Spatial Segregation of γ-Secretase and Substrates in Distinct Membrane Domains*

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γ-Secretase facilitates the regulated intramembrane proteolysis of select type I membrane proteins that play diverse physiological roles in multiple cell types and tissue. In this study, we used biochemical approaches to examine the distribution of amyloid precursor protein (APP) and several additional γ-secretase substrates in membrane microdomains. We report that APP C-terminal fragments (CTFs) and γ-secretase reside in Lubrol WX detergent-insoluble membranes (DIM) of cultured cells and adult mouse brain. APP CTFs that accumulate in cells lacking γ-secretase activity preferentially associate with DIM. Cholesterol depletion and magnetic immunosolation studies indicate recruitment of APP CTFs into cholesterol- and sphingolipid-rich lipid rafts, and co-residence of APP CTFs, PS1, and syntaxin 6 in DIM patches derived from the trans-Golgi network. Photoaffinity cross-linking studies provided evidence for the preponderance of active γ-secretase in lipid rafts of cultured cells and adult brain. Remarkably, unlike the case of APP, CTFs derived from Notch1, Jagged2, deleted in colorectal cancer (DCC), and N-cadherin remain largely detergent-soluble, indicative of their spatial segregation in non-raft domains. In embryonic brain, the majority of PS1 and nicastrin is present in Lubrol WX-soluble membranes, wherein the CTFs derived from APP, Notch1, DCC, and N-cadherin also reside. We suggest that γ-secretase residence in non-raft membranes facilitates proteolysis of diverse substrates during embryonic development but that the translocation of γ-secretase to lipid rafts in adults ensures processing of certain substrates, including APP CTFs, while limiting processing of other potential substrates.

Sequential processing of amyloid precursor protein (APP)1 by β- and γ-secretases releases the 39–42-amino acid-long β-amyloid (Aβ) peptides, which accumulate in the brains of aged individuals and patients with Alzheimer disease (AD) (1). The major β-secretase in neurons is an aspartyl protease termed BACE-1, which cleaves APP within the luminal domain, generating the N terminus of Aβ (2). The C terminus of Aβ is generated by intramembranous cleavage of APP C-terminal fragments (CTFs) by γ-secretase, a multiprotein complex made of four essential components, presenilin (PS) 1 (or PS2), nicastrin, PEN-2, and APH-1 (3). Mutations in PSEN1 and PSEN2, encoding multipass membrane proteins PS1 and PS2, respectively, co-segregate with the majority of cases of autosomal dominant familial early-onset AD (4). Familial AD-linked PS1 and PS2 variants elevate the production of highly fibrillogenic Aβ42 peptides (1). A role for PS1 in Notch function was first discovered in Caenorhabditis elegans screens and involves intramembranous cleavage of the Notch receptor, analogous to APP processing (5, 6). Nicastrin is a type I membrane protein independently identified as a novel component of the GLP-1/Notch signaling pathway in C. elegans early embryos and in the biochemical characterization of proteins that interacted with PS1 (7, 8). Multitransmembrane protein APH-1 and the two-transmembrane protein PEN-2 were also identified in C. elegans screens as genes essential for Notch signaling (9, 10). The four components of the γ-secretase interact early during biosynthesis and cooperatively exit the ER. Nicastrin then undergoes complex glycosylation, and PS1 undergoes endoproteolysis to generate N- and C-terminal derivatives to generate the functional γ-secretase (3).

Compelling evidence from the transition state analog inhibitors show that γ-secretase is a transmembrane aspartyl protease, and two Asp residues essential for γ-secretase activity have been localized to the transmembrane domains 6 and 7 of PS1 (11). In contrast to the complex nature of the functional γ-secretase assembly, intramembranous cleavage by γ-secretase shows poor sequence specificity and does not appear to involve strict rules for substrate recognition. However, efficient cleavage by γ-secretase requires the removal of the extracellular domain by juxtamembrane proteolysis, leaving membrane-bound CTFs (12). Consequently, several type I membrane proteins that undergo ectodomain shedding, mediated by a set of metalloproteases termed “α-secretases,” are also substrates for intramembranous cleavage by the γ-secretase. To date, known γ-secretase substrates include APP homologs, Notch1 and homologs, Notch ligands Jagged2 and Delta, ErbB4, CD44, low density lipoprotein receptor-related protein, N- and E-cadherins, nectin-1α, deleted in colorectal cancer (DCC), p75 neuretin receptor, syndecan 3, etc. Exactly how such diverse

