Presenilin 1 Regulates Pharmacologically Distinct γ-Secretase Activities

IMPLICATIONS FOR THE ROLE OF PRESENILIN IN γ-Secretase Cleavage*

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M. Paul Murphy‡§, Sacha N. Uljon§, Paul E. Fraser‡, Abdul Fauq‡, Hilary A. Lookingbill‡, Kirk A. Findlay§, Tawnya E. Smith‡, Patrick A. Lewis‡, D. Chris McLendon‡, Rong Wang§, and Todd E. Golde‡**

From the ‡Mayo Clinic Jacksonville, Department of Pharmacology, Jacksonville, Florida 32224, ¶The Rockefeller University, Laboratory for Mass Spectrometry, New York, New York 10021, and §The University of Toronto, Tanz Neuroscience Building, 6 Queen’s Park Crescent, Toronto, Ontario M5S 3H2, Canada

Presenilins (PSs) are polytopic membrane proteins that have been implicated as potential therapeutic targets in Alzheimer’s disease because of their role in regulating the γ-secretase cleavage that generates the amyloid β protein (Aβ). It is not clear how PSs regulate γ-secretase cleavage, but there is evidence that PSs could be either essential cofactors in the γ-secretase cleavage, γ-secretase themselves, or regulators of intracellular trafficking that indirectly influence γ-secretase cleavage. Using presenilin 1 (PS1) mutants that inhibit Aβ production in conjunction with transmembrane domain mutants of the amyloid protein precursor that are cleaved by pharmacologically distinct γ-secretases, we show that PS1 regulates multiple pharmacologically distinct γ-secretase activities as well as inducible α-secretase activity. It is likely that PS1 acts indirectly to regulate these activities (as in a trafficking or chaperone role), because these data indicate that for PS1 to be γ-secretase it must either have multiple active sites or exist in a variety of catalytically active forms that are altered to an equivalent extent by the mutations we have studied.

The 4-kDa amyloid β protein (Aβ) deposited in Alzheimer’s disease (AD) is a normally secreted proteolytic product of the amyloid β protein precursor (APP) (1–3). Generation of Aβ from APP requires two sequential proteolytic events: an initial cleavage at the amino terminus of the Aβ sequence referred to as β-secretase (4) and a subsequent cleavage at the carboxyl terminus known as γ-secretase. Recently, a membrane-bound aspartic protease has been implicated as a β-secretase (5–8). However, the protease(s) responsible for γ-secretase cleavage have not been identified. In addition, a third proteolytic activity referred to as α-secretase cleaves within the Aβ sequence to release a large secreted derivative (sAPP), thus precluding formation of full-length Aβ. In mammalian cells, at least two members of the ADAM family (a disintegrin and metalloprotease) can contribute to the α-secretase activity (9, 10). Although full-length APP is not cleaved by γ-secretase, APP carboxy-terminal fragments (CTF) generated through cleavage of APP by either α- or β-secretase are both substrates for γ-secretase, with cleavage of CTFs releasing a peptide referred to as p3 (Aβ17-40 or Aβ17-42) (1–3).

γ-Secretase-catalyzed cleavages are of particular interest for a number of reasons. First, they are unusual in that the cleavage site of the substrate is predicted to lie within the transmembrane domain (TMD). Rather than primary amino acid sequence, position of the γ-cleavage site with respect to the membrane appears to be the prime determinant of cleavage, with the length of the luminal TMD determining that position (11). Whether γ-secretase actually cleaves residues within the membrane is a controversial topic. To date, there is no definitive evidence showing that any protease can cleave bonds when they are buried within a TMD. Second, altered γ-secretase cleavage is implicated in the development of AD (reviewed in Ref. 12). FAD-linked mutations in APP, presenilin 1 (PS1), and PS2 alter γ-secretase activity by increasing the amount of a minor Aβ species, the more amyloidogenic Aβ42, without significantly altering total Aβ production. Significantly, both PS1 knockout and presenilin aspartate mutants decrease γ-secretase cleavage, but it is not known if this is a direct or indirect effect (13–15). Third, APP CTF are not the only substrate for γ-secretase activity: CTF generated after ligand-induced cleavage of the extracellular domain of Notch are cleaved at residues near the cytoplasm/membrane junction by a γ-secretase-like activity (16–18). Significantly, this γ-secretase activity appears necessary for Notch signaling and also appears to be regulated by PSs. Finally, because γ-secretase cleavage is the final step in the generation of Aβ, it remains a major therapeutic target for strategies designed to lower Aβ production. Thus, γ-secretase is not only an unusual proteolytic activity, but its activity has important biological consequences both with respect to the pathogenesis and treatment of AD and for cell biology in general.

