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Neuronal β-amyloid generation is independent of lipid raft association of β-secretase BACE1: analysis with a palmitoylation-deficient mutant

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Abstract

β-Secretase, BACE1 is a neuron-specific membrane-associated protease that cleaves amyloid precursor protein (APP) to generate β-amyloid protein (Aβ). BACE1 is partially localized in lipid rafts. We investigated whether lipid raft localization of BACE1 affects Aβ production in neurons using a palmitoylation-deficient mutant and further analyzed the relationship between palmitoylation of BACE1 and its shedding and dimerization. We initially confirmed that BACE1 is mainly palmitoylated at four C-terminal cysteine residues in stably transfected neuroblastoma cells. We found that raft localization of mutant BACE1 lacking the palmitoylation modification was markedly reduced in comparison to wild-type BACE1 in neuroblastoma cells as well as rat primary cortical neurons expressing BACE1 via recombinant adenoviruses. In primary neurons, expression of wild-type and mutant BACE1 enhanced production of Aβ from endogenous or overexpressed APP to similar extents with the β-C-terminal fragment (β-CTF) of APP mainly distributed in nonraft fractions. Similarly, β-CTF was recovered mainly in nonraft fractions of neurons expressing Swedish mutant APP only. These results show that raft association of BACE1 does not influence β-cleavage of APP and Aβ production in neurons, and support the view that BACE1 cleaves APP mainly in nonraft domains. Thus, we propose a model of neuronal Aβ generation involving mobilization of β-CTF from nonraft to raft domains. Additionally, we obtained data indicating that palmitoylation plays a role in BACE1 shedding but not dimerization.

Introduction

Cerebral accumulation of β-amyloid protein (Aβ) is a specific neuropathological hallmark of Alzheimer's disease (AD) and is considered central to AD pathogenesis (Hardy and Selkoe 2002). Aβ is a hydrophobic peptide composed of ~40–43 amino acids derived from proteolytic processing of amyloid precursor protein (APP). In amyloidogenic processing, APP is initially cleaved by the β-secretase, BACE1, and the resultant β-C-terminal fragment (β-CTF) subsequently cleaved by γ-secretase complexes to generate Aβ (De Strooper 2010). BACE1 is a membrane-bound protease abundantly expressed in neurons in the brain (Vassar et al. 1999) that undergoes several posttranslational modifications including glycosylation, phosphorylation, and palmitoylation (Citron 2004; Stockley and O'Neill 2008). BACE1 deletion abolishes Aβ production in mice without overt abnormalities and the protein is implicated in AD pathogenesis, making it an important therapeutic target for AD (Citron 2004; Ohno 2008; Stockley and O'Neill 2008).

Lipid rafts are distinct membrane domains characterized by high concentrations of cholesterol and glycosphingolipids (Laude and Prior 2004). Recent studies have identified lipid rafts as important sites for the generation and accumulation of Aβ (Cordy et al. 2006; Araki 2010; Rushworth and Hooper 2010; Vetrivel and Thinakaran 2010). BACE1 and

γ-secretase complexes are partially and mainly localized in lipid rafts, respectively (Riddle et al. 2001; Wahrle et al. 2002; Cordy et al. 2003; Ehehalt et al. 2003; Vetrivel et al. 2004; Urano et al. 2005; Hur et al. 2008). Previous reports indicate that association of BACE1 with lipid rafts promotes Aβ production, supporting the importance of this process in Aβ generation. S-Palmitoylation of membrane proteins plays important functional roles in protein–protein interactions, folding, trafficking, and association with lipid rafts (Charollais and Van der Goot 2009). Vetrivel et al. (2009) showed that BACE1 is S-palmitoylated at four C-terminal cysteine residues, and its lipid raft localization is regulated by palmitoylation but has no direct impact on Aβ production in murine cell lines. Owing to these controversial findings, the issue of whether lipid raft association of BACE1 plays an important role in Aβ production in neurons remains unclear at present.

Mature BACE1 is partly cleaved in the extracellular domain to generate soluble BACE1 that is released extracellularly (Benjannet et al. 2001; Hussain et al. 2003; Murayama et al. 2005). However, the physiological significance of this BACE1 shedding remains to be established. In addition, BACE1 appears to exist as a homodimer in the native state (Westmeyer et al. 2004; Schmechel et al. 2004). We are yet to determine whether BACE1 shedding and dimerization are affected by its palmitoylation.

In this study, we sought to clarify whether lipid raft localization of BACE1 affects Aβ production in neurons. For this purpose, we employed human neuroblastoma cells stably expressing wild-type or mutant BACE1 lacking the palmitoylation modification, as well as rat primary cortical neurons expressing these forms of BACE1 via recombinant adenoviruses. Our findings provide evidence that BACE1 cleavage of APP and Aβ generation is independent of lipid raft association of the protease in neurons and suggest that BACE1 cleaves APP mainly in nonraft domains. Data from further analyses indicate that palmitoylation plays a role in BACE1 shedding but not dimerization.

Materials and Methods

Antibodies

Four BACE1 antibodies were used: rabbit polyclonal antibody (NBA) raised against the N-terminal part of BACE1 (Murayama et al. 2005), mouse monoclonal anti-BACE1 ectodomain antibody (MAB9311, R&D Systems, Minneapolis, MN), and rabbit polyclonal anti-BACE1 C-terminal antibodies (M-83, Santa Cruz Biotechnology, Santa Cruz, CA; AB5832, Millipore, Billerica, MA). Two rabbit polyclonal antibodies against the C-terminus of APP, designated AC24 (Estus et al. 1992) and R37 (Kametani et al. 1993), were employed. Mouse monoclonal 1D4 antibody to the rhodopsin tag (VSKTETSQVAPA) (Hodges et al. 1988) was obtained from University of British Columbia. Rabbit polyclonal antibodies against flotillin-1 were purchased from IBL (Gunma, Japan) and Sigma-Aldrich (St Louis, MO). Mouse monoclonal anti-β-actin antibody was acquired from Sigma. Horseradish peroxidase-conjugated secondary antibodies against mouse or rabbit IgG were purchased from GE Healthcare (Piscataway, NJ).

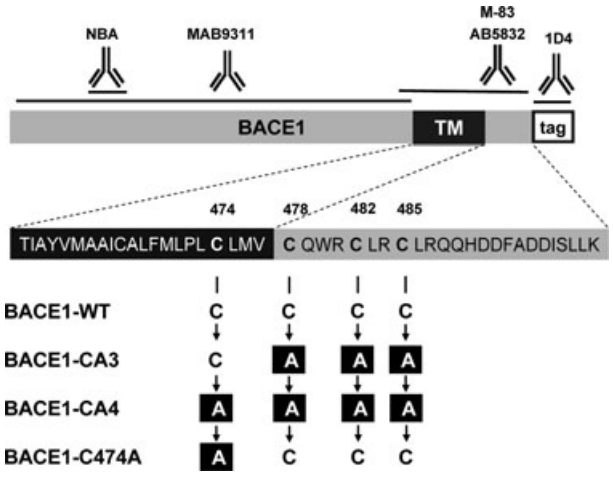

Figure 1. Schematic illustration of BACE1 protein. BACE1 is palmitoylated at four cysteine residues (Cys474, 478, 482, and 485) in the transmembrane (TM) and C-terminal cytoplasmic domains. BACE1 mutants with three, four, and one Ala substitutions are designated as BACE1-CA3, BACE1-CA4, and BACE1-C474A, respectively. The locations of the epitopes for antibodies used in this study are also depicted.

cDNA constructs

Human BACE1 cDNA fused with a C-terminal rhodopsin tag was subcloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA). This plasmid was generously provided by Dr. Michael Farzan (Farzan et al. 2000). Mutant BACE1 cDNAs (BACE1-CA3, BACE1-CA4, and BACE1-C474A with three, four, and one Cys-Ala substitutions, respectively) were generated (Fig. 1) using the GeneEditor^{TM} in vitro mutagenesis system (Promega, Madison, WI), according to the manufacturer's instructions. The resultant cDNAs were verified by sequencing.

Establishment of neuroblastoma cells stably expressing BACE1

Human neuroblastoma SH-SY5Y cells were maintained in a humidified atmosphere of 5% CO_2/95% air in Dulbecco's modified Eagle's medium (DMEM) mixed at a 1:1 ratio with Ham's F-12 medium supplemented with 10% fetal bovine serum. Wild-type or mutant BACE1 cDNA was transfected into human neuroblastoma SH-SY5Y cells and stable transformants selected with 400 μg/mL G418, as described previously (Takeda et al. 2004; Murayama et al. 2006).

