figure 5). This sensitive, membrane-based γ-secretase assay provides a system to study the biochemical properties of the enzyme under conditions that are very close to those in intact cells.

When evaluating γ-secretase as a therapeutic target for Alzheimer's disease, one of the key issues is whether Aβ40 and Aβ42 are the products of the same protease or different proteases. Both the time course and the substrate dependence of Aβ40 and Aβ42 γ-secretase activity are remarkably similar (Figures 1 and 2). These kinetic data suggest that Aβ40 and Aβ42 activities have similar K_m values for the C99 substrate and confirm that C99 availability is the rate-determining factor for γ-secretase cleavage under normal conditions. Aβ40 and Aβ42 also have similar broad pH-

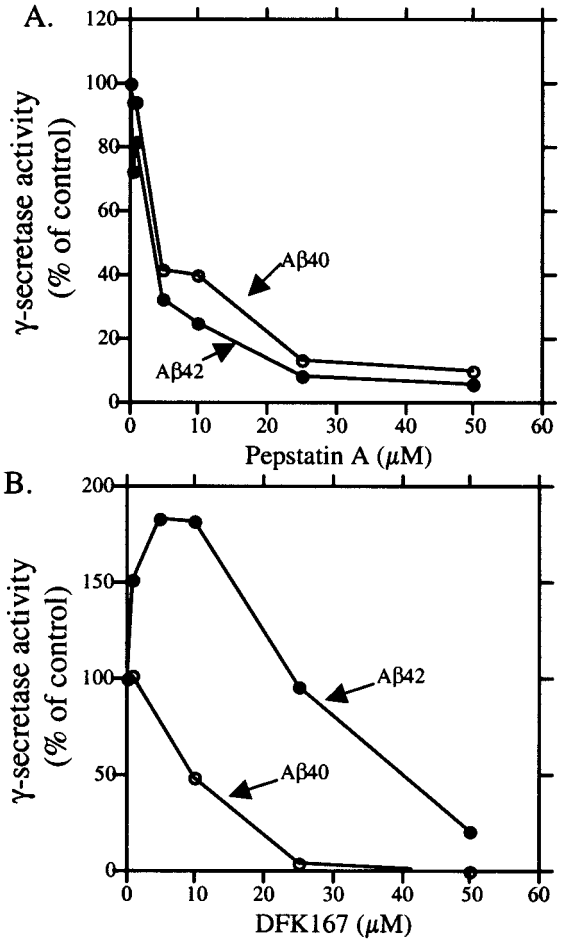

FIGURE 6: Effect of protease inhibitors on γ-secretase activity in membranes. Aβ40 and Aβ42 γ-secretase reactions were carried out in the presence of the specified protease inhibitors. (A) Effect of pepstatin A on Aβ40 and Aβ42 γ-secretase activity. (B) Effect of DFK167.

dependent profiles. Although these data do not definitively resolve the issue of whether one or two enzymes are responsible for Aβ40 and Aβ42 γ-secretase activities, they are consistent with the involvement of a single γ-secretase in both Aβ40 and Aβ42 production. The involvement of one γ-secretase in generating both Aβ40 and Aβ42 is also supported by the recent finding that both activities can be solubilized from membranes and recovered in a complex with PS1 by purification with an anti-PS1 antibody (*14*).