Many disparate roles have been hypothesized to account for...
the observed effects of PSs on γ-secretase cleavage. Recent studies of γ-secretase activity in cells derived from PS1 knock-out mice have implicated PS1 in the regulation of intracellular trafficking (19), as cofactors for γ-secretase activity (13), or as γ-secretases themselves (14). The latter notion that PSs may be γ-secretase gained further support from studies demonstrating that mutation of either of two aspartate residues potentially lying in opposing transmembrane domains in both PS1 and PS2 decrease γ-secretase activity, presumably through a dominant negative mechanism (14, 15). It was thus proposed that PSs may be novel intramembranous proteases with the aspartates functioning as the catalytic residues; alternatively, PSs may function as di-aspartyl cofactors for γ-secretase activity (14). Consistent with this hypothesis, treatment of cultured cells with either pepstatin (11), a prototypic aspartyl protease inhibitor, or a difluoroketone compound, which inhibits aspartyl proteases (20), reduces Aβ production to a similar extent as seen in PS1 knockout cell lines. However, in all of these cases Aβ production is not completely abolished, indicating that more than one protease likely contributes to γ-secretase-catalyzed cleavages, a notion suggested by numerous studies showing differential inhibition of the γ-40 and γ-42 activities (Refs. 11, 21–24, and this study).

Because γ-secretase is one of the major therapeutic targets in AD, it is essential to determine whether PSs are γ-secretases or whether they alter γ-secretase activity in some other fashion. It has been pointed out that one of the major problems with the “PSs-as-γ-secretases” hypothesis is the discrepancy between the predominantly ER localization of PSs and the subcellular sites of the majority of γ-secretase activity in the more distal secretory pathway (Golgi) and endosomal system (25). In this study, we illustrate another aspect of presenilin regulation of the γ-secretase pathway (Golgi) and endosomal system (25).

**EXPERIMENTAL PROCEDURES**

**Inhibitor Synthesis**—Boc-Gly-Val-CHO was synthesized using standard peptide bond-forming reactions. Thus, the dipeptide Boc-Gly-Val-OBn (made by BOP/HOBt/DIEA/DMF-mediated coupling of Boc-Gly-OH with H-Val-OBn) was saponified and then coupled with the Weinreb valine amide (H-Val-(OMe)(Me), Aldrich) using BOP/HOBt/DIEA/DMF to yield the Weinreb tripeptide Boc-Gly-Val-Val-(OMe)(Me). The diisobutylaluminum hydride reduction of this product furnished, after silica gel purification, the inhibitor Boc-Gly-Val-Val-(OMe)(Me) in good overall yield.

**Mutant PS Constructs**—Each PS mutant was constructed by generating two polymerase chain reaction fragments from wild type (wt) PS1 cDNA template. The 5’-segment was produced using a forward primer to generate an HindIII site at the 5’-end of PS1 and a specific reverse primer from the actual site of mutation, which also created a class II restriction site (BonB1). The BonB1 site is removed by the restriction enzyme leaving the final sequence unaltered except for the specific desired mutation. A similar strategy was used to generate the 3’-end of the cDNA, terminating in a BamHI site. Following the appropriate restriction digests, the pieces were assembled by triple ligation in the pAG3hyg vector (11). All mutations were verified by sequencing (primer sequences are available on request). In this manner we generated four PS1 aspartate mutants (D257A or E, D385A or E; D → A/E), the PS ins254–6 and ins386–8 (PS “out”) mutant, which contains amino acid insertions of SVY at position 256 and FIF at position 388 (duplicating the amino- and carboxyl-terminally adjacent 3 residues, respectively), and ΔTM1-2, which contains a deletion from amino acids 81–154 (inclusive).

**Generation of Pooled Stable Lines**—70% confluent 6-well plates of either Chinese hamster ovary (CHO), human embryonic kidney 293 (HEK), or human neuroglioma (H4) cells were transfected with 1 μg of plasmid DNA preincubated with 3 μl of FuGene 6 transfection reagent (Roche Molecular Biochemicals) in serum-free OptiMEM (Life Technologies, Inc.) overnight. Media were then replaced with either Ham’s F-12 (CHO cells), Dulbecco’s modified Eagle’s (HEK cells), or OptiMEM (H4 cells) supplemented with 10% fetal bovine serum (HyClone) and 800 μg/ml Hygromycin B (Calbiochem). After 10–14 days, selection was reduced to 200 μg/ml. Expression levels were monitored periodically by immunoblotting throughout the course of the experiment, and have been maintained for multiple passages. Transient transfections were performed in an identical manner, albeit without hygromycin selection. All cell lines were maintained at 37 °C under 5% CO2.

