Presenilins and γ-Secretase Inhibitors Affect Intracellular Trafficking and Cell Surface Localization of the γ-Secretase Complex Components*

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The intramembranous cleavage of Alzheimer β-amyloid precursor protein and the signaling receptor Notch is mediated by the presenilin (PS, PS1/PS2)–γ-secretase complex, the components of which also include nicastrin, APH-1, and PEN-2. In addition to its essential role in γ-secretase activity, we and others have reported that PS1 plays a role in intracellular trafficking of select membrane proteins including nicastrin. Here we examined the fate of PEN-2 in the absence of PS expression or γ-secretase activity. We found that PEN-2 is retained in the endoplasmic reticulum and has a much shorter half-life in PS-deficient cells than in wild type cells, suggesting that PSs are required for maintaining the stability and proper subcellular trafficking of PEN-2. However, the function of PS in PEN-2 trafficking is distinct from its contribution to γ-secretase activity because inhibition of γ-secretase activity by γ-secretase inhibitors did not affect the PEN-2 level or its egress from the endoplasmic reticulum. Instead, membrane-permeable γ-secretase inhibitors, but not a membrane-impermeable derivative, markedly increased the cell surface levels of PS1 and PEN-2 without affecting that of nicastrin. In support of its role in PEN-2 trafficking, PS1 was also required for the γ-secretase inhibitor-induced plasma membrane accumulation of PEN-2. We further showed that γ-secretase inhibitors specifically accelerated the Golgi to the cell surface transport of PS1 and PEN-2. Taken together, we demonstrate an essential role for PSs in intracellular trafficking of the γ-secretase components, and that selective γ-secretase inhibitors differentially affect the trafficking of the γ-secretase components, which may contribute to an inactivation of γ-secretase.

Mutations in genes encoding presenilins (PS1 and PS2)† are responsible for the majority of early onset familial Alzheimer disease (1, 2). Extensive studies in the past few years, focusing on the mechanisms by which PS mutations cause Alzheimer disease, have demonstrated that PS is a critical component of γ-secretase (3–7). γ-Secretase is an unusual protease catalyzing the intramembranous cleavage of amyloid precursor protein (APP) (8) as well as other type I membrane proteins including Notch (9), the ErbB4 receptor tyrosine kinase (10), CD 44 (11), nectin-1α (12), E-cadherin (13), and low density lipoprotein receptor-related protein (LRP) (14).

Presenilins are synthesized as immature holoproteins residing almost exclusively in the endoplasmic reticulum (ER) (15–17). Nascent full-length PS molecules undergo endoproteolysis to generate N- and C-terminal fragments (NTF and CTF) that associate with each other as heterodimers (18, 19). Accumulated evidence suggests that the PS NTF and CTF heterodimer is the catalytic component of γ-secretase (5, 6, 9, 20–22). Biochemical characterization demonstrates that the PS heterodimer principally exists in a high molecular weight complex (19, 23). Recent genetic and biochemical studies in Caenorhabditis elegans, Drosophila, and mammals show that the PS1 complex contains at least three other membrane proteins including nicastrin (Nct), APH-1, and PEN-2 (24–26). The four γ-secretase components physically interact, and deficiency of any one of them impairs γ-secretase activity (26–30). Cellular reconstitution studies strongly suggest that a complex consisting of PS1, Nct, PEN-2, and APH-1 is sufficient for γ-secretase activity (30–32). The precise functions of Nct, APH-1, and PEN-2 have not been fully elucidated. Recent studies including ours suggest that PEN-2 mediates endoproteolysis of PS1, whereas APH-1 and Nct play regulatory roles in maintaining the stability of PS1 and the complex (26, 30, 33–35).

