γ-Secretase Activity Is Present in Rafts but Is Not Cholesterol-Dependent†

Satoko Wada,‡ Maho Morishima-Kawashima,‡ Yue Qi,‡ Hiroaki Misono,‡,§ Yukiko Shimada,‖ Yoshiko Ohno-Iwashita,‖ and Yasuo Ihara*,‡

Department of Neuropathology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan, and Biomembrane Research Group, Tokyo Metropolitan Institute of Gerontology, 35-2 Sakae-cho, Itabashi-ku, Tokyo 173-0015, Japan

Received May 28, 2003; Revised Manuscript Received August 30, 2003

ABSTRACT: Cholesterol has been claimed to be involved in the generation and/or accumulation of amyloid β protein (Aβ). However, the underlying molecular mechanisms have not been fully elucidated yet. Here, we have investigated the effect of membrane cholesterol content on γ-secretase activity using Chinese hamster ovary cells stably expressing β-amyloid precursor protein (APP) and either wild-type or N141I mutant-type presenilin 2. Cholesterol was acutely depleted from the isolated membrane by methyl-β-cyclodextrin, and Aβ production was assessed in a cell-free assay system. Reduced cholesterol did not significantly alter the amounts of Aβ produced by either total cell membranes or cholesterol-rich low-density membrane domains. Even its extremely low levels in the latter domains did not affect Aβ production. This indicates that the membrane cholesterol content does not directly modulate the activity of γ-secretase. To ascertain that γ-secretase resides in cholesterol-rich membrane domains, low-density membrane domains were further fractionated with BCθ (biotinylated θ-toxin nicked with subtilisin Carlsberg protease), which has recently been shown to bind selectively to rafts of intact cells. The membrane domains purified with BCθ did indeed produce Aβ. These observations indicate that the γ-cleavage required for generating Aβ occurs in rafts, but its activity is virtually cholesterol-independent.

Amyloid β-protein (Aβ)[1] is the major component of senile plaques observed in the brains of patients affected by Alzheimer's disease (AD) and in those from many cognitively normal, aged individuals. Aβ is generated through sequential cleavages from β-amyloid precursor protein (APP), a type I membrane protein. β-Secretase (β-site amyloid precursor protein-cleaving enzyme; BACE) cleaves first at the amino terminus of Aβ (*1*), generating β-carboxyl-terminal fragment of APP (β-CTF). This, in turn, is cleaved by γ-secretase in the middle of its transmembrane domain (γ-cleavage) and also at the cytoplasmic membrane boundary (ε-cleavage), concomitantly producing Aβ of ∼4 kDa and γ-CTF of ∼6 kDa (mainly CTF50−99), respectively (*2*−*4*). The former is eventually secreted into the extracellular space, and the latter appears to move to the nucleus (*5*). The γ-secretase is presumed to be a high-molecular-weight multiprotein complex that consists of presenilin (PS) 1 or 2 and other components, and at least four components are known for now to be required for the enzymatic activity (*6*−*8*). However, there is another pathway, in which APP is cleaved by α-secretase in the middle of the Aβ region to generate α-cleaved CTF (α-CTF), thereby precluding the production of Aβ. Thus, α-secretase and β-secretase may compete for their substrate, APP, and appear to have a reciprocal functional relationship. Specifically, if α-secretase is activated, Aβ production decreases, but if β-secretase is activated, Aβ production increases.

Several retrospective epidemiological studies suggest a correlation between cholesterol and AD. There may be a correlation between serum cholesterol levels and the prevalence of AD. Some recent studies have claimed that administration of statins reduces the incidence of AD and mild cognitive impairment (*9*). In vitro experiments with cultured cells showed that reducing cholesterol by administration of statin and/or methyl-β-cyclodextrin (MβCD) decreased the levels of intracellular and secreted Aβ (*10*−*12*). Further, in vivo, simvastatin reduces the levels of Aβ in both the brain and cerebrospinal fluid (CSF) (*12*). Since α-CTF is increased and β-CTF is reduced by cholesterol-reducing agents, the Aβ-lowering effects of statins and MβCD are considered to be due to the combination of enhanced α-cleavage and decreased β-cleavage. But this does

† This work was supported in part by a grant-in-aid for Scientific Research on Priority Areas, Advanced Brain Science Project, from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and a grant-in-aid for Science Research (C) from Japan Society for the Promotion of Science (JSPS), Japan.

* Address correspondence to this author: Tel +81-3-5841-3541; fax +81-3-5800-6852; e-mail yihara@m.u-tokyo.ac.jp.

‡ University of Tokyo.

§ Present address: Department of Biochemistry and Cell Biology, State University of New York at Stony Brook, Stony Brook, NY 11794.

‖ Tokyo Metropolitan Institute of Gerontology.

[1] Abbreviations: Aβ, amyloid β-protein; AD, Alzheimer's disease; APP, β-amyloid precursor protein; BACE, β-site amyloid precursor protein-cleaving enzyme; BCθ, biotinylated θ-toxin nicked with subtilisin Carlsberg protease; BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; CHO, Chinese hamster ovary; CSF, cerebrospinal fluid; CTF, carboxyl-terminal fragment; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; LDM, low-density membrane; MβCD, methyl-β-cyclodextrin; MES, 2-(*N*-morpholino)ethanesulfonic acid; MT, mutant-type; NTF, amino-terminal fragment; PBS, phosphate-buffered saline; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PS, presenilin; SDS, sodium dodecyl sulfate; Tris/tricine, tris(hydroxymethyl)aminomethane/*N*-tris(hydroxymethyl)methylglycine; WT, wild-type.

10.1021/bi034904j CCC: $25.00
Published on Web 11/01/2003

not exclude the possibility that cholesterol depletion might have an additional effect on γ-cleavage. This is possible because γ-secretase is believed to be situated deep in the membrane, and an alteration in the cholesterol content of the membrane may have an effect on its conformation and thereby affect its enzymatic activity. One example is sonic hedgehog, the autoprocessing of which is inhibited by cholesterol deprivation (*13*).

We sought to isolate the effect of membrane cholesterol on γ-secretase by depleting cholesterol directly from the fractionated membrane. Our results shown here clearly demonstrate that a reduction in membrane cholesterol does not suppress γ-secretase activity. We initially thought that the total cell membrane might interfere via unknown mechanisms with cholesterol extraction from particular membrane domains that contain γ-secretase. To exclude the possibility, we prepared a CHAPSO-insoluble low-density membrane (LDM) fraction that was able to produce Aβ. Even in these LDM domains, cholesterol depletion had no effects on the production of Aβ. Furthermore, to confirm that Aβ is produced in rafts, cholesterol-rich membrane domains were purified from the LDM fraction by use of biotinylated θ-toxin nicked with subtilisin Carlsberg protease (BCθ), which has recently been shown to bind selectively to rafts (*14*), and assessed for Aβ production. The purified cholesterol-rich microdomains demonstrated γ-secretase activity, indicating that Aβ production can take place in rafts.

EXPERIMENTAL PROCEDURES

Cells. Chinese hamster ovary (CHO) cell lines stably coexpressing wild-type (WT) human APP751 and WT human PS2, N141I mutant-type (MT) human PS2, WT human PS1, or M233T MT human PS1 (termed here WT PS2, MT PS2, WT PS1, or MT PS1 cells, respectively) (*15*) were used. Cell culture was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 250 μg/mL Zeocin, and 200 μg/mL G418.

Antibodies. The monoclonal antibodies against Aβ used here were 6E10 (raised against Aβ1−17, for assessing total Aβ; Signet Laboratories, Dedham, MA), BA27 (raised against Aβ1−40, specific for Aβ40), and BC05 (raised against Aβ35−43, specific for Aβ42). The specificity of the latter two antibodies was described in detail previously (*16*). UT421 (raised against the 20-residue carboxyl-terminal cytoplasmic domain of APP; a gift of Dr. T. Suzuki, Hokkaido University) was used for the detection of APP and APP-CTFs (*17*). C4 (raised against the 30-residue cytoplasmic domain of APP) was used for immunoprecipitation of APP γ-CTF. Presenilin-2 (raised against residues 324−335 of human PS2; Oncogene, Cambridge, MA) or 2972N (raised against residues 1−75 of human PS2) (*18*) was utilized for the detection of PS2-CTF or -NTF, respectively. The monoclonal antibodies against caveolin, flotillin, and calnexin were purchased from Transduction (Lexington, KY).