Primary neuronal cultures

Primary neuronal cultures were prepared as described earlier (Brewer et al. 1993; Araki et al. 2001), with minor modifications. Cerebral cortices were removed from rat embryos at embryonic day 17, in Hank's Balanced Salt Solution (without Ca^{2+} and Mg^{2+}) and the meninges discarded. Cortical tissues were minced with surgical blades and dissociated via trituration using fire-polished Pasteur pipettes. Cells were plated on a poly-L-lysine-coated 6-well plate or 6-cm dish in Neurobasal medium containing B27 supplements (BD Biosciences, San Jose, CA).

Generation of recombinant adenoviruses

Recombinant adenoviruses were prepared using an Adenovirus Dual Expression Vector Kit (Takara Bio, Shiga, Japan). Briefly, BACE1-WT, BACE1-CA4, and human wild-type and Swedish mutant APP695 (Takeda et al. 2004) cDNA fragments were blunted and inserted into the SwaI site of the cosmid vector pAxCAwtit2 to generate pAxCAwtit2-BACE1-WT, pAxCAwtit2-BACE1-CA4, pAxCAwtit2-APP, and pAxCAwtit2-swAPP, respectively. After digesting with BspT107I, recombinant cosmids were transfected into HEK293 cells using Lipofectamine 2000 (Invitrogen). Recombinant adenoviruses were screened and propagated according to the manufacturer's instructions.

^{3}H-Palmitic acid labeling

^{3}H-palmitic acid (PA) labeling was performed essentially using a previously described method (Magee et al. 1995). In brief, cells plated on 6-cm dish were labeled with 0.2 mCi ^{3}H-PA for 5 h, and collected with a cell scraper. Cells were lysed with RIPA (radioimmunoprecipitation assay) buffer, and extracts immunoprecipitated with 1D4 antibody and protein G-agarose. Immunoprecipitates were treated with Laemmli sample buffer, followed by SDS-PAGE. Gels were soaked in DMSO for 30 min twice and 20% (w/w) diphenyloxazole in DMSO for 3 h, and rinsed with water for 1 h. After drying, gels were analyzed using fluorography.

Immunoblot analysis

Immunoblot analyses were performed as described previously (Takeda et al. 2004; Murayama et al. 2006). Cells were lysed in RIPA buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitors. Proteins were separated on 8% polyacrylamide gels and blotted onto polyvinylidene difluoride membranes. Blots were blocked in phosphate-buffered saline (PBS) containing 0.05% Tween-20 and 5% nonfat-dried milk, and probed with the appropriate antibodies, followed by secondary horseradish peroxidase-conjugated anti-rabbit or mouse IgG. Signals were detected with enhanced chemiluminescence reagents (Perkin-Elmer, Boston, MA), and the resulting images analyzed with a LAS-1000 (Fuji Film, Tokyo, Japan) image analyzer.

Lipid raft isolation

Sucrose density gradient ultracentrifugation was performed as described previously (Yoon et al. 2007; Oda et al. 2010). Briefly, cell pellets were disrupted by 10 strokes through a 25-G needle in TNE buffer (25 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA) containing protease inhibitors. An equal volume of 2% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate) in TNE buffer was mixed and incubated on ice for 30 min. Cell extracts were mixed with TNE buffer containing sucrose to yield a final concentration of 45% (w/v) sucrose, and the mixture adds to the bottom of an ultracentrifuge tube. TNE buffers containing 35% and 5% sucrose were successively and carefully layered over CHAPS cell extracts. Samples were spun at 4°C for 14–16 h at 190,000 *g* in the SW60 rotor (Beckman, Fullerton, CA). In total, 10 fractions were collected in 0.285 mL volumes from the top to bottom. Equal volumes of each fraction were subjected to SDS-PAGE and immunoblotting.

Aβ measurement

Primary neurons were cultured on a 6-well plate for 7 days and infected with recombinant adenoviruses at a multiplicity of infection of ~10. One day after infection, the whole medium was changed, and the amounts of Aβ40 and Aβ42 in 24 h-conditioned media measured using sandwich ELISA kits (Wako, Osaka, Japan) (Suzuki et al. 1994; Araki et al. 2001). Briefly, samples and Aβ standard solutions were applied to 96-well plates coated with BNT77 overnight at 4°C, and incubated with horseradish peroxidase-conjugated BA27 or BC05 for 2 h at room temperature. Bound enzyme activity was measured using the TMB microwell peroxidase substrate system (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

Immunocytochemistry

Primary neurons cultured on cover slips were fixed with 4% paraformaldehyde in PBS. Fixed cells were permeabilized and blocked with 0.3% Triton X-100 and 1% FBS in PBS, and incubated with 1D4 antibody for 1 h, followed by DyLight649-conjugated anti-mouse IgG (Jackson Immuno-Research Laboratories, Bar Harbor, ME) for 1 h. For double immunolabeling, cells were subsequently stained with anti-flotillin1 antibody (Sigma, St. Louis, MO, USA) and Alexa488-conjugated anti-rabbit IgG (Invitrogen). Specimens were examined with a Leica TCS SP2 MP confocal microscope system (Leica Microsystems, Heidelberg, Germany).

Immunoprecipitation

Soluble-BACE1

SH-SY5Y cells expressing BACE1 were cultured on 6-cm dishes and grown overnight in serum-free DMEM/F12 containing N2 supplements (BD Biosciences). Conditioned media were harvested, mixed with NP-40 (0.1%), Tris, pH 8 (10 mM), NaCl (150 mM), and protease inhibitors, and incubated overnight at 4°C with anti-BACE1 ectodomain antibody (MAB9311) and protein G-agarose (Murayama et al. 2005). Immunoprecipitated materials were subjected to immunoblot analysis with BACE1 N-terminal (NBA) or C-terminal (M-83) antibodies.

APP CTF

Fractions from lipid raft isolation experiments were diluted 10 times with TNE buffer and used for immunoprecipitation with anti-APP antibodies (AC24). Immunoprecipitated materials were subjected to Tris/Tricine SDS-PAGE and immunoblot analysis with anti-APP (R37).

Blue native polyacrylamide gel electrophoresis

Blue native-PAGE (BN-PAGE) was performed as described previously (Schägger and von Jagow 1991). Membrane and cytosolic fractions of SH-SY5Y cells expressing BACE1 were separated using a previously described method (Murayama et al. 2006). The extracts were applied onto BN-PAGE (4–16%), and transferred onto PVDF membranes. Blots were destained for 1 h in distilled water/methanol/acetic acid (60%/30%/10%) and subjected to immunoblotting with 1D4 antibodies.

Statistical analysis

All results are presented as means ± SEM. Statistical analyses were performed with Student's *t*-test or one-way analysis of variance (ANOVA), followed by Bonferroni multiple comparison test with a threshold of $P < 0.05$.

Results

BACE1 palmitoylation occurs mainly at four cysteine residues in the C-terminal region

We investigated S-palmitoylation of BACE1 using human neuroblastoma cells stably expressing BACE1. cDNA of wild-type BACE1 (BACE1-WT) or mutant BACE1 (BACE1-CA3 or BACE1-CA4 with three or four Cys–Ala substitutions, respectively) (Fig. 1) was transfected into SH-SY5Y cells and stable transfectants (designated SH-BACE1-WT, SH-BACE1-CA3, and SH-BACE1-CA4 cells) were established. Western blot analysis revealed that all transfected cells expressed equal levels of BACE1 (Fig. 2a). Next, we evaluated palmitoylation of BACE1 using ^{3}H-PA labeling. As shown in Figure 2b, the ^{3}H-PA-labeled BACE1 level was reduced to 23% and 2% in BACE1-CA3- and BACE1-CA4-expressing cells, respectively, compared to BACE1-WT cells. These findings indicate that palmitoylation of BACE1 is abolished in the BACE1-CA4 mutant, confirming that BACE1 is mainly modified at the four cysteine residues in the C-terminal region.

