Association of active γ-secretase complex with lipid rafts

Yasuomi Urano,[*,†] Ikuo Hayashi,[§] Noriko Isoo,[§] Patrick C. Reid,[*,†] Yoshikazu Shibasaki,[†] Noriko Noguchi,[†] Taisuke Tomita,[§] Takeshi Iwatsubo,[§] Takao Hamakubo,[1,*,†] and Tatsuhiko Kodama[†]

Department of Molecular Biology and Medicine* and Laboratory for Systems Biology and Medicine,[†] Research Center for Advanced Science and Technology, The University of Tokyo, Tokyo 153-8904, Japan; and Department of Neuropathology and Neuroscience,[§] Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo 113-0033, Japan

Abstract Cholesterol has been implicated in the pathogenesis of Alzheimer's disease (AD). Although the underlying mechanisms are not yet clear, several studies have provided evidence for the involvement of cholesterol-rich lipid rafts in the production of amyloid β peptide (Aβ), the major component of amyloid deposits in AD. In this regard, the γ-secretase complex is responsible for the final cleavage event in the processing of β-amyloid precursor protein (βAPP), resulting in Aβ generation. The γ-secretase complex is a multiprotein complex composed of presenilin, nicastrin (NCT), APH-1, and PEN-2. Recent reports have suggested that γ-secretase activity is predominantly localized in lipid rafts, and presenilin and NCT have been reported to be localized in lipid rafts. In this study, various biochemical methods, including coimmunoprecipitation, in vitro γ-secretase assay, and methyl-β-cyclodextrin (MβCD) treatment, are employed to demonstrate that all four components of the active endogenous γ-secretase complex, including APH-1 and PEN-2, are associated with lipid rafts in human neuroblastoma cells (SH-SY5Y). Treatment with statins, 3-hydroxy-3-methylglutaryl-CoA-reductase inhibitors, significantly decreased the association of the γ-secretase complex with lipid rafts without affecting the distribution of flotillin-1. This effect was partially abrogated by the addition of geranylgeraniol. These results suggest that both cholesterol and protein isoprenylation influence the active γ-secretase complex association with lipid rafts.—Urano, Y., I. Hayashi, N. Isoo, P. C. Reid, Y. Shibasaki, N. Noguchi, T. Tomita, T. Iwatsubo, T. Hamakubo, and T. Kodama. **Association of active γ-secretase complex with lipid rafts. *J. Lipid Res.* 2005.** 46: **904–912.**

Supplementary key words Alzheimer's disease • statin • cholesterol • isoprenylation • presenilin • nicastrin • APH-1 • PEN-2

It is well established that the abnormal generation and deposition of amyloid β peptides (Aβs) is a pathologic hallmark of Alzheimer's disease (AD). Aβ is generated by two sequential proteolytic cleavage steps from the β-amyloid precursor protein (βAPP) (reviewed in refs. 1–3). βAPP is initially cleaved by β-secretase, which has been identified as a β-site APP-cleaving enzyme (4), followed by the subsequent intramembrane proteolysis of the membrane-bound C-terminal fragment (βCTF, C99) catalyzed by γ-secretase. In an alternative pathway, βAPP is cleaved by α-secretase within the Aβ domain and the remaining CTF (C83) is also cleaved by γ-secretase to release the nonamyloidogenic p3 peptide.

Recent studies suggest that the activation of γ-secretase requires the formation of a stable high-molecular-weight multiprotein complex, which includes an endoproteolysed and fragmented form of presenilin, nicastrin (NCT), APH-1, and PEN-2 (5). These four transmembrane proteins are presumed to be indispensable for γ-secretase activity, because their coexpression enables reconstitution of the γ-secretase activity (5–9), whereas the absence of even one results in the absence of γ-secretase activity and defects in the expression and/or maturation of the remaining partners (5, 6). γ-Secretase is known to be responsible for the intramembranous cleavage of other type I transmembrane proteins, such as Notch, CD44, and E-cadherin (5).

There is growing evidence that cholesterol is linked to the development of AD. For example, the ε4 allele of apolipoprotein E, which is a major apolipoprotein in the brain, has been identified as an important risk factor for AD (10). Several epidemiological studies have suggested that the use of 3-hydroxy-3-methylglutaryl-CoA-reductase inhibitors (statins) to treat hypercholesterolemia may reduce the risk of dementia (11–13). In vivo studies have demonstrated that high-cholesterol diets can increase Aβ levels in rabbits (14) and in an AD mouse model (15). Treat-

Manuscript received 31 August 2004 and in revised form 19 January 2005.

Published, JLR Papers in Press, Feburary 16, 2005.
DOI 10.1194/jlr.M400333-JLR200

Abbreviations: Aβ, amyloid β peptide; AD, Alzheimer's disease; βAPP, β-amyloid precursor protein; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; CTF, C-terminal fragment; DRM, detergent-resistant membrane; LPDS, lipoprotein-deficient serum; MβCD, methyl-β-cyclodextrin; NCT, nicastrin; NTF, N-terminal fragment.

[1] To whom correspondence should be addressed.
e-mail: hamakubo@lsbm.org

ment with cholesterol synthesis inhibitors can lower the Aβ levels in guinea pig (16) and in the AD mouse (17). Cholesterol depletion also inhibits Aβ generation in hippocampal neurons (16, 18) and increases α-secretase activity in cultured cells (19). Thus, it has been reported that cholesterol depletion by statins and/or cholesterol-extracting agents appears to inhibit the amyloidogenic pathway while inducing the alternative nonamyloidogenic pathway (20).

Several lines of evidence suggest the involvement of lipid rafts in β- and γ-cleavage of βAPP (21–25). Lipid rafts are membrane microdomains enriched in cholesterol and sphingolipids, and proteins can be selectively included or excluded from these microdomains (26). Lipid rafts have been shown to function as a concentrating platform for a variety of signal transduction molecules (27, 28). It has been reported that the proteins relevant to Aβ generation, including presenilin, NCT, and a small portion of βAPP, localize in lipid rafts (21–25, 29–32). It has also been reported that β-secretase localizes in lipid rafts and that cholesterol depletion abrogates this localization (22). Ehehalt et al. (21) have reported that βAPP exists in two pools, one associated with lipid rafts, in which β-cleavage occurs, and another outside of lipid rafts, where α-cleavage occurs. Recent reports have suggested that γ-secretase activity is predominantly localized in lipid rafts and that cholesterol can directly regulate the γ-secretase activity in isolated lipid rafts (24). Thus, lipid rafts offer a structural platform for examining the effect of cholesterol on Aβ generation. Here, we report that the four endogenous components of the γ-secretase complex, including APH-1 and PEN-2 in SH-SY5Y cells, are associated with detergent-resistant membranes (DRMs). We also show that statin treatment affects the association of the active form of the γ-secretase complex with lipid rafts.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium, fetal bovine serum, MβCD, lipoprotein-deficient serum (LPDS), and geranylgeraniol were purchased from Sigma. L-685,458 and kifunensine were obtained from Calbiochem. Hybond-ECL nitrocellulose membrane and protein G Sepharose were purchased from Amersham. Complete protease inhibitor cocktail was from Roche Diagnotics. All other reagents were purchased from Wako, unless otherwise specified.