**Western Blotting**—Lysates were prepared in ice-cold 1% Triton X-100/BSA (pH 8.0) + PIC, and the insoluble material was separated by high speed centrifugation (20,000 × g). Lysates (10–15 μg of total protein, by bicinchoninic acid assay) were separated on 10–20% Tris-Tricine gels, transferred to polyvinylidene difluoride membranes, blocked overnight with 5% non-fat dried milk/TBS (pH 7.4)/0.05% Tween 20, and immunoblotted. PS1 was visualized with rabbit anti-PS1 loop and/or anti-PS1N antibodies (each at 1:1000 dilution) (27). For APP, blots were probed with anti-CT20 antibody (1:500 dilution).

**Metabolic Labeling**—Confluent 6-well plates of pooled stable HER293 cells or HEKwt cells were labeled for 2 h with 200 μCi/well [3H]methionine/cysteine. For inducible α-secretase experiments, cells were treated with 1 μg of phorbol 12,13-dibutyrate (PDBu), and serum-free conditioned media were collected after 6 h. Total APP was immunoprecipitated with Ab207/protein-G-agarose beads, separated on 10% Tris-Tricine gels, and dried, and exposed to a low energy phosphor screen for 7 days. Data were analyzed using a Storm PhosphorImager (Molecular Dynamics) and ImageQuant™ software. For analysis of CTF, APP, transmembrane CTFs, and disulfide-disulfide disulfide structures were subjected to mass spectrometry analysis as described (26). The amount of Aβ was standardized (to correct for transfection efficiency) to sAPP levels measured in the same sample using an Ab207 competitive ELISA with plates coated with 60 ng/well purified, recombinant sAPP (11). Aβ40 and Aβ42 were measured by BAN50/BA27 and BAN50/BC05 ELISA, respectively. All measurements were performed in duplicate. For mass spectrometry, conditioned serum-free media from APP-transfected CHO cells was immunoprecipitated with 4g/μl protein-A-G plus-agarose beads and subjected to matrix-assisted laser desorption/time-of-flight mass spectrometry analysis as described above.

**RESULTS**

**γ-40 and γ-42 Secretase Activities Are Pharmacologically and Spatially Distinct**—To further establish the existence of pharmacologically distinct γ-secretase cleavage sites, we examined the effects of a substrate-based (γ40-site) aldehyde inhibitor (Boc-glycine-valine-valinal; GVV) on Aβ production and APP CTF. When tested on transiently transfected 293T cells overexpressing APP695NL, GVV increased APP CTF by pharmacologically distinct fashion (data not shown) and selectively inhibited Aβ cleavage at sites other than 42, as measured both by ELISA (Fig. 1A) and (IP/MS) (Fig. 1B). The selective inhibition of cleavages other than Aβ42 by GVV is qualitatively similar to the effects on Aβ production observed with pepstatin (11), other peptide aldehydes (23), and some difluoroketone compounds (21); however, GVV appears to be even more selective than those previously reported with doses that inhibit Aβ40 by >90% while not inhibiting Aβ42 production. Significantly, we have recently observed selective inhibition of Aβ40 production by GVV, pepstatin, and other peptide aldehyde inhibitors in an in vitro system. This study demonstrates that Aβ production is not completely abolished, indicating that more than one protease likely contributes to γ-secretase-catalyzed cleavages, a notion suggested by numerous studies showing differential inhibition of the γ-40 and γ-42 activities (Refs. 11, 21–24, and this study).
vivo assay of γ-secretase activity, indicating that the selectivity is not simply an issue of differential cell penetration of the inhibitor. Thus, the elevations in Aβ42 production seen at low concentrations are best explained by an increased availability of APP CTF to a distinct γ-42 activity that is less sensitive to inhibition by GVV.

Subsequent analysis of the effects of GVV treatment on γ-secretase cleavage in APP TMD mutants by ELISA (data not shown) and IP/MS (Fig. 1B) indicates that these mutants possess a variety of GVV-sensitive and -insensitive sites, further indicating that multiple proteolytic activities are involved in γ-secretase cleavage (11). Interestingly, these data also revealed that those GVV-sensitive sites were closer to the luminal side of the APP TMD, whereas those GVV-insensitive sites were more distal. Thus, it appears that we can monitor at least two γ-secretase activities, a “γ-40 activity,” which cleaves residues more proximal to the luminal side and a γ-42 activity, which cleaves residues more distal to the luminal side.