Lipid raft distribution of BACE1 depends on palmitoylation in neuroblastoma cells

Next, we evaluated the role of palmitoylation in lipid raft distribution of BACE1 in SH-SY5Y cells via sucrose density gradient centrifugation. Immunoblot analysis of individual fractions showed that the raft marker, flotillin-1, was present in fraction 4, indicative of the fractionation of lipid raft components. Although a proportion of BACE1 was recovered in fraction 4, the majority of the protein was present in high-density membrane fractions (fractions 7–10) (Fig. 3a). The percentages of BACE1 distributed in the raft fraction per total BACE1 in all fractions for BACE1-WT, BACE1-CA3, and BACE1-CA4 cells were 15.6%, 11.4%, and 5.8%, respectively (Fig. 3a). These results indicate that lipid raft distribution of BACE1 depends on its palmitoylation in SH-SY5Y cells.

Since the distribution patterns of BACE1 in SH-BACE1-CA3 and SH-BACE1-CA4 cells were markedly different, we examined the effect of palmitoylation at cysteine 474 alone. For this purpose, we established SH-SY5Y cells stably expressing BACE1-C474A (designated SH-BACE1-C474A cells). No significant differences were observed in the percentage of BACE1 within the raft fraction between SH-BACE1-WT and SH-BACE1-C474A cells (Fig. 3b), suggesting that palmitoylation specifically at cysteine 474 does not have a major impact on lipid raft targeting of BACE1.

Lipid raft association of BACE1 depends on palmitoylation in primary cortical neurons

We further evaluated lipid raft localization of BACE1 in rat primary cortical neurons infected with recombinant adenoviruses expressing BACE1-WT or BACE1-CA4. Upon sucrose density gradient fractionation, lipid rafts were recovered mainly in fraction 4, where flotillin-1 was predominantly present. A proportion of BACE1 was recovered in fraction 4, while the majority was present in high-density nonraft fractions (fraction 8–10) (Fig. 4a). The percentage of BACE1 in the raft fraction was 14% and 3% in neurons expressing BACE1-WT and BACE1-CA4, respectively (Fig. 4b), confirming that lipid raft association of BACE1 is dependent on its palmitoylation in primary neurons as well.

A proportion of APP was detected in fraction 4, with the majority recovered from nonraft fractions. The percentages of APP in the raft fraction were similar for SH-BACE1-WT- and SH-BACE1-CA4-expressing cells (Fig. 4c), suggesting

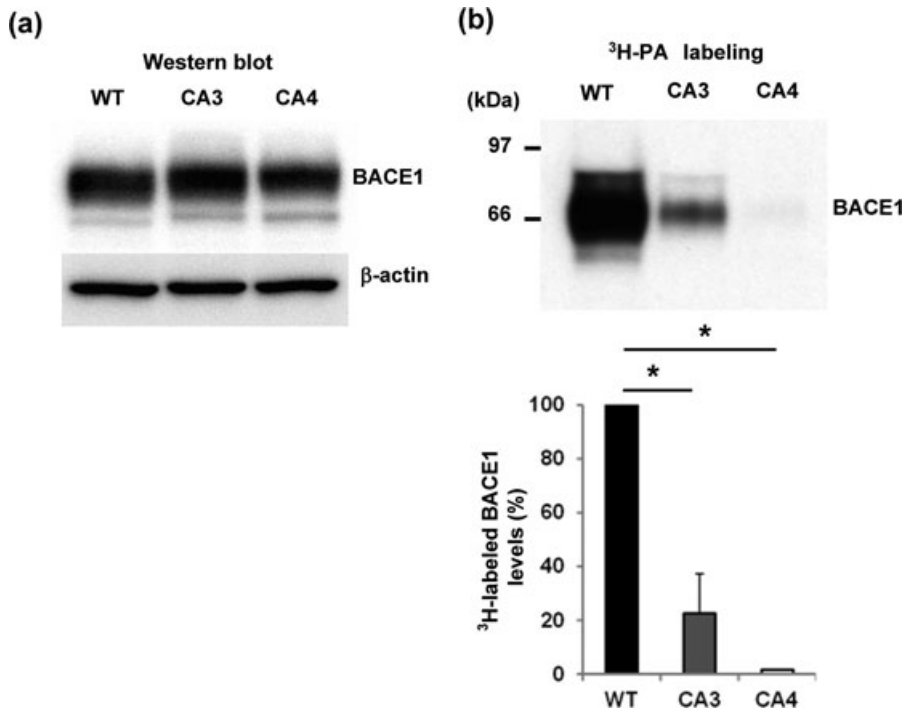

Figure 2. Four cysteine residues in BACE1 are palmitoylated. (a) Cell lysates of SH-BACAE1-WT, SH-BACE1-CA3, and SH-BACE1-CA4 cells were analyzed by immunoblotting with 1D4 antibody. (b) Cells were labeled with ^{3}H-palmitic acid, and lysates analyzed by immunoprecipitation with 1D4. The relative levels of ^{3}H-labeled BACE1 were calculated and graphed. Data are presented as means ± SEM from two independent experiments. *$P <$ 0.01.

that raft distribution of BACE1 does not directly affect that of APP.

We additionally evaluated the cellular localization of BACE1 using double immunofluorescence staining. Immunostaining with 1D4 revealed localization of BACE1 in both soma and neurites. There were no appreciable differences in the staining patterns between BACE1-WT- and BACE1-CA4 (Fig. 4d). Upon double labeling with 1D4 and anti-flotillin-1, flotillin-1 immunoreactivity was observed as punctate staining that partially overlapped that of 1D4. The extent of the colocalization of 1D4 and flotillin-1 immunoreactivities appeared reduced in neurons expressing BACE1-CA4, relative to those expressing BACE1-WT (Fig. 4d), consistent with the results of biochemical fractionation.

Aβ production is not influenced by raft association of BACE1 in neurons

Since lipid rafts appear to represent an important site for amyloidogenic processing of APP by BACE1 (Cordy et al. 2006; Araki 2010; Rushworth and Hooper 2010; Vetrivel and Thinakaran 2010), we analyzed the secretion of Aβ from primary neurons overexpressing BACE1-WT or BACE1-CA4. On Western blots, neurons expressed comparable levels of BACE1-WT and BACE1-CA4 (Fig. 5a). BACE1-WT and BACE1-CA4 enhanced secretion of both Aβ40 and Aβ42 to similar extents (by approximately 80%), compared to control cells infected with empty adenovirus (Fig. 5b and c).

To determine the effects of BACE1-WT and BACE1-CA4 on Aβ production from overexpressed APP, neurons were infected with both recombinant wild-type APP and BACE1 adenoviruses. Compared to neurons expressing APP only, mature APP levels were significantly diminished in those expressing APP plus BACE1-WT or APP plus BACE1-CA4, suggesting that a significant proportion of APP is cleaved by BACE1 (Fig. 5d). Consistently, neurons coexpressing APP and either BACE1-WT or BACE1-CA4 secreted ∼6.5-fold higher amounts of Aβ40 and ∼2.5-fold or ∼2.9-fold higher amounts of Aβ42, respectively, than those expressing APP alone (Fig. 5e and f). Together, BACE1-WT and BACE1-CA4 exerted similar Aβ-promoting effects, suggesting that β-cleavage of APP does not depend on raft localization of BACE1.

β-CTF is predominantly localized in nonraft membrane domains

To evaluate the β-cleavage of APP in raft and nonraft domains, we performed Western blot analysis of APP CTF.

