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Identification of Phospholipid Scramblase 1 as a Novel Interacting Molecule with β-Secretase (β-Site Amyloid Precursor Protein (APP) Cleaving Enzyme (BACE))*

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β-Site amyloid precursor protein (APP)-cleaving enzyme (BACE) is an integral membrane aspartic proteinase responsible for β-site processing of APP, and its cytoplasmic region composed of 24 amino acid residues has been shown to be involved in the endosomal localization of BACE. With the yeast two-hybrid screening, we found that the cytoplasmic domain of phospholipid scramblase 1 (PLSCR1), a type II integral membrane protein, interacts with the cytoplasmic region of BACE. In cultured cells, BACE and PLSCR1 were colocalized in the Golgi area and in endosomal compartments, whereas they were co-redistributed in late endosome-derived multivesicular bodies when treated with U18666A, suggesting that both proteins share a common trafficking pathway in cells. Co-immunoprecipitation analysis showed that both proteins form a protein complex at an endogenous expression level in the human neuroblastoma SH-SY5Ycells, and the dileucine residue of the BACE tail is also revealed to be essential for the physical interaction with PLSCR1 *in vitro* and *in vivo*. Moreover, both BACE and PLSCR1 were localized in a low buoyant lipid microdomain in SH-SY5Y cells. The dileucine-defective BACE mutant was also fractionated into the lipid microdomain, but much less stably than wild-type BACE. Taken together, our current study suggests the functional involvement of PLSCR1 in the intracellular distribution of BACE and/or recruitment of BACE into the detergent-insoluble lipid raft.

The amyloid β-peptide (Aβ),[1] a principal constituent of senile plaques, is a major hallmark of familial Alzheimer's disease (AD). Aβ peptides are generated from a type I membrane glycoprotein, amyloid precursor protein (APP) (1), by proteolytic events that involve the participation of β- and γ-secretases (2, 3). A number of studies on familial AD have shown that mutations in genes of APP, presenilin-1 or -2, affect APP processing and result in increases in the total levels of Aβ, especially Aβ42. Aβ42 is known to form amyloid fibrils more readily than Aβ40 (4, 5), and its overproduction may thus accelerate plaque formation, leading to early onset AD (6) and sporadic AD as well. Thus, Aβ formation has been the subject of considerable interest as a key event of AD.

A proteinase with β-secretase activity has recently been cloned and is referred to as BACE (beta-site of APP-cleaving enzyme) (7, 8). The cells develop β-site cleavage activity when BACE is ectopically expressed, and the recently established BACE-deficient mouse shows considerably diminished β-secretase activity, indicating that BACE functions as a major β-secretase *in vivo* (9). Intracellular sites for β-secretase activity have been investigated, and at least three sites including endoplasmic reticulum/intermediate compartments (10), Golgi/trans Golgi network (TGN) (11), and endosomal compartments (12, 13) have been reported. When BACE is ectopically expressed, it is mainly detected in the intracellular compartments including Golgi apparatus, TGN, endosomes, and the plasma membranes (14, 15). Recent studies also revealed that a pathogenic Glu^{11}-site cleavage of Aβ is increased with the limited expression of BACE-furin chimeric protein in TGN (16), and sialyltransferase, a TGN-resident protein, was also found to be another substrate of BACE *in vivo* (17). These results suggest that pathogenic cleavage with BACE could occur in TGN. Although the cytoplasmic region of BACE has been shown to be essential for the intracellular localization of BACE (14), the molecular mechanisms of BACE trafficking between TGN, endosomes, and plasma membrane remain to be elucidated.

In addition, recent analyses of the Aβ formation have uncovered a novel regulatory mechanism for APP processing. Caveolae in the plasma membrane constitute a microdomain that has a unique lipid composition with a high content of both cholesterol and glycosphingolipids. In the brain, the caveolae-like microdomain has been referred to as a detergent-insoluble glycolipid membrane complex (DIG) (18). Brain-derived DIG, or so-called lipid raft, has been reported to accumulate APP (19), presenilin-1 and -2, and Aβ peptides (20). Moreover, BACE has also been shown to be localized in the lipid microdomain in cultured cells in a cholesterol-dependent manner (21). Therefore, DIGs represent putative sites where amyloid biogenesis or transport takes place, and cholesterol metabolism is

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[1] The abbreviations are: Aβ, amyloid β-peptide; AD, Alzheimer's disease; APP, amyloid precursor protein; BACE, β-site APP-cleaving enzyme; PLSCR1, phospholipid scramblase 1; WT, wild-type; TGN, trans Golgi network; DIG, detergent-insoluble glycolipid membrane complex; CI-MPR, cation independent mannose-6-phosphate receptor; GFP, green fluorsecent protein; EGFP, enhanced GFP; DSP, dithiobis[succinimidyl propionate]; PNS, post-nuclear supernatant; lum, lumenal.

strongly assumed to be associated with the APP processing events.

The present study showed that the cytoplasmic tail of BACE binds directly to phospholipid scramblase 1 (PLSCR1), another component of the plasma membrane lipid microdomain (22). The interaction required a dileucine repeat in BACE ($Leu^{499,500}$), which was required for the endocytic transport of BACE (14). Coprecipitation experiments also showed that BACE forms a protein complex with PLSCR1 in SH-SY5Y cells, a neuroblastoma cell line. We also demonstrated that BACE and PLSCR1 are fractionated in the DIGs at an endogenous expression level in SH-SY5Y cells. These findings strongly suggest that PLSCR1 is a binding partner of BACE *in vivo* and provide a functional implication of PLSCR1 in the intracellular trafficking of BACE.