All four γ-secretase components are synthesized in the ER and travel through the secretory pathway. Following endoproteolysis, PS1-NTF and -CTF remain as a heterodimer and localize predominantly in the Golgi and, to a lesser extent, at the cell surface and in endocytic compartments (36–40). Newly synthesized Nct undergoes maturation/glycosylation through the secretory pathway in a PS1-dependent manner. Its “mature,” highly glycosylated form that interacts with PS1 accumulates mainly in the Golgi and can be transported to the cell surface (38, 41–44). On the other hand, endogenous PEN-2 and APH-1 have been described as localizing to the ER and Golgi (33, 45). It has been reported that a significant portion of Aβ is fragment; Nct, nicastrin; ER, endoplasmic reticulum; TGN, trans-Golgi network; WT, wild type; 2×KO, double knockout; E5, embryonic stem; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
generated in the Golgi/trans-Golgi network (TGN) (46–48), although other cellular compartments including the ER (46, 48–50), endosomes (49, 51), and the plasma membrane (38, 52) may also be involved.

In addition to the essential role of PS in γ-secretase activity, numerous reports have assigned additional physiological functions to PS, including roles in calcium homeostasis, skeletal development, neurite outgrowth, apoptotic amaptic plasticity and tumorgenesis (53, 54). Still, details of the molecular and cellular mechanisms underlying the multiple biological roles of PS remain unknown. One of the attractive hypotheses regarding the multifunctionality of PS is that it regulates the intracellular trafficking of critical proteins involved in such processes. Indeed, recent studies from several groups including ours have revealed a role for PS1 in regulating intracellular trafficking/maturation of APP and Nct (34, 38, 55–58). Furthermore, PS1 deficiency also significantly affects trafficking of the tyrosine kinase receptor TrkB (56), as well as the dendritic outgrowth-promoting protein intercellular adhesion molecule-5 (ICAM-5)/telecephalin (59).

Despite providing support for a role in protein trafficking, the above mentioned studies offered little information on the relevance of the enzymatic activity of γ-secretase (the catalytic sites of which may reside within PS molecules (21, 22)) to the role of PS in protein trafficking. Recently, several highly selective and potent inhibitors of γ-secretase activity have been identified. These include transition state analog inhibitors, such as inhibitor X (L-685,458) (22, 60), and non-transition state small compounds, such as compound E (61).

Among them, some have been shown to bind directly to the PS1 complex (22, 61), but the possibility that these γ-secretase inhibitors may affect the integrity or trafficking of the PS complex has not been examined. In the present study we investigated the effects of PS deficiency and pharmacological inhibition of γ-secretase activity on intracellular trafficking of the PS complex components.

MATERIALS AND METHODS

Cell Cultures—Mouse neuroblastoma (N2a) cells were maintained in medium containing 50% Dulbecco’s modified Eagle’s medium and 50% Opti-MEM, supplemented with 5% fetal bovine serum (Invitrogen). N2a cells overexpressing PS1, PEN-2, and APPswe (N2a PSS15) were maintained under the selection of 0.2 mg/ml G418 and 0.4 mg/ml hygromycin—guest on July 25, 2018http://www.jbc.org/Downloaded from

PEN-2 Is Distinct from PEN-2—Polyclonal antibody PNT2 against PS1 KO ES cells. Comparable levels of labeled PEN-2 were detected after 15 min of labeling, indicating an unchanged biosynthetic rate in WT and PS2 KO cells. The majority of the newly synthesized PEN-2 remains stable for at least 8 h in WT cells, whereas the level of PEN-2 falls below the detectable range after 4 h chase in 2×KO cells (Fig 1A). These data indicate that PSs are required for the stability of nascent PEN-2 molecules.

