

\( \gamma\)-Secretase Exists on the Plasma Membrane as an Intact Complex That Accepts Substrates and Effects Intramembrane Cleavage*\\[S]

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Research on Alzheimer’s disease led to the identification of a novel proteolytic mechanism in all metazoans, the presenilin/-\( \gamma\)-secretase complex. This unique intramembrane-cleaving aspartyl protease is required for the normal processing of Notch, Jagged, \( \beta\)-amyloid precursor protein (APP), E-cadherin, and many other receptor-like proteins. We recently provided indirect evidence of \( \gamma\)-secretase activity at the cell surface in HeLa cells following inhibition of receptor-mediated endocytosis. Here, we directly identify and isolate \( \gamma\)-secretase as an intact complex (Presenilin, Nicastrin, Aph-1, and Pen-2) from the plasma membrane, both in overexpressing cell lines and endogenously. Inhibition of its proteolytic activity allowed cell surface \( \gamma\)-secretase to be captured in association with its plasma membrane-localized APP substrates (C83 and C99). Moreover, non-denaturing isolation of the intact enzyme complex revealed that cell surface \( \gamma\)-secretase can specifically generate amyloid \( \beta\)-peptide from an APP substrate and similarly cleave a Notch substrate. These data directly establish the proteolytic function of \( \gamma\)-secretase on the plasma membrane, independent of a hypothesized substructure trafficking role. We conclude that presenilin/-\( \gamma\)-secretase exists as a mature complex at the cell surface, where it interacts with and can cleave its substrates, consistent with an essential function in processing many adhesion molecules and receptors required for cell-cell interaction or intercellular signaling.

Presenilin (PS)\(^1\) was originally identified as a gene in which missense mutations lead to an aggressive, dominantly inherited form of early-onset Alzheimer’s disease (1). Subsequent studies in many laboratories support the hypothesis that PS functions as the catalytic subunit of the \( \gamma\)-secretase complex (2), an unprecedented intramembrane aspartyl protease that similarly cleaves a Notch substrate. These data directly establish the proteolytic function of \( \gamma\)-secretase on the plasma membrane, independent of a hypothesized substructure trafficking role. We conclude that presenilin/-\( \gamma\)-secretase exists as a mature complex at the cell surface, where it interacts with and can cleave its substrates, consistent with an essential function in processing many adhesion molecules and receptors required for cell-cell interaction or intercellular signaling.

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\(^1\) The abbreviations used are: PS, presenilin; APP, \( \beta\)-amyloid precursor protein; DAPT, \( \alpha\)-(N-\( N\)-(3,5-difluorophenacetyl)-\( L\)-alanyl)-S-phenylglycine \( \tau\)-butyl ester; CHAPOS, 3\( \beta\)-(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid; PAS, protein A-Sepharose; NTF, NH\(_2\)-terminal fragment; CTF, COOH-terminal fragment; ELISA, enzyme-linked immunosorbent assay; PGA, protein-G-agarose.

amyloid \( \beta\)-peptide (A\( \beta\)). It is now known that PS requires three other integral membrane proteins to effect cleavage within the lipid bilayer (3–5), namely Nicastrin (Nct) (6), Aph-1 (7), and Pen-2 (8). \( \gamma\)-Secretase cleaves APP and numerous other type 1 membrane proteins within their transmembrane domains in a process known as regulated intramembrane proteolysis (9), in which an obligatory first cleavage (e.g. \( \beta\)-secretase cleavage of APP in the case of A\( \beta\) generation) sheds most of the ectodomain and allows the substrate to undergo subsequent intramembranous cleavage.

An increasing number of \( \gamma\)-secretase substrates has been identified, including Notch, ErbB4, E-cadherin, Delta/Jagged, nectin-1a, CD44, and LRP (reviewed in Ref. 10), implicating presenilin/-\( \gamma\)-secretase in many normal cellular processing events. PS1 knockout mice show perinatal mortality (11, 12), and genetic deletion of both PS1 and its mammalian homologue, PS2, produces embryonic lethality (13), highlighting the requirement for PS during embryogenesis. Interestingly, most of the known \( \gamma\)-secretase substrates function in intercellular communication or adhesion. Moreover, disruption of PS function perturbs cell-cell interactions, whereas overexpression of PS enhances them (14–16). PS may also be involved in cell-extracellular matrix interactions (17).

