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Presenilin-dependent γ-secretase processing of β-amyloid precursor protein at a site corresponding to the S3 cleavage of Notch

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The presenilin (PS)-dependent site 3 (S3) cleavage of Notch liberates its intracellular domain (NICD), which is required for Notch signaling. The similar γ-secretase cleavage of the β-amyloid precursor protein (βAPP) results in the secretion of amyloid β-peptide (Aβ). However, little is known about the corresponding C-terminal cleavage product (CTFγ). We have now identified CTFγ in brain tissue, in living cells, as well as in an *in vitro* system. Generation of CTFγ is facilitated by PSs, since a dominant-negative mutation of PS as well as a PS gene knock out prevents its production. Moreover, γ-secretase inhibitors, including one that is known to bind to PS, also block CTFγ generation. Sequence analysis revealed that CTFγ is produced by a novel γ-secretase cut, which occurs at a site corresponding to the S3 cleavage of Notch.

INTRODUCTION

Alzheimer's disease (AD) is the most abundant neurodegenerative disorder worldwide. Senile plaques, composed of amyloid β-peptide (Aβ), appear to be a major pathological alteration in the brain of AD patients (Selkoe, 1999). Almost all familial AD (FAD) associated mutations affect the generation of Aβ by increasing the production of the highly amyloidogenic 42 amino acid variant (Selkoe, 1999). Aβ is produced from the β-amyloid precursor protein (βAPP) by endoproteolysis. At least two proteolytic activities are required for Aβ generation. β-secretase (BACE) mediates the N-terminal cleavage producing a membrane-associated C-terminal fragment (CTFβ) of βAPP (Vassar and Citron, 2000). The resulting CTFβ is the immediate precursor for the intramembraneous γ-secretase cut. This cleavage is facilitated by the presenilins (PSs) PS1 and PS2, and there is evidence that PSs themselves could be unusual aspartyl proteases, which mediate the γ-secretase cut (Wolfe *et al.*, 1999; for review see Steiner and Haass, 2000; Wolfe and Haass, 2001). γ-secretase cleavage results in the production of Aβ and the generation of the small hydrophobic p7 (Haass and Selkoe, 1993) also called CTFγ. Recent observations indicate that a CTFγ-like fragment is indeed generated during βAPP processing (McLendon *et al.*, 2000; Pinnix *et al.*, 2001). However, it is still unknown whether the γ-secretase cut occurs exclusively after position 40 or 42 of Aβ. A cleavage of the β-amyloid domain further C-terminal would be consistent with the site 3 (S3) cleavage of Notch (Schroeter *et al.*, 1998), which like the βAPP cleavage occurs by a PS-dependent proteolytic activity (De Strooper *et al.*, 1999). The identification of the initial cleavage site is of great importance, since γ-secretase inhibition is thought to be a major approach for therapeutic intervention.

In order to identify the precise γ-secretase cleavage sites of βAPP we have now used an *in vitro* assay for CTFγ generation (McLendon *et al.*, 2000; Pinnix *et al.*, 2001). This assay produced sufficient amounts of CTFγ to allow its further biochemical characterization and may be very useful to monitor the purification of the γ-secretase activity. We demonstrate that CTFγ is generated in a PS-dependent manner. Moreover, CTFγ

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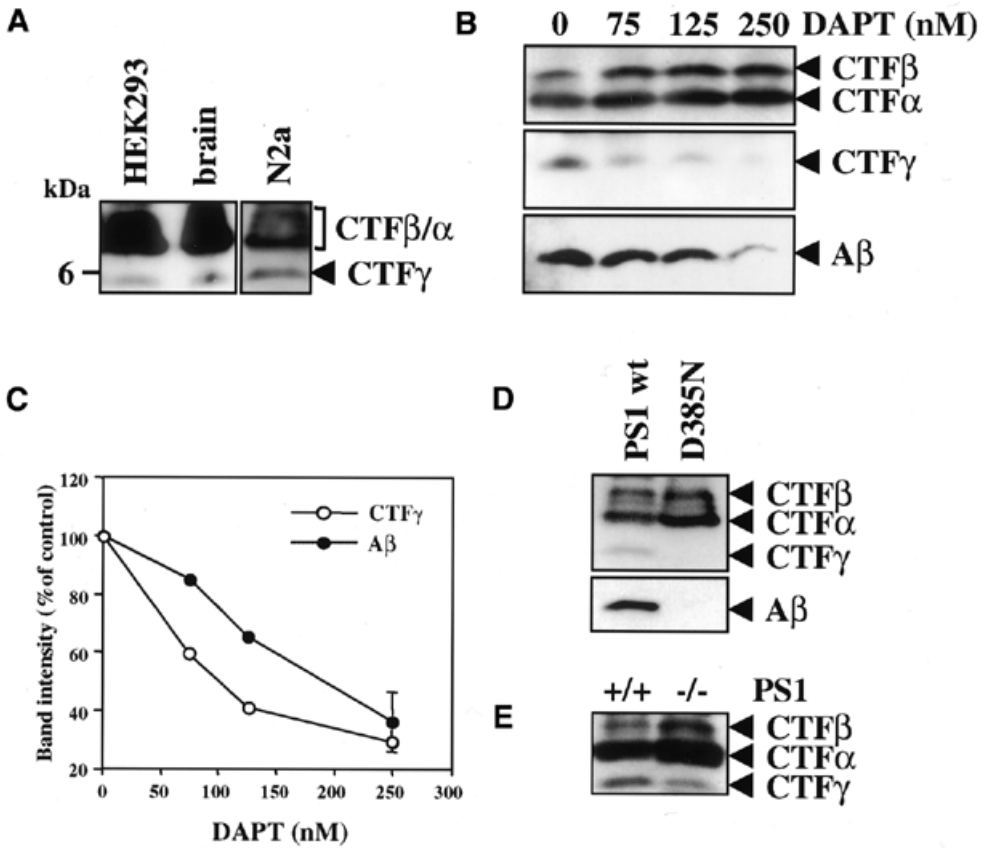

