Amyloid β-Protein Precursor Juxtamembrane Domain Regulates Specificity of γ-Secretase-dependent Cleavages

Zhao Ren, Dale Schenk, Gurigbal S. Basi, and I. Paul Shapiro

From Elan Pharmaceuticals, Inc., South San Francisco, California 94080

Amyloid β-protein (Aβ), the major component of cerebral plaques associated with Alzheimer disease, is derived from amyloid β-protein precursor (APP) through sequential proteolytic cleavage involving β- and γ-secretase. The intramembrane cleavage of APP by γ-secretase occurs at two major sites, γ and ε, although the temporal and/or mechanistic relationships between these cleavages remain unknown. In our attempt to address this issue, we uncovered an important regulatory role for the APP luminal juxtamembrane domain. We demonstrated in cell-based assays that domain replacements in this region can greatly reduce secreted Aβ resulting from γ-cleavage without affecting the ε-cleavage product. This Aβ reduction is likely due to impaired proteolysis at the γ-cleavage site. Further analyses with site-directed mutagenesis identified two juxtamembrane residues, Lys-28 and Ser-26 (Aβ numbering), as the critical determinants for efficient intramembrane proteolysis at the γ-site. Consistent with the growing evidence that ε-cleavage of APP precedes γ-processing, longer Aβ species derived from the γ-cleavage-deficient substrates were detected intracellularly. These results indicate that the luminal juxtamembrane region of APP is an important regulatory domain that modulates γ-secretase-dependent intramembrane proteolysis, particularly in differentiating γ- and ε-cleavages.

Accumulation of brain β-amyloid is the major pathological feature of Alzheimer disease. The generation of Aβ2 from APP is a complex process requiring successive cleavages by two proteases, β- and γ-secretase (1). β-Secretase is a membrane-bound aspartyl protease that cleaves APP on its luminal portion (2–5), producing a C-terminal fragment consisting of 99 amino acids (C99/β-CTF). The β-CTF can be subsequently cleaved by γ-secretase at two major sites within the transmembrane domain (TMD), γ and ε, generating Aβ and an intracellular fragment known as APP intracellular domain (AICD) (6, 7). Alternatively, an α-secretase-dependent processing of APP results in a shorter α-CTF/C83 fragment that can undergo similar cleavages (1). γ-Secretase is also known to cleave Notch, CD44, and other type I transmembrane proteins (8). The amino acid sequence requirement for γ-secretase-dependent cleavage seems relatively relaxed, depending more on the size of the extracellular domain of a substrate than the recognition of specific sequences (9). The Notch processing resembles that of APP, with two homologous γ-secretase cleavage sites, S4 and S3, positioned in the middle of the TMD and near the cytoplasmic leaflet, respectively (10, 11). Notchβ and Notch intracellular domain are the two cleavage products, with the latter being an important transcriptional activator (12).

γ-Secretase is a multisubunit aspartyl protease that consists of at least four different membrane proteins, presenilin (PS), nicastrin, Aph-1, and Pen-2 (8). PS is thought to be the catalytic subunit of the holoenzyme, containing two conserved intramembrane aspartate residues essential for substrate cleavage (13, 14). The precise mechanisms by which γ-secretase recognizes and cleaves its substrates remain elusive, partly because these proteolytic events occur within a hydrophobic environment of membrane lipid bilayer. It has been shown that certain hydrophobic residues within the TMD of APP play a key role in determining cleavage efficiency as well as specificity (15, 16). Other substrate domains are also implicated in regulating γ-secretase-dependent proteolysis. Studies using transition-state analogue γ-secretase inhibitors support the existence of substrate–enzyme binding sites beyond the TMD (17, 18). Because the cytoplasmic tail of APP is known to have little direct impact on its cleavage by γ-secretase (19), these data seem to suggest a role for the APP luminal domain in substrate–enzyme interaction and/or catalysis. Consistent with this view, numerous studies have shown that the alternative processing of APP as well as Notch luminal domain by different secretases could significantly alter their downstream cleavages by γ-secretase (20–22). The importance of the APP luminal domain is further confirmed by a recent study in which certain luminal juxtamembrane mutations were shown to inhibit Aβ generation (23). Finally, Shah et al. (24) demonstrated that the free N termini of APP and Notch luminal juxtamembrane stubs (exposed upon ectodomain shedding) are crucial for substrate recruitment by γ-secretase through their direct interactions with Nicastrin. Collectively, these studies provide ample evidence that the luminal juxtamembrane domain of γ-secretase substrate plays an important role in γ-secretase-mediated proteolysis. However, until now, it has not been investigated whether alterations in this region can differentially affect γ- and
e-cleavages, a potential mechanism that may be exploitable for developing safe γ-secretase inhibitors for AD therapy.

In the present study we examined the significance of APP luminal juxtamembrane domain in γ-secretase-mediated proteolysis. Through a series of mutagenesis experiments using a C99-derived substrate that contains a Gal4/VP16 (GVP) signaling domain, we were able to evaluate the effects of juxtamembrane mutations on multiple cleavage events within the same substrate. Our data show that, similar to the wild type C99, C99-GVP is a functional γ-secretase substrate cleavable at both γ- and ε-sites. Domain swap as well as point mutations in its luminal juxtamembrane domain led to significant reduction in secreted Aβ without affecting AICD production. The divergent effects of these mutations on γ- and ε-cleavages further support a modulatory role for the APP luminal juxtamembrane domain in cleavage specificity in addition to its known function in substrate recognition and recruitment.