Although many \( \gamma\)-secretase substrates are thought to be processed at or near the cell surface, it has been reported that PS resides principally in the endoplasmic reticulum, trans-Golgi network, and intermediate compartments (18–20). This apparent discrepancy between PS localization and \( \gamma\)-secretase activity, referred to as the “spatial paradox” (19, 20), has led some investigators to question whether and how presenilin can mediate \( \gamma\)-secretase cleavage of plasma membrane receptors. In this regard, some studies have detected a population of PS molecules on the plasma membrane using surface biotinylation (17, 21, 22). In addition, PS has been detected at or near the cell surface by immunofluorescence or immunoelectron microscopy (14–17, 22–27), and fully glycosylated (i.e. mature) Net has also been observed at the surface by biotinylation (22, 28, 29).

However, the other two obligatory \( \gamma\)-secretase members, Aph-1 and Pen-2, have not yet been localized, and most importantly, no evidence of an assembled, active enzyme complex at the cell surface has been achieved. In addition, whereas some studies have suggested a trafficking function for PS at the surface (22, 30–32), definitive proof of a proteolytic function for \( \gamma\)-secretase on the plasma membrane has yet to be demonstrated.

\( \gamma\)-Secretase cleavage of the APP derivatives, C83 and C99, at the cell surface would have additional significance in terms of targeting therapeutic compounds that inhibit the production or increase the clearance and degradation of A\( \beta\) in aged humans and patients with Alzheimer’s disease. Therefore, we undertook a systematic analysis of the entire \( \gamma\)-secretase complex and its activity on APP and Notch substrates at the cell surface. In this article, we provide direct evidence for a role of
γ-secretase in the proteolysis of substrates on the plasma membrane, in accord with an essential function in processing various receptors and adhesion molecules required for cell-cell interaction and intracellular signaling. Cell surface cleavage of γ-secretase substrates that are involved in intercellular adhesion suggests a potential role for γ-secretase in the structural disassembly of cell-cell adhesions, in addition to its role in releasing intracellular signaling fragments. Furthermore, our data demonstrate the involvement of the cell surface in Aβ generation, suggesting that inhibitors of β- or γ-secretase targeted to the exterior of the cell may prove efficacious.

MATERIALS AND METHODS

Cell Culture and Cell Lysate Preparation—γ-30 cells (5) were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, penicillin/streptomycin, G418, puromycin, zeocin, and hygromycin. HeLa dynamin K44A cells were grown as described previously (33). Tetracycline was present in all media to maintain gromycin. HeLa dynamin K44A cells were grown as described previously (33) with the mouse monoclonal antibody M2-agarose. Surface biotinylated γ-secretase was selected from this pool of total cellular γ-secretase using a second streptavidin-agarose pulldown of FLAG-peptide elutions of the M2-agarose-immunoprecipitated samples. 10 μM DAPT was added to some samples prior to the addition of C100-FLAG or N100-FLAG substrates and phospholipids as a control. Biotinylated HeLa cell lysates were precipitated with streptavidin-agarose directly for activity assays.

ELISA—ELISA for Aβ X-40 was performed as described previously (29). Surface biotinylated mature Nct, holo-PS, and the PS NTF (data not shown). In addition, we specifically recovered the PS COOH-terminal fragment (CTF) (Fig. 1). Each biotinylated protein co-migrated with its corresponding control. Biotinylated HeLa cell lysates were precipitated with streptavidin-agarose directly for activity assays.

Quantitative Western Blotting—PS NH2-terminal fragment (NTF) levels in M2-agarase immunoprecipitations and in M2-agarase/streptavidin-agarose sequential precipitations were quantified by densitometry using ImageQuant software (Amersham Biosciences). Standard curves for PS NTF were generated by Western blotting 2-fold dilutions of M2-agarase/streptavidin-agarose precipitates. The fraction of cell surface-associated PS NTF was quantified using the standard curve generated from Western blots of serial 2-fold dilutions of PS NTF from M2-agarase immunoprecipitations.

RESULTS

Direct Biotinylation of Each γ-secretase Complex Member at the Cell Surface—First, we attempted to demonstrate the presence of each γ-secretase complex member on the plasma membrane using surface biotinylation. Nct and PS have previously been biotinylated at the cell surface (17, 21, 22, 28, 29), and we repeated those results here. The recent elucidation of the topologies of Aph-1 and Pen-2 (36, 37) suggested that these proteins could also be biotinylated in their extracellular domains. Aph-1 contains one primary amine group available for biotinylation at the free amino terminus of the protein, which protrudes into the luminal space. The internal lysines are all facing the cytoplasmic side and are not available for biotinylation. Pen-2 is oriented with both its amino and carboxyl termini facing the luminal side, thus presenting its free amino terminus as well as two lysines at positions 11 and 17 for possible biotinylation.

