Proteolysis of Chimeric β-Amyloid Precursor Proteins Containing the Notch Transmembrane Domain Yields Amyloid β-like Peptides*

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γ-Secretase is an unusual intramembranous protease that has been reported to cleave the β-amloid precursor protein (APP) near the middle of its transmembrane domain (TMD) but cleave Notch near the cytoplasmic end of its TMD. To ascertain whether the TMD sequence of the substrate determines where γ-secretase cleaves and whether the region just before the TMD participates in recognition by the enzyme, we expressed chimeric human APP molecules containing either the TMD or pre-TMD regions of Notch or other transmembrane proteins. APP chimeras bearing either the Notch or the amyloid precursor-like protein-2 TMD released similar amounts of ~4-kDa amyloid β-peptide (Aβ)-like peptides as did intact APP. Mass spectrometry revealed that the principal Aβ-like peptide ended at residue 40, indicating cleavage at the middle of the Notch TMD in the chimera. Generation of Aβ-like peptides was significantly decreased when the APP TMD was replaced by those of SREBP-1 or human epithelial growth factor receptor 3. Replacement of the APP pre-TMD region (Aβ 10–28) with that of SREBP-1 increased generation of Aβ-like peptides, while those of human epithelial growth factor receptor 3 or amyloid precursor-like protein-2 decreased it. We conclude that γ-secretase can cleave near the middle of the Notch TMD, that Aβ-like peptides may arise during Notch processing, and that the pre-TMD sequence of the substrate influences recognition or binding by the enzyme.

γ-Secretase is an unusual protease that cleaves the β-amyloid precursor protein (APP) within its single transmembrane domain as one of two proteolytic scissions required to generate amyloid β-peptide (Aβ). This processing event occurs normally throughout life and is augmented in patients with autosomal dominant forms of Alzheimer’s disease. Recent studies of presenilins 1 and 2 (PS1 and PS2) suggest that they are strong candidates for the active site of the γ-secretase enzyme. Deletion of PS1 in mice markedly decreases APP processing at the γ-secretase cleavage site (1), and deletion of both PS1 and PS2 completely abolishes γ-secretase activity (2, 3). Mutation of either of two conserved intramembranous aspartate residues (Asp257 and Asp385) in PS1 markedly decreases cellular γ-secretase activity (4), and mutation of the transmembrane (TM) aspartates in both PS1 and PS2 abolishes it (5). Deleting PS1 or mutating one of these critical TM aspartates also blocks the proteolytic release of the Notch intracellular domain (6–11), a critical step in the Notch signaling pathway. Furthermore, immunoprecipitation of PS1 has been shown to co-precipitate APP (12) and Notch (13), even at endogenous protein levels, indicating a physical interaction between PS1 and these γ-secretase substrates. Finally, transition state analogue inhibitors specifically targeted to the active site of γ-secretase bind directly to PS1 (14, 15). Taken together, these findings strongly suggest that presenilin contains the active site of γ-secretase and that APP and Notch are γ-secretase substrates.

Previous studies have suggested that the substrate requirements for proper γ-secretase cleavage of APP are relatively relaxed, depending more on the hydrophobicity of the cleavage region than its specific amino acid sequence (16, 17). For example, single amino acid substitutions in the TM domain (TMD) of APP only altered the cleavage location within the TMD and did not inhibit the cleavage by γ-secretase (16, 17). A serial mutation, deletion, and insertion study of the APP TMD indicated that γ-secretase cleavage specificity is primarily determined by the location of the cleavage site with respect to the membrane boundaries rather than by the specific sequence (18). Substituting residues 38–47 or 39–56 of the Aβ domain with a TM sequence from the epidermal growth factor receptor, human epithelial growth factor receptor (HER)-3, still yielded a ~4-kDa Aβ peptide (19), reflecting loose sequence specificity in the region carboxy-terminal to the γ-secretase cleavage.

It is possible that substrate recognition and/or cleavage by γ-secretase also require sequences not immediately adjacent to the cleavage site. For example, γ-secretase cleavage was not abrogated by removing the entire region of APP following the TMD (20), suggesting that the APP sequence immediately before (luminal to) the TMD might help direct cleavage specificity. Moreover, discrete amino acid substitutions 4 residues C-terminal to the Aβ42 cleavage site (but still within the TMD) were shown in recombinant and native systems to increase cleavage at residue 42 over that at residue 40, thus demonstrating effects of regions downstream of the actual cleavage site (18).