Experimental Procedures

Plasmid Construction—A pcDNA3.1-C99 plasmid similar to the previously described SPA4CT-LE construct (25) was generated by a PCR method. The APP signal peptide was fused to the N terminus of the C99 fragment via the dipeptide leucine-glutamic acid linker. The method to generate the pcDNA3.1-C99GVP construct has been previously described (26). Briefly, an Ascl site was introduced immediately 3′ of the nucleotides encoding the triple-lysine membrane anchor of C99, where the GVP coding sequence was subsequently inserted in-frame. To make the juxtamembrane chimeras, a 19-residue luminal juxtamembrane domain in C99GVP (amino acids 606–625 in APP695) was replaced by corresponding 19-residue luminal juxtamembrane domain in C99GVP (amino acids 606–625 in APP695) was replaced by corresponding amino acids 674–693), Notch1 (amino acids 1716–1734), or sterol regulatory element binding protein-1 (SREBP1; amino acids 6469–674), generating C99GVP-APLP2, C99GVP-Notch1, and C99GVP-SREBP1, respectively. All three chimeras were constructed by using a two-stage PCR method with two pairs of overlapping primers. Additional domain swap chimeras retaining the pre-TMD GSK3 β motif of APP, namely C99GVP-APLP2G, C99GVP-Notch1G, and C99GVP-SREBP1G, were generated in a similar fashion with a different set of primers. The C99GVP-SLSS quadruple mutant was also constructed with the same PCR method. Point mutations within the luminal juxtamembrane domain (i.e. C99GVP-G25S, -S26L, -N27S, and -K28S as well as APP695-S26L and -K28S) were generated using QuikChange (Stratagene) site-directed mutagenesis. All cDNAs were verified by sequencing. The Aβ and Aβ-like peptides generated from C99GVP and various mutant substrates were numbered with reference to the first N-terminal residue (Asp-1) of the Aβ peptide.

Antibodies—Polyclonal antibody against the last 20 amino acids of APP was purchased from Sigma and used at 1:20,000 for Western blots. The polyclonal AICD neo-epitope antibody was raised against a VMLKKK conjugate and produced in-house. Monoclonal anti-VP16 (Santa Cruz Biotechnology) was used at 1:500 for Western blots. Monoclonal antibodies 2H3 (specific to Aβ4–7), 2G3 (specific to Aβ33–40), and 21F12 (specific to Aβ30–42) have been described previously (27).

Characterization of the AICD neo-epitope antibody is detailed in supplemental Fig. S1.

Cell Culture and Transient Transfection—Human embryonic kidney 293 (HEK 293) cells (ATCC) were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum and 50 units/ml penicillin and streptomycin (37 °C, 5% CO2). All transfections were carried out in 6-well tissue culture plates (Costar). Three plasmids, pGSE1B-luc (200 ng), pCMV-β-gal (100 ng), C99GVP or various mutants (200–400 ng), were added together to each well. FuGENE6 reagent (Roche Applied Science) was used according to the manufacturer’s protocol for the transient transfection of adherent cells. Transfected cells were reseeded onto 12-well and/or 96-well plates (Costar) 16 h post-transfection; fresh media were added either with or without γ-secretase inhibitors. The cells and conditioned media were harvested 48 h post-transfection for analysis.

Inhibitor Treatment of Transfected HEK Cells—The transfection state analogue γ-secretase inhibitor L-685,458 (Sigma) and the peptidomimetic inhibitor DAPT (28) were dissolved in Me2SO to make 20 mM stocks. Inhibitors were added to HEK cultures at the indicated final concentration, and the treated cells were harvested 48 h post-transfection. The metalloprotease inhibitor TAPI-1 (Calbiochem) was used at a final concentration of 40 μM. The Aβ-degrading enzyme inhibitors bactitracin (Calbiochem) and phosphoramidon (Calbiochem) were used at final concentrations of 1 mg/ml and 40 μM, respectively. All inhibitor treatment was performed in triplicate and repeated at least three times.

Western Blot Detection of the Substrates and AICD—Forty-eight hours after transient transfection, HEK cells grown in 12- or 6-well tissue culture plates were washed with cold Tris-buffered saline (TBS) and homogenized in 1 ml of lysis buffer (0.1% SDS, 0.5% deoxycholate, and 1% Nonidet P-40 in TBS) with a protease inhibitor mixture (Sigma). All samples were solubilized at 4 °C for 1 h and cleared by centrifugation at 14,000 × g for 30 min. Aliquots of the supernatant were boiled for 5 min in Laemmli sample buffer and resolved on 10–20% Tris-Tricine gels and subsequently permeabilized with 0.2% Triton X-100 in PBS for 10 min. C99GVP and chimera substrates as well as AICD-GVP were detected by incubating the samples sequentially with polyclonal anti-VP16 for 2 h and rhodamine-conjugated secondary antibody (Jackson Laboratory) for 1 h. All staining was visualized on a Bio-Rad MRC 1024ES confocal microscope (Bio-Rad) and captured with a coupled CCD camera.