Fig. 1. Identification of *in vivo* produced CTFγ in human cells and mouse brain. (**A**) Membrane fractions of HEK 293 cells stably transfected with Swedish mutant βAPP_{695} (swAPP) were analyzed by combined immunoprecipitation/immunoblotting with antibody 6687 to the C-terminus of βAPP. Three βAPP CTFs were detected (CTFβ, CTFα and the ~6 kDa CTFγ). The same βAPP CTFs including the ~6 kDa CTFγ were also observed in mouse brain as well as in N2a cells transiently transfected with swAPP. (**B**) The γ-secretase inhibitor DAPT inhibits CTFγ production. HEK 293 cells stably transfected with swAPP were treated with the indicated concentrations of DAPT for 4 h. Upper and middle panel, membrane fractions were prepared and analyzed for βAPP CTFs as in (A). Increasing concentrations of DAPT led to a build up of CTFβ and CTFα (upper panel) with a concomitant significant block of CTFγ generation (middle panel). Note that exposure time for CTFβ and CTFα (upper panel) was shorter than that for CTFγ (middle panel). Lower panel, conditioned media were analyzed for secreted Aβ by combined immunoprecipitation/immunoblotting with antibodies 3926/6E10. Note the dose-dependent reduction of Aβ generation by DAPT treatment. (**C**) Quantitation of CTFγ and Aβ generation in the presence of γ-secretase inhibibitor DAPT. HEK 293 cells stably transfected with swAPP were treated with the indicated concentrations of DAPT and analyzed for CTFγ and Aβ as in (B). Bars represent the mean ± SE of three independent experiments. Note that error bars within the symbols are too small to be displayed. (**D**) CTFγ production is dependent on biologically active presenilins. Upper panel, membrane fractions from control cells expressing wild-type PS1 or cells stably expressing PS1 D385N were analyzed as in (A). Expression of the non-functional PS1 D385N variant increases CTFβ and CTFα and significantly reduces CTFγ production. Lower panel, conditioned media were analyzed for secreted Aβ as described in (B). Expression of the non-functional PS1 D385N variant severely reduces Aβ production. (**E**) Reduced CTFγ production in the absence of PS1. Membranes from $PS1^{+/+}$ or $PS1^{-/-}$ mouse embryonic fibroblasts transiently transfected with swAPP were analyzed for CTFγ as in (A).

begins at amino acid 50 of the β-amyloid domain, a site that corresponds to the S3 cleavage of Notch.

RESULTS

PS-mediated γ-secretase cleavage results not only in the generation of soluble Aβ but also in the generation of a βAPP C-terminal fragment (CTFγ) representing the counterpart of Aβ. To prove whether this occurs in living cells, C-terminal fragments of βAPP were immunoprecipitated from membrane fractions of human embryonic kidney 293 (HEK 293) cells stably transfected with βAPP_{695} carrying the Swedish mutation (swAPP) (Citron *et al.*, 1992). This revealed the presence of an ~6 kDa C-terminal fragment migrating below the major βAPP CTFs generated by α- and β-secretase (Figure 1A). A similar CTF was also found in homogenates of mouse brain as well as in N2a cells transiently transfected with swAPP (Figure 1A).

