Presenilin-1 uses phospholipase D1 as a negative regulator of β-amyloid formation

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Presenilin (PS1/PS2) is a major component of γ-secretase, the activity that mediates proteolysis of β-amyloid precursor protein to generate β-amyloid (Aβ). Here we demonstrate that PS1, through its loop region, binds to phospholipase D1 (PLD1), thereby recruiting it to the Golgi/trans-Golgi network. Overexpression of wild-type PLD1 reduces Aβ generation. Conversely, down-regulation of endogenous PLD1 by small hairpin RNA elevates Aβ production. The Aβ-lowering effect of PLD1 is independent of its ability to promote vesicular budding of β-amyloid precursor protein. The data indicate that overexpression of PLD1 decreases, and down-regulation of PLD1 increases, the catalytic activity, and the association of the subunits, of γ-secretase.

β-amyloid precursor protein | γ-secretase complex activity | negative regulator | protein interaction | trans-Golgi network

Most early-onset, familial Alzheimer's disease (FAD) cases are linked to mutations in presenilin (PS1/PS2) genes (1). PS is a major component of γ-secretase, the activity that mediates proteolysis of β-amyloid precursor protein (βAPP) to generate β-amyloid (Aβ) (2, 3). Expression of autosomal dominant variants of PS results in increased $Aβ_{42}$ production, leading to amyloid plaque deposition in the brains of patients and early onset of disease (3–5). PS interacts with three other membrane proteins (nicastrin, APH1, and PEN2) to form high-molecular-weight complexes with γ-secretase activity that cleave type I membrane proteins including βAPP and Notch-1 (5–11). The demonstration that coexpression of these four proteins in *Saccharomyces cerevisiae*, which lacks endogenous γ-secretase activity, is sufficient to reconstitute γ-secretase activity (12) confirms that these four molecules are the major core components of the γ-secretase complex. Much attention in the Alzheimer's disease field is being devoted to the characterization of γ-secretase and identification of factors that regulate its activity.

The lipid composition of cellular membranes appears to regulate the production of Aβ species (13, 14). For example, it was reported that phosphatidylcholine and sphingomyelin increase γ-secretase activity without changing cleavage specificity (13). Interestingly, disordered metabolism of membrane phospholipids has been reported in Alzheimer's disease (15, 16). In the present study, a possible role for phospholipase D1 (PLD1), a phospholipid-modifying enzyme, in regulating γ-secretase activity has been explored.

Results

PS1, Through Its Loop Region, Interacts with and Recruits PLD1 to the Golgi/Trans-Golgi Network (TGN). To investigate the modulation of PS1-regulated βAPP processing and trafficking (5–11, 17–21), a variety of factors known to be important in regulating protein trafficking were examined, including Rab11, RhoA, and PLD family and Arf family members. Among these factors, only PLD1 showed an interaction with PS1. The interaction between PS1 and PLD1 was demonstrated by coimmunoprecipitation (co-IP) from wild-type (wt) ES cell lysates (Fig. 1*a*). To confirm the specificity of the interaction, lysates derived from *PS1/PS2*-deficient ES cells were used, and no co-IP signals were detected. In addition, no co-IP signals were detected between PS1 and PLD2, the other PLD family member (Fig. 1*a Bottom*), or between PS1 and Rab11.

We next investigated the region of PS1 that interacts with PLD1. Antibodies specific for the PS1 loop region or the PS1 N-terminal fragment (NTF) were separately included in a permeabilized cell-free system derived from ES wt cells, as a means of interfering with the association of PS1 and PLD1. Anti-rabbit IgG antibodies were used as a control (Fig. 1*b*, lane 1). After incubation, membranes were lysed, and the interaction between PS1 and PLD1 was determined by co-IP. The results show that the amount of PLD1 associated with PS1 was decreased in the presence of PS1 loop antibody but was unchanged in the presence of PS1 NTF antibody (Fig. 1*b*).

The subcellular localization of PLD1 in PS1wt cells was compared with that in PS1-deficient cells by immunofluorescence microscopy studies. PLD1 in wt cells was localized in cytosol and in the Golgi/TGN, whereas PS1 deficiency resulted in a diffuse, mostly cytosolic, distribution of PLD1 (Fig. 1*c*; note lack of colocalization with the TGN marker, γ-adaptin). These observations indicate that the interaction of PLD1 with a specific region of PS1 may be required for PLD1 recruitment to the Golgi/TGN.

