

PS1 N- and C-terminal fragments form a complex that functions in APP processing and Notch signaling

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Presenilin proteins play critical roles in the proteolytic processing of both Notch and amyloid precursor protein (APP). Presenilin itself undergoes endoproteolytic processing to generate an N-terminal and C-terminal fragment. As demonstrated previously, overexpression of presenilin 1 (PS1) holoprotein does not change the levels of the N-terminal and C-terminal fragments (NTF and CTF). When we co-express the PS1 NTF and CTF, marked increases in the cellular levels of these fragments are seen. By coexpressing the PS1 NTF and CTF, we demonstrate conclusively that a noncovalent complex of the NTF and CTF is the active species of presenilin. However, although the PS1 NTF/CTF complex is necessary for γ -secretase activity, it is not sufficient. Independent overexpression of the PS1 NTF and CTF was also used to show that the Asp-257 and Asp-385 mutations in PS1 decrease A β production by a direct effect on γ -secretase activity and not by the inhibition of PS1 endoproteolysis.

Three genetic loci have been linked to familial Alzheimer's disease (FAD): amyloid precursor protein (APP), presenilin 1 (PS1), and presenilin 2 (PS2). The extracellular amyloid plaques that are a hallmark of the disease are composed of the amyloid- β s A β 40 and A β 42, which are produced by the proteolysis of APP. Mutations in APP associated with FAD either selectively increase the more amyloidogenic form of A β , A β 42, or increase production of both A β 40 and A β 42. Similarly, FAD mutations in PS1 and PS2 have been found to increase selectively A β 42 production. Thus, all genetic loci associated with Alzheimer's disease identified to date point to a role for A β production in the disease process.

A β production requires the activity of two enzymes. The first is β -secretase (BACE), which cleaves APP at the N terminus of A β . The molecular identity of this enzyme was revealed recently to be a single transmembrane domain aspartyl protease (1–5). The second enzyme, γ -secretase, cleaves APP within its single transmembrane domain at a site that will become the C terminus of A β . The PSs, which are multipass transmembrane proteins, are required for γ -secretase activity (6). A β production is reduced severely in cells derived from PS1 knockout mice and abolished completely in cells derived from the double PS1/PS2 knockout (7, 8).

There is now mounting evidence that if PS is not itself γ -secretase, it is associated intimately with a protein that has this catalytic activity. Mutation of two aspartate residues in two adjacent transmembrane domains of PS1 or PS2 severely reduced A β production, leading to the speculation that PS is itself an unusual aspartyl protease (9). More recently, selective γ -secretase inhibitors have been shown to cross-link to PS (10–12). However, even if PSs are the elusive γ -secretase, there is some evidence that suggests that they do not act in isolation. For example, these proteins are found in high molecular weight complexes (13, 14), their abundance is regulated carefully by an undescribed cellular component (15), and some PS mutations differentially affect A β generation and Notch signaling (16).

PSs are also known to facilitate Notch signaling (17–21). Notch receptors are single transmembrane domain proteins that undergo a proteolytic processing event in response to ligand that ultimately liberates the Notch intracellular domain (NICD) from the membrane-bound protein (22–24). The NICD then translo-

cates to the nucleus to affect downstream gene expression. A role for PS in regulating Notch activity was first indicated by the identification of a PS homolog in *Caenorhabditis elegans*, *sel-12*, which was shown to facilitate Notch signaling (17). In the absence of PS, the cleavage of Notch within the transmembrane domain that liberates the NICD does not occur with great efficiency (21). Moreover, the aspartate mutations in PS1 described above decrease NICD production, and some γ -secretase inhibitors also inhibit NICD production (25). These data further support a role for PSs in intramembranous proteolysis and may indicate that γ -secretase activity is responsible for both APP and Notch processing.