To elucidate further the structural requirements for γ-secretase recognition and cleavage of substrates such as Notch, APP,
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and an APP homologue, amyloid precursor-like protein (APLP)-2 (21), we performed domain-swapping experiments using membrane-inserted chimeric substrates rather than site-directed mutants of APP. Our results indicate that the Notch TMD within an APP milieu is a good γ-secretase substrate, as expected from studies demonstrating that Notch itself is a good γ-secretase substrate. Surprisingly, the principal cleavage site when in the context of APP is near the middle of the Notch TMD, similar to that of APP itself, rather than at the reported Notch cleavage site (22), suggesting that cleavage specificity may be influenced by surrounding sequences within the substrate. In support of this conclusion, we show that the sequence just N-terminal to the TMD plays a role in the recognition or binding of substrate by γ-secretase.

MATERIALS AND METHODS

Chimeric DNA Construction—Human APP695 cDNA containing the “Swedish” mutations K596N/M597L (SW-APP), which enhances β-secretase cleavage at Aβ Asp-1, was used as a template, and replacement sequences were generated by PCR (Fig. 1). The entire TMD of SW-APP i.e. residues 626–648) was replaced by the TMD of APLP-2 (aa 694–716) (yielding SW-m-APLP-2), HER-3 (aa 642–664) (SW-m-HER-3), SREBP-1 (aa 488–509) (SW-m-SREBP-1), or human Notch-1 (aa 1735–1757) (SW-m-Notch). In another set of chimeras, the 19–20 aa region just N-terminal to the TMD of SW-APP695 (aa 606–625), including the α-secretase cleavage site, was replaced by the corresponding region of either APLP-2 (aa 674–693) (yielding SW-α-APLP-2), HER-3 (aa 622–641) (SW-α-HER-3), SREBP-1 (aa 469–487) (SW-α-SREBP-1), or human Notch-1 (aa 1716–1734) (SW-α-Notch). The chimeric DNAs were then subcloned into PCDneo vector (Promega). Certain point mutations within the Notch TMD (SW-m-NotchV40A, L41G, L42A, V49L, and 29G+) were generated from the SW-m-Notch chimeric cDNA by site-directed mutagenesis. The 29G+ construct contains the Notch TMD with a glycine at its N terminus, thus increasing the length of the Notch TMD and allowing it to match precisely that of the APP TMD. The fidelity of all mutant genes was confirmed by DNA sequencing. For clarity, all Aβ or Aβ-like peptides generated from SW-APP and its various chimeras are numbered from the first N-terminal residue (Asp-1) of the Aβ peptide.

Transient Transfection of COS Cells—Transfection of COS cells was performed according to instructions for the LipofectAMINE transfection reagent (Invitrogen). Cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. Plasmids encoding SW-APP or the chimeric DNAs (9 µg for each 10-cm dish, 3 µg for each 6-cm dish, and 1 µg for each well of a six-well plate) were introduced into COS cells. After transfection, cells were changed to fresh medium (5 ml for each 10-cm dish, 2.5 ml for each 6-cm dish, and 1.5 ml for each well of a six-well plate) and conditioned for 48 h. For Aβ ELISA or immunoprecipitation (IP)/Western blotting (WB), short-term conditioned media were collected, and Aβ-sensitive sandwich ELISA using antibodies 266 (against Aβ), 3D6 (against Aβ), 2G3 (against Aβ), and 21F12 (specific to Aβ) was used for detection (26).

γ-Secretase Inhibitor Treatment of Transfected COS Cells.—The well-differentiated duodenoileum peptidomimetic γ-secretase inhibitor MW115 or an inactive control compound (MW124) of closely similar structure (compounds 11 and 12, respectively, in Ref. 23) were dissolved in DMSO to a 25 mM final concentration. The inhibitors were added to the COS cultures after transfection to final concentrations as noted throughout, and these were then cultured for ≤48 h. The final DMSO percentage in the media was ≤0.2%.

Antibodies—Polyclonal antibodies C7 to the last 20 residues of Aβ (4, 24) and 207 to the first 100 residues of Aβ (25) were described previously. Monoclonal antibodies 3D6 (specific to Aβ 1–15, 206 (specific to Aβ 13–28), 203 (specific to Aβ 35–40), and 21F12 (specific to Aβ 30–42), used for Aβ ELISA, were kindly provided by P. Seubert and D. Schenk (Elan Pharmaceuticals, Inc.) (26).