EXPERIMENTAL PROCEDURES

Cell Cultures and Transfection—HeLa cells were cultured in α-MEM (Invitrogen) containing 8% fetal bovine serum supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin. HEK293 (clone JCRB9068, provided from the Human Science Research Resources Bank) and human neuroblastoma SH-SY5Y cells (23) were maintained in Dulbecco's modified Eagle's medium supplemented with 4.5 g/liter glucose and 10% fetal bovine serum. For the transfection of HeLa and HEK293 cells, the FuGene-6 reagent (Roche Molecular Biochemicals) and LipofectAMINE Plus (Invitrogen) were used, respectively, following the manufacturer's recommended protocols. Wild-type and transfected HeLa cells were also cultured in the presence or absence of 3 μg/ml U18666A (BIOMOL Research Laboratory).

Cloning, Plasmids, and Sequence Analysis—Wild-type BACE cDNA was generated from human brain cDNA (Clontech) and human placenta cDNA using PCR. The DNA fragment obtained was cloned into pcDNA3 (Invitrogen) and pcDNA-EGFP expression vectors to construct pHB1-full and pHB1-EGFP, respectively. Epitope tagging of PLSCR1 with 3xHA was carried out with subcloning of the PLSCR1 open reading frame to the pEF-BOS-3HA vector. Site-directed mutagenesis of the BACE tail (amino acids 478–501) was carried out by PCR using following primers containing the mutation sites: 5′-gcgctcgagtcacttcgccgcggagat-3′, 5′-ggcctcgagtcacttcagcagggcgatgtc-3′, and 5′-ggcctcgagtcactcagcaggtcgatgtc-3′. The cytoplasmic tails of BACE2 (amino acids 495–518), mouse CI-MPR (amino acids 2316–2482), and human APP695 (amino acids 649–695) were cloned into pGBD-C1 using the following primer pairs: 5′-gccgaattccggtgtcagcgtcgcccc-3′ and 5′-ggcctcgagtcatttccagggatgtct-3′, 5′-gccgaattccataagaaggagagaagg-3′ and 5′-ggcctcgagttagatgtgtaagagggt-3′, and 5′-gccgaattcaagaagaaacagtacaca-3′ and 5′-ggcctcgaggttctgcatctgctcaa-3′, respectively. The deletion plasmids of pGAD10-PLSCR1-full, 290, 156, and 118 were constructed by subcloning of the amplified DNA fragments into pGAD10. The PCR primers used for construction of pGAD10 plasmids are as follows: 5′-ggcctcgagctaccacactcctgattt-3′ (full-as), 5′-ggcctcgagtttcattttaacatcaaggtc-3′ (290-as), 5′-ggcctcgagtggcccacagcaatttcg-3′ (156-as), 5′-ggcctcgagttccagaagttcaatttg-3′ (118-as).

Yeast Two-hybrid Screening—The DNA fragment corresponding to the BACE cytoplasmic region was inserted into the pGBD-C1 vector to construct pGBD-HB1cyt. PJ69–4A cells (kindly provided from Dr. Philip James, University of Wisconsin; Ref. 24), harboring pGBD-HB1cyt were transformed with a HeLa cDNA library (Clontech), and Ade^{+}-positive clones were obtained, and the library clones were subsequently subjected to DNA sequencing analysis after the reintroduction into PJ69–4A/pGBD-HB1cyt. One of the obtained clones, pKM2–8 was found to harbor a full-length open reading frame of the *PLSCR1* gene.

In Vitro Binding Assay—DNA fragments encoding the cytoplasmic domains of wild-type or mutant BACE tail were introduced into the pGEX6P-1 vector (Amersham Biosciences), and full-length PLSCR1 open reading frame amplified from pKM2–8 by the PCR method was subcloned into the pMAL-C2 vector (New England Biolabs) for the bacterial expression of the GST and MBP chimeric proteins, respectively. After purification with glutathione-Sepharose 4B (Amersham Biosciences) or amylose resin (New England Biolabs) columns, the beads containing GST fusion proteins and the eluted MBP-PLSCR1 protein were subjected to the following *in vitro* binding assay. GST beads containing ~1 μg of each GST chimeric protein were incubated with 5 μg of MBP-PLSCR1 in a binding buffer (25 mM phosphate buffer (pH 7.2), 0.5 mM $CaCl_2$, 150 mM NaCl, 0.5% Triton X-100, 10 mM β-mercaptoethanol, 1× protease inhibitor mixture (Roche Molecular Biochemicals), and 1 mM phenylmethylsulfonyl fluoride) at 4 °C for 2 h. The beads were then washed extensively with binding buffer, and the bead-bound proteins were subjected to immunoblot analysis with anti-GST or anti-MBP antibodies.