Whether PS is itself γ -secretase or associated closely with the activity, the ultimate identification of γ -secretase will rely on reconstitution of the constellation of proteins necessary to cleave APP to generate A β . All components will need to be overexpressed individually and recombined *in vitro* or overexpressed together in an *in vivo* system lacking γ -secretase activity. This complicated undertaking is confounded by some regulatory aspects of PS biology. PSs undergo endoproteolytic cleavage between transmembranes 6 and 7 to generate an N- and C-terminal fragment (NTF and CTF, respectively) (15). The NTF and CTF remain stably associated with each other in a high molecular weight complex (13). Because significant levels of PS NTF and CTF are detected in native neurons while PS holoprotein is virtually undetectable, it is widely assumed that the NTF and CTF are the active components of PS. This has not been demonstrated formally, however. Conclusive identification of the NTF/CTF complex as the active species of PS is an essential step in efforts to identify and reconstitute the components of the γ -secretase complex. Unfortunately, it is difficult to manipulate the cellular levels of the NTF and CTF, because overexpression of PSs in cells leads to the accumulation of the holoproteins, whereas NTF and CTF levels are tightly regulated at a fairly constant level (15). This may explain why overexpression of wild-type PS1 does not affect A β production.

To address these issues and as a first step toward the purification of the individual components required for γ -secretase activity, we report the reconstitution *in vivo* of coexpressed PS NTF and CTF fragments. We demonstrate that we can achieve overexpression of the NTF and CTF and that, when coexpressed, these two fragments retain PS activity in two different assays. Our results also provide strong evidence that PS alone does not constitute γ -secretase.

Materials and Methods

C. elegans Genetics. We used standard methods for culturing *C. elegans* (26). *sel-12(ty11)* results in an early truncation of the

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Abbreviations: FAD, familial Alzheimer's disease; APP, amyloid precursor protein; PS, presenilin; A β , amyloid β -peptide; NICD, Notch intracellular domain; NTF, N-terminal fragment; CTF, C-terminal fragment; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; Ni-NTA, nickel-nitrilotriacetic acid.

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protein and will be described in detail elsewhere (33). Briefly, the *ty11* lesion is a W134STOP missense mutation resulting in a protein that is truncated before the third transmembrane domain. Strains were grown at 20°C.

NTF and CTF Expression Constructs. PS1 NTF was amplified by using the primers PS1-NR TAAGCGGCGCCTATGTTGAGGAG-TAAATG and PS1-NF GATGCGGCCGCATGACAGAGT-TACCTGCA. PS1 CTF was amplified by using the primers PS1-CR GCAGCGGCCGCCTAGATATAAAAATTGATGG and PS1-CF CATGCGGCCGCATGGTGTGGTTGGT-GAATA. The PCR product was digested with *NotI* and cloned into ps12Ex (described in ref. 27), a *sel-12* expression vector.

PS NTFs and CTFs containing D257N or D385A were PCR-amplified (using the primers described above) from the full-length mutant sequences in pcDNA3.1, which were derived from wild-type PS1 by using the site-directed mutagenesis kit (Stratagene). All clones were confirmed by DNA sequencing. PS1 NTFs and CTFs then were released from the ps12Ex vector and cloned into pcDNA3.1 (Invitrogen) for assays in tissue culture cells.

All *C. elegans* transgenic animals were made by injection of test constructs into *sel-12(ty11)* at a concentration of 20 µg/ml with 100 µg/ml pRF4[rol-6(su1006)] as a cotransformation marker. F₁ Rollers were picked, and transgenic lines were established. To assess rescue, L₄ roller progeny were cloned to individual plates and scored for the subsequent 2 days for the ability to lay eggs. Because so few *sel-12(ty11)* animals lay any eggs at all, animals were scored as Egl+ if they laid over 30 eggs.

Antibodies. Antibody G2-10 and G2-11 were obtained from Konrad Beyreuther (University of Heidelberg, Germany). Biotin-4G8 was purchased from Senetek (St. Louis, MO). Antibody 14 recognizes the N-terminal structure of PS1 and was obtained from Huaxi Xu (Rockefeller University, NY). Antibody NB, which recognizes the C-terminal structure of PS1, was obtained from Paul Fraser (University of Toronto, Canada).

Aβ Production in HEK293 Cells. HEK293 cells were cotransfected with APP695 carrying both Swedish and London mutations (APP^{sw-lon}), PS1 NTFs, and CTFs by using LipofectAMINE Plus (GIBCO) according to manufacturer instructions. Conditioned media were collected, and Aβ₄₀ and Aβ₄₂ were measured by using an electrochemiluminescence-based immunoassay as described previously (28, 29).