PLD1 Inhibits Aβ Production. Because PS1 regulates βAPP processing to generate Aβ, we next examined the effects of PLD1 on Aβ generation in N2a cells expressing FAD PS1 mutant ΔE9. Overexpression of PLD1 reduced the levels of secreted and intracellular Aβ to 61% and 57% of controls, respectively, and increased the levels of soluble βAPPα (sβAPPα) and β-C-terminal fragment (βCTF) by 73% and 46%, respectively (Fig. 2*a*). The level of full-length βAPP was not significantly changed by overexpression of PLD1. Surface-enhanced laser desorption/ionization mass spectrometry analysis of media from PS1ΔE9 cells (Fig. 2*b*) and from PS1wt cells (data not shown), after overexpression of PLD1, showed a proportional decline in $Aβ_{1–38}$, $Aβ_{1–40}$, and $Aβ_{1–42}$.

Conversely, down-regulation of PLD1 by small hairpin RNA (shRNA) (≈72% reduction in PLD1 protein levels) in PS1ΔE9

Conflict of interest statement: No conflicts declared.

Abbreviations: FAD, familial Alzheimer's disease; PS, presenilin; βAPP, β-amyloid precursor protein; Aβ, β-amyloid; PLD, phospholipase D; TGN, trans-Golgi network; wt, wild type; co-IP, coimmunoprecipitation; NTF, N-terminal fragment; sβAPP, soluble βAPP; CTF, C-terminal fragment; shRNA, small hairpin RNA; NICD, Notch intracellular domain; IP, immunoprecipitation; HA, hemagglutinin.

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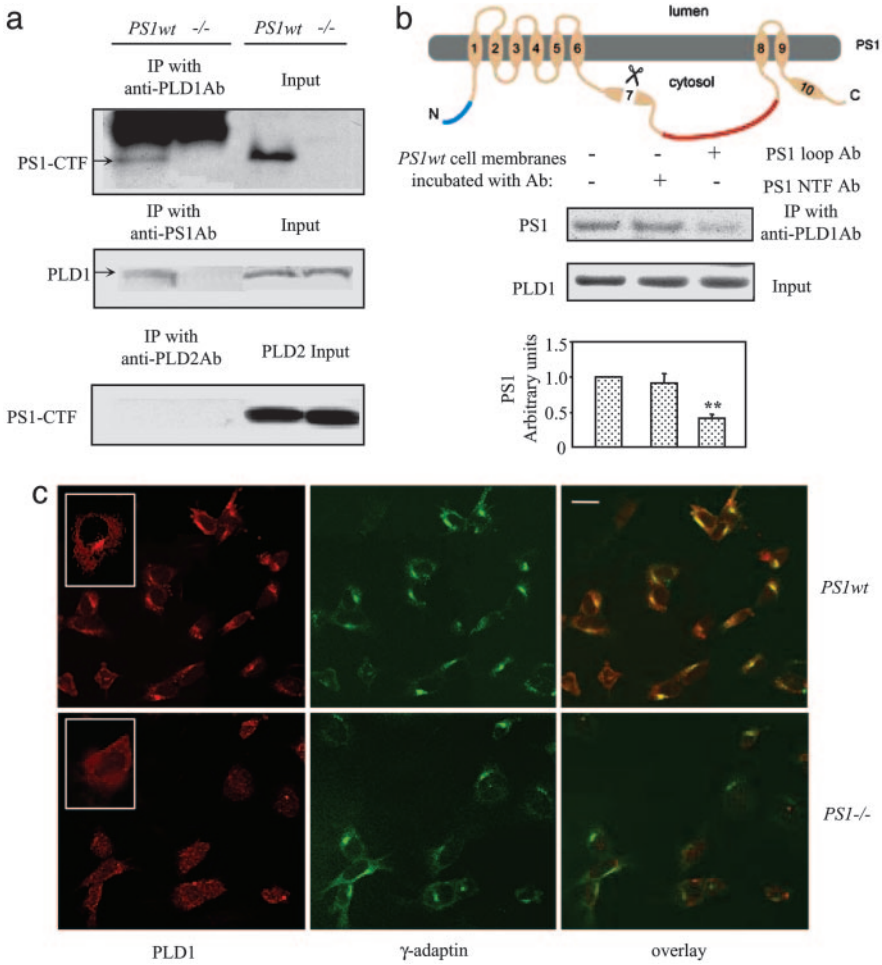

