Characterization of a Presenilin-mediated Amyloid Precursor Protein Carboxyl-terminal Fragment γ

EVIDENCE FOR DISTINCT MECHANISMS INVOLVED IN γ-SECRETASE PROCESSING OF THE APP AND Notch1 TRANSMEMBRANE DOMAINS*

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A variety of investigations have led to the conclusion that presenilins (PS) play a critical role in intramembranous, γ-secretase proteolysis of selected type I membrane proteins, including Notch1 and amyloid precursor protein (APP). We now show that the generation of the S3/Notch intracellular domain and APP-carboxyl-terminal fragment γ (CTFγ) derivatives are dependent on PS expression and inhibited by a highly selective and potent γ-secretase inhibitor. Unexpectedly, the APP-CTFγ derivative is generated by processing between Leu-645 and Val-646 (of APP95), several amino acids carboxyl-terminal to the scissile bonds for production of amyloid β protein peptides. Although the relationship of APP-CTFγ to the production of amyloid β protein peptides is not known, we conclude that in contrast to the highly selective PS-dependent processing of Notch, the PS-dependent γ-secretase processing of APP is largely nonselective and occurs at multiple sites within the APP transmembrane domain.

Presenilin 1 and 2 (PS1 and PS2)† are polytopic membrane proteins that are mutated in the majority of pedigrees with early-onset FAD. Compelling evidence has emerged supporting a role for PS in intramembranous γ-secretase processing of the type I membrane proteins, including APP (1, 2) and Notch1 (3). γ-Secretase processing of a set of membrane-tethered APP-CTFs results in the production of Aβ peptides that are secreted and subsequently deposited in brains of patients with Alzheimer’s disease. On the other hand, γ-secretase processing of the Notch1 derivative, termed S2/NEXT, releases the intracellular domain (S2/NICD), a transcriptional coactivator that associates with CBF1/Su(H)/Lag1 (4–6). The observation that Aβ and S3/NICD production are completely eliminated in cells with compound deletions of PS1 and PS2 (7, 8), and the demonstration that PS1 and PS2 can be photocross-linked to γ-secretase inhibitors (9), have led to the conclusion that PS are the molecules that execute intramembranous cleavage of APP and Notch1. Furthermore, PS shares limited homology with the prepilin peptidases, a family of bacterial aspartyl proteases (10), and PS binds to pepstatin, an aspartyl protease inhibitor (11). Although compelling, several observations have questioned the veracity of this model. First, and despite reports showing PS1 to be resident in late compartments, including endosomes and plasma membranes (12–14), we and others have revealed that PS are preponderantly localized in the endoplasmic reticulum and associated compartments, sites that are incompatible with the known cellular sites for γ-secretase cleavage of APP and Notch1 that include the plasma membrane, Golgi, and endosomes (15, 16). Second, endocytosis and recycling of APP-CTFs is a major pathway for generating Aβ (17), but production of the Notch S3/NICD does not require endocytic trafficking of S2/NEXT (18). Third, γ-secretase has contrasting substrate specificities for processing within the APP (19, 20) and Notch (5) TM domains, and γ-secretase processing of the APP TM domain occurs at heterogeneous sites (19, 20) whereas γ-secretase cleavage of Notch1 appears to generate a single S3/NICD species (5).

In the present report, we examine the production of γ-secretase-generated APP and Notch1 derivatives. We now document, both in living cells and cytosol-free membrane preparations, the generation of APP-CTFγ derived from membrane-tethered APP-CTFs. In parallel, we document the production of the Notch S3/NICD in these preparations. The production of the APP-CTFγ and S3/NICD derivatives are dependent on PS1 expression and inhibited by a γ-secretase inhibitor. Unexpectedly, the APP-CTFγ derivative produced in these reactions is generated by endoproteolytic processing at a site several amino acids carboxyl-terminal to the scissile sites for production of Aβ peptides but interestingly, occurs at a site immediately proximal to the analogous site for production of S3/NICD. Although the relationship of APP-CTFγ to the production of Aβ peptides is not known, we conclude that in contrast to the highly selective PS-dependent proteolysis of the Notch TM domain, the PS-dependent γ-secretase processing of APP is largely nonselective and occurs at multiple sites within the APP TM domain.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfection, and Immunoblot—Cell culture, transfection, and immunoblot were performed as described (21–23). Myc-tagged Notch derivatives were detected using 9E10 (21). Full-length APP and