The γ-30 Chinese hamster ovary cell line, which stably over-expresses human PS1, Aph-1-HA, and FLAG-Pen-2 in the presence of high amounts of endogenous Net, has markedly elevated γ-secretase activity (5). We biotinylated intact γ-30 cells and precipitated biotinylated surface proteins using streptavidin-agarose beads. Following surface biotinylation and pull-downs, we recovered biotinylated mature Net, holo-PS, and the PS NTF produced by endoproteolysis of PS (38) only in the biotinylated sample pulled down with streptavidin beads (Fig. 1), results are consistent with earlier reports (17, 21, 22, 28, 29). We likewise recovered the PS COOH-terminal fragment (CTF) (data not shown). In addition, we specifically recovered biotinylated Aph-1-HA and FLAG-Pen-2 from the cell surface (Fig. 1). Each biotinylated protein co-migrated with its corre-
elution of 2% digitonin. We verified that the low pH treatment allowed consistent with the presence of the intact, mature complex at the cell surface. Biotinylated holo-PS is present because we used an antibody, 1563, targeted against PS for immunoprecipitation and does not represent incorporation of holo-PS into fully assembled γ-secretase complexes.

We next analyzed the 1563-precipitated, pH-eluted γ-secretase by blue native PAGE. We found a strong biotinylated band migrating slightly larger than the 232-kDa marker using 1/20th of the total immunoprecipitated sample from a confluent 10-cm dish of γ-30 cells (Fig. 2B). This band migrated at a size for γ-secretase previously reported by our laboratory (5). The biotinylated band co-precipitated on the native gel with Nct, Aph1-HA, and FLAG-Pen2 from the same samples probed with specific antibodies. These results demonstrate robust biotinylation of the intact, 4-component γ-secretase complex at the cell surface in γ-30 cells.

Endogenous, Intact γ-Secretase Resides at the Cell Surface in HeLa Cells—We next asked if we could find the enzyme complex on the plasma membrane under endogenous conditions. We had previously used HeLa cells to obtain indirect evidence for γ-secretase proteolytic activity at the cell surface (33). HeLa cells were biotinylated as described above, and lysates were prepared in 1% digitonin lysis buffer. We immunoprecipitated with 1563 and eluted intact γ-secretase using the low pH elution.

Specific anti-biotin reactive bands were detected in the biotinylated 1563 immunoprecipitate lane, with no signals in control samples. The biotin-positive bands co-precipitated with co-immunoprecipitated Nct, PS NTF, and Aph1-HA (Fig. 3A). However, we were unable to consistently detect a biotinylated band that co-precipitated with endogenous Pen2 (Fig. 3A, left panels), despite the clear co-immunoprecipitation of Pen2 with PS (Fig. 3A, right panels). This result is consistent with the very low level of biotinylated FLAG-Pen2 detectable by straight biotinylation in the overexpressing γ-30 cells (Fig. 1, lane 2) and with our difficulty in detecting a biotinylated band that co-precipitates with FLAG-Pen2 following 1563 immunoprecipitation in these cells (Fig. 2A). These data suggest that the N terminus of Pen2 is buried and not easily accessible for surface biotinylation. We showed above in Fig. 2 the presence of holo-PS at the cell surface in the overexpressing γ-30 cell line. However, we have been unable to detect any endogenous holo-PS at the cell surface in HeLa cells at any time, and have obtained no evidence of its existence at the cell surface endogenously.

Using blue native PAGE, we observed a biotin-positive band migrating slightly larger than the 232-kDa marker only in the biotinylated 1563 immunoprecipitate (Fig. 3B). This band, which represents 1/6th of the immunoprecipitate from a confluent 10-cm plate of HeLa cells, was immunoreactive for Nct, PS, and Aph1. No suitable antibody for detecting endogenous Pen2 on blue native PAGE was available. Overall, we believe that the biotin-positive band co-precipitated with Nct, PS, and Aph1 on blue native PAGE demonstrates that the endogenous γ-secretase complex resides at the surface of normal, untransfected cells.