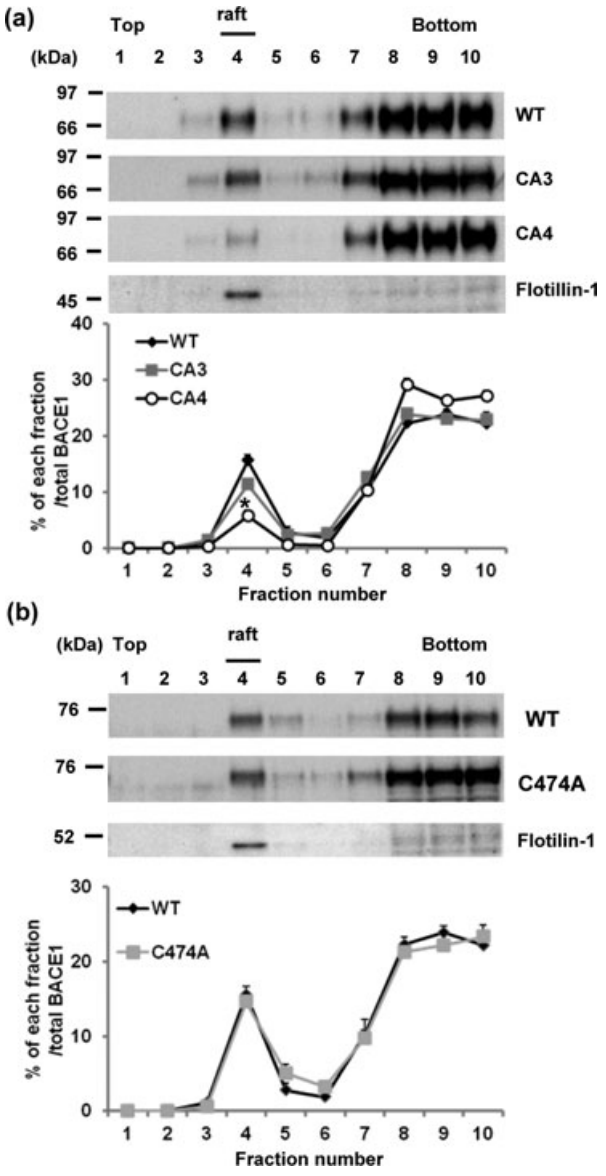

Figure 3. Lipid raft distribution of BACE1 depends on palmitoylation in neuroblastoma cells. (a) SH-BACE1-WT, SH-BACE1-CA3, and SH-BACE1-CA4 cells were subjected to sucrose density gradient fractionation, as described in section Materials and Methods. Each fraction was analyzed by Western blotting with 1D4 or anti-flotillin-1 (a marker protein for lipid rafts). The BACE1 level in each fraction was quantified and expressed as a percentage per total BACE1 level in whole fractions. (b) Similar analysis performed using SH-BACE1-WT and SH-BA-C474A cells. Data are presented as means ± SEM from three independent experiments. $^{*}P < 0.01$, compared with SH-BACE1-WT cells; $^{\#}P < 0.05$, compared with SH-BACE1-CA3 cells.

Western blots of RIPA lysates revealed that levels of β-CTF and β′-CTF (derived from alternative BACE1 cleavage of APP between Tyr10 and Glu11 within the Aβ region) were remarkably increased and those of α-CTF (derived from α-secretase cleavage of APP between Lys16 and Leu17 within the Aβ region) decreased in neurons expressing APP plus BACE1-WT or APP plus BACE1-CA4, compared to those expressing APP alone (Fig. 6a). Subsequently, we examined the distribution of APP CTF in raft and nonraft fractions following sucrose density gradient fractionation. Immunoprecipitation–Western blot analysis revealed that the majority of β-CTF and β′-CTF was recovered in nonraft fractions (fractions 8–10) of neurons expressing APP plus BACE1-WT or APP plus BACE1-CA4, whereas only low levels were present in the raft fraction (fraction 4). No differences in the localization pattern of CTFs were observed between neurons expressing BACE1-WT and BACE1-CA4 (Fig. 6b).

Next, we evaluated the β-cleavage of APP by endogenous BACE1. For this purpose, primary neurons overexpressing Swedish mutant APP, a preferred substrate of BACE1, via recombinant adenoviruses were treated with a γ-secretase inhibitor DAPT (*N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-(*S*)-phenylglycine *t*-butyl ester) (Dovey et al. 2001) that augments the levels of APP CTF. The distributions of endogenous BACE1 and APP CTF were then analyzed following sucrose density gradient fractionation. Bands of BACE1 were observed in both raft and nonraft fractions and faint bands probably representing dimeric BACE1 were additionally detected in nonraft fractions (Fig. 6c). Higher levels of β-CTF and β′-CTF were obviously recovered in nonraft fractions than in the raft fraction (Fig. 6d). These results suggest that β-cleavage of APP by overexpressed as well as endogenous BACE1 occurs mainly in nonraft fractions.

Shedding of BACE1 is regulated by palmitoylation

Next, we focused on the effects of palmitoylation on BACE1 shedding. As reported previously (Murayama et al. 2005), immunoprecipitation–Western blot analysis of conditioned media of SH-BACE1-WT cells revealed two bands corresponding to soluble BACE1 (sol-BACE1) and full-length BACE1 (FL-BACE1) (Fig. 7a). Compared to SH-BACE1-WT cells, the FL-BACE1 level appeared increased, while that of sol-BACE1 was decreased in media of SH-BACE-CA4 cells (Fig. 7a). Quantitative analysis disclosed a significant increase in the FL-BACE1/sol-BACE1 ratio in the media of SH-BACE1-CA4 cells relative to SH-BACE1-WT cells (Fig. 7b).

BACE1 shedding was additionally analyzed in primary neurons expressing BACE1-WT or BACE1-CA4. The FL-BACE1/sol-BACE1 ratio in the conditioned media was significantly increased in BACE1-CA4-expressing neurons, compared to BACE1-WT-expressing neurons (Fig. 7c and d). Altogether, the results suggest that BACE1 shedding is positively regulated by palmitoylation.

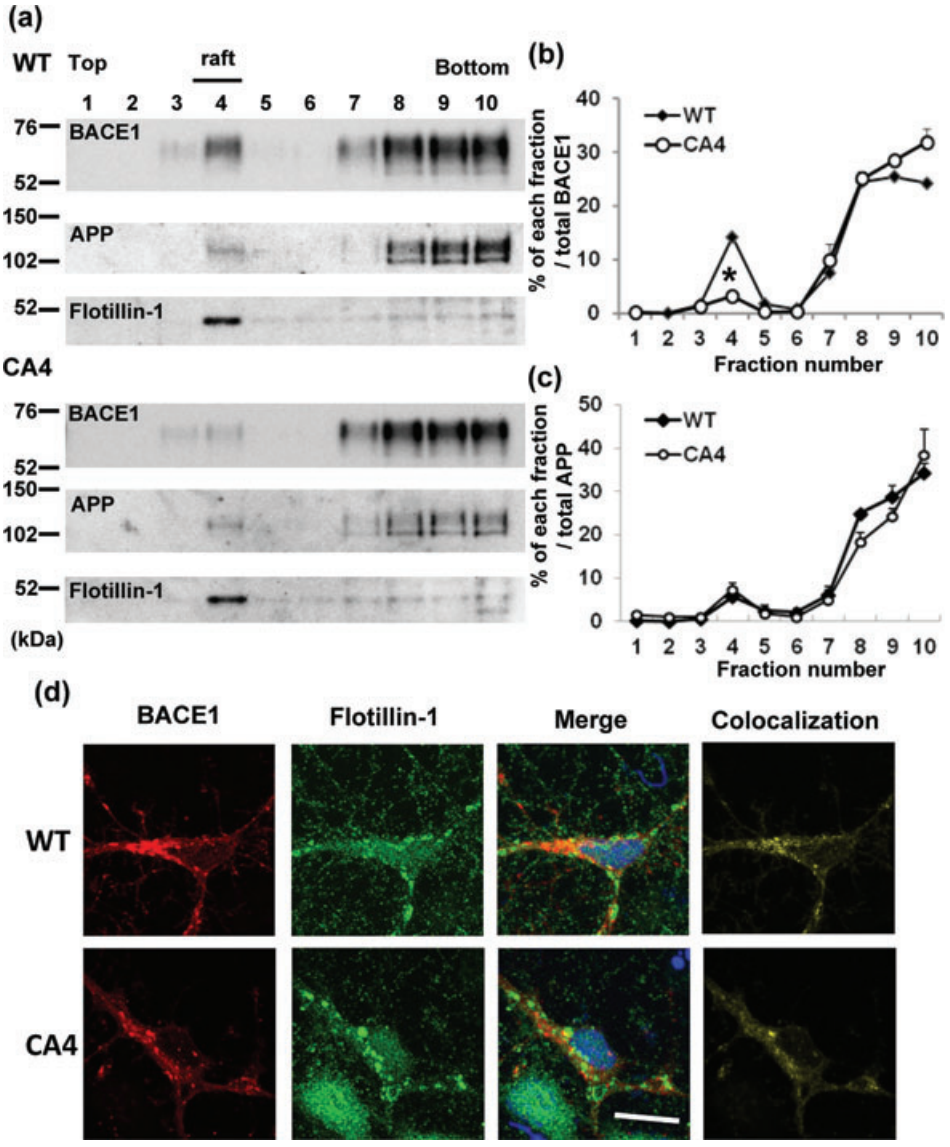

Figure 4. Lipid raft distribution of BACE1 depends on palmitoylation in primary rat cerebral cortical neurons. (a) CHAPS extracts of cultured neurons infected with recombinant adenoviruses expressing BACE1-WT or BACE1-CA4 were fractionated via sucrose density gradient fractionation, and analyzed by Western blotting with 1D4, anti-APP (R37), or anti-flotillin-1 antibodies. (b, c) The relative levels of individual fractions were quantified as for Fig. 3 and graphed. Data are presented as means ± SEM from three independent experiments. $*P < 0.01$. (d) Neurons grown on cover slips were infected with recombinant adenoviruses as above, fixed, and analyzed via double immunofluorescence staining with 1D4 (red) and anti-flotillin-1 (green). Nuclei were visualized with DAPI (4′, 6′-diamidino-2-phenylindole) staining (blue). Scale bar, 10 μm.