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†The abbreviations used are: PS, presenilin; Aβ, amyloid β protein; APP, amyloid precursor protein; CTF, carboxyl-terminal fragment; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometer; NICD, Notch intracellular domain; PAGE, polyacrylamide gel electrophoresis; TM, transmembrane; NEXT, Notch extracellular truncation.

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APP-CTFs were detected by CT15 (22). For detection of Aβ, conditioned medium was immunoprecipitated with 2D6 antibody (23) and immunoblotted with 2D6.

**Pulse-Chase and Immunoprecipitation**—N2aWT.7 or D385A.16 cells were labeled for 15 min with 750 μCi/ml [35S]methionine (PerkinElmer Life Sciences). Cells were either harvested after pulse or chased for 45 min at 37 °C in the presence of 0.5 mM cold t-methionine (Life Technologies, Inc.). Conditioned media were collected, centrifuged briefly, and immunoprecipitated with 4G8. Cell pellets were treated with 250 μl of 3% SDS in phosphate-buffered saline containing 10 μg/ml β-mercaptoethanol and subjected to vortexing and heating at 95 °C for 10 min, followed by sonication and centrifugation at 100,000 × g for 10 min. The supernatants were diluted 1:4, adjusted to a final concentration of 2% Triton X-100 and 190 mM NaCl, 20 mM Tris-Cl, pH 8.8, and 2 mM EDTA, and subjected to immunoprecipitation with 369 or 4G8.

**Purification of Membrane Fractions and in Vitro γ-Secretase Assay**—Purification of membrane fractions from cultured cells was performed as described (24). Briefly, cell pellets from ten 10-cm dishes were resuspended in Buffer A (250 mM sucrose, 20 mM HEPES, pH 7.4) containing protease inhibitor mixture. Post-nuclear supernatant was layered onto 30% Percoll solution and subjected to high speed centrifugation. Cellular membranes were resuspended in 14% iodixanol and overlaid with 12 and 6% iodixanol. Following centrifugation at 52,000 × g for 90 min, membranes were collected from each interface. Collected membranes were incubated at 4 or 37 °C for 60 min, and the reactions were terminated by the addition of Laemmli sample buffer. In the γ-secretase inhibitor experiments, the reaction tubes were preincubated at 4 °C for 10 min with 100 mM L-685,458 (25) prepared in Me₂SO, or an equivalent concentration of Me₂SO, and then incubated at 37 °C for 60 min.

**Mass Spectrometric Analysis**—The reaction samples were centrifuged at 100,000 × g for 1 h to separate the membrane-bound and soluble APP-CTFs. CTFγ from the soluble fraction was immunoprecipitated with CT15 and protein A-agarose and analyzed by MALDI-TOF MS (26).

## RESULTS

**Cellular Production of S3/NICD and APP-CTFγ Is PS1-dependent**—We demonstrated previously that a Notch6-myc-green fluorescent protein chimera is subject to PS1-dependent γ-secretase processing; this processing event is stimulated either by addition of extracellular ligand or by depletion of extracellular calcium (21). To assess the role for PS1 in the production of an APP carboxyl-terminal derivative, termed APP-CTFγ, that would be generated following intramembranous proteolysis by γ-secretase, we performed Western blot analysis of detergent lysates from PS1-deficient fibroblasts transiently cotransfected with cDNA encoding APPmyc and either wild type PS1 or a PS1 aspartate variant (D385A) that has been shown to reduce Aβ secretion (27, 28) (Fig. 1A). As expected, upon transfection of APPmyc cDNA, −12-kDa CTFmyc and −14-kDa CTFmyc appear that are significantly elevated over the −10-kDa endogenous CTFo seen in untransfected cells (Fig. 1A, compare lanes 1 and 2). On the other hand, coexpression of human wild type PS1 with APPmyc reduces the levels of these −12- and −14-kDa CTFmyc species, commensurate with the accumulation of a novel −7-kDa CTFmyc species, which we propose is CTFmyc and an endogenous CTFo and Aβ are indicated at right. *, APP-CTFmycγ.