Fig. 1. PS1, through its loop region, interacts with and recruits PLD1 to the Golgi/TGN. (*a*) Protein lysates from ES cells derived from PS1/PS2wt and $PS1^{-/-}/PS2^{-/-}$ mice were immunoprecipitated with anti-PLD1 antibody followed by immunoblotting with anti-PS1 loop antibody, or vice versa. Ten percent of total protein lysate was loaded as input. Alternatively, ES cells overexpressing HA-tagged PLD2 were lysed, and protein lysates were immunoprecipitated with HA antibody followed by immunoblotting with anti-PS1 loop antibody. (*b*) The interaction between PS1 and PLD1 was assayed in a cell-free system derived from ES cells (PS1/PS2wt) with added anti-PS1 NTF antibody (epitope in blue), anti-PS1 loop antibody (epitope in red), or anti-rabbit IgG antibody used as a control. After incubation, the cell-free system was diluted into IP buffer and immunoprecipitated by anti-PLD1 antibody followed by immunoblotting with anti-PS1 antibody. Graph shows mean ± SE of three experiments. **, $P < 0.001$; Student's *t* test. (*c*) Fibroblasts derived from $PS1^{+/+}$ and $PS1^{-/-}$ mice were immunolabeled for endogenous PLD1 by antibody P1-P4 followed by rhodamine-conjugated secondary antibody (red fluorescence). (*Insets*) Enlargement of typical cells. γ-adaptin was used as a marker for Golgi apparatus and TGN (green fluorescence). Overlays represent digitally merged images. Yellow fluorescence indicates colocalization of PLD1 with γ-adaptin. (Scale bar, 10 μm.)

cells (Fig. 2*c*) increased the levels of secreted and intracellular Aβ by 117% and 274%, respectively, and decreased the level of βCTF by 83% (Fig. 2*c*). The levels of full-length βAPP and sβAPPα were not significantly changed by down-regulation of PLD1 in these cells. In PS1wt cells, PLD1 shRNA increased secreted and intracellular Aβ levels to 163% and 247%, respectively, and reduced the level of βCTF by 89%.

PLD1 Inhibits γ-Secretase Activity via a Mechanism Distinct from Its Effect on βAPP Trafficking. We further explored the mechanism responsible for the Aβ-lowering effects of PLD1 by taking advantage of the fact that overexpression of wt PLD1 increased trafficking of βAPP, whereas the catalytically inactive mutant PLD1 (K898R) failed to do so (22). Overexpression of catalytically inactive PLD1 (K898R) in FAD PS1 mutant cells reduced intracellular and secreted Aβ levels by 28% and 65%, respectively (Fig. 3*a*), under conditions in which this mutant PLD1 failed to affect βAPP trafficking (22). In addition, this mutant PLD1 retained the ability of wt PLD1 to interact with PS1 CTF (Fig. 3*b*). These results indicate that PLD1 suppression of Aβ generation can occur in the absence of any effect on βAPP trafficking from the TGN.

Inhibition of γ-secretase activity results in inhibition of the S3 cleavage of Notch-1, another substrate of γ-secretase (23). Overexpression of PLD1 in PS1ΔE9 cells potently inhibited generation of the Notch intracellular domain (NICD) to ≈30% of control (Fig. 3*c*). The effects of PLD1 on Notch cleavage were similar to its effects on βAPP metabolism. However, PLD1 did not affect vesicular budding of Notch from the TGN (Fig. 3*d*), in contrast to its stimulation of βAPP trafficking (22).

PLD1 Disrupts the Association of γ-Secretase Components. Insight into the mechanism by which PLD1 inhibits the cleavage of βAPP to Aβ came from examining the interactions of γ-secretase components (PS1, PEN2, APH1, and nicastrin) under conditions of overexpression or down-regulation of PLD1. Overexpression of wt PLD1 resulted in decreased association of PEN2 with PS1 CTF, PS1 NTF, nicastrin, and APH1 (Fig. 4*a*). A reduced association between PEN2 and each of these components was also found in cells expressing PLD1 K898R (al-