Membrane Preparation from HEK293 Cells. Wild-type or PS1 NTF- and CTF-transfected HEK293 cells were harvested and resuspended in buffer A containing 20 mM Hepes, pH 7.5, 50 mM KCl, 2 mM EDTA, 2 mM EGTA, and Complete™ protease inhibitor tablets (Roche Biochemicals). The cells were lysed in a nitrogen bomb at 600 psi (1 psi = 6.89 kPa). The cell lysate was centrifuged at 800 × g for 10 min to remove nuclei and large cell debris. The supernatant was centrifuged at 100,000 × g for 1 h. The membrane pellet was washed once in buffer A and re-collected by centrifugation at 100,000 × g for 1 h. The membranes were resuspended in buffer A plus 10% glycerol, flash-frozen in liquid N₂, and stored at -70°C before use.

Solubilization and Measurement of γ-Secretase Activity. Membranes were resuspended in 20 mM Hepes, pH 7.0, 150 mM KCl, 2 mM EDTA, 2 mM EGTA, and 1% 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO, Calbiochem) at 2 mg/ml and solubilized at 4°C for 1 h. The solubilized membranes were centrifuged at 100,000 × g for 1 h, and the supernatants were collected. To measure γ-secretase activity, 5 µg of solubilized membrane was incubated at 37°C for 90 min in

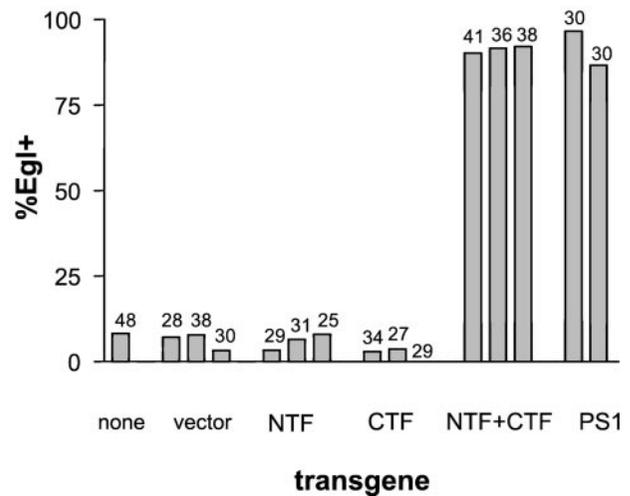


Fig. 1. PS1 NTF + CTF can rescue the *sel-12(ty11)* egg-laying defect. Transgenic lines were generated carrying the constructs indicated on the horizontal axis. The PS1 transgene indicates rescuing activity from PS1 holoprotein. Each bar represents an individual transgenic line. The number above the bars is the number of animals scored. The vertical axis represents the level of rescuing activity. 100% rescuing activity is defined as all animals exhibiting egg-laying competence as seen in the wild type. Therefore, the level of rescuing activity is relative to the expression of endogenous wild-type *sel-12*.

a 50-µl reaction containing 50 mM Tris-HCl, pH 7.0, 2 mM EDTA, 0.2% CHAPSO, and C99 substrate isolated from Sf9 cells. The amount of Aβ₄₀ generated in this reaction was determined by using the same electrochemiluminescence method as that used for the conditioned media.

Isolation of PS1 NTF and CTF Complex from Sf9 Cells. PS1 NTF and CTF were cloned into the pFAST vector (GIBCO), and baculoviruses were generated by using the Bac-to-Bac kit (GIBCO) according to manufacturer instructions. A tag of 6-His was engineered at the C terminus of PS1 CTF for purification. Sf9 insect cells were coinfecting with PS1 NTF- and CTF-His baculovirus; the membranes were isolated and solubilized from these cells by using a protocol similar to that for HEK293 cells as described above. The PS1 NTF/CTF complex was isolated by incubating the solubilized membrane extract with nickel-nitrilotriacetic acid (Ni-NTA) agarose at 4°C overnight with rocking. The agarose beads were then spun-down, and the proteins were eluted with buffer containing 200 mM imidazole.

General Methods. Protein concentration was determined with protein assay dye (Bio-Rad) by using BSA as standard. Western blots were developed by using ECL™ reagents (Amersham Pharmacia Biotech) according to manufacturer instructions.