APP C83 and C99 Are Associated with the γ-Secretase Complex at the Cell Surface—γ-Secretase is a protease with the highly unusual characteristic of being associated with low levels of its substrates (e.g., C83 and C99), even when its protease activity is not being inhibited (39). An apparent explanation for this phenomenon is that the enzyme has an initial docking site for its substrates that is distinct from the active site (35). A conformational change in substrate or protease may then allow
FIG. 2. Cell surface biotinylation of intact γ-secretase. γ-Secretase was immunoprecipitated from surface-biotinylated γ-30 cells using the PS NTF antibody, 1563, in 1% digitonin lysis buffer, which keeps the γ-complex intact. Immunoprecipitated proteins were eluted using a brief 100 mM glycine, pH 2.8, elution and analyzed for co-immunoprecipitated and biotinylated proteins. A, analysis by SDS-PAGE after immunoprecipitation with 1563 (to PS1) reveals the co-precipitation of Nct, Aph-1, and Pen-2 (right panels). Probing with an anti-biotin antibody reveals the presence of biotinylated proteins that co-migrate with Nct, PS, Aph-1, and Pen-2 (left panels). B, analysis of the 1563-precipitated complex by blue native PAGE reveals the presence of a biotinylated complex migrating slightly larger than 232 kDa (left panel) that contains Nct, PS, Aph-1, and Pen-2 (right panels), thus representing γ-secretase. Controls of precipitation with beads alone or mock biotinylation show no background signal (left panel). PGA, protein G-agarose.
access of the scissile bond to the catalytic region of the enzyme. We took advantage of this evidence that γ-secretase can be captured with its substrates to ask whether C83 and C99 associate with γ-secretase directly at the cell surface.

First, we demonstrated that we could directly biotinylate C83 and C99 at the cell surface in γ-30 cells (Supplemental Materials Fig. S1). We detected biotinylated C83 and C99 by immunoprecipitating with the affinity purified COOH-terminal APP antibody, C9, and then probing against biotin. We further showed that treatment of γ-30 cells with the γ-secre-

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**Fig. 3. Cell surface biotinylation of endogenous γ-secretase in HeLa cells.** γ-Secretase was immunoprecipitated from surface-biotinylated HeLa cells and eluted as described in the legend to Fig. 2. A, analysis by SDS-PAGE after immunoprecipitation by 1563 (to PS1) reveals the co-precipitation of Nct, Aph-1, and Pen-2 (right panels). Probing with an anti-biotin antibody reveals the presence of biotinylated proteins that co-migrate with Nct, PS, and Aph-1 (left panels). Biotinylated endogenous Pen-2 was not detectable. B, analysis of the 1563-precipitated complex by blue native PAGE reveals the presence of a biotinylated complex migrating slightly larger than 232 kDa and containing Nct, PS, and Aph-1, thus representing γ-secretase. No suitable antibody for endogenous Pen-2 detection on blue native PAGE was available. Controls of precipitation with beads alone or mock biotinylation show no background signal. *, nonspecific band that does not appear consistently.
tase inhibitor, DAPT, increased total cellular pools of C83 and C99 and also increased the levels of surface biotinylated C83 and C99. These data are consistent with previously published data that finds C83 and C99 at the cell surface (28, 31, 32).

We next asked if we could co-immunoprecipitate biotinylated C83 and C99 using an antibody, X81, targeted against PS NTF. We treated cells with DAPT to increase C83 and C99 association with \(\gamma\)-secretase and, importantly, also to select for C83 and C99 associated with proteolytically active \(\gamma\)-secretase. We verified that DAPT treatment specifically increased C83 and C99 levels (Fig. 4A). Western blotting of X81 co-immunoprecipitates revealed substantially increased C83 and C99 co-precipitation in DAPT-treated samples as compared with control. B, a small pool of surface-biotinylated C83 and C99 is captured with \(\gamma\)-secretase following inhibition with DAPT. Antibody X81 was used to immunoprecipitate PS, followed by a sequential IP of co-precipitating APP COOH-terminal fragments (C83 and C99) with antibody C9. Controls were precipitation with PAS beads alone or after mock biotinylation. All 3 samples were blotted with antibiotin (left) or C9 (right). The holo-PS doublet represents conformers of the holo-PS protein. The collapse of these forms on other blots is likely due to variations in denaturation prior to PAGE or to lack of optimal resolution, and we have no current evidence that these phenomenon are biologically important.