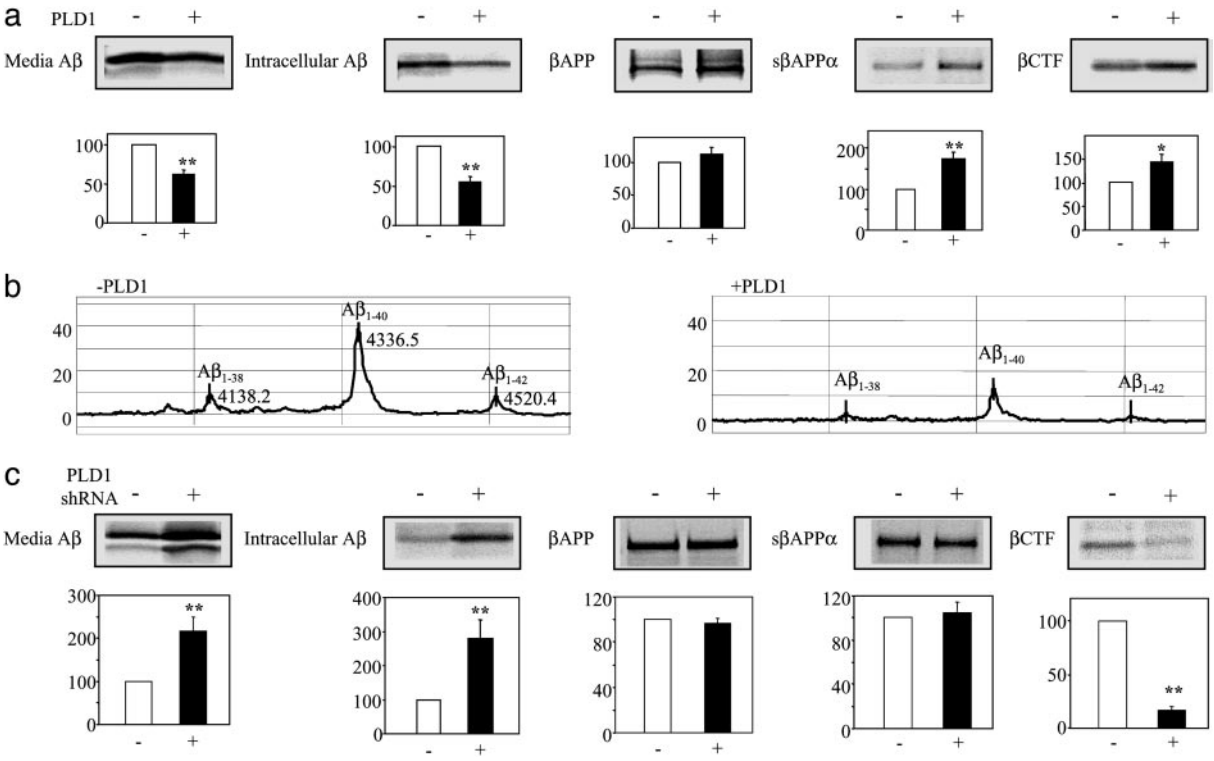

Fig. 2. PLD1 inhibits Aβ production. (*a*) N2a cells expressing PS1ΔE9 were transiently transfected with PLD1wt or mock cDNA. Secreted Aβ, intracellular Aβ, full-length βAPP, sβAPPα, and βCTF levels were compared. *, $P < 0.01$; **, $P < 0.001$; Student's *t* test. (*b*) Surface-enhanced laser desorption/ionization mass spectrographic analysis comparing $Aβ_{1–38}$, $Aβ_{1–40}$, and $Aβ_{1–42}$ secreted from ΔE9 cells transfected with PLD1wt or mock cDNA. (*c*) N2a cells expressing PS1ΔE9 were transiently transfected with control shRNA or PLD1 shRNA. Secreted Aβ, intracellular Aβ, full-length βAPP, sβAPPα, and βCTF levels were compared. **, $P < 0.001$; Student's *t* test.

though to a lesser extent compared with PLD1wt), indicating that this catalytically inactive PLD1 mutant can also interrupt the association of γ-secretase components. Down-regulation of PLD1 by shRNA increased the association of γ-secretase components (Fig. 4*b*). These results support the notion that regulation of PS1–γ-secretase activity by PLD1 occurs through protein–protein interactions and is independent of PLD1 catalytic activity.