Results

Rescue of *sel-12(ty11)* by PS1 NTF and CTF. *sel-12* mutant animals are egg-laying-defective (17). It has been shown previously that human PS1 and PS2 can functionally substitute for *sel-12* (27, 30) and confer egg-laying competence on these animals. We used this *sel-12* rescue assay to assess the function of the NTF and CTF of PS1 when expressed individually or when coexpressed, independent of the production of PS1 holoprotein.

As shown in Fig. 1, PS1 NTF or PS1 CTF alone have no ability to functionally substitute for *sel-12*. However, when the two fragments are coexpressed in the same animal, they exhibit a robust rescuing activity. This rescuing activity is comparable to what is seen when human PS1 holoprotein is expressed in this

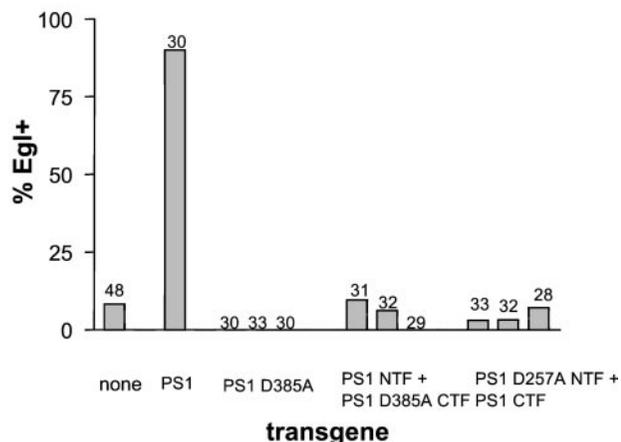


Fig. 2. PS1 NTF + CTF aspartate mutants have no rescuing activity. Transgenic lines were generated carrying the constructs indicated on the horizontal axis. The PS1 transgene indicates rescuing activity from the PS1 holoprotein. Each bar represents an individual transgenic line. The number above the bar is the number of animals scored. The vertical axis represents the level of rescuing activity.

same assay system (27). These results confirm the idea that a complex of the PS1 NTF and CTF comprises the active protein.

Aspartate Mutations on PS1 NTF and CTF Do Not Confer Rescuing Activity. We took advantage of the robust nature of the *sel-12* rescuing assay to address an outstanding issue concerning PS1 function. Wolfe *et al.* (9) have shown that mutation of either aspartate residue 257 in transmembrane 6 or aspartate residue 385 in transmembrane 7 of PS1 leads to a loss of A β production. They postulated that PS1 is an unusual diasparyl protease and that these aspartate residues are the catalytic residues. These mutations also prevent endoproteolysis of PS1 such that the NTF and CTF are not generated. If indeed the complex of the PS1 NTF and CTF is the active species, the aspartate mutations could reduce A β production by reducing γ -secretase catalytic activity and/or by preventing PS1 endoproteolysis.

Our system of expressing the PS1 NTF and CTF separately and then recombining the fragments *in vivo* allows us to distinguish between these two possibilities. As shown in Fig. 2, when wild-type PS1 NTF is coinjected with PS1 CTF D385A, no rescuing activity is observed. Similarly, when wild-type PS1 CTF is coinjected with PS1 NTF D257A no rescuing activity is observed. These results suggest that the loss of activity of the aspartate mutants of PS1 is not caused by the failure of these proteins to undergo endoproteolysis but is caused by the substitution of the aspartate residues that may have more direct consequences for PS activity.

Coexpression of PS1 NTF and CTF Leads to the Formation of a Functional Complex But Not Increased γ -Secretase Activity in Cells. In a complimentary approach to the *in vivo* studies in *C. elegans*, we next assessed the effect of the expression of PS1 NTF and CTF on A β production. We measured γ -secretase activity by using an *in vitro* assay with the enzyme extracted from either wild-type HEK293 cells or HEK293 cells cotransfected with PS1 NTF and CTF. As shown in Fig. 3A, coexpression of PS1 NTF and CTF does result in a substantial increase in the production of these fragments. However, almost identical substrate-dependent γ -secretase activity is seen in these membranes as compared with that of wild type (Fig. 3B).