Luciferase Reporter Gene Assay—Luciferase reporter assays were carried out 48 h post-transfection. Cells seeded on 96-well plates (BD Biosciences) were washed once with phosphate-buffered saline and harvested in 20 μl of reporter lysis buffer (Promega) per well. After adding 100 μl of luminescent sub-
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strate (Promega), the luciferase activity was measured with a MicroLumatPlus microplate luminometer (Berthold Technologies). The β-galactosidase activity was measured similarly using a luminescent β-galactosidase substrate (BD Biosciences). As a control for transfection efficiency and general effect on transcription, the luciferase activity was normalized by the β-galactosidase activity measured on a duplicate plate. All measurements were done in triplicate, and similar experiments were repeated at least three times.

Immunoprecipitation (IP) and Western Blot Detection of Aβ—Total Aβ peptides in conditioned medium or cell lysate were immunoprecipitated at 4 °C overnight with 4 μg of the 2H3 antibody followed by incubation with 50 μl of a 50% protein G-Sepharose (GE Healthcare) slurry for 1 h and 3 washes in the same lysis buffer. Bound proteins were eluted in Laemmli sample buffer by heating at 70 °C for 5 min and resolved on 10−20% Tris-Tricine SDS-PAGE or the modified Tris-Tricine/8 M urea gels (29). After transferring onto nitrocellulose membranes (Invitrogen), the membranes were heated to 98 °C for 5 min in phosphate-buffered saline, immunostained with the 2H3 antibody, and visualized with Super-signal West Pico chemiluminescent substrate (Pierce). Each experiment was repeated at least three times.

Aβ ELISA—ELISAs used to quantify different Aβ species have been described previously (27). The Aβ40 and Aβ42 peptides in the samples were captured onto 2G3 or 21F12 antibody-coated plates, respectively, and detected with a biotinylated 2H3 antibody. The fluorescence signal generated from a streptavidin-alkaline phosphatase conjugate (Roche Applied Science) was measured with a CytoFluor microplate reader (Applied Biosystems). Synthetic Aβ40 or Aβ42 peptides (Anaspec) were used to generate standard curves. All measurements were done in triplicates.

RESULTS

C99GVP Undergoes Normal γ-Secretase-dependent Cleavages—γ-Secretase-dependent cleavage of C99/β-CTF at the γ- and ε-sites generates Aβ and the highly labile AICD, respectively. To facilitate the measurement of both cleavages, we engineered a C99GVP substrate in which a GVP signaling domain was inserted immediately after the triple-lysine anchor of C99 transmembrane region (Fig. 1A). In addition, the APP signal peptide was fused to its N terminus through a leucine-glutamate dipeptide bridge to maintain correct intracellular trafficking and membrane insertion. Cleavage of C99GVP by γ-secretase releases an Aβ-like peptide with the extra LE residues on its N terminus (30) as well as an AICD-like fragment (AICD-GVP) that can transactivate a luciferase reporter through its GVP domain (Fig. 1A). To confirm that C99GVP is a functional γ-secretase substrate, we transfected it into HEK 293 cells and characterized its expression and processing. As shown in Fig. 1B (top 3 panels, lanes 1 and 2, solid arrow), in the absence of γ-secretase inhibitors, the full-length C99GVP was readily detected by VP16 and APP antibodies as well as the 2H3 antibody that recognizes an N-terminal epitope (Fig. 2A). Interestingly, another band with slightly faster mobility was also detected by the former two (Fig. 1B, middle 2 panels, lanes 1 and 2, solid arrowhead) but was unrecognizable by 2H3 (Fig. 1B, top panel, lanes 1 and 2), indicating that it is an N-terminal-truncated fragment of C99GVP. Because previous reports have shown substantial stabilization of AICD after fusing with various protein tags (31, 32), it is likely that this faster migrating band might be the ε-cleavage product AICD-GVP. Indeed, this fragment is about 4 kDa smaller than the full-length C99GVP, a difference expected for the putative AICD-GVP. We then tested whether this truncated fragment of C99GVP is sensitive to treatment with γ-secretase inhibitors L-685,488 (33) and DAPT (28). As shown in Fig. 1B (middle 2 panels, lanes 3–6), this putative AICD-GVP was abolished after exposure to a high concentration of either inhibitor. In addition, the disappearance of this putative AICD fragment coincided with a significant stabilization and accumulation of the full-length C99GVP (Fig. 1B, top 3 panels, lanes 3–6). We then probed the same blot with an AICD neo-epitope antibody reactive to the free N terminus resulting from ε-cleavage of APP. As expected, it detected a band co-migrating with the putative AICD-GVP fragment that was also sensitive to γ-secretase inhibitor treatment (Fig. 1B, bottom panel). Finally, to examine the subcellular distribution of C99GVP, transiently transfected COS-7 cells were immunostained with the VP16 antibody. In the presence of DAPT, C99GVP showed a largely homogenous expression profile with no apparent nuclear staining (Fig. 1F, bottom right panel). In contrast, a prominent nuclear signal was observed without DAPT treatment, consistent with robust AICD-GVP generation and nuclear translocation (Fig. 1F, top right panel).

γ-Secretase inhibitor treatment not only led to the abolishment of AICD-GVP but also the emergence of a new protein fragment whose electrophoresis mobility is intermediate between that of C99GVP and AICD-GVP (Fig. 1B, middle 2 panels, lanes 3–6, open arrowhead). Interestingly, this nascent species was not recognizable by the 2H3 antibody (Fig. 1B, top panel, lanes 3–6), suggesting that it is an N-terminal-truncated C99GVP derivative as well. Considering the robust α-secretase activity in the host cells, this fragment is most likely C83GVP, a cleavage product from the C99GVP substrate. In support of this view, concomitant treatment with the α-secretase inhibitor TAPI-1 largely abolished this intermediate species (data not shown).