To prove that this polypeptide indeed represents the γ-secretase-generated CTFγ, we treated HEK 293 cells stably transfected with swAPP with the previously described γ-secretase inhibitor DAPT (Dovey *et al.*, 2001). As shown in Figure 1B and C, concomitant with an increase of βAPP CTFβ and CTFα (Figure 1B, upper panel) a dose-dependent inhibition of CTFγ generation was observed (middle panel). This was further confirmed by the immunoprecipitation of Aβ from the conditioned media of these cells, which consistent with previous results (Dovey *et al.*, 2001)

also revealed a dose-dependent reduction of Aβ generation (Figure 1B, lower panel and C). Similar results were also obtained with N2a cells (data not shown). To further prove the PS dependence of this cleavage, we immunoprecipitated βAPP and its proteolytic fragments from cells expressing PS1 D385N. As shown previously (Steiner *et al.*, 1999; Wolfe *et al.*, 1999), PS1 D385N acts like a dominant-negative mutation that inhibits the biological function of PSs required for the γ-secretase cleavage of βAPP. As expected we observed an increase of βAPP CTFβ and CTFα (Figure 1D, upper panel) as well as severely reduced Aβ production (Figure 1D, lower panel), demonstrating an inhibition of γ-secretase cleavage. Moreover, generation of CTFγ was also strongly reduced (Figure 1D, upper panel). To further demonstrate the involvement of PS in CTFγ generation, we analyzed its production in swAPP-transfected embryonic fibroblasts derived from $PS1^{+/+}$ and $PS1^{-/-}$ mice. As shown in Figure 1E, concomitant with an increase of CTFβ and CTFα, reduced amounts of CTFγ were observed in the absence of PS1 (see also Figure 2F). Taken together, these results indicate that the observed low molecular weight C-terminal cleavage product of βAPP is indeed produced by the authentic PS-dependent γ-secretase activity. However, rather small amounts of this fragment accumulate *in vivo* most likely due to the very rapid degradation of this fragment. A very similar phenomenon is also observed for NICD, which is degraded by the proteasome (De Strooper *et al.*, 1999). We therefore attempted to generate CTFγ in an *in vitro* assay, which could allow the efficient stabilization of this fragment by the use of a variety of protease inhibitors (McLendon *et al.*, 2000; Pinnix *et al.*, 2001). Membranes from HEK 293 cells stably expressing swAPP were incubated at 37°C for 0–2 h to allow accumulation of CTFγ, chilled on ice and centrifuged at 100 000 *g*. The pellet (P100) and the supernatant (S100) fraction were analyzed for the presence of βAPP CTFs. CTFα and CTFβ were predominantly observed within the P100 fraction (Figure 2A, upper panel), whereas CTFγ was significantly enriched in the S100 fraction (Figure 2A, lower panel). The predominant accumulation of CTFγ within the soluble fraction demonstrates that this fragment is released from the membrane. As described above (Figure 1) small amounts of CTFγ were also detected in the membrane fraction (P100) (Figure 2A, upper panel). However, due to the inhibition of proteolytic breakdown the *in vitro* assay allows the detection of large amounts of released CTFγ. Prolonged incubation led to the generation of robust levels of CTFγ (Figure 2A, lower panel). The maximum production of CTFγ was observed after approximately 1–2 h (Figure 2A). To prove whether the *in vitro* generated CTFγ is indeed the product of an authentic PS-dependent γ-secretase cut, the membrane fractions were incubated in the presence of two previously described γ-secretase inhibitors, DAPT (Dovey *et al.*, 2001) and CM256 (Esler *et al.*, 2000). As shown in Figure 2B and C both γ-secretase inhibitors efficiently reduced *in vitro* generation of CTFγ. Similarly, DAPT significantly reduced CTFγ production when membranes from N2a cells were investigated in the *in vitro* assay (Figure 2D). Moreover, CTFγ generation was also significantly reduced when membranes were isolated from HEK 293 cells co-expressing swAPP and functionally inactive PS1 D385N (see above) (Figure 2E). The remaining production of CTFγ was almost completely inhibited by the addition of the γ-secretase inhibitor DAPT (Figure 2E). Finally, membranes from embryonic fibroblasts derived from $PS1^{+/+}$ and $PS1^{-/-}$ mice that were stably transfected with $βAPP_{695}$ were analyzed for *in vitro* CTFγ generation. As shown in Figure 2F, CTFγ generation was significantly reduced in the absence of PS1 and almost completely inhibited by the addition of DAPT. Taken together, these results demonstrate that the *in vitro* assay produces very robust levels of CTFγ in a PS- and γ-secretase-dependent manner.