**PS1 Mutants That Inhibit γ-Secretase Cleavage**—In addition to the aspartate mutants reported by Wolfe et al., we have...
identified two additional PS1 mutants that inhibit γ-secretase (Fig. 2). The first mutant, PS1 ins254–6+ins386–8 (PS1 out), was generated to assess whether location of the aspartate residues relative to the membrane was an important functional element. In this mutant, the aspartates are not mutated, but their positions shifted by two 3-amino acid insertions within the transmembrane domain, potentially moving both Asp-257 and Asp-385 toward the cytoplasm and out of phase with the putative catalytic site. In the second mutant, ∆TM1-2, the first two transmembrane domains of PS1 are deleted, theoretically preserving the topology of the remaining protein. The inhibitory effect of this mutant on γ-secretase was identified during a screen for PS1 deletion mutants that altered Aβ production.

We examined the effects of PS1 D→A/E, ∆TM1-2, and PS1 out mutants on the γ-secretase cleavages in three different cell lines: H4, HEK, and CHO. In each of these, we established pooled stable lines expressing wild type PS1 or the PS1 mutants, and then assessed APP processing and Aβ production after transient transfection with various APP expression constructs. To validate this system of transient transfection of APP into pooled stable PS1 lines, we performed extensive experiments on pooled stable lines expressing the FAD-linked PS1 mutants M139V and E280G. An example of this validation for H4 cells is shown in Fig. 3. In these experiments, both M139V and E280G are overexpressed (Fig. 3A) and increase Aβ42 production without significantly altering total Aβ production (Fig. 3B). Fig. 3 also shows the results obtained for PS1 mutants that inhibit γ-secretase activity in H4 cells. In lines expressing the ∆TM1-2 mutant, limited endoproteolysis of the smaller holoprotein (Fig. 3A) is indicated by the presence of a truncated amino-terminal fragment migrating at ~23 kDa. The use of pooled stable lines prevents us from precisely determining both the extent of cleavage and replacement of endogenous PS1. However, it is clear that overexpressed PS1 to some extent replaces endogenous PS1 (as indicated by decreased levels of endogenous PS1 NTF in the case of ∆TM1-2), and aspartate mutants are cleaved less efficiently (D257E shown). This was confirmed by blotting with a human-specific PS1 antibody (Chemicon; not shown). Significantly, following transient transfection with APP695NL, Aβ production (total, Aβ40 and Aβ42) is greatly curtailed in these cells (Fig. 3B), and both the ∆TM1-2 and PS1 out mutants (not shown) were at least as effective at inhibiting Aβ production as were the aspartate mutations.

To further investigate the effect of these PS1 mutants on γ-40 and γ-42 activities, pooled stable CHO and HEK cells overexpressing either PS1wt or one of the mutant constructs (Fig. 4) were transiently transfected with either APP695NL or the APP TMD mutants I637P, T639K, ins625–628, or del625–628. PS1 expression, total Aβ, sAPP, and APP CTF were analyzed, transferred, and probed with a combination of anti-PS1-loop/anti-PS1-N. As expected, PS1 holoprotein from ∆TM1-2 is noticeably smaller than wild type PS1. Replacement of endogenous PS1 is not complete in this cell line. Also, ∆TM1-2 is cleaved in H4 cells (smaller ~23 kDa ∆TM1-2 derived NTF marked with an asterisk), whereas D257E is not cleaved efficiently. B, ELISA analysis of secreted Aβ from H4 cells transfected with APP. The reduction in Aβ total, Aβ40, and Aβ42 is equivalent for both D257E and ∆TM1-2 (* t test versus PS1wt: \( p < 0.05 \)). Two PS1 FAD mutants, E280G and M139V, are shown as positive controls for the pooled stable approach and ELISA measurement of Aβ42.
functional high molecular weight complexes. To explore this possibility, we examined digitonin lysates from PS1wt, ΔTM1-2, and D385A cell lines by glycerol density gradient centrifugation. To facilitate analysis, lysates from cells expressing the PS1 wild-type protein were used as an internal control and combined with an equal amount of lysate from the ΔTM1-2 cells. Analysis of these gradients revealed that the full-length ΔTM1-2 was incorporated into a defined complex with an apparent molecular mass of 150–250 kDa (Fig. 6). Similar results were obtained for the D385A cell lines (data not shown). Both mutant proteins were distributed in a comparable manner to that of the PS1wt holoprotein. In these gradients, the endoproteolytic fragments overlapped with the holoproteins, making it difficult to discern if the mutant complex was completely matured. Although additional studies may be required to ascertain whether this represents a fully functional complex, the mutants were not abnormally distributed and appeared to be handled similar to the wild-type protein.