Although overexpression of PS1 NTF and CTF did not increase A β production, it is possible that overexpression of the fragments did not result in the formation of a functional

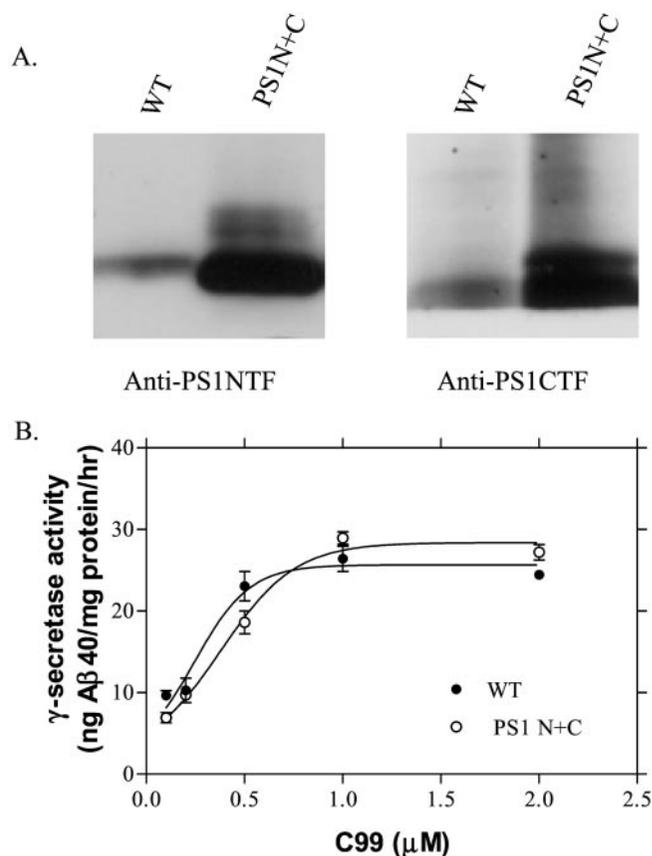


Fig. 3. Overexpression of PS1 NTF and CTF in HEK293 cells does not enhance γ -secretase activity. HEK293 cells were transiently transfected with PS1 NTF and CTF. The membranes were isolated and solubilized as described in *Materials and Methods*. (A) Western blots of PS1 NTF and CTF in control cells and PS1 NTF- and CTF-cotransfected cells. WT, wild type; PS1N+C, PS1 NTF/CTF complex. (B) γ -Secretase activity in membranes isolated from control and PS1 NTF- and CTF-cotransfected cells. The data presented here are representative of three independent transfection experiments.

complex. To address this possibility, first we assessed whether the coexpressed NTF and CTF physically interact. Sf9 insect cells were coinjected with baculovirus carrying PS1 NTF and His-tagged CTF. When the detergent-solubilized membrane fraction was passed through a Ni-NTA column, PS1 NTF copurified with His-tagged CTF (Fig. 4), suggesting that the two fragments form a complex in cells.

To test whether the complex of PS1 NTF and CTF in a coexpression system has functional γ -secretase activity, we expressed PS1 NTFs and CTFs bearing FAD mutations. As reported previously, the overexpression of PS1 carrying a FAD mutation has no significant effect on APPs α and A β 40 production (Fig. 5A and B) but dramatically increases the production of A β 42 (Fig. 5C and D). The FAD mutation Y115H on the NTF also considerably increases A β 42 production when coexpressed with a wild-type PS1 CTF (Fig. 5). The expression of NTF carrying Y115H alone has no effect on A β 42 production. This same result is also seen when the FAD mutation L392V on the CTF is coexpressed with a wild-type PS1 NTF. Furthermore, when FAD mutations are present on both NTF and CTF, the increase in A β 42 production is even more significant, consistent with the finding that PS1 FAD mutations have an additive effect on A β 42 production (31). Taken together, these data suggest that the PS1 NTF and CTF can form a functional complex and participate in γ -secretase activity in human cells but that over-