Cell lines and culture

Human SH-SY5Y neuroblastoma cells were obtained from The American Type Culture Collection. Wild-type and presenilin $1^{-/-}$/presenilin $2^{-/-}$ mouse embryonic fibroblasts (33) were kindly provided by Drs. W. Annaert and B. De Strooper. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (Gibco-BRL) at 37°C in 5% CO_2. For the inhibition of γ-secretase activity, cells were incubated with 2 μM L-685,458 for 18 h. For cholesterol depletion experiments, cells were incubated with or without 2 mM MβCD in serum-free media for 30 min at 37°C. In statin treatment experiments, cells were washed with PBS, and then cultured in Dulbecco's modified Eagle's medium supplemented with 5% LPDS and 250 μM sodium mevalonate in the absence or presence of statins (50 μM compactin from Dr. A. Endo or 5 μM pitavastatin from Kowa Co., Ltd.) for 48 h. In statin and geranylgeraniol treatment experiments, cells were cultured with 5% LPDS and 10 μM pitavastatin in the absence or presence of 10 μM geranylgeraniol for 48 h. For the inhibition of mannosidase I, cells were treated in the absence or presence of 1 μg/ml kifunensine for 48 h.

Isolation of DRMs

All steps were carried out at 4°C. Confluent cells were washed and scraped into ice-cold PBS and resuspended in 200 μl buffer R (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA) containing 2% 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO) and Complete protease inhibitor cocktail. After a 20 min incubation on ice, the cell lysate was adjusted to 45% sucrose and placed in the bottom of a 5 ml Beckman ultra-clear centrifuge tube and overlaid with 3 ml 35% sucrose in buffer R, followed by 1 ml 5% sucrose in buffer R. After centrifugation at 100,000 *g* for 16 h at 4°C in a Beckman SW55 rotor, 10 fractions (500 μl) were collected from the top of the gradient.

Immunoblotting and antibodies

Samples were applied to SDS-PAGE using 8% Tris-Glycine gels or 15% Tris-Tricine gels, and transferred to Hybond-ECL at 75V for 2 h. Blots were probed with the following antibodies: Antibodies against presenilin 1 N-terminal fragment (NTF) (anti-G1Nr2; 1:2000), presenilin 1 CTF (anti-G1L3; 1:3000), presenilin 2 CTF (anti-G2L; 1:5000), and PEN-2 (anti-PNT3; 1:2000) were described previously (9, 34, 35). Antibodies against NCT (N-19, Santa Cruz; 1:5000), APH-1aL (Covance; 1:2000), APH-1aL/S (Covance; 1:1000), APH-1b (Covance; 1:1000), C99 (6E10, Chemicon; 1:1000), flotillin-1 (BD Bioscience; 1:2000), calnexin (BD Bioscience; 1:1000), PDI (BD Bioscience; 1:500), and EEA-1 (BD Bioscience; 1:3000) were purchased commercially. Bound antibodies were detected with species-specific secondary antibodies conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories; 1:10,000) and SuperSignal West Dura Extended Duration Substrate (Pierce).

In vitro γ-secretase assay and coimmunoprecipitation

In vitro γ-secretase assays using each gradient fraction and quantitation of Aβ by two-site ELISA were performed as previously described, with some modifications (6, 9, 36). Purified recombinant C100-Flag/Myc/His (1 μM) was incubated with each fraction in 1 × γ buffer [HEPES buffer containing 5 mM EDTA, 5 mM 1,10-phenanthroline, 10 μg/ml phosphoramidon, 0.1% phosphatidylcholine (Avanti), Complete protease inhibitor cocktail] adjusted to 0.25% CHAPSO at 4 or 37°C for 6 h. The reaction was stopped by boiling for 2 min. The samples were centrifuged, and the supernatants were analyzed by two-site ELISA. For coimmunoprecipitation, fraction 3, representing the DRM, and fraction 9, representing the detergent-soluble fraction, were precleared with protein G Sepharose for 2 h at 4°C, incubated with anti-G1L3 or control rabbit IgG overnight, followed by reaction with protein G Sepharose for 3 h at 4°C. After washing 3 times with buffer R containing 1% CHAPSO, immunoprecipitates were eluted in 1 × SDS-PAGE sample buffer, and were then analyzed by immunoblotting.

Cholesterol content analysis of DRM fractions

Lipids were extracted from each fraction by 2 vols of chloroform-methanol (2:1, v/v). The upper aqueous phase was removed,

and the resulting lower chloroform phase was dried under nitrogen gas. Lipids were redissolved in the eluent of an HPLC. Free cholesterol was measured with an HPLC equipped with a UV detector at 210 nm as previously described (37). An LC-8 column (4.6 × 250 mm, 5 μm particle size, Supelco, Tokyo) and isopropyl alcohol-acetonitrile (70:30, v/v) as an eluent were used at a flow rate of 1.0 ml/min. Protein concentrations were measured by BCA protein assay kit (Pierce).

RESULTS

Endogenous γ-secretase complex is associated with DRM in active forms

To confirm that endogenous γ-secretase complex is present in lipid rafts in cultured cells, we used SH-SY5Y cells, a human neuroblastoma cell line, to isolate DRM by a discontinuous sucrose density gradient method using 2% CHAPSO as the nonionic detergent. Under these experimental conditions, the use of Triton X-100 is inappropriate, because it results in the inactivation of γ-secretase activity (38). CHAPSO has been previously used with efficiency similar to that of Triton X-100 (39) for the preparation of DRM enriched in presenilin without affecting γ-secretase activity (24). SH-SY5Y cells were cultured in complete medium containing 10% fetal bovine serum, and the 2% CHAPSO lysate was subjected to sucrose density gradient centrifugation.