After confirming AICD-GVP generation in HEK cells, we then tested its ability to transactivate a luciferase reporter gene that contains Gal4 response elements in the upstream activation sequence. As shown in Fig. 1C, no appreciable signal was detected from cells transfected with the reporter gene alone, whereas co-expressing an active form of GVP resulted in strong transactivation, thus confirming the specificity of this reporter assay. Robust signals, comparable to that of the GVP control, were also observed for cells cotransfected with C99GVP (Fig. 1C). Importantly, γ-secretase inhibitor treatment led to dose-dependent decrease of luciferase activity only in the C99GVP transfected (Fig. 1C), indicating that C99GVP-induced reporter transactivation is γ-secretase-dependent. Some residual luciferase activity remained in the presence of excess γ-secretase inhibitors even though identical treatment completely abolished AICD production as measured by Western blot (Fig. 1B). This discrepancy likely results from the extraordinary sensitivity and nonlinear signal output of this assay (26,
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FIGURE 1. C99-GVP is a functional γ-secretase substrate undergoing physiological cleavages. A, schematic view of γ-secretase-dependent processing of C99GVP and the luciferase reporter assay measuring AICD production. The two proteolytic sites (γ and ε) within the TMD region are depicted as well as the position (open arrow) where signal peptide (SP) is cleaved upon protein maturation. After cleavages at the γ-secretase sites, the intracellular domain containing the GVP domain (AICD-GVP) translocates into nucleus and initiates luciferase expression under control of the upstream activation sequence (UAS) promoter. LE, leucine-glutamate. B, AICD-GVP is generated from C99GVP in a γ-secretase-dependent manner. Transiently transfected HEK cells treated without (lanes 1 and 2) or with γ-secretase inhibitor L-685,458 (lanes 3 and 4) or DAPT (lane 5 and 6) for 24 h were harvested 48 h post-transfection and Western blotted with the following antibodies: 2H3, anti-VP16, anti-APP, or anti-AICD neo-epitope. AICD-GVP (solid arrowheads) was readily detectable in cells subjected to no treatment (lane 1) or treated with Me2SO (DMSO, lane 2). No AICD-GVP was detected after treatment with L-685,458 at 1 µM (lane 3) or 20 µM (lane 4) nor with DAPT at 1 µM (lane 5) or 20 µM (lane 6). Stabilization of AICD-GVP occurred after inhibitor treatment (lanes 3–6). Open arrowheads indicate C83GVP, the N-terminal-truncated fragment of C99GVP after α-secretase-dependent cleavage (lanes 3–6). C, γ-secretase inhibitors block luciferase reporter transactivation in a dose-dependent manner. The effect L-685,458 and DAPT was analyzed in HEK 293 cells transiently transfected with a luciferase reporter. Both inhibitors caused a dose-dependent reduction in luciferase activity in C99GVP co-transfected cells but not in cells co-expressing a constitutively active GVP. Data are the mean ± S.D. luminescence units of three independent experiments. *, p < 0.01 versus reporter-only control; **, p < 0.05 versus C99GVP-transfectants subjected to no inhibitor treatment. D, γ-secretase inhibitors block secreted Aβ. Conditioned media from transiently transfected cells treated with Me2SO, L-685,458 (5 µM), or DAPT (5 µM) for 24 h were collected, and Aβ40 (hatched bars) and Aβ42 (open bars) were measured by sandwich ELISAs (top panel). The capture/detection antibody pairs were 2G3/2H3 and 21F12/2H3, respectively. Data are the mean ± S.D. of three independent experiments. Total secreted Aβ (bottom panel) in the same conditioned media was IP/Western-blotted with the 2H3 antibody. *, p < 0.01 versus Aβ40 from C99GVP-transfectants treated with Me2SO; **, p < 0.01 versus Aβ42 from the same group. E, γ-secretase inhibitors show equal Aβ-inhibition potency with C99GVP and a native substrate. Dose-response curves were derived from C99GVP or APP-transfected cells treated with serially diluted L-685,458 (left panel) or DAPT (right panel). The Aβ40 level in conditioned media was measured by ELISA and expressed as percentage of Me2SO-treated control. Inhibitor concentrations are shown in log scale. The inserted tables show the calculated IC50 values. F, subcellular distribution of C99GVP and AICD-GVP in transiently transfected COS-7 cells. The robust nuclear (N) staining of AICD-GVP in Me2SO-treated cells was abolished after DAPT (5 µM) treatment, and a more homogeneous expression profile of C99GVP was revealed.

Next, we characterized Aβ generation from C99GVP. Wild type HEK cells and the mock-transfection control secreted little Aβ into the conditioned media (Fig. 1D, bottom panel, lane 1). In contrast, transient expression of C99GVP led to robust Aβ
production, as measured by IP/Western blot (Fig. 1D, bottom panel, lane 2) and ELISAs that detect Aβ40 and Aβ42 species, respectively (Fig. 1D, top panel). Consistent with previous reports, Aβ40 (210.8 ± 19.2 pm) is the major secreted species, whereas Aβ42 (39.1 ± 6.4 pm) only accounts for a small fraction (15.7 ± 2.5%) of the total Aβ (Fig. 1D, top panel). γ-Secretase inhibitor treatment completely abolished Aβ secretion (Fig. 1D, top panel and bottom panel, lanes 3 and 4). Finally, we compared the Aβ-lowering potency of two inhibitors using either C99GVP or the wild type APP as substrate. As determined by ELISA, the respective IC50 values for the two substrates are essentially identical (Fig. 1E).