Membrane Preparation. A total membrane fraction for the cell-free Aβ production assay was prepared as described previously (*15*). Briefly, cultured cells were washed twice with ice-cold phosphate-buffered saline (PBS), scraped into PBS, and collected by centrifugation. The pellets were homogenized in buffer A (20 mM Pipes, pH 7.0, 140 mM KCl, 0.25 M sucrose, 5 mM EGTA) with a glass/Teflon homogenizer. The homogenates were centrifuged at 800*g* for 10 min to remove nuclei and cell debris. The resulting postnuclear supernatants were centrifuged at 100000*g* for 1 h. The pellets were suspended in buffer A and centrifuged again. The resulting membrane pellets were suspended in buffer B [buffer A containing a cocktail of protease inhibitors (5 mM 1,10-phenanthroline, 1 mM thiorphan, 0.1 mM diisopropyl fluorophosphate, 0.1 mM phenylmethyl sulfonyl fluoride, 5 μg/mL N^{α}-*p*-tosyl-L-lysine chloromethyl ketone, 1 μg/mL antipain, and 1 μg/mL leupeptin)] to give a final protein concentration of 2.5 mg/mL and were stored at −80 °C until use.

Isolation of Detergent-Insoluble LDM Domains. An LDM fraction was obtained as described (*19*) with some modifications. Cells were homogenized in ~5 volumes of 10% sucrose in MES-buffered saline (25 mM MES, pH 6.5, and 150 mM NaCl) containing 1% CHAPSO and various protease inhibitors. The homogenate was adjusted to 40% sucrose by addition of an equal volume of 70% sucrose in MES-buffered saline, placed at the bottom of a ultracentrifuge tube, and overlaid with 4 mL of 35% sucrose and 4 mL of 5% sucrose in MES-buffered saline. The discontinuous gradient was centrifuged at 39 000 rpm for 20 h at 4 °C on an SW 41 Ti rotor (Beckman, Palo Alto, CA). An interface at 5/35% sucrose (fraction 2), each layer containing 5%, 35%, or 40% sucrose (fractions 1, 3, and 4, respectively), and the pellet (fraction P) were carefully collected. An aliquot from each fraction was subjected to Western blotting. Fraction 2 was centrifuged again after dilution with MES-buffered saline. The resultant pellet was washed twice with buffer A, resuspended in buffer B to give a final protein concentration of 1 mg/mL, and subjected to the cell-free Aβ production assay.

Cholesterol Depletion. The total membrane and LDM fractions were incubated with various concentrations of methyl-β-cyclodextrin (MβCD; Sigma, St. Louis, MO) at 4 °C for up to 1 h to deplete cholesterol. The reaction mixtures were centrifuged at 100000*g* for 1 h. The amounts of free cholesterol in the pellet were determined using the Determinor L FC kit (Kyowa, Tokyo, Japan). Those of phospholipids were quantified by the phospholipid C-test WAKO kit (Wako, Osaka, Japan), which assesses the levels of phosphatidylcholine and sphingomyelin according to the phospholipase D and cholineoxidase method.

Cell-Free Aβ Production Assay. Membrane fractions suspended in buffer B were incubated at 37 °C for the various times indicated. In some experiments, membrane fractions were preincubated in the presence of β- or γ-secretase inhibitor on ice for 15 min, with the final concentrations of dimethyl sulfoxide (DMSO, solvent for the inhibitor) being kept at 1%, and then incubated at 37 °C. The sample including 1% DMSO alone was used as a control. The reaction was terminated by placing the sample tube on ice and immediately adding chloroform/methanol (2:1). After extraction of lipids with chloroform/methanol, the protein residues were extracted with formic acid and subjected to quantitative Western blotting for Aβ.

Western Blotting and Quantification. For Western blotting, the proteins separated on an SDS−polyacrylamide gel were transferred onto a polyvinylidenedifluoride (PVDF) membrane (Immobilon; Nihon Millipore Ltd, Yonezawa, Japan). The bound antibodies were detected by enhanced chemilu-

minescence (ECL) (Amersham Pharmacia Biotech, Buckinghamshire, U.K.). For the detection of Aβ and APP-CTF, the proteins were separated on a 16.5% Tris/tricine gel and transferred to a nitrocellulose membrane (pore size 0.22 μm; Schleicher & Schuell, Keene, NH), as described previously (*20*). The blotted membrane was immersed in boiling PBS for 5 min to enhance immunoreactivity, followed by labeling with the antibodies to Aβ or APP. The blots were developed by an ECL system, and the signals were detected on a LAS-1000plus luminescent image analyzer (Fuji Film, Tokyo, Japan). Scanned images were quantified for Aβ by use of Image Gauge software (Fuji Film) with defined amounts of synthetic Aβ1−40 or 1−42 as a control.

BCθ-Affinity Purification of Cholesterol-Rich Domains. BCθ was prepared as described previously (*21*). The LDM fraction (fraction 2) obtained by sucrose density gradient centrifugation was spun, and the resultant pellet was washed twice and suspended in PBS containing 2 mM EGTA and various protease inhibitors to give a final protein concentration of 0.5 mg/mL. Added to the suspensions were 10 μg/mL BCθ in PBS containing 0.1% bovine serum albumin (BSA) or PBS containing 0.1% BSA alone as a control, and the suspensions were then incubated at 4 °C for 1 h. Following addition of streptavidin-coated magnetic beads (Dynal, Oslo, Norway), the samples were further incubated overnight at 4 °C with gentle agitation. The beads were recovered by a magnet (Dynal), washed twice, and then resuspended in the SDS sample buffer for Western blotting or in PBS containing 2 mM EGTA and protease inhibitors for the cell-free Aβ production assay. On the Western blots, BCθ was localized by using peroxidase-conjugated streptavidin (Boehringer Mannheim, GmbH, Germany) or anti-θ toxin antibody (*22*). It should be noted that BCθ was eluted from the streptavidin beads only with formic acid but not with the SDS sample buffer. For some experiments, the LDM fraction was treated with MβCD and centrifuged at 100000*g* for 30 min before BCθ-affinity purification.

Other Methods. Protein concentrations were determined with the bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL) in the presence of 1% SDS. β-Secretase inhibitors, GL189 and APP β-secretase inhibitor, were purchased from Calbiochem (San Diego, CA). Specific γ-secretase inhibitors, L-685,458 and DFK-167, were purchased from Calbiochem and Enzyme Systems Products (Livermore, CA), respectively.

RESULTS

Effects of Cholesterol Depletion on γ-Secretase Activity in the Total Cell Membrane. To determine whether γ-secretase activity depends on the cholesterol content of the membrane, cholesterol was depleted from the total cell membrane preparations (see Experimental Procedures), and γ-secretase activity of the membranes was assessed by the cell-free Aβ production assay. In this assay system, Aβ production depends exclusively on γ-secretase and preexisting β-CTF. Specific γ-secretase inhibitors, DFK-167 and L-685,458, at appropriate concentrations profoundly suppressed the production of Aβ in this cell-free system (*15*). The addition of specific β-secretase (BACE) inhibitors did not suppress Aβ production, suggesting that the activity of β-secretase is undetectable in this system (*23*). The pH (7.0) of the reaction mixture is far from the optimal pH for β-secretase (*1*), and its activity may have been completely inhibited. In either case, this suggests that the production and supply of β-CTF are not increased during incubation in this assay system. Regarding α-secretase activity, it should be inhibited by EGTA and phenanthroline, both of which are included in this cell-free system, because α-secretase is a metalloprotease. We chose CHO cells stably coexpressing WT APP 751 and WT or N141I MT PS2 (termed WT PS2 or MT PS2 cells, respectively) that are well characterized in this laboratory. Both cell lines produce and secrete large amounts of Aβ. While WT PS2 cells produce mostly Aβ40, MT PS2 cells produce predominantly Aβ42. Thus, altered γ-cleavage at the Aβ40 or Aβ42 site can be readily monitored by use of a pair of these cell lines.

The total membrane fraction was prepared from WT or MT PS2 cells and treated with methyl-β-cyclodextrin (MβCD). Unlike other cholesterol-binding reagents, MβCD is strictly surface-acting: it is incorporated into the membrane and selectively depletes membrane cholesterol (*24*). Cholesterol content of the membrane decreased as the concentrations of MβCD increased (Figure 1A, left). When the membrane from WT PS2 cells was treated with 30 mM MβCD, the levels of free cholesterol were reduced by ∼40%, whereas the levels of phospholipid were unaltered, as reported previously (*25*) (Figure 1A, right). MT PS2 membranes showed very similar alterations in the lipid profile by the same treatment (data not shown).