Because PSs are essential for the maturation and trafficking of Nct (41), we next examined whether PSs affect the trafficking of PEN-2. Using a well established sucrose density gradient fractionation method, we have shown that the majority of endogenous PEN-2 molecules are localized in the Golgi (33), a subcellular compartment where the PS1 N- and C-terminal fragments and mature Nct also reside (36, 39–41). The same pattern of PEN-2 distribution was confirmed in ES WT cells (Fig 1B). Considering a reduced level of PEN-2 in ES cells lacking both PS1 and PS2 (2×KO cells), more 2×KO cell homogenates were subjected to sucrose density gradient fractionations to ensure a reasonable comparison of PEN-2. We found that in PS 2×KO cells PEN-2 localizes mainly in the fractions corresponding to the ER (Fig 1B). Consistent with our previous report, Nct also fails to mature in PS-deficient cells, and the immature forms of Nct accumulate in the ER (Fig 1B). These results suggest that the presence of PSs is essential for PEN-2 to achieve posttranslational stability and to exit the ER, providing additional evidence for the trafficking function of PSs.

The Function of PSs in Regulating ER to Golgi Transport of PEN-2 Is Distinct from γ-Secretase Activity—Recent evidence suggests that PS1 (or PS2) is the catalytic component of the γ-secretase complex (5, 6, 9, 20–22). Hence we investigated whether, similar to PS deficiency, inactivation of PS-dependent γ-secretase activity also affects the subcellular localization of PEN-2. As shown in Fig. 2, incubation of WT ES cells for 16 h with compound E, a potent γ-secretase inhibitor known to efficiently inhibit Aβ production (61), does not alter the ER and Golgi localization pattern of endogenous PEN-2, PS1 or Nct.
Furthermore, the maturation of Nct was also not affected (Fig. 2). These data demonstrate that the trafficking function of PSs is independent of γ-secretase activity. The data also suggest that γ-secretase inhibitors may not target γ-secretase complexes prior to their maturation and assembly upon reaching the TGN (see below).

**γ-Secretase Inhibitors Promote the Plasma Membrane Localization of PEN-2 and PS1 Fragments but Not That of Nct**—Two components of the γ-secretase complex, glycosylated Nct and, to a lesser extent, PS1 fragments, have been detected at the plasma membrane (37, 38, 41). Although expected, the existence of PEN-2 at the plasma membrane has not previously been demonstrated. In our cell surface biotinylation experiments, a minor but detectable amount of PEN-2 was found biotinylated at the cell surface (Fig. 3A, lane 2). Conversely, the cytosolic protein γ-adaptin is not biotinylated (Fig. 3A, top left).
Panel A: As a positive control, a relatively high level of Na,K-ATPase, a plasma membrane channel protein, appears at the cell surface of N2a cells (Fig. 3A, lane 2).

A plasma membrane localization of the γ-secretase complex would be consistent with the site of S3 cleavage of Notch by γ-secretase (9, 37). Having demonstrated that pharmacological inhibition of γ-secretase activity by specific inhibitors has no apparent effect on PEN-2 trafficking from the ER to the TGN, we next examined whether inhibition of γ-secretase activity affects cell surface localization of PEN-2. To this end, N2a cells were incubated for 16 h with control vehicle Me₂SO, γ-secretase inhibitor X (L-685,458) or compound E prior to cell surface biotinylation. Strikingly, cell surface levels of PEN-2 and fragments of PS1 increased significantly (by ~7-fold for PEN-2 and 5-fold for both PS1 NTF and CTF (Fig. 3B)) after treatment of cells with γ-secretase inhibitors (Fig. 3A, compare lanes 3 and 4 with lane 2). However, the level of plasma membrane-associated Nct was reduced slightly (~10–20%, Fig. 3B) upon inhibitor treatment (Fig. 3A, compare lanes 3 and 4 with lane 2).

To rule out the possibility that γ-secretase inhibitors alter membrane protein levels nonspecifically, the level of plasma membrane-bound Na,K-ATPase was examined and found to be unaffected by the inhibitors (Fig. 3A, compare lanes 2 and 3 with lane 4). It is notable that cell surface APP is not affected by the inhibitors (Fig. 3A, compare lanes 2 and 3 with lane 4). Similar results were also observed in ES cells (data not shown), indicating that the effect of γ-secretase inhibitor treatment on surface levels of γ-secretase components is not unique to N2a cells.