BACE1 dimerization is not affected by palmitoylation

Finally, we investigated whether palmitoylation affects the homodimer formation of BACE1. Extracts of the membrane fractions of SH-BACE1-WT, SH-BACE1-CA3, and SH-BACE1-CA4 cells were separated using BN-PAGE, followed by Western blotting. For SH-BACE1-WT cells, a band with mass of ~160 kDa instead of the expected mass of ~70 kDa reacted with the 1D4 antibody, confirming that BACE1 exists as a homodimer under native conditions (Fig. 8a and b). Similarly, only the band representing dimeric BACE1 was observed in SH-BACE1-CA3 and SH-BACE1-CA4 cells (Fig. 8b). Therefore, BACE1 dimer formation appeared to be unaffected by palmitoylation.

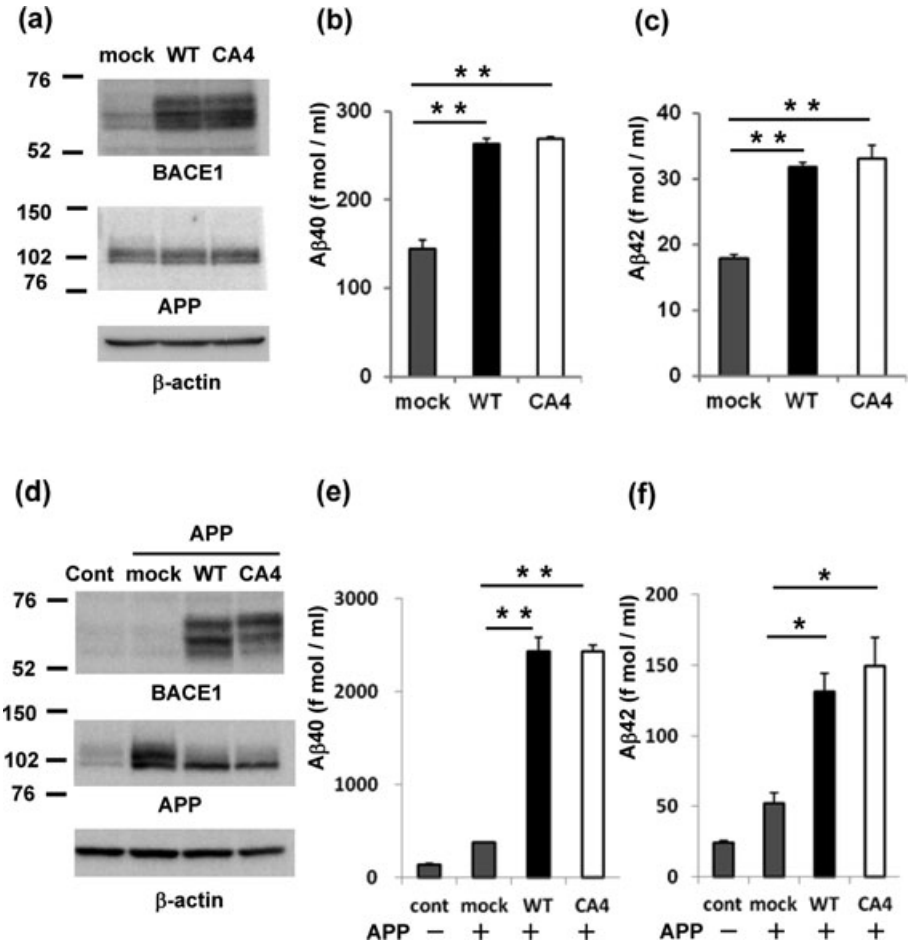

Figure 5. Neuronal Aβ production is not influenced by raft association of BACE1. (a–c) Cultured neurons were infected with recombinant adenoviruses expressing BACE1-WT, BACE1-CA4, or empty adenoviruses (mock). (a) Cell lysates were analyzed by Western blotting using NBA antibody for BACE1 or R37 antibody for APP. The amounts of Aβ40 (b) and Aβ42 (c) in conditioned media were determined with sandwich ELISA as described in section Materials and Methods. (d–f) Neurons were infected with recombinant APP adenoviruses plus empty adenoviruses or recombinant adenoviruses expressing BACE1-WT or BACE1-CA4. Western blotting (d) and Aβ measurement (e, f) were performed as above. BACE1-WT or BACE1-CA4 had comparable effects on the production of Aβ40 and Aβ42. Values represent means ± SEM from three separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

Discussion

The issue of whether lipid raft association of BACE1 plays a significant role in neuronal Aβ production remains controversial. We therefore sought to clarify this issue, as its resolution would shed light on the mechanisms underlying BACE1 regulation and assist in the development of effective disease-modifying therapeutics for AD. Using neuroblastoma cells expressing wild-type BACE1 (BACE1-WT) or mutant BACE1 (BACE1-CA3, BACE1-CA4, and BACE1-C474A), we confirmed that BACE1 is palmitoylated at four Cys residues in the juxtamembrane and C-terminal regions, in accordance with data from a recent study by Vetrivel et al. (2009). Raft localization of BACE1-CA4 without palmitoylation sites was markedly reduced in both neuroblastoma cells and primary cerebral cortical neurons, indicating that raft targeting of BACE1 is palmitoylation dependent. Our subsequent analysis revealed that BACE1-WT and BACE1-CA4 promoted neuronal production of Aβ40 and Aβ42 from endogenous and overexpressed APP to similar extents. These results clearly indicate that BACE1 raft localization does not affect Aβ production in neurons, in agreement with a previous report (Vetrivel et al. 2009) in which BACE1-null fibroblasts and mouse N2a neuroblastoma cells were used for experiments. Furthermore, we observed that both β-CTF and β′-CTF metabolites derived from BACE1 processing were mainly localized in the nonraft fractions of neurons expressing APP and either BACE1-WT or BACE1-CA4. We additionally observed that β-CTF derived from APP cleavage by