To examine whether cholesterol depletion affects the membrane level of β-CTF, an immediate substrate for γ-secretase, the membrane fractions treated with MβCD were centrifuged. The resultant pellet and supernatant were assessed for β-CTF. We found that β-CTF was not released from the membrane by ∼40% cholesterol reduction (data not shown). The membranes treated with various concentrations of MβCD were incubated at 37 °C and assessed for Aβ production. Without this treatment, the production of Aβ40 and Aβ42 proceeded linearly until 20 min, as described previously (*15*) (data not shown). In the membrane prepared from WT PS2 cells, Aβ40 composed ∼70% of the total Aβ produced (Figure 1B,C). On the other hand, the membrane prepared from N141I MT PS2 produced Aβ42 predominantly and marginal levels of Aβ40. Cholesterol depletion with MβCD did not alter significantly either the amounts of total Aβ production (assessed with 6E10) by the membranes from WT and MT PS2 cells or the proportions of Aβ40/Aβ42 produced (Figure 1B,C). Very similar results were obtained with the membranes from WT and MT PS1 cells (data not shown). These results indicate that the cholesterol content of the total cell membrane does not affect γ-secretase activity.

Effects of Cholesterol Depletion on γ-Secretase Activity of LDM Domains. One of the sites for Aβ generation within the cell is assumed to be LDM domains, the cholesterol-enriched membrane microdomains, which are known as rafts (*26*). To investigate the effect of cholesterol depletion on the well-defined membrane domains, LDM domains were prepared from the cells and assessed for γ-secretase activity. Because Triton X-100 inactivates γ-secretase (although it is most commonly used for purification of LDM domains)-(*27*), we tried several detergents, including 1% CHAPSO, 1% CHAPS, and 1% Lubrol, and also sodium carbonate. Of these, 1% CHAPSO was the most efficient for both purifica-

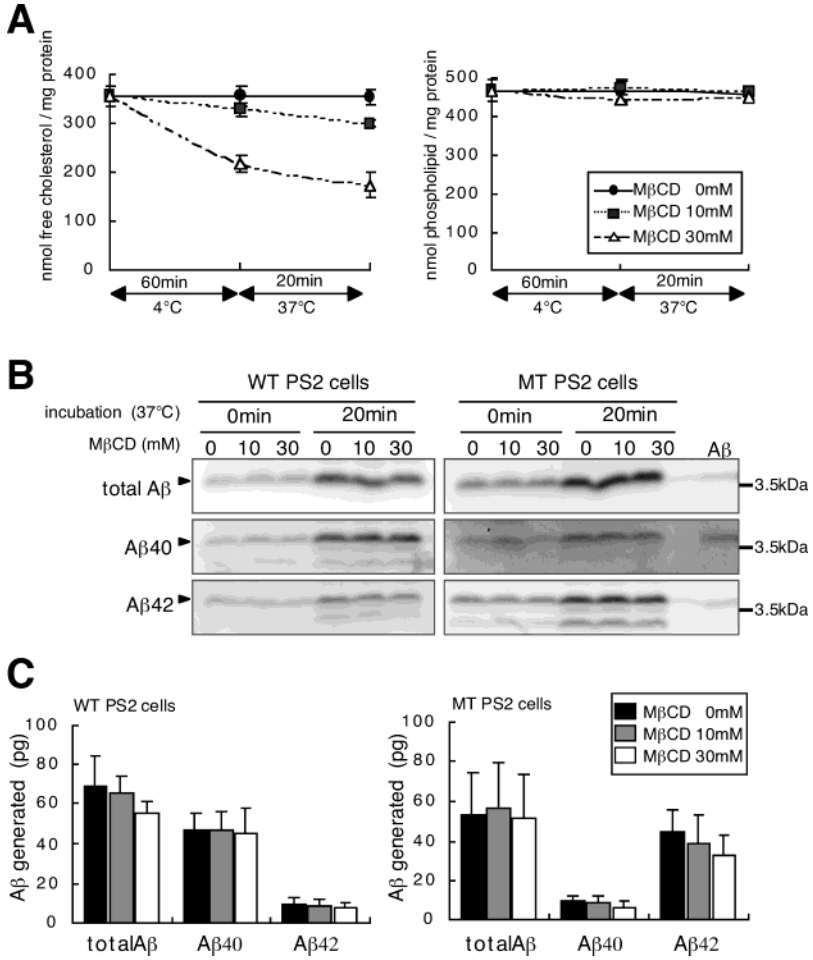

FIGURE 1: Cholesterol depletion has no effects on the γ-secretase activity of the total cell membrane. The membrane fractions were prepared from CHO cells stably expressing APP and either WT or MT PS2 and were treated with methyl-β-cyclodextrin (MβCD) at 4 °C for 60 min. The treated membranes were incubated at 37 °C for another 20 min for determination of Aβ production. (A) Levels of cholesterol in the total membranes were reduced by treatment with 10 and 30 mM MβCD at 4 °C for 60 min and to a small extent by incubation at 37 °C for 20 min (left panel), but levels of phospholipid remain unaltered (right panel). With 30 mM MβCD, the membrane cholesterol content decreased ~40%. (B) Western blots of cell-free generated Aβ. Cholesterol depletion by up to ~40% had no effects on Aβ generation by the membranes from WT PS2 (left panel) or MT PS2 cells (right panel). Neither the total amount of Aβ nor the proportion of each Aβ species was affected. The bands of faster mobilities (most discernible in the bottom panels) likely represent amino-terminally truncated Aβ, because they are not labeled by 6E10. Synthetic Aβ (10 pg) was loaded on the rightmost lane as an authentic control. (C) Quantification by Western blotting of generated Aβ. The level of each Aβ is expressed in picograms (25 μg of protein was loaded per lane). Solid, shaded, and open columns represent nontreatment, 10 mM, and 30 mM MβCD treatment, respectively. Bars indicate SEM ($n = 3$).

tion of LDM domains and maintenance of their γ-secretase activity. With sodium carbonate, many membrane-spanning proteins derived from endoplasmic reticulum (ER) were found in the LDM fraction.

By use of sucrose density gradient centrifugation in the presence of 1% CHAPSO, flotillin and caveolin, well-known LDM markers, floated up and were exclusively localized in fraction 2 at the interface of 5/35% sucrose (Figure 2A). Calnexin, an ER marker, was mostly localized to the CHAPSO-solubilized membrane fraction (fraction 4). Most of the CTF and NTF of PS2 and a fraction of APP and β-CTF, all essential components of the γ-secretase complex, were recovered in fraction 2 (Figure 2A and data not shown). Profiles for protein distribution after density gradient fractionation were very similar between WT and MT PS2 cells. The levels of free cholesterol and phospholipid per milligram of protein in fraction 2 were 2.7- and 2.1-fold higher than in the total cell membrane, respectively (Figure 2B). The ratio of free cholesterol/phospholipid (phosphatidylcholine and sphingomyelin) was also higher in fraction 2 (molar ratio 0.98) than that in the total cell membrane (molar ratio 0.74). Furthermore, Triton X-100-insoluble LDM fraction prepared from CHO cells showed a similar cholesterol/phospholipid ratio when assessed by phosphorus determination (data not shown), and this ratio corresponds to that obtained by another group (*28*). Taken together, our results indicate that the protocol used here produces cholesterol-enriched membrane fraction. Fraction 2 from both WT and MT PS2 cells exhibited similar levels of cholesterol enrichment.

We next characterized the γ-secretase activity of LDM domains in the cell-free system. On incubation, the LDM fraction (fraction 2) produced Aβ, which was inhibited by specific γ-secretase inhibitors, DFK 167 at 100 μM or L-685,458 at 600 nM (Figure 2C). This indicates that Aβ generation by the LDM fraction is dependent on γ-secretase. As expected, the LDM fraction exhibited higher γ-secretase activity per milligram of protein than the total cell membranes from both WT and MT PS2 cells. The production of both Aβ40 and Aβ42 proceeded linearly until 10 min (data not shown). Therefore, we used a reaction mixture with a