We then used the *in vitro* assay to isolate sufficient amounts of CTFγ to allow the determination of its N-terminus by radiosequencing. HEK 293 cells stably co-expressing swAPP and wild-type PS1 were metabolically labeled with [^{35}S]methionine. Radiolabeled CTFγ was generated *in vitro* as described above. After centrifugation, radiolabeled CTFγ was immunoprecipitated from the supernatant fraction and subjected to automated Edman degradation (Haass *et al.*, 1992). Surprisingly, this revealed a major peak of radioactivity in fraction 2 and not in fractions 9 and 11 as one would have expected for a CTFγ beginning at position 41 or 43 of the β-amyloid domain (Figure 3A). This indicates that CTFγ is generated by a proteolytic cleavage between amino acids 49 and 50 of the β-amyloid domain (Figure 3A). The peak of radioactivity in fraction 2 thus corresponds to methionine 51. Consistent with a proteolytic fragment starting at valine 50, a second peak of radioactivity was obtained at position 32. Cleavage of βAPP after amino acid 49 is of particular interest, since that additional γ-secretase cut occurs at a site corresponding to the S3 cleavage site of Notch. S3 cleavage of mouse Notch1 has been shown to occur within or close to the transmembrane domain (TM) between glycine 1743 and valine 1744 (Schroeter *et al.*, 1998) (see Figure 4).

While substitution of valine 1744 significantly reduces the efficiency of S3 cleavage (Schroeter *et al.*, 1998; Huppert *et al.*, 2000) it is unclear whether S3 cleavage requires a specific recognition sequence (Struhl and Adachi, 2000) (see Discussion). We then investigated whether CTFγ generation may be dependent on valine 50 and changed this residue to glycine. However, as shown in Figure 3B, cell lines stably expressing swAPP V50G still produced CTFγ *in vivo* as well as in the *in vitro* assay. Thus, these data suggest that CTFγ generation may not be absolutely dependent on valine 50 of the β-amyloid domain.

The results above could indicate that CTFγ is produced by sequence-independent exoproteolytic trimming. In order to investigate this possibility, we co-migrated recombinant CTFγ fragments beginning either at amino acid 50 (rCTFγ50) or at amino acid 43 (rCTFγ57) of the β-amyloid domain with CTFγ produced in living HEK 293 cells. As expected, *in vivo* generated CTFγ co-migrated with rCTFγ50, whereas rCTFγ57 migrated at a higher molecular weight than authentic CTFγ (Figure 3C). Together with the experiments described in Figure 1, this confirms that a major PS-dependent cut of βAPP occurs C-terminal of the authentic γ-secretase cleavage after amino acids 40 and 42. Moreover, this also demonstrates that the truncated CTFγ observed *in vivo* is not generated by exoproteolytic trimming since the rCTFγ57 fragment should be trimmed as well under these conditions.

DISCUSSION

Biochemical characterization of CTFγ surprisingly revealed a major cut after amino acid 49 of the β-amyloid domain of βAPP (Figures 3 and 4). This cleavage is fully dependent on biologically active PSs (Figures 1 and 2). Moreover, two independent γ-secretase