We next performed a time course study and revealed that compound E is able to stimulate the plasma membrane accumulation of PEN-2 and PS1-NTF in as little as 10–20 min, whereas plasma membrane-bound Nct remains unchanged up to 2 h (Fig. 3C). This rapid cell surface accumulation of PS1-NTF and PEN-2 after inhibitor treatment suggests that the effect of the inhibitors on the PS complex is likely posttranslational.

Panel B: γ-Secretase Inhibitor-induced Cell Surface Accumulation of PEN-2 Requires PS1—Because PS1 is necessary for plasma membrane delivery of Nct (41) and ER to Golgi transport of PEN-2 (Fig. 1B), we examined whether PS1 is also required for the inhibitor-induced plasma membrane accumulation of PEN-2. Given that the cell surface PEN-2 is undetectable in PS1 and PS2 double knockout cells (ES 2×KO cells) (data not shown), we compared cell surface PEN-2 in PS1 heterozygous (PS1+/−) and homozygous (PS1−/−) knockout fibroblast cells. In contrast to PS1+/− cells (Fig. 4, lanes 1–3), inhibitor treatment for 16 h exhibits no effect on the level of cell surface PEN-2 in PS1 homozygous knockout cells (PS1−/−) (Fig. 4, lanes 4–6), demonstrating that γ-secretase inhibitor-induced cell surface accumulation of PEN-2 requires PS1. This observation is consistent with our previous finding that Nct fails to reach the cell surface in the absence of PS1 (41) and further supports a role for PS1 in protein trafficking.

Panel C: γ-Secretase Inhibitors Target the Intracellular PS1 Complex—Both inhibitor X and compound E have been shown to directly target PS1 molecules (22, 61). To determine whether the cell surface accumulation of PEN-2 and PS1 resulted from altered trafficking of the intracellular PS1 complex or the cell surface PS1 complex, we used a membrane-impermeable derivative of inhibitor X, termed MRL631.2 Recently, MRL631 has been shown to abolish the γ-cleavage (the S3 cleavage) of Notch without any significant effect on Δ9 production in vivo, which is consistent with the notion that Notch cleavage occurs at the plasma membrane and is therefore sensitive to MRL631, whereas Δ9 is generated predominantly within the secretory compartments, mainly the Golgi/TGN (46–48). Interestingly, treatment of N2a cells with MRL631 at 2 µM, a concentration known to abolish Notch cleavage, fails to increase the level of plasma membrane-bound PEN-2 and PS1-CTF. Cell surface Nct is also unaffected or in some experiments slightly reduced (Fig. 5). These data suggest a model in which γ-secretase inhibitors directly target the intracellular PS1 complex (most likely the ones in the Golgi/TGN) resulting in differential cell trafficking.

![Figure 3](https://example.com/fig3.png)

**Fig. 3.** γ-Secretase inhibitor treatment results in plasma membrane accumulation of PS1 and PEN-2 but not that of Nct. A, N2a cells were treated with either dimethyl sulfoxide (DMSO), inhibitor X (X), or compound E (E) for 16 h, and cell surface biotinylation experiments were performed. One dimethyl sulfoxide-treated sample was not subjected to biotinylation and therefore was used as a negative control. Cells were lysed, and biotinylated cell surface proteins were affinity precipitated by streptavidin-agarose beads and analyzed by Western blotting. γ-Adaptin was used as a negative control for non-surface protein. PEN-2 was detected by PNT2 antibody, and Nct was detected by SP716 antibody. PS1-CTF and PS1-NTF were detected by anti-PS1Loop and Ab14 antibodies, respectively. APP was detected by antibody 369. Na,K-ATPase α was used as a control for cell surface proteins. B, quantification data of three independent experiments performed as in A. D, E, and X represent dimethyl sulfoxide, compound E, and inhibitor X, respectively. Standard errors were presented. C, kinetic studies of plasma membrane-bound PEN-2, PS1-NTF, and Nct after compound E treatment. N2a cells were incubated with either dimethyl sulfoxide or compound E (3 µM) for various times, and then surface-bound PEN-2 and PS1-NTF were detected by surface biotinylation and Western blotting using PNT2 and Ab14 antibodies. Nct was detected by SP716 antibody.
surface trafficking of PS1 fragments and PEN-2 compared with Nct.

