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-SY5Y cells, and the dileucine residue of the BACE tail is also revealed to be essential for 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), 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 (β-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 Glu11-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 caveola-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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PLSCR1, and 1 mM phenylmethylsulfonyl fluoride. After centrifugation at 100,000 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 mM NaCl, 0.2 M Na2CO3 (pH 11.0), 2 mM urea, or 1% Triton X-100 on ice for 30 min. After incubation, each sample was centrifuged at 100,000 x 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 pHBl1-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 100 mM phosphate buffer (pH 7.0) 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 buffer and 1 mM phenylmethylsulfonyl fluoride. The cell lysates were then centrifuged at 100,000 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 mM NaCl, 0.2 M Na2CO3 (pH 11.0), 2 mM urea, or 1% Triton X-100 on ice for 30 min. After incubation, each sample was centrifuged at 100,000 x 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.

Flotation of Detergent-insoluble Lipid Microdomains—After washing with phosphate-buffered saline twice, the cells were harvested and suspended in buffer X (50 mM phosphate buffer [pH 7.5], 150 mM NaCl, 1 mM CaCl2, 5 mM MgCl2, 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 1/2 volume of 1× 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 the expression of BACE and PLSCR1. The total membrane fraction that contains 10 mg of proteins was further subjected to immunoprecipitation of BACE or PLSCR1, as described (22, 28).

Floation 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. 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).
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. 1A, 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. 1B-D). The Ser<sup>298</sup> residue, which has been shown to be phosphorylated in vitro 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 APP<sup>695</sup>, CI-MPR, or BACE2 were found to be incompetent to bind with the BACE tail (Fig. 1B–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 lysed, and endogenous PLSCR1 was immunoprecipitated with an anti-PLSCR1 antibody. Wild-type but not AA-mutant BACE was coprecipitated with PLSCR1 (Fig. 2C).

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. 2D, 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.

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,
was subjected to immunoblotting as a control.

respectively. 5% of immunoprecipitated PLSCR1 from the cell lysate and PLSCR1 with anti-BACE-cyt and anti-PLSCR1 (4D2) antibodies, the immunoprecipitates were subjected to immunoblotting of BACE were lysed, BACE was immunoprecipitated (IP) concentrations of DSP on ice for 30 min. After quenching of DSP, cells were harvested and treated with the indicated fractions. Each fraction was prepared from SH-SY5Y cells, the membranes were incubated in the absence or presence of 0.3 mM DSP, and endogenous BACE and PLSCR1 were fractionated in the lipid microdomain at an endogenous expression level in a neuronal cell line. SH-SY5Y human neuroblastoma cells were treated with a non-ionic 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 lipid microdomain, which contain known DIG proteins, at the peripheral punctate structures, partial colocalization of both proteins was observed (Fig. 3A, g–i), suggesting colocalization at these organelles. In addition, at the peripheral punctate structures, partial colocalization of both proteins was observed (Fig. 3A, g–i), suggesting 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. 3A, 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. 3A, 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. 3B), and the BACE-positive multivesicular bodies were also co-labeled with a monoclonal antibody against a late endosome-specific lipid, laosibiosphatidic 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, a-secretase activity, and BACE accumulate in detergent-insoluble, cholesterol-rich, 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 non-ionic 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 flotillin-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-
sequence of the dileucine residues of the BACE tail in recruit-
ment 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-associ-
ated BACE-AA varies in each experiment, whereas BACE-WT,
PLSCR1, and caveolin-1 were reproducibly fractionated in the
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 express-
ing 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. 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

FIG. 4. Co-fractionation of BACE and PLSCR1 in the low buoy-
ant lipid microdomain. A, SH-SY5Y neuroblastoma cells were har-
vested 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 im-
munoprecipitated 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 immu-
noblot analysis. As shown in B and C, HEK293 cells transiently ex-
pressing BACE-WT (B) or BACE-AA (C) were subjected to the DIG
preparation carried out as in panel A. Each fraction was used for immu-
noblotting 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.
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, we observed 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 microdomains termed 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 (495DD496) 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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