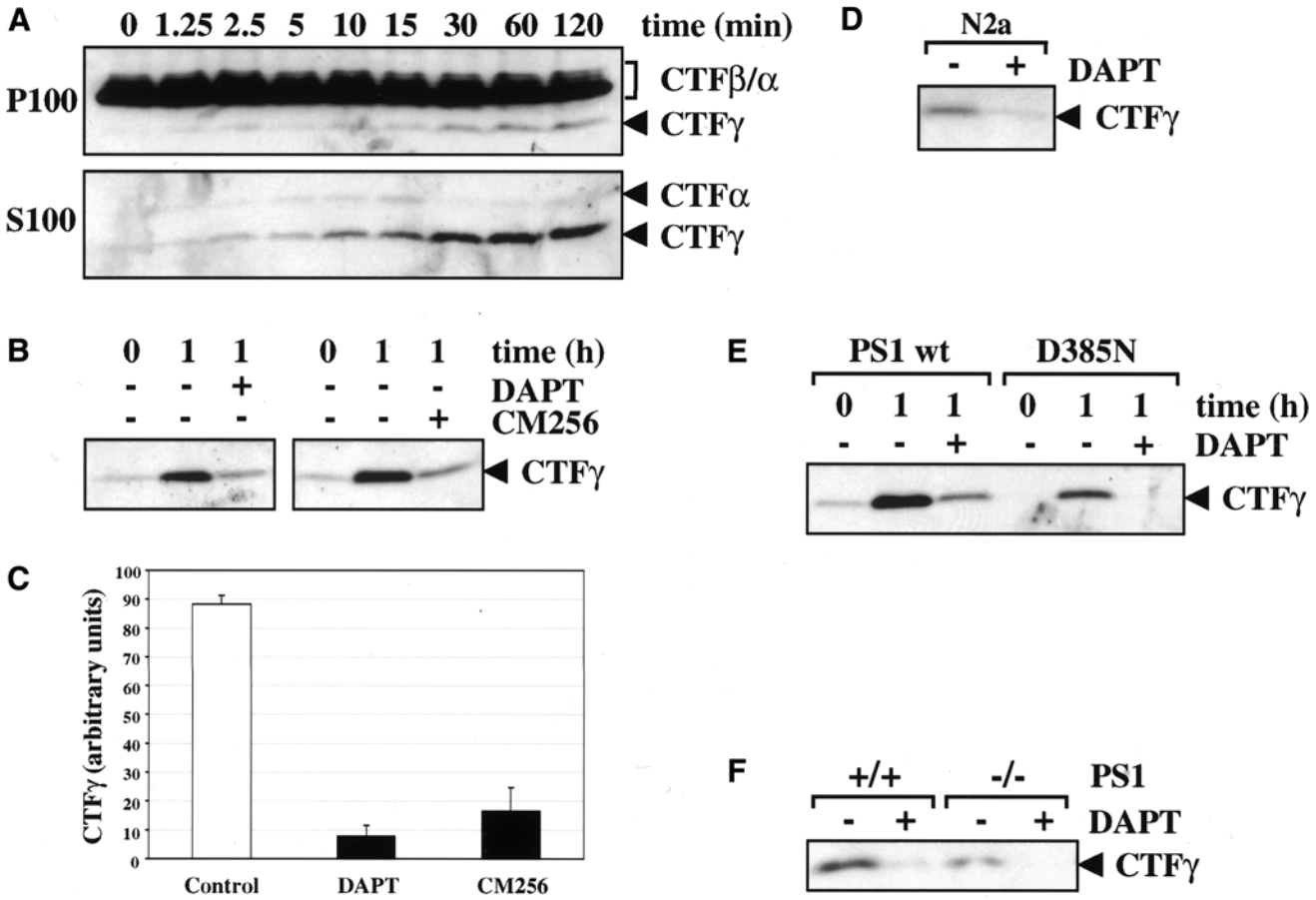

Fig. 2. *In vitro* generation of CTFγ. (**A**) Time-dependent *in vitro* production of CTFγ. Membrane preparations from HEK 293 cells stably transfected with swAPP were incubated at 37°C for the indicated time points. The reaction mixes were then separated in a pellet fraction (P100; upper panel) and a soluble fraction (S100; lower panel) by ultracentrifugation. These fractions were immunoblotted with antibody 6687. Note the selective accumulation of CTFγ in the S100 (lower panel) fraction after 1–2 h incubation time. Small amounts of CTFγ were detected in the P100 fraction. (**B**) Two independent γ-secretase inhibitors (DAPT and CM256) inhibit the *in vitro* production of CTFγ. Membrane preparations were incubated with (+) or without (–) 250 nM DAPT (left panel) or 50 μM CM256 (right panel) at 37°C for the indicated time. The reaction mixes were then subjected to ultracentrifugation and the S100 fractions were analyzed as in (A). Note that both inhibitors significantly reduce CTFγ generation. (**C**) Quantitation of CTFγ generation in the presence of γ-secretase inhibitors. Membrane preparations were treated with γ-secretase inhibitors as in (B). Bars represent the mean ± SE of three independent experiments. (**D**) The γ-secretase inhibitor DAPT inhibits the *in vitro* production of CTFγ from membranes derived from N2a cells. Membrane preparations were incubated at 37°C for 1 h with (+) or without (–) 250 nM DAPT, and CTFγ was analyzed as in (B). (**E**) *In vitro* generation of CTFγ depends on biologically active PSs. Membrane preparations derived from HEK 293 cells stably co-expressing swAPP and wild-type PS1 or biologically inactive PS1 D385N were incubated in the presence (+) or absence (–) of 250 nM DAPT as in (B). After termination of the *in vitro* reactions, CTFγ was identified as in (B). Note the inhibition of CTFγ production in membrane preparations derived from cells expressing the biologically inactive PS1 D385N mutation as well as the reduction of the remaining *in vitro* CTFγ production by the γ-secretase inhibitor DAPT. (**F**) Reduced *in vitro* generation of CTFγ in the absence of PS1. Membrane preparations from $PS1^{+/+}$ or $PS1^{-/-}$ mouse embryonic fibroblasts stably transfected with βAPP_{695} were incubated for 1 h at 37°C in the presence (+) or absence (–) of 250 nM DAPT, and analyzed for CTFγ as in (A). Note the inhibition of CTFγ production in membrane preparations derived from $PS1^{-/-}$ cells as well as the further reduction of the remaining *in vitro* CTFγ production by the γ-secretase inhibitor DAPT.