Antibodies, Immunofluorescence Microscopy, and Immunoelectron Microscopy—To raise the antibodies against the BACE cytoplasmic (BACE-cyt; Cys^{478} to Lys^{501}) or lumenal (BACE-lum; Asp^{48} to Thr^{80}) region, bacterially expressed GST fusion protein that contains each region was injected to rabbits. Anti-human BACE monoclonal antibody (MAB5308) and anti-APP antibody 22C11 were purchased from Chemicon. Anti-caveolin-1 polyclonal antibody and anti-flotilin-1 antibodies were purchased from Santa Cruz Biotechnology, and BD Transduction Laboratories, respectively. Anti-cation independent mannose 6-phosphate receptor (CI-MPR) polyclonal antibody was used for a TGN marker as reported previously (25), and anti-Lamp1 monoclonal antibody was provided from the Developmental Studies Hybridoma Bank. Indirect immunofluorescence microscopy was performed as described elsewhere (26) with a confocal laser scanning microscope (LSM-510, Zeiss). For immunoelectron microscopy, HeLa cells cotransfected with pHB1-EGFP and pEF-BOS-3HA-PLSCR1 were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 15 min on ice and further fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 1 h at 4 °C. The cells were then scraped and subjected to immunoelectron microscopic procedures using anti-GFP antibody (Invitrogen) and anti-hemagglutinin antibody (3F10, Roche Molecular Biochemicals), as described elsewhere (27).

Differential Solubilization—HEK293 cells stably expressing BACE were homogenized by passing through the 27-gauge needle for 25 strokes in phosphate-buffered saline containing 1× protease inhibitor mixture and 1 mM phenylmethylsulfonyl fluoride and centrifuged at 700 × *g* for 5 min to generate a post-nuclear supernatant (PNS) fraction. The PNS fraction was separated to six aliquots, and each aliquot was treated with buffer to a final concentration of 1 M NaCl, 0.2 M Na_2CO_3 (pH 11.0), 2 M urea, or 1% Triton X-100 on ice for 30 min. After incubation, each sample was centrifuged at 100,000 × *g* for 1 h, and the resultant pellet and supernatant fractions were subsequently subjected to immunoblotting using anti-BACE-lum and anti-PLSCR1 (4D2) antibodies.

Cross-linking and Immunoprecipitation—HeLa cells transfected with pHB1-full or pHB1-AA were washed with ice-cold phosphate-buffered saline twice, harvested, and resuspended in buffer X (50 mM phosphate buffer (pH 7.5), 150 mM NaCl, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 5% glycerol, and 1× protease inhibitor mixture (Roche Molecular Biochemicals)). Then, a chemical cross-linker, dithiobis[succinimidyl propionate] (DSP, Pierce), was added at the concentrations indicated and incubated on ice for 30 min. After termination of the cross-linking by the addition of ⅕ volume of 1 M Tris-HCl (pH 7.6), BACE, or PLSCR1 was immunoprecipitated with anti-BACE (MAB5308) or anti-PLSCR1 (4D2) antibodies as described elsewhere (22, 28). The precipitates were subsequently subjected to immunoblotting. For coprecipitation of endogenous BACE and PLSCR1, ~1×10^8 of SH-SY5Y cells were homogenized by passing through the 27-gauge needle for 30 strokes in phosphate-buffered saline (pH 7.4) containing 1× protease inhibitor mixture (Roche Molecular Biochemicals) and 1 mM phenylmethylsulfonyl fluoride. After centrifugation at 700 × *g* for 5 min to generate a PNS, the PNS was further centrifuged at 100,000 × *g* for 1 h to generate a total membrane fraction. The total membrane fraction that contains ~10 mg of proteins was further subjected to immunoprecipitation of BACE or PLSCR1, as described (22, 28).

Flotation of Detergent-insoluble Lipid Microdomains—After washing with phosphate-buffered saline twice, the cells were harvested and suspended in buffer L (150 mM NaCl, 50 mM phosphate buffer (pH 7.2), 1% Lubrol WX (Serva Electrophoresis), 1× protease inhibitor mixture) and incubated for 30 min on ice. The cell lysates were then adjusted to a final concentration of 45% sucrose by the addition of an equal volume of 90% sucrose and placed at the bottom of an Ultracentrifuge tube and then overlaid with 35 and 5% sucrose solutions containing phosphate-buffered saline. The discontinuous sucrose gradient was centrifuged at 160,000 × *g* for 20 h in an SW41 rotor (Beckman Instruments) and fractionated from the top of the gradient with Piston gradient fractionator (BioComp Instruments). An equal volume of each fraction was used for immunoblotting or immunoprecipitation. For preparation of the lipid microdomain from SH-SY5Y cells, 3×10^8 cells were used, and endogenous BACE was immunoprecipitated from each fraction with anti-BACE antibody (MAB5308) and detected by immunoblotting using MAB5308.

RESULTS

Phospholipid Scramblase 1 Interacts with the Cytoplasmic Domain of BACE—As shown previously, the short cytoplasmic tail of BACE is essential for its endosomal distribution in cells (14). To better understand the molecular mechanism of BACE trafficking, BACE tail-interacting proteins were searched with the yeast two-hybrid method. From a HeLa cDNA library, we obtained a cDNA encoding the phospholipid scramblase 1 (*PLSCR1*) gene. PLSCR1 was first identified from the erythrocyte plasma membrane and shown to mediate calcium dependent transbilayer movement of membrane phospholipids *in vitro* (29–31). To gain information on the binding region of PLSCR1, several deletion mutants were constructed and assayed for interaction with BACE tail by the yeast two-hybrid system. As depicted in Fig. 1*A*, a segment of PLSCR1 that interacts with BACE appeared to map to the N-terminal half of PLSCR1. We also found that substitution of the dileucine at the C terminus of the BACE tail to alanine drastically reduced the molecular interaction in the two-hybrid system and the *in vitro* binding assay using the recombinant proteins (Fig. 1, *B–D*). The Ser^{498} residue, which has been shown to be phosphorylated *in vivo* and involved in the regulation of the endosomal transport of BACE (15), was not essential for the interaction with PLSCR1. Moreover, the cytoplasmic tails of other type I integral membrane proteins such as APP695, CI-MPR, or BACE2 were found to be incompetent to bind with the BACE tail (Fig. 1, *B–D*). These results suggest that the molecular interaction between BACE and PLSCR1 is specific and dependent on the C-terminal dileucine residues of the BACE tail.