![Fig. 4](http://www.jbc.org/)

**Fig. 4.** Cell surface association of C83 and C99 with \(\gamma\)-secretase. \(\gamma\)-30 cells were treated with MeSO vehicle alone or DAPT in MeSO for 4 h. A, DAPT treatment selectively increased the total cellular levels of C83 and C99, while total cellular levels of Nct, PS, Aph-1, and Pen-2 were unaffected. DAPT treatment markedly enhanced the co-immunoprecipitation of C83 and C99 with presenilin using the PS NTF antibody X81, whereas co-immunoprecipitation of Nct, Aph-1, and Pen-2 were unaltered as compared with control. B, a small pool of surface-biotinylated C83 and C99 is captured with \(\gamma\)-secretase following inhibition with DAPT. Antibody X81 was used to immunoprecipitate PS, followed by a sequential IP of co-precipitating APP COOH-terminal fragments (C83 and C99) with antibody C9. Controls were precipitation with PAS beads alone or after mock biotinylation. All 3 samples were blotted with antibiotin (left) or C9 (right). The holo-PS doublet represents conformers of the holo-PS protein. The collapse of these forms on other blots is likely due to variations in denaturation prior to PAGE or to lack of optimal resolution, and we have no current evidence that these phenomenon are biologically important.

To capture biotinylated C83 and C99 with \(\gamma\)-secretase, we immunoprecipitated PS with X81 from surface-biotinylated \(\gamma\)-30 cells treated with DAPT. We eluted the co-immunoprecipitated proteins in conditions that disrupt protein-protein interactions, and then immunoprecipitated C83 and C99 with C9. Western blotting revealed the recovery of biotinylated C83 and C99 in the DAPT-treated samples that co-migrated with total co-immunoprecipitated C83 and C99 (Fig. 4B). We could not detect co-immunoprecipitated biotinylated C83 and C99 in samples treated with MeSO alone (data not shown). This result is in accord with the low levels of total biotinylatable C83 and C99 in cells (Supplemental Materials Fig. S1).

The Cell Surface Pool of \(\gamma\)-Secretase Is Functionally Active on Both APP and Notch Substrates in \(\gamma\)-30 Cells—Detecting biotinylated C83 and C99 complexed with \(\gamma\)-secretase at the cell surface following DAPT treatment suggests the existence of proteolytically active \(\gamma\)-secretase on the plasma membrane. As direct verification, we performed \(\gamma\)-secretase activity assays using biotinylated \(\gamma\)-secretase isolated from the cell surface. To perform these experiments, we developed a sequential pull-down protocol to select first for \(\gamma\)-secretase using M2-agarose immunoprecipitation (against FLAG-Pen-2) and second for cell surface proteins using streptavidin-agarose (Supplemental Materials Fig. S2). We performed in vitro activity assays on these samples using the well characterized \(\gamma\)-secretase sub-
strate C100-FLAG (35, 41), and measured de novo Aβ x-40 generation by ELISA. To confirm that we were measuring γ-secretase activity, we treated some samples with 10 μM DAPT. As shown in Fig. 5A, we found a very robust generation of Aβ x-40 peptide from samples specifically containing cell surface γ-secretase. These samples generated 27 ± 12 ng/ml Aβ x-40 (mean ± S.D.), whereas controls of biotinylated PAS pulldowns had 0.08 ± 0.17 ng/ml, and mock biotinylated streptavidin-agarose pulldowns had 1.1 ± 0.5 ng/ml. This proteolytic activity was inhibited by a mean of 87% by 10 μM DAPT (to 3.6 ± 0.38 ng/ml), proving that we are measuring bona fide γ-secretase activity. These data represent 6 sets of samples generated in three independent experiments (p < 0.005).

In parallel, we Western blotted for AICD-FLAG, the complementary product of the γ-secretase cleavage reaction, in the same samples we analyzed for Aβ x-40 by ELISA. As shown in a representative Western blot (Fig. 5A), we observed the specific generation of AICD-FLAG in the biotinylated streptavidin-agarose pulldown lane. These results reveal that the cell surface γ-secretase in γ-30 cells is proteolytically active on APP substrates. We next performed in vitro activity assays using the well characterized substrate, N100-FLAG, derived from the Notch protein (41), and analyzed results by Western blotting. Just as seen with C100-FLAG, N100-FLAG was specifically cleaved to generate NICD-FLAG in the biotinylated streptavidin-agarose pulldown lane, with little to no background in controls (Fig. 5B). Notably, DAPT treatment inhibited the generation of NICD-FLAG. Thus, cell surface-derived γ-secretase is competent to cleave multiple single transmembrane substrates.