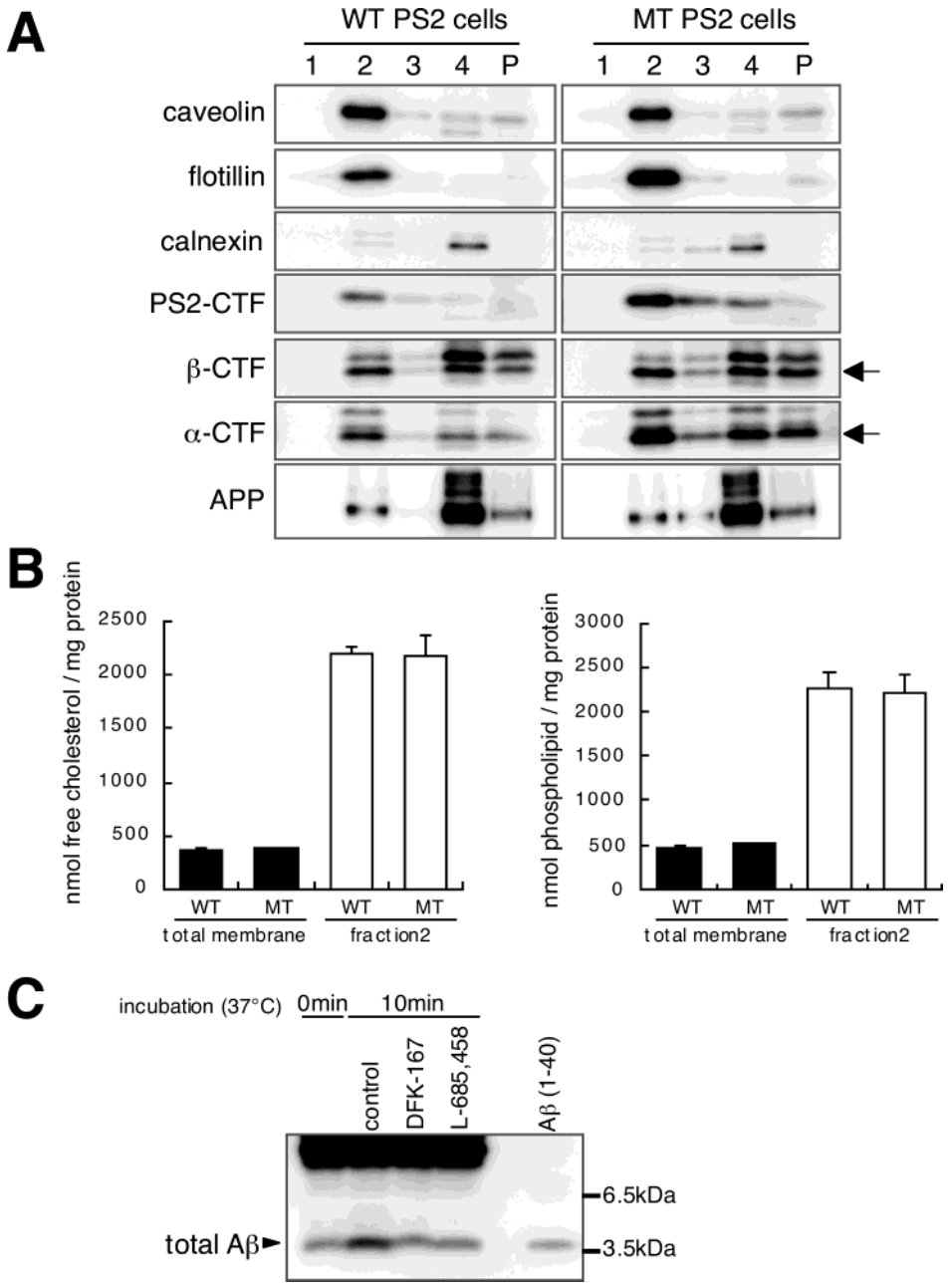

FIGURE 2: Isolation of LDM domains by sucrose density gradient centrifugation. (A) CHO cells stably expressing APP and WT (left panel) or MT PS2 (right panel) were homogenized in MBS containing 1% CHAPSO and were subjected to sucrose density gradient (5/35/40% sucrose discontinuous gradient) centrifugation. Equivalent aliquots from each were subjected to Western blotting, and probed with specific antibodies to cellular organelles. Fraction 2 from the 5/35% interface represents LDM domains and is characterized by the presence of flotillin and caveolin. It should be noted that small to substantial proportions of PS2-CTF and APP α-CTF (labeled with UT421; arrow) and β-CTF (labeled with 6E10; arrow) floated to the same interface. 6E10 (raised against Aβ1−17) labels two bands, which are also labeled with UT421 (raised against the 20-residue carboxyl-terminal cytoplasmic domain of APP). On the basis of the mobility of an authentic control, it is assumed that the lower band labeled with 6E10 is β-CTF and the upper band is a longer fragment that extended beyond the amino-terminus of β-CTF (see Figure 3B). (B) Levels of cholesterol (left panel) and phospholipid (right panel) in the total membrane (solid columns) and LDM domains (open columns; fraction 2). Their levels do not differ significantly between the two stable transfectants. Fraction 2 is rich in cholesterol and phospholipid. Bars indicate SEM ($n = 3$). (C) γ-Secretase activity in LDM fraction and the effect of γ-secretase inhibitors. After preincubation on ice for 15 min, LDM fraction (fraction 2) was incubated at 37 °C for indicated times in the presence or absence of a specific γ-secretase inhibitor, DFK 167 at 100 μM or L-685,458 at 600 nM, and subjected to Western blotting with 6E10. Synthetic Aβ (20 pg) was loaded on the rightmost lane as an authentic control. The LDM fraction exhibits Aβ production, which is inhibited by DFK 167 or L-685,458.

lower protein concentration (1 mg/mL) for the Aβ production assay and quantified the Aβ amount produced after a 10 min incubation. The proportions of Aβ40/Aβ42 produced in the LDM fraction were very similar to those in the total cell membranes: Aβ40 amounted to ∼70% of the total Aβ produced by the LDM fraction from WT PS2 cells, and Aβ42 was the predominant species produced by the LDM fraction from MT PS2 cells (Figure 3C,D).

To deplete cholesterol, the LDM fraction was treated with MβCD at 4 °C for 60 min and then subjected to the cell-free Aβ production assay. Treatment with 50 mM MβCD decreased the level of cholesterol in the LDM fraction ∼50%,