Direct Interaction between BACE and PLSCR1 in Vivo—From its primary structure, PLSCR1 is predicted to be an integral membrane protein with a single hydrophobic stretch at the C terminus. Moreover, recent studies revealed that PLSCR1 receives phosphorylation and palmitoylation at the N-terminal region *in vivo* (32, 33), suggesting that PLSCR1 is a type II membrane protein. As shown in Fig. 2, a differential solubilization experiment showed that both BACE and PLSCR1 were solubilized only with detergent treatment but not with other reagents such as alkali, high salt, or urea. This result indicates that PLSCR1 is an integral membrane protein. We next examined the interaction of BACE and PLSCR1 *in vivo* by means of co-immunoprecipitation experiments. HeLa cells expressing wild-type BACE (BACE-WT) were incubated in the absence or presence of DSP, a membrane-permeable chemical cross-linker. After quenching, cells were harvested, and BACE-WT was immunoprecipitated by a specific antibody. As shown in Fig. 2, ~1.5% of cellular PLSCR1 was coprecipitated with BACE in the absence of DSP, and precross-linking increased the amount of coprecipitated PLSCR1 up to ~6.5% of total PLSCR1. Next, we examined the molecular interaction between endogenous PLSCR1 and expressed wild-type or dileucine motif mutant (AA-mutant; BACE-AA) BACE. HeLa cells transfected with BACE-WT or BACE-AA were lyzed, and endogenous PLSCR1 was immunoprecipitated with an anti-PLSCR1 antibody. Wild-type but not AA-mutant BACE was coprecipitated with PLSCR1 (Fig. 2*C*).

To further confirm the *in vivo* interaction between BACE and PLSCR1 at an endogenous expression level, total membrane fractions prepared from neuroblastoma SH-SY5Y cells were subjected to immunoprecipitation analysis. As shown in Fig. 2*D*, PLSCR1 was coprecipitated with BACE in the absence of the cross-linker, and a higher amount of PLSCR1 was coprecipitated in the presence of DSP. These results strongly suggest that BACE forms a protein complex with PLSCR1 *in vivo*, and the interaction depends on the dileucine residues of the BACE tail.

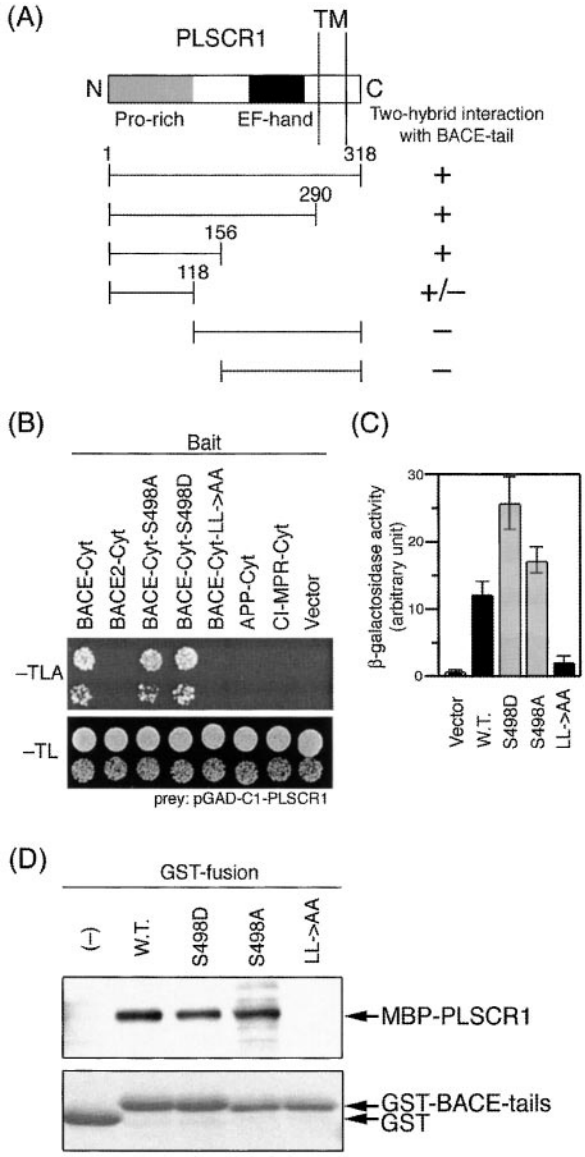

FIG. 1. **Yeast two-hybrid assay of the interaction between BACE tail and PLSCR1.** In *A*, the primary structure of PLSCR1 is illustrated. The proline-rich region (*Pro-rich*) and Ca^{2+} binding domain (*EF-hand*) are indicated by *gray* and *black boxes*, respectively. PJ69–4A cells harboring pGBD-BACE-tail were transformed with PLSCR1 deletion constructs and their interactions assayed. *TM*, transmembrane region. *B*, interaction between PLSCR1 and BACE tail mutants. PJ69–4A cell harboring pGAD-C1-PLSCR1 was transformed with pGBD-C1-BACE-tail mutants (S498A, S498D, or L499, 500A, indicated as *LL* → *AA*) or pGBD vectors harboring cytoplasmic tails of BACE2, APP695, or CI-MPR and examined for growth on SD (−Trp-Leu-Ade) (indicated as −*TLA*) and SD (−Trp-Leu) (indicated as −*TL*) plates. *C*, β-galactosidase activity of the transformants shown in *panel B*. *D*, *in vitro* binding of PLSCR1 with BACE tails. Interaction of MBP-PLSCR1 with the GST-BACE-tail was examined as described under "Experimental Procedures." The bound MBP-PLSCR1 was detected with an anti-MBP antibody.