APP Juxtamembrane Alteration Inhibits Aβ but Not AICD—It has been reported that certain APP luminal juxtamembrane
mutations could drastically alter Aβ secretion (23); however, their effect on AICD production remains unknown. To gain more insights into the luminal juxtamembrane function in γ-secretase-dependent proteolysis, we made juxtamembrane chimeras. A 19-residue sequence preceding the TMD of C99GVP was replaced by its topological counterparts from other transmembrane proteins, including APLP2, Notch1, and SREBP1 (Fig. 2A). The resulting chimeras, C99GVP-APLP2, C99GVP-Notch1, and C99GVP-SREBP1, expressed comparably in HEK cells as detected by 2H3 and APP antibodies (Fig. 2B, top and middle panel), whose epitopes remain unaltered. AICD-GVP production was largely unaffected as well (Fig. 2B, middle panel). In addition, the subcellular distribution of these chimeras also resembled that of C99GVP (Fig. 2G). Finally, the signaling assay revealed that all three chimeras elicited robust reporter activity indistinguishable from the C99GVP control (Fig. 2C). These data demonstrate that domain swap mutations in the luminal juxtamembrane region of C99GVP have minimal impact on its e-cleavage by γ-secretase.

Next, we measured the amount of Aβ-like peptides generated from the juxtamembrane chimeras. Similar to the C99GVP control, C99GVP-SREBP1 gave rise to abundant total Aβ (Fig. 2B, bottom panel) as well as Aβ40 and Aβ42 (Fig. 2D). In contrast, C99GVP-APLP2 and C99GVP-Notch1 generated little Aβ, as shown by Western blot (Fig. 2B, bottom panel). ELISA analyses revealed similar reductions in both Aβ40 (Fig. 2D) and Aβ42-like species (Fig. 2E). C99GVP-APLP2 had a 96.5 ± 1.3% decrease in Aβ40 and a 90.4 ± 2.5% drop in Aβ42, respectively, whereas C99GVP-Notch1 showed a near 90% reduction in both species. These observations are largely in agreement with a previous study examining similar mutants derived from the full-length APP substrate (23). Because the steady-state level of all three chimeras is comparable (Fig. 2B, top two panels), the significant difference in secreted Aβ cannot be attributed to any discrepancy in substrate expression. We also examined the interactions between γ-secretase and various substrates by communoprecipitation. Interestingly, the juxtamembrane chimeras and wild type C99GVP bound equally well to PS1 (Fig. 2F), suggesting that the juxtamembrane replacements did not disrupt enzyme-substrate interaction.

Reduction in Aβ Secretion Is Likely a Result of Impaired γ-Cleavage—To exclude the possibility that some non-γ-secretase-dependent mechanisms might be responsible for the aforementioned Aβ lowering, we investigated the potential involvement of other relevant factors. Enhanced γ-secretase cleavage of C99GVP-APLP2 and C99GVP-Notch may inhibit Aβ secretion by decreasing substrate level through N-terminal truncations (i.e. to C83GVP-like fragments). However, our data show the level of their intact forms was equivalent to that of C99GVP (Fig. 2B, top two panels). In fact, the two chimeras appear resistant to α-secretase processing, evident by their reduced N-terminal truncation (Fig. 2B, middle panel, open arrowhead). Consistent with this low susceptibility to α-secretase cleavage, TAPI-1 treatment did not markedly enhance Aβ40 secretion from either chimera (data not shown) as compared with the C99GVP control (Fig. 3A). In fact, similar Aβ reduction (97.5 ± 1.5 and 93.5 ± 2.2% for C99GVP-APLP2 and C99GVP-Notch1, respectively) was observed after TAPI-1 treatment (Fig. 3A).

Various Aβ-degrading enzymes can also reduce secreted Aβ levels by increasing its turnover (36). To rule out this possibility, we measured Aβ in the presence of potent inhibitors targeting multiple Aβ-degrading enzymes. Treatment with phosphoramidon and bacitracin did slightly enhance Aβ accumulation in the C99GVP control (Fig. 3B) as well as in other groups (data not shown). However, Aβ40-like peptides generated from the C99GVP-APLP2 and C99GVP-Notch1 chimeras remained significantly lower (97.1 ± 1.6 and 92 ± 3.4%, respectively) (Fig. 3B). Therefore, the selective Aβ reduction observed for both chimeric substrates is not a result of increased Aβ degradation.

Extracellular secretion of Aβ-like peptides after γ-secretase-dependent cleavage of Notch1 or APLP2 has been well documented (37, 38). It is, thus, unlikely that the juxtamembrane domain exchanges between these substrates will dramatically alter secretion of the resulting γ-cleavage products. Consistent with this argument, we found no intracellular accumulation of