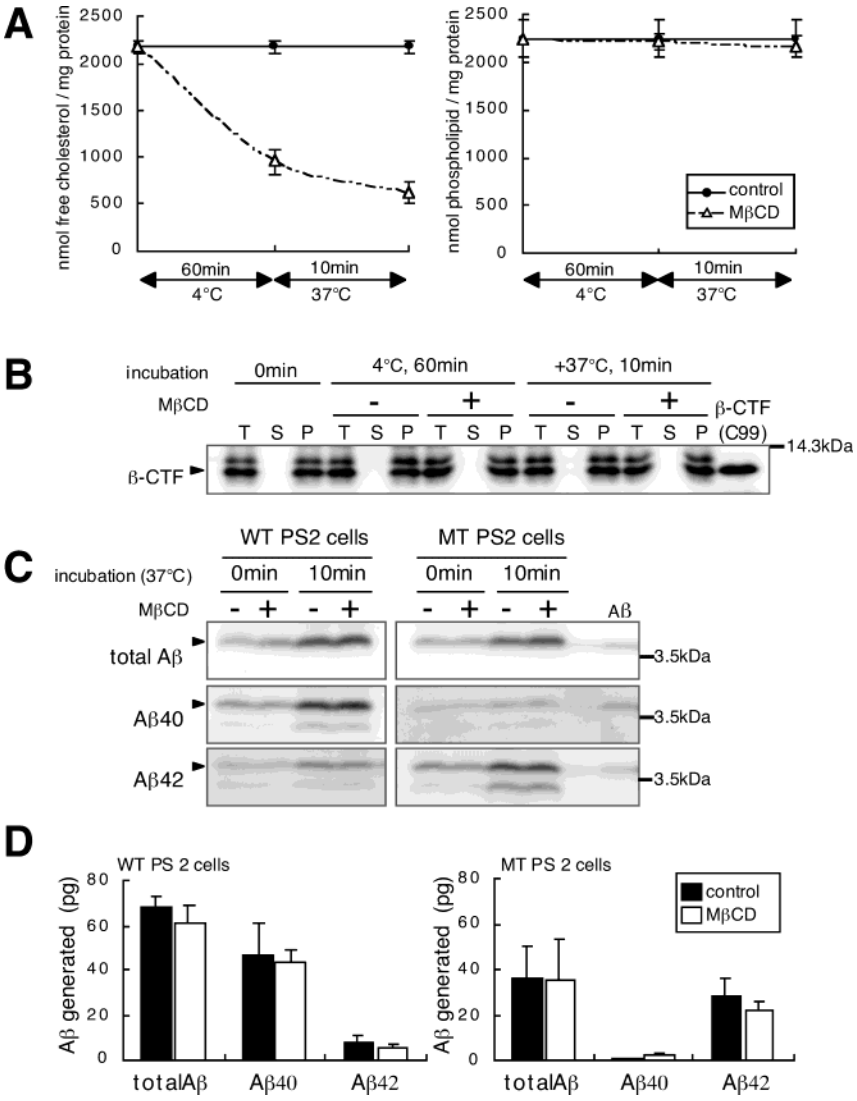

FIGURE 3: No effects of cholesterol depletion on the γ-secretase activity of LDM domains. The LDM fraction from each cell line was treated with MβCD at 4 °C for 60 min to deplete cholesterol and was incubated at 37 °C for another 10 min for cell-free Aβ generation. (A) With 50 mM MβCD, the cholesterol levels in the LDM fraction decrease ~50% (left panel), while the phospholipid levels remained unaltered (right panel). (B) No effects of MβCD treatment are discernible on the partition of β-CTF. LDM fractions (T), treated with or without 50 mM MβCD, were centrifuged and the resulting membrane pellet (P) and the remaining supernatant (S) were subjected to Western blotting with 6E10. Cell lysates prepared from CHO cells overexpressing β-CTF (C99) of APP were loaded in the rightmost lane as a positive control for β-CTF. β-CTF (indicated by arrowhead) was found exclusively in the pellet but not in the supernatant. (C) Western blots for cell-free generated Aβ. No effects of cholesterol depletion on the generation of Aβ are discernible in either WT or MT PS2 cell-derived LDM domains. Synthetic Aβ (10 pg) was loaded on the rightmost lane as an authentic control. (D) Quantification by Western blotting for generated total Aβ and Aβ40/42. The level of each Aβ is expressed in picograms (12.5 μg of protein was loaded per lane). Solid and open columns represent untreated and MβCD-treated membranes, respectively. Bars indicate SEM ($n = 3$).

while the levels of phospholipid were unaltered (Figure 3A). LDM fractions treated with 50 mM MβCD were centrifuged, and both the resulting membrane pellet and the remaining supernatant were subjected to Western blotting. β-CTF was found exclusively in the pellet, the amount of which was equivalent to the original amount found in the total reaction mixture (Figure 3B). Cholesterol reduction with MβCD by ~60% did not affect significantly either the amounts of total Aβ produced by the LDM fraction or the proportions of Aβ40/Aβ42 produced (Figure 3C,D). These results were obtained for LDM fractions from both WT and MT PS2 cells. Similar results were obtained with the MβCD-treated LDM fraction from WT and MT PS1 cells (data not shown).

Although certain contents of cholesterol in the membrane are required to maintain integrity of living cells, membrane cholesterol was further depleted to examine the effect of its extremely low levels on γ-secretase activity. After treatment with 50 mM MβCD at 4 °C for 60 min, the LDM fraction was pelleted, resuspended in buffer B, and treated similarly once again. This treatment decreased the level of cholesterol in the LDM fraction by ~90% (Figure 4A). However, such profound cholesterol reduction affected significantly neither the amounts of total Aβ produced by the LDM fraction nor the proportions of Aβ40/Aβ42 produced (Figure 4B,C).

Because it has recently been reported that β-secretase activity is present in rafts (*29*), the effect of BACE inhibitors (GL189 and APP β-secretase inhibitor) on the cell-free Aβ production was again evaluated. As observed for the total cell membranes, Aβ production by the LDM fraction was unaffected by these inhibitors (data not shown). As mentioned above, it is also possible that the pH of the reaction mixture may inhibit completely the activity of β-secretase.

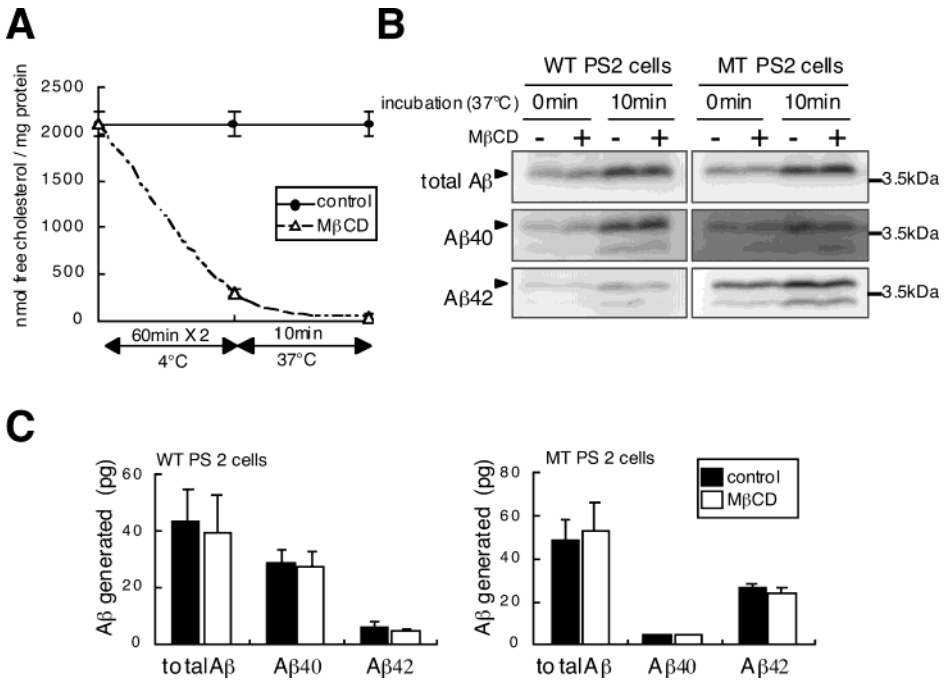

FIGURE 4: Extremely low cholesterol levels have no effects on γ-secretase activity in the LDM domains. Following treatment with 50 mM MβCD at 4 °C for 60 min, the LDM fraction was centrifuged. The resulting pellet was resuspended in buffer B and treated again with 50 mM MβCD at 4 °C for 60 min to further deplete cholesterol. Thus-treated LDM fraction was incubated at 37 °C for another 10 min for assessing cell-free Aβ generation. (A) Twice successive treatments with MβCD decreased the cholesterol levels in the LDM fraction by ~90%. (B) Western blots for cell-free generated Aβ. Profound cholesterol reduction did not affect significantly the amounts of Aβ generated by either WT or MT PS2 cell-derived LDM domains. (C) Quantification by Western blotting for generated total Aβ and Aβ40/42. The level of each Aβ is expressed in picograms (12.5 μg of protein was loaded per lane). Solid and open columns represent untreated and MβCD-treated membranes, respectively. Bars indicate SEM ($n = 4$).

Thus, the amounts of produced Aβ should accurately reflect γ-secretase activity alone. These observations indicate that cholesterol depletion in LDM domains has no effects on γ-secretase activity.

Cholesterol-Rich Microdomains Purified by BCθ Have γ-Secretase Activity. To confirm that Aβ is produced in the cholesterol-rich membrane domains, such microdomains were further purified from the LDM fraction by use of BCθ and examined for γ-secretase activity. Perfringolysin O (θ-toxin), a bacterial cytolysin produced by *Clostridium perfringens*, binds specifically to cholesterol in the membrane, depending on the cholesterol contents (*30*), and forms porelike structures, leading to cytolytic effects (*31*, *32*). Like θ-toxin, BCθ, a protease-nicked and biotinylated derivative of θ-toxin, retains the ability to bind to membrane cholesterol; however, it lacks pore-forming activity and is therefore a powerful probe for intact cells (*21*, *33*). Accordingly, BCθ has been shown to bind selectively to rafts of intact cells (*14*), and has been used to visualize the distribution of rafts on the plasma membrane (*33*−*35*).

The LDM fraction prepared from WT PS2 cells was incubated with BCθ. BCθ-bound cholesterol-rich microdomains were collected by use of streptavidin-coated magnetic beads. Most of the proteins in the LDM fraction were recovered on BCθ-bound beads (data not shown). Raft markers such as flotillin and caveolin were found mostly in the BCθ-bound fraction and scarcely in the unbound fraction (Figure 5A). Other raft residents including Gβ (β subunit of heterotrimeric G protein), src, and fyn were also recovered exclusively in the BCθ-bound fraction (unpublished data). When LDM domains were treated with 50 mM MβCD prior to BCθ-affinity purification, only BCθ, but not flotillin and caveolin, was recovered by streptavidin beads (Figure 5B), indicating that BCθ did not bind any more to MβCD-treated LDM domains as shown previously (*14*). This also validates that BCθ binds selectively to the cholesterol-rich membrane domains.