BACE Is Colocalized with PLSCR1 in Vivo—As reported previously, ectopically expressed BACE is localized in the post-Golgi organelles including the Golgi apparatus, TGN, endosomes, and plasma membrane (14). Although PLSCR1 has been reported to be localized mainly in the plasma membrane (34), the accurate distribution remains uncertain. Thus, we examined the intracellular localization of PLSCR1 and BACE precisely by immunofluorescent microscopy. Endogenous PLSCR1 was localized mainly in the plasma membrane,

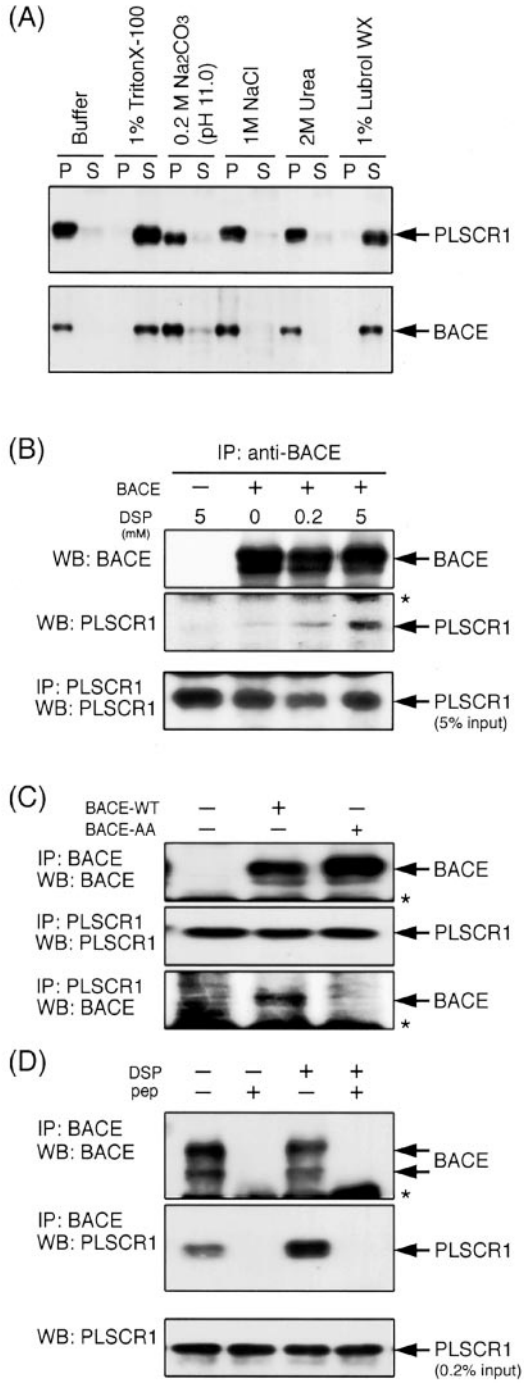

FIG. 2. *A*, differential solubilization of PLSCR1. PNS from HEK293 cells transfected with pcDNA-HB-full was treated with the reagents indicated on ice for 30 min and then centrifuged at 100,000 × *g* for 1 h to generate the precipitated (*P*) and supernatant (*S*) fractions. Each fraction was subjected to immunoblotting of BACE and PLSCR1 with anti-BACE-lum and anti-PLSCR1 (4D2) antibodies, respectively. *B*, co-immunoprecipitation of BACE and PLSCR1. HEK293 cells transiently expressing BACE were harvested and treated with the indicated concentrations of DSP on ice for 30 min. After quenching of DSP, cells were lysed, BACE was immunoprecipitated (*IP*) with MAB5308, and the immunoprecipitates were subjected to immunoblotting of BACE and PLSCR1 with anti-BACE-cyt and anti-PLSCR1 (4D2) antibodies, respectively. 5% of immunoprecipitated PLSCR1 from the cell lysate was subjected to immunoblotting as a control. *WB*, Western blot. *C*, dileucine-dependent interaction of BACE and PLSCR1. HEK293 cells transiently expressing BACE-WT or BACE-AA were lysed, and endogenous PLSCR1 was immunoprecipitated with anti-PLSCR1 monoclonal antibody 4D2. The immunocomplex was subjected to immunoblotting of BACE (MAB5308) and PLSCR1 (4D2). As shown in *D*, endogenous BACE interacts with PLSCR1 in neuroblastoma cells. Total membrane fraction was prepared from SH-SY5Y cells, the membranes were incubated in the absence or presence of 0.3 mM DSP, and endogenous BACE was immunoprecipitated with anti-BACE tail antibody (MAB5308) in the absence or presence of excess amount of the BACE tail synthetic peptide (*Pep*). The immunocomplex was subsequently subjected to immunoblotting of BACE (MAB5308) and PLSCR1 (4D2). 0.2% of membrane fraction used for immunoprecipitation of BACE was subjected to immunoblotting of PLSCR1 (4D2) as a control. *Asterisks* indicate IgG.