**γ-Secretase Inhibitor Treatment Specifically Accelerates Golgi/TGN to Plasma Membrane Transport of PEN-2 and PS1 Fragments**—The failure of MRL631 to raise cell surface levels of PEN-2 and PS1 fragments suggests that membrane-permeable γ-secretase inhibitors may accelerate the delivery of PEN-2 and PS1 fragments to the plasma membrane. To test this possibility, we first assessed whether newly synthesized PS1 fragments can be detected on the cell surface. WT and PS1-/-/PS2-/- (2×KO) ES cells were subjected to pulse-chase and biotinylation analysis (see “Materials and Methods”), an approach that allows newly synthesized proteins to exit synchronously from the TGN to the cell surface. Briefly, newly synthesized proteins were labeled by [35S]methionine at 37 °C and accumulated in the TGN by chasing at 20 °C and then released in the absence or presence of compound E for 90 min to resume their trafficking from the TGN to the plasma membrane. After biotinylation and affinity precipitation using streptavidin-agarose beads and visualized by autoradiography. As shown in Fig. 6A, after release of the TGN block, a band just below 30 kDa (the molecular mass of PS1-NTF is 27 kDa) appeared in ES WT cells, but not in ES 2×KO cells, suggesting that this ~30-kDa band represents authentic endogenous PS1-NTF. In addition, this band is specifically enhanced after compound E treatment, which is consistent with our above observations.

We further carried out more detailed dynamic studies on the plasma membrane delivery of PS1-NTF and PEN-2 after compound E treatment. To increase sensitivity, N2a PSP15 cells stably expressing PS1, PEN-2, and APP were used. In this set of experiments, we observed a marked increase in the plasma membrane delivery of both PEN-2 and PS1-NTF by inhibitor treatment in a time-dependent manner. The inhibitor, however, does not affect the TGN to cell surface transport of APP (Fig. 6B).

**DISCUSSION**

Our study demonstrates the following: 1) PSs are required for maintaining the stability and proper subcellular localization of PEN-2; 2) abrogation of γ-secretase function in WT cells by membrane-permeable γ-secretase inhibitors, but not a membrane-impermeable derivative, markedly increases cell surface levels of PEN-2 and PS1 without affecting that of Nct; 3) this increase results from the accelerated transport of PEN-2 and PS1 from the Golgi to the cell surface; 4) plasma membrane accumulation of PEN-2 resulting from γ-secretase inhibitor treatment requires the presence of PS1.

It is speculated that, like other membrane-associated complexes, the proper assembly of a functional PS-γ-secretase complex should involve multiple and precisely regulated cellular events. Studies using gene inactivation and gene knockdown approaches reveal that the stability and maturation of each component of the γ-secretase complex relies on its other members (see Ref. 28 for review). It is becoming clear that proper assembly of nascent PS1, Nct, PEN-2, and APH-1 as a complex within the ER is a prerequisite for posttranslational maturation, stabilization, and trafficking of the complex to its final destination. In support of this view, our previous studies revealed the requirement of PS1 for the proper trafficking and maturation of Nct (41). Similarly, here we report that, in the absence of PS1, PEN-2 is retained in the ER. Because the half-life of PEN-2 in the absence of PS is still quite long, the failure in PEN-2 trafficking to the Golgi is likely not due to its rapid degradation in the ER. Rather, the prolonged residence in the ER because of PS deficiency may render PEN-2 vulnerable to degradation presumably by the ER-associated protein degradation machinery. To support this notion, we performed an experiment in which PEN-2 degradation in PS-deficient cells was partially prevented by using the proteasome inhibitor MG132 and found that PEN-2 still fails to exit the ER (data not shown). Recently, the observation that PEN-2 can be degraded
by the ER-associated proteasome degradation machinery was reported by other groups during the preparation of our manuscript (65, 66).