IP/WB—Aβ peptides secreted into media were immunoprecipitated at 4 °C overnight with 3D6 monoclonal antibody (1:800) and protein G plus A-agarose (Calbiochem). Immunoprecipitates were washed for 20 min at 4 °C in a lysis buffer of 50 mM Tris, pH 7.6, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, and a protease inhibitor mixture (5 µg/ml leupeptin, 5 µg/ml aprotinin, 2 µg/ml peptatin A, and 0.25 mM phenylmethylsulfonyl fluoride (Sigma)) and then washed in lysis buffer containing 0.1% SDS. The samples were washed again in lysis buffer, eluted in Laemmli sample buffer, heated at 70 °C for 5 min, separated by 16% SDS-PAGE, and transferred to nitrocellulose membrane (Millipore Corp.). The membrane was heated to 98 °C for 5 min in PBS, blocked with the 3D6 antibody, and detected with a Supersignal kit (Pierce) according to the instructions of the supplier.

Visualization of APP Chimeric Holoproteins and Their C-terminal Fragments—COS cells were solubilized in lysis buffer (see above) and centrifuged at maximum speed in an Eppendorf centrifuge for 2 min to remove cellular debris. The samples were mixed with Laemmli buffer, cooled at 4 °C for 5 min, resolved on SDS-PAGE on 10% or 4–20% Tris-glycine or Tris-Tricine gels (Novex), transferred to nitrocellulose, and immunoblotted with C7 antibody.

Mass Spectrometric Analysis of Aβ-like Peptides—After transfection, COS cells were allowed to recover in normal medium containing 10% serum overnight and then changed to reduced serum medium (Opti-MEM (Invitrogen)) and conditioned for 48 h. Conditioned media were then concentrated to 1.5 ml of conditioned medium using monoclonal Aβ antibody, 3D6, and protein G plus A-agarose beads (Oncogene Science, Inc., Cambridge, MA) and analyzed using a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (Voyager-DE STR BioSpectrometry work station; PerSeptive Biosystems), as described (27). Spectra were calibrated using bovine insulin as an internal mass calibrator.

Aβ ELISAs—Aβ in conditioned medium was quantified by sandwich ELISA (see Fig. 1) using monoclonal 266 as capture antibody for all TMD replacements or either monoclonal antibodies 2G3 or 21F12 as capture antibody for all of the α-secretase region (pre-TM) replacements. In both cases, antibody 3D6 was used for detection (26).

RESULTS

The Notch TM Domain in APP Chimeric Molecules Can Be Cleaved by γ-Secretase—γ-Secretase/presecretin is required for intramembranous proteolytic processing of Notch to release its cytoplasmic domain (NICD), which is then involved in crucial cell fate decisions during development in all metazoans (6, 7). The reported Notch cleavage site is near the C-terminal end of its TMD (22), while APP releases Aβ peptides by γ-secretase cleavages at or near the middle of its TMD, mainly after position 40 or 42 of the Aβ peptide (Fig. 1A). To assess further the cleavage site in the Notch TMD and whether the Notch TMD gives rise to Aβ-like peptides, chimeric SW-APP expression constructs containing the wild-type human Notch TMD were made (Fig. 1B), either without or with certain point mutations (see below) and then transiently expressed in COS cells. SW-APP was chosen, because this isoform undergoes increased β-secretase cleavage, yielding more C99 as a substrate for γ-secretase and thus substantially enhancing the detection of any Aβ peptides generated (28). Conditioned media were collected, immunoprecipitated with a N-terminal specific Aβ antibody (3D6), and Western blotted with the same antibody, which recognizes Aβ 1–5, an epitope not altered by the mutants created in this study. We replaced the 24-residue TMD of SW-APP with the 23-residue Notch TMD, unless indicated otherwise.