whereas positive signals were also detected in the perinuclear Golgi area, which were also positive for CI-MPR immunofluorescence in HeLa cells (Fig. 3*A*, *a–c*). In HeLa cells transiently expressing BACE-EGFP, BACE and PLSCR1 were also colocalized in the Golgi compartment and at the cell surface (Fig. 3*A*, *g–i*), suggesting colocalization at these organelles. In addition, at the peripheral punctate structures, partial colocalization of both proteins was observed (Fig. 3*A*, *g–l*), suggesting the localization of PLSCR1 in endosomal compartments. Essentially, similar staining patterns were observed in other cultured cell lines, such as human embryonic kidney (HEK293) and human neuroblastoma (SH-SY5Y) cell lines.[2]

Intracellular Trafficking of BACE and PLSCR1 via a U18666A-sensitive Route—To clarify the routes in which the two proteins are trafficking, HeLa cells expressing BACE-EGFP were treated with U18666A. U18666A is a class II amphiphile and known to cause the selective accumulation of low density lipoprotein-derived cholesterol in late endocytic compartments and blockage of the outward protein transport from the endosomal compartments (35, 36). As shown in Fig. 3, U18666A treatment induced a drastic co-redistribution of BACE-EGFP and PLSCR1 to the perinuclear large punctate structures (Fig. 3*A*, *m–r*). As has been shown previously, these structures are positive for immunofluorescence of CI-MPR (35), suggesting that the compartments are derived from late endosomes (Fig. 3*A*, *d–f*). For further analysis of the structures, immunoelectron microscopic analysis was carried out with the HeLa cells transiently cotransfected with BACE-EGFP and hemagglutinin-tagged-PLSCR1 expression vectors. U18666A treatment revealed that these proteins were colocalized in multivesicular bodies (Fig. 3*B*), and the BACE-positive multivesicular bodies were also co-labeled with a monoclonal antibody against a late endosome-specific lipid, lisobisphosphatidic acid (LBPA, 36),[2] suggesting that the compartments are derived from the late endosomes (36, 37). Furthermore, the redistribution of PLSCR1 was restored 4 h after washing out of U18666A.[2] These results suggest that both BACE and PLSCR1 are actively trafficking through the overlapped pathway, including late endosomal compartments.

BACE Is Cofractionated with PLSCR1 in the Lipid Microdomain—Recent studies have suggested that APP and its processing activities, namely presenilins, α-secretase activity, and BACE accumulate in detergent-insoluble, cholesterol-enriched, membrane microdomains called DIGs (21, 38, 39). PLSCR1 was also reported to be a component of lipid raft in human oral epithelial carcinoma (22). We next examined whether BACE and PLSCR1 could be fractionated in the lipid microdomain at an endogenous expression level in a neuronal cell line. SH-SY5Y human neuroblastoma cells were treated with a nonionic detergent Lubrol WX, and low buoyant, lipid-associated proteins were separated by flotation in a discontinuous sucrose gradient. As shown in Fig. 4, BACE and PLSCR1 were fractionated in the DIG fractions, which contain known DIG proteins such as flotilin-1 and APP. Lamp-1, a late endosomal integral membrane protein, was not fractionated in DIGs, as reported previously (40). Next, to examine the functional con-

[2] S. Kametaka, unpublished results.