In contrast to the interaction between PS1 and PLD1 (Fig. 1*a*), no interaction was detected between PLD1 and PEN2 (Fig. 4*c*)

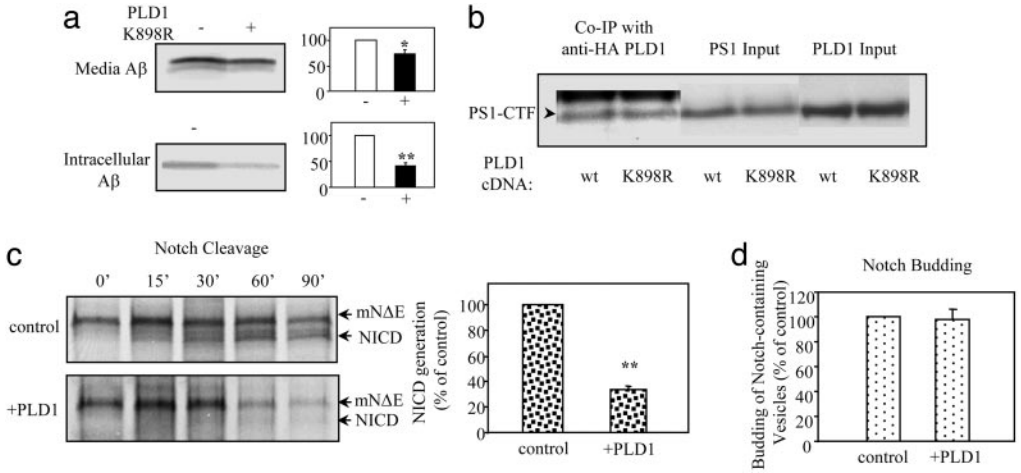

Fig. 3. PLD1 inhibits γ-secretase activity via a mechanism distinct from its effect on βAPP trafficking. (*a*) N2aΔE9 cells were transiently transfected with PLD1 mutant K898R or mock cDNA, and secreted and intracellular Aβ levels were compared. *, $P < 0.01$; **, $P < 0.001$; Student's *t* test. (*b*) ES PS1/PS2 wt cells were transiently transfected with HA-tagged PLD1wt or K898R cDNA. The interaction between PS1 and PLD1 was assayed by IP with anti-HA antibody followed by immunoblotting with anti-PS1 loop antibody. (*c*) N2aΔE9 cells overexpressing myc-tagged mNotchΔE (mNΔE) were cotransfected with either PLD1 cDNA or mock cDNA, followed by pulse labeling with [^{35}S]methionine at 37°C for 30 min, and were chased for 15–90 min. mNotchΔE and its cleavage product, NICD, were immunoprecipitated with anti-myc antibody followed by SDS/PAGE and autoradiography. **, $P < 0.001$; Student's *t* test. The graph shows quantification of NICD generation at 90 min of incubation. (*d*) Cells were permeabilized and incubated at 37°C for 60 min to allow the formation of post-TGN vesicles. mNotchΔE in nascent vesicles was immunoprecipitated by anti-myc antibody followed by SDS/PAGE and autoradiography. Results are expressed as percentage of controls.