brane-bound intracellular domain; TNF, tumor necrosis factor; wt, wild type; AD, Alzheimer disease; Tricine, N-[2-hydroxy-1,1-bis(3-hydroxy- methyl)ethyl]glycine; mAb, monoclonal antibody; PS, presenilin; MβCD, methyl-β-cyclodextrin.
sets of molecules residing in multiple subcellular sites are recognized by the γ-secretase is not well understood. Similarly, with the exception of Notch signaling, there is only limited information available on the functional consequence downstream of intramembranous cleavage of several γ-secretase substrates.

We reported recently on the cholesterol-dependent association of each of the γ-secretase components with lipid rafts in post-Golgi and endosome membranes enriched in syntaxin 6, syntaxin 13, and VAMP4 (13). Lipid rafts are cellular membrane microdomains rich in cholesterol and sphingolipids (14). These specialized detergent-insoluble microdomains (DIM) contribute to trafficking of proteins and lipids in the secretory and endocytic pathways by regulating vesicle sorting and formation (15). In addition, lipid rafts serve as versatile platforms wherein certain plasma membrane receptors and kinases regulate signal transduction (14). There is growing evidence to suggest that amyloidogenic processing by secretases may also be compartmentalized in lipid rafts by targeting BACE-1, γ-secretase, and APP to DIMs (13, 16–20). Most interestingly, only a small fraction of endogenous APP is found in DIMs, raising concerns whether γ-secretase is active exclusively within ordered lipid raft domains or not. Furthermore, the role of lipid rafts in intramembranous cleavage of other γ-secretase substrates has not been investigated.

In this study we investigated the distribution of select γ-secretase substrates in cholesterol-rich raft domains isolated by sucrose density gradient fractionation of detergent-insoluble membranes. We report that the majority of APP CTFs and active γ-secretase resides in lipid rafts in cultured cells and adult mouse brain. On the other hand, CTFs of Notch1, Jagged2, and DCC are predominantly found in non-raft membranes. While addressing this apparent discrepancy, we found that PS1, APP, and other γ-secretase substrates, including DCC and N-cadherin, localize to non-raft membranes in developing embryonic brain. We propose that spatial segregation of γ-secretase within cholesterol- and sphingolipid-rich ordered lipid domains limits access to certain γ-secretase substrates in the adult.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Mouse N2a neuroblastoma stable cell lines were cultured in 45% Dulbecco's modified Eagle's medium and 50% Opti-MEM (Invitrogen) supplemented with 5% fetal bovine serum. The stably transfected N2a cell line Swe.10 expressing c-Myc-tagged human APP695 harboring "Swedish" double mutation (APPSwe), N2a cell line Wt.11 co-expressing APPSwe and wild-type (wt) PS1, and pooled stable N2a transfectants co-expressing APPSwe and wt PS1 or PS1 ΔD355A have been described previously (21–23). Wild-type and PS1(−/−)/PS2(−/−) embryonic fibroblasts, NIH 3T3 cells stably expressing human Jagged2, and PS1(−/−)/mouse embryonic fibroblasts stably expressing Notch1−/−6mycGFP were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (24–26). For γ-secretase inhibitor treatments, cells grown to 70% confluency were treated either with Me2SO, 10 μM compound E (CompE), or 1 μM L-685,458 for 18 h. For depleting cholesterol, confluent cultures were treated either with Me2SO, 10 nM compound E (CompE), or 1 μM L-685,458 for 18 h. For depleting cholesterol, confluent cultures were treated either with Me2SO, 10 nM compound E (CompE), or 1 μM L-685,458 for 18 h. For depleting cholesterol, confluent cultures were treated either with Me2SO, 10 nM compound E (CompE), or 1 μM L-685,458 for 18 h.