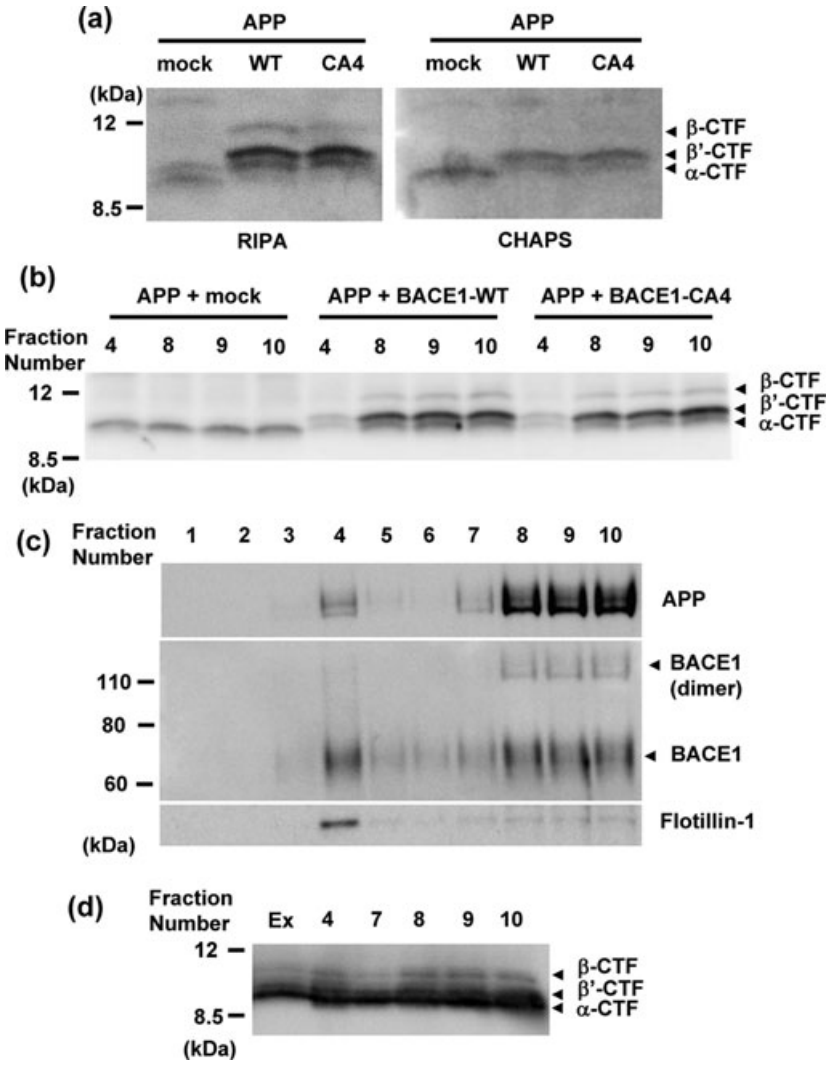

Figure 6. Predominant localization of APP CTF in nonraft domains of neurons coexpressing APP and BACE1. (a) RIPA or CHAPS extracts of neurons coexpressing APP and either mock or BACE1-WT or BACE1-CA4 were subjected to Tris/Tricine SDS-PAGE and immunoblotting with anti-APP. We observed remarkable increases in the levels of β-CTF and β'-CTF in both neurons expressing BACE1-WT and BACE1-CA4. (b) CHAPS extracts in (a) were fractionated using sucrose density gradient centrifugation, and raft (4) and nonraft (8-10) fractions analyzed by immunoprecipitation–Western blot analysis with anti-APP, as described in section Materials and Methods. The majority of β-CTF and β'-CTF was recovered in nonraft fractions of both neurons expressing BACE1-WT and BACE1-CA4. Each diagram is representative of two independent experiments with essentially identical results. (c) Neurons expressing Swedish mutant APP were treated with 0.5 μM DAPT for 1 day, and subjected to sucrose density gradient fractionation, as in (b). Each fraction was analyzed by Western blot analysis with anti-APP, anti-BACE1 (AB5832), and anti-flotillin-1. (d) CHAPS extracts (Ex) and raft (4) and nonraft (7-10) fractions in (c) were analyzed by immunoprecipitation–Western blot analysis with anti-APP, as above. β-CTF and β'-CTF were mainly recovered in nonraft fractions. Each diagram is representative of three experiments with essentially identical results.

endogenous BACE1 also distributes mainly in nonraft fractions of neurons expressing Swedish mutant APP. These data support the view that BACE1 cleavage of APP occurs mainly in the nonraft domains in neurons.

A mutant form of BACE1 (BACE1-GPI) in which transmembrane and cytoplasmic domains are replaced with glycosylphosphatidylinositol (GPI) anchor attachment signal that preferentially localizes in lipid rafts was reported to exhibit increased β-cleavage activity (Cordy et al. 2003). However, a recent study has revealed that the increase in Aβ secretion under the condition of BACE1-GPI expression is mainly due to reduced APP cleavage at the β'-site, compared with wild-type BACE1 (Vetrivel et al. 2011). It is likely that β-site cleavage efficiency of BACE1 is essentially unaltered by its association with lipid rafts. Based on the present and earlier results, we propose a dynamic model of neuronal Aβ generation, as illustrated in Fig. 9. First, APP is cleaved by BACE1 mainly in nonraft domains, generating β-CTF. Next, β-CTF mobilizes from nonraft to raft domains through unknown mechanisms, where it is finally cleaved by γ-secretase, resulting in Aβ production. Thus, identification of the specific factors involved in the transport of β-CTF from nonraft to raft domains is an important subject of focus for future research.

Endosomes and the trans-Golgi network are important organelles for the production of Aβ and β-CTF. A previous study (Vetrivel et al. 2009) presented evidence that palmitoylation of BACE1 does not regulate its distribution in these organelles or at the cell surface. Thus, BACE1-WT and BACE1-CA4 likely exert β-cleavage activity in nonraft domains of the same subcellular sites. Interestingly, the β'-CTF level was higher than that of β-CTF in neurons coexpressing APP and BACE1. Consistently, a recent study has indicated that β'-cleavage is a major processing event that occurs with human

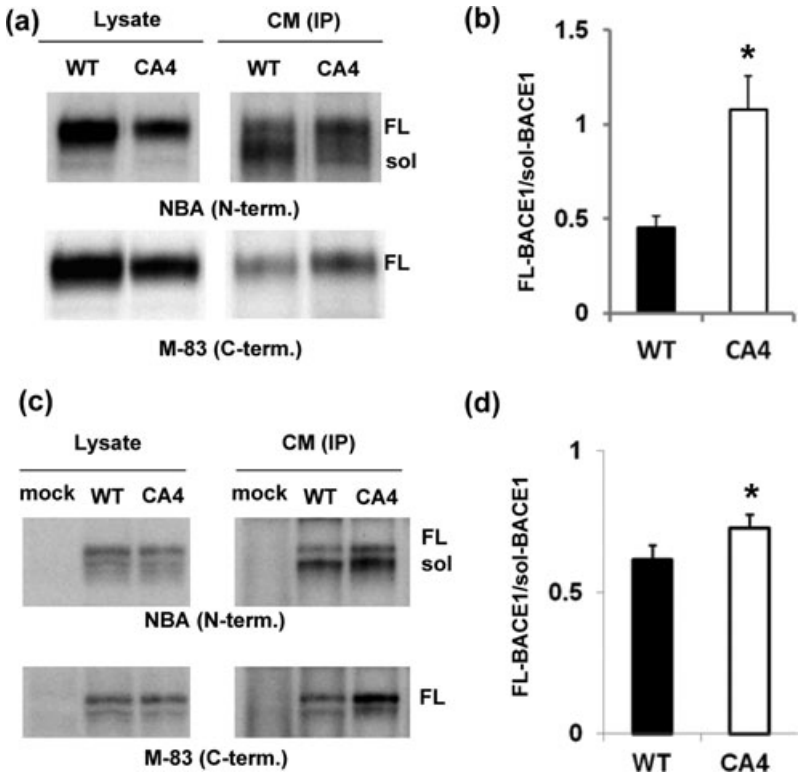

Figure 7. BACE1 shedding is regulated by palmitoylation. (a) Conditioned media of SH-BACE1-WT or SH-BACE1-CA4 cells were analyzed by immunoprecipitation–Western blot analysis, as described in section Materials and Methods. Two bands corresponding to FL-BACE1 and sol-BACE1 were detected with the NBA antibody, while only the upper FL-BACE1 band was recognized with the M83 antibody. (b) Band intensities of FL-BACE1 and sol-BACE1 (a) were quantitated and the FL-BACE1/sol-BACE1 ratios calculated. (c, d) Conditioned media of neurons infected with recombinant adenoviruses expressing BACE1-WT or BACE1-CA4 were analyzed as for (a) FL-BACE1/sol-BACE1 ratios were calculated and graphed as for (b). Data are presented as means ± SEM from three independent experiments. $^*P < 0.05$.

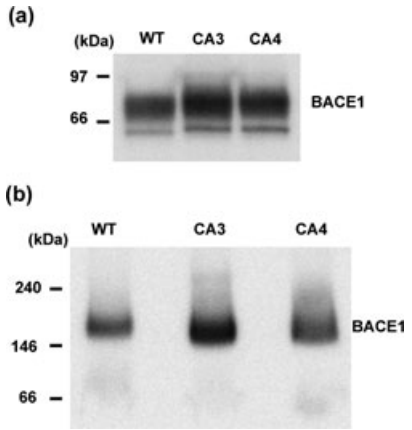

Figure 8. BACE1 dimerization is not affected by palmitoylation. Extracts of membrane fractions of SH-BACE1-WT, SH-BACE1-CA3, or SH-BACE1-CA4 cells were separated using SDS-PAGE (a) or BN-PAGE (b), and analyzed via Western blotting with 1D4. On BN-PAGE, only a ~160-kDa band most likely corresponding to dimeric BACE1 was observed in all three cell types. Each diagram is representative of two independent experiments that gave essentially identical results.

APP in neuronal cultures (Zhou et al. 2011). It is possible that β'-CTF is generated via cleavage of β-CTF by BACE1, since Vetrivel et al. (2011) showed that BACE1 readily processes β-CTF into β'-CTF in COS cells.