![Fig. 1. Detection of APP-CTFγ](image-url)
FIG. 2. PS-dependent generation of NICD and APP-CTFγ. A and B, cysteol-free membranes purified from P3T1 fibroblasts that express Notch6-α-myc-green fluorescent protein and were transiently transfected with APP-α-myc were incubated at 37°C for 60 min in the presence of 10 mN EDTA. Reactions were separated on 5% Tris-glycine SDS-PAGE to detect Notch1 derivatives (A, lanes 1–3) and 16% Tris-Tricine SDS-PAGE to detect APP-CTFs (B, lanes 4–6). Separated samples were immunoblotted using 9E10 for Notch1 derivatives and CT15 for APP-CTFs. Production of both ~115-kDa S3/NICD and ~6-kDa APP-CTFγ was observed upon incubation (lanes 2 and 5) but was fully inhibited by γ-secretase inhibitor, L-685,458 (lanes 3 and 6). TMIC, Notch transmembrane and intracellular domains; WB, Western blotting.

In Vitro Generation of NICD and APP-CTFγ—To develop a biochemically tractable system to examine γ-secretase processing of Notch and APP, we isolated membrane fractions from P3T1 fibroblasts that express the Notch-green fluorescent protein chimera and examined EDTA-induced γ-secretase processing (Fig. 2A). We show that incubation of these membranes at 37°C led to the production of the S3/NICD fragment (Fig. 2A, lanes 1 and 2) and that this reaction was inhibited by addition of the γ-secretase inhibitor L-685,458 to 100 nM (Fig. 2A, lane 3). In parallel, we assessed the production of a previously described ~6-kDa CTFγ derived from γ-secretase cleavage of endogenous ~10-kDa CTFα (29, 30). We detected the CTFγ only after incubation at 37°C (Fig. 2B, lanes 1 and 2), and this reaction was also inhibited by addition of the γ-secretase inhibitor (Fig. 2B, lane 3). In titration experiments using varying concentrations of L-685,458, we have demonstrated that the in vitro generation of S3/NICD and APP-CTFγ are equally sensitive to the inhibitor with an IC50 of ~50 pM, findings that suggest that these derivatives are generated by similar, if not identical, γ-secretase activities.

Biochemical Characterization of CTFγ—As the levels of endogenous APP-CTFγ generated in the fibroblasts were extremely low, we chose to analyze the CTFγ fragment generated in membranes from a cell line (N2aWT11) that stably expresses APP-α-myc and wild type PS1. Incubation of membranes at 37°C for 60 min from N2aWT11 cells leads to unchanged levels of full-length APP but a diminution in levels of the ~12- and ~14-kDa CTFγ derivatives. Commensurate with reduction in levels of these CTFγ species was the appearance of an ~7-kDa CTFγ derivative, similar to the PS1-dependent CTFγ fragment observed in living cells (Fig. 3A, lanes 1 and 2; see Fig. 1B). With the assumption that the CTFγ fragment might be extruded into the cytoplasmic compartment in a manner similar to the S3/NICD fragment of Notch, we prepared membrane and soluble fractions. The ~12- and ~14-kDa CTFγ fragments were membrane-bound prior to, or after, incubation at 37°C (Fig. 3B, lanes 2 and 4), and as expected, the CTFγ fragment was found exclusively in the soluble fraction (Fig. 3B, lane 6). To establish the identity of the CTFγ fragment in the soluble fraction, we immunoprecipitated the peptide with CT15 and subjected recovered material to MALDI-TOF MS (Fig. 3C). We observed principal peptides of m, 7341.3 and 7110.4, which includes the mass of the 12-amino acid myc epitope tag; the prominent m, 7341.3 peptide is generated by endoproteolytic cleavage between Leu-645 and Val-646 (of APPα-myc), whereas the m, 7110.4 peptide is generated by cleavage between Met-647 and Leu-648 (Fig. 3C).