In an attempt to determine whether inactivation of PS function by γ-secretase inhibitors might affect the fate of γ-secretase components in secretory compartments, we treated cells with a transition-state analog (inhibitor X) or a non-transition state small molecule (compound E) γ-secretase inhibitor. We observed a marked increase in the amount of PS1-derived NTF and CTF being transported to the cell surface. Additionally, PEN-2 was also targeted to the cell surface upon treatment of cells with γ-secretase inhibitors in a PS1-dependent manner. Using pulse-chase and biotinylation experiments we demonstrated that these inhibitors specifically accelerated the TGN to the plasma membrane delivery step of PS1 and PEN-2 trafficking (Fig. 6). TGN is a key compartment involved in various cellular functions such as protein sorting, secretory granule formation, and endoproteolysis of prohormones and transmembrane proteins, including APP (67). A recent study demonstrated that the TGN is one of the major organelles for the assembly of the functional PS complex (68). Several signal transduction pathways have been known to affect Aβ generation by impacting secretory vesicle biogenesis from the TGN (67, 69–71). Here we report that γ-secretase inhibitor treatment specifically accelerated PS1 and PEN-2 trafficking from the TGN to the cell surface but not from the ER to the Golgi. Together with the observation that there are no changes in cell surface levels of PS1 and PEN-2 upon treatment by a membrane-impermeable γ-secretase inhibitor, we propose that γ-secretase inhibitors target PS1 complexes (or PS1/PEN-2 subcomplexes, see below) in the Golgi/TGN. Once bound by γ-secretase inhibitors, the PS1 molecules (together with PEN-2) may tend to get incorporated into vesicles budding from the TGN either by loss of their retention ability or conformation-induced recognition by the TGN sorting machinery.

Although γ-secretase inhibitor treatment causes accelerated cell surface delivery of PEN-2 and PS1, we detected little change in the levels of cell surface Nct, another integral component of the γ-secretase complex. This observation of the distinct trafficking of PS1/PEN-2 and Nct upon inhibitor treatment suggests that γ-secretase inhibitors may target a minimal assembly composed of PEN-2 and PS1 in the Golgi and consequently facilitate their packaging into secretory vesicles and thus accelerate their transport to the cell surface. This notion is consistent with a recently proposed model of subcomplexes in the biogenesis/assembly of functional γ-secretase: PEN-2 may exist in a mini-complex with PS1 (72), whereas Nct and APH-1 associate with each other forming a Nct-APH-1 “subcomplex” (73, 74). In fact, it has recently been reported that γ-secretase components exist as multiple forms of membrane-bound complexes (75). Alternatively, it is conceivable that γ-secretase inhibitors may target PS molecules within the active, four-component complex localized to the Golgi, leading to complete or partial disassembly of the complex. In this scenario, Nct is retained in the Golgi, whereas the “free” PEN-2-PS mini-complex is transported by default anterograde trafficking to the cell surface. One cannot distinguish at present which is the correct model. Further experiments characterizing the effect of γ-secretase inhibitors on disassembly of the complex are necessary to fully explain our observations. Although it is not clear how the inhibitors affect the γ-secretase complex at the molecular level, it is conceivable that these inhibitors alter the conformation as well as the trafficking property of PS1 through a direct interaction with PS1 (this interaction has previously been reported (60, 61, 64)), which in turn affects the proper composition and/or trafficking of γ-secretase components.

Taken together, our results strongly support an essential role for PSs in intracellular trafficking of individual γ-secretase components during biogenesis as well as following their assembly into a functional multimeric complex. Our findings also suggest that highly selective inhibitors of γ-secretase, which target the intracellular PS1 complex, can cause altered localization/trafficking of some of its components, a novel functional mechanism for γ-secretase inhibitors.
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