In BACE1-expressing cell medium, soluble as well as full-length BACE1 are detected. We showed previously that treatment with a metalloprotease inhibitor TAPI-1 reduces BACE1 shedding but increases full-length BACE1 release, suggesting that these two events are interrelated physiological processes (Murayama et al. 2005). In the present study, we showed that the FL-BACE1/sol-BACE1 ratio was significantly increased in the media of SH-SY5Y cells and primary neurons expressing BACE1-CA4, compared to those expressing BACE1-WT. These results suggest that BACE1 shedding is reduced in the absence of BACE1 palmitoylation, although the underlying mechanism is yet to be established. It is unlikely that lack of raft localization of BACE1 is related to reduced shedding because metalloproteases responsible for BACE1 shedding, such as ADAM10, are predominantly distributed in nonraft fractions (Kojro et al. 2001). Palmitoylation of BACE1 may affect the physical interactions between BACE1 and its sheddases and promote BACE1 shedding.

BACE1 exists as a homodimer under native conditions. Dimer formation could be influenced by BACE1 posttranslational modifications such as palmitoylation. Previous studies have indicated that dimerization of BACE1 is inhibited in bHEK cells treated with cerulenin, which has an inhibitory effect on palmitoylation (Parsons and Austen 2005), implying a role of palmitoylation in BACE1 dimerization. However, BN-PAGE analysis disclosed no differences in dimer

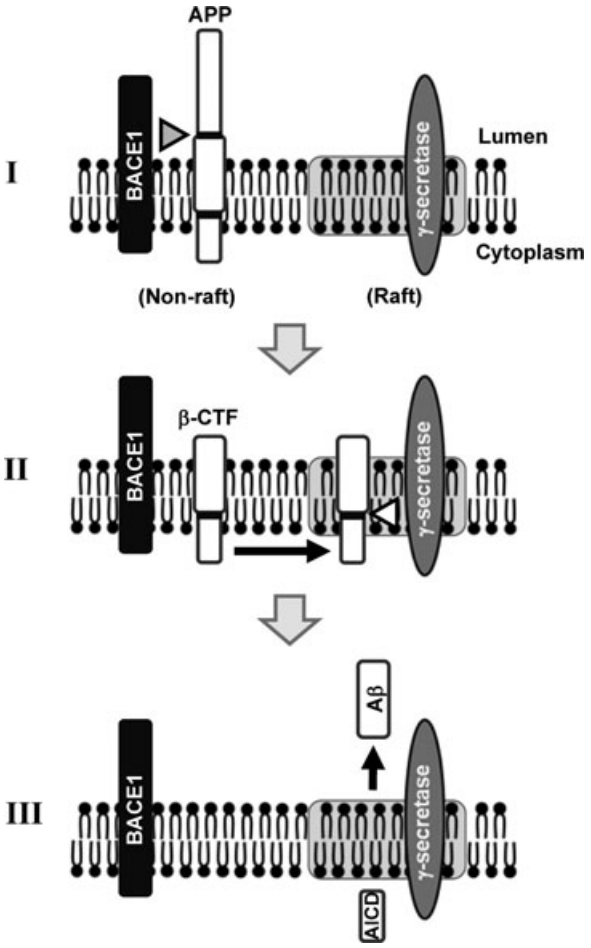

Figure 9. Hypothetical model for APP processing. APP processing in neurons is possibly a dynamic process involving three steps. (I) APP is cleaved by BACE1 mainly in nonraft domains, generating β-CTF. (II) β-CTF is transported from nonraft to raft domains via unknown mechanisms. (III) β-CTF is cleaved by γ-secretase in raft domains, generating Aβ and AICD (APP intracellular domain).

formation between BACE1-WT and BACE1-CA4 proteins. Thus, it appears that palmitoylation of BACE1 does not directly influence dimerization.

It is likely that BACE1 in lipid rafts has functional roles other than processing of APP. BACE1 cleaves a number of substrates, including neuregulins, p-selectin glycoprotein ligand-1, β-subunits of voltage-gated sodium channels, and lipoprotein receptor-related protein (Lichtenthaler et al. 2003; von Arnim et al. 2005; Wong et al. 2005; Hu et al. 2006; Willem et al. 2006; Kim et al. 2007). It is plausible that BACE1 functions to cleave these membrane proteins that are localized in lipid rafts. In our immunocytochemical analysis of BACE1-expressing neurons, BACE1 immunoreactivity was observed throughout neuronal processes, implying activity in the metabolism of specific synaptic proteins.

The mechanisms underlying regulation of BACE1 activity in neurons are complex (Stockley and O'Neill 2008). We and others have demonstrated that reticulon (RTN) proteins such as RTN3 and RTN4-B/C interact with BACE1 and inhibit its β-cleavage activity (He et al. 2004; Murayama et al. 2006). Our preliminary data indicate that RTN3 and RTN4-B/C mostly distributed in nonraft domains (data not shown) where they appear to regulate BACE1. It is important to make further investigations of nonraft membrane domains as a possible site of physiological regulation of BACE1. The hypothetical model of APP processing involves mobilization of β-CTF. However, the transport mechanism of β-CTF between nonraft and raft domains remains to be elucidated; resolving this issue would provide a basis for the identification of new therapeutic targets for AD.

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References

Araki, W. 2010. Beta- and gamma-secretases and lipid rafts. Open Biol. J. 3:316–320.

Araki, W., K. Yuasa, S. Takeda, K. Takeda, K. Shirotani, K. Takahashi, and T. Tabira. 2001. Pro-apoptotic effect of presenilin 2 (PS2) overexpression is associated with down-regulation of Bcl-2 in cultured neurons. J. Neurochem. 79:1161–1168.

Benjannet, S., A. Elagoz, L. Wickham, M. Mamarbachi, J. S. Munzer, A. Basak, C. Lazure, J. A. Cromlish, S. Sisodia, F. Checler, *et al.* 2001. Post-translational processing of beta-secretase (beta-amyloid-converting enzyme) and its ectodomain shedding. The pro- and transmembrane/cytosolic domains affect its cellular activity and amyloid-beta production. J. Biol. Chem. 276:10879–10887.

Brewer, G. J., J. R. Torricelli, E. K. Evege, and P. J. Price. 1993. Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. J. Neurosci. Res. 35:567–576.

Charollais, J., and F. G. Van Der Goot. 2009. Palmitoylation of membrane proteins (Review). Mol. Membr. Biol. 26:55–66.

Citron, M. 2004. Beta-secretase inhibition for the treatment of Alzheimer's disease – promise and challenge. Trends Pharmacol. Sci. 25:92–97.

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.

Cordy, J. M., N. M. Hooper, and A. J. Turner. 2006. The involvement of lipid rafts in Alzheimer's disease. Mol. Membr. Biol. 23:111–122.

De Strooper, B. 2010. Proteases and proteolysis in Alzheimer disease: a multifactorial view on the disease process. Physiol. Rev. 90:465–494.

Dovey, H. F., V. John, J. P. Anderson, L. Z. Chen, P. de Saint Andrieu, L. Y. Fang, S. B. Freedman, B. Folmer, E. Goldbach, E. J. Holsztynska, *et al*. 2001. Functional gamma-secretase inhibitors reduce beta-amyloid peptide levels in brain. J. Neurochem. 76:173–181.

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.

Estus, S., T. E. Golde, T. Kunishita, D. Blades, D. Lowery, M. Eisen, M. Usiak, X. M. Qu, T. Tabira, B. D. Greenberg, *et al*. 1992. Potentially amyloidogenic, carboxyl-terminal derivatives of the amyloid protein precursor. Science 255:726–728.

Farzan, M., C. E. Schnitzler, N. Vasilieva, D. Leung, and H. Choe. 2000. BACE2, a beta -secretase homolog, cleaves at the beta site and within the amyloid-beta region of the amyloid-beta precursor protein. Proc. Natl. Acad. Sci. USA 97:9712–9717.

Hardy, J., and D. J. Selkoe. 2002. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science 297:353–356.

He, W., Y. Lu, I. Qahwash, X. Y. Hu, A. Chang, and R. Yan. 2004. Reticulon family members modulate BACE1 activity and amyloid-beta peptide generation. Nat. Med. 10:959–965.

Hodges, R. S., R. J. Heaton, J. M. Parker, L. Molday, and R. S. Molday. 1988. Antigen-antibody interaction. Synthetic peptides define linear antigenic determinants recognized by monoclonal antibodies directed to the cytoplasmic carboxyl terminus of rhodopsin. J. Biol. Chem. 263:11768–11775.