Flotillin-1, a raft-associated protein in neuronal cells, was recovered in the low density DRM fractions (**Fig. 1A**, fractions 2–4), whereas calnexin and PDI, both ER markers, and EEA-1, an early endosomal marker, were recovered in the CHAPSO-soluble fractions (Fig. 1A, fractions 9 and 10, and data not shown). As has been reported by others, presenilin 1 NTF, presenilin 1 CTF, and presenilin 2 CTF were observed in fractions 2–4, the same fractions as flotillin-1 (Fig. 1A) (24, 25, 29). In addition, the other essential components of the γ-secretase complex, i.e., NCT, APH-1aL, and PEN-2 were also found in the DRM fractions. In accordance with a previous observation (32), the

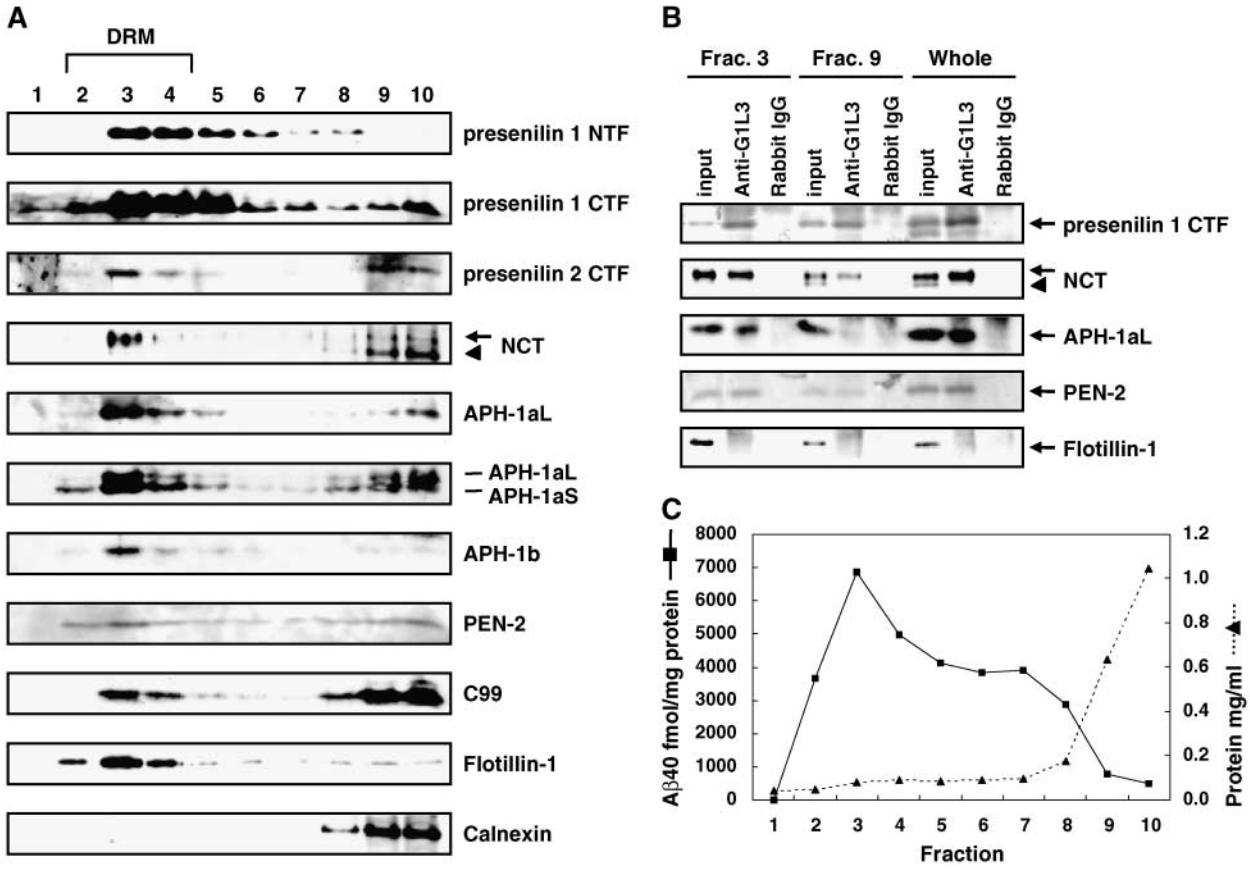

Fig. 1. The active form of γ-secretase complex is associated with detergent-resistant membranes (DRMs). A: SH-SY5Y cells were lysed in buffer R containing 2% 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO), and the cell lysate was fractionated by a discontinuous sucrose density gradient as described in Materials and Methods. Fractions were subjected to SDS-PAGE and immunoblotting with antibodies against presenilin 1 N-terminal fragment (NTF), presenilin 1 C-terminal fragment (CTF), presenilin 2 CTF, nicastrin (NCT), APH-1aL, 1aL/S, 1b, PEN-2, C99, flotillin-1, and calnexin. The arrow indicates the mature form of NCT, and the arrowhead indicates the immature form of NCT. The C99 panel shows the anti-C99 immunoblot of fractions from the cells pretreated with a γ-secretase inhibitor, L-685,458. B: Coimmunoprecipitation of presenilin 1 CTF, NCT, APH-1aL, and PEN-2 from DRM. Fraction 3 (Frac. 3), fraction 9 (Frac. 9), and whole-cell lysate were subjected to immunoprecipitation with anti-G1L3 (anti-presenilin 1 CTF) or rabbit IgG. Immunoprecipitates were analyzed by SDS-PAGE and immunoblotting using antibodies against presenilin 1 CTF, NCT, APH-1aL, PEN-2, and flotillin-1. C: In vitro γ-secretase assay from each fraction. Levels of Aβ40, generated from the recombinant C100 by in vitro coincubation with each fraction, were evaluated by two-site ELISA.