The Cell Surface Pool of Endogenous γ-Secretase Is Functionally Active—We next searched for endogenous γ-secretase activity at the cell surface in HeLa cells. To this end, we precipitated biotinylated HeLa cell lysates directly with streptavidin-agarose and performed in vitro activity assays. Biotinylated samples that were specifically precipitated with streptavidin-agarose generated a mean of 10.6 ± 2.3 ng/ml Aβ x-40 in 24 h (Fig. 5). In comparison, control pulldown samples had levels of Aβ x-40 below the limits of detection. Biotinylated streptavidin-agarose pulldown samples treated with 10 μM DAPT generated 1.6 ± 0.5 ng/ml of Aβ x-40, representing an 85% inhibition of Aβ x-40 generation. These data were highly consistent in eight sets of samples from four independent experiments (p < 0.00001). The levels of Aβ x-40 generated by cell surface γ-secretase in γ-30 cells (above) are not comparable on a direct quantitative basis to those generated in HeLa cells, as we used different preparations of phosphatidylethanolamine, phosphatidylcholine, and C100-FLAG substrate in generating each set of data. In the complementary assay, Western blotting revealed the specific generation of AICD-FLAG in the biotinylated streptavidin-agarose pulldown samples, but no detectable AICD-FLAG in either control pulldown or DAPT-treated samples (Fig. 6).

Estimating the Fraction of Cellular γ-Secretase at the Cell Surface—We next estimated the fraction of total cellular γ-secretase that resides at the cell surface via two methods. First, we quantified Aβ x-40 generation in in vitro γ-secretase assays using ELISA; and second, we compared the levels of co-immunoprecipitated PS NTF from specific resin pulldowns using quantitative Western blotting. In the first approach, we
estimate the fractional γ-secretase activity at the cell surface; in the second approach, we estimate the fractional γ-secretase protein level at the cell surface. Using γ-30 cells, we first selected for γ-secretase by immunoprecipitating with M2-agarose. We considered this material to represent 100% of total cellular γ-secretase. We then eluted γ-secretase using excess FLAG peptide and performed a second pulldown with streptavidin-agarose. We considered the latter material to represent the total recoverable cell surface γ-secretase.

We performed activity assays using C100-FLAG on both the M2-agarose samples and the M2-agarose/streptavidin-agarose samples. We normalized Aβ x-40 levels from each M2-agarose/streptavidin-agarose sample to the levels from the corresponding M2-agarose samples. We found that the recoverable cell surface pool of γ-secretase had an activity level of 6.1% ± 1.6% of the total cellular γ-secretase. These data represent 9 sets of samples from three independent experiments.

For the second method (quantitative densitometry), we normalized the levels of PS NTF in the M2-agarose/streptavidin-agarose samples to standard curves generated by serial 2-fold dilutions of the corresponding M2-agarose samples. We found that the recoverable cell surface levels of PS NTF averaged 6.5% ± 1.4% of the total cellular PS NTF complexed with FLAG-Pen-2. Thus, the fractional activity and fractional protein levels of γ-secretase at the cell surface are in very close agreement, allowing us to conclude that γ-secretase at the cell surface represents ~6–7% of the total cellular γ-secretase in γ-30 cells under the conditions of our assay.

**DISCUSSION**

Presenilin-mediated intramembrane proteolysis was originally identified for APP and Notch, one a single transmembrane protein of unknown function and the other a well studied surface receptor critical for intercellular signaling in metazoans. Subsequently, it has become apparent that a variety of type 1 integral membrane proteins undergo proteolysis within the lipid bilayer by γ-secretase. Indeed, it may well turn out that a large number of proteins that undergo ectodomain shedding by α-secretase-like proteases (“sheddases”) become substrates for PS/γ-secretase (42). Therefore, γ-secretase, which is highly conserved in evolution and has been documented down to slime molds, may be a kind of “housekeeping” protease that turns over hydrophobic, membrane-embedded protein segments. In addition, some of the cytoplasmic fragments released by this processing have themselves evolved to confer a variety of important signaling functions.

In view of the multifaceted and vital biological implications of this unusual processing mechanism, it becomes important to establish definitively whether the PS/γ-secretase complex exists on the plasma membrane, can accept receptor substrates there, and is then proteolytically active. Here, we present several lines of evidence that demonstrate conclusively: 1) that Aph-1 and Pen-2 are present on the cell surface; 2) that the four components form an intact enzyme complex at the surface that can be biotinylated and co-immunoprecipitated; 3) that the substrates derived from APP (C83 and C99) can complex with the enzyme at the cell surface; and 4) that γ-secretase isolated from the cell surface can cleave both Notch and APP substrates.