FIGURE 3. Juxtamembrane domain swap-induced Aβ reduction is likely caused by impaired γ-cleavage. A, the effect of an α-secretase inhibitor on secreted Aβ40. Conditioned media from cells treated with Me2SO (DMSO, gray bar) or 40 μM TAPI-1 (black bars) were collected and analyzed by ELISA specific for Aβ40. Data are expressed as the percentage of the TAPI-1-treated C99GVP control. *, p < 0.01; **, p < 0.05. B, the effect of Aβ-degrading enzyme inhibitors on secreted Aβ40. Conditioned media from cells treated with Me2SO (gray bar) or 40 μM phosphoramidon plus 1 mg/ml bacitracin (checkered bars) were collected and analyzed by ELISA specific for Aβ40. Data are expressed as percentage of the inhibitor-treated C99GVP control; *, p < 0.01.
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Aβ40 or Aβ42-like species in cells transfected with either juxtamembrane chimera using sensitive ELISA measurements (data not shown). However, when we analyzed 2H3 immunoprecipitates from cell lysates using a urea-gel system (29), Aβ-like species co-migrating with the synthetic Aβ1–46 chimera peptide were detected in C99GVP-APLP2 (Fig. 3C, lane 4) or C99GVP-Notch1 transfectant (data not shown). The multiple bands observed likely reflect N-terminal heterogeneity of these species. In contrast, no such peptides were detected in the C99GVP control (Fig. 3C, lane 2). These intracellular Aβ peptides are reminiscent of the longer Aβ species described in recent reports (29, 39). This finding is consistent with a scenario that, when γ-cleavage is inhibited in C99GVP-APLP2 or C99GVP-Notch1 chimera, longer Aβ species can still be generated after normal ε- and ζ-cleavages but fail to be secreted extracellularly due to increased hydrophobicity (29, 39). Therefore, the diminished Aβ secretion from both C99-APLP2 and C99-Notch1 chimeras is most likely caused by impaired γ-cleavage rather than γ-secretase-independent mechanisms.

Mapping APP Juxtamembrane Residues Critical for γ-Cleavage—To better define the precise sequence determinants of γ- versus ε-cleavage selectivity, we first examined the significance of certain polar residues within the luminal juxtamembrane region of APP. Specifically, we made alanine substitutions of the residues arginine-histidine-aspartate (Aβ5–7), histidine-histidine-glutamine-lysine (Aβ13–16), and glutamate-aspartate (Aβ13–16) within C99GVP. None of these mutations led to significant change in secreted Aβ as shown earlier for the C99GVP-APLP2 and C99GVP-Notch1 chimeras (data not shown). There was little change in AICD production and reporter transactivation as well (data not shown). Subsequently, we evaluated certain residues immediately preceding the TMD partly because of their physical proximity to the intramembrane cleavage sites. In C99GVP as well as the full-length APP, the four amino acids N-terminal to the TMD are GSNK. We, therefore, investigated their role in Aβ generation by retaining this tetrapeptide motif in a new set of chimeras, named C99GVP-APLP2*, C99GVP-Notch1*, and C99GVP-SREBP1*, respectively (Fig. 4A, top panel). The expression profile of these new chimeras was comparable with that of the C99GVP control (Fig. 4A, lower panels). In addition, little change was observed for AICD production (Fig. 4A, bottom panel) as well as AICD-GVP-mediated reporter transactivation (Fig. 4B). However, in marked contrast to their predecessors, the GSNK-containing C99GVP-APLP2* and C99GVP-Notch1* chimeras demonstrated robust Aβ production indistinguishable from the C99GVP control (Fig. 4, C and D). As expected, the C99GVP-SREBP1* chimera also maintained normal Aβ secretion (Fig. 4, C and D). These results clearly revealed an important role for the GSNK motif in γ-cleavage and Aβ production. To further confirm this finding, we made another mutant, C99GVP-SLSS, in which the GSNK motif of C99GVP was substituted with a corresponding SLSS sequence from APLP2 (Fig. 5A, top panel). This mutation led to a marked reduction (~97%) in secreted Aβ (Fig. 5, B and C) but little change in AICD production (Fig. 5A, bottom panel) and reporter transactivation (Fig. 5D). These findings, along with the data obtained from the original juxtamembrane chimeras, unequivocally demonstrate that even subtle alteration in the APP luminal juxtamembrane domain could lead to profound changes in γ-cleavage.

Finally, we investigated the contribution of individual amino acids within the GSNK motif by mutating each of the four residues to their counterpart in APLP2 (Fig. 5A, top panel). The point mutants, namely C99GVP-G25S, -S26L, -N27S, and -K28S, express comparably in HEK cells (Fig. 5A, lower panels). There was also little difference in their respective AICD production (Fig. 5A, bottom panel) and signaling activity (Fig. 5D), again demonstrating equivalent ε-cleavage. However, substantial decrease in secreted Aβ was observed for both C99GVP-S26L and C99GVP-K28S mutants. The S26L mutation led to a 65.7 ± 8.5% reduction in total Aβ and a 52.7 ± 2.3% drop of Aβ40 (Fig. 5, B and C), whereas the K28S substitution resulted in an even more substantial (~90%) decrease in both measurements (Fig. 5, B and C). Similar reductions in secreted Aβ40 (i.e. 46.2 ± 6.8% by the S26L mutation and 87.3 ± 3.1% by the K28S mutation) were also observed when these mutations were introduced into a full-length, non-chimeric APP695 substrate (Fig. 5E). In contrast, the other two point mutations, G25S and N27S, showed no obvious effect on secreted Aβ (Fig. 5, B and C). Together, these data indicate that Lys-28 and Ser-26 are two critical residues in the APP luminal juxtamembrane domain, the substitution of which could selectively inhibit γ- but not ε-cleavage.