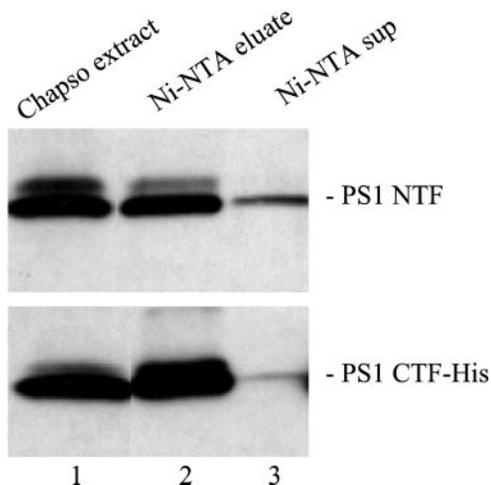


Fig. 4. PS1 NTF coelutes with PS1 CTF-His from Ni-NTA agarose resin. Sf9 cells were coinfecting with PS1 NTF and PS1 CTF-His baculovirus. Membranes were isolated and solubilized in 1% CHAPSO as described in *Materials and Methods*. The solubilized membrane extract was incubated with Ni-NTA agarose, and proteins bound to the resin were eluted. Western blots were developed by using antibody 14 for PS1 NTF and antibody NB for PS1 CTF. Lane 1, proteins from membrane extracts; lane 2, proteins eluted from Ni-NTA beads; lane 3, the supernatant after removing the Ni-NTA agarose.

expression of the wild-type fragments alone is not sufficient to increase γ -secretase activity and A β production.

Discussion

We used an *in vivo* assay in *C. elegans* to demonstrate that coexpression of PS1 NTF and CTF fragments could rescue the *sel-12* mutant phenotype as effectively as PS1 holoprotein and thereby function in LIN-12/NOTCH signaling. We also showed that the same PS1 NTFs and CTFs form a functional complex in HEK293 cells and can participate in APP processing. These studies provide further evidence that a noncovalent complex of the NTFs and CTFs is indeed the active constituent of PSs. Indirect support for this conclusion also comes from the studies described in refs. 10–12, which showed that γ -secretase inhibitors label the PS1 NTF and CTF but not the holoprotein.

In cells overexpressing the reconstituted PS1 NTF and CTF, as in cells overexpressing the holoprotein, an increase in γ -secretase activity is not observed. One explanation for these results is that PSs are part of a larger complex that constitutes γ -secretase, and the other members of the complex are limiting. Because of the limitations of PS biology, it was not possible previously to overexpress the functional NTF and CTF from PS, making it difficult to interpret the role of PS and its mutations in γ -secretase activity. Because we have shown that overexpression of the wild-type PS1 NTF and CTF does not lead to an increase in γ -secretase activity, we can now conclude that PS1 does not act alone as the γ -secretase enzyme.

These studies also allowed us to address the nature of the FAD mutations. The observations that simple overexpression of functional PS1 NTF and CTF does not result in increased γ -secretase activity indicates that the increased A β production in cells expressing the PS1 FAD mutations does not result from a simple gain of activity of an independently acting γ -secretase. This result suggests that PS1 acts as part of a γ -secretase complex and that the FAD mutations in PS1 serve to activate other components of the enzyme complex. In support of this notion, the transmembrane protein nicastrin has been identified recently as a component of a PS1 complex (14). Full characterization of the molecular nature of the γ -secretase enzymatic activity will await identification of all components of the complex.