In this work, we find intact, active γ-secretase on the plasma membrane at endogenous levels of protein expression. Whereas we do not detect biotinylated endogenous Pen-2 co-immunoprecipitating with PS nor have a suitable Pen-2 antibody on blue native PAGE, we believe that Pen-2 is present for the following reasons. First, biotinylation of Pen-2 is very difficult, as evidenced by the very weak biotinylation of a FLAG-tagged form of Pen-2 in the overexpressing γ-30 cell line. Second, the endogenous complex migrates at the same apparent molecular weight as the complex from γ-30 cells. Finally, the endogenous cell surface complex is proteolytically active. The inclusion in each experiment of a set of negative controls, including mock precipitations with protein A or protein G beads, mock biotinylations, and probing for control proteins, ensured that the results achieved were specific. We were also able to perform quantitative assays of γ-secretase activity that included enzyme inhibition with a widely validated γ-secretase inhibitor (DAPT), again ensuring the accuracy of the results. We found that the two most extensively characterized substrates of PS/γ-secretase, APP and Notch, were similarly processed by the plasma membrane-derived enzyme complexes. The approach described here could be extended to the study of other recently identified γ-secretase substrates to determine whether they have closely similar or disparate degrees of proteolysis by the surface enzyme as a fraction of total cellular γ-secretase activity.

We detected biotinylated C83 and C99 at the cell surface in γ-30 cells and found that treatment with DAPT increased their cell surface levels. Three previously published studies also reported increased cell surface C83 and C99 using PS mutants that are proteolytically inactive (28, 31, 32). Interestingly, these studies also reported that holo-APP levels at the cell surface were increased in cell lines expressing mutant PS. Kaether et al. (22) further reported that APP internalization from the cell surface was decreased following DAPT treatment,

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2 S. Zorca, L. Kreppel, and A. Kimmel, personal communication.
and that total surface APP was increased. In our experiments, we did not see a similar rise in biotinylated cell surface holo-APP levels. Under these conditions, in which the processing of γ-secretase substrates was inhibited by DAPT, we found that a fraction of cell surface C83 and C99 was associated with PS. We used the γ-secretase inhibitor to target a pool of γ-secretase that has proteolytic activity. Use of this inhibitor allows us to selectively capture γ-secretase with C83 and C99 molecules that would have otherwise cleaved, i.e. we are affecting a pool of γ-secretase that has proteolytic function. Our results using DAPT coupled with biotinylation firmly establish that C83 and C99 selectively capture

Here, we extend these results by finding C83 and C99 directly complexed with a trafficking mutant, dynamin K44A (33). In those dynamin-mutant cells, virtually all C99 molecules were retained at the cell surface. Here, we extend these results by finding C83 and C99 directly complexed with γ-secretase at the cell surface in the absence of any perturbation of trafficking. In concert with earlier data (47), available evidence suggests that a major pathway for APP processing requires presentation of the molecule at the cell surface, and that this allows APP to specifically enter a proteolytic pathway that generates Aβ. Our previous work (33) suggested that γ-secretase cleavage can also occur following endocytosis, resulting in intracellular generation of Aβ and either its retention in intracellular compartments or its release from recycling endosomes, a conclusion that is again consistent with earlier work (48). This balance of intracellular versus cell surface Aβ generation could be affected by the half-life of APP at the cell surface, as modulated, for example, by interactions with adaptor proteins such as the Mints and Fe65, and also by the levels and dynamic regulation of γ-secretase at the cell surface. The presentation of APP at the cell surface prior to the cleavages that generate Aβ and p3 predicts a cell surface signal that initiates proteolysis of APP. Interestingly, the presence of PS at the cell surface could be dependent on extracellular signals from cell-cell contacts or by ECM substrates (14–17, 24) and may further affect the balance of secreted versus intracellular Aβ.