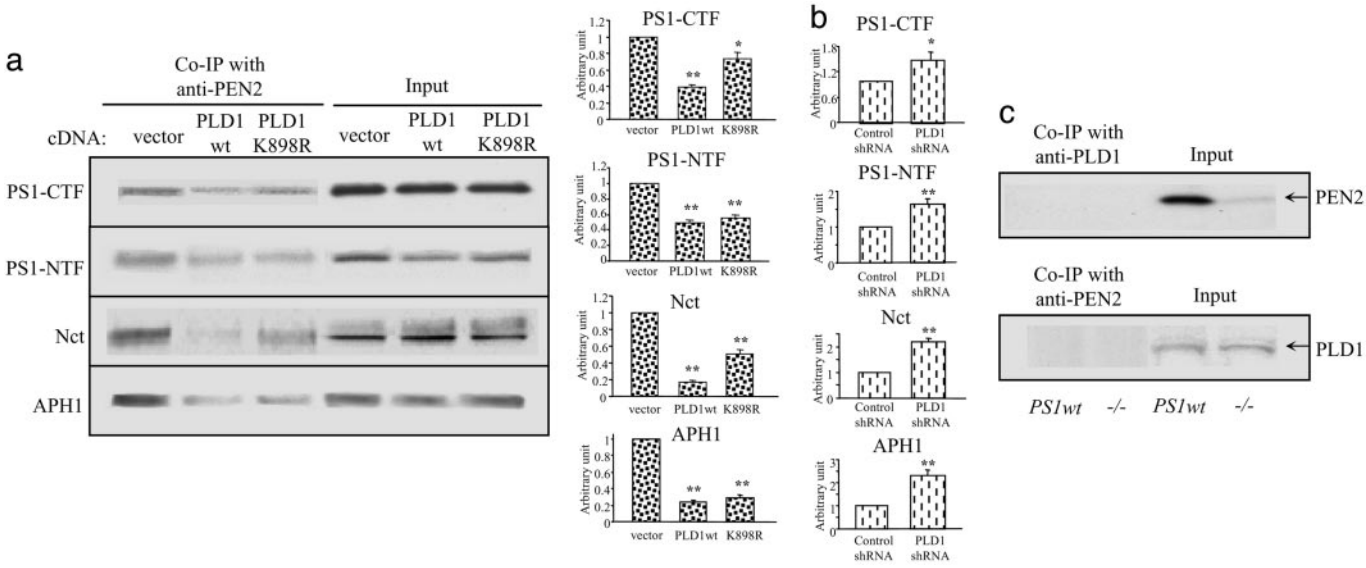

Fig. 4. PLD1 disrupts the association of γ-secretase components. (*a*) Protein lysates from *PS1/PS2* wt ES cells transfected with PLD1wt, PLD1 K898R, or mock cDNA were immunoprecipitated with anti-PEN2 antibody. The precipitates were subjected to SDS/PAGE and immunoblotting with anti-PS1 loop antibody (for PS1 CTF), Ab14 (for PS1 NTF), Ab716 [for nicastrin (Nct)], or anti-APH1 antibody. Ten percent of total protein lysates was loaded and shown as input lanes. Graphs show means ± SE of three experiments. *, $P < 0.01$; **, $P < 0.001$; Student's *t* test. (*b*) Protein lysates from *PS1/PS2* wt ES cells transfected with PLD1 shRNA or control shRNA were immunoprecipitated with anti-PEN2 antibody. The precipitates were subjected to SDS/PAGE and immunoblotting as described for *a*. Graphs show means ± SE of three experiments. *, $P < 0.01$; **, $P < 0.001$; Student's *t* test. (*c*) Protein lysates from $PS1^{wt}$ and $PS1^{-/-}$ ES cells were immunoprecipitated with anti-PLD1 antibody and immunoblotted with anti-PEN2 antibody, or vice versa.

or between PLD1 and other γ-secretase components (data not shown). Therefore, it seems likely that the effects of PLD1 on the association of γ-secretase components result from a direct interaction with PS1 rather than through other components of the γ-secretase complex.

Discussion

It has been established that PSs and three other proteins (nicastrin, APH1, and PEN2) are essential for γ-secretase cleavage of βAPP (5–11). Although these four components represent the core activity of γ-secretase, it seemed possible that additional proteins might modify the activity of this complex. Here we report that PS1 interacts with and recruits PLD1 to the Golgi/TGN membranes. The PS1-mediated recruitment of PLD1 to the Golgi/TGN appears to play a negative role in the actions of PS1 (Fig. 5). Our data suggest that PLD1 elicits two distinct effects: promotion of βAPP trafficking and inhibition of γ-secretase activity. The promotion of βAPP trafficking requires intact catalytic activity of PLD1 (22), whereas the inhibition of γ-secretase activity does not.

It is believed that regulation of βAPP trafficking has an indirect effect on γ-secretase processing of βAPP by affecting substrate availability (18). However, the inhibition of γ-secretase processing of βAPP by PLD1 appears to be independent of any effects on βAPP trafficking. The catalytically inactive form of PLD1 (K898R), which failed to influence βAPP trafficking from the TGN, reduced Aβ generation. In addition, PLD1 inhibited Notch cleavage without affecting budding of Notch-containing vesicles from the TGN. Our data further suggest that PLD1 disrupts the proper association of γ-secretase complex components. PLD1 interacted with PS1 but not with other γ-secretase components (PEN2, nicastrin, and APH1). The interaction between PLD1 and PS1 may elicit a conformational change in PS1, which could prevent it from forming an active γ-secretase complex, and thus reduce the quantity of active γ-secretase available to cleave βAPP.

It has been reported that metabolism of phospholipids is disrupted in Alzheimer's disease brains (15, 16). FAD mutations reduced the ability of PLD to generate phosphatidic acid in cells, and PLD1 was depleted from cytosolic vesicles in these cells (22). In addition, recent studies show that $A\beta_{40}$ and $A\beta_{42}$ regulate cholesterol and sphingomyelin metabolism, respectively, whereas pathological PS mutations result in altered levels of cholesterol as well as sphingomyelin (24).