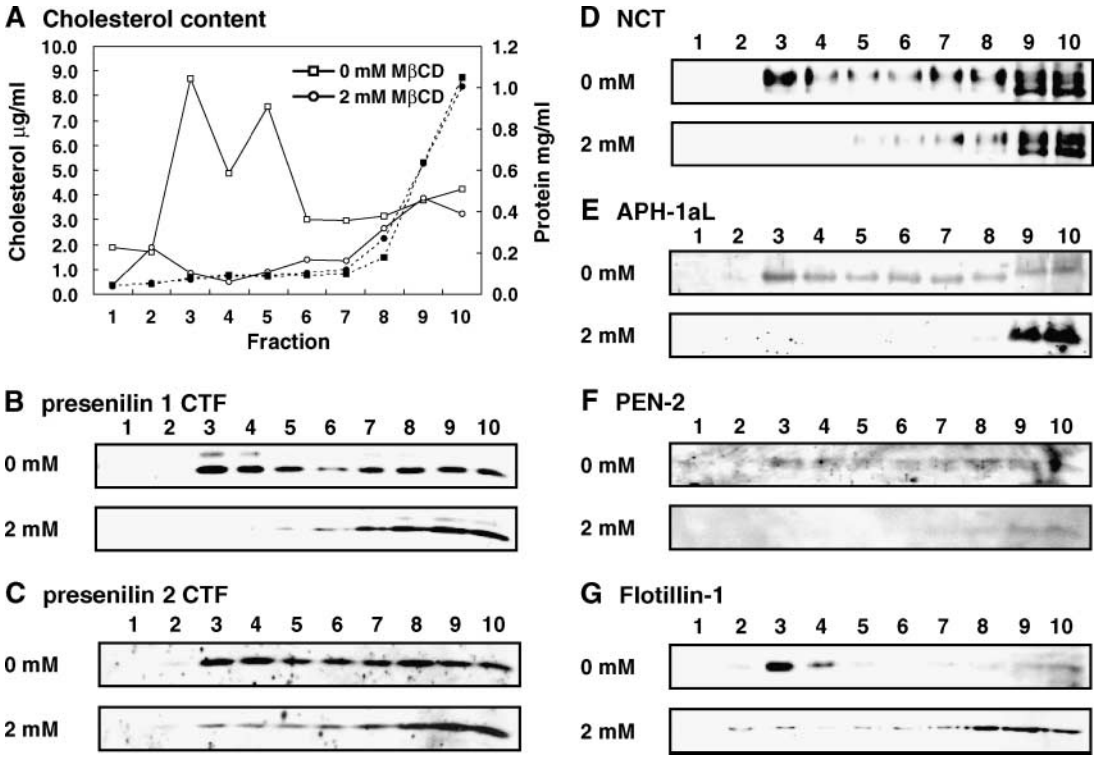

Fig. 2. The γ-secretase complex shifts from DRM to the high density fraction upon treatment with MβCD. SH-SY5Y cells were incubated with (square) or without (circle) 2 mM MβCD in serum-free media for 30 min at 37°C, and then DRMs were prepared as described in Materials and Methods. Fractions were subjected to measurements of the cholesterol content (open symbol) and the protein concentration (closed symbol) (A), and were immunoblotted with anti-presenilin 1 CTF (B), anti-presenilin 2 CTF (C), anti-NCT (D), anti-APH-1aL (E), anti-PEN-2 (F), and anti-flotillin-1 (G).

majority of NCT observed in DRM was in the mature form (Fig. 1A). A recent report (40) indicated that two APH-1a splice forms, APH-1aL and APH-1aS, and APH-1b exist in mammalian cells, and each APH-1 variant is a constituent of six distinct active γ-secretase complexes that contains either presenilin 1 or presenilin 2 as the catalytic subunit. We therefore examined the localization of APH-1aS and APH-1b in addition to APH-1aL. In SH-SY5Y cells, APH-1aS and APH-1b were also observed in DRM as well as APH-1aL (Fig. 1A). To examine the localization of the substrate of γ-secretase, βCTF/C99, which is undetectable under normal conditions, a specific γ-secretase inhibitor (L-685,458) was used. L-685,458 treatment resulted in the accumulation of βCTF/C99 in DRM as well as in the detergent-solu-

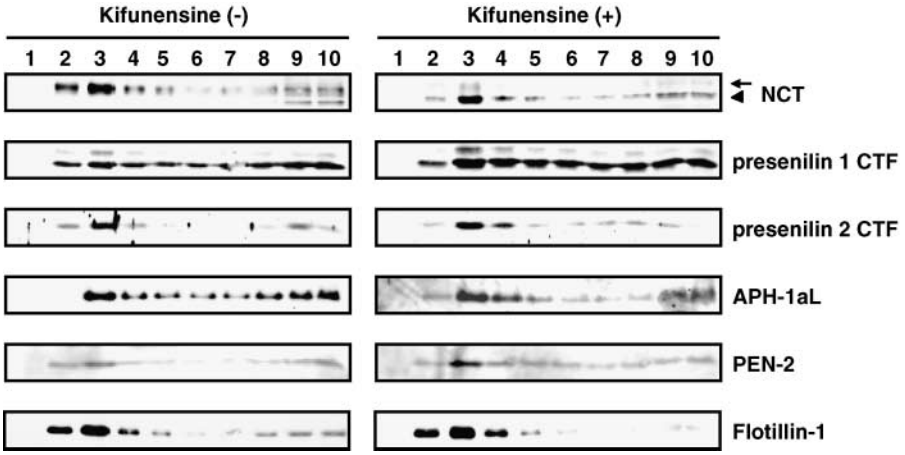

Fig. 3. The distribution of the γ-secretase complex into lipid rafts is not influenced by kifunensine treatment. SH-SY5Y cells were treated in the absence (−) or the presence (+) of 1 μg/ml kifunensine for 48 h at 37°C, and then DRMs were prepared as described in Materials and Methods. Fractions were subjected to SDS-PAGE and immunoblotting with antibodies against presenilin 1 CTF, presenilin 2 CTF, NCT, APH-1aL, PEN-2, and flotillin-1. The arrow indicates the mature form of NCT, and the arrowhead indicates the immature form of NCT.

ble fraction (Fig. 1A). There was no change in the composition of the γ-secretase complex in DRM with this treatment (data not shown).

We next investigated whether these γ-secretase components retained γ-secretase activity in DRM. Coimmunoprecipitation analysis with an antibody against presenilin 1 CTF showed that mature NCT, APH-1aL, and PEN-2 were coimmunoprecipitated with presenilin 1 from DRM (fraction 3), but flotillin-1 was not (Fig. 1B). Using recombinant C100 as a substrate, we assessed γ-secretase activity in all fractions, and the activity was normalized to the protein content. As shown in Fig. 1C, a high specific activity of γ-secretase was detected in DRM, consistent with a previous report (24). Small amounts of residual γ-secretase activity were observed in the middle fractions 5–8 from our DRM preparation (Fig. 1C). This activity is probably attributable to the small amounts of the γ-secretase complex observed in fractions 5–8 after DRM preparation, as confirmed by immunoblotting (Fig.1A). These results indicate that the four essential components form the active γ-secretase complex in DRM.

Cholesterol is an essential component of lipid rafts, and depletion of cholesterol by MβCD, a cholesterol-sequestering reagent, is commonly used to disrupt lipid rafts, resulting in lipid raft proteins becoming solubilized upon detergent treatment (27). Therefore, we treated SH-SY5Y cells with 2 mM MβCD for 30 min. This treatment caused the reduction of cholesterol in DRM (**Fig. 2A**), and resulted in the partial solubilization of flotillin-1 (Fig. 2G). Under these conditions, presenilin 1 CTF, presenilin 2 CTF, mature NCT, APH-1aL, and PEN-2 shifted from DRM to fractions 9 and 10 (Fig. 2B–F). These results demonstrate that