PS2-CTF and PS2-NTF were found mostly in the BCθ-bound fraction (Figure 5A). α-CTF and β-CTF were also recovered mostly in the BCθ-bound fraction (Figure 5C, 0 min). In contrast, most of the γ-CTF was found in the unbound fraction. Regarding Aβ, approximately 60% was found in the BCθ-bound fraction and ~40% remained to be in the unbound fraction (Figure 5C, lower left panel, 0 min). We next assessed γ-secretase activity of the BCθ-bound or unbound fraction in the cell-free system. Each BCθ-bound or unbound fraction was incubated at 37 °C for 30 min and assessed for Aβ production. The BCθ-bound fraction did indeed produce Aβ and γ-CTF (Figure 5C,D). The proportions of Aβ40/Aβ42 produced were similar to those in the LDM fraction (data not shown). In contrast, Aβ production was undetectable in the BCθ-unbound fraction [Figure 5C-(lower left panel),D]. To confirm that Aβ production in the BCθ-bound fraction is due to γ-secretase, the effects of two specific γ-secretase inhibitors (DFK 167 and L-685,458) were examined. Each inhibitor, DFK 167 at 100 μM and L-685,458 at 600 nM, inhibited Aβ production by the BCθ-bound fraction (Figure 5E). These data indicate that the cholesterol-rich microdomains have γ-secretase activity.

DISCUSSION

Cholesterol depletion in the membrane disrupts rafts (*36*, *37*), and at the same time reduces Aβ production of the living cells (*10*−*12*). Thus, it is possible that cholesterol is involved

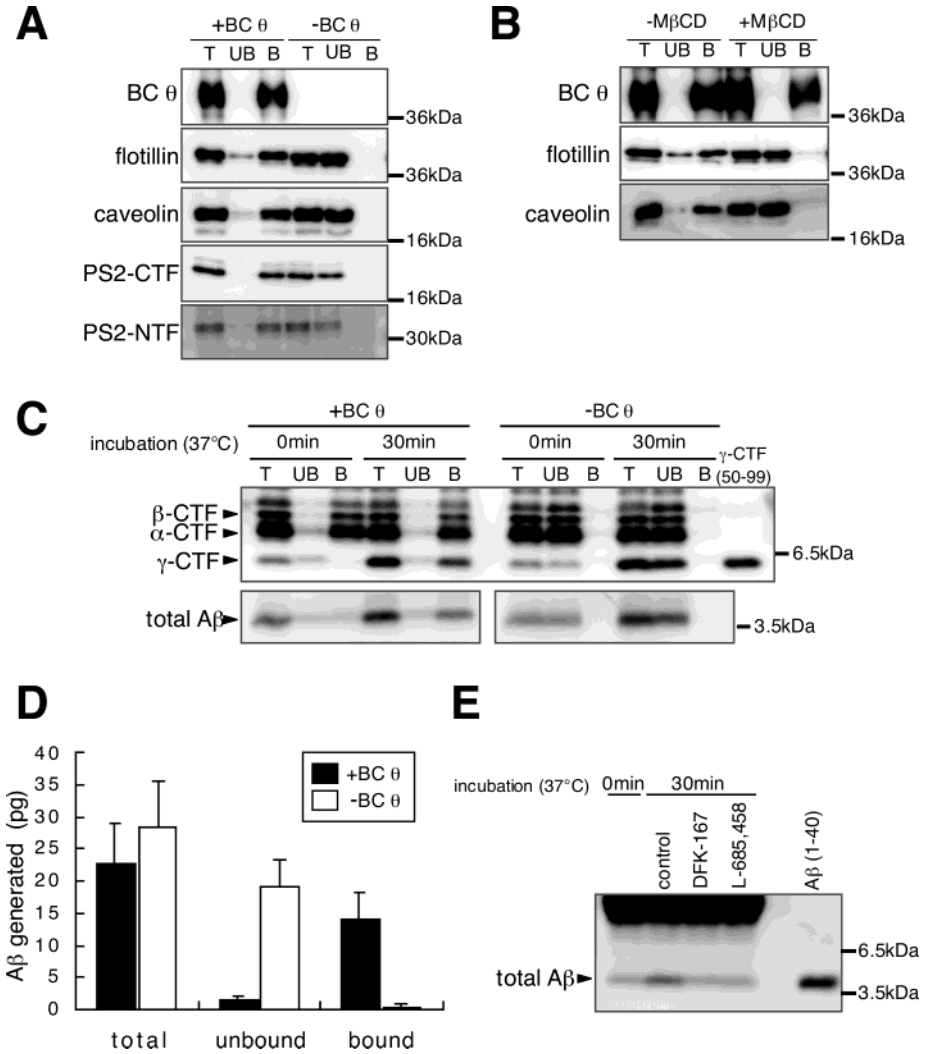

FIGURE 5: Cholesterol-rich domains were further purified from the LDM fraction of the WT PS2 cell homogenate by use of BCθ and assessed for γ-secretase activity. (A) The LDM fraction (T, total) was incubated with or without BCθ, which binds exclusively to cholesterol-rich domains, and was separated into BCθ-bound (B) and unbound fractions (UB). BCθ was labeled with peroxidase-conjugated streptavidin. The majority of flotillin and caveolin, raft markers, in the LDM fraction were recovered in the bound fraction in the presence of BCθ. PS2-CTF and PS2-NTF were also found mostly in the BCθ-bound fraction. (B) Prior to the incubation with BCθ, the LDM fraction was treated with 50 mM MβCD to deplete cholesterol. When treated, flotillin and caveolin remained in the BCθ-unbound fraction, and only BCθ was recovered by streptavidin beads, indicating that cholesterol-rich domains are disrupted by the treatment. (C) Following BCθ fractionation, each fraction was assessed for γ-secretase activity. Each fraction was incubated at 37 °C for 30 min for cell-free Aβ generation. The BCθ-bound fraction generated Aβ and γ-CTF. An authentic γ-CTF (CTF50−99) was prepared as follows; Membrane fraction prepared from CHO cells stably expressing human APP751 was incubated at 37 °C for 30 min. γ-CTF produced (mostly CTF50−99) was immunoprecipitated with C4 and confirmed by mass spectrometric analysis. (D) Quantification by Western blotting of Aβ produced by the BCθ-bound and unbound fractions. The level of Aβ is expressed in picograms (6.25 μg of protein was loaded per lane for total, and the corresponding amount of each fraction was loaded per lane for bound and unbound fractions). Solid and open columns represent BCθ-treated and untreated reaction mixtures, respectively. Bars indicate SEM ($n = 3$). (E) Aβ production in the BCθ-bound fraction depends on γ-secretase. BCθ-bound fraction was preincubated with DFK 167 at 100 μM, L-685,458 at 600 nM, or DMSO (control) on ice for 15 min and subjected to incubation at 37 °C for 10 min for cell-free Aβ generation. Synthetic Aβ (50 pg) was loaded on the rightmost lane as an authentic control. Aβ production is inhibited by either DFK 167 or L-685,458.

in the formation of an appropriate raft environment for Aβ production rather than in modulation of the enzymatic activity. In the cholesterol-rich rigid membrane environment, the γ-secretase complex and β-CTF could cluster and interact freely with each other. In other words, the effects of cholesterol-reducing reagents on Aβ generation by living cells may result from aberrant protein and/or membrane trafficking rather than direct modulation of γ-secretase activity. In fact, various biological activities appear to be regulated by membrane lipids including cholesterol. For example, treatment of cells with U18666A, a cholesterol transport-interfering agent, causes Aβ accumulation in Rab7-positive late endosomes (*38*, *39*). This effect may be explained by retarded protein sorting caused by retention of cholesterol in endosomal/lysosomal compartments. Similarly, γ-secretase accumulates in Rab7-positive vesicular organelles and produces a larger amount of Aβ, which is retained by the organelles (*39*). Thus, from a functional point of view, domain sorting and subcellular compartmentalization of cholesterol, or even the distribution of the membrane lipid, may be more important rather than cellular bulk levels of cholesterol.

Consistently with our results, Wahrle et al. (*40*) also observed that γ-secretase activity is present in the low buoyant density cholesterol-rich membrane fraction. In contrast to our results, however, they reported that the activity

was cholesterol-dependent. An explanation for the discrepancy between the two results is not presently clear. A notable difference is that they treated the cells with a γ-secretase inhibitor prior to harvest. Using the parent APP-transfected CHO cells, two of the present authors (H.M. and Y.I.) initially made an observation suggesting that the cellular cholesterol levels can modulate γ-cleavage of APP. When the cells were treated with 25-hydroxycholesterol, which reduces the levels of cholesterol and elevates those of sphingomyelin, the levels of intracellular Aβ significantly decreased, while those of β-CTF increased. This ultimate effect is very similar to that of a potent γ-secretase inhibitor. However, we found no suppression of Aβ production by membranes isolated from 25-hydroxycholesterol-treated cells. Thus, these data raise the possibility that the treatment of in vitro cultured cells with a certain inhibitor prior to cellular fractionation may perturb additional cellular (especially lipid) parameters to compensate for aberrant metabolism, and as a result thus-prepared membranes may not necessarily represent the membrane under normal metabolic conditions. In other words, the γ-secretase activity would be modulated or influenced by many factors that may be involved mostly in membrane integrity.