**Lipid Raft Isolation**—Lipid rafts were isolated from Lubrol WX lysates of cultured cells by discontinuous flotation density gradients as described previously (13). PS1Loop mouse embryonic fibroblasts stably expressing Notch1−/−6mycGFP were washed with Hanks' buffered salt solution and treated with 10 mM EDTA for 30 min to induce Notch cleavage (26) prior to lipid raft isolation. Lipid rafts were isolated from mouse forebrain as described previously (35) with some modifications. Briefly, 200–300 mg of tissue from brains of 12-month-old wt mice or E14–16 embryos generated by intercrossing C57Bl/6J PS1(−/−) mice (36) were homogenized with a glass-Teflon homogenizer in 2 ml of buffer A containing 20 mM Tris-HCl (pH 7.4), 50 mM NaCl, 250 mM sucrose, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture (Sigma). After passage through a 25-gauge needle three times, homogenates were spun at 960 × g for 10 min at 4 °C. The supernatant was collected, and the pellet was resuspended in 1 ml of buffer A, which was passed through a 25-gauge needle five times and centrifuged at 20,000 × g for 15 min. The supernatant was then adjusted to 0.5% Lubrol WX, 25 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 5 mM EDTA and mixed at 4 °C for 20 min. The lysate was then adjusted to 45% final concentration of sucrose and centrifuged as described previously (13).

**Immunosolubilization of Raft Patches Using Magnetic Beads—Monoclonal syntaxin 6 antibody was bound to Dynabeads M-280 beads pre-activated with 10 μg/ml anti-mouse IgG (Dynal) according to the manufacturer's instructions. Antibody OKT8 (recognizes CD8α) was used as negative control to establish the specificity of the immunosolubilization procedure. Syntaxin 6 containing raft patches were immunosolubilized from pooled lipid raft fractions as described previously (13).**

**Photoaffinity Cross-linking—Flotation density gradient fractions 4 and 5 (raft) or 6–12 (non-raft) were pooled and incubated with 1 μM L-852,505, a photoactive, biotinylated derivative of the aspartyl protease transition state analog of L-685,458 (37), at 4 °C for 1 h followed by irradiation with 365 nm UV light, using a Stratalinker (Stratagene), on ice for 90 min. Biotinylated proteins were captured with streptavidinagarose beads (Pierce) at 4 °C and eluted by incubation in Laemmli sample buffer. Eluted proteins as well as 1/40th of the input were fractionated by SDS-PAGE and analyzed by Western blot analysis with aPS1Loop antibody.

**RESULTS**

Association of γ-Secretase Components and APP CTFs in Lubrol-resistant Lipid Rafts in the Adult Brain—Previous studies have suggested that processing of APP to the amyloid-β peptide occurs predominantly in lipid rafts and that BACE-1 is the rate-limiting enzyme in this process. In order to gain insights into membrane subdomains involved in γ-secretase processing of APP CTFs, we isolated detergent-insoluble membrane microdomains (DIMs) by discontinuous flotation density gradient centrifugation of mouse brain lysates prepared in Lubrol WX. Lubrol WX-resistant buoyant light density membranes, enriched in the classical lipid raft-associated protein flotillin-2 (38), were found in fractions 4 and 5, at the interface between 5 and 35% sucrose. Consistent with previous reports, we found that ∼20% of mature BACE-1 and the vast majority of PS1, mature nicastrin, and PEN-2 were present in these raft fractions (Fig. 1). In agreement with previous reports, less than 5% of full-length mature APP was recovered in Lubrol WX-resistant fractions (17, 20, 39), whereas the majority of full-length mature and immature APP was found in the denser, non-raft fractions 9–12 (Fig. 1). Most interestingly, about 70% of APP C-terminal fragments (CTFs) were recovered in the DIM fractions (Fig. 1). The above results suggest that a small fraction of full-length APP partitions into lipid rafts, whereas APP CTFs preferentially associate with lipid raft microdomains in the adult mouse brain.
Similarly, in lipid raft fractions as compared with non-raft fractions (Fig. 2). Examination of endogenous APP CTFs both in the raft and non-raft fractions (Fig. 2). Flotation gradient analysis showed that similar to wt PS1-derived N-terminal fragment, a fraction of PS1 D385A variant was recovered in Lubrol WX-resistant DIM fractions 4 and 5 (Fig. 3). Further analyses revealed the presence of endogenous mature nicastrin, APH-1, and PEN-2 in lipid raft fractions in PS1 D385A cells, indicating that the mutation of PS1 Asp-385 residue did not impair localization of the catalytically inactive PS1 molecule or other γ-secretase components in lipid raft microdomains (Fig. 3). As described previously, APP CTFs accumulate in D385A cells due to the absence of γ-secretase activity (40). As predicted from the PS1−/−/PS2−/− findings described above, the majority of APP CTFs in D385A cells partitioned into lipid raft fractions 4 and 5 (Fig. 4). Similarly, the majority of CTFs derived from endogenous wt APP in stable HEK293 cells expressing PS1 D385A also accumulated in lipid raft fractions, whereas less than 10% of full-length mature glycosylated APP partitioned into lipid raft microdomains (data not shown). Together, these results demonstrate that APP CTFs accumulate to a greater extent in Lubrol-resistant lipid raft microdomains in cells expressing the catalytically inactive PS1 D385A mutant. Most interestingly, we also observed the raft accumulation of a small fraction of mature full-length APP in PS1 D385A cells, indicating the potential effects of dominant-negative PS1 expression on selective turnover or processing of raft-associated full-length APP.