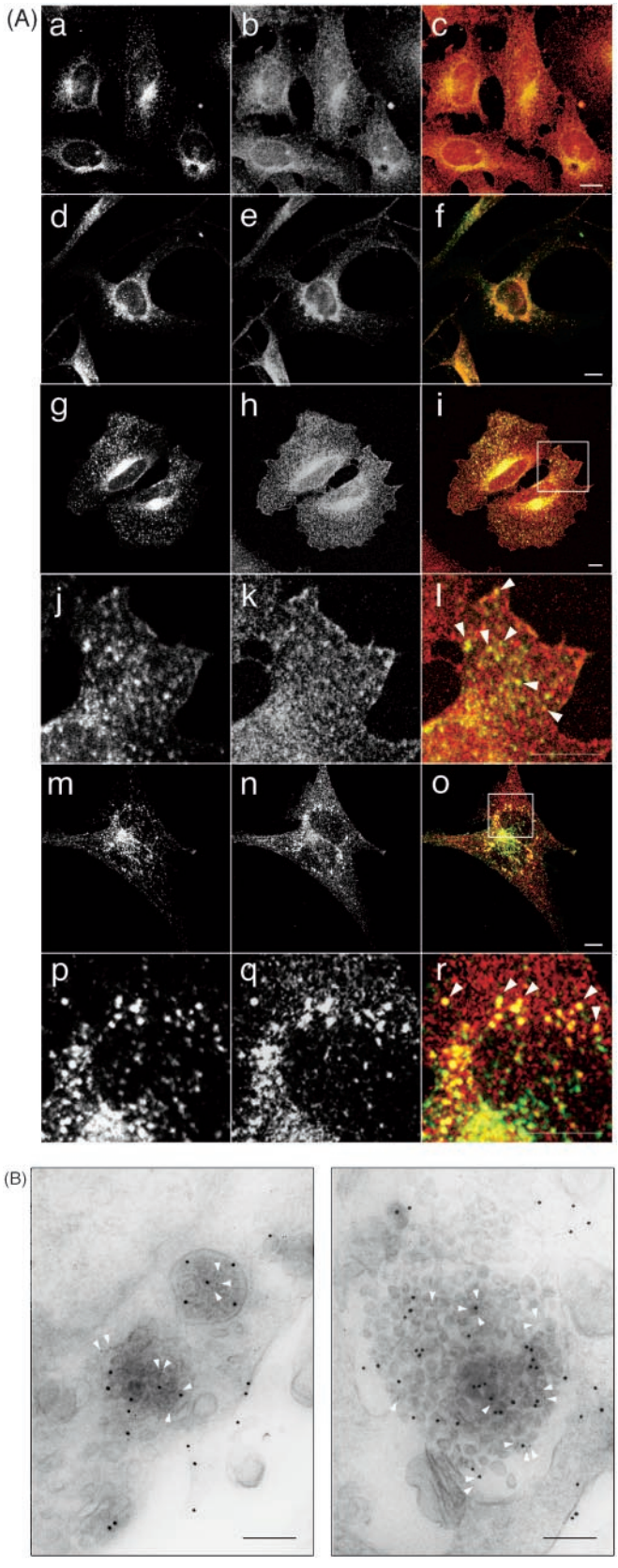

FIG. 3. **Co-localization of BACE and PLSCR1.** As shown in *A*, HeLa cells (*a–f*) or HeLa cells transiently expressing BACE-EGFP (*g–r*) were incubated for 20 h in the absence (*a–c* and *g–l*) or presence (*d–f* and *m–r*) of 3 μg/ml U18666A. After fixation and permeabilization, the cells were labeled with anti-CI-MPR (*a* and *d* and *green image* in *c* and *f*) and anti-PLSCR1 (*b*, *e*, *h*, *k*, *n*, and *q* and *red image* in *c*, *f*, *i*, *l*, *o*, and *r*). Localization of BACE-EGFP is shown in *g*, *j*, *m*, and *p* (*green image* in *i*, *l*, *o*, and *r*). Merged images are shown (*c*, *f*, *i*, *l*, *o*, and *r*). The *boxed* areas indicated in *i* and *o* are shown in higher magnification in *j–l* and *p–r*, respectively. *Scale bars*, 10 μm. As shown in *B*, HeLa cells expressing BACE-EGFP and 3HA-PLSCR1 were treated with U18666A and examined by immunoelectron microscopy using anti-GFP (15 nm-gold) and anti-hemagglutinin (5 nm-gold: *arrowheads*) antibodies. *Scale bar*, 200 nm.

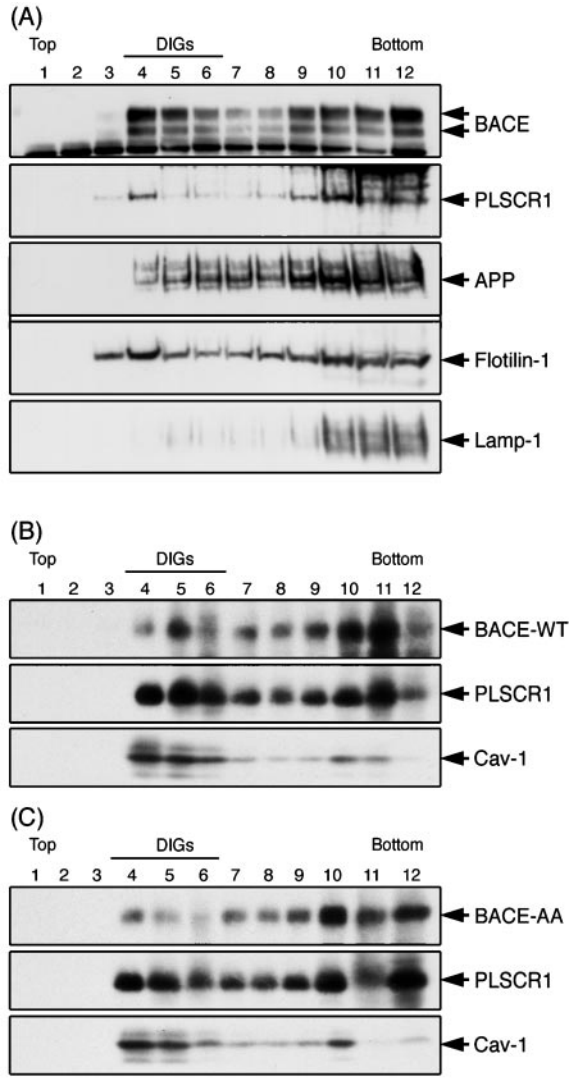

FIG. 4. **Co-fractionation of BACE and PLSCR1 in the low buoyant lipid microdomain.** *A*, SH-SY5Y neuroblastoma cells were harvested and treated with 1% lubrol WX on ice for 30 min and subjected to sucrose discontinuous density gradient centrifugation as described under "Experimental Procedures." From each fraction, BACE was immunoprecipitated with anti-BACE antibody (MAB5308) and detected by immunoblotting using MAB5308. The *asterisk* indicates IgG. For detection of other proteins, each fraction was directly used for immunoblot analysis. As shown in *B* and *C*, HEK293 cells transiently expressing BACE-WT (*B*) or BACE-AA (*C*) were subjected to the DIG preparation carried out as in *panel A*. Each fraction was used for immunoblotting with anti-BACE, anti-PLSCR1, or anti-caveolin-1 (*Cav-1*) antibody. Fraction 4 contains the 5–35% interface of sucrose gradient, and the detergent insoluble lipid microdomain fractions are indicated as DIGs.

sequence of the dileucine residues of the BACE tail in recruitment of BACE into the lipid microdomain, HEK293 cells stably overexpressing BACE-WT or BACE-AA were subjected to DIG preparation. PLSCR1 was fractionated into the DIG fractions as well as wild-type BACE (Fig. 4). Although BACE-AA was also fractionated in the DIG fractions, the ratio of DIG-associated BACE-AA varies in each experiment, whereas BACE-WT, PLSCR1, and caveolin-1 were reproducibly fractionated in the

DIG-fraction through five independent experiments. These results suggest that the BACE-PLSCR1 molecular interaction is not essential for the recruitment of BACE into DIGs, but it may be involved in the stable association of BACE with the lipid microdomain.