In summary, our studies have demonstrated that PS1 recruits PLD1 to the Golgi/TGN membrane and that PLD1 antagonizes PS1-mediated cleavage of βAPP. Overexpression of PLD1 results in dissociation of the γ-secretase complex and in decreased production of Aβ, whereas down-regulation of PLD1 leads to increased association of γ-secretase subunits and to accumula-

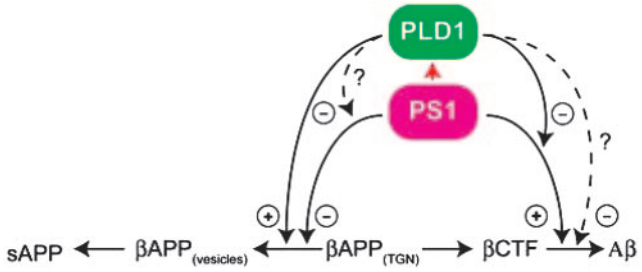

Fig. 5. Model of the mechanisms by which PLD1 may antagonize PS1 regulation of βAPP metabolism and stimulate βAPP trafficking from the TGN. PS1 is required for the recruitment of PLD1 to the Golgi/TGN membranes (red arrowhead). The recruited PLD1 serves, in two ways, as a negative regulator of PS1 function. First, PS1 inhibits budding of βAPP-containing vesicles from the TGN, whereas PLD1 stimulates this process through a mechanism that is at least partially independent of PS1. Second, PLD1, through a direct interaction with PS1 and disruption of association of the γ-secretase subunits, inhibits the ability of the protease to cleave βCTF to generate Aβ. The present data do not exclude the possibility (*i*) that PLD1 might also antagonize PS1 inhibition of βAPP trafficking by its ability to bind directly to PS1, or (*ii*) that PLD1 might also inhibit Aβ generation through an action independent of its inhibitory effect on PS1 (dashed arrows). This scheme is based on the present study and that of Cai *et al.* (22).

tion of Aβ. The discovery that it is possible to affect the rate of PS1-mediated βAPP metabolism by modulating PLD1 expression levels suggests the development of novel therapeutic approaches for delaying or preventing Alzheimer's disease.

Methods

Cell Lines. Mouse N2a neuroblastoma cells doubly transfected with cDNAs encoding human βAPP harboring the "Swedish" mutant (βAPPswe) and human wt PS1 or FAD PS1 mutants (3, 25) were maintained in medium containing 50% DMEM and 50% Opti-MEM, supplemented with 5% FBS, antibiotics, and 200 μg/ml G418 (Invitrogen). Immortalized $PS1^{-/-}$ fibroblasts (26) were maintained in DMEM supplemented with 10% FBS and antibiotics. Blastocyst-derived wt and $PS1^{-/-}/PS2^{-/-}$ cells have been described previously (27).

Transfection of cDNA Constructs and RNA Interference. Transient transfections of cDNA encoding wt human phosphocholine-specific PLD1 (28) or the catalytically inactive mutant K898R (29) were performed by using FuGENE-6 transfection reagent (Roche Diagnostics). Coexpression of multiple cDNA constructs was achieved by cotransfection of equal amounts of DNA, resulting in comparable levels of protein expression. shRNA-mediated targeting of PLD1 using pSuper (30, 31) (sequence, CTGGAAGATTACTTGACAA) and control shRNA (pSuper-luciferase, CGGAATACTTCGATTCAAG) were used. Transfection of shRNA was performed by using FuGENE-6 transfection reagent. Levels of PLD1 were determined by Western blot by using antibody AE596, which recognizes the PLD1 C-terminus (32).