PS1 Aspartate Mutants do Not Augment Inducible α-Secretase Cleavage—Although a role of PSs in regulating γ-secretase activity is well established, several studies also suggest that PS1 may regulate the phorbol ester-inducible α-secretase cleavage that appears to be carried out by members of the ADAM family of metalloproteases (30–32). Two studies showed that overexpression of PS1 augments inducible α-secretase cleavage of APP, whereas overexpression of FAD-linked mutant PS1 does not (31, 32). Additionally, PS1 knockout abolishes inducible α-secretase cleavage of APP (30). To determine whether PS1 aspartate mutants also inhibit inducible α-secretase cleavage, wild-type CHO and HEK lines were compared with those overexpressing PS1wt, M139V, Asp-257, or D385E treated with 1 μM of the phorbol ester compounds phorbol...
12-myristate 13-acetate or PDBu for 4–6 h. CHO cells were transiently transfected with APP695wt, and sAPP was measured by ELISA (not shown), whereas endogenous levels of sAPP were evaluated in HEK cells following metabolic labeling with [35S]methionine and IP with Ab207 (Fig. 7). As is the case with the PS1 FAD-linked M139V mutation, the PS aspartate mutants abolish the effect of PS overexpression on phorbol ester treatment.

**Discussion**

The study of γ-secretase activity using the substrate-based inhibitor GVV further extends and confirms our previous observations that there are at least two distinct γ-secretase activities (11): a γ-40 activity, which cleaves more proximal to the ectodomain of APP and is more sensitive to these inhibitors, and a γ-42 activity, which cleaves more distal residues and is less sensitive. Based on these data we have explored the regulation of these distinct γ-secretase activities by PS1 mutants.

As previously reported, we have found that expression of PS1 aspartate mutants inhibit γ-secretase cleavage of APP CTF (33). In addition, we have identified two other PS1 mutants whose expression inhibits Aβ production. Both PS1 out and ΔTM1-2 mutants inhibit γ-secretase as effectively or more effectively than the PS1 wild type and the FAD-linked M139V mutant. The PS1 mutants used in this study decrease both the γ-secretase and PS2 is the other, because knockout of either PS1 (which reduces both Aβ40 and Aβ42 equally) or PS2 (which has no effect on either Aβ species) does not specifically alter γ-40 or γ-42 activity (13, 34).

Of course, it is still possible that PS1 is γ-secretase, but for this to be the case then they must have either multiple active sites or multiple active conformations. In any case, one of these sites or conformations must then be responsible for the γ-40 activity and the other for the γ-42 activity. Given that PSs have no homology to any known protease, if PSs contain the active site(s) of γ-secretase then they represent a truly unprecedented class of proteolytic enzyme. Furthermore, based on some of the same criteria that have lent credence to the PS-as-γ-secretase hypothesis (e.g. PS1 knockout and the effect of aspartate mu-

![Fig. 6. Mutant PS1 is incorporated into high molecular weight complexes.](image)

**Fig. 6.** Mutant PS1 is incorporated into high molecular weight complexes. Glycerol velocity gradient centrifugation of ΔTM1-2. Digitonin lysates were separated on a linear glycerol gradient and SDS-polyacrylamide gel electrophoresis as described (29). The incorporation of the ΔTM1-2 PS1 mutant into high molecular weight complexes was essentially unchanged from that of PS1wt.

![Fig. 7. PS1 mutations attenuate inducible γ-secretase activity.](image)

**Fig. 7.** PS1 mutations attenuate inducible γ-secretase activity. A, pooled stable HEK293 cell lines were labeled, treated with PDBu (+) or vehicle alone (−), and serum-free-conditioned media was collected and endogenous sAPP was immunoprecipitated with Ab207. The experiment was performed twice, in triplicate; a representative 7-day phosphorimage is shown. B, sAPP bands from PDBu-treated cells were quantified, expressed as a percentage of control sAPP, and compared with PS1wt values by analysis of variance followed by Dunnett’s test. There were no significant differences between the two experiments, and constitutive levels of sAPP did not differ between the various cell lines. Overexpression of PS1wt potentiated the effect of PDBu on sAPP secretion, whereas overexpression of mutant forms of PS1 (both aspartate mutant and the FAD-linked M139V) did not (* = p < 0.05; ** = p < 0.01).
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M. Paul Murphy, Sacha N. Uljon, Paul E. Fraser, Abdul Fauq, Hilary A. Lookingbill, Kirk A. Findlay, Tawnya E. Smith, Patrick A. Lewis, D. Chris McLendon, Rong Wang and Todd E. Golde

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