DISCUSSION

PLSCR1 Is a β-Secretase Binding Partner—A series of recent functional analyses revealed that the C-terminal cytoplasmic region is required for the correct intracellular localization of BACE (14, 15). Although the mutational analysis of the BACE tail uncovered that the BACE tail contains several sites for post-translational modification and signals for intracellular trafficking of BACE (14, 15, 41), the molecular mechanisms responsible for the tail-dependent cellular trafficking of BACE were still unknown. In the current study, our yeast two-hybrid screening implicated PLSCR1 as a novel BACE interacting molecule. PLSCR1 was first identified as a plasma membrane protein that has phospholipid scrambling activity *in vitro* (29, 30). PLSCR1 was also shown to physiologically and functionally interact with epidermal growth factor receptors and other cell surface growth factor receptors, as well as with intracellular kinases that are known to be activated by these receptors. Nevertheless, the biological function of PLSCR1 in growth factor-regulated proliferation and differentiation remains to be completely elucidated (22, 32, 42). We showed here that BACE forms a protein complex with PLSCR1 in neuronal cells under normal conditions, and the dileucine residues of the BACE tail were revealed to be essential for the physical interaction with PLSCR1 *in vitro* and *in vivo*. Generally, cytoplasmic dileucine residues of integral membrane proteins can be utilized as an endosomal retention signal or an internalization signal from the plasma membrane (43). In the case of BACE, indeed, the BACE-AA mutant in which the dileucine residues are substituted with alanine accumulated on the plasma membrane.[2] A previous study also revealed that the dileucine mutant exhibited defects in internalization from the plasma membrane and is more readily recycled to the cell surface from endosomal compartments (14). These results allow us to imply that PLSCR1 is involved in the dileucine-dependent transport of BACE.

U18666A-dependent Redistribution of BACE and PLSCR1—As has been shown previously, BACE is endocytosed from the plasma membrane in a dileucine residue-dependent manner (14). Moreover, our photobleaching analysis in living cells showed that BACE-EGFP in the peripheral area can be transported back to the perinuclear Golgi area,[2] suggesting that BACE is dynamically trafficking between the Golgi area and plasma membrane, presumably via endosomal compartments. The present study also demonstrates that PLSCR1 is localized in intracellular organelles besides the plasma membrane. Furthermore, U18666A treatment led to the redistribution of PLSCR1 in perinuclear punctate structures, which were positive for BACE-EGFP (Fig. 3) but only slightly positive for BACE-AA-EGFP.[2] This finding suggests that BACE is transported to the late endosomal compartments and that the dileucine residues are required for targeting of BACE to late endosomes from the cell surface. Our immunoelectron microscopic observations supported the notion that BACE and PLSCR1 are colocalized in the enlarged, multivesicular endosomes in U18666A-treated cells. Although the molecular mechanism of how U18666A perturbs the endosomal transport remains unclear, the findings herein suggest that PLSCR1 moves in the intracellular compartments via a pathway, which overlaps with that of BACE.

PLSCR1 Is a Raft-resident Protein as Well as BACE in Neuronal Cell Lineage—Thus far, a series of studies focusing on the relationship between Aβ formation and cellular cholesterol have revealed that cholesterol is required for Aβ production (44). Moreover, the correct intracellular distribution of cholesterol is also important for Aβ metabolism (45), and in addition, the major components required for APP processing, such as presenilin-1 and -2, APP, α-secretase activity, and BACE, have been fractionated into the cholesterol-enriched, low buoyant lipid microdomain called DIGs (20, 38, 39, 46, 47). Moreover, we recently reported that PLSCR1 is also a component of lipid microdomain (22, 33). In this study, we showed that both BACE and PLSCR1, as well as APP, are components of the lipid microdomains in a neuronal cell line, SH-SY5Y cells, at an endogenous expression level. Furthermore, interestingly, BACE-WT, but not BACE-AA was reproducibly fractionated in the DIG fractions, suggesting that the dileucine residues may be involved in efficient recruitment of BACE into DIGs or in stable retention of BACE in the lipid microdomain. Although the physiological function of PLSCR1 is still unclear, it was shown that recombinant PLSCR1 protein solely has a phospholipid-scrambling activity *in vitro* (30, 31). This leads to an implication that PLSCR1 might alter the local composition or topology of plasma membrane phospholipids so as to influence the process of endocytosis of BACE and/or potentially other cell surface components, such as the epidermal growth factor receptor (22). Recently, a family of GGA adapter proteins has been shown to directly interact with the BACE tail in a dileucine-dependent manner (48, 49). Moreover, the molecular property of the GGA-BACE interaction was found to be different from that of the PLSCR1-BACE interaction; GGAs require both the acidic amino acid cluster (^{495}DD^{496}) and dileucine in the BACE tail for physical interaction (49), whereas PLSCR1 does not require the acidic cluster.[2] At present, the functional consequence of these adapter molecules on BACE trafficking is also unclear. Further functional analysis of the BACE interacting molecules will clarify the complex molecular mechanisms responsible for the intracellular trafficking of BACE and regulatory mechanisms of Aβ formation.

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