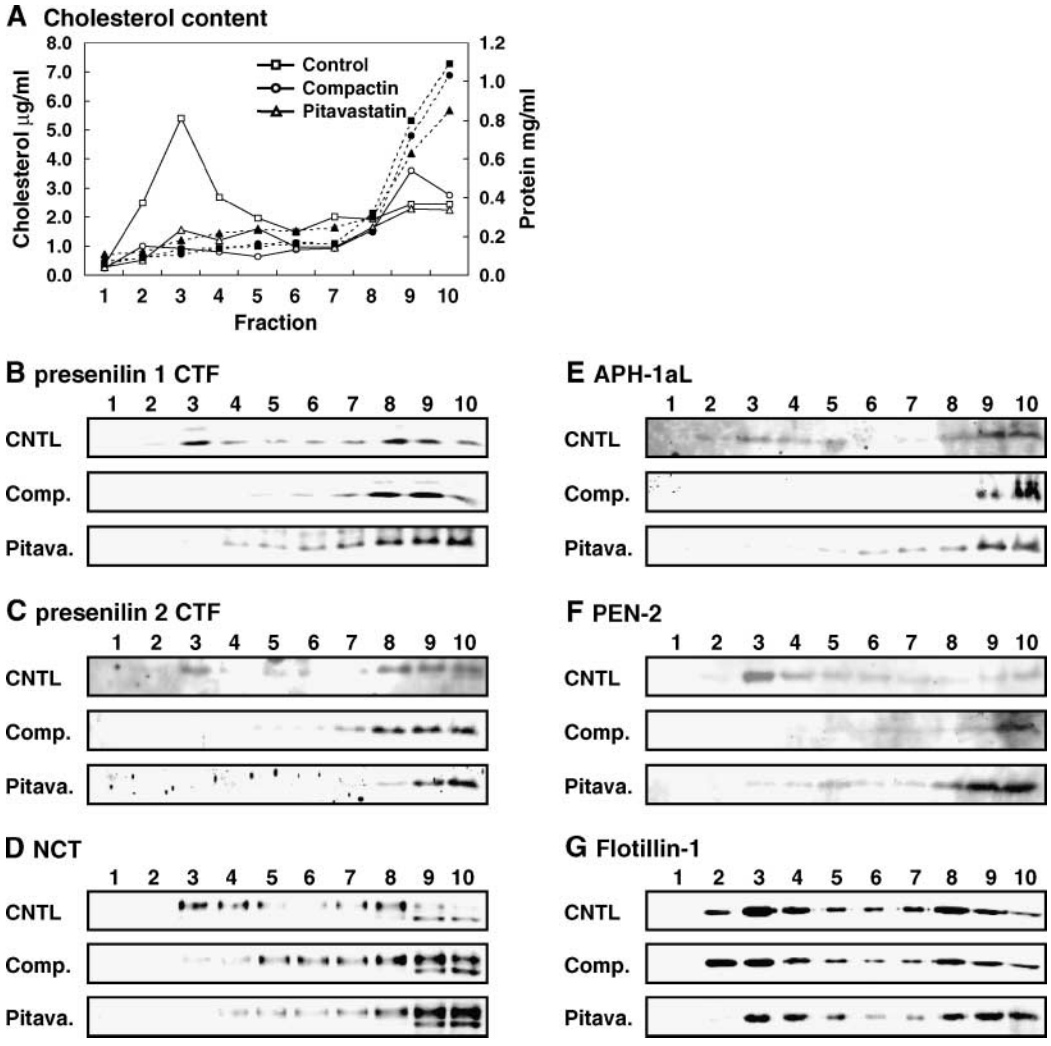

Fig. 4. Treatment with statin results in the distributional change of the γ-secretase complex location from DRM to the detergent-soluble fraction. SH-SY5Y cells were cultured in medium supplemented with 5% lipoprotein-deficient serum (LPDS) and 250 μM sodium mevalonate in the absence (control, square) or the presence of 50 μM compactin (circle) or 5 μM pitavastatin (triangle). After a 48 h incubation at 37°C, the cells were collected, and then DRMs were prepared as described in Materials and Methods. Fractions were subjected to measurements of the cholesterol content (open symbol) and protein concentration (filled symbol) (A), and were immunoblotted with anti-presenilin 1 CTF (B), anti-presenilin 2 CTF (C), anti-NCT (D), anti-APH-1aL (E), anti-PEN-2 (F), and anti-flotillin-1 (G).

the γ-secretase complex is localized in the cholesterol-rich lipid microdomain.

The maturation of NCT is associated with γ-secretase complex assembly (33, 41–43) and can be blocked by kifunensine, a mannosidase I inhibitor (33, 43). In the presence of kifunensine, immature-like NCT was increased in SH-SY5Y cells, and was also observed in DRM (**Fig. 3**). In addition, the distributions of presenilin 1, presenilin 2, APH-1aL, and PEN-2 were unchanged upon kifunensine treatment.

We analyzed the association of NCT, APH-1aL, and PEN-2 with lipid rafts in presenilin $1^{-/-}$/presenilin $2^{-/-}$ mouse embryonic fibroblasts (33, 44–46). When 2% CHAPSO lysates from wild-type or presenilin 1/presenilin 2-deficient cells were subjected to sucrose density gradient centrifugation, flotillin-1 and all four γ-secretase components were recovered in DRM in wild-type cells (data not shown), similar to the distribution in SH-SY5Y cells, whereas in presenilin 1/presenilin 2-deficient cells, only APH-1aL was recovered in DRM (data not shown). Thus, the localization of NCT and PEN-2, but not APH-1aL, to lipid rafts depends on presenilin expression.

γ-Secretase components shifted from DRM to nonraft by statin treatment

We next tested the effects of statin treatment, a 3-hydroxy-3-methylglutaryl-CoA-reductase inhibitor, on the distribution of the γ-secretase complex. Cells were cultured in medium supplemented with LPDS to reduce intracellular cholesterol stores, and sodium mevalonate (250 μM) in the absence or presence of statins. It has been previously reported that under these conditions, statin treatment prevents the production of Aβ in cultured cells (16, 18, 19, 21). SH-SY5Y cells were treated with compactin, a widely used statin, or pitavastatin (47), a novel and robust statin, for 48 h, followed by DRM isolation. Under control conditions, i.e., in medium supplemented with LPDS and mevalonate in the absence of statins, a significant amount of cholesterol was found in DRM (**Fig. 4A**). In contrast, statin treatment reduced the cholesterol content in DRM (Fig. 4A). In the results of immunoblotting, the distribution of flotillin-1 did not dramatically change upon treatment with statins (Fig. 4G). In regard to the γ-secretase complex, the shifts of presenilin 1 CTF, presenilin 2 CTF, mature NCT, APH-1aL, and PEN-2 from DRM to the detergent-soluble fraction were observed upon statin treatment (Fig. 4B–F). Statin treatment did not affect the expression level of the γ-secretase complex or the maturation of NCT. In addition to their cholesterol-lowering effects, statins have pleiotropic effects attributed to inhibition of protein isoprenylation (48). To define the effects of statins on the distributional change of the γ-secretase complex resulting from the blockage of isoprenylation, cells were treated with or without geranylgeraniol. Geranylgeraniol can serve as the major substrate for isoprenylation in the presence of a statin, and is more cell-permeable than geranylgeranylpyrophosphate (49). Addition of geranylgeraniol partially abrogated a shift of presenilin 1 CTF from DRM to detergent-soluble fraction upon statin treatment (**Fig. 5**).