**DISCUSSION**

A series of genetic and pharmacological approaches have revealed that PS are required for γ-secretase processing of Notch1 and APP (31). In the case of Notch1, a membrane-tethered S2/NEXT is subject to processing by γ-secretase, leading to the generation of a cytoplasmic fragment, S3/NICD (4). APP is processed by α- and β-secretases to generate a set of membrane-tethered CTFs, and these derivatives are subsequently processed by γ-secretase to generate a spectrum of secreted Aβ peptides (see below). However, the residual CTFγ resulting from γ-secretase processing of the CTfal and -β, has been elusive. Very recently, McLendon et al. (29) and Pinnix et al. (30) reported on the generation of a CTFγ derivative in isolated membranes from brain or cultured cells. This derivative was not biochemically characterized, but its production was inhibited by high concentrations of pepstatin A, MG132, and a substrate-based difluoroketone (30).

To these latter observations, the present report offers several novel insights relevant to γ-secretase processing of the Notch S2/NEXT and the APP-CTFs. First, we document that a peptide corresponding to APP-CTFγ can be detected in lysates of transfected cells and that the production of this derivative is dependent on PS1 expression and largely eliminated by expression of the dominant negative D385A PS1 variant. Second, we establish that both the APP-CTFγ and S3/NICD can be generated in cytosol-free membranes and that the production of these derivatives is inhibited by a γ-secretase inhibitor. Third, and most surprising, is our demonstration using MALDI-TOF MS that the prominent in vitro-derived APP-CTFγ is generated by proteolysis between the 21st (Leu-645) and 22nd (Val-646) amino acids of the 24-amino acid APP TM domain. Notably, the P1’ Val-646 is one residue more carboxyl-terminal to the P1’ Val that is necessary for Notch processing (5). Our studies are consistent with recent results from Sastre et al. (32) and Gu et al. (33).

Having established the identity of CTFγ, we are now faced with the conundrum that the processing sites for generation of this CTF are distinct from the scissile sites for Aβ40 or Aβ42 peptides, which occur between the 12th (Val-636) and 13th (Ile-637) or 14th (Ala-638) and 15th (Thr-639) amino acids, respectively. Taken together with our present data showing that CTFγ production is dependent on PS1 expression, and inhibited by a selective γ-secretase inhibitor that also blocks production of Aβ, we offer three mechanistic scenarios.

The first of these is that CTFγ is derived by secondary endoproteolysis of the CTF57 or CTF59 derivatives that are the residual fragments from proteolysis at the scissile bonds that generate Aβ. We consider this unlikely, as we have detected neither the CTF57 nor CTF59 derivatives in in vitro pulse-chase studies nor in kinetic studies in cytosol-free membrane preparations. The second possibility is that proteolysis between Leu-645 and Val-646 is obligatory for subsequent endo- or exoproteolytic activity events necessary for generating Aβ40/42 peptides. This notion can be tested by assessing CTFγ and Aβ production in cells expressing APP variants with mutations surrounding the scissile site. In this regard, and in view of the finding that a V1744G substitution at the P1’ site in the Notch blocks NICD production and Notch activity (34), Sastre et al. (32) have recently examined CTFγ production in membranes.
Characterization of APP-CTFγ

A.

B.

C.

D.