inhibitors that were both described to efficiently block Aβ40 and Aβ42 generation (Esler *et al.*, 2000; Dovey *et al.*, 2001) also inhibited the cleavage after amino acid 49 of the β-amyloid domain (Figures 1 and 2). Thus, it appears likely that this cleavage occurs by the γ-secretase itself. Although cleavage by another protease that is dependent on γ-secretase can not formally be excluded, our results rather suggest that γ-secretase mediates at least three different cuts within the TM of βAPP (Figure 4). The finding of an additional γ-secretase cut close to the predicted border of the TM may indicate that the cytoplasmic tail of βAPP requires 'shedding' before/during it undergoes the final γ-secretase cut after positions 40/42 of the β-amyloid domain. Such a 'shedding' event would be very similar to the essential ectodomain shedding of γ-secretase substrates (Struhl and Adachi, 2000). Alternatively, γ-secretase may cut first at position 40/42 of the β-amyloid domain followed by a second cleavage after position 49, releasing CTFγ from the membrane. However, since neither an Aβ49 species nor a CTFγ starting at position 41/43 of the β-amyloid domain has been found, our data may indicate simultaneous cleavage at all three sites.

Interestingly, γ-secretase cleavage at position 49 of the β-amyloid domain is located at a position that corresponds to the

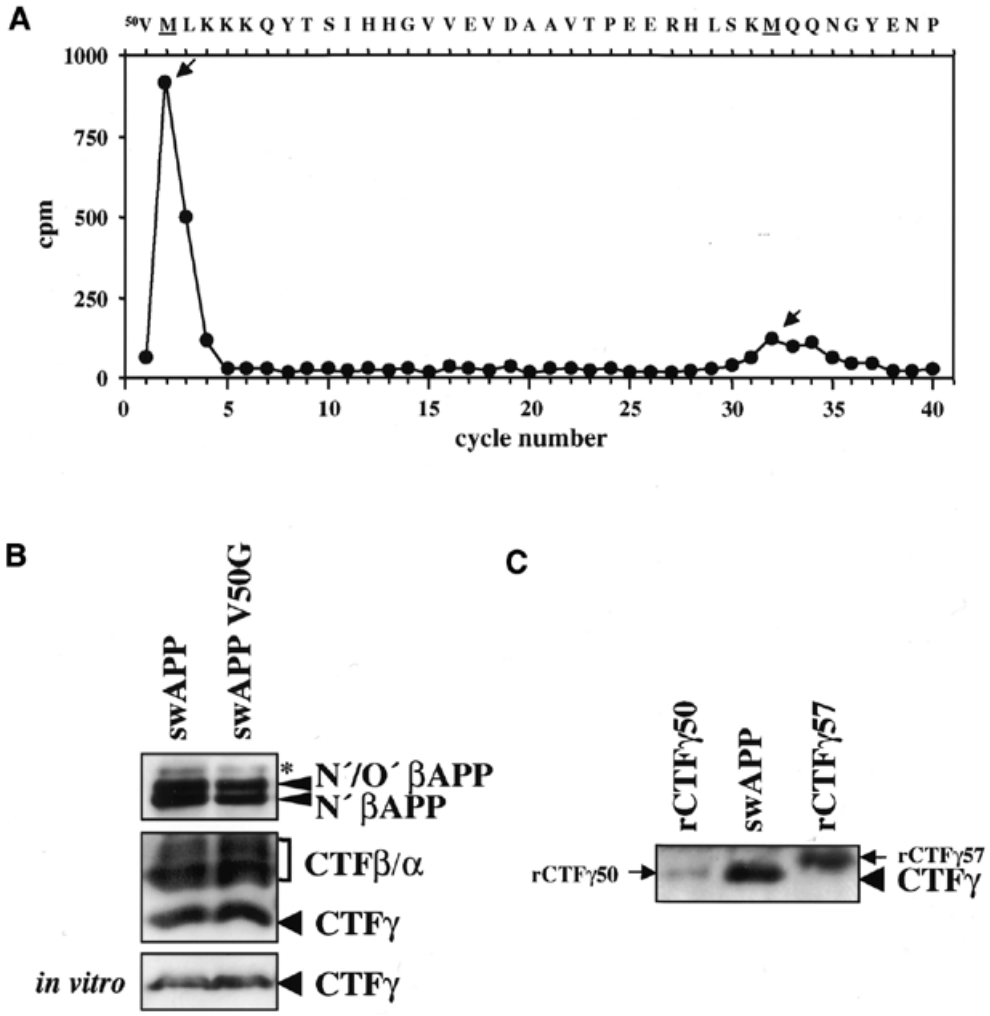

Fig. 3. Identification of a novel γ-secretase-dependent cleavage site. (**A**) Radiosequencing of CTFγ. CTFγ generated *in vitro* from membrane preparations of [^{35}S]methionine-labeled HEK 293 cells stably co-expressing swAPP and wild-type PS1 was subjected to radiosequencing. A major peak of radioactivity was observed at cycle 2 and a second peak at cycle 32 of the Edman degradation. The corresponding amino acid sequence of CTFγ starting at valine 50 is shown above. The same result was obtained in independent sequencing runs. (**B**) Mutation of valine 50 of the β-amyloid domain does not interfere with CTFγ generation. Upper and middle panel, membrane preparations of HEK 293 cells stably overexpressing swAPP or swAPP V50G were analyzed for the levels of βAPP holoprotein, CTFβ, CTFα and CTFγ by combined immunoprecipitation/immunoblotting with antibody 6687. The asterisk denotes the mature form of endogenous $βAPP_{751}$. Lower panel, membrane preparations were incubated at 37°C for 1 h, and CTFγ was analyzed from S100 fractions by immunoblotting with antibody 6687. (**C**) Detection of a truncated CTFγ in living HEK 293 cells stably overexpressing swAPP. Membrane fractions of HEK 293 cells stably overexpressing swAPP or cell lysates of HEK 293 cells transiently transfected with cDNA encoding a recombinant CTFγ starting either at amino acid 50 (rCTFγ50) or at amino acid 43 (rCTFγ57) were analyzed by combined immunoprecipitation/immunoblotting with antibody 6687 to the C-terminus of βAPP. Note that the *in vivo* produced CTFγ migrates faster than rCTFγ57 recombinant fragment.