Cholesterol-dependent Accumulation of APP CTFs in Lipid Rafts of Cells Treated with γ-Secretase Inhibitors—It has been reported previously that APP CTFs accumulate on the cell surface and other intracellular organelles in cells lacking PS1 expression or γ-secretase activity (43–45). In order to determine whether accumulation of APP CTFs in lipid rafts in cells lacking γ-secretase components is directly related to the lack of γ-secretase activity, we turned our attention to pharmacological γ-secretase inhibitors. L685,458 and CompE represent two classes of inhibitors that have been characterized; L685,458 is
a transition state analog, and CompE is a small molecule inhibitor (46, 47). Most interestingly, both inhibitors have been successfully cross-linked to PS1, demonstrating that PS1 is the direct target of either of these highly potent \( \gamma \)-secretase inhibitors. Treatment of stable N2a cell line expressing human APPswe (Swe.10) with L685,458 or CompE results in marked accumulation of APP CTFs without discernible change in the levels of full-length APP. Flocculation gradient centrifugation revealed the preponderance of APP CTFs in Lubrol-resistant lipid raft fractions, whereas the vast majority of immature and mature full-length APP was recovered in non-raft fractions (Fig. 4, A and B). Together, these results indicate that upon pharmacological inhibition of the \( \gamma \)-secretase activity, the substrate APP CTFs accumulate in Lubrol-resistant microdomains.

We considered the possibility that the topographical relationships between APP CTFs and distinct lipid domains may not be adequately preserved when native membranes are disturbed by the biochemical detergent extraction and fractionation methods. To address this issue, we incubated Swe.10 cells with Me2SO, a drug known to selectively deplete biological membranes of cholesterol. Cholesterol plays an important role in stabilizing lipid raft microdomains, and depletion of cholesterol disrupts lipid raft integrity and imparts detergent solubility on certain raft-associated proteins, including \( \gamma \)-secretase components and PrP (13, 48, 49). Incubation of CompE-treated Swe.10 cells with Me2SO prior to raft isolation caused the displacement of APP CTFs from the low density Lubrol WX-resistant membrane domains (Fig. 4B) to the denser Lubrol WX-soluble membrane fractions, providing evidence that APP CTF-rich buoyant membranes indeed represent \textit{bona fide} cholesterol-rich lipid rafts. In addition to APP CTFs, we also observed the accumulation of a small fraction (5–10%) of mature full-length APP in lipid raft fractions, which is also sensitive to Me2SO treatment. Thus we conclude that in the absence of \( \gamma \)-secretase processing, a significant fraction of APP CTFs associates with lipid rafts in a cholesterol-dependent manner.