FIG. 3. Identification of γ-cleavage site of APPswe. A, membrane fractions from N2a WT.11 cells were prepared by two-step sequential gradients as described under "Experimental Procedures." The in vitro reaction was performed for 45 min at either 4 or 37 °C. APP species were detected with antibody CT15. B, CTFγ was generated as described above, and samples were centrifuged at 100,000 × g for 1 h. The supernatant and membrane pellet fractions were recovered and subject to Western blotting with antibody CT15. CTFγ (indicated by small arrows) was detected in the supernatant fraction but not in membrane pellet. Total, the in vitro reaction sample; Sup, supernatant fraction. C. CTFγ was prepared from supernatant fraction as described above and immunoprecipitated with CT15 antibody. The immunocomplex was analyzed by mass spectrometry. Two distinct peptides were detected. D, schematic presentation of γ cleavage sites within the APP transmembrane domain. The major site is denoted with a bold arrow between Leu-645 and Val-646 of APP695, whereas a second cleavage site is denoted with a small arrow between Met-647 and Leu-648. The sites for generation of Aβ40 and Aβ42 are also indicated.

prepared from cells expressing APP with a V646G substitution. Surprisingly, the production of CTFγ was unimpaired. Clearly, a more rigorous mutational analysis is required before a conclusion can be drawn about the relationship between the generation of CTFγ and Aβ production. The third alternative is that proteolysis giving rise to Aβ does not require prior generation of the CTFγ or vice versa. In this scenario, the membrane-tethered APP-CTFs are randomly processed at multiple sites. In view of the multiplicity of Aβ-related peptides with termini at 34, 37, 38, 39, 42, and 43 that are invariably detected (19, 20, 26), and the demonstration that extensive mutagenesis of the APP TM domain have little effect on the production of Aβ-related peptides (19, 20), we would argue that the PS-dependent γ-secretase activity is largely nonselective. We would offer the proposal that the APP TM domain is subject to processing by a presently unidentified membrane-resident, multicatalytic protease similar to the proteosome. We envision that the function of this protease would be to introduce nicks in TM segments of many membrane proteins, resulting in slippage of the residual TM segments into either the cytoplasmic or luminal space wherein the entire domain is subject to the proteolytic activities resident within those compartments. In this vein, it is equally conceivable that regulated forms of this protease class would be responsible for generating cytosolic fragments that play critical roles in nuclear signaling events (35). The recent demonstration of a potential nuclear signaling role for the cytoplasmic domain of APP (36) underscores the importance of understanding the mechanisms responsible for intramembranous processing of APP.

Despite the differences in the precise sites of proteolysis and sequence requirements within the APP and Notch TM domains, we have shown that L-685,458 is an equally potent inhibitor of the reaction(s) that generate Notch S3/NICD and CTFγ (see Ref. 21 and this study).2 These data would imply that if γ-secretase is a single entity, it must be highly unusual. In this regard, it is not clear how expression of PS1 harboring a D257A substitution is still capable of generating Aβ but fails to generate Notch S3/NICD (28). Similarly, expression of FAD-linked L166P PS1 variant or the experimental L286E or L286R PS1 mutants leads to overproduction of Aβ42 but fails to generate S3/NICD (37).3 Finally, recent studies have revealed that processing of APP and Notch1 can be discriminated by a JLK family of non-peptidic inhibitors (38). These inhibitors block Aβ production but have very little, if any, effect on production of S3/NICD (38). Collectively, these experiments prove unequivocally that γ-secretase processing of APP and Notch can be dissociated, leading us to conclude that the catalytic activities responsible for processing these substrates are not one in the same. Thus, whereas PS are critical for regulating γ-secretase activities, it is our view that these polypeptides are unlikely to be the sole effectors of intramembranous proteolysis of APP and Notch1. In the final analysis, it will be critical to develop in vitro reconstitution systems with purified components to establish the role(s) of PS and their interacting components in facilitating γ-secretase processing of APP and Notch1.

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