Hu, X., C. W. Hicks, W. He, P. Wong, W. B. Macklin, B. D. Trapp, and R. Yan. 2006. Bace1 modulates myelination in the central and peripheral nervous system. Nat. Neurosci. 9:1520–1525.

Hur, J. Y., H. Welander, H. Behbahani, M. Aoki, J. Franberg, B. Winblad, S. Frykman, andL. O. Tjernberg. 2008. Active gamma-secretase is localized to detergent-resistant membranes in human brain. FEBS J. 275:1174–1187.

Hussain, I., J. Hawkins, A. Shikotra, D. R. Riddell, A. Faller, and C. Dingwall. 2003. Characterization of the ectodomain shedding of the beta-site amyloid precursor protein-cleaving enzyme 1 (BACE1). J. Biol. Chem. 278:36264–36268.

Kametani, F., K. Tanaka, T. Ishii, S. Ikeda, H. E. Kennedy, and D. Allsop. 1993. Secretory form of Alzheimer amyloid precursor protein 695 in human brain lacks beta/A4 amyloid immunoreactivity. Biochem. Biophys. Res. Commun. 191:392–398.

Kim, D. Y., B. W. Carey, H. Wang, L. A. Ingano, A. M. Binshtok, M. H. Wertz, W. H. Pettingell, P. He, V. M. Lee, C. J. Woolf, *et al*. 2007. BACE1 regulates voltage-gated sodium channels and neuronal activity. Nat. Cell. Biol. 9:755–764.

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.

Laude, A. J., and I. A. Prior. 2004. Plasma membrane microdomains: organization, function, and trafficking. Mol. Membr. Biol. 21:193–205.

Lichtenthaler, S. F., D. I. Dominguez, G. G. Westmeyer, K. Reiss, C. Haass, P. Saftig, B. De Strooper, and B. Seed. 2003. The cell adhesion protein P-selectin glycoprotein ligand-1 is a substrate for the aspartyl protease BACE1. J. Biol. Chem. 278:48713–48719.

Magee, A. I., J. Wootton, and J. de Bony. 1995. Detecting radiolabeled lipid-modified proteins in polyacrylamide gels. Methods Enzymol. 250:330–336.

Murayama, K. S., F. Kametani, and W. Araki. 2005. Extracellular release of BACE1 holoproteins from human neuronal cells. Biochem. Biophys. Res. Commun. 338:800–807.

Murayama, K. S., F. Kametani, S. Saito, H. Kume, H. Akiyama, and W. Araki. 2006. Reticulons RTN3 and RTN4-B/C interact with BACE1 and inhibit its ability to produce amyloid beta-protein. Eur. J. Neurosci. 24:1237–1244.

Oda, A., A. Tamaoka, and W. Araki. 2010. Oxidative stress up-regulates presenilin 1 in lipid rafts in neuronal cells. J. Neurosci. Res. 88:1137–1145.

Ohno, M. 2008. Beta-Secretase as a prime therapeutic target for Alzheimer's disease: a perspective from mouse model studies. Pp. 1–25 *in* W. Araki, ed. Recent advances in the biology of secretases, key protease in Alzheimer's disease. Research Signpost, Kelara.

Parsons, R. B., and B. M. Austen. 2005. Protein lipidation of BACE. Biochem. Soc. Trans. 33:1091–1093.

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.

Rushworth, J. V., and N. M. Hooper. 2010. Lipid rafts: linking Alzheimer's amyloid-beta production, aggregation, and toxicity at neuronal membranes. Int. J. Alzheimers Dis. 2011:603052.

Schagger, H., and G. von Jagow. 1991. Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. Anal. Biochem. 199:223–231.

Schmechel, A., M. Strauss, A. Schlicksupp, R. Pipkorn, C. Haass, T. A. Bayer, and G. Multhaup. 2004. Human BACE forms dimers and colocalizes with APP. J. Biol. Chem. 279:39710–39717.

Stockley, J. H., and C. O'Neill. 2008. Understanding BACE1: essential protease for amyloid-beta production in Alzheimer's disease. Cell Mol. Life Sci. 65:3265–3289.

Suzuki, N., T. T. Cheung, X. D. Cai, A. Odaka, L. Otvos, Jr., C. Eckman, T. E. Golde, and S. G. Younkin. 1994. An increased

percentage of long amyloid beta protein secreted by familial amyloid beta protein precursor (beta APP717) mutants. Science 264:1336–1340.

Takeda, K., W. Araki, and T. Tabira. 2004. Enhanced generation of intracellular Abeta42 amyloid peptide by mutation of presenilins PS1 and PS2. Eur. J. Neurosci. 19:258–264.

Urano, Y., I. Hayashi, N. Isoo, P. C. Reid, Y. Shibasaki, N. Noguchi, T. Tomita, T. Iwatsubo, T. Hamakubo, and T. Kodama. 2005. Association of active gamma-secretase complex with lipid rafts. J. Lipid Res. 46:904–912.

Vassar, R., B. D. Bennett, S. Babu-Khan, S. Kahn, E. A. Mendiaz, P. Denis, D. B. Teplow, S. Ross, P. Amarante, R. Loeloff, *et al*. 1999. Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. Science 286:735–741.

Vetrivel, K. S., and G. Thinakaran. 2010. Membrane rafts in Alzheimer's disease beta-amyloid production. Biochim. Biophys. Acta 1801:860–867.

Vetrivel, K. S., H. Cheng, W. Lin, T. Sakurai, T. Li, N. Nukina, P. C. Wong, H. Xu, and G. Thinakaran. 2004. Association of gamma-secretase with lipid rafts in post-Golgi and endosome membranes. J. Biol. Chem. 279:44945–44954.

Vetrivel, K. S., X. Meckler, Y. Chen, P. D. Nguyen, N. G. Seidah, R. Vassar, P. C. Wong, M. Fukata, M. Z. Kounnas, and G. Thinakaran. 2009. Alzheimer disease Abeta production in the absence of S-palmitoylation-dependent targeting of BACE1 to lipid rafts. J. Biol. Chem. 284:3793–3803.

Vetrivel, K. S., A. Barman, Y. Chen, P. D. Nguyen, S. L. Wagner, R. Prabhakar, and G. Thinakaran. 2011. Loss of cleavage at beta'-site contributes to apparent increase in beta-amyloid peptide (Abeta) secretion by beta-secretase (BACE1)-glycosylphosphatidylinositol (GPI) processing of amyloid precursor protein. J. Biol. Chem. 286:26166–26177.

von Arnim, C. A., A. Kinoshita, I. D. Peltan, M. M. Tangredi, L. Herl, B. M. Lee, R. Spoelgen, T. T. Hshieh, S. Ranganathan, F. D. Battey, *et al.* 2005. The low density lipoprotein receptor-related protein (LRP) is a novel beta-secretase (BACE1) substrate. J. Biol. Chem. 280:17777–17785.

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.

Westmeyer, G. G., M. Willem, S. F. Lichtenthaler, G. Lurman, G. Multhaup, I. Assfalg-Machleidt, K. Reiss, P. Saftig, and C. Haass. 2004. Dimerization of beta-site beta-amyloid precursor protein-cleaving enzyme. J. Biol. Chem. 279:53205–53212.

Willem, M., A. N. Garratt, B. Novak, M. Citron, S. Kaufmann, A. Rittger, B. DeStrooper, P. Saftig, C. Birchmeier, and C. Haass. 2006. Control of peripheral nerve myelination by the beta-secretase BACE1. Science 314:664–666.

Wong, H. K., T. Sakurai, F. Oyama, K. Kaneko, K. Wada, H. Miyazaki, M. Kurosawa, B. De Strooper, P. Saftig, and N. Nukina. 2005. Beta Subunits of voltage-gated sodium channels are novel substrates of beta-site amyloid precursor protein-cleaving enzyme (BACE1) and gamma-secretase. J. Biol. Chem. 280:23009–23017.

Yoon, I. S., E. Chen, T. Busse, E. Repetto, M. K. Lakshmana, E. H. Koo, and D. E. Kang. 2007. Low-density lipoprotein receptor-related protein promotes amyloid precursor protein trafficking to lipid rafts in the endocytic pathway. FASEB J. 21:2742–2752.

Zhou, L., N. Brouwers, I. Benilova, A. Vandersteen, M. Mercken, K. Van Laere, P. Van Damme, D. Demedts, F. Van Leuven, K. Sleegers, et al. 2011. Amyloid precursor protein mutation E682K at the alternative β-secretase cleavage β'-site increases Aβ generation. EMBO Mol. Med. 3:291–302.