SW-APP and chimeric SW-APP containing either wild-type (SW-m-Notch) or mutant (SW-m-NotchV40A, L41G, L42A, V49L, and 29G+) Notch TMDs were all cleaved, and the resultant Aβ-like peptides were secreted into the media and detected by IP/WB using the Aβ N-terminal antibody, 3D6 (Fig. 2, A and B). The amounts of Aβ-like peptides generated from the various chimeras differed modestly, as determined by a sensitive sandwich ELISA using antibodies 266 (against Aβ 19–28) and 3D6 (against Aβ 1–5) (Fig. 2C). The immunoreactive epitopes of these Aβ-like peptides are outside of the APP sequence that had been replaced. A mutation in the Notch TMD reported to inhibit Notch cleavage markedly (i.e. Val49 to Leu (SW-m-NotchV49L) (22, 29) did not prevent cleavage by γ-secretase. The amounts of Aβ-like peptides released from SW-m-Notch- and SW-m-NotchV49L-transfected cells were about the same and only modestly (but statistically significantly) lower than those from SW-APP (Fig. 2C). By Aβ ELISA, the amount of Aβ-like peptides secreted from SW-m-Notch transfected cells (1068 ± 125 pg/ml) was about 70% of that from the SW-APP transfectants (1565 ± 148 pg/ml) (n = 17, p <
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Heterogeneous TMD and Pre-TMD APP Chimeras Can Generate Aβ-like Peptides—Single amino acid substitutions placed into the APP TMD have been reported to change the efficiency and precise location of cleavage within the TMD but not block cleavage per se (16, 17). Moreover, the locations of the cleavage sites were found to be near the middle of the TMDs in a serial mutation, deletion, and insertion study of the APP TMD (18). It has also been reported that the luminal and cytosolic domains of APP are not absolutely required for γ-secretase processing (20, 31). As described above, SW-m-Notch chimeric protein is cleaved by γ-secretase despite substantial sequence differences between the APP and Notch TMDs. To understand whether the specific TM region (m) sequence or the region just N-terminal to the TM (α secretase cleavage region) plays any role in γ-secretase recognition and cleavage, we carried out domain-specific monoclonal antibody, 3D6, was used to immunoprecipitate Aβ-like peptides (see “Materials and Methods”). The molecular masses of various Aβ-like peptides were measured by using a matrix-assisted laser desorption ionization time-of-flight mass spectrometer. These masses were then used to identify the Aβ-like peptides produced from each chimera (Fig. 3). The relative peak intensity was used to determine the relative abundance of Aβ-like peptides. All Aβ-like peptides are numbered from the first N-terminal residue (Asp-1), known to result from β-secretase cleavage of the SW-APP molecule (30).

The mass spectra of Aβ peptides produced from SW-APP showed that Aβ1–40 was the major Aβ species, as expected, and that minor species included 1–38 > 1–37 > 1–39 = 1–42 > 1–34 = 1–33, as ranked by relative peak intensity (Fig. 3B). The mass spectral patterns of the Aβ-like peptides released from SW-m-Notch- and SW-m-NotchV49G-transfected cells were very similar (Fig. 3, C and D). The major Aβ-like peptide species was again 1–40, closely followed by 1–39, 1–38, 1–28, 1–32, and 1–37 in that order of intensity. Unlike with SW-APP, we detected no species corresponding to Aβ1–42 in the conditioned media of SW-m-Notch and SW-m-NotchV49G transfectants (Fig. 3). The Val to Gly mutation near the C terminus of the Notch TMD did not prevent the cleavage, despite the reported observation that this mutation prevents the cleavage of Notch that generates the NICD (29). The major cleavage site determined by mass spectrometry occurred just C-terminal to residue 40, the same as that seen for SW-APP. However, the relative amounts of the minor γ-secretase cleavages seen in SW-APP and its Notch TMD chimera were somewhat different; the ratio of major to minor peaks was reduced, Aβ1–39 was the second most common cleavage site, and there was some cleavage at 1–28. These results indicate that the sequence specificity of the cleavage in the SW-m-Notch TMD is subtly altered, although the main cleavage still occurs at or near the middle of the TMD.

A γ-Secretase Inhibitor Blocks the Intramembranous Cleavage of SW-APP and SW-m-Notch with a Similar IC_{50}—We performed experiments with a γ-secretase inhibitor to determine whether the cleavages of APP and APP-Notch chimeras share a similar pharmacological profile. After transfection with SW-APP or SW-m-Notch cDNAs, COS cells were treated for 48 h with compound 115, a well characterized difluoroketone γ-secretase inhibitor (6, 23), at a dose near its reported IC_{50} for Aβ inhibition (25 μM) or else with an inactive analogue (compound 124) having a closely similar structure. Aβ-like peptides in the conditioned media were detected by Aβ ELISA (Fig. 4A). Compound 115 produced ~50% inhibition of Aβ generation in both SW-APP and SW-m-Notch transfectants. In four dose-response experiments, the concentrations of inhibitor needed for 50% inhibition were similar (~25 μM), although the IC_{50} for SW-m-Notch was slightly higher (33.83 ± 8.47 μM) than for SW-APP (23.43 ± 4.44 μM) (Fig. 4B). The average inhibition at 25 μM is 57 ± 6% (S.E.) for SW-APP and 32 ± 14% (S.E.) for SW-m-Notch. This difference is not significant (p > 0.05, n = 4).