Here, we used BCθ, which binds selectively to rafts of living cells, to purify rafts from the LDM fraction further. The BCθ-purified membrane domains demonstrated γ-secretase activity. The involvement of rafts in Aβ generation has recently been reported by Ehehalt et al. (*29*). They showed that antibody cross-linking induces copatching of APP and β-secretase (BACE1) and increases the production of Aβ in a cholesterol-dependent manner. Inhibition of endocytosis reduces the generation of Aβ, but this is overcome by artificial raft clustering via antibody cross-linking. On the basis of these observations, they concluded first that APP and β-secretase are located in distinct rafts and second, following endocytosis, that these distinct rafts merge to promote β-cleavage and produce a greater amount of β-CTF. Currently we do not know whether the endocytosis that combines separate rafts together also enhances γ-cleavage. Our experience in the cell-free system (*23*) suggests that γ-cleavage may not proceed efficiently in endosomes because of their acidic environment. Thus, during recycling of endosomes that is associated with production of β-CTF, γ-cleavage may occur at the surface of the membrane, and the resulting Aβ may be coupled with secretory machinery and released into the extracellular space.

Subcellular fractionation showed repeatedly that γ-secretase activity or active presenilin complex cofractionates with Golgi markers (*41*, *42*), thus suggesting that the Golgi cisternae are the site of Aβ production. On the other hand, PS1/2 were shown to be predominantly located within early secretory compartments such as ER and the intermediate compartment (*43*, *44*). Thus, there appears to be a gap between the subcellular localization of PS1/2 and the compartment involved in Aβ production. However, a recent report showed that only a small portion of PS1 appears to be involved in the active γ-secretase complex (*45*). With BCθ as a probe for rafts, a very careful immunoelectron microscopic study has recently been performed (*35*). BCθ labels mostly plasma membrane, especially fillopodialike structures and vesicles outside the cell surface (exosomes), and internal vesicles of multivesicular bodies but rarely Golgi cisternae. This indicates that at least a fraction of active γ-secretase is located in rafts at the cell surface and could explain several observations indicating that γ-cleavage must occur at the cell surface. This is consistent with the observation by Kaether et al. (*46*) that PS1 is targeted as a biologically active complex with mature nicastrin to the plasma membrane of living cells.

In addition to APP, several other type 1 membrane proteins have been reported to be cleaved within their transmembrane domains by γ-secretase. These include Notch (*47*), ErbB4 (*48*), E-cadherin (*49*), low-density lipoprotein receptor-related protein (LRP) (*50*), CD44 (*51*), Nectin-1α (*52*), and Delta1 and Jagged2 (*53*). There are many observations that suggest that for these substrates γ-cleavage takes place in the plasma membrane. The Notch receptor undergoes proteolytic processing in the ectodomain within the trans-Golgi network by a furin-like protease (*54*), and it forms a mature heterodimeric receptor that accumulates on the cell surface (*55*). Its ligand binding on the cell surface triggers further sequential proteolytic processing in the extracellular juxtamembrane region by tumor necrosis factor α-converting enzyme (TACE) (*56*, *57*), and subsequently, the membrane-tethered derivative is cleaved within the membrane by γ-secretase. Thus it is reasonable to assume that through ligand binding Notch is cleaved by γ-secretase on the plasma membrane. In stable cell−cell adhesion, the E-cadherin−β-catenin complex in the cadherin-based adherens junctions (CAJ) is anchored to the actin cytoskelton via α-catenin (*58*). PS1 forms a complex with components of CAJ at the cell surface in Madin−Darby canine kidney (MDCK) cells (*59*). γ-Cleavage of E-cadherin, stimulated by apoptosis or calcium influx, promotes dissociation of the CAJ components from the cytoskeleton and their release into the soluble cytosol (*48*). Thus, it is likely that γ-cleavage of E-cadherin occurs at the plasma membrane. CD44 is mainly localized to filopodia of the plasma membrane and is normally colocalized with a small amount of PS1 (*60*). The 12-*O*-tetradecanoylphorbol 13-acetate (TPA) treatment promotes the intramembranous cleavage of CD44, which leads to redistribution of PS1 to the ruffling areas of the plasma membrane. Thus, the present observation provides a firm basis for the assumption that γ-cleavage occurs at the plasma membrane.

Together, the evidence shows that rafts are very unusual sites, where all the important mechanisms of AD converge—that is, essential components of Aβ production, β-CTF, BACE1, and γ-secretase, and presumably the necessary environment for Aβ aggregation, cholesterol, and GM1 ganglioside. Thus, the function of rafts during aging and AD warrants focus upon in future research.

ACKNOWLEDGMENT

We are grateful to Dr. E. H. Koo, University of California, San Diego, for providing the APP751 CHO cells; Dr. C. Haass, Ludwig-Maximilians-University, for providing 2972N antibody; and T. Suzuki, Hokkaido University, for providing UT421.

REFERENCES

1. Vassar, R., Bennet, 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.
2. Gu, Y., Misonou, H., Sato, T., Dohmae, N., Takio, K., and Ihara, Y. (2001) *J. Biol. Chem. 276*, 35235−35238.