DISCUSSION

γ-Secretase cleaves APP, Notch, and other type I membrane proteins at multiple sites within their TMDs, but the precise temporal and mechanistic relationships between these proteolytic events remain elusive. This has hindered the development of safe γ-secretase inhibitors because of the central concern that inhibiting γ-secretase to lower Aβ production in AD could also adversely affect other crucial physiological functions. Indeed, significant toxic effects of γ-secretase inhibitors, caused by Notch signaling blockade, have been described in animal studies (40–42). With more and more physiologically relevant γ-secretase substrates being identified, it is of great importance to understand the intricate relationships between these proteolytic events and uncover molecular mechanisms that differentiate various substrates and/or alternative cleavages. In the present study we attempt to address these questions by examining the γ-secretase-dependent cleavage of a C99GVP substrate and various juxtamembrane chimeras. The advantage of this approach is that the signaling potential of the engineered GVP domain provides us with a sensitive measurement of the e-cleavage event. In addition, this modification also facilitates the biochemical detection of the otherwise extremely labile AICD by significantly increasing its stability. With these modified substrates, it is possible to simultaneously examine the effect of juxtamembrane mutations on both γ- and e-cleavages.

We found that alterations in the APP luminal juxtamembrane domain can selectively impair γ-cleavage while leaving e-cleavage intact. This result reveals a previously unknown role for the luminal juxtamembrane domain in regulating γ-secretase-mediated proteolysis at distant sites beyond the TMD.

Both Notch1 and APLP2 substrates are well documented to undergo γ- and ε-like intramembrane cleavages that are
believed to be largely sequence-independent (10, 11, 37). In this regard, our finding that C99GVP-APLP2 and C99GVP-Notch1 juxtamembrane chimeras failed only in H9253- but not H9280-cleavage is perplexing. However, the concept that H9253- and H9280-cleavages can be differentially regulated is also strongly supported by previous studies examining the effects of certain APP and presenilin mutations. A number of mutations in the APP transmembrane region were shown to have distinct effects on the two cleavage events (43, 44). Similarly, certain pathogenic presenilin mutations are known to increase the levels of amyloidogenic Aβ42 while suppressing the H9280-cleavage of APP as well as the homologous Notch S3 cleavage (45–47). It is not clear how these mutations cause the aforementioned changes, although conformational alterations have been proposed as the most likely underlying mechanisms. Indeed, unique conformational changes of presenilin have been observed either as a result of particular PS mutation or caused by differential interaction with mutant substrates (44, 48). Some allosteric γ-secretase inhibitors are reported to have divergent effects on γ- and ε-cleavages as well. For instance, certain non-steroid anti-inflammatory drugs selectively modulate γ-cleavage of APP without affecting AICD or Notch intracellular domain production (49). Importantly, these non-steroid anti-inflammatory drugs also lead to a conformational change of presenilin opposite to that induced by pathogenic mutations (50). Therefore, it will be of great interest to test whether the γ-cleavage-deficient mutants identified in this study can induce distinctive conformational changes of presenilin opposite to that induced by pathogenic mutations (50). According to a model proposed by Tian et al. (51), γ-secretase substrates need to move from the initial docking site on the enzyme complex to the catalytic center and unwind their α-helical TMD structure during this process to ensure efficient cleavage. It is plausible that juxtamembrane mutations may cause conformational changes that in turn interfere with substrate movement or helix unwinding, thus leading to preferential inhibition of γ-cleavage.