Fig. 1. A, schematic of the Aβ region of APP (in dark green, underlined in the expanded view), showing the principal β-, α-, and γ-secretase cleavage sites. APP cleaved by β-secretase generates a large N-terminal ectodomain fragment, APP-β, and the C-terminal C99 fragment. APP cleaved by γ-secretase generates the N-terminal APP-α and the C-terminal C83 fragment. The “Swedish” mutation (KM→NL) at the β-secretase site is indicated. Epitopes (black bars) of antibodies used to detect APP, APPα, and Aβ by ELISA and IP/Western blotting are shown. B, amino acid sequences of the Aβ regions of chimeric DNA molecules. Chimeric DNAs were generated by PCR using SW-APP695 as template (Aβ region is in boldface type). Two regions of SW-APP695, the TMD (m) or the region just prior to the TMD including the α-secretase hydrolysis site (α) were replaced by analogous regions of APLP-2, HER-3, SREBP-1, or human Notch-1.
replacements using the corresponding regions of several integral membrane proteins (APLP-2, SREBP-1, HER-3, and Notch-1) (Fig. 1B).

Levels of Aβ-like peptides released from SW-m-APLP-2- and SW-α-SREBP-1-transfected cells were similar or higher than in the SW-APP transfec tant (Fig. 5A). In contrast, levels of Aβ-like peptide released from SW-α-Notch (not shown), SW-α-APLP-2, SW-α-HER-3, SW-m-HER-3, and SW-m-SREBP-1 cells were all substantially decreased (Fig. 5A). The expression levels of full-length SW-APP and these various chimeras were roughly similar in multiple experiments, except for SW-m-SREBP-1, which consistently yielded very little holoprotein (Fig. 5B). Thus, any small differences in the amounts of the expressed holoproteins could not account for the substantial differences in the amounts of the derived Aβ-like peptides. Differences in the general protein turnover of the C-terminal fragments generated by the various chimeras could affect availability of these substrates for γ-secretase processing. However, close inspection of the steady-state levels of C99 derived from the chimeras (Fig. 5B) revealed that they were in reasonable agreement with the respective amounts of Aβ (Fig. 5A), except for SW-α-HER-3. This HER-3 replacement of the α-region generated an extra cleavage site, yielding a ~22-kDa peptide in addition to very large amounts of C-terminal-like fragments, some of which were presumably generated by α- and/or β-secretases (Fig. 5B). Importantly, the levels of total APP (IP with 207, blot with 8E5) and APPβ (IP with 192SW, blot with 8E5) secreted into condition media after α-region replacements with the corresponding SREBP-1 and HER-3 sequences were not significantly changed from those of SW-APP (Fig. 5C). Therefore, any minor alteration of α-secretase processing of these chimeras could not account for the major changes in the production of Aβ-like peptides.

Interestingly, the APLP-2 TMD is more homologous to the APP TMD than is that of Notch (Fig. 1B), but the γ-secretase cleavage pattern of SW-m-Notch (Fig. 3C) was closer to that of SW-APP (Fig. 3B) than SW-m-APLP-2 (Fig. 5D), as revealed by mass spectral analysis. While the major cleavage site for SW-m-Notch was at the position corresponding to Aβ40 (Fig. 3C), the major cleavage site of SW-m-APLP-2 shifted to the Aβ43 position, with only minor cleavages detected at the 40- and 42-positions (Fig. 5D). SW-α-SREBP-1 generated a 4-kDa Aβ-like peptide with the major cleavage occurring at residue 40 (Fig. 5E). Taken together, these various results suggest that not all hydrophobic TMDs can successfully replace the APP TMD and thus that the conformation of a TMD or the total effects of side chain residues presented to the TMD may influence the composition of γ-secretase products. Furthermore, the sequence immediately N-terminal to the TMD also plays a role in the recognition and/or cleavage of substrate by γ-secretase. 

**Fig. 2.** Aβ-like peptides are secreted from SW-m-Notch chimeric transfectants. COS cells were transfected with the indicated chimeric SW-APP cDNAs containing the wild type or mutant Notch TMD. In the sequences, *underlining* indicates the wild type Aβ region, and *boldface type* indicates the substituted Notch TMD. Point mutations placed within the Notch TMD are indicated. A and B, Aβ-like peptides secreted into media were detected by IP with antibody 3D6, separation on 16% Tricine gels, and WB with 3D6. Two representative gels are shown. C, Aβ ELISA of conditioned media from the transfectants shown in A. Antibody 266 (to Aβ13–28) was used for capture and antibody 3D6 (to Aβ1–5) for detection. Means ± S.D. of n = 5 independent experiments is shown. D, transfected cells were solubilized in lysis buffer after collection of the above media, separated on 4–16% Tris-glycine gels, and blotted with APP antibody C7. Full-length APP and the α- and β-secretase-generated products, C83 and C99, are indicated. We did not clearly detect a C89 product, perhaps because the endogenous β-secretase in COS cells has little cleavage activity at this site in APP.
DISCUSSION