3. Sastre, M., Steiner, H., Fuchs, K., Capell, A., Multhaup, G., Condron, M. M., Teplow, D. B., and Haass, C. (2001) *EMBO Rep. 9*, 835−841.
4. Weidemann, A., Eggert, S., Reinhard, F. B., Vogel, M., Paliga, K., Baier, G., Masters, C. L., Beyreuther, K., and Evin, G. (2002), *Biochemistry 41*, 2825−2835.
5. Kimberly, W. T., Zheng, J. B., Guenette, S. Y., and Selkoe, D. J. (2001) *J. Biol. Chem. 276*, 40288−40292.
6. Takasugi, N., Tomita, T., Hayashi, I., Tsuruoka, M., Niimura, M., Takahashi, Y., Thinakaran, G., and Iwatsubo, T. (2003) *Nature 422*, 438−41.
7. Edbauer, D., Winkler, E., Regula, J. T., Pesold, B., Steiner, H., and Haass, C. (2003) *Nat. Cell Biol. 5*, 486-488.
8. Kimberly, W. T., LaVoie, M. J., Ostaszewski, B. L., Ye, W., Wolfe, M. S., and Selkoe, D. J. (2003) *Proc. Natl. Acad. Sci. U.S.A. 100*, 6382−6387.
9. Wolozin, B., Kellman, W., Rousseau, P., Celesis, G. G., and Siegel, G. (2000) *Arch. Neurol. 57*, 1439−1443.
10. Simons, M., Keller, P., De Strooper, B., Beyreuther, K., Dotti, C. G., and Simons, K. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 6460−6464.
11. Kojro, E., Gimpl, G., Lammich, S., Marz, W., and Fahrenholz, F. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 5815−5820.
12. Fassbender, K., Simons, M., Bergmann, C., Stroick, M., Lutjohann, D., Keller, P., Runz, H., Kuhl, S., Bertsch, T., von Bergmann, K., Hennerici, M., Beyreuther, K., and Hartmann, T. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 5856−5861.
13. Guy, R. K. (2000), *Proc. Natl. Acad. Sci. U.S.A. 97*, 7307−7312.
14. Waheed, A. A., Shimada, Y., Heijnen, H. F., Nakamura, M., Inomata, M., Hayashi, M., Iwashita, S., Slot, J. W., and Ohno-Iwashita, Y. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 4926−4931.
15. Qi, Y., Morishima-Kawashima, M., Sato, T., Mitsumori, R., and Ihara, Y. (2003) *Biochemistry 42*, 1042−1052.
16. Suzuki, N., Cheung, T. T., Cai, X. D., Odaka, A., Otvos, L., Jr., Eckman, C., Golde, T. E., and Younkin, S. G. (1994) *Science 264*, 1336−1340.
17. Tomita, S., Kirino, Y., and Suzuki, T. (1998) *J. Biol. Chem. 273*, 6277−6284.
18. Walter, J., Capell, A., Grunberg, J., Pesold, B., Schindzielorz, A., Prior, R., Podlisny, M. B., Fraser, P., Hyslop, P. S., Selkoe, D. J., and Haass, C. (1996) *Mol. Med. 2*, 673−691.
19. Morishima-Kawashima, M., and Ihara, Y. (1998) *Biochemistry 37*, 15247−15253.
20. Morishima-Kawashima, M., Osima, N., Ogata, H., Yamaguchi, H., Yoshimura, M., Sugihara, S., and Ihara, Y. (2000) *Am. J. Pathol. 157*, 2093−2099.
21. Iwamoto, M., Morita, I., Fukuda, M., Murota, S., Ando, S., and Ohno-Iwashita, Y. (1997) *Biochim. Biophys. Acta 1327*, 222−230.
22. Iwamoto, M., Ohno-Iwashita, Y., and Ando, S. (1990) *Eur. J. Biochem. 194*, 25−31.
23. Sato, T., Dohmae, N., Qi, Y., Kakuda, N., Misonou, H., Mitsumori, R., Maruyama, H., Koo, E. H., Haass, C., Takio, K., Morishima-Kawashima, M., Ishiura, S., and Ihara, Y. (2003) *J. Biol. Chem. 278*, 24294−24301.
24. Pitha, J., Irie, T., Sklar, P. B., and Nye, J. S. (1988) *Life Sci. 43*, 493−502.
25. Hansen, G. H., Immerdal, L., Thorsen, E., Niels-Christiansen, L. L., Nystrom, B. T., Demant, E. J., and Danielsen, E. M. (2001) *J. Biol. Chem. 276*, 32338−32344.
26. Lee, S. J., Liyanage, U., Bickel, P. E., Xia, W., Lansbury, P. T., Jr, and Kosik, K. S. (1998) *Nat. Med. 4*, 730−734.
27. Li, Y. M., Lai, M. T., Xu, M., Huang, Q., DiMuzio-Mower, J., Sardana, M. K., Shi. X. P., Yin, K. C., Shafer, J. A., and Gardell, S. J. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 6138−6143.
28. Gkantiragas, I., Brugger, B., Stuven, E., Kaloyanova, D., Li, X. Y., Lohr, K., Lottspeich, F., Wieland, F. T., and Helms, J. B. (2001) *Mol. Biol. Cell 12*, 1819−1833.
29. Ehehalt, R., Keller, P., Haass, C., Thiele C., and Simons, K. (2003) *J. Cell. Biol. 160*, 113−123.
30. Ohno-Iwashita, Y., Iwamoto, M., Mitsui, K., Kawasaki, H., and Ando, S. (1986) *Biochemistry 25*, 6048−6053.
31. Mitsui, K., Sekiya, T., Nozawa, Y., and Hase, J. (1979) *Biochim. Biophys. Acta 554*, 68−75.
32. Rossjohn, J., Feil, S. C., McKinstry, W. J., Tweten, R. K., and Parker, M. W. (1997) *Cell 89*, 685−692.
33. Fujimoto, T., Hayashi, M., Iwamoto, M., and Ohno-Iwashita, Y. (1997) *J. Histochem. Cytochem. 45*, 1197−1205.
34. Hagiwara, H., Kogure, S. Y., Nakamura, M., Shimada, Y., Ohno-Iwashita, Y., and Fujimoto, T. (1999) *Biochem. Biophys. Res. Commun. 260*, 516−521.
35. Mobius, W., Ohno-Iwashita, Y., van Donselaar, E. G., Oorschot, V. M., Shimada, Y., Fujimoto, T., Heijnen, H. F., Geuze, H. J., and Slot, J. W. (2002) *J. Histochem. Cytochem. 50*, 43−55.
36. Keller, P., and Simons, K. (1998) *J. Cell. Biol. 140*, 1357−1367.
37. Kabouridis, P. S., Janzen, J., Magee, A. L., and Ley, S. C. (2000) *Eur. J. Immunol. 30*, 954−963.
38. Yamazaki, T., Chang, T. Y., Haass, C., and Ihara, Y. (2001) *J. Biol. Chem. 276*, 4454−4460.
39. Runz, H., Rietdorf, J., Tomic, I., de Bernard, M., Beyreuther, K., Pepperkok, R., and Hartmann, T. (2002) *J. Neurosci. 22*, 1679−1689.
40. Wahrle, S., Das, P., Nyborg, A. C., McLendon, C., Shoji, M., Kawarabayashi, T., Younkin, L. H., Younkin, S. G., and Golde, T. E. (2002) *Neurobiol. Dis. 9*, 11−23.
41. Greenfield, J. P., Tsai, J., Gouras, G. K., Hai, B., Thinakaran, G., Checler, F., Sisodia, S. S., Greengard, P., and Xu, H. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 742−747.
42. Xia, W., Ray, W. J., Ostaszewski, B. L., Rahmati, T., Kimberly, W. T., Wolfe, M. S., Zhang, J., Goate, A. M., and Selkoe, D. J. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 9299−9304.
43. Annaert, W. G., Levesque, L., Craessaerts, K., Dierinck, I., Snellings, G., Westaway, D., George-Hyslop, P. S., Cordell, B., Fraser, P., and De Strooper, B. (1999) *J. Cell. Biol. 147*, 277−294.
44. Cupers, P., Bentahir, M., Craessaerts, K., Orlans, I., Vanderstichele, H., Saftig, P., De Strooper, B., and Annaert, W. (2001) *J. Cell. Biol. 154*, 731−740.
45. Lai, M. T., Chen, E., Crouthamel, M. C., DiMuzio-Mower, J., Xu, M., Huang, Q., Price, E., Register, B., Shi, X. P., Donoviel, D. B., Bernstein, A., Hazuda, D., Gardell, S. J., and Li, Y. M. (2003) *J. Biol. Chem. 278*, 22475−22481.
46. Kaether, C., Lammich, S., Edbauer, D., Ertl, M., Rietdorf, J., Capell, A., Steiner, H., and Haass, C. (2002) *J. Cell. Biol. 158*, 551−561.
47. De Strooper, B., Annaert, W., Cupers, P., Saftig, P., Craessaerts, K., Mumm, J. S., Schroeter, E. H., Schrijvers, V., Wolfe, M. S., Ray, W. J., Goate, A., and Kopan, R. (1999) *Nature 398*, 518−522.
48. Ni, C. Y., Murphy, M. P., Golde, T. E., and Carpenter, G. (2001) *Science 294*, 2179−2181.
49. Marambaud, P., Shioi, J., Serban, G., Georgakopoulos, A., Sarner, S., Nagy, V., Baki, L., Wen, P., Efthimiopoulos, S., Shao, Z., Wisniewski, T., and Robakis, N. K. (2002) *EMBO J. 21*, 1948−1956.
50. May, P., Reddy, Y. K., and Herz, J. (2002) *J. Biol. Chem. 277*, 18736−18743.
51. Lammich, S., Okochi, M., Takeda, M., Kaether, C., Capell, A., Zimmer, A. K., Edbauer, D., Walter, J., Steiner, H., and Haass, C. (2002) *J. Biol. Chem. 277*, 44754−44759.
52. Kim. D. Y., Ingano, L. A., and Kovacs, D. M. (2002) *J. Cell. Biol. 277*, 49976−49981.
53. Ikeuchi, T., and Sisodia, S. S. (2003) *J. Biol. Chem. 278*, 7751−7754.
54. Logeat, F., Bessia, C., Brou, C., LeBail, O., Jarriault, S., Seidah, N. G., and Israel, A. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 8108−8112.
55. Blaumueller, C. M., Qi, H., Zagouras, P., and Artavanis-Tsakonas, S. (1997) *Cell 90*, 281−291.
56. Mumm, J. S., Schroeter, E. H., Saxena, M. T., Griesemer, A., Tian, X., Pan, D. J., Ray, W. J., and Kopan, R. (2000) *Mol. Cell 5*, 197−206.
57. Brou, C., Logeat, F., Gupta, N., Bessia, C., LeBail, O., Doedens, J. R., Cumano, A., Roux, P., Black, R. A., and Israel, A. (2000) *Mol. Cell 5*, 207−216.
58. Nathke, I. S., Hinck, L., Swedlow, J. R., Papkoff, J., and Nelson, W. J. (1994) *J. Cell. Biol. 125*, 1341−1352.
59. Georgakopoulos, A., Marambaud, P., Efthimiopoulos, S., Shioi, J., Cui, W., Li, H. C., Schutte, M., Gordon, R., Holstein, G. R., Martinelli, G., Mehta, P., Friedrich, V. L., Jr., and Robakis, N. K. (1999) *Mol. Cell 4*, 893−902.
60. Murakami, D., Okamoto, I., Nagano, O., Kawano, Y., Tomita, T., Iwatsubo, T., De Strooper, B., Yumoto, E., and Saya, H. (2003) *Oncogene 22*, 1511−1516.

BI034904J