\textbf{APP\( \beta \)-CTFs Co-reside with Syntaxin 6 in DIMs—The finding described above imply that DIMs are the likely platforms where \( \gamma \)-secretase processing of APP occurs. Recently, we characterized the co-residence of components of the \( \gamma \)-secretase complex with syntaxin 6 in Golgi/Trans-Golgi network/endosome lipid raft membrane microdomains (13). Hence we predicted that in the absence of \( \gamma \)-secretase processing, APP CTFs likely accumulate in DIMs of intracellular organelles where \( \gamma \)-secretase resides. To test this idea directly, we carried out antibody-mediated magnetic immunopurification of syntaxin 6 containing DIM patches from a pool of Lubrol WX-resistant DIM fractions from Swe.10 cells treated with Me2SO or 5 mM M\( \beta \)CD for 2 h at 37 °C prior to fractionation. Note that DIM accumulation of APP CTFs is sensitive to cholesterol depletion. C, pooled fractions 4 and 5 from the indicated cell lines were fractionated on 16.5% Tris-Tricine gels and sequentially probed with APP C-terminal polyclonal antibody 369 and mAb 26D6 (raised against epitopes 1–12). Antibody 369 reacts with \( \beta \), \( \beta ^\prime \), (11)-, and \( \alpha \)-CTFs, whereas mAb 26D6 only reacts with \( \beta \)-CTF. D, DIM fractions from Swe.10 cells treated with CompE were incubated with magnetic beads coated with syntaxin 6 or ORT8 antibody. Bound DIMs were analyzed by Western blotting using 369, mAb 26D6, and PS1\( \gamma \). An aliquot of the input (1/30th volume) was also fractionated in the same gel for comparison. Note that APP CTFs, but not full-length APP, co-reside with syntaxin 6 in DIMs.
in non-raft input relative to its abundance in the raft input, we considered the possibility that photoprobe labeling of PS1 CTF in non-raft fractions was beyond the limit of detection. To address this issue, we repeated this experiment by using raft and non-raft preparations from N2a cells overexpressing PS1, and we increased the amount of non-raft input. Following phototivation, readily discernible levels of PS1 CTF were labeled by L-852,505 in both raft and non-raft preparations (Fig. 5B). Nevertheless, a comparison of photoprobe-labeled CTF to input indicated preferential labeling of CTF in DIMs. Finally, to confirm the presence of active γ-secretase in brain DIMs, we incubated raft and non-raft preparations from adult mouse brain with the L-852,505 photoprobe. As shown in Fig. 5C, the large majority of labeled PS1 CTF was found in the raft fraction. The above findings suggest that the preponderance of active γ-secretase complex is present in DIMs of untransfected fibroblasts and adult mouse brain.

**Non-raft Localization of Notch1 and Jagged2 CTFs**—Although the studies described above are consistent with the presence of active γ-secretase and APP CTFs in Lubrol-resistant DIMs, these results are insufficient to conclude that γ-secretase processing of all substrates occurs exclusively within DIMs. Therefore, we set out to determine directly whether other γ-secretase substrates also associate with DIMs. Similar to our characterization of APP CTFs, we asked whether the inhibition of γ-secretase activity leads to DIM accumulation of Notch C-terminal derivative termed S2/NEXT (Notch extracellular truncation), which is the immediate precursor for γ-secretase processing (50). For these studies, we employed a mouse embryonic fibroblast line stably expressing a chimeric full-length Notch1–6mycGFP molecule (26). In this fibroblast line, immunoblotting with mAb 9E10 detects ~350-kDa full-length Notch and ~80-kDa furin cleaved Notch CTF termed S1/TMIC (transmembrane-bound intracellular domain). Treatment with EDTA stimulates sequential cleavage of S1/TMIC by an ADAM protease and γ-secretase, resulting in robust production of membrane-tethered C-terminal derivatives S2/NEXT and S3/NICD (Notch intracellular domain), respectively (26).

We decided to employ the ligand-independent, Ca2+-depletion paradigm to facilitate S2 and S3 cleavage of Notch (51). We treated stably transfected Notch-6mycGFP fibroblasts with EDTA for 20 min and analyzed lysates by flotation gradient centrifugation. As shown in Fig. 6A, the majority of full-length Notch and a greater fraction of TMIC and NEXT polypeptides (which migrate as a close doublet in our gel system) were associated with detergent-soluble heavier fractions (fractions 8–12), and about 15–20% of TMIC/NEXT polypeptides were present in DIM fractions (fractions 4 and 5). As expected, preincubation of Notch1–6mycGFP cells with CompE resulted in complete loss of NICD and a proportional increase in the levels of TMIC/NEXT polypeptides. Most interestingly, we observe a consistent increase in the TMIC/NEXT polypeptide intensity in the non-raft fractions, whereas the signal intensity of TMIC/NEXT in DIM fractions remains largely unaffected (Fig. 6A, right panel). Furthermore, in contrast to TMIC/NEXT polypeptides, endogenous APP CTFs preferentially accumulated in DIM fractions of Notch1–6mycGFP fibroblasts pretreated with CompE. It should be noted that although the majority of PS1 and mature nicastrin was found in the raft fractions, it is still plausible that the low levels of PS1/γ-secretase in the non-raft fractions are catalytically active in promoting intramembranous proteolysis of Notch 1 CTFs. In any event, our results clearly demonstrate differential accumulation of APP and Notch1 CTFs in raft versus non-raft fractions of fibroblasts.