Numerous studies indicate that a γ-secretase highly similar to that which processes APP is also responsible for the apparent intramembranous cleavage of Notch to release its intracellular domain (NICD), allowing the latter to signal in the nucleus (6, 8, 10, 32, 33). In accord, both Notch and APP have been shown to interact with PS1 (12, 13, 34). PS1 and PS2 appear to contain the active sites of γ-secretase (4, 5, 14, 15). Interestingly, the activities of PS1 in cleaving APP and Notch can be differentially modulated by artificial mutations introduced at residue Leu286 (35), suggesting that PS1-mediated γ-secretase activity is complex, involving interactions with various substrates and/or modulators, both at the active site and elsewhere within the enzyme.

Two outstanding questions about the unusual mechanism of γ-secretase are where within their respective TMDs the APP and Notch substrates are cleaved and how similar these cleavage events are. To address these questions and further characterize the relationship of these substrates, we replaced the TMD of APP with that of Notch and certain other integral membrane proteins. Our results show that an APP-m-Notch chimera is readily cleaved by γ-secretase at similar positions to those cleaved in APP itself, despite the significant sequence divergence of the two TMDs. Our mass spectrometry data clearly show that γ-secretase cleaves the Notch TMD at or near its middle (i.e. after residue 40) in these chimeric molecules. Mutations at the reported downstream cleavage site (SW-m-NotchV49G) that markedly decrease or abolish NICD release from full-length Notch constructs (22, 29) did not prevent cleavage in our chimeras. Thus, either the context of the TMD (i.e. the flanking sequences) determines the specificity of γ-secretase cleavage, or the previously reported Notch cleavage site near the cytoplasmic face is not the sole cleavage site.

After these experiments were completed, several laboratories reported the detection of a γ-secretase-generated cytoplasmic fragment of APP itself, referred to as the AICD (36–39). A portion of the AICD was shown to enter the nucleus in complex with Fe65, a known APP cytoplasmic binding partner (36). These results complement recent functional evidence that AICD can participate with Fe65 and the Tip60 histone acetylase complex in the transactivation of

![Figure 3](image_url) Mass spectral analysis of Aβ-like peptides secreted by SW-APP and its chimeric protein containing the Notch TMD. Representative spectra are shown of Aβ-like peptides produced by COS cells transfected with SW-APP (B), SW-m-Notch (C), or SW-m-NotchV49G DNA (D) (see “Materials and Methods”). Spectra are normalized to the most abundant Aβ-like peptide species in each medium sample (set at 100%), and peaks in the spectra are labeled with their masses and the corresponding Aβ-like peptide length, counting from the first N-terminal residue (Asp-1). The peaks from mock-transfected cells (A) are background peaks.

![Figure 4](image_url) Secretion of Aβ-like peptides from cells expressing a SW-m-Notch chimera is reduced by a γ-secretase inhibitor. A, SW-APP or SW-m-Notch chimeric cDNA-transfected COS cells were treated for 48 h with a 25 μM concentration of a difluoroketone peptidomimetic inhibitor (115) or an inactive analogue (124). Aβ-like peptides secreted into media were quantified by Aβ ELISA. Data are means ± S.E. of five independent experiments. Values for 115 (25 μM) are significantly decreased versus vehicle alone (p < 0.05); values for 124 (25 μM) are not significantly different from vehicle alone. B, COS cells transfected with SW-APP (●) or SW-m-Notch (○) chimeras were treated for 48 h with increasing concentrations of inhibitor 115.
heterologous nuclear reporter genes (40). Interestingly, the AICD generated from APP appears to begin at valine 50 near the cytoplasmic face of the APP TMD, a position homologous to the valine at which NICD is reported to begin (22, 39). When these findings are considered together with our mass spectrometry on the Aβ-like peptides generated from the APP-m-Notch chimera, two possibilities emerge: that Notch and APP are cleaved at their valine 50 positions followed by secondary cleavages near the middle of the TMDs, or vice versa. We favor the latter sequence, because (a) mutagenesis suggests that γ-secretase is directed to cleave APP at the middle of its TMD (17, 18) and (b) the two intramembranous aspartates in PS that may represent the active site of γ-secretase (4) are themselves predicted to be in the middle of the respective PS TMDs (41).