Co-IP. To detect association between PLD1 and PS1, cells were lysed in immunoprecipitation (IP) buffer containing 0.5% Nonidet P-40, followed by IP with PLD1 antibody AE596, SDS/PAGE, and immunoblotting with anti-PS1 loop antibody, which recognizes an epitope within the hydrophilic loop domain of PS1. Alternatively, cell lysates were immunoprecipitated with anti-PS1 loop antibody followed by immunoblotting with PLD1 antibody. ES cells overexpressing hemagglutinin (HA)-tagged PLD2 were also lysed in IP buffer as described, followed by IP with HA antibody (Roche), SDS/PAGE, and immunoblotting with anti-PS1 loop antibody. In some experiments, ES cells overexpressing HA-tagged PLD1wt or mutant K898R were lysed as described, followed by IP with HA antibody (Roche), SDS/PAGE, and immunoblotting with anti-PS1 loop antibody. In some experiments, anti-PS1 loop antibody, anti-PS1 NTF antibody, or normal rabbit IgG (used as control) was added to the permeabilized cell-free system. After 90 min of incubation, the TGN membranes were collected and solubilized with IP buffer containing 0.5% Nonidet P-40. Lysates were immunoprecipitated with anti-PLD1 antibody AE596, followed by immunoblotting with anti-mouse PS1 antibody (Chemicon). For γ-secretase complex association, cells were lysed in IP buffer containing 0.5% digitonin, followed by IP using anti-PEN2 antibody CR8 (Covance, Richmond, CA) and immunoblotting with Ab14 (which recognizes a PS1 N-terminal epitope), anti-PS1 loop antibody, Ab716 (which recognizes a nicastrin C-terminal epitope), or APH1 antibody (Zymed).

Immunofluorescence Confocal Microscopy. For staining of PLD1, cultured PS1wt or $PS1^{-/-}$ fibroblast cells were fixed with 4% formaldehyde and permeabilized by ice-cold methanol. Next, cells were incubated with antibody against PLD1 [P1-P4 antibody (32); 1:2,000 dilution].

Preparation of Permeabilized Cells. ES wt cells or N2a PS1ΔE9 cells were permeabilized as described (18). Broken cells (cell-free system) were washed and incubated in a final volume of 300 μl containing 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 110 mM KCl, cytosol (30 μg of protein) prepared from N2a cells (14, 33), an energy-regenerating system (final concentrations of 1 mM ATP, 0.02 mM GTP, 10 mM creatine phosphate, and 80 μg/ml creatine phosphokinase), and a complete protease inhibitor mixture.

Measurement of Nascent Secretory Vesicles in Permeabilized Cells and IP. After incubation of cell-free systems, vesicle and membrane fractions were separated by centrifugation at $1.14 \times 10^4 \times g$ for 30 s at 4°C in a Brinkman centrifuge. Vesicle (supernatant) and membrane (pellet) fractions were diluted with IP buffer (50 mM Tris·HCl, pH 8.8/150 mM NaCl/6 mM EDTA/2.5% Triton X-100/5 mM methionine/5 mM cysteine/1 mg/ml BSA), immunoprecipitated by using anti-βAPP C-terminal antibody 369 (34, 35) or anti-myc antibodies (Santa Cruz Biotechnology), and analyzed by SDS/PAGE. Each experiment was performed at least three times. Band intensities were analyzed and quantified by using NIH IMAGEQUANT software, version 1.61.

Aβ Detection. After transient transfection of PLD1 (wt or K898R) or mock cDNA, N2a cells expressing PS1 variants were labeled with [^{35}S]methionine [500 μCi/ml (1 Ci = 37 GBq)] for 4 h at 37°C, and medium was collected for detection of Aβ and sβAPPα levels by sequentially immunoprecipitating with 4G8 (Signet Laboratories, Dedham, MA) and MAB348 (Roche) antibodies. Intracellular Aβ levels were determined by boiling cell pellets in 3% SDS at 95°C for 5 min followed by sonication. Then samples were immunoprecipitated with 4G8 antibody, and full-length βAPP, βCTF, and Aβ were resolved by SDS/PAGE and autoradiography.

Surface-Enhanced Laser Desorption/Ionization Mass Spectrometry. Medium was harvested from N2a Swe/ΔE9 cells transiently transfected with PLD1 or control cDNA. Antibody 6E10 or mouse anti-IgG antibody (ICN) was ligated to Ciphergen PS10 protein chips by using the Ciphergen 8-well bioprocessor accessory according to the manufacturer's instructions.

Vesicle Budding of Notch from the TGN and Notch-1 Cleavage Assays in N2a Cells. N2a PS1wt cells were transiently transfected to overexpress mNotchΔE (truncated Notch-1, lacking most of the Notch extracellular domain with a C-terminal myc tag) (36) and/or PLD1. Then cells were permeabilized and incubated at 37°C for 0–60 min. The budding of mNotchΔE-containing vesicles was determined by IP with anti-myc antibody followed by SDS/PAGE and autoradiography. For detection of Notch cleavage, cells were pulse labeled for 30 min with [^{35}S]methionine (500 μCi/ml) and chased for 15–120 min at 37°C. mNotchΔE and NICD were detected in cell lysates by IP using anti-myc antibody (Santa Cruz Biotechnology).

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