We further extended our analysis to Jagged2, one of the
RAFT AND NON-RAFT DISTRIBUTION OF γ-SECRETASE AND SUBSTRATES

Notch1 ligands that has been shown to undergo γ-secretase processing (25, 52). Flotation density gradient analysis of raft and non-raft distribution of γ-secretase and substrates in embryonic brain. Since γ-secretase plays an essential role in mammalian embryonic development, we performed a series of studies using brain tissue from PS1+/− embryos harvested at E15.5. First, we examined the distribution of Notch1 using a C-terminal-specific antibody. Immunoblot analysis of fractions from embryonic brain homogenates that were subjected to flotation gradient centrifugation showed predominant non-raft distribution of full-length Notch1 as well as Notch1 C-terminal derivatives TMIC and NEXT polypeptides both in PS1+/− and PS1−/− embryonic brain (Fig. 7). As expected from previous reports, the polypeptide corresponding to NICD was not detectable in PS1−/− embryonic brain lysates. These results are consistent with our findings from stably transfected fibroblasts described above. Next, we turned our attention to other γ-secretase substrates that have been reported to play important roles in the nervous system. We recently reported that DCC, the netrin-1 receptor, is subject to proteolysis within the ectodomain segment and that the residual membrane-tethered DCC “stub” is subsequently processed by γ-secretase to generate DCC-intracellular domain (53, 54). In PS1−/− embryonic brain, full-length DCC and the vast majority of DCC CTF were recovered in non-raft fractions with only a minor fraction of each species present in DIM raft fractions (Fig. 7). In PS1−/− embryonic brain, wherein γ-secretase activity is greatly reduced, we readily observed an increase in the levels of DCC CTF. Furthermore, the relative abundance of DCC CTF in the non-raft fractions of PS1−/− embryonic brain was higher when compared with that of PS1+/− embryonic brain. We also examined the distribution of N-cadherin, another γ-secretase substrate (55), and found that the majority of full-length N-cadherin and its CTFs were present in non-raft fractions of embryonic brain. Consistent with the lack of γ-secretase cleavage, we observed increased levels of CTFs in non-raft fractions of PS1−/− embryonic brain compared with that of the PS1+/−. The above results suggest that γ-secretase processing of DCC and N-cadherin CTFs occurs in non-raft fractions in embryonic brain.

As we had observed in the embryonic brain, the majority of TMIC peptides was present in the non-raft fractions of adult mouse brain (Fig. 7). However, and in contrast to the findings in embryonic brain where we failed to detect any TMIC in raft fractions, we observed low levels of TMIC in the raft fractions from adult brain (Fig. 7). These findings raise the possibility that lipid raft association of proteins could markedly differ between embryonic and adult brain tissue. Hence, we examined the raft/non-raft distribution of full-length APP and APP CTFs in embryonic brain lysates, and we found that the vast majority of full-length APP molecules was predominantly associated with non-raft membranes, consistent with the results from analysis of the adult brain. Most interestingly, and in contrast to the adult brain in which the vast majority of APP CTFs was present in raft fractions (see Fig. 1), we found that significant amounts of APP CTFs were present in non-raft fractions in embryonic brain (Fig. 8).

Further evaluation of embryonic brain samples revealed the surprising finding that the large majority of PS1 and nicastrin also resided in non-raft domains amenable to solubilization in Lubrol WX. Immunoblotting with raft marker flotillin-2, and raft-associated t-SNARE syntaxin-6, documented that reproducible recovery of PS1 and APP in non-raft fractions of embryonic brain did not result from the absence of Lubrol WX-resistant DIM in embryonic brain tissue or inadvertent disruption of DIM domain integrity during the biochemical isolation procedure (Fig. 8). These results are in accord with γ-secretase processing of substrates in both raft and non-raft subdomains of cellular membranes during embryonic development.