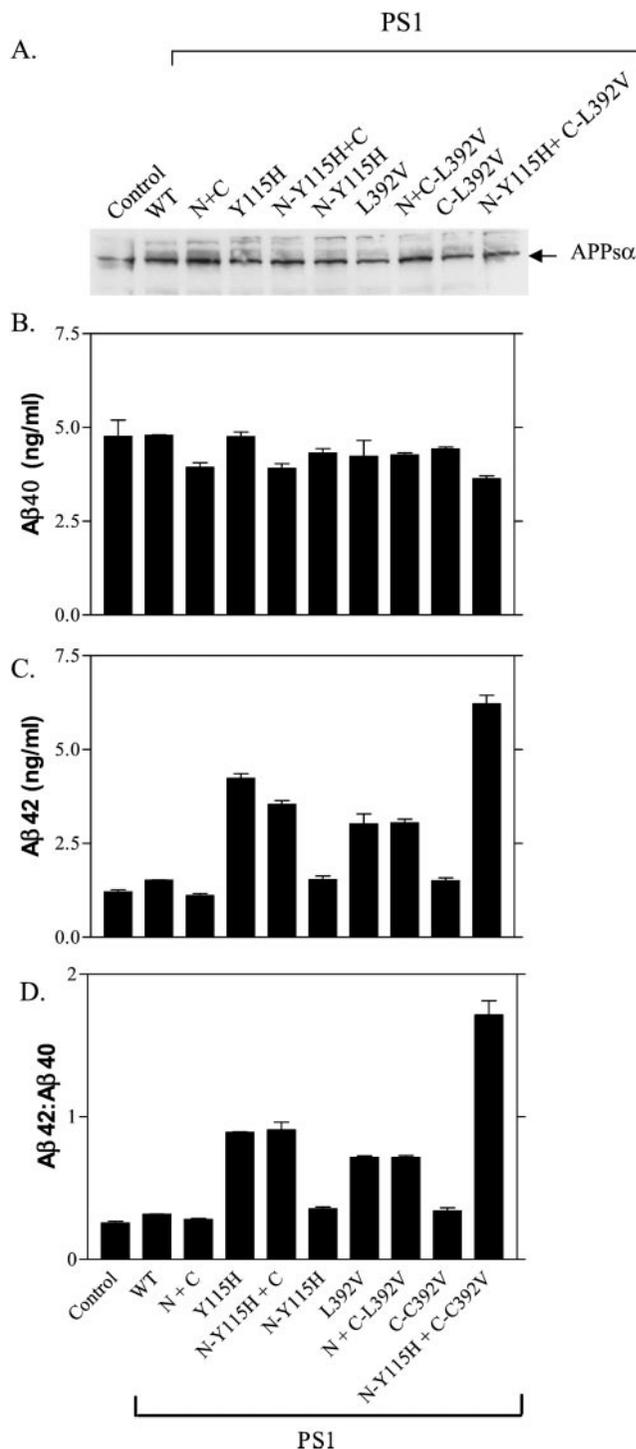


Fig. 5. Coexpression of PS1 NTF and CTF carrying FAD mutations potentiate A β 42 production in HEK293 cells. HEK293 cells were transiently transfected with APPsw-Ion and PS1 constructs as specified. A β 40 and A β 42 levels in conditioned media were determined as described in *Materials and Methods*. The data presented here are representative of more than three independent transfection experiments. (A) Western blot analysis of APPs α secretion in conditioned media as detected by antibody W02. WT, wild type; N+C, PS1 NTF/CTF complex. (B) Secreted A β 40 in conditioned media. (C) Secreted A β 42 in conditioned media. (D) A β 42/A β 40 ratio in conditioned media.

In our study, A β 42 production is elevated by coexpression of PS NTF carrying the Y115H mutation with a wild-type CTF or a wild-type PS NTF with CTF carrying a C392V mutation but not

by the expression of NTF-Y115H or CTF-C392V alone. Previously it was reported that human PS1 NTF does not form a complex with endogenous murine CTF, whereas a chimeric PS1/PS2 polypeptide forms a stable PS1 NTF-PS2/CTF complex (32). In these latter experiments, it is possible that the disparate timing of expression of the human PS1 NTF and the murine PS1 CTF interferes with their association. Taken together, the results suggest that proteolysis of PS1 into the NTF and CTF and association of the NTF and CTF with each other precedes incorporation into the γ -secretase complex.

Coexpression of PS1 NTF and CTF permitted us to address the effect of the aspartate mutations on PS. We were able to separate the endoproteolysis and γ -secretase effects of these mutations on PS activity by engineering the mutations into the coexpressed fragments. We demonstrated that the effect of the aspartate mutations is not caused by an inhibition of PS endoproteolysis but is caused by a more direct effect on γ -secretase activity, perhaps by directly effecting catalytic residues.

Our finding that coexpressed PS1 NTFs and CTFs function both in Notch signaling and $A\beta$ production conclusively shows that the PS fragments are the active components of the protein. These studies fulfill the first step toward reconstitution of γ -secretase activity *in vitro*. An initial requirement for this process is to be able to express large quantities of the active components of PS. We have now demonstrated that the PS1 NTF and CTF can be overexpressed stably in tissue culture cells resulting in protein levels that are significantly higher than those achieved from expression of the PS1 holoprotein. Although reconstitution of γ -secretase is likely to be an arduous task involving a number of membrane-bound components, functional overexpression of the PS1 NTF/CTF complex is a first step toward that end.

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