The treatment of cells with the vesicle-trafficking inhibitor BFA completely blocks Aβ secretion from cells. However, membranes isolated from BFA-treated cells displayed higher γ-secretase activity than membranes isolated from control cells (Figure 5C,D). BFA is known to block anterograde vesicle transport from ER to Golgi and causes the redistribution of proteins in a newly merged ER/Golgi compartment that is formed as a consequence of Golgi to ER retrograde transport (*24*). Inhibition of Aβ secretion from intact cells has been reported previously, and suggests that Aβ generated intracellularly must traverse an intact secretory pathway to be released into media and/or that an intact secretory pathway is required for APP and γ-secretase to co-localize and generate Aβ. Since membranes isolated from BFA-treated cells produce Aβ, it is not likely that BFA treatment prevents the co-localization of APP and γ-secretase. The fact that more Aβ is generated in membranes derived from BFA-treated cells suggests that both Aβ40 and Aβ42 γ-secretase cleavage can occur in ER and/or Golgi, and that in the newly merged ER−Golgi compartment formed after BFA treatment the enzyme is accessible to more substrate and consequently produces more Aβ. Previously many subcellular compartments have been reported as the sites of γ-secretase cleavage, including ER, Golgi, trans-Golgi network (TGN), and endosomes and lysosomes (*25*−*27*). Some of these studies point to separate locations for Aβ40 and Aβ42 production, implicating more than one enzyme as being involved. While our data do not exclude the possibility of additional compartment(s) in which the γ-secretase reaction occurs, an ER/Golgi localization of γ-secretase is consistent with the primarily ER/Golgi localization of the presenilins (*28*), which are known to be closely associated with γ-secretase activity.

Various protease inhibitors have been shown to modulate γ-secretase activity in two distinct fashions in intact cells (*16*, *17*, *21*−*23*). While pepstatin A inhibited Aβ40 and Aβ42 γ-sceretase activity with similar potency, peptide aldehyde and difluoroketone protease inhibitors inhibit Aβ40 production at a wide range of concentrations, increase Aβ42 production at low concentrations, and inhibit Aβ42 production at high concentrations (Figure 6). Although the differential effects of peptide difluoroketone and peptide aldehyde protease inhibitors on Aβ40 and Aβ42 secretion have attracted much interest, to date it has been difficult to rule out the possibility that the increase of Aβ42 production is due to nonspecific effects on other metabolic pathways in intact cells. Therefore, our finding that these inhibitors potentiate Aβ42 production in a cell-free system is of particular interest. The fact that the membrane-based assay is free of cytosolic activity suggests that these compounds act directly on γ-secretase rather than other cellular processes such as proteasome-mediated protein degradation, which has been suggested to contribute to the increase of Aβ42 production (*17*, *29*). A protease inhibitor designated as compound N (*16*) has been reported that enhances Aβ42 production without affecting that of Aβ40, further suggesting that the stimulation of Aβ42 and the inhibition of Aβ40 are independent events. Taken together, these data provide strong evidence that all these protease inhibitors act directly on γ-secretase and the capacity for up-regulation of Aβ42 production is an intrinsic characteristic feature of γ-secretase. However, the definitive proof that these inhibitors indeed act directly on γ-secretase requires showing their effects on purified γ-secretase or site-directed labeling of γ-secretase with these inhibitors.

The mechanism for the selective increase of Aβ42 production by these small molecule protease inhibitors is not clear. The finding that PS1 and PS2 are the major molecular targets of γ-secretase inhibitors (*11*, *12*) raises the possibility that these compounds may increase Aβ42 γ-secretase activity by the same mechanism as presenilin FAD mutations. Since the more than 70 PS1 FAD mutations are distributed throughout the protein structure (*1*), it is unlikely that the selective increase of Aβ42 caused by these mutations is a consequence of modification at the catalytic site of γ-secretase. FAD mutations and some γ-secretase inhibitors may have an allosteric effect on PS1 and PS2 that either directly alters γ-secretase activity or indirectly alters the interaction of PS1 and PS2 with other components in the γ-secretase complex. The potential allosteric modulation of γ-secretase activity

by small molecules may open up the opportunity for developing inhibitors with distinct mechanisms. However, stimulation of Aβ42 production could be a potential liability of molecules aimed at inhibiting γ-secretase activity via an allosteric mechanism. It is possible to circumvent this problem by developing mechanism-based γ-secretase inhibitors targeted at the catalytic site, such as pepstatin A and L685,458 (*14*). On the other hand, active site targeted protease inhibitors are mostly peptidomimetics that generally have poor oral bio-availability. Clearly, elucidation of the molecular identity and biochemical mechanism of γ-secretase activity will be a critical factor for the success of developing γ-secretase inhibitors for AD therapy.

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