We concluded our experiments with in vitro γ-secretase cleavage assays that demonstrate the biological activity of cell surface γ-secretase. In γ-30 cells, we estimate that 6% of the total cellular γ-secretase activity resides at the cell surface. In excellent agreement, we find that ~6.5% of γ-secretase protein resides at the cell surface, as judged by quantitative Western blotting for PS NTF. Kaether et al. (22) reported that ~3.3% of PS CTF resides at the cell surface, as determined via quantitative Western blotting of PS CTF, a result rather close to our value. Our data provide the first estimate of cell surface γ-secretase levels based on an enzymatic assay, thereby focusing on its functional role in proteolysis versus in a putative trafficking function at the plasma membrane. While our estimates are based on cleavage activity assays in vitro, the actual fraction of total cellular Aβ production at the cell surface in vivo depends on the relative surface concentrations of both substrate and enzyme and their rates of flux to and from the plasma membrane. It is relevant to our data that another protease, TACE, known to cleave certain substrates specifically at the cell surface (including APP and Notch) occurs, like presenilin, predominantly within intracellular compartments at steady-state (49). Thus, finding only a small fraction of γ-secretase at the cell surface does not preclude the functional importance of this pool. Whereas the steady-state levels of γ-secretase measured at the cell surface represent a minority of the total cellular γ-secretase, we do not know what fraction of γ-secretase traffics through the cell surface and how rapidly it does so. It will be interesting to study the kinetics of γ-secretase endocytosis, which we predict to be rapid, based on the apparently high steady-state levels within intracellular compartments suggested by immunocytochemistry. Moreover, one will require an estimate of the kinetics of γ-secretase cleavage following the initial substrate binding step relative to the kinetics of endocytosis, as cell surface γ-secretase-C99 complexes are dynamic and could be internalized in part prior to the γ-cleavage.

Emerging data suggest that γ-secretase is still a valid candidate for pharmacological inhibition in the treatment of Alzheimer’s disease. The Aβ kinase inhibitor, Gleevac, and a related molecule have been shown to lower Aβ40 and Aβ42 in neuronal cultures and in guinea pig brain in vivo without inhibiting Notch cleavage (50). We have recently reproduced this finding.\(^3\) This selectively signifies that an FDA-approved drug that lowers Aβ in the mammalian brain can spare Notch processing. In view of these findings, it is likely that other small molecules will be found to show such selectivity. Moreover, several papers have confirmed and extended the discovery of Weggen and co-workers (51) that certain approved non-steroidal anti-inflammatory drugs (e.g. ibuprofen) can selectively lower Aβ42 and raise Aβ38 levels without affecting production of Aβ40 and without significantly interfering with Notch signaling (52, 53). We recently reported that this effect of a non-steroidal anti-inflammatory drug can be directly observed in highly purified γ-secretase preparations in vitro, indicating that the target is the presenilin-γ-secretase complex itself (54). Our current findings suggest that a γ-secretase inhibitor need not necessarily penetrate the cell surface to be effective as a treatment for Alzheimer’s disease.

Overall, our data provide direct and compelling evidence for a proteolytically active pool of γ-secretase on the plasma membrane. Moreover, establishing that the mature presenilin-γ-secretase complex can interact with and cleave its substrates at the cell surface strengthens the concept that a common property of γ-secretase substrates is that they perform functions at the plasma membrane, including cell adhesion and intercellular signaling. Finally, cell surface γ-secretase activity suggests that γ-secretase may play an active role in the structural disassembly of cell-cell adhesions, as well as the release of intracellular signaling fragments.

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REFERENCES

1. Sherrington, R., Rogaev, E. I., Liang, Y., Rogaeva, E. A., Levesque, G., Ikeda, M., Chi, H., Lin, C., Li, G., Holman, K., Tsuda, T., Mar, L., Fonsin, J.-F., Brun, A. C., Montesi, M. P., Sorbi, S., Rainero, I., Pinassi, L., Nee, L., Chumakov, I., Pollen, D. A., Roses, A. D., Fraser, P. E., Rommens, J. M., and St. George-Hyslop, P. H. (1995) Nature 375, 754–760
2. Wolfe, M. S., Xia, W., Otsazewski, B. L., Dehl, T. S., Kimberly, W. T., and Selkoe, D. J. (1999) Nature 399, 513–517
3. Ed Bauer, D., Winkler, E., Regula, J. T., Tesold, B., Steiner, H., and Haass, C. (2003) Nat. Cell Biol. 5, 486–488
4. Takasugi, N., Tomita, T., Hayashi, I., Tsuuroku, M., Niimura, M., Takahashi, Y., Thinakaran, G., and Iwatsubo, T. (2003) Nature 422, 438–441

\(^3\) P. Fraering, W. Ye, and D. Selkoe, unpublished data.
γ-Secretase Exists on the Plasma Membrane as an Intact Complex That Accepts Substrates and Effects Intramembrane Cleavage
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