**FIGURE 4.** The GSNK motif in APP juxtamembrane domain is important for normal γ-cleavage. A, expression profile of the modified juxtamembrane (JMD) chimeras that retain the GSNK motif. Sequences of C99GVP and the chimeras are shown, with the replaced residues highlighted by color (top panel). Cell lysates from transiently transfected HEK cells treated with MeSO or 5 μM DAPT were immunoblotted with 2H3 (middle panel) or APP antibody (bottom panel). The open arrowhead indicates the C83GVP-like fragments derived from C99GVP or the chimeras. B, the modified chimeras show robust reporter transactivation mediated by AICD-GVP. Cells treated with MeSO (gray bars) or 5 μM DAPT (black bars) were assayed for their luciferase signal 48 h post-transfection. Data are shown as percentage transactivation activity compared with the MeSO-treated C99GVP control. C, the modified chimeras show normal Aβ secretion. Conditioned media from the MeSO-treated cells were collected and IP/Western-blotted with 2H3 antibody (bottom panel). The quantification (top panel) was performed by densitometry using a synthetic Aβ40 peptide as standard. Data are expressed as percentage of the C99GVP control. D, the modified chimeras show normal Aβ40 secretion. Conditioned media from MeSO (gray bars)- or DAPT (black bars)-treated cells were collected and analyzed by ELISA specific for Aβ40. Data are expressed as percentage of the MeSO-treated C99GVP control.
FIGURE 5. Mapping juxtamembrane residues critical for efficient γ-cleavage. A, expression profile of the mutant substrates that contain point mutations in the GSNK motif. Sequences of C99GVP and the point mutants are shown, with the substituted residues highlighted in red (top panel). Cell lysates from transiently transfected HEK cells treated with Me2SO or 5 μM DAPT were immunoblotted with 2H3 (middle panel) or anti-APP antibody (bottom panel). The open arrowhead indicates the C83GVP-like fragments derived from C99GVP or the mutants. B, differential effect of point mutations on total secreted Aβ. Conditioned media from Me2SO-treated cells were collected and IP/Western-blotted with 2H3 antibody (bottom panel). The quantification (top panel) was performed by densitometry, and data are expressed as the percentage of the C99GVP control. *, p < 0.01. C, differential effect of point mutations on secreted Aβ40. Conditioned media from Me2SO (gray bars) or DAPT (black bars)-treated cells were collected and analyzed by ELISA specific for Aβ40. Data are expressed as percentage of the Me2SO-treated C99GVP control. *, p < 0.01. D, AICD-GVP-mediated reporter transactivation is not altered by the point mutations. Cells treated with Me2SO (gray bars) or 5 μM DAPT (black bars) were assayed for their luciferase signal 48 h post-transfection. Data are shown as percentage transactivation activity compared with the Me2SO-treated C99GVP control. E, the effect of point mutations on secreted Aβ40 derived from a full-length APP substrate. Conditioned media from cells transfected with wild type APP695 or the mutants were collected and analyzed by ELISA specific for Aβ40 (top panel). Cell lysates from each group were Western blotted for APP expression using the anti-APP antibody (bottom panel). Mock-transfected cells were also included as control. Data are expressed as percentage of the wild type APP695 group. *, p < 0.01.
The interactions between γ-secretase complex and its substrates are likely mediated through multiple binding sites. It has been reported that the transition-state γ-secretase inhibitors fail to disrupt the binding between presenlin and the endogenous C83/C99 substrates (17, 52). This observation was later corroborated by imaging studies and further expanded to the Notch substrate (18, 53). Shah et al. (24) recently showed that Nicastrin interacts with the free N termini of several substrates (exposed after ectodomain shedding) through a critical aspartate residue in its extracellular domain. Importantly, this binding proves to be crucial for efficient substrate recruitment and cleavage by γ-secretase (24). All these data support the existence of a substrate binding/docking site(s) in γ-secretase complex, although their precise locations remain largely unresolved. It is conceivable that the APP luminal juxtamembrane domain might be part of the region involved in substrate binding/docking, a subtle change of which could lead to significant alterations in subsequent proteolytic events. This hypothesis makes particular sense in the context of lipid membranes where the water-containing active site of γ-secretase is likely sequestered from the hydrophobic environment. In this regard, it is interesting to notice that the two juxtamembrane residues critical for γ-secretase cleavage, Ser-26 and Lys-28, are polar and basic amino acids, respectively. Conservative substitution of either one, as in the case of C99GVP-SREBP1, caused no apparent change in normal proteolysis at the γ-secretase site. This hypothesis is corroborated by imaging studies and further expanded to the γ-secretase site of known substrates (59). The effect of γ-secretase inhibitors, which can preferentially inhibit the pathogenic γ-secretase-dependent Notch cleavage at the S3 and S4 peptidomonomers (54). It is, thus, conceivable that potential conformational changes in the juxtamembrane region of APP may also impact its binding with γ-secretase complex and subsequent proteolytic events. More rigorous experiments are currently under way to test these hypotheses.

In the present study we also found that when γ-secretase cleavage was selectively inhibited, longer Aβ species were generated after normal proteolysis at the e-site. This observation is consistent with recent reports documenting the existence of such novel species. Aβ peptides that end at the e-site (i.e. Aβ48/49) or the newly defined intermediate ζ-site (i.e. Aβ46) were detected in a broad range of biological samples (29, 39, 55). These discoveries have profound implications in sorting out the temporal relationship between γ- and e-cleavages. The fact that only longer Aβ but not longer AICD was identified, even after extremely rigorous attempts, suggests that e-cleavage of APP precedes γ-cleavage. Consistently, it has been shown that Aβ40/42 can be generated from heterologously expressed longer Aβ peptides whose C terminal end at the e-cleavage site (56). Similarly, Lefranc-Jullien et al. (57) demonstrated that APPe (APP with a C-terminal truncation at the e-cleavage site) could undergo normal γ-cleavage upon ectodomain shedding. These observations suggest a sequential relationship between e- and γ-cleavage of APP. Furthermore, according to a recent report, γ-secretase-dependent Notch cleavage at the S3 and S4 sites also proceeds in a similar sequential fashion (58), thus supporting the notion this might be a general principle applicable to all other γ-secretase substrates as well.

γ-Secretase-dependent substrate cleavages at multiple sites are extremely complex processes, which are not only involved in the pathogenesis of AD but also prove crucial for normal physiological functions. It is conceivable that the precise regulation of these alternative cleavage events may require additional factors. Indeed, a recent report identified TMP21, a member of the p24 cargo protein family, as a novel modulator of γ-secretase activity (59). Under physiological conditions, this protein selectively inhibits APP processing at the γ-site without affecting e-cleavage of known substrates (59). The effect of TMP21 seems similar in nature to that resulting from APP luminal juxtamembrane mutations, thus raising the possibility that TMP21 might be part of a complex mechanism that mediates juxtamembrane mutation-induced γ-cleavage inhibition. Future experiments are needed to address this hypothesis. A genetic study in Drosophila revealed that another protein, crumbs, may have the opposite effect of TMP21 and suppress γ-cleavage (60). Based on these observations, an intriguing scenario emerges in which the balance between γ and e cleavages can be modulated by the opposing actions of these and perhaps other yet to be identified regulators. The present study along with a larger number of existing reports strongly supports the concept that the separation of γ- and e-cleavages can be regulated through a variety of mechanisms that involve γ-secretase components, substrates, and functional modulators. These findings should provide a theoretical rationale as well as practical guidance for the development of selective γ-secretase inhibitors, which can preferentially inhibit the pathogenic cleavage without compromising normal biological functions.

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