DISCUSSION

Several lines of evidence suggest a role for lipid rafts in amyloidogenic processing of APP. In this study we characterized the raft versus non-raft distribution of CTFs derived from APP and other select γ-secretase substrates (Notch1, Jagged2, DCC, and N-cadherin) and report several novel insights regarding γ-secretase processing. First, loss of γ-secretase processing leads to preferential association of APP CTFs with DMs. Association of APP CTFs is sensitive to depletion of cholesterol by using methyl-β-cyclodextrin, strongly suggesting...
their recruitment into cholesterol- and sphingolipid-rich lipid rafts. By using a photo-cross-linking approach, we also demonstrate the preponderance of active $\gamma$-secretase in lipid raft fractions of cultured cells and adult mouse brain. These findings implicate lipid rafts as the principal sites in cellular membranes where $\beta$-amyloid is generated. Second, we show that the large majority of CTFs generated by ectodomain cleavage of Notch1, Jagged2, DCC, and N-cadherin remain Lubrol WX-soluble when $\gamma$-secretase cleavage is inhibited (in cultured cells) or absent (in $PS1^{-/-}$ embryonic brain), indicating that these substrates are likely cleaved by $\gamma$-secretase in non-raft membrane domains. Third, we show that significant amount of PS1, nicastrin, and APP CTFs remain Lubrol WX-soluble in the embryonic brain. Together, these results suggest that in cultured cells and in adult brain the majority of active $\gamma$-secretase and APP CTFs is sequestered in lipid rafts away from several other $\gamma$-secretase substrates, and that catalytic levels of $\gamma$-secretase are sufficient to facilitate processing of substrates such as Notch1 and Jagged2 in non-raft membranes.

The functional significance of lipid rafts in regulating cellular signaling is well established (reviewed in Ref. 14). For example, tumor necrosis factor (TNF) receptor 1 is recruited to lipid rafts within minutes after TNF binding, and this translocation is essential for NF-$\kappa$B activation (56). Disruption of the lipid raft sensitizes cells to TNF receptor 1-induced cell death by blocking ubiquitination of adaptor proteins in the TNF receptor 1 signaling complex and preventing NF-$\kappa$B activation. Similarly, immunoglobulin E signaling and T-cell antigen receptor signaling in immune cells involves redistribution into lipid rafts and clustering of raft components (14). The examples above also address serious concerns regarding the relationship between lipid ordered domains in living cells and light buoyant density membranes resulting from extraction of cells in cold non-ionic detergents. First, whereas detergent insolubility can be an inherent property of certain proteins, differential behavior of surface receptors following ligand binding argues in favor of their dynamic association with detergent-resistant membrane microdomains in live cells during signal transduction. Second, experimental manipulations that are used to deplete cholesterol in cellular membranes modulate the functional outcome of ligand-induced signaling, strongly suggesting the involvement of cholesterol-rich lipid raft microdomains in spatial control of signaling in live cells. Thus, cholesterol-dependent association with light buoyant density membranes is an acceptable criterion for raft localization of proteins.

There has been considerable debate regarding the levels of full-length APP that partitions into lipid rafts (20, 57–59). We find that only a small fraction of endogenous full-length APP becomes associated with Lubrol WX-resistant raft fractions in mouse brain and in cultured cell lines. In marked contrast, loss of $\gamma$-secretase activity, resulting from a lack of PS1/PS2 expression, expression of a dominant-negative PS1 mutant, or exposure to highly potent $\gamma$-secretase inhibitors, leads to preferential accumulation of APP $\alpha$- and $\beta$-CTFs in Lubrol WX-resistant fractions. Data from wt adult mouse brain demonstrate that raft association of $\alpha$- and $\beta$-CTFs does not depend on compromised $\gamma$-secretase activity.

**FIG. 7.** Non-raft association of Notch1, DCC, and N-cadherin CTFs. Brain tissue from 12-month-old adult mouse or E15.5 embryos were homogenized in 0.5% Lubrol WX lysis buffer and subject to flotation sucrose density gradient centrifugation. Aliquots of the gradients were analyzed by Western blotting with antibodies against C terminus of Notch1, DCC, N-cadherin, and syntaxin 6. Notch cleavage products S1/TMIC (indicated by filled arrowhead), S2/NEXT (open arrowhead), and S3/NICD (open circle) are indicated. Note that in $PS1^{-/-}$ embryos S3/NICD is absent, and the levels of S2/NEXT as well as DCC and N-cadherin CTFs are higher relative to wt embryonic brain.