The cleavage of our Notch chimera was inhibited by a well characterized γ-secretase inhibitor, with a similar IC50 to that for APP processing. The addition of a glycine just N-terminal to the Notch TMD or the mutation of the Notch TMD at aa 40, 41, 42, or 49 did not prevent the cleavage of the SW-Notch chimeric protein. These results extend earlier evidence that the primary structure of the TMD is not crucial for γ-secretase recognition and cleavage, although it can change the efficiency of the cleavage (16–19). We examined this issue further by substituting the TMDs of APLP-2, SREBP, or HER-3 for that of APP. Here, only the TMD of the close APP homologue, APLP-2, underwent Aβ-like cleavage, although the major cleavage site was closer to the N terminus of the TMD than occurs with APP itself. The SW-m-SREBP-1 chimera could not be expressed at sufficient levels to determine whether this TMD can serve as a substrate of γ-secretase. However, earlier work has shown that the unusual metalloprotease (site 2 protease) responsible for the intramembranous cleavage of SREBP does not mediate APP processing (42). Our results thus indicate that some hydrophobic TMDs cannot be cleaved by a γ-secretase-like mechanism. The conformation of the TMD, not just its primary structure, may be the principal determinant for γ-secretase recognition and cleavage.

We have also implicated the region of APP just N-terminal to the TMD as having a role in recognition and/or cleavage of APP. Chimeric molecules with substitutions of the α-region (i.e. the 19–20 residues immediately N-terminal to the TMD) revealed that this region is also important for proper γ-secretase processing. Replacement of the Aβ10–29 region of APP with the corresponding sequence of the growth factor receptor HER-3 (yielding SW-α-HER-3) generated a large amount of the C99- and C83-like fragments (Fig. 5B), but very little of this was cleaved to form Aβ-like peptides. On the other hand, replacement of this region of APP (Aβ10–28) with the corresponding SREBP-1 sequence (yielding SW-α-SREBP-1) gener-
ated more Aβ-like peptides (Fig. 5A). There were no significant increases in the secretion of total APP, and APP*β from this chimeric protein (Fig. 5C). Mass spectral analysis showed that the γ-secretase cleavage pattern was very similar to that of APP (i.e., with a major cleavage at 40 and a minor cleavage at 42) (Fig. 5E). These data suggest that the pre-TM domain plays a hitherto unrecognized role in the ability of γ-secretase to recognize and properly cleave its substrate. The fact that the α-region (Aβ 10–28) replacements did not substantially alter β-secretase cleavage is consistent with published data indicating that deletion of Aβ 5–9 or Aβ 9–12 still allowed generation of Aβ, p3, and APPs, whereas mutations at or immediately adjacent to the β-secretase cleavage site had profound effects (20).

In conclusion, the picture emerging from these and previous mutagenesis studies (16–19) is that γ-secretase is relatively promiscuous regarding the substrate sequence within the lipid bilayer but that it preferentially cleaves at or near the middle of the TMD. Moreover, the cytoplasmic tail of the substrate is dispensable for proper γ-secretase cleavage (20), and the large ectodomain must be shed before the intramembranous scission can occur (43). While the principal determinant of cleavage efficiency and specificity seems to be the conformation and size of the TMD itself, a sequence in the pre-TMD region helps regulate this cleavage. Whether the latter region contains a recognition or binding site for the protease or simply serves to alter subtly the conformation of the intramembranous portion remains to be seen.

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Lichtenthaler, S. F., Fang, W., Grimm, H., Uljon, S. N., Masters, C. L., and Beyreuther, K. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 3055–3058.

Murphy, M. P., Hickman, L., Eckman, C. B., Uljon, S. N., Wang, R., and Golde, T. E. (1999) J. Biol. Chem. 274, 2637–2644.

Schroeter, E. H., Kissingler, J. A., and Kepan, B. (1998) Nature 393, 382–386.

Wolfe, M. S., Xia, W., Moore, C. L., Leatherwood, D. D., Ostaszewski, B., Rahmati, T., Donkor, I. O., and Selkoe, D. J. (1999) Biochemistry 38, 4720–4727.

Podlisny, M., Tolan, D., and Selkoe, D. J. (1991) Am. J. Pathol. 138, 1423–1435.

Lowery, D. E., Pasternack, J., Gonzalez-DeWhitt, P. A., Zurcher-Neely, H., Tomich, C. C., Altman, R. A., Fairbanks, M. B., Heinrikson, R. L., Younkin, S. G., and Greenberg, B. D. (1991) J. Biol. Chem. 266, 19842–19850.

Johnson-Wood, K., Lee, M., Motter, R. H., Ru, K., Gordon, G., Barbour, R., Khan, K., Gordon, M., Tan, H., Games, D., Lieberburg, I., Schenk, D., Seubert, P., and McConlogue, L. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 1550–1555.