S3 cleavage of Notch (Schroeter *et al.*, 1998) (Figure 4). Both cleavages are PS dependent and can be blocked by γ-secretase inhibitors (De Strooper *et al.*, 1998, 1999). Thus, it is likely that γ-secretase cleavage at position 49 of the β-amyloid domain and S3 cleavage of Notch are mediated by the same PS-dependent enzyme. Previously it was reported that the efficiency of S3 cleavage of mouse Notch1 is significantly reduced by mutations of valine 1744 at the S3 cleavage site (Schroeter *et al.*, 1998; Huppert *et al.*, 2000) (see also Figure 4). Consistent with this finding, mice homozygous for a *Notch1* gene variant carrying the V1744G mutation display an embryonic lethal phenotype resembling that of *Notch1* gene ablated mice (Huppert *et al.*, 2000). Although certain S3 mutants clearly reduce the efficiency of S3 cleavage, evidence was presented that γ-secretase-like S3 cleavage of Notch may be independent of the amino acid sequence at the cleavage site (Struhl and Adachi, 2000). Consistent with this finding, we demonstrate that mutagenesis of valine 50 of the β-amyloid domain to glycine does not abolish generation of CTFγ. This may indicate that TMs of Notch with mutations at S3 and TMs of βAPP with mutations at the site corresponding to S3 may be differentially recognized by a PS-dependent γ-secretase/S3 protease activity.

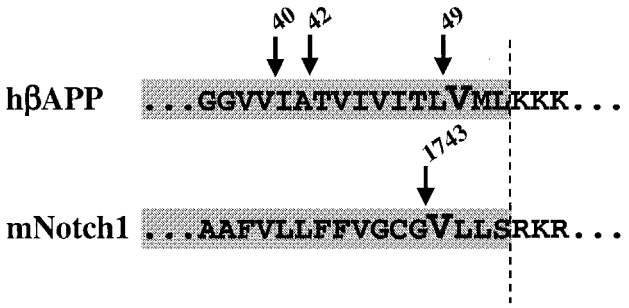

Fig. 4. Topologically similar PS-dependent γ-secretase/S3 protease cleavages of βAPP and Notch. Human βAPP is cleaved by γ-secretase in a PS-dependent manner after positions 40, 42 and 49 of the β-amyloid domain. Mouse Notch1 is cleaved PS-dependent at S3 after amino acid 1743 (Schroeter *et al.*, 1998). Note the similar location of the γ-secretase cleavage site at position 49 of the β-amyloid domain and the S3 cleavage site of Notch. The gray box represents the transmembrane domain; the dashed line the proposed membrane border.

Speculation

Finally, the identification of CTFγ *in vivo* may also raise the interesting possibility that this fragment similar to NICD may have a biological function in signal transduction. Based on the striking similarity of the biological mechanisms involved in the generation of NICD and CTFγ, as well as potentially similar functions in signal transduction, we therefore propose the term AICD for the amyloid precursor protein intracellular domain.

METHODS

Cell lines, cell culture and transient transfection. HEK 293 cell lines were generated and cultured as described (Steiner *et al.*, 2000). Mouse neuroblastoma N2a as well as immortalized embryonic fibroblast cells derived from PS1$^{+/+}$ or PS1$^{-/-}$ mice were cultured as described for HEK 293 cells. cDNA transfections were carried out using DOTAP (Roche) according to the supplier's instructions.

cDNA constructs. cDNA constructs encoding recombinant CTFγ50 or CTFγ57 were generated by PCR and cloned into pcDNA3 vector (Invitrogen). Start codons were introduced at the 5′ end of these cDNA constructs. The cDNA encoding swAPP V50G was constructed by PCR-mediated mutagenesis and cloned into pcDNA3 vector (Invitrogen).