DISCUSSION

Several epidemiological studies have suggested that cholesterol lowering by statin treatment is beneficial for some AD patients (11–13). In vitro and in vivo studies have further demonstrated that Aβ generation is decreased by treatment with these lipid-lowering drugs (16–19). These observations led us to study the relationship between the γ-secretase complex and lipid rafts. As has been demonstrated in several reports, the four essential components of the γ-secretase complex affect each other's stability, localization, and maturation, and the relative abundance of each component is rate limiting for the assembly of the active enzyme (5). Therefore, exogenously expressed proteins may not adequately represent the physiological state of γ-secretase in the membrane. In this study, we evaluated endogenously expressed proteins in the neuroblastoma cell line SH-SY5Y and in nonneuronal mouse embryonic fibroblasts. When the cells were lysed with CHAPSO and examined by sucrose density gradient ultracentrifugation, active presenilin forms (presenilin CTF and presenilin NTF) and mature NCT were recovered in DRM as well as in the CHAPSO-soluble fractions, consistent with previous reports (24, 25, 29, 32). In addition, we now report that each of the endogenous APH-1 variants and endogenous PEN-2 are also located in the same DRM fractions as the presenilin fragments. In vitro γ-secretase assay and coimmunoprecipitation results together showed that essential γ-secretase components in lipid rafts formed the active γ-secretase complex. MβCD

Fig. 5. Geranylgeraniol partially abrogates the distributional change of presenilin 1 location in pitavastatin-treated cells. SH-SY5Y cells were cultured in medium supplemented with 5% LPDS and 10 μM pitavastatin in the absence or presence of 10 μM geranylgeraniol. After a 48 h incubation at 37°C, the cells were collected, and then DRMs were prepared as described in Materials and Methods. Fractions were subjected to SDS-PAGE and immunoblotting with antibodies against presenilin 1 CTF and flotillin-1.

pretreatment resulted in the shifts of γ-secretase components from DRM to the soluble fraction, confirming that the active γ-secretase complex localizes in cholesterol-rich lipid rafts.

The majority of NCT observed in lipid rafts was found in the mature form (32). Inhibition of NCT maturation by kifunensine treatment resulted in the incorporation of immature NCT into the γ-secretase complex, and did not influence the distribution of presenilin 1, presenilin 2, APH-1aL, or PEN-2 into lipid rafts. These data are consistent with the observation that kifunensine treatment does not affect Aβ production or the cell surface localization of NCT (33, 43). On the other hand, presenilin 1/presenilin 2 ablation caused the dissociation of NCT from lipid rafts. It has been reported that in the absence of presenilin 1/presenilin 2, NCT and PEN-2 are retained in the endoplasmic reticulum (33, 44–46). Taken together, these results suggest that the maturation of NCT is not essential for the targeting of the γ-secretase complex to lipid rafts, but the localization of NCT and PEN-2 in lipid rafts is dependent on presenilin expression. Under the same conditions, a significant amount of APH-1aL was associated with lipid rafts in the absence of presenilin 1/presenilin 2. Therefore, there may be a presenilin-independent trafficking pathway for APH-1 to lipid rafts, suggesting that APH-1 may play an important role in the raft association of the γ-secretase complex.

Inhibition of 3-hydroxy-3-methylglutaryl-CoA-reductase by statin treatment induced a shift in the location of γ-secretase components from lipid rafts to the detergent-soluble fraction. However flotillin-1, a well-characterized raft-resident protein, did not shift upon statin treatment. In addition, flotillin-1 was not found to be coimmunoprecipitated by the anti-presenilin 1 antibody, suggesting that flotillin-1 and γ-secretase may be associated with different rafts. Other possibilities include that they may behave differently in terms of their partitioning into cholesterol-rich membrane microdomains, or that γ-secretase is more rapidly recycled between membranes than is flotillin-1 (27). Recently, it has been reported that flotillin-1 DRM association in Jurkat T cells and U937 cells was resistant to MβCD treatment, suggesting that the different sensitivities to cholesterol are considered to be the most plausible mechanism (50).

In addition to their effects on cellular cholesterol levels, statins are known to exert pleiotropic effects through the blockage of isoprenylation (48). In the presence of pitavastatin in LPDS, addition of geranylgeraniol partially abrogated the shift of presenilin 1 from DRM to detergent-soluble fraction. This observation suggests that protein isoprenylation may also play an important role in the association of the γ-secretase complex with lipid rafts. Wahrle et al. (24) have reported that cholesterol-dependent γ-secretase activity localizes in the buoyant cholesterol-rich membrane microdomain. In a conflicting report, Wada et al. (25) observed that γ-secretase activity in lipid rafts does not depend on cholesterol. The findings presented here suggest that the γ-secretase complex association with lipid rafts is influenced by cholesterol and protein isoprenylation, and may affect the efficiency of contact with its substrate in lipid rafts.

Statin treatment has been reported to reduce the generation of Aβ in vitro (16, 18, 19, 21). In these reports, the cells were treated with medium containing the statin, LPDS as a serum, and a small amount of sodium mevalonate for 48–72 h. Ehehalt et al. (21) suggested that the clustering of the βAPP and β-secretase contained in lipid rafts occurs during endocytosis and that β-cleavage of βAPP is carried out in endosomes. It has been previously shown that cholesterol depletion abrogates the localization of β-secretase in lipid rafts (22). Therefore, the reduction of Aβ generation by statin treatment may depend on the inaccessibility of β-secretase in lipid rafts and/or a decrease in the clustering of the βAPP and β-secretase rafts (16, 18, 20–23). The results presented here demonstrate that the γ-secretase complex and C99 also colocalize in lipid rafts, and that the association of γ-secretase with lipid rafts is affected by statin treatment. Our results suggest that the reduction in Aβ production upon statin treatment may be due in part to the dissociation of the γ-secretase complex from lipid rafts. In the future, it will be important to determine whether statin treatment can induce a similar distributional change of the γ-secretase complex in neuronal cells in vivo.

The association of the active γ-secretase complex with lipid rafts and its relation to cholesterol metabolism shown here will provide new insights for understanding the mechanism of Aβ generation in AD.

The authors are grateful to Takeda Chemical Industries for the kind provision of the Aβ ELISAs, Drs. W. Annaert and B. De Strooper for generously providing mouse embryonic fibroblasts, Dr. A. Endo for the kind offer of compactin, and Dr. K. Boru of Pacific Edit for review of the manuscript. This study was supported in part by Grant-In-Aid for Science Research (B2) number 16310147, by special coordination funds for promoting science and technology, by the 21st Century Center of Excellence Program from the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government, and by the Program for Promotion of Fundamental Studies in Health Sciences of the Pharmaceuticals and Medical Devices Agency, Japan. T.T. is a recipient of postdoctoral fellowships for research abroad from the Japan Society for the Promotion of Science.

REFERENCES

1. Haass, C. 2004. Take five-BACE and the gamma-secretase quartet conduct Alzheimer's amyloid beta-peptide generation. *EMBO J.* **23:** 483–488.
2. Selkoe, D. J. 2002. Deciphering the genesis and fate of amyloid beta-protein yields novel therapies for Alzheimer disease. *J. Clin. Invest.* **110:** 1375–1381.
3. Haass, C., and H. Steiner. 2002. Alzheimer disease gamma-secretase: a complex story of GxGD-type presenilin proteases. *Trends Cell Biol.* **12:** 556–562.
4. Vassar, R. 2001. The beta-secretase, BACE: a prime drug target for Alzheimer's disease. *J. Mol. Neurosci.* **17:** 157–170.