Wang, R., Sweeney, D., Gandy, S. E., and Sisodia, S. S. (1996) J. Biol. Chem. 271, 31894–31902.

Citron, M., Oltersdorff, T., Haass, C., McConlogue, L., Hung, A. Y., Seubert, P., Vigo-Pelfrey, C., Lieberburg, I., and Selkoe, D. J. (1992) Nature 362, 672–674.

Huppert, S. S., Le, A., Schroeler, E. H., Mumm, J. S., Saxena, M. T., Milner, L. A., and Kopan, R. (2000) Nature 405, 966–970.

Citron, M., Vigo-Pelfrey, C., Teplow, D. B., Miller, C. S., Schenk, D., Sisodia, S. S., Winblad, B., Vettier, N., Landfester, L., and Selkoe, D. J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 11993–11997.

Bunnell, W., Pham, H. V., and Glabe, C. G. (1998) J. Biol. Chem. 273, 31947–31955.

Mumm, J. S., Schroeter, E. H., Saxena, M. T., Griesemer, A., Tian, X., Pan, D. J., Ray, W. J., and Kopan, R. (2000) Mol. Cell. 5, 197–206.

Song, W., Nadeau, P., Yuan, M., Yang, X., Shen, J., and Yankner, B. A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 6959–6963.

Xia, W., Ray, W., Ostaszewski, B. L., Rahmati, T., Kimberly, W. T., Wolfe, M. S., Zang, J., Goate, A. M., and Selkoe, D. J. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 9290–9304.

Kulic, L., Walter, J., Multhaup, G., Teplow, D. B., Baumeister, R., Romig, H., Capell, A., Steiner, H., and Goate, A. M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 5918–5923.

Kimberly, T. W., Zheng, B. J., Guenette, S. Y., and Selkoe, D. J. (2001) J. Biol. Chem. 276, 40288–40292.

Gu, Y., Misonou, H., Sato, T., Doehnau, N., Takio, K., and Ihara, Y. (2001) J. Biol. Chem. 276, 35235–35238.

Capell, A., Lai, M. T., Huang, Q., Castro, J. L.,和, DiMuzio-Mower, J., Rassar, R., Bennett, B., Luo, Y., and Selkoe, D. J. (2000) J. Neurochem. 75, 583–593.

REFERENCES

1. De Strooper, B., Saffig, P., Craessaerts, K., Vanderstichele, H., Guhde, G., Anneta, W., Von Figura, K., and Van Leuven, F. (1998) Nature 391, 387–390.

2. Herreman, A., Serneels, L., Anneta, W., Collen, D., Schoonjans, L., and De Strooper, B. (2000) Nat. Cell Biol. 2, 461–462.

3. Zhang, Z., Nadeau, P., Song, W., Donoviel, D., Yuan, M., Bernstein, A., and Yankner, B. A. (2000) Nat. Cell Biol. 2, 463–465.

4. Wolfe, M. S., Xia, W., Ostaszewski, B. L., Diehl, T. S., Kimberly, W. T., and Selkoe, D. J. (1999) Nature 398, 513–517.

5. Kimberly, T. W., Xia, W., Rahmati, T., Wolfe, M. S., and Selkoe, D. J. (2000) J. Biol. Chem. 275, 3173–3178.

6. De Strooper, B., Anneta, W., Cuperus, P., Saffig, P., Craessaerts, K., Mumm, J. S., Schroeter, E. H., Schrijvers, V., Wolfe, M. S., Ray, W. J., Goate, A., and Kopan, R. (1999) Nature 398, 518–522.

7. Struhl, G., and Greenwald, I. (1999) Nature 398, 522–525.

8. Steiner, H., Duff, K., Capell, A., Romig, H., Grim, M. G., Lincon, S., Hardy, J., Yu, X., Picciano, M., Fichteler, K., Citron, M., Kopan, R., Pascali, B., Keck, S., Baeder, M., Tomita, T., Iwatsubo, T., Baumeister, R., and Haass, C. (1999) J. Biol. Chem. 274, 28669–28673.

9. Ray, W. J., Yao, M., Mumm, J., Schroeter, E. H., Saffig, P., Wolfe, M., Selkoe, D. J., Kopan, R., and Goate, A. M. (1999) J. Biol. Chem. 274, 36801–36807.

10. Berezovska, O., Jack, C., McLean, P., Aster, J. C., Hicks, C., Xia, W., Wolfe, M. S., Kimberly, W. T., Weinmaster, G., Selkoe, D. J., and Hyman, B. T. (2000) J. Neurochem. 75, 583–593.