**FIG. 8.** Non-raft association of PS1 and APP CTFs in embryonic mouse brain. Raft and non-raft distribution of PS1, nicastrin, APP, flotillin-2, calnexin, and $\gamma$-adaptin in flotation density gradient fractions from E15.5 embryonic brains were analyzed by Western blotting. Note the predominant non-raft localization of PS1, nicastrin, and APP CTFs. mat, mature; imm, immature.
One plausible explanation for the accumulation of β-CTF in rafts is that BACE cleaves full-length APP mainly within lipid rafts, and once generated, β-CTFs continue to remain associated with lipid raft microdomains until they are processed by γ-secretase. There is sufficient evidence to indicate the presence of mature BACE and amyloidogenic processing of APP by BACE in lipid rafts (17, 18, 39, 60). For example, antibody-induced co-patching of APP and BACE-1 at the cell surface promotes Aβ production in a cholesterol-dependent manner (17). Direct evidence demonstrating the presence of α-secretase(s) or α-secretase processing of APP in lipid rafts is still lacking. However, based on zinc metalloprotease-mediated α-secretase-type shedding of PrP, a glycosylphosphatidylinositol-anchored plasma membrane protein localized to lipid rafts (61), we cannot formally rule out the possibility of α-secretase cleavage of APP within lipid rafts. Alternatively, accumulation of α-CTF within lipid rafts upon inhibition of γ-secretase activity might suggest that APP CTFs have the intrinsic property to segregate into cholesterol- and sphingolipid-rich microdomains regardless of the ectodomain cleavage of APP in raft or non-raft membranes.

By extending our previous studies on raft association of γ-secretase components (13), we performed photoaffinity cross-linking studies using a biotinylated, benzophenone-derivatized γ-secretase inhibitor and report the predominant localization of active γ-secretase in lipid raft microdomains in cultured cell lines and in adult mouse brain. Nevertheless, this direct labeling approach also reveals detectable levels of active γ-secretase in non-raft fractions of neuroblastoma cells overexpressing human PS1. Most interestingly, the dominant-negative PS1 variant lacking the critical aspartate residue Asp-385 (40) also localizes to DIMs, suggesting that PS1 complex is recruited into lipid raft microdomains independent of the catalytic potential of the γ-secretase complex. Thus, although the large majority of each of the four components of the γ-secretase is associated with lipid rafts in cultured cells, the residence in raft is neither required for γ-secretase activity nor indicative of functional γ-secretase.

Our results show that CTFs derived from Notch1, Jagged2, DCC, and N-accluderin mainly accumulate in Lubrol WX-soluble membrane fractions in the absence of γ-secretase activity, indicating that these substrates are likely processed by γ-secretase in non-raft regions of cellular membranes. It is somewhat surprising that in embryonic brain, the majority of PS1 and nicastrin, as well as APP CTFs, remains Lubrol WX-soluble. These findings support the notion that γ-secretase cleavage is not restricted to proteins that are localized within lipid rafts. Furthermore, predominant non-raft localization of PS1 and nicastrin in embryonic brain may be indicative of efficient γ-secretase processing of diverse substrates such as Notch1 and DCC that are known to play essential roles during embryonic development (62). These results also suggest that in the adult tissue, signaling resulting from regulated intramembrane proteolysis of certain substrates is regulated by efficient partitioning of active γ-secretase in lipid raft domains. How this developmental regulation is achieved is a focus of our future investigations. One possibility that might explain our findings is that protein and lipid components of raft domains are not sufficiently stabilized in embryonic brain, rendering them susceptible to solubilization in Lubrol WX. Changes in lipid composition and organization have been documented during in vitro differentiation of cultured neurons (63). However, raft association of full-length DCC, syntaxin 6, and flotillin-2 seems to be largely unaffected in embryonic brain, arguing against this notion of “immature” status of lipid raft microdomains in embryonic brain tissue. Nevertheless, it is clear that γ-secretase complex and APP CTFs have some yet unidentified properties that allow them to get recruited and remain associ-
Raft and Non-raft Distribution of γ-Secretase and Substrates