5. De Strooper, B. 2003. Aph-1, Pen-2, and nicastrin with presenilin generate an active gamma-secretase complex. *Neuron.* **38:** 9–12.
6. Takasugi, N., T. Tomita, I. Hayashi, M. Tsuruoka, M. Niimura, Y. Takahashi, G. Thinakaran, and T. Iwatsubo. 2003. The role of presenilin cofactors in the gamma-secretase complex. *Nature.* **422:** 438–441.
7. Edbauer, D., E. Winkler, J. T. Regula, B. Pesold, H. Steiner, and C. Haass. 2003. Reconstitution of gamma-secretase activity. *Nat. Cell Biol.* **5:** 486–488.
8. Kimberly, W. T., M. J. LaVoie, B. L. Ostaszewski, W. Ye, M. S. Wolfe, and D. J. Selkoe. 2003. Gamma-secretase is a membrane protein complex comprised of presenilin, nicastrin, Aph-1, and Pen-2. *Proc. Natl. Acad. Sci. USA.* **100:** 6382–6387.
9. Hayashi, I., Y. Urano, R. Fukuda, N. Isoo, T. Kodama, T. Hamakubo, T. Tomita, and T. Iwatsubo. 2004. Selective reconstitution and recovery of functional gamma-secretase complex on budded baculovirus particles. *J. Biol. Chem.* **279:** 38040–38046.
10. Strittmatter, W. J., A. M. Saunders, D. Schmechel, M. Pericak-Vance, J. Enghild, G. S. Salvesen, and A. D. Roses. 1993. Apolipoprotein E: high-avidity binding to beta-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease. *Proc. Natl. Acad. Sci. USA.* **90:** 1977–1981.
11. Jick, H., G. L. Zornberg, S. S. Jick, S. Seshadri, and D. A. Drachman. 2000. Statins and the risk of dementia. *Lancet.* **356:** 1627–1631.
12. Wolozin, B., W. Kellman, P. Ruosseau, G. G. Celesia, and G. Siegel. 2000. Decreased prevalence of Alzheimer disease associated with 3-hydroxy-3-methyglutaryl coenzyme A reductase inhibitors. *Arch. Neurol.* **57:** 1439–1443.
13. Rockwood, K., S. Kirkland, D. B. Hogan, C. MacKnight, H. Merry, R. Verreault, C. Wolfson, and I. McDowell. 2002. Use of lipid-lowering agents, indication bias, and the risk of dementia in community-dwelling elderly people. *Arch. Neurol.* **59:** 223–227.
14. Sparks, D. L., S. W. Scheff, J. C. Hunsaker III, H. Liu, T. Landers, and D. R. Gross. 1994. Induction of Alzheimer-like beta-amyloid immunoreactivity in the brains of rabbits with dietary cholesterol. *Exp. Neurol.* **126:** 88–94.
15. Refolo, L. M., B. Malester, J. LaFrancois, T. Bryant-Thomas, R. Wang, G. S. Tint, K. Sambamurti, K. Duff, and M. A. Pappolla. 2000. Hypercholesterolemia accelerates the Alzheimer's amyloid pathology in a transgenic mouse model. *Neurobiol. Dis.* **7:** 321–331.
16. Fassbender, K., M. Simons, C. Bergmann, M. Stroick, D. Lutjohann, P. Keller, H. Runz, S. Kuhl, T. Bertsch, K. von Bergmann, M. Hennerici, K. Beyreuther, and T. Hartmann. 2001. Simvastatin strongly reduces levels of Alzheimer's disease beta-amyloid peptides Abeta 42 and Abeta 40 in vitro and in vivo. *Proc. Natl. Acad. Sci. USA.* **98:** 5856–5861.
17. Refolo, L. M., M. A. Pappolla, J. LaFrancois, B. Malester, S. D. Schmidt, T. Thomas-Bryant, G. S. Tint, R. Wang, M. Mercken, S. S. Petanceska, and K. E. Duff. 2001. A cholesterol-lowering drug reduces beta-amyloid pathology in a transgenic mouse model of Alzheimer's disease. *Neurobiol. Dis.* **8:** 890–899.
18. Simons, M., P. Keller, B. De Strooper, K. Beyreuther, C. G. Dotti, and K. Simons. 1998. Cholesterol depletion inhibits the generation of beta-amyloid in hippocampal neurons. *Proc. Natl. Acad. Sci. USA.* **95:** 6460–6464.
19. Kojro, E., G. Gimpl, S. Lammich, W. Marz, and F. Fahrenholz. 2001. Low cholesterol stimulates the nonamyloidogenic pathway by its effect on the alpha-secretase ADAM 10. *Proc. Natl. Acad. Sci. USA.* **98:** 5815–5820.
20. Simons, M., P. Keller, J. Dichgans, and J. B. Schulz. 2001. Cholesterol and Alzheimer's disease: is there a link? *Neurology.* **57:** 1089–1093.
21. Ehehalt, R., P. Keller, C. Haass, C. Thiele, and K. Simons. 2003. Amyloidogenic processing of the Alzheimer beta-amyloid precursor protein depends on lipid rafts. *J. Cell Biol.* **160:** 113–123.
22. Riddell, D. R., G. Christie, I. Hussain, and C. Dingwall. 2001. Compartmentalization of beta-secretase (Asp2) into low-buoyant density, noncaveolar lipid rafts. *Curr. Biol.* **11:** 1288–1293.
23. Cordy, J. M., I. Hussain, C. Dingwall, N. M. Hooper, and A. J. Turner. 2003. Exclusively targeting beta-secretase to lipid rafts by GPI-anchor addition up-regulates beta-site processing of the amyloid precursor protein. *Proc. Natl. Acad. Sci. USA.* **100:** 11735–11740.
24. Wahrle, S., P. Das, A. C. Nyborg, C. McLendon, M. Shoji, T. Kawarabayashi, L. H. Younkin, S. G. Younkin, and T. E. Golde. 2002. Cholesterol-dependent gamma-secretase activity in buoyant cholesterol-rich membrane microdomains. *Neurobiol. Dis.* **9:** 11–23.
25. Wada, S., M. Morishima-Kawashima, Y. Qi, H. Misono, Y. Shimada, Y. Ohno-Iwashita, and Y. Ihara. 2003. Gamma-secretase activity is present in rafts but is not cholesterol-dependent. *Biochemistry.* **42:** 13977–13986.
26. Simons, K., and E. Ikonen. 1997. Functional rafts in cell membranes. *Nature.* **387:** 569–572.
27. Simons, K., and D. Toomre. 2000. Lipid rafts and signal transduction. *Nat. Rev. Mol. Cell Biol.* **1:** 31–39.
28. Ikonen, E. 2001. Roles of lipid rafts in membrane transport. *Curr. Opin. Cell Biol.* **13:** 470–477.
29. Parkin, E. T., I. Hussain, E. H. Karran, A. J. Turner, and N. M. Hooper. 1999. Characterization of detergent-insoluble complexes containing the familial Alzheimer's disease-associated presenilins. *J. Neurochem.* **72:** 1534–1543.
30. Bouillot, C., A. Prochiantz, G. Rougon, and B. Allinquant. 1996. Axonal amyloid precursor protein expressed by neurons in vitro is present in a membrane fraction with caveolae-like properties. *J. Biol. Chem.* **271:** 7640–7644.
31. Lee, S. J., U. Liyanage, P. E. Bickel, W. Xia, P. T. Lansbury, Jr., and K. S. Kosik. 1998. A detergent-insoluble membrane compartment contains A beta in vivo. *Nat. Med.* **4:** 730–734.
32. Murphy, M. P., P. Das, A. C. Nyborg, M. J. Rochette, M. W. Dodson, N. M. Loosbrock, T. M. Souder, C. McLendon, S. L. Merit, S. C. Piper, K. R. Jansen, and T. E. Golde. 2003. Overexpression of nicastrin increases Abeta production. *FASEB J.* **17:** 1138–1140.
33. Herreman, A., G. Van Gassen, M. Bentahir, O. Nyabi, K. Craessaerts, U. Mueller, W. Annaert, and B. De Strooper. 2003. Gamma-secretase activity requires the presenilin-dependent trafficking of nicastrin through the Golgi apparatus but not its complex glycosylation. *J. Cell Sci.* **116:** 1127–1136.
34. Tomita, T., R. Takikawa, A. Koyama, Y. Morohashi, N. Takasugi, T. C. Saido, K. Maruyama, and T. Iwatsubo. 1999. C terminus of presenilin is required for overproduction of amyloidogenic Abeta42 through stabilization and endoproteolysis of presenilin. *J. Neurosci.* **19:** 10627–10634.
35. Tomita, T., S. Tokuhiro, T. Hashimoto, K. Aiba, T. C. Saido, K. Maruyama, and T. Iwatsubo. 1998. Molecular dissection of domains in mutant presenilin 2 that mediate overproduction of amyloidogenic forms of amyloid beta peptides. Inability of truncated forms of PS 2 with familial Alzheimer's disease mutation to increase secretion of Abeta42. *J. Biol. Chem.* **273:** 21153–21160.
36. Takahashi, Y., I. Hayashi, Y. Tominari, K. Rikimaru, Y. Morohashi, T. Kan, H. Natsugari, T. Fukuyama, T. Tomita, and T. Iwatsubo. 2003. Sulindac sulfide is a noncompetitive gamma-secretase inhibitor that preferentially reduces Abeta 42 generation. *J. Biol. Chem.* **278:** 18664–18670.
37. Kritharides, L., W. Jessup, J. Gifford, and R. T. Dean. 1993. A method for defining the stages of low-density lipoprotein oxidation by the separation of cholesterol- and cholesteryl ester-oxidation products using HPLC. *Anal. Biochem.* **213:** 79–89.
38. Li, Y. M., M. T. Lai, M. Xu, Q. Huang, J. DiMuzio-Mower, M. K. Sardana, X. P. Shi, K. C. Yin, J. A. Shafer, and S. J. Gardell. 2000. Presenilin 1 is linked with gamma-secretase activity in the detergent solubilized state. *Proc. Natl. Acad. Sci. USA.* **97:** 6138–6143.
39. Schuck, S., M. Honsho, K. Ekroos, A. Shevchenko, and K. Simons. 2003. Resistance of cell membranes to different detergents. *Proc. Natl. Acad. Sci. USA.* **100:** 5795–5800.
40. Shirotani, K., D. Edbauer, S. Prokop, C. Haass, and H. Steiner. 2004. Identification of distinct gamma-secretase complexes with different APH-1 variants. *J. Biol. Chem.* **279:** 41340–41345.
41. Yang, D. S., A. Tandon, F. Chen, G. Yu, H. Yu, S. Arawaka, H. Hasegawa, M. Duthie, S. D. Schmidt, T. V. Ramabhadran, R. A. Nixon, P. M. Mathews, S. E. Gandy, H. T. Mount, P. St George-Hyslop, and P. E. Fraser. 2002. Mature glycosylation and trafficking of nicastrin modulate its binding to presenilins. *J. Biol. Chem.* **277:** 28135–28142.
42. Kimberly, W. T., M. J. LaVoie, B. L. Ostaszewski, W. Ye, M. S. Wolfe, and D. J. Selkoe. 2002. Complex N-linked glycosylated nicastrin associates with active gamma-secretase and undergoes tight cellular regulation. *J. Biol. Chem.* **277:** 35113–35117.
43. Shirotani, K., D. Edbauer, A. Capell, J. Schmitz, H. Steiner, and C. Haass. 2003. Gamma-secretase activity is associated with a conformational change of nicastrin. *J. Biol. Chem.* **278:** 16474–16477.
44. Leem, J. Y., S. Vijayan, P. Han, D. Cai, M. Machura, K. O. Lopes,

M. L. Veselits, H. Xu, and G. Thinakaran. 2002. Presenilin 1 is required for maturation and cell surface accumulation of nicastrin. *J. Biol. Chem.* **277:** 19236–19240.
45. Bergman, A., E. M. Hansson, S. E. Pursglove, M. R. Farmery, L. Lannfelt, U. Lendahl, J. Lundkvist, and J. Näslund. 2004. Pen-2 is sequestered in the endoplasmic reticulum and subjected to ubiquitylation and proteasome-mediated degradation in the absence of presenilin. *J. Biol. Chem.* **279:** 16744–16753.
46. Steiner, H., E. Winkler, D. Edbauer, S. Prokop, G. Basset, A. Yamasaki, M. Kostka, and C. Haass. 2002. PEN-2 is an integral component of the gamma-secretase complex required for coordinated expression of presenilin and nicastrin. *J. Biol. Chem.* **277:** 39062–39065.
47. Kajinami, K., N. Takekoshi, and Y. Saito. 2003. Pitavastatin: efficacy and safety profiles of a novel synthetic HMG-CoA reductase inhibitor. *Cardiovasc. Drug Rev.* **21:** 199–215.
48. Takemoto, M., and J. K. Liao. 2001. Pleiotropic effects of 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibitors. *Arterioscler. Thromb. Vasc. Biol.* **21:** 1712–1719.
49. Crick, D. C., D. A. Andres, and C. J. Waechter. 1997. Novel salvage pathway utilizing farnesol and geranylgeraniol for protein isoprenylation. *Biochem. Biophys. Res. Commun.* **237:** 483–487.
50. Rajendran, L., M. Masilamani, S. Solomon, R. Tikkanen, C. A. Stuermer, H. Plattner, and H. Illges. 2003. Asymmetric localization of flotillins/reggies in preassembled platforms confers inherent polarity to hematopoietic cells. *Proc. Natl. Acad. Sci. USA.* **100:** 8241–8246.