Antibodies. The polyclonal antibodies 6687 to the last 20 C-terminal amino acids of βAPP (Steiner *et al.*, 2000) and 3926 to Aβ1–42 (Wild-Bode *et al.*, 1997) have been described. The monoclonal antibody 6E10 to Aβ1–17 was obtained from Senetek.

Inhibition of γ-secretase activity. γ-secretase activity was inhibited using DAPT (Dovey *et al.*, 2001) and CM256, previously designated compound 1 (Esler *et al.*, 2000). γ-secretase inhibitors were diluted from stock solutions in dimethyl sulfoxide to the concentrations described.

Analysis of secreted Aβ. Aβ was immunoprecipitated from conditioned media with antibody 3926, separated on 10–20% Tris–Tricine gels (Invitrogen), detected by immunoblotting with antibody 6E10 using a chemiluminescent detection system (Tropix) and quantified by densitometric scanning.

Analysis of CTFγ *in vivo*. CTFγ was analyzed by combined immunoprecipitation/immunoblotting with antibody 6687 of membrane extracts from stably transfected HEK 293 cells or from mouse brain. Briefly, homogenates of cells or brain tissue were prepared in hypotonic buffer (10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA) containing 1× protease inhibitors (PI) (Complete; Roche) as described (Steiner *et al.*, 1998). Following homogenization, membranes were isolated from the post-nuclear supernatant (PNS) by centrifugation at 16 000 *g* for 45 min at 4°C. The membranes were resuspended in RIPA buffer (10 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% cholic acid, 0.1% SDS, 5 mM EDTA, 1× PI), cleared by a spin at 16 000 *g* for 10 min at 4°C, and subjected to immunoprecipitation with antibody 6687. Following SDS–PAGE on 10–20% Tris–Tricine gels (Invitrogen) CTFγ was analyzed by immunoblotting using enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech) and quantified by densitometric scanning. To analyze expression of recombinant CTFγ50 or CTFγ57, lysates were prepared with RIPA buffer 48 h after transfection, subjected to immunoprecipitation with antibody 6687 and analyzed as above.

Generation and analysis of CTFγ *in vitro*. CTFγ was generated *in vitro* from membrane preparations of the indicated cell line following previously described procedures (McLendon *et al.*, 2000; Pinnix *et al.*, 2001). Cells were resuspended (0.5 ml/10 cm dish) in homogenization buffer (10 mM MOPS pH 7.0, 10 mM KCl, 1× PI) and homogenates and a PNS were prepared as described (Steiner *et al.*, 1998). Membranes were pelleted from the PNS by centrifugation for 20 min at 16 000 *g* at 4°C, washed with homogenization buffer and resuspended (50 µl/10 cm dish) in assay buffer (150 mM sodium citrate pH 6.4, 1× PI). To allow generation of CTFγ, samples were incubated at 37°C for the indicated time points in a volume of 25 µl/assay. Control samples were kept on ice. After termination of the assay reactions on ice, samples were separated into pellet (P100) and supernatant (S100) fractions by ultracentrifugation for 1 h at 100 000 *g* at 4°C. Following SDS–PAGE on 10–20% Tris–Tricine gels (Invitrogen), CTFγ was analyzed by immunoblotting with antibody 6687 using enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech) and quantified by densitometric scanning.

Radiosequencing of CTFγ. Confluent swAPP-transfected HEK 293 cells in 10 cm dishes were radioactively labeled with 1.4 mCi/dish [^{35}S]methionine/[^{35}S]cysteine (Promix; Amersham Pharmacia Biotech)/dish for 4 h in methionine-free MEM. CTFγ was then generated *in vitro* from membrane preparations as described above except that the assay reactions were separated into pellet (P16) and supernatant (S16) fractions by centrifugation at 16 000 *g* for 30 min at 4°C. After isolation of CTFγ from S16 by immunoprecipitation with antibody 6687, immunocomplexes were separated by SDS–PAGE on 10–20% Tris–Tricine gels (Invitrogen) and blotted onto a PVDF membrane. After audioradiography, the CTFγ band was excised and subjected to radiosequencing by automated Edman degradation as described (Haass *et al.*, 1992).

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NOTE ADDED IN PROOF

Consistent with our findings, we learned that Ihara and colleagues (Gu *et al., J. Biol. Chem.,* in press) as well as Weidemann and Beyreuther (personal communication) and Sisodia and colleagues (personal communication) found cleavage of βAPP after position 49